METABOLIC ALTERATIONS IN GENETICALLY SELECTED *DROSOPHILA* STRAINS WITH DIFFERENT LONGEVITIES

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ABSTRACT

Sometime ago we obtained biomarker data suggesting that the earliest determining event in the expression of the extended longevity phenotype in our selected strains of *Drosophila* took place early in adult life at about 5-7 days of age. In a later series of experiments we documented that our La and Lb long lived strains underwent a specific up-regulation of the antioxidant defense system (ADS) genes and enzymes. This led to a reduction in oxidative damage and an extended longevity. In the current work, we assayed the activity of 17 metabolically important enzymes in 5-7 day old flies of 13 strains variously selected for different longevity. We conclude that the two sets of replicated long-lived strains have an altered metabolic pattern (relative to normal-lived animals) which is consistent with an increased flux through the pentose shunt and an enhanced NADP⁺ reducing system to support the increased activity of the ADS enzymes. This result can be interpreted as a shift of energy expenditure from reproduction to somatic maintenance. We conclude that theories based on differential energy allocations appear to empirically explain, at least in part, the mechanisms underlying the transformation of a normal longevity phenotype to an extended longevity phenotype.

INTRODUCTION

The whole point of doing transgenic or selection experiments designed to alter the longevity of a model organism such as *Drosophila melanogaster* is finally to alter its complement of enzymatic, regulatory or structural proteins (proteome) in such a way so as to allow the investigators to identify those key proteins essential to the expression of the new longevity phenotype. Different theories require significantly altered expression of particular proteins, and those laboratory interventions which result in the predicted alterations are cited as evidence in support of the particular theory. There are many theories of aging (1). Some of these have been disproven (e.g., error catastrophe), others have been strengthened (e.g., oxidative stress). Recent work has suggested that theories based on genetic or metabolic

principles may be united at a deep biological level by their alteration of energy metabolism. This particular point of view was phrased in modern evolutionary terms as the disposable soma theory of Kirkwood (2) or the optimality theory of Partridge and Barton (3). These theories posit that extended longevity can arise only when the organism's energy metabolism is altered in such a way so as to allow the reallocation of energy from an emphasis on reproduction to an emphasis on somatic maintenance, and that there are different optimal allocation values in different environments.

Some years ago, we and others performed selection experiments in *Drosophila* based in part on the antagonistic pleiotropy theory and in part on the central role of reproduction in modulating the organism's longevity (3-6). The enhanced expression of the antioxidant defense system (ADS) genes and proteins in our long-lived strains (generically termed the 'Wayne State strains') has been cited as evidence favoring the oxidative stress or free radical theory of aging (7-10).

Beginning with our initial work in selection for longevity (4, 6), we have done replicate selection for extended longevity (La, Lb and 2La, 2Lb strains), normal longevity (Ma, Mb and Ra, Rb strains) and shortened longevity (2Ea, 2Eb strains). In an independent endeavor, a strain highly resistant to paraquat (PQR) was generated by direct selection (11). All of these strains were derived from our normal-lived Ra or Rb strains. These control strains were deliberately kept under non-selective conditions so as to maintain (more or less) their original state (6) while the Ma and Mb strains were kept under selective conditions for a normal lifespan. Our initial assumption that these R animals represented an optimal mix of life history characteristics is supported by recent mathematical modeling (12). In addition, we used the La and Lb strains to generate by reverse selection the normal-lived RevLa and RevLb strains (8, 9).

During the course of these experiments, we also assayed all 13 of our selected longevity strains, spanning long-, short- and normal-lived strains, for their specific activity of 17 different metabolic enzymes involved in the glycolytic, citric acid, and pentose shunt pathways. Only a portion of these data have been presented elsewhere and were consistent with our other work showing that extended longevity in our selected strains is dependent on an early up-regulation of the ADS genes (8, 9, 12).

However, that partial analysis was not sufficient to answer the question as to whether the changes in metabolically important enzyme activity patterns are consistent or not consistent with the basic arguments of

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these energy allocation theories. We believe such theories make four testable predictions in the context of our selection experiments:

- First, each of the several different longevity phenotypes should be accompanied by different but consistent patterns of changes in various metabolic enzymes.
- Second, selection for longevity should yield repeatable results.
- Third, use of replicate or sister lines should enable the identification of enzyme changes due to selection from those due to chance alone.
- Fourth, and most important, the differences observed in the patterns of change of various metabolic enzymes should be interpretable in terms of the reallocation of energy expenditure from growth and reproduction to somatic maintenance. We now analyze that database of 17 enzymes in 13 strains with the goal of testing these predictions.

RESULTS

Longevity:

Figure 1 presents representative survival curves for the 13 strains analyzed in this report. The replicate longevity phenotypes are statistically identical and the replicate strains within each set seem to follow the same mortality kinetics. Thus each different selection protocol seems to have yielded replicate identical longevity phenotypes, which are statistically different from those generated by the other selection protocols. Our selection protocols are effective. Note that the major difference between the several sets of strains resides not in their early survival pattern but rather in the time of onset of their senescent phase. We have shown elsewhere that the time of onset of senescence is closely coupled to the presence or absence of an early up-regulation of the antioxidant defense system (ADS) genes and the concomitant decrease in oxidative damage (8, 9). Presumably, senescence begins when the level of oxidative damage reaches some sort of functional threshold (10).

Figure 1: Survival curves of the 13 strains analyzed in this report. There are six pairs of replicate strains for four different longevity phenotypes (long-lived = La, Lb, 2La, 2Lb; reverted long-lived = RevLa, RevLb; normal-lived = Ra, Rb, Ma, Mb; short-lived = 2Ea, 2Eb) and one non-replicated strain selected for paraquat resistance (PQR). See text for details.

Allelic Changes and Selection:

We assayed 23 enzymes across our 13 different longevity strains to determine if selection had altered their electrophoretic mobility. Fourteen enzymes show no change in this regard (AK-1, CAT, GDH, GPI, GR, MDH, ME, NADHdia, NADPHdia, PGM-1, PGM-3, PNP, SOD, and XDH). Nine enzymes showed changes in their electrophoretic mobility and these are detailed in Table 1. When compared to the Ra or Rb strains, selection for extended and/or shortened longevity altered the isoenzyme pattern, either qualitatively or quantitatively in these cases. A qualitative change relative to the appropriate R strain was noted for all these enzymes except HK. For two of the enzymes (ICD, PGD), selection resulted in alleles being expressed in some strains which are not present in the appropriate Ra or Rb progenitor strains. The most reasonable interpretation of this phenomenon is that the 'missing' alleles are present in the progenitor population but at a low frequency (i.e., less than 1 animal out of 80 sampled). Their obvious presence in the selected lines suggests the power of selection on these alleles.

Table 1:

Strain Lifespan		Allelic Status of Enzyme in Each Strain									
		ADH	EstD	G6PDH	G3PDH	HK	ICD	LDH	PGD	SOD	
La	Long	SS	SF	SS	FF	SF	sF	Sf	FF	Sf	
Lb	Long	SS	SF	SS	Sf	SF	SF	Sf	FF	SS	
2La	Long	SF	SF	sf	FF	SF	Sf	Sf	FF	SS	
2Lb	Long	SF	SF	sf	FF	SF	Sf	Sf	FF	SS	
RevLa	Intermed.	SF	SS	SS	SF	Sf	Sf	Sf	FF	Sf	
RevLb	Intermed.	Sf	SS	SS	SF	Sf	Sf	Sf	FF	Sf	
Ra	Normal	SF	SF	SS	SF	Sf	Ss'	Sf	SS	SS	
Rb	Normal	Sf	SF	SS	SF	Sf	Ss'	Sf	FF	SS	
Ma	Normal	SF	SF	sf	FF	SF	SS	Sf	FF	Sf	
Mb	Normal	SF	SF	sf	SF	SF	SS	Sf	FF	Sf	
2Ea	Short	Sf	SF	sf	SS	Sf	Ss'	Sf	SS	Sf	
2Eb	Short	SF	SF	sf	SS	Sf	Ss'	Sf	SS	Sf	
PQR	Increased Mean Only	SF	SF	sf	SF	Sf	Sf	Sf	SS	Sf	

Notes: The slow allele of each enzyme is designated by S and the fast allele by F. s' or f' indicate minor bands migrating slower or faster than the main S or F band. Lower case letters indicate lower levels of an enzyme. See Materials and Methods for enzyme abbreviations.

Specific Activity Changes and Selection:

The actual data showing the mean ($\pm$ standard deviation (sd)) of the 17 enzymes assayed in the 13 strains are shown in Figure 2. There are some enzymes which show no apparent differentiation between the 13 strains (e.g., GDH), some which appear to have higher activities in the shorter-lived strains (e.g., NADPHdia) and others which appear to have higher activities in the longer-lived strains (e.g., GPI). Understanding the meaning of these differences requires a statistical analysis.

We did a multivariate MANOVA of strains vs. enzymes. The omnibus F value, calculated by Wilkes lambda, was 43.557 (hypo df=187, error df=98), $P < 0.001$. A *post hoc* multiple comparison of the 17 enzymes in the 13 strains was done using a Bonferroni correction. Only

Figure 2:

Figure 2: (continued)

Figure 2: (continued)

Figure 2: The mean specific activity $\pm$ std.dev. for each of the 17 enzymes assayed is shown for the 13 strains surveyed in this report. Note that the data for the RevLa and RevLb strains was combined in this assay. See text for details.

those 125 comparisons in which the experimental strain showed a statistically significant difference relative to its progenitor strain were retained. They were then examined for concordance by comparing and retaining only those pairs which showed statistically significant differences in both the *a* and *b* sister strains. Only biologically important comparisons were made, as follows: The Ra values were compared to those for the Rb, Ma, La, 2La, 2Ea and PQR strains; the Rb values were compared to those for the Mb, Lb, 2Lb, and 2Eb strains. The RevLab values were compared to those of the La and Lb strains as they were its progenitors. Those enzymes which changed in a concordant manner are of special interest in the current context and are summarized in Table 2 and below. We have two replicate sets of long lived strains and thus we analyze the Lab and 2Lab pairs separately. It appears as if the two sets follow different metabolic strategies. Our observations are as follows:

- There are five enzymes which change concordantly in the Lab strains. Significant increases in mean activity are shown by AK, G6PDH, HK, ICD, and PGM; while only NADPHdia shows a significant decrease in specific activity relative to the Rab strains.
- An overlapping but different set of six enzymes show significant concordant changes in the 2Lab strains. Significant increases in mean activity are shown by HK, NADHdia, PGM and XDH. Two enzymes (GAPDH and NADPHdia) show significant decreases in specific activity relative to the Rab strains.
- HK, PGM and NADPHdia are the only enzymes which show significant and concordant changes in all four long-lived replicate strains.
- Selecting a long lived strain for a shorter longevity (i.e., RevL strains of Table 2) leads to significantly reduced relative activities for seven enzymes (GAPDH, GPI, G6PDH, G3PDH, HK, MDH, and ME). NADPHdia shows a significant increase in mean activity in response to this reverse selection protocol. Note that the overall response of the RevL strains to selection is almost the converse of the response of the L strains.
- Using selection to maintain a normal life span in the M strains as described above leads to a significant

increase in mean activity for GAPDH and XDH, relative to the R strains. Six enzymes show a significant decrease in relative mean activity: GPI, G6PDH, G3PDH, ICD, ME and NADHdia. Although the life spans of the two sets (M and R strains) of normal lived strains are similar (but not identical: Kaplan Meier log-rank test =15.14, 1 df., $P=0.0001$), their metabolic profile is quite different. The stock maintenance protocols alter the stock's genetic and biochemical profile as well as its longevity.

- Selection for a shortened life span leads to a significant decrease of metabolic enzyme activities relative to the controls for four enzymes: GAPDH, G6PDH, ME, and NADHdia. No enzyme activities are increased by this selection protocol.
- A direct selection for paraquat resistance (PQR strain) led to significant relative increases in specific activity for ADH, AK, G3PDH, HK, PGM and XDH. A significant relative decrease in specific activity was noted for NADHdia and PGD. These data may represent an over-estimate of the real situation since there are no replicate strains with which to identify chance events from selection effects.

Table 2: Enzymes which show concordant significant differences in the various selected strains relative to their progenitor strains

Type of Change	STRAIN TYPES					
	L	2L	RevL	M	2E	PQR
Increase	AK G6PDH HK ICD PGM	HK NADHdia PGM XDH	NADPHdia	GAPDH XDH	—	ADH AK G3PDH HK PGM XDH
Decrease	NADPHdia	NADPHdia GAPDH	GPI G6PDH G3PDH HK ICD MDH ME	GPI G6PDH G3PDH ICD ME NADHdia	GAPDH G6PDH ME NADHdia	ME NADHdia PGD

In Table 3, we compare the enzyme profiles of the Ra and Rb replicate normal-lived strains. These strains were each derived from a common wild type progenitor strain, and they are maintained under a non-selective regime as described (6). Their survival curves are statistically similar (Figure 1). Even so, there are observable differences in their metabolic enzyme profile (Figure 2). There are four enzymes (ADH, G3PDH, MDH and PGM) which are significantly higher in the Ra strain relative to the Rb strain. The other enzyme are statistically equivalent. We previously showed that the two strains differed in their ADS gene and enzyme activities as well (9), thus supporting the idea that replicate strains derived from the same population and expressing identical longevity phenotypes need not be biochemically nor genetically identical.

Table 3: The two progenitor Ra and Rb strains are similar but not identical

COMPARISON	ENZYMES
Ra = Rb	AK, GAPDH, GDH, GPI, G6PDH, HK, ICD, LDH, ME, NADHdia, NADPHdia, PGM, XDH
Ra > Rb	ADH, G3PDH, MDH, PGD
Ra < Rb	--none--

DISCUSSION

Electrophoretic Changes As A Reflection of Gene Frequency Changes:

The electrophoretic mobility data is summarized in Table 1. We note that in eight cases (out of a potential of 18 cases (e.g., 9 enzymes x 2 strain families) , the changes induced in electrophoretic mobility by our forward selection (i.e., R→L) were reversed by our reverse selection (i.e., L→RevL). We conclude that the changes in electrophoretic mobilities summarized in Table 1 are adaptive and reflect the outcome of selective pressures on the organism. Selection alters the organism at the genetic level.

Specific Activity Changes As A Reflection of Shifts in Energy Metabolism:

The significant specific activity changes summarized in Table 2 are, with the exception of the PQR strain, the result of concordant changes in replicate strains. These are presumably the result of the selection pressures which generated the different longevity phenotypes (Figure 1). We note that each of the different longevity phenotypes are accompanied by its own unique metabolic enzyme profile (Table 2). These observations support the first three predictions associated with our interpretation of the disposable soma theory. The most important prediction, however, has to do with the nature of the metabolic strategies adopted by the several strains, and we now turn our attention to this problem.

Long-lived Strains:

We have conveniently summarized the nine enzymes responsible for the altered metabolic profile of the Lab and 2Lab strain sets, and their functions in Table 4. We interpret these data as suggesting that the Lab and 2Lab strain sets are following two different metabolic strategies.

The Lab strains are likely to have a higher flux through the pentose shunt. This generates increased levels of NADPH which is used for the detoxification of peroxides formed after the dismutation of superoxide by CuZnSOD (15). We have shown elsewhere that the ADS gene and enzyme levels are significantly elevated in the Lab strains relative to the Rab controls (7-9). It follows that elevated ADS activities require correspondingly elevated NADPH levels if the enzymes are to function effectively. These can be produced by the pentose shunt. Classic insect biochemistry studies have shown that insects usually have a deficient NADPH reoxidizing system (16); thus the activity of the pentose shunt depends on the rate of reoxidation of NADPH. The down-regulation of NADPHdia would release the NADP⁺ dependent inhibition of G6PDH, thereby increasing the metabolic flux through the pentose shunt, and increasing the reduction of the NADP⁺ produced by the ADS enzymes. This scenario would require a higher G6PDH activity, which is observed. ICD not only regulates the flux through the TCA cycle but also generates NADPH. The enzymes glucose-6-phosphate dehydrogenase (G6PDH) and isocitrate dehydrogenase (ICD) both generate NADPH and both have been implicated

TABLE 4: Comparison of Metabolic Profile of Lab and 2Lab Long-Lived Strains

Enzyme	Notes on Function of Enzyme	Strain & Relative Activity Level
AK	increases cAMP feedback control of enzyme processes, increases AMP → ADP production, may be involved in lipid biosynthesis	Lab + 2Lab =
HK	increased G6DP formation & thus energy production from glucose metabolism	Lab + 2Lab +
PGM	increases the conversion of 3PG to 2PG and thus increases rate of ATP formation	Lab + 2Lab +
NADHdia	increases the level of NADH relative to NAD ⁺	Lab = 2Lab +
GAPDH	increase NADH levels and thus ATP levels	Lab = 2Lab -
G6PDH	rate limiting enzyme which regulates the flux through pentose shunt in conjunction with NADP ⁺	Lab + 2Lab =
ICD	one of three rate limiting enzymes regulating the flux through TCA cycle; increases NADH levels	Lab + 2Lab =
XDH	increases level of uric acid which is a non-enzymatic ROS scavenger	Lab = 2Lab +
NADHdia	increases NADPH levels and decreases NADP ⁺ levels. An increased activity would inhibit G6PDH activity and thus inhibit flux through pentose shunt. A decreased activity would release the NADPH dependent inhibition of G6PDH and thus increase the flux through pentose shunt	Lab - 2Lab -

Notes: +, -, or = indicates alteration of mean specific activity relative to that of Rab

in influencing life span in *Drosophila* by other investigations (17, 18). We conclude that the Lab strains have extended their longevity by utilizing a metabolic strategy based on the significant up-regulation of the ADS system (particularly CuZnSOD and MnSOD in the case of the La strains(8) or catalase in the case of the Lb strains (9)), coupled with the significant up-regulation of the pentose shunt in both cases.

Since the La animal is known to have the same mean daily metabolic rate (based on direct measurement of oxygen consumption) as does the Ra animal (19, 20), then the extra energy needed for this improved somatic maintenance must be obtained at the expense of some other variable. The strategy appears to have deleterious effects on reproduction, the daily fecundity of the La females being significantly reduced and delayed in early adult life (see Fig. 8 of ref. 21). (However, the reader is advised to see ref. 22 for an updated re-analysis of these and related unpublished data, and the additional conclusions to be drawn from it.) Mathematical modeling of the Ra strain's metabolic energy expenditures on reproduction and somatic maintenance suggest that decreasing the energy spent on egg production would significantly increase life span (12). In the case of the La female, increasing the flux through the

pentose shunt and using the reduced NADPH generated therein to oxidize the ADS enzymes instead of being used to generate more ATP, represents an increase in the amount of energy being used for somatic maintenance. Given the fact that the mean daily metabolic rate of the La animal is identical to that of the Ra animal, then this increased energy flux devoted to ADS operation can only be obtained by diverting it from some other category of bodily energy expenditure. Taken together, the evidence presented here supports the idea that the metabolic enzyme changes we observe in the Lab strains reflect a shift of energy from reproduction to somatic maintenance.

This strategy supports an improved somatic maintenance as evidenced by the decreased levels of oxidative damage and the concomitant delayed onset of senescence (8). A similar hypothesis was used to explain the extended longevity of virgin female medflies (23). The 2Lab strains do not appear to have an increased flux through the pentose shunt. In fact, they are likely to have a significantly reduced flux through this pathway and apparently depend on an increased flux through the glycolytic and TCA cycle. There is no indication that these strains have an increased NADPH production from this source. The 2Lab strains do have a significant increase in their XDH activity. This enzyme produces uric acid which can function as a non-enzymatic ROS scavenger. Presumably these may function as a NADP⁺ reducing system for the elevated ADS genes presumably involved in these strains, in much the same manner as ascorbic acid is known to function in the reduction of oxidized glutathione or Vitamin E (24). We conclude that the 2Lab strains have extended their longevity by utiliz-

ing a metabolic strategy based on the significant up-regulation of the ADS system as evidenced by their significant resistance to oxidative stress (25) coupled with a significant increase in uric acid to function as the required NADP⁺ reducing system.

Both the Lab and 2Lab strategies are consistent with a shift of energy metabolism from the optimal energy allocations observed in the Ra strain balance of reproduction vs somatic maintenance (12) to a new energy allocation altered in favor of increased somatic maintenance (via high ADS) and decreased reproduction (21, Fig. 8). A schematic outline of this energy shift is shown in Figure 3.

Reverted Longevity Strains:

The RevLab strains show a significant decrease in activity, relative to their Lab progenitors, of those enzymes probably involved in up-regulation of the pentose shunt and/or glycolysis (Tables 3 and 5). We have shown elsewhere that the reverse selection protocol selectively reversed the enhanced ADS activities characteristic of the Lab strains (8,9). If the ADS activities were the main target of the reversed selection protocol, then a correlated response would have been the down-regulation of the NADP⁺ reducing systems required for the efficient operation of the ADS. We conclude that the RevLab strains have reduced their longevity by down-regulating their ADS activities and the supporting NADP⁺ reducing systems. In addition, the lower G3PDH levels, usually very high in insect flight muscle, suggest that the RevLab strains might have a reduced ability to use glycolytically derived NADH to drive oxidative respiration in the mitochondria. If so, their flight ability should be impaired

Selection for Metabolically Efficient Energy carriers is a Necessary Precondition for the Expression of the Extended Longevity Phenotype: A Possible Scenario

Figure 3: A diagram of the supposed interactions connecting the observed metabolic alterations with the expression of the extended longevity phenotypes. The premise of the scheme is that selection for increased ADS enzyme activity does not take place in an autonomous manner but rather requires the simultaneous selection of alleles which can alter the organism's metabolism in such a manner so as to supply the cofactors necessary to initiate and maintain high enzyme activities. The figure depicts one such set of probable interactions. See text as well as reference 22 for details.

relative to that of the Lab strains which are known to have significantly enhanced flight duration (26).

Our observations are consistent with the RevLab strains undergoing a selection-induced shift of energy metabolism favoring somatic maintenance in their Lab progenitor strains to one favoring growth and reproduction as in the Rab strains.

Normal-lived Strains:

The normal-lived Mab strains are kept under selective pressure for a normal life span as described above. Although both the Mab and Rab strains are reproduced at a mean age of 15 days, the particular selection protocol used for the Mab strains has yielded a significantly shorter life span than does the deliberately non-selective regime used for the Rab strains (Figure 1). Their enzyme pattern is also altered (Table 3). Relative to the Rab strain, the Mab animals are likely to generate less NADPH and are more likely to rely heavily on the glycolytic-TCA pathway for energy (see Tables 3 and 5). The lowered NADPH levels are likely to lead to a consequent down-regulation of their ADS genes relative to the Rab animal. Their up-regulated XDH activity is known to be NAD⁺ dependent and can be interpreted as a strategy of upholding some somatic maintenance in the absence of extensive ADS up-regulation. We also conclude that the wrong choice of a control strain, such as choosing the M type over the R type, may account for the discrepancies reported in various experiments (e.g., 27)

Short-lived Strains:

The 2Eab strains have been selected for short life and have a significantly shortened longevity relative to the Rab strain (Kaplan Meier survival analysis, log rank test = 9.22, df = 1, P = 0.0024). They show only decreases in their metabolic enzyme activity profile (Table 2). There is no indication that they are enhancing their basic glycolysis-TCA based metabolism in any way. There is no indication they are doing anything extraordinary to enhance their somatic maintenance in any way.

Stress-resistant Strains:

The PQR strain is known to have an increased P450 activity coupled with decreased ADS activities (11). It has purchased its ability to detoxify dangerous compounds at the cost of preventing oxidative damage. Their up-regulated XDH activity is known to be NAD⁺ dependent and can be interpreted as a strategy of upholding some somatic maintenance in the absence of extensive ADS up-regulation. The increases seen in the other five enzymes are consistent with an elevated flux through the glycolysis-TCA pathway but not through the pentose shunt.

Data From Other Strains:

Mocket et al (27) used highly inbred lines derived from sister strains to those discussed herein and concluded that those flies expressed no increased ADS activities although they lived long. We suggest that the inbreeding procedure drastically changed the genetic compo-

sition of their flies relative to ours, which have been deliberately kept as a mildly outbred population. We have empirical evidence showing that inbreeding often eliminates the paraquat resistance of inbred long-lived strains (Arking and Hwangbo, in preparation). In addition, mathematical modeling has demonstrated that our Ra strain has the optimal energy allocations predicted by theory (12). We interpret this fact as indicating that highly inbred animals may not be the best experimental models for every experiment. Altering the genetic composition of the animals via inbreeding may well alter the genome to as great an extent as does selection (Table 1), and the sampling problem inherent to inbreeding may be responsible for the difference in results. We do not dispute the data of Mockett et al (27) but we do believe that their animals and ours are no longer directly comparable. We are able to explain the existence of their apparently paradoxical results (22 and in preparation). We also believe that the appropriate comparison for a long-lived animal is not the short-lived animal but rather an optimally balanced normal-lived strain (6, 12).

The B and O strains of Rose (5) were selected in the same manner as ours but used a different progenitor population. The mechanisms apparently responsible for the extended longevity in these strains are different from the ones we identify in our strains. There is no contradiction in the data. Different genetic inputs from the two different progenitor strains could probably account for the different outcomes. There is more than one way to make a fly live long, as our own La and Lb strains demonstrate. But both the O strains and the L strains apparently use these different mechanism in the context of shifting energy utilization in some way from reproduction to somatic maintenance (28). At a deep biological level, these different strains are using different tactics to pursue the same fundamental strategy.

CONCLUSION

Our biomarker data (29) suggested that the earliest determining event in the expression of the extended longevity phenotype took place early in adult life at about 5-7 days of age. Other data (7-9) documented the specific up-regulation of the ADS genes and enzymes at this age. We assayed the activity of 17 metabolically important enzymes in 5-7 day old flies of 13 strains variously selected for different longevities. We conclude that the two sets of replicated long-lived strains have an altered metabolic pattern (relative to normal-lived animals) which is consistent with an increased flux through the pentose shunt and an enhanced NADP⁺ reducing system to support the increased activity of the ADS enzymes. This result can be interpreted as a shift of energy expenditure from reproduction to somatic maintenance. We conclude that theories based on differential energy allocations, such as the disposable soma theory of Kirkwood (2), appear to empirically explain, at least in part, the mechanisms underlying the transformation of a normal longevity phenotype to an extended longevity phenotype. A more detailed analysis of how these transformations may take place is

presented elsewhere (22).

EXPERIMENTAL PROCEDURES

Strains

Strains were raised as previously described (6). The results of our initial selection for long-lived (La and Lb), normal-lived (Ra and Rb, Ma and Mb) and short-lived (Ea and Eb) strains were previously reported (6). A replicate selection for long-lived (2La and 2Lb) and short-lived (2Ea and 2Eb) strains was done about one year and five years later respectively. The 2La and 2Lb strain longevity data have been reported (8, 9). All of these strains were derived from the Ra or Rb strain by a direct selection for delayed female fecundity as described (6). The Ra strain was later used as the progenitor for a direct selection of paraquat resistance (PQR strain) and these data have been reported elsewhere (11). Finally, the La and Lb strains were used as the progenitors of strains reverse selected for early female fecundity (RevLa and RevLb strains), and these data have been presented elsewhere (8, 9). The following strains are reported in this communication: La, Lb, 2La and 2Lb long-lived strains; Ra, Rb, Ma and Mb normal-lived strains; 2Ea and 2Eb short-lived strains; RevLa and RevLb reverse-selected normal-lived strains; and a paraquat-resistant PQ R strain. The Ma and Mb strains are maintained at a normal longevity by selection for reproduction at 15 days of adult life. In contrast, the Ra and Rb strains are maintained in a non-selective environment by breeding them at an adult age between 4 and 30 days which is chosen by a random number generator for each generation. Neither the Ma and Mb nor the 2Ea and 2Eb longevity or enzyme data have been presented elsewhere. This is the first integrated analysis of the entire database of the 13 strains.

Enzyme Assays:

For determination of the 17 metabolic enzymes listed in Table 1, 5-7 day old flies were collected simultaneously from all strains and processed as a group. Animals were transferred to clean empty bottles, chilled on ice for several minutes and 80 mixed sex adults (1:1 male:female ratio) were placed in a tared microcentrifuge tube, weighed to within 0.1 mg and a homogenate done by hand using plastic pestles and phosphate-buffered saline with 0.01M 2-mercaptoethanol at a volume of 2 ul/mg wet weight of flies. The homogenate was flash frozen at -70° C. All homogenates were simultaneously thawed and centrifuged at 12,000 rpm for 15 minutes at 4° C. The supernatants were removed, avoiding the lipid surface layer, and aliquoted to Teriyaki plates and stored under liquid N₂ vapor before use. Plates were thawed and kept on ice during handling and were not refrozen. After thawing, the enzyme activity in the extracts was assayed using an adaptation of the procedure for colorimetric assay of the enzyme activity in isozyme electrophoresis. The 2 ml reaction mix contained 8 mg MTT (dimethylthiazole), 10 mg NAD or NADP (as required), 5-10 mg substrate, 50 ul of a 1 mg/ml solution of Meldola Blue (6-dimethylamino-2,3-benzophenoxazine),

50 ul of 1M MgCl₂ (as required), and specific substrate reagents for each reaction as specified by (13). All reagents were from Sigma, and the reaction mix was prepared fresh. The thawed extracts were further diluted 1:20 in phosphate buffered saline and kept on ice. Reaction mixtures were aliquoted to 96 well plates at 100 ul per well and 20 ul of homogenate was added in triplicate, mixed and allowed to react at room temperature for a time previously determined to incorporate the linear portion of the enzyme activity curve. Reactions were terminated by the addition of 100 ul acidic isopropanol containing 1% (v/v) concentrated HCl. Samples were pipetted thoroughly to dissolve the formazan crystals and promptly read on a BioTech Eliza Plate Reader at 595 nm. All readings were blanked against wells containing everything except the specific substrate. The protein content of each sample was determined using the dye binding assay (kit # 500-0002, BioRad Labs, Richmond, CA). The data from the three replicates of each sample was tallied and enzyme activity expressed as the mean (± std. dev.) absorbance units/ug protein/minute. For logistical reasons, animals from the RevLa and RevLb strains were combined (1:1) for the enzyme activity assays; thus the RevL data presented here represents the mean of the two strains.

Electrophoresis and Staining of Enzymes:

Frozen extracts were thawed, mixed, placed on cold packs and 2ul samples were loaded onto Electrophoresis Systems™ Corning Universal[®] precast gel which is a 1 mm thick 1% Agarose and 1.3% Barbitol buffer gel (Ciba Corning Diagnostics Corp., Alameda, CA 94502 USA) specially made for isoenzyme analysis. Gels were loaded into Corning Electrophoretic Cell Covers with cold water jackets to keep gel temperatures low during run time in order to prevent denaturation, placed on the cell base with reservoirs filled with 80 ml cold Authentikit™ SAB 8.6 buffer containing sodium barbitol, barbitol, NaCl, EDTA and sucrose octaacetate. Gels were electrophoresed for 20-45 minutes, empirically determined depending on the enzyme being tested, at 160V using an E-C Apparatus Corp. power supply. After electrophoresis, gels were rinsed briefly in distilled water before being stained for specific enzymes. Staining solutions were prepared according to outlines provided by Innovative Chemistry, using the reaction mix buffer solution. Most of the enzymes tested utilized a 2 ml reaction mix containing the following basic components: 8 mg MTT, 10 mg NAD or NADP as required, 5-10 mg substrate, 50 ul of a 1 mg/ml solution of Meldola Blue (6-dimethylamino-2,3-benzophenoxazine), 1 mg PMS (phenazine methosulfate), 50 ul of 1M MgCl₂ as required, and specific substrate reagents for each reaction as specified by Harris and Hopkinson (1978). All reaction mixes were prepared fresh just prior to staining of gels and were added to the gel surface uniformly and allowed to incubate at either room temperature or 37°C, depending on the enzyme, until specific enzyme bands appeared. Gels were washed for 30 minutes in distilled water with three changes of rinse solution and dried in the dark overnight at room temperature.

Enzyme Abbreviations:

The 22 enzymes assayed and the abbreviations used to refer to them are as follows: ADH, alcohol dehydrogenase; AK, adenylate kinase; CAT, catalase; EstD, esterase-D; GDH, glucose dehydrogenase; G6PD, glucose-6-phosphate dehydrogenase; GPI, glucose phosphate isomerase; GAPDH, glyceraldehyde phosphate dehydrogenase; GPD, glycerol-3-phosphate dehydrogenase; GR, glutathione reductase; PNP, purine nucleotide phosphorylase; HK, hexokinase; ICD, isocitrate dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; ME, malic enzyme; NADHdia, NADH diaphorase; NADPHdia, NADPH diaphorase; PGM, phosphoglucomutase; PGD, phosphogluconate dehydrogenase; SOD, CuZn superoxide dismutase; and XDH, xanthine dehydrogenase.

Statistical Analysis:

The statistical package SPSS (v. 7) was used for all calculations. We performed multivariate analysis and a post hoc multiple comparison of means using a Bonferroni correction as described. Standard statistical procedures and precautions were followed (14).

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