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Biochemical analysis of genetic differences in the growth of *Drosophila*

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1. INTRODUCTION

The biochemical analysis of genetically determined differences in growth provides a valuable approach to the study of development generally. By judicious selection for different parameters of growth, it is often possible to create large differences which reveal developmental interrelations which would otherwise be exceedingly difficult to detect. The present paper is concerned with the application of this approach to a number of different selected lines of *Drosophila melanogaster* Meig., which differ greatly in adult weight and duration of the larval period. Wet weight, protein, RNA and DNA content per animal have been determined, at successive stages from the egg to adult, on ten lines and also the unselected stock from which they were derived. Comparison of growth, in terms of the absolute and relative amounts of these primary constituents, has revealed differences in composition which can be related to characteristic differences in the response to selection, especially the developmental origin of correlated and uncorrelated changes in the duration of the larval period and adult body size.

The different strains have been created by selecting for either large or small body size, or longer or shorter development time, on different, chemically defined, axenic media (Robertson, 1963, 1964). According to the nature of the diet, the duration of the larval period was either unchanged or correlated to a variable degree with changes in body size. Such contrasts have been shown (Robertson, loc. cit.) to reflect differences in the time taken to reach a so-called 'critical size', early in the third instar (Bakker, 1959; Robertson, 1963), the attainment of which is defined by the ability of the larva to pupate even though it is no longer allowed to feed.

Large lines, with a longer development time, reach a larger critical size at a relatively later time, while in lines with unaltered development time the critical size is reached at the same time as in the unselected foundation stock. Presumably the critical size coincides with a significant shift in hormonal balance which governs

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growth and the onset of the differentiation of adult tissues. Thus selection for apparently the same trait, on different diets, may lead to well-defined differences in growth which offer unique material for biochemical analysis.

Before this aim could be realized, it was first necessary to work out sufficiently accurate and reproducible methods of estimating the various constituents for this rather difficult material. A full account of the methods and the results of estimating protein, lipids, DNA, total RNA and also its composition, for successive stages in the unselected population, has been presented elsewhere (Church & Robertson, in press). The present paper is concerned with the application of these methods to the study of the substantial differences between the selected lines.

2. MATERIAL AND METHODS

(i) *General*

All the lines have been derived by mass selection from the Pacific population of *Drosophila melanogaster* which has been maintained for several years as a large cage population. The procedure for axenic culture was the same as that described by Robertson (1960). The chemically defined diets used for selection included either Medium C of Sang (1956) or various modifications which involved reduction of either the yeast nucleic acid content from the usual 0·4 to 0·075%, or of protein, in the form of casein, from 5 to 2%, or of the available phospholipid. In the latter case, lecithin was omitted and choline, at the rate of 30 mg./l., was added instead. Choline cannot entirely replace lecithin for maximum growth rate in *Drosophila* (Sang, 1956); the amount added is well below the concentration (150 mg./l.) at which further increase in choline leads to no improvement in growth rate.

Selection procedure and the early responses to selection of most of the lines dealt with here have been reported earlier (Robertson, 1964). Selection was either for longer or shorter thorax length, which is highly correlated with body weight, or for longer or shorter development time. Size and development time were recorded in the manner outlined by Robertson (1964). The larval period was taken as the total time from hatching from the egg to eclosion of the adult, minus 4·3 days, which represents the duration of the pupal period in the stock studied and which has not been noticeably affected by selection.

Critical sizes were determined by removing larvae from the food at successive intervals, weighing them, and transferring them to moist food-free containers to determine whether they could pupate and eclose. The size at which 50% could eclose was taken as the estimate of 'critical size'. It is important to handle larvae with care to avoid injury and hence erroneous estimates of critical size.

The development time in the egg was also recorded. Flies were supplied with abundant food under conditions favouring oviposition, allowed to lay freely overnight and then eggs were collected over half-hour periods, kept at 25°C. and inspected to determine the average time of hatching.

The following symbols are used to refer to the various lines. The first letter or pair of letters, i.e. L, S, F, or Sl refers to the character and direction of selection, i.e.

large or small body size, fast or slow development time respectively. The second letter refers to diet: O, R, P or C refers to the optimal medium or media deficient in either ribonucleic acid, protein or choline respectively.

(ii) *Biochemical estimations*

Considerable effort has been devoted to the technical problem of estimating the various constituents. This has included comparison of alternative procedures and tests of repeatability. With the methods used here (Church & Robertson, in press), replicate tests on aliquots of the same material led to coefficients of variation of 2–3% for both protein and RNA and 2–5% for DNA.

For estimating protein, a modification of the procedure of Lowry, Rosebrough, Farr & Randall (1951) was used and was shown to yield excellent agreement with micro-Kjehldahl determinations of total protein, after dialysis of homogenates to remove uric acid. DNA was estimated by the diphenylamine reaction of Dische (1930), as modified by Burton (1956), after the tissue had been treated in various ways to remove contaminating material which could, otherwise, bias the estimates (Church & Robertson, in press). For RNA, a modification of the procedure of Schneider, Hogeboom & Ross (1950) was used and RNA was estimated as the difference between the estimates for total nucleic acid content and the diphenylamine estimate of DNA. Since differences in base ratio between the nucleic acid which is to be estimated and the nucleic acid used as standard may lead to error (Levenbook, Travaglini & Schultz, 1958), all the DNA and RNA estimates refer to standards made from purified DNA and RNA extracted from *Drosophila* (Church, in press; Church & Robertson, in press).

3. RESULTS

(i) *Comparison of lines*

Body size

Table 1 shows the deviation of the lines from unselected, averaged over generations 10–15, for thorax length and the larval period, of flies on the various alternative axenic media. The values are expressed on log scales, so the differences approximate to percentage differences. There is great variation in the presence or absence of correlated change. Thus, on low protein, approximately 20% differences in favour of either large or small body size are accompanied by either no difference in development time (LP) or 10% reduction (SP). With selection for shorter development time, a 5% reduction in time is accompanied by a 10% reduction of body size, while, with selection for longer time, on the other hand, a 10% increase in larval period is without effect on body size. Thus selection for the same character in opposite directions may lead to contrary evidence of correlation between the traits.

On the low RNA medium the same phenomenon is apparent in selection for large or small size but here it is the large line which shows the correlation and the small line which does not. In selection for shorter and longer development time there is a correlated change in both cases.

Finally, in the large lines, selected on either the optimum or the low choline

Table 1. *Average deviation from unselected of selected lines—body size and duration of the larval period on axenic diets*

Selection	$3\log_e$ thorax length	$\log_e$ larval life
	Low protein	
Small size (SP)	−0·21**	−0·10**
Large size (LP)	+0·20**	−0·01
Short development (FP)	−0·13**	−0·05*
Long development (SlP)	0·02	0·10**
	Low ribonucleic acid	
Small size (SR)	−0·17**	−0·02
Large size (LR)	0·18**	0·09**
Short development (FR)	−0·09**	−0·05*
Long development (SlR)	0·12**	0·15**
	Low choline	
Large size (LC)	0·24**	0·04
	Optimum axenic	
Large size (LO)	0·22**	0·04 *

* and ** indicate significance of differences at the 0·05 and 0·01 levels of probability.

medium, more than 20% increase in body size is without significant change in development time. Thus, of the five large lines, only two, both selected on low RNA, show any evidence of correlated change in development time.

Earlier work (Robertson, 1963, 1964) has provided similar evidence of variable incidence of correlation, which reflects changes in either growth rate or in the period during which larval growth is effective. The likelihood of presence or absence of correlated change in development time, when body size is selected for, is very unequal. Generally, and especially under optimal conditions, lack of correlation is the rule, but selection on certain diets, especially those deficient in RNA, regularly leads to a correlation. Hence effective selection for alternative kinds of developmental change is influenced by the diet, presumably due to differences in the expression and/or genetic behaviour of the segregating differences. There is also evidence (Robertson, 1964) that continued selection establishes stability of expression when the lines are provided with different diets.

(ii) *The analysis of differences*

One of the main reasons for the biochemical analysis was to answer the question: Are there differences in composition which correspond to the differences in growth which can be inferred from the study of body size and duration of larval period alone? And, if so, what light does this shed on the developmental origin of the growth differences?

The data comprise records of average wet weight, protein, DNA and RNA per individual at successive periods of life from the egg to adult for animals grown on the

optimum axenic diet. The characteristic deviations from unselected shown by the lines on the media used for selection, are either as well or almost as well expressed on the optimum axenic diet, which was, therefore, the obvious choice of culture medium for these comparisons since it also eliminates possible confusion due to differences in DNA content between flies grown axenically or on live yeast (Church & Robertson, in press). Replicate samples of larvae, varying from fifty per sample first instar to three per sample late larvae, were removed from the medium at 6-hourly intervals from the time of hatching from the egg to about 72 hours, after which time 12-hourly intervals were generally used. The larvae were washed in insect Ringer and then 60% sucrose, in which they float, before transferring them to moist black filter paper.

On the optimum axenic medium, the larval period is prolonged by 24–26 hours, due, apparently, to delayed penetration of the agar surface by the young larvae; this is not a problem on live yeast medium since there is yeast on the surface. But once the larvae penetrate the medium they grow as fast as on live yeast and reach virtually the same average size (Church & Robertson, loc. cit.). In the present data, the duration of this initial delay has been estimated from the growth curves of the unselected controls in any given test, particularly by reference to the time to reach maximum wet weight, pupation and also the time of the second and third moults, which can be inferred from discontinuities in the protein–time curve and by inspection. All the data have been corrected by subtracting this estimated delay from the observed times. Many tests and also the culture in the same tubes of Pacific control flies, marked with the white-eye gene, have supported the validity of this procedure. The growth rate of individuals carrying the white-eye mutant has been shown to be indistinguishable from that of wild-type individuals.

Before presenting the data, we must first consider the kind of comparison likely to be most revealing.

(*a*) *General*

There is no single, generally accepted method for the analysis of growth data and the procedure chosen will depend on the aspect under study. Perhaps the most unambiguous criterion is the DNA content per individual, since DNA replication is intimately related to growth generally. Hence, DNA has been taken as a primary index of growth and differences in the other constituents have been related to the DNA content. Since the diploid complement is constant (Vendrely, 1955), differences in content in tissues made up of diploid cells can be equated to differences in cell number, while constancy of DNA, accompanied by differences in protein, can be attributed to differences in cell size. In young adult *Drosophila* it is generally accepted that most cells are diploid, as all polytene larval cells degenerate during imaginal development except for Malpighian tubules and some adipose cells (Makino, 1938; Bodenstein, 1950). Hence the distinction that adult cells are generally diploid can be applied with reasonable confidence, qualified, of course, by some doubt as to the relative contribution of polytene cells to the total DNA content and also how far strains are alike in this respect.

But in purely larval tissue the situation is reversed since larval growth is predominantly by increase in cell size accompanied by polyteny (Bodenstein, 1950), and this raises an interesting problem which does not seem to have been discussed. If, following Davidson & Leslie (1950, 1951) we take the ratio of protein to DNA as an index of cell size, we may expect to find differences in the ratio in the polytene larval cells. Will we find parallel differences for diploid adult and for polytene larval cells? Is the protein to DNA ratio influenced by whether the DNA is distributed between diploid cells or restricted to fewer polytene cells, or is the ratio of protein to diploid DNA complement the important index? We may expect the present data to bear on this problem.

(*b*) *Ontogenetic comparisons*

Since there are substantial differences between the lines in the duration of the larval period, this raises the difficulty of how best to compare them, especially at later stages of larval growth during which the imaginal discs are growing and making their separate, but undetected, contribution to the estimates of total DNA, RNA and protein, with the possibility that the proliferation rate of the imaginal discs may differ between lines. Ideally, comparison at the end of each instar would be suitable but this is laborious for so many lines. Therefore, apart from general comparisons of larval growth, attention has been focused on the biochemical composition at the critical size compared with that of the adults. The critical size has been estimated for each line and can be regarded as an ontogenetic landmark which will be fully representative of the growth of purely larval tissue. The time of the second moult has also been noted and shown to be highly correlated ($r = 0{\cdot}97$) with the time of the critical size. Also, differences in composition between the lines at the second moult lead to the same conclusions as those derived from differences at the critical size, which has, however, been given major emphasis because it has been more precisely determined and because of its probable significance with respect to hormonal balance. The degree of correlation between larvae and adults in the deviation from unselected of protein, RNA and DNA will show whether pre- and post-critical growth follow the same pattern or diverge. Since the imaginal discs, which give rise to most of the adult tissue, proliferate at the end of larval life, it is conceivable that growth of the discs and growth of purely larval tissues may be partly independent and that appropriate selection might demonstrate this.

(*c*) *Growth curves*

The range of body size between the lines is substantial—extending to a maximum difference of 45% of the wet weight of the unselected flies. Figures 1*a* and *b* show the wet weight increase for the different lines. To avoid the confusion of overlapping graphs, the four lines SR, LO, LC and LP, which do not differ from the unselected in development time, are shown separately. The growth of the control unselected individuals is shown for each set of curves. All the lines show the same basic pattern of growth to a maximum wet weight in late larval life, at about 72 hours at 25°C. in the unselected Pacific, followed by a decline in wet weight to pupation. In all data

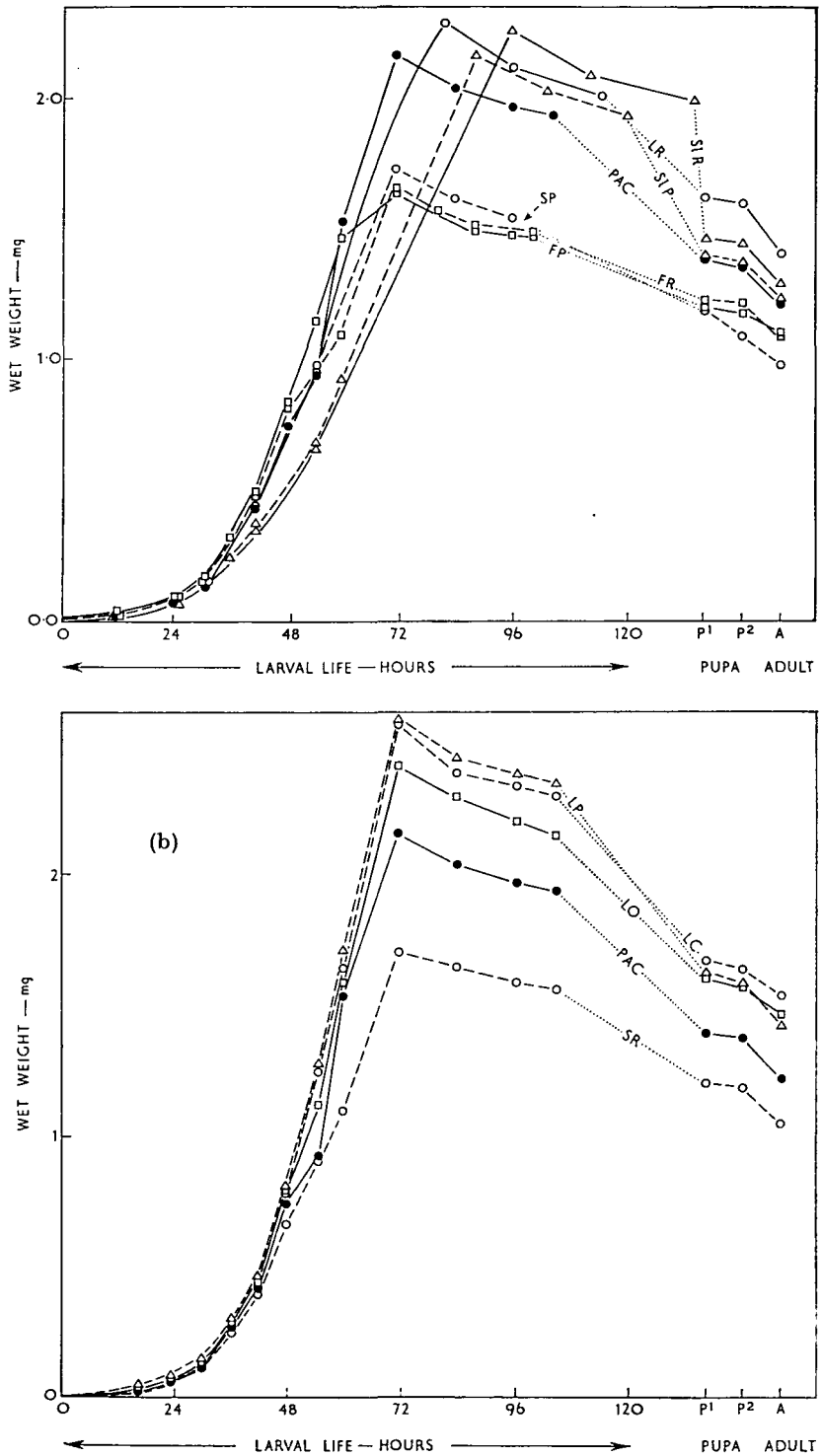

Fig. 1*a*, *b*. Average wet weight per individual during growth of the selected lines and the unselected stock. The figures for pupa and adult are shown on an arbitrary time scale. P_1 and P_2 refer to estimates made within 5 hours of pupation (eversion of the spiracles) and within 20 hours of eclosion respectively. All data refer to animals grown on the optimal axenic medium.

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presented, both sexes are represented until the sexes could be easily separated, at 54 hours. Thereafter, including adults, only females are represented. Males follow a similar pattern of growth approximately 25% below the females (Church & Robertson, in press). Only minor changes occur in the pupal period and the further decline in the adults is due to the discarded puparium. The greater part of the decline, after maximum wet weight, represents water loss when the larva ceases

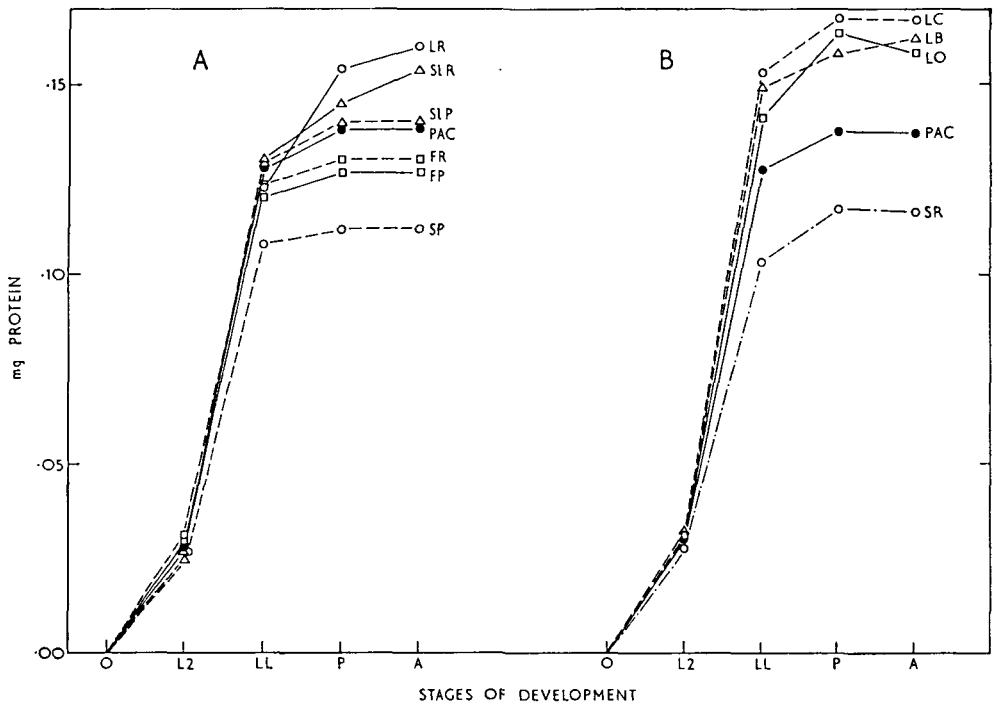

Fig. 2. Average protein content per individual of the selected lines and the unselected stock at corresponding stages of development. L_2 refers to the time of the second larval moult; LL refers to late larval life, approximately 20 hours before pupation. P and A refer to 5-hour-old pupa and newly emerged adult female respectively. All data are from animals grown on the optimal axenic medium.

feeding and leaves the medium before pupation. It is obvious from inspection of the graphs, that early growth rate tends to be inversely related to differences in the duration of the larval period.

There is a very high correlation between protein content and wet weight and so the growth curves for protein follow the same course as for wet weight except for the end of larval life, when water loss leads to a decline in wet weight. The resemblances and differences between the lines can be most easily comprehended by comparing the protein content at corresponding ontogenetic stages. Figure 2 shows the protein per individual at the second moult, in late larval life, 20 hours before pupation, in the early pupa and in the newly emerged adult.

The curves show that changes in protein, after the late larval stage, are minor in almost all lines and the unselected controls. But there are three exceptions, LR

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SlR and LO. All these lines show a substantial increase in protein content after the late larval stage, extending into pupal life in LR and SlR while in LO there appears to be a decline in the pupal period. Such differences suggest that the sequence of growth rates in corresponding stages, as defined above, are subject to genetic modification so that the proportion of total growth effected in different stages is not necessarily constant.

Table 2. *Percentage deviations of the selected lines from unselected in composition at the critical size and in adults, and also in the time taken to reach the critical size or complete larval development*

Geno-type	Larva				Adult			
	Time	DNA	Protein	RNA	Time	DNA	Protein	RNA
SP	−9	−7	−40	−23	−9	−1	−19	−9
LP	0	1	6	18	1	1	19	20
FP	−9	−6	−30	−22	−5	−11	−9	−6
SlP	12	1	9	0	10	−1	1	0
SR	0	−3	−23	−16	1	−1	−16	−11
LR	11	2	16	8	10	6	16	10
FR	−9	−5	−33	−15	−5	−10	−6	−4
SlR	12	4	12	12	17	4	12	3
LC	0	0	12	13	−3	0	19	18
LO	0	4	16	8	2	6	15	12

(d) Comparison between composition in larvae and adults

Table 2 shows the percentage deviation from unselected at the critical size and in adults with respect to DNA, protein, RNA and also the times to reach the critical size and the total larval period. The data may be considered from several points of view:

(i) Larval relations. Figure 3 shows the relationship between the DNA content and the time to reach the critical size. There is a highly significant correlation ($r = 0{\cdot}76$). The regression of percent deviation of DNA on time works out at $0{\cdot}37 \pm 0{\cdot}10$.

Figure 4 shows the parallel plot for the deviation of protein from unselected, in percentage terms, against percent deviation in time. Two features are to be noted. Firstly, the lines which reach the critical size at the same time as the unselected show large differences in protein content so that the differences between the extremes is about as great as where development time differs. Secondly, the lines which take longer or shorter time to reach the critical size have larger or smaller protein content, but there is an asymmetrical relationship with respect to the differences in protein associated with positive or negative changes in development time. Thus, the regression of percent protein deviation on percent of time deviation, is $3{\cdot}74 \pm 0{\cdot}84$ for the small fast lines (FP, FR and SP) and $1{\cdot}00 \pm 0{\cdot}41$ for the large slow lines (LR, SlR, SlP). It is evident, therefore, that protein and DNA are subject to considerable independence with respect to the relative deviation from the corresponding levels in the unselected.

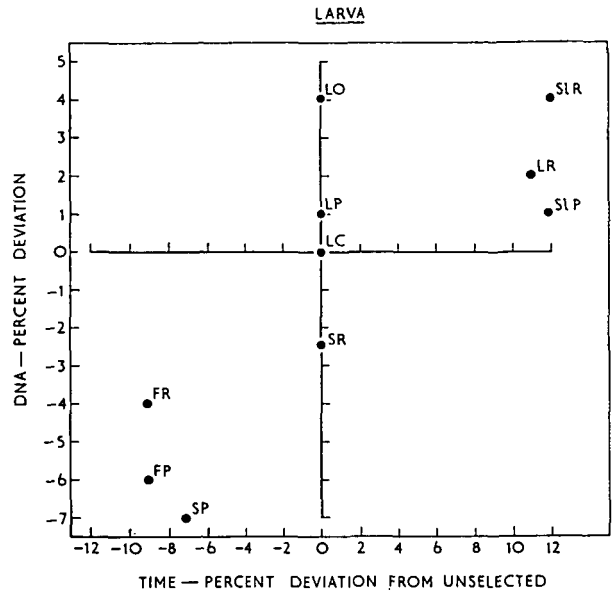

Fig. 3. The relationship between average DNA content per individual of the selected lines at the critical size and the time to reach this stage. Both estimates are expressed as percentage deviations from unselected.

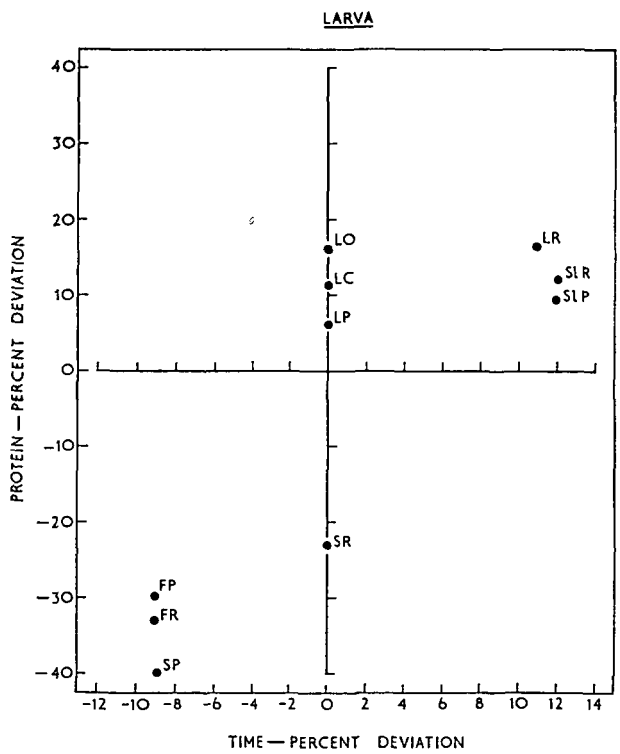

Fig. 4. The relationship between average protein content per individual of the selected lines, at the critical size, and the time to reach this stage. Both estimates are expressed as percentage deviations from unselected.

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The next question to ask is whether there are differences in the rate of synthesis of DNA in growth up to the critical stage. For this test it is convenient to take the ratio of DNA content in a given line to the content in the unselected stock at the same larval age. In the ratios shown in Fig. 5 for the 0–48 hour period, there are apparently three groups; generally either faster or slower DNA synthesis, or which resemble the unselected. The faster rate is found in the small fast-developing lines (SP, FR and SR), the slow rate in the large lines with the longer development time (LR, SlR, SlP) while the lines with the same development time as the unselected (SR, LO, LC, LP) have about the same rate of DNA synthesis. In the first group, especially, the

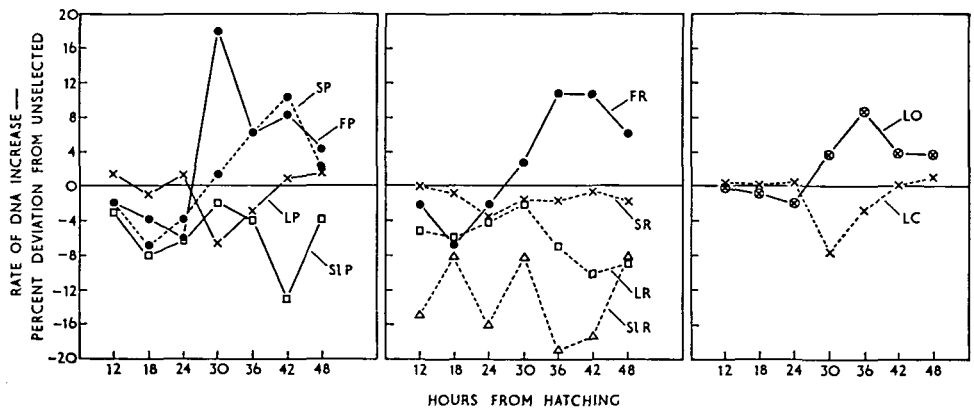

Fig. 5. The DNA content per individual up to 48 hours of larval life of the lines selected on different media. The values are expressed as percentage deviations from the corresponding values of the unselected stock. All estimates are derived from animals grown on the optimum axenic medium.

relative rate is lower at the very beginning of development up to 18–24 hours, i.e. during the first instar, but during the second instar the deviations from the unselected become well established. It is conceivable that the fluctuations, most noticeable in SlR and to a lesser extent also in SlP and FP, reflect cycles of DNA synthesis, similar to that often encountered in tissue culture.

To provide an average figure of the relative differences in rate, the ratios have been averaged for the estimates in the 24–48 hour period and have been plotted against the DNA content at the critical size in Fig. 6. There is a highly significant negative correlation ($-0{\cdot}81$) between the average early rate of DNA synthesis and the actual DNA content at the critical size.

The combined evidence from the interrelations between DNA content at critical size, the time to reach this stage, and the differences in the rate of DNA synthesis during the second instar suggest that the rate of DNA replication is a primary parameter of larval growth, especially with respect to the positive correlation between body size and the duration of the larval period. The protein content, on the other hand, is subject to wide variation independent of both DNA content and the time to reach the critical size.

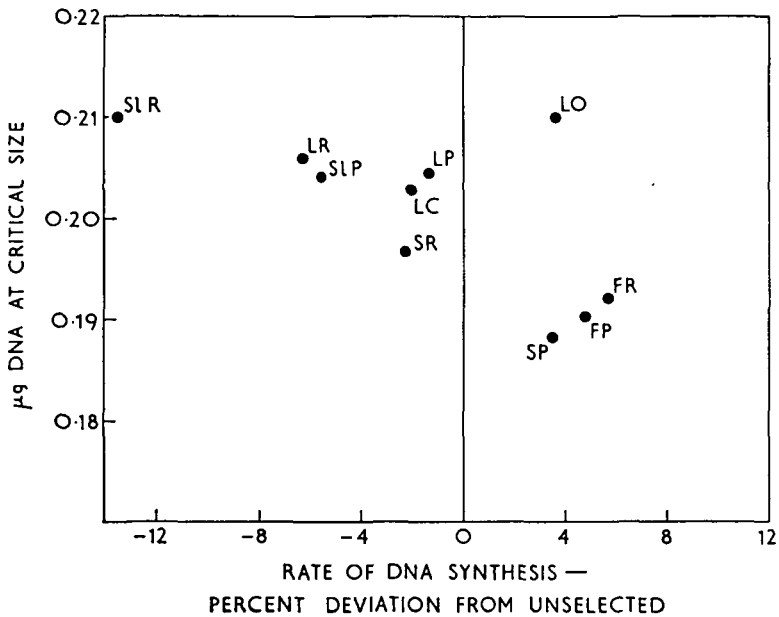

Fig. 6. The relation between DNA content at the critical size and the percentage deviation from unselected of the DNA content per individual, averaged for the period 24–48 hours of larval life.

It is of further interest to know whether selection has influenced the fraction of the total larval period or of adult DNA or protein accounted for by growth to the critical size. Table 3 shows the relevant figures classified into the two groups according to whether or not development time to the critical size differs from that of the

Table 3. *The ratios of the time taken to reach critical size to the total larval period and also of DNA and protein content at critical size to the corresponding content in adults*

Genotypes	Percentage at critical size		
	Time	DNA	Protein
	Size and time correlated		
FP	43·7	21·1	23·2
FR	44·5	20·5	23·8
SP	46·6	18·8	22·3
Unselected	45·6	19·9	31·2
LR	47·2	19·3	31·3
SlP	48·0	20·3	33·6
SlR	48·5	20·0	31·0
	Size and time uncorrelated		
SR	45·7	16·8	28·4
LP	45·4	19·8	28·0
LC	45·7	20·1	28·9
LO	47·6	19·6	31·6

unselected. For development time there is a significant tendency, in the small fast lines, for growth to the critical size to account for less (44·9%) of the total than in the large slow lines (47·9%). For DNA the values are close to the control value of 20% and there is no evidence of any systematic difference. For protein there is a distinct indication that the fraction is least in the small and fast lines (average 23·1%) and

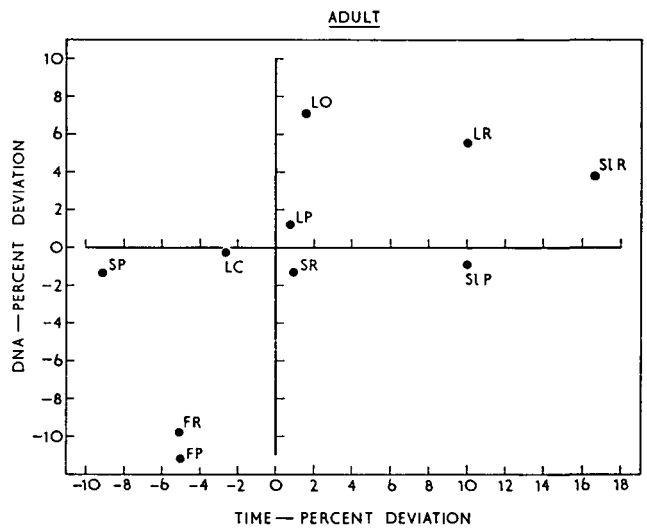

Fig. 7. The relationship between average DNA content per individual newly emerged adult female of the selected lines and the total duration of larval life. Both estimates are expressed as percentage deviations from unselected.

greatest in the large and slow lines (average 32·0%), while in the other lines the average is 29·6%; so there is a general tendency for the fraction of growth, or protein content, accounted for by growth to the critical size, to be greater as the critical size is delayed. But, compared with the major differences in final size, such differences are comparatively small.

(*e*) *Adult relations*

Figure 7 shows the plot for DNA of the percentage deviation from the content in the unselected against the percentage deviation in total larval period. Comparing this figure with Fig. 4, which shows the corresponding values at the critical size, we note that the relation between DNA and time are about the same for all the lines except SP and SlP. SP, although it has the fastest development time of the lines, has a DNA content close to that of the unselected, whereas, in the larvae, the DNA level is relatively much lower. At the critical size the DNA content in SlP is appreciably higher than in the controls whereas in adults it is not significantly different, although the proportional deviation in development time is about the same. The correlation between the two traits for all the lines is 0·75 and the regression works out at $0{\cdot}70 \pm 0{\cdot}22$.

With respect to protein (Fig. 8), adults and larvae display a similar pattern,

except that the percentage reduction of the small and the fast lines is lower in adults than in larvae, while adult SlP flies do not differ in protein content from the unselected.

Inspection of Table 2 shows that in adult FP and FR, the deviation from unselected is relatively great for DNA and less for both protein and RNA, and the two lines are very similar in their behaviour. SP resembles them in the relations at the critical

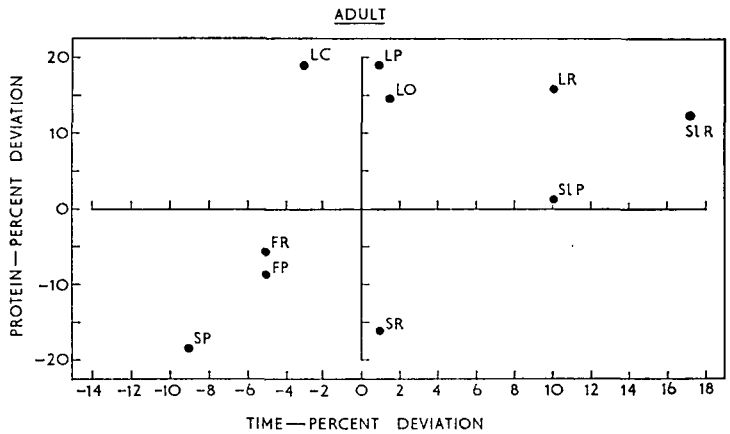

Fig. 8. The relationship between protein content per individual newly emerged adult female of the selected lines and the total duration of larval life. Both estimates are expressed as percentage deviations from unselected.

size but differs in the adult, as noted above. In the large and slow lines, LR and SlR, there is good agreement in percentage deviation from unselected in both larvae and adults, but SlP, on the other hand, which resembles them as larvae, differs as an adult and is like the unselected stock in all but development time.

Such contrasts between larvae and adults imply that the relative deviation from unselected, which is established in the growth of purely larval tissue, generally, but not always, holds for adult tissues as well. It may also be significant that the apparent disproportionate reduction in adult protein content in SP, compared with FR and FP, which are so similar to SP as larvae, may be related to the fact that SP was selected for small adult size while the others were not. In SlP the failure to maintain in adults the early correlation between body size and development time may be related to the selection for development time. Such contrasts could be interpreted in terms of independent changes in the growth of the imaginal discs.

(*f*) *Cell size and number*

If it is assumed that adult cells are mostly diploid and that differences in the protein/DNA ratio are correlated with differences in cell size, then differences in the composition of the adults can be formally described in terms of changes of cell size and/or number. Table 4 shows the ratios, for adults and critical size larvae, of protein and RNA to DNA, in terms of percentage deviation from the corresponding control values. The percentage deviations for DNA are listed as well. The lines are grouped according to the presence or absence of changes in development time.

With respect to the adults, the lines which differ in development time, namely FP, FR, LR, SlP and SlR, show only minor differences in either the protein/DNA or the RNA/DNA ratios. Only SP has a lower apparent cell size which reflects the disproportionate reduction in adult protein content which has already been noted.

Table 4. *The ratio of protein and RNA to DNA in terms of percentage deviation from unselected, compared with percentage deviation of DNA content*

Geno-type	Adults			Larvae		
	DNA	Protein/DNA	RNA/DNA	DNA	Protein/DNA	RNA/DNA
			Size and time correlated			
SP	−2	−18	−8	−7	−35	−17
FP	−11	3	5	−6	−26	−8
FR	−10	4	6	−5	−29	−8
LR	6	2	4	2	−14	0
SlP	−1	−2	1	1	8	0
SlR	4	8	−1	4	8	17
			Size and time uncorrelated			
SR	−1	−15	−10	−3	−21	−25
LO	6	9	6	4	18	0
LP	1	18	19	1	6	17
LC	0	20	21	0	8	0

The DNA values suggest that the greatest reduction in cell number has occurred in the fast lines and the greatest increase in the line LR. In the other group, with unchanged time of development, there are large differences in the ratios, apparently indicating differences in cell size, and only LO shows an appreciable increase in cell number reflected, of course, in a lower cell size or protein/DNA ratio. There is a high correlation between the deviations for protein and for RNA (0·89) while the regression of the RNA/DNA deviation on the protein/DNA deviation is $0{\cdot}48 \pm 0{\cdot}09$. For the adults, therefore, it appears, as a general rule, that correlated changes in development time and body size are associated with changes in cell number, while changes in size without change in development time are due to changes in cell size.

Similar comparisons are also shown for larvae at the critical size. For the DNA deviations, there is a fairly high correlation of 0·69 between the corresponding deviations for larvae and for adults. The regression of adult percent deviation on the corresponding deviation for larvae is $0{\cdot}90 \pm 0{\cdot}34$. Thus there is a strong similarity between adult and larval tissues in relative DNA content although, in the former, the DNA is mainly distributed between diploid and, in the latter, mainly between polytene cells. But the correlation is by no means complete and there are well-defined differences between the two stages in the small and fast lines.

In the protein/DNA ratios there are large differences between larvae and adults, especially in those lines which differ in development time and which are comparatively like the controls in protein/DNA and RNA/DNA ratios. This is most striking

in the two lines selected for fast development, FP and FR, in which the protein/DNA ratio is 26–29% below the level of unselected in larvae and 3–4% greater in adults. The RNA/DNA ratio shows a parallel contrast. For the larvae in this group,the correlation between the protein and the RNA deviation is 0·93 while the regression RNA/DNA upon protein/DNA, in terms of percent deviations, is $0{\cdot}74 \pm 0{\cdot}11$. In the other group the general trend is similar in larvae and adults but there is considerable variation in the values of the particular ratios.

Although it has been suggested that differences in protein/DNA ratio may reflect differences in average cell size, it must be clearly recognized that this inference can only be accepted as a first approximation on current evidence. In homogeneous diploid tissue differences in this ratio may be interpreted with reasonable confidence as due to differences in cell size but here we are dealing with the contribution of a variety of tissues to the total estimates of protein and DNA per animal. The protein/DNA content in such tissues is likely to vary widely and their separate contribution to the totals may also differ greatly between lines. The same qualification holds for the RNA/DNA ratio, with the further likelihood that the relative RNA content will reflect difference in hormonal activity and physiological state, both of which may differ between lines for any particular tissue. Hence the interpretation of growth differences in terms of changes in cell size and/or number is more in the nature of a working hypothesis than an established inference.

(g) Timing of differences

It has been shown in Fig. 5 that there are clear differences between the lines in the rate of DNA synthesis early in the second instar from 24–30 hours. There is, however, additional evidence of characteristic differences in the growth parameters at even earlier stages. It has been shown elsewhere (Church & Robertson, loc. cit.), that although the constituents RNA, DNA and protein rapidly increase after the larvae hatch from the egg, the amounts of RNA and protein relative to DNA undergo a regular cycle of change. For RNA the ratio is high at hatching, due to synthesis during embryogenesis, and the RNA/DNA curve rapidly reaches a peak at 24 hours in the Pacific unselected stock, after which it declines sharply to a comparatively low level which is maintained thereafter. The protein/DNA ratio follows a closely similar pattern, except that it is displaced in time so that the peak is reached about 36 hours later in the unselected Pacific stock. Consideration of the RNA/DNA ratio is therefore sufficient for present purposes.

Figure 9 shows the curves of the RNA/DNA ratio for the two groups of lines while Table 5 gives the observed time to reach the peak in the lines and the ratio of this time to the time to reach the critical size. For the unselected stock and the lines with constant development time, except LO, the peak occurs at the same time and the ratio to the time to critical size is 0·5. In the other group there is a high correlation beween the time to reach the peak and the time to critical size. The absolute times of the peak range from 11 hours in SP to 54 hours in SlR and the ratios range from 0·26 in SP to 0·95 in SlR, i.e. in the largest line, with the longest development time, the peak almost coincides with the critical size. However, the main point to

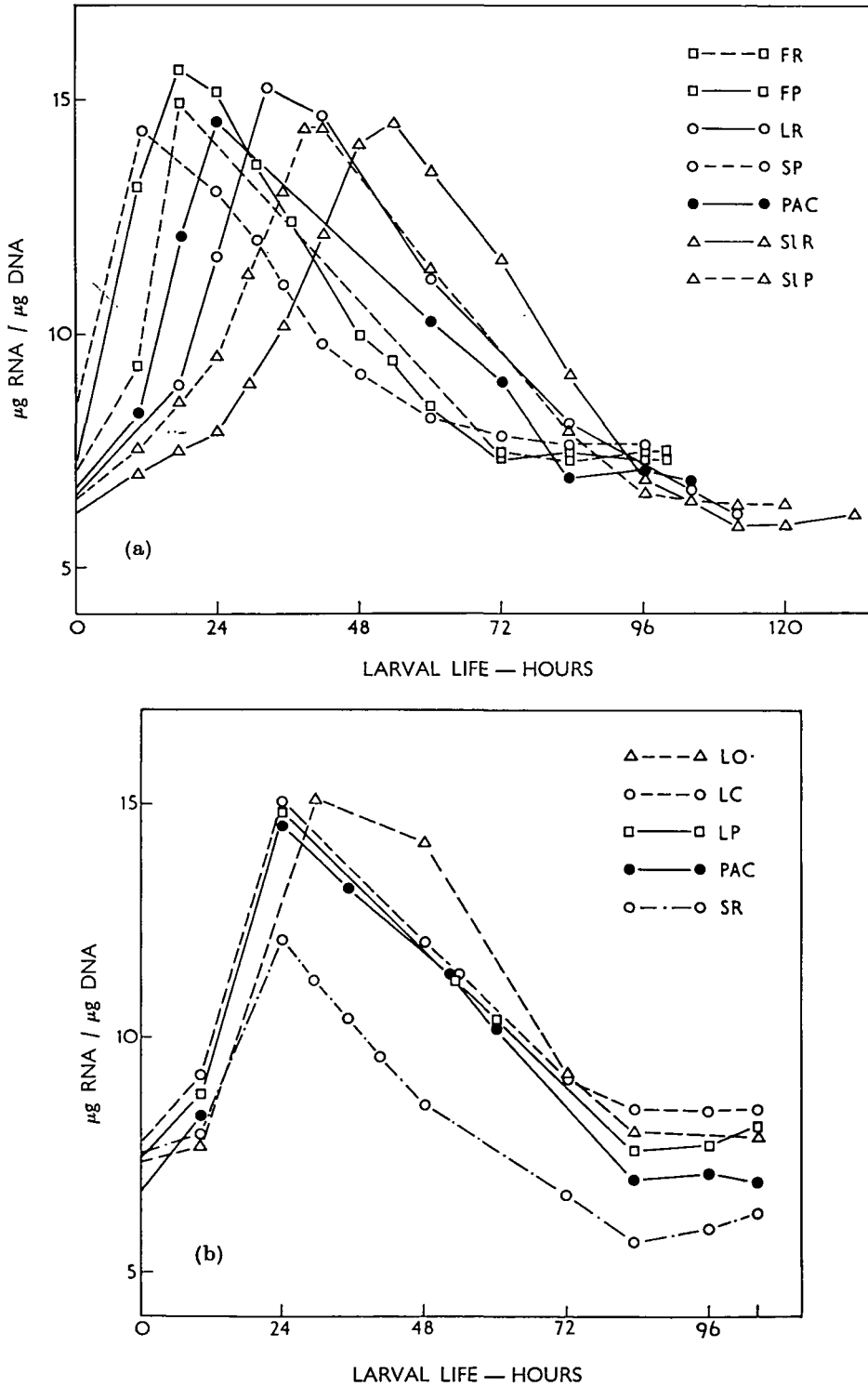

Fig. 9. The ratio of average RNA (μg) content per individual to average DNA content (μg) at successive stages of larval life. (*a*) and (*b*) refer respectively to the lines with and without correlated changes in size and time.

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Table 5. *The time to reach peak RNA/DNA*

Genotype	Time to maximum ratio (hours)	Ratio of time to critical age time
	Size and time correlated	
SP	11·0	0·26
FP	17·5	0·45
FR	17·5	0·40
Unselected	24·0	0·50
LR	33·0	0·61
SlP	42	0·76
SlR	54	0·95
	Size and time uncorrelated	
SR	24	0·50
LP	24	0·50
LC	21	0·50
LO	29	0·62

note is that the metabolic differences are apparent from the earliest time the larva leaves the egg and represent a continuation of synthetic differences established very early in embryogenesis (Church & Robertson, loc. cit.).

The time taken for newly laid eggs to hatch has been recorded for all the lines and the values compared with the interval of time between hatching from the egg and

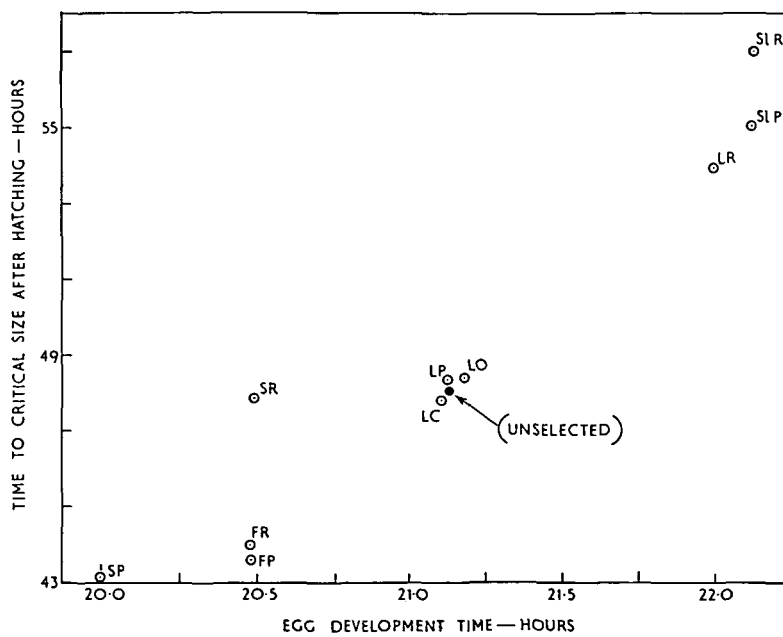

Fig. 10. The relation between the average time taken by the eggs laid by females of different lines to hatch and the time to reach the critical size after hatching. All flies were provided with the conditions most favourable for uninterrupted oviposition.

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attainment of the critical size. Figure 10 shows there is a strong positive association between the time to complete development in the egg and the time from hatching to the critical size; the correlation coefficient is 0·94. It is possible that the maternal contribution to cytoplasm may be partly responsible for such differences, but it is improbable that it is entirely so. Thus we can follow the establishment of the characteristic growth relations which distinguish the selected lines back to the earliest stages of larval growth.

4. DISCUSSION

The variable incidence of correlation between the duration of the larval period and adult body size was the point of departure for these experiments. It is now clear that such variation is closely related to differences in DNA content at corresponding ontogenetic stages, especially in larvae. The relationship is also evident in the adults, but is not so well defined, due apparently to divergence in growth rates in larval and imaginal disc tissues in some instances. The protein and RNA content, which are themselves highly correlated, can vary within wide limits without alteration of the time to the critical size and to pupation and, in this case, the DNA content is either unchanged or only slightly so, with the exception of one line, LO. The association between development time and DNA content is also related to the rate of DNA synthesis in early larval life, especially in the second instar. The rate of increase tends to be negatively correlated with the absolute DNA content at the critical size, so that lower rates are associated, not only with longer time to reach the critical size, but also a higher DNA content at this stage as well, and vice versa. There is a very high correlation between the duration of development in the egg and the duration of the time to the critical size after hatching. Hence, the genetic differences responsible for this syndrome of effects appear to operate from the earliest stage of larval growth.

If the critical size represents arrival at a certain threshold of hormonal concentration or a particular hormonal balance, it appears that DNA synthesis is closely geared to the hormonal system generally. The ratio of DNA content at critical size to that of the adults is virtually constant, while the corresponding ratios for time and protein content vary, although not greatly. The constancy of the ratio for DNA could be regarded as further evidence of a relationship between DNA synthesis, hormonal activity and the timing of ontogenetic events.

At present, the hormonal control of insect development is imperfectly understood and hence speculation about possible interrelations must be recognized as such. It might be thought that correlated changes in body size and development time are primarily due to variation in the rate of secretion of the growth and moulting hormone and/or juvenile hormone and that such variation influences DNA synthesis. But such a hypothesis meets difficulties if it is the hormone concentration in tissues and haemolymph, which determines the attainment of the critical size, since the concentration should differ in large or small lines in which the DNA content and rate of synthesis is unchanged but which differ greatly in their rate of protein and RNA increase. Alternatively, responsibility for differences in timing might be

transferred to differences in the threshold of tissue sensitivity with or without changes in rates of hormone secretion. Such alternatives by no means exhaust the possibilities. Although we cannot yet account for the syndrome of effects, it would be in line with current thinking if the genetic differences primarily affected hormonal activity which, in turn, controlled DNA synthesis and the particular DNA levels at which various ontogenetic stages are reached.

Since a positive correlation between the time to critical size and the DNA content holds generally for all the lines examined, whereas the protein and RNA content may vary widely without change in either development time or DNA, it appears that larval growth tends to follow one or other of two alternative developmental pathways. In adults, the contrast has been tentatively interpreted in terms of changes in either cell size or cell number. But it appears that the essential criterion of difference may be the ratio of protein or RNA to diploid DNA equivalents, irrespective of whether cell division has taken place or not.

These contrasts have been produced by selection on different diets deficient in either ribonucleic acid, protein or choline. Earlier evidence (Robertson, 1963, 1964) has shown that on various other diets changes in body size generally occur without change, or with only minor changes in the duration of the larval period. Hence, in so far as growth by cell division or cell increase, or, at least, with constancy or variation in the protein/DNA ratio represent alternative routes, the probability of which will be followed is very unequal under most environmental conditions. To produce changes entirely or predominantly by cell division, rather special conditions seem necessary. Selection on diets deficient in ribonucleic acid especially favour changes of this kind. *Drosophila* is unable to synthesize adenylic acid (Sang, 1959) and it is conceivable that deficiency of ATP may particularly restrict changes in cell size or the protein/DNA ratio.

It is at present unclear how far there may be metabolic imcompatibility between the alternative developmental pathways in which an equal change in body mass may be effected. A study of the changes in body size and development time during the course of long-continued selection of the present lines has shown different relations between the two traits at different stages of selection. Thus, the early relationship between adult size and larval development time (Robertson, 1964) in LP and SR disappeared after five to seven generations. In FR selection for faster development time led to larger body size for the first five generations, after which a reversal occurred so that body size declined rapidly as development time decreased. In LP and SR, especially, development time gradually returned to the normal value. On the other hand, some lines, e.g. FP, SlR and LR, showed a well-marked positive correlation between the two traits over the entire period of selection.

Such contrasts suggest that the composition of the genetic variance with respect to which of the alternative routes will be favoured may change during selection. The composition of the variance will be influenced by change in environment and by change in the genetic background which, by itself, may influence the likelihood of selecting for alternative kinds of effect. In addition, natural selection will be operating, especially by favouring a return to the normal length of development. In the

present experiments a new batch of casein was used at generation 9 or 10 during the selection and minor differences in its composition could have contributed to the sequential changes in the relations between the two traits.

Although it remains to be determined how far changes which leave the protein/DNA ratio unchanged and alter development time are genetically independent from those which alter the ratio and leave development time unchanged, it is clear that, in the unselected wild population and on most diets on which selection has been carried out, variation in growth rate, without change in development time, accounts for most of the genetic variation in body size which can be detected in such populations. Reduction of the duration of the larval period is notoriously difficult in *Drosophila* (Sang & Clayton, 1957; Clarke, Maynard-Smith and Sondhi, 1961) at least under the prevailing conditions to which the species is adapted. Present experience has shown that correlated changes, which reduce both size and time of development, are particularly subject to gene-environment interaction so that the expression of such effects is greater on the axenic diet of selection than on the live yeast medium. The difficulty in reducing development time and the normal lack of correlation between body size and duration of larval development no doubt reflect the importance of the latter as a parameter of fitness. It has already been minimized by natural selection, but genetic variation, which influences this trait, can be exposed and utilized by suitable control of the diet and selection.

With respect to the general stability of average body size in a population, the stability or canalization of the size and time relations has been accorded a major role (Robertson, 1964) and there is no reason to depart from this view, although it can now be formulated more materially in terms of the rate and extent of DNA synthesis.

The present, more extensive, data require modification of an earlier hypothesis. Robertson (1963, 1964) suggested that the presence or absence of correlation between body size and development time is due to different genetic changes which influence, respectively, either growth to the critical size or growth thereafter, in the period when nutritional change is without effect on time of pupation. Although there are instances of growth differences between earlier and later stages of larval growth, the original interpretation must be modified in the light of the current evidence that the absolute critical size is correlated with that of the adult, whether or not there are changes in development time, and also that the intrinsic differences in growth operate from early stages of development. Although the developmental interpretation has been corrected, this in no way affects the general inferences which have been derived from the variation in genetic correlation between the two traits (Robertson, 1964).

There is evidence, in the percentage deviation from controls for RNA, DNA and protein in larvae and adults (Table 2), that the pre- and post-critical growth may diverge. Perhaps it is not surprising that this should be so since the proliferation of the imaginal discs in late larval life, when changes in diet no longer affect the time to pupation, at least provides a possible basis for independence of genetic effects on growth. Naturally, such independence between the two systems would be subject

to severe limitations, of which the most obvious is the upper limit imposed by the total growth of larvae on the growth of adult tissue. Hence selection for larger adult size is unlikely to provide evidence of divergence. In the present material, in SlP, selected for slow development time, it appears that extended larval growth has led to larger individuals at the critical size. This difference is not maintained in the growth of the imaginal discs since the adults have the same body size as that of the unselected adults. Also, the small line SP, which so closely resembles the lines FP and FR in the larval stage, differs from them as an adult in having a relatively smaller protein and RNA content—although a relatively higher DNA content. The relatively lower protein and RNA is consistent with the continued pressure of selection for small size in SP but absence of such in the lines FP and FR, which are selected for shorter development time.

The comparisons of protein and DNA content and the ratio of protein to DNA in adults, suggest that changes uncorrelated with development time may involve changes in cell size while correlated changes involve change in cell number. As noted earlier it is an open question as to how far this inference can be applied to all or most tissues. However, taken at face value, it apparently conflicts with the inferences derived from the analysis of the cell size and number relations in the wing (Robertson, 1959*a*, *b*, *c*). The earlier work showed that changes in body size, due to selection under optimum conditions, in which we do not normally find much evidence of correlation between size and time, were associated with constancy of wing cell size. This apparent discrepancy suggests that the cell size and number relations in the wing may differ from those in the bulk of the tissue which chiefly determine the estimates of total protein and DNA. Of course, even if the wing tissue were totally discrepant with the rest, this would not be detected in the whole estimates since the contribution of the wing to the total is small.

The cellular relations in the wing can be changed by selection, without affecting wing or body size (Robertson, loc. cit.), while relative wing size can be changed without affecting wing cell size (Robertson, 1961). Also, there is good evidence (Robertson, unpublished), from experiments in which flies have been grown on various media which reduce body size, that wing cell size is frequently held constant on different diets which alter both wing and body size. It is therefore evident that the wing comprises a complex system with its own set of rules relating change in cellular composition to the growth and metabolism of the body generally and the manner in which particular kinds of genetic change is expressed. This conclusion does not affect the earlier inferences, which have been derived from the analysis of cellular changes in the wing, and the characteristic association between genetic behaviour and cellular make-up. But it does suggest that one way to advance our understanding of this complex situation would be to compare, in suitable genotypes, at successive stages of development in different organs, the protein and DNA content, to discover how far similar changes in gross size are realized by parallel or dissimilar changes in cell size and number. To further this aim work is now in progress to determine the cell size and number relations in the imaginal discs of genetically different lines.

SUMMARY

1. Lines of *Drosophila melanogaster*, which differ greatly in body size and/or development time, have been created by selecting for either large or small body size or longer or shorter development time on different chemically defined axenic media. The ten lines studied here have been selected either on the optimum medium or on media deficient in either protein, ribonucleic acid or choline.

2. The lines show a variable degree of correlation between adult size and the duration of the larval period. Selection for one trait may involve a positively correlated change in the other or have no such effect. There is a fairly regular association between the composition of the diet and the presence or absence of evidence for genetic correlation.

3. The biochemical composition of the various lines, and also the unselected stock from which they were derived, has been compared at successive stages from egg to adult in animals grown on the optimum axenic diet. Records of wet weight, protein, RNA and DNA content per individual have revealed differences in composition which can be releated to characteristic differences in the response to selection.

4. Comparisons between the individual content of the various constituents at the critical size, i.e. at the time in early third instar when larvae can complete development even if no longer allowed to feed, and in newly emerged adults indicate a high positive correlation between DNA content and the time to reach the critical size and also the time to pupation. Protein and RNA content, on the other hand, may vary within wide limits without alteration of the development time. The association between DNA content and development time which underlies the correlation between body size and development time, is related to the rate of DNA synthesis in early larval life, such that the rate of synthesis tends to be negatively correlated with the absolute DNA content at the critical size and in the adult.

5. Since the protein/DNA ratio is comparatively unchanged in lines with correlated changes in body size and development time, but varies widely in lines in which development time is unaltered, the contrasts may reflect, in adults at least, differences in respectively cell number or cell size.

6. An earlier hypothesis of the origin of correlated and uncorrelated changes in body size and development time has been modified. It appears that the absolute critical size is positively correlated with the size of the adult, whether or not changes in development time have occurred. The intrinsic differences in growth operate from very early stages of development. This has been demonstrated in the differences in RNA/DNA ratio in early larval life and also in a high correlation between the time to complete development in the egg and the time to reach critical size after hatching from the egg.

7. There is also evidence, in the relative deviations from unselected of the various estimated constituents, of partial independence of growth between early and late larval life, and this may reflect differences in the growth of the imaginal discs.

8. The data are discussed in relation to earlier experimental evidence relating to the growth of *Drosophila*. The point is made that judicious selection for appropriate

parameters of growth will often create differences great enough to reveal developmental interrelations which would otherwise be difficult to detect.

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