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# Biophysical and biological meanings of healthspan from *C. elegans* cohort

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ABSTRACT

Lifespan among individuals ranges widely in organisms from yeast to mammals, even in an isogenic cohort born in a nearly uniform environment. Needless to say, genetic and environmental factors are essential for aging and lifespan, but in addition, a third factor or the existence of a stochastic element must be reflected in aging and lifespan. An essential point is that lifespan or aging is an unpredictable phenomenon. The present study focuses on elucidating the biophysical and biological meanings of healthspan that latently indwells a stochastic nature. To perform this purpose, the nematode *Caenorhabditis elegans* served as a model animal. *C. elegans* fed a healthy food had an extended healthspan as compared to those fed a conventional diet. Then, utilizing this phenomenon, we clarified a mechanism of healthspan extension by measuring the single-worm ATP and estimating the ATP noise (or the variability of the ATP content) among individual worms and by quantitatively analyzing biodemographic data with the lifespan equation that was derived from a fluctuation theory.



## 1. Introduction

Despite an isogenic cohort with the same birth and a nearly uniform environment, the lifespan among individuals varies widely, as shown in model organisms from yeast to mammals [1–6]. Needless to say, the genetic factors as well as the environmental factors are essential for aging and lifespan; however, the above fact implies that a third factor, or the existence of stochastic element, must be reflected in lifespan or aging [7–10]. Thus, to analyze demographic data precisely and conveniently, the author has derived the lifespan equation from a fluctuation theory based on the third factor [10–14]. Also, the author maintains that aging biologically or physically holds the process of individuation which is one of the basic ideas in analytical psychology [15].

An essential point is that lifespan or aging is an unpredictable phenomenon. Indeed, the research regarding the predictors of lifespan has shown that lifespan could be predicted only in part [16–19].

With the background above, the present study focuses on elucidating the biological meaning of healthspan. For this purpose, the nematode *Caenorhabditis elegans* served as a model animal, for high analytical precision would be required in this research. According to the author's experiences, *daf-16* mutants were especially chosen as an experimental material because the biodemographic curves of this mutant strain could be fitted well with a single component when using the lifespan equation [11,12,14]. The variability, or noise, of the ATP content and size was given as the coefficient of variation among individuals, i.e., the standard deviation divided by the mean.

Usually, researchers using *C. elegans* culture use the *Escherichia coli* strain OP50 as a food source. However, this bacterial strain is pathogenic and shortens *C. elegans'* lifespan as compared with other bacterial strains [20,21]. Likewise, such longevity was also realized in the case of *C. elegans'* fed UV-killed *E. coli* OP50 as a healthy food [11,22,23]. In this work, we considered the biophysical and biological meanings of healthspan by quantitatively analyzing a mechanism of healthspan extension with the lifespan equation, using worms fed healthy food.

## 2. Materials and methods

### 2.1. C. elegans strains, culture conditions, and lifespan assays

In the present work, *daf-16(mu86)* mutants in *C. elegans* were used, maintained, and manipulated under standard conditions [24]. The nematode strain and the *E. coli* strain OP50 used in this work were provided by the Caenorhabditis Genetics Center. Synchronized animals were prepared as described previously [12]. The *daf-16* mutants were continuously cultured at 25 °C after being hatched at 20 °C. During egg laying, parental hermaphrodite

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animals were transferred to new plates every day at 25 °C to prevent contamination of progeny. The age denotes the time after hatching (0 = the time of hatching). Statistical analysis was conducted and figures were illustrated using KaleidaGraph software (version 4.1, Hulinks Inc., Japan). Error bars represent the standard error of the mean (SEM), unless otherwise noted. $p$ values were calculated using the unpaired Student's $t$-test.

### 2.2. Single-worm ATP and size measurements

The entire ATP content in a single worm was measured by using luciferase-mediated bioluminescence, as written previously [14]. To estimate the ATP noise, the number of worms used in each point was 10–14. The individual worms were randomly selected to exclude the experimenters' arbitrariness. The noise level of inevitable system error in measurements, or the measurement error, was 0.05 ($n$ = 10), which was obtained from the same sample.

To investigate the influence of the ATP concentration rather than the ATP molecules as a noise effect, the length of individual worms was measured. Usually, a volume is proportional to the length raised to the 3rd power. The length of *C. elegans* from larva to adult highly correlated with the volume of *C. elegans* because of the geometric similarity of the body formation [25]. The relationship between volume (nl) and length (μm) is well fitted by the following expression: volume = $1.09 + 1.11 \times 10^{-9} \times \text{length}^3$ and correlation coefficient ($r$) = 0.951. The noise level of the measurement error for the length was 0.02 ($n$ = 10), which was obtained from the same sample.

### 2.3. Culture of C. elegans on the E. coli OP50 strain killed by UV irradiation

As described in our previous paper [11], bacteria killed by UV irradiation were obtained by controlling their exposure to UV light (254 nm). After hatching, synchronized L1 larvae in a bacteria-free solution were spread out on the UV-treated bacterial lawn on NGM plates. To continuously grow *C. elegans* on bacteria killed by UV, this treatment was repeated daily from the young adulthood of *C. elegans* until the measurement of each strain was nearly ended.

### 2.4. The lifespan equation derived from the fluctuation theory

The fraction survival to age $x$ is given by $l_x = 100(N_x/N_0)\%$ as the percent of survival, where $N_0$ and $N_x$ are the initially set population of animals and the number of animals alive at age $x$, respectively. As a practical matter in the analysis of *C. elegans* data on aging only, note that $N_0$ means the finally scored number that subtracts the number of abnormal deaths from the initially set population. We derived the lifespan equation from the stochastic fluctuation theory as described in a previous paper [10].

First, as the simplest case in which individuals are genetically and environmentally homogeneous and compose a cohort, we derived the following solution:

$$l_x = l_0(x,t_0) + (100 - l_0(x,t_0))e^{-(x-t_0)^2/z^2} \text{ and}$$
$$l_0(x,t_0) = \begin{cases} 100\% & \text{at } x < t_0 \\ 0\% & \text{at } x \geqslant t_0, \end{cases} \quad (1)$$

where $z^2 = 4Dt_0$. $D$ and $t_0$ represent the fluctuation constant and the onset of biodemographic aging, respectively. In that time, we assumed from theoretical necessity that animals do not die until $x = t_0$ in the diffusion equation. The $t_0$ is statistically determined by fitting experimental points with Eq. (1). Here, note that it is a measure for a population, but the measure is not determined for individuals. Also animals cultured under identically controlled circumstances, including temperature, nutrients, and lack of predators, were assumed to die of a single natural cause. As a biological meaning of $z$, we have reported that the inverse of $z$ is approximately proportional to the exponential decline rate constant ($\lambda$) in the respiratory activity with aging; $1/z \approx \lambda/2$ [10]. The mean lifespan, $\langle x_d \rangle$, and the maximum lifespan, $\omega$, are given as follows:

$$\langle x_d \rangle = t_0 + (\sqrt{\pi}/2)z, \quad (2)$$

$$\omega = t_0 + \left(\sqrt{\ell n(100/c^*)}\right)z, \quad (3)$$

where $c^*$ is a certain constant (see our paper of 2007 regarding the details) and the logarithm in Eq. (3) represents the natural logarithm.

For the case of n causes of death, the extended version of Eq. (1) has been concretely described in our paper [13]. That expression could be effectively applied to a heterogeneous case that was generated even in genetically and environmentally homogeneous cohorts of the same birth. A two-component case is especially given by:

$$l_x = l_1(x) + l_2(x) = l_{01}(x,t_{01}) + (l_{01} - l_{01}(x,t_{01}))e^{-(x-t_{01})^2/z_1^2} + l_{02}(x,t_{02}) + (l_{02} - l_{02}(x,t_{02}))e^{-(x-t_{02})^2/z_2^2}, \quad (4)$$

$$\langle x_d \rangle = \frac{1}{100}\left\{(l_{01}t_{01} + l_{02}t_{02}) + \frac{\sqrt{\pi}}{2}(l_{01}z_1 + l_{02}z_2)\right\},$$

$$z_i = \sqrt{4D_i t_{0i}}, \quad l_{0i}(x,t_{0i}) = \begin{cases} l_{0i} & \text{at } x < t_{0i} \\ 0 & \text{at } x \geqslant t_{0i} \end{cases},$$

where $i$ = 1 or 2 and $l_{01} + l_{02} = 100\%$.

## 3. Results

### 3.1. Biodemographic analyses of the daf-16 mutant cohort

Two survival curves for *daf-16* mutant cohorts fed different food sources are merged in Fig. 1A. The lifespan of worms fed the UV-killed *E. coli* OP50 strain tends to be longer than that of worms fed the normal (or living) *E. coli* OP50 strain. Each survival curve was statistically analyzed, as indicated in Fig. 1B and C, by using the lifespan equation with a single component, Eq. (1), or two components, Eq. (4). As summarized in Table 1, in many independent experiments, the two components were revealed only in the case fed UV-killed bacteria; besides, the first mode in the two components was negligibly small (<5%). Probably the existence of two components may be due to the lack of uniformity (or approximate homogeneity) of the environment when using a solid medium. Thus, although the reason remains unclear, the second mode in the case fed UV-killed bacteria was comparable to the data in the case fed living bacteria, below, except for the mean lifespan.

The mean lifespan, $t_0$, and $z$ of worms fed normal bacteria were statistically significantly different as compared with those of worms fed UV-killed bacteria, as shown in Table 1. However, interestingly, both maximum lifespans were statistically identical.

### 3.2. Measurements of the ATP content per single worm and the ATP noise as a function of age

Using Bristol N2 (wild type) and *daf-16* mutants in our previous work [14], we proved that there is a strong correlation between $t_0$ and the noise of the ATP content among individuals. The ATP content per animal serves as an indicator to characterize a stochastic nature among individuals. An ATP measurement of a single animal, whose protocol was specially provided, gives us confidence that the ATP can easily rapidly be measured with high precision and reliability. Thus, we utilized this as one technique for evaluating

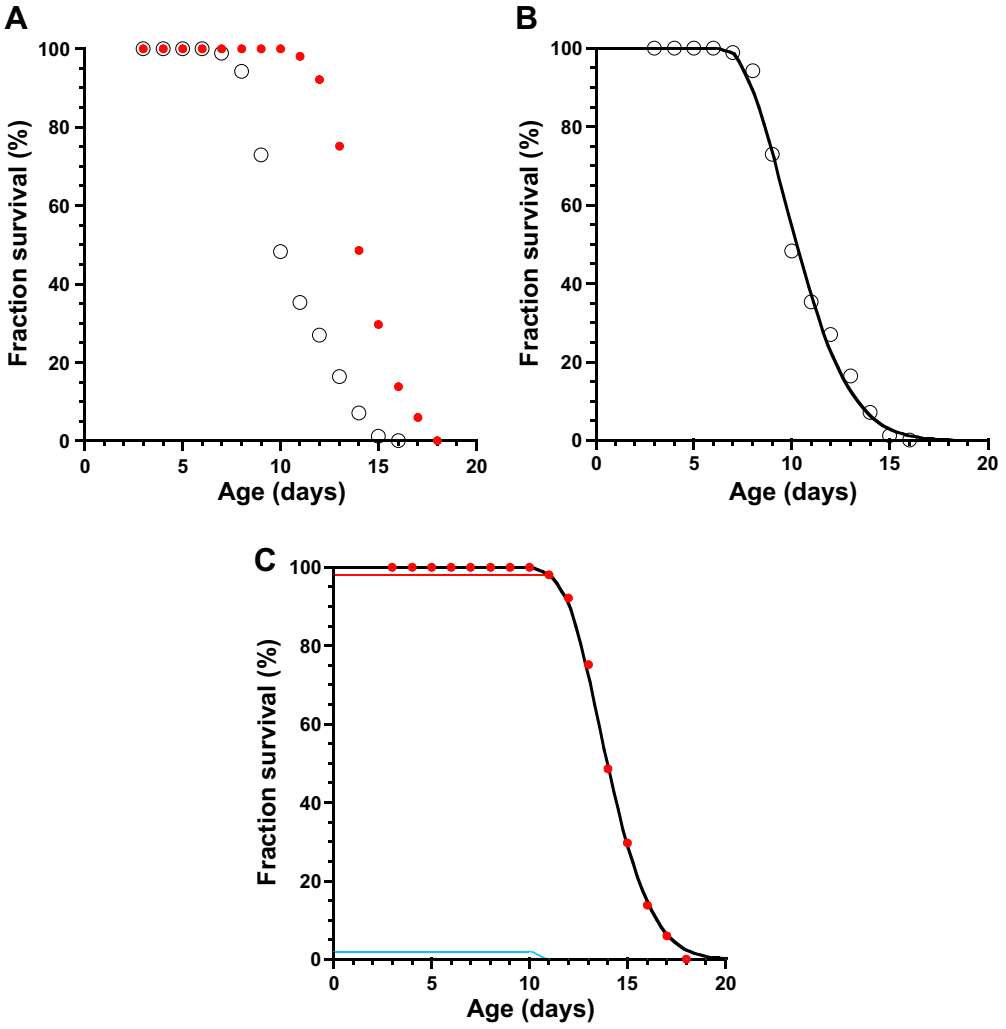


**Fig. 1.** Biodemographic data and analyses by the lifespan equation. (**A**) Survival curves of the *daf-16* mutants cultured on normal or UV-killed *E. coli* OP50 strains as a food source. The worms were transferred daily to fresh plates. Symbols used are as follows: open circles, the case fed normal bacteria (85); red-filled circles, the case fed UV-killed bacteria (101); the numbers in parentheses represent the number of scored worms. Raw experimental values were fitted by the non-linear least squares method with the lifespan equation, Eq. (1) or (4). The survival curves in the case fed normal bacteria could be fitted well with a single component, but those in the case fed UV-treated bacteria had to be analyzed with two components. Blue, red, and bold curves represent, respectively, the 1st mode, the 2nd mode, and the theoretically reconstructed mode (=1st mode + 2nd mode) that fit the original law data. The fitting parameters for normal bacteria in (B) were $t_0 = 6.5$ and $z = 4.51$, where the correlation coefficient was $r^2 = 0.995$. On the other hand, the case fed UV-killed bacteria is shown in (C). The fitting parameters of the 1st mode were $l_{01} = 2.0$, $t_{01} = 10$, and $z_1 = 0.34$, while those of the 2nd mode were $l_{01} = 98.0$, $t_{02} = 11$, $z_2 = 3.63$, and $r^2 = 0.999$. The statistically determined fitting parameters for all survival experiments are listed in Table 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

noise (or variability among individuals). To verify whether the previous conclusion can be reproduced under different conditions, we further tested using a *daf-16* mutant cohort feeding on UV-killed bacteria.

Noise-data points of the ATP content of each animal that were obtained under a different food source were merged in Fig. 2A. The ATP noise showed an increasing tendency from around $t_0$ with aging. This result is consistent with our previous conclusion. As the author has remarked in his 2013 paper, it seems as if the timing of noise amplification synchronized with the onset time of biodemographic aging ($t_0$). In other words, this interpretation implies that the produced timing of $t_0$ may be driven by such noise amplification. On the other hand, the mean values of the ATP content per animal exponentially declined with aging after maturation (Fig. 2B), and this finding is also compatible with our previous result. This declining feature is in good agreement with that of oxygen consumption rates [10,11]. Since both quantities (ATP and respiration) are coupled in the mitochondria, as remarked previously, this consistency seems to be quite reasonable.

### 3.3. Evaluation of the ATP concentration per single worm and its noise

To reduce the ATP content per single worm to the ATP concentration of an entire body, we measured the length of each worm (as one size variable) just before measuring the ATP content. Then the total volume of individual worms was approximately reduced on the basis of the geometric similarity that is naturally established in *C. elegans* [25].

As seen in Fig. S1A, the peak of size (at day 7) for the worms fed UV-killed bacteria shifted approximately two days later than that (at day 5) in the case fed normal bacteria. This clearly reflects the delay of development and/or growth. Interestingly, the size noise indicated a similar profile to the ATP noise, as shown in Fig. S1B; that is, the timing of the amplification of the length noise synchronized to that of $t_0$. This result suggests that there may be a correlation between the ATP content and size. However, a statistically significant correlation between these two is not recognized, as shown in Fig. S1E and F. Strangely, however, the amplification timing of the ATP concentration noise could strongly correlate with

**Table 1**
Statistical and analytical values for biodemographic data in cases fed normal or UV-killed *E. coli* OP50 strains as a food source.

| | Normal *E. coli* | UV-killed *E. coli* |
|---|---|---|
| $t_0$ or $t_{02}$ | 6.4 ± 0.1 (5) | ***10.8 ± 0.3 (4) |
| $z$ or $z_2$ | 5.0 ± 0.3 (5) | **3.4 ± 0.1 (4) |
| $\omega$ | 16.6 ± 0.7 (5) | *17.8 ± 0.3 (4) |
| $\langle x_d \rangle$ | 11.3 ± 0.3 (5) | ***14.1 ± 0.3 (4) |
| $l_{02}$ | ND | 95.6 ± 1.3 (4) |
| $D$ or $D_2$ | 0.99 ± 0.10 (5) | 0.27 ± 0.01 (4) |

$t_0$, $z$, $\omega$, and $\langle x_d \rangle$ are the onset of biodemographic aging, a parameter related with the aging rate in the lifespan equation (Eq. (1) or (4)), the maximum lifespan, and the mean lifespan, respectively. These are given as mean ± SEM, where the parentheses represent the number of independent experiments.
ND is "not detected."
* $p = 0.17$, where $p$ values were calculated using the unpaired Student's $t$-test.
** $p < 0.01$, where $p$ values were calculated using the unpaired Student's $t$-test.
*** $p < 0.0001$, where $p$ values were calculated using the unpaired Student's $t$-test.

the timing of $t_0$, as seen in Fig. S1C, where the ATP concentration denoted the ATP content divided by the total volume, which was given as the third power of the length of individual worms, as described in detail in Section 2.2. This result undoubtedly indicates that the ATP content per animal hardly correlates with the individual's size.

## 4. Discussion

### 4.1. Biophysical and biological meanings of healthspan

Szewczyk et al. [26] cultured worms in a chemically defined liquid medium that is an axenic food source as a healthy diet and found that development slows, fecundity declines, lifespan increases, lipid and protein stores decrease, and gene expression changes as compared to worms fed a bacterial diet.

The extension of the healthspan with a healthy food for UV-killed bacteria (Fig. 1A) is mainly due to that of the $t_0$ term in Eq. (2). Interestingly, the maximum lifespan of the two, despite the different healthspan, was statistically identical. The similar results have been obtained in wild-type *C. elegans* [11]. The other parameter ($z$) included in Eq. (2) was smaller in the worms fed the healthy food as compared with those fed the pathogenic diet. As the healthspan is made up of two terms as expressed in Eq. (2), such an extension of healthspan can be interpreted as the reason that the extension of the first term, including $t_0$ in Eq. (2), exceeds the shortening of the second term, including $z$. The shortening of $z$ means a rapid increase of the aging rate because $z$ is in inverse proportion to the aging rate [10,11]. In other words, the rapid increase in the aging rate means the decrease of the accidental element. In a case where the genetic factors changed, such as in the long-lived *daf-2* mutant we examined, however, both $t_0$ and $z$ of *daf-2* became much larger than did those of the wild type [13]. Likewise, genetic factors and caloric restriction, the consumption of fewer calories, extend both the mean and the maximum lifespans in flies [27] and mice [28,29] as well as *C. elegans* [30]. Accordingly, as far as the genetic factors and calories do not change, we infer that an extension of $t_0$ would dominantly contribute to an extension of the healthspan. This has to be one of the likeliest mechanisms for the extension of the healthspan with the decrease of the accidental factor.

### 4.2. The cause of noise itself and the biological role of noise amplification in relation to aging and lifespan

The $t_0$ value in a *daf-16* mutant cohort fed UV-killed bacteria was longer (about four days) than that in the case fed normal

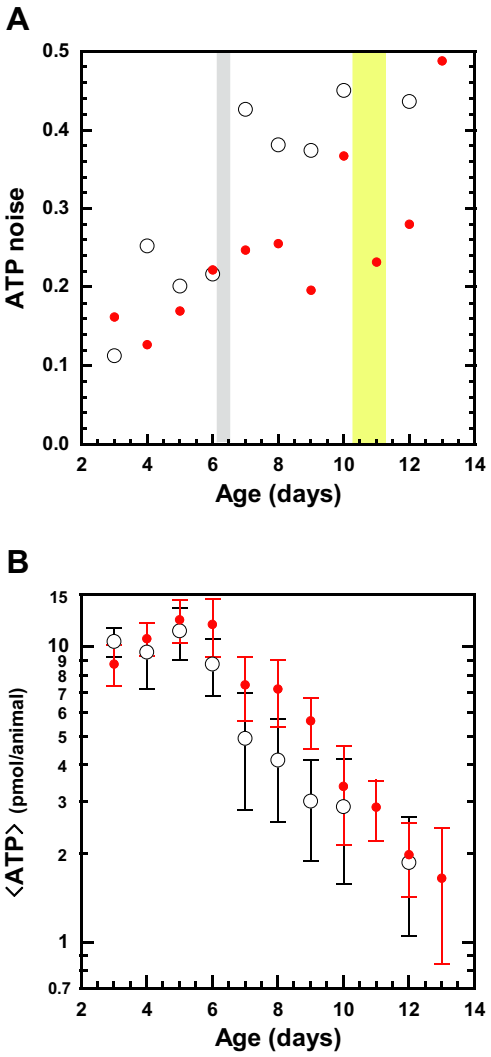


**Fig. 2.** Amplification of the ATP noise and decline of the averaged ATP content with aging in a *daf-16(mu86)* mutant cohort. (A) Noise of the ATP content with age. This experiment was repeated three times, yielding similar results. The $t_0$ values (mean ± 95% confidence interval) for the case fed normal bacteria were 6.4 ± 0.2 days (gray area, $n = 5$), while those for the case fed UV-killed bacteria were 10.8 ± 0.5 days (yellow area, $n = 4$). (B) The averaged ATP content per animal as a function of age for the raw data of (A). The averaged ATP content per animal is shown in the semi-logarithm with age. The error bars represent the standard deviation of the mean (SD). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

bacteria, as seen in Table 1. We maintained previously that $t_0$ may be related to growth and/or development. Indeed, the peak of size in the case fed UV-killed bacteria deviated by approximately two additional days than that in the case fed living bacteria (Fig. S1A). In addition, as if the timing of the ATP noise amplification in the case fed the UV-killed bacteria would respond to the $t_0$ lengthening, it deviated by approximately four additional days as compared to the case fed the living bacteria. This result also supports the previous suggestion that the timing of $t_0$ may be driven by noise amplification.

From the above discussion, it seems reasonable to accept that lifespan and aging are closely related to noise. Then, naturally, anyone must question the cause of noise itself or noise amplification. Perhaps the simplest answer is attributed to the variability of the gene expression. Gene expression differs among cells, individuals, and populations; it is usually thought to be a major determinant of phenotypic variation. According to the most recent report [31],

however, protein levels appear to be heritable molecular phenotypes that exhibit considerable variation between individuals, populations, and sexes in humans. Variation in messenger RNA expression is not a perfect surrogate for protein expression because the latter is influenced by an array of post-transcriptional regulatory mechanisms, and, empirically, the correlation between protein and mRNA levels is generally modest.

Besides, in microorganisms, noise in gene expression is known to give rise to cell-to-cell variability in protein concentrations. In mammalian cells, protein levels also vary, and individual cells differ widely in their responsiveness to uniform physiological stimuli. From this point of view, Spencer et al. [32] showed that naturally occurring differences in the levels or states of proteins regulating receptor-mediated apoptosis are the primary causes of cell-to-cell variability in the timing and probability of death in human cell lines. This finding seems to be closely related to our result showing the strong correlation between noise amplification and the timing of $t_0$.

For another factor, an epigenetic effect, producing noise, Fraga et al. [33] found that although twins are epigenetically indistinguishable during the early years of life, older monozygous twins exhibit remarkable differences in their overall content and genomic distribution of 5-methylcytosine DNA and histone acetylation.

In another cause of noise for non-coding small RNAs of the micro-RNA class (miRNA), Pincus et al. [17] showed that lifespan is at least in part epigenetically determined. Neither genetic nor environmental factors fully account for the variability in individual longevity: genetically identical invertebrates in homogenous environments often experience no less variability in lifespan than do outbred human populations. Such variability is often assumed to result from stochasticity in damage accumulation over time. However, Pincus et al. maintained that two miRNAs act upstream in insulin/IGF-1-like signaling (IIS) and other known longevity pathways, and that these microRNAs appear to not only report on but are also likely to determine longevity. In addition, they suggested that fluctuations in early-life IIS, due to variation in these miRNAs and from other causes, might determine an individual's lifespan. Likewise, Lucanic et al. [34] found that miRNA abundance was highly variable between individual worms raised under identical conditions and that expression variability generally increased with age. They suggested that miRNA expression levels vary between individuals, that at least one of these miRNAs has a dramatic influence on aging, and that there is considerable phenotypic variation in aging animals.

Consequently, the above facts suggest that a cause of noise is not always unique, and also that the cause of noise amplification may not be determined uniquely. It seems likely that we should not be restricted by the law of causality, but should rather focus on discovering a quantitative relation between noise and other factors. Actually, we worked according to this viewpoint and found a strong correlation between the timing of noise amplification and the timing of $t_0$. In future research regarding lifespan and aging, it seems that we must change our way of thinking to an approach from the law of causality. If we turn our eyes to the process of individuation, then it becomes important to focus not on the *average* lifespan but on *individual* lifespan (or life), even in worms. The future scientific way of finding out a quantitative relation between noise and genetic factors and between noise and environmental factors must be targeted as an essential strategy.

## 5. Conclusion

In summary, we found that the healthspan of *C. elegans* cultured with a healthy food for UV-killed bacteria was extended beyond that of worms fed living bacteria, but the maximum lifespan did not statistically change. Analyzing the demographic data with the lifespan equation, we elucidated that the extension of the healthspan is mainly due to that of the $t_0$ (the onset of biodemographic aging). We also found that the noise amplification of the ATP content per single animal is synchronized with the timing of the $t_0$. Thus, the problem of how noise as a third factor is related to aging and lifespan is very essential in an isogenic cohort with the same birth in a uniform environment. In this work, we especially noted that aging research is very important to investigate from the viewpoint of *individuality*.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.08.037.

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