Molecular basis of extended longevity in selected *Drosophila* strains

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We review the data which led us to conclude that the antioxidant defense system (ADS) is responsible for the extended longevity of our La strain of *Drosophila melanogaster*. A synthetic wild caught strain was initially subjected to indirect selection for extended longevity. The response was rapid, yielding ca. 40% increases in mean and maximum lifespan by the F22 generation. Theory testing showed that the rate of living theory as classically described did not apply to our animals. So we used biomarker analysis to identify the critical age at which the extended longevity phenotype is first expressed; the initial delay in the onset of senescence takes place early, at day 5 of adult life. The first biomarker differentially affected in the long lived and control strains was paraquat resistance. This suggests that resistance to oxidative stress might be an operative mechanism. Paraquat resistance was found to be the only biomarker robustly associated with extended longevity in a number of different strains. Measurement of the ADS gene mRNA prevalence showed that the long-lived strains show a differential up-regulation of several ADS genes beginning at day 5 of adult life. These changes in mRNA prevalence are accompanied by significant increases in ADS enzyme activities. This differential gene expression continues throughout the lifespan. Long-lived animals subjected to reverse selection for longevity show corresponding decreases to control levels in their ADS gene expression patterns. These data suggest that the ADS genes probably play an important and possibly causal role in the expression of extended longevity. Regulatory mutants which specifically alter ADS gene expression also alter longevity. Possible interactions of the ADS with the organism's other defense systems are discussed.

IN 1985, Francois Jacob asked why, when organisms possessed a marvelously complex process which reliably guided their development from a single cell to a functional adult, the adult forms should not be able to simply maintain the structures they had so laboriously constructed but instead underwent the degenerative processes of aging and senescence[1]. The work done in response to this 'simple' question has progressed to the point where we may now see the outlines of the answer: Organisms age because there is no evolutionary reason for them to maintain their stress defense and repair mechanisms once their reproductive period is nearly over[2]. But knowing the ultimate cause of aging does not necessarily address the proximate causes[3], and it is towards the 'how' of aging that we shall devote the remainder of our attention.

Many laboratory manipulations can lead to significant lifespan extension[4,5]. All of these extended longevity phenotypes are associated with an increased resistance to various environmental stresses. Parsons[6] pointed out that evolutionary considerations predict that selection for stress resistance should always be accompanied by changes in longevity. Evolutionary theory predicts the existence of multiple proximal aging mechanisms[7], yet it is clear that all of the laboratory models described to date involve some form of stress resistance. The variation resides in the type of stress resistance mechanism employed by each of these different model systems.

In this review of the molecular biology of aging mechanisms in our selected strains of *Drosophila melanogaster*, we will first review the data which has led us to regard the antioxidant defense mechanisms as being responsible for the extended longevity characteristic of our long-lived strains. Independently derived long-lived strains generated by other laboratories probably rely on other mechanisms[8]. We will then discuss some more recent information which delineates the interactions of the antioxidant defense system (ADS) with other aspects of the animal's phenotype.

Selection

We decided to use artificial selection to generate long-lived strains of flies, with the eventual goal of comparing them to their normal-lived progenitor strains in order to deduce which processes had been significantly altered and, therefore, might play an important role in the expression of the extended longevity phenotype. We chose to use freshly caught wild strains, not inbred laboratory strains, as the progenitors of our long-lived strains. As a matter of convenience, our selection regime was based on a direct selection for delayed female fecundity coupled with an indirect selection for extended

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longevity[9]. Selection was rapidly successful, significant increases in longevity being noted by the F9 and 40–50% increases in both mean and maximum lifespan observed by the F22 (refs 9, 10). It should be noted that the normal-lived progenitor R strains were maintained under conditions such that they were not subjected to selection[9]; thus their lifespan has remained more or less constant[11]. It is our practice to compare the long-lived L strains only to the normal-lived R strains. Other laboratories compare the L strains to their strains selected for short life. This latter practice is valid only if one assumes that the processes involved in extended longevity are identical to but opposite in sign to those involved in shortened longevity. We have reason to believe that this assumption is not correct; hence we compare only the selected strain to the control strain.

Theory testing and biomarkers

Having generated long-lived strains, we began to use them in comparative studies designed to uncover the important processes involved. This process also allowed us to do critical theory testing, since we could now test the predictions of classic theories of aging in a quantitative manner. It was this motivation which led us to measure the lifetime age-specific metabolic rates of the R and L strains under a variety of ambient temperatures[12]. We concluded that the long life of the L strain was not due to a significantly lowered metabolic rate relative to the R strain, and those experiments led us to conclude that the rate of living theory as classically described did not apply to our animals[12,13]. The ability to critically test theories justified in our minds the efforts we expended in generating the selected strains in the first place.

This result still left us with the task of determining first, if the L strain's extended longevity was due to a slower aging rate, a delayed onset of senescence, or to some other process; and, secondly, what types of mechanisms were actually involved. We chose to answer the first part of the question by measuring the animals' age-specific loss of performance in several unrelated, and therefore, presumably independent processes[14]. We concluded that the L strain animals lived long because of a delayed onset of senescence, and that this delayed onset was tightly coupled to events that began at about day 5–7 of adult life. Furthermore, although we did not know the nature of this event, we had logical grounds to suspect that it probably involved resistance to oxidative stress since the first biomarker which was differentially affected in the two strains was that of paraquat (PQ) resistance. This bipyridyl herbicide generates free radicals in the cell and is used as an index of resistance of oxidative stress[15]. The precision of this conclusion from the biomarker data, which allowed us to identify both the timing and the process, justified the use of the biomarker approach as opposed to a random casting about for plausible causes, most of which might not be actually affected in these flies.

Genetic studies: Chromosome localization

We then wanted to move from a strain-based analysis to a gene-based analysis. The first step in this process was to perform a controlled chromosome replacement study in which we constructed 27 isochromosomal lines generated from the 1st, 2nd and 3rd chromosomes (c1, c2, c3) of the R and L strains. The longevity of each geneotype and sex was measured. The analysis of this and other data led us to the following conclusions[16]. First, the genes essential to the expression of the extended longevity phenotype are located only on the L strain c3 and map somewhere to the left of *ebony* (i.e., on c3L and the proximal portion of c3R). Second, these genes are recessive. Third, there exists a complex pattern of epistasis such that the longevity enhancing genes on c3 are repressed by (unknown) genes on c2, which can themselves be repressed by (unknown) genes on c1. Thus, the c3 genes can be expressed either in the mutual presence of L-type c1 and c2, or in the absence of the L-type c2. The existence of c2 regulatory genes has been verified by Graf and Ayala[17], Bewley and Laurie-Ahlberg[18], and our recent mutant work described below. Fourth, larval density can also modulate the c3 gene expression, the critical period for its application being from 60 to 120 h after egg lying[19,20].

Recent work (Arking, unpublished) has shown that the larval density effect can be removed without any effect on the extended longevity phenotype. Selection on acidic media allowed us to generate constitutive long-lived L lines with phenotypes essentially identical to that of the original inducible lines. This observation suggests that the density effect is not essential for extended longevity. All our recent genetic work is based on these constitutive lines and not on inducible lines.

The regulatory circuits derived from these studies are shown in Figure 1.

Genetic studies: Differential gene expression

Knowing that PQ resistance was the earliest biomarker altered in the L strain suggested that it might play a causal role in the expression of the extended longevity phenotype (ELP)[14]. This supposition was strengthened by the finding that PQ resistance was the only biomarker which was robustly correlated with ELP in each of our several long-lived strains (Figure 2 and Table 1). We interpreted these findings as strongly supporting the existence of a mechanism imparting early adult resistance

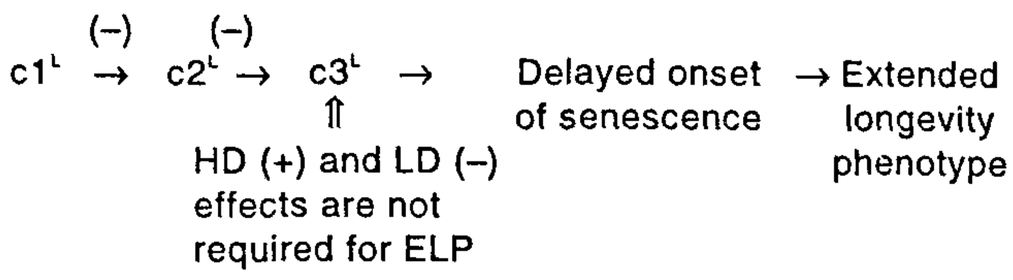

Figure 1. A summary of the chromosomal and environmental interactions involved in the expression of the extended longevity phenotype. Loci on the L-type c1 (c1L) and c2 (c2L) interact with c3L, both positively (c1) and negatively (c2), respectively, such that c1 represses c2, which in turn, represses c3. The recessive genes on c3L are absolutely required for the expression of the delayed onset of senescence and the extended longevity phenotype, both of which may be fully expressed in the mutual presence and mutual absence of c1L and c2L. The positive and negative roles of larval density are indicated by HD (high density) and LD (low density); their roles are now known to be non-essential as indicated in the figure. Based on data presented in refs 16, 20 and 32, as well as the current text.

Table 1. Values of lipid, glycogen, desiccation and paraquat resistance expressed relative to controls*

Strain	Lipid content	Glycogen content	Desiccation resistance	Paraquat resistance
R_A	1.000	1.000	1.000	1.000
$2E_A$	0.939	1.322	0.829	1.062
M_A	0.826	1.289	1.091	1.198
$2L_A$	0.588	1.861	1.064	2.000
L_A	0.667	1.816	1.059	2.176
R_B	1.000	1.000	1.000	1.000
$2E_B$	1.012	1.376	1.089	nd
M_B	0.789	1.281	1.146	1.395
$2L_B$	0.696	2.089	1.306	2.395
L_B	0.682	1.300	1.350	2.549

*The data are taken from Table 7 of reference 24. Each value represents μq lipid or μg glycogen/mg wet weight, or the mean survival time (h) for desiccation or paraquat resistance for each strain expressed relative to the value for the Ra or Rb progenitor strain, as appropriate. Note that the only consistent and significant relative increases associated with extended longevity are those connected with paraquat resistance.

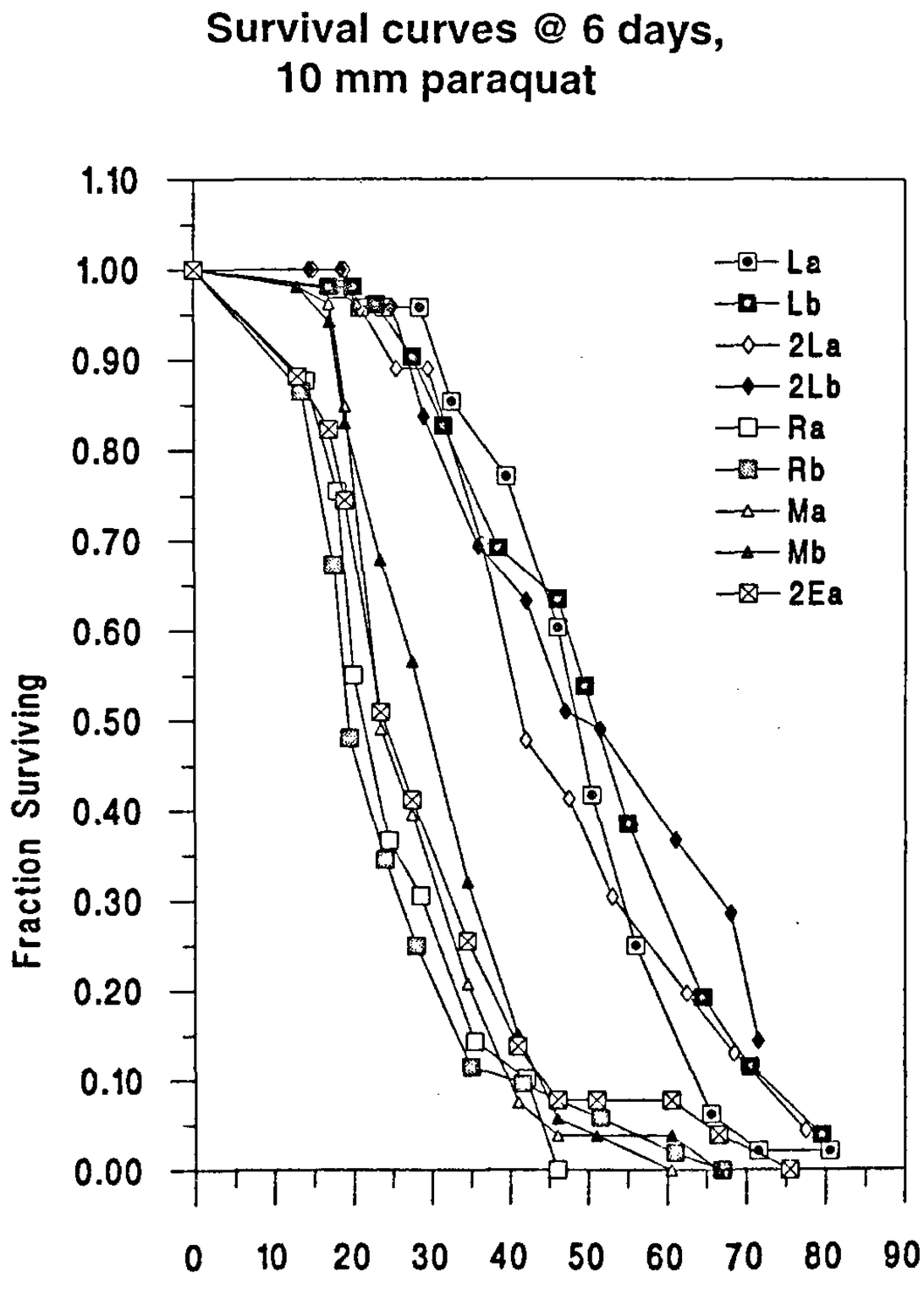

Figure 2. The paraquat resistance of nine different longevity lines, expressed as a survival curve of animals subjected to 10 mM PQ under standard conditions. The data are based on the testing of 50–100 animals per strain. The L/2L long-lived animals have significantly different survival curves than do the shorter-lived R, M, or 2E strains as determined by the Kolgomorov–Smirnov test. The L and 2L strains do not differ from each other, nor do the R, M, and 2E strains differ from one another. The descriptions of the different strains are given in ref. 24, from which this figure is taken with permission.

to oxidative stress as an integral part of the processes leading to the expression of the ELP.

Direct evidence to support these statements was obtained by measurement of the mRNA prevalence of a number of different known antioxidant genes[21]. The data (Figure 3) show that, in the 5-day-old adults, there is a significant increase in the prevalence of mRNA of three of the four ADS genes examined (the GST values are borderline significant) in the L strain relative to the R strain controls. In addition, there are significant relative increases in ADS enzyme activities in the L strain as well. No such significant changes were observed in the expression patterns of non-ADS genes used as controls (Figure 3). Incidentally, all of the ADS genes examined (Figure 3) are located on c3 within the region to the left of *ebony*, as previously specified by our earlier mapping experiments mentioned above.

We have recently examined the patterns of ADS gene expression throughout the adult life span of both our La and Lb long-lived strains[22,23]. These two strains are replicate sister strains, each derived from an aliquot of the original R strain[9]. Two points are quite clear in the data of Figure 4. First, the enhanced expression of ADS genes noted in the early La adult[21] continues throughout adult life such that the CuZnSOD (copper–zinc superoxide dismutase) mRNA levels peak at about day 30 and remain at a substantial excess relative to their Ra progenitor control values. Second, the La and Lb strains have strikingly different patterns of CuZnSOD mRNA prevalence relative to their Rb progenitor control values. The La animals show a peak value of CuZnSOD mRNA in midlife at about 30 days and thereafter, while the Lb animals show a late life peak at 60 days in their CuZnSOD prevalence. In absolute terms, the Lb animals have higher CuZnSOD values than do the La animals,

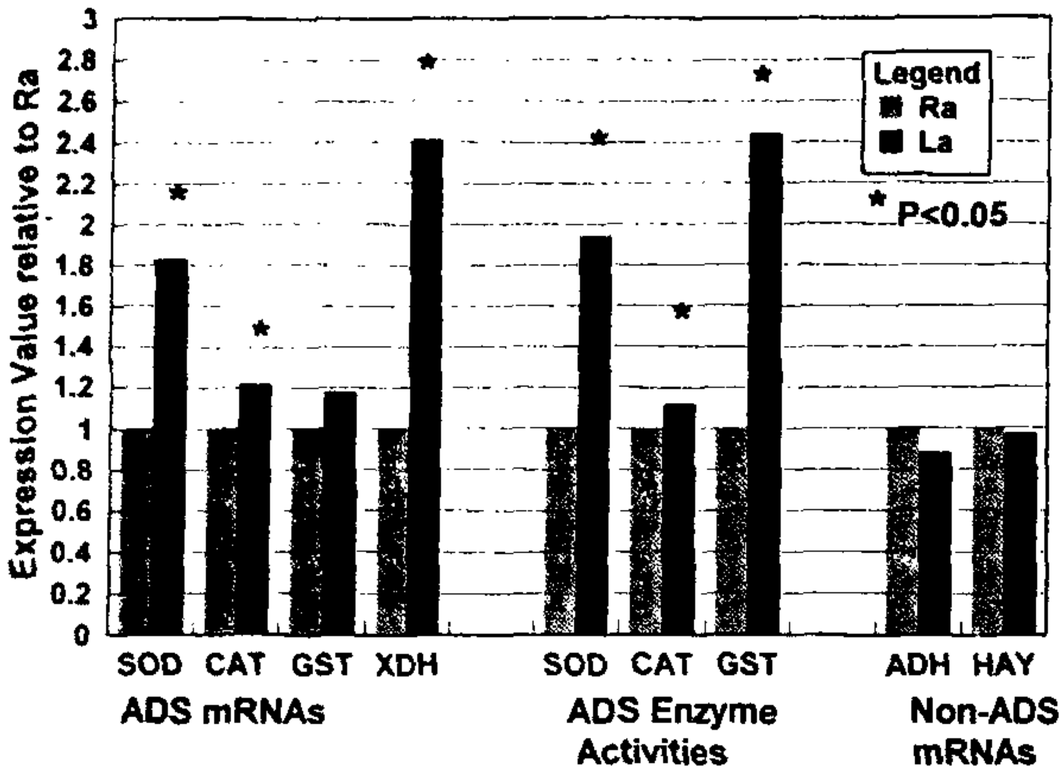

Figure 3. A summary of the data on ADS gene expression in five-day-old Ra and La adults as presented in ref. 21. That age was used since the biomarker data (see text) suggested that the event(s) associated with a delayed onset of senescence began at this time. The CuZnSOD (SOD), catalase (CAT), glutathione-S-transferase (GST), and xanthine dehydrogenase (XDH) are examples of ADS genes; the alcohol dehydrogenase (ADH) and haywire (HAY) are examples of non-ADS genes used as internal controls. The data are expressed as mRNA prevalence units probed RNA/units rRNA, or units enzyme activity/minute/μg protein, as appropriate, for the La relative to the Ra values. Note the difference in expression between the ADS and non-ADS genes.

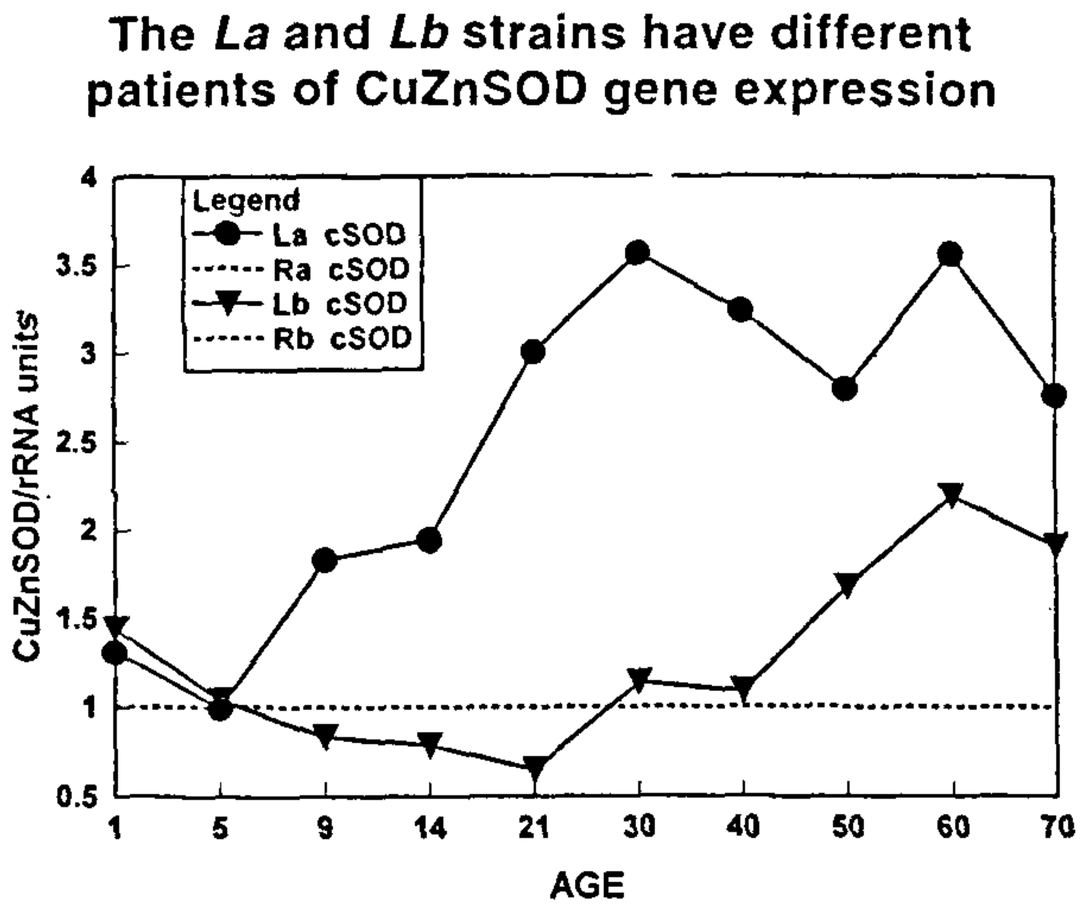

Figure 4. The CuZnSOD mRNA prevalence patterns throughout the adult lifespan of the La and Lb strains are presented relative to the value of their progenitor Ra or Rb strain, respectively. Note the obvious differences between the two sister strains. See text for further discussion. Taken from data presented in refs 22 and 23.

but the difference in the relative data comes about because the Rb animals have substantially higher CuZnSOD prevalence values than do the Ra controls. In retrospect, the three to four generation long adaptation of the original R strain population to laboratory culture was probably sufficient to bring about significant differences in gene frequency between the supposedly 'identical' replicate Ra and Rb strains. The data for catalase and for manganese-SOD follow the same pattern as described above.

This unexpected situation suggests that replicate strains with statistically identical longevity phenotypes may have non-identical molecular phenotypes, a phenomenon we have discussed previously[24]. Hence averaging data across replicates may in fact obscure the very information we are trying to discern. It also suggests that other antioxidant factors may be operative during the early life period of the long-lived Lb strain animals, since these animals have low ADS levels during the early life period when it is in fact robust and resistant to oxidative stress (Figure 2).

No consistent changes in ADS gene expression were observed during the larval or pupal stages, thereby reinforcing the idea that the developmental stages do not have a direct relationship to adult longevity[21].

In addition to this direct evidence of coordinately upregulated strain-specific patterns of ADS gene expression in our long lived strains, we have obtained some

Table 2. Change in ADS and non-ADS levels as a result of forward and reverse selection*

Strain	Mean longevity (days)	Non-ADS enzyme, relative level, day 5	ADS (CuZnSOD) mRNA levels, day 30
R	43.2	1.00	1.000
L	70.2	1.06	3.006
Rev-L	53	0.94	0.466
Mean change		+ 6%, −9%	+200%, −85%

*The data are taken from ref. 23. The non-ADS values represent the mean enzyme activity/μg protein for 16 enzymes associated with carbohydrate metabolism in the 5-day-old L strains (forward selection) or the Rev-L strains (reverse selection), each expressed relative to the value of these enzymes in the progenitor R strains. The ADS mRNA represents the mean CuZnSOD units/rRNA units for each strain at day 30 (age of peak value), again expressed relative to the value of the R strain. ADS, antioxidant defense system.

data indicating that the L strain animals have lower levels of oxidative damage relative to their controls[22,23]. Thus, there seems to be a close correlation between enhanced resistance to oxidative stress, lower levels of oxidative damage, and increased longevity, as predicted by the oxidative stress theory of aging[7,25].

It seemed to us that evidence of a causal, or at least an important, role of the ADS genes in bringing about the expression of the ELP could be obtained via properly constructed selection experiments. Our original selection experiment involved the generation of the L strains from their corresponding R strains and yielded evidence for the involvement of the ADS as noted here. This, of course, constitutes correlative data. We decided to

complete the circle of logic by reverse selecting L strain animals for short life and assaying the resulting shorter-lived Rev-L strain animals for ADS activity. Our prediction was that such animals should have a significantly decreased level of ADS activity. The net result of such forward (R→L) and reverse (L→Rev-L) selection should be large changes in the levels of molecules coded by genes that are causally involved in the expression of longevity in these strains, as compared with modest changes in the levels of molecules coded by genes that are not causally involved in this process. The actual data (Table 2) supports this prediction: a heterogeneous group of 16 enzymes catalysing various steps of the glycolytic and citric acid cycles show a modest 6 to 9% change as a function of selection while the level of CuZnSOD mRNA shows directional changes of an order of magnitude larger[22,23]. We suggest that these data support our contention that the ADS genes and their gene products play an important and probably causal role in the expression of longevity in these strains. In the absence of explicit evidence, these findings cannot be generalized to the species as a whole since selected long-lived strains generated by other laboratories appear to depend on other, non-ADS, mechanisms[8]. This strongly suggests that independently derived strains may have different molecular mechanisms that give rise to the same longevity phenotype[4,5].

Genetic studies: Mutant analysis

We decided to use mutational methods to identify the unknown genes on c2 which regulated the activity of the c3 ADS genes and presumably affected the expression of the ELP (Figure 1). We adopted an indirect approach in which we generated homozygous single-shot P-element mutants on the c2 chromosome of the La strain, exposed them to PQ under our standard conditions, and retained only those mutants which altered the PQ resistance by ±3 standard deviations from the mean values characteristic of the La strain. These extreme mutants were then assayed for their levels of CuZnSOD and catalase activity, and only those which significantly altered the ADS enzyme activity in the direction predicted by the mutant's performance on the PQ test were retained for future study. Thus we did not select for mutants affecting longevity directly but rather we screened directly for mutants affecting resistance to oxidative stress and ADS gene expression levels, and thus with an indirect effect on longevity. These studies are still in progress, but we expect that the four PQ resistant and twelve PQ sensitive mutants we have isolated will prove to be interesting in their own right as well as serving as an entry into the genetic analysis of the system regulating ADS gene expression and thereby longevity. In the meantime, however, it is reasonable to suggest that the existence of these mutants is consistent with the predictions of our earlier chromosome substitution studies summarized in Figure 1, as well as with the independent results of Graf and Ayala[17], and Bewley and Laurie-Ahlberg[18] indicating the existence of c2 mutants regulating the activity of the c3 ADS genes.

Genetic studies: Interactions with other systems

Given all of the above data, we expected that Ra strain animals directly selected for resistance to PQ should have high levels of ADS gene products coupled with a significantly increased mean and maximum life span. We have done this experiment[26]. After 24 generations of selection, the resulting PQ-resistant (PQR) strain animals show a significant four-fold increase in their ability to resist PQ under standard conditions. This PQ resistance is coupled with an increase in their mean longevity but not in their maximum longevity. The existence of this unexpected phenotype meant that our prediction was not verified. It also meant that our understanding of the system was not complete. However, continued investigation revealed important mechanistic differences between the PQR and La strain animals. The enhanced resistance of the PQR strain is not due to an increase in their ADS activities but rather to a significant increase in their cytochrome P450 levels (Table 3). The cytochrome P450 system is a highly conserved gene family which constitutes one of the organisms' main defense against harmful endogenous and exogenous substances. Presumably, it was the application of two different selection regimes for PQ resistance (indirect vs. direct) that permitted the Ra genome to respond in two different ways: direct selection giving rise to PQ resistance based on detoxification of the exogenous stressor molecule; and indirect selection giving rise to PQ resistance based on the ability to detoxify the harmful metabolic byproducts (free radicals) of the exogenous stressor molecule. Each strategy of PQ resistance has its own effect on longevity. In addition, the data in Table 3 also show that there exists a reciprocal interaction between the two systems such that the animal can apparently increase its

Table 3. Reciprocal interactions between cytochrome P450 and ADS*

Strain	Relative ADS levels	Relative P450 levels
La	1.75	0.93
Ra	1.00	1.00
PQR	0.85	2.05

*Data taken from ref. 26. Each value represents the levels in the 5-day-old animal of the ADS level as represented by the CuZnSOD mRNA/rRNA value, or the nM P450 enzyme/mg microsomal protein value; each expressed relative to the value in the Ra strain. ADS, antioxidant defense system.

levels of one protective system only at the expense of the other. The P450 system and the ADS system are reciprocally interconnected, an observation which we intend to pursue vigorously in the hope of deciphering the nature of the linkages involved. Others have also noted the pleiotropic interconnectedness of metabolic systems[22].

When confirmed in detail by our ongoing experiments, this interaction of the two systems may well provide some insight into important evolutionary questions and molecular mechanisms. Many workers have noted that the long-lived flies are more robust and fecund than are their normal lived progenitors[28]. Why then is long life not a common phenotype in the wild? The mutation accumulation and antagonistic pleiotropy concepts are valuable hypotheses which address these questions[2]. Unfortunately, some of the trade-offs suggested by these theories are not fully supported by empirical evidence: a decreased early fecundity seems not to be a constant aspect of long life in all strains; older animals may actually have a lower age-specific mortality and higher life expectancy than younger cohorts; and so forth. We have noted elsewhere that an increased developmental mortality seems to be a common feature of several independently generated long-lived strains[29]. We believe that the lower cytochrome P450 levels in the La animals (Table 3), and consequently a probable lower level of protection against harmful exogenous substances in the food, might well be related to their significantly higher level of developmental mortality – a level sufficiently high to explain the otherwise puzzling greater fitness of the R animals.

Since we first described this system[10] we have deployed a variety of behavioral, physiological, genetic, and molecular tests designed to elucidate answers to the interesting questions regarding longevity extension and gene expression in *Drosophila*. In 1989, we pointed out that the general habit of measuring aging changes as a function of time was not accurate[30]. We shall learn something substantive about the aging process only when we can substitute the operation of the actual physiological mechanisms in place of time. The data presented here suggest that the ADS systems plays a critically important role in this process in our strains, and that its up-regulation is necessary for the expression of the extended longevity phenotype[21]. However, more work needs to be done. For example, the correlations between ADS gene expression and oxidative damage patterns need to be further strengthened. The reality of the process is more complex than our simple hypotheses have so far envisioned. In addition, the complexities of the interactions of the ADS system with other systems such as the P450 are beginning to be deciphered. Although we have much to learn about the operation of these molecular and physiological systems, we believe that we are now somewhat closer to our goal of removing time from the description of aging than we were some years ago.

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