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TITLE:

Quantification of Macronutrients Intake in a Thermogenetic Neuronal Screen Using *Drosophila* Larvae

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KEYWORDS:

Drosophila, larvae, thermogenetic neuronal screen, larval feeding behavior, macronutrient balancing, protein, carbohydrates, food intake assay, food dye, colorimetric quantification

SUMMARY:

Described here is a protocol that enables the colorimetric quantification of the amount of food eaten within a defined interval of time by *Drosophila melanogaster* larvae exposed to diets of different macronutrient quality. These assays are conducted in the context of a neuronal thermogenetic screen.

ABSTRACT:

Foraging and feeding behaviors allow animals to access sources of energy and nutrients essential for their development, health, and fitness. Investigating the neuronal regulation of these behaviors is essential for the understanding of the physiological and molecular mechanisms underlying nutritional homeostasis. The use of genetically tractable animal models such as worms, flies, and fish greatly facilitates these types of studies. In the last decade, the fruit fly *Drosophila melanogaster* has been used as a powerful animal model by neurobiologists investigating the neuronal control of feeding and foraging behaviors. While undoubtedly valuable, most studies examine adult flies. Here, we describe a protocol that takes advantage of the simpler larval nervous system to investigate neuronal substrates controlling feeding behaviors when larvae are exposed to diets differing in their protein and carbohydrates content. Our methods are based on a quantitative colorimetric no-choice feeding assay, performed in the context of a neuronal thermogenetic-activation screen. As a read-out, the amount of food eaten by larvae over a 1 h interval was used when exposed to one of the three dye-labeled diets that differ in their protein to carbohydrates (P:C) ratios. The efficacy of this protocol is demonstrated in the context of a neurogenetic screen in larval *Drosophila*, by identifying candidate neuronal

populations regulating the amount of food eaten in diets of different macronutrient quality. We were also able to classify and group the genotypes tested into phenotypic classes. Besides a brief review of the currently available methods in the literature, the advantages and limitations of these methods are discussed and, also, some suggestions are provided about how this protocol might be adapted to other specific experiments.

INTRODUCTION:

All animals depend on a balanced diet to acquire the necessary amounts of nutrients for survival, growth, and reproduction[1]. The choice of what and how much to eat is influenced by a multitude of interacting factors related to the internal state of the animal, like the satiety level, and environmental conditions, such as food quality[2-5]. Protein and carbohydrates are two major macronutrients and its balanced intake is essential to sustain animals' physiological processes. Therefore, the understanding of the neural mechanisms controlling feeding behaviors and sustaining a balanced intake of these macronutrients is extremely relevant. This is because life history traits like lifespan, fecundity, and metabolic health are directly affected by the levels of protein intake intake[6-10].

The use of simpler more tractable organisms that exhibit evolutionarily conserved feeding habits with complex animals, including mammals, is essential to this type of studies. Importantly, these simpler animal models provide a good opportunity to dissect complex biological questions in a costly, ethically and technically more effective context. In the last decades, *Drosophila*, with its powerful genetic toolkit, intricate and stereotypical behavior and conserved architecture of peripheral and nutrient-sensing mechanisms with mammals, has been a fruitful model for behavioral neurobiologists[11]. Ultimately, the hope is that by understanding how food intake is regulated in this animal, with a simpler nervous system, we can then begin to untangle neuronal malfunctions underlying human eating disorders.

The study of neuronal substrates for feeding behaviors is deeply dependent on being able to simultaneously measure animals' food intake while manipulating their neuronal activity. Due to the minimal quantities of food ingested, quantifying the amount of food eaten by flies is extremely challenging, and all methods currently available present significant limitations. Thus, the gold standard is to use a combination of complementary methodologies[12]. Adult flies have been historically favored as a genetic and behavioral model. Nevertheless, *Drosophila* larvae, also offer opportunities to investigate neuronal substrates encoding feeding behavior. The larval central nervous system (CNS), with around 12,000 neurons, is significantly less complex than that of the adult, which contains approximately 150,000 neurons. This lower complexity is not only numerical but also functional, since larval behaviors rely on simpler locomotive functions and sensory systems. Despite the apparent simplicity of their nervous systems, larvae still exhibit complete feeding behaviors, and some methods to quantify food ingestion in *Drosophila* larvae have been described[5,13-15]. By pairing with manipulations of neuronal activity, *Drosophila* larvae can constitute a highly tractable model for understanding the neural regulation of food intake.

Provided here is a detailed protocol to quantify food intake in larvae exposed to diets of different macronutrient quality. The diets, so-called macronutrient balancing diets, differed in the protein

and carbohydrates contents, specifically with respect to the protein to carbohydrate (P:C) ratios: 1:1 (protein-rich diet), 1:4 (intermediate diet), and 1:16 (protein-poor diet), as shown in **Figure 1A**. Briefly, a quantitative no-choice feeding assay was established using these three isocaloric sucrose-yeast (SY)-based diets dyed with a blue food dye. Because yeast extract and sucrose were used as protein and carbohydrate sources, and both contain carbohydrates, variation in the P:C ratios was obtained by changing the balance of these two components, as previously described[16] and as indicated in **Figure 1B**. A schematic overview of the protocol, showing the main experimental steps, is available in **Figure 2**.

This protocol was established with the aim of investigating the role of specific neuronal populations on the regulation of larval feeding levels in diets of different P:C ratios and in the context of a thermogenetic neuronal screen. A well-characterized neurogenetic tool was used from the Transient Receptor Potential (TRP) family: *Drosophila* Transient Receptor Potential channel (dTRPA1), which is a temperature and voltage-gated cation channel, allowing the firing of action potentials when ambient temperatures rise above 25 ˚C[17]. To express the dTRPA1 transgene, we took advantage of the Gal4 lines based on *cis*-regulatory regions from the *Drosophila* genome, established in the Rubin laboratory, in the context of the FlyLight project at Janelia Research Campus[18,19].

Although the protocol, here described, has been established in the context of an activation screen, it can be easily adapted by the experimenter to other specific needs or interests, namely to perform a suppression screen using the temperature sensitive neuronal silencer ShibireTS[20], in alternative to dTRPA1. This and other adaptions are discussed in the protocol and discussion sections.

PROTOCOL:

1. Preparation of the sucrose-yeast (SY) diets

1.1. Weigh all the dry ingredients (agar, yeast, sucrose) for the macronutrient balancing and L3 rearing diets. The amounts in grams for each of the ingredients needed to prepare 1 L of food are indicated in **Figure 1B.**

NOTE: Take into account that approximately 13 mL of food is needed to fill a 60-mm Petri dish.

1.2. Dissolve all ingredients in sterile distilled water (use approximately 50% of the total volume of water needed to prepare the food) and stir the medium for 5-10 min.

1.3. Autoclave for 50 min.

1.4. After allowing the mediums to cool down, add nipagin and propionic acid solutions to the diets, at 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%. Complete the total volumes with distilled water.

1.5. Carefully pour the food diets to 60 mm Petri dishes, so that the quantity of food poured is approximately the same in each one of the plates. Label the plates with the P:C ratios of the diets.

NOTE: Prepare the macronutrient balancing diets on the day of the feeding assay. If not possible, store the prepared diets at 4 ˚C, in a sealed container, for a maximum duration of 3 days. Longer storage periods render the diet too dry and hard, and the larvae cannot burrow into the medium.

2. Genetic cross of parental lines

NOTE: Use the Gal4/UAS system[21] to set up the genetic crosses. In this protocol, in order to activate neuronal function in specific neuronal populations, female virgins of the UAS dTRPA1 line[17] were used and crossed to males from the Janelia Gal4 lines (**Figure 2A**). The genetic control used was the progeny of a cross between the dTRPA1 line and an "empty GAL4" line, which carries Gal4 in the vector used to generate the Rubin Gal4 collection but with no regulatory fragment present (attP2)[22]. To promote the neuronal suppression, a UAS line encoding ShibireTS[20] can be used, instead of dTRPA1.

2.1. Set up 60 mm embryo collection cages with L3 rearing diet plates, supplemented with some active yeast paste.

2.2. Transfer the adult UAS dTRPA1 female virgins and Janelia Gal4 males, aged 5-8 days, to the embryo collection cages and allow the mating to occur for 24-48 h, at 25 °C, with 60% humidity and a 12:12 light-dark cycle (**Figure 2A**). For 60 mm embryo collection cages, use around 100 virgin females and 30 males per cross.

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. Preparation of third-instar larvae (L3)

3.1. Transfer the mated adult flies to fresh L3 rearing diet plates and allow the egg-laying to occur for 3-4 h, at 25 °C (**Figure 2B**). Make sure that all the plates are labeled with the genotype, P:C ratio of the diet and date of the egg lay.

NOTE: To save time, perform the egg-laying directly into the L3 rearing diet, which avoids extra handling of the eggs. In the case of small-scale genetic screenings, optimization of the egg-laying can be obtained by using apple juice agar plates.

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

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.

3.3. Estimate the number of eggs per plate and keep the larval density to a maximum of 200 embryos per plate. This estimation can be done by counting the number of embryos in one quarter of the plate.

NOTE: An overcrowded plate will delay larval development and affect larval feeding behaviors.

3.4. Incubate the L3 rearing plates at 18 ˚C (permissive temperature), 60% humidity and a 12:12 light-dark cycle, and allow the larvae to grow for 9 days (**Figure 2B**).

3.5. On the ninth day after egg laying (AEL), collect three groups of 10 L3 from each of the genotypes (and for the replicates) to be tested. Additionally, collect groups of 10 L3 for the "zero-dye food" control. Ensure that the larvae collection is done during equivalent time periods of the day used to do the egg lay (e.g., if the egg laying occurred between 10am-2pm, collect the larvae during the same period of time 9 days AEL) and is performed, as gently as possible, by using forceps #5 or a featherweight forceps. Directly transfer the larvae as indicated in next step (3.6).

NOTE: 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.

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.**

NOTE: The collection of L3 to plastic boats containing water or 1x Phosphate-buffered saline (PBS), is important to keep larvae well hydrated before the start of the feeding assay. This is especially important if several experimental L3 groups from different genotypes are being collected at the same time. Keep track of the collection order for each group, so differences in the duration of food-deprivation for each group are minimized. The use of plastic boats in this step facilitates the optional step 4.3 as it enables larvae to float directly into the water bath.

4. Thermogenetic activation and no-choice feeding assay

NOTE: It is recommended to perform the feeding assays at approximately the same time of the day to minimize possible variations related to the circadian rhythms. Also, always run the control experiments (the progeny of the "empty Gal4" line crossed to UAS dTRPA1 and the "zero-dye food" larvae), in parallel with the genotypes of interest.

4.1. Set up an incubator to 30 ˚C (non-permissive temperature) and keep high levels of humidity (at least 65%) to avoid larval dehydration during the assay.

4.2. Before starting the feeding assay, equilibrate the assay plates' temperature by warming them at 30 ˚C for 30 min.

4.3. (Optional) Heat-shock the experimental larvae for 2 min in a 37 ˚C water bath. Perform this step with the animals in the plastic weight boats containing some water.

NOTE: The aim of this step is to intensify the neuronal activation by promoting the firing of the neurons since the beginning of the feeding assay.

4.4. Keep multiple timers set for 1 h ready. The number of timers to be used depends on the number of experimental groups being tested and on the experimenter's skillfulness level on handling larvae.

NOTE: The use of multiple timers is critical to keep the duration of the assay consistent for all the genotypes.

4.5. Carefully drain the water from the plastic boats and, using a moistened soft brush, gently transfer the L3 groups from the boats to the center of the assay plates. Put the plates' lids back and start a timer for each plate (or group of plates) to maintain accurate 1-h feeding sessions.

4.6. Allow the larvae to feed for 1 h, at 30 ˚C, in the dark (**Figure 2C**).

NOTE: The performance of the assay in the dark is important to control for differences in the visual cues across diets, as the diets will differ in tones, even though they contain the same dye concentration.

4.7. Stop the feeding assay by transferring the plates to an ice bath. Press down the ice as much as possible to provide a stable surface for the plates.

NOTE: Cold temperatures will promote the end of the feeding by inhibiting burrowing and digging behaviors. Most of the larvae will surface the food plates after some minutes, facilitating their recovery in the following steps.

5. Food dye extraction

5.1. Prepare 2 mL microtubes for each group of 10 L3 tested, containing approximately the same amounts of 0.5 mm-glass beads (enough to fill the bottom portion of the microtube) and 300 µL of ice-cold methanol. Keep the microtubes in the cold, using a bench cooler.

CAUTION: Methanol is highly flammable and toxic. Follow all safety procedures recommended for handling this reagent, including working in a well-ventilated area and wearing nitrile gloves.

NOTE: The use of methanol is important to fix the larval samples and avoid melanization reactions

in the cuticle.

5.2. Using #5 or featherweight-forceps, carefully recover the groups of 10 L3 from the feeding assay plates and transfer them to the lids of the assay plates containing some water. Rinse the larvae to remove any food debris on their bodies while gently handling the larvae to avoid any injuries. Keep a record of the number of larvae recovered for each genotype per replicate, so that the mean amount of food intake per larva can be quantified.

NOTE: Injured larvae should be discarded as they will have melanized cuticle, being unsuitable for colorimetric quantification.

5.3. Transfer the L3 groups to the 2 mL microtubes prepared in 5.1.

5.4. Lyse the larval tissues to extract the food dye from the guts by a mechanical lysis method using a tissue lyzer and glass beads added in step 5.1. (if a tissue lyzer is not available, use a homogenizing pestle). Preferentially, perform this step at 4 °C (**Figure 2D**).

NOTE: The duration of this step will depend on the equipment used. Using a conventional tissue lyser, 1 min extraction is sufficient. In case of time constrictions, the protocol can be paused in the end of this step and proceeded later. Store the samples at -20 °C.

5.5. Transfer the extracts to clean 1.5 mL microtubes, by directly inverting the 2 mL microtubes onto the new 1.5 mL microtubes. If performed gently, most of the glass beads will stay at the bottom of the 2 mL microtube.

5.6. Clear the cellular debris by centrifuging the extracts, at a maximum speed for 10 min, at 4 °C.

5.7. Collect the supernatants to clean 1.5 mL microtubes. If cellular debris are still visible in the supernatants, repeat steps 5.6 and 5.7.

6. Colorimetric quantification of food consumption

6.1. Prepare standard solutions, to generate a calibration curve, by performing serial 1:2 dilutions in methanol of a starting blue dye solution. As blank, use methanol only. The concentration of the standards is dependent on the animals' levels of food intake.

NOTE: In the case of the pilot screen presented here, 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.

6.2. Transfer 100 µL of the experimental samples (obtained in step 5.7), standards and blank

(step 6.1) to the wells of a 96-well microplate and measure the absorbance at 600 nm, using a plate reader (**Figure 2E**). To remove the background absorbance, measure the absorbance of extracts obtained from larvae fed on food without blue-dye as a "zero" for the larval extracts ("zero-dye food control).

6.3. Generate a standard curve and correlate the absorbance values obtained for the samples from each experimental larval group with the amount of food intake (volume in mL). Find the average food consumption per larva by taking into account the number of larvae collected for each group in the step 5.2

REPRESENTATIVE RESULTS:

Drosophila larvae regulate their protein intake at the cost of ingesting excess carbohydrates[23] (schematic plot in **Figure 2E**). Actually, this prioritization of protein intake has been observed in many other animals and is called the protein leveraging[24,25].

Taking advantage of this robust feeding behavioral response, a behavior-based screen was designed aiming to identify neuronal populations involved in macronutrient balancing. A no-choice feeding assay was established, which consisted of allowing groups of L3 (10 individuals per group) to feed ad libitum for 1 hour and under neuronal thermogenetic-activation conditions using dTRPA1, in three isocaloric (248 Cal/L) food-dyed diets containing specific P:C ratios (1:1, 1:4 and 1:16) (**Figure 1** and **Figure 2C**). As a read-out, the mean amount of food eaten in the macronutrient diets of different P:C ratios was used. Taking advantage of the Gal4/UAS system[21] and using some of the Janelia Gal4 lines from the FlyLight Project[18,19], the expression of dTRPA1 was induced in specific neuronal populations.

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. This experimental approach demonstrated that activating distinct populations of neurons significantly affected macronutrient balancing in third-instar larvae (**Figure 4**, **Table 1**). The feeding pattern observed for the control line (attP2) demonstrates the effectiveness of the method by showing an expected compensatory increase of food intake by larvae tested in lower P:C ratio diets (grey dots and line in **Figure 4**). Moreover, a significant interaction between the genotypes and the diet was found, which means that the thermogenetic-activation of specific neuronal populations changes the way larvae regulate their food intake in response to the macronutrient quality of the diet.

The feeding patterns of the genotypes tested in the three macronutrient balancing diets (1:1, 1:4, 1:16) are shown by the colored dots and lines in **Figure 4** and the statistical analysis are available in **Table 1**.

In the activation screen, in total, 36 Janelia Gal4 lines known to be sparsely expressed in the larval nervous system were tested. 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).

Across all three diets, R12E06 ate significantly more food than control animals. In addition, it overcompensated the increase in food intake on the intermediate and low protein diets, as indicated by a significant difference in the interaction term between food intake and P:C ratio of the diet (**Table 1**). R22H01 ate significantly more than controls but did not differ in the macronutrient balancing response (**Table 1**). R14B11, R19G11, R21B06, R29C02 and R48F09 larvae ate little amounts of food and lost the ability to compensate for the poor macronutrient quality of the diet available (as indicated by the significant interaction terms between food intake and P:C ratio of the diet, **Table 1**). Finally, R45D11 larvae ate significantly more in the protein-rich diet containing a P:C ratio of 1:1 than in the intermediate and in the protein-poor diets (1:4 and 1:16), which is the opposite of what one would expect on the low protein diets.

Therefore, our methods allowed us to classify the experimental larvae, from each genotype, into phenotypic classes related to the total amount of food eaten and ability to prioritize protein intake by overconsuming in the diets of low P:C ratio. Five phenotypic classes were established for the experimental animals (**Figure 5**): 1 – "Eat a lot" (more than the control animals) and overcompensate for protein dilution; 2 – "Eat a lot but compensate normally"; 3 – "Eat little (less than the control) but compensate"; 4 – "Eat little and do not compensate"; 5 – "Eat aberrantly" (more in protein-rich and intermediate diets than in the protein-poor diet). Additionally, for each of these phenotypic classes and genotypes, we show the GFP patterns in the central nervous systems of third-instar larvae. This information was obtained from the publicly available imaging data in the FlyLight Project online platform, where one can get access to the expression patterns of all the Rubin Gal4 lines of interest [26].

FIGURE AND TABLE LEGENDS:

Figure 1: The sucrose-yeast (SY) diets used in our protocol. (**A**) The blue dots represent the isocaloric (248 calories/L) macronutrient balancing diets used in the feeding assay, which differ in the protein to carbohydrate (P:C) ratios: 1:1, 1:4 and 1:16. The beige dot represents the diet used to rear the experimental third-instar larvae (L3), which contained a P:C ratio of 1:2 and a caloric density of 495 calories/L. (**B**) Detailed composition and nutritional information of the sucrose-yeast (SY) based diets. The components are the same for all the diets: agar, sucrose and yeast. The amount in grams of the components needed to prepare 1 L of diet is shown. Note that 1% (v/v) of blue dye must be added to the macronutrient balancing diets and to the L3 rearing diet nipagin and propionic acid solutions must be added to a final concentration (v/v) of 3% and 0.3%, respectively.

Figure 2: Schematic representation of the main steps involved in our protocol (**A**) Genetic cross of parental lines taking advantage of the Gal4/UAS system. The cross between the Rubin Gal4 lines and the UAS line encoding dTRPA1, allows the thermogenetic activation of specific neuronal populations in the larval central nervous system. (**B**) Preparation of the experimental third-instar larvae (L3). The parental females were allowed to lay eggs for 3-4 h and the larval staging occurs at the permissive temperature (18 ˚C) for 9 days. Optional is the heat shock at 37 ˚C for 2 min

before the feeding assay. (**C**) Thermogenetic activation of the neuronal function and no-choice feeding assay for 1 h at the non-permissive temperature (30 ˚C). Three groups of 10 experimental L3 from each genotype were allowed to feed in each one of the macronutrient balancing diets containing specific protein to carbohydrates (P:C) ratios (1:1, 1:4 and 1:16). (**D**) Food dye extraction. Mechanical lysis of larvae, using a tissue lyser, to extract the blue food dye. (**E**) Food intake quantification. Colorimetric quantification of the mean amount of food eaten per larva by quantifying food dye concentration in the larval extracts. The absorbance of the experimental samples, standards and "zero" was measured at 600 nm (blue), using a 96-well plate reader.

Figure 3: Differences between second (L2) and third-instars *Drosophila* larvae (L3). The L2 and L3 can be easily distinguished by the observation of spiracles under a stereomicroscope. The anterior spiracles of L2 are club-like, while in L3 are branched. Other characteristics may help to distinguish the two instars but are subjective and less reliable. The posterior spiracles of L3 have a dark orange ring at their tip, which is lacking or weakly present in the L2. The trachea is thicker in L3 larvae. Illustration by Marisa Oliveira.

Figure 4: Amount of food eaten per larva under neuronal thermogenetic-activation conditions in three macronutrient balancing diets containing specific protein to carbohydrates (P:C) ratios. Mean levels of amount of food eaten per larva (mL) in 3 macronutrient balancing diets containing the specific P:C ratios of 1:1, 1:4 and 1:16. Groups of 10 third-instar larvae, from each genotype, were allowed to feed during 1 hour, under neuronal thermogenetic-activation conditions, using dTRPA1, at 30 ˚C. The genotypes tested (larval progenies from the genetic crosses between the Rubin Gal4 lines and the UAS dTRPA1 line) are indicated by dots and lines of different colors. As a genetic control (indicated in grey), the larval progeny from a cross between the "empty Gal4" line (attP2) and UAS dTRPA1 were used. The names given to the genotypes, indicated in the legend, were related to the "Rubin GAL4" lines used.

Figure 5: Grouping the lines tested in 5 main phenotypic classes. The phenotypic classes indicated by numbers were based on the combination of the phenotypes observed in terms of total amount of food eaten and ability to maintain the protein intake prioritization response: 1 - eat a lot (more than the control animals) and were able to compensate for protein dilution by overeating; 2 – eat a lot and were not able to compensate; 3 - eat little (less than the control) but compensate; 4 – eat little and were not able to compensate; and 5 - an extra phenotypic class, that were called "aberrant", in which the larvae didn't behave as expected in response to the macronutrient dilution of protein content in the diet, eating more in protein-rich and intermediate diets than in the protein-poor diet. For each genotype, the GFP expression pattern in the central nervous systems of third-instar larvae is shown. This imaging data of the Rubin Gal4 lines used in this assay was extracted from the publicly available FlyLight Project online platform[26].

Table 1: ANOVA table for the effect of neuronal thermogenetic-activation and macronutrient quality of the diet available on the amount of food intake. A linear model was fitted in order to determine the genotypes exhibiting a feeding behaviour significantly different than the control animals.

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.

DISCUSSION:

With this protocol, one could test the ability of larvae under thermogenetic-activation of specific neuronal populations to regulate the intake levels of protein and carbohydrates, two major macronutrients, when exposed to diets of different P:C composition. This method was tested in the context of a larval preliminary screening aiming to identify neuronal populations associated with the control of food intake across diets of different macronutrient quality. This work also contributes to demonstrating that *Drosophila* larvae are valuable animal models for investigating the neuronal basis of feeding behaviors associated to nutrient homeostasis.

In addition to the information provided as notes in the protocol, we would like to further discuss some important aspects. As in any behavioral assay, measures must be taken by the experimenter to minimize the variation associated to animal behavior. A very important aspect that one should keep in mind is related to the importance of obtaining developmentally synchronized animals. The use of early L3 larvae that are well synchronized in their developmental stage, will decrease the behavioral variations exhibited by the animals during the feeding assay[27]. Synchronizing larvae is achieved by short egg lays and by controlling the density of larvae in the cultures. Do not use longer periods of egg-laying than the ones we indicate in the protocol (3-4 hours). Also, controlling the larval density to a maximum number of 200 animals per plate will avoid developmental delays and eliminate additional variation in feeding behavior. Please, note that the 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. Please, take into consideration that the stress induced to the animals during larval handling can also negatively impact behavior. Try to be as gentle as possible, by using a soft and water-moistened brush. Finally, keep in mind that a high number of replicates generates a more reliable dataset.

As in any experimental protocol, our methods present some limitations. Using a colorimetric method to quantify food intake based on the accumulation of a food-dye in animals' guts involves some precautions related to the duration of the assay. For adult flies it was demonstrated that there is a significant risk of reaching a steady state for dye accumulation, in which the rate of egestion equals the rate of intake, reducing the accuracy of the method[28]. Although there is no evidence of this happening in larvae, we decided to perform a feeding assay with a maximum duration of 60 min. 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]. The sensitivity of food-dying methods is also relatively low when small amounts of food are consumed, which

significantly reduces the resolution among genotypes exhibiting very low levels of food intake. We set up our feeding assays using a no-choice paradigm. Only one diet type is available to each experimental group of larvae, which doesn't allow animals to independently regulate the levels of proteins and carbohydrates consumption. Furthermore, because we use chemically undefined diets, it is hard to keep control of nutrients concentrations that might directly affect the patterns of larval feeding. To overcome these issues, or to confirm and further dissect hits found on a preliminary screen, the experimenter might want to consider the possibility of establishing a precise and controlled experimental nutritional context, by using defined synthetic (holidic) mediums[10] and setting up food choice assays as previously described[30]. While using a protocol involving thermogenetic neuronal modulation, it is important to consider that the necessary temperature shifts might directly affect animals' behavioral outputs. A complementary use of optogenetic approaches would be interesting to control for temperature-induced false positives, but the use of optogenetics in the context of larval feeding assays is technically challenging, since feeding larvae spend most of the time burrowed in the food substrate.

Nevertheless, several strengths of our experimental approach can be enumerated. The simplicity and relatively high throughput of our method allow the quantification of food intake for several genotypes when exposed to different nutritional conditions. Feeding behaviors in the larval stage are more readily quantifiable than in adult flies, enabling the generation of better functional readouts. It is also less challenging to establish feeding assays resembling the natural environment in larvae than it is for adults, as it has been previously discussed[31]. Furthermore, when compared to other previously established methods to quantify feeding in larvae, namely the ones based on manual counting the number of mouth hook contractions during a certain period of time[32], our colorimetric method enables genetic screening studies on larger scales. Some other methods are simply based on scoring the proportion of larvae with dyed-food in their guts, not allowing an accurate quantification of food intake levels[33,34]. Concerning the neurogenetic control of neuronal function, the fact that TRPA1 transgene is inactive at 18 ˚C ensures that neuronal activity is not affected throughout larval development. This ensures that the experimental neuronal activation will be performed exclusively during the feeding assay and not during the larval development. Additionally, we would like to mention, one more time, that our protocol can be easily adapted to specific needs and interests of the experimenter. For example, the suppression of the neuronal function, instead of activation, can be easily obtained by substituting the dTRPA1 for a UAS line encoding the temperature sensitive neuronal silencer ShibireTS[20]. Also, if the feeding levels exhibited by the experimental larvae are very low, making it hard to quantify food intake, it is possible to perform an extra step of 30 min larval starvation before the feeding assay (before the steps in section 4 of the protocol), as previously described[15]. This food-deprivation step can be particularly interesting if you are investigating modulators of hunger-driven behaviors. Finally, in previous studies, using quantitative colorimetric methods, it was shown that labeling food with blue-dye has no influence on feeding[12]. Nevertheless, we think that the use of complementary, more accurate and sensitive methods, like the radiolabeling of the food[12], in more advanced stages of a study, aiming to confirm or further dissect hits found during preliminary stages would be a good complement of our method and should be considered by the experimenter. For all these reasons, we believe in the attractiveness of our methods to perform genetic screens (especially primary screens) aiming to identify neuronal populations

involved in the assembling of neuronal circuits encoding feeding behaviors.

As a final note, we would like to mention the fact that thousands of larval Gal4 lines established in Janelia Research Campus are publicly available, at Bloomington Drosophila Stock Center and a large amount of information about larval[26] and adult[19] CNS expression patterns is also publicly accessible at the FlyLight Image Database (http://www.janelia.org/gal4-gen1). These resources make it possible to elaborate putative structure-function neuronal maps of the neurons regulating feeding behavior in *Drosophila* larvae. This is possible by integrating the phenotypic information generated in neuronal screens with the expression patterns of the drivers used. We believe our methods constitute a valid approach to generate preliminary neuronal maps for feeding behaviors associated to macronutrient balancing in the *Drosophila* brain.

ACKNOWLEDGMENTS:
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DISCLOSURES:
The authors have nothing to disclose.

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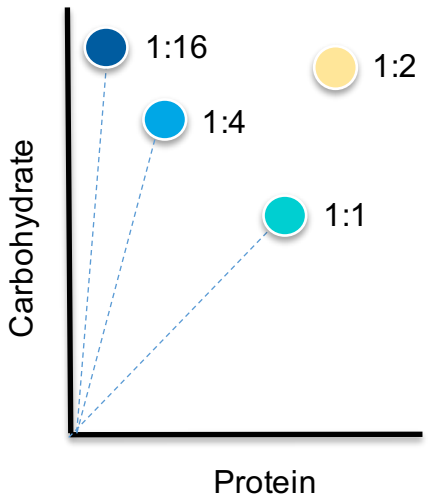
Figure 1 Experimental diets used to grow L3 larvae and in the macronutrient balancing quantification

A **Protein to carbohydrate (P:C) ratios and caloric contents of the diets**



Macronutrient balancing diets

- Protein-rich (1:1 and 248 calories/L)
- Intermediate (1:4 and 248 calories/L)
- Protein-poor (1:16 and 248 calories/L)

Third-instar (L3) rearing diet

- 1:2 and 495 calories/L

B **Composition of the sucrose-yeast (SY) diets**

	Nutritional Composition (g) in 100 g of food		Amount of Components (g) for 1L of food			
	Protein	Carbohydrates	1:1	1:4	1:16	L3 diet
Agar	0	0	10	0	0	10
Sucrose	0	100	8.54	0	8.54	50
Yeast	45	33	71.11	32	23.47	100

Figure 2

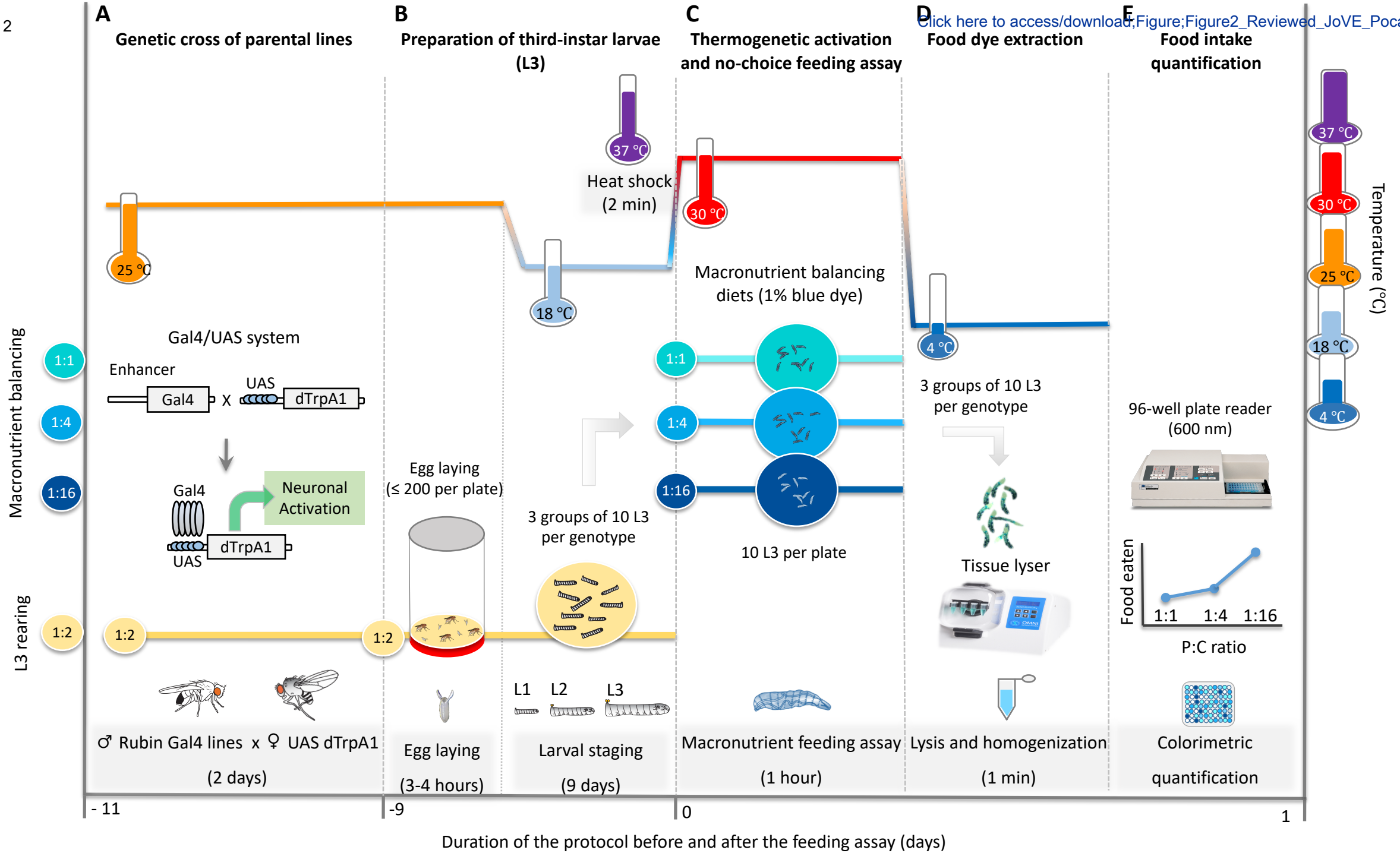


Figure 3

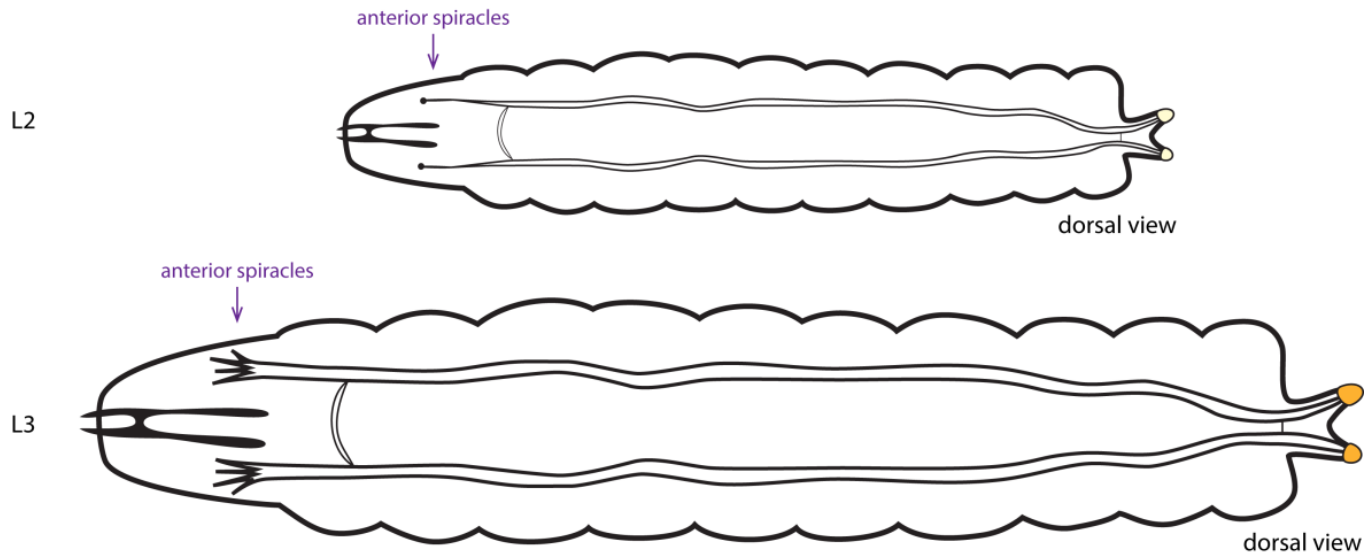


Figure 4

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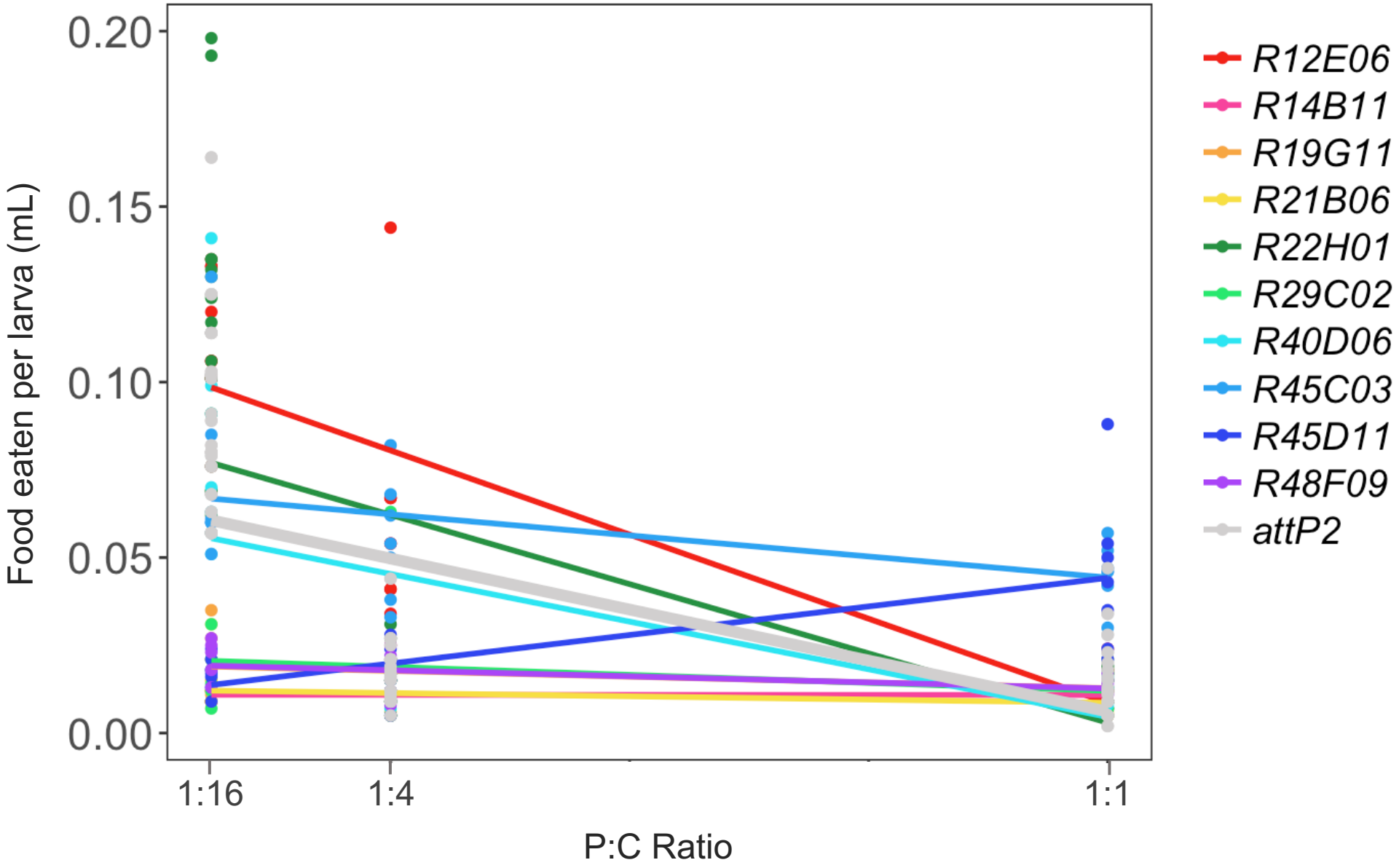

Figure 5

Table 1

Anova Table (Type II tests)

Response: Concentration/L3

	Sum Sq	Df	F value	Pr(>F)
Food	0.086832	1	113.5358	< 2.2e-16
Genotype	0.078443	10	10.2567	9.762e-15
Food : Genotype	0.064038	10	8.3733	6.416e-12
Residuals	0.215673	282		

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Summary Table (coefficients below are compared to the attP control genotype):

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.064245	0.004316	14.886	< 2e-16
Food	-0.058117	0.007206	-8.066	2.10e-14
Genotype R12E06	0.040243	0.008961	4.491	1.03e-05
Genotype R14B11	-0.053347	0.014361	-3.715	0.000245
Genotype R19G11	-0.044880	0.010788	-4.160	4.23e-05
Genotype R21B06	-0.051912	0.009363	-5.544	6.79e-08
Genotype R22H01	0.017682	0.007296	2.423	0.016004
Genotype R29C02	-0.043102	0.011113	-3.879	0.000131
Genotype R40D06	-0.005341	0.009876	-0.541	0.589102
Genotype R45C03	0.004064	0.009876	0.412	0.680997
Genotype R45D11	-0.052579	0.009876	-5.324	2.08e-07
Genotype R48F09	-0.044612	0.011362	-3.926	0.000108
Food : Genotype R12E06	-0.037763	0.015440	-2.446	0.015067
Food : Genotype R14B11	0.058054	0.027100	2.142	0.033031
Food : Genotype R19G11	0.051532	0.017726	2.907	0.003937
Food : Genotype R21B06	0.054403	0.015689	3.467	0.000607
Food : Genotype R22H01	-0.020863	0.012377	-1.686	0.092979
Food : Genotype R29C02	0.048996	0.018714	2.618	0.009317
Food : Genotype R40D06	0.003804	0.016550	0.230	0.818371
Food : Genotype R45C03	0.034117	0.016550	2.061	0.040177
Food : Genotype R45D11	0.090661	0.016550	5.478	9.53e-08
Food : Genotype R48F09	0.051184	0.019045	2.688	0.007625

Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 0.02765 on 282 degrees of freedom

Multiple R-squared: 0.516, Adjusted R-squared: 0.4799

F-statistic: 14.31 on 21 and 282 DF, p-value: < 2.2e-16

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Table 2

Code Name	Genotype	Associated Gene
TRPA1	w[*] ; P{UAS-TrpA1(B).K}attP2 / TM6B, Tb[1]	
J02 ("empty")	w[1118] ; P{GAL4.1Uw}attP2	
J08	w[1118] ; P{GMR12E06-GAL4}attP2	net (CG11450)
J12	w[1118] ; P{GMR14B11-GAL4}attP2 / TM3, Sb[1]	dnc (CG32498)
J17	w[1118] ; P{GMR19G11-GAL4}attP2	CG33696
J20	w[1118] ; P{GMR21B06-GAL4}attP2	oa2 (CG6919)
J22	w[1118] ; P{GMR22H01-GAL4}attP2	fru (CG14307)
J31	w[1118] ; P{GMR29C02-GAL4}attP2	Ptp69D (CG10975)
J41	w[1118] ; P{GMR40D06-GAL4}attP2	cnc (CG17894)
J46	w[1118] ; P{GMR45C03-GAL4}attP2	kni (CG4717)
J47	w[1118] ; P{GMR45D11-GAL4}attP2	pnt (CG17077)
J51	w[1118] ; P{GMR48F09-GAL4}attP2	dpr8 (CG32600)

Origin	BDSC Stock Number
Bloomington	26264
Janelia	68384
Janelia	NA
Janelia	49255
Janelia	48864
Janelia	49857
Janelia	49001
Janelia	48088
Janelia	48616
Janelia	47936
Janelia	49563
Janelia	50377

Table of Materials

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Name of Material/Equipment	Company	Catalog Number
1.5 mL microtubes	Sarstedt AG & Co.	72.690.001
10xPBS	Nytech	MB18201
2.0 mL microtubes	Sarstedt AG & Co.	72.695.500
60 mm petri dishes	Greiner Bio-one, Austria	628161
96 well microplates	Santa Cruz Biotechnology	SC-204453
Agar	Pró-vida, Portugal	
Bench cooler	Nalgene, USA	Labtop Cooler 5115-0032
Blue food dye	Rayner, Billingshurst, UK	
Cell disruption media	Scientific Industries, Inc.	888-850-6208
Dish weight boats	Santa Cruz Biotechnology	SC-201606
Embryo collection cage for 60 mm petri dishes	Flystuff, Scientific Laboratory Supplies, UK	FLY1212 (59-100)
Featherweight forceps	BioQuip Products, USA	4750
Fly food for stocks maintenance		
Forceps #5	Dumont	0108-5-PS
Incubator	LMS Ltd, UK	Series 2, Model 230
Incubator	Percival Scientific, USA	DR36NL
Janelia lines	Janelia Research Campus	
Macronutrient balancing diets		
Methanol	VWR	CAS number: 67-56-1
Nipagin (Methyl 4-hydroxybenzoate)	Sigma-Aldrich	H5501
Nitrile gloves	VWR, USA	
Refrigerated centrifuge	Eppendorf, Germany	5804 R / Serial number: 5805CI364293
Rubin Gal4 ines	Janelia Research Campus	
ShibireTS UAS line	Bloomington Drosophila Stock Center	BDSC number: 66600
Soft brushes		
Spectrophotometer plate reader	Thermo Fisher Scientific	Multiskan Go 51119300
Stereo microscope	Nikon	1016625
Sucrose	Sidul, Portugal	
Third-instar larvae (L3) rearing diet		
Timer		

Tissue lyzer / bead beater	MP Biomedicals, USA	FastPrep-24 6004500
TRPA1 UAS line	Bloomington Drosophila Stock Center	BDSC number: 26264
Water bath	Sheldon Manufacturing Inc., USA	W20M-2 / 03068308 / 9021195
Yeast extract	Pró-vida, Portugal	

Comments/Description

(0.5 mm glass beads)

1 L food contains: 10 g Agar, 100 g Yeast Extract, 50 g Sucrose, 30 mL Nipagin, 3 mL propionic acid
Standard tips, INOX, 11cm
For thermogenetic feeding assay (30◦C)
To stage larvae (19◦C)
Detailed information in Table 2
Composition and nutritional information in Figure 1

Stoks available at Bloomington Drosophila Stock Center
Provided by Carlos Ribeiro Group
For sorting anaesthetised fruit flies

Composition and nutritional information in Figure 1

Expresses TrpA1 under UAS control; may be used to activate neurons experimentally at 25 ◦C

51% Protein, 15% Carbohydrate

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Gonçalo M. Poças:

In 2015, I was awarded my PhD in Neuroscience from Universidade Nova de Lisboa (NOVA), where I studied the molecular mechanisms underlying neurodegeneration in the context of Parkinson's and Huntington's diseases. My most significant contribution was to demonstrate that alpha-Synuclein and Huntingtin maintain a physical and functional interaction in the nervous system that may be relevant for the evolution of these pathologies. Currently, I am conducting my post-doctoral studies in the School of Biological Sciences at Monash University and ITQB-NOVA, where I am investigating the neuronal regulation of foraging and feeding behaviors.

Christen K. Mirth:

I was awarded my PhD in 2002 from University of Cambridge (UK) and conducted my post-doctoral training at the University of Washington, USA (2003-2008), and Janelia Research Campus, USA (2008-2010). In 2010, I took up an appointment as a Group Leader at the Instituto Gulbenkian de Ciência (Portugal). In November 2015, I was recruited to the School of Biological Sciences at Monash University and in 2017, was awarded an ARC Future Fellowship. Work in my lab aims to uncover the molecular mechanisms through which diet and nutrition modify animal development to affect traits, like body size and shape, and how evolution acts on these mechanisms to generate phenotypic diversity.

Pedro M. Domingos:

My long-term scientific goal is to understand the molecular mechanisms that regulate neurodegeneration. During my post-doctoral training at the Rockefeller University, I have begun investigating this problem, by focusing on the role of the Unfolded Protein Response (UPR) in a model of retinal degeneration in *Drosophila*. I have continued this work as an independent investigator at ITQB-NOVA, currently leading a group of 6 people, including 3 PhD students and 2 postdocs.

Script on R for Statistical Analysis of Representative Results Data

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