Staging the metamorphosis of *Drosophila melanogaster*

By S. PAUL BAINBRIDGE AND MARY BOWNES[1]

From the Department of Molecular Biology, University of Edinburgh

SUMMARY

A sequence of 51 visible changes is described during the course of metamorphosis in *Drosophila melanogaster*, and a series of 24 convenient stages is defined for use in the experimental analysis and exploitation of this part of the insect life cycle. The duration of each stage is estimated and times are suggested for batch collections of symphasic animals.

INTRODUCTION

The imaginal discs of *Drosophila* have been widely used in the study of animal development because they exhibit a wide difference between the time of cell determination and that of the expression of the determined state which results in the formation of the adult cuticle. The embryo is the site of the formation of the discs; the larval instars are the sites of cellular proliferation and the source of the discs for experimentation; and the adult bears the results of such experiments in the form of cuticle markers, either on its surface or on metamorphosed implants in the abdomen; so these phases of development are well known. But the events of metamorphosis have generally received less attention, possibly because of the technical difficulties involved. In the cyclorrhaphous Diptera, including *Drosophila*, the last larval cuticle is tanned and retained throughout metamorphosis as a hard case inside which the developing imaginal hypoderm is at first extremely delicate. In addition, much of the larval structure disintegrates as the various organ systems are histolysed and reconstituted. Nevertheless, 'pupation' has recently attracted attention because during this period the realization of the determined state is achieved.

Methods for staging a continuous process have been developed for other systems, e.g. Bownes (1975), for *Drosophila* embryos; Nieuwkoop & Faber (1967), for *Xenopus* tadpoles. No method exists for *Drosophila* pupae, however, although *timed* animals were employed by Robertson (1936) and Bodenstein (1950) to construct a timecourse of the anatomical changes seen in sectioned material. This takes no account of differences in rate of development between

[1] *Authors' address:* Department of Molecular Biology, University of Edinburgh, Kings Buildings, Mayfield Road, Edinburgh EH9 3JR, Scotland.

individuals and between the sexes. Rate studies on timed animals were reviewed by Ashburner & Thompson (1978). Bliss (1926) noted four staging criteria but found their timing to be erratic. Several authors recorded the effect of a diurnal rhythm upon eclosion and the greater time required by males to reach eclosion (e.g. Powsner, 1935; Kalmus, 1940; Bakker & Nelissen, 1963).

This paper presents a practical system for the staging of living prepupae and pupae and a method for collecting some batches of animals all in the same stage from timed plates without laborious hand sorting. However, it must be emphasized that staging is useful precisely because it eliminates variation due to stock and culture condition differences, and therefore any timings adopted from our data must be checked first and, if necessary, modified accordingly. Where possible our teminology follows that recommended by Fraenkel & Bhaskaran (1973) for cyclorrhaphous dipterans in general.

It is hoped that this staging method will facilitate a precise approach to the study of metamorphosis together with its underlying molecular mechanisms regulating gene expression.

MATERIALS AND METHODS

All observations were made using an Ore-R stock of D. *melanogaster* maintained on yeasted Lewis's medium at 25 ± 0.1 °C and exposed to a 12:12 h day/night cycle until collected for use in this study unless otherwise stated in the text. The colour of the 'yellow body', identified according to Bodenstein (1950), was checked on Instant Drosophila Food (Carolina Biological Supply Co.). The Malpighian tubules are identified according to Wessing & Eichelberg (1978).

(a) *Establishing the sequence of events and some extreme timings*

White prepupae were picked from the sides of culture bottles at random times throughout the day/night cycle and placed in pupation plates consisting of sealed Petri dishes lined with moist filter paper. Collections continued for about 20 min, after which time some individuals had become pale brown and were discarded. Any larvae still able to crawl were also discarded. The plates were stored in the dark and withdrawn for observation at various times throughout metamorphosis. Time zero was therefore puparium formation (P.F.) + 20 min. max. Records of observations were made at 30 min. intervals and the data were pooled. The flies remained fairly synchronous during puparation, becoming less so as pupation proceeded. 256 observations were made of 44 plates, each of which contained about 25 flies. In a separate experiment we determined the viability under these conditions to be 97 % (total 946).

The typical sequence of events (i.e. visible changes) was assembled, together with a range of times at which each event had been seen to occur. The animals were examined on a white background using a Wild dissecting microscope with lateral illumination close to the stage, interposing the tip of a pair of watch-

maker's forceps between the lamp and the puparium to cast a shadow over any feature which may be obscured by surface reflexion but which would show up with light scattered inside the animal – this was particularly true of the Malpighian tubules and the meconium. The puparia were wetted for observation.

(b) Durations of stages by frequency in a population of continuous age distribution

Five mature males and five mature females were placed in each of four fresh culture bottles and kept in constant darkness to encourage continuous egg laying. On days 11–14 mixed populations of prepupae and pupae could be seen on the sides of the bottles. Adult flies were etherized, sexed, staged and counted. Prepupae and pupae were then removed, washed, staged and counted. Staging was performed on a random sample from a bottle's total population and lasted no more than 1 h, because development continued during this time. Living animals were employed since freezing and fixation altered their coloration and other staging characteristics.

(c) The photography

Animals were collected, washed and staged as above (b). The characteristics employed here as staging criteria may be observed through the puparium, but to facilitate the taking of photographs this cuticle is best removed from pupae. The dissections and subsequent photography were performed on animals submerged in 1 % agar in a tissue culture dish. A Wild dissecting microscope was used with a × 50 objective and fitted with a Photoautomat MPS 55 and camera attachment. Illumination was from above using Volpi Intralux Fibre Optics 150H against a dark field.

OBSERVATIONS AND RESULTS

(a) The sequence of visible events during metamorphosis

The following list of 52 numbered events is presented in the order most typical of animals observed under the conditions defined above. Each stage is given both a numerical and a verbal designation, and each is preceded by the change(s) diagnostic of its beginning. Extreme times at which events have been observed to occur in individual animals are given where available (Ext.). Note that these are not extreme stage *durations* from the pooled observations, (c.f. Table 3). Numbered changes 1–47 are indicated on Figs. 1–6 for which the following list constitutes a running legend. A developmental series of photographs of whole animals is shown (Figs. 7–12) and photographic details of staging criteria are given (Figs. 22–31).

Fig. 1   Fig. 2
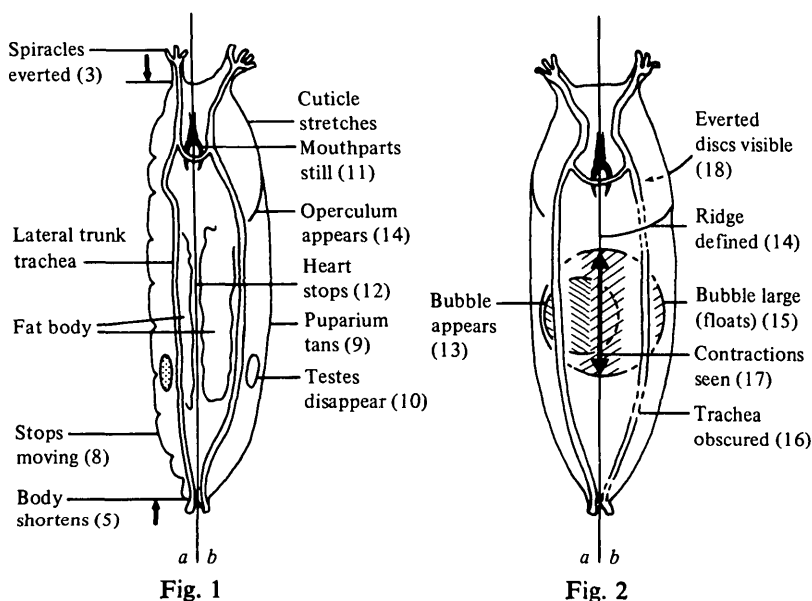
Figs. 1–6. Diagrammatic representation of the visible events observed during meta-morphosis. Segments of individual figures are presented in reading order: *a, b, c, d*. Not all changes are employed as criteria for staging. Numbers in parentheses correspond to the list of visible changes presented in the text. Staging criteria are further illustrated by photographs (Figs. 7–31). Figs. 1–5 are dorsal views, Fig. 6 ventral.

Fig. 1. Stages L1 (Post-feeding) to P2 (Brown puparium). The left side shows half of the un-contracted late third instar larva, drawn the same length as its other half a few hours later, which is shown on the right. Thus the boundary divides the drawing unequally, and the two halves are not to scale.

Fig. 2. Stages P3 (Bubble prepupa) to P4 (Buoyant). The bubble which appears within the abdomen is shown firstly small and often to one side of the animal, and secondly large and visible dorsally. Contracting muscles are seen stretched across the surface of the bubble.

Third-instar larva

(1) Stops feeding, climbs bottle wall:

   Stage L1 (Post-feeding) (Fig. 1)

(2) Stops crawling (reversible until 5);

(3) Everts anterior spiracles:

   Stage L2 (Spiracles everted) (Fig. 1)

(4) Withdraws three apparent anterior segments;

(5) Body shortens;

(6) Sticks to wall of culture bottle:

Prepupa

Stage P1 (White puparium) (Figs. 1, 7)

(7) Posterior spiracles and ridge between anterior spiracles tan orange (Ext. 0–½ h);

(8) Stops wriggling completely (Ext. 1 h);

(9) Puparium becomes brown to the unaided eye (Ext. 20 min–1 h):

Stage P2 (Brown puparium) (Fig. 1)

(10) Male gonads become less distinct; (may still be faintly distinguished in stage P3) (Ext. 0–3 h);

(11) Oral armature stops moving permanently (Ext. 1–5 h);

(12) Dorsal medial abdominal contractions stop, (i.e. heart stops pumping) (Ext. 1–6¼ h);

(13) Gas bubble becomes visible within the abdomen (Ext. 3 h). (It is not known how this bubble forms; but it appears to have tracheal associations when dissected out, and it fails to develop in submerged prepupae):

Stage P3 (Bubble prepupa) (Figs. 2, 22)

(14) Ridge of operculum becomes distinct (Ext. 3–6 h):

Fraenkel & Bhaskaran (1973) place the larval-pupal apolysis at this point (4–6 h); i.e. the puparium becomes separated from the underlying epidermis, starting anteriorly. The resulting stage is termed the *Crypto-cephalic pupa*. (However, see Figs. 24, 25. The movements of gas associated with pupal morphogenesis seem to be involved in the final shedding by the prepupa of the larval endocuticle, and so the larval/pupal apolysis is not *strictly* completed until a few seconds before head eversion (P4 (ii)). The cryptocephalic pupa is then to be regarded as only a transient stage if *physical separation* of successive cuticles is taken into account, i.e. stage P4 (ii) represents pupation.)

(15) Becomes positively buoyant (Ext. 6½–7 h):

Stage P4(i) (Buoyant) (Figs. 2, 23)

(16) Lateral trunk tracheae become obscured (Ext. 6½–8 h);

(17) Dorsal medial abdominal contractions observed, (also ventral; mid-intestinal peristalsis (Robertson, 1936)) (Ext. 9½–13½ h);

(18) Everted leg and wing discs become visible (translucent);

(19) Bubble appears in posterior portion of puparium, displacing the pupa anteriorly, and the bubble within the abdomen disappears (Ext. 12–13½ h):
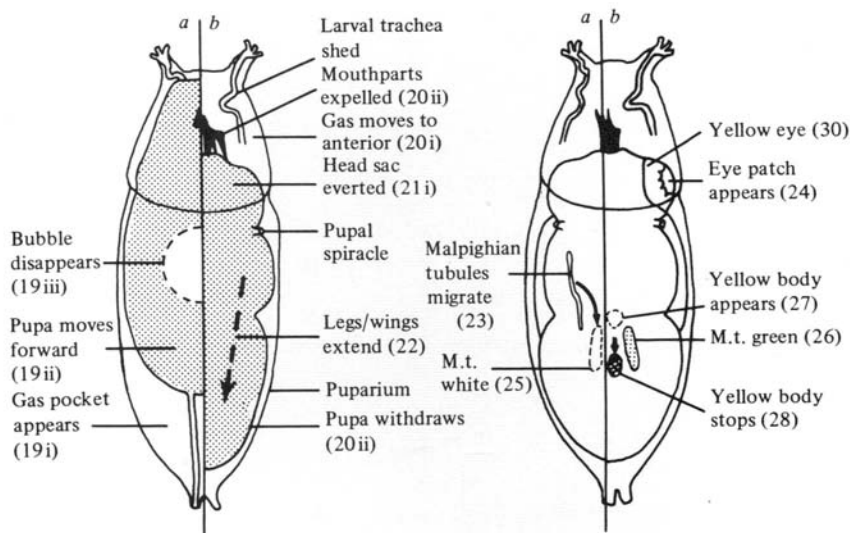
Fig. 3   Fig. 4

Fig. 3. Stages P4 (Moving bubble) to P5 (Malpighian tubules migrating). The pupa is shown in two positions within the puparium, firstly at the anterior end during shedding of the posterior tracheal linings and secondly in its final position after displacement of gas to the anterior end (see also Figs. 24–26) and head eversion. All tracheal connexions with the outside of the puparium are broken. The arrow represents the ventral extension of the appendages by hydrostatic pressure. The pupal spiracle marks the insertion of the lateral trunk trachea in the larva/pupa.

Fig. 4. Stages P5 (Malpighian tubules migrating) to P7 ('Yellow body'). The white enlarged initial segments of the anterior Malpighian tubules (M.t.) are first visible in the thorax but move through the constriction into the abdomen where they may at first be completely obscured. Later, they become faintly visible again and eventually turn green. The 'yellow body' appears at the thorax–abdomen junction as a dark shadow and is most prominent after moving to lie between the M.t.'s.

Stage P4(ii) (*Moving bubble*) (Figs. 3, 24–26)

(20) Bubble is gradually displaced to the anterior portior of the puparium and the pupa withdraws to the posterior end (Ext. 12–13½ h);

(21) Imaginal head sac is everted and the oral armature of the larva is expelled (Ext. 12–13½ h):

Phanerocephalic pupa

Stage P5(i) (*Malpighian tubules migrating*) (Fig. 4)

(22) Legs and wings achieve their full extension along the abdomen (Ext. 12½–13½ h);

(23) Enlarged initial segments of anterior pair of Malpighian tubules move from thorax into abdomen;

(24) Translucent patch lacking adhering fat body cells becomes discernible in the middle of the eye region;

(25) Pair of white Malpighian tubules becomes visible dorsally in the abdomen when viewed in the shadow of forceps (Ext. 15–48 h):

Stage P5(ii) (*White Malpigian tubules*) (Figs. 5–7; Figs. 4, 8–10)

(26) Malpigian tubules become prominent and green (Ext. 13–48 h):

Stage P6 (*Green Malpigian tubules*) (Fig. 4)

(27) Dark green 'yellow body' appears between the anterior ends of the two Malpighian tubule segments, mid-dorsally at the anterior of the abdomen (Ext. 25–46 h):

Stage P7 (' *Yellow body*') (Fig. 4)

(28) The 'yellow body' moves back between the Malpighian tubules:
     Fraenkel & Bhaskaran (1973) place the pupal-adult apolysis at this point (34–50 h), i.e. the transparent pupal cuticle separates from the underlying epidermis, starting posteriorly. The resulting stage is termed the *Pharate adult;*

(29) Eye cup becomes yellow at its perimeter (Ext. 43–47 h):

Stage P8 (*Yellow-eyed*) (Figs. 5, 11, 27)

(30) Pale yellow pigmentation spreads inwards across the eye;
(31) Eye becomes bright yellow;
(32) Eye colour changes to amber (Ext. 49–57 h):

Stage P9 (*Amber*) (Fig. 5, 12)

(33) Eye colour darkens to deep amber;
(34) Eyes become very pale pink (Ext. 71–78 h):

Stage P10 (*Red-eye Bald*) (Fig. 5)

(35) Eyes become bright red;
(36) Orbital and ocellar bristles and vibrissae darken:

Stage P11(i) (*Head bristles*) (Fig. 5)

(37) Dorsal thoracic micro- and macrochaetes become visible (Ext. $72\frac{1}{2}$–77 h):

Stage P11(i) (*Thoracic bristles*) (Figs. 6, 28, 29)

(38) 'Tips' of folded wings become grey:
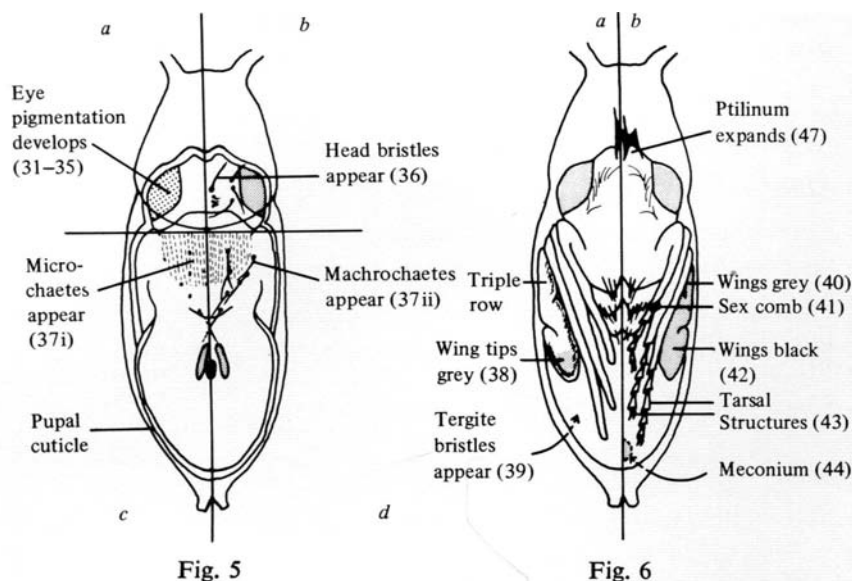
3-2

Fig. 5.

Fig. 6.

Fig. 5. Stages P8 (Yellow-eyed) to P11 (Thoracic bristles). Pigmentation of the eye begins with yellowing of the perimeter. The rest of the eye colours (P8) and later darkens to amber (P9). The perimeter followed by the whole eye becomes pink at the beginning of P10 and this colour darkens to the mature bright red of adults (P10).

Fig. 6. Stages P11 to P15 (Meconium). The 'distal' part of the folded wing blade becomes grey (P12(i)), then the whole blade darkens (P12(ii)). The wings then undergo further darkening to appear black when folded, usually beginning with the 'distal' part again (P13). Tanning of the sex comb in P12(ii) allows one to separate the sexes from now on instead of sexing prepupae. Tarsal claws do not darken until P14 when the legs may also begin to twitch. The meconium appears dorsally as a green patch (P15(i)) which persists until eclosion (P15(ii)). Then, the ptilinum, an extensible sac between the eyes, is expanded by hydrostatic pressure from the contracting abdomen. The operculum breaks open anteriorly, the fly emerges and the ptilinum contracts while the abdomen shortens and widens (A1). The wings are unfurled (A2) and the banding of the abdomen becomes distinct (A3).

Stage P12(i) (Wing tips grey) (Figs. 6, 13–15)

(39) Bristles on abdominal tergites become visible, (Ext. 73–97 h):

(40) Wings become grey (Ext. 73–78 h):

Stage P12(ii) (Wings grey) (Fig. 6)

(41) Sex comb darkens;

(42) Wings darken to black (Ext. 75–86 h):

Stage P13 (Wings black) (Figs. 6, 16–18, 30, 31)

(43) Tarsal bristles darken and claws become black:

Stage P14 (Mature bristles) (Figs. 6, 19, 20)

(44) Green patch (the meconium) appears dorsally at the posterior tip of the abdomen (Ext. 87–103 h):

Stage P15(i) (Meconium) (Figs. 6, 21)

(45) Malpighian tubules and 'yellow body' become obscured by tanning of the tergites (Ext. 90–103 h);
(46) Legs are seen to twitch: flies able to walk if the puparium is removed prematurely (Ext. 90 h);
(47) Ptilinum expands by hydrostatic pressure, opening the operculum (Ext. 93–c. 105 h):

Stage P15(ii) (Eclosion)

(48) Eclosion is completed:

Exarate adult

Stage A1 (Newly eclosed) (Fig. 30)

(49) Wings unfold, flatten and harden;
(50) Abdomen becomes broader:

Stage A2 (Wings extended)

(51) Abdominal tergites tan to shiny brown:

Stage A3 (Tergites tanned)

Two other species of Drosophila were checked for the presence of the stages listed above. *D. simulans* (sibling species) is very similar to *D. melanogaster* except for the site of metamorphosis (on the food) which makes it impossible to collect L1 (post-feeding) as wandering larvae. *D. pseudo-obscura* (obscura group) also shows this character to a lesser extent, but some individuals do move away from the food. However, the diagnostic feature of Stage P7 is lacking in this species since the 'yellow body' is not observed until P8 and even at P9 has still to move into position between the M.t.'s. In both spp. the larval male gonads and the sex comb(s) (two in *D. pseudo-obscura*) are available as criteria for sexing individuals. In general then, the stages of metamorphosis in related species are similar to *D. melanogaster* though their proportional durations may differ.

(b) *Estimation of durations of stages L1–A2 by frequency*

Flies were scored from cultures of four ages, namely 11, 12, 13 and 14 days. When the culture contained both F1 adults in stage A3 and post-feeding larvae (stage L1) it was deemed acceptable. Since laying commenced on day 0 and eclosion on day 10, this composite population approximated to a continuous age distribution, and mean percentage frequencies were then obtained for each
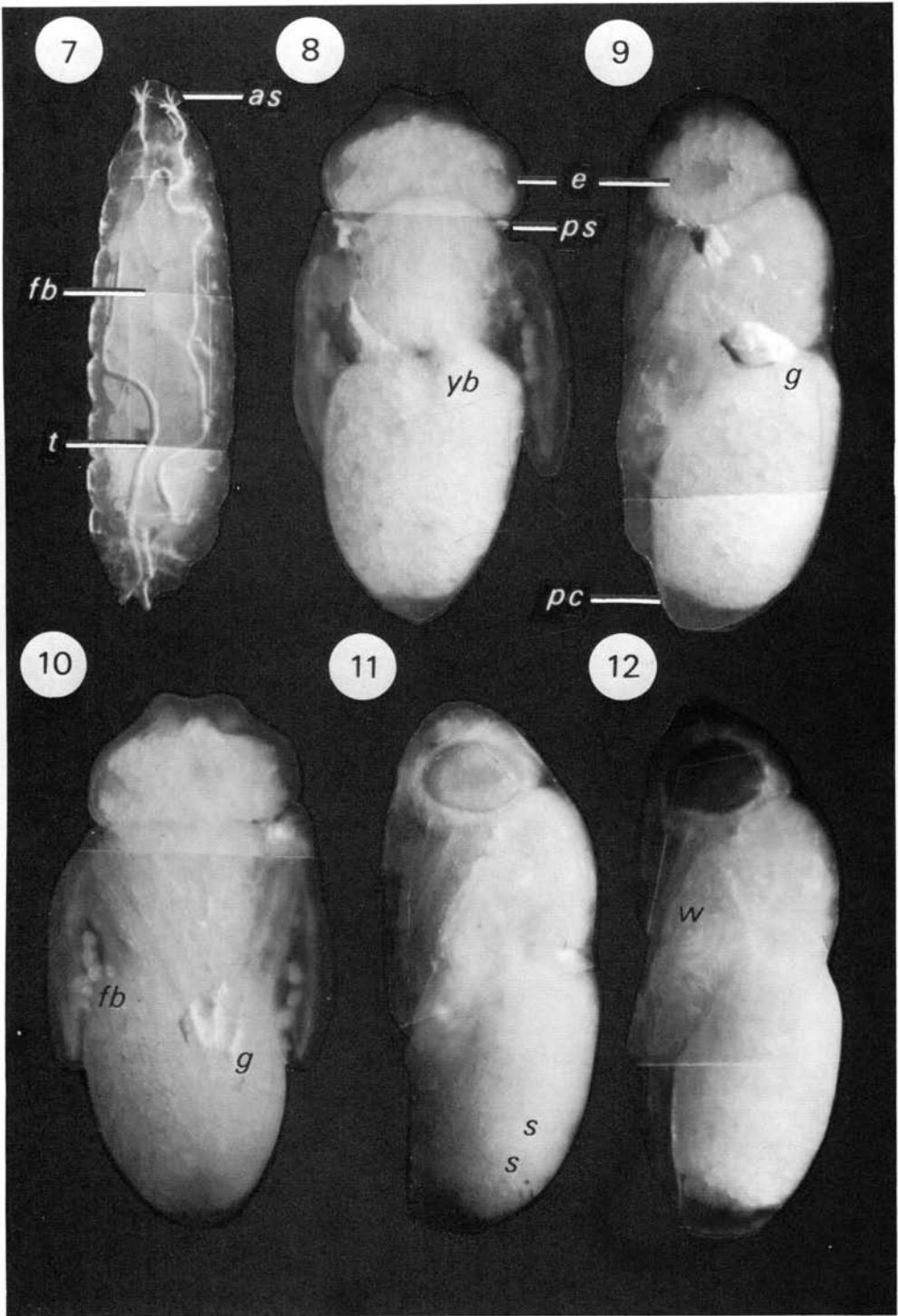
stage within it. Table 1 shows the sampling procedure and the proportions L:P:A involved.

Table 2 shows the observed frequencies of each stage in the various cultures and their calculated means. However, in order to relate the frequencies to a known time-span (P.F. to eclosion), the scores for all stages (L1–A2) are expressed as percentages of the total P1–P15 taken from Table 1 (*). The time taken from P.F. to eclosion was estimated from the observation of pupation plates. The mid-point of the range of eclosion times was about 100 h, that for females being 98 ± 5 h, and that for males 102 ± 4 h, giving a difference of 4 h which is in agreement with that reported by Powsner (1935), i.e. $3 \cdot 7$ h at 25 °C. So for a mixed-sex population the approximate duration of the period P1–P15 may be taken to be 100 ± 7 h. If we now assume that the size of any given stage in a random sample is directly related to the time spent in that stage, then a 1 % stage size indicates a duration for that stage of 1 h. This principle is employed in Table 3 to describe a timecourse for metamorphosis.

### (c) *Times for harvesting batches of symphasic animals*

The timecourse obtained from stage frequencies may be compared with timed extremes of stage durations which have been derived from pupation plate data and are shown in Table 3. For the most part these extremes will lie on either side of the frequency-ranges. Timecourses may also be calculated for males and females separately and these, when compared, reveal periods during which we can expect animals of both sexes to be in the same stage. This comparison is made in Table 3. A dash indicates that no such overlap occurs. We now consider the general timecourse and limit the durations of its component stages using both the extreme timings and the predicted male/female overlaps. In this way we define periods of development during which a high proportion of the animals


Fig. 7. Female of stage P1 (white puparium), dorsal view. Everted anterior spiracle (*as*); fat body (fb); lateral trunk trachea (*t*). Scale smaller then subsequent figures – contraction of the cuticle is incomplete.

Figs. 8–10. Stage P5(ii) (White Malpighian tubules), dorsal, lateral and ventral aspects respectively, after removal of the puparium. Pupal morphogenesis is complete. Fat body cells (*fb*) have entered the wing pouches by hydrostatic pressure and line the hypoderm except in the middle of the eye (*e*). Malpighian tubules must be viewed in shadow at this stage. The first signs of the 'yellow body' (*yb*) are visible here but it will move posteriorly by stage P7. The pupal spiracles (*ps*) were severed from the anterior larval spiracles at head eversion (P4/P5). Pupal cuticle (*pc*); gas pockets (*g*) adhering to *pc*.

Fig. 11. Stage P8 (Yellow-eyed), lateral view. Yellow pigment defines the extent of the compound eye. Abdominal segment margins (*s*) would not be distinct through the puparium.

Fig. 12. Stage P9 (Amber), lateral view. Eye colour has darkened. The wing is visibly folded within its sleeve of pupal cuticle (*w*).
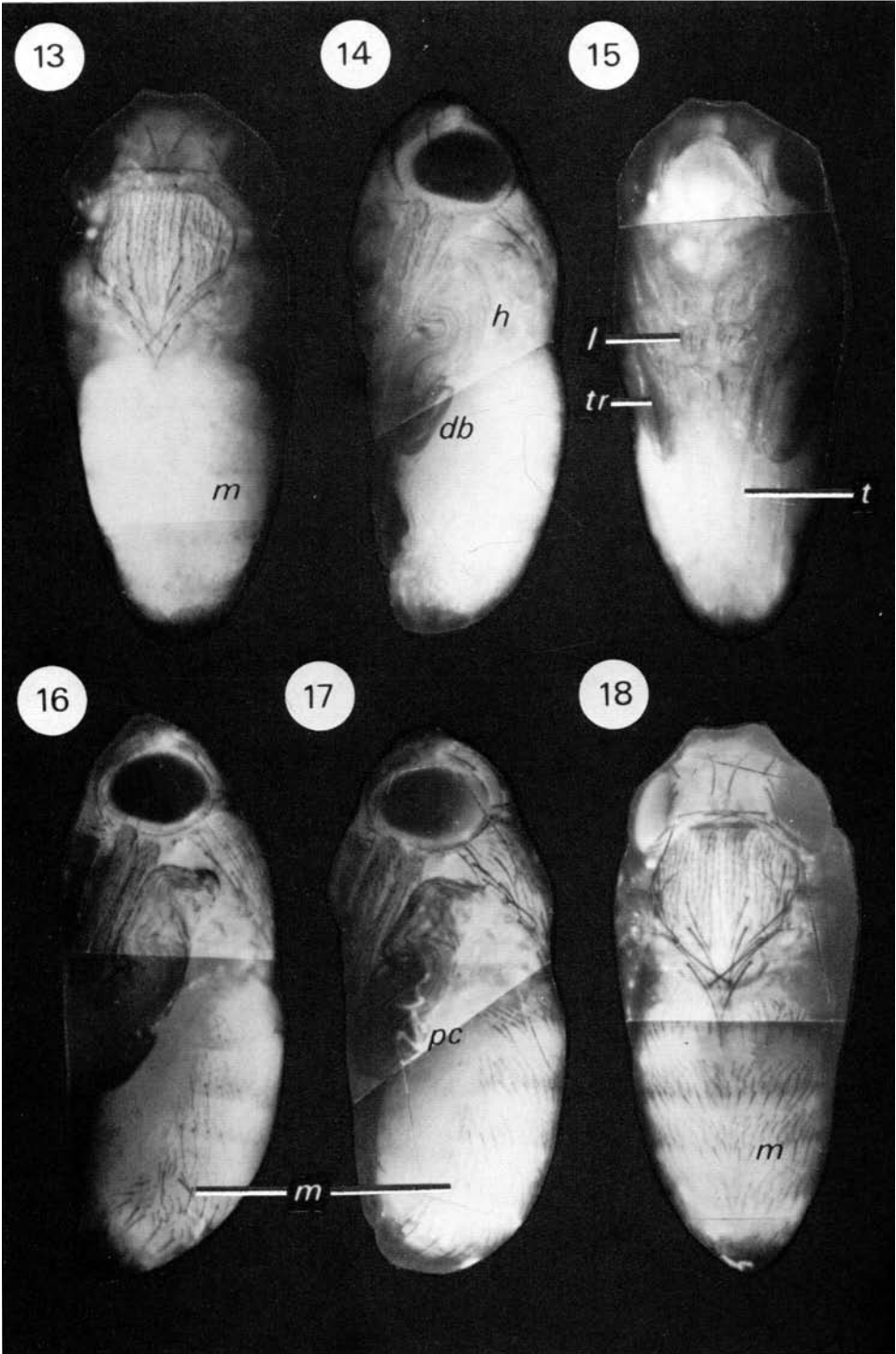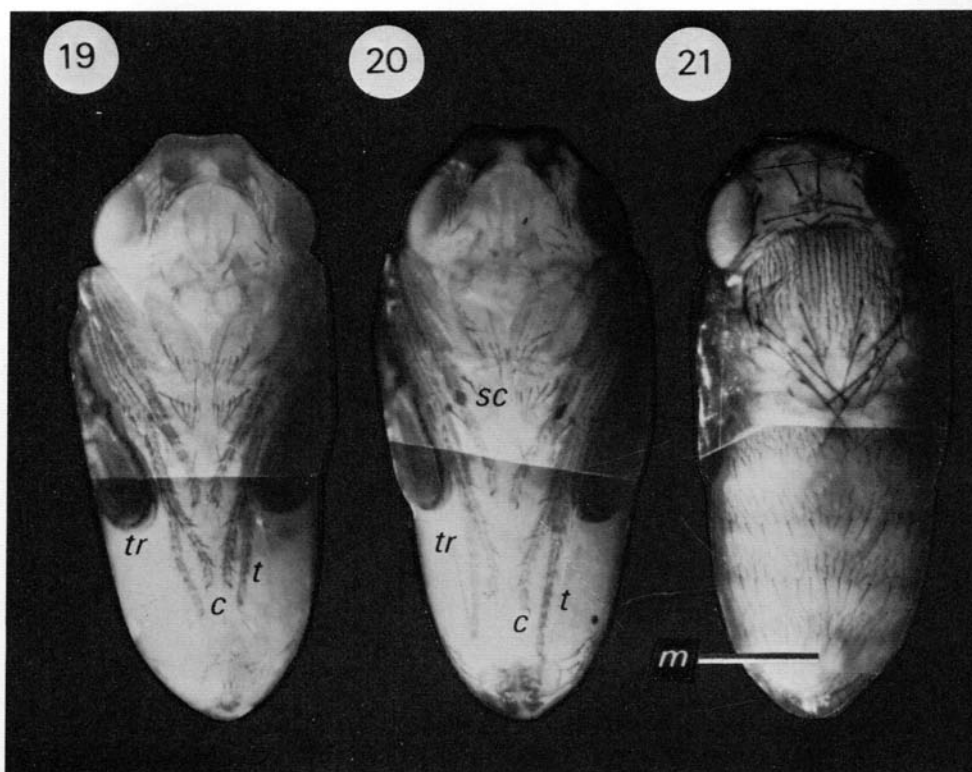
Figs. 19 and 20. Stage P14 (Mature bristles), female and male ventral aspects respectively. Tarsal bristles (*t*), claws (*c*), sex comb (*sc*) and triple row (*tr*) are black. The genital disc derivatives are obscured by the posterior spiracular papillae until the puparium is removed.

Fig. 21. Stage P15(i) (Meconium), dorsal view. The meconium (*m*) is a pale green patch. It will be expelled soon after eclosion (A1) or sometimes inside the puparium during eclosion (P15(ii)). It is composed of fluid derived from the 'yellow body' (Robertson, 1936).

Figs. 13–15. Stage P12(i) (Wing tips grey), dorsal, lateral and ventral views respectively. Macrochaetes along the posterior margins of the abdominal tergites are beginning to darken (*m*). The wing hinge region (*h*) is untanned while the distal blade has darkened (*db*). Triple row (*tr*); dark proximal leg bristles (*l*); untanned tarsi (t).

Fig. 16. Early stage P13 (Wings black), lateral view. The wing has darkened further. Macrochaetes of tergites 2 to 6 (*m*).

Figs. 17 and 18. Late stage P13 (Wings black), lateral and dorsal views respectively. The whole wing is deeply tanned. After the pupal-adult apolysis, the degradation of the pupal cuticle (*pc*) continues. Here it has torn during removal of the puparium. At eclosion it is left inside the puparium. The Malpighian tubules and yellow body are no longer prominent. Tergite macrochaetes are very dark (*m*).
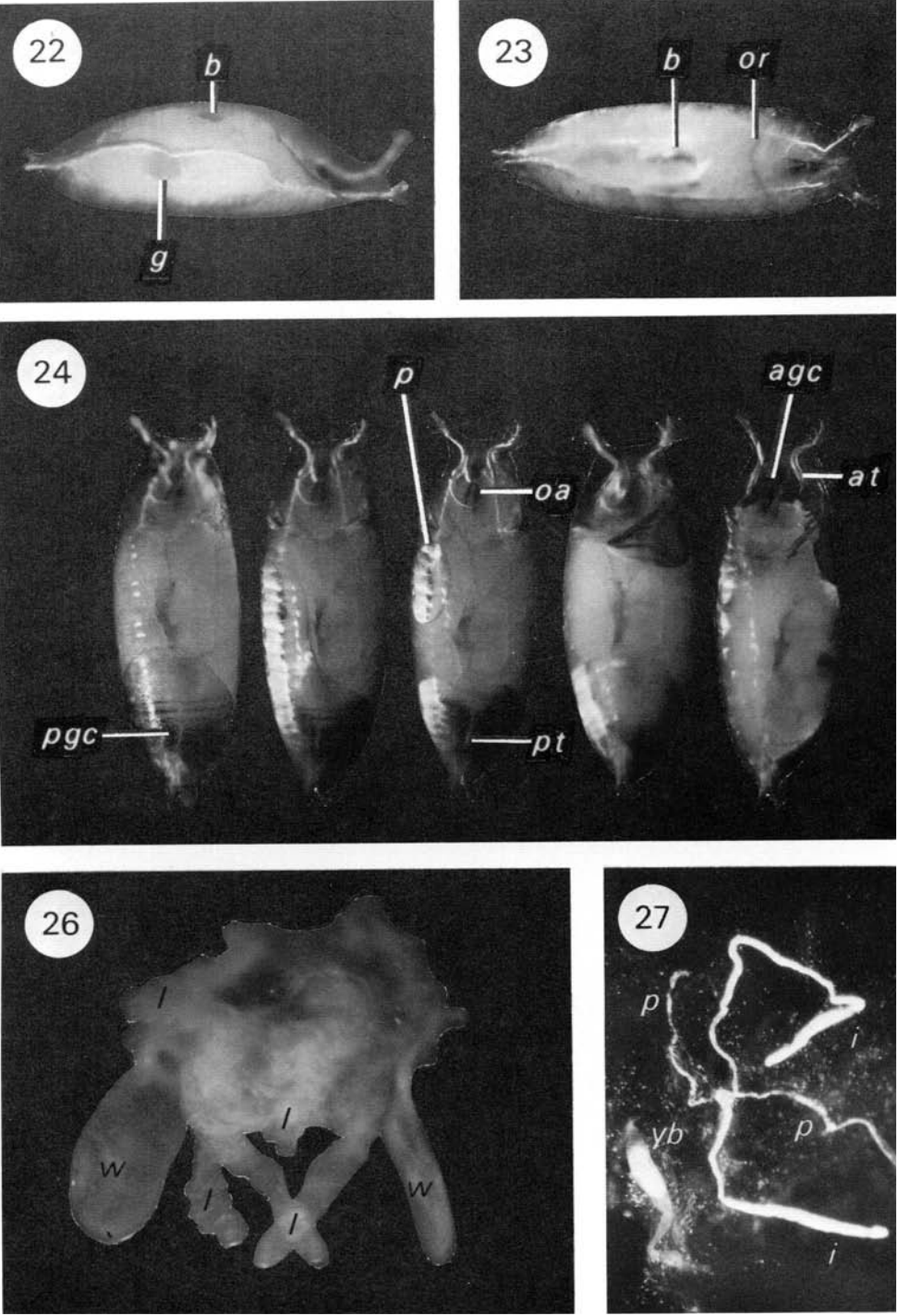
collected together at P.F. may be expected to be in the same stage, i.e. symphasic. Such 'safe' collection periods are indicated at Fig. 32.

Note that some stages cannot be obtained homogeneously by timing alone, (e.g. P6); and that in any case no sub-stages (i or ii) may be reliably obtained without there first being a visual check performed. So large symphasic batches of animals may be collected by plating prepupae at the beginning of P1, maintaining them at 25 °C in continuous darkness, and harvesting stages P2–P14 at the times shown. It must be emphasized, however, that these absolute times may vary between stocks, so, at least initially, batches must be checked and times modified if necessary. It is often convenient to segregate males and females as larvae or prepupae and time them separately, but still hand-sorting is required for the short stages P11(i)–P13. Note also that after P2 no sexing of flies may be done until P12(ii), though the females are generally larger than the males, so we recommend prior sexing of prepupae in stage P1 for experimental analysis of these stages. This is best done with wet animals on black filter paper. Some difficulty may be encountered at first in drawing the line between two stages, e.g. P8 (Yellow-eyed) and P9 (Amber). To avoid ambiguity in the resulting batches it is best to sort the plate into three sub-groups and discard the animals of intermediate colour. Subdivision of stages may also be performed given a large enough number of flies by employing an internal standard within a given

Fig. 22. The appearance of the translucent male gonads (g) of the larva/prepupa, seen here still visible in Stage P3 (Bubble prepupa) and distinct from the gas bubble (b). The female gonads are indistinguishable from fat body. Lateral view.

Fig. 23. The gas bubble (b) in an animal from Stage P4(i) (Buoyant), lying between and beneath the lobes of the fat body. The lateral trunk tracheae are still intact and, since head eversion has yet to occur, the pupa is cryptocephalic. Opercular ridge (*or*). Dorsal view.

Figs. 24–26 show pupal morphogenesis of the head and thorax in Stage P4(ii) (Moving bubble).

Fig. 24. Unequal time-lapse series of gas bubble movements within the puparium, and head eversion. Dorsal view. The first three photographs represent 1 min, and the whole process takes from 2–10 mins. Note the absence of a gas bubble. Posterior gas chamber (*pgc*); posterior tracheal lining (*pt*); gas pocket (*p*); larval oval armature (*oa*); anterior tracheal lining (*at*); anterior gas chamber (*agc*).

Fig. 26. The state of the thoracic imaginal discs towards the end of pupal morphogenesis, Stage P4(ii). The complex of fused discs with its continuous layer of pupal cuticle has been dissected out of the puparium. Evertion of legs (*l*) and wings (*w*) by hydrostatic pressure is incomplete. In the intact pupa, the anterior region is translucent while the abdominal hypoderm, of larval origin, is opaque.

Fig. 27. Displayed visceral dissection of the pupa shown at Fig. 11 to show the Malpighian tubules and 'yellow body' in Stage P8 (Yellow-eyed). Dissection performed in 1 % agar. Enlarged initial segments (*i*) of the anterior pair (*p*) of Malpighian tubules join the more slender posterior pair which are invisible in the intact animal. The 'yellow body' (*yb*) is the imaginal mid-intestine containing the larval mid-intestine, gastric caecae and proventriculus (Bodenstein, 1950).
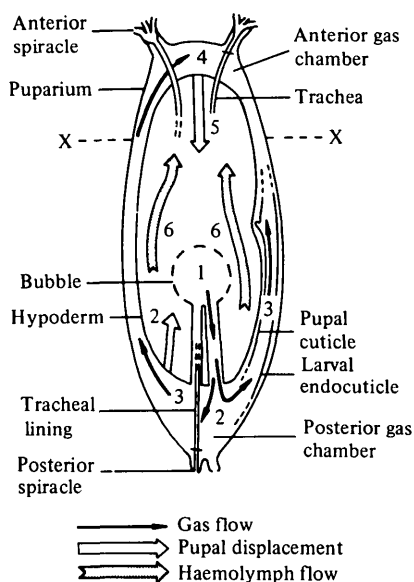
Fig. 25. Diagrammatic interpretation of the morphogenetic events shown at Fig. 24. Numbered arrows represent the sequence of events. (1) The bubble within the animal disappears as (2) the posterior gas chamber develops inside the puparium and the animal moves accordingly toward the anterior spiracles. As a result, the posterior linings of the lateral trunk tracheae are stretched across the chamber and so are shed (Robertson, 1936; Whitten, 1957a, b). Tracheal connexions are schematic, and gas may also exit through more anterior abdominal ecdysial openings (Whitten, 1957a). (3) The gas is shunted forward by slow wriggling using the larval abdominal muscles. Small pockets of gas pass between the pupal cuticle and the larval endocuticle or lining of the puparium (formerly called the 'prepupal cuticle') (Robertson, 1936; Poodry & Schneiderman, 1970), thus effecting the final physical step in the larval/pupal apolysis. The pupal cuticle is already continuous and rather tough. (4) The increase in size of the anterior gas chamber is accompanied by the backward-displacement of the pupa (5) and the withdrawal of the anterior tracheal linings. These and subsequent changes are independent of all the spiracles (Robertson, 1936) and the pupa now has no tracheal connexions with the puparium. Pupal spiracles mark the sites of connexions to the larval anterior spiracular papillae, (see Fig. 8). (6) By sudden muscular contractions, haemolymph and dissociated larval fat body is pumped into the folded head sac which then everts into the anterior gas chamber. The larval oral armature is expelled and lies ventrally inside the puparium. X-X marks the level of the antennal hypoderm within the puparium. The leg and wing discs, which are already evaginated, evert more slowly and extend along the abdomen during stage P5(i) to reach their pupal positions (see Fig. 10).

plate: e.g. 'early P9' may be collected when most of the members of the plate are still in P8, and similarly 'late P8' when most are already in P9. With practice, colour sorting may become sufficiently reproducible, though subjective, to allow of the collection of 'mid-stage x' in addition to 'early' and 'late' by sub-sorting of a plate. It is not convenient, however, to *sub-time* within a given stage, due to the large number of P1s required to yield a small group of synchronous animals later on.

DISCUSSION

The narrowness of the 'safe' periods (Fig. 32) where all flies are in the same stage for a period of time confirms that staging rather than timing is essential to studies of *Drosophila* metamorphosis since the members of a timed batch are unlikely to fall unequivocally into one developmental stage. It is possible that the extreme timings are partly due to the effect of the diurnal rhythm, as the prepupae were withdrawn from a day/night cycle and had therefore pupariated under these conditions. It seems likely, however, that the diurnal rhythm induces a block in the latter part of pupation: thus some flies eclose before the meconium becomes visible, while others retain this character but do not eclose for some time. But certainly the difference between males and females is established early, to the extent that, if pupae are segregated on the basis of early or late accession to P5 (i.e. time of head eversion), then they are also segregated sexually with an accuracy of about 70 %. The effects of the day/night cycle are decreased in the estimation of stage durations by frequency, as both oviposition and P.F. took place in the dark to avoid gating.

It is interesting to note that, whilst the timing and frequency data generally correspond, there is one short stage, that of bubble displacement and head eversion (P4(ii)) which is exceptional (see *Table 3). On the basis of frequency the duration of this stage is found to be 0·2 h, i.e. 12 min, which agrees with several observations of the process in action. It is predicted to begin at 8 h post-P.F., and repeated timings have shown P4(ii) to begin no earlier than 12 h at 25 °C. However, this is of no practical significance since P4(ii) can be most conveniently collected at 18 °C, 24–25 h after P.F. Here we assume that morphogenetic and other events employed in staging are not unrelated to the underlying biochemistry and so will not be uncoupled from the metabolism of the organism when metamorphosis occurs at other temperatures. Should the assumption prove to be justified, we may exploit other temperatures to obtain all stages at convenient times of day. Table 4 shows factors by which durations P1–15 at 18, 22 and 29 °C are shown in the literature to differ from that at 25 °C, (Ludwig & Cable, 1933; Powsner, 1935; Davidson, 1944). If we further assume that the proportions of time devoted to the various stages will remain constant, then these factors may be used, together with Table 3, to predict collection times for stages at other temperatures if these are more convenient; but now hand sorting becomes more than ever essential.

The abdominal bristles are found to tan unreliably in stage P12(i), and they have been observed to appear asynchronously across the abdomen, e.g. they appeared (tanned) first across the second tergite, then along the flanks of the abdomen, then dorsally. This may be related to Trepte's observations (1980) on *Sarcophaga barbata*, where the chromosome puffing pattern of bristle cells and the associated process of bristle differentiation were found to be autonomous in different regions of the body surface. In *Drosophila* the tanning of these
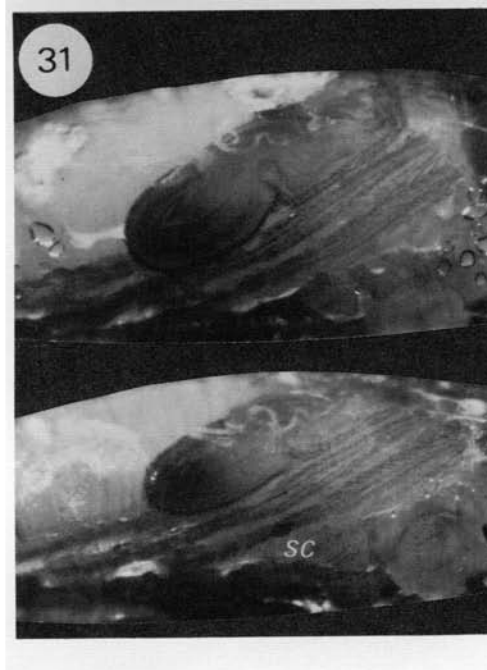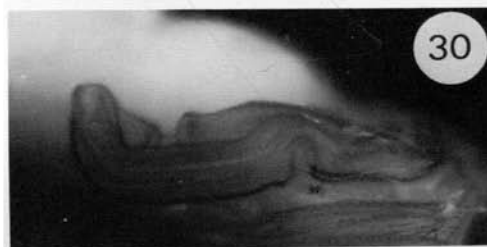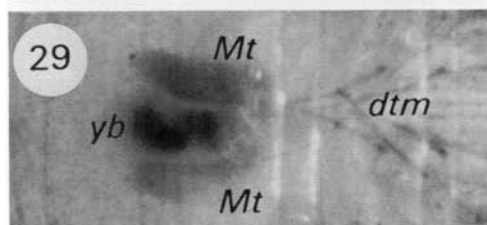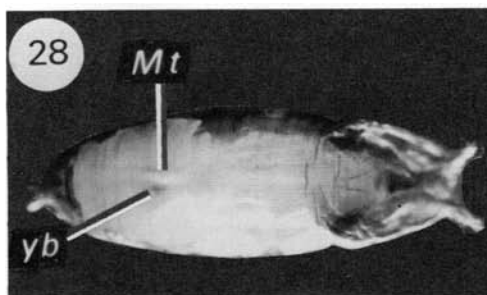
Table 1. *Sampling procedure*

Days after oviposition begins:			11	12	13	14	Mean
L1–A3 (total = 2286)			367	688	373	858	571·5
Sample size (= 100 %)			159	140	196	119	
Classes within sample	L %		3·1	5·7	2·5	1·7	3·25 %
	P %		93·1	88·6	71·5	44·5	74·4 %
	(Total*)		(148)	(124)	(140)	(53)	
	A %	♀	3·2	2·9	18·4	30·3	13·7 %
		♂	0·6	2·8	7·6	23·5	8·6 %
		Total	3·8	5·7	26·0	53·8	22·3 %

* These values become 100 % in Table 2, q.v.

bristles cannot be employed as a strict diagnostic feature for staging. The tanning of the tarsi is less variable but not unique to stage P13, although the tanning in the leg does appear to follow a sequence in which the tarsi are tanned last. This is in contrast to the order of acquisition of differentiative competence in the leg disc, (distoproximal to medial; Schubiger, 1974) and the sequence of bristle differentiation in the pupal leg, (distal to proximal; authors' observations).

The staging system presented in this paper constitutes a more precise approach to the problems of insect metamorphosis than has previously been available to *Drosophila* workers, who have used timed batches of animals, often of mixed sexes. The visible changes described here presumably do reflect the underlying metabolic state of the animal, so symphasic individuals may be assumed to be reasonably synchronous in metabolic terms, though they may well be of widely differing chronological ages. The hormonal basis of morphogenesis and differentiation could now be readily investigated by assaying hormone titres of the various stages to produce a profile for each sex (in preparation). Protein synthesis may be examined, e.g. in the tanning of cuticle and in those tissues – brain,

Fig. 28. The relationship between the Malpighian tubules (*Mt*) and the 'yellow body' (*yb*) *in situ*. Dorsal view, stage P11(ii) (Thoracic bristles). These structures are by now distinctly green. Note also the orbital and ocellar bristles, and the dark (i.e. red) eyes. Reflexions are due to gas trapped beneath the puparium.

Fig. 29. The mature relationship between the green Malpighian tubules (*mt*) and the almost black 'yellow body' (*yb*) in their most prominent condition in a stage P11(ii) animal before they begin to be obscured. Dorsal thoracic macrochaetes (*dtm*).

Fig. 30. The folding of the imaginal wing before eclosion. The puparium and pupal cuticle of the pharate adult shown at Fig. 16 (Stage P13 (Wings black)) have been removed, allowing the wing blade to unfurl prematurely. Compare Fig. 31.

Fig. 31. Comparison of the sexes after the darkening of the male-specific sex comb (*sc*) in stage P12(ii). Ventrolateral views, early stage P13 (Wings black). The wings are blackening distoproximally. Droplets of water line the puparium.

Table 2. *Frequencies of stages L1–A2, expressed as percentages of total P (i.e. P1–P15; see Table 1*)*

Day after oviposition begins:		11	12	13	14	Mean %
Total P		148	124	140	53	
Stage:						
L1	(Post-feeding)	2·0	5·6	3·6	3·8	3·8
L2	(Spiracles everted)	1·4	0·8	0	0	0·5
P1	(White puparium)	0	0·8	2·1	3·8	1·7
P2	(Brown puparium)	1·4	3·2	2·1	5·7	3·1
P3	(Bubble prepupa)	0·7	2·4	0·7	0	1·0
P4(i)	(Buoyant)	3·4	1·6	4·3	0	2·2
P4(ii)	(Moving bubble)	0	0	0·7	0	0·2
P4		3·4	1·6	5·0	0	2·4
P5(i)	(M.T. migrating)	0·7	0	0	3·8	1·1
P5(ii)	(White M.T.)	8·8	7·3	12·1	9·4	9·4
P5		9·5	7·3	12·1	13·2	10·5
P6	(Green M.T.)	14·9	27·4	10·7	1·9	13·7
P7	('Yellow body')	23·6	16·1	2·1	5·7	11·9
P8	(Yellow-eyed)	20·3	19·4	5·7	3·8	12·3
P9	(Amber)	17·6	12·9	14·3	11·3	14·0
P10	(Red eye bald)	0·7	0·8	1·4	13·2	4·0
P11(i)	(Head bristles)	0	0·8	0·7	3·8	1·3
P11(ii)	(Thoracic bristles)	0	0	0·7	1·9	0·7
P11		0	0·8	1·4	5·7	2·0
P12(i)	(Wing tips grey)	0	0·8	0·7	1·9	0·9
P12(ii)	(Wings grey)	0·7	1·6	2·1	0	1·1
P12		0·7	2·4	2·8	1·9	2·0
P13	(Wings black)	2·7	0	2·8	7·6	3·3
P14	(Mature bristles)	4·1	2·4	15·0	16·9	9·6
P15(i)	(Meconium)	0·7	1·6	20·7	9·4	8·1
P15(ii)	(Eclosion)	0	0·8	0·7	0	0·4
P15		0·7	2·4	21·4	9·4	8·5
A1	(Newly eclosed)	0	0	4·2	3·8	2·0
A2	(Wings extended)	0	0·8	8·6	45·3	13·7
					Total P =	100 %

fat body and Malpighian tubules – which survive metamorphosis. Finally, the involvement of hormones and other factors in the control of gene expression may be studied in relation to the secretion of both pupal and adult cuticles, the latter having well-characterized surface markers whose sequential appearance may be followed in successive stages. The clustering of stages at P10–P12 is fortunate since by this time asynchrony is most significant.

Table 3. *Timecourse of metamorphosis estimated by stage frequencies*

| P1–P15 =	100 h		(Timed extremes (h) for comparison)	98 h (♀) Cumulative percentages Stage ends (h)	102 h (♂) Cumulative percentages Stage ends (h)	∴ ♀/♂ overlap (h)
	% ≡ h	Cumulative percentages ≡ timecourse				
Stage:						
(L.III)		−4.3		−4.2	−4.4	
L1	3.8	−0.5		−0.5	−0.5	
L2	0.5	0		0	0	
P1	1.7	+1.7		+1.6	+1.7	0–1.6
P2	3.1	4.8	⅓–3	4.6	4.9	1.7–4.6
P3	1.0	5.8	3–7	5.6	5.9	4.9–5.6
P4(i)	2.2	8.0		7.8	8.2	5.9–7.8
P4(ii)	0.2	8.2*	12–13½*	8.0	8.4	—
P4	2.4	8.2	6½–13½	8.0	8.4	5.9–8.0
P5(i)	1.1	9.3		9.1	9.5	8.4–9.1
P5(ii)	9.4	18.7		18.3	19.1	9.5–18.3
P5	10.5	18.7	12½–48	18.3	19.1	8.4–18.3
P6	13.7	32.4	13–46	31.7	33.1	19.1–31.7
P7	11.9	44.3	25–47	43.4	45.2	33.1–43.4
P8	12.3	56.6	43–57	55.5	57.7	45.2–55.5
P9	14.0	70.6	49–78	69.2	72.0	57.7–69.2
P10	4.0	74.6	71–77	73.1	76.1	72.0–73.1
P11(i)	1.3	75.9		74.4	77.4	—
P11(ii)	0.7	76.6	72½–	75.1	78.1	—
P11	2.0	76.6		75.1	78.1	—
P12(i)	0.9	77.5		76.0	79.0	—
P12(ii)	1.1	78.6	73–	77.1	80.1	—
P12	2.0	78.6		77.1	80.1	—
P13	3.3	81.9		80.3	83.5	(80.1–80.3)
P14	9.6	91.5		89.7	93.3	83.5–89.7
P15(i)	8.1	99.6	87–(100+)	97.6	101.6	93.3–97.6
P15(ii)	0.4	100.0	93–106	98.0	102.0	—
P15	8.5	100.0		98.0	102.0	93.3–98.0
A1	2.0	102.0		100.0	104.0	
A2	13.7	115.7		113.4	118.0	104–113.4

* See Discussion.
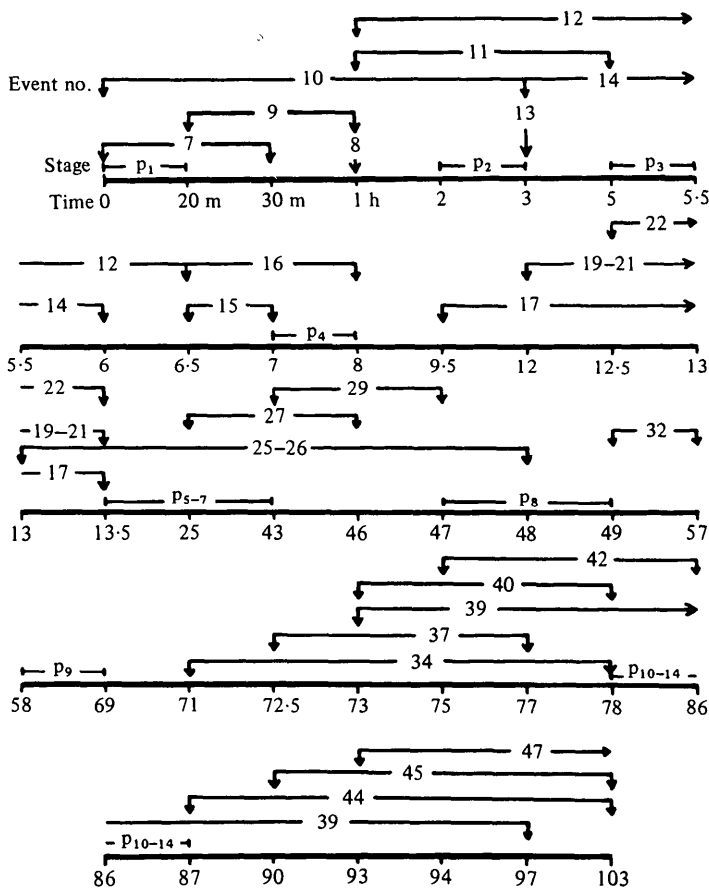
Fig. 32. Suggested periods for symphasic batch collections. Extreme times for numbered visible changes during metamorphosis are displayed against a non-linear time scale. The numbers refer to the order in the text, and arrows indicate the ranges over which these events were seen to occur in the pooled observations of pupation plates (mixed males and females). Where there is no overlap between staging criteria, 'safe' periods are predicted for the collection of homogeneous batches of animals, and these are restricted further where appropriate by the estimated male and female timecourses calculated from stage frequencies (Tables 2 and 3), to give bands of time when asynchronous males and females might be expected to be largely of the same appearance, i.e. symphasic. Due to the clustering of stages beyond P10 and the increasing asynchronization of the animals, this is impossible for a given stage beyond P9. Thus a visual check is always required, (see text) and stages P5-7, 10-15 must be hand-sorted from non-uniform batches. Some events are not respected, e.g. eye colour (no. 29) supersedes the appearance of the Malpighian tubules (nos. 25/26) in predicting entry into stage P8. Note that strictly this chart may not be used to age untimed pupae, which may very well lie beyond the safe periods chronologically.

Table 4. *Durations of period from P1–P15 inclusive at four temperatures, estimated from the literature on timing of metamorphosis*

Temperature (°C)	Factor	Estimated durations (h)	
		Female	Male
		(our conditions)	(our conditions)
25	× 1	98	102
	(reported)	(calculated)	(calculated)
18	× 2·07	203*	211*
22	× 1·31	128	133
29	× 0·79	77*	80*

* These eclosion times are in rough agreement with our observations of eclosion at these temperatures.

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