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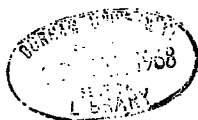
SOME ASPECTS OF THE PHYSIOLOGY OF AGEING IN THE BLOWFLY, CALLIPHORA ERYTHROCEPHALA, MEIG.

by

M. A. Tribe, B.Sc. Dip.Ed.

Being a thesis presented in candidature for the degree of Doctor of Philosophy of the University of Durham, July 1967.

University College,
Durham.



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GLOSSARY

ADP	adenosine-5-diphosphate
ATP	adenosine-5-triphosphate
ATP-ase	adenosine-5-triphosphatase
BSA	bovine serum albumin (fraction V)
c/s	cycles per second
DNP	2:4 dinitrophenol
DNP factor	the rate of Mg^{2+} activated ATP-ase activity in the presence and in the absence of DNP
EDTA	ethylene diamine tetra-acetate (sodium salt)
Lx_{10}, etc.	indicate time at which 10 per cent of flies in a series survived, etc.
NAD^+	nicotine-adenine dinucleotide (oxidised form)
NADH	nicotine-adenine dinucleotide (reduced form)
P_i	inorganic phosphate
P:O	P:O ratio (number of molecules of phosphate esterified per atom of oxygen consumed)
QO_2	oxygen uptake expressed as μl per hour and per mg. protein or per mg. dry weight.
R.C.I.	respiratory control index (the rate of oxidation in the presence and in the absence of ADP)
Tris	tris-(hydroxymethyl)-aminomethane

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SUMMARY

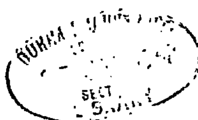
Life tables were constructed for four lines of *Calliphora erythrocephala* Meig., under different dietary conditions.

All the survival curves obtained from these tables followed the typically rectangular shape of an ageing population, except for males and females which had been reared from underfed larvae and subsequently given a sugar/water diet only.

Whilst all flies could survive quite well on sugar/water only, the addition of protein (liver) to the diet significantly increased longevity in all cases. The mean survival time of wild type S males being 55.3 ± 2.0 days, and females 49.9 ± 2.2 days, with Lx_{10} in both cases at approximately 85 days. However, a liver/water diet only was found to be little better than water alone. Protein added to a sugar/water diet at the 60 per cent survival level increased longevity significantly ($P < 0.001$), although its action was not immediately apparent, and in about 30 per cent of the flies there was no improvement in survival. Liver added to a sugar/water diet at the 20 per cent survival level did not significantly alter the life span of survivors.

These results are considered as supporting the threshold idea of ageing proposed by Maynard Smith (1962).

Determinations of the effect of age on the physiological performance of wild type S were carried out on male blowflies under three different experimental conditions, (1) at rest, (2) 'free moving', and (3) in flight.



Mean oxygen consumption for young flies at rest was about 1.6 μl O_2/mg. wet wt./hr. . This value rose significantly in old flies to be about 2.0 μl O_2. In 'free-moving' flies the oxygen consumption ranged between 2.4 μl O_2 on the day of eclosion to 8.9 μl O_2 maximum at 7 days of age, and then fell progressively with age to 3.9 μl O_2 at 80 days. Changes in oxygen consumption of 'free-moving' flies were correlated closely with their activity.

Flies on the day of emergence flew for short periods only, and had low wing-beat frequencies (100 c/s). There was a progressive rise with increasing age in both wing-beat frequency and oxygen consumption in flight, until at 50 days they were 184 c/s and 60 μl O_2/mg. wet wt./hr. respectively. After 4 days of age the average flight duration was 6 to 7 minutes, which after about 60 days had fallen to 2 to 3 minutes. As flies aged it also became progressively more difficult to elicit the 'flight reflex'.

In all experiments there was a decline in efficieny and physiological performance as a function of age, but the physiology of very young flies was considerably affected by developmental rather than age-related changes.

The increase in oxygen consumption in very old flies at rest suggests a loss of efficiency, and this point is discussed in relation to work reported on changes in the rate of protein synthesis in old *Drosophila*.

Measurements of oxygen consumption were made on preparations of blow-fly thoracic flight muscle as a function of age, under four different experimental conditions.

Results in all cases showed a significant increase in oxygen uptake with age from a value of about 10 to 11 μl O_2/mg. dry weight muscle/hour in preparations from 7 day-old flies to between 18-19 μl O_2/mg. dry weight muscle/hour in preparations from 85 day-old flies.

These results confirmed earlier findings with the whole animal at rest.

Measurements made with 2 per cent serum albumin in the medium suggested that partial in vivo uncoupling was taking place in old tissues, which could change the ADP/ATP ratio, and as a consequence increase the QO_2.

In vitro experiments on isolated sarcosomes measuring (1) oxidative phosphorylation, (2) respiratory control, and (3) ATP-ase activity, confirmed that partial uncoupling of oxidative phosphorylation was taking place, but unlike the tissue studies no increase in QO_2 values was observed. Results showed a significant reduction in P:O ratios with increasing age using α-glycerophosphate as substrate, from 1.7 in young flies (8 to 10 days old) to 1.2 in old flies (70 to 90 days old). Similarly, stimulation of ATP-ase activity by 2:4 dinitrophenol was much greater in young flies (producing high DNP factors) than in old flies. Also better respiratory control was obtained with young sarcosomal preparations than in old preparations, although it appeared that the time lag before addition of ADP was a critical factor in producing any stimulation.

A Coulter Counter analysis showed marked changes in the size distribution and possibly total number of sarcosomes throughout the life span of the imago. In young imagoes (0 to 3 days of age), there was a high percentage

of small sarcosomes (1.8 μ diameter), whereas in older flies (50 to 85 days old) there was an increase in the frequency of larger sarcosomes (2.5 to 3.0 μ), although in 85 day preparations there was also an increase in the number of small sarcosomes. These changes indicated that it is the alterations in the sarcosomal ultrastructure which are primarily responsible for partial uncoupling taking place in the sarcosomes from old flies.

Other possible factors affecting ATP synthesis and utilisation during senescence, particularly protein synthesis, are also discussed.

The results obtained with isolated sarcosomes are directly related to the observations made on the whole animal, and are considered to be responsible for the decline in activity and the deterioration in flight performance.

Chapter 1.

GENERAL INTRODUCTION

The main objectives of this work on Calliphora erythrocephala were twofold. First, by constructing life tables, to establish whether this insect exhibited senescence, and secondly to follow the age-related changes associated with a specific physiological function, namely respiration.

The definition or measurement of ageing is difficult because of the complex interaction of biological and environmental factors involved in this process. Senescence, or the biology of ageing, has been succinctly defined by several leading authorities. For example, Medawar (1952) defines senescence "as that change of the bodily faculties and sensibilities and energies which accompanies ageing, and which renders the individual progressively more likely to die from accidental causes of random incidence".

Comfort (1956, 1964) gives the following definition: "Senescence is a deteriorative process. What is being measured, when we measure it, is a decrease in viability and an increase in vulnerability ..... Senescence shows itself as an increasing probability of death with increasing chronological age. The study of senescence is the study of the group of processes, different in different organisms, which lead to this increase in vulnerability".

Strehler's definition (1962) of senescence as "the changes which occur generally in the postreproductive period and which result in a decreased survival capacity on the part of the individual organism", is essentially similar to those above.

All three definitions consider ageing to be a deteriorative process. One in which there is a progressive loss of vigour with the passage of time. In other words, as an organism ages it becomes more susceptible to both extrinsic and intrinsic factors, which in turn lead to a failure in homeostasis. The increased probability of death with increasing age can be expressed as the force of mortality (μ). Its value at any given age (t) is given by the following negative differential equation,

$$\mu_t = -\frac{dn}{dt} \cdot \log_e . n,$$

where n is the number of individuals which have survived to age t. If there is an increase in the force of mortality with age, then ageing processes must be occurring, provided, of course, that old individuals are not being subjected to more severe conditions than young ones, (Comfort, 1964; Maynard Smith, 1966).

The difference between a population of animals dying in the wild, and a population of female Drosophila subobscura exhibiting senescence under laboratory conditions at 20°C is shown in Figures 1 (a) and (b) (data after Maynard Smith, 1962). In (a) the population is not subject to senescence, but rather to mortality brought about by all random events. The decline, therefore, in number is logarithmic, and the force of mortality constant. These animals have died from random causes which would have killed them at any age. On the other hand, Figure 1 (b) shows the characteristic, rectangular-shaped survival curve, which is typical of a population showing senescence, and within which the force of mortality increases throughout life.

An alternative method of demonstrating senescence in a given species of animal is to measure a particular physiological function in individuals of different ages, or better, the same individuals at different ages. The difference between the two methods is given in Figure 2 (data after Hollingsworth and Bowler, 1966). It may be said that the form of the life table is the result of the effects of the biological and environmental factors which affect survival within a population of animals, whereas the relative significance of the various factors implicated in ageing, can best be assessed by physiological studies.

The present research, therefore, was undertaken with both these methods of measurement in mind, for although the life table relates to differences in random events between individuals, it tells little about the time course of specific age-related changes in individuals.

Man's interest in ageing goes back into early history, and has led to a variety of theories to account for, and combat, the ageing process, see Comfort (1964).

Earlier than 1950, most theories tended to be metaphysical in character, and as a result there appeared to be little chronological progress.

From the early literature, the work of Minot (1908, 1913) deserves attention, since he related senescence to the decline in growth. Minot considered in fact that senescence was the direct result of cellular differentiation. This idea was elaborated by the experimental studies of Child (1915), who showed that cellular differentiation and senescence were reversible (rejuvenescence) in planarians.

Perhaps the next major contribution to the biology of ageing, came in two books written by Pearl. The "Biology of Death" (1922) and perhaps the more important "The Rate of Living" (1928). The "rate of living" theory suggested that several factors, particularly temperature, directly accelerated or retarded developmental and metabolic processes, which in turn could either increase or postpone senescence. Pearl, and several authors subsequently, came to the conclusion that the life span of poikilotherms in particular, was temperature-dependent. There is no doubt that temperature profoundly affects the life span of poikilotherms since, by raising the temperature, the life span is shortened. However, as a generalisation, the 'rate of living' theory has been challenged by Clarke and Maynard Smith (1961), particularly with regard to the rate of ageing. In Drosophila subobscura at least, the rate of ageing is independent of temperature between 15°C and 30°C, Clarke and Maynard Smith (1961), and Hollingsworth (1966). As a result, a "threshold" theory of ageing is preferred by these authors for poikilotherms. For evidence in support of this theory, see Chapter 3.

A further question of importance, raised by Bidder (1932), is whether all animals age, or whether ageing is apparent in only some animals? Bidder pointed out that in several fish there was no ground for suspecting that mortality ever increased with increasing age. He therefore suggested that ageing was directly related to the evolution of determinate growth and final absolute size. It might be possible, therefore, to distinguish between two categories of vertebrates, one in which the life span is fixed,

as in mammals, and the other where it is not fixed, as in fish.

Again, it now seems likely, from the work of Comfort (1960, 1961), that some fish at least, do show senescence like other vertebrates.

Currently, three principle theories of ageing are accepted, and these are reviewed in full by several authors, (Lansing, 1952; Szilard, 1959; Strehler, 1962; Maynard Smith, 1962, 1966, and Comfort, 1964).

Briefly, the theories may be classified as ageing changes taking place in (i) 'inert' cellular materials, in (ii) dividing cells, and (iii) through loss of irreplaceable cells. The agencies which bring about these changes can be divided into two categories.

(1) Intrinsic factors

These determine the time of onset, the course, and the duration of senescence. For example, the cessation of growth leads to a failure in replacing regressing cells, and as a result might also lead to a breakdown in the efficiency of existing cells. Alternatively, depletion of essential cellular materials, or an accumulation of harmful substances, could have serious consequences for the organism, and thus precipitate senescence.

(2) Extrinsic factors (environmental 'wear and tear')

These factors cumulatively increase the vulnerability and decrease the viability of an organism. For example, Szilard (1959) proposed that radiation induces somatic mutation, the accumulating effects of which lead to cell death. On genetic grounds, this theory has been criticized

by Maynard Smith (1959). However, the fact that radiation usually shortens the life span of most animals is not disputed, although it is still not clear whether it does so by causing the same deteriorative (senescent) changes to occur more quickly, or whether it causes an additional kind of deteriorative change, peculiar to radiation. In some instances in female Drosophila, specific radiation doses actually prolong longevity by decreasing fecundity, Lamb (1964).

Other extrinsic factors of importance include temperature (discussed above), and diet. In rats, as in several other animals, the classical feeding experiments of McCay and his associates (1939, 1941 and 1956) have shown that the life span may be altered dramatically by changes in the quantity and the quality of the diet. It will be shown later (Chapter 3) that this observation is equally true of Calliphora.

Lastly, under this heading, pathogenic and parasitic organisms must be considered for they reduce vigour, and as a result shorten the life span, although it is questionable whether they increase the rate of ageing.

With so many possibilities, it seems most likely that senescence and eventual death are brought about as a result of the interaction of many factors, which may well be different in different organisms, rather than by a single limiting ageing process. The mere synchrony of the ageing changes taking place in various organs and organ systems of mammals, for example, would however indicate a single or central controlling process. But as Maynard Smith (1962) points out, just because different ageing processes are synchronised, it does not prove that they are physiologically inter-dependent,

since selection would be expected to produce synchrony.

Strong support for a 'multiple' theory of ageing comes from the experiments of Krohn (1962, 1966), on 'heterochronic' transplantations of ovaries and oocytes in mice. Most of Krohn's results support the idea that organ systems age independently, and also that specific types of cells (in this case oocytes), do not in themselves follow the same ageing programme of the whole organ, of which they are part.

However, even if the 'multiple' theory of ageing is correct, which seems likely, it is still not clear whether cells age because they are in old organisms, or whether organisms age because their cells are old.

The extensive nature of the literature concerned with ageing in Insects, (see Clark and Rockstein, 1964), reflects the value of these short-lived, easily propagated animals in ageing studies. Many authors have studied ageing in Diptera, particularly in Drosophila. However, few of these studies have been extended to the physiological and biochemical levels, since most of them have been concerned with the construction of life tables. The only life table previously reported for Calliphora, are those by Feldham-Muhsam and Muhsam (1945). Their life table suggested that ageing in Calliphora is essentially different from ageing in other Diptera.

It was therefore decided to construct life tables for Calliphora erythrocephala to establish the basis for the differences between this species and other Diptera. It was also decided to follow the changes in respiration at the whole animal, tissue, and cellular levels as a function of adult age.

From the work reported here on *Calliphora*, it is hoped that, by following the changes in a specific physiological function, it may be possible to draw some generalised conclusions concerning some of the fundamental changes which occur during the ageing process.

Chapter 2.

GENERAL MATERIALS AND METHODS

The choice of animal.

The blowfly, Calliphora erythrocephala, Meig., was chosen for this study for a number of reasons.

First, most Diptera lend themselves very readily to ageing studies, because of their comparatively short life span, and their availability in large numbers. These factors are of major importance when considering the ratio of time spent to productivity of research.

Secondly, laboratory stocks of Calliphora were readily available from a number of sources, and although these stocks are not genetically as pure as some lines of Drosophila, the 12 year old, wild type, mass-mated, laboratory culture used in most of the present work, was considered to be a sufficiently pure line, although stress has been laid on animals of known genotype.

Third, in common with all Diptera, the imago fails to grow further by cell division, with the exception of the gonads. This means that at a constant temperature, and after a period of post-emergence maturation, a fly is for a time in a steady state condition. A period, during which all its physiological systems are most efficient. Any failure or deterioration in the steady state condition with the passage of time is, by definition, an ageing process. It is possible to measure some of these physiological processes directly, (see Chapter 4), and thus gain an insight into the ageing changes taking place within a fixed number of regressing cells.

Methods for rearing and maintaining flies.

1. Housing the flies.

Blowflies for life-tables and physiological studies were kept in metal framed cages, covered with muslin, measuring 18 x 18 x 18 inches, similar to the design proposed by Parkin and Green (1958), see Figure 3. The bottoms of the cages inside were covered with corrugated paper, as flies were found to be more able to right themselves on the uneven surface. The cages in turn were kept in wooden constant temperature cabinets, measuring 6 x 2 x 2 feet. The cabinets were continuously illuminated by fluorescent lighting, the temperature being thermostatically maintained at $24^{\circ}C \pm 0.5^{\circ}$. The relative humidity in the cabinets was not controlled, but frequent recordings of relative humidity showed it to be 50 per cent $\pm$ 10 per cent.

2. Culture methods.

Eggs were obtained from stock cages, the females being supplied with fresh ox liver for oviposition. The eggs were incubated at $24^{\circ}C$ in a separate incubator and the young larvae transferred to a larger piece of fresh liver (about 100 larvae per 100 grams of liver) in large, deep sided, glass, crystallising dishes covered over with muslin. The dishes also contained sufficient peat moss or sawdust to facilitate pupation. After pupation the decaying liver was removed and the pupae transferred into wide-necked conical flasks (750 ml capacity), containing a little fresh sawdust or peat moss, and plugged with a cotton wool stopper. The sawdust was not permitted to become absolutely dry during pupation, and the contents of the flask were gently shaken from time to time, to permit circulation of

air to the pupae. During the whole developmental process the temperature was maintained at $24^{\circ} \pm 0.5^{\circ}C$, the time taken from egg to imago being 12-14 days.

For most of the experiments reported in this thesis, a wild type culture of Calliphora was used. These flies were reared from pupae originally obtained from the Pest Infestation Laboratories, Slough, Bucks., where they had been kept in a mass culture for over 10 years. They are referred to in the life table and subsequent studies as wild type S.

Three other genotypically different lines were also used in the life table studies, and are reported in detail in Chapter 3. The methods for rearing and maintaining these flies were the same as those described for wild type S.

Chemical reagents.

All chemical reagents used in this work were of the highest purity commercially, and all solutions were made up in glass-distilled, deionised water.

Chapter 3.

LIFE TABLES FOR CALLIPHORA ERYTHROCEPHALA AND THE EFFECT OF DIET ON LONGEVITY

INTRODUCTION

Previously, life tables for Calliphora erythrocephala had been cited by Feldham - Muhsam and Muhsam (1945). From their findings it would appear that survival curves for Calliphora are somewhat different from those constructed for other Diptera, for example, Drosophila melanogaster (Loeb and Northrop, 1917; Pearl and Parker, 1922; Gonzalez, 1923); Drosophila subobscura (Clarke and Maynard Smith, 1955; Hollingsworth and Bowler, 1966); Musca vicina (Feldham - Muhsam and Muhsam, 1945); Musca domestica (Rockstein and Lieberman, 1958). The diagonal survival curves (type-C as proposed by Pearl and Miner, 1935) for Calliphora as described by Feldham-Muhsam and Muhsam (1945), resemble more closely the type of survival curve expected from a population in the wild, instead of the more rectangular or negatively skew curves (A and B types of Pearl and Miner) obtained for other Diptera so far investigated, with the exception of the vg mutant of D. melanogaster (Gonzalez, 1923). Consequently, it was considered necessary to evaluate the work of Feldham-Muhsam and Muhsam on C. erythrocephala before commencing physiological studies, for the diagonal shape of Feldham-Muhsam and Muhsam's survival curves indicate that their populations of Calliphora were not dying of senescence (Comfort, 1964).

There is a large literature for a variety of animals, demonstrating that nutrition is an important factor in modifying longevity. Many authors have shown that underfeeding both in the developmental and later stages, actually prolongs life, for example in Planaria dorotocephala (Child, 1915); rotifers (Rahm, 1923); Daphnia longispina (Ingle, Wood and Banta, 1937); Dermacentor variabilis (Bishopp and Smith, 1938); laboratory rats, (McCay, Maynard, Sperling and Osgood, 1941; Ross, 1961).

On the other hand, Kopec (1928), found that whilst controlled restricted feeding may prolong the life of the developmental stage, food restriction of adult D. melanogaster never increases the imaginal life span. Alpatov (1930) reared small images from experimentally underfed larvae, but found that the longevity of these adults was not significantly different from those reared under standard conditions.

Again, it is difficult to evaluate the role played by the addition of protein to the diet of adult insects. Haydak (1953) for example, showed that although longevity of three different species of cockroaches decreased with increasing levels of protein, it also increased to a certain extent at very low protein levels. Haydak found too that the requirements of the nymphal form were not the same as those of the adult. Liles and Delong (1960) have shown in Aedes aegypti, that the addition of protein to an otherwise sugar/water diet reduced the adult life span.

However, Rockstein (1957) found that protein (KLIM) in the diet of M. domestica significantly prolonged the life of females, but did not significantly alter the life span of males.

In *C. erythrocephala* Sedee (1956) has made a thorough study of dietary requirements in the larva, particularly regarding intermediary protein metabolism. Strangeways-Dixon (1961, 1962) has discussed the relationships between nutrition, hormones and reproduction in female *C. erythrocephala*, but the effect of the addition of protein to the diet of males has not been clarified in the literature. Fraenkel (1940) for example, suggests that protein is only essential for the development of eggs and does not increase the duration of life.

Another dietary feature, which may be of importance, but which has not been examined in this study, is the effect of vitamins on longevity. In *Calliphora*, the importance of the water soluble B vitamins has been established for the larvae, in order to meet the quantitative requirements for growth and development, Sedee (1956). The importance of B vitamins in the imago has not been established. Investigations by Greenberg and Burkman (1963) on the effect of B vitamins and a mixed bacterial flora on the longevity of germ-free adult houseflies, *Musca domestica* showed that there was no increase in longevity.

What does appear to be true, is that the dietary needs of the adult are not readily distinguishable from nutrient reserves, because Sang and King (1961) found that with *Drosophila melanogaster*, the adult requirement of vitamins was largely dependent on the status of the larval supply.

Life tables for *Calliphora erythrocephala* were constructed therefore for the following reasons.

(1) To establish whether *Calliphora* exhibits senescence under controlled laboratory conditions, by the definition given in Chapter 1.

(2) To establish the importance of diet, and particularly of protein in all stages of the life history, and its consequent effect on longevity.

(3) As a basis for the assessment of the physiological and biochemical studies to be reported later.

MATERIALS AND METHODS

The genotypes of the blowflies used in construction of the life tables.

Four genotypically different lines of *Calliphora erythrocephala* were employed in this investigation.

(1) Wild type S, as reported in Chapter 2.

(2) The mutant form "chalky-eye" or *c*, were cultured from pupae obtained from Dr. H. Langer, of the University of Würtzburg, Germany (see Langer, 1962). This mutation arose spontaneously in 1960 from a stock of *Calliphora* in Berlin. The expression of the gene is seen in both males and females, (see Figure 4) and in some generations of flies there is quite a high reverse mutation rate (1 to 2 per cent) back to the wild type.

(3) The mutant form "white-eye" or *w*, were cultured from pupae obtained from Miss H. Tscharntke of the University of Munich. This mutation

of Calliphora was first described by Tate (1947). The expression of the gene is sex-linked and sex-limited to females, the males appearing phenotypically as the wild type. This particular stock proved to be very inviable, and great difficulty was experienced in maintaining the culture and also in producing sufficient numbers for a life table at 24°C. There was also a greater percentage (12 per cent) of deformed imagoes at eclosion, compared with the wild type S or o-mutant forms (2 to 3 per cent). Such deformed imagoes were not used for study from any stock.

(4) For one life table a fertilised female was caught several miles from Durham and allowed to ovipost on liver in the laboratory. Survival curves were constructed from her F_1 progeny and referred to as wild type D.

Construction of life tables.

Flies were reared and maintained by the standard method (Chapter 2) at 24°C, except that in the "underfed experiments" reported here, about 300 young larvae were placed on 100 grams of liver. Treatment was made more effective by using smaller crystallising dishes. The difference in the size of the flies can be seen in Figure 4.

Sufficient flies for a life table were taken off on the same day of eclosion as far as possible, but where numbers had to be made up over 2 or 3 days, coloured cellulose dope markers were used on the back of the thoraces to facilitate accurate daily readings from the time of eclosion.

Unless otherwise stated, flies were given water, together with cane sugar moistened with a little water to cake it. When given fresh liver, (in most cases once a week), it was covered with a layer of perforated Parafilm, allowing feeding and egg laying to take place, but preventing flies sticking to the liver. Although liver is chemically complex, it is the protein content which is considered as the important dietary constituent in this study.

Usually between 60 and 100 flies were placed in each cage at a time.

In the present study males have been chosen for most of the work, as the physiology of females is complicated by the stress of egg laying (see Figure 13).

The following definitions and abbreviations have been used in the text:

Mortality - the number of individuals dying in a stated interval of time, divided by the number of individuals alive at the start of that period.

The concept of the force of mortality has already been discussed in Chapter 1 of this thesis.

Lx_{50}, Lx_{20} etc. indicate times at which 50 per cent, or 20 per cent, etc., of flies in a series survived.

RESULTS

Figures 5 and 6 show the effect of diet on longevity of wild type S males and females respectively. It can be seen that liver added to a sugar/water diet caused a marked increase in survival. On the other hand,

the omission of sugar from the diet caused a marked fall in survival. In fact, a liver/water diet alone was little better than water only (see Figure 5). The mean survival times for wild type S males and females on the various diets are not significantly different from each other, see Table 1. Mortality curves for wild type S males, fed on sugar/water and sugar/liver/water diets (Figure 7) show typically an increase in the force of mortality with age.

Figure 8 shows similarly constructed survival curves for c-mutant males. It can be seen that sugar and liver in the diet had the same survival values as in the wild type S males. However, the mean survival times of c-mutant male flies on a sugar/water diet, 25.3 ± 0.93 days, and on a sugar/liver/water diet, 44.5 ± 1.25 days, were significantly less ($P < 0.001$) than those of similarly fed wild type S males. On the other hand, c-mutant females, with a mean life span of 51.3 ± 3.94 days, were comparable with wild type S females on a sugar/liver/water diet, see Table 2. The c-mutant males fed liver/water only, lived longer, 7.2 ± 0.29 days, compared with the wild type S males, 5.6 ± 0.23 days, ($P = 0.001$). This may be because viability in the developmental stages of the c form is lower than in the wild type S and consequently a smaller number of larger pupae are produced, presumably with greater food reserves, and thus the imagoes produced are more able to tolerate an inadequate diet.

In contrast, both w-mutant males and females lived significantly shorter than any of the other genotypes investigated, on a sugar/liver/water diet. Females, too, with a mean life span of 43.39 ± 2.29 days lived significantly longer than males, 35.52 ± 1.9 days ($P = 0.01$), see Table 3 and Figure 9.

Figures 10 and 11 show the effect of adding liver to the diet of sugar/water fed flies at Lx_{60}, in the wild type S males and c-mutant males respectively. The effect of the liver-protein on survival was delayed to about the same point in each case, and in fact in only about 30 per cent of the flies was any improvement in longevity observed. The improvement in survival in both strains of flies can readily be seen from the sudden decrease in the force of mortality, see Figures 7 and 12. However, when liver was added to a sugar/water only diet as late as Lx_{20}, no significant improvement was observed, although four flies lived for about 70 days, as against the maximum age of 60 days reached by flies fed sugar/water only.

It can be seen from Tables 1 and 2 that the ratios Lx_{10}/Lx_{50} increase with the addition of liver to the diet at Lx_{60} from 1.55 to 2.47 in wild type S and from 1.35 to 2.50 in c-mutant.

Figure 13 shows the survival curves of male and female wild type S, reared from experimentally underfed larvae, and which were given either sugar/water or sugar/liver/water as imagoes. These underfed flies weighed at eclosion 20 $\pm$ 3.14 mg, as compared with 46.8 $\pm$ 5.43 mg for flies reared under standard conditions. This experiment differed from the last in that the males and females were not segregated. Where no liver was added to the diet, the mean survival times of males, 18.9 $\pm$ 1.0 days, and of females 16.8 $\pm$ 1.1 days, were not significantly different ($P = 0.15$), see Table 1. Underfed wild type S males segregated from females and fed on a sugar/water diet had a mean survival time of 19.9 $\pm$ 0.89 days, not significantly different from the mean survival time of males kept with females.

The mean survival times of males and females fed on sugar/liver/water diet were, however, significantly different from each other, the males surviving longer, 35.5 ± 2.47 days as compared with females 29.4 ± 2.19 days ($P = 0.05$).

As the results presented here differ markedly from those of Feldham-Muhsam and Muhsam (1945), it was considered necessary to construct life tables from the F_1 progeny (wild type D) of a female caught in the wild. The survival curves of the males and females which were kept together and fed sugar and water only, are shown in Figure 14. The mean survival time of females 52.8 ± 1.52 days is significantly longer than that of males 46.8 ± 1.47 days ($P < 0.01$), see Table 4. The rectangualr shape of the survival curves are consistent with those of the laboratory stocks.

Although in general, in insects, females live longer than males, (see Clark and Rockstein, 1964), in the four lines of *Calliphora* investigated here, only two (wild type D, and *w*-mutant) showed significant differences in survival between the sexes. In the other two lines (wild type S and *c*-mutant) no significant differences were observed. The results are summarised in Table 5.

DISCUSSION

The results of the life table studies indicate quite clearly that ageing does occur in adult *Calliphora* under controlled laboratory conditions. It is difficult therefore to account for the results obtained by Feldham-Muhsam and Muhsam (1945) compared with those presented here.

First, the mean survival times of their stocks (F_1 progeny of flies caught in the wild) kept at 18°C and fed on a sugar/liver/water diet were shorter even than the mean survival times of the wild type D males and females, kept at 24°C and fed sugar/water only, see Table 4.

Secondly, Feldham-Muhsam and Muhsam did not segregate male and female flies, but this would only affect the survival time of females and not males, see Table 1.

It is well known from the work of Loeb and Northrop (1917) and subsequent authors that the life span of imagoes and larvae is temperature-dependent. Feldham-Muhsam and Muhsam conducted their experiments (imago only) at 18°C, whereas in the present study the temperature at all stages in the life history was kept at 24°C. It would be expected therefore that the mean survival time of Feldham-Muhsam and Muhsam's flies would be greater than those reported here. This was not the case. Even the relative humidity, which in the present investigation was 50 per cent ± 10 per cent, compared with the controlled 60 per cent relative humidity adopted by Feldham-Muhsam and Muhsam, is unlikely to account for the very significant differences in the two sets of results, especially when one compares the differences in mean survival times at 40 per cent and 60 per cent relative humidity (27°C) obtained by Feldham-Muhsam (1944) for M. vicina.

Thirdly, Feldham-Muhsam and Muhsam report high initial mortality. In this work high initial mortality was only observed in flies which had been underfed as larvae (see Figures 10 and 13).

The diagonal shape of Feldham-Muhsam and Muhsam's survival curves probably reflects the interaction of several factors. Some flies may have been underfed as larvae, and overcrowding may have been an important factor, for they kept their flies in comparatively small cages. It was shown by Pearl, Miner and Parker (1927) that there was an optimum population density for D. melanogaster, below and above which the specific death rates were higher than at the optimum. Although the optimum population density for Calliphora is not known precisely, it seems quite possible that the number of flies to a given cage-volume may be an important stress factor contributing to mortality, particularly in the initial stages after eclosion when the number of flies is greatest. Differing genetic constitution of the stocks could equally well be the dominant factor determining the differences between present results and those of Feldham-Muhsam and Muhsam. However, this seems less likely when it is realised that the results given above are consistent, in that the shape of the survival curves is rectangular, although the genotype of the wild type S, wild type D, c-mutant and w-mutant flies must obviously be different. A fourth factor, which cannot be overlooked is the possibility that Feldham-Muhsam and Muhsam's flies were unhealthy.

Returning to the survival curves shown above, the superior fitness of the wild type D males and females (F_1 from female caught in the wild) can readily be seen in Figure 14. This agrees with the inbred nature of the wild type S stock which have been kept in mass culture for many years. The inbreeding initially took place when factors such as diapause were

eliminated from the stock. The c-mutant and w-mutant are still shorter lived, particularly the latter. Several factors might have contributed to the protracted survival:

(i) A direct gene effect.

(ii) Some inbreeding in the course of maintaining the mutant stock, for Clarke and Maynard Smith (1955) showed that inbreeding significantly affected longevity in Drosophila subobscura.

(iii) The use of a rearing temperature above optimum (22°C as proposed by Tate, 1948; Tscharntke, 1966), to which the inbred lines would be more susceptible, Maynard Smith (1956).

The importance of the quality (Sedee, 1956) and the quantity of the larval diet cannot be understated, for it seems as though survival in the 4 days following eclosion is determined by the food reserves carried over from larval feeding. Therefore, to ensure that large healthy flies are obtained with a normal expectation of life, an adequate diet must be given to the larval stage. This is contrary to the findings of Alpatov (1930) for D. melanogaster, who showed that small flies reared from experimentally underfed larvae had a life span which was not significantly different from flies reared from "normal" larvae.

Regarding the diet of the imago, in all cases where liver was added to the basic sugar/water diet, a significant increase in mean survival time occurred. This is contrary to the suggestion of Fraenkel (1940), who stated that "Addition of protein is essential only for the development of the eggs, as it does not increase the duration of life and there is, indeed, evidence

that it may have the contrary effect". In fact it was found that the addition of protein after 27 days on a sugar/water only diet, caused a marked decrease in the force of mortality in both wild type S and c-mutant males. Hover, this improvement was not immediately apparent. It seems that the processes leading up to death in about 25-30 per cent of the population are irreversible at this stage, and that for these flies the force of mortality increases. For the remaining 30 per cent of survivors, the addition of liver to the diet retards the ageing processes which finally lead to death. It was not surprising therefore that the addition of liver to an otherwise sugar/water diet at Lx_{20} did not significantly lengthen the life span of survivors. The results of these experiments would seem to be evidence supporting a "threshold" theory of ageing, as proposed by Maynard Smith (1962). In this case, when liver is added to the diet at Lx_{60}, 30 per cent of the flies have already passed the threshold and are in the dying phase, and only those which have not yet reached the threshold gain any benefit from the protein added to the diet.

Nevertheless, the most beneficial amount of protein in the diet of Calliphora for maximum longevity has still to be determined. As mentioned previously, Haydak (1953) showed that when cockroaches were given a very high protein diet (91 per cent), longevity was reduced and noticeable anatomical and physiological changes occurred. For example, there was a considerable increase in urate production, accompanied by extensive deposition of fat, leading to swollen abdominal and other organs. There is a parallel to be drawn here with the present work; where flies were given

no alternative to a liver diet, in all cases studied survival was protracted. The very high force of mortality following eclosion in these flies suggests that, although failure of the excretory system to deal with the high protein intake may be a contributory factor, the main agency was an inability to utilise protein, or its component amino acids, as a respiratory substrate. A liver/water-only diet was found to be little better than water alone (see Figure 5).

The superior survival of flies on a sugar/liver/water diet supposes that the addition of protein enhances replacement and repair or retards tissue protein breakdown. The failure of 30 per cent of the individuals to respond to additional protein at Lx_{60} after a sugar/water diet only, might perhaps suggest that ageing is primarily due to a breakdown in synthetic mechanisms (Maynard Smith, 1962), and for 30 per cent of the flies which were in the dying phase, no benefit was obtained from the added protein.

Chapter 4.

PHYSIOLOGICAL STUDIES ON THE WHOLE INSECT IN RELATION TO AGE

INTRODUCTION

In the previous chapter, life tables showed that Calliphora exhibited senescence under controlled laboratory conditions, and that longevity was dramatically affected by diet.

Temperature is another factor which profoundly affects the life span of Insects. For example, Loeb and Northrop (1917) and subsequent authors have shown that the life span of poikilotherms is temperature-dependent. Pearl (1928) proposed a 'rate of living' theory to account for this phenomenon, although more recently Clarke and Maynard Smith (1961) and Hollingsworth (1966) have shown that the rate of ageing is independent of temperature in Drosophila subobscura. Clarke and Maynard Smith (1961) in fact suggest that there are two phases of ageing, first an 'ageing phase', followed by a 'dying phase'. They found the 'ageing phase' to be approximately independent of temperature from 15° to 30°C, but the 'dying phase' highly temperature-dependent. At a threshold between the two phases the insect ceased to be able to maintain the steady state condition and death followed.

From the physiological studies which have been made the experimental data available for the decline in various functional capacities with age are understandably more complete in man than in any other animal. Data

concerning the physiological performance of invertebrate animals in relation to age are particularly sparse. Only a few such investigations have been carried out amongst the Arthropoda. Ingle, Wood and Banta (1937) showed that starvation increased longevity in Daphnia longispina and at the same time produced a marked reduction in the heart beat. Williams, Barness and Sawyer (1943) investigated changes in wing-beat frequency and utilization of glycogen in Drosophila as an aspect of the physiological process of ageing. Levenbook and Williams (1956) similarly demonstrated changes in mitochondrial cytochrome c in relation to ageing and wing-beat frequency of the blowfly, Phormia regina.

The following studies were undertaken to obtain a clearer picture of some of the physiological changes which occur at the individual level, in relation to the increase in age. The work reported is concerned with oxygen consumption as a measure of metabolic rate in relation to age under three different experimental conditions, (i) at rest, (ii) 'free moving', and (iii) in flight.

MATERIALS AND METHODS

Blowfly cultures.

As the physiology of female flies is markedly affected by egg-laying, segregated male flies only were used in these investigations.

The wild type S male blowflies used in these experiments were reared and maintained under standard conditions at 24°C (Chapter 2), on a sugar/liver/water diet. Under such conditions the mean life expectancy was 55.3 ± 3.5

days and 90 per cent death at approximately 12 weeks, (Chapter 3).

Measurement of oxygen uptake.

(i) With flies at rest

Oxygen consumption was determined by the conventional Warburg technique using 25 ml flasks unless otherwise stated. In all cases the water in the bath was maintained at 24°C (± 0.1°C).

The following procedure was employed to obtain an estimate of basal metabolic rate. Samples of between 8 and 24 individuals were selected from an ageing population of 100 male flies. The flies were lightly anaesthetized with carbon dioxide, weighed, and placed in perforated 'Lilly' gelatin capsules (size No. 1), with a small cotton-wool plug. The capsules were only large enough to take a single fly, and the cotton-wool plug was adjusted to prevent the fly from struggling. The capsules were then placed singly in the manometric flasks, the centre wells of which contained 0.3 ml 10% potassium hydroxide and a filter paper wick (Whatman No. 40); the gas phase was air. The flasks and manometers were mounted in the water bath and shaken slowly. The stop taps were closed after 15 minutes thermo-equilibration, and readings were then taken every 10 minutes for at least 1 hour. When struggling did occur it was obvious from the manometric records as oxygen uptake was not linear, such records (less than 5 per cent) were excluded.

Measurement of oxygen uptake.

(ii) With flies under free-moving conditions.

A further 80 male flies were used for experiments involving the changes with age in both activity and oxygen consumption under 'free-moving' conditions. For 'free-moving' measurements flies were lightly anaesthetized and weighed as before and then placed singly in manometric flasks. The flies were free to move within the flasks and readings were taken for at least 1 hour after allowing 30 to 45 minutes for the flies to adapt to the flasks. Manometer readings obtained in these experiments were not usually linear unless a fly remained at rest in the flask, consequently a line of best fit was calculated by the method of least squares.

Measurement of activity.

Measurements of activity were determined as follows. Single flies were placed in the activity chambers and allowed to adapt to the conditions for about 1 hour. Continuous records were then taken over 24 hours, under continuous illumination in a constant temperature room at 24°C (± 0.5°C). Activity was measured by the number of times a fly interrupted the infra-red beam, and this in turn was recorded on a four-channel 'Rustrak' event recorder running at a chart speed of 3 inches/hour. The activity chambers consisted of 4 inch diameter thin glass Petri dish bottoms inverted on a fine mesh metal gauze base, over a Perspex bath containing sugar solution. Thus a fly could feed anywhere in the chamber during its confinement. The infrared beam, which was directed across the diameter of the chamber, was

aligned with the phototransistor by mounting both on a metal optical bench. Sensitivity was adjusted by correct focusing of the light source with a system of lenses. Details of the apparatus are given in Figure 15, modified after Brown (1959).

Measurement of oxygen uptake in flight.

To measure oxygen consumption in flight, each fly was carefully anaesthetized with ether and weighed. Anaesthetization by carbon dioxide as used in the previous experiments was found to be unsatisfactory because flies recovered too quickly before thermo-equilibration was complete. After weighing, a $\frac{1}{2}$ inch length of 5 A fuse wire with a small eye at one end was attached to the back of the fly's thorax by means of cellulose dope. The animal was then mounted by means of a hook at the other end of the wire, plus a drop of cellulose dope, onto a thin glass rod projection inside a 60 ml flask. The off-centred well of the experimental flask contained 0.5 ml of 20% caustic potash together with a filter paper wick. The manometers and flasks were clipped simultaneously into position onto a large glass-sided tank. The water in the tank was maintained at 24°C (± 0.1°C). To reduce the time for thermo-equilibration to a minimum of 3 minutes, all experiments were carried out in a constant temperature room at 24°C. After thermo-equilibration the manometer taps were closed and the two manometers read. Spontaneous flight was observed 4 to 5 minutes after careful anaesthetization, at which time a stop watch was started and the manometers read again. Thereafter further readings were taken at every minute of

continuous flight. When flight ceased, the stop watch was stopped and the final manometric readings taken. Flies which flew for less than 2 minutes were not included in the results presented here (see Figure 18), because normally the uptake of oxygen is proportionately much higher at the beginning of the flight period. Results are expressed as $\mu l\ O_2$/mg wet wt./hour to conform with most of the literature, although no fly flew for an hour. Oxygen uptake was found to be more or less linear over most of the flight period, but in all cases both wing-beat frequency and oxygen consumption fell off towards the end of flight, see Figures 21 and 19.

Fraenkel (1932) suggested that flight was stimulated under these conditions by the so-called 'flight reflex' and refers to such flight as 'true flight'. Pringle (1965), however, refers to such flight as 'anaesthetic flight' ('Rauschflug').

Measurement of wing beat frequency.

A simultaneous record of wing-beat frequency with oxygen consumption in flight was attempted, as carried out by Chadwick and Gilmour with *Drosophila* (1940) and Yurkiewicz and Smyth (1966) with *Phaenica sericata*, but found to be impractical with the larger flasks and water bath used here. However, in some instances wing-beat frequency was recorded from some flies after removal from the flasks, and such records were found to correspond with the wing-beat frequencies obtained for flies of the same age exclusively flown for this purpose. Wing-beat frequency was recorded separately by the stroboscopic method. A Dawe 'Stroboflood' type 120 IE was used in this study in conjunction with an 'Ediswan' L.F. Oscillator type R666, calibrated electronically.

Records were taken from anaesthetized flies of the same age and experimental units as used for oxygen consumption in flight. All flight times were taken with a stop watch, but only those readings lasting for more than 15 seconds were kept for records.

Measurements of wing loss and flight response.

Records of percentage wing loss with age of survivors were kept for 70 flies, together with the percentage of flies tried at each age group which gave no flight response. The results are shown in Figure 22.

RESULTS

Oxygen uptake at rest.

Figure 16 shows a survival curve for adult male *Calliphora* at 24°C. The negatively skew curve, type B, as proposed by Pearl and Miner (1935), is typical of an ageing population, Comfort (1964).

Figure 16 and Table 6 also show the results of oxygen consumption of flies at rest. Readings were taken on the first and fourth days after eclosion and thereafter at weekly intervals up to 90 per cent death of the population.

The mean oxygen consumption at rest over the whole life span was found to be 1.67 µl O_2/mg wet wt./hour, which compares very favourably with the value of 1.7 µl O_2 obtained by Thomsen (1949) with allectomized adult *Calliphora*. The initial rise to a value of 1.9 µl O_2 on the 4th day after eclosion may be attributed to the continuing developmental changes taking place at the cellular level during the first week. For example, there are changes in sarcosomal size and weight Watanabe and Williams (1953);

Levenbook and Williams (1956); Rockstein and Bhatnagar (1965). From the seventh to the forty-second day there was no significant difference in oxygen consumption at rest, which remained relatively constant at a value of 1.5 µl O_2/mg wet wt/hour. However, between the forty-second and sixty-third day this rose to a maximum of 2.3 µl O_2, followed by a slight fall in the final weeks of life to a value of 1.9-2.0 µl O_2. This rise in oxygen consumption in the older age groups is statistically significant ($P = 0.001$). The rise in oxygen consumption over the last 6 weeks was not due to any change in the relative weights of old flies, see Table 7.

Oxygen uptake under 'free-moving' conditions and the relationship with activity.

Table 8(i) and Figure 17 in contrast show the oxygen consumption of 'free-moving' flies. Here there was a sharp rise in oxygen consumption from the time of eclosion to a peak at the end of the first week of 8.9 µl O_2/mg wet wt./hour, falling only slightly between the fourteenth and forty-second days of life. After the forty-second day there was a more gradual decline to a value of 4.0 µl O_2 in the latter weeks of life. This general trend can be correlated with the changes in activity (see Table 8(ii) Figure 17 also), but here the decline was more pronounced, and there was a significant difference between the first 6 weeks and the last 6 weeks of life ($P < 0.001$).

Records of activity for each 2-hourly period within the 24 hours of the experiment were also kept. Individual flies showed a certain amount of variation, but taking a mean of all the flies at each 2-hourly interval,

no significant changes in activity took place over 24 hours, except for flies placed in the chambers on the morning of eclosion, where activity was minimal for the first few hours. This is interesting as it is confirmed by the results obtained for oxygen consumption of 'free-moving' flies on the morning and afternoon of the first day. The differences, which are significant ($P = 0.05$), are shown in Figure 17.

Oxygen uptake in flight.

Figure 18 and Table 9 show the results for oxygen consumption in flight with age. Owing to the short flight times or lack of flight response under the experimental conditions mentioned above, readings from flies less than 1 week old and more than 60 days old were difficult to obtain. In older flies wing loss was a further factor.

The general trend was towards a slight increase in mean values of oxygen consumption from 48-62 µl O_2/mg wet wt./hour as flies became older, although it was only possible to demonstrate a significant difference between the mean values for the first and eighth weeks ($P < 0.05$). In these experiments, however, far less variability was observed than in those reported by Davis and Fraenkel (1940) for *Lucilia sericata*. For example, very few flies had oxygen consumption rates greater than 80 µl O_2/mg.wet wt./hour or less than 25 µl O_2/mg wet wt./hour, and in all cases where flies flew for periods of more than 10 minutes, oxygen consumption was always between 40 µl O_2 and 65 µl O_2/mg wet wt/hour. The results are more in agreement with those obtained by Yurkiewicz and Smyth (1966) for the sheep blowfly *Phaenicia sericata*,

although the somewhat higher values presented here probably represent a real difference between moving and stationary flies.

The uptake of oxygen per minute of flight by the longest-flying flies in each age group is shown in Figure 19. It can be seen that there is in general a higher rate of oxygen uptake at the beginning of flight (about 1.2-1.3 µl O_2/min/mg wet wt.), followed by a period of linear uptake (about 0.8-1.0 µl O_2/minute), which gradually falls to about 0.6-0.5 µl O_2 in the last minutes of continuous flight. Assuming uptake to be linear throughout the whole of the flight period this would give a value of about 1 µl O_2/mg wet wt./minute, as indicated by the dotted line.

Wing-beat frequency.

Table 10(i) and Figure 20 show the change in wing-beat frequency with age. The results were very similar to those obtained by Levenbook and Williams (1956) for *Phormia regina*, Chadwick (1953) for *Drosophila*, and Yurkiewicz and Smyth (1966) for *Phaenicia sericata*. There was a marked change in wing-beat frequency during the first week of life from a mean value of 100 cycles/second (c/s) on the first day to a mean value of 147 c/s on the seventh day, at 24°C. Thereafter the change was less well marked up to a frequency between 160-170 c/s as flies became older. Flies 50 to 60 days old exhibited higher wing-beat frquencies (180 c/s) than all flies preceding them, but the wing-beat frequencies obtained from flies 70 to 80 days old were slightly lower (175 c/s). The lowest wing-beat frequency recorded was 90 c/s on the first day, and the highest recorded 220 c/s on the forty-ninth day.

As with oxygen consumption, the wing-beat frequency was taken at every minute of continuous flight. The typical records of the longest fliers selected from various groups are shown in Figure 21. The pattern of wing-beat frequency confirms the findings of oxygen consumption per minute of flight, i.e. a high initial frequency, followed by a relatively steady period with a tendency to fall off at the end of flight.

Duration of flight and wing loss.

Table 10(ii) and Figure 21 also show the mean flight times as a function of age, obtained from all flies which flew, including those which flew for only short periods. It can be seen that flight times were short both at the beginning, during the first week, and at the end of adult life.

The short flight times observed in old flies can be accounted for to some extent by partial or complete wing loss. There appears to be a correlation (see Figure 22) between wing loss and the ability to elicit the 'flight reflex' under the conditions stated.

DISCUSSION

It was previously stated that the mean oxygen uptake at rest was 1.67 µl O_2/mg. wet wt./hour, for a fly weighing about 48 mg, and the mean value in flight was 55.1 µl O_2/mg. wet wt./hour. This represents a thirty-threefold increase from the resting state. These results may be compared with those obtained by Davis and Fraenkel (1940) for *Lucilia sericata*, a fly weighing about 31 mg, where "the oxygen uptake of adult blowflies which

were <u>not</u> at rest" was between 2.0 and 3.0 µl O_2/mg wet wt./hour, and the uptake in flight 95.58 µl O_2/mg wet wt./hour. From these data, Davis and Fraenkel conclude "that the ratio between the rest and flight metabolism is in the region of 1:100". However, on examination of their results, the mean oxygen consumption by the Warburg method was 61.25 µl O_2, whereas the mean value obtained by the Barcroft method was 112.35 µl O_2. The former value approximates more closely to the results presented here. The higher rates which they obtained may be explained by the fact that they ran their experiments at a higher temperature (27°C), and that in 27 out of 30 flights by the Warburg method, their flies flew for 5 minutes or less. The fact that they apparently made no distinction between male and female flies, nor did they consider the age of the flies, could have given rise to the greater variability between their results and those presented here.

Increases in respiratory rate for other insects of comparable size have been quoted by Jongbloed and Wiersma (1934), for hive bees, a fiftyfold increase, and von Buddenbrock (1939), a fortyfold increase for the hoverfly, *Eristalis*. A more recent account by Yurkiewicz and Smyth (1966) for the sheep blowfly *Phaenicia sericata* shows the oxygen consumption in flight by 7- to 10-day-old male flies, mean weight 28 mg, to be 27.14 µl O_2/mg wet wt./hour at 25°C. As they give no value for oxygen uptake at rest it is not possible to give a ratio between metabolism at rest and in flight. In common with the present work, Yurkiewicz and Smyth found that oxygen consumption, expressed per hour, decreased with longer periods of continuous flight to exhaustion.

Proportionally, the thirty-threefold increase given here holds true as the insect becomes older, for although oxygen uptake at rest increased, so also did the uptake in flight. This higher rate of oxygen uptake in flight may however be influenced by the increase in wing-beat frequency, which in turn was higher in older age groups. The higher wing-beat frequency is probably indicative of some wing loss, since wing shortening increases wing-beat frequency (Sotavalta, 1952). The mean wing-beat frequency was approximately 150 to 160 c/s throughout life, and the general rise in the rate of the wing-beat frequency from the time of eclosion is in agreement with other investigators.

The interesting points which emerge in relation to age and flight performance are as follows. First, there seems to be little doubt that the changes in flight ability and wing-beat frequency during the first week of adult life are due to changes in size, weight, chemical composition and possibly number of flight muscle sarcosomes. Watanabe and Williams (1953) found that there were changes in the sarcosomal size of Drosophila during the first week of adult life, but concluded that there were no further changes with age. Levenbook and Williams (1956) showed that there were changes in the dry weight of sarcosomes from Phormia regina, but found no significant changes in number. Rockstein and Bhatnagar (1965), on the other hand, found significant changes in both size and number of sarcosomes with age in Musca domestica. These changes in sarcosomal size and distribution have been confirmed with Calliphora, and will be reported in greater detail in Chapter 6 of this thesis.

This dramatic change in the sarcosomes during the first week is a maturation process, and would seem to be responsible for the relatively high percentage (25-30 per cent) of young flies which would not elicit the 'flight reflex'.

Secondly, that as a fly becomes older, its ability to fly declines. This was reflected in the 'flight reflex' response (Figure 22) where over 50 per cent of the flies tried at 49 days of age failed to elicit the response, and at 77 days 80 per cent failed to do so. Thirdly, the flight times became shorter after 9 weeks of adult life (Figure 20). Fourthly, it should be noted that there was a significant difference in oxygen uptake in flight between the first and eighth weeks (Figure 18), which conformed with the general trend found in animals at rest.

Although there is a correlation between wing loss and the ability of a fly to elicit the 'flight reflex' (Figure 22), the two factors are not absolutely interrelated. For example, some old flies with fairly extensive wing loss have been observed to fly quite frequently, whereas others with no wing loss were quite incapable of flight. This inability to fly, despite intact wings, was common among very young flies. On the other hand, some very old flies, 80 to 90 days of age, with almost complete wing loss were still observed to elicit the 'flight reflex' although they were unable to fly normally within the cages.

These results suggest, therefore, that there are age-related changes taking place in the flight muscle itself, which are to some extent independent of wing loss.

From the biochemical viewpoint, there are deteriorative changes taking place in the efficiency of flight muscle sarcosomes (see Chapter 6), and also in certain 'key' enzyme systems. For example, Rockstein and Brandt (1963) found that there was a decline in α-glycerophosphate dehydrogenase activity between 24 to 48 hours prior to the actual onset of wing loss and an initial decline in mitochondrial ATP-ase activity in male flies (Musca domestica). Clark and Rockstein (1964) also showed a decline in cytochrome c oxidase activity after the onset of wing loss. These authors suggested that the age-related changes which they observed were part of a larger integrated pattern of biochemical senescence, which precedes the failure of flight. Their results will be discussed again in the light of the present findings on isolated flight muscle sarcosomes (Chapter 6).

Again, Rockstein and Bhatnagar (1965) found that both sarcosome number and size changed with age in Musca, and that the changes were the same in both normal flies and flies de-allated at eclosion.

The decline in physiological performance with age is more obvious from the results obtained with 'free-moving' flies (Figure 17).

During the early part of the first week, oxygen consumption and activity of the imagoes were comparatively low, due to developmental rather than ageing changes. Both activity and oxygen consumption correlate well, and the differences seen in Figure 17 may well reflect the differences in the duration of respective experiments. After 8 weeks (approximately the mean life span), there was a 90 per cent drop in activity, and a 50 per cent drop in oxygen consumption. The 'plateau' up to 6 weeks, followed by a

more gradual fall in oxygen consumption, is very similar to the pattern of events observed by Bowler and Hollingsworth (1966) for Drosophila subobscura, but records of activity over 24 hour periods showed a much more pronounced decline from the first week.

One of the postulated causes of ageing is that irreplaceable cells are lost in senescent animals (Comfort, 1964). Evidence for such loss of cells in insects, where in the adult there is no cell replacement (except in the reproductive organs), has been cited by Hodge (1894) and Rockstein (1950) for brain cells of the honey bee. If such cell loss is widespread amongst insects, then one might expect that there would be a reduction in oxygen consumption in the ageing animal, if the oxygen consumed by each cell remains the same throughout life. There is certainly evidence that oxygen uptake declines with age in some animals, from reports by Hopkins (1930) for molluscan adductor muscle, by Reiner (1947) for rat brain homogenate, and by Pearse (1936) for liver, kidney, and heart homogenate (rat). In man, however, Shock, Watkin and Yiengst (1954) found no decrease in oxygen uptake per litre of intracellular fluid with age.

Considering these points, the data presented in Figure 16 and Table 6, for oxygen consumption of flies at rest was most unexpected. Although one would expect a decline in oxygen consumption with age, the results showed a significant rise. The surprising nature of this result posed the question whether the same phenomenon could be demonstrated using isolated tissues and mitochondria. Subsequent work on isolated flight muscle tissue, which will be reported in the next chapter, has indeed supported the results described here for the whole animal. As the values for oxygen consumption

at rest are very close to the basal metabolic rate, this can only mean that the fly becomes less efficient with age, requiring the expenditure of more energy later in life to maintain functional integrity.

The following questions emerge from these results: What is the underlying cause for the changes in respiratory physiology? Does the cause lie in the respiratory apparatus itself, or is it due to changes which are taking place in other physiological processes, or both?

Changes in flight muscle sarcosomes, and in certain important enzyme systems have been reported above, which would suggest that part, if not all of the answer lies in the respiratory apparatus itself. On the other hand, Clarke and Maynard Smith (1966), working with Drosophila subobscura, have shown that protein synthesis increases very markedly in old flies. Also Bowler and Hollingsworth (1966) have shown that old Drosophila subobscura are unable to adapt to temperature. They have suggested that this is because of an inability to make the correct enzymes required by the new temperature regime. This in turn suggests that the increased protein synthesis reported by Clarke and Maynard Smith may well be a failure to synthesize correct proteins, an idea which is further supported by the work of Harrison and Holliday (1967).

In an attempt to answer some of the questions raised here, the logical course of inquiry seemed to require a more thorough investigation of the respiratory physiology at the tissue and mitochondrial level.

Chapter 5.

AGE RELATED CHANGES IN THE RESPIRATORY PHYSIOLOGY OF BLOWFLY FLIGHT MUSCLE

INTRODUCTION

It was shown in the previous chapter that respiratory measurements on the ageing blowfly imago, under different experimental conditions, showed a progressive decline in physiological performance. Unexpectedly, there was an increase in oxygen consumption in old flies at rest, indicating a rise in basal metabolic rate.

Although most reports indicate a decline in physiological performance with age, there appear to be further instances where physiological activity as such actually increases in senescent animals. For example, Clarke and Maynard Smith (1966) found that protein synthesis as measured by the rate at which labelled leucine was incorporated into protein, increased significantly with age in _Drosophila subobscura_. Such results were unexpected in the light of earlier work, Clarke and Maynard Smith (1961), and Maynard Smith (1963). Again, Wulff, Samis and Falzone (1966) obtained a similar increase in protein synthesis in old rats.

In an attempt to clarify some of the changes in the respiratory physiology of the whole animal, an investigation was made into the respiratory activity of isolated tissues.

Flight muscle tissue was thought to be most suitable for these experiments for the following reasons.

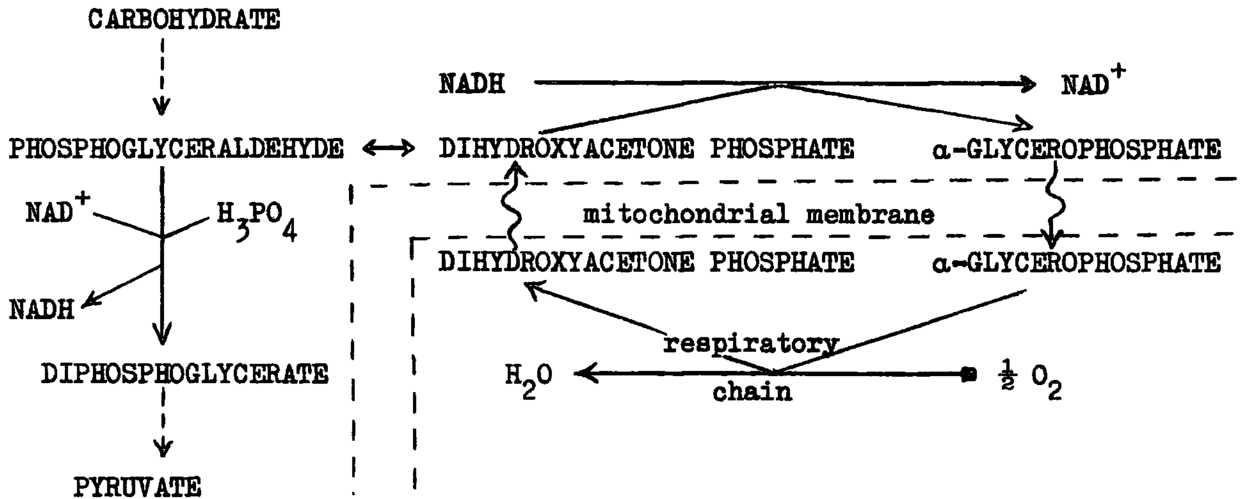
(1) The tissue, which lies entirely within the thorax, is compact, and contributes about 19 per cent to the wet weight of the fly.

(2) Flight muscle tissue is capable of maintaining a high metabolic rate, although from the present results and those obtained by Clegg and Evans (1961), this high level is not approached in isolated tissue experiments.

The energy requirements for flight are met by the large number of giant mitochondria or sarcosomes, lying like chains of beads between the muscle fibrils.

(3) Unlike mammalian muscle, the oxygen supply to the muscle fibres is a direct one, through a well developed tracheal system. Also the muscle does not fatigue as a result of oxygen debt and accumulation of anaerobic by-products such as lactic acid, but simply through lack of available substrate for oxidation. The reason for this basic difference between mammalian and insect muscle is primarily due to the development in the latter of an α-glycerophosphate cycle. From the intra-mitochondrial breakdown of α-glycerophosphate to dihydroxyacetone phosphate by flavin-linked α-glycerophosphate dehydrogenase, electrons are transferred directly to the cytochrome system, accompanied by two phosphorylations (ADP to ATP). The mitochondrial membrane is permeable to both α-glycerophosphate and dihydroxyacetone phosphate, but not to NADH. Dihydroxyacetone phosphate passes out of the mitochondrion into the cytoplasm, where its presence enables the NAD-linked α-glycerophosphate

dehydrogenase to reoxidise NADH, produced in the oxidation of the glycolytic cycle. At the same time dihydroxyacetone is converted into α-glycerophosphate, which can thus enter the mitochondrion to begin the cycle again. The system is in fact a shuttle for the oxidation of extramitochondrial NADH, without the necessity of passing through the tricarboxylic acid cycle, (Sacktor 1955). The significance of this rather detailed account will become more apparent in Chapter 6, and for convenience is summarised below.



The α-glycerophosphate cycle

MATERIALS AND METHODS

Blowfly cultures.

Both segregated male and female wild type S imagoes were used for these experiments.

Methods for rearing and maintaining the flies were as described

previously in Chapter 2.

Survival data was based on the results obtained for flies fed on a sugar, liver and water diet at 24°C, and also for some experiments on a sugar and water diet only at 24°C. Under these two dietary conditions it was noted that there was no significant difference in survival between the sexes, (Chapter 3).

Identification of the principal haemolymph sugars.

Samples of haemolymph were removed from flies after the procedure adopted by Hudson (1958). The front pair of legs were cut off at the base of the coxae, and the insect gently squeezed at the thorax to produce two small droplets of haemolymph at the cut surfaces. The droplets were then quickly taken up in a graduated micro-pipette (about 3-4 μl) and expelled into 1 ml of 5 per cent trichloracetic acid to precipitate protein. The portions of the supernatant were then used for spotting on chromatograms. The sugars present in the haemolymph were determined by thin layer chromatography in a developing solvent combination of n-butanol - ethanol - acetone - water (5:4:3:2 v/v). The spots were identified by spraying with aqueous silver nitrate in acetone, and subsequent treatment with 6N ammonium hydroxide followed by momentary exposure to hydrogen sulphide, after Trevelyan, Proctor and Harrison (1950). The principal sugars were found to be glucose and trehalose.

The incubation media.

As a result of the haemolymph sugar determinations, four different incubation media were used in the determination of oxygen uptake by flight

muscle tissue:

Medium 1. A saline essentially similar in concentration to that used by Bodenstein (1946) (Bodenstein's saline contains 128 mM $NaCl$, 4.7 mM KCl, 2 mM $CaCl_2$ and 10 mM Na_2HPO_4/KH_2PO_4 at pH 7.3), buffered to pH 7.3 with 10 mM Tris/HCl.

Medium 2. As in 1 above, but with the addition of 30 mM trehalose.

Medium 3. As in 1 above, but with the addition of 30 mM glucose.

Medium 4. As in 3 above, but with 2% bovine serum albumin added.

Preparation of the flight muscle tissue.

The 'half thorax' preparations of flight muscle were similar to those first described by Clegg and Evans (1961). Flies were immobilised by cooling to $0^{o}C$ and the wings and legs removed. The abdomen was then severed from the thorax at the scutellum; the head was carefully pulled from the thorax taking with it the remains of the alimentary tract. The thorax was then cut in half longitudinally by means of a sharp razor blade. The leg muscle, nerve cord and lower portion of the thoracic cuticle were removed, and the 'half thorax' preparation placed in an ice cold dish containing the appropriate incubation medium. When sufficient preparations had been collected (usually for six flasks at a time), four 'half thoraces' were blotted gently and placed into each Warburg flask. The 25 ml flasks contained 2.5 ml of the incubation medium, and 0.2 ml of 20% caustic potash in the centre well, together with a filter paper wick to absorb

carbon dioxide. The time taken between the preparation of the tissues and the first reading on the manometer for six flasks at a time was between 35 and 40 minutes.

Measurement of oxygen uptake.

Oxygen uptake was determined by conventional Warburg manometry at 24°C. After 10 minutes thermoequilibration, readings were taken at 5 minute intervals for at least an hour with constant shaking. At the end of the observation period, the flasks and manometers were removed from the water bath and the half thoraces removed from each flask. The flight muscle tissue was then carefully dissected from the cuticle on pre-weighed pieces of aluminium foil, and dried in the oven at 110°C for an hour or more to constant weight. All results are expressed as µl O_2/mg dry wt. muscle/hour (QO_2), although two sets of values have been calculated, (i) from the first half hour of observations and (ii) from the first hour of observations. Reasons are given below.

RESULTS

Experiments with sugar/liver/water fed flies.

Table 11 shows the results obtained with 'half thorax' preparations selected at stated intervals from three consecutive generations of ageing imagoes. Most of the results were obtained using medium 2, and these are presented in Figure 23.

It can be seen that oxygen uptake by tissues from young flies was between 10 and 11 µl O_2/mg dry wt. muscle/hour, rising to 13 to 14 µl O_2

in 70 to 75 day preparations, and finally to between 18 and 19 µl O_2 in 85 day preparations. Oxygen uptake was observed to be linear with time for all preparations up to 50 days of age. Thereafter, there was an increasing tendency for preparations to show higher rates of uptake in the first half hour of readings. This became progressively more marked in 70 to 85 day preparations, where after very high readings during the first half hour (20-25 µl O_2/mg dry wt. muscle/hour), uptake decreased and finally stopped after 70-90 minutes, (occasionally earlier in a few instances). Mathematically this could be described as a monotonically increasing function, where with increase in time there is a decrease in oxygen uptake. In consequence the results have been calculated from (i) the first half hour of readings, and (ii) over the whole hour. The various differences in the observations mentioned above are shown in Figure 24.

An analysis of variance was carried out on the data presented in Table 11. This showed that the increase in oxygen uptake with age is statistically significant at the 1 per cent level.

In the first series of experiments, medium 2 was used, because previous reports had shown that trehalose was metabolised directly by the thoracic muscle of several flying insects. For example in Musca domestica, Sacktor (1955); in the locust, Bücher and Klingenberg (1958), and the blowfly Phormia regina, Clegg and Evans (1961). However, when glucose was used as substrate (medium 3), the results obtained were very similar to those with trehalose (Table 11). In the experiments without substrate (medium 1), the results again were similar, though somewhat lower throughout, see Table 11.

These latter values emphasise the point that usually sufficient endogenous substrate is available for continued respiration, and that additional substrate does little to improve oxygen uptake. This is in agreement with the results obtained by Clegg and Evans (1961).

The unusual non-linear uptake of oxygen exhibited by 70-80 day preparations did, however, pose the question whether there might be a substrate deficiency after about 30 to 40 minutes incubation in the flasks. A control series of experiments was therefore set up, whereby additional substrate was added from the side arm of the flasks after 40 minutes incubation. This, however, failed to restore the oxygen uptake to its former level, see Figure 25.

A further possibility was that partial uncoupling of oxidative phosphorylation was taking place in the flight muscle sarcosomes of old preparations, which in turn could give rise to the observed elevation in oxygen uptake (see discussion below). Under these conditions the tissues might fail to meet their energy requirements, since less ATP would be available, and accordingly they would die more quickly, as the observations indicated. A fourth medium containing 2% bovine serum albumin was therefore used with some old tissue preparations, since there is copious evidence in the literature to show that BSA stimulates oxidative phosphorylation and inhibits uncoupling in insect mitochondria, Lewis and Slater (1954), Sacktor, O'Neil and Cochran (1958), Cochran (1961), van den Bergh (1962), and Balboni (1965). The results of the experiments with serum albumin present gave an hourly rate of oxygen uptake which was little different from those obtained with the other media, except that the values calculated from the first half

hour's observation approach very closely the values obtained over the whole hour. This was due to the fact that after a comparatively high rate of oxygen uptake during the first 10-20 minutes in most preparations, oxygen uptake was linear for longer periods of time, in comparison to preparations without serum albumin. The effect can be seen in Figure 24. From these results it seems unlikely that BSA is completely inhibiting the uncoupling effect in old preparations, because of the permeability factor involved with pieces of muscle tissue.

Experiments with tissues from sugar/water fed flies.

Measurements of oxygen uptake were also made on tissues from sugar/water fed flies. As these flies came from the same generation as the sugar/liver/water fed flies, but separated from them at eclosion, the different results are directly comparable. At 7 days there were no observable differences, but thereafter at 28, 42 and 50 days, lower QO_2 values were obtained throughout for tissues from sugar/water fed flies only. The values are given in Table 12, where only the 28-day readings are significantly different from each other.

DISCUSSION

From the results presented above for sugar/liver/water fed flies and the results of the earlier studies with the whole animal at rest, the higher rate of oxygen uptake is not only an intrinsic property of senescent muscle tissue in vitro, but also a feature of the tissues in vivo.

There are very few records in the literature dealing with oxygen uptake by invertebrate muscle as a function of age. Hopkins (1930), reported that there was a fall off in oxygen uptake in molluscan adductor muscle with age. However, although this may well be the case with molluscan muscle, Hopkins did not present any life tables for his animals, and it is possible therefore that his observed changes were not complete in that no observations were made on really senescent animals. In the present work, for example, slightly lower values were obtained with 42 and 56 day preparations, compared with the other results.

The main problem, however, was to find an explanation for the higher rates of oxygen consumption observed in old preparations. Statistical evidence had shown that increase in oxygen consumption is related to increase in age, whereas evidence from the literature seemed to indicate otherwise. For example, Rockstein (1950) reported a loss of irreplaceable cells in the honey bee with age; Rockstein and Bhatnagar (1965) with *Musca domestica* and experiments (Chapter 6) with *Calliphora* had shown that changes occur in the size distribution and total number of flight muscle mitochondria with age. Rockstein and Brandt (1963) also showed that there was a decline in activity of certain important enzyme systems with age in *Musca*. All these features would be expected to produce a decrease rather than an increase in oxygen consumption.

However, when considering biochemical inefficiency during senescence, it does not necessarily follow that there will be an observable decline in the activity of one system or another. An increase in physiological

activity is quite possible and capable of explanation in a number of ways, as shown by Clarke and Maynard Smith (1966).

Several explanations seemed possible to account for the increase in oxygen consumption with age, presented above.

1. That ATP production is less efficient in old tissues. This could be brought about either by changes in the ultrastructure of the sarcosomes, or by a failure of the sarcosomes to be produced or repaired.

2. That other cellular processes are directly or indirectly responsible for the observed increase in oxygen consumption. For example, if the demand by other cellular processes exceeded the capacity for ATP to be produced, then this would lead to a reduction in ATP level and a consequent imbalance in the ADP/ATP ratio.

3. That all or some of the possibilities mentioned in 1. and 2. above, contribute to the increase in oxygen consumption found in old tissues.

From the results given above it appeared that partial in vivo uncoupling was taking place in the flight muscle mitochondria of senescent flies. If the mitochondria have a definite total work capacity, partial in vivo uncoupling would impose a severe strain on the 'respiratory control' system by raising the ADP/ATP ratio with a consequent increase in QO_2, since the link between oxidation and phosphorylation is compulsory, as demonstrated by Lardy and Wellman (1952). In insect sarcosomes the occurence of an endogenous uncoupler has been demonstrated by Wojtczak and Wojtczak (1960), and the enzymatic nature of its generation by Lewis and Fowler (1960).

However, it must be added that Lewis and Fowler found very little endogenous uncoupler in fresh tissue, although the blowflies which they used were appreciably younger than the 70 - 85 day flies under consideration in the present work. Nevertheless, although partial in vivo uncoupling does appear to take place, it seems unlikely from the evidence to be presented in Chapter 6, that release of long chain fatty acid is the principal cause of the deterioration, but rather the changes in the ultrastructure of the sarcosomes themselves. In these particular experiments, however, release of some long chain fatty acids may have contributed to sarcosomal uncoupling, since Lewis and Fowler (1960) showed that the fatty acids were released during the isolation procedure, by crushing the thoraces. It is possible, therefore, that some fatty acids were released in the preparation of the muscle tissue for these experiments, and that the addition of serum albumin had a particularly beneficial effect on old tissues.

Preliminary investigations into the extraction of fatty acids from intact thoraces of old and young flies have so far suggested that very little, if any, fatty acids are released. However, this is perhaps a topic for further research. If, however, there does prove to be an increase in the production of endogenous uncoupler within senescent tissues, one must presume from the work of Björntrop, Ells and Bradford (1964), that the concentration of fatty acids released is critical, otherwise a lowering of the QO_2 would be expected.

Nevertheless, we are still left with several fundamental problems. Even if partial in vivo uncoupling is taking place in the mitochondria

of senescent imagoes, is this a deteriorative process per se, or is it the effect (or perhaps the cause) of other deteriorative processes which are taking place during senescence?

An imbalance between cellular systems, such as an increase in protein synthesis, or even a degradation of protein, might well impose extra demands for ATP, which in turn could give rise to the observed elevation in oxygen consumption.

To try to clarify this situation, investigations were made into the in vitro properties of isolated sarcosomes, and these results are reported in the next chapter.

The results shown in Table 12 demonstrate differences in the respiratory physiology of flies fed on a 'normal' diet and those fed on a deficient diet. Clearly, as pointed out in Chapter 3, the absence of protein from the diet seemingly causes the imago to age and thus die much earlier than the same insect on a sugar/protein/water diet. The suggestion was that the addition of protein enhances replacement and repair and retards tissue protein breakdown. If the only consideration was that ageing is accelerated in protein deficient flies, then one might expect that the rate of oxygen uptake by tissues from these flies would be higher than their counterparts at the same age on a sugar/liver/water diet, especially between 20 and 50 days. The results, in fact, might be similar to those obtained for 70 day sugar/liver/water flies presented above. The reverse, however, is true. Oxygen consumption is somewhat lower in tissues from sugar/water fed flies.

This suggests a fundamental difference at the tissue and mitochondrial level between the two types, and thus reflects a fundamental difference in the ageing process in these two groups.

Perhaps sugar/water fed flies are dying because enzymes and other essential proteinaceous cellular components are simply not available after the larval reserves have been used up, whereas in sugar/liver/water fed flies proteins can be synthesized, but with an increase in frequency of the 'wrong type' as the fly becomes older?

Both could be a cause of ageing, but one may bring about death much more quickly than the other.

The answers to these questions are as yet unresolved, and certainly provide thought for future research.

Chapter 6.

CHANGES IN SIZE, NUMBER, AND RESPIRATORY EFFICIENCY OF ISOLATED FLIGHT MUSCLE SARCOSOMES

INTRODUCTION

The results presented in the previous two chapters indicated that marked changes were taking place in the respiratory physiology of the blowfly in relation to age. Since mitochondria are the 'powerhouses' of energy (ATP) production, it was important to try to establish what changes were occurring in these cellular organelles, which might account for some of the observations made on the whole animal and with the isolated tissue experiments.

The giant mitochondria or sarcosomes of thoracic flight muscle tissue, as pointed out previously, are capable of very high energy output, sufficient to meet the demands made by flight. Any significant changes in the efficiency, structure, size and number of these giant mitochondria will therefore have a profound effect on flight performance, and may perhaps reflect the overall pattern of changes taking place at the cellular level.

Because of the importance of the sarcosomes in insect flight, and their accessibility for experimental work, there have been several reports citing changes in the size and number of sarcosomes in relation to the age of the fly; for example, Watanabe and Williams (1953), Levenbook and Williams (1956) for *Drosophila funebris* and *Phormia regina*, and Rockstein and Bhatnagar (1965) for *Musca domestica*. The dramatic increase in the size of flight muscle sarcosomes, which takes place in the first week after eclosion in the imago

is considered by these authors as a maturation process, and is responsible for the rapid elevation in the wing beat frequency during this period. For comparison see Figure 20 of this thesis.

On the other hand, there are very few reports in the literature dealing with respiratory efficiency of sarcosomes in relation to age. Van den Bergh (1962) found no significant decline in QO_2 values or P:O ratios with houseflies from 0 to 21 days of age, using in turn α-glycerophosphate, pyruvate + malate and succinate as substrates. These observations cover the period up to the mean life span in this insect (data from Rockstein and Lieberman, 1958). Rockstein and Brandt (1963), and Clark and Rockstein (1964), however, have shown a decline in the activity of certain 'key' enzymes in the flight muscle apparatus, which they relate directly to wing loss in male houseflies.

It was against this rather sparse background that the present studies were undertaken.

In many respects, experiments with isolated sarcosomes are unsatisfactory, because isolation procedure markedly affects morphological integrity and functional behaviour, as demonstrated by Balboni (1965) and Carney (1966). Nevertheless, if the limitations of the technique are borne in mind, a comparative study can still be made with sarcosomes isolated from flies of various ages, provided that experimental procedure is rigidly standardised. Considerable care is needed, because sarcosomes can be 'aged' by grinding during isolation, or by allowing sarcosomes to stand for long periods of time after isolation, see Lewis and Slater (1954) and van den Bergh (1962). Such treatments have the effect of reducing the efficiency, or irreversibly

uncoupling oxidative phosphorylation, as shown by reductions in the P:O ratios, Lewis and Slater (1954), and by effectively altering ATP-ase activity, as shown by a decrease in the DNP factor, van den Bergh (1962).

If changes do occur in the ultrastructure and in number of sarcosomes, together with a reduction in the efficiency of oxidative phosphorylation and the ATP-P_i exchange reaction with increasing age of the fly, then these would account for some of the features associated with the physiological decline previously discussed in the whole animal. In order to test this hypothesis, four different measurements were made on sarcosomes in vitro from flies of different ages:

(1) A Coulter Counter analysis of size and total numbers of sarcosomes.

(2) Measurements of oxidative phosphorylation with intact sarcosomes.

(3) Respiratory control experiments.

(4) Mg^{2+} stimulated and DNP/Mg^{2+} stimulated ATP-ase activity.

With regard to the points made above, the choice of substrate is important when working with intact sarcosomes. The Krebs cycle intermediates are unsuitable as substrates, because there is a permeability barrier to them across the mitochondrial membrane which can be very variable. Much higher QO_2 values for example are obtained with sonicated or disrupted mitochondria than with intact mitochondria, van den Bergh and Slater (1962). The choice of substrate therefore for these experiments appeared to rest between the two end-products of glycolysis, pyruvate or α-glycerophosphate.

The latter was chosen as it is much more temperature-stable than pyruvate. The metabolic pathways and phosphorylations from α-glycerophosphate oxidation have been explained in detail in the Introduction to Chapter 5.

MATERIALS AND METHODS

Blowfly cultures.

Segregated wild type S males and females were used in this study. Rearing the cultures was as described in Chapter 2, the imagoes were kept at 24°C and given a sugar/liver/water diet.

Flies of both sexes were selected at different ages from four consecutive generations. Approximately 400 imagoes were initially present in each generation.

Isolation of sarcosomes.

Flight muscle sarcosomes were isolated after the procedure used by Lewis and Slater (1954) and Lewis and Fowler (1960).

Unless otherwise stated, 30 flies were immobilised by cooling at 0°C, and the heads and abdomina removed as quickly as possible. The thoraces were collected in an ice-cold tube (0° to 2°C), and then transferred to a small glass cylinder as used by Greville, Munn and Smith (1965). The cylinder was surrounded by crushed ice and contained 2 ml of sucrose medium (1). The thoraces were very gently crushed in the cylinder for 2 minutes with a cold, loosely-fitting, flat-footed glass rod. The resulting pulp was then transferred with a further 1 ml of sucrose medium to a funnel lined with

four layers of fairly coarse muslin, which had been previously boiled in distilled water and rinsed with cold isolation medium. The muscle pulp was squeezed forcibly through the muslin and the filtrate collected in a centrifuge tube surrounded by crushed ice. The filtrate was centrifuged at 100 g for 4 minutes at 0°C in a 'Mistral' 2L centrifuge (M.S.E.) to remove particles of chitin, and the supernatant again centrifuged at 2200 g for 10 minutes at 0°C. The pellet of sarcosomes was resuspended in ice-cold serum albumin free sucrose medium (2), and re-centrifuged at 2200 g for 10 minutes at 0°C. The resulting pellet of washed sarcosomes was finally re-suspended in 0.75 ml of ice-cold KCl medium (3) to give about 1 ml of suspension. The total time taken for the isolation procedure was 50 minutes. 30 thoraces normally gave about 0.4 mg sarcosomal protein/0.05 ml (i.e. per flask).

Isolation media used.

(1) 0.32 M sucrose, plus 0.01 M EDTA, plus 2 per cent bovine serum albumin, buffered to pH 7.3 with 0.01 M Tris/HCl.

(2) The same medium without serum albumin.

(3) 0.15 M KCl, plus 0.001 M EDTA at pH 7.3.

Coulter Counter technique.

In these experiments, male flies only were selected for use in groups of 10, and all were from the same generation. Normally three determinations were made at each age group. All flies were previously weighed (reasons given below) and then the sarcosomes were isolated by the procedure given

above, producing 1 ml of sarcosomal suspension in the KCl medium (3) above. Aliquots of 0.01 ml were taken from the suspension and diluted to 100 ml in a volumetric flask with 0.15 M KCl. The saline, which is isotonic with insect haemolymph, had previously been twice filtered through a fine millipore filter under pressure. This treatment maintained the background count on the Coulter Counter to a minimal level.

A Coulter Counter, Model A with a 50μ aperture was calibrated to the saline electrolyte (KCl) employed here, and was then used to size and count simultaneously all particles greater than 0.9μ. The instrument, which employs the principle of electrical gating (Mattern, Brackett and Olson, 1957), measures the volume of each particle in 0.05 ml samples of the diluted suspension passing through the gate. Since blowfly flight muscle sarcosomes are virtually spherical, the instrument can be used to measure the diameter of each particle directly. Duplicate counts were made at each size setting on the instrument to compensate for polarity on the switch. From the counts a sigmoid-shaped size distribution curve was drawn, and the relevant per centage size data extrapolated.

The Coulter Counter technique for counting and sizing large numbers of small particles is therefore much quicker and more accurate than other methods which employ graticuled slides and laborious manual counting techniques.

In addition, samples of the diluted sarcosomal suspension were also examined under oil-immersion using a calibrated Watson 'shearing-image' eyepiece, to check size and confirm that isolation and subsequent treatment through the Coulter Counter had no serious deleterious effect on sarcosomal morphology.

From the Coulter Counter data, it is possible to calculate the total number of sarcosomes per thorax. However, the actual count obtained on the instrument assumes that extraction efficiency of the sarcosomes is 100 per cent. This is obviously not so. In order to obtain the total number of sarcosomes per thorax, the extraction efficiency of each sample must be taken into account, and the count from the Coulter corrected accordingly.

From the wet weight data for male flies given previously in this thesis (Table 7), and the data of Levenbook and Williams (1956), and Watanabe and Williams (1951), the amount of sarcosomal protein per fly can be calculated:

(a) The average body weight of male Calliphora is normally about 48 mg, but this must be determined for each sample of flies.

(b) In different insect species the flight muscle-mass makes up a constant proportion (about 19%) of the total body weight.

(c) Sarcosomes make up 40 per cent of the muscle-mass wet weight.

(d) The dry weight of the sarcosomes is 21 per cent of their wet weight.

(e) Protein makes up 60 per cent of the sarcosomal dry weight, although this is not quite true for very young flies (1 to 3 days old).

The amount of sarcosomal protein, calculated from these data is 0.46 mg/fly. The amount of sarcosomal protein actually present in the sarcosomal suspension can be estimated by the Folin-phenol method of Lowry, Rosebrough, Farr, and Randall (1951). The efficiency of extraction can then be calculated. For most preparations the extraction efficiency was found to be 60 to 70 per cent, although with preparations from young flies extraction efficiency

calculated on the data above was as low as 47 per cent. This is partly due to an artefact, because slightly less protein is present in the sarcosomes of flies 1 to 3 days old, Lewis (personal communication, 1967). The method is really a measure of the extraction efficiency of protein, and therefore faulty in this respect. Nevertheless, the total number of sarcosomes per thorax calculated by this method is still considered to be more accurate than estimations based on counts from graticule slides.

Determination of sarcosomal protein.

Sarcosomal protein was determined by the Folin-phenol method of Lowry, Rosebrough, Farr and Randall (1951), using bovine serum albumin (Fraction V) as a standard.

Reagents: (i) A mixture of 0.5 ml 1% (w/v) copper sulphate and 0.5 ml 2% (w/v) sodium potassium tartrate in 50 ml of 2% (w/v) sodium carbonate in 0.1 N NaOH. (Folin solution A).

(ii) Diluted Folin-Ciocalteau phenol reagent: 4 ml diluted to 10 ml with water (Folin solution B).

(iii) A standard protein solution: 1 mg/ml bovine serum albumin.

Procedure: Duplicate blanks (distilled water 0.2 ml), duplicate standards (0.1 ml protein plus 0.1 ml distilled water), and duplicates of the unknown protein solution (0.05 ml protein solution plus 0.15 ml water) were set up in spectrophotometer cuvettes. 3 ml of Folin solution A were added to each cuvette and mixed thoroughly. The solutions were allowed to stand for 20 minutes at room temperature. 0.3 ml of Folin solution B

was then added to each cuvette, giving a blue colouration of variable intensity. After mixing, the solutions were allowed to stand for 1 hour at room temperature. The optical density (absorbance) of the blue colour was then read at 750 mμ on a Hilger-Watt spectrophotometer (Hilger-Gilford).

Standard protein curve.

The bovine serum albumin used as standard for this curve was 98-99 per cent pure protein (Sigma). Different quantities of pure protein were carefully weighed out, and made up into solutions. Serial dilutions were then made, and duplicate (identical) samples taken for (i) a micro-Kjeldahl determination of protein-nitrogen, (ii) a determination of optical density (absorbance) at 280 mμ (u/v) on a Hilger-Watt spectrophotometer, and (iii) an estimation of protein by the Folin-phenol method outlined above.

Table 13 and Figure 26 summarises for comparison the results obtained from the three methods.

Because of the many technical difficulties experienced with the micro-Kjeldahl method, protein determination by the spectrophotometric method was the method used, since it was found to be more convenient when a large number of samples had to be handled.

Measurement of oxidation and phosphorylation.

Oxygen uptake was determined by conventional Warburg manometry at 24°C, using 8 ml flasks.

Reaction medium: the reaction medium contained 60 mM DL α-glycerophosphate, 30 mM phosphate buffer (Sörensen) at pH 7.3, 30 mM glucose, 5 mM $MgCl_2$, 2 mM ADP, 1 mM EDTA, 200 K.M units hexokinase, 20 mM Tris/HCl pH 7.3, 50 mM KCl and 2 per cent bovine serum albumin (when added). The addition of 0.05 ml sarcosomal suspension made the total volume up to 1 ml.

The centre well of the flasks contained 0.15 ml 10 per cent KOH, and an acid washed filter paper wick (Whatman No. 40) to absorb carbon dioxide. The total incubation time was 25 minutes, and the gas phase was air. Oxygen uptake was linear with time, and total uptake was calculated by extrapolation, Slater and Holton (1954). The reaction was stopped at 25 minutes by the addition of 1 ml ice-cold 25 per cent trichloracetic acid (TCA), and the flasks placed immediately on ice in a refrigerator at 0° to $2^{o}C$ to prevent acid hydrolysis of ATP. The contents of the vessels were centrifuged in the cold, and inorganic phosphate in the supernatant estimated after the method of Fiske and Subbarow (1925). 20 μgm/ml KH_2PO_4 (Sigma) was used as standard.

Corrections for endogenous oxidation and phosphorylation have not been made, since the amounts were always exceedingly small (usually zero), in agreement with van den Bergh (1962) and Lewis (personal communication 1966).

Fiske and Subbarow estimation of inorganic phosphate.

A standard phosphorus curve was made by serial dilution of the 20 μgm/ml KH_2PO_4 solution (see Figure 27). The estimation of inorganic phosphate in solution was carried out as follows.

Clean test tubes were set up to accommodate reagent blanks, standards, controls and unknowns. 0.1 ml samples of the control and unknowns were

pipetted into the tubes and made up to 5 ml with distilled water. 1 ml of acid-molybdate solution (0.5 ml 5 N H_2SO_4 and 0.5 ml 2.5% ammonium molybdate) was then added to each tube, followed by 0.25 ml of Fiske and Subbarow reducing agent. After careful shaking the solutions were allowed to stand for 10 minutes at room temperature. Samples were then transferred to cuvettes. At 15 minutes exactly, the optical density (absorbance) of the blue colouration was measured at 660 mμ on a Hilger-Watt spectrophotometer.

Measurement of respiratory control.

The standard reaction medium was used, except that 0.1 mM ATP was present in place of glucose, ADP and hexokinase, to prevent the oxidase systems from being irreversibly inactivated, Holton, Hülsmann, Myers and Slater (1957). Glucose, hexokinase and ADP (at pH 7.3) were added from the side arm of the Warburg flasks (a) after 10 minutes incubation (albumin absent from the medium) and (b) after 6 minutes incubation (albumin present in reaction medium).

The respiratory control index (RCI) is the ratio of the respiratory rates in the absence and in the presence of ADP.

Measurement of ATP-ase activity.

For these experiments, only 15 thoraces were used at a time from a single ageing population of 200 male flies, giving about 0.2 to 0.3 mg sarcosomal protein/0.05 ml.

ATP-ase activity was determined after Myers and Slater (1957).

The reaction medium contained 75 mM KCl, 0.5 mM EDTA, 50 mM sucrose, 1.5 mM $MgCl_2$ and 2 mM ATP. The pH (7.3) was fixed by the addition of 0.3 ml of 0.25 M Tris/acetate buffer per 1.2 ml of reaction medium. In addition 0.1 mM 2:4 dinitrophenol solution (DNP) was present in half the samples.

The flasks were set up in a water bath at 24°C to thermoequilibrate. One flask measured magnesium (Mg^{2+}) stimulated ATP-ase activity, and the other Mg^{2+}/DNP stimulated ATP-ase activity concurrently. Each flask contained a total volume of 5 ml reaction medium. The reaction was started by the addition of sarcosomal suspension (0.05 ml suspension per ml. of reaction medium). The contents of the flask were stirred gently throughout the experiment. At intervals of 5 minutes, 1 ml aliquots were withdrawn from each flask and pipetted directly into centrifuge tubes containing 1 ml of ice-cold 25 per cent TCA. The contents were centrifuged in the cold, and the amount of inorganic phosphate liberated determined as above by the method of Fiske and Subbarow (1925).

The DNP factor is the ratio of Mg^{2+} stimulated ATP-ase activity in the absence and in the presence of DNP.

Chemicals:

Adenosine-5'-diphosphate (ADP), adenosine-5'-triphosphate (ATP), hexokinase (type III from yeast), DL α-glycerophosphate (α-GP), bovine serum albumin (Fraction V), and tris(hydroxymethyl)aminomethane (Trizma base) were all obtained from Sigma Chemical Co. Ltd. ADP, ATP, hexokinase and bovine serum albumin (Fraction V) were stored desiccated at -20°C.

Ethylenediaminetetraacetic acid (di-sodium salt) (EDTA), 2:4 dinitrophenol (DNP), and other standard chemicals were of AnalaR grade from British Drug Houses.

RESULTS

Coulter Counter analysis.

A statistical analysis within samples from specific age groups showed no significant differences between them but an analysis of variance between samples at different ages showed a significant increase in the mean sarcosomal size with increasing age, ($P = 0.05$). The results are presented in Table 14 and Figure 28. It can be seen from these data that in the 4 hour-old and 3 day-old imagoes there is a high proportion of small sarcosomes (1 to 2 μ range). Between 7 and 30 days a greater percentage of sarcosomes falls within the 1.5 to 3 μ range. In older flies, however, there is not only an increase in the frequency of large sarcosomes, but also an increase in the number of small sarcosomes, particularly in 85 day-old flies.

Estimates of the total number of sarcosomes per thorax are also given in Table 14. These results show a reduction in number by about 20 per cent, in agreement with the results obtained by Rockstein and Bhatnagar (1965). However, care should be taken in interpreting these results too precisely. Firstly, small errors are introduced when correcting for extraction efficiency. Secondly, from microscope observations, it appeared that there were either fragments of mitochondria, or a number of much smaller mito-

chondria in preparations from old flies, which were below the detectable size of the Coulter Counter used here. To compensate for loss of number, an estimation of the solid volume below detectable size has been made, after the method of Eckhoff (1966). The corresponding frequencies below 1 μ in 50, 65 and 85 day flies have been accordingly marked with an asterisk.

Oxidative phosphorylation

Table 15 gives the mean QO_2 values (μl O_2/mg protein/hour) and the P:O ratios (ratio of molecules of P_i esterified per atom of oxygen utilised), at selected ages throughout the life span of the imago. An analysis of variance showed a significant correlation at the 0.1 per cent level, between the decrease in P:O ratios and increasing age. The mean P:O values as a function of age are presented in Figure 29, together with the regression lines of the two variables.

The number of flask determinations (n) given in Table 15 represents the combined results of samples which were actually divided into two sets at each age group. 2 per cent serum albumin was added to the reaction medium in one half of the samples, in the other half it was absent. As can be seen from Table 16, there was no significant difference between the two sets of readings, implying that the serum albumin added initially to the isolation medium was sufficient to stimulate oxidative phosphorylation and that serum albumin added to the reaction medium had little further effect.

In another series of experiments, serum albumin was eliminated altogether from the isolation medium. Subsequently, when 2 per cent serum albumin was added to the reaction medium there was a slight improvement in

the P:O ratios, and at the same time QO_2 values were lowered, Table 17. However, the P:O ratios obtained in these experiments were all considerably lower than in the first series of experiments where 2 per cent serum albumin was initially present in the isolation medium.

These experiments completely support the findings of van den Bergh and Slater (1962). High P:O values were obtained only when serum albumin was present in the isolation medium, but had little further effect when added to the reaction medium. Again the concentrations of sucrose and KCl in the isolation media were found to be critically important, but the concentrations of KCl (10 mM to 100 mM), and Tris (10 to 50 mM) in the reaction medium could be varied without changing the respiratory rates, using α-glycerophosphate as substrate. Van den Bergh and Slater also found no change in respiratory rate by varying the phosphate concentration (10 to 50 mM) of the reaction medium.

Respiratory control.

Table 18 shows the results of the first series of investigations made into respiratory control by sarcosomes extracted from flies of three representative age groups. When ADP was added from the side arm after 10 minutes incubation, respiratory control could only be demonstrated with preparations from young flies (8 days old). With 30 day flies, the results obtained were inconclusive, and with 60 day flies respiratory control could not be demonstrated at all. The QO_2 values on addition of ADP were much lower (450 as against 700 to 800) than in the experiments for oxidative

phosphorylation. The reason for these low values was not entirely clear. However, it seemed that the design of the experiment (ie. ADP added later) was sufficient to account for the different observations. Carney (1966) for example has shown that the QO_2 values and respiratory stimulation on the addition of ADP can be appreciably reduced, the longer the time lag before addition of ADP, particularly with comparatively low sarcosomal concentrations.

The respiratory control experiments were repeated with serum albumin added to the reaction medium, and the time interval before addition of ADP reduced to 6 minutes incubation. The respiratory control ratios and QO_2 values were as a result much improved, Table 19. Respiratory control could be demonstrated in 7 and 10 day old preparations (R.C.I. 1.55), in 20 day old preparations (R.C.I. 1.48) and in 45 day old preparations (R.C.I. 1.42). In 65 day preparations however respiratory control was not conclusive (R.C.I. 1.14). It appeared therefore from these results that the time lag before addition of ADP to the reaction was a critical factor. This was confirmed by setting up a pair of control flasks with 7 day preparations, in which ADP was present in the reaction medium at the beginning of the reaction. The mean QO_2 value obtained was 700.

The respiratory control ratios with young flies were comparatively low with α-glycerophosphate as substrate, in agreement with many other workers, Klingenberg and Bücher (1959); Gregg, Heisler and Remmert (1960); Stegwee and van Kammen-Wertheim (1962); van den Bergh and Slater (1962); Cochran (1963); and Balboni (1965).

<u>ATP-ase activity</u>.

In vitro Mg^{2+} stimulated and Mg^{2+}/DNP stimulated ATP-ase activity in relation to the age of the fly is shown in Table 20. A statistical analysis was carried out on the 5, 10, 15 and 20 minute readings separately with age. The results showed a significant reduction in the DNP factor with increasing age at the 5 minute ($P = 0.001$) and 10 minute ($P = 0.02$) readings only. The data is also set out in Figure 30, where the overall trend is marked by a line of best fit (all readings).

The results therefore confirm the findings for oxidative phosphorylation in that the DNP factors are high in young flies, especially at the 5 and 10 minute readings, indicating a 'tighter degree' of coupling present. Sarcosomes from old flies on the other hand, do not exhibit such high initial DNP factors. The DNP factor therefore provides a useful measure of the efficiency and intactness of the sarcosomes in vitro, and thus of the extraction procedure, van den Bergh (1962).

The values obtained for P_i release (Table 20) correspond closely with the values obtained by van den Bergh (1962) for <u>Musca domestica</u>. However, Clark and Rockstein (1964), also working with <u>Musca</u> obtained very much lower values for P_i release for all age groups.

DISCUSSION

The results of in vitro studies carried out on isolated sarcosomes from the blowfly show quite conclusively that respiratory efficiency declines significantly as a function of age. Reductions in the P:O ratios and DNP factors indicate clearly that oxidation and phosphorylation are either

partially uncoupled or less tightly coupled in old sarcosomes. This situation may have arisen as a result of the isolation procedure, because as Carney (1966) points out, isolation procedure markedly affects the morphological integrity and functional efficiency of sarcosomes, causing them to shrink or swell according to the medium used. Nevertheless, it seems unlikely that the isolation procedure alone is responsible for the observed deterioration in efficiency, because care was taken to ensure that all sarcosomes were subjected to the same experimental conditions. However, if the isolation procedure does adversely affect the sarcosomes it does so differentially, having a more pronounced effect on sarcosomes from older flies. This then in itself indicates that age related changes are taking place in the sarcosomes, which in turn support the previous findings from the tissue respiration studies.

Assuming that this is the case, then the question is what agencies are responsible for the breakdown in efficiency?

Several factors may be contributing to the decline in ATP production by sarcosomes from old flies.

(1) The production or release of greater quantities of endogenous uncoupling agents.

(2) Lysosomal activity.

(3) Changes in the size and number of sarcosomes with age, together with changes in their ultrastructure.

(4) An increase in ATP-ase activity in vivo.

(5) A greater demand by other cellular processes for ATP, exceeding the capacity for ATP synthesis.

To take these points in order. First, from the work of Lewis and Fowler (1960) it is known that with intact thoraces of Calliphora, endogenous uncoupling agents in the form of long chain fatty acids, are not present in the thorax of the living insect (7 to 10 days old), but are only released when the thorax is crushed during the isolation process. This does not however preclude the possibility that greater quantities of endogenous uncoupler are present in the old fly. However, it seems a less likely explanation for the decline in respiratory efficiency of the sarcosomes, because Lewis and Fowler showed that the uncoupling effects of the inhibitory substances extracted from crushed thoraces could be completely reversed by the addition of serum albumin to the medium. Previously, Sacktor, O'Neill and Cochran (1958) and many other authors had demonstrated the stimulatory effect of serum albumin on oxidative phosphorylation and in consequence it was always used in the isolation of sarcosomes in the present work. If more endogenous uncoupler is released in tissues from old flies, then one would expect that the P:O ratios obtained with old flies would be of the same order as the P:O ratios obtained with sarcosomes from young flies, as the activity of the inhibitor should be completely reversed with serum albumin present. However, this was not the case, although as reported in the previous chapter, the addition of serum albumin did have a beneficial effect on oxygen uptake of senescent flight muscle tissue.

There seem to be two explanations. The first is that if old sarcosomes are subjected to the effects of partial uncoupling for a sufficiently long period of time in vivo, as compared to the relatively short period of time in vitro,

then the endogenous uncoupler may have a more permanent effect on the sarcosomal ultrastructure, which can not be substantially reversed by the addition of serum albumin. Alternatively, if an endogenous uncoupler is present in the flight muscle tissue of old flies, it may not be in the form of long chain fatty acids. If this is so, then serum albumin, which has a strong affinity for fatty acids, may not combine so readily with other endogenous uncouplers, and in consequence would be incapable of restoring oxidative phosphorylation to the high rate obtained with sarcosomes from young flies.

Another possible cause for the decrease in respiratory efficiency of flight muscle sarcosomes is lysosomal activity.

There is evidence that there is an increase in the number of lysosomes with age, and that these organelles play an important role in physiological cell death, Brandes, Bertini and Smith (1965), Duncan (1966). It also seems that the lysosomal membrane, which normally retains the powerful hydrolytic enzymes in an inactive form, becomes more easily ruptured with age thus releasing enzymes for autolytic purposes. It may be therefore, that during the crushing of thoraces from old flies (however gently), some hydrolytic enzymes are released with deleterious effects. Sawant, Desai and Tappel (1964) for example, report that there are lysosomal enzymes in rat liver which can degrade some components (insoluble and soluble protein, lipid and cytochrome c) of the mitochondria. This activity can be directly related to in vivo autolysis of liver tissue. Lysosomes also appear to be involved

in the disorganisation (Ashford and Porter, 1962; Moe and Benke, 1962), and in the turnover of mammalian liver mitochondria, (Fletcher and Sanadi, 1961).

The main objection is that there is no evidence in the literature that lysosomes are present in any quantity, or are especially active in old skeletal muscle. Kohn (1966) reports that although lipofuchsin granules are present in old mammalian muscle, he was unable to isolate any protease associated with them capable of degrading major structural proteins.

The test which needs to be done, but has not been carried out in the present work, is to examine histochemically the acid phosphatase activity in flight muscle tissue from young and old flies. This would give some indication of the extent of lysosomal activity in senescent flies.

Although in vitro studies do not permit a complete insight into the in vivo situation, changes in sarcosomal size and total numbers have been demonstrated in Calliphora in relation to age. Similar changes have also been found in other Diptera by Watanabe and Williams (1953) with Drosophila funebris and Phormia regina, by Levenbook and Williams (1956) with Phormia regina, and by Rockstein and Bhatnagar (1965) with Musca domestica. Levenbook and Williams (1956) also recorded a threefold increase in dry weight and an increase in cytochrome c titre in the sarcosomes of Phormia during the first week of adult life. From the point of view of the in vitro studies reported above, the decrease in total number of sarcosomes is irrelevant, but it is

of considerable importance in the physiology of the whole fly. If, as the results indicate, there is a loss of 20 per cent in the total number of sarcosomes per thorax, then this should have a profound effect on flight performance. The number of mitochondria is often indicative of functional activity, Lehninger (1964). From observations on the activity of the whole fly, this statement in general appears to be true, with the exception of the increase in oxygen uptake by flies at rest.

A size analysis of blowfly sarcosomes in relation to age showed that there was a marked increase in the mean diameter of the sarcosomes during the first week of adult life, and thereafter a more gradual increase in the frequency of large sarcosomes together with an increase in the frequency of small sarcosomes after 50 days of age. These observations are most noticeable in 65 and 85 day preparations (Figure 28). The increase in size may be brought about in a number of ways. Either by accumulation of the growing matrix mass and cristae size number, or by association with smaller mitochondria (Clark, 1957), or again by the mitochondria swelling, Payne (1946). This latter observation may be particularly relevant here, because the decrease in number could be directly related to the swelling phenomenon in the 65 and 85 day preparations presented above. Payne's observations on the anterior pituitary of senescent fowls showed just this. A further cause for the decrease in number of sarcosomes may be as a result of disintegration, Andrew (1956). This process might be operating quite independently or perhaps as a result of the swelling effect. Again, the present results also indicate that disintegration is playing an important

part in the reduction in sarcosomal number, particularly as there was an increase in the number of particles less than 0.9μ in old flies. It appears then that the changes in size, maturation, and eventual lysis of blowfly sarcosomes continues throughout the life of the imago. The cycle of events in itself may not necessarily be an index of ageing (Hess and Lansing, 1954), but the ascendancy of the breakdown processes in old flies may be making an important contribution to senescence.

Changes in size and presumably ultrastructure will also have a very important bearing on the functional efficiency of sarcosomes in both in vivo and in vitro states. From the experiments of Packer (1961), it is known that mitochondria undergo swelling and contraction cycles which are related to their respiratory state, (for a fuller discussion see Lehninger, 1964). Consequently, any changes in shape or reduction in size of the sarcosomes is likely to have a profound effect on the spatial arrangement of electron chain particles and the juxtaposition of enzymes and substrates. Changes such as these are presumably responsible for the artificial 'ageing' of sarcosomes by grinding the thoraces during the isolation procedure or subjecting sarcosomes to severely hypo- or hypertonic media. The result is irreversible uncoupling (unaffected by serum albumin) and gives rise to low P:O ratios.

Although it is important to establish possible causes for a breakdown in ATP synthesis, it is equally important to consider the changes in vivo leading to an increase in demand for ATP. Removal of ATP is usually brought

about as a result of energy requirements within the cell, although little is known about the extent of ATP-ase activity in vivo during senescence. Greater demands however by other cellular processes for ATP, exceeding the capacity for ATP production will tend to reduce the ATP levels. This may be redressed by increasing the rate of phosphorylation of ADP to ATP. If the demand is fairly excessive over a comparatively long period of time, then it may well impose a severe strain on the ATP synthetic mechanisms, with a consequent breakdown in efficiency, eventually leading to death.

Much of the recent evidence points to the fact that protein synthesis increases in a wide variety of organisms during senescence. Clarke and Maynard Smith (1966) have shown that protein synthesis, as measured by the rate at which labelled [^{3}H]-leucine was incorporated into protein increased with age in Drosophila subobscura. A similar result has been obtained with the locust by Heslop (1967, personal communication) using [^{14}C]-valine. Wulff, Samis and Falzone (1966) have also shown that the specific activity of liver protein of old rats (815-817 days old) is approximately 70 per cent higher than the specific activity of liver protein of young rats (116-118 days old), both at 15 and at 30 minutes after intraperitoneal injection of labelled leucine. Similarly, Moore, Kocun and Umbreit (1966) have shown that protein synthesis, as directed by endogenous messenger RNA increases with age in cell-free extracts of Streptococcus faecalis, with an accompanying increase in the membrane bound ribosomes.

Increases in protein synthesis such as the examples outlined above require additional energy supplies, and it would not be surprising therefore to find an increase in basal metabolism in these organisms.

Chapter 7.

GENERAL DISCUSSION

The life tables presented in Chapter 3 have shown that Calliphora ages in a similar way to other Diptera, and that the genotype profoundly affects the life span, as had been previously shown for inbred and outbred lines of Drosophila subobscura by Clarke and Maynard Smith (1955). In Calliphora, for example, the wild type D flies were much more vigorous than the wild type S, and these flies in turn survived longer than both the c-mutant and w-mutant forms.

The effect of diet, too, in both the larval and adult stages profoundly influenced longevity. Liver, added to a sugar and water diet was found to be essential for maximum survival at 24°C. Indeed, when liver was added later at Lx_{60}, to a population of flies on a sugar/water diet only, it still had a beneficial effect on the survival of approximately 30 per cent of the population, whereas the life span was considerably protracted on a sugar/water diet only.

The decline in vitality observed in the physiological measurements on the whole animal, compares with the increase in mortality with age, as is evident from the life tables. For example, it was shown that the overall activity of flies, especially flight performance, had fallen to a very low level at about 60 days of age, a period just beyond the mean survival time of these insects. The marked increase in activity, in wing beat frequency,

in sarcosomal protein and sarcosomal size, during the first week after emergence of the adult, are all considered to be part of a maturation process continued over from the pupal stage.

Observations on the oxygen consumption of the whole animal at rest on the other hand, showed that there was an increase in basal metabolic rate with an increase in age. Although this was perhaps surprising, the same increase was confirmed in experiments with isolated flight muscle tissue. This finding indicated that the rise in oxygen consumption was not necessarily the result of a central control system (hormonal or nervous), but rather an intrinsic property of senescent tissues. In addition, the tissue experiments demonstrated that serum albumin, to some extent, had a stimulatory effect on the respiration of old tissues, and suggested that the increased rate of oxygen uptake found in flight muscle tissue of old flies was due to changes in the mitochondria, rather than the indirect effect of other factors on the mitochondria.

Different measurements of the respiratory efficiency of isolated mitochondria indicated that less ATP is available for use by flight muscle fibres as flies increase in age. Experiments showed that there were significantly lower P:O ratios and DNP factors, and poorer respiratory control in sarcosomes from old flies. If the same ATP level is to be maintained, and yet a temporary imbalance between the ADP/ATP ratio exists, this will tend to increase the rate of phosphorylation of ADP to ATP, by the self-regulating mechanisms of the cell, since there is obligatory coupling between oxidation and phosphorylation. This in turn will lead to

an increase in the QO_2, until uncoupling agencies, whether structural or chemical, bring about a complete breakdown in the ATP producing system. The increase in oxygen consumption observed in old blowflies at rest, and the increase in QO_2 by intact tissues from senescent flies, is considered to be a direct result of the process outlined above. The fact that no increase was observed in the QO_2 values in isolated sarcosomes with increase in age demonstrates a difference between in vitro and in vivo states.

However, with an increase in the oxygen uptake of the whole animal at rest, there will also be a greater demand for substrate to provide the 'fuel' for ATP synthesis. There are therefore two alternatives: either the fly will feed more frequently to maintain the same level of activity (an energy consuming process in itself), or it will substantially reduce activity. Results showed that in old flies there is a significant reduction in activity. In addition, as the ATP synthetic mechanisms become less efficient during senescence, a fly will eventually be unable to carry out the more energy demanding activities such as flight. The point at which this 'threshold' in physiological performance is reached will vary from fly to fly. This has been confirmed by the results of experiments on flight performance in relation to age. However, the critical ATP level for flight is difficult to establish. Heslop and Ray (1964), for example, found that young male houseflies, which have just recovered from anoxia, were still able to fly quite well, when the ATP level in the thorax was only about 50 per cent of that in the normal fly. If this is also true of _Calliphora_, is the ATP level more critical in old flies than young flies, and if not, what additional factors are contributing to the failure of flight?

In Musca domestica, Rockstein and Brandt (1963) and Clark and Rockstein (1964) have suggested that wing loss and biochemical inefficiency of the wing muscles are intricately related. So much so that they found an observable decline in certain important enzyme systems (α-glycerophosphate dehydrogenase and ATP-ase) 24 to 48 hours prior to the actual onset of wing loss. The decline in other enzyme systems (cytochrome c oxidase) immediately followed wing loss. They conclude that the actual possession of wings in some way encourages biochemical senescence ('biocheminescence').

Although this may well be the case in Musca and possibly in some other Diptera, the suggestion does not entirely fit with the observations made on old blowflies (Calliphora). Wing loss, which occurs with greater frequency in old flies, and the ability to provide sufficient energy for flight, are equally important, and in the majority of flies occur concurrently. But this does not necessarily imply that the two factors can not determine the ability to fly quite independently. As was mentioned previously (Chapter 4), many young flies and several old flies with wings intact would not elicit the 'flight reflex' response, whereas a few very old flies (80 - 90 days) with complete wing loss, were still able to elicit the 'flight reflex' response, although quite incapable of true flight through wing loss. This strongly suggests that in Calliphora at least, the two phenomena can account quite separately for failure of flight, but as expected they will occur more frequently together in old flies. Wing loss and biochemical inefficiency of the flight muscle tissue, may in fact be an example of synchrony of events brought about by natural selection, but as was pointed out by Maynard Smith (1962), mere synchrony does not prove them to be physiologically inter-

dependent, since selection would be expected to produce synchrony.

The changes in sarcosomal size and number, together with the accompanying changes in respiratory efficiency are therefore seen to be directly responsible for the observable decline in activity and physiological performance in the senescent blowfly.

Many questions, however, remain unanswered. In particular, the underlying cause or causes for this deterioration have yet to be established. Indeed, it is not known what contribution is made by changes in other cellular organelles. Recent work on protein synthesis in Drosophila, for example, (Clarke and Maynard Smith, 1966), has shown that old flies have a higher rate of protein turnover than young flies, which as both Orgel (1963) and Harrison and Halliday (1967) suggest, may well be a failure of cells to maintain accurate protein synthesis, and thus in turn be a fundamental cause of senescence. Clearly, any increase in the demand for ATP by other metabolic processes in senescent animals could place further stress on inefficient mitochondria, and thus precipitate death more quickly.

TABLE 1

Survival data for segregated and non-segregated males and females, wild type S, fed on different diets.

Diet	No.	Males and females seg./ non-seg.	Mean survival time in days	S.D.	Lx_{50} (days)	Lx_{10} (days)	Lx_{10}/Lx_{50}
S/L/W male	145	seg.	55.3 ± 2.03	24.43	57.3	87.5	1.53
female	100	seg.	49.9 ± 2.23	22.30	47.5	79.0	1.66
S/W male	145	seg.	29.2 ± 0.87	10.50	27.5	42.5	1.55
female	100	seg.	27.9 ± 0.81	8.13	26.3	39.0	1.49
L/W male	75	seg.	5.6 ± 0.23	2.02	4.8	8.3	1.74
female	100	seg.	5.9 ± 0.29	2.95	4.3	10.3	2.41
S/W to Lx_{60} then L every 4 days male	75	seg.	34.1 ± 1.81	15.67	27.5	67.8	2.47
S/W to Lx_{20} then L every 4 days male	85	seg.	30.7 ± 1.16	10.70	29.4	40.3	1.37

continued/...

TABLE 1 (continued)

Diet	No.	Males and females seg./ non-seg.	Mean survival time in days	S.D.	Lx_{50} (days)	Lx_{10} (days)	Lx_{10}/Lx_{50}
Underfed as larvae, S/W male	102	seg.	19.9 ± 0.89	8.97	21.3	28.5	1.34
Underfed as larvae, S/W male	100	non-seg.	18.9 ± 1.0	9.97	20.5	29.8	1.45
female	100	non-seg.	16.9 ± 1.1	11.13	17.0	31.5	1.85
Underfed as larvae, S/L/W male	50	non-seg.	35.5 ± 2.47	17.46	41.5	55.0	1.32
female	50	non-seg.	29.4 ± 2.19	15.49	25.0	53.0	2.12

S - Sugar, W - Water, L - Liver

TABLE 2

Survival data for segregated *c*-mutant males and females fed on different diets

Diet	No.	Males and females seg./non-seg.	Mean survival time in days	S.D.	Lx_{50} (days)	Lx_{10} (days)	Lx_{10}/Lx_{50}
S/L/W male	194	seg.	44.5 ± 1.25	17.35	45.3	65.8	1.45
female	32	seg.	51.3 ± 3.94	21.95	55.0	74.0	1.35
S/W male	58	seg.	25.3 ± 0.93	7.09	24.5	33.0	1.35
L/W male	30	seg.	7.2 ± 0.29	2.17	6.8	9.8	1.45
S/W to Lx_{60} then L every 4 days male	75	seg.	34.4 ± 2.10	18.16	27.0	67.5	2.50

TABLE 3

Survival data for w-mutant of *Calliphora erythrocephala* at 24°C on a sugar, liver and water diet. Males and females not segregated.

Diet	No.	Mean survival time in days	S.D.	Lx_{50} (days)	Lx_{10} (days)	Lx_{10}/Lx_{50}
S/L/W female	63	43.39 ± 2.29	18.07	41.5	72	1.73
male	68	35.52 ± 1.90	15.56	31	61	1.97

TABLE 4

Survival data for non-segregated males and females wild type D, fed on sugar and water only.

Diet	No.	Males and females non-seg.	Mean survival time in days	S.D.	Lx_{50} (days)	Lx_{10} (days)	Lx_{10}/Lx_{50}
S/W male	70	non-seg.	46.8 ± 1.47	12.26	46.8	60.0	1.28
female	70	non-seg.	52.8 ± 1.52	12.72	52.3	68.0	1.30

TABLE 5

Comparison of mean survival times between females and males from four different lines of *Calliphora* at 24°C.

Type	No.	Diet	Mean survival time in days	P values between sexes
Wild type D				
female	70	S/W	52.8 ± 1.52	P = 0.01
male	70		46.8 ± 1.47	
Wild type S				
female	100	S/L/W	49.9 ± 2.23	P = 0.1
male	145		55.3 ± 2.03	
c-mutant				
female	32	S/L/W	51.3 ± 3.94	P = 0.1
male	194		44.5 ± 1.25	
w-mutant				
female	63	S/L/W	43.39 ± 2.29	P = 0.01
male	68		35.52 ± 1.90	

TABLE 6

Means and standard errors of oxygen uptake by male blowflies at rest

Age (days)	No.	µl O_2/mg wet wt. per hr
1	24	1.60 ± 0.06
4	20	1.90 ± 0.04
7	24	1.20 ± 0.08
14	24	1.61 ± 0.20
21	9	1.41 ± 0.37
28	21	1.54 ± 0.18
35	18	1.56 ± 0.11
42	14	1.48 ± 0.08
49	16	1.93 ± 0.16
56	15	1.88 ± 0.09
63	14	2.26 ± 0.19
70	16	2.14 ± 0.20
77	12	2.10 ± 0.15
84	8	1.92 ± 0.21

TABLE 7

Mean weights and standard errors of male flies from an ageing population of 50.

Age (days)	No.	Weight (mg)
1	50	47.44 ± 0.84
7	50	47.93 ± 0.93
14	49	49.10 ± 0.71
21	43	46.83 ± 0.67
28	41	46.52 ± 0.78
35	40	-
42	39	45.97 ± 0.57
49	38	47.14 ± 0.74
56	29	46.72 ± 0.79
63	21	46.81 ± 1.17
70	15	48.10 ± 1.39
77	8	45.81 ± 1.68
84	5	45.70 ± 1.80

Readings taken at the same time each week.

TABLE 8 (i)

Means and standard errors of oxygen uptake by male blowflies under 'free-moving' conditions

Age (days)	No.	µl O_2/mg wet wt. per hr
1 a.m.	12	2.37 ± 0.61
1 p.m.	10	4.77 ± 0.93
4	22	6.37 ± 0.73
7	24	8.86 ± 1.38
14	24	6.86 ± 1.13
21	28	8.49 ± 1.10
28	17	7.83 ± 0.98
35	15	8.43 ± 1.04
42	11	7.19 ± 1.56
49	14	4.73 ± 0.70
56	14	4.92 ± 0.97
63	12	6.36 ± 1.42
70	12	4.00 ± 0.62
77	10	3.86 ± 0.99
84	7	3.91 ± 1.49

TABLE 8 (ii)

Activity - means and standard errors of the number of times flies interrupted the infra red beam

Age (days)	No.	Activity
1	8	1046 ± 384
7	10	3988 ± 538
14	10	2423 ± 568
21	8	2401 ± 551
28	8	1692 ± 370
35	8	1263 ± 411
42	8	924 ± 382
49	8	1209 ± 377
56	8	230 ± 57
63	9	192 ± 96
70	8	371 ± 111
77	5	165 ± 95
84	4	87 ± 49

TABLE 9

Means and standard errors for oxygen consumption of male blowflies in flight

Age (days)	No.	μl O_2/mg wet wt. per hr
7	20	48.3 ± 2.71
14	25	53.4 ± 2.22
21	20	50.1 ± 3.91
28	19	56.3 ± 4.13
35	5	51.1 ± 2.87
42	6	57.9 ± 6.43
49	5	61.2 ± 6.65
56	6	62.2 ± 4.78

TABLE 10 (i)

Mean wing-beat frequencies of male blowflies with age

Age (days)	No.	cycles/sec.
1	7	100.3 ± 2.3
2	9	113.6 ± 2.3
3	6	134.2 ± 5.8
4	6	124.8 ± 3.5
7	7	147.3 ± 2.3
14	6	162.0 ± 3.1
21	6	162.4 ± 2.6
28	6	168.5 ± 1.9
35	6	161.0 ± 2.0
42	5	168.5 ± 2.6
49	6	180.1 ± 4.7
60	4	179.6 ± 6.1
70	5	172.5 ± 4.0
77	2	178.4 ± 5.1

TABLE 10 (ii)

Mean flight times for all flies investigated, including those which flew for short periods only

Age (days)	No.	Mean flight times (min.)	(sec.)	(min.)
1	7	1	12	± 0.29
2	9	2	42	± 0.49
3	6	2	22	± 1.73
4	6	1	56	± 0.33
7	27	5	45	± 1.13
14	31	6	14	± 0.95
21	28	5	33	± 0.70
28	28	5	30	± 0.69
35	15	8	34	± 2.74
42	11	5	53	± 1.43
49	10	4	39	± 0.75
56	7	8	36	± 1.67
60	4	3	36	± 1.29
70	5	2	40	± 1.59
77	2	1	40	± -

TABLE 11

Means and standard errors of oxygen uptake (QO_2) by 'half thorax' preparations of flight muscle tissue with age at 24°C.

(i) Values calculated from the first half hour of readings.

(ii) Values calculated from the first hour of readings.

Medium	Substrate	Age (days)	Samples n	QO_2 (µl O_2/mg dry wt. muscle/hr) (i)	(ii)
1.	None	7	6	10.05 ± 0.45	10.05 ± 0.45
		56	3	6.76 ± 0.37	6.50 ± 0.35
		75	4	15.27 ± 0.61	13.09 ± 0.51
2.	Trehalose 30mM	2	12	11.56 ± 0.39	11.56 ± 0.39
		8	12	11.06 ± 0.36	11.06 ± 0.36
		14	12	10.12 ± 0.47	10.12 ± 0.47
		21	14	11.20 ± 0.59	11.20 ± 0.59
		28	12	10.82 ± 0.58	10.82 ± 0.58
		42	10	9.20 ± 0.65	9.20 ± 0.65
		56	10	9.94 ± 0.72	9.57 ± 0.77
		70	11	14.60 ± 1.17	12.85 ± 0.93
		85	6	18.87 ± 0.91	16.76 ± 0.56
3.	Glucose 30mM	2	8	10.70 ± 0.25	10.70 ± 0.25
		56	3	11.36 ± 1.55	10.35 ± 1.46
		75	7	16.44 ± 1.40	14.12 ± 1.53
4.	Glucose 30mM + 2% BSA	75	9	16.50 ± 1.35	15.30 ± 1.03

TABLE 12

Differences in the mean QO_2 values between tissues from sugar/water and sugar/liver/water fed flies from the same generation at 24°C.

Medium 2 used for incubation

Age (days)	S/L/W No.	S/L/W QO_2	S/W No.	S/W QO_2	P values
28	12	10.82 ± 0.58	10	7.99 ± 0.42	0.01
42	10	9.20 ± 0.65	8	7.99 ± 0.62	0.2
50	-	-	5	8.42 ± 0.98	0.3
56	10	9.94 ± 0.72	-	-	

TABLE 13

A comparison of methods for determining standard protein solutions

Protein standards made up	Spectrophotometric method at 280 mμ	Micro-Kjeldahl determinations
	(Absorbance units)	
1.0 mg/ml	0.606	1.001 mg/ml
2.0 mg/ml	1.208	1.968 mg/ml
2.5 mg/ml	1.504	2.390 mg/ml
3.0 mg/ml	1.830	-
5.0 mg/ml	2.662	4.860 mg/ml

TABLE 14

A Coulter Counter analysis of size and number of sarcosomes in relation to age

Size in Microns	AGE IN DAYS.							
	0.167	3	7	15	30	50	65	85
	PERCENTAGES OF TOTALS							
below 1	1.75	1.50	None	None	Negligible	2.00 *	2.75 *	4.00 *
1.0 - 1.5	40.25	40.50	17.50	7.00	12.25	17.50	13.50	27.50
1.5 - 2.0	33.25	25.50	26.75	23.00	26.00	18.00	12.00	17.50
2.0 - 2.5	14.75	14.25	27.75	31.00	29.00	18.50	12.25	14.75
2.5 - 3.0	5.00	8.25	15.00	23.50	16.50	16.25	13.00	12.75
3.0 - 3.5	3.00	4.50	7.00	8.00	7.25	11.50	13.00	9.25
3.5 - 4.0	1.25	2.75	2.75	3.75	3.50	6.25	11.50	4.75
4.0 - 5.0	0.50	2.00	1.75	2.25	3.00	6.50	12.50	6.00
5.0 - 6.0	0.25	0.50	1.25	1.25	1.50	1.75	5.25	2.50
above 6.0	None	0.25	0.25	0.25	1.00	1.75	4.25	1.00
Total numbers per Thorax x 10^8	4.5	4.1	4.8	4.7	4.2	3.1	3.1	3.4

TABLE 15

Mean QO_2 values and P:O ratios of sarcosomes extracted from male and female flies as a function of age

Days	No.	QO_2	Range	P:O	Range	mg protein/ flask
3	14	809	(908-605)	1.61	(1.88-1.42)	0.32
8	13	847	(1184-568)	1.68	(1.86-1.55)	0.41
10	20	810	(929-662)	1.73	(1.90-1.56)	0.39
14	18	821	(1036-576)	1.53	(1.78-1.21)	0.40
20	19	804	(965-745)	1.50	(1.62-1.34)	0.43
30	14	792	(856-721)	1.48	(1.59-1.36)	0.42
40	14	874	(1120-596)	1.43	(1.56-1.26)	0.42
60	8	672	(800-526)	1.40	(1.51-1.23)	0.42
70	6	838	(1038-678)	1.21	(1.43-0.83)	0.44
80	8	854	(937-740)	1.34	(1.40-1.21)	0.38
90	8	802	(863-708)	1.26	(1.38-1.13)	0.40

TABLE 16

Mean P:O ratios with and without 2% BSA in the reaction medium

Days	P:O +BSA	P:O -BSA
3	1.61	1.62
8	1.74	1.63
10	1.75	1.72
14	1.58	1.49
20	1.51	1.50
30	1.48	1.47
40	1.46	1.40
60	1.40	1.40
70	1.14	1.29
80	1.36	1.32
90	1.26	1.26

TABLE 17

Results obtained when serum albumin was absent from the isolation medium. Mean QO_2 values and P:O ratios with and without 2% BSA added subsequently to the reaction medium

Days	No.	QO_2 +BSA	QO_2 -BSA	P:O +BSA	P:O -BSA	mg protein/ flask
6	6	807	992	0.72	0.45	0.40
24	4	802	1017	0.46	0.32	0.44
50	4	710	1015	0.52	0.36	0.42

Respiratory control by blowfly sarcosomes as a function of age of the fly

TABLE 18

ADP added after 10 minutes incubation
(Mean values given)

Days	No.	QO_2 -ADP	QO_2 +ADP	R.C.I.	mg protein flask
8	6	264	406	1.54	0.38
30	8	302	315	1.04	0.40
60	6	256	232	Not demonstrated	0.38

TABLE 19

ADP added after 6 minutes incubation

(Mean values given)

Days	No.	QO_2 -ADP	QO_2 +ADP	R.C.I.	Range	mg protein/ flask
3	4	413	572	1.38	(1.33-1.45)	0.30
7	3	392	605	1.54	(1.43-1.73)	0.35
10	7	365	574	1.57	(1.50-1.79)	0.37
20	3	450	665	1.48	(1.38-1.63)	0.36
45	3	336	476	1.42	(1.41-1.43)	0.36
65	3	386	441	1.14	(1.07-1.23)	0.37

TABLE 20

Magnesium stimulated ATP-ase activity of sarcosomes in relation to age of the fly

Age (days)	Time (minutes)	μMoles P_i/mg protein -DNP	+DNP	DNP factor
1	5	1.74	3.96	2.28
	10	2.48	6.40	2.58
	15	4.16	10.20	2.46
	20	4.98	11.08	2.22
7	5	1.98	5.94	3.00
	10	3.93	9.40	2.39
	15	5.74	11.08	1.93
	20	6.38	11.74	1.84
	45	9.81	12.95	1.32
14	5	2.28	5.00	2.20
	10	4.17	7.50	1.80
	15	5.67	8.66	1.53
	20	6.78	9.23	1.36
21	5	1.81	4.31	2.38
	10	3.41	5.32	1.56
	15	4.42	6.71	1.52
	20	5.81	7.66	1.32
	45	10.85	12.44	1.15

continued/...

TABLE 20 (contd.)

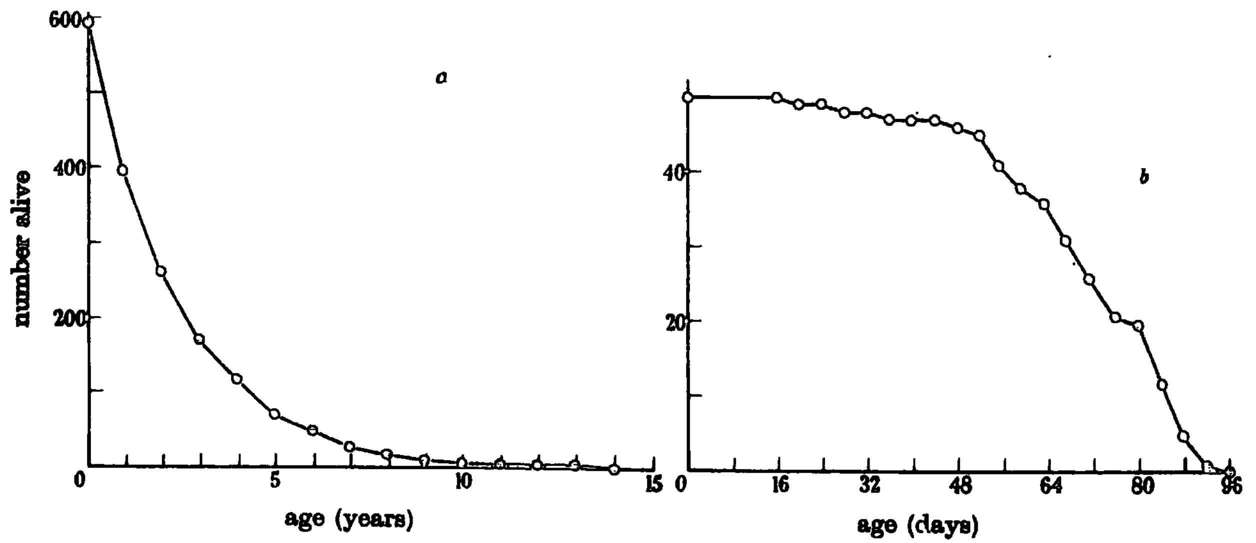
Age (days)	Time (minutes)	Moles P_i/mg protein -DNP	+DNP	DNP factor
35	5	1.50	3.90	2.60
	10	3.05	5.60	1.83
	15	4.85	6.85	1.42
	20	5.45	7.40	1.36
	45	8.40	9.55	1.14
50	5	1.85	4.03	2.18
	10	3.90	6.60	1.69
	15	4.94	7.61	1.54
	20	5.55	7.83	1.41
60	5	2.10	3.11	1.48
	10	3.48	5.75	1.65
	15	4.58	7.24	1.58
	20	5.67	7.75	1.37
	45	7.96	10.04	1.26
72	5	1.23	1.82	1.48
	10	3.02	4.11	1.36
	15	3.56	5.38	1.51
	20	5.28	7.48	1.42
	45	8.68	10.84	1.25
80	5	1.46	2.14	1.47
	10	3.20	4.90	1.53
	15	5.14	7.08	1.38
	20	6.78	8.07	1.19
	45	9.32	10.90	1.17

Figure 1 Survivorship curves for

(a) Lapwings in the wild,

(b) *Drosophila subobscura* females in the laboratory at 20°C.

(data from Maynard Smith 1962)



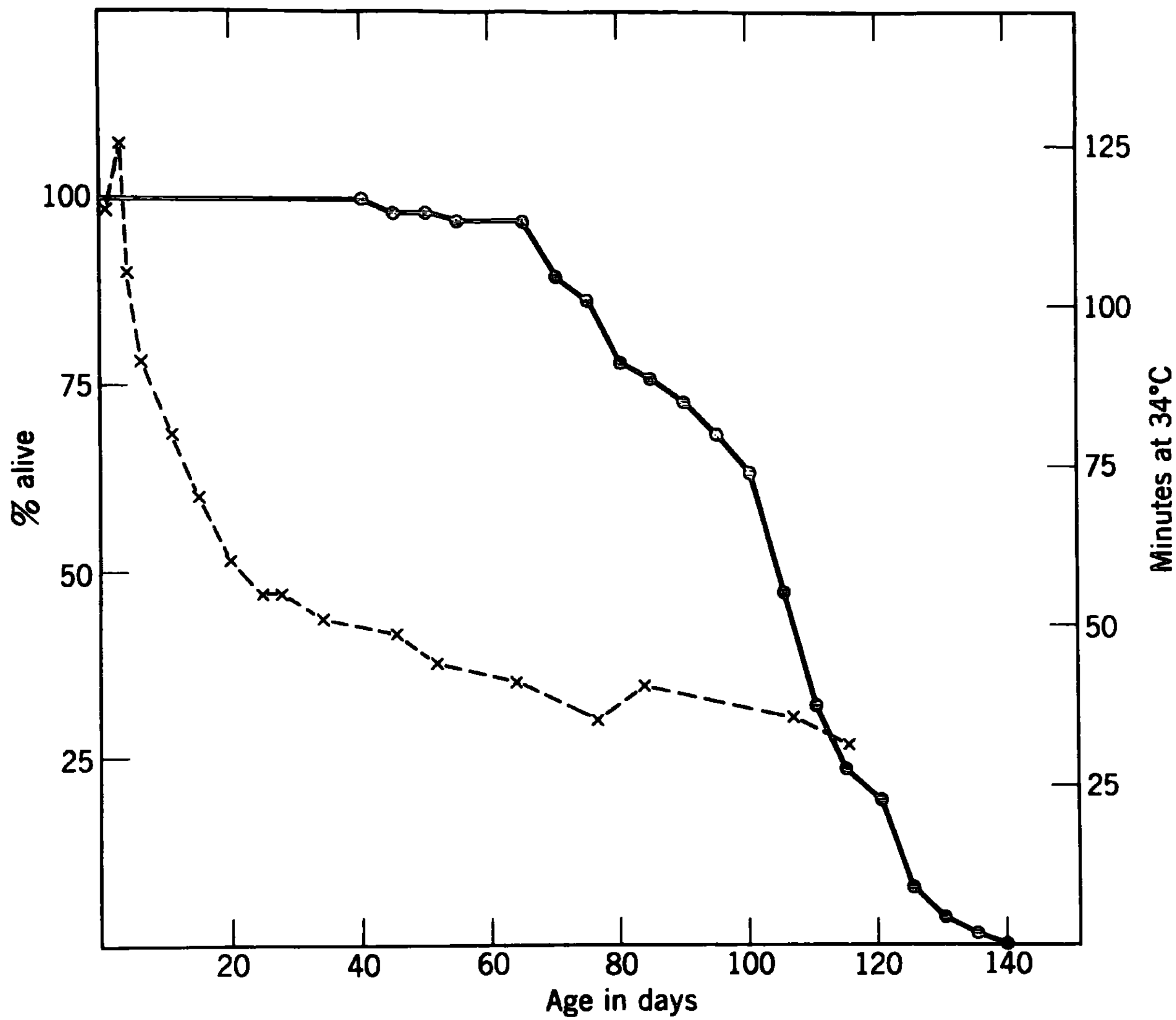
600
400
200
0
number alive
a
5
10
15
age (years)
b
40
20
0
16
32
48
64
80
96
age (days)

Figure 2 Two methods of measuring ageing in male Drosophila subobscura

—o—o— % survivors

x--x--- survival time in minutes at 34°C in dry air.

(data after Hollingsworth and Bowler 1966)



% alive
100
75
50
25
Minutes at 34°C
125
100
75
50
25
20
40
60
80
100
120
140
Age in days

Figure 3

Blowfly cages and the constant temperature cabinets.

Figure 4

Male *c*-mutant flies

Female *c*-mutant flies

Demonstrating the difference in size between imagoes bred from 'normally' fed and 'underfed' larvae.

Figure 5

Survival curves for female wild type S. (A) Given water only. (B) Given liver and water only. (C) Given sugar and water only. (D) Given sugar, liver and water.

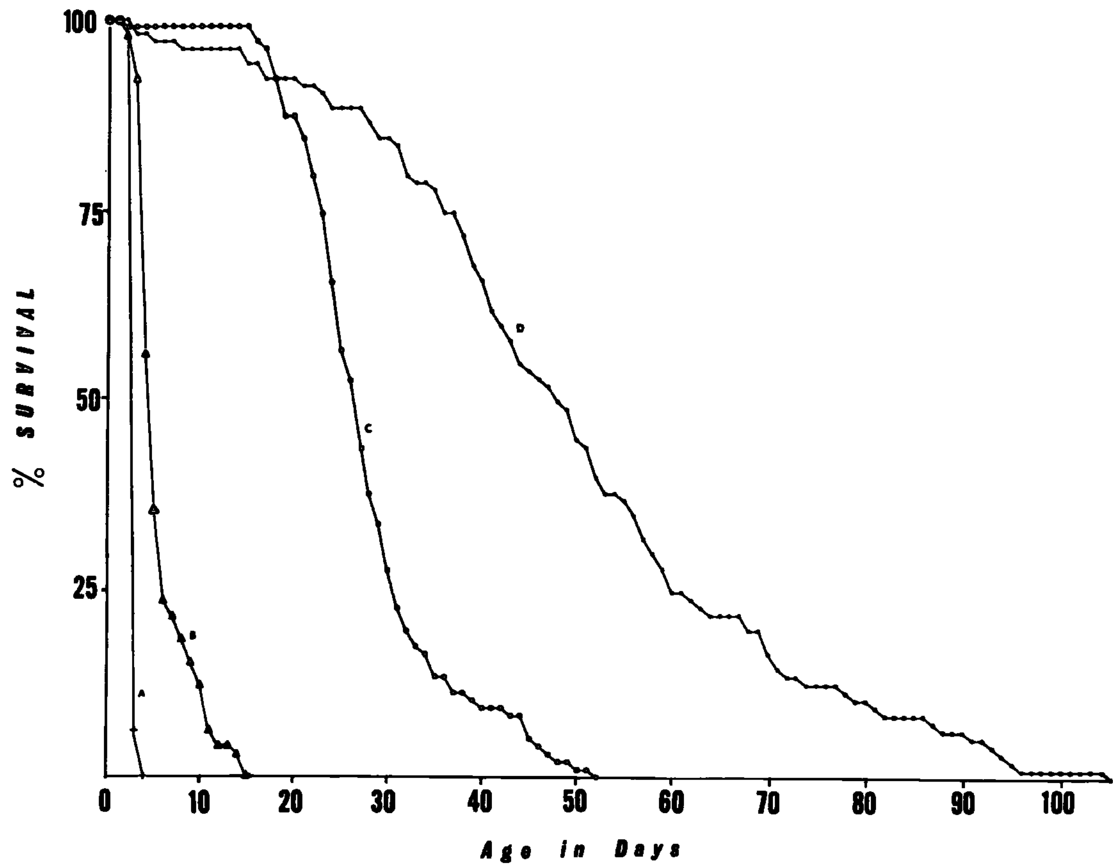
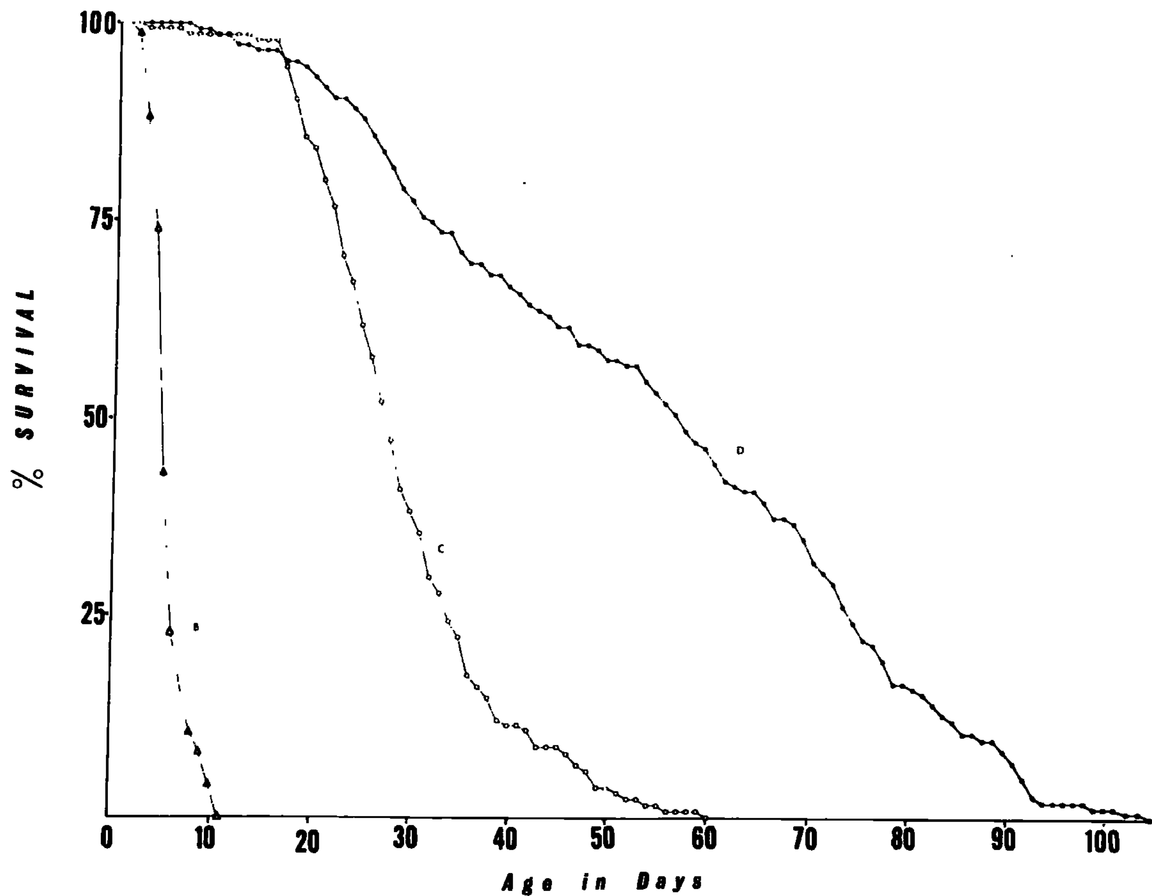

100
75
50
25
% SURVIVAL
0
10
20
30
40
50
60
70
80
90
100
Age in Days
A
B
C
D

Figure 6

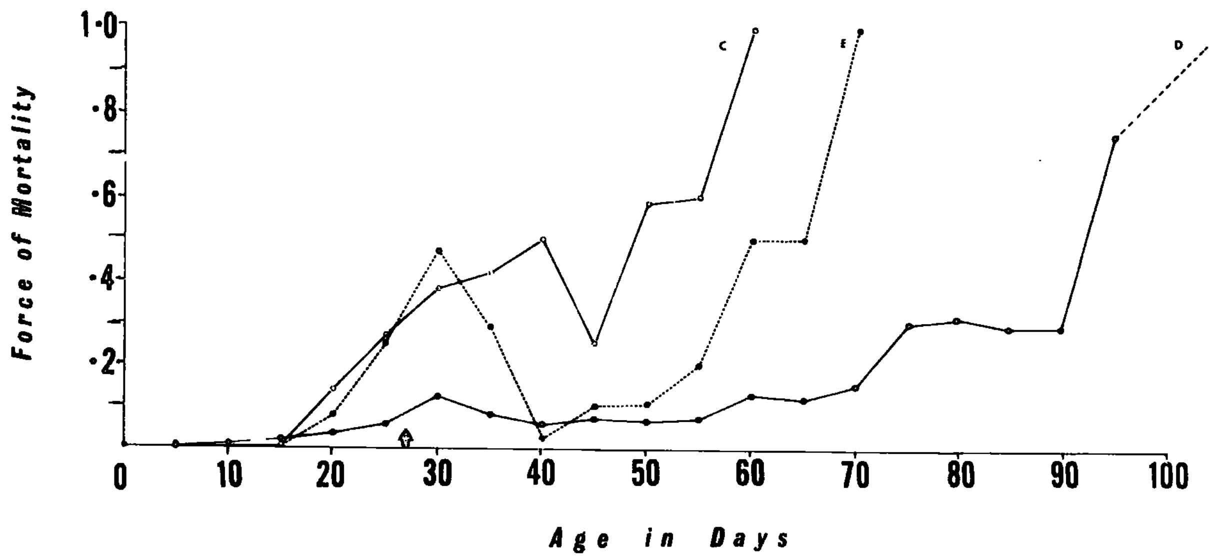
Survival curves for male wild type S. (B), (C), and (D) as in Figure 5.



100
75
50
25
% SURVIVAL
0
10
20
30
40
50
60
70
80
90
100
Age in Days
B
C
D

Figure 7

Mortality of male wild type S. (C) sugar/water only. (D) sugar/liver/water. (E) sugar/water to Lx_{60} then liver added every 4 days to diet.

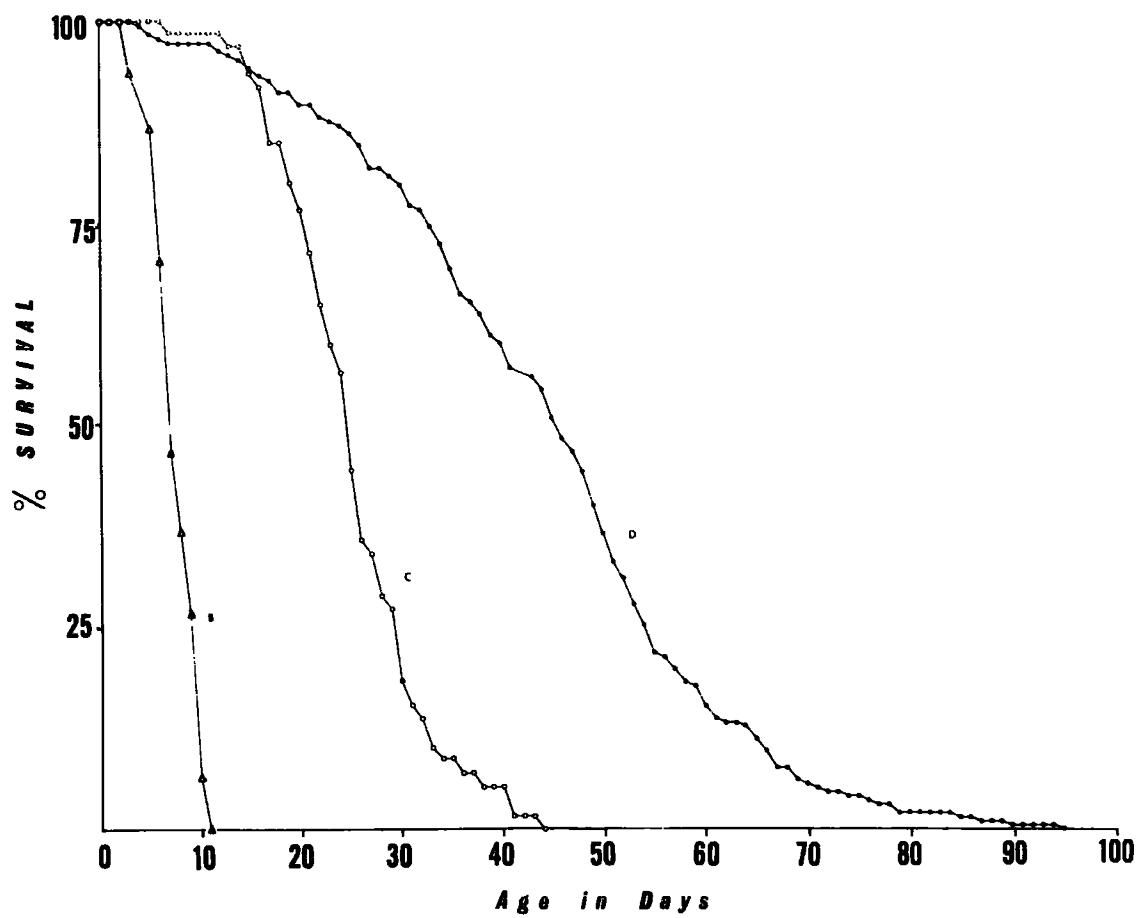


1·0
·8
·6
·4
·2
0
10
20
30
40
50
60
70
80
90
100
C
E
D
Force of Mortality
Age in Days

Figure 8

Survival curves for male c-mutant.

(B), (C), and (D) as in Figure 5.



100
75
50
25
% SURVIVAL
0
10
20
30
40
50
60
70
80
90
100
Age in Days
C
D

Figure 9

Survival curves for non-segregated male (M) and female (F) *w*-mutant flies on a sugar, liver, water diet.

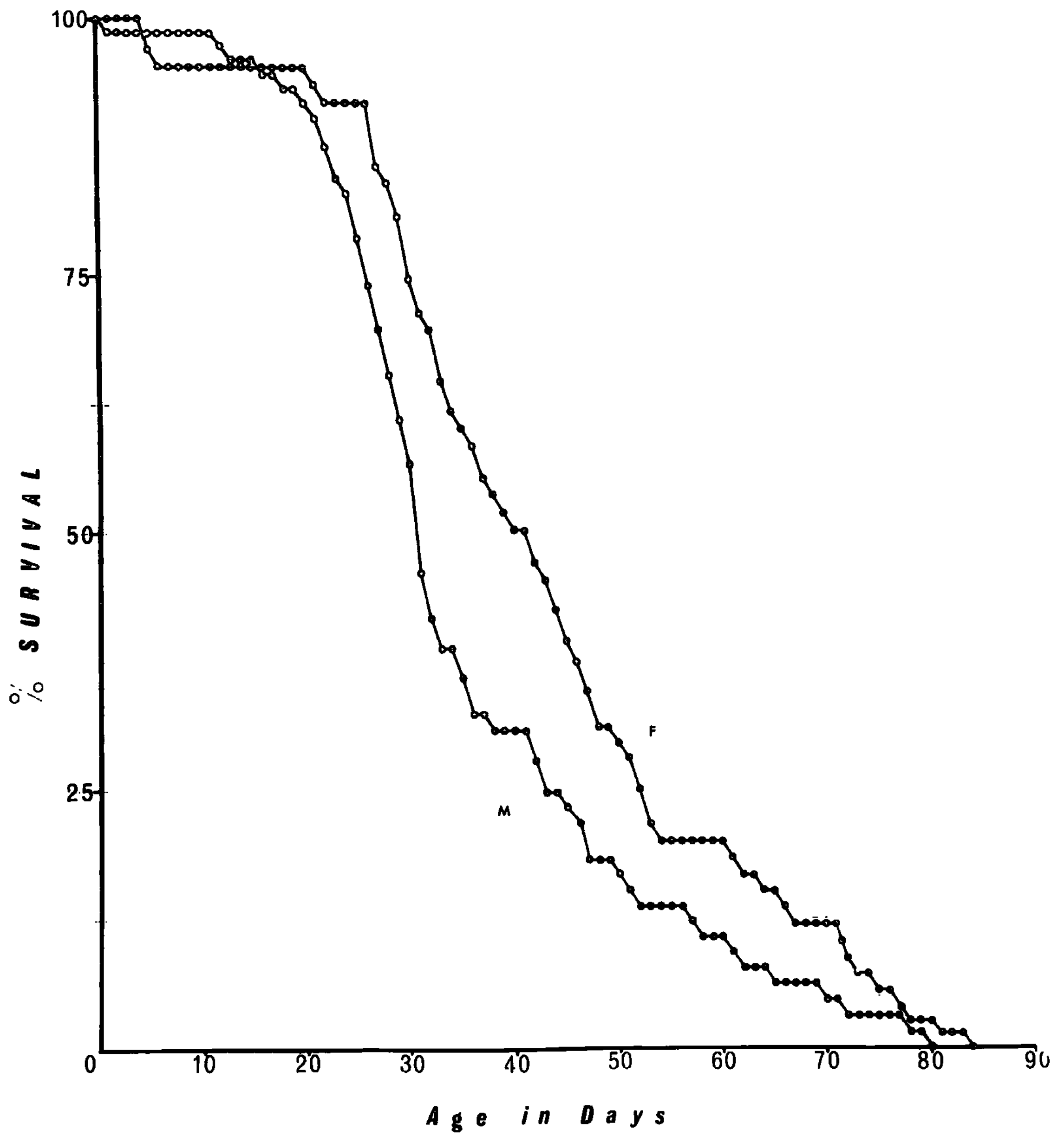

100
75
50
25
% SURVIVAL
0
10
20
30
40
50
60
70
80
90
Age in Days
F
M

Figure 10

Survival curves for male wild type S. (C) as in Figure 5. (E) given sugar and water to Lx_{60} (indicated by the arrow), then liver added to the diet every 4 days thereafter. (F) males reared from underfed larvae, segregated from females and given sugar/water diet only.

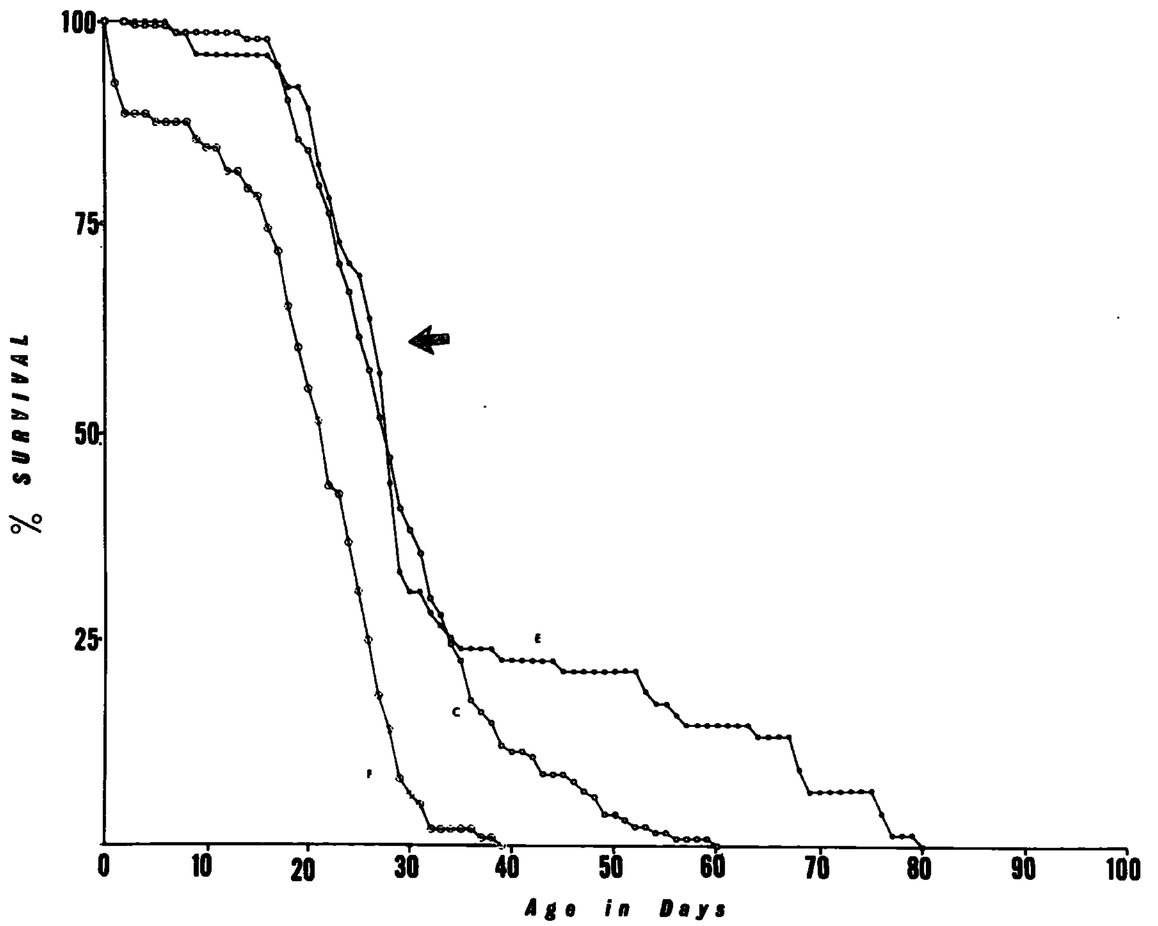

100
75
50
25
% SURVIVAL
E
C
F
0
10
20
30
40
50
60
70
80
90
100
Age in Days

Figure 11

Survival curves for male c-mutant.

(C) and (E) as in Figure 10.

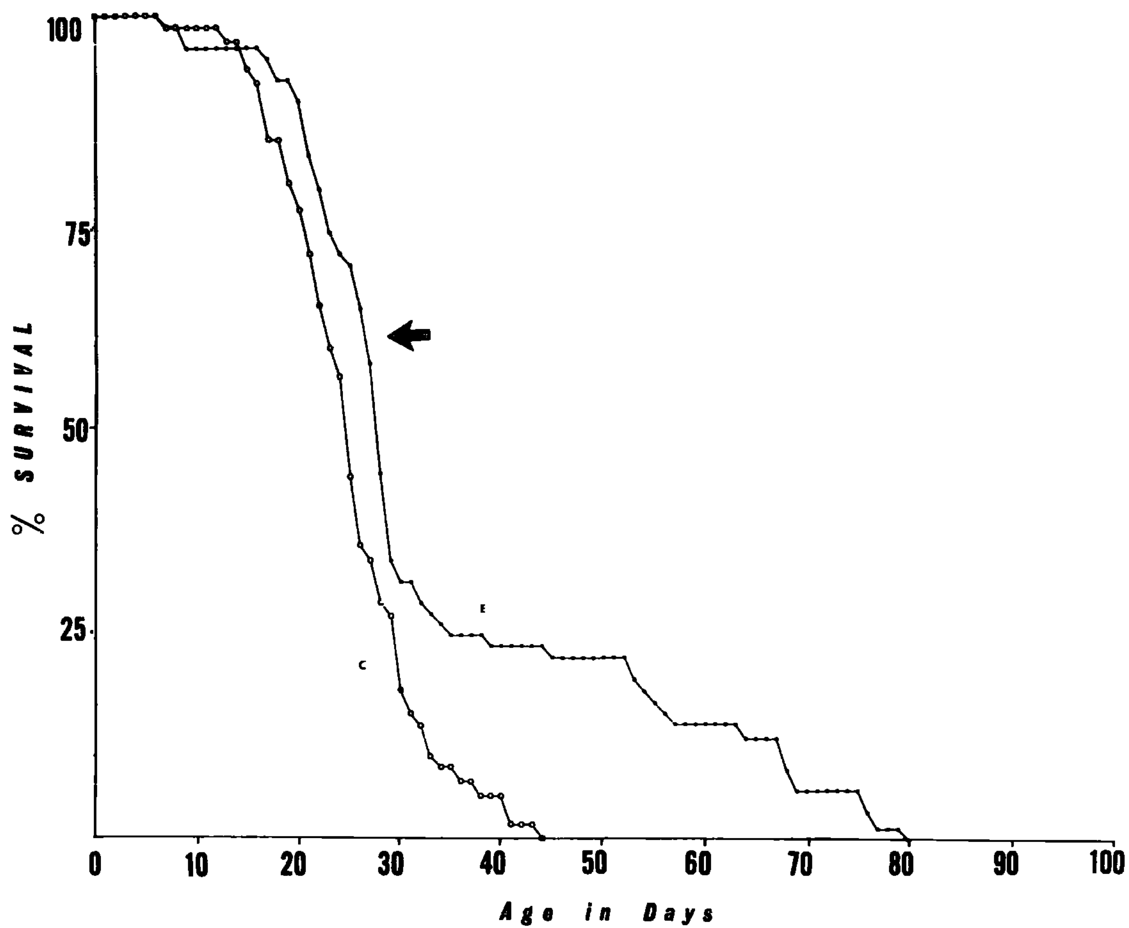
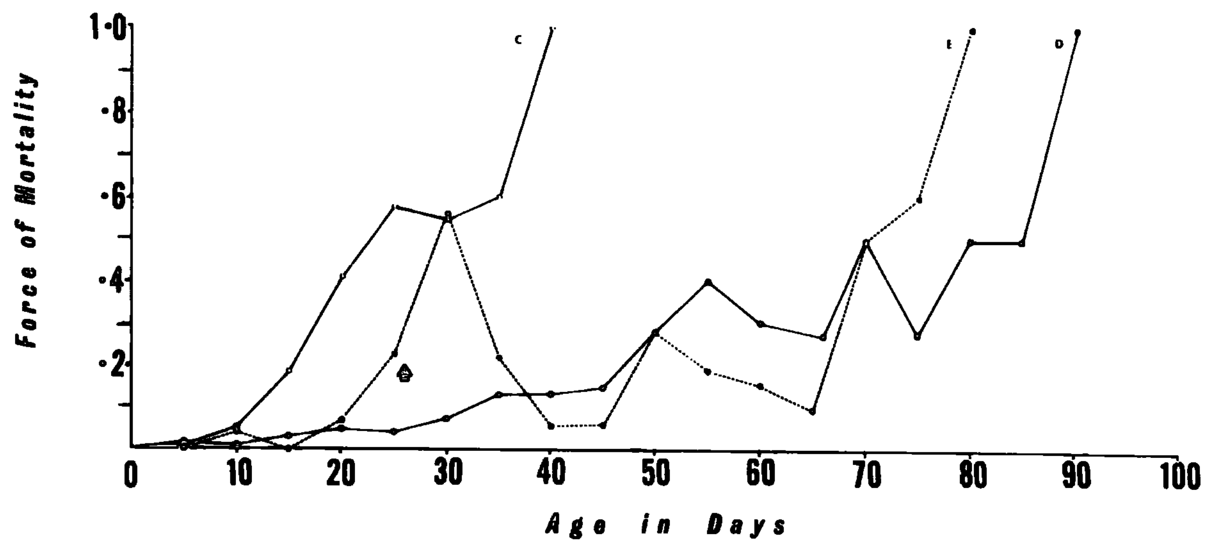

100
75
50
25
% SURVIVAL
E
C
0
10
20
30
40
50
60
70
80
90
100
Age in Days

Figure 12

Mortality of male c-mutant.

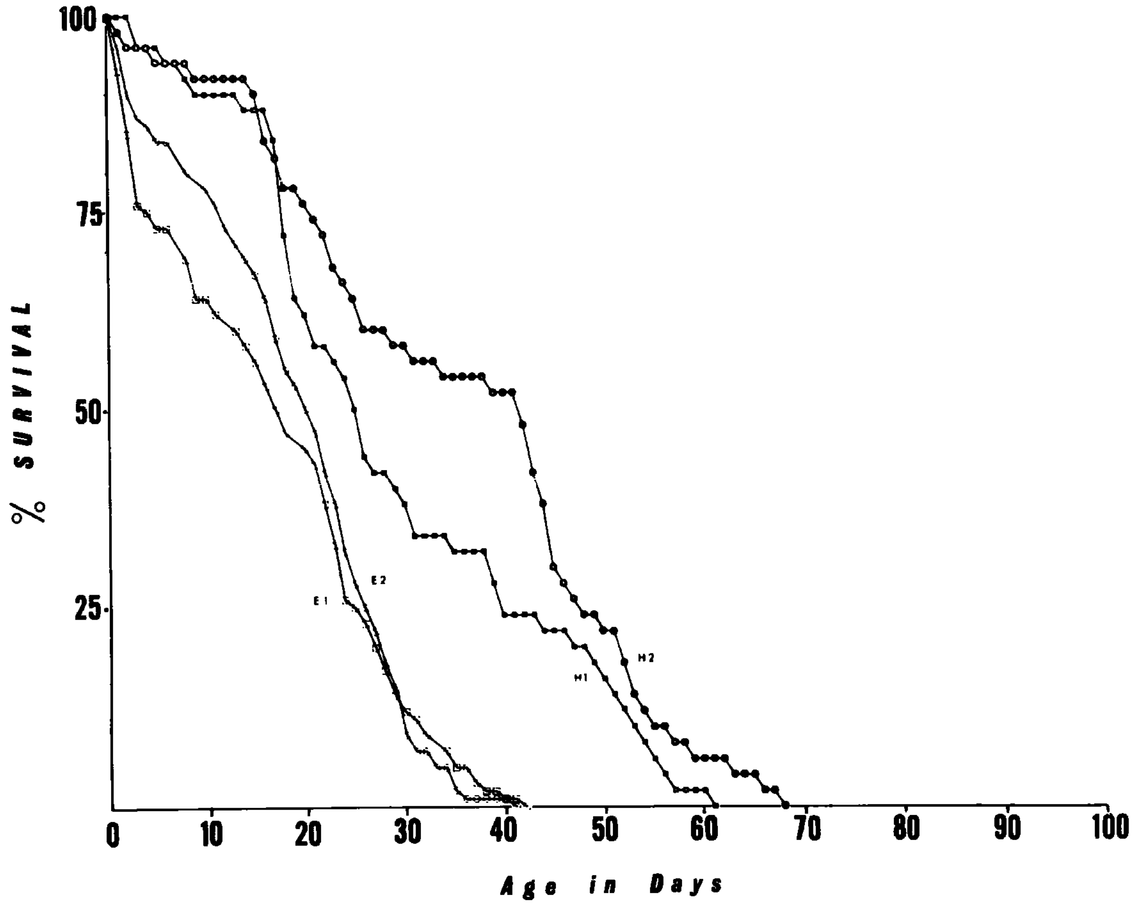
(C), (D), and (E) as in Figure 7.



Force of Mortality
1·0
·8
·6
·4
·2
0
10
20
30
40
50
60
70
80
90
100
Age in Days
C
E
D

Figure 13

Survival curves for male and female wild type S. (E1) female and (E2) male, reared from underfed larvae, sexes not segregated and fed on a sugar/ water diet only. (H1) female and (H2) male, reared from underfed larvae, sexes not segregated, but fed sugar/liver/water.



100
75
50
25
% SURVIVAL
0
10
20
30
40
50
60
70
80
90
100
Age in Days
E1
E2
H1
H2

Figure 14

Survival curves for male and female wild type D (F1 progeny) (C1) male and (C2) female, sexes not segregated, given sugar/water only.

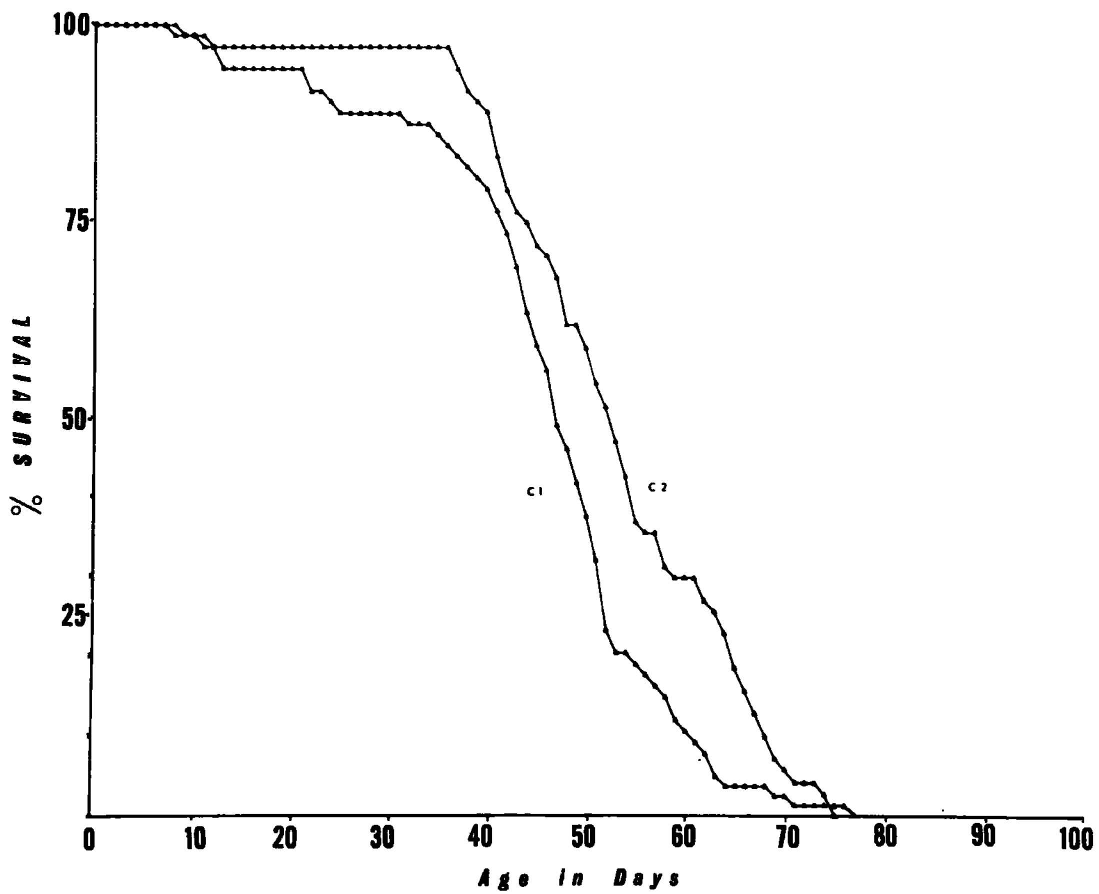

100
75
50
25
0
10
20
30
40
50
60
70
80
90
100
% SURVIVAL
Age in Days
C1
C2

Figure 15

The activity recorder.

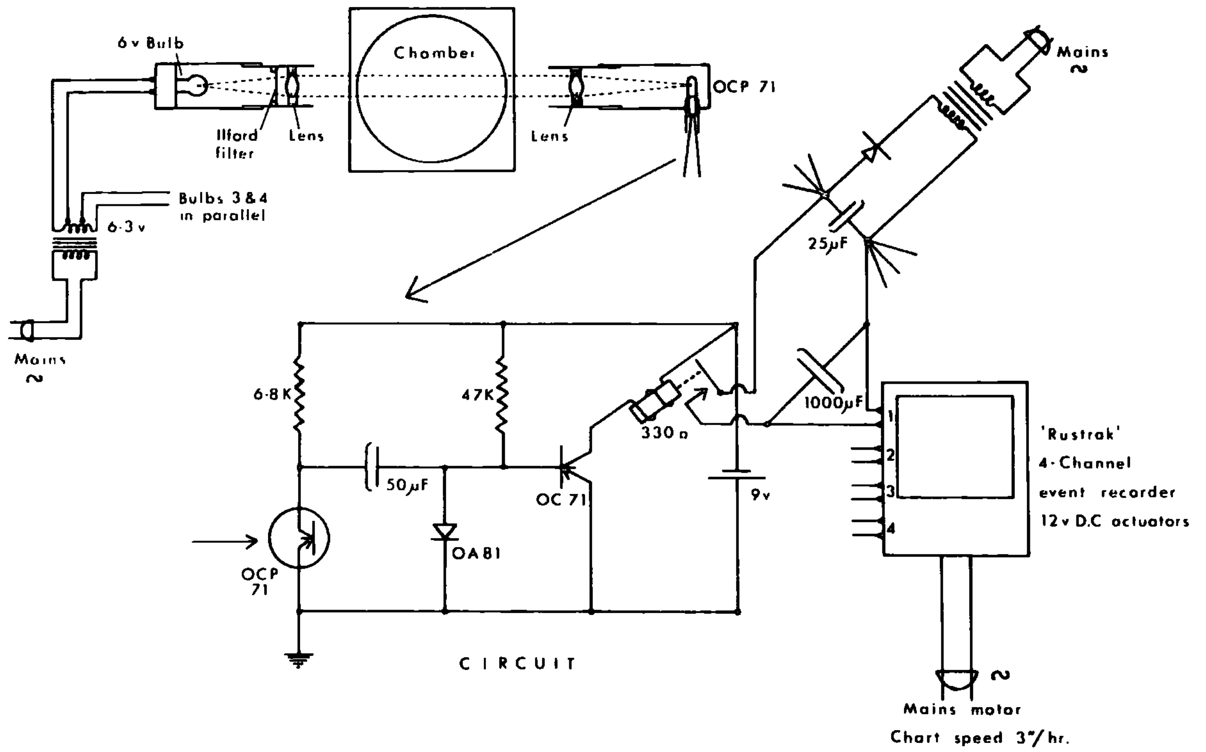
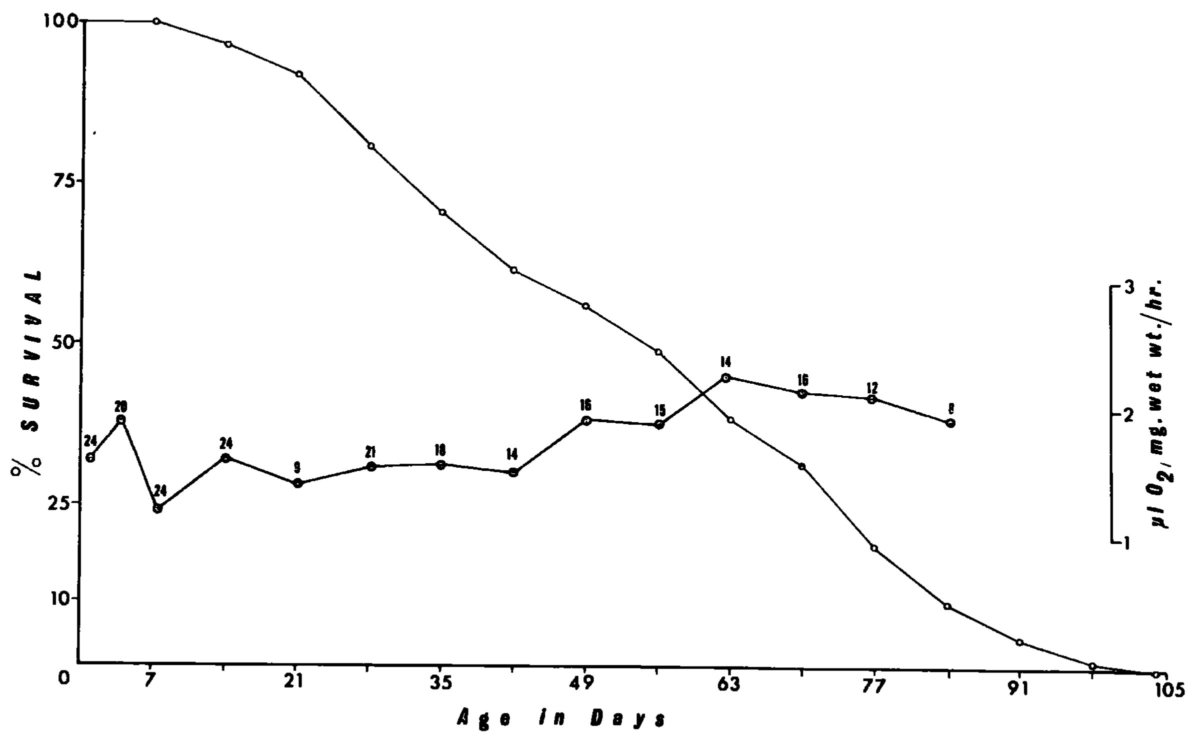

6 v Bulb
Chamber
OCP 71
Mains
Ilford filter
Lens
Lens
Bulbs 3 & 4 in parallel
6·3 v
25μF
Mains
6·8 K
47K
330 Ω
1000μF
50μF
OC 71
9 v
OA 81
OCP 71
'Rustrak' 4-Channel event recorder 12 v D.C actuators
1
2
3
4
CIRCUIT
Mains motor
Chart speed 3"/hr.

Figure 16

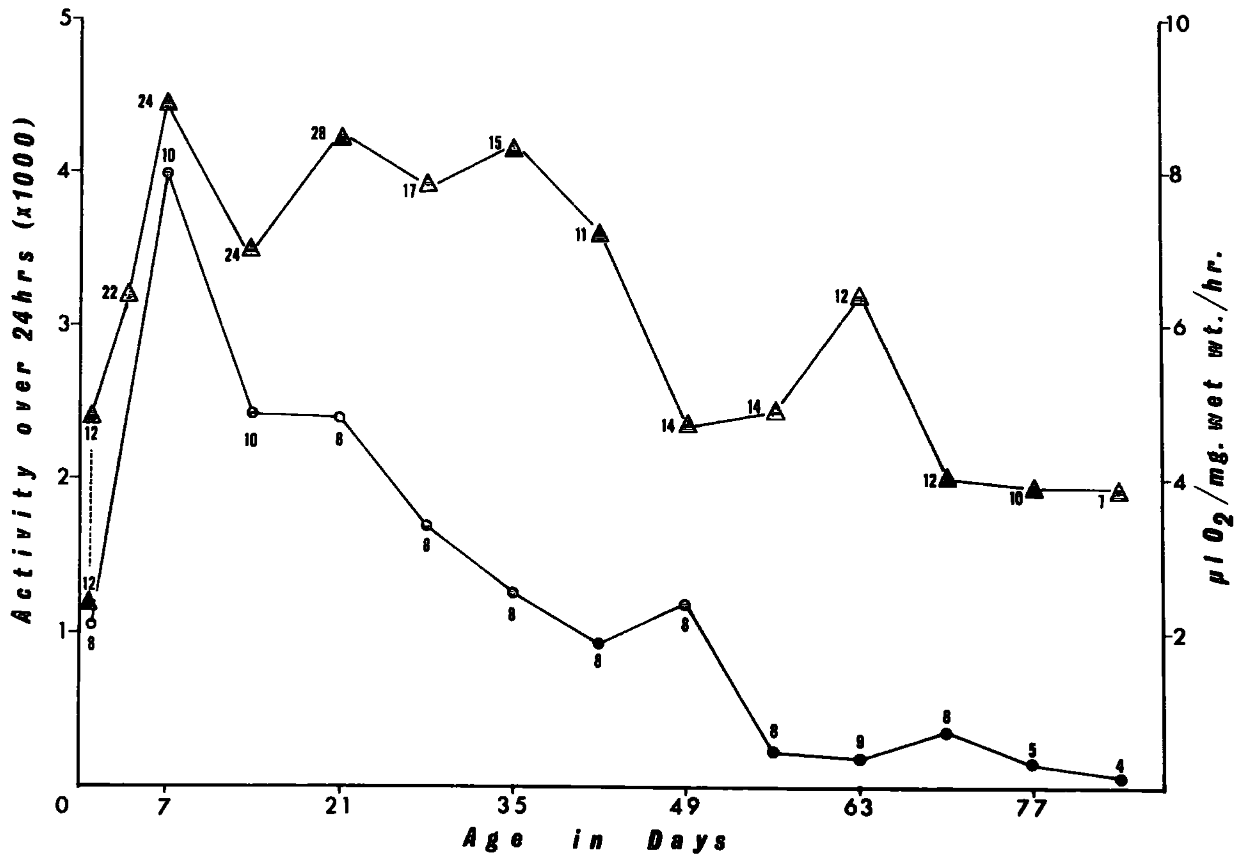
A survival curve for male blowflies at 24°C, —⊖——⊖—, and oxygen consumption at rest, —o——o—. Numbers against each point indicate the number of flies selected.



100
75
50
25
10
0
% SURVIVAL
7
21
35
49
63
77
91
105
Age in Days
3
2
1
μl O2/mg. wet wt./hr.
24
20
24
24
9
21
18
14
16
15
14
16
12
8

Figure 17

Records of activity and oxygen consumption of male blowflies under 'free-moving' conditions at 24°C. Activity, —o——o—; O_2 uptake, —Δ——Δ—. Numbers against each point indicate the number of flies selected.



Activity over 24hrs (x1000)
μl O_2/mg. wet wt./hr.
Age in Days
0
7
21
35
49
63
77
1
2
3
4
5
2
4
6
8
10
24
10
28
15
17
24
11
22
12
12
14
14
12
10
7
12
12
8
10
8
8
8
8
8
8
8
9
8
5
4

Figure 18

Oxygen consumption in flight at 24°C. Numbers against each point indicate the number of flies flown successfully under the stated conditions.

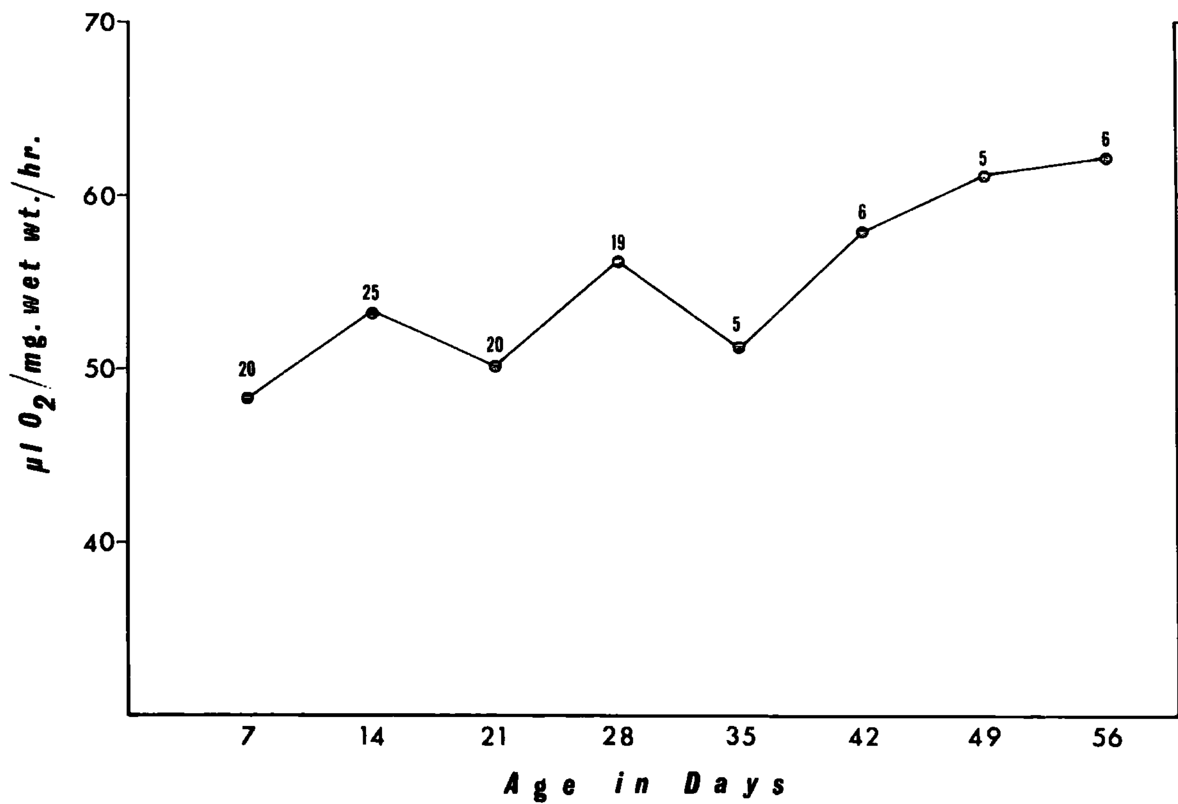

70
60
50
40
μl O_2/mg. wet wt./hr.
20
25
20
19
5
6
5
6
7
14
21
28
35
42
49
56
Age in Days

Figure 19

Oxygen uptake/minute of continuous flight by the longest-flying insects in each selected age group. Different symbols represent different flies.

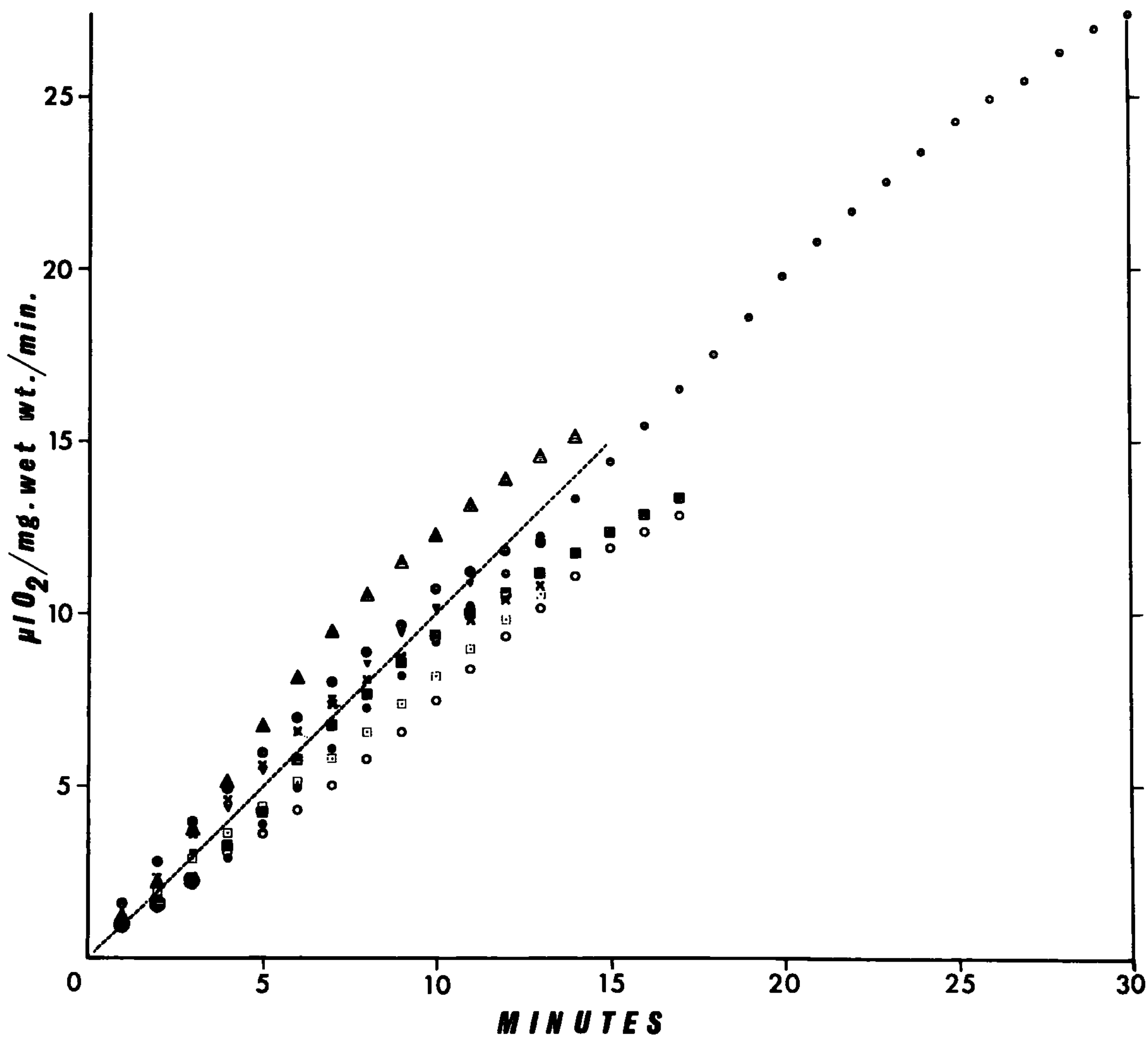
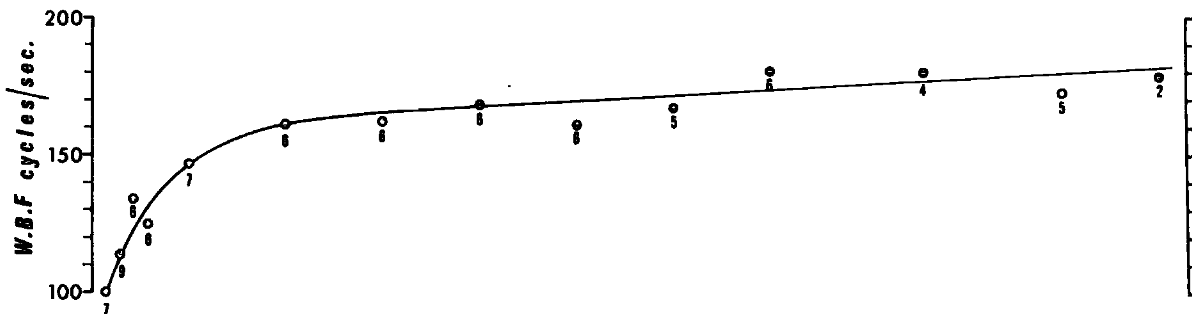

25
20
15
10
5
0
5
10
15
20
25
30
μl O2/mg. wet wt./min.
MINUTES

Figure 20

Variations of -

Mean wing-beat frequencies and flight times of male blowflies with age at 24°C. Numbers against each point indicate the number of flies selected.


200
150
100
W.B.F cycles/sec.
7
9
6
6
7
6
6
6
6
5
6
4
5
2

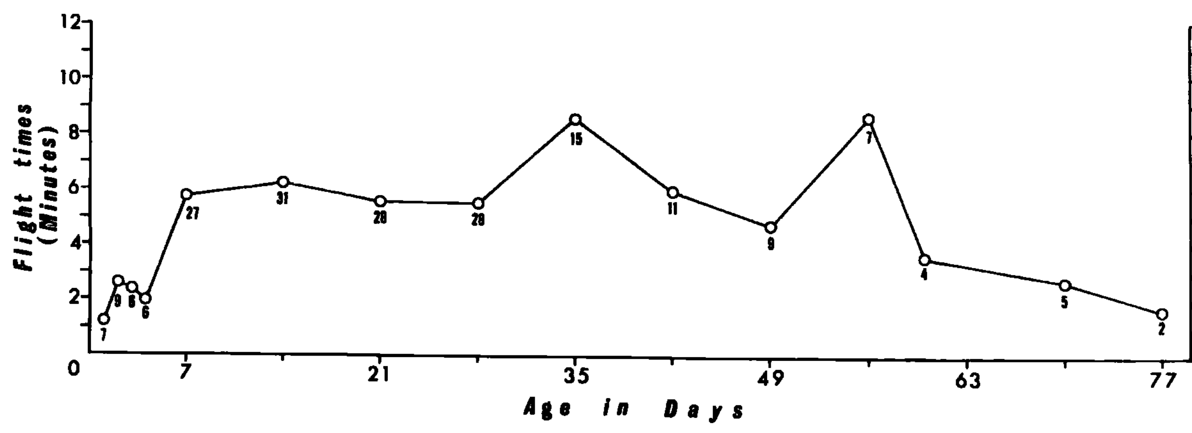
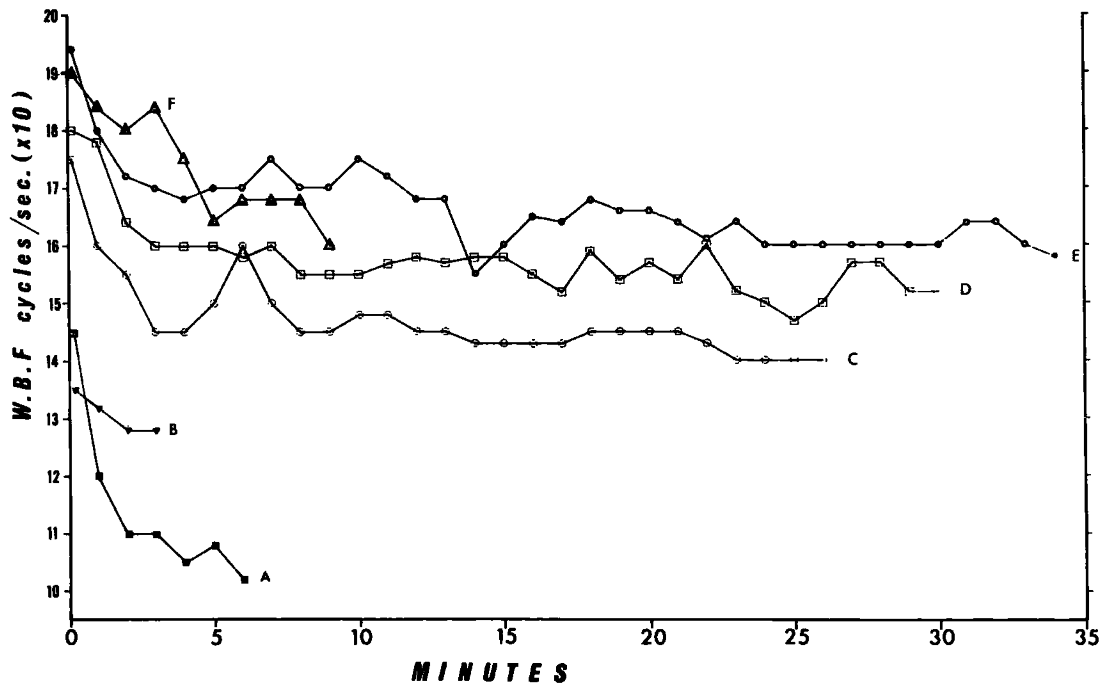

12
10
8
6
4
2
0
Flight times (Minutes)
7
9
6
6
27
31
28
28
15
11
9
7
4
5
2
7
21
35
49
63
77
Age in Days

Figure 21

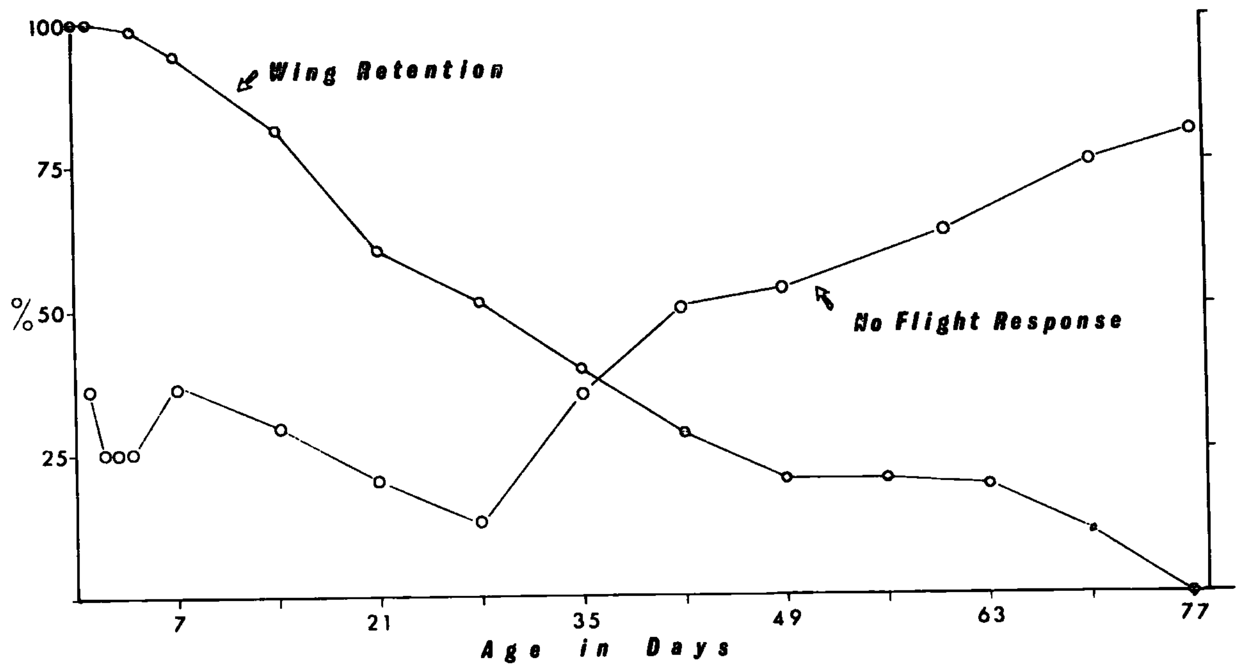
Wing-beat frequency per minute of continuous flight by the longest-flying insects in selected age groups. A - 1st day; B - 4th day; C - 7th day; D - 14th day; E - 35th day; F - 70th day.



W.B.F cycles/sec.(x10)
MINUTES
20
19
18
17
16
15
14
13
12
11
10
0
5
10
15
20
25
30
35
A
B
C
D
E
F

Figure 22

Per cent wing retention of male blowflies kept at 24°C, —o——o—; percentage of flies tried in experiments, which failed to elicit a flight response, —o——o—.



100
75
%50
25
Wing Retention
No Flight Response
7
21
35
49
63
77
Age in Days

Figure 23

Mean values for oxygen uptake by flight muscle tissue preparations in relation to age at 24°C (substrate 30 mM trehalose). The dotted line indicates the difference between hourly as against half-hourly rates in old preparations.

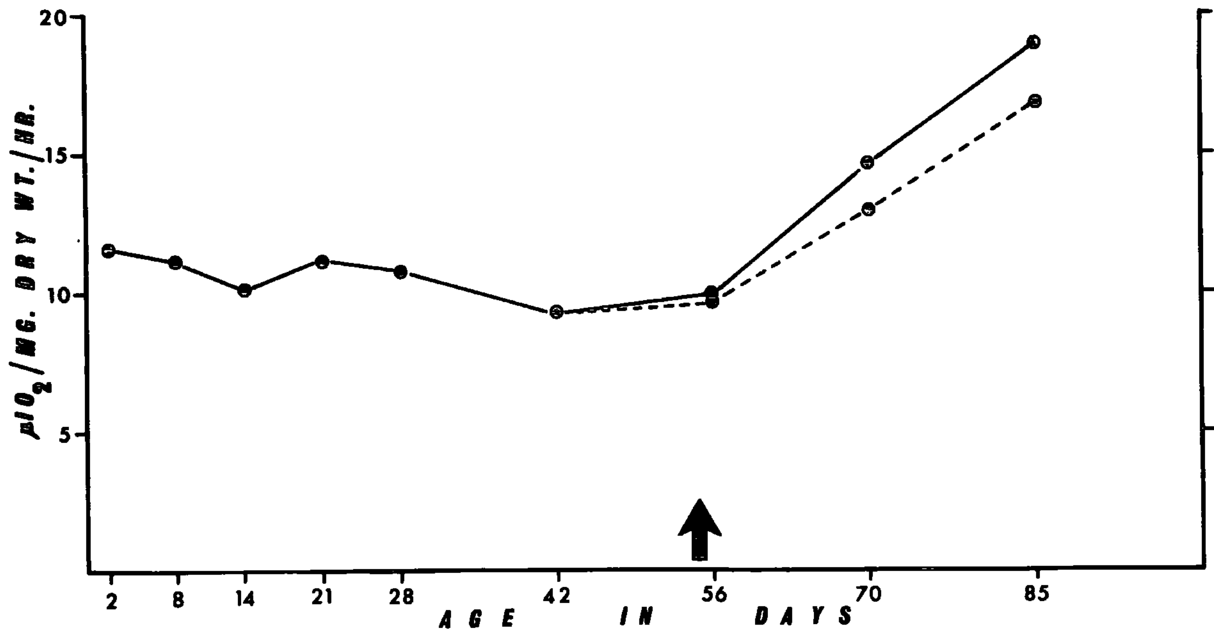

20
15
10
5
μlO2/MG. DRY WT./HR.
2
8
14
21
28
42
56
70
85
AGE IN DAYS

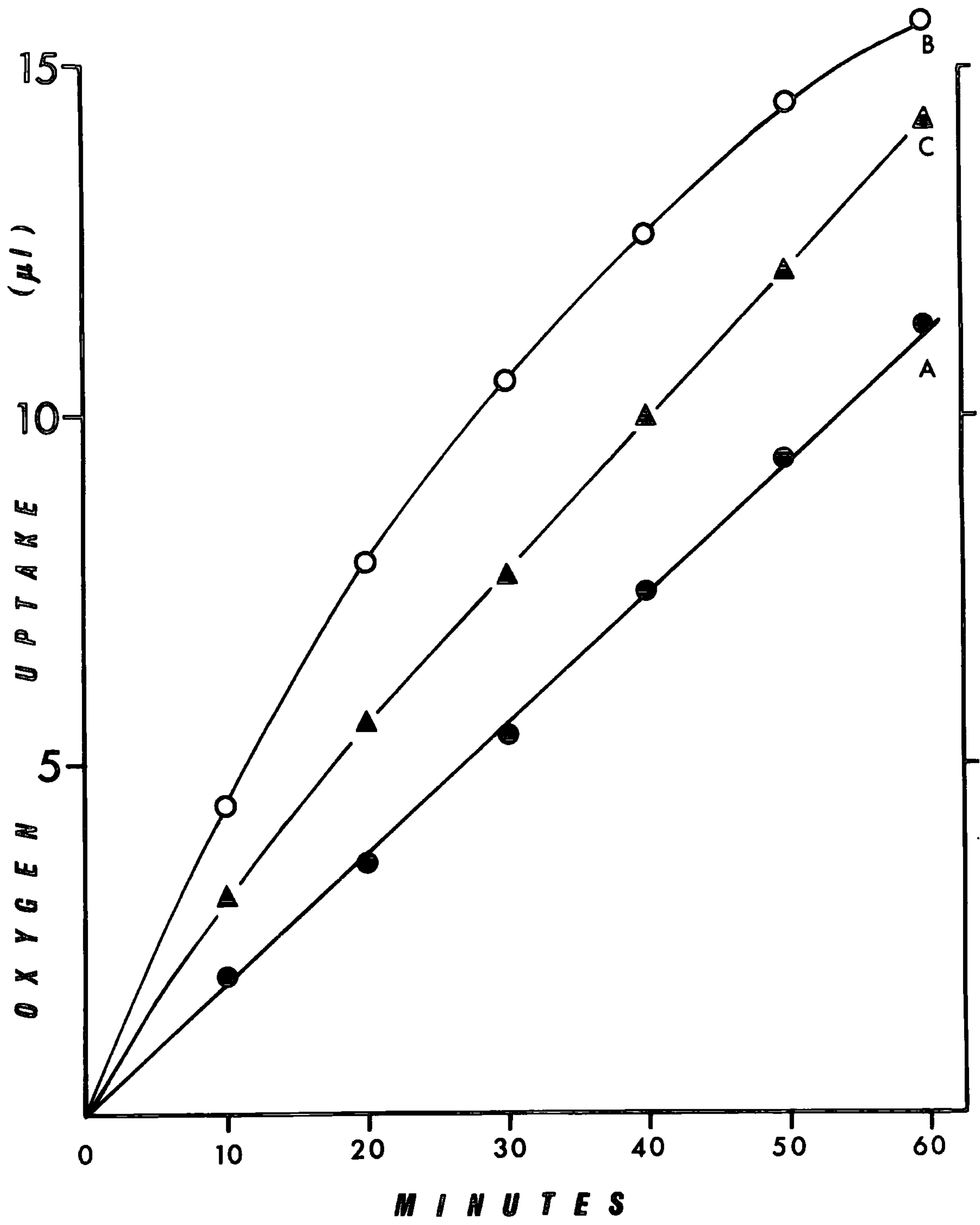
Figure 24

Representative examples of oxygen uptake by flight muscle preparations at 24°C (10 minute intervales shown)

A - from 7 day-old preparations (-BSA)

B - from 85 day-old preparations (-BSA)

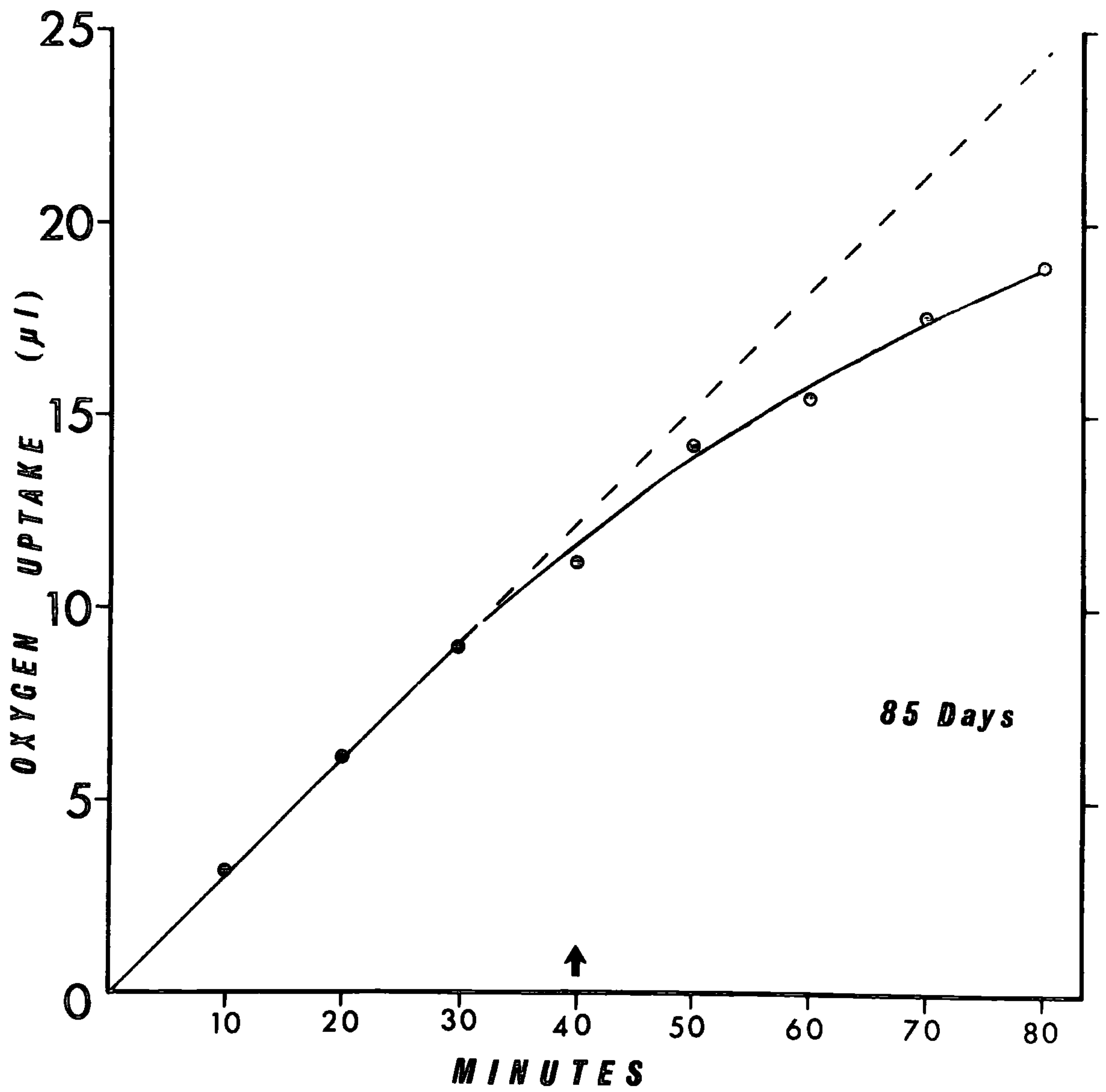
C - from 75 day-old preparations (+BSA)



OXYGEN UPTAKE (µl)
15
10
5
0
10
20
30
40
50
60
MINUTES
A
B
C

Figure 25

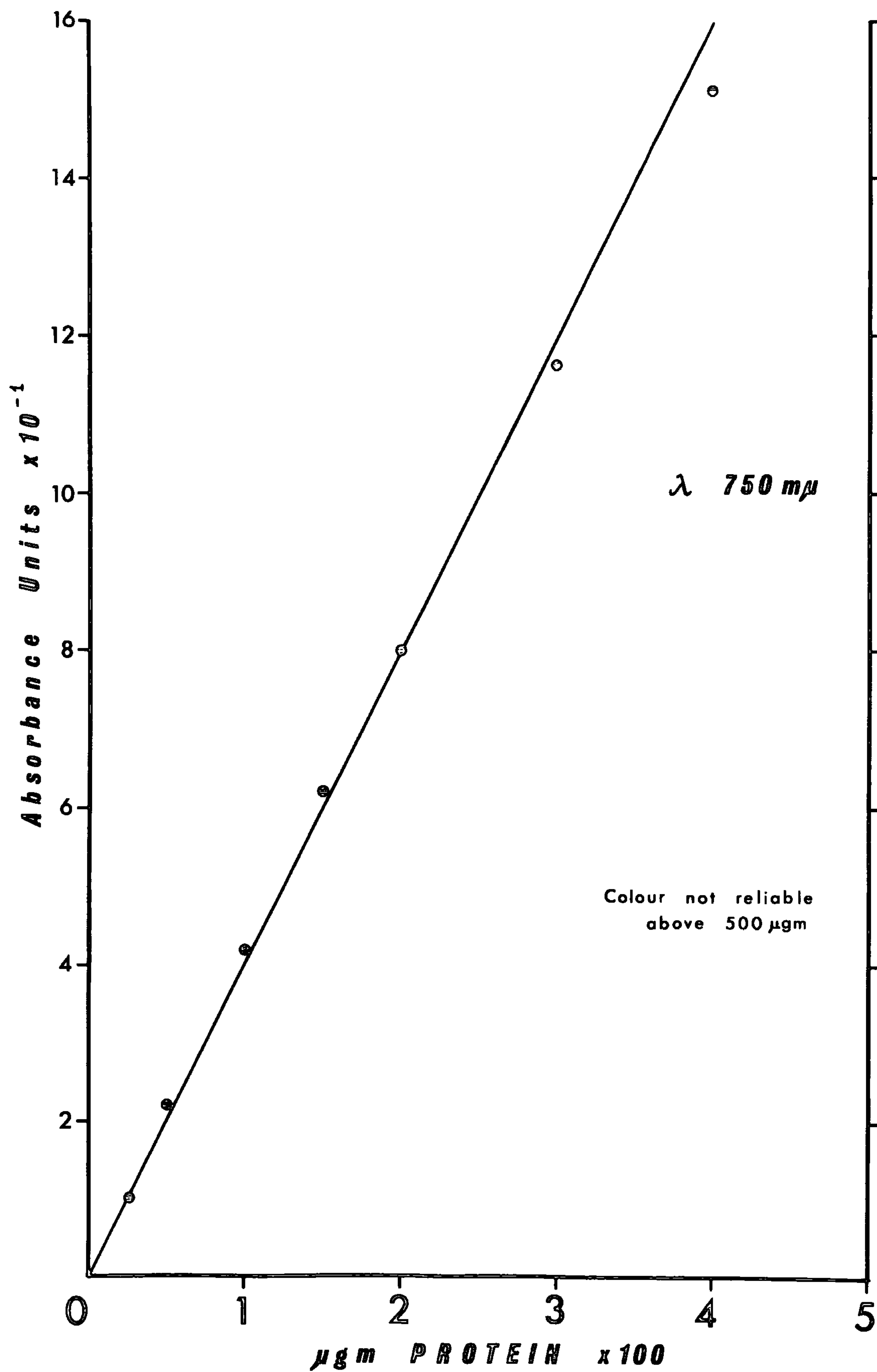
The effect of additional substrate on the oxygen uptake of senescent (85 day-old) tissue preparations. The dotted line indicates the theoretical time course of the reaction if the substrate was a limiting factor.



25
20
15
10
5
0
OXYGEN UPTAKE (μl)
85 Days
10
20
30
40
50
60
70
80
MINUTES

Figure 26

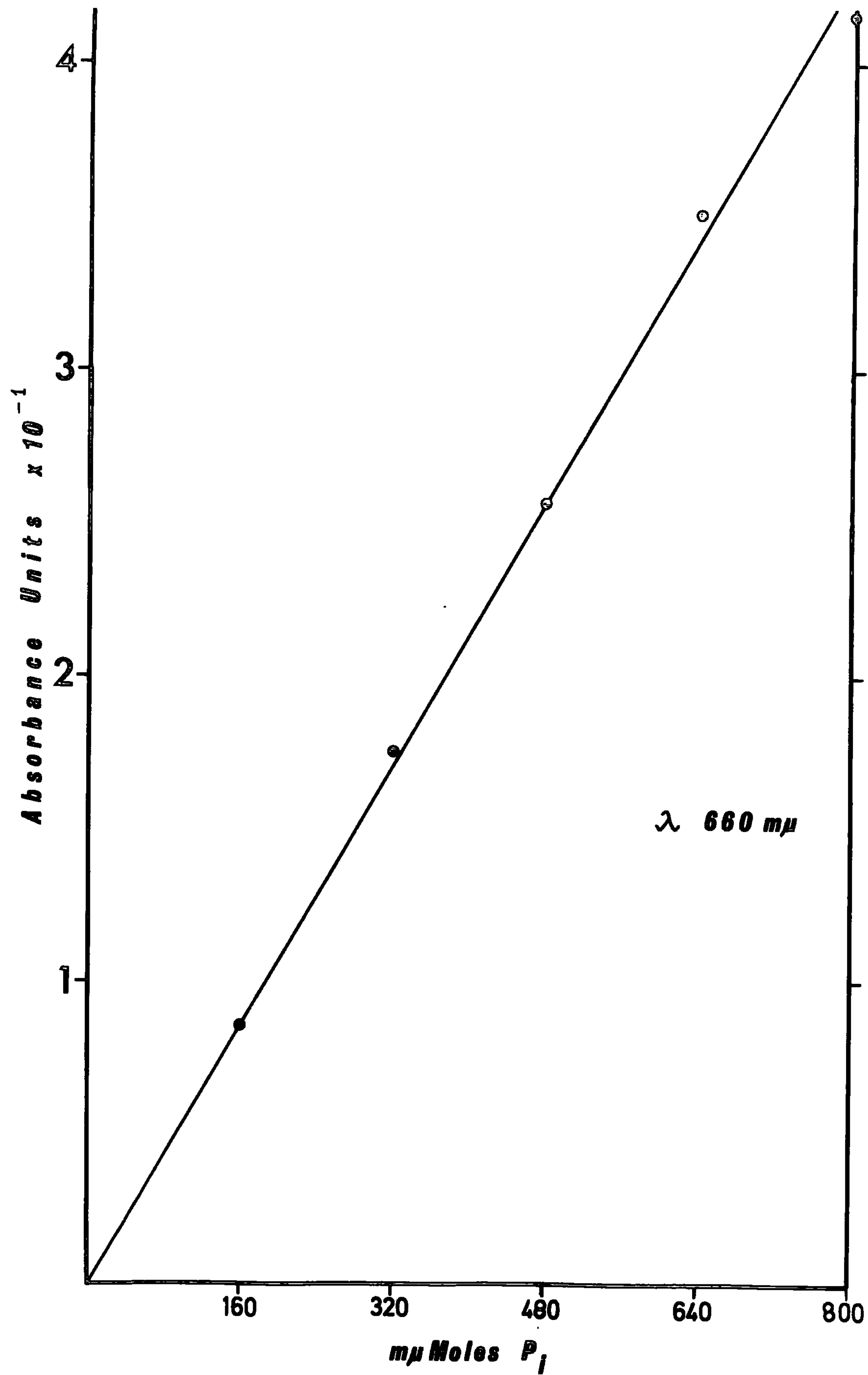
Standard protein curve obtained with the Folin-phenol reagent. (BSA as standard protein).



16
14
12
10
8
6
4
2
0
1
2
3
4
5
Absorbance Units x 10⁻¹
λ 750 mμ
Colour not reliable
above 500 μgm
μgm PROTEIN x100

Figure 27

Standard phosphorus curve obtained by the method of Fiske and Subbarow. (20 μgm/ml KH_2PO_4 solution as standard).



Absorbance Units x 10⁻¹
4
3
2
1
λ 660 mμ
160
320
480
640
800
mμ Moles P_i

Figure 28

A histogramic representation of the percentage size distribution of sarcosomes with age. Mean sarcosomal diameters are also given.

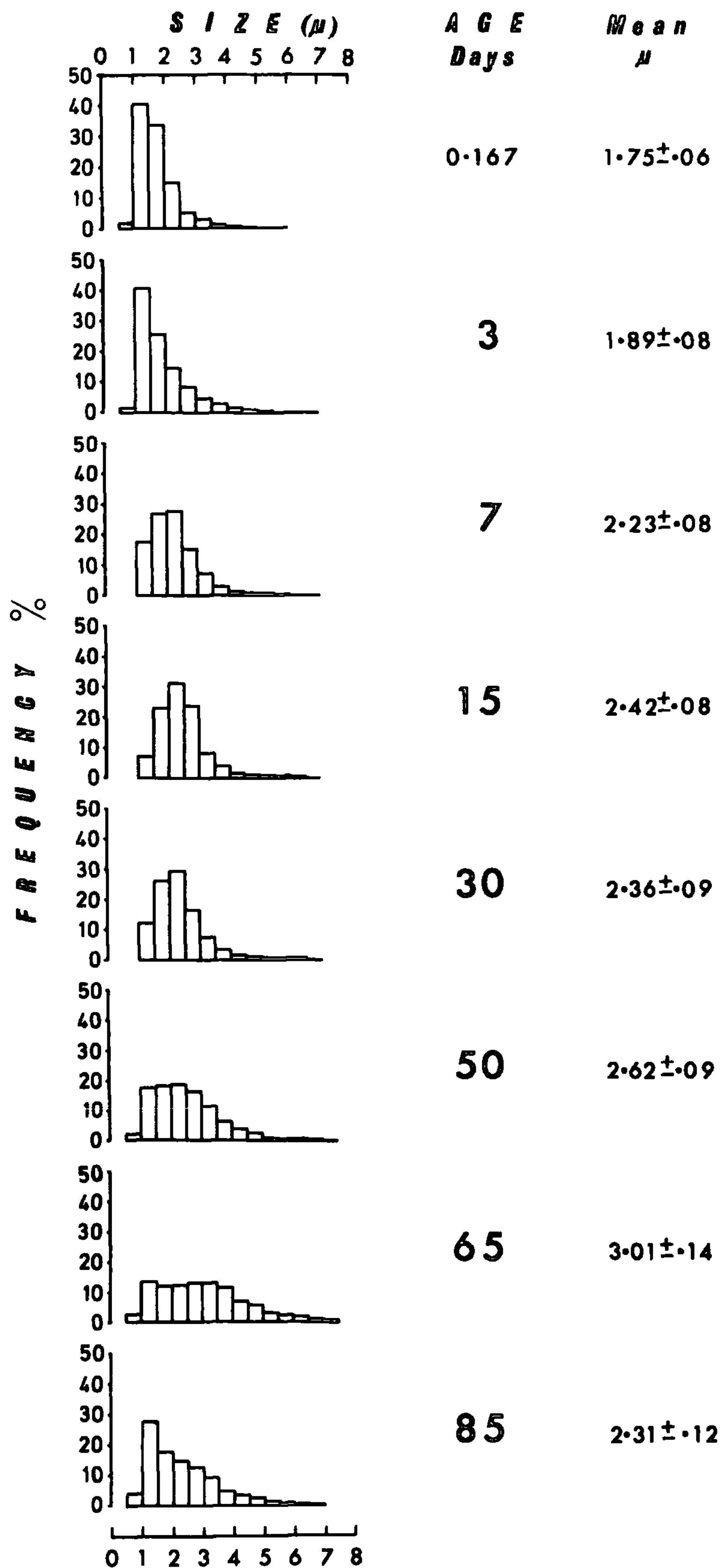

SIZE (μ)
AGE Days
Mean μ
FREQUENCY %
0·167
1·75±·06
3
1·89±·08
7
2·23±·08
15
2·42±·08
30
2·36±·09
50
2·62±·09
65
3·01±·14
85
2·31±·12

Figure 29

Mean P:0 values in relation to age of the fly.

The regression lines of the two variable are

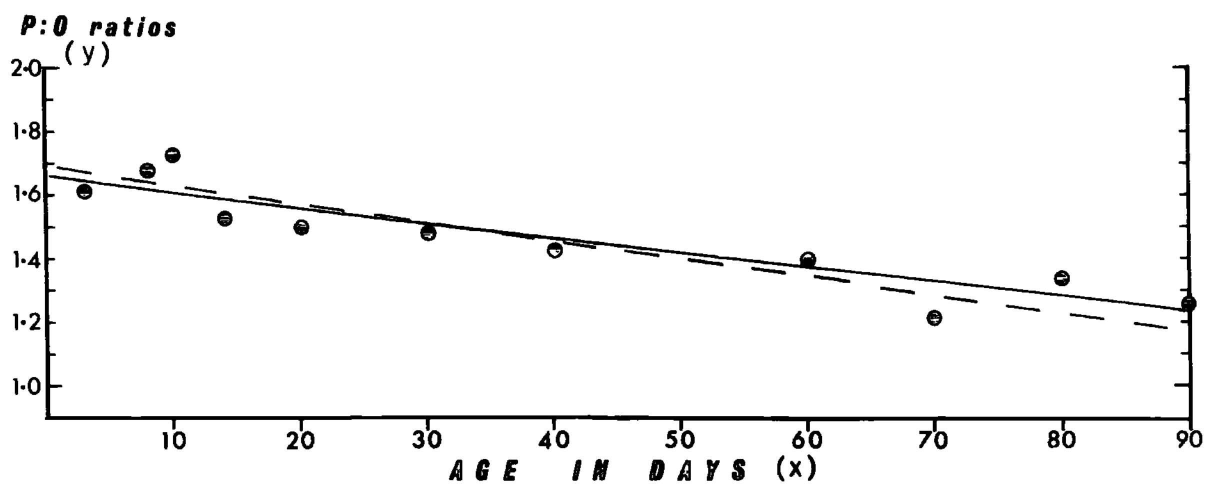
given: x on y ———,

y on x -----.

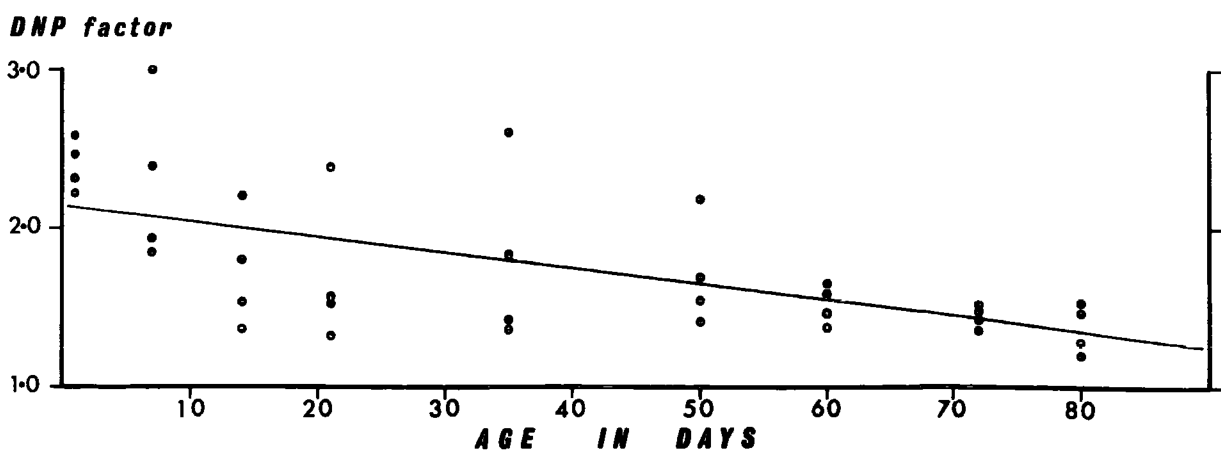
Figure 30

DNP factors at 5 minute intervals at various ages.

A line of best fit marking the general trend is drawn.



P:0 ratios
(y)
2·0
1·8
1·6
1·4
1·2
1·0
10
20
30
40
50
60
70
80
90
AGE IN DAYS (x)



DNP factor
3·0
2·0
1·0
10
20
30
40
50
60
70
80
AGE IN DAYS

REFERENCES

ALPATOV, W. (1930). Experimental studies on the duration of life. XIII. The influence of different feeding during the larval and imaginal stages on the duration of life in the imago of D. melanogaster. Am. Nat. 64, 37-55.

ANDREW, W. (1956). The mitochondria of the neuron. Int. Rev. Cytol. 5, 147-169.

ASHFORD, T.P., and PORTER, K.R. (1962). Cytoplasmic components in hepatic cell lysosomes. J. cell. Biol. 12, 198-202.

BALBONI, E.R. (1965). Influence of preparative procedure on the oxidative activity of honeybee flight muscle sarcosomes. J. Insect Physiol. 11, 1559-1572.

BIDDER, G.P. (1932). Senescence. Brit. Med. J. ii, 5831.

BISHOPP, F.C., and SMITH, C.N. (1938). The American dog-tick, eastern carrier of Rocky Mountain spotted fever. Circ. U.S. Dep. Agric. 478, 1.

BJÖRNSTROP, P., ELLS, H.A., and BRADFORD, R.H. (1964). Albumin antagonism of fatty acid effects on oxidation and phosphorylation reactions in rat liver mitochondria. J. Biol. Chem. 239, 339-344.

BODENSTEIN, D. (1946). Investigation on the locus of action of DDT in flies (Drosophila). Biol. Bull. Woods Hole. 90, 148-157.

BOWLER, K., and HOLLINGSWORTH, M.J. (1965). The effect of inbreeding on temperature acclimatisation in Drosophila subobscura. Genet. Res. 6, 1-12.

BOWLER, K., and HOLLINGSWORTH, M.J. (1966). A study of some aspects of the physiology of ageing in Drosophila subobscura. Exp. Gerontol. 2, 1-8.

BRANDES, D., BERTINI, F., and SMITH, E.W. (1965). Role of Lysosomes in cellular lytic processes. II. Cell death during holocrine secretion in sebaceous glands. Exp. and Molec. Path. 4, 245-265.

BROWN, R.H.J. (1959). A simple activity recorder. J. Insect Physiol. 3, 125-126.

BÜCHER, T. (1965). Formation of Insect Flight Muscle. Aspects of Insect Biochemistry. Biochem. Soc. Symposia. 25. Academic Press N.Y.

CARNEY, G.C. (1966). The effects of different isolation media on the respiration and morphology of housefly sarcosomes. J. Insect Physiol. 12, 1093-1103.

CHADWICK, L.E., and GILMOUR, D. (1940). Respiration during flight in Drosophila repleta Woolaston. Oxygen consumption considered in relation to the wing rate. Physiol. Zöol. 13, 398-410.

CHADWICK, L.E. (1953). The motion of wings. In Insect Physiology (Ed. Roeder, K.) pp. 577-614. John Wiley, New York.

CHILD, C.M. (1915) Senescence and Rejuvenescence. Chicago University Press.

CLARK, S.L. (1957). Cellular differentiation in the kidneys of newborn mice studied with the electron microscope. J. biophys. biochem. Cytol. 3, 349-362.

CLARK, A.M., and ROCKSTEIN, M. (1964). Ageing in Insects. In Physiology of Insecta (Ed. by Rockstein, M.) pp. 227-281. Academic Press, New York.

CLARKE, J.M., and MAYNARD SMITH, J. (1955). The genetics and cytology of Drosophila subobscura. XI. Hybrid vigour and longevity. J. Genet. 53, 172-180.

CLARKE, J.M., and MAYNARD SMITH, J. (1961). Two phases of ageing in Drosophila subobscura. J. Exp. Biol. 38, 679-684.

CLARKE, J.M. and MAYNARD SMITH, J. (1961). Independence of temperature of the rate of ageing in Drosophila subobscura. Nature, Lond. 190. 1027-1028.

CLARKE, J.M. and MAYNARD SMITH, J. (1966). Increase in the rate of protein synthesis with age in Drosophila subobscura. Nature, Lond. 209, 627-629.

CLEGG, J.S., and EVANS, D.R. (1961). The physiology of blood trehalose and its function during flight in the blowfly. J. Exp. Biol. 38, 771-793.

COCHRAN, D.G. (1961). The influence of serum albumin on oxidative phosphorylation in mitochondria from cockroach muscle. Proc. 11th int. Congr. Ent. 1 663-667.

COCHRAN, D.G. (1963). Respiratory control in cockroach muscle mitochondria. Biochim. Biophys. Acta 78, 393-403.

COMFORT, A. (1954). Biological aspects of Senescence. Biol. Rev. 29, 204-329.

COMFORT, A. (1960). The effect of age on growth resumption in fish (Lebistes) checked by food restriction. Gerontologia, 4, 177-186.

COMFORT, A. (1961). The expected rate of senescence and age-dependent mortality in fish. Nature, Lond. 191, 822-823.

COMFORT, A. (1964). Ageing: the biology of senescence. Routledge & Kegan Paul. London.

DAVIS, R.A., and FRAENKEL, G. (1940). The oxygen consumption of flies during flight. J. Exp. Biol. 17, 402-407.

DUNCAN, C.J. (1966). Properties and stabilization of the lysosomal membrane. Nature, Lond. 210, 1229-1230.

ECKHOFF, R.H. (1966). Determination of the solid volume percentage below detectable size in Coulter Counter analysis. Nature, Lond. 210, 765-766.

FRAENKEL, G. (1932). Untersuchungen über die Koordination von Reflexen und automatischnervosen Rhythmen bei Insekten. I. Die Flugreflexe der Insekten und ihre Koordination. Z. vergl. Physiol. 16, 371-393.

FRAENKEL, G. (1940). Utilisation and digestion of carbohydrates by the adult blowfly. J. Exp. Biol. 17, 18-29.

FELDHAM-MUHSAM, B. (1944). Studies on the Ecology of the Levant House Fly (M. domestica vicina. Macq.) Bull. ent. Res. 35, 53-67.

FELDHAM-MUHSAM, B., and MUHSAM, H.V. (1945). Life-tables for Musca vicina and Calliphora erythrocephala. Proc. zool. Soc. Lond. 115, 296-305.

FISKE, C.H., and SUBBAROW, Y. (1925). The colourimetric determination of phosphorus. J. biol. Chem. 66, 375-400.

FLETCHER, M.J., and SANADI, D.R. (1961). Turnover of rat-liver mitochondria. Biochim. Biophys. Acta 51, 356-360.

GREENBERG, B., and BURKMAN, A.M. (1963). Effect of B-vitamins and a mixed flora on the longevity of germ-free adult houseflies, Musca domestica. J. cell. Comp. Physiol. 62, 17-22.

GONZALEZ, B.M. (1923). Experimental studies on the duration of life of certain mutant genes of Drosophila melanogaster. Am. Nat. 57, 289-296.

GREGG, C.T., HEISLER, C.R., and REMMERT, L.F. (1960). Oxidative phosphorylation and respiratory control in housefly. Biochim. Biophys. Acta. 45, 561-570.

GREVILLE, G.D., MUNN, E.A., and SMITH, D.S. (1965). Observations on the fragmentation of isolated flight-muscle mitochondria from Calliphora erythrocephala (Diptera). Proc. Roy. Soc. B. 161, 403-420.

HARRISON, B.J., and HOLLIDAY, R. (1967). Senescence and the fidelity of protein synthesis in Drosophila. Nature, Lond. 213, 990-992.

HAYDAK, M.H. (1953). Effect of diet on uric acid in the fat body of Blattella. Ann. ent. Soc. Am. 46, 547-560.

HESLOP, J., and RAY, J.W. (1964). Glycerol-1-phosphate metabolism in the housefly (Musca domestica L) and the effects of poisons. Biochem. J. 91, 187-195.

HESLOP, J. (1967). Increase of protein synthesis with age in the locust. (Personal communication).

HESS, A., and LANSING, A.I. (1954). The fine structure of young and old spinal ganglia. J. Gerontol. 9, 361.

HODGE, C.F. (1894-95). Changes in human ganglion cells from birth to senile death. Observations on man and honeybee. J. Physiol. 17, 129-134.

HOLLINGSWORTH, M.J. (1966). Temperature and the rate of ageing in Drosophila subobscura. Exp. Gerontol. 1, 259-268.

HOLLINGSWORTH, M.J., and BOWLER, K. (1966). The decline in the ability to withstand high temperature with increase in age in Drosophila subobscura. Exp. Gerontol. 1, 251-257.

HOLTON, F.A., HÜLSMANN, W.C., MYERS, D.K., and SLATER, E.C. (1957). A comparison of the properties of mitochondria isolated from liver and heart. Biochem. J. 67, 579-594.

HOPKINS, H.S. (1930). Age differences and the respiration of muscle tissue of mollusks. J. exp. Zool. 56, 209-240.

HUDSON, A. (1958). The effect of flight on the taste threshold and carbohydrate utilisation of Phormia regina. J. Insect Physiol. 1, 293-304.

INGLE, L., WOOD, T.R., and BANTA, A.M. (1937). A study of the longevity, growth, reproduction and heart rate in Daphnia longispina as influenced by limitations in quantity of food. J. exp. Zool. 76, 325-352.

JONGBLOED, J., and WIERSMA, C.A.G. (1934). Der Stoffwechsel der Honigbiene während des Fliegens. Z. vergl. Physiol. 21, 519-533.

KLINGENBERG, M., and BÜCHER, T. (1959). Flugmuskelmitochondrien aus Locusta migratoria mit Atmungskontrolle. Biochem. Z. 331, 312-333.

KOHN, R.R. (1966). In Topics in the biology of Aging. (Ed. Krohn, P.L.) p. 158. John Wiley, N.Y.

KOPEC, S. (1928). On the influence of intermittent starvation on the longevity of the imaginal stage of Drosophila melanogaster. J. Exp. Biol. 5, 204-211.

KROHN, P.L. (1962). Heterochronic transplantation in the study of ageing. Proc. Roy. Soc. B. 157, 128-147.

KROHN, P.L. (1966). Transplantation and Ageing. In Topics in the biology of Aging. (Ed. Krohn, P.L.) pp. 125-148.

LAMB, M. (1964). The effects of radiation on the longevity of female Drosophila subobscura. J. Insect Physiol. 10, 487-497.

LANGER, H. (1962). A new eye colour mutation in Calliphora erythrocephala. Meig. Nature, Lond. 194, 111-112.

LANSING, A.I. (1952). Cowdry's Problems of Ageing. 3rd. Ed. Williams and Wilkins, Baltimore, Maryland.

LARDY, H.A., and WELLMAN, H.J. (1952). Oxidative phosphorylations: role of inorganic phosphate and acceptor systems in control of metabolic rates. J. biol. Chem. 195, 215-224.

LEHNINGER, A.L., and RAY, B.L. (1957). Oxidation-reduction state of rat liver mitochondria and the action of thyroxine. Biochim. Biophys. Acta 26, 643-644.

LEHNINGER, A.L. (1964) The Mitochondrian. Benjamin Inc. Press, New York.

LEVENBOOK, L., and WILLIAMS, C.M. (1956). Mitochondria in the flight muscle of insects. III. Mitochondrial cytochrome c in relation to ageing and wing beat frequency of flies. J. gen. Physiol. 39, 497-512.

LEWIS, S.E., and SLATER, E.C. (1954). Oxidative phosphorylation in insect sarcosomes. Biochem. J. 58, 207-217.

LEWIS, S.E., and FOWLER, K.S. (1960). Nature and origin of an inhibitor of oxidative phosphorylation present in freshly isolated sarcosomes from blowfly thoraces. Biochim. Biophys. Acta 38, 564-565.

LEWIS, S.E. Personal communications (1965, 1966, 1967).

LILES, J., and DELONG, D.M. (1960). The longevity and productivity of adult male and female *Aedes aegypti* when reared separately and together on three different diets. Ann. ent. Soc. Am. 53, 277-280.

LOEB, J., and NORTHROP, J.H. (1917). On the influence of food and temperature on the duration of life. J. biol. Chem. 32, 103-121.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L., and RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. J. biol. Chem. 193, 265-275.

MATTERN, C.F.T., BRACKETT, F.S., and OLSON, B.J. (1957). Determination of number and size of particles by electrical gating: blood cells. J. appl. Physiol. 10, 56-70.

MAYNARD SMITH, J. (1958). The effects of temperature and of egglaying on the longevity of *Drosophila subobscura*. J. Exp. Biol. 35, 832-842.

MAYNARD SMITH, J. (1959). A theory of ageing. Nature, Lond. 187, 10-18.

MAYNARD SMITH, J. (1962). The causes of ageing. Proc. Roy. Soc. B. 157, 115-127.

MAYNARD SMITH, J. (1963). Temperature and the rate of ageing in poikilotherms. Nature, Lond. 199, 400-402.

MAYNARD SMITH, J. (1966). Theories of Ageing and protein synthesis. In Topics in the biology of Aging. pp. 1-35. Academic Press, N.Y.

MEDAWAR, P.B. (1952). An unsolved problem of biology. H.K. Lewis. London.

MINOT, C.S. (1908). The problem of age, growth and death; a study of cytomorphosis, based on lectures at the Lovell Institute, March 1907. London.

MINOT, C.S. (1913). Moderne probleme der Biologie. Jena.

MOE, H., and BEHNKE, O. (1962). Cytoplasmic bodies containing mitochondria, ribosomes and rough-surfaced endoplasmic membranes in the epithelium of the small intestine of newborn rats. J. cell. Biol. 13, 168-170.

MOORE, L.D., KOCUN, F.J., and UMBREIT, W.W. (1966). Cell free protein synthesis: Effects of age and state of ribosomal aggregation. Science, N.Y. 154, 1350-1352.

MYERS, D.K., and SLATER, E.C. (1957). The enzymic hydrolysis of adenosine triphosphate by liver mitochondria. Biochem. J. 67, 559-572 and 572-579.

McCAY, C.M., MAYNARD, L.A., SPERLING, G., and BARNES, L.L. (1939). Retarded growth, life span, ultimate body size and age changes in the albino rat after feeding diets restricted in calories. J. Nutrit. 18, 1-13.

McCAY, C.M., MAYNARD, L.A., SPERLING, G., and OSGOOD, H.S. (1941). Nutritional requirements during the latter half of life. J. Nutrit. 21, 45-60.

McCAY, C.M., POPE, F., and LUNSFORD, W. (1956). Experimental prolongation of life. Bull. N.Y. Acad. Med. 32, 91-101.

ORGEL, L.E. (1963). The maintenance of the accuracy of protein synthesis and its relevance to ageing. Proc. Nat. Acad. Sci. Wash. 49, 517-521.

PACKER, L. (1960). Metabolic and structural states of mitochondria - I. Regulation by ADP. J. biol. Chem. 235, 242-249.

PACKER, L. (1961). II. Regulation by phosphate. J. biol. Chem. 236, 214-220.

PARKIN, E.A., and GREEN, A.A. (1958). Ch. 69. In Handbook of Laboratory Animals. Univ. Fed. Animal Welfare, London.

PAYNE, F. (1946). The cellular picture in the anterior pituitary of normal fowls from embryo to old age. Anat. Rec. 96, 77-91.

PEARSE, J.M. (1936). Age and tissue respiration. Am. J. Physiol. 114, 255-260.

PEARL, R. (1922). The Biology of Death. Monographs in Experimental Biology. J.B. Lippincott, Philadelphia and London.

PEARL, R. (1928). The Rate of Living. Knof, New York.

PEARL, R., MINER, J.R., and PARKER, S.L. (1927). Experimental studies on the duration of life XI. Density of population and life duration in Drosophila. Am. Nat. 61, 289-318.

PEARL, R., and MINER, J.R. (1935). Experimental studies on the duration of life XIV. The comparative mortality of certain lower organisms. Quart. Rev. Biol. 10, 60-79.

PEARL, R., and PARKER, S.L. (1922). Experimental studies on the duration of life. II. Hereditary differences in duration of life in line-bred strains of Drosophila. Am. Nat. 56, 174-187.

PRINGLE, J. W. (1965). Locomotion: Flight. In The Physiology of Insecta. 2. (Ed. Rockstein, M.) pp. 283-329. Academic Press, N.Y.

RAHM, P.G. (1923). Beiträge zur Kenntnis der Moosfauna. Z. allg. Physiol. 20, 1-12.

REINER, J.M. (1947). The effect of age on carbohydrate metabolism of tissue homogenates. J. Geront. 2, 315-319.

ROCKSTEIN, M. (1950). The relation of cholinesterase activity to change in cell number with age in the brain of the adult worker honey bee. J. cell. comp. Physiol. 35, 11-24.

ROCKSTEIN, M. (1957). Longevity of male and female houseflies. J. Gerontol. 12, 253-256.

ROCKSTEIN, M. and LIEBERMAN, H.M. (1958). Survival curves for male and female houseflies (Musca domestica. L). Nature, Lond. 181, 787-788.

ROCKSTEIN, M., and BHATNAGAR, P.L. (1965). Age changes in size and number of the giant mitochondria in the flight muscle of the common housefly. J. Insect Physiol. 11, 481-491.

ROCKSTEIN, M., and BHATNAGAR, P.L. (1966). Duration and frequency of wing beat in the aging housefly, Musca domestica. Biol. Bull. Woods Hole. 131, 479-486.

ROCKSTEIN, M., and BRANDT, K.F. (1963). Enzyme changes in flight muscle correlated with aging and flight ability in the male housefly. Science, N.Y. 139, 1049-1051.

ROSS, M.H. (1961). Length of life and nutrition in the rat. J. Nutrit. 75, 197-210.

SACKTOR, B. (1955). Cell structure and the metabolism of insect flight muscle. J. Biophys. Biochem. Cytol. 1, 29-46.

SACKTOR, B., O'NEILL, J.J., and COCHRAN, D.G. (1958). The requirement for serum albumin in oxidative phosphoryltation of flight muscle mitochondria. J. biol. Chem. 233, 1233-1235.

SANG, J.H., and KING, R.C. (1961). Nutritional requirements of axenically cultured Drosophila melanogaster adults. J. Exp. Biol. 38, 793-809.

SAWANT, P.L., DESAI, I.D., and TAPPEL, A.L. (1964). Digestive capacity of purified lysosomes. Biochim. Biophys. Acta. 85, 93-102.

SEDEE, P.H.J.W. (1956). Dietetic requirements and intermediary protein metabolism of the larva of Calliphora erythrocephala. Ph.D. Thesis. University of Utrecht.

SHOCK, N.W., WATKIN, D.M., and YIENGST, M.J. (1954). Age changes in renal function and basal oxygen consumption as related to total body water. Fed. Proc. 13, 136.

SLATER, E.C., and HOLTON, F.A. (1954). Oxidative phosphorylation coupled with the oxidation of α-ketoglutarate by heart muscle sarcosomes. Biochem. J. 56, 28-40.

SOTAVALTA, O. (1952). The essential factor regulating the wing stroke frequency of insects in wing mutilation and loading experiments and in experiments at substmospheric pressure. Suomal. eläin-ja Kasvit. Seur. van eläin, Julk. 15, 1-67.

STEGWEE, D., and van KAMMEN-WERTHEIM, A.R. (1962). Respiratory chain metabolism in the colorado potato beetle. I. Respiration and oxidative phosphorylation in sarcosomes from active beetles. J. Insect Physiol. 8, 117-126.

STRANGEWAYS-DIXON, J. (1961). The relationship between nutrition, hormones and reproduction in the blowfly, Calliphora erythrocephala. Meig. I. Selective feeding in relation to the reproduction cycle, the corpus allatum volume and fertilisation. J. Exp. Biol. 38, 225-235.

STRANGEWAYS-DIXON, J. (1962). III. The corpus allatum in relation to nutrition, the ovaries, innervation, and the corpus cardiacum. J. Exp. Biol. 39, 293-306.

STREHLER, B.L. (1962). Time, Cells and Aging. Academic Press. N.Y.

SZILARD, L. (1959). On the nature of the ageing process. Proc. Nat. Acad. Sci. Wash. 45, 30-45.

SZILARD, L. (1959). A theory of ageing. Nature, Lond. 184, 958-961.

TATE, P. (1947). 'White-eyed' sex-limited mutation of the blowfly Calliphora erythrocephala. J. Genet. 48, 176-182.

THOMSEN, E. (1949). Influence of the corpus allatum on the oxygen consumption of adult Calliphora erythrocephala. J. Exp. Biol. 26, 137-149.

TSCHARNTKE, H. (1966). Personal Communication.

TREVELYAN, W.E., PROCTOR, D.P., and HARRISON, J.S. (1950). Detection of sugars on paper chromatograms. Nature, Lond. 166, 444-445.

van DEN BERGH, S.G. (1962). Respiration and energy production in the flight muscle of the housefly, Musca domestica. Ph.D. Thesis. University of Amsterdam.

van DEN BERGH, S.G., and SLATER, E.C. (1962). The respiratory activity and permeability of housefly sarcosomes. Biochem. J. 93, 128-136.

von BUDDENBROCK, W. (1939). Grundriss der vergleichenden Physiologie, Bd. II. Gebrüder Bornträger, Berlin.

WATANABE, M.I., and WILLIAMS, C.M. (1953). Mitochondria in the flight muscle of insects - II. Effects of the medium on the size, form and organisation of isolated sarcosomes. J. gen. Physiol. 37, 71-89.

WILLIAMS, C.M., BARNESS, L.A., and SAWYER, W.H. (1943). The utilisation of glycogen by flies during flight and some aspects of the physiological ageing of Drosophila. Biol. Bull. Woods Hole. 84, 263-272.

WOJTCZAK, L., and WOJTCZAK, A.B. (1960). Uncoupling of oxidative phosphorylation and inhibition of ATP-P_i exchange by a substance from insect mitochondria. Biochim. Biophys. Acta. 39, 277-286.

WULFF, V.J., SAMIS, H.V., and FALZONE, J.A. (1966). In Advances in Gerontological Research Vol. 2. (Ed. Strehler, B.L.) Academic Press. N.Y.

YURKIEWICZ, W.J., and SMYTH, T. (1966). Effects of temperature on oxygen consumption and fuel utilisation in the sheep blowfly. J. Insect Physiol. 12, 403-408.