Video Article

Methods for Staging Pupal Periods and Measurement of Wing Pigmentation of *Drosophila guttifera*

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Abstract

Diversified species of *Drosophila* (fruit fly) provide opportunities to study mechanisms of development and genetic changes responsible for evolutionary changes. In particular, the adult stage is a rich source of morphological traits for interspecific comparison, including wing pigmentation comparison. To study developmental differences among species, detailed observation and appropriate staging are required for precise comparison. Here we describe protocols for staging of pupal periods and quantification of wing pigmentation in a polka-dotted fruit fly, *Drosophila guttifera*. First, we describe the method for detailed morphological observation and definition of pupal stages based on morphologies. This method includes a technique for removing the puparium, which is the outer chitinous case of the pupa, to enable detailed observation of pupal morphologies. Second, we describe the method for measuring the duration of defined pupal stages. Finally, we describe the method for quantification of wing pigmentation based on image analysis using digital images and ImageJ software. With these methods, we can establish a solid basis for comparing developmental processes of adult traits during pupal stages.

Video Link

The video component of this article can be found at https://www.jove.com/video/56935/

Introduction

Some of the morphological traits of *Drosophila* are diversified among species[1,2,3,4,5]. We can approach the question of how morphological diversity arises by comparing the mechanisms of generation of these morphologies. Examples of such morphologies are larval trichomes, adult sex combs, external genital apparatus, abdominal pigmentation, and wing pigmentation[6,7,8,9,10,11,12,13,14,15]. To study morphological differences among adults, observation and analysis of the pupal stages are important, because the fate of adult traits is determined in the late larval stages and subsequent morphogenesis proceeds during the pupal period.

In developmental biology studies of *Drosophila melanogaster*, "hours APF" (hours after pupal formation) is the common method to indicate a pupal stage[16]. This system employs absolute time after pupal formation and is very convenient for routine experiments. However, developmental speed may differ among pupae, and may be affected by slight genetic, epigenetic or microenvironmental differences, and therefore having the same absolute time after pupal formation does not guarantee that pupae are at the same developmental stage. In many cases, stages defined by morphological features are preferable for comparing multiple individuals. Especially, a comparison between species requires precise staging and comparison among corresponding (homologous) stages.

Bainbridge and Bownes[17] recognized 20 pupal stages (P1 to P15(ii)) based on morphological features of *Drosophila melanogaster* pupae. This staging is the most widely used system of morphological developmental staging[18]. In a previous study, we performed pupal staging of *Drosophila guttifera* to establish a basis for wing pigmentation studies[19]. *D. guttifera* has a black polka-dot pattern on its wings and is one of the model species for wing pigmentation formation[20]. Although we referred to the morphological criteria described in the Bainbridge and Bownes' research[17], we directly measured stage durations by serial observations[19], instead of using Bainbridge and Bownes' estimation of stage durations from observed frequency. Here we describe the method of pupal staging and measurement of durations of pupal stages of *Drosophila* used in Fukutomi *et al*[19].

To study the developmental mechanism of wing pigmentation, we need to know when in pupal or adult stages the pigmentation occurs. Fukutomi *et al*.[19] quantified optical densities (ODs) of pigmentation during pupal and adult stages by image analysis of wing images. The pigmentation of *Drosophila* wings is thought to be caused by accumulation of black melanin[21]. For quantification of ODs, gray-scale images and ImageJ software (https://imagej.nih.gov/ij/)[22] were used. To recognize and quantify the spot-specific pigmentation (ΔOD), we subtract the OD outside of a spot

from the OD inside of a spot. To make this method reproducible and objective, the places of OD measurement should be determined using wing veins as landmarks. In this article, we describe in detail this method of quantification of wing pigmentation in *Drosophila guttifera*.

Protocol

1. Fly stock

1. Use *Drosophila guttifera* for all of the following protocols.
2. Use plastic vials (diameter 25 mm x height 96 mm) and cellulose plugs (diameter 23 mm x height 26 mm) for stock maintenance. Use a standard cornmeal/sugar/yeast/agar food and follow a publication described three other alternative recipes for this species[2].
   NOTE: *D. guttifera* (stock number 15130-1971.10) is provided by the Drosophila Species Stock Center at the University of California, San Diego. Although *D. guttifera* belongs to the *immigrans-tripunctata* radiation, which is distantly related to *D. melanogaster* within the genus[23], it has many biological properties in common with *D. melanogaster*. Accordingly, this protocol can be applied for many *Drosophila* species, although some species require specific food and/or technical tips to maintain them[2].

2. Observation of pupa and definition of pupal stages

NOTE: The pupa for observation is taken from the fly stock maintained with a 12:12 h light/dark cycle at 25 °C. Bainbridge and Bownes[17] described a low risk of moving *D. melanogaster* pupae from the original place of pupation onto a piece of moistened tissue paper (97% survival of 946 moved pupae). *D. guttifera* pupae can be prepared by essentially the same method.

1. Place healthy adults of *Drosophila* on fresh food (standard cornmeal/sugar/yeast/agar food) in a plastic vial (diameter 25 mm x height 96 mm) and let them lay eggs. Wait 7 days to obtain late 3rd instars.
2. Place 1 mL of standard cornmeal/sugar/yeast/agar food into each 1.5 mL microtube. Make 3 pinholes with 2 mm spacing in the lids of microtubes by penetrating with a push-pin to allow breathing.
3. Centrifuge the microtubes with food for 7 s in a mini microcentrifuge (860 x g).
4. Invert and tap vials to remove all adults from the vial.
5. Pour 5 - 10 mL of ddH_2O (or reverse osmosis water) into the vial.
6. Pour out larvae with water into a plastic Petri dish (diameter 90 mm x height 15 mm). Identify late 3rd instars by their large body size (3 - 4 mm in length).
7. Gently move late 3rd instar larvae with forceps into the microtubes with food (10 larvae / microtube). Incubate them overnight at 25 °C.
8. Move newly formed pupae onto a piece of tissue paper that has been moistened by ddH_2O and placed in a plastic Petri dish (diameter 35 mm x height 10 mm).
9. Place the Petri dish in a moist chamber (containing 10 mL of ddH_2O in the bottom), and wait until the pupae develop to the desired stage.
10. Move pupae onto a moistened piece of tissue paper in a plastic Petri dish (diameter 60 mm x height 15 mm).
    NOTE: The definition of stages can mostly be made based on the stages of *D. melanogaster*[17]. Typically, the pupal period of *Drosophila* can be categorized into P1 - P15(ii), although some modifications of stage definition would be required depending on the species used. If possible, removing the puparium enables precise and detailed observation. See details below (Step 3).
11. Observe pupae under a stereo microscope. Take photographs using a digital camera attached to the stereo microscope.

3. Removing puparium

NOTE: Pupae of *Drosophila* are covered by a structure called the puparium. An insect of Muscomorpha (flies) does not shed its larval cuticle at pupation; instead, it hardens the cuticle after apolysis, and uses it as a protective cover of the pupa, the puparium[24]. A pupa residing inside a puparium has a true pupal cuticle, which is very soft and fragile. Before apolysis takes place around P4(ii), epithelia and puparium are attached together, and therefore removing the puparium without damage is very difficult. After P5, removing the puparium is laborious, but useful for morphological observation and definition of pupal stages. The process is carried out as follows.

1. Affix a piece of double-sided tape on a piece of paper towel.
2. Place a pupa on the double-sided tape ventral side up (**Figure 1A**).
3. Locate the space between the anterior side of the puparium and the internal pupa. Grasp and remove the puparium around this gap using forceps, and expose the anterior side of the head of the pupa.
4. Insert the tip of a forceps by moving it parallel to the anterior-posterior axis. Lift the tip of the forceps to locally break the puparium. Repeat this action until the breakage reaches the posterior part of the puparium. Ensure that a gap is also formed between the puparium and pupal legs, and break the ventral side of the puparium and minimize the damage to the internal pupa (**Figure 1B**).
5. After breaking the puparium as much as possible, take out the pupa using a fine paintbrush (#5/0) (**Figure 1C**).
6. Place the pupa on a piece of tissue paper that has been moistened with ddH_2O and placed in a plastic Petri dish (diameter 60 mm x height 15 mm). Take photographs as soon as possible because the exposed pupa is vulnerable and easily becomes dry.
   NOTE: Pupae without a puparium are not suitable for measuring durations of pupal periods (Step 4), because stress (such as desiccation) and physical damage might interfere with the normal development.

4. Measuring durations of pupal stages

1. Prepare pupae for measuring durations of pupal stages as described in Step 2. Collect pupae of 1 - 2, 2 - 3, and 3 - 4 days after pupal formation (20 pupae each). Give individual identification numbers (1 - 60) to pupae for identification. Continue collecting newly formed pupae during the following steps, to obtain 20 more young pupae. Give individual identification numbers (61 - 80) to the newly formed pupae. Place a pupa on a piece of ddH_2O-moistened tissue paper in a well (3.9 cm^2) of a 12-well cell culture plate (1 pupa/well, 12 pupae/plate). Fill the

inter-well space of plates with ddH_2O to maintain humidity, put the lids on, and place the plates in 25 °C, constant light (24:0 h light/dark) conditions.
NOTE: Pupae without a puparium are not suitable for measuring durations of pupal periods. Please do not remove the puparium.
2. Observe morphological features including body color, bristles, Malpighian tubules and yellow body of all pupae once every 30 min, and record observed stages (P1 - P15(ii), based on the references[17,19]) in a tally sheet.
3. Continue recording over four straight days (96 h) by a rotating shift of three (or more) persons.
4. Count numbers of records of each particular stage, and average them (average count of observations / pupae). Then multiply them by 0.5 (h), resulting in the estimated lengths of stages (h).

5. Measurement of intensity of black spots on a wing

NOTE: The intensity of black spots on a pupal or adult wing can be quantified by measuring optical density (OD). A glass filter with known ODs (stepped density filter) is used for calibration[25], so that one can calculate the OD of a particular area from a digital image of a wing. The OD in a spot and the OD outside of the spot are measured, and the latter is subtracted from the former to obtain the intensity of the spot (ΔOD). Here, we describe the method of dissection, measurement and calculation of ΔOD. This procedure can be done after Step 2, independent from Step 3 and Step 4. Once one has performed Step 2 and understands all pupal stages, one can directly start or repeat Step 5.

1. Image preparation
    1. Prepare a new pupa of a focal stage as described in Step 2. Remove the anterior part of a puparium with forceps. Take out the pupa using forceps and place it into phosphate buffered saline (PBS, **Table 2**) in a plastic Petri dish (diameter 35 mm x height 10 mm).
    2. Cut the basal joint of a wing (basal joint is the narrow proximal part of the wing). As the wing is folded, place it into a plastic Petri dish (diameter 35 mm x height 10 mm) filled with ddH_2O to extend it by osmotic pressure (the wing unfolds by itself).
    3. Collect newly eclosed adults once every 10 min from a stock vial. Anesthetize a fly with CO_2 using a CO_2 anesthetizing pad, confirm anesthetization by immobility and cut the basal joint of a wing.
    4. Place 10 µL of PBS on a glass slide, place the wing there, and cover with a cover slip (18 mm x 18 mm).
    5. Turn on the light of the stereo microscope. Set the light to be at maximum level. Set the objective lens 11.5X. Set the diaphragm to be the most open state. Turn on the camera. Set the camera to be (ISO: 100, mode SHQ 3136 x 2352 pixels, shutter speed: 1/20 s). Focus on the sample by moving the focus knob of the microscope.
    6. Push the shutter button of the remote-control unit to take an image. Take 3 images per wing, each of which must be centered on a campaniform sensillum, longitudinal vein spot or posterior crossvein, positioning the distal part of the wing on the left side and the anterior part of the wing on the upper side.

2. Calibration
    1. Take images of 9 parts of a stepped density filter using the same camera settings used to obtain the wing image.
    2. Initiate ImageJ software (https://imagej.nih.gov/ij/)[22].
    3. Click **File | Open |** and select one of the images of the stepped density filter.
    4. Click **Image | Type | 8-bit |** to convert the image to an 8-bit image.
    5. Click **Edit | Selection | Specify |** and check **Oval** and **Centered** column. Write 100 (pixels) in **Width** column, 100 (pixels) in **Height** column, 1568 in **X coordinate** column and 1176 in **Y coordinate** column. Click **OK**.
    6. Click **Analyze | Measure|**. The "mean grey value" of selected areas are measured.
    7. Repeat 5.2.3. to 5.2.6. for the 8 remaining images.
    8. Click **Analyze | Calibrate** and select **Rodbard**[25] in **Function** column and write the following number in the right column in the middle (0.04, 0.336, 0.632, 0.928, 1.224, 1.52, 1.816, 2.112, 2.408; these numbers depend on the densities of the stepped density filter).
    9. Check **Global calibration** column and click **OK**.
    NOTE: By performing this procedure, "mean grey value" is converted to "optical density (OD)" using the Rodbard function. After this step, optical density can be calculated for a particular selected area in ImageJ software.

3. Choosing area of measurements
NOTE: Spots are typically associated with landmarks, such as campaniform sensilla, longitudinal vein tips, and crossveins. These and other landmarks on a wing can be used to choose the region of measurements. Here, an example in *D. guttifera* (**Figure 2**) is described.
    1. Definition of Point A, campaniform sensillum spot.
        1. Open an image in which a campaniform sensillum spot is at the center of the image. Click **Image | Type | 8-bit |** to convert the image to an 8-bit image.
        2. Click **Rectangle** in **Area Selection Tools** and draw a rectangle. Set the upper left vertex of the rectangle so that it is attached to the posterior line of the third longitudinal vein and more distal from a campaniform sensillum spot. Set the right side of the rectangle so that it is located to the right of the campaniform sensillum spot.
        3. Click **Edit | Selection | Add to Manager |**. Check **Show All** column.
        4. Click **Angle tool** in **Line Selection Tools**. Draw the first line on the posterior line of third longitudinal vein. Set the left endpoints of the line on the vertex of the rectangle drawn in Step 1. Draw the second line on the upper side of the rectangle. Press the "m" key to measure the angle between the two lines drawn in this step. Click the window of the image on the screen of the computer.
        5. Click **Edit | Selection | Add to Manager |**.
        6. Click **Rectangle** in **Area Selection Tools** and draw a rectangle of approximately 1/9 the size of the image window. Click **Edit | Selection | Rotate |**. Write the minus degrees of the angle measured in Step 5.3.1.4. in **Angle** column and click **OK** to rotate the rectangle drawn in this step.
        7. Move the rectangle drawn in Step 5.3.1.6. by using the arrow keys. Set the endpoint of the posterior line of the second longitudinal vein on the left side of the rectangle and the lower side of the rectangle attached to the posterior line of the third longitudinal vein. Confirm that a perpendicular line from the end point of the second longitudinal vein to the posterior line of the third longitudinal vein is drawn in this procedure. Define the foot of the perpendicular line as Point A (**Figure 2A**).

8. Record the x coordinate and the y coordinate of Point A, indicated below **Area Selection Tools** when placing the cursor on Point A.

2. Definition of Point B, longitudinal vein tip spot
    1. Open an image in which a longitudinal vein tip spot is at the center of the image. Click **Image | Type | 8-bit |** to convert the image to an 8-bit image.
    2. Repeat the same procedure described in Step 5.3.1. (Definition of Point A, campaniform sensillum spot) to find Point A in the image.
    3. Click **Edit | Selection | Add to Manager |**.
    4. Click **Rectangle** in **Area Selection Tools** and draw a rectangle. Set the upper left vertex of the rectangle at the end point of the posterior line of the third longitudinal vein.
    5. Click **Edit | Selection | Add to Manager |**.
    6. Click **Angle tool** in **Line Selection Tools**. Draw the first line to connect Point A and the end point of the posterior line of the third longitudinal vein. Define this line as Line A. Draw the second line on the upper side of the rectangle drawn in Step 5.3.2.4. Press "m" key to measure the angle between the two lines.
    7. Click **Edit | Selection | Add to Manager |**.
    8. Click **Rectangle** in **Area Selection Tools** and draw a rectangle of approximately 1/9 the size of the image window. Click **Edit | Selection | Rotate |**. Write the minus degrees of the angle measured in Step 5.3.2.6. in **Angle** column and click **OK** to rotate the rectangle drawn in this step. Move the rectangle by using the arrow keys. Set the upper side of the rectangle so that it is attached to Line A and the end point of the anterior line of the fourth longitudinal vein so that it is on the left side of the rectangle.
    9. Click **Edit | Selection | Add to Manager |**.
    10. Click **Rectangle** in **Area Selection Tools** and draw a rectangle of approximately 1/9 the size of the image window. Click **Edit | Selection | Rotate |**. Write the minus degrees of the angle measured in Step 6 in **Angle** column and click **OK** to rotate the rectangle drawn in this step. Move the rectangle by using the arrow keys. Set the lower left vertex of the rectangle so that it is at the upper left vertex of the rectangle drawn in Step 5.3.2.8., resulting in obtaining the perpendicular line from the end point of the anterior line of the fourth longitudinal vein to Line A. Define the intersection point of the perpendicular line and the posterior line of the third longitudinal vein as Point B (**Figure 2B**).
    11. Record the x coordinate and the y coordinate of Point B, indicated below **Area Selection Tools** when placing the cursor on Point B.

3. Definition of Point C, posterior crossvein spot
    1. Open an image in which a posterior crossvein spot is at the center of the image. Click **Image | Type | 8-bit |** to convert the image to an 8-bit image.
    2. Define Point C as the posterior-most point of the anterior line of the fourth longitudinal vein in the intersection area of the posterior crossvein and the fourth longitudinal vein (**Figure 2C**).
    3. Record the x coordinate and the y coordinate of Point C, indicated below **Area Selection Tools** when placing the cursor on Point C.

4. Definition of Point D, control area
    1. Open an image in which a campaniform sensillum spot is at the center of the image. Click **Image | Type | 8-bit |** to convert the image to an 8-bit image.
    2. Click **Straight** in **Line Selection Tools** and draw a line connecting the end point of the anterior line of the second longitudinal vein and the end point of the posterior line of the fourth longitudinal vein. Define Point D as the crossing point of this line and the posterior line of the third longitudinal vein (**Figure 2D**).
    3. Record the x coordinate and the y coordinate of Point D, indicated below **Area Selection Tools** when placing the cursor on Point D.

4. Measurements
    1. Open one of the images of wing spots (for measurement of Point A, open the image with Point A in the center). Click **Image | Type | 8-bit |** to convert the image to an 8-bit image.
    2. Click **Rectangle** in **Area Selection Tools** and draw a rectangle of approximately 1/9 size of the image window.
    3. Click **Edit | Selection | Specify |**. Check **Oval** and **Centered** column. Write 100 (pixels) in **Width** and **Height** columns, write the x coordinates of Point A (or Point B or Point C) and D in **X coordinate** column and write the y coordinates of Point A (or Point B or Point C) and D in **Y coordinate** column. Click **OK**.
    4. Click **Analyze | Measure |**. If the calibration described in 5.2 has already been finished, ODs are indicated in **Mean** column.
    5. Calculate ΔODs by subtracting OD of Point D from ODs of Points A, B, and C.
    NOTE: Point D is in a transparent part of the wing and does not include pigmentation, and therefore is suitable for a background control.

Representative Results

The pupal period of *D. guttifera* is divided into 17 stages (P1 - P15(ii); images of three representative stages (P1, P5 - 6, P10) are shown **Figure 3**, and all 17 stages are illustrated in **Figure 4**). Although Bainbridge and Bownes[17] recognized 20 stages in *D. melanogaster*, some of these stages could not be applied to *D. guttifera*. The order of two developmental events, the appearance of the yellow body (mass of shed cells within the midgut[26]) and the timing of Malpighian tubules turning green, are not strictly controlled in *D. guttifera*, and hence we could not separate P5(i), P5(ii) and P6. Also, unlike in *D. melanogaster*, the timing of blackening of thoracic and abdominal bristles was synchronized, and therefore we could not separate P11(i) and P11(ii)[19].

We could measure the length of pupal stages of *D. guttifera* (Table 3, from Fukutomi et al.[19]). The entire pupal period is approximately 20 h longer than that of *D. melanogaster* at 25 °C[17]. We calculated ΔODs of areas around a campaniform sensillum, longitudinal vein tip and posterior crossvein. Here, we show the ODs and ΔODs in adults 7 days after eclosion (**Table 4**). By comparing the data of multiple stages, we found that stage P12(i) is the timing of onset of pigmentation, and that pigmentation is completed by 24 h after eclosion (**Figure 5**, from the original measurements used in Fukutomi *et al.*[19]).

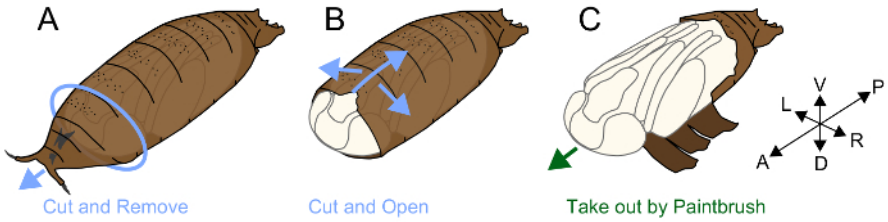

Figure 1. Illustration of removing puparium. (A) Place a pupa ventral side up on a piece of double-sided tape. Remove the anterior part of the puparium. (B) Break the puparium with forceps from the ventral side. (C) After breaking the puparium, take out the pupa using a paintbrush. Please click here to view a larger version of this figure.

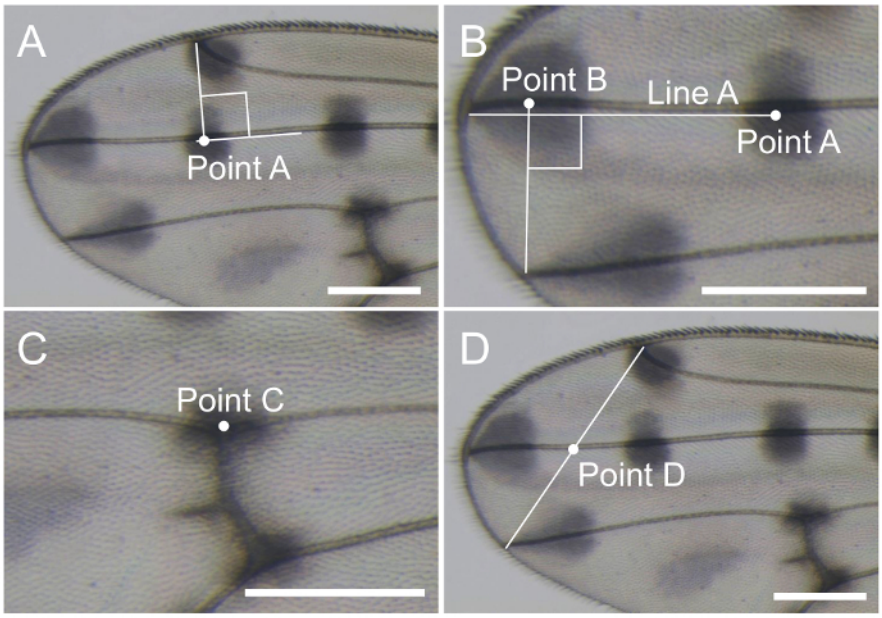

Figure 2. Definition of area for measuring pigmentation. (A) Point A for a spot associated with a campaniform sensillum. (B) Point B for a spot associated with a longitudinal vein tip. (C) Point C for a spot associated with a posterior crossvein. (D) Point D for a control area. Scale bars indicate 250 µm. Please click here to view a larger version of this figure.

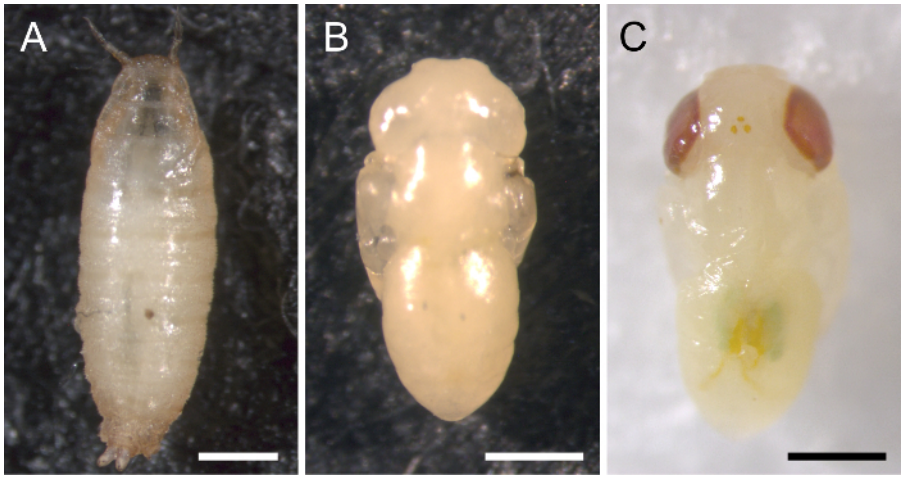

Figure 3. Examples of defined pupal stages. (A) Pupa of stage P1 covered with puparium. (B) Pupa of stage P5 - 6. (C) Pupa of stage P10. The puparia are removed before observation in (B) and (C). Scale bars indicate 500 µm. Please click here to view a larger version of this figure.

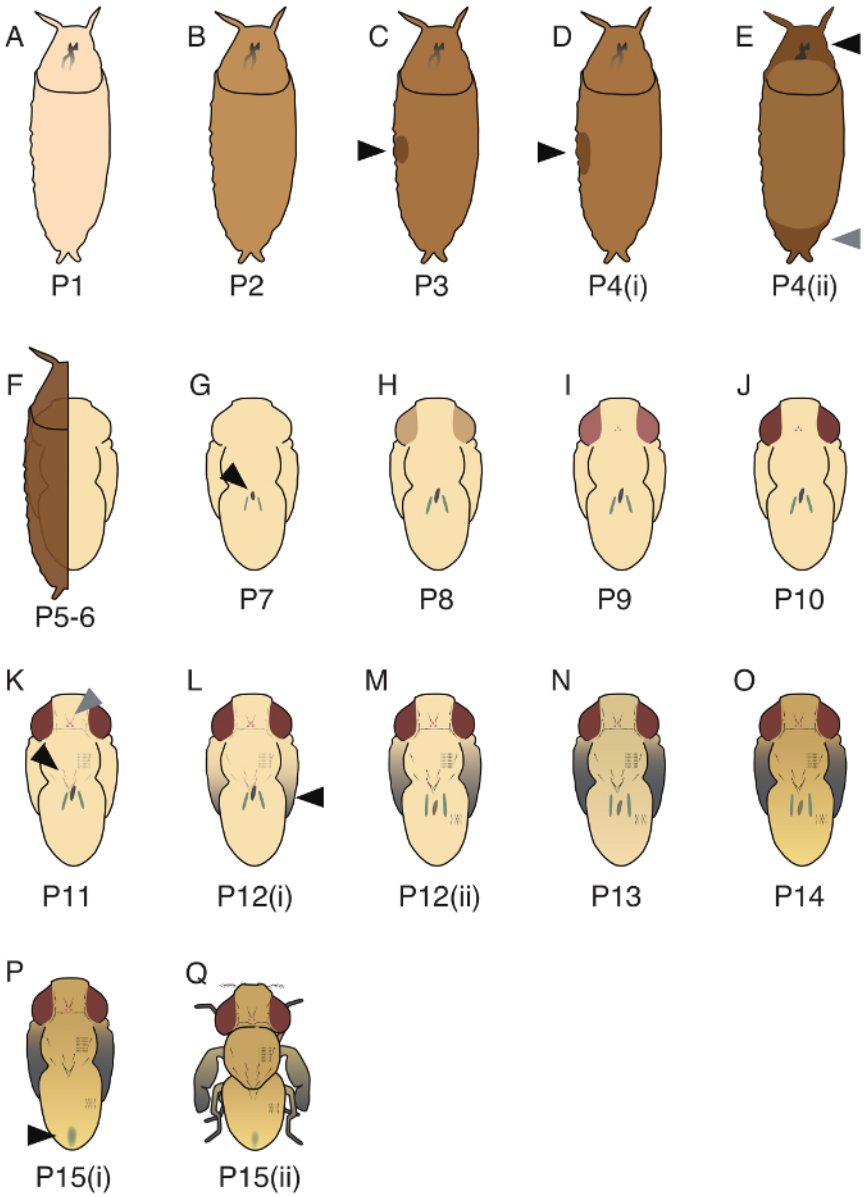

Figure 4. Illustrations of 17 pupal stages identified in *D. guttifera*. (A) P1, the puparium is white. (B) P2, the color of the puparium is light brown. (C) P3, a bubble is observed in the lateral side (black arrowhead). (D) P4(i), the bubble is larger than that in P3 (black arrowhead), and the pupa is buoyant in PBS. (E) P4(ii), a gap is observed in the anterior part (black arrowhead) and the posterior part (gray arrowhead). (F) P5 - 6, Malpighian tubules migrate (difficult to see if covered by puparium). The pupal shape is formed by pupal epithelium and pupal cuticle. (G) P7, the yellow body can be observed in the dorsal side (black arrowhead). (H) P8, the eyes are yellow. (I) P9, the eyes are amber. (J) P10, the eyes are red. (K) P11, Orbital and ocellar bristles (gray arrowhead), vibrissae, thoracic macrochaetae (black arrowhead), and tarsal bristles are black and visible. (L) P12(i), the tips of wings are gray. (M) P12(ii), all parts of the wings are gray (black arrowhead). (N) P13, the wings are completely black. (O) P14, the head and the legs are completely darkened. (P) P15(i), the meconium can be observed on the dorsal abdomen (black arrowhead). (Q) P15(ii), the fly is eclosing. Details of these stages were described in Fukutomi *et al.*[19] Please click here to view a larger version of this figure.

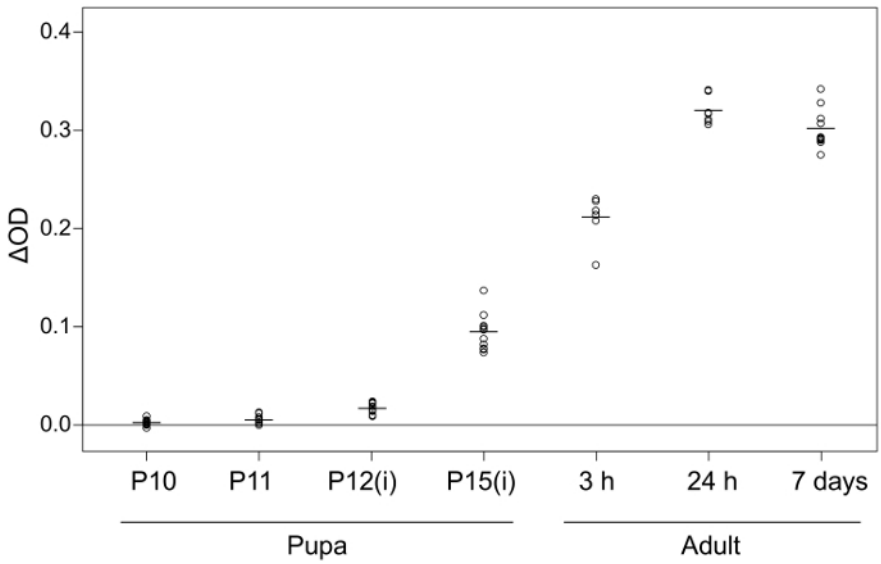

Figure 5. Development of pigmentation around a companiform sensillum on a wing. Circles indicate individual ΔODs, and horizontal bars indicate averages. P10: n = 10, P11: n = 10, 12(i): n = 10, P15(i): n = 11, 3 h: n = 8, 24 h: n = 7, 7 days: n = 10. Please click here to view a larger version of this figure.

Component	
White soft sugar	51.6 g
Corn flour	172.4 g
Corn grits - C	86.4 g
Dry beer yeast	106 g
Agar powder	35.28 g
ddH_2O	4000 mL
Boil for 30 min and leave to cool down to 70 °C.	
Add 4 g of butyl p-hydroxybenzoate dissolved in 40 mL of ethanol.	
Mix well and pour 9 mL each into plastic vials (diameter 25 mm x height 96 mm).	

Table 1. The composition of standard cornmeal/sugar/yeast/agar food.

Component	**Amount**
NaCl	80 g
KCl	2 g
$Na_2HPO_4 \cdot 12H_2O$	29 g
KH_2PO_4	2 g
ddH_2O	up to 10 L
	set pH 7.4

Table 2. The composition of PBS (1X).

Stage	Mean of duration (h)	s.d.	n
P1 - 2	1.7	0.65	16
P3	2.1	0.65	16
P4(i)	2.1	1.69	19
P4(ii)	0.3	0.28	29
P5 - 6	5.0	3.07	30
P7	31.9	7.22	46
P8	9.6	2.81	57
P9	10.9	2.66	55
P10	11.7	2.96	39
P11	4.4	2.81	44
P12(i)	1.1	0.76	44
P12(ii)	2.0	0.70	43
P13	2.2	0.68	10
P14 - 15(i)	28.6	2.75	10
P15(ii)	1.4	0.87	10
Total	**121.7**		

Table 3. Measured durations of pupal stages of *D. guttifera*.

	OD					ΔOD		
	Campaniform sensilum	Longitudinal vein tip	Posterior crossvein	Control		Campaniform sensilum	Longitudinal vein tip	Posterior crossvein
Individual	(Point A)	(Point B)	(Point C)	(Point D)		(Point A - Point D)	(Point B - Point D)	(Point C - Point D)
1	0.549	0.484	0.515	0.256		0.293	0.228	0.259
2	0.529	0.489	0.516	0.254		0.275	0.235	0.262
3	0.546	0.48	0.533	0.255		0.291	0.225	0.278
4	0.583	0.496	0.566	0.255		0.328	0.241	0.311
5	0.523	0.479	0.528	0.235		0.288	0.244	0.293
6	0.572	0.509	0.546	0.265		0.307	0.244	0.281
7	0.568	0.511	0.56	0.256		0.312	0.255	0.304
8	0.56	0.507	0.562	0.27		0.29	0.237	0.292
9	0.551	0.485	0.569	0.259		0.292	0.226	0.31

Table 4. Measured ODs and ΔODs of *D. guttifera* adults 7 days after eclosion.

Discussion

We describe here the protocols for definition of pupal stages, removing the puparium for detailed observation, measuring durations of pupal stages, and measurement of intensity of black spots on a wing in *D. guttifera*. These protocols can be applied for many *Drosophila* and related fly species, especially species with wing pigmentation.

In-depth observation and description of more detailed developmental events would enable further subdivision of stages. In many cases, a developmental event requiring dissection or sectioning of a pupa is not suitable for stage definition, because one has to kill a pupa for staging, and further use of that individual is difficult. For use of a new *Drosophila* species, one should employ developmental events distinguishable from outside of the puparium as the first step. Depending on the purpose of the study, one can then further subdivide stages based on particular organogenesis or other developmental events.

For interspecific comparison of multiple stages, a potential difficulty is an inversion of the order of developmental events among species (heterochrony[27]). For example, in *D. melanogaster*, the Malpighian tubule becomes green and then the yellow body becomes visible, whereas this order can be inverted in some pupae of *D. guttifera*[19]. In such a case, strict comparison between homologous stages is difficult. Depending on the phenomenon of interest, one may need to re-define or subdivide a particular stage based on a developmental event. For example, we

can roughly select pupae of P5 - 6 and do interspecific comparison of gene expressions in pupal wings using wing morphology as an indicator of developmental timing[14].

Typically, it takes 10 - 30 min to remove the puparium. If one wants to observe a short stage, pupae should be prepared taking into account the time that passes during puparium removal. For example, if one wants to observe P12(i) of *D. guttifera*, which has only 1.1 h duration, preparing pupae at P11 would give a good result.

In our protocol, moistened tissue paper is used for the background of pupal images. Depending on the stage of observation and structures one wants to show in a figure, one can use white or black tissue paper. For the P3 to P4(ii) transition, the position of the bubble in pupa is important for distinguishing stages, and black tissue paper helps to observe the position of the bubble. For the stages after P5, white tissue paper is better because it helps to observe the yellow body, eye color, bristles, and body color.

Bainbridge and Bownes[17] estimated the duration of pupal stages from their frequency of appearance. Their staging table is the most widely used one for *D. melanogaster*[18]. For their method, they prepared four food bottles containing five adult females and five adult males and kept them in the dark, and then pupae were randomly taken out from one bottle each at 11, 12, 13, and 14 days after the onset of egg laying. They counted the number of pupae in particular stages, and calculated the averages. The length of each pupal stage could be estimated based on the data of the total duration of the pupal period and these frequency data. One problem with this method is that it can not be used to estimate the precise duration of pupal stages if developmental timing tends to be synchronized among pupae.

In fact, we tried Bainbridge and Bownes's method in *D. guttifera*, and we obtained biased data because of synchronization among pupae. We could not identify the cause of this phenomenon, but some possibilities are 1) pupae retained circadian rhythm from their young stage and/or 2) they reacted to the exposures to light that occurred at observation. Therefore, we decided to measure actual stage duration by direct observation. This minimizes bias caused by circadian rhythm.

The method described here is a method to quantify extra accumulation of melanin in spots compared to their surrounding control area (ΔOD), by subtracting the OD of the control area from the OD of the spot area. This method was inspired by a method for quantifying nuclear DNA content by Feulgen staining and image analysis (densitometry[28,29]). As one of the potential problems for applying this method to wing pigmentation, ΔOD can be a negative value, especially when a pupa is very young and has almost no pigmentation. In later stages, there could be some pigmentation in the control area. The use of the simple OD of the spot area itself might be appropriate instead of ΔOD depending on the purpose of the study. In the case of *D. guttifera,* use of the simple OD instead of ΔOD did not change the tendency of the data or the conclusions of the study.

Disclosures

The authors have no conflict of interest.

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References

1. Carson, H. L., Hardy, D. E., Spieth, H. T., Stone, W. S. The evolutionary biology of the Hawaiian Drosophilidae. In: Hecht, M. K., Steere W. C. (eds) *Essays in evolution and genetics in honor of Theodosius Dobzhansky.* Springer (Meredith Corporation), New York. 437-543 (1970).
2. Markow, T. A., O'Grady, P. M. *Drosophila: a guide to species identification and use.* Academic Press, New York. (2006).
3. Patterson, J. T. *The Drosophilidae of the southwest.* University of Texas Publication, **4313**, 7-216 (1943).
4. Setoguchi, S., Takamori, H., Aotsuka, T., Sese, J., Ishikawa, Y., Matsuo, T. Sexual dimorphism and courtship behavior in *Drosophila prolongata*. *J Ethol.* **32** (2), 91-102 (2014).
5. Werner, T., Jaenike, J. *Drosophilids of the Midwest and Northeast.* River Campus Libraries, University of Rochester, Rochester (2017).
6. Arnoult, L. et al. Emergence and diversification of fly pigmentation through evolution of a gene regulatory module. *Science.* **339** (6126), 1423-1426 (2013).
7. Camino, E. M., Butts, J. C., Ordway, A., Vellky, J. E., Rebeiz, M., Williams, T. M. The evolutionary origination and diversification of a dimorphic gene regulatory network through parallel innovations in *cis* and *trans*. *PLoS Genet.* **11** (4), e1005136 (2015).
8. Gompel, N., Prud'homme, B., Wittkopp, P. J., Kassner, V. A., Carroll, S. B. Chance caught on the wing: *cis*-regulatory evolution and the origin of pigment patterns in *Drosophila*. *Nature*. **433** (7025), 481-487 (2005).
9. Glassford, W. J. et al. Co-option of an ancestral Hox-regulated network underlies a recently evolved morphological novelty. *Dev. Cell* . **34** (5), 520-531 (2015).
10. Koshikawa, S. Enhancer modularity and the evolution of new traits. *Fly.* **9** (4), 155-159 (2015).
11. Koshikawa, S. et al. Gain of *cis*-regulatory activities underlies novel domains of *wingless* gene expression in *Drosophila*. *Proc Natl Acad Sci U S A*. **112** (24), 7524-7529 (2015).
12. McGregor, A. P. et al. Morphological evolution through multiple *cis*-regulatory mutations at a single gene. *Nature.* **448** (7153), 587-90 (2007).
13. Tanaka, K., Barmina, O., Kopp, A. Distinct developmental mechanisms underlie the evolutionary diversification of *Drosophila* sex combs. *Proc Natl Acad Sci U S A.* **106** (12), 4764-4769 (2009).
14. Werner, T., Koshikawa, S., Williams, T.M., Carroll, S.B. Generation of a novel wing colour pattern by the Wingless morphogen. *Nature.* **464** (7292), 1143-1148 (2010).

15. Wittkopp, P. J. et al. Intraspecific polymorphism to interspecific divergence: genetics of pigmentation in *Drosophila. Science.* **326** (5952), 540-544 (2009).
16. Lawrence, P. A., Morata, G. Compartments in the wing of Drosophila: a study of the engrailed gene. *Dev Biol.* **50** (2), 321-337 (1976).
17. Bainbridge, S. P., Bownes, M. Staging the metamorphosis of *Drosophila melanogaster. J. Embryol. Exp. Morphol.* **66**, 57-80 (1981).
18. Ashburner, M., Golic, K.G., Hawley, R.S. *Drosophila: A Laboratory Handbook.* Second Edition. Cold Spring Harbor Laboratory Press, New York. (2005).
19. Fukutomi, Y., Matsumoto, K., Agata, K., Funayama, N., Koshikawa, S. Pupal development and pigmentation process of a polka-dotted fruit fly, *Drosophila guttifera* (Insecta, Diptera). *Dev Genes Evol.* **227** (3), 171-180 (2017).
20. Koshikawa, S., Fukutomi, Y., Matsumoto, K. *Drosophila guttifera* as a model system for unraveling color pattern formation. In Sekimura T and Nijhout HF (ed) *Diversity and evolution of butterfly wing patterns: an integrative approach.* Springer, New York. in press (2017).
21. True, J. R., Edwards, K. A., Yamamoto, D., Carroll, S. B. *Drosophila* wing melanin patterns form by vein-dependent elaboration of enzymatic prepatterns. *Curr Biol.* **9** (23), 1382-1391 (1999).
22. Schneider, C. A., Rasband, W. S., Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods.* **9** (7), 671-675 (2012).
23. Izumitani, H. F., Kusaka, Y., Koshikawa, S., Toda, M. J., Katoh, T. Phylogeography of the Subgenus *Drosophila* (Diptera: Drosophilidae): evolutionary history of faunal divergence between the Old and the New Worlds. *PLoS ONE.* **11** (7), e0160051 (2016).
24. Resh, V. H., Cardé, R. T. *Encyclopedia of Insects.*, 2nd Edition, Academic Press, Burlington. (2009).
25. DeLean, A., Munson, P. J., Rodbard, D. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am J Physiol.* **235** (2), E97-102 (1978).
26. Robertson, C.W. The metamorphosis of *Drosophila melanogaster*, including an accurately timed account of the principal morphological changes. *J Morphol.* **59** (2), 351-399 (1936).
27. McKinney, M.L., McNamara, K. *Heterochrony: the evolution of ontogeny.* Plenum Press, New York. (1991).
28. Hardie, D. C., Gregory, T. R., Hebert, P. D. From pixels to picograms: a beginners' guide to genome quantification by Feulgen image analysis densitometry. *J Histochem Cytochem.* **50** (6), 735-749 (2002).
29. Koshikawa, S., Miyazaki, S., Cornette, R., Matsumoto, T., Miura, T. Genome size of termites (Insecta, Dictyoptera, Isoptera) and wood roaches (Insecta, Dictyoptera, Cryptocercidae). *Naturwissenschaften.* **95** (9), 859-867 (2008).

