

A More Standardized Method for Imaging and Measuring *Drosophila* Thorax Length Using ImageJ

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[Abstract] Thorax measurements are important metrics of *Drosophila* fitness and are often correlated with key life-history traits, such as lifespan and fecundity. Therefore, thorax length is a widely used measure in studies of genetic variation and plasticity in response to environmental alterations. However, the lack of fixed landmarks means that thorax measurements can sometimes be inconsistent and highly variable. Here, we outline a dorsal bristle-guided method of thorax measurements, which we could use to draw a reliable and repeatable line from the thorax scutellum to the anterior thorax edge. We analyzed and compared this bristle-guided marker method to a non-landmark-based thorax measurement in a set of measures of female and male *D. melanogaster*. We found that the bristle-guided method exhibited significantly less variation between technical replicates than the non-landmark-based measure.

Keywords: Thorax, *Drosophila*, Morphometrics, Measurements, Phenotypic plasticity

[Background] Thorax measurements in *Drosophila melanogaster* are an important and widely used metric in studies of life history, plasticity, and heritability (e.g., Imasheva et al., 1999; Bergland et al., 2008; Lafuente et al., 2018). This is due to the well-established relationship between thorax size and metrics of fitness, such as fecundity and lifespan (Hasson et al., 1992). Conventional thorax measurements in many studies choose to measure from the tip of the scutellum to the edge of the thorax. However, unlike for other sets of morphometric measurements (e.g., those made on the wings, in which the veins and vein junctions make clear anchor points and landmarks), the thorax provides fewer obvious fixed and identifiable edge points. This can make thorax measurements more challenging and potentially unstandardized across different studies. For example, the angle of measurements may be arbitrary, which can introduce high variation between replicated measurements. Here, we outline a protocol for making consistent thorax length measurements based upon a fixed bristle point situated at the anterior end of the thorax when viewed laterally. This fixed point allows for a repeatable angle to be drawn from the scutellum, thereby significantly improving the consistency across replicated measurements when compared to a non-landmark-guided approach.

Materials and Reagents

1. Slides (clear glass ground edges, 25.4 × 76.2 mm, 1-1.2 mm thick) (Thermo Fisher, catalog number: 7101)
2. Pipette Tips (20 µl Bevelled TipOne® Filter Tip, sterile) (Starlab, catalog number: S1120-1810)
3. *Drosophila melanogaster*

We used the wild-type Dahomey strain in this protocol, originating from a population collected in West Africa in the 1970s (Puijk and De Jong, 1972).

Equipment

1. Camera (GT Vision, GXCAM HiChrome-S)
2. Dissection Microscope (Leica, model: MZ 7.5)
3. Superglue (Fix All Crystal) (Soudal, catalog number: DoP 230212)
4. Forceps (Watkins and Doncaster No. 5 superfine stainless steel)
5. 1 mm graticule
6. Computer (Mac or PC)

Software

1. ImageJ (version 1.53 at time of writing, <https://imagej.nih.gov/ij/index.html>)
2. GX Capture (version 8.5, <https://www.gtvision.co.uk/GX-Capture-Camera-Control-Image-Capture-Storage-Annotation-Enhancement-Analysis-FREE>)
3. R (version 4.0.2, <https://www.r-project.org/>) (R Core Team, 2020)

Procedure

A. *Drosophila melanogaster* mounting

1. Before mounting, remove both wings below the hinge using forceps so that the wings do not obscure the view of the thorax.
2. Label a microscope slide with the id of each sample to be measured.
3. Place a small (~5 µl) drop of superglue onto the microscope slide using a 20 µl pipette tip. Each slide can be used for three samples spread across the length of the slide (Figure 1A).
4. Mount flies in the superglue. Use the brush to gently manipulate the fly in the superglue to position it on its side, ensuring a clear lateral view of the thorax. Ensure the head is not obscuring the anterior end of the thorax; move the head away from the body if necessary (Figure 1B).



Figure 1. Preparation of *Drosophila melanogaster* specimens for measurements. A. *D. melanogaster* with the wings removed are superglued to a microscope slide on their side. Each individual is placed onto ~5 µl of superglue and manipulated using a brush until they are angled correctly. Individuals are spread apart, ensuring that photographs can be taken of each for measurement. B. A magnified image of a *D. melanogaster* male on its side taken under a microscope lens. The individual is superglued to the slide and ready for imaging.

B. Imaging

1. Set up the imaging station using an eyepiece camera connected to the GX Capture imaging software loaded up onto a computer.
2. Set the dissection microscope to 5× magnification.
3. Take an initial picture of a 1 mm scale graticule for calibration purposes. An image of the graticule should be taken at the start of every imaging session or any time the magnification is altered for any reason.
4. Adjust the microscope focus depth in each instance so that the tip of the scutellum and the dorsal bristles are visible and in sharp focus. Ensure that the microscope is always kept at a 5× magnification.
5. Take and save a picture of each sample using the GX Capture software default image settings.

C. Measuring

1. Load the ImageJ software.
2. Load the 1 mm graticule image and draw a straight line across the length of the graticule.
3. Calibrate ImageJ.
 - a. Click Analyze → Set Scale on the ImageJ toolbar.
 - b. Change Unit of Length from “pixel” to “mm;” this sets the number of pixels that compose a length of 1 mm.
 - c. Tick the box that says global and click okay. By ticking “global,” the scale will be applied across all images opened in the current ImageJ session.
4. Load your desired thorax image.
5. Draw a straight line from the most posterior tip of the scutellum to the anterior edge of the thorax, angling the measurement line through the point where the dorsal bristles at the anterior edge of the thorax stop (Figures 2, 3). This point will be just behind the prescutal lobe. There may be

some dorsal bristles that carry on further forward below the prescutal lobe (Figure 2A) we suggest not to use these bristles as it can be difficult to clearly distinguish their end point.

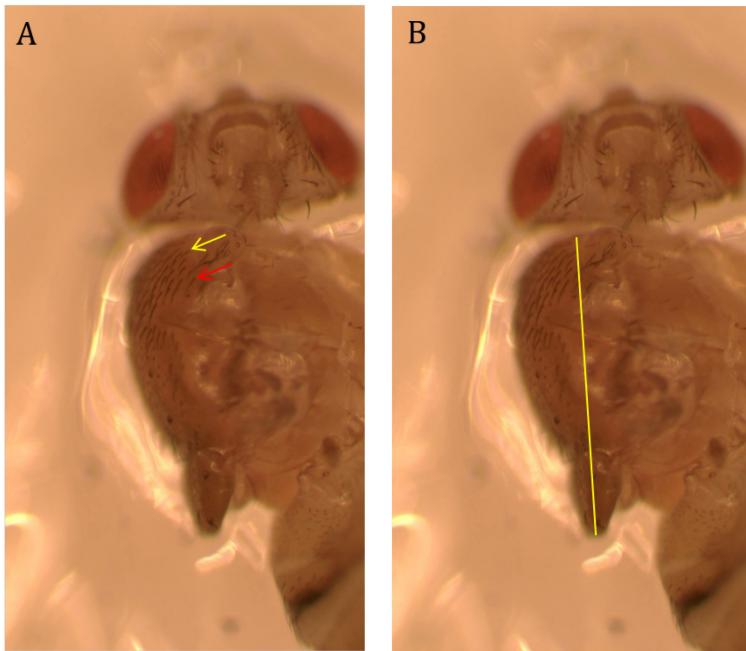


Figure 2. Female thorax illustrating the fixed bristle point and measurement line. A. The fixed bristle point near the edge of the thorax is indicated by the yellow arrow and is used to draw the measurement line. We suggest that the measurement line not be drawn against the bristles indicated by the red arrow below the prescutal lobe as it is often difficult to see their distinct end point (5 \times magnification). B. Measurement line drawn from the tip of the scutellum to the edge of the thorax, passing through the edge of the bristle indicated by the yellow arrow (5 \times magnification).

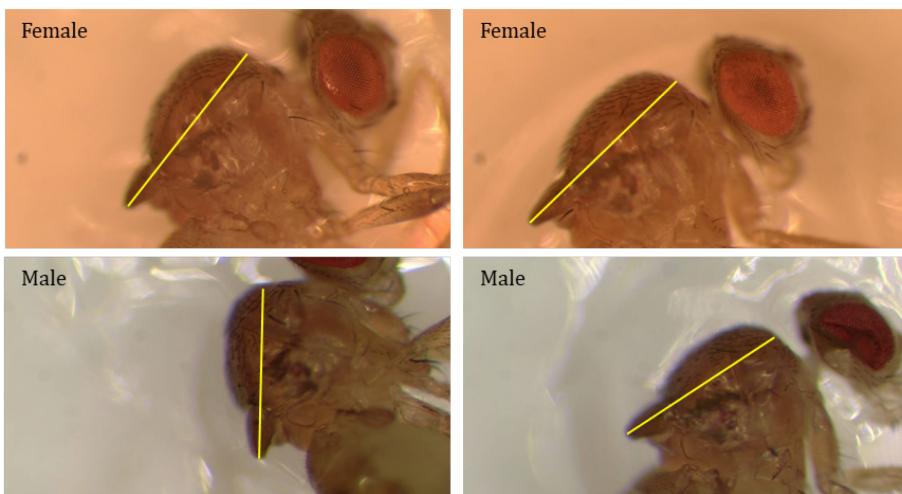


Figure 3. Bristle-guided measurements of male and female *D. melanogaster* thoraxes. Two bristle-guided measurements of individual female (top) and male (bottom) thoraxes are

shown here, demonstrating the consistency with which these landmarks can be identified (5 \times magnification).

6. Click Analyze → Measure to record the measurement. ImageJ will display the length of the drawn line in the units specified by the user in the “Set Scale” box above.
7. Draw the line and take the measurement a total of three times per thorax image. This is recommended every time a measurement is done to minimize variation arising from the drawing of the measurement line. Additionally, the replicated sets of measurements can be performed by different observers to test for any variation in placing the measurement line. The mean value of the three replicated measurements can then be calculated to give a final thorax length measurement.

Data analysis

To test for the consistency of this method against methods that do not use the fixed landmarks above, we measured male and female thoraxes using two different methods. First, we measured each thorax using the described bristle-based protocol described above. Second, we measured each thorax using a non-landmark-based method that did not use a fixed point at the edge of the thorax. This involved drawing a straight line from the tip of the scutellum to the midpoint of the scutellum and extending this line until it reached the edge of the thorax (Figure 4). Each measurement was taken three times, and the mean was calculated.

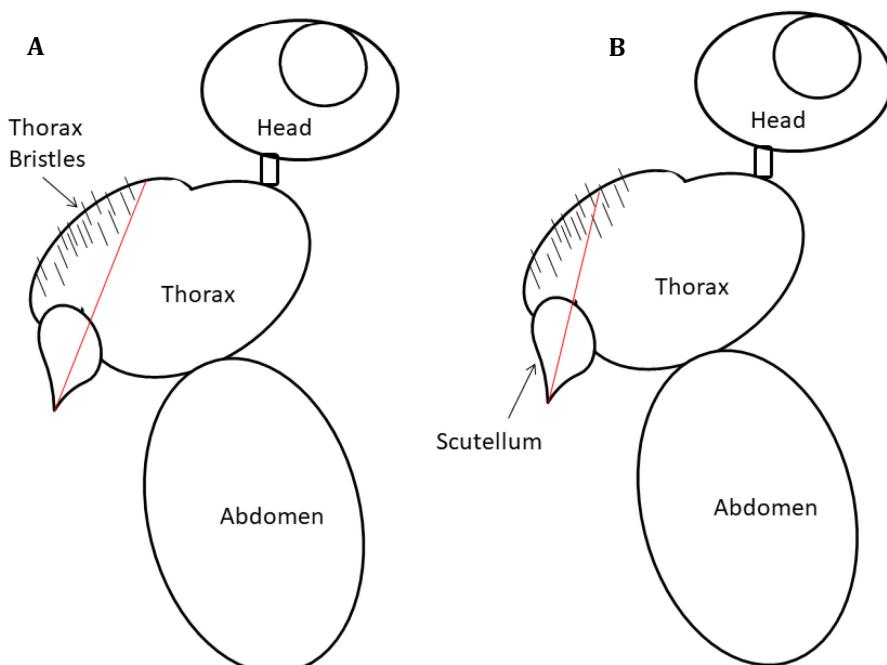


Figure 4. Schematic diagram of bristle-guided (A) and scutellum-guided (B) measurements. Illustrated parts of a *Drosophila* individual in lateral view are labelled. The

measurement line drawn for each technique is in red. A. In the bristle-guided measurement, the measurement line is drawn from the tip of the scutellum to the edge of the thorax at the point where the dorsal bristles stop. B. In the scutellum-guided measurement, the line is drawn from the tip of the scutellum, bisects through the midpoint of the scutellum, and carries on to the edge of the thorax.

Statistical analysis was carried out in R version 4.0.2 (R Core Team, 2020). We used the coefficient of variation (standard deviation/mean×100) to quantify variation between the three technical replicate measures for each thorax image. We then used a paired Wilcoxon rank sum test (wilcox.test in R) to analyze the difference in coefficient of variation between the bristle and the scutellum methods for each sex. Finally, we used a Spearman correlation coefficient to test whether there was a correlation between high variation in the bristle method and high variation in the scutellum method in the same samples. We found the bristle method to be significantly less variable between technical replicates than the scutellum method in both sexes (female thoraxes: $V = 1095$, $P = 0.0005318$, $n = 87$; male thoraxes: $V = 1312$, $P = 0.00129$, $n = 92$) (Figure 5A). However, there was no correlation between high variation in the bristle method and high variation in the scutellum method within the same samples in either the male ($r_s = -0.008507654$, $P = 0.9358$) or female ($r_s = -0.04373243$, $P = 0.6875$) measurements (Figure 5B). We therefore advocate the use of the bristle-guided method as the less prone to technical variation.

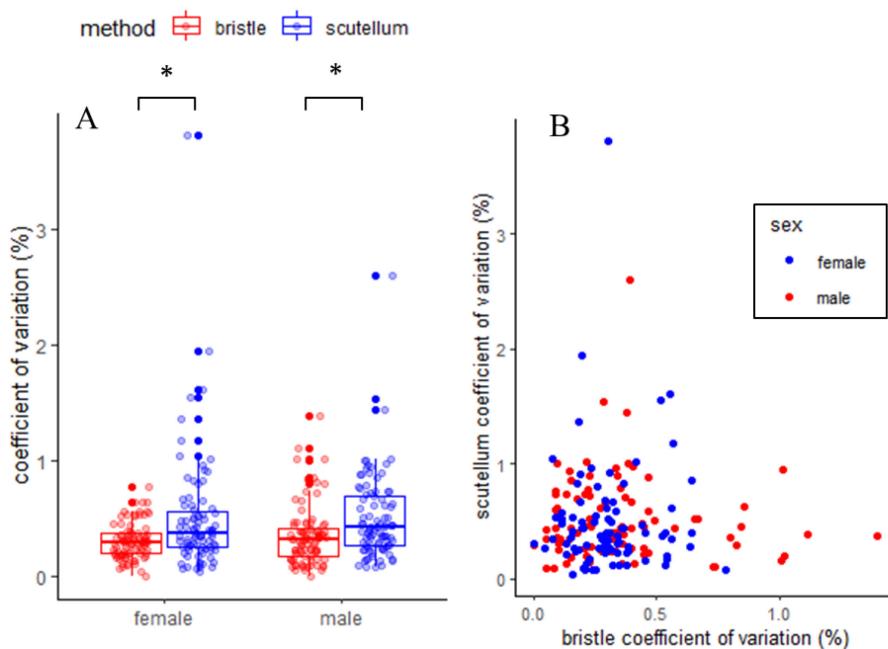


Figure 5. Variation between replicate measures of thorax length in male and female *D. melanogaster* using two different methods. Each data point represents the percentage coefficient of variation of three technical replicates. In total, 87 female and 92 male thoraxes were measured using each method. A. For both males and females, the bristle-guided method

produced significantly less variation between replicates than the scutellum-guided method in male and female measurements. Asterisks indicate a significance of $P < 0.05$ between treatments. B. No correlation was found between a high coefficient of variation of the bristle method and a high coefficient of variation of the scutellum method for male or female measurements.

Raw data: Data available via Figshare, DOI: 10.6084/m9.figshare.13547186.

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Author contributions: EKF and SL conducted the research, collected and analyzed the data; EKF, SL, and TC wrote the paper; all authors contributed to the final draft.

Competing interests

The authors declare no conflicts of interest.

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