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Technique Notes

A high-throughput spectrophotometric assay of adult size in *Drosophila* that facilitates microbial and biochemical content analysis.

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Abstract

Large-scale laboratory experiments often necessitate the processing of numerous samples in little time, their long-time storage and the joint analysis of morphological, biochemical, and microbiological features. Combining different types of assays is often not compatible with classical methods to estimate size in adult *Drosophila* flies. We therefore designed a new spectrophotometric assay for the high-throughput estimation of adult size in *Drosophila* that facilitates microbial and biochemical content analysis. The new method uses optical density at 202 nm of single fly homogeneates as size proxy. We tested the method in a variety of *Drosophila* populations - including wild caught flies - and compared its explanatory power with two classical size estimates: wet-weight and wing-length. It was also used to control for size when comparing the fat content of different fly populations. Results show fly homogenate optical density is an powerful size proxy that may be used for both male and female flies.

Insect size is a phenotype that responds to genetic and environmental factors and affects important life-history traits and, therefore, fitness (Partridge, *et al.*, 1987). In adult *Drosophila* flies, size can be assayed by several methods; the full body can be weighed wet or dried, its size estimated by dissecting and measuring wings, thorax, or leg segments (David, *et al.*, 1994; Partridge, *et al.*, 1994). Each of these methods has practical limitations. The fresh (*i.e.*, wet) weight of animals changes according to recent food and water intake as well as egg and feces production. Besides obtaining fresh weight implies the prompt manipulation of assayed individuals, which can be too time-consuming when many insects must be processed simultaneously. Measuring dry weight is freed from water intake variations but prevents the study of insect microbial content as the drying process is lethal to numerous species of the microbiota. Fly biologists have a long tradition of measuring wing-length as a proxy of insect size. This is convenient as it does not alter body content (*i.e.*, does not harm microbial symbionts). However, dissection, like wet weighing, can take too long to process numerous individuals in a given timeframe. Besides, environmental factors such as temperature during development can alter the relative sizes of wings and body (David, *et al*., 1997; Partridge, *et al*., 1994).

For an experiment on the symbiosis between microbes and flies we had to simultaneously estimate the size of adult flies, count the number of live bacteria and yeast cells they contained, and assay biochemical content (*e.g.*, triglycerides content). None of the methods listed above enabled processing a number of flies that sufficed to keep track with the work-load of an experiment that involved hundreds of flies. We therefore designed an alternative sizing method based on spectrophotometry. In brief, the method consists in homogenizing adult flies in a liquid, split the sample in several sub-samples, some of them with glycerol, and store at -80°C. It is later possible to thaw each sub-sample and measure its optical density (*i.e.*, our size-proxy), plate and count the number of live microbes (sub-samples frozen with cold-protecting glycerol), and perform biochemical analyses.

In the process of designing this protocol, we identified key steps to ensure size estimates were meaningful. First, we had to identify a wavelength where the relationship between sample concentration and optical density was linear. Second, we validated the measure in a variety of *D. melanogaster* samples, from the lab and from the wild. Eventually, we tested the method with a mock study on the relationship between size (*i.e.*, homogenate optical density) and fat reserves.

Choice of wave-length for fly homogeneate assay

The first step of our procedure was to identify a wave length at which optical density varies linearly with sample concentration. To this aim we prepared 10 fly homogenates in PBS in 1.5 mL centrifugation tubes. Flies originated from a stock-culture of the Oregon-R strain. Each of the 10 samples of adult flies was homogenized with a pestle and serially diluted so that we could measure Optical Density (OD) pure, or at a concentration of 0.5, 0.25, and 0.125. The diluted sub-samples were then separated in four: two sub-samples were analyzed immediately while two others were analyzed 4 hours later (samples were kept at room temperature ±21°C). This step was necessary to ensure delays between sample preparation and analysis did not alter measures.

A volume of 2 µl of each sub-sample was placed in one of sixteen measure locations of a µdrop plate (Thermo Scientific, # N12391) and its optical density read in a Multiskan GO spectrophotometer (Thermo Scientific, # N10588). The µdrop device enables analysis of smaller samples than traditional 96 or 384 well plates and is often used for nucleic-acids quantification. We tested optical densities at ten different wavelengths: 200, 202, 204, 206, 208, 210, 215, 220, 225, and 230 nm.

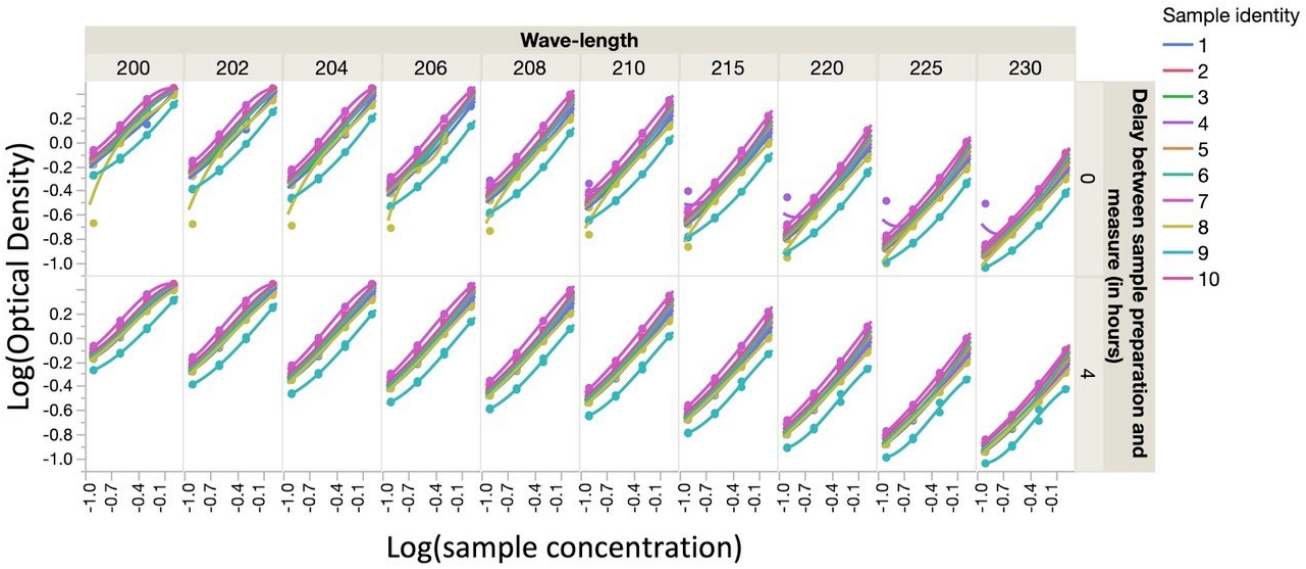

Figure 1. Relationship between sample concentration and homogenate optical density at several wave-lengths. Two series of measurements were run 4 h apart.

Visual exploration (Figure 1) of the data revealed the relationship between sample concentration and optical density was somewhat linear when both variables were log-transformed. In order to choose the wavelength at which linearity was best and test for the effect of time between sample preparation and optical density measurement we used linear models. Models had *Log*(*Optical Density*) as response variable and *Log*(*concentration*) and *Sample identity,* as well as their interaction, as factors. We fitted separate models for each combination and time between sample preparation and assay (*i.e.*, 0 h or 4 h). The only two factors to be significant in all models were *Sample identity* and *Log*(*Concentration*) (all $P < 0.0001$); but the interaction *Log*(*Concentration*)**Sample identity* was significant at wave-length superior to 206 nm, suggesting short wavelengths were better candidates. Similarly, models of data acquired 4 h post sample preparation consistently had slightly better R^2 than when samples were analyzed right after preparation (*e.g.*, at 202 nm, after 0 h $R^2 = 97.8$ while after 4 h $R^2 = 99.6$). In order to choose the best wavelength among those that were visually satisfying, we analyzed the residuals of the models' linear fits. For each wavelength, we saved the residuals of the models with *Sample identity* and *Log*(*Concentration*). We reasoned the best wavelengths would be the ones which residuals would have the least standard-deviation. The two wavelengths that fitted

this criterion best were 202 and 204 nm (s.d. = 0.0157 and s.d. = 0.0165, respectively, while all other wavelength had standard-deviations above 0.019). From thereon, we chose to work exclusively at 202 nm.

Table 1. Fly samples used for the comparison of homogenate OD with wet weight, wing length and triglycerides content.

Sample name	Description
Oregon-R old	Flies from the Oregon-R strain. Old individuals from vial set up more than 2 weeks before collection.
Oregon-R young	Flies from the Oregon-R strain. Young individuals from vial set up less than 2 weeks before collection.
Wild caught	Wild-caught flies from Southern France.
Wild-type 1	Wild-type population of unknown origin. Adult flies of unknown age.
Hemiclone-type	Mix of adult flies of unknown age from lines used for hemiclone analyses.

Comparison of homogenate OD with wet weight and wing length

The second step of protocol design was to compare how the new proxy compared to classical size estimates, namely wet weight and wing length. To this aim we assayed individual flies, both males and females, from various age and origin (Table 1). In particular we were cautious to include wild-caught adult flies, lab-reared flies from distinct genetic backgrounds, and flies of different ages but from the same background. Males and females were treated separately.

Individuals were anaesthetized with CO_2, individually weighed, and had their wings removed and placed on a microscope slide. Wing length was measured with a stereo-microscope. The average of the two wings was used for further analysis. Right after the wings were removed, individuals were frozen at -80°C in PBS. Several days after freezing the samples were thawed and homogenized in a Tissue Lyser II (Qiagen, #85300) for 30 s at 30 Hz with Ø3 mm glass balls, centrifuged for 30 s at 2000G. Optical density of 2 µL of supernatant was then read on spectrophotometer µDrop device.

Our first approach was to neglect population differences and relate each of our two size predictors (*i.e.*, wing length and OD) to wet-weight. In other words, we assumed wet weight was an adequate size estimator and compared the performance of two size proxies. To this aim we used linear models with *Log*(*wet weight*) as response variable and the size proxies as explanatory factors. We found that both wing length and homogenate OD were good predictors of wet weight variation and revealed differences among sexes. In males, homogenate OD explained 71.5% and wing length 57% of Log(wet weight) variance. In females, homogenate OD explained 39.2% and wing length 51.3% Log(wet weight) variance. Linear models explaining Log(wet weight) with both OD and wing length as factors showed each metric conveys different information as proportion of explained variance was improved for both males and females (R^2 males = 77.1%; R^2 females = 68.9%; in both cases the two factors were highly significant).

In a second stage, we investigated differences among fly populations and sex. Response variable remained *Log(wet weight*), factors were *Sex*, *Population,* and either *Wing length* or *Log*(*OD*), as well as all possible interactions between the 3 factors the initial model contained. After a step-wise deletion of non-significant terms, models based on wing length or homogenate OD provided similar information (Table 2) and explained a similar proportion of wet weight variance: 82% with Log(OD) and 83.6% with wing length. We note none of the interactions comprising sex and OD or sex and wing length were significant - and even though lack of significant difference must not be interpreted as proof of similarity - the above result suggests that the general relationship between the two size estimates and wet weight may be robust relative to fly sex. More importantly, interactions between population and the two size estimates were both significant (Table 2). In both cases the interaction was driven by the steeper slope of the *Hemiclone-type* population (wing length t = 2.68, P = 0.009; OD t = 2.32, P = 0.023) and the flatter slope of the *Wild-type 1* population in the case of wing length (t = -2.52, P = 0.014). Size estimate*population interactions suggest estimates may provide unreliable data when comparing different populations. It is however not possible to know whether population differences

are due to genetic or environmental factors (flies from the same populations may have been sampled in separate vials; however, this information was lost). Comparisons based on wing length and homogenate OD are therefore more accurate for insects from the same populations and reared in similar conditions than for samples from very different origins.

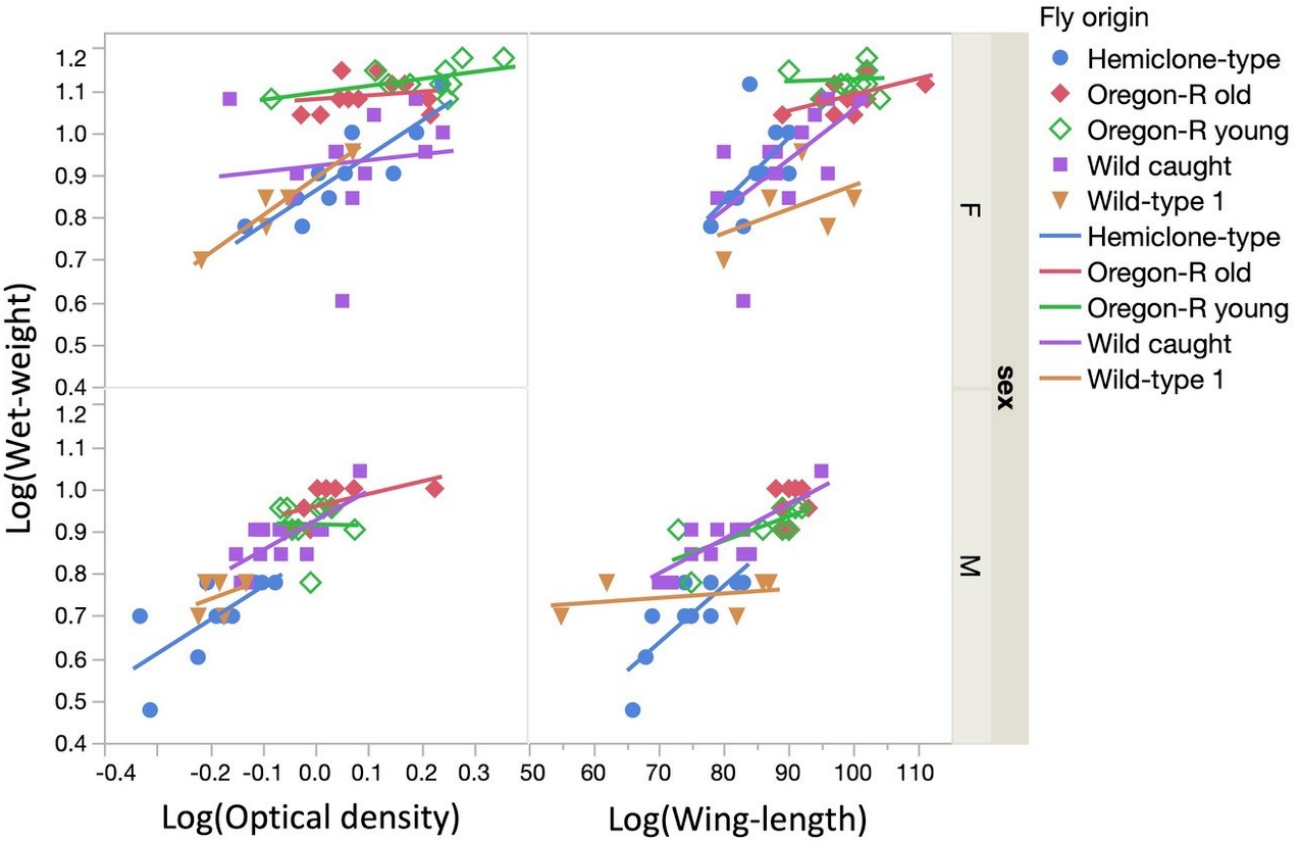

Figure 2. Relationship between either homogenate OD or wing length and wet weight.

Table 2. Statistical models explaining Log (wet weight) as a function of fly sex, population and either Log (homogenate optical density) or wing length.

Size proxy	Factor	DF	F Ratio	P > F
Adult fly homogenate Optical Density Log-transformed)				
AIC= -221.1	Population	4.80	12.7	< 0.0001
R^2= 0.820	Sex	1.80	24.4	< 0.0001
	Population * Sex	4.80	3.18	0.0177
	Log (OD)	1.80	9.34	0.0030
	Population * Log (OD)	4.80	3.32	0.0142
Wing length				
AIC= -229.7	Population	4.80	12.9	< 0.0001
R^2= 0.836	Sex	1.80	9.81	0.0024
	Population * Sex	4.80	3.77	0.0073
	Wing length	1.80	32.1	< 0.0001
	Wing length * Population	4.80	3.89	0.0062

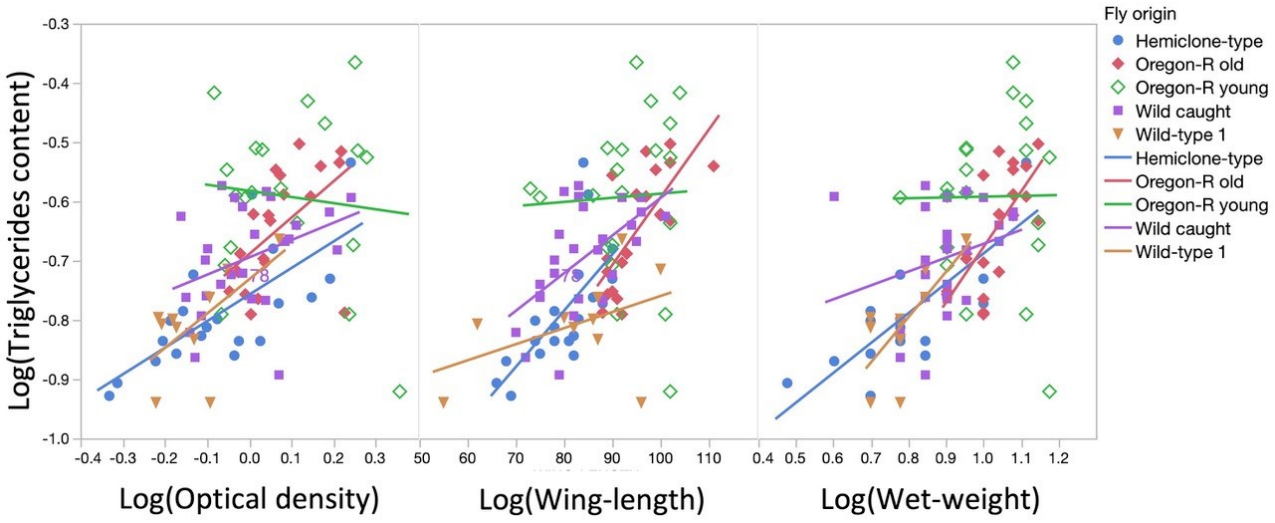

Figure 3. Relationship between either homogenate OD, wing length or wet weight and triglyceride content.

Table 3. statistical models explaining triglycerides content (log-transformed) as a function of fly sex, population and one of three estimators.

Size proxy	Factor	DF	F Ratio	P > F
Adult fly homogenate Optical Density (Log-transformed)				
AIC= -167.8	Population	4.85	8.35	< 0.0001
R^2= 0.527	Sex	1.85	6.50	0.0126
	Log (OD)	1.85	2.50	0.1171
	Population * Log (OD)	4.85	2.73	0.0339
Wing length				
AIC= -169.4	Population	1.85	7.52	< 0.0001
R^2= 0.534	Sex	1.85	1.46	0.2297
	Wing length	4.85	8.18	0.0053
	Wing length * Population	4.85	2.19	0.0766
Wet weight (Log-transformed)				
AIC= -165.8	Population	4.84	4.91	0.0013
R^2= 0.532	Sex	1.84	3.56	0.0623
	Log (wet weight)	1.84	4.66	0.0336
	Population * Log (wet weight)	4.84	2.28	0.0668

Case study: explaining adult fly fat content with size-estimates

The protocol evaluated in this manuscript was developed to enable the high-throughput processing of samples and the streamlining of size, microbial, and biochemical assays. In order to evaluate the relevance of homogenate OD as size proxy in this context, we analyzed the triglyceride content of the fly samples described above (Table 1). Triglycerides are the main form of fat storage in insects. Triglyceride concentration was measured following a classical biochemical method initially described by Clark and Gellman (1985). In brief, homogenate was incubated with Triglycerides reagent (Sigma Aldrich, #T2449) for 20 minutes at 25°C, centrifuged to clear-off particles, incubated again with Free Glycerol Reagent (Sigma Aldrich, #F6428) for 20

more minutes at 25°C, and read at 540 nm in 96-wells flat-bottom microplates (Thermo Scientific, #269620). We then used linear models to model triglyceride content variation in response to size, sex, and population. We compared three types of models with either *Log*(*OD*), *Wing length*, and *Log*(*wet weight*) as size estimate, *Sex* and *Population* as discreet factors, and all second order interactions.

All three size proxies produced similar results (Figure 3). Concentration in triglycerides was explained by size, sex, population and a close to 5% p-value interaction between size and population (Table 3). Comparison of models AIC and R^2 suggest using wing-length as size proxy may give best results, but differences are minimal. We also note that with all three size proxies the interaction term was driven by the flatter slope of the *Oregon-R young adults* population ($t < -2.43$, $P < 0.017$ in three cases).

Conclusions

Homogenate OD appeared a size proxy of similar explanatory power as wing-length and wet-weight. A caveat of this new method is that the nature of the molecules quantified at 202 nm is unknown. However, all other size measurements available convey incomplete information as the concept of size is in itself the simplification of a multi-dimensional phenomenon (*e.g.*, water content may or may not be a relevant parameter, morphology may change independent of volume). Depending on intended use, size may be best estimated by one of the many proxi available. The method we propose here is best suitable when many samples need to be processed at once, and microbial or biochemical content must be analyzed, too.

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References: Clark, A.G., and W. Gellman 1985, Dros. Inf. Serv. 61: 190; David, J. *et al.,* 1994, Genetics Selection Evolution 26: 229; David, J.R. *et al.,* 1997, Journal of Thermal Biology 22: 441-451; Partridge, L. *et al.,* 1994, Evolution 48: 1269-1276; Partridge, L. *et al.,* 1987, Animal Behaviour 35: 468-476.

Service Announcement:

The Elgin Lab fly room will be closed May 31, 2019, with the flies being euthanized. Over the years we have generated many unique *Drosophila melanogaster* lines. Most of these contain P-element constructs with a visible reporter of Position Effect Variegation (PEV), most often an *hsp70*-driven *white* gene, designed to report on the local chromatin environment. Lines currently in our collection are listed on our webpage – https://sites.wustl.edu/elginlab – under stocks, in association with the paper that describes the generation and characterization of the particular lines in greater detail (usually including determining the insertion site of the P-element reporter). PEV lines in the collection include those with insertions into pericentric heterochromatin, telomere-associated regions, the fourth chromosome, and the Y chromosome. **All lines are available now through May 2019**, when the Elgin Drosophila lab will be closed. Please direct requests to Jo Wuller at wuller@wustl.edu, with a cc to Sarah Elgin (selgin@wustl.edu).