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Research article

Heating stress patterns in *Caenorhabditis elegans* longevity and survivorship

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Abstract

Survival data from *Caenorhabditis elegans* strain TJ1060 (*spe-9*; *fer-15*) following brief exposure to 35 °C have been investigated. Three experiments with 3-day-old worms were conducted with heat duration ranging between 0 and 12 hours. A statistically significant increase in life expectancy was observed in the groups heated for less than 2 hours, as compared to the unheated control groups. In different experiments P-values for the observed life spans under the hypothesis that heating has no influence on longevity were $P < 0.004$ after 0.5 hour heat, $P < 0.012$ after 1 hour heat and $P < 0.055$ after 2 hours of heating. A biphasic survival model with Gamma distributed frailty has been constructed to describe the survival of worms after heating. The increase in the remaining life expectancy is determined by more effective protection by heat-induced substances in the ages yanger than 27 days. The unheated control group demonstrated acquired heterogeneity of frailty with chronological age while the heat-induced substances defend the worms in a universal way and protect against the development of frailty.

Introduction

Survival and mortality are important indicators of aging – the accumulation of changes in organism that increase the risk of death. Investigation of survival and mortality may clarify the role of different environmental and genetic factors in aging, detect biologically meaningful processes and indicate interventions that may affect the aging process(es). Recently, several experiments involving the response to stress in genetic variants of a variety of invertebrate species have been conducted to investigate longevity and the control of aging. The range of effective interventions includes reports that in some insects (Sohal and Allen 1984) and in *Caenorhabditis elegans* (Johnson and Hartman 1986) low doses of radiation appears to slow aging and prolong life span, that hypergravity postpones aging in *Drosophila melanogaster* (Le Bourg and Minois 1999), and the observation that dietary restriction has antiaging action (Masoro 1998). Recent publications emphasise the role of metabolic capacity and resistance to stress in determining life span in *C. elegans* and other species (Larsen 1993; Vanfleteren 1993; Lithgow et al. 1995; Johnson et al. 1996; Martin et al. 1996; Jazwinski 1998).

The problem of survival modeling in *C. elegans* has been considered by many authors (Brooks et al. 1994; Vaupel et al. 1994; Easton 1997; Vanfleteren et al. 1998). In these publications, authors investigated the deviations of experimental survival and mortality curves from the classical Gompertz Law (Gompertz 1825) and suggested that differential mortality could result from a change either in population heterogeneity or from changes at the level of the individual organism. Other studies have emphasised the importance of different stress factors on longevity

and survival (Johnson et al. 1996; Walker et al. 1998) and the relationship between stress resistance and longevity (Larsen 1993; Lithgow et al. 1995; Murakami and Johnson 1996, 1998).

In this study we investigate the influence of thermal stress applied at the beginning of the nematode life on subsequent longevity. The consideration is based on the concept of life protection, which includes either the ability to defend the organism from the external negative influences, or the possibility to make reparations of accumulated damages. This concept characterizes the ability to restore the initial conditions of homeostasis after a challenge (that is, a change in condition due to internal or external energy fluctuations) considered by Strehler and Mildvan (1960). The hypothesis about linear loss with age of maximum value for the ability to restore initial conditions, called vitality (Strehler and Mildvan 1960), is used to construct mathematical model for survival after heating stress and to estimate changes in protective abilities of nematode organism with increasing chronological age. This approach gives simple and convenient mathematical formulation of aging and survival processes in normal and in stress environment, which allows to use formal statistical techniques for model identification and hypotheses testing.

Materials and methods

Heating stress experiments

Caenorhabditis elegans worms (strain TJ1060 (*spe-9(hc88ts)* I; *fer-15(b26ts)* II) were raised on solid medium for three days on NGM plates prespotted with E.coli at 25.5 °C and were therefore sterile. These worms were selected for pilot study before making large population experiments. The response of these mutants to heat shock was never studied before. At three days of age, the worms were divided into ten groups and exposed to 35 °C for 0, 1, 2, 4, 6, 8, 10, 12, 16 or 24 hours. Worms were then permitted to recover for 24 hours at 20 °C and transferred to liquid survival medium at 20 °C for the reminder of their lives. Starting at day five, the numbers of alive and dead worms were counted daily for all groups. At the lower doses (1 through 4 hours of heat) no worms died. At the highest doses, (16 and 24 hours) no worms survived heat exposure. In the results section this experiment is addressed as experiment A. Two additional experiments with the same strain, addressed later as experiments B and C, were conducted at different time using the same experimental design. In these experiments worms were exposed to 35 °C for 0, 0.5, 1, 2, 3, 4, 6, 8, 10 hours and for 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8 hours, allowed to recover as before and in experiment C were put onto agar plates for the rest of their lives. In both experiments B and C the numbers of alive and dead worms were counted not daily but with different intervals.

Data analysis

Remaining life expectancy after heating. The mean value for remaining life span after heating has been estimated by the formula

$$LE = \frac{1}{N}\sum_j \frac{x_j + x_{j-1}}{2} d_j - x_0$$

where d_j is the number of worms which died in the age interval (x_{j-1}, x_j), x_0 – age of observations start. This formula is the mean value for grouped life spans with an uncertainty correction of half an grouping interval length. N denotes the total number of worms in the experiment $N = \sum_j d_j$. The standard error for LE estimate is given by the formula

$$SE = \sqrt{\frac{1}{N} VE}$$

where variance VE for the remaining life span is estimated by the expression

$$VE = \frac{1}{N-1}\sum_j \left(\frac{x_j + x_{j-1}}{2} - LE - x_0 \right)^2 d_j.$$

Test for significance of the remaining life expectancy increase. Comparison of estimates for remaining life expectancies in control group and a group exposed to the heat treatment was made by calculating the normalized statistics

$$z = (LE_1 - LE_0)/\sqrt{SE_1^2 + SE_0^2},$$

where LE_0, LE_1, SE_0 and SE_1 are remaining life expectancy estimates and standard errors for these estimates in the control group and the group exposed to heat. Statistic z has asymptotically normal distribution with mean 0 and variance 1 under the hypotheses H_0: remaining life expectancy does not depend on the heat treatment. The P-value for accepting or

rejecting hypothesis H_0 against the hypothesis H_1: heat prolongs the remaining life expectancy, is calculated by

$$P = 1 - \Phi(z),$$

where $\Phi(z)$ is the cumulative distribution function for the standard Normal distribution with mean 0 and variance 1.

Test for equality of the survivor functions. The nonparametric log-rank test (Kalbfleisch and Prentice 1980) has been used to test the difference in survival curves without heating and after heating. In general, the *log-rank* test is used to test hypotheses about the proportionality of hazard functions in the two populations. In this study, we applied this procedure to test the hypothesis: heating does not change the survival curve, which is a specific case for the proportion between hazards equal 1.

Biphasic model for survival of individual organisms. On the level of the individual organism, the probability of surviving x days after heating have been modelled using the concept of vitality – the energy capacity of an individual organism at a given age (Strehler and Mildvan 1960). This model relates the age pattern of mortality with the decline of homeostatic capacity to respond to environmental stresses. The simple and convenient mathematical formulation of this relationship makes this model extremely useful in the studies of the effects of stress on aging and survival. In accordance with this model death occurs if the energy demand to stay alive in response to the environmental challenges exceeds vitality. The value of the energy demand is high if the environmental conditions are challenging, i.e., not good for subsequent survival. This value is low if the life protecting system operates efficiently. The latter means that the increase in energy demand during life under fixed environmental conditions may reflect the loss of damage protection and repair potential on the cellular level. Under three additional assumptions:

- vitality declines linearly with age
- environmental challenges in terms of demand for energy expenditures are random with a Boltzmann distribution
- average demand for energy expenditures required to stay alive is independent of age

Strehler and Mildvan derived the Gompertz law for survival as a function of age. Mathematically the parameters of the Gompertz model of mortality $\mu(x) = a*\exp(bx)$ are related to the vitality and energy expenditure characteristics by the relationships

$$a = K\exp(-V_0/\varepsilon), \quad b = V_0 B/\varepsilon,$$

here ε is the average demand for energy expenditures to stay alive and K is a quotient of proportionality between the frequency of environmental changes and mortality. Vitality deceleration with time is expressed in the form $V(x) = V_0(1 - Bx)$.

A simple way to reflect changes in protective ability of the organism with age is to consider a biphasic survival model. In this model the average demand for energy expenditures can change with age in a step-wise mode, increasing at a time point x^* from an initial level ε_0 to a secondary level ε_1. The probability of surviving x days after heating is calculated in the Appendix.

Biphasic model for survival in heterogeneous population. To take into account the differences between organisms one can use the conception of frailty, which reflects the differences in mortality between members in population. The reason for this difference can be of genetics, environmental, developmental and so on nature. Mathematically it is convenient to operate with gamma-distributed frailty and proportional hazard model (Vaupel et al. 1979)

$$H(x, z, \varepsilon) = zH_0(x, \varepsilon)$$

where z is frailty, a random variable having a Gamma distribution with mean 1, and $H_0(x)$ is an underlying hazard, corresponding to an 'average' organism in the population. In the biphasic survival model for a population, the frailty variance in the first phase for $x < x^*$ can be different from the frailty variance in the second phase when $x \geq x^*$. This allows the modeling of experiments on genetically homogeneous populations with initially negligible variance of frailty. During development and early adult life, different individuals are exposed to different variations in the environment, which can cause accumulation of heterogeneity expressed in the second phase of the life span as a new value for the frailty variance. The final form of the biphasic survival function for heterogeneous populations as well the likelihood function, used in the parameters estimation, are given in the Appendix.

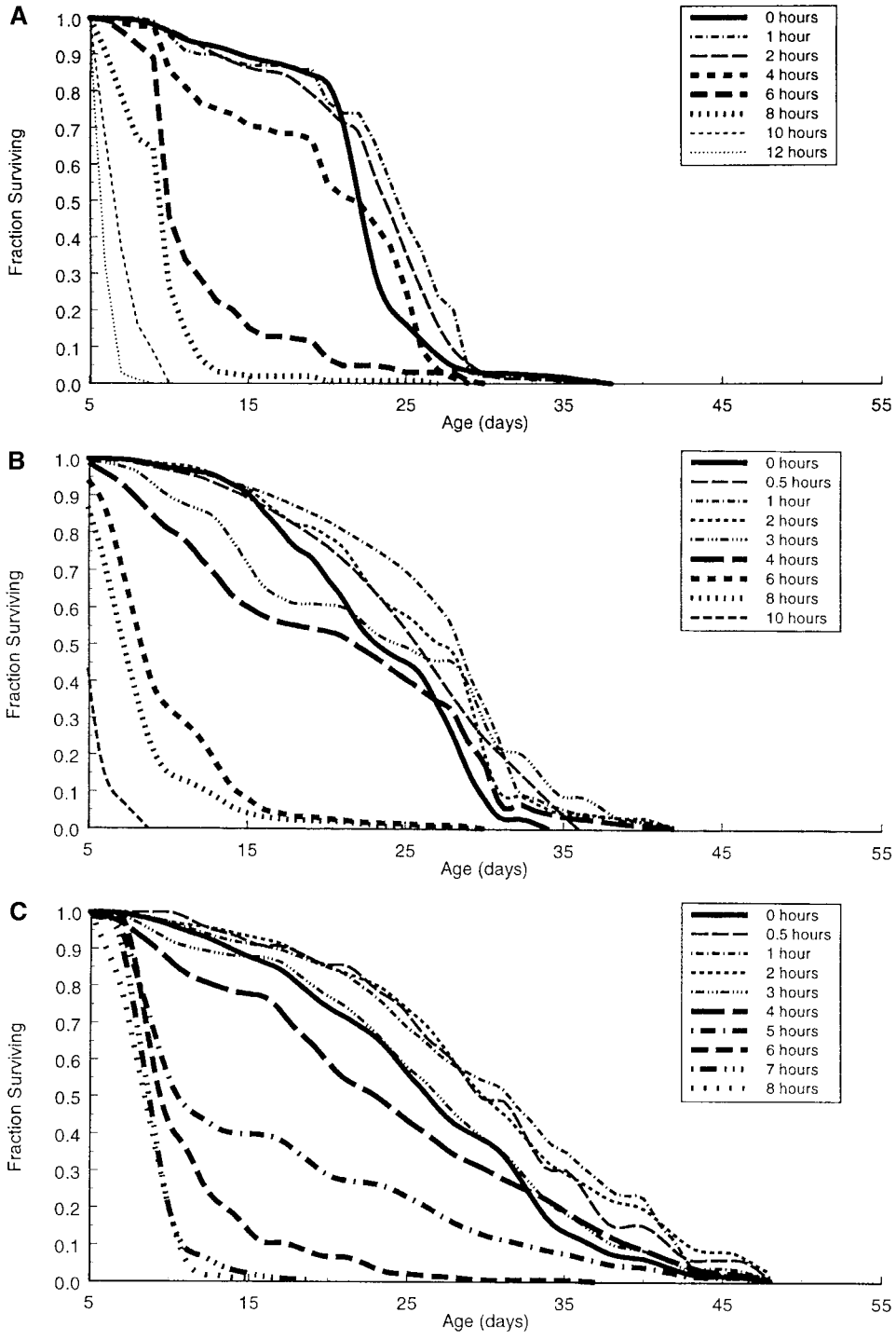

Figure 1. (A) Proportion by age of survived worms in experiment A after different heating treatment. (B) Proportion by age of survived worms in experiment B after different heating treatment. (C) Proportion by age of survived worms in experiment C after different heating treatment.

Table 1. Remaining life expectancy in the three experiments after different periods of heat treatment.

Experiment	Heat duration (hours)	Total deaths after heating	Number of dead worms during heating	*LE* (days)	Percent to control	*SE* (days)
A	0	137	0	16.6	100.0	0.4
	1	100	0	18.2	109.6	0.6
	2	152	0	17.6	106.0	0.4
	4	133	1	14.6	88.0	0.6
	6	164	1	6.8	41.0	0.4
	8	152	14	4.2	25.3	0.2
	10	198	63	1.8	10.8	0.09
	12	178	121	0.8	4.8	0.08
B	0	174	0	18.5	100.0	0.4
	0.5	186	0	20.0	108.6	0.4
	1	189	0	22.0	119.2	0.5
	2	139	1	20.8	112.4	0.5
	3	90	1	18.7	101.1	1.0
	4	164	1	15.8	85.3	0.7
	6	200	5	5.1	27.8	0.3
	8	200	25	3.6	19.3	0.2
	10	199	145	0.9	5.1	0.08
C	0	174	0	20.9	100.0	0.6
	0.5	187	0	24.5	117.2	0.6
	1	188	0	24.8	118.4	0.7
	2	180	0	24.9	119.1	0.7
	3	193	0	21.3	102.0	1.7
	4	187	0	18.5	88.5	0.8
	5	210	2	10.9	52.2	0.7
	6	209	3	5.9	28.2	0.3
	7	210	1	3.6	17.1	0.1
	8	242	10	3.0	14.6	0.08

Results

Figures 1A–1C present proportions of survived worms by age in experiments A, B and C after different heating treatments. From the figures one can see changes in survival after different heating treatment.

The heating treatment affects remaining life expectancy after heat treatments (LE), which have been estimated for different duration of heat in all three experiments with the same strain. The results are presented in Table 1. From the table, one can see a positive role of mild heat. In all three experiments the statistical significance of life expectancy increase after heating up to 3 hours was supported by small P-values ($P < 0.06$), calculated under the hypotheses that remaining life expectancy is independent on the heat treatment. Heating longer than 3 hours decreases the remaining life expectancy in all three experiments, because debilitation overwhelms the positive role of heating.

Figure 2 presents the remaining life expectancy after heating as the percent of the life expectancy in the control group. The 95% confidence intervals are presented in the figure as well. The curves demonstrate similar patterns of hormetic effect in all three experiments, for heat exposure shorter than 3 hours. Since major effects (hormesis and debilitation) look similar in all three experiments we restricted ourselves by the modeling and statistical analysis of data obtained in experiment A.

The proportion of survival by age in the control group and groups exposed to heat in experiment A

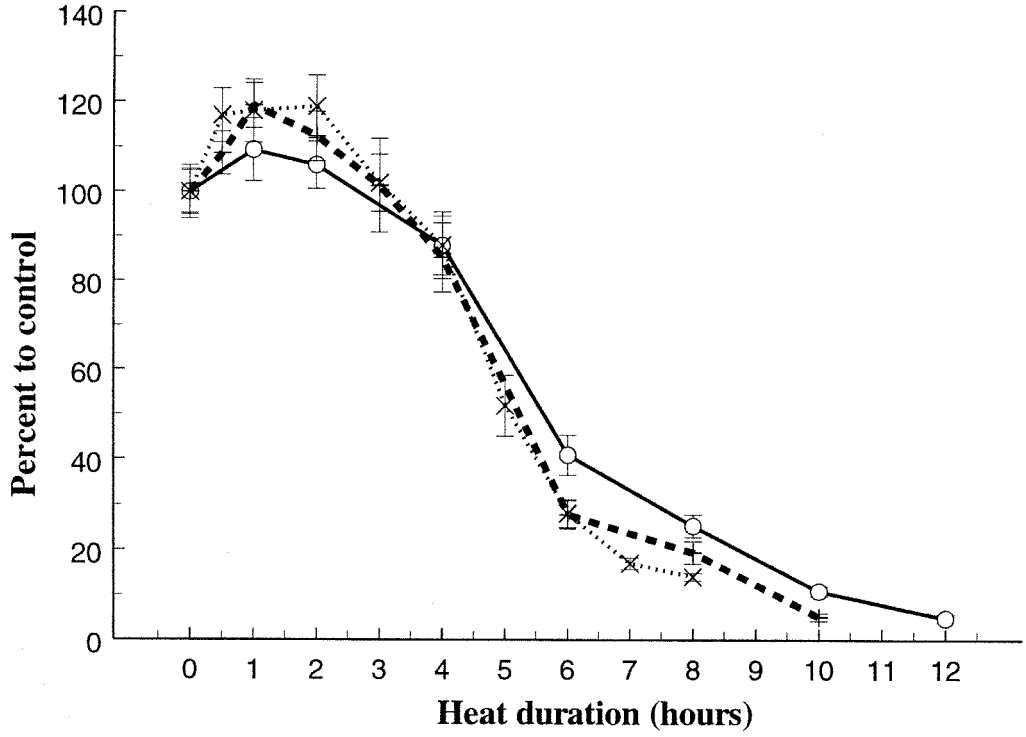

Figure 2. Remaining life expectancy after heating in the three experiments as percents of the remaining life expectancy in the control group (experiment A, solid line and 'O'; experiment B, dashed line and '+'; experiment C, dotted line and 'X').

are presented in Figure 3. In the figure, one can see the similarity between survival in the control and in heat-stressed groups for ages younger than 19 days and older than 27 days. For ages younger than 19 days hormetic effect after heating is expected, but statistically insignificant because of low mortality level at this age interval. At ages 19–27 days the effect is statistically significant and at ages older than 27 days data show no statistically significant influence of heating on survival, which could be the result of small number of survivors till these ages. To test these conclusions the log-rank test was used in the three age intervals: (5–19) days, (19–27) days and (27–37) days. Table 2 presents the *P*-values for this test, calculated if there is no difference between survival curves in the control and the heated groups. Large *P*-values for interval (5–19) can be explained by the fact that for the young ages the number of deaths is small and hormetic effect is not statistically significant. The small *P*-values obtained for the age interval (19–27) days show that moderate heating changes survival statistically significant while large *P*-values for (27–37) days interval could be interpreted as if the right tales of the survival curves do not depend on the heating. This hypothesis should be tested with larger population of worms.

Table 2. P-values for testing hypothesis that in experiment A heating produces no difference in survival in different age intervals.

Age interval (days)	5–19	19–27	27–37
1 hour heating	0.25	0.0015	0.29
2 hours heating	0.18	0.00003	0.12

The increase in life expectancy after moderate heat and differences in survival curves can be interpreted to be the result of the action of an additional life-protection system. This system could be based on heat-induced substances, such as heat shock proteins and/or antioxidants. The similarity between the three curves in Figure 3 for ages less than 19 days is consistent with multiple interpretations. First, young animals have strong intrinsic life support systems, which protect the organism as or more effectively than do the novel heat-induced substances or (2) that the vitality of young worms is such that increased frailty

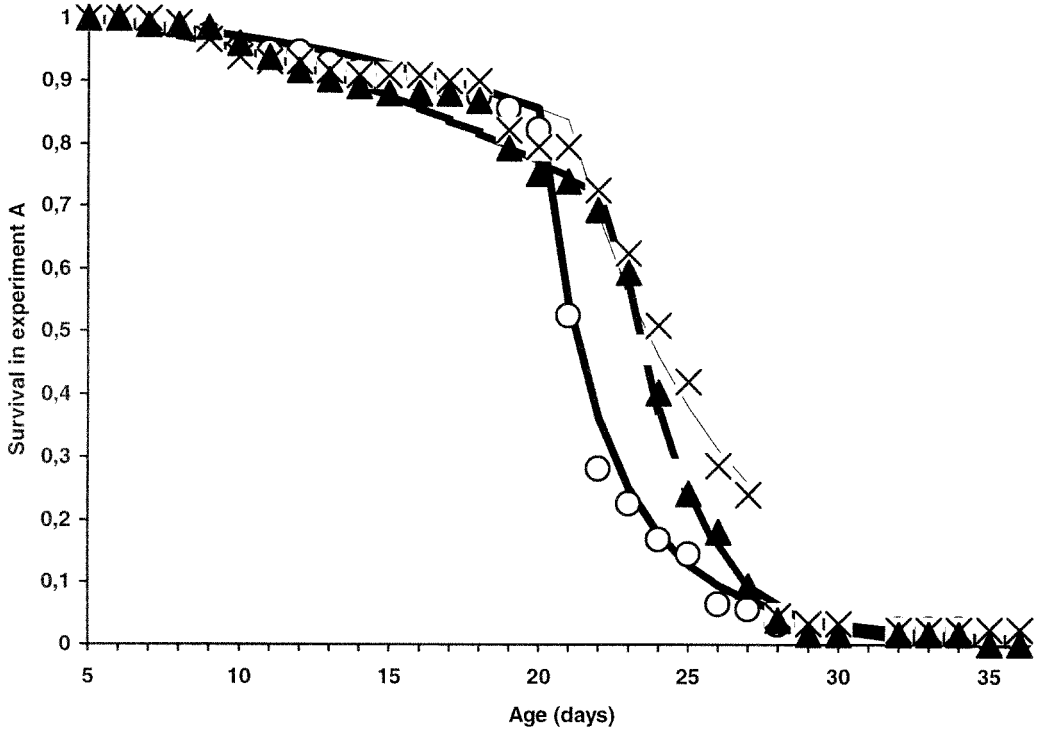

Figure 3. Survival proportions and modeled survival in experiment A *Survival proportions*: control group, 'O'; 1 hour heating, 'X'; 2 hours heating, '▲'; *Modeled survival*: control group (solid line); 1 hour heating (thin solid line); 2 hours heating (long dashed line).

before 19 days is not detectable. Only during the high mortality period after 19 days does this intrinsic life-support system become inefficient, allowing heat-induced protection mechanisms to play the major role in protection. After 27 days, too few worms are alive to discern whether survival differs and the three survival curves again become similar.

A quantitative analysis of the changes in survival after heat shock can be interpreted using the biphasic survival model. Figure 3 presents experimental and modelled survival in experiment A after 0, 1 and 2 hours of heating. The modelled survival was calculated using maximum likelihood estimates for parameters x^*, ε_0, ε_1, σ_0^2, σ_1^2 for different duration of heating. The maximum likelihood estimates were obtained by maximization the log-likelihood function presented in Appendix. The estimated model parameters for different duration of heating are presented in Table 3. From the first line of the table one can see that without heating the estimate for initial level of the mean energy demands ε_0 corresponds to 0.12 relative units. In the second phase, which starts after age x^*, estimated as 19.7 days, the estimate for the secondary level of the mean energy demands ε_1, changes to 0.26 relative units. Estimates σ_0^2, σ_1^2 for frailty variance in the first and the second phases show that the initially homogeneous population ($\sigma_0^2 = 0.0001$) became heterogeneous in the second phase of the life span ($\sigma_1^2 = 0.34$). The significance of the increase in the variance of frailty is justified by the small P-value ($P = 0.003$) for the *log-likelihood* ratio test (Cox and Oakes 1984) under hypothesis H_0: $\sigma_0^2 = \sigma_1^2$.

Table 3. Biphasic model parameters estimates for different duration of heat in experiment A.

Heat duration (hours)	ε_0	ε_1	x^* (days)	σ_0^2	σ_1^2	P-value (H_0: $\sigma_0^2 = \sigma_1^2$)
0	0.12	0.26	19.7	0.0001	0.34	0.003
1	0.12	0.20	21.6	0.0001	0.0001	0.91
2	0.12	0.24	22.7	0.0001	0.0001	0.89

The modeling results show that in both groups exposed to heat, the initial levels of mean energy demand ε_0 were similar to the level in the control group. At a subsequent age, dependent on the duration of heat treatment, the mean energy demand jumps to

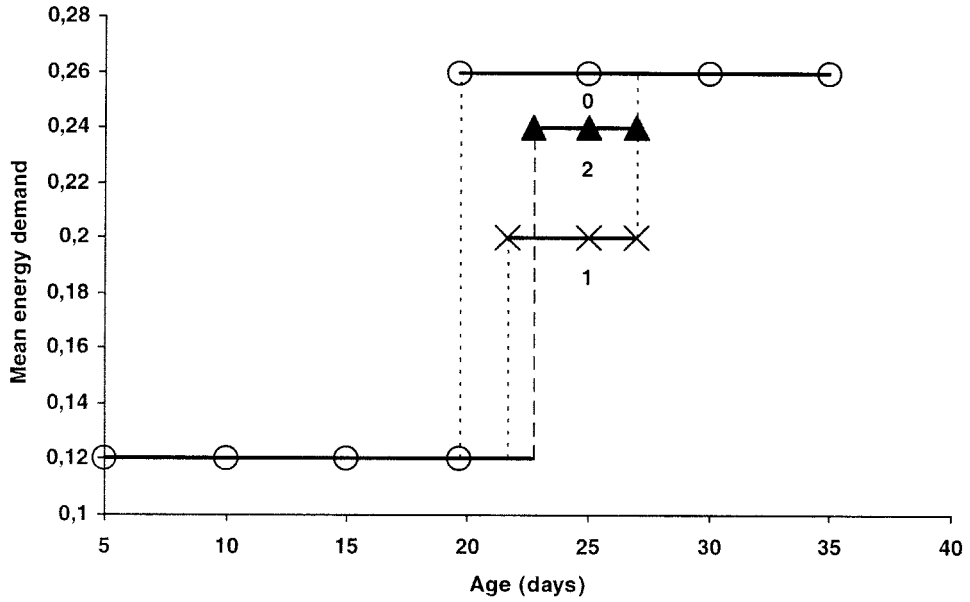

Figure 4. Modeled mean energy demand (relative units) in experiment A (control group, 'O'; 1 hour heating, 'X'; 2 hours heating, '▲').

a level ε_1, which is less than the level in the control group, but increasing with heat duration.

An interesting observation is that in the groups exposed to heat, the frailty variance does not change in the second phase. This is justified by the large *p*-value for *log-likelihood* ratio test under hypothesis H_0: $\sigma_0^2 = \sigma_1^2$, presented in the last column in Table 3. These results mean that the heat-induced system(s) may be more universal life protectors than the intrinsic protection and repair mechanisms. The efficiency of this heat-induced protection slightly depends on the individual features of the protecting organism.

After 27 days of age, in both groups exposed to heat, the mean energy demand jumps to the level of the control. Figure 4 presents mean energy demand estimates in all groups with age.

Discussion

The results of analysis of survival data from stress experiments together with the analysis of the results of other studies of aging and survival of nematode worms allow us to suggest possible mechanism of aging and survival, and to describe the role of stress in this process. The mechanism has the following features:

- Survival in the control group shows a biphasic mode with regard to energy demands. It may be interpreted as relatively high protection of the organism at the beginning of life and lower protection at older ages.
- Mild heat stress at the beginning of life activates production of additional protection or repair processes, which lead to hormetic effects, i.e. an increase in the remaining life expectancy after heat treatment.
- These heat-induced processes are especially effective in the second phase of the life span in the period after 19 days of age. After age 19 days more than 80 animals in the groups with the duration of heating shorter than 4 hours are still alive. The log-rank test shows significant decrease in mortality after this age for this number of animals. Further investigation of this effect may involve measurements of biological processes including expression of heat shock genes and antioxidant producing genes (Tatar 1999). Experiments with *Drosophila* show that the maximum stress resistance effect is observed later than maximum expression of heat shock protein genes (Dahlgaard et al. 1998).
- This protection is seen in a genetically homogeneous group of worms, demonstrating acquired heterogeneity in the second phase of life, if no heat treatment is applied. The additional protection, induced by heating early in life, converts the population to homogeneity. This inference is the result of statistical modeling. No prior assump-

tions about heterogeneity in the two phases of the life span have been made in the analysis.

The above observations are the results of statistical analysis and mathematical modeling using survival data. To obtain a direct biological justification of the inference made in this article it is necessary to add biochemical measurements of concentrations of antioxidants and heat shock proteins in the tissues of animals in different age groups to survival data. Especially important would be the biological confirmation of the existence of the second phase in survival, which is characterized by low effectiveness of endogenous protective factors. The comparative analysis of survival data observed in three stress experiments conducted in different time shows that survival curves characterized by the same controlled experimental conditions look different. This may be the result of influence of some uncontrolled factors. For example, there is an evidence that the shape of the survival curve in nematodes is affected by the quality of food (Klass 1977; Wilson 1994).

The results of our analysis suggest an idea that the role of genetic and environmental factors in the determination of life span and stress resistance can be reinvestigated using the concept of life protection. This approach could be especially productive when additional information about environmental conditions; the quality of food; and about activity of biological defense mechanisms in worms under stress, is available.

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Appendix

The probability of survival and likelihood function

The cumulative Gompertz hazard is given by

$$\begin{aligned} H(x,\varepsilon) &= \frac{a}{b}(\exp(bx)-1) \\ &= \frac{K\exp(-V_0/\varepsilon)}{V_0B/\varepsilon}(\exp(V_0Bx/\varepsilon)-1. \end{aligned}$$

Let x^* be the age where the survival pattern changes in accordance with the biphasic model. Then in the absence of hidden heterogeneity survival function will be determined by cumulative hazard $H(x, \varepsilon_0)$ at the interval $[0,x^*]$. At the interval $[x^*,x]$ the cumulative hazard at age x becomes $H(x, \varepsilon_0) + H(x - x^*, \varepsilon_1)$. Finally, survival function may be described as

$$S(x) = \begin{cases} \exp(-H(x,\varepsilon_0)) & x < x^* \\ \exp(-H(x^*,\varepsilon_0) - H(x-x^*,\varepsilon_1)) & x \geq x^* \end{cases}$$

Standard calculations associated with introduction of hidden gamma-distributed frailty in proportional hazard model (Vaupel et al. 1979) yield the following expression for survival function

$$S(x) = \begin{cases} (1+\sigma_0^2 H(x,\varepsilon_0))^{\frac{-1}{\sigma_0^2}} & x < x^* \\ (1+\sigma_0^2 H(x^*,\varepsilon_0))^{\frac{-1}{\sigma_0^2}} (1+\sigma_1^2 H(x-x^*,\varepsilon_1))^{\frac{-1}{\sigma_1^2}} & x \geq x^* \end{cases}$$

where σ_0^2 and σ_1^2 are the frailty variances in the first and second phases of the life span, respectively. Values V_0, B, K, x^*, ε_0, ε_1, σ_0^2, σ_1^2 are considered as parameters and can be estimated from the experimental data by maximization of the log-likelihood function

$$LnL = \sum_j (d_j \ln p_j + (n_j - d_j)\ln(1-p_j))$$

where n_j is the number of worms which were alive in the beginning of the jth day after heating, $p_j = 1 - S(j)/S(j-1)$ is the probability of death during the jth day under condition to survive till this age, and $S(0) = 1$.

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