- Heat mixture 1 in a stove or a microwave oven, stirring some times to prevent clumping. Let it boil, stir the mixture, and boil again.
- Separately, prepare mixture 2 in a blender.
- After boiling mixture 1 twice, add the blended mixture 2 and heat again, stirring some times. Let it boil three times.
- Remove it from the heat source and transfer it to clean vials.
- Let the medium cool for some hours, protected from dust and other contaminants.

After the medium is cool, it is advisable to scratch the surface with a clean spatula to stimulate oviposition and add a previously sterilised folded piece of filter paper, to control excessive moisture and provide a perching and pupation site.

The mixture of simple and complex carbohydrate sources results in a highly nutritive medium, fulfilling dietary requisitions for more exigent species and allowing the development of well-fed third instar larvae for salivary glands preparations. Karo® and Yoki® were successfully used as corn syrup. Methylparaben (Nipagin®) is a mold inhibitor. In this recipe ethanol is not used (as some recipes advise to improve mold inhibition) to avoid high concentrations of ethanol in the medium, which some species may not tolerate. In spite of this, as the recipe is boiled several times, proliferation of mold has not been a problem. The prepared vials with medium can be stored for a few days.

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A comparison of feeding rate methods in *Drosophila melanogaster* indicates that consumption is influenced by body size.

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Abstract

Dietary restriction, a decrease in nutrient intake without malnutrition, has been shown to increase life span in many species and is highly linked to feeding behavior. Although *Drosophila melanogaster* is an excellent model organism to study the effects of dietary restriction on life span and associated traits, measuring feeding rate in this organism is particularly challenging. Several methods have been used to estimate feeding rate in *Drosophila melanogaster*, but it remains unclear which method is most precise. We examined the effectiveness of two popular methods that label media with blue dye or radioactive isotopes to quantify food uptake. We found that the radioactive label assay was more precise than the blue dye assay and likely most useful for comparing the effects of different treatments (genotypes, diets) on feeding rates. We found that the relationship between feeding rate and dietary treatment depends on the size of the fly, so we also suggest incorporating body size as a covariate in data analysis to improve the accuracy of feeding rate estimates.

Introduction

Dietary restriction (DR), reducing nutrient intake or specific components of the diet without malnutrition, is known to increase life span in a diverse range of organisms (reviewed in Katewa and Kapahi,

2010). *Drosophila melanogaster* has been an important model in many of these studies (Chippindale *et al*., 1993; Chapman and Partridge, 1996; Pletcher *et al*., 2002; Mair *et al*., 2003). One drawback of using flies in DR experiments is that it is difficult to control for the degree of DR achieved. Unlike vertebrate studies where the amount of food available can be controlled and the amount consumed easily measured, most *Drosophila* experiments are carried out on solid media where the amount of food available is unlimited. Traditional DR treatments using flies dilute or otherwise manipulate the concentration of nutrients in the medium. A critical assumption of this approach is that there is uniform consumption across treatments (*e.g*., different diets or by different genotypes). However, flies may manipulate their feeding behavior when faced with different diets. Uncoupling the effects of DR and feeding rate on focal phenotypes is important for interpreting the results of such studies. Several experimental approaches have been developed to quantify feeding rate in *Drosophila* to determine if flies partially or fully compensate for lowered nutrient levels with increased consumption (reviewed in Tatar, 2011). Some studies find common results of compensatory feeding in diet-restricted flies compared to unrestricted flies (Carvalho *et al*., 2005; Mair *et al*., 2005; Ja *et al*., 2007; Wong *et al*., 2009); however, other studies report conflicting results of no compensatory feeding (Wong *et al*., 2008, 2009), which may be confounded by differences in the type of assay used. As a result, the relationship between feeding rate and the effects of DR remain unclear. In this study we attempt to evaluate two existing methodologies that measure feeding rate of *Drosophila melanogaster* on solid medium and suggest an additional step in analysis to improve upon the precision of one method.

Several feeding assays have been developed, but each one has several limitations. One method estimates feeding rate as the frequency of flies observed with their proboscis extended and inserted into the medium for a given amount of time (Mair *et al*., 2005). Although this method allows data collection without disturbing the flies with vial transfers, it does not account for the total volume of food ingested, and not all proboscis extensions result in uptake (Carvalho *et al*., 2005). Another method utilizes a capillary feeder (CAFE) to accurately quantify the volume of food ingested by flies (Ja *et al*., 2007). This method has many benefits, including its accuracy and the fact that it permits repeated measurements throughout a fly's lifetime. A major criticism is that CAFE requires a liquid diet (Wong *et al*., 2009), which differs from the typical solid medium used in many DR studies.

Two other commonly used approaches measure food intake by labeling the food with either a visible dye or a radioactive isotope tracer to quantify consumption. The dye assay requires transferring flies to new vials and measuring feeding rate for 30 minutes, which separates ingestion from egestion (Wong *et al*., 2008). One drawback of this approach is the physical disturbance of transferring flies and the exposure to fresh food may alter the short-term feeding rate (Mair *et al*., 2005; Wong *et al*., 2009). Additionally, feeding rate data are obtained only in a "snapshot in time". As such, the consumption estimates are influenced by the fly's condition at the time of measurement and likely vary with the time of day. Likewise, the data may be influenced by behavioral differences in daily feeding patterns, especially if different genotypes are being compared that differ in this trait.

An advantage of the radioactive label assay is that it allows flies to consume labeled media for 24 hours (Carvalho *et al*., 2005) and so minimizes the "snapshot in time" problem. However, measuring consumption over the longer term is criticized for confounding absorption and elimination rates (Wong *et al*., 2008). Additionally, the amount of isotope incorporated in a fly depends on its body's capacity to retain the label (Wong *et al*., 2008), which could be particularly concerning in DR studies, because flies on restricted diet treatments may have larger gut capacities (Wong *et al*., 2008). However, when used in feeding rate studies, radiolabels like ^{32}P and ^{14}C in dietary media were found to accumulate in fly tissues in a nearly linear fashion for up to 72 hours when flies are fed on labeled medium. Because this assay ceases at 24 hours, long before a saturation plateau would occur, it is not likely that body capacity limits the rate of label uptake (Carvalho *et al*., 2005; and see Carvalho *et al*.'s reply in Wong *et al*., 2008).

In this study, we compare feeding rate results of the blue dye and radioactive labeling assays to determine which test is a more precise feeding rate method to complement *Drosophila* DR studies. We compared the results of these assays using flies maintained on either a high or low yeast diet which had previously been shown to produce differences in life span and reproduction (Skorupa *et al*., 2008). We carried out the blue dye assay at two different times of day to test the hypothesis that diurnal patterns of feeding rate influence the results of the blue dye assay. We also used two different genotypes of flies from the Genetic

Reference Panel (DGRP) (Mackay *et al*., 2012) to compare genotype specific responses to these treatments. Finally, we attempted to improve the precision of the radioactive label assay by accounting for the effect of body size on feeding rate.

Methods

Stocks

Newly eclosed virgin females of two genotypes from the *Drosophila melanogaster* Genetic Reference Panel (DGRP) (Mackay *et al*., 2012): DGRP_161 and DGRP_774 were collected and placed with age-matched males from line DGRP_774. Individual pairs were maintained in vials containing either high yeast (HY) or low yeast (LY) media. The media consisted of 200 g (HY) or 50 g (LY) baker's yeast (Lesaffre Yeast Corporation, Milwaukee, WI), 50 g sucrose (Domino, Baltimore, MD), 15 g agar (Moorhead & Co, Inc., Rocklin, CA), 3 ml propionic acid (Fisher, USA), and 15 ml 20% methyl paraben (in 95% EtOH) (Mallinckrodt Baker, Inc., Phillipsburg, NJ) for every 1000 ml distilled water. The diets used in this study, modeled after Skorupa *et al*. 2008, differed only in yeast concentration (20% HY and 5% LY). As the primary protein source for the flies, yeast is a major dietary component influencing life history traits like life span and fecundity (Skorupa *et al*., 2008). Flies were maintained on these diets for one week before they were used to evaluate the effectiveness of the two different feeding rate assays.

Blue Dye Assay

The blue-dye assay used standard 5% (wt/vol) blue food coloring (McCormick, Hunt Valley, MD) to label the HY or LY medium in a manner similar to reference (Wong *et al*., 2009). Five females from either line DGRP_161 or DGRP_774 were transferred into vials containing 2% agar to starve for two hours to stimulate feeding. They were then placed on either HY or LY medium and allowed to feed for 30 minutes starting from the first observed proboscis extension. These five flies were then homogenized in a microcentrifuge tube with 0.5 ml distilled water. For each line and diet combination, this method was repeated using both dyed food and non-dyed food to control for genetic variation in fly pigment and variation in medium color (HY is darker than LY) that may influence color intensity measurements. The supernatant of flies fed the non-dyed medium was then used as a blank for spectrophotometric analysis of the corresponding genotype/diet category of flies fed the dyed medium. The blue color intensity of each tube was quantified using a spectrophotometer (SmartSpec Plus Spectrophotometer, BioRad, USA at OD 600 nm) to estimate the average feeding rate of the five homogenized females in each of two replicate tubes for each category. This assay was carried out over four consecutive days at 10 am and 2 pm to detect variation in circadian patterns that may affect the results of experiments using this assay. In total this assay used 80 flies to create 16 samples allowing 2 replicate absorbance measurements for each genotype and diet combination. These were compared to 80 additional flies that were used as spectrophotometric blanks.

Radioactive Label Assay

The radioactive labeling assay was modeled after Carvalho *et al*. (2005), and used the radioactive isotope [α-^{32}P]dATP (Perkin Elmer, Boston, MA, catalog # BLU512H25OUC) to label HY and LY food. Because variation in maximum gut capacity between flies may influence their absolute feeding rate, we measured the thorax length of each female prior to experimentation to estimate body size. This was then used as a covariate in the statistical analysis of feeding rate. Both diets were labeled with 20% radioactive isotopes. We heated 20 mls of each diet to a smooth liquid state before adding 40 μCi of [α-^{32}P]dATP. While still in a liquid state, 1 ml of the mixture was pipetted into individual vials and 1 ml of each diet was pipetted into scintillation vials for initial isotope counts. The initial isotope level in each scintillation vial was then measured in an LS 6500 Multi-purpose scintillation counter (Beckman Coulter, Inc., USA). Once the mixture in the vials solidified, individual females were transferred into each vial for a 24 hour period. Flies were transferred onto 2% agar for thirty minutes to allow them to clean their exterior of any radioactively labeled food (Carvalho *et al*., 2005), and then frozen on dry ice for five minutes before being placed into individual scintillation vials with 15 ml scintillation fluid to enhance the isotope measurement. Isotope levels within each individual fly were quantified as above using a scintillation counter. The volume of food ingested by each fly

(in µl) was then calculated by first converting the initial food isotope count from CPM/ml to CPM/µl and then dividing the CPM isotope count in each individual fly the following day by this value. To ensure that no radioactivity was present in the atmosphere or in the food prior to labeling it, isotope levels of individual females maintained on unlabeled food for 24 hours in the same location as the experimental flies were measured in a similar fashion. Radioactivity in these control flies was found to be negligible (*i.e*., under 30 CPM per fly), so they were not considered in any further analyses. In total, five replicates were conducted per line for each diet over two days yielding 40 values from 40 flies. An additional four control flies were used to assess background radioactivity, one reared on HY and one on LY for both days.

Statistical Analysis

All statistical analyses were completed using the PROC GLM procedure in SAS version 9.2 (SAS Institute, Cary, NC). Analysis of variance (ANOVA) was used to evaluate the influence of genotype, diet, and time of day on feeding rate for the blue dye assay using the model:

$$y = \mu + l + d + t + l*d + l*t + l*d*t + \varepsilon$$

where y is the volume of food ingested in ml, μ is the mean, l is the effect of genotype (line), d is the effect of diet, t is the effect of the time of day, $l*t$ is the effect of the line*diet interaction , $l*t$ is the interaction between line and time of day, $l*d*t$ is the three-way interaction term, and ε is the effect of error.

Analysis of covariance (ANCOVA) was used to evaluate the data from the radioactive label assay in a similar fashion. The full model was run and non-significant interaction terms were dropped. The final model used for ANCOVA was:

$$y = \mu + bs + g + d + g*d + bs*d + \varepsilon$$

where bs is the covariate body size as estimated by thorax length in mm, $bs*d$ is the interaction term between body size and diet, and all other variables are defined as above.

Results

Differences in feeding behavior are evident when using radioactive labels but not evident when using blue dye method

When the blue dye assay was used we found no significant differences in feeding rate between genotypes ($p = 0.4650$), diets ($p = 0.4292$), time of day ($p = 0.6836$), or any interactions between main effect terms, but some general trends were apparent. Females of both genotypes tended to eat more on HY (mean ± 1SE) (3.5869 ± 0.4909 absorbance units AU / 5 flies) than LY (2.9245 ± 0.5830 AU / 5 flies) medium (Figure 1 a and b), and line DGRP_774 (3.5609 ± 0.5037 AU / 5 flies) ate more food overall than line DGRP_161 (2.9505 ± 0.5762 AU / 5 flies). Although females of line DGRP_161 showed a difference in feeding rate between the diets of 0.25 AU and 0.04 AU at 10 am and 2 pm, respectively, our statistical power was too small to detect the difference ($1 - B = 0.118$). Likewise, line DGRP_774 showed a 1.95 AU difference in feeding rate between the diets at 10 am and a 0.4 AU difference at 2 pm; however, the power was also too small to detect this difference ($1 - B = 0.142$). We also observed differences in circadian patterns of feeding rate between the genotypes, but this was not significant using this assay ($p = 0.1127$). Females of line DGRP_774 ate more in the afternoon (4.3000 ± 0.3000 AU / 5 flies *vs*. 4.0 ± 0 AU / 5 flies) on both diets, but the power was too low to detect this difference ($1 - B = 0.076$). Line DGRP_161 ate more in the morning (3.9510 ± 0.0490 AU / 5 flies *vs*. 2.0965 ± 1.9035 AU / 5 flies), and again the power was too low to detect this difference ($1 - B = 0.102$). The lack of significance in this analysis is due to a large amount of variation in the data. A power calculation reveals a minimum of 11 samples per diet and time of day would have been needed to detect differences in feeding rates between the diets at a power of 0.80 with an alpha value of 0.05. This equates to a minimum of 220 and 180 flies for lines DGRP_161 and DGRP_774, respectively, for feeding rate measurements using the blue dye assay. In addition, doubling this sample size would be required to obtain the flies used as spectrophotometric blanks in absorbance analysis. The demands of sample size and number of flies required to obtain sufficient power using this method is a major drawback in the use of this methodology.

The radioactive assay identified the same general trends in feeding rate as the blue dye assay, but was able to detect significant differences in feeding rates between the dietary treatments. This difference is likely a reflection of the larger sample size and statistical power achieved by the ability to measure individual flies

using the radioactive label method. Flies ate significantly more on HY (9.3745 ± 0.5917 µl/fly) than LY (5.9437 ± 0.4400 µl/fly) ($p = 0.0134$), and this pattern did not differ by genotype ($p = 0.5845$) (Figure 1c). Line DGRP_774 showed a higher but non-significant ($p = 0.5556$) feeding rate (8.1723 ± 0.5795 µl/fly) than line DGRP_161 (7.1459 ± 0.6999 µl/fly) (Figure 1c). There is no need to examine differences in circadian feeding patterns in this assay, because it encompasses a 24 hour sampling window. Although statistical analysis using ANCOVA revealed no significant effect of body size as a main effect ($p = 0.9697$), we did see a significant interaction between diet and body size ($p = 0.0088$). Our data indicate that smaller flies did not alter feeding rates between diets, whereas larger flies fed more on HY than on LY (Figure 2).

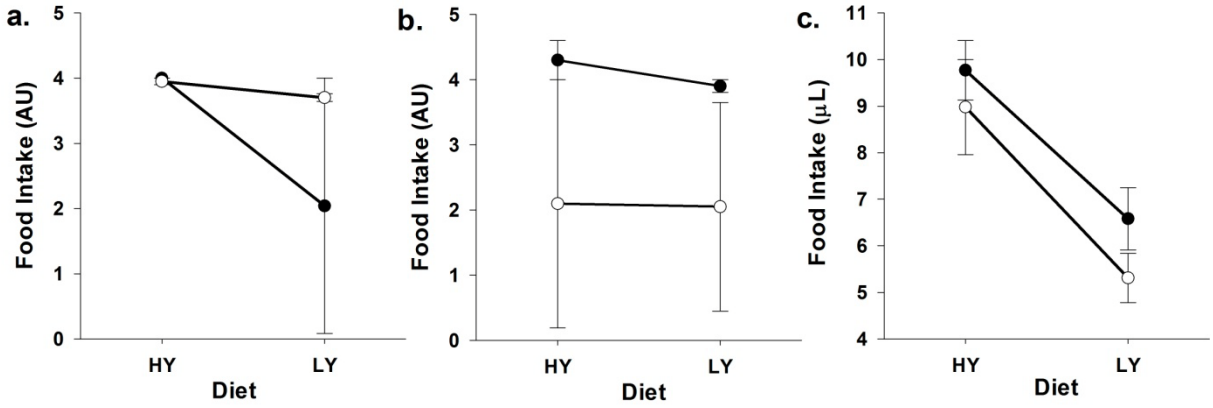

Figure 1. Radioactive label assay is more precise than the blue dye assay, even before correcting for body size. Both graphs show the effect of diet on feeding rate for each genotype using (a and b) the blue dye assay (AU = absorbance units for 5 flies total over 30 minutes) and (c) the radioactive labeling assay (µl food consumed in 24 hours per fly). Open circles represent line DGRP_161 and closed circles represent line DGRP_774. Blue dye assay reveals no significant relationship at (a) 10 am ($p = 0.4319$) nor (b) 2 pm ($p = 0.8951$), whereas (c) the radioactive label assay indicates a significant difference in feeding rate between diets, averaged over both lines ($p = 0.0003$). Data points indicate mean food intake ± 1 SE. The blue dye assay yields much higher error rates than the radioactive assay. Note that blue dye data include 5 flies total in each sample ($n = 2$ per diet and line combination) and radioactive label data indicate individual flies ($n = 10$ per diet and line combination).

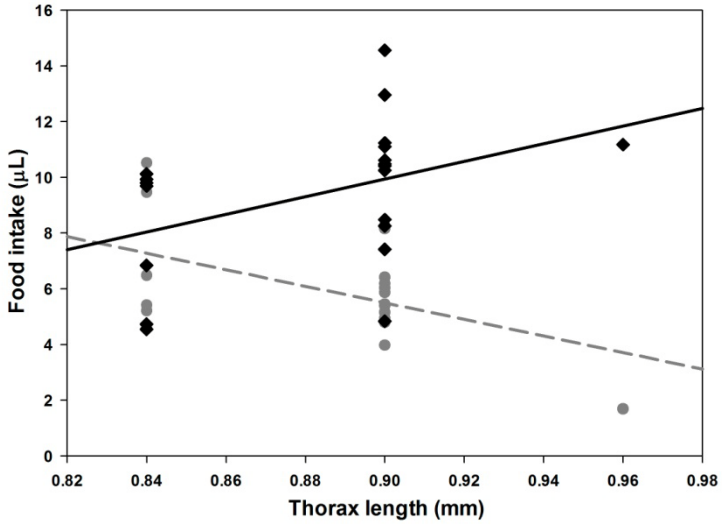

Figure 2. Effect of diet on feeding rate depends on body size. Points indicate feeding rate of individual flies as measured by [α-^{32}P]dATP tracer levels converted to µl of food consumed. Gray circles and dashed line indicate LY and black diamonds and solid line indicate HY. Linear regression shows that small flies showed little to no difference in consumption between diets whereas larger flies ate more on a HY diet than a LY diet ($p = 0.0339$).

Discussion

High degree of error leads to difficulty detecting variation in feeding behavior using the blue dye assay

While both methods used in this study are useful for estimating feeding rates, the radioactive assay was superior to the blue dye assay for detecting significant differences between treatments. The blue dye assay is advantageous in that it is a more cost and time effective method. Compared with the radioactive label assay, food coloring is inexpensive. Additionally this assay is complete in less than three hours while it takes two days to complete the radioactive isotope assay. However, the blue dye assay was more labor intensive and led to high experimental variance that could be influenced by procedural sources such as vial transfers, human error, and aggregated sampling. Vial transfers at the start of each trial cause physical disturbances to the flies that may alter normal feeding patterns (Wong *et al*., 2009), and the short length of this assay may not be enough time for flies to recover. Human error is also inevitable, because careful observation is required to detect the first proboscis extension from any of 5 flies in each vial to mark the start of the 30 minute feeding period. This usually took 10-30 minutes to occur, and the difficulty of tracking 5 flies in a vial limited the sample size possible for each experimental session to only four vials per person each time the assay is carried out. Additionally, this assay required a large number of flies for each data point, because the level of blue dye in the fly homogenate is not adequately detected with fewer than five flies in each sample. Each sample also required a control homogenate to serve as a spectrophotometric blank, so a total of 10 flies are required to create one data point, and each data point potentially produces a high degree of measurement error, because it assumes relatively equal feeding rates among flies within a vial.

Several other factors may also contribute to the high experimental variance of the blue day assay. Using proboscis extensions as a starting point to measuring food intake is likely inaccurate, because every extension does not result in food intake (Carvalho *et al*., 2005). Including proboscis extensions in the assay may bias the sample toward inflated feeding rates, and removing this step of the assay would be a less laborious methodology that could allow a larger sample size collection; however, it would also lower averaged feeding rate measurements by including flies that did not attempt to feed at all (having a zero feeding rate). Another pitfall of this method is that flies were required to starve for two hours before the assay, which may alter normal feeding rate if flies "binge" after starving (Farhadian *et al*., 2012). Additionally, feeding rate must be measured after just thirty minutes of feeding, because the food ingested by the fly could be excreted after this time frame and it would be impossible to separate ingestion from egestion (Wong *et al*., 2008). Finally, in accordance with previous research (Wong *et al*., 2009), our results indicate that flies vary feeding rate with circadian patterns, and this behavioral pattern varies with genotype. Because the blue dye assay is brief, it is susceptible to increased experimental variance due to differences in circadian behavior patterns.

Improved radioactive label assay yields precise, repeatable results in detecting differences in feeding rates

Although the radioactive label assay is more costly and time consuming for each replicate, its benefits as a method to measure feeding rate outweigh the pitfalls. Human error is reduced by eliminating the proboscis extension observations, and circadian behaviors are controlled for by allowing flies to feed undisturbed for 24 hours. This 24 hour feeding session is acceptable for this assay because labeled food is not only ingested, but also metabolically processed and the radioactive label maintained in the tissues of the fly rather than being excreted (Carvalho *et al*., 2005). Another benefit of the radioactive label assay is that it evaluates the feeding rate of individual flies rather than groups of five flies at a time. This translates to larger sample sizes within each replicate block. These factors allow for a much more accurate sampling of the population and more efficient data acquisition.

Previously used radioactive labeling methods have been criticized in that the amount of isotope detected may vary by the body capacity of the flies, so results may actually indicate the volume of food a fly is capable of housing rather than the rate of food intake (Carvalho *et al*., 2005; Wong *et al*., 2009). We accounted for this problem by estimating the body size of each fly with a thorax length measurement. We used thorax length as a proxy to estimate gut size, because it is relatively easy to measure on live flies. Other measures, such as crop size, are more accurate estimates of gut size, but require the flies to be sacrificed, and so would have to be completed after the flies feed on the radioactive food. This is problematic because of the

amount of time required to dissect and measure the crop of each individual fly before isotope measurements and the potential loss of isotopes to any surfaces used in the dissection process.

Using body size as a covariate in our statistical analysis revealed that, although body size itself did not significantly influence feeding rate as a main effect, it did show a significant interaction with diet, meaning that the relationship between feeding rate and diet depends on the size of the fly (Figure 2). Including a body size estimate would likely increase the precision of the blue dye assay as well (Wong *et al*., 2008), but would have to be aggregated for the number of flies used in each spectrophotometric measurement, thus introducing further variation compared to the radioactive label assay that measures individual flies.

This study was conducted to identify a feeding rate assay that would best detect differences in feeding rate between samples of flies. Thus, our goal was to determine which method yielded the most precise estimates of feeding rate. While our results suggest that the radioactive label assay best satisfies our goal of precision, the blue dye and other assays may be more ideal than the radioactive label assay for other research goals such as high-throughput capabilities, or cost-effectiveness. It is also important to note that many aspects of these two assays are procedurally different. For example, the blue dye assay measures only a thirty minute sample of feeding and requires starvation before the assay, whereas the radioactive label assay measures a 24 hour sample with no starvation. For this reason, we cannot compare absolute feeding rates across assays, but rather, we compare the ability of each assay to detect differences in feeding rates, if one exists.

Conclusions

Overall, the radioactive assay was a more effective and precise method of measuring feeding rate in *Drosophila melanogaster* than the blue dye assay. In addition, this study indicates that body size is an important variable to consider in feeding rate measurements and other assays involving dietary treatments because consumption appears to be dependent on body size. We suggest that future experiments either are limited to size-matched individuals or that body size be measured to account for the effects of body size variation on feeding rates.

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