

## THE EFFECT OF A BIOLOGICAL CLOCK ON THE DEVELOPMENTAL RATE OF *DROSOPHILA* PUPAE

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### INTRODUCTION

During the last 15 years many of the studies on diurnal, or circadian, rhythms have centred on the eclosion rhythm of *Drosophila*. In this insect the majority of adults emerge from the pupal case over a period of 3 or 4 hr. during the light phase of a light:darkness cycle.

Pittendrigh (1954) suggests that eclosion is strongly inhibited during the dark period and that at dawn this inhibition is removed. That is, he suggests that pupal development may be completed at any hour of the night, but as the actual eclosion cannot take place until dawn a large number of flies will emerge at the one time.

There does not, however, appear to be any evidence that pupal development is in fact completed at all times of the night, and furthermore, it is very difficult to apply the Pittendrigh interpretation to strains of *Drosophila* which exhibit dual emergence peaks. A study of the course of pupal development has therefore been made in an attempt to establish the factors affecting the time of eclosion.

### METHODS

Two strains of *Drosophila melanogaster* were used, a wild-type, hereafter designated 'wild', and a mutant strain 'straw' obtained from the Department of Genetics, Cambridge University.

The two strains differ in the time at which adult eclosion occurs when the insects are kept in a 12 hr. light:12 hr. darkness cycle. The strain 'straw' shows only one major peak of eclosion, at the beginning of the light period, whereas 'wild' shows two major peaks, one at the beginning and one towards the end of the light period.

Adults were allowed to oviposit, over a period of 24 hr., in culture bottles containing a standard amount of food medium. Towards the end of the third instar the larvae were sexed and only males were used in the present observations. In every case the original sexing was checked by finally sexing the adults; females were occasionally found to have been included. It was not possible to tell from which pupae such females had come, but no difference was found in the results obtained from these samples and those from entirely male samples.

As the larvae underwent the final moult, or shortly afterwards, the resultant prepupae were placed on damp filter-paper in Petri dishes. The filter-paper had previously been marked out in small numbered squares so that the progress of individual pupae could be recorded throughout their development. The Petri dishes and culture

bottles were kept in cabinets in which the light cycle could be controlled, and in which the temperature was maintained at 26° C.

Larvae and pupae were treated in one of four different ways:

(1) Larvae and pupae were kept in 12 hr. bright light:12 hr. dim light from the time of hatching until the eclosion of the adult.

(2) Larvae were kept in 12 hr. bright light:12 hr. dim light until the last larval moult, and were then transferred to continuous darkness. Batches were withdrawn every hour, without light being admitted to the remaining samples. The withdrawn samples were discarded after examination.

(3) Larvae and pupae were kept in 12 hr. light:12 hr. darkness. Samples were observed *in situ* during the light period, but during the dark period samples were withdrawn and discarded after examination as above.

(4) Larvae were kept in 12 hr. light:12 hr. darkness until the last larval moult, and were then kept in continuous darkness and treated as in (2) above.

In all cases observations were made every hour, and the pupae were observed *in situ*, with the aid of a hand-lens, whenever the lights were on in the cabinets. The cabinets opened at the top, with the lights on the side walls, so that pupae could be observed without moving them relative to the lights.

In order to measure the progress of development of the pupae a number of 'markers' were selected which could be recognized with the aid of a hand-lens, even in the dim light of the dim light:bright light cycle. Four of the 'markers' proved to be useful in these studies; they are (a) head eversion, (b) the appearance of yellow eye-pigmentation, (c) the beginning of pigmentation of the costal region of the wing, (d) the eclosion of the adult. A fifth 'marker', the beginning of red eye-pigmentation was used in a few studies. Each of the first four 'markers' represents the beginning or end of a 'stage' of development.

The markers which involve colour recognition, that is eye and wing pigmentation, introduce an element of subjective recognition, although they are both very clear. Precautions were taken against possible bias in their recognition by the use of mixed and coded samples in which between 70 and 100 individual pupae, all at different stages of development, were being observed at the same time. There was no possibility of the observer remembering, over the period of observation (several days), in which stage each individual had been in any preceding hour, let alone at what time it had reached the beginning of that stage, which might have been any time up to 54 hr. earlier.

The time taken to complete each stage was measured for pupae entering the stage at each particular hour of the day; for each hour of entry at least 45 pupae were included in the sample. In all, more than 8000 pupae were observed over the 2 years in which the experiments were carried out.

## RESULTS

### *Developmental rates in strain 'straw'*

I. *Duration of the stage beginning with head eversion and ending with the appearance of yellow eye coloration.*

(a) *Larvae and pupae in 12 hr. bright light:12 hr. dim light.* The results are summarized in Fig. 1; in this figure the time of day at which each sample (containing at

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least 50 pupae) entered the stage is plotted against the number of hours taken to complete the stage. It will be noticed that within nearly all the samples two distinct developmental periods occur, but that the variation about these peaks is limited to  $\pm 1$  hr. (with the exception of line L + 5 which will be discussed later).

The appearance of two distinct developmental periods can be related to the method of observation; one example will be considered in detail. The majority (70%) of the

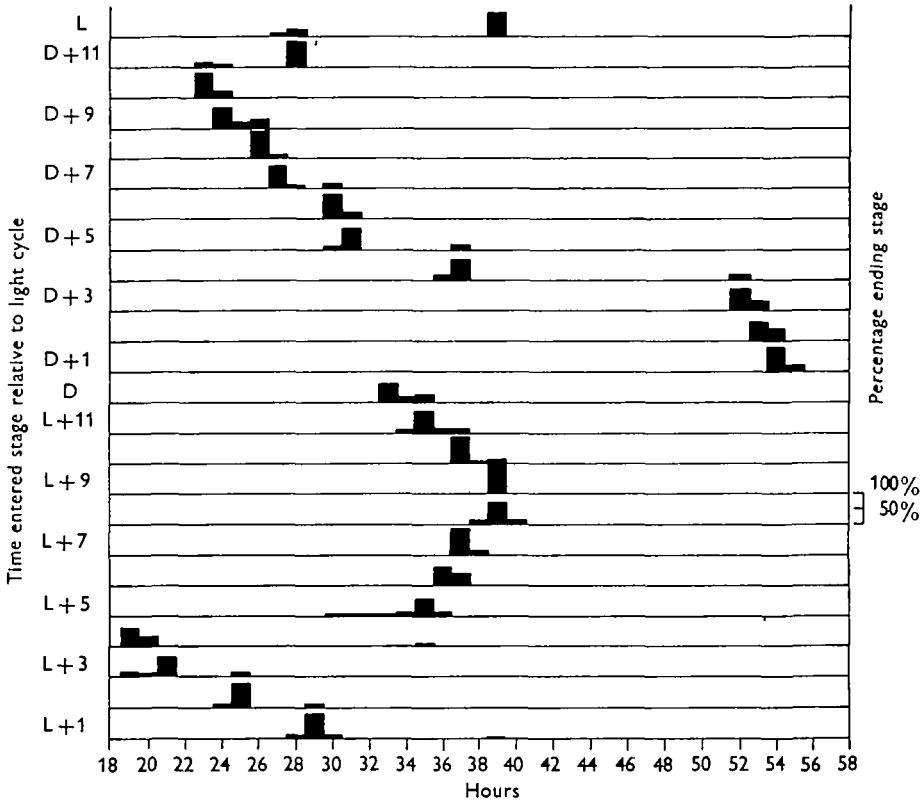


Fig. 1. The number of hours taken by pupae of the strain 'straw', to complete the stage 'head eversion to yellow eye' when pupae enter the stage at different times in a 12 hr. bright light: 12 hr. dim light cycle. The letters on the ordinate, D + 5, L + 5, etc., indicate the number of hours after the dim light (D) or bright light (L) was switched on.

pupae recorded as having entered the stage 5 hr. after the beginning of darkness (line D + 5) took 31 hr. to complete the stage; the majority (66%) of the pupae recorded as having entered the stage an hour earlier (line D + 4) took 37 hr. to complete the stage. Unless, by chance, the time of observation coincided precisely with the time of the change in developmental rate it would be expected that some of the pupae observed entering the stage at D + 5 hr. would show the developmental period typical of the D + 4 samples: the results show that 18% of the D + 5 sample took 37 hr. to complete the stage.

In the line D + 5 it will also be noticed that 12% of the pupae took 30 hr. to complete the stage, 1 hr. less than the majority. The variation of 1 hr. about the peaks can also be predicted as a consequence of the method of observation. That pupae taking

exactly the same length of time to complete the stage could be recorded as having taken either 30 or 31 hr. may be seen by reference to the theoretical case illustrated in Fig. 2. In this example pupa A enters the stage 10 min. after the observation at D + 4 hr., and is therefore not recorded until D + 5 hr., whereas pupa B enters the stage just before the observation at D + 5 hr. and is therefore judged to have entered the stage

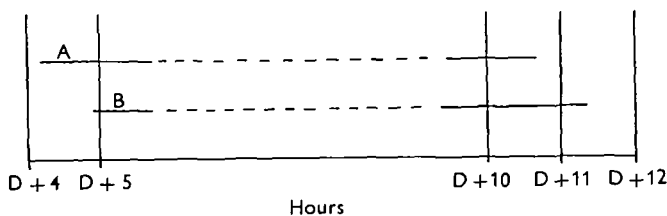


Fig. 2. Theoretical example showing how two pupae with identical developmental periods could be recorded as differing by 1 hr.

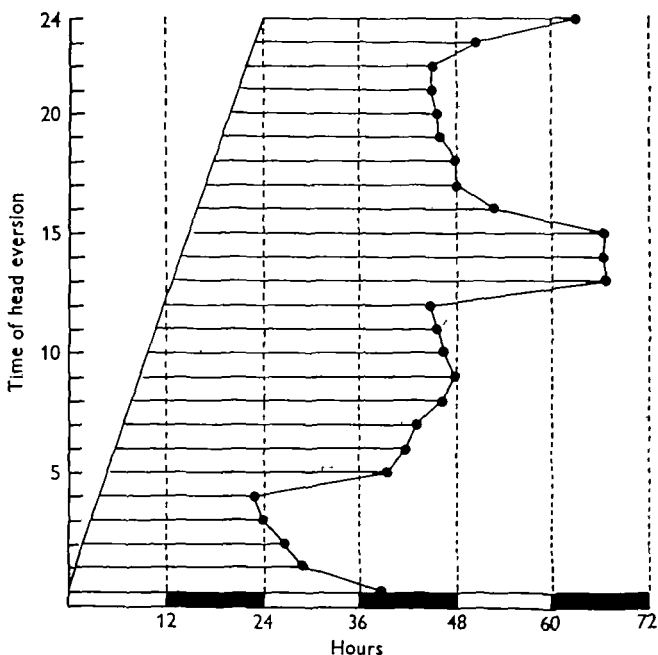


Fig. 3. The time taken for development of pupae of the strain 'straw' to complete the stage 'head eversion to yellow eye' when the stage is entered at different times in the light cycle. Bright light indicated by white box below abscissa, dim light by black box.

at the same time as pupa A, although the latter has already been developing for 40 min. If both pupae took 30.5 hr. to complete the stage, the end would be recorded as D + 11 on the next day for A but as D + 12 for B: that is a difference of 1 hr. would appear in their developmental periods. It seems likely, therefore, that the actual variation about the main peaks is less than the observed  $\pm 1$  hr. (still with the exception of line L + 5).

In the case of line L + 5, 79% of the 98 pupae observed completed the stage in

$35 \pm 1$  hr., but the times taken for the remaining 21 % ranged between 30 and 33 hr.; this is the only case in which a wide range of developmental periods occurred.

These results show quite clearly that the duration of the stage beginning with head eversion, and ending with the appearance of yellow eye coloration, is affected by the time of day, relative to the light cycle, at which head eversion occurs. The range of developmental periods shown by pupae entering the stage at different hours of the day is considerable. The most rapid development occurs when head eversion has taken place 4 hr. after the beginning of the light period, when the stage takes 19 hr.; the slowest development occurs when head eversion has taken place 1 hr. after the beginning of darkness, when the stage takes 54 hr.

In Fig. 3 the results are replotted to show the developmental periods in relation not only to the time of the beginning of the stages, but also to the light cycle to which the pupae are exposed throughout the stage; only the major peaks are marked for each sample. There does not seem to be any correlation between the length of the developmental period and the number of bright light:dim light cycles, or to any sequence of these cycles, but only to the light conditions during the first hour or two of the stage.

(b) *Larvae and pupae in 12 hr. light:12 hr. darkness, and larvae in 12 hr. light:12 hr. darkness followed by constant darkness.* The results obtained under both these conditions are identical with those obtained in the conditions described in the previous section, when larvae and pupae were kept in 12 hr. bright light:12 hr. dim light throughout their development.

Since identical developmental periods were recorded when the pupae were in constant darkness or in a light cycle, it appears that the factors which determine the rate of development are not immediately dependent on the prevailing environment, and their time relationships can be maintained in constant darkness.

## II. *Duration of the stage yellow eye to costal wing pigmentation, and the stage wing pigmentation to eclosion*

(a) *Larvae in 12 hr. bright light:12 hr. dim light.* The results are summarized in Fig. 4*a, b*. The variation is not shown in these figures, but again two distinct developmental periods occur within each sample entering a stage at a particular time of day; the minor peak is again assumed to represent the period typical of the pupae entering the stage in the preceding hour. The variation about the major peak is again limited to  $\pm 1$  hr.

A striking feature of these results is that the range of developmental periods for both these stages follows curves which are identical in form with each other, and with that for the stage 'head eversion to yellow eye'. The mean value of each sample in the stage 'yellow eye to wing pigmentation' is approximately 82 % of that shown by the equivalent sample in the previous stage. The mean value for each sample in the stage 'wing pigmentation to eclosion' is approximately 126 % of that of the equivalent sample in the stage 'yellow eye to wing pigmentation'.

(b) *Larvae in 12 hr. bright light:12 hr. dim light, followed by constant darkness.* When the pupae were kept in constant darkness it was not possible to observe the time of the beginning of the stages 'yellow eye to wing pigmentation' or 'wing pigmentation to eclosion' since, once the observation was made the sample had been exposed to light and had to be discarded. The time of prepupation, however, was known, and

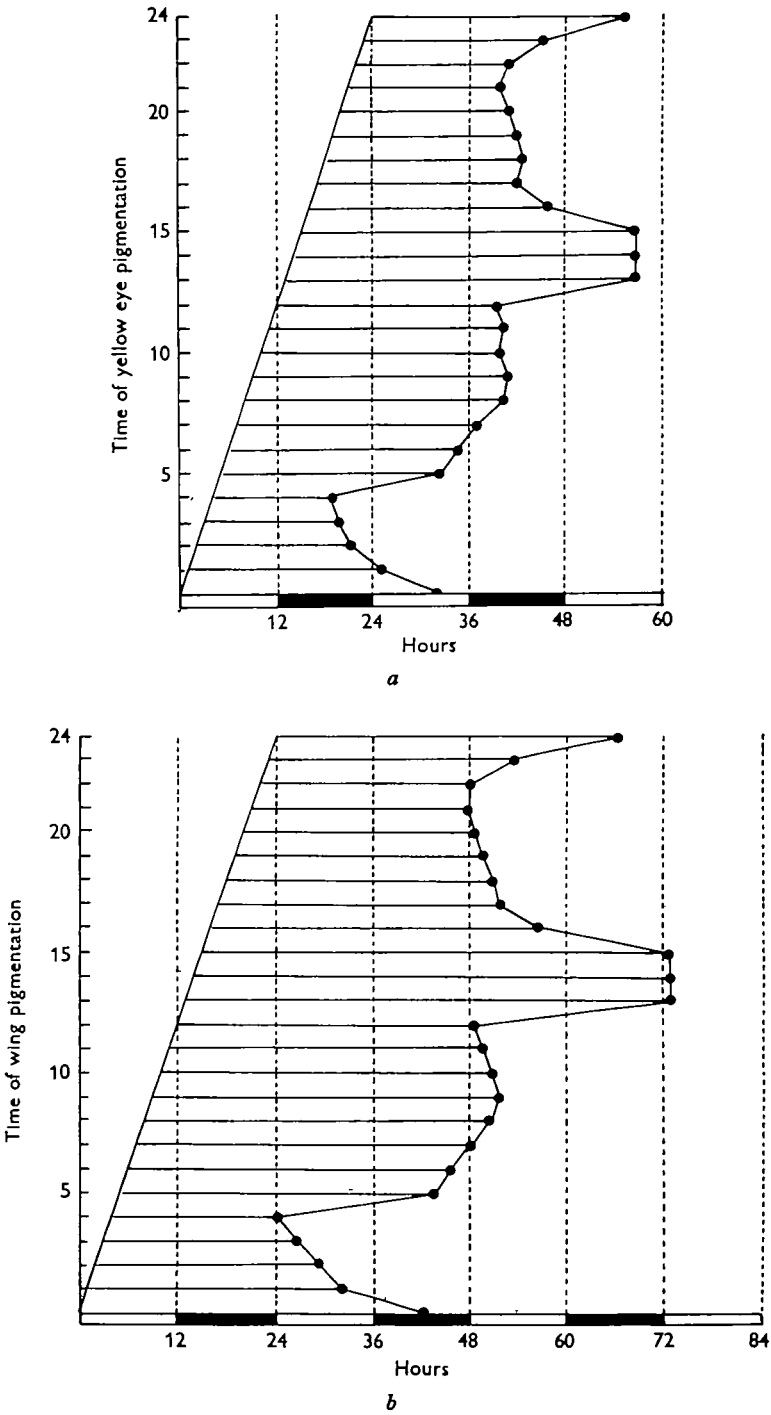


Fig. 4. The time taken for pupae of the strain 'straw' to complete the stages (a) 'yellow eye to wing pigmentation', (b) 'wing pigmentation to eclosion', when the stages are entered at different times in the light cycle.

the results described in the previous section show that the time taken to reach yellow eye-pigmentation is the same in constant darkness as in a light cycle. Hence from the time of prepupation it is possible to calculate the time of 'yellow eye', and the observation of the time of wing pigmentation allows calculation of the period of the stage. It is clear from the results that the same developmental periods are involved whether the pupae are in a light cycle or in constant darkness. Using similar calculations it is also found that constant darkness does not affect the periods involved in completing the stage 'wing pigmentation to eclosion'.

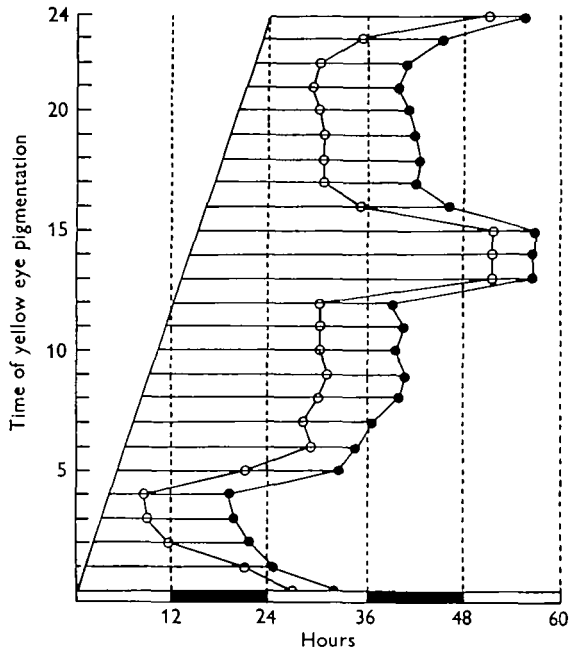


Fig. 5. The time within the stage 'yellow eye to wing pigmentation' at which red eye pigmentation appears in the strain 'straw'. ○, Appearance of red eye; ●, appearance of wing pigmentation.

### III. Duration of stages yellow eye to red eye, and red eye to wing pigmentation

The time-interval curve for the stage 'yellow eye to red eye' is of the same form as those of the stages already discussed, as can be seen when the time at which red eye is reached is plotted on the curve for the stage 'yellow eye to wing pigmentation' (Fig. 5). Although the time at which yellow eye is reached determines the time of red eye pigmentation the appearance of this pigmentation (or the factors which cause its appearance) does *not* influence the time to reach wing pigmentation. It can be seen from Fig. 5, for instance, that when red eye is reached 4 hr. after the beginning of the light period (samples at 7, 13 hr.) either 9 or 5 hr. may be taken to reach wing pigmentation; when red eye is reached 9 hr. after the beginning of darkness (samples at hr. 1, 5) either 3 or 13 hr. are taken to reach wing pigmentation. It is concluded, therefore, that this particular 'marker', red eye, does not signify the beginning of a stage of development controlled by the environmental light cycle.

*Developmental rates in strain 'wild'*I. *Duration of stage head eversion to yellow eye*

(a) *Larvae and pupae in 12 hr. bright light:12 hr. dim light.* The results are summarized in Fig. 6 in which the number of hours taken to complete the stage is plotted against the time of day at which each sample (of at least 50 pupae) entered the stage.

As with the strain 'straw' two distinct developmental periods occur within the majority of samples, and the variation about these peaks, with one exception, is

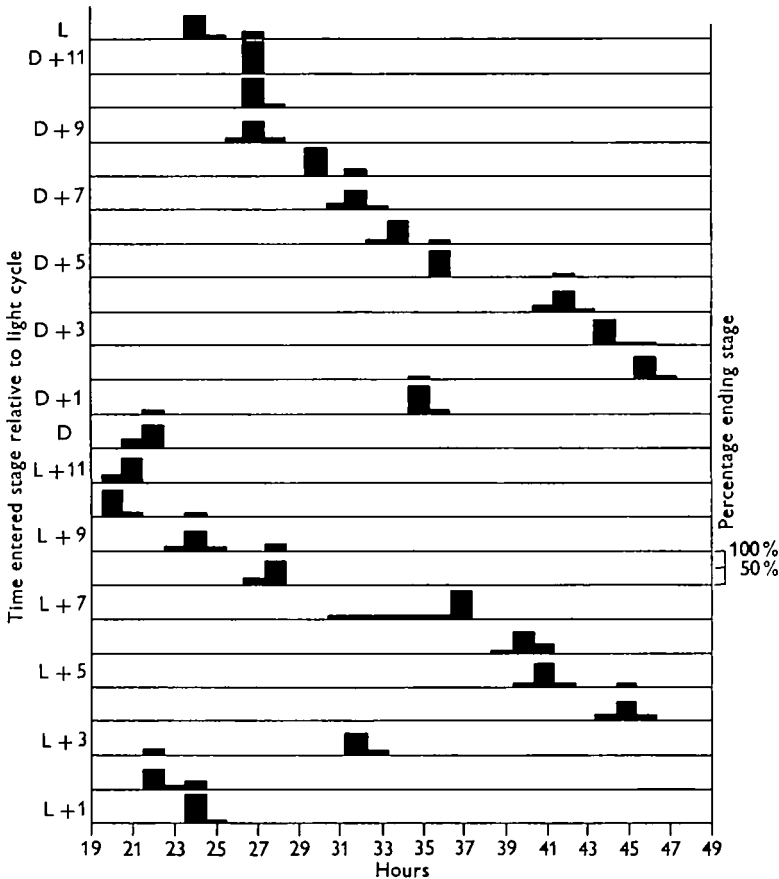


Fig. 6. The number of hours taken by pupae, of the strain 'wild', to complete the stage 'head eversion to yellow eye' when pupae enter the stage at different times in a 12 hr. bright light:12 hr. dim light cycle. The letters on the ordinate, D+5, L+5, etc., indicate the number of hours after the dim light (D) or bright light (L) was switched on.

$\pm 1$  hr. The fact that, as in 'straw', there is just one exception to the very close limits of variation is worthy of note. In this one exceptional case (line L+7), of the pupae undergoing head eversion 7 hr. after the beginning of the light period 82% complete the stage in 37 hr., but the remaining 18% show developmental periods ranging from 31 to 36 hr.

The results for all pupae are replotted in Fig. 7 in order to show the developmental



periods in relation to the light cycle to which the pupae are exposed throughout the stage. Again there does not appear to be any correlation between the length of the developmental period and the number or sequence of bright light:dim light cycle, but only to the light conditions during the first hour or two of the stage.

(b) *Larvae in 12 hr. light: 12 hr. darkness followed by constant darkness.* The results obtained under these conditions are identical with those obtained when the light cycle was maintained throughout pupal development.

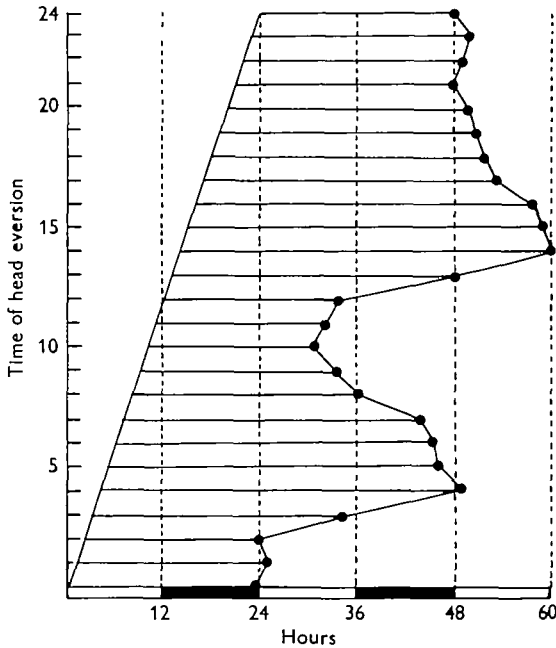


Fig. 7. The time taken for development of pupae of the strain 'wild' to complete the stage 'head eversion to yellow eye' when the stage is entered at different times in the light cycle.

## II. *Duration of stages yellow eye to costal vein pigmentation, and costal vein pigmentation to eclosion.*

The results obtained when pupae are kept in 12 hr. bright light: 12 hr. dim light are summarized in Fig. 8a, b. Two distinct developmental peaks occur within each sample entering a stage at each hour of the day: the minor peak is again assumed to represent members of the sample showing the developmental rate typical of the previous hour, and it is ignored in these figures. The variation about the major peak is limited to  $\pm 1$  hr.

The periods involved in the entire group of pupae again follow the same curve, in both stages, as that for the stage beginning with head eversion. The mean value of each hourly sample in the stage 'yellow eye to wing pigmentation' is approximately 86% of that for the equivalent pupae in the preceding stage (compare 82% in 'straw'); for the stage 'wing pigmentation to eclosion' the values are approximately 119% of those for the equivalent pupae in the stage 'yellow eye to wing pigmentation' (compare 126% in 'straw').

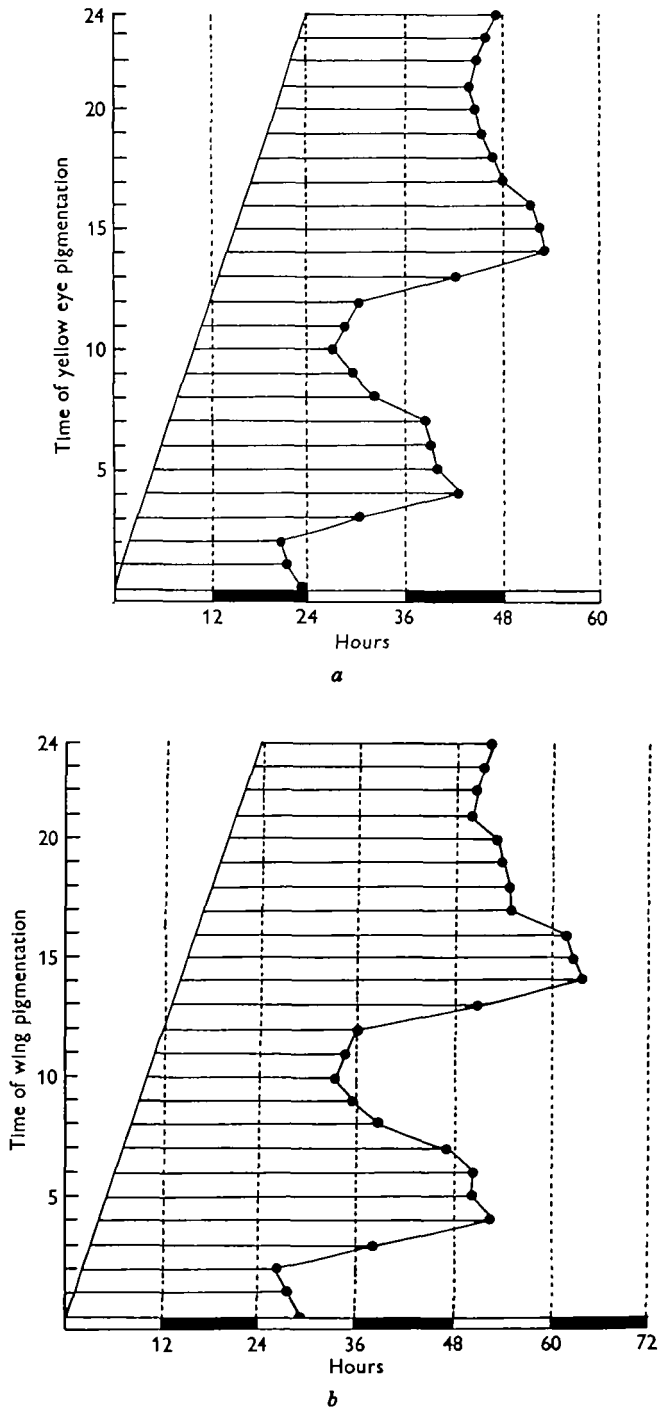


Fig. 8. The time taken for development of pupae of the strain 'wild' to complete the stages (a) 'yellow eye to wing pigmentation', (b) 'wing pigmentation to eclosion', when the stages are entered at different times in the light cycle.

*Summation of stages*

(a) *Strain 'Straw'*

Fig. 9 shows the developmental periods, throughout the entire development, of pupae when head eversion begins at successively later hours of the day in a 12 hr. light: 12 hr. darkness cycle.

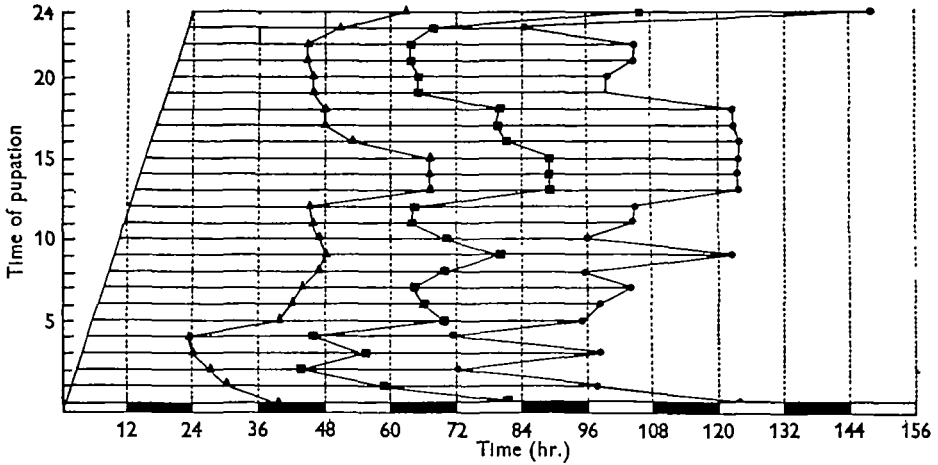


Fig. 9. The time taken for the entire pupal development of the strain 'straw' when head eversion occurs at different times in the light cycle. ▲, Time of 'yellow eye'; ■, time of 'wing pigmentation'; ●, time of eclosion.

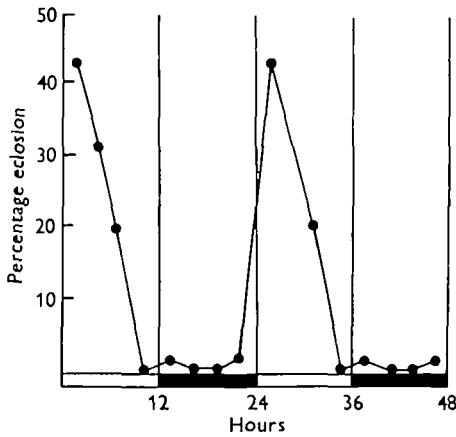


Fig. 10. The percentage of adults of the strain 'straw' emerging at different times of day, calculated by summing the daily emergence shown in Fig. 9.

It can be seen from these results that, because of the very wide range of developmental rates, dependent upon the time within the light cycle at which each stage begins, a population in which the larvae all pupate within a 24 hr. period will continue to produce adult flies over a 5 day period.

If the number of days from pupation is ignored, and only the time of day at which

eclosion occurs is considered (Fig. 10), it can be seen that the majority of flies emerge in the first 3 hr. of the light period. The number of flies emerging decreases during the later hours of the light period, and only a very small number (16% of the total) emerge at night.

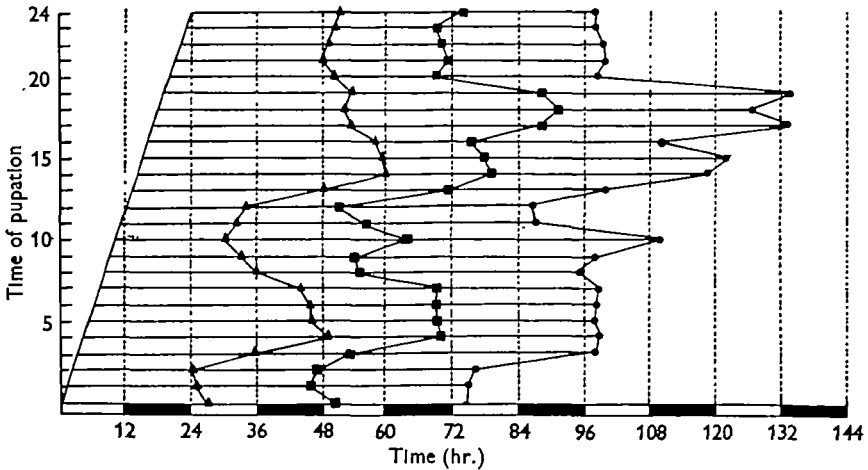


Fig. 11. The time taken for the entire pupal development of the strain 'wild' when head eversion occurs at different times in the light cycle. ▲, Time of 'yellow eye', ■, time of 'wing pigmentation'; ●, time of eclosion.

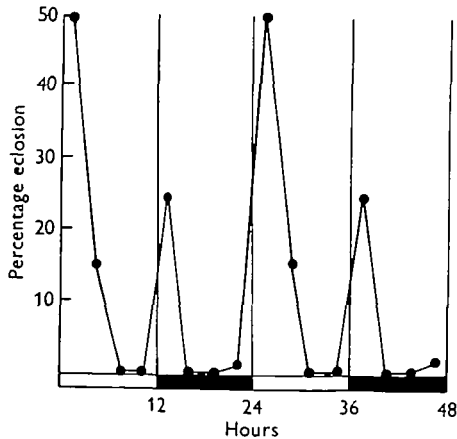


Fig. 12. The percentage of adults of the strain 'wild' emerging at different times of day, calculated by summing the daily emergence shown in Fig. 11.

Since prepupation occurs at all hours of the day, even in a 12 hr. light:12 hr. darkness cycle, and head eversion occurs  $12 \pm 1$  hr. later, the results shown in Fig. 9 represent to some extent the picture which would be obtained from a breeding culture of *Drosophila*. In such a culture there would be an overlap in the time of eclosion of larvae reaching the prepupal stage on successive days; for instance rapidly developing pupae which pupated 2 days later than slowly developing pupae might in fact emerge 3 days earlier than the latter. Therefore, in a breeding culture the rhythm of eclosion shown in Fig. 10 would be apparent.

These results have, however, been obtained by selecting samples of prepupae to give equal weight to individuals pupating at each hour of the day. It may be that in normal populations a greater number of insects pupate at one time of the day than at others, through the operation of a rhythm of egg-laying, hatching or development in the larvae. Preliminary experiments suggest that the development of the larvae is affected by the light:darkness cycle, and that in an entire population a greater proportion of flies would emerge at the beginning of the light period than is shown in Fig. 10.

(b) *Strain 'wild'*

Fig. 11 shows the developmental periods, throughout the entire development, of pupae when head eversion occurs at successively later hours of the day in a 12 hr. light:12 hr. darkness cycle. Pupae of this strain which have undergone pupation during one 24 hr. period may emerge as adults from 3 to 5 days later. When the time of day at which insects emerge as adults, regardless of the actual days, is plotted, it can be seen (Fig. 12) that there are two peaks of eclosion, one at the beginning of the light period, and the other at the beginning of the dark period.

DISCUSSION

It is quite clear from the results that the rate of pupal development is critically influenced by the environmental light cycle. Furthermore, since the rate of development of pupae kept in constant darkness is correlated with the timing of the 24 hr. light cycle to which they are exposed as larvae, the rate of development must be influenced by an 'internal clock' system of the type concerned in the control of diurnal, or circadian, rhythms.

The beginning and end of the stages of development described in this paper have been measured in terms of the appearance of characters which could be recognized visually. Two of these characters define major changes in the insect, as head eversion and eclosion represent the beginning and end of the pupal phase. The other characters are correlated with changes in pigmentation, and may, in themselves, not be particularly significant to the course of development. It may be that they appear concurrently with important biochemical changes or changes in the internal anatomy.

At the time of yellow eye-pigmentation the wing muscle becomes striated, the pupal abdominal muscles histolyse, and the secretion of the adult cuticle begins. At the time of pigmentation of the wing the rest of cuticle and the testis begin to pigment and the imaginal abdominal muscles become striated.

The oxygen consumption of developing *Drosophila* pupae has been measured by Wolsky (1938), and it is noteworthy that the major changes in the level of oxygen uptake coincide with head eversion and with the appearance of eye and wing pigmentation. The curve is U-shaped and oxygen uptake falls to the minimum at the time of head eversion, begins to rise again at the time of eye pigmentation and reaches its peak at the time of wing pigmentation. Wolsky suggests that the curve reflects changes in the pupal enzyme systems.

Development of insects is largely controlled by hormones, but the part played by hormones in the sequence of events in pupal development is still uncertain. Pupation

itself is well known to be under hormonal control but it is not known whether a second burst of hormonal activity coincides, in *Drosophila*, with the beginning of the rise in the respiratory curve. The ring gland, however, breaks down during the stage 'head eversion to yellow eye' and is re-formed in the adult position during the stage 'yellow eye to wing pigmentation', so it seems possible that the hormone titre increases during the latter stage.

Thus it seems likely that the pattern of development found in the present work is a property of a sequence of metabolic changes in the developing pupa, rather than of the particular anatomical markers chosen.

The very close correlation between the time-interval curves for the three stages of each strain is of particular interest. The results do not give any information about the processes involved in the rate-determination, but it seems unlikely that any simple change in either availability of substrate or production of some metabolite or hormone could give rise to identical curves of such complex form. Pupae entering a stage at successive hours may show very different rates of development; a later entry may result in either a sharp increase or a sharp decrease in the time for completion of the stage. In other cases only a very slow change in rate occurs over many hours. These two facts suggest that the competence of the tissue may be involved, as well as less drastic changes in the metabolic state of the tissue, and in the hormone level. Changes of competence with time are well-known in embryology, but the control of the timing of competence by an internal clock system related to a diurnal rhythm has not, as far as is known, been suggested hitherto. Further studies are at present being made of the tissues involved.

In previous studies of the eclosion rhythm of *Drosophila* (Brett, 1955; Pittendrigh, 1954, 1958, 1959, 1960; Bakker & Nelissen, 1963) attention has been confined to the time of eclosion, and it has been assumed that the eclosion of fully developed pupae is delayed during the dark period. The observations recorded here do not strictly preclude this, but they do make it seem extremely unlikely. The time-interval curves for all three stages take the same form, in spite of the fact that in some stages the majority of the pupae reach the end of the stage during the dark period, whereas in others they reach the end during the light period. The results show that the time of completion of a stage is dependent on the phasing of the time of entry relative to the light:darkness cycle, and is independent of the light:darkness conditions at the end of the stage.

The final eclosion rhythm is dependent on whatever processes control the developmental rate throughout the pupal stage. The eclosion rhythm is a population effect, and does not reflect the phasing of individuals to a dawn eclosion. Individuals can, and do, undergo eclosion at nearly all hours of the day and night, but the majority emerge at dawn because of the summation effect of circadian rhythms of development at earlier stages.

#### SUMMARY

1. A study of the course of pupal development in two strains of *Drosophila melanogaster* has been made in an attempt to establish the factors affecting the time of adult eclosion.
2. The time taken to complete three stages of pupal development has been measured for pupae entering each stage at each particular hour of the day when insects were kept

in 12 hr. light: 12 hr. darkness, 12 hr. bright light: 12 hr. dim light, or in continuous darkness.

3. The duration of each stage, in both strains, is affected by the time of day, relative to the light cycle, at which the stage is entered. The duration of each stage for pupae kept in continuous darkness is affected by the time of day at which the stage is entered, relative to the light cycle to which they had been exposed as larvae.

4. The time-interval curves for all three stages of any one strain take the same form.

5. Because of the very wide range of developmental rates, dependent upon the time within the light cycle at which each stage begins, a population in which the larvae all pupate within a 24 hr. period will continue to produce adult flies over several days.

6. The eclosion rhythm is a population effect and does not reflect the phasing of individuals to a dawn eclosion; the majority of adults emerge at dawn because of the summation effect of circadian rhythms of development at earlier stages.

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