

Aging can be genetically dissected into component processes using long-lived lines of *Caenorhabditis elegans*

(biomarkers/development/reproduction/life-history traits/quantitative genetics)

THOMAS E. JOHNSON

Department of Molecular Biology and Biochemistry, University of California, Irvine, CA 92717

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ABSTRACT The aging process has been dissected by analysis of genetic variants of the nematode *Caenorhabditis elegans*. Long-lived recombinant inbred lines were generated; some of these lines have mean and maximum life spans up to 70% longer than wild type. Longer life results from a slowing of the characteristic exponential increase in mortality rate that is typical of aging populations in all species. The length of developmental periods and the length of the reproductive period are unrelated to increased life span. Lengthened life is due entirely to an increase in postreproductive life span. Development, reproduction, and life span are each under independent genetic control. General motor activity decays linearly with chronological age in all genotypes. The decay in general motor activity is correlated with and a predictor of life span, suggesting that both share at least one common rate-determining component.

The study of metazoan aging has been a complex, often misleading, and largely intractable area for experimental research. Analyses are hampered by the complexity of the aging process in which many molecular, cellular, and organ systems display remarkable changes, many of which are progressive with chronological age (1-3). Distinguishing which of these so-called "biomarkers of aging" are causal and which instead are products of the aging process has been difficult (2) due, in part, to the general lack of experimental methods for manipulating the aging process. Consequently, many hypotheses concerning the primary cause(s) of senescence remain untested or only indirectly tested (4-6).

Since the most widely assayed and relevant consequence of aging is a limited life span, stocks of *Caenorhabditis elegans* have been generated that have significant, easily measured lengthening of maximum life span for use in examining the mechanisms of aging (7, 8).

As a model for the analysis of the aging process, *C. elegans* offers significant advantages, including a 20-day life span, a 3-day life cycle, and an excellent system for genetic analysis (8). This paper describes the use of these genetic variants to separate processes frequently associated with aging.

MATERIALS AND METHODS

Strains, Media, and Analysis of Life Span. Stocks were maintained as described by Brenner (9). Analysis of life span and generation of recombinant inbred (RI) lines have been described (7, 8).

Measurement of Spontaneous Movement. Ten hermaphrodites of each stock were individually maintained in wells of a 24-well microtiter plate containing 0.6 ml of survival medium (7) and were assayed every 2 days throughout life. Spontaneous movement was assayed by transferring an

individual hermaphrodite to a fresh 4-cm plate containing 10 ml of NGM (9) and counting movement cycles for 1 min. Data recorded were forward oscillations (backwardly directed waves), backward oscillations (forwardly directed waves), and changes of direction [omega waves (see ref. 10 for an explanation of nematode behavior)].

Length of Development. Length of embryonic development was determined by measuring the period from the two-cell stage to hatching at 16°C. Ten two-cell stage eggs were dissected out of fecund hermaphrodites of the appropriate genotype and followed until hatch; 16°C was chosen for convenience of assay. The time of molting was determined by monitoring pharyngeal lethargus (11) using a Nikon Microphot equipped with DIC optics. Fifty worms from a larger population were assayed, approximately hourly, throughout the period of larval development. The time point at which fewest worms were pumping was taken as the midpoint for each molt and is reported here. The time of 50% fecundity was similarly determined.

Age-Specific Fecundity. Ten individuals of each genotype were cloned to 24-well CoStar plates in 0.6 ml of survival medium (7) and were transferred every 24 hr to fresh medium. The number of progeny in each well was counted 2 days later. Individuals who died due to internal egg hatch were not included in the analysis (7).

RESULTS

Life Span of RI Lines. The RI lines have been assayed several times for length of life. In typical experiments mean life spans of these RI lines vary 3-fold, ranging from 13.8 days to 37.9 days (Fig. 1A). Maximum life spans are also altered. Compared with the wild-type, N2, strain, shorter (17 days) and up to 63% longer (63 days) maximum life spans are observed. There is an increase in life expectancy at all chronological ages as demonstrated by the strong positive correlations between the mean and the 90th percentile, the 95th percentile, or maximum life span (Fig. 1B). These correlations are not trivial, because a higher mean life span can result either from decreased early life mortality or from an increase in maximum life span.

Analysis of Mortality Rates. Mortality rates of humans (12) and other species increase exponentially with chronological age (13, 14). This general characteristic leads to the rectangular shape of population survival curves, which is often taken as a characteristic of a population undergoing normal aging (13). The shapes of the survival curves of large populations of selected RI lines and of the two parental genotypes vary slightly between lines, but all are rectangular (Fig. 2A and C).

The age-specific mortality rate in the parent stocks and in these RI lines was examined to see if mortality increases

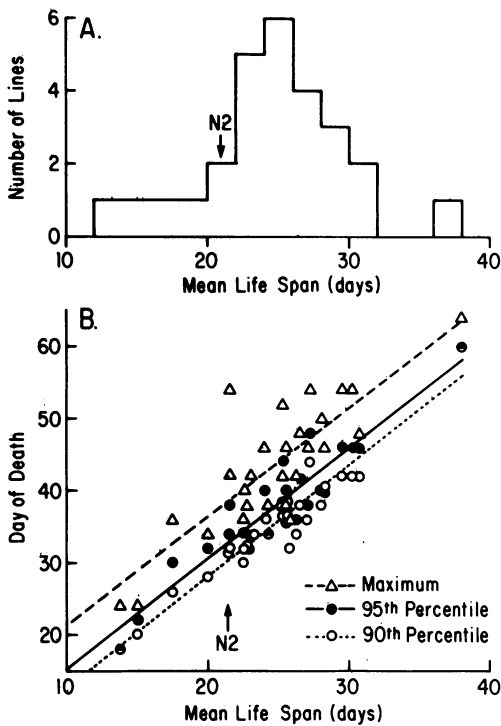


FIG. 1. Life spans of hermaphrodites from RI lines. (A) Mean life spans of 27 RI lines. Data are the average of two survival experiments, each containing 50 nematodes. The entire experiment involved the assay of 2950 nematodes; 2206 died of natural causes. (B) Regression of mean life span (same nematodes described in A) on either maximum life span, the 95th percentile of life span, or the 90th percentile of life span. Mean life span is highly correlated ($P \ll 0.001$) with maximum life span ($r = 0.83$), the 95th percentile of life span ($r = 0.93$), and the 90th percentile of life span ($r = 0.96$).

exponentially with chronological age. The form of the mortality curve is closely approximated by the equation:

$$\mu_x = \mu_0 e^{\alpha x},$$

commonly referred to as the Gompertz equation (13). μ_x , the mortality rate at age x , is a function of the mortality rate at birth, μ_0 (sometimes referred to as the basal mortality rate), and of α , the Gompertz mortality rate, a parameter expressing how fast the logarithm of the mortality rate increases with age (14).

In wild-type and RI strains, the Gompertz mortality rate increases exponentially with increasing chronological age. This is most clearly seen when plotting mortality rate against chronological age on a semilogarithmic scale (Fig. 2 B and D).

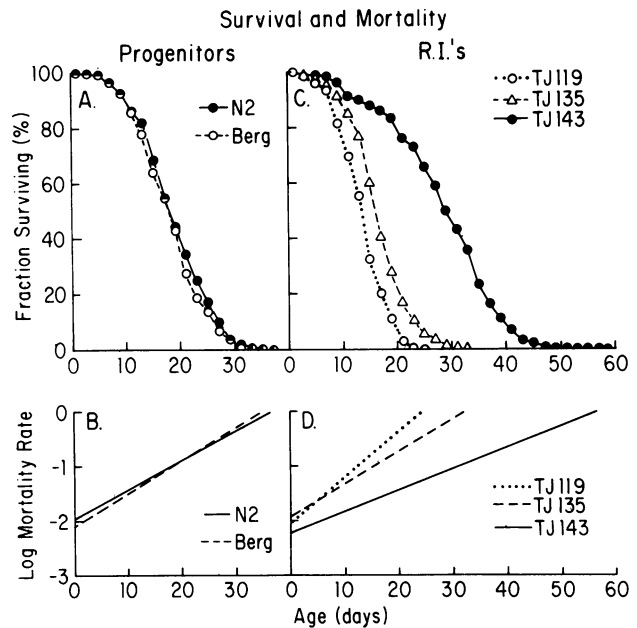


FIG. 2. Survival data (A and C) and mortality rates (B and D) for the parental and three RI lines. (A and B) Survival data obtained as described in Fig. 1 except that each curve is the result of assays on 400 nematodes, 200 in each of two experiments, and that survival assays were performed every 12 hr. Subgroups of the same genotype were tested for consistency (7). One sample of 50 hermaphrodites (TJ143) showed significant differences in survival from the other three TJ143 subgroups and was excluded from this analysis; however, inclusion of that sample does not noticeably affect the results (see Table 1). (B and D) Plots of age-specific mortality rates versus chronological age. Age-specific mortality rates were calculated for each 2-day period throughout life using SPSS subprogram Survival. Lines are weighted regression estimates (SPSS). (A and B) Survival curves and mortality data for the parental stocks N2 and Bergerac BO (Berg). (C and D) Survival curves and mortality data for TJ119, TJ135, and TJ143. TJ143 is one of the longest-lived stocks generated.

Because increased mean life expectancy could result from a lower basal mortality rate (μ_0) or from a slower rate of increase in mortality (α) (or to alterations in both), how these components varied in the RI lines was assessed. Gompertz mortality rate varies between RI lines (Fig. 2 B and D; Table 1). The longest-lived stock TJ143 has a consistent increase in mean and maximum life span (Table 1) and a highly significant decrease (by a factor of 1.5) in Gompertz mortality rate (Table 1). In another stock (TJ119) a significant increase in Gompertz mortality rate was consistently observed along with a shorter mean and maximum life span. No significant change in initial vulnerability was observed.

Table 1. Weighted, age-specific mortality data

Genotype	Mean life span,* days		r^\dagger	Total number	Gompertz parameter, log[probability of death per day]	
	Exp. 1	Exp. 2			Gompertz mortality rate‡	Basal mortality rate
N2	18.1 ± 0.3	20.0 ± 0.7	0.756	328	0.053 ± 0.05	-1.93
Bergerac BO	17.9 ± 0.5	18.6 ± 0.5	0.899	333	0.060 ± 0.03	-2.09
TJ119	12.7 ± 0.4	14.5 ± 0.4	0.812	234	0.083 ± 0.08	-2.05
TJ135	18.1 ± 0.4	15.6 ± 0.4	0.842	356	0.060 ± 0.04	-1.93
TJ142	—	25.5 ± 0.8	0.864	351	0.040 ± 0.04	-2.28
TJ143	29.6 ± 0.7	27.3 ± 0.9§	0.866	317	0.039 ± 0.03	-2.23

Data are also presented in Fig. 2.

*±SEM.

†Correlations are of the logarithm of the mortality rate with age and are highly significant ($P \ll 0.001$).

‡±95% confidence interval.

§Excluding one sample containing 34 worms (see legend to Fig. 2).

The increases in mean and maximum life spans in TJ143 are observed in many repeats, as are the relative changes in life spans of the other RI lines. Nevertheless, small but statistically significant differences in mean life span are observed between measures of the same lines repeated at different times (e.g., Table 1). Such variations are caused by uncontrolled environmental effects (7, 8). This type of environmental effect is common in analysis of quantitative traits (15) and will be explored in detail elsewhere.

Specification of Senescence of General Motor Activity. Older nematodes display the same set of body movements as do newly molted adult hermaphrodites (16, 17). Spontaneous movement, a measure of general motor activity, decreases linearly with chronological age in the wild type, N2 (17). Spontaneous movement was monitored longitudinally on 10 individual hermaphrodites in four selected RI lines and their wild-type progenitors. Spontaneous movement decreased linearly with chronological age in each of the six lines in each of two separate experiments (Fig. 3A).

The x intercept of spontaneous movement was correlated with, and a good predictor of, mean and maximum life span in both experiments (Fig. 3B and C). This could be explained by the presence of a common physiological process specifying the age-related loss of general motor activity and age-specific mortality rate.

Specification of Development. Development of *C. elegans* occurs in two physically distinct phases. If extended life span is specified by genes that also specify length of development, we would expect increases in the length of either or both of these phases. Embryonic development, which consists of a series of rapid determinate cell divisions, cell migration, and cellular differentiation (18), was monitored by isolating two-cell embryos and monitoring these embryos until hatch. Very little variation in the length of the embryonic period was observed (Fig. 4A); the RI lines were all intermediate to N2 and Bergerac BO. Although by statistical criteria N2 had the

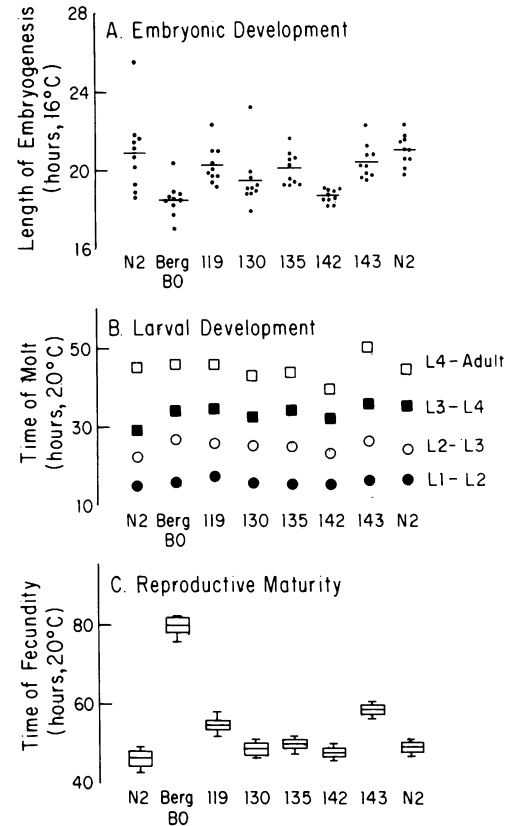


FIG. 4. Duration of development. (A) Length of embryonic development; horizontal lines represent the means. (B) Times of larval molts. (C) Time of 50% fecundity. Shown here are Tukey box plots (19) illustrating the 10th, 25th, 50th, 75th, and 90th percentiles of fecundity.

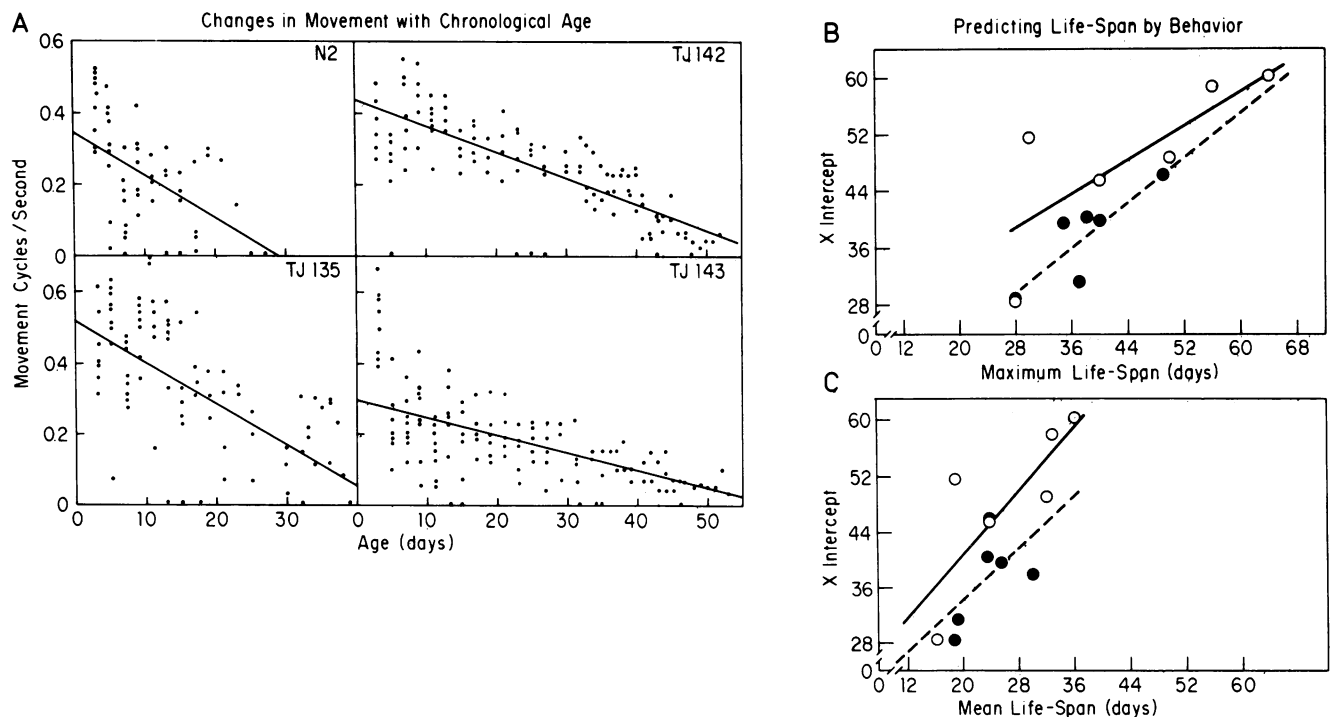


FIG. 3. (A) Spontaneous movement at different chronological ages for the N2 and three representative RI lines: TJ135, TJ142, and TJ143. Hermaphrodites that died prematurely due to internal egg hatch were not used in this compilation (7), but the data differ only slightly if these hermaphrodites are used. Lines are regression lines calculated using SPSS subprogram One-way. (B and C) Regression of the x intercept of age-specific, spontaneous movement on maximum (B) or mean (C) life span. The expected time of x intercept was determined as in A. Between the x intercept and maximum life span, correlations were 0.77 (\circ) and 0.91 (\bullet), significant at the 0.05 and 0.01 levels, respectively. Correlations between the x intercept and mean life span were 0.74 (\circ) and 0.61 (\bullet), both nonsignificant.

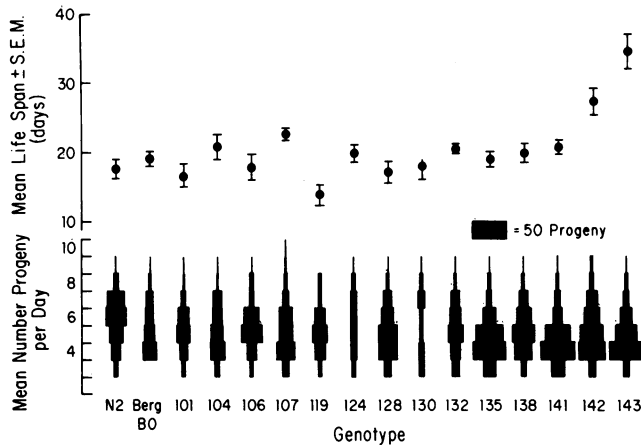


FIG. 5. Number of progeny produced on each day of reproduction, length of the reproductive period, and mean life span.

longest embryonic period, this difference amounted to only 60–90 min over the 20-hr period and may be biologically insignificant.

Larval development, a period of substantial growth that is punctuated by four successive larval molts (20), was followed in a total of 14 RI lines and in the parental stocks. Genetic variation among the RI lines in the rate of larval development was observed but there was no correlation between the length of any larval period and life span (Fig. 4B). The usual end point of development is reproductive maturity. Again there was genetic variation among the RI lines; the time of 50% fecundity for the Bergerac BO parental strain was more than 24 hr later than that of N2, but again there was no correlation between the length of fecundity and life span (Fig. 4C). Thus, the genes specifying length of life do not function to specify the rates at which development occurs.

Specification of Reproductive Senescence. Reproduction in the wild type, N2, begins soon after the fourth and final larval molt, whereas that of Bergerac is delayed more than 24 hr after this molt (Fig. 4B and C). For N2, reproduction reaches a maximum 3 days later and falls off quickly, reaching zero by 9 days of age (Fig. 5).

If the length of the reproductive period is specified by the same genes that specify life span, one might expect that long-lived lines would have longer reproductive periods than wild type and that short-lived lines might have shorter periods. There is no correlation between the length of the reproductive period and life span (Fig. 5). All of the RI lines, except 107, become sterile on day 10. I conclude that the genes specifying length of the reproductive period are independent of those specifying length of life.

DISCUSSION

Interrelationships Between Components of Senescence in *C. elegans*. The data described here, when combined with previous communications and observations, lead to several conclusions about the genetic specification of senescence (Fig. 6). First, senescence does not begin until development is complete. This conclusion is based on the observation that there is a strict correspondence of 1 day of additional life for each day of developmental arrest (21, 22) and the finding that the loci specifying organismic longevity in the RI lines do not specify development rate. At least three processes normally associated with aging in *C. elegans* (mortality, behavioral senescence, and reproductive senescence) are delayed by prolonging development (21). The simplest explanation for this observation is that at least one process, completed by the

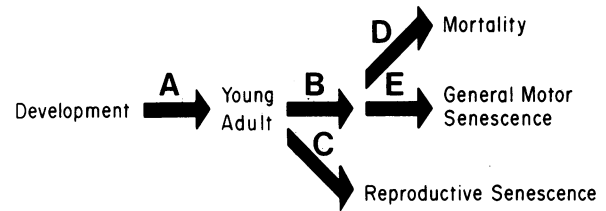


FIG. 6. Diagram describing the order of dependency of events in senescence of *C. elegans*. Arrows indicate dependency relationships.

end of development, is required before the aging process begins.

Second, since general motor activity decays more slowly in long-lived lines than in short-lived lines (Fig. 3A), common events specify the rate of decay of general motor activity and longevity (Fig. 6, arrow B). Third, since the long-lived strains have no change in reproductive life span (Fig. 5), reproductive senescence is specified independently of life span and general motor senescence (Fig. 6, arrow C). Arrows D and E are included in Fig. 6 to account for independent genetic specification of some aspects of general motor activity and organismic longevity (8, 9).

Several other observations are pertinent to the lack of relationship between reproductive senescence and mean life span. First, although significant correlations were observed between age-specific fecundity or total fecundity and length of life, in a larger study of RI lines (N. L. Foltz and T.E.J., unpublished data), there was no correlation between the length of the reproductive period and organismic life span. Second, in a series of studies on single gene mutants that dramatically lengthen life span, it has been observed that the overall length of the reproductive period is not altered, although there is a dramatic change in total fecundity (D. B. Friedman and T.E.J., unpublished data). Third, the life spans of several temperature-sensitive, gonad-defective mutants that completely eliminate progeny production are not significantly different from wild type (8). Finally, it has been generally accepted that sperm number limits fecundity of *C. elegans* hermaphrodites (23).

An earlier study on general motor senescence in *C. elegans* (17) detected no correlations between any of three parameters describing the rate of decline of stimulated movement and age at death. In contrast, it is shown here that the x intercept of age-specific spontaneous movement predicts life expectancy of the RI lines (Fig. 3B and C). These observations are not inconsistent; there are three differences between experiments. First, spontaneous movement was observed here, not stimulated movement as in the earlier study. Second, the assay conditions of this investigation were different from those of Bolanowski *et al.* (17) but were also highly reliable, showing 79% retest reliability. Third, and most importantly, in this study significant correlations were observed, not within single genotypes, confirming the observations of Bolanowski *et al.* (17), but rather among genotypes. Some comparisons between worms within a single genotype did show significant correlations between the x intercept of spontaneous movement and life span, but this was not uniformly observed (M. L. Keller and T.E.J., unpublished data). These findings show that special environmental variance of spontaneous movement [V_{Es} (15)] among individuals of the same genotype is large enough to add an additional component of variance obscuring the genetic covariance between movement and life span among individuals of the same genotype. Studies comparing populations of genetically identical individuals, such as those presented here, have the advantage shared by all studies on RI lines: this component of variation is eliminated (24).

Rationale for Studying Senescence in Long-Lived Strains. Many experimental methods have been used in an attempt to dissect the aging process by altering life span and thereby, presumably, altering the rate of aging. Methods have included caloric restriction, treatments with drugs of known and inferred action (4–6), comparative studies between related species (1, 2, 6), and the isolation of genetic stocks with altered life spans (25–29).

Shorter life span, typical of most mutant strains in sexually outcrossing species, stems from metabolic and developmental changes that result from typical mutational events (8, 26, 29) as well as from inbreeding depression (26, 30), which is an almost inevitable consequence of mutant isolation, where recessive mutations must be detected in the homozygous state. In contrast, *C. elegans* does not display inbreeding depression for life span (7, 8) or other life-history traits (unpublished observations). This fact may result from the self-fertilizing mode of hermaphrodite reproduction in *C. elegans*, which leads to completely inbred strains, even in the wild (7).

Some authors have isolated and studied short-lived genotypes in an effort to gain an understanding of the aging process (26, 29, 31). However, a defect in any organ or cellular system might lead to a premature death that would be completely unrelated to the mechanisms normally responsible for aging. This same criticism has been made (29) of attempts to directly study “normal” human aging by studying genetically determined progeroid syndromes leading to premature senescence.

In contrast, genetic variants having longer life have altered those processes that limit the normal life span. Indeed, if we accept the premise that organismic senescence results from metabolic and physiological processes that enfeeble the homeostatic mechanisms of the organism and that death results from the inability of one or more metabolic or physiological systems to maintain homeostasis as a result of organismic senescence, then any protocol that lengthens life must somehow alter those rate-determining processes that specify senescence in the normal individual. Only two other instances of an increase in maximum life span, both in *Drosophila*, have been reported (27, 28).

Criteria for Establishing the Existence of a Process Specifying Aging. There may be potential problems in using life-span extension as the sole criterion for change in the basic aging rate. In the absence of a better understanding of the aging process, however, appropriate criteria for detecting such a change remain elusive. One way of establishing such criteria is by demonstrating that manipulations that prolong life also slow processes displaying age-related functional changes, biomarkers of aging (32). Identified here is a physiological system, general motor ability, in which the time of complete loss of functional ability covaries with life span (Fig. 3 B and C). This could mean that life span and general motor ability are specified by a common process or processes. (These might be termed aging?) By examining other behavioral, physiological, and molecular processes in genetic lines that have different life expectancies, one can ascertain whether the potential marker of biological age is specified coordinately with life span. Though such correlations cannot prove causality, a lack of correlation is telling evidence that the molecular process under study is not itself a determinant of length of life.

This approach—the demonstration of functional linkage between two processes—confirms the existence of dependence relationships. Further analyses of this system can serve to link other events to this putative “aging process” and to identify events that are unlinked. This sort of bootstrap approach in which genetic, morphological, molecular, and physiological analyses are simultaneously applied to a bio-

logical system has been quite successful in the genetic analysis of other biological processes (33).

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