Experiments on the effects of cultural conditions, increasing age on respiratory rates, weight and resistance to metabolic inhibitors on *Drosophila melanogaster* males of the mutant, vestigial ebony

by

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I, Alan E. Phillips declare that this thesis is my own work and composition, and that it has not been submitted for any other degree or qualification.

C O N T E N T S

Section 1. LENGTH OF LIFE

Introduction

During the course of previous experiments in this laboratory it became apparent that one of the major factors affecting the survival of male vestigial ebony *Drosophila melanogaster*, was a tendency for their vestigial wings to stick to the solid piece of bakers' yeast which was placed in each culture tube. This tendency was even more obvious in flies reared at 27° C., when exceptionally long vestiges were common (Morgan, Bridges & Sturtevant, 1925). It was therefore of interest to discover just what effect this source of mortality had on the survival curve of these insects, as constructed from the results of previous experiments.

Other workers (Pearl, 1928; Clarke & Maynard Smith, 1961) have reported using drops of yeast suspension instead of solid pieces of yeast. Using this method Clarke & Maynard Smith (1961) reported that unless flies were given fresh food at frequent intervals, the insects did not survive. In this case it was recommended that flies be given fresh food every two days. Although this method offered less chance of the flies sticking in the yeast, it did also suggest that there was some factor concerned with the food which had an adverse effect on the survival of the flies. It was therefore possible that the survival curves obtained for flies reared under these conditions were not the same as those which could be obtained under optimal culture conditions.

During the course of these experiments further reading disclosed a report by Loeb & Northropp (1916), in which they stated that the only foodstuff necessary for the survival of Drosophila melanogaster was live yeast. Moreover they stated that, provided larvae were reared in cultures containing live yeast, then on being transferred to fresh tubes immediately after eclosion, they would carry over sufficient yeast for survival. Flies were therefore reared and maintained as suggested by Loeb & Northropp, and were changed to fresh tubes every two or three days. In no case was yeast added after eclosion.

Study of the mortalities of flies involved in all the experiments showed that the death rate for flies given fresh food at three day intervals greatly exceeded that of flies changed at two day intervals. This suggested that the adverse effects as revealed in the work of Clarke & Maynard Smith, were still present. This effect might be due to an accumulation of carbon dioxide, or some other gas derived from the metabolism of the live yeast, within the closed tubes. Reference to a report by Raymond Pearl (1928) showed that flies cultured in bottles secured with gauze tops, lived longer than flies cultured in bottles with solid caps. It was therefore decided that the use of gauze caps, coupled with the method of maintenance as suggested by Loeb & Northropp might improve the culture conditions. A small experiment was therefore conducted along these lines.

Materials and methods

Flies which were bred in half-pint milk bottles were taken from the stocks held in this laboratory. The vestigial ebony mutant of Drosophila melanogaster was chosen for the purposes of comparison with previous results and for the shorter life expectancy of this mutant, compared to the wildtype (Gonzales, 1923). Ease of handling the adult fly was also a prime consideration in the selection of this mutant.

In Series 1 of the experiments, larvae were reared on maize-agar medium according to the standard Edinburgh recipe as used in this laboratory. The only difference was that food was made in a double boiler and excess was stored under refrigeration until required. It was then reheated and used as food in 7/8" x 3" culture tubes. Rearing bottles were cleared daily and pairs of newly hatched males were placed in 7/8" x 3" culture tubes, containing food to which one drop of yeast suspension was added. Males only were used in all experiments, as some flies were used for respirometry experiments. Flies were deemed to have hatched on the day of clearance and all ages were calculated on this basis. Where flies were used for respirometry a special note was taken to ascertain the effect of experimentation on their survival potential.

All flies were changed to fresh food on Monday, Wednesday, and Friday. Thus there were periods of three days, two days, and

two days between changes. All deaths were deemed to have occurred on the day that they were discovered. That is, on the day of the next change to fresh food. Tubes were secured with "Oxoid" solid metal test-tube caps.

In Series 2 of the experiments, flies were maintained as before, in pairs in 7/8" x 3" glass tubes secured with solid "Oxoid" test-tube caps. Flies were changed to fresh food and tubes on the same rota as Series 1, but in this series no yeast was added to the food. Dates of eclosion and death were calculated as for the previous series.

In Series 3 conditions were identical with those of Series 2, except for the method of closing the tubes. For this series stoppers were made from a piece of brass tubing of 1" diameter, over one end of each piece of which was soldered a piece of 40 mesh/inch brass gauze.

All experiments were performed in a constant temperature room at 27° C. The variation in temperature was a maximum of 1° C. Parents, eggs, larvae, and imagoes were all maintained throughout life at this temperature.

Results

Series 1.

In this series involving 1513 flies, the maximum length of life attained by one fly was forty days. The mean life span was calculated as the age at which 50% of the flies remained alive, and was found to be between 14 and 15 days. The average life span, calculated as for the previous experiments conducted in this laboratory, (i.e. total number of days lived by all the flies divided by the total number of flies established), was found to be 16.93 days.

The number of flies dying per day for each two day change in this series, was found to be 2.28, whereas the number of flies dying per three day change period was found to be 4.25. This represents an increment of 86.4% in the mortality rate.

Series 2.

The maximum length of life attained in this series was fifty-seven days, although this was once more attained by only one fly out of the 585 established. This was seven days more than the next longest lived. The mean life span was calculated as 24 days, and the average life-span as 24.26 days.

The death rate for two day changes in this experiment was 46% of that in Series 1, and for three day changes 34% of that in Series 1.

Series 3.

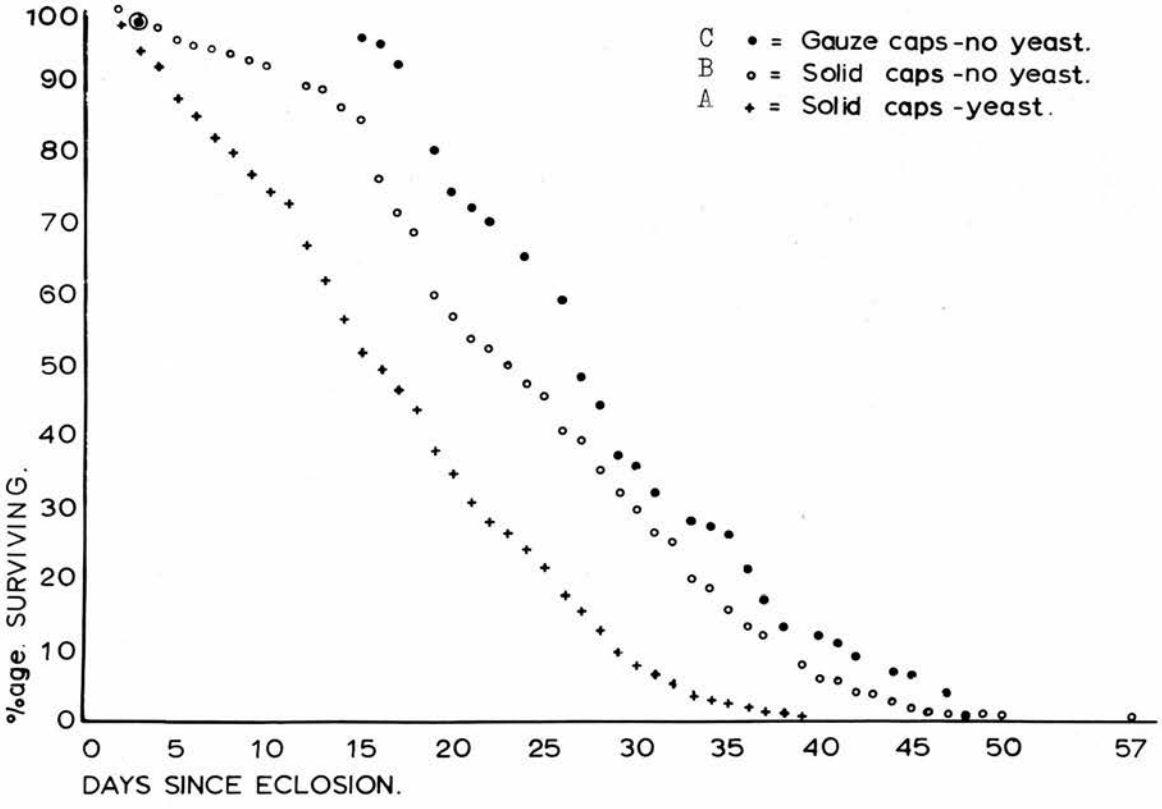
From a total of only 99 flies, three flies survived to 48 days. The mean length of life was 27 days and the average life span was 28.23 days. The death rate per day for two day changes was 1.0, and for three day changes was 1.09. This represents an increment of 9.0%. These death rates represented 95% and 75% respectively compared with the two and three day changes of Series 2, and 44% and 26% of Series 1.

The results are summarized in Table 1 and the survival graphs shown in Graphs A, B, and C.

TABLE I.

	Series I. (Solid caps + yeast)	Series II. (Solid caps - yeast)	Series III. (Gauze caps: no yeast)	Previous results with solid caps + solid yeast. 2 day changes
Number established	1513	585	99	-
Max. life span	40	57	48	34
Mean life span $\left(\frac{\text{Total no. days lived}}{\text{Total no. flies}}\right)$	14.5	24	27	-
Av. life span (50% dead)	16.93	24.26	28.23	18.96
Av. number dying per 2 day change period	2.28	1.05	1.0	-
Av. number dying per 3 day change period	4.25	1.45	1.09	-
Ratio of deaths per 2 day changes Series I: II: III.	2.28	1.05	1.0	-
Ratio of deaths per 3 day changes Series I: II: III.	3.90	1.33	1.0	-
Increases in deaths/day 3 day over 2 day changes for each individual series	86.4%	34.29%	9.0%	-

GRAPHS A, B, C.


C • = Gauze caps-no yeast.
B ∘ = Solid caps -no yeast.
A + = Solid caps -yeast.
%age. SURVIVING.
100
90
80
70
60
50
40
30
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10
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0 5 10 15 20 25 30 35 40 45 50 57
DAYS SINCE ECLOSION.

TABLE II.

Effect of Carbon Monoxide poisoning and age on survival time

Age at day of experiment	No. of flies	Number of days survived after poisoning with carbon monoxide and number of flies surviving																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
13	8				1		1					1		2		1			2			
17	7		2					1				1			1				1			1
19	3		2										1									
24	8		3					2		1		1			1							
26	6		4			2																
28	6			6																		
31	4		2		2																	
Total flies dying		-	13	6	3	2	1	3	-	1	-	3	1	2	2	1	-	-	3	-	-	1

Discussion

In the previous length of life experiments conducted in this laboratory, all flies were changed to fresh food on a strict two day rota. The average length of life, calculated on the basis of the total number of days lived divided by the total number of flies established, was 18.96 days. The temperature in each set of experiments was the same. The results obtained from the current set of experiments are not therefore strictly comparable with the previous experiments, since, in this latter series the flies were changed on Monday, Wednesday, and Friday, i.e. three, two, and two days.

It is obvious from Series 1, that if flies are changed to fresh food every three days, the number dying between changes is 86.4% more than the numbers dying between each two day change. In this series the average length of life for all flies, calculated on the same basis as above was 16.93 days. The difference of approximately two days or 10.7% could possibly be due to the effect of the three day change periods.

The results of Series 2 show that the work of Loeb & Northropp, demonstrating that the addition of yeast, other than that carried over by the flies from the breeding bottles, was unnecessary, is repeatable and that the conclusions drawn were valid. The average length of life attained in conditions exactly comparable to Series 1, except that no

yeast was added to the feeding bottles, was 24.26 days. This represents an increase of 43.3% over the average life span achieved in Series 1. This increase occurred in spite of the fact that flies were changed to fresh food at three day intervals as well as two day intervals, although the difference in mortality rate between the three day and two day changes was much less in this series than in Series 1, 34.29% as compared with 86.4%. This suggests that the omission of the yeast suspension minimised the difference in mortality rate found between the longer and the shorter change periods.

In Series 3, the difference in mortality rate between three and two day changes has been further reduced to only 9.0%. This can only be the result of the introduction of the perforated tops as all other conditions were similar to those of Series 2. The average life span of the flies was this time calculated as 28.23 days, which represents an increase of 66.7% over that achieved in Series 1 and 16.4% over that in Series 2.

It would appear therefore that the experiments with no added yeast more nearly approach optimum culture conditions than do those employing yeast, whilst those with gauze show a further benefit to the average length of life. This is, in the latter respect, as might be expected from the work of Raymond Pearl (1928).

Both Series 1 and Series 2 showed a significant increase in the number of flies dying when there was a period of three days between changes, when compared with the number dying in the time between two day changes (86.4% and 34.29% respectively). The difference in these proportions between Series 1 and Series 2, is attributable only to the presence of the yeast suspension as all other conditions were strictly comparable. Series 3 showed little difference between the numbers dying between three and two day periods between changes (9%) and in this experiment the only difference from the conditions in Series 2 was that the caps securing the bottles were perforated.

If the work of Loeb & Northropp is taken as a guide, there was a small amount of yeast present in both Series 2 and Series 3, carried over on the feet of the animals when changed, and that this small amount was sufficient to sustain life. It would seem therefore that the cause of the shorter life span of the animals in Series 1, may be directly linked with an excess of yeast. An alternative reason may be due to a deterioration of the food as a result of the activity of the yeast. There would appear to be a fairly strong suggestion that this excess causes a condition which by the third day becomes highly toxic, to a large proportion of the flies. This condition still occurs, but to a lesser degree in Series 2, whereas in Series 3 the effect appears to have almost disappeared. This would suggest that the free diffusion of the atmosphere through the lid reduces the toxicity of the condition within the tube.

Further to this it would seem that it is probably the products of the metabolism of the yeast which give rise to the lethal condition within the tube, although it is possible that the yeast multiplies sufficiently rapidly, in Series 1, to cover the surface of the food and so to cause partial starvation, which older flies might be less well equipped to resist. This I consider to be unlikely. If however the products of the metabolism of the yeast are to blame, then it can only be attributable to alcohol, acetic acid, carbon dioxide, or any combination of these three. In the absence of any measurements of these it would appear most likely from the results of Series 3 that gases are the important agent. The work of Northropp (1926) might suggest that the most likely cause is carbon monoxide gas.

Time did not permit an analysis of either the atmosphere within the tube, or of any pH changes, if any, in the food. As a result, the conclusion as to the cause of the toxicity within the tube can only be conjecture.

Lack of time also prevented the performance of a series of tests to find the effect of the gauze tops on the length of life and on the number of flies dying, when yeast is added as in Series 1. However Pearl (1928) does point out that gauze tops used in this type of experiment permitted and animals to live longer than those

kept in vials secured with a cotton wool plug. This again may support the idea that the metabolic products of the yeast cause the environment to become lethal, at least to the weaker flies, and may further support the suggestion that carbon dioxide accumulation may be the real cause.

The maximum length of life attained by one fly in the previous experiments performed in this laboratory, was thirty-four days at 27° C. In Series 1 of these latter experiments, the maximum length of life attained by one fly was forty days, in Series 2 fifty-seven days by one fly and in Series 3, forty-eight days by three flies. Raymond Pearl (1928) reported that the maximum length of life achieved by vestigial males was forty-three days at 25° C. In the foregoing series of experiments, conducted at 27° C, Series 1 produced no flies to achieve this age. Series 2 produced 3.59% of all flies to exceed forty-four days of life, and Series 3 16.2%. It has been observed that flies raised at 27° C. would live for a shorter time than those reared at 25° C. This shorter life span would be expected of the mutant ebony as opposed to pure vestigial as employed by Pearl (Pearl & Parker, 1921). Bearing these two facts in mind it would appear that the culture conditions appertaining to Series 2, and to an even greater extent to Series 3, were more nearly optimal to permit flies to reach their maximal life span, than were those employed in previous experiments in this laboratory and by other

workers. This would also suggest that the manifestations of "ageing" in this animal are seriously affected by the culture conditions to which they are subjected.

A small number of flies was studied to discover if gassing with carbon monoxide affected the length of time that the experimental flies survived after experimentation. In all forty-two flies of varying ages were studied, and it was observed that no fly over the age of twenty-four days at the time of experiment, survived more than five days. Twelve of the sixteen such flies survived no more than three days. It is possible, owing to the fact that bottles were changed every two, or three days, that no fly exceeded the dying period referred to by Maynard Smith (1962).

Bearing in mind these facts, the study of the survival curves (graphs A, B, and C) may show more clearly the effect of the culture conditions upon the signs of ageing shown by this animal. Graph A (solid caps and yeast suspension) approximates to the exponential. This would suggest a constant force of mortality being exerted on the animals. Graph B shows a slow death rate for the first fifteen days of life, after eclosion and thereafter becomes comparatively steep, until the last few survivors at about forty days, whereupon the curve is very much more gradual. This approximates more closely to the rectilinear survival curve which would be ideal if all flies

had the same potential longevity, and if culture conditions were optimal. Graph C is an even closer approximation to the rectilinear survival curve and is completely different from that proposed for vestigial flies by Raymond Pearl (1928), who suggested that it was diagonal.

These graphs suggest that before any survival curves are taken as factual, a great deal of experiment into culture conditions and their effects is necessary.

Conclusions:-

(1) that culture conditions affect the length of survival of male Drosophila melanogaster:

(2) that an excess of yeast is possible in the culture tubes:

(3) that this excess yeast causes a toxic condition in the tube or the food:

(4) that this toxicity is most likely due to metabolic products from the yeast: (May be carbon dioxide, alcohol or acetic acid.)

(5) that flies carry over enough yeast for survival when they are transferred for rearing to culture bottles:

(6) that perforated tops to the tube permit flies to survive longer:

(7) that the conditions under which flies are maintained in Series 3 are more nearly optimal than in either Series 1 or 2: (No added yeast and perforated tops.)

(8) that the survival curve constructed under these conditions for vestigial ebony flies approaches more closely the rectangular than had previously been reported:

(9) that results of ageing experiments previously performed under sub-optimal conditions must be considered suspect.

(10) that old flies are less able to withstand the effects of carbon monoxide poisoning than young flies.

Section 2. EFFECT OF AGE ON WEIGHT

Introduction

Many workers (e.g. Hunter, 1964 etc.) have quoted the results of their researches into respiratory rates of Drosophila and other animals as $\mu l/O_2/hr.$ mg. wet weight. This led to the thought that age itself might be correlated with weight in some way. This possibility was born out by the fact that actuarial tables for humans always allow an increase in weight with age. A small experiment involving ten flies was therefore performed.

Materials and methods

Ten male Drosophila melanogaster vestigial ebony flies were established in individual identifiable bottles, under exactly the same conditions as for the length of life studies Series 1. The flies were weighed individually each day on a ten milligram torsion balance and then returned to their bottles. It was necessary to anaesthetise the flies in order to weigh them, but no fly was killed by the ether and, as suggested by Pearl (1928), it was expected that no damage was done by the anaesthetic. Weighings were carried out as nearly as possible at the same time each day. Culture bottles were changed on Monday, Wednesday, and Friday as for the length of life studies.

Results

Weights of individual flies throughout life are shown in Table III. The weights at eclosion varies from 0.42 mg. to 1.04 mg. and at death from 0.50 mg. to 0.95 mg., although the fly achieving this weight suffered from a distended abdomen. If this latter fly is ignored, as being abnormal, then the weights at death varied from 0.50 mg. to 0.78 mg., a much smaller variation than at eclosion.

The average daily weights (Table IV) were calculated until the number of flies surviving dropped to below five. Animal number three was excluded from these calculations as the bloated abdomen, which became apparent on day 23, might possibly have been caused by a condition affecting the fly's metabolism from birth. These averages were tabulated against age in days from eclosion (Table IV).

TABLE III.

Individual weight records

Fly	Weight at eclosion	Maximum weight achieved	Minimum weight achieved	Difference as a % of weight at eclosion	Weight at death	Age at death
1	0.66 mg.	0.76 mg.	0.62 mg.	21.2%	0.67 mg.	26 days
2	0.64 mg.	0.83 mg.	0.58 mg.	39.1%	0.60 mg.	29 days
3	0.42 mg.	0.99 mg.*	0.42 mg.	135.7%	0.95 mg.	26 days
4	1.04 mg.	1.04 mg.	0.61 mg.	41.3%	0.62 mg.	29 days
5	0.96 mg.	0.96 mg.	0.69 mg.	28.1%	0.78 mg.	18 days
6	0.52 mg.	0.61 mg.	0.47 mg.	26.9%	0.50 mg.	24 days
7	1.02 mg.	1.02 mg.	0.64 mg.	37.3%	0.75 mg.	12 days
8	0.76 mg.	0.76 mg.	0.50 mg.	34.2%	0.58 mg.	18 days
9	0.78 mg.	0.78 mg.	0.56 mg.	28.2%	0.62 mg.	18 days
10	0.52 mg.	0.77 mg.	0.52 mg.	48.1%	0.63 mg.	26 days

* Animal had a bloated abdomen.

TABLE IV.

Age v. average weight

Age in days	Average weight in mg.	No. of flies in each sample
1	0.767	9
2	0.669	9
3	0.659	9
4	-	-
5	-	-
6	0.700	9
7	0.652	9
8	0.630	9
9	0.663	9
10	0.610	9
11	-	-
12	0.674	9
13	0.680	8
14	0.670	8
15	0.651	8
16	0.648	8
17	0.630	8
18	0.659	8
19	-	-
20	0.634	5
21	0.634	5
22	0.614	5
23	0.652	5
24	0.634	5

Discussion

The first thing that is obvious from these results is that, as might be expected, male Drosophila melanogaster vestigial ebony flies do not all emerge from larvae at the same weight although, theoretically, the availability of foodstuffs and the culture conditions are equal for all. However, by the second day after eclosion the weights are much nearer uniformity. On the first day after eclosion the heaviest fly was exactly twice the weight of each of the two lightest, whereas by the second day the heaviest was only 1.4 times as heavy as the lightest. Some flies, in this period lost weight and others gained. There is no obvious reason for this levelling off of weights. It was however the case that all flies under 0.7 mg. on the first day gained weight whilst those over 0.7 mg. on the first day lost weight. Again the reason for this is not immediately obvious.

In the case of individual flies the main tendency appeared for there to be a loss in weight with increasing age, although flies No. 1 and 10 did actually gain weight, e.g. No. 10:- weight at eclosion + 1 day = 0.52 mg; weight at death 0.63 mg: increase = 21%. Fly No. 1 showed only a small difference of 2% between weights at eclosion + 1 day and at death. Fly No. 3 was again ignored because it was feared that its "illness" would cause spurious results. The loss of weight shown by the other flies was not a constant amount and varied between 40% and 4%.

The average of the weights of the flies still alive each day reflects this tendency to lose weight and also reflects the daily changes in the weight of each fly. It is obvious that the weight of each fly varies daily over a fairly wide range. The reason for this can only be guessed at this stage. Although the flies were weighed each day at approximately the same time, it is possible that each fly feeds, and, perhaps more important, evacuates its gut, randomly throughout the day and throughout its life. It is therefore within the bounds of possibility that a fly may have a completely empty gut one day when weighed, a half-full one the next time and a full one at the time of the third weighing. It is however unlikely that, in the present culture conditions, any fly would at any time have a completely empty gut. The individual results confirm that this variation occurs with each fly.

Whilst it must be stated that these are the results obtained from an extremely small sample, it does seem that there is a tendency for flies to lose weight throughout life whereas actuarial tables would suggest the reverse situation in Man. (Actuarial Tables of Prudential Assurance Co.) Before any firm conclusions could be drawn however, as to the validity of the assumptions made from these results, a much larger experiment would have to be performed.

One conclusion can however be drawn from this "pilot" experiment and that is that increasing and decreasing weight does not afford a

parameter for measuring physiological age in this species, and that changes of metabolic rate of flies with time cannot be explained by the reduction in metabolising mass. In this metabolising mass protein may be replaced by water and therefore little change in weight would be observed although a marked change in metabolic rate might result.

Conclusions

(1) Weight changes with age are erratic and irregular.

(2) Weight tends to decrease with age.

(3) Changes are not adequate to account for changes in metabolic rate, found with increasing age.

(4) Changes in weight are too irregular to act as a parameter of physiological age.

Section 3. INDIVIDUAL RESPIRATORY RATES

Introduction

Bowler & Hollingsworth (1966) reported a decline in the respiration rate of *Drosophila subobscura* with increasing age. This confirms observations made in this laboratory on individual adult male *Drosophila melanogaster*.

These latter observations, made with a view to correlating respiration rate and hence metabolic rate with the work of Maynard Smith (1963) and of Clarke & Maynard Smith (1961), left several points unanswered. The range of results obtained for oxygen consumption per fly was far greater than anything obtained by other workers who had studied various *Drosophila* species (Hunter, 1964). It was necessary therefore to repeat these experiments to confirm that the wide range of results obtained previously, was not due to faulty methods and then, having confirmed this, to try to explain the differences.

Two possible raasons for this difference between my results and those obtained by others were readily apparent. Firstly, in nearly every case, except for example Orr 1937, repiratory rates were obtained from a batch of flies and then the average rate per fly obtained by dividing the total amount of O_2 consumed by the number

of flies in the experiment. The very nature of such experiments might well mask any extremes of individual respiration rate. Secondly, in all the experiments reported, the flies were subjected to restraint, whereas those used for experiments in this laboratory were allowed a much greater freedom, although they were not entirely free. This restriction of movement might well prevent extremes of respiratory rate occurring for any of several possible reasons.

In the previous experiments conducted in this laboratory it was found that there was often a marked increase in respiratory rate, immediately prior to death. This increase might well correspond with the significant increase in the rate of protein metabolism reported to occur in Drosophila subobscura with increasing age (Clarke & Maynard Smith, 1966). During the course of this work therefore, any further evidence was sought, as also was evidence to support the apparent cyclical nature of individual daily respiratory rates, as found in previous experiments. This cyclical activity appeared to have a period of approximately three days and was most noticeable when the respiratory rate of individual flies was plotted as oxygen consumption per fly against number of days prior to death. For this latter purpose the respiratory rate of one individual adult fly was measured continually throughout its life.

Materials and methods

(a) Experimental animals

These were *Drosophila melanogaster* vestigial ebony males. The original culture being taken from the departmental stock which had been inbred for a minimum of thirty generations. In the original experiments (1964-5) these had been chosen because *Drosophila subobscura*, as used by Maynard Smith, were not readily available and also because the shorter life-span of *melanogaster* was an advantage, as was the further shortening of the life-expectancy due to the mutant vestigial (Pearl & Parker, 1921). Males only were used as Maynard Smith (1963) has shown that egg-laying has an effect on the life-span of the animals.

Where experiments were carried out to the respiration rates of batches of flies restricted in their movements against the rates of batches not restricted, simultaneous experiments were performed using wildtype male flies so that a further comparison might be made between wildtype and vestigial. In all comparison experiments, flies of equal age were used.

(b) Food

This was prepared according to the "Edinburgh *Drosophila* medium" recipe used successfully in this department for many years. Each fresh batch of food was boiled in a double boiler for two hours. Any surplus was put hot into a "Kilner" jar and stored in a refrigerator until required, when it was reheated in the double boiler for a minimum of twenty minutes before being poured into bottles or tubes. Any bottles or tubes complete with food but not in use were capped with butter muslin and stored in the refrigerator. One drop of Bakers' yeast suspended in distilled water was added to each tube and three drops to each ½ pint milk bottle, before flies were put into them.

(c) Culture methods

Larvae were reared until eclosion in half-pint milk bottles secured by butter-muslin tops. Bottles were then cleared at daily intervals and the males separated from the females. A pair of males was then put into each 3" x 1" diameter tube and a rolled piece of "Kimwipe" added before the tube was closed with a plain aluminium "Oxoid" rimless test-tube cap. Both rearing bottles and tubes were maintained at 27° C. throughout life, either in an incubator or a constant-temperature room. Humidity was maintained

in the oven by an open dish of tap water and in the constant-temperature room by keeping the sink full of tap-water. Tubes were changed on Monday, Wednesday, and Friday. The date of eclosion of the flies was taken to be that of the day when the bottle was cleared.

(d) Respirometers

(1) When batches of flies were being used, all respiratory rate measurements were made in standard Warburg respirometers. In this instance the flasks were all of approximately 20 ml capacity. If flies were loose in these flasks, the 0.2 ml of 10% KOH in the centre-well of the flask was protected by a metal gauze cylinder which slipped over the outside wall of the well and over the piece of rolled "Kimwipe" which was used to absorb the KOH. In other experiments the flies were restricted by a No. 1 gelatine pharmaceutical capsule in which 10 pin-pricks were made at each end or in a small brass gauze capsule. In neither case was it necessary to protect the centre-well by a gauze cylinder. Flies were anaesthetised before being placed in capsules or flasks and allowed to revive before recordings were started. This usually took 15-20 minutes. As these experiments were not conducted in a constant temperature room, this time also allowed for acclimatisation of the flies and equilibration of the apparatus in the water bath.

(2) Where individual flies were being used it was necessary to employ a more sensitive variation of the Warburg technique. Various methods were tried before a satisfactory one was found.

First of all a method was sought which would allow the use of the standard 20 cc flask but which would confer much greater sensitivity on the manometer. For this purpose the flask was almost filled with wax and its capacity determined. Even with very small flask volumes it was found that the required sensitivity was not obtainable.

Secondly a method suggested by Burk & Hobby (1954) was tried which was based on Hooke's 9th paradox.

In the first series of trials based on this method, metal rods of known diameter were used, together with an introduced air bubble. This method which proved too sensitive and extremely difficult to operate was therefore discarded in favour of their second method which for purposes of magnification of the movement of the meniscus employs different fluids of known density. After various trials the fluid found to be most suitable was secondary butyl acetate which had a density of 0.864 at 27° C compared with that of the Brodie's fluid which was measured at 1.034 at 27° C.

This gave a magnification factor as follows:-

$$M = \frac{D}{D - d\left(1 - \frac{a}{A}\right)}$$

$$= 4.9.$$

During experiments with individual flies, which had small respiratory rates it was necessary, in order that repeatable readings might be made, to reduce the capacity of the flask. For this purpose "Quickfit" stoppers were modified to fit the manometer ends. The stoppers used were about 10 cc capacity before modification. The neck of each stopper was drilled to allow the passage of gas. As the necks of the stoppers were too wide to fit the necks of the manometers, brass collars were made which closed this gap. These had to be grooved so that a gas channel could be made patent when required. All parts were assembled and made gas-tight by lanolin. The final capacity of the new flasks was determined and in each case was close to 7 ml.

Readings of the meniscus interface were made with the aid of a small hand lens so that accuracy to 0.5 mm. could be maintained. The error represented in actual respiratory rate would therefore be of the following magnitude:-

Assume a flask constant of 0.71 (as per flask No. 6)

Magnification factor = 4.90

∴ $\frac{1}{2}$ mm. error in reading would represent

$$\frac{0.5 \times 0.71}{4.90} \ \mu l \ O_2$$

$$= \underline{0.073 \ \mu l}.$$

In nearly every case the respiratory rate was in excess of 1.50 µl/h. Therefore the maximum percentage error introduced by this would be:-

$$\frac{0.073}{1.50} \times \frac{100}{1} = \frac{7.3}{1.5} = \underline{\underline{4.86\%}}$$

Results

(a) Continued recording throughout life of respiratory rate of individual fly

In this case the results obtained for the respiratory rate of the individual fly varied considerably from 1.16 µl O_2 per hour on day 10 to 7.53 µl per hour on day 11. These results also showed a series of cyclical variations similar to those previously obtained. In the day prior to death, the respiratory rate of the fly achieved a level of 27.33 µl per hour. (Graph J.)

The length of time required to set up these individual recordings was so great as to preclude multiple experiments, which would have excluded any other experimentation or cultivation of flies.

(b) Random individual respiratory rates

Individual respiratory rates of flies of known age were ascertained from animals taken randomly from the bulk stocks. The purpose was to check the range of rates possible and not the fluctuations throughout life. As will be seen from Graph D considerable variation was found from less than 1 µl per hour to over 20 µl per hour per fly.

(c) Comparison of the respiration rates of loose and encapsulated flies

Five experiments were performed with wild type Drosophila melanogaster males in which ten flies were loose in a respirometer flask and compared with ten flies encapsulated in another flask. The results were:-

Series A. TABLE V.

µl O_2/hr/fly average in capsuls	Av. µl O_2/hr/fly loose	% increase - loose capsule
2.76	2.99	8.3
1.91	3.70	93.7
1.97	2.48	25.9
3.30	4.30	30.3
1.64	3.82	132.9

The results of three of these experiments are shown in graph E.

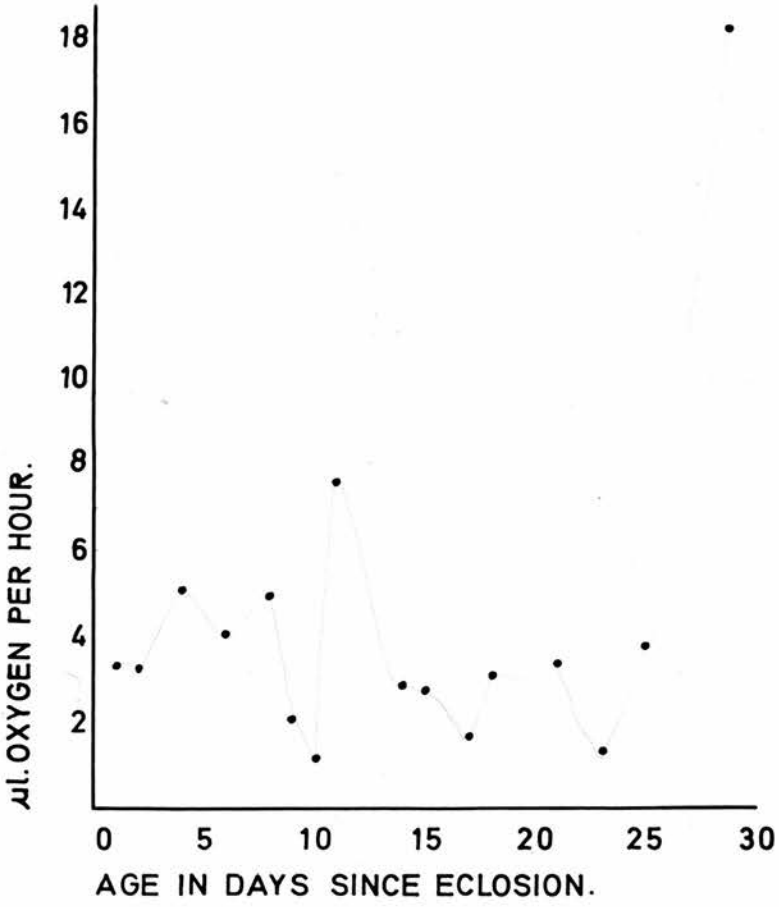
Six similar experiments were performed with vestigial males - again all flies were of equal chronological age.

Series B. TABLE VI.

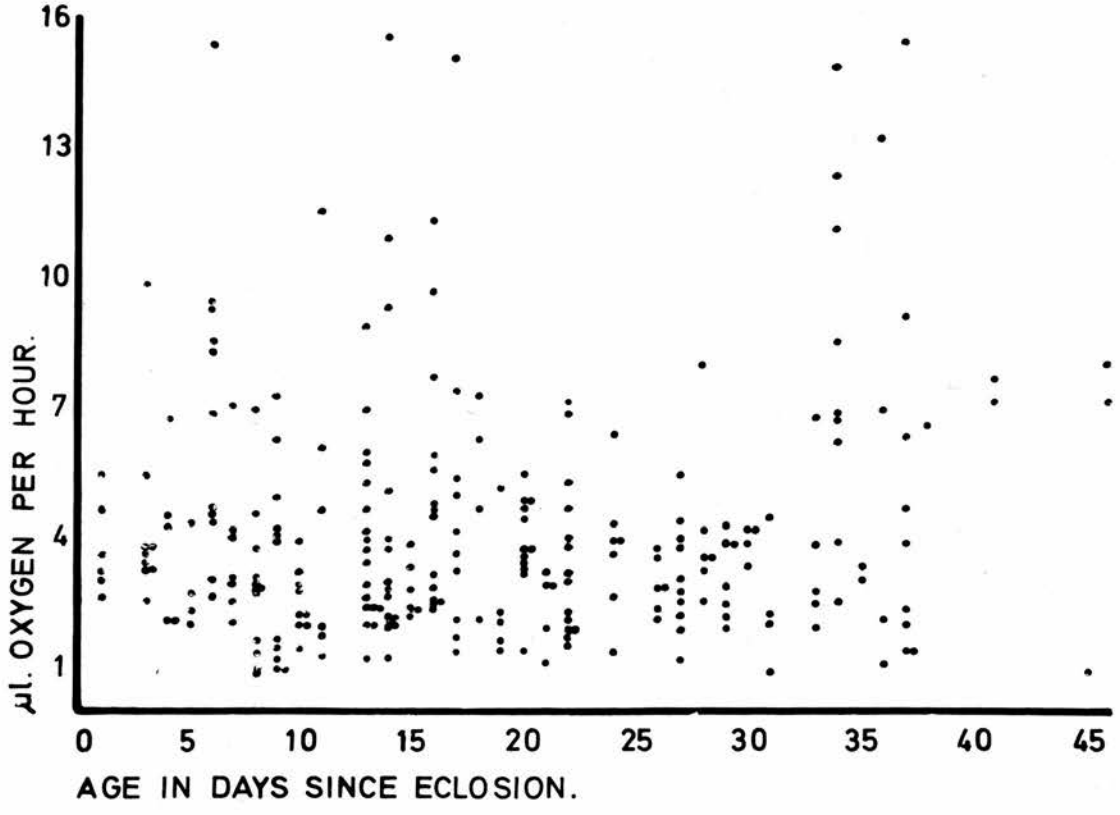
Av. µl O_2/hr/fly in capsule	Av. µl O_2/hr/fly loose	% increase
2.40	2.73	13.75
2.18	2.85	30.70
1.85	3.01	62.70
2.24	2.57	9.82
2.89	3.12	7.96
2.50	3.20	28.00

The results of three of these experiments are shown in graph F.

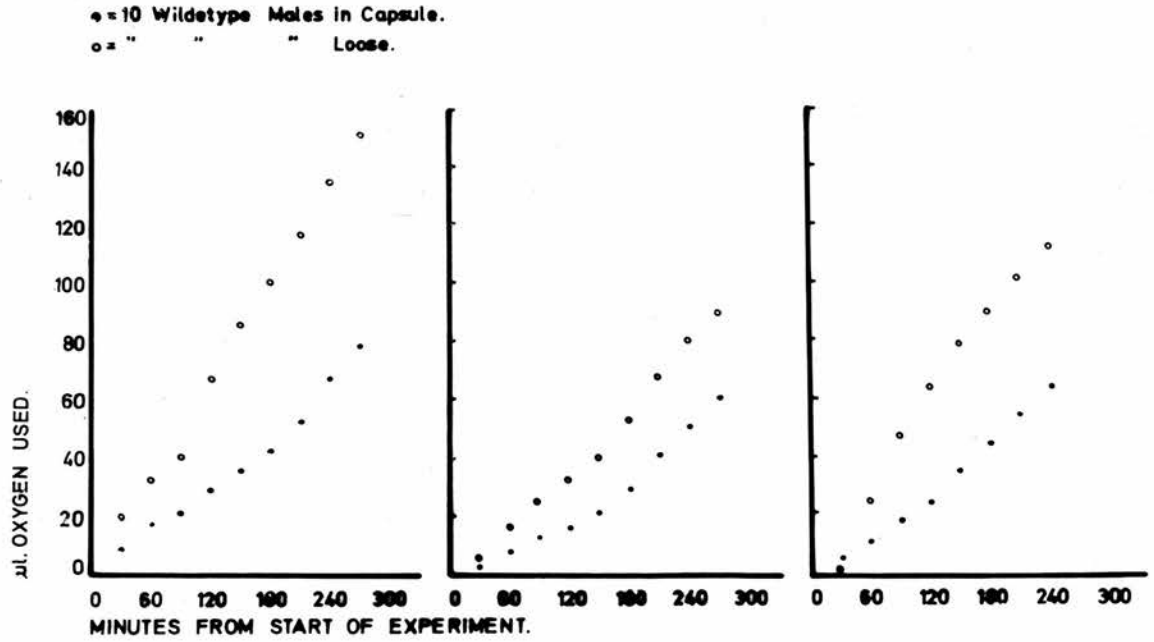
GRAPH J.


18
16
14
12
10
8
6
4
2
µl. OXYGEN PER HOUR.
0
5
10
15
20
25
30
AGE IN DAYS SINCE ECLOSION.

GRAPH D.

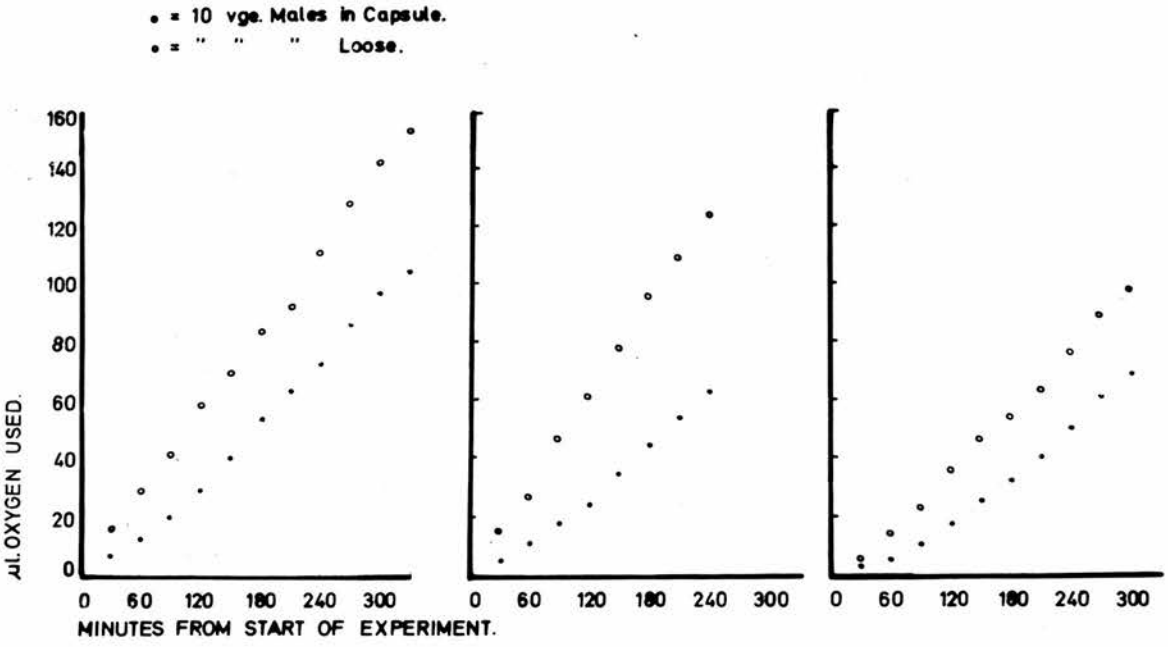

16
13
10
7
4
1
µl. OXYGEN PER HOUR.
0
5
10
15
20
25
30
35
40
45
AGE IN DAYS SINCE ECLOSION.

GRAPH E.



wild type

GRAPH F.



vestigial ebony

In the case of the wild type flies the smallest average respiratory rate was 0.50 of the largest in the capsules and 0.56 in the loose. For vestigial the figures were 0.64 and 0.77.

Discussion

As may be seen from the results obtained, the variation in respiration rate throughout its life of this one fly is fairly wide and would also suggest a cyclical variation over a period of three to four days. Although the results obtained from one fly are, in themselves, of little significance they do add confirmation to the results obtained previously in this laboratory, by other means. This in itself would suggest that the previous results were not the outcome of a fault in the method and that both variations are real. The most obvious explanation of this rhythmical variation is that it is a rhythm in the activity of the animal, specifically of the physical activity, since reproductive behaviour is ruled out.

The sudden extreme rise in respiration rate, manifest on the day of death, may also be taken as confirming the result found previously in this laboratory. In these previous experiments 67% of flies reared at 27° C showed this marked rise immediately prior to death. The other 33% may not have had their respiration rates checked at exactly the right physiological moment. A similar indication of rise in metabolic rate towards death has been found by

Maynard Smith (1966) in an increase in the rate of protein catabolism. It would be of interest to ascertain whether this might be an indication of an escape from control of the animals' total metabolism immediately prior to death, although it is appreciated that such an escape, if present, need not be the direct cause of death, but only a sign of such a cause.

In comparison of average results for flies both loose and in capsules, as flies were all of the same age, any variation in oxygen consumption between the flies in the capsules and those loose in the flasks cannot be ascribed to a metabolic difference due to age. The only conditions which varied in the experiments were those attached to the capsule or freedom.

In all cases, whether wild type or vestigial, there was an increase in the average respiration rate per fly when the rates for the loose flies was compared with that of those in capsules. This increase varied in both sets of experiments over a fairly wide range, although the variation of individual rates within each category was not excessive.

This increase, taken into consideration with the individual variations within each category, would suggest that the flies which were loose in the respirometer had a basically higher respiratory rate than those in the capsules, e.g. series A 1.64 µl/hr/fly :

2.46 µl/hr/fly, but that despite this basically higher rate, there was a decided increase in the respiratory rate of the loose flies.

This increase might well be due to increased activity and may more nearly approach the respiratory rate of flies in the wild. However, some movement was possible for the flies within the capsule so that the results here do not reflect anything approaching a Basal Metabolic Rate, whereas the experiments performed by other workers where the fly is restricted completely in its movements (Orr, 1937) may do so. Even in this case however, one cannot be sure that this reflects a B.M.R. as the fly may well be struggling against the restriction.

From this, therefore, it would seem that measurements of respiratory rates of flies must be made in much more standard conditions and that differences in values found by different workers may well reflect differences in degrees of freedom permitted to the animal.

It was thought necessary to check that the differences in respiratory rates was not due to the particular animals in each experiment. Therefore animals which had been encapsulated on one day were loose for the next day and those that had been loose were encapsulated. In each case the free flies showed a higher respiration rate than those in the capsule, thus obviating the above possibility.

Conclusions

(1) There is strong evidence to suggest a wide range in the variations of daily respiratory rate for each individual fly.

(2) There is evidence of a sudden increase in respiratory rate immediately prior to death.

(3) Supporting evidence was found for the cyclical variation in individual respiratory rates throughout life.

(4) Flies allowed free movement during the measurement had a higher respiratory rate than those restricted in their movement.

(5) All work on respiratory and metabolic rates of *Drosophila* should be performed under standard conditions to facilitate comparisons.

Section 4. RESPONSE TO METABOLIC INHIBITORS

Introduction

The fact that ageing, as a phenomenon, may be considered from either a chronological or a physiological point of view, has presented one of the main problems besetting its investigation. In the case of Drosophila melanogaster, a fly which has reached an age of nine days since eclosion may die on the tenth day and so at nine days be considered to have completed 9/10ths of its physiological ageing. A similar fly aged nine days may live to twenty days and be thought therefore to have completed only 9/20ths of its physiological ageing. Although both flies are the same chronological age they are obviously not at the same physiological point (or age) in their life cycle.

Raymond Pearl (1928) tried to explain this difference in the lifespan of two siblings by his "Rate of Living" theory which said, basically, that each fly was born with a innate store of "vitality" which it could expend at a rate characteristic of itself. Any difference in the rate at which this "vitality" was exhausted would therefore explain the difference in lifespan of two such animals. Recently doubts have been cast on this theory by the work of Maynard Smith and his associates. The result of this and other considerations (Comfort, 1964) is that a method is being sought,

which will measure the physiological age of an animal at any given chronological age. The method used previously in this laboratory was based on the premise that there might well be a regular change in metabolic rate with increasing physiological age and that such a change in rate might be directly linked with a measurable regular change in respiratory rate. In order to test this hypothesis, the individual respiratory rates of male vestigial ebony Drosophila melanogaster were measured continually throughout life from eclosion to death. The results of these determinations showed that although there was a decline in respiration rate with increasing age, this decline was not sufficiently regular to afford a parameter for the measurement of physiological age in this species.

The work of Muir, De Kock, De Kock and Inkson (1959), on the effect of metabolic inhibitors on rat brain tissue slices of varying ages, together with that of Bliss & Broadbent (1935) on the effect of hydrogen cyanide gas on whole Drosophila melanogaster, suggested to me that the response of the whole animal to metabolic inhibitors might well show a linear change with increasing age. Such a change might possibly be reflected in the respiratory rate and the experiments were therefore designed to test this.

Materials and methods

Carbon monoxide gas was considered to be the most suitable metabolic inhibitor both for ease of administration and for control of amount supplied to the animals. As recorded by Darcy Gilmour (1965) this gas combines with the terminal cytochrome in the electron transport chain of insect mitochondria and as a result inhibits cellular respiration at that point. The reaction, reversible as a result of light stimulation, was therefore less likely to prove fatal.

First gas mixtures of carbon monoxide and pure oxygen were made in the laboratory but determination of the exact concentrations was found to be difficult so further supplies were purchased as mixtures of known composition from Messrs. Hilger & Watts, I.R.D., Ltd. It was considered that the high concentration of oxygen in the mixture might also affect the respiratory rates of the experimental animals. A small experiment was therefore performed in order to compare the respiratory rate of Drosophila melanogaster vestigial males in air with that of the same animals in pure oxygen. As a result of these experiments and the problem of accurate determination of concentrations of carbon monoxide in laboratory-made mixtures, it was decided to purchase cylinders of 5% and 10% carbon monoxide in air. Both mixtures had a guaranteed accuracy of $\pm$ 5% CO content.

Experimental animals and conditions

The animals used were the same flies as those used in individual respiratory rate measurements in air. Experiments on the response to metabolic inhibitors and pure oxygen were performed immediately following the individual determinations in air.

Measurements

Measurements were made in the same 7 cc Warburg flasks as for the previous individual respiratory rate in air determinations. Flies were not removed from the flasks between experiments. Because of the design of the small flasks the entire respirometer had to be removed from the water bath for the purpose of administering the gas. Each flask, containing a fly, was given a 15 sec flushing with the appropriate gas mixture. The flask stoppers were then closed and the flasks returned to the water bath at 27° C as quickly as possible. Each flask was allowed thirty minutes equilibration time in the water bath before readings were made. In all cases readings were made at fifteen minute intervals with the aid of a hand lens.

As mentioned previously the recovery from carbon monoxide poisoning is accelerated by light and it was therefore deemed necessary to perform all experiments in the dark and all readings with aid of a hand torch. In order to obtain as exact a comparison as possible with the individual respiratory rates of flies in air,

these latter experiments were also performed in darkness. Another advantage accruing from this was thought to be that flies might be less active in the dark than the light and that the respiratory rate might therefore more nearly approach a basal figure.

One series of experiments was performed using 10% carbon monoxide in air mixture, one series with 5% carbon monoxide in air and a small series using pure oxygen. In all cases flies were of known age and determinations were made on adult flies of varying ages from eclosion to death.

Results

Response to pure oxygen

Twenty male vestigial flies were used for each experiment and for ease of expression the results were expressed as the average amount of oxygen consumed per fly per hour.

TABLE VII. Average number of µl. O_2 per fly per hour

	Air	Oxygen
Expt. 1	3.11	2.63
" 2	3.11	2.56
" 3	2.81	2.99
" 4	3.16	2.58
" 5	2.46	2.42
Total O_2 consumption per 100 flies	14.65 x 20 µl. O_2	13.18 x 20 µl. O_2

$\therefore$ Ratio $\frac{O_2}{air}$ = 0.899

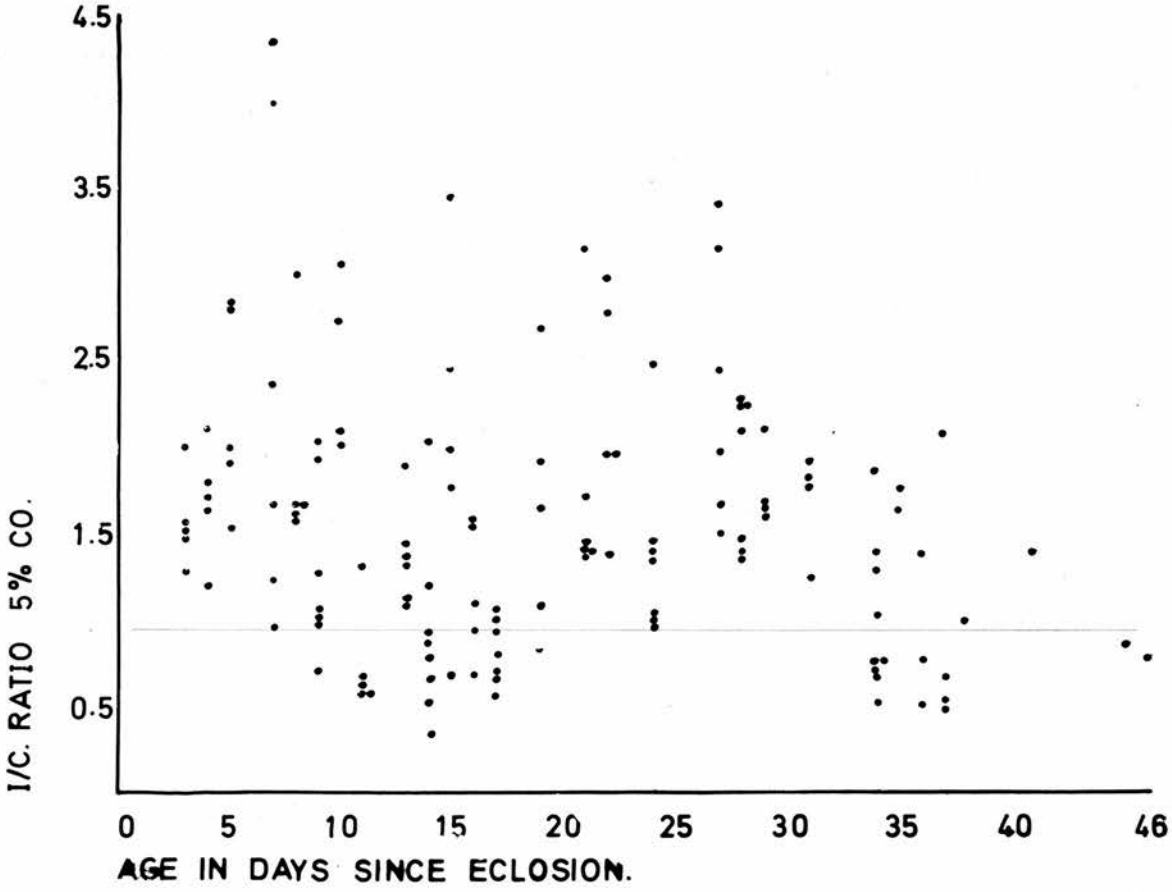
$\therefore$ Inhibition 10%

Graph G shows the results of the determinations of the effect of 5% carbon monoxide in air mixture on 120 male vestigial ebony flies. Of these 25 flies exhibited some degree of inhibition, 7 flies showed no effect, and 88 showed stimulation, i.e. 21% inhibited; approx. 6% no effect; approx. 73% stimulation.

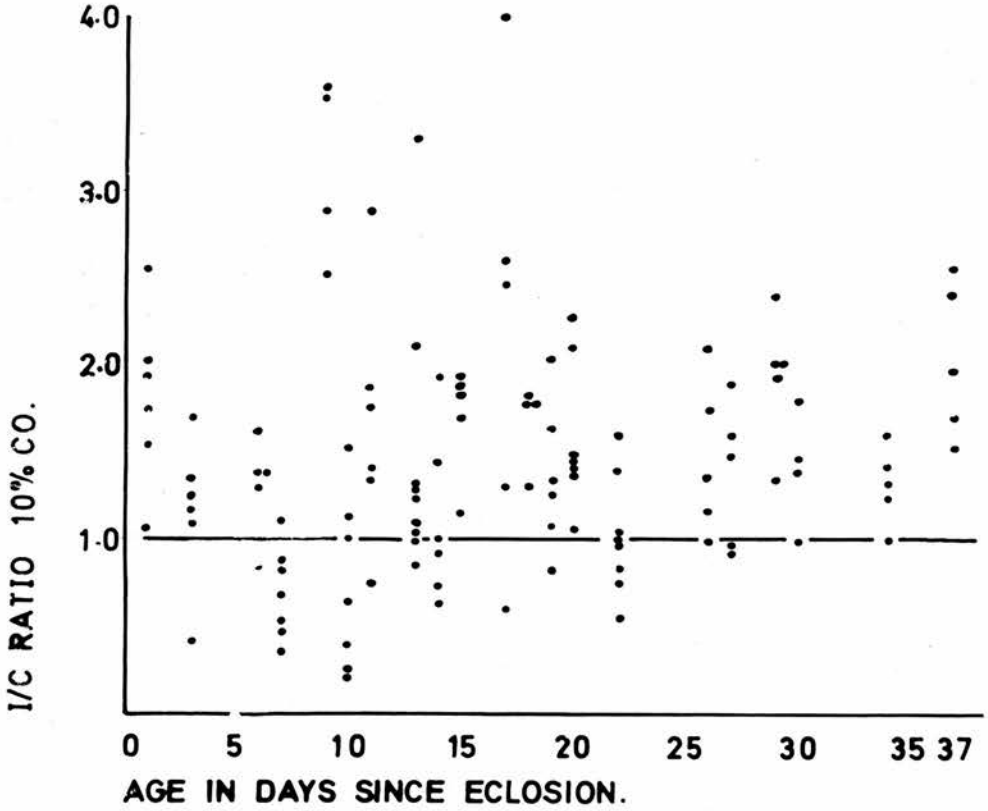
Graph H shows the results of the experimental series which used 10% carbon monoxide in air. Of a total of 143 flies examined, 32 showed inhibition, 2 no effect, and 109 stimulation, i.e. 22.3% inhibited; 1.4% no effect and 77.3% stimulation.

In this latter series the graph shows a decrease in the number of flies that suffered an inhibition in respiratory rate with increasing age until no inhibition occurred after 27 days from eclosion. In the case of the 5% carbon monoxide series inhibition occurred until 46 days from eclosion.

GRAPH G.


4.5
3.5
2.5
1.5
0.5
I/C. RATIO 5% CO.
0
5
10
15
20
25
30
35
40
46
AGE IN DAYS SINCE ECLOSION.

GRAPH H.


4·0
3·0
2·0
1·0
I/C RATIO 10% CO.
0
5
10
15
20
25
30
35 37
AGE IN DAYS SINCE ECLOSION.

Discussion

Effect of pure oxygen

The results of this experimental series as shown in Table (VII) suggest that an inhibition in respiratory rate of approximately 10% occurs when flies are subject to pure oxygen. If the one result showing stimulation is discounted the inhibition in respiratory rate would be approximately 14%.

These figures whilst they may not be taken as conclusive proof of inhibition actually occurring were sufficient to suggest that it would be advisable to discount the measurements made using carbon monoxide in pure oxygen. Therefore only results of observations made using the Hilger & Watts mixtures have been taken as worthy of consideration.

The abberrant result might, it is thought, be explained with reference to the work previously performed in this laboratory and already mentioned. Specific notice was taken previously of the sudden increase in respiratory rate of flies immediately prior to death. Such a condition occurring in one of the flies in Expt. 3 would perhaps be sufficient to cause the small increase of approximately 6%. A fairly intensive burst of activity by one or more of these flies might also account for this latter result.

The results of the experiments shown in graph G. show that the effect of the administration of a mixture of 5% carbon monoxide in air to male Drosophila melanogaster of the mutant vestigial ebony is one of stimulation. For each fly that is inhibited, 3.5 flies are stimulated. The expectation was that, with increasing age the stimulatory effect would be greater. However reference to the graph shows that this was not the effect obtained. The results of administering 5% carbon monoxide on all young flies below the age of 9 days was stimulation. Between ten and twenty days there was a considerable amount of inhibition, approximately 38.5%. Again between twenty and thirty-three days there was no inhibition but from thirty-four to forty-six days there was inhibition in 54.5% of cases. In the two cases where flies survived beyond forty days there was inhibition.

Provided the findings of workers previously cited are accepted as a basis from which extrapolations to whole animals may be made, it would seem that the concentration of carbon monoxide in these mixtures is too low to have any serious effect on the respiratory rate of the whole animal.

The results of the similar set of experiments with 10% (Graph H) carbon monoxide show a different trend where the effect of the inhibitor on animals of increasing age is more pronounced.

In this series inhibition occurs from the second day until the twenty-seventh day. After that the effect is one of stimulation. This is what one would expect. That is that young tissue might exhibit inhibition when exposed to respiratory inhibitors which would stimulate older tissues. It would seem therefore to be probable that at this concentration of metabolic inhibitor the whole animal mimics the response that one would obtain from tissue slices of organs of other animals. However the effect of the inhibitor is not constant for each animal. The $^{i}/_{c}$ ratio varies considerably even on the thirty-seventh day. As this is the case it is obvious that the metabolic response is insufficiently constant at this concentration of carbon monoxide to provide a parameter for the measurement of physiological age in male Drosophila melanogaster of the mutant vestigial ebony.

Comparison of graphs G and H would suggest that a further series of experiments should be performed with concentrations in the regions of $17\frac{1}{2}$ and 30% carbon monoxide. Provided the mixture is kept below a lethal level, it is possible that a more consistent result might be obtained.

The lack of consistency in the results obtained with 5% carbon monoxide mixture might be expressed as a measure of the ability of the animal's tissue to recover from the effect on the cytochrome chain. As previously mentioned this reaction is reversible under the influence of light. If the 5% mixture has such a small effect

then it is possible that the reaction at this concentration is more easily reversible. Even though the experiment was performed in the dark, it was almost impossible to exclude all light from the laboratory. The amount of light which did enter was perhaps sufficient to catalyse the dissociation reaction. With the greater concentration of carbon monoxide in the 10% mixture the amount of inhibitor combined with terminal cytochrome may well be greater. Therefore the small amount of light entering the laboratory might well not have been sufficient to catalyse such a high proportion of dissociation reaction. It is not clear if each animal recovers at the same rate. If animals do recover at different rates then this might explain the different $^{i}/_{c}$ ratios obtained for each animal. This difference could well be reinforced by the different effect, if any, of different physiological age.

The results of the 10% carbon monoxide series would suggest that further experimentation along these lines might produce results which would enable determination of the physiological age of the animal. If possible a series should be performed on individual animals continuously throughout adult life. By this means the $^{i}/_{c}$ ratio for individual animals throughout life might be determined. As will be seen from graph H there are several results which show an $^{i}/_{c}$ ratio of 1. This could possibly represent the change-over point from inhibition to stimulation and might well indicate a well

determined physiological age of the animal. It is not known at just what physiological age this change could occur, but ideally it would be at the half-way mark if the "rate of living" theory of Raymond Pearl were correct. From the results shown in the graph it would seem highly unlikely that this was the half-way mark in the adult life span but perhaps more closely approaching the end. The work of Maynard Smith, previously mentioned, would suggest that a more likely stage in the life cycle represented by an $^{i}/_{c}$ ratio of 1.0 might be the point at which the irreversible dying period commences. Graph G also shows several points where the $^{i}/_{c}$ ratio is either 1.0 or a very close approximation thereof. This would further stress this possibility.

A check was kept on several animals, but not all, as to the length of life after gassing. Several died three days after being subjected to 10% carbon monoxide. After twenty-six days, no fly survived more than five days after being subject to 10% carbon monoxide. The number of animals watched after experiment was small and therefore this is only suggesting lines of further experiment which might not confirm the above suggestions.

Conclusions

(1) Results of the 5% carbon monoxide series suggest that this method of respiratory inhibition is too irregular to provide a means of measuring physiological age.

(2) Results of the 10% carbon monoxide series suggest a more positive stimulatory effect with increasing age.

(3) This reaction to 10% carbon monoxide mixture is not sufficiently regular under the conditions of the experiment to provide a means of measuring the physiological age of this species.

(4) It is suggested that the response to 10% carbon monoxide/air mixture of a single animal throughout life might well repay investigation.

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