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# Stressors and antistressors: how do they influence life span in *HER-2/neu* transgenic mice?

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**Abstract**

The purpose of this study is to investigate possible influences of different stressors (saline injections, light deprivation and constant light regimen) and geroprotectors (Epitalon and melatonin) on survivals of female *HER-2/neu* transgenic mice. We propose a semi-parametric model of heterogeneous mortality (frailty model) for the analysis of the experimental data. In this model, we assume that treatment influences parameters of both frailty distribution and baseline hazard. The unique design of the experiments makes it possible to compare the effects on survival produced by different treatments in terms of changes in population heterogeneity and underlying hazard. Parameters of the model help to describe the possible influences of various stressors, geroprotectors, and their dosage on the life span of laboratory animals. The proposed model helps to advance our understanding of the effects—such as debilitation, longevity hormesis and incomplete hormesis—which occur in the population as a result of different treatments.


*Keywords:* Aging; Frailty model; Mortality; Stress; Survival patterns

## 1. Introduction

The *HER-2/neu* transgenic mice bear the oncogene which encodes a 185 kDa (p 185) receptor protein that belongs to the epidermal growth factor receptor family involved in organogenesis and epithelial differentiation (Andrechek et al., 2000). Amplification and mutation of *HER-2/neu* plays a pathogenetic role in several malignancies, including carcinoma of the breast, ovary and uterus (Chan et al., 1999; Weinstein et al., 2000). Overexpression of *ErbB-2/HER-2/neu* occurs in 15–40% of human breast cancers (Jones and Stern, 1999). Its appearance is correlated with poor prognosis and is therefore an important target for physiological investigation and therapeutic intervention (Weinstein et al., 2000). This makes *HER-2/neu* transgenic mice an important model in cancer prevention research.

The series of experiments aiming to investigate the development of spontaneous mammary adenocarcinomas in *HER-2/neu* transgenic mice under different treatments were conducted at Laboratory of Carcinogenesis and Aging, N.N. Petrov Research Institute of Oncology (St. Petersburg, Russia). The effects produced by the stressors (saline injections, light deprivation and constant light regimen) and geroprotectors (Epitalon and melatonin) on mean latent period, cumulative and total number of tumors per mice, as well as up or down regulation of *HER-2/neu* gene expression were described in several publications (Anisimov et al., 2002a,b,2003; Baturin et al., 2001). The effect on life span was also mentioned, but the issues related to longevity hormesis were not addressed in those previous studies.

The purpose of the present study is to investigate possible influences of different stressors and geroprotectors mentioned above on the survival of female *HER-2/neu* transgenic mice. For most of the factors considered, experiments with two different doses of the treatment were carried out and a group of untreated mice served as a control.

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We suggest a semi-parametric heterogeneous mortality model for the analysis of experimental survival functions. This approach is based on the modification of fixed frailty model (Vaupel et al., 1979), and was initially developed to test hypotheses about the presence of hidden heterogeneity in a population (Yashin et al., 1996). The semi-parametric representation of the model allows us to avoid the widely used but biologically unjustified technical assumption of a parametric form for the underlying hazard. In this model, the treatment applied influences parameters of both frailty distribution and baseline hazard. The model allows us to capture the effects of debilitation or adaptation and the non-linear transformation of frailty in response to the treatment applied. We show that, thanks to its flexibility, the proposed model can reproduce all essential features of the survival patterns.

The unique design of the experiments makes it possible to compare the effects on survival produced by the different treatments. The model's different parameters help to describe possible influences of various stressors, geroprotectors, and their dosage on the life span of transgenic mice. This study is an attempt to engage more sophisticated mathematical models in gerontological research. The proposed model helps to advance our understanding of the effects, which occur in the population under different treatments: for example, debilitation (permanent increase in the risk of death as a result of stress-induced damage), longevity hormesis (the induction of a stress response in an individual organism, reducing the risk of death after the stress), and incomplete hormesis (the inter section of survival curves in the stressed and control groups).

## 2. Materials and methods

### 2.1. Experiments on laboratory animals

Homozygous *HER-2/neu* transgenic mice obtained from Charles River (Hollister, CA) by the Italian National Research Center for Aging were housed and bred in the Laboratory of Carcinogenesis and Aging. Mice were kept 5–7 in polypropylene cages (30×21×10 cm) under a standard light/dark regimen (12/12 h), if not exposed to a regimen treatment, at temperature 22±2 °C, and received standard laboratory chow (Anisimov et al., 2002b) and tap water ad libitum.

At the age of 2 months, 10 groups of mice were subjected to different treatments and one group served as control.

Mice in the first and second groups were subcutaneously injected with 0.1 ml of 0.9% normal saline. The first group was subjected to a course treatment—5 consecutive days every month—and the second group was subjected to a constant treatment—5 consecutive days every week.

The mice of the third and the fourth groups subcutaneously received 1.0 μg of Epitalon (synthesized tetrapeptide Ala-Glu-Asp-Gly with high biological activity) dissolved in 0.1 ml of saline. The third group was subjected to a course treatment and the fourth group to a constant treatment as described above. The Epitalon was synthesised in St. Petersburg, in the Institute of Bioregulation and Gerontology, by E.I. Grigoriev and was 99.8% pure. Treatments with saline and Epitalon are described in detail in Anisimov et al. (2002a,b).

The fifth and sixth groups were given melatonin (Sigma Chemical Co., St Louis, MO) dissolved in tap water (20 mg/l) during the night (from 18.00 to 09.00 h) five times monthly (course treatment) or five times weekly (constant treatment). Melatonin was dissolved in several drops of 96% ethanol and diluted with sterile tap water to the stated concentration. A fresh melatonin solution, which is stable in water solution for 6 months, was prepared three times a week. These two treatments are described in detail in Anisimov et al. (2003).

The seventh group was subjected to light deprivation using the methodology described by Anisimov et al. (1994). The next two groups were subjected to constant light treatment. Mice in the eighth group were exposed to electric lamps (75 W, 200 V, Russia) with illumination of 300 lux at the bottom of the cages at the distance 1.7 m. Mice in the ninth group were exposed to two luminescent lamps LB-40-2 (Russia) with illumination of 2500 lux at the bottom of cages at a distance of 1.5 m. An illumination check was performed weekly with the luxmeter U-116 (GOST-14841, Russia).

The last, 10th group, was subjected to a mixed treatment. Mice were exposed to a constant light regimen with illumination of 300 lux (as group 8) and were also given melatonin dissolved in tap water (20 mg/l) five times weekly (as group 6). Constant light regimens and mixed treatment are described in Baturin et al. (2001)

### 2.2. Statistical methods

For each experimental group, including the control group, empirical estimates of mortality rates at the age of $j$ days were calculated using the ratio

$$q_j = \frac{d_j}{n_{j-1}},$$

where $d_j$ is the number of dead mice observed during the $j$th day of life and $n_{j-1}$ is the number of mice alive at the end of the previous day. The Kaplan–Meier estimates of experimental conditional survival functions (Kalbfleisch and Prentice, 1980) were calculated as the cumulative product:

$$S_j = \prod_{i=x^*}^{j}(1-q_i), \quad x^* = 150.$$

The log-rank test statistic (Cox and Oakes, 1988) was used to test the null hypothesis that the applied treatment produced no difference in the survival of the experimental populations.

For every experimental group the Cox's regression model (Cox, 1972) was used to estimate relative risk of death under the treatment compared to the control group

$$h(t,z) = h_0(t)\exp(z\beta_k), \quad k = 1,2,\ldots,10,$$

where $h(t, z)$ and $h_0(t)$ denote the conditional hazard and baseline hazard rates, respectively, $\beta_k$ is the unknown parameter for each treatment group, and $z$ takes values 0 and 1, being an indicator variable for two samples—the control and treatment group.

Then we specified a heterogeneous mortality model for the treatment groups of each experiment, and estimated parameters of the model from the data using the maximum likelihood procedure.

### 2.3. Heterogeneous mortality model

In our case the model of heterogeneous mortality is a frailty model. For every individual in the population the risk of death is proportional to the unobserved characteristic called frailty or heterogeneity variable. We assume frailty to be gamma-distributed with mean 1 and variance $\sigma^2$. Detailed description of the model and derivation of its semi-parametric representation are given in Appendix A. The main distinctive feature of our model in comparison to other gamma frailty models (Klein, 1992; Nielsen et al., 1992) is that we assume that treatment influences parameters of both frailty distribution and baseline hazard. Denoting conditional survival function (given $x \geq x^* = 150$) for the control group as $S_c$ and survival functions for the treatment groups as $S_k$, $k=1, 2, \ldots, 10$ we can write an expression for the survival under treatment as follows (Appendix A):

$$S_k(x) = \Big(1 + r_k\gamma_k(S_c(x)^{-\sigma^2} - 1) + \gamma_k r_k \sigma^2 \frac{\alpha_k}{\beta_k}(e^{\beta_k(x-x^*)} - 1)\Big)^{-1/\gamma_k\sigma^2}.$$

One can see that the model has four unknown parameters $\alpha_k$, $\beta_k$, $r_k$, $\gamma_k$ that are specific to each experimental group and its treatment and one parameter $\sigma^2$ that is common to all groups—the frailty variance in the control group. Dependence of the baseline and treatment groups' survival patterns on the model's parameters is shown in Fig. 1.

Parameter $\sigma^2$ indicates the presence of heterogeneity in the control population. Different baseline survival patterns which can resume in the same survival function for the control group, depending on heterogeneity of the latter, are presented in Fig. 1a. If $\sigma^2 \to 0$, the control group becomes homogeneous, and $S_c \to S_0$ (Appendix A). With an increase of the frailty variance the survival function for the control group shifts to the right along the age axis with a noticeable increase of the tail.

Effects of changes in the baseline hazard, controlled by parameters $\alpha$ and $\beta$, are presented in Fig. 1b–d. If $\beta = 0$ in the additive part of hazard for the treatment group $f(x) = a\exp(\beta x)$, changes in parameter $\alpha$ reflect permanent (constant) decrease or increase of the baseline hazard, producing rectangularization or derectangularization of the survival curve, respectively, depending on whether $\alpha$ is greater or less than zero (Fig. 1b). In our study we call these effects debilitation or adaptation, depending on increase or decrease of baseline hazard. It can be seen in Fig. 1b that constant debilitation and adaptation do not influence the 'tail' of the survival curve.

Parameter $\beta$ describes the amplification or disappearance of the $\alpha$-effect, according to whether $\beta$ is greater or less than zero. For each effect small value of $\alpha$ was fixed. Vanishing debilitative and adaptive effects are shown in Fig. 1c. A decrease of negative $\beta$ draws the survival curve for the treatment group closer to the survival curve for the control group. Vanishing effects of debilitation and adaptation also do not shift the tail of survival function. Amplified debilitation and adaptation are shown in Fig. 1d. An increase of positive $\beta$ shifts the survival curve to the left along the age axis (compared to the control group) in case of amplified debilitation, and to the right in case of amplified adaptation. In both cases the tail of survival curve moves in the same direction. The shifts produced are not parallel; they resemble rotation around the initial level of debilitation or adaptation.

Effects of changes in the frailty distribution are presented in Fig. 1e–f. An increase or decrease in mean of the frailty distribution produces nearly parallel shift of the survival curve along the age axis with respective lengthening/shortening of its tail (Fig. 1e). Parameter $r < 1$ shows an increase in the average robustness, while $r > 1$ indicates an accumulation of frail individuals in the population. Parameter $\gamma \neq 1$ shows an increase ($\gamma > 1$) or decrease ($\gamma < 1$) in the population heterogeneity. These effects influence mostly the tail of survival function (Fig. 1f).

This model, unlike traditional gamma frailty model (Vaupel et al., 1979), allows us to avoid the widely used but biologically unjustified technical assumption of a parametric form for the underlying hazard. Moreover, using estimated frailty variance and survival function for the non-treated (control) group we estimate the baseline survival (from Eq. (A2) in Appendix A, see Fig. 1a). We call this representation semi-parametric because a non-parametric estimator for $S_c(x)$ (e.g. the Kaplan–Meier estimator) can be used in the representation of $S_k(x)$. In order to have a smooth curve, we approximate $S_c(x)$ using the Gamma–Gompertz model in our calculations:

$$S_c(x) = \left(1 + \sigma^2 \frac{\alpha}{\beta}(e^{\beta x} - 1)\right)^{-\frac{1}{\sigma^2}}.$$

To apply this model to the analysis of impacts produced by different treatments on the survival of transgenic mice, let us assume that the possible changes in the baseline hazard and the individuals' frailties happened during the age interval

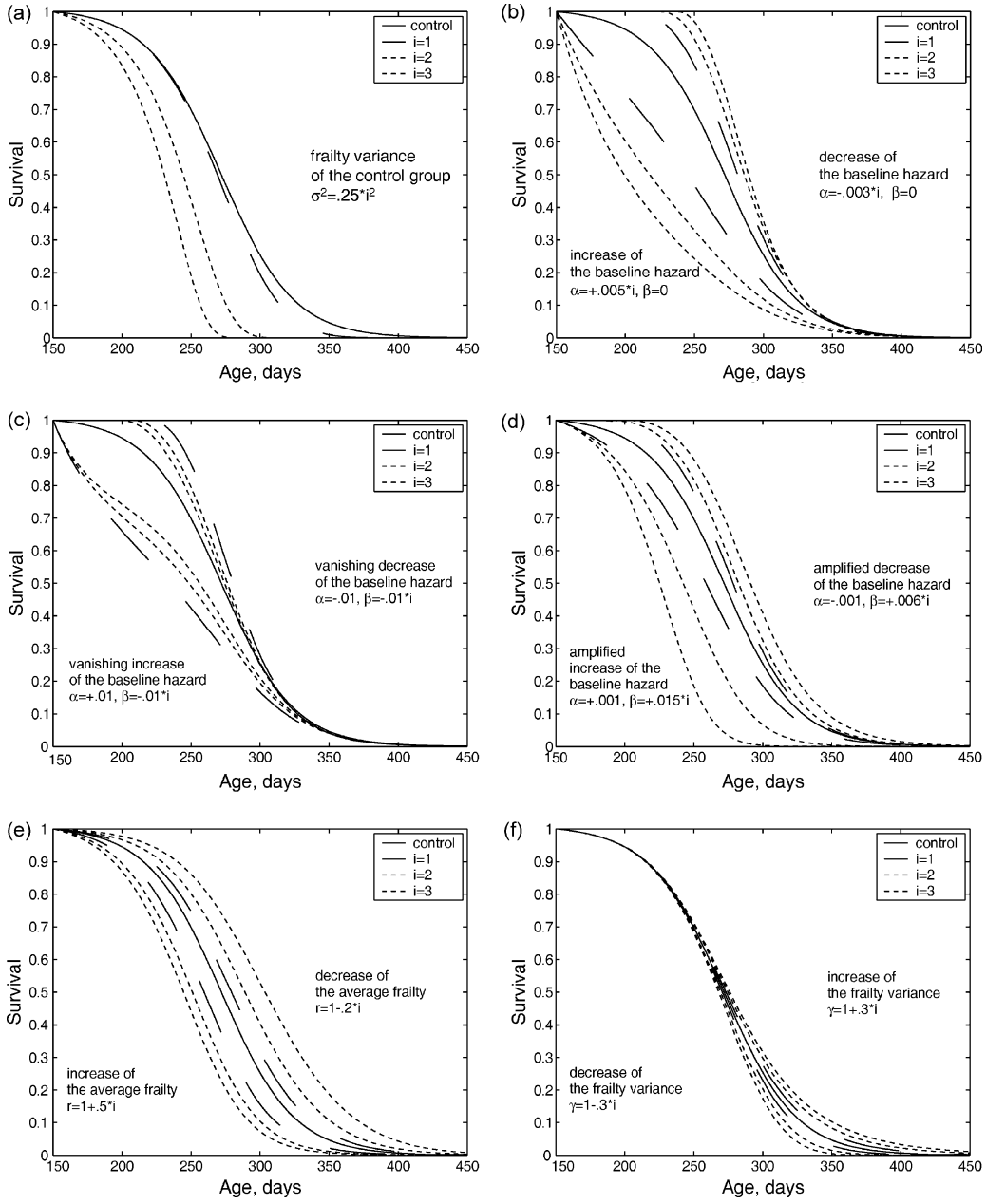


Fig. 1. Different baseline (a) and treatment groups survival patterns (b–f) depending on changes of the model's parameters.

from 60 (2 months) to 150 days. This assumption enables us to exclude from consideration the process of selection because there were no deaths observed during these 3 months of treatment either in control or in the experimental groups.

### 2.4. Parameters estimation procedure

To obtain the estimates of the model parameters for each experimental data set, the observations of life spans in all treatment groups in the experiment were used simultaneously. The maximum likelihood approach was implemented and parameters were estimated using a nonlinear optimization procedure (Fletcher, 1987). Because the structure of the data corresponds to the number of dead and alive mice during discrete time periods, log-likelihood function is derived from the binomial distribution, where binomial probabilities depend on model parameters

$$\text{Log Lik} = \sum_j (m_j\, ln(q_j) + (n_j - m_j)\ln(1 - q_j)),$$

where $m_j$ is the number of deaths on day $j$ of life, and $n_j$ is the number of individuals which were alive on day $j-1$. Values $q_j$ are related to survival functions for the stressed groups by the relationship:

$$q_j = 1 - \frac{S(j+1)}{S(j)}.$$

Confidence intervals for parameter estimates were calculated using the bootstrap method (Davison and Hinkley, 1997).

## 3. Results

### 3.1. Empirical results

Table 1 summarizes empirical estimates of conditional mean, standard deviation, minimum and maximum life span for the groups of mice subjected to different stressors and antistressors. The percentage of tumor-bearing mice is also presented.

A significant increase in average life span compared to the control group was observed in groups subjected to course saline and constant Epitalon injections, light deprivation, and constant light treatments of different illumination. Constant saline and course melatonin treatments as well as course Epitalon injections and mixed treatment produced no significant effect on average life span compared to the control group. Constant melatonin treatment led to a significant decrease in the average life span as well as in the mean life span of the last 10% of survivors. Course saline injections, light deprivation, constant light of both illuminations, and mixed treatment all significantly increase the average life span of the last 10% of survivors. The constant saline and melatonin treatments led to a decrease of average life span of the last 10% of survivors. The remaining treatments produced no significant effect on the tail of survival distribution compared to the control group.

Details concerning the effects produced by all these treatments on the development of spontaneous mammary adenocarcinomas, mean latent period, cumulative number and the number of tumors per mice, as well as up or down regulation of *HER-2/neu* gene expression has been already studied (Anisimov et al., 2002b,2003; Baturin et al., 2001).

Although this paper is dedicated to analysis of survival data, it should be noticed that the prolongation of life span associated with course saline injections, light deprivation and constant light treatment with 2500 lux illumination was accompanied by an increase in the proportion of tumor-bearing mice in the populations of transgenic mice. Constant saline treatment not only shortened the average life span of the last 10% of survivors, but also increased the proportion of mice with tumors. Course Epitalon and melatonin treatments did not influence life span but the number of tumor-bearing mice decreased in both experimental groups compared to the intact mice. Constant melatonin treatment reduced the number of mice with tumors, even though it negatively influenced the life span. The only treatment which increased life span whilst also reducing the number of tumor-bearing mice was Epitalon given subcutaneously five times every week. Constant light of 300 lux illumination and mixed treatment produced no effect on the proportion of tumor-bearing mice.

The Kaplan–Meier estimates for the survival functions in the groups of female *HER-2/neu* mice subjected to different treatments are presented in Fig. 2.

It can be seen (Fig. 2a) that light stress with subcutaneous saline injections produced hormetic effect on longevity. Course saline treatment shifted the survival curve of mice to the right along the age axis. The difference between survival distributions in this group and in the control group is significant, with a $p$-value of 0.0218. Although the $p$-value is not small enough, hormetic effect is pronounced in significance of difference between stressed and the control group in mean life span and mean life span of the last 10% of survivors. With an increase of the stress load (constant treatment) positive effect on survival vanished. Survival distributions in this group and in the control group are

Table 1
Descriptive statistics for life spans and the number of tumor-bearing mice in the groups of female *HER-2/neu* transgenic mice subjected to different treatments

| Experiment | Nb.[a] | Mean LS[b] | STD[c] | Median LS | Min LS | Max LS | Age90[d] | Mean LS10[e] | % of TBM[f] |
|---|---|---|---|---|---|---|---|---|---|
| Intact control | 30 | 281.2 (±8.1) | 44.5 | 275 | 223 | 391 | 351 | 372.0 (±11.6) | 76.7 (23) |
| Saline (5tm) | 29 | 309.4 (±10.4)* | 56.1 | 308 | 224 | 431 | 393 | 405.7 (±12.7)* | 79.3 (23) |
| Saline (5tw) | 24 | 289.3 (±9.3) | 45.7 | 300 | 190 | 360 | 340 | 350.0 (±10.0)** | 87.5 (21) |
| Epitalon (5tm) | 25 | 270.6 (±8.5) | 42.5 | 270 | 180 | 376 | 331 | 347.3 (±14.4) | 72.0 (18) |
| Epitalon (5tw) | 24 | 327.7 (±6.6)* | 32.2 | 320 | 290 | 410 | 380 | 395.0 (±15.0) | 54.2 (13)** |
| Melatonin (5tm) | 27 | 270.6 (±7.9) | 41.2 | 266 | 212 | 376 | 314 | 354.3 (±20.2) | 66.7 (18) |
| Melatonin (5tw) | 22 | 244.1 (±9.4)** | 44.0 | 249 | 165 | 327 | 296 | 313.5 (±13.5)** | 59.1 (13) |
| Light deprivation | 24 | 321.2 (±11.5)* | 56.1 | 317 | 230 | 473 | 418 | 452.0 (±21.0)* | 83.3 (20) |
| 300 lux | 28 | 320.4 (±16.0)* | 84.8 | 310 | 198 | 657 | 402 | 494.0 (±81.7)* | 75.0 (21) |
| 2500 lux | 28 | 361.3 (±24.2)* | 128.3 | 316 | 260 | 737 | 565 | 675.7 (±55.4)* | 96.4 (27)* |
| 300 lux + mlt (5tw) | 27 | 288.1 (±11.4) | 59.5 | 277 | 174 | 397 | 394 | 395.0 (±1.0)* | 77.8 (21) |

*Significant increase ($p$-value < 0.01); **significant decrease ($p$-value < 0.01).
[a] Nb.: number of mice.
[b] LS: life span.
[c] STD: standard deviation.
[d] Age90: age at which 90% of the population is dead.
[e] LS10: life span of the last 10% of survivors.
[f] TBM: tumor-bearing mice.

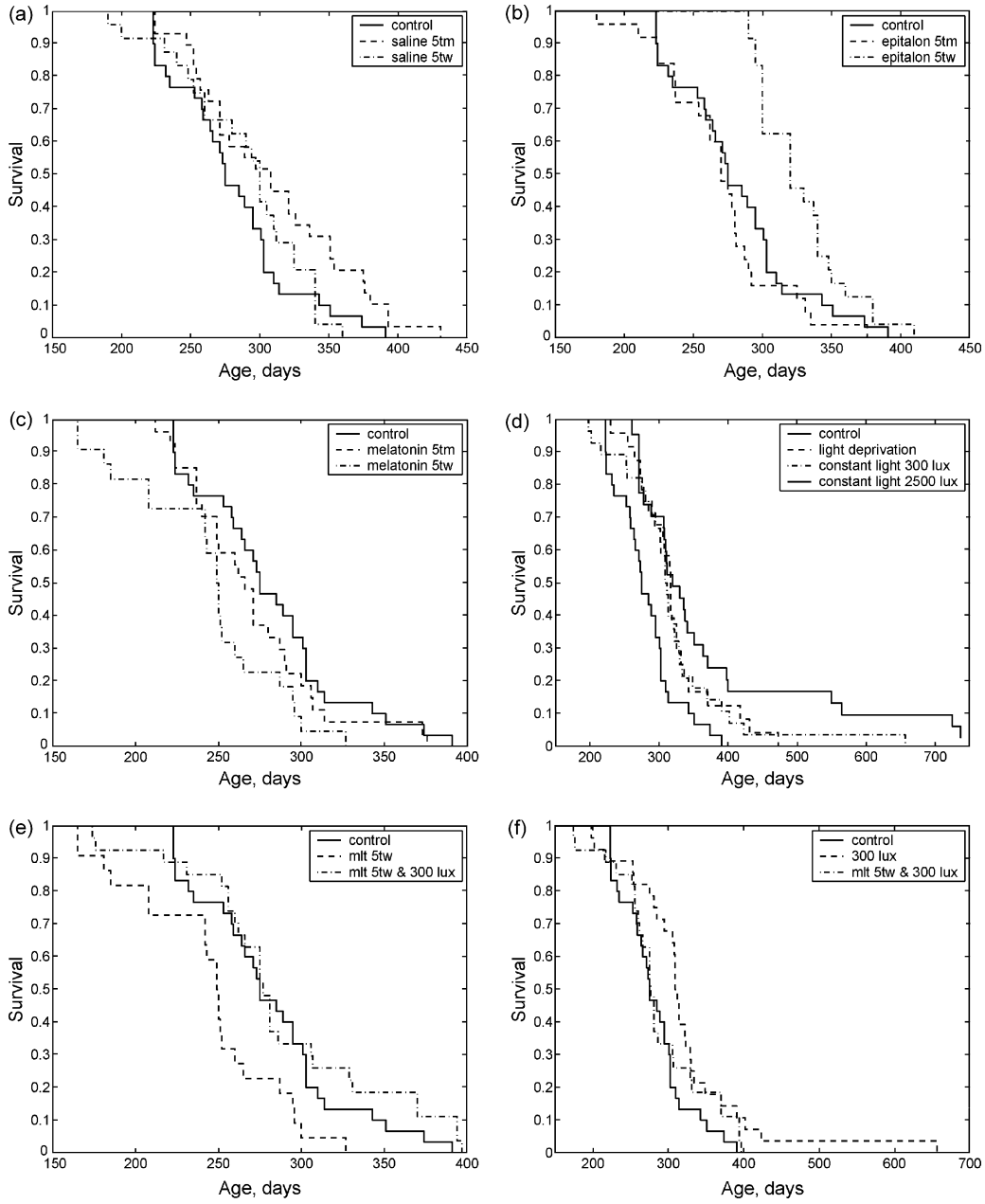


Fig. 2. Kaplan–Meier estimates for the survival functions of female *HER-2/neu* transgenic mice subjected to different treatments, grouped by type of treatment, and compared to the control population.

identical, with probability 0.606, although survival curve in the stressed group has a significantly shorter tail.

A low dosage of antistressor Epitalon (course treatment) produced no effect on life span; the difference between this and the control group is insignificant ($p$-value is equal to 0.356). Constant treatment produced a rectangularization of the survival curve with a slight increase in the mean life span of the last 10% of survivors (Fig. 2b, Table 1). According to the log-rank test, the difference in survival distributions is significant ($p$-value equals to 0.0024).

Melatonin, which is said to be antistressor, produced no significant effect on survival ($p$-value equals to 0.335) at the lower dosage (course treatment), moreover survival for this group is actually slightly lower than in the control group (Fig. 2c). Constant melatonin treatment shifted the survival curve of the mice to the left along the age axis compared to the control group; this difference between empirical survival functions is significant ($p$-value is equal to 0.00455). As described in Anisimov et al. (2003), the adverse effect of melatonin on life span may be unique to the transgenic model used.

Changes in light/dark regimen to dark/dark or light/light are considered to be stressful. But all these treatments produced a hormetic effect on the longevity of these *HER-2/neu* mice (Fig. 2d). Survival functions for stressed groups are shifted to the right along the age axis compared to

the control group. $p$-values for the difference between stressed and the intact group are 0.00791 for light deprivation, 0.00978 for constant light 300 lux, and 0.000611 for the constant light 2500 lux. The tails of survival functions under constant light treatment lengthened dramatically.

Fig. 2e and f represents survival for the group subjected to the mixed treatment with a stressor (constant light regimen with illumination of 300 lux) and antistressor (melatonin five times weekly) compared to survival under anti stress-treatment (Fig. 2e), stress-treatment (Fig. 2f), and to the control group. According to the log-rank test, the difference between survivals under mixed treatment and the control group is insignificant ($p$-value equals to 0.314); survival function for the treatment group nevertheless has a significantly longer tail than that of the control group. It seems that the hormetic effect on longevity produced by constant light regimen compensated for the harmful effect of melatonin, but on the other hand, constant light promoted development of mammary adenocarcinomas, which we assume was suppressed by melatonin treatment. The role of melatonin in mixed treatment is to return survival values and the spontaneous tumorigenesis to the level of the control group, which otherwise would be increased under the constant light regimen.

### 3.2. Modeling results

#### 3.2.1. Cox's regression

Estimated parameter values of the Cox's proportional hazard model as well as relative risk, standard errors, and $p$-values for different treatment groups are presented in Table 2.

One can see from this table that course saline, constant epitalon treatments, light deprivation and constant light regimen decreased significantly the relative risk of death in *HER-2/neu* transgenic mice. Constant melatonin treatment significantly increased the relative risk of death, while effects of the other treatments were estimated as non-significant. However, it is shown in Table 1 that constant saline treatment shortened significantly the average life span of the last 10% of survivors and mixed treatment prolonged life of long living individuals.

Table 2
Parameter estimates of the Cox's regression model for the groups of female *HER-2/neu* mice subjected to different treatments

| | $\beta$ | exp($\beta$) | se($\beta$) | $p$ |
|---|---|---|---|---|
| Saline 5tm | −0.621 | 0.537 | 0.276 | 0.024 |
| Saline 5tw | −0.114 | 0.892 | 0.282 | 0.69 |
| Epitalon 5tm | 0.248 | 1.28 | 0.276 | 0.37 |
| Epitalon 5tw | −0.79 | 0.454 | 0.282 | 0.0051 |
| Melatonin 5tm | 0.253 | 1.29 | 0.269 | 0.35 |
| Melatonin 5tw | 0.815 | 2.26 | 0.298 | 0.0062 |
| Light deprivation | −0.75 | 0.472 | 0.290 | 0.0095 |
| 300 lux | −0.711 | 0.491 | 0.277 | 0.01 |
| 2500 lux | −0.95 | 0.387 | 0.288 | 0.00097 |
| 300 lux + mlt 5tw | −0.273 | 0.761 | 0.277 | 0.32 |

Table 3
Fit of different models to the experimental survivals

| | $r, \sigma^2$ | $\alpha, r, \sigma^2$ | $\alpha, \beta, r, \gamma, \sigma^2$ |
|---|---|---|---|
| −Log Lik | 1375.18 | 1369.71 | 1334.57 |
| $p$-value | $2.22\times10^{-6}$ | $3.17\times10^{-7}$ | |

#### 3.2.2. Heterogeneous mortality model

In order to describe effects produced by treatments on frailty distribution and baseline hazard, several specifications of heterogeneous mortality model were considered. The first one deals with effects such as increase of average robustness or accumulation of frail individuals in the population. In the second, changes in mean frailty are accompanied by debilitative or adaptive effect. The third takes into account the opportunity of changes in population heterogeneity during the treatment in addition to debilitation or adaptation and changes of the mean of the frailty distribution. All models are nested, so respective hypotheses were tested using the likelihood ratio statistics. Table 3 summarizes the results of the fit of different models to experimental data sets. For all experimental survival functions, the best model, according to the likelihood ratio test, corresponds to debilitation (or adaptation), changes in average frailty and heterogeneity.

Parameters of the model for all experiments are presented in Table 4. Estimated parameters of the Gamma–Gompertz survival for the control group are: $\alpha = 4\times10^{-4}$ ($3\times10^{-4}$, $5\times10^{-4}$), $\beta = 3.7\times10^{-2}$ ($3.5\times10^{-2}$, $3.8\times10^{-2}$), and $\sigma^2 = 9.3\times10^{-1}$ ($9.2\times10^{-1}$, $9.4\times10^{-1}$).

One can see (Table 4) that parameter $\sigma^2$, common to all groups, significantly greater than zero. This confirms that the observed populations are heterogeneous. Fig. 3 shows estimates for conditional baseline hazard and survival function for female *HER-2/neu* transgenic mice. It can be seen from the lengthening of the tail of survival curve for the control group, compared to the baseline survival (Fig. 3a), that the control population of mice contained some robust individuals, whose chances of survival were higher. Due to heterogeneity of the control population we observed the leveling off of the hazard rates at advanced ages (Fig. 3b). It can also be seen that the baseline hazard deviates from the Gompertz law and decelerates with age.

Further interpretation of the estimated parameter values of the heterogeneous mortality model gives us an insight into the differences in effects produced by the stressors and antistressors in population of female *HER-2/neu* transgenic mice. The fit of the model to each experimental data set is shown in Fig. 4.

Both saline treatments produced amplified debilitation (parameters $\alpha$ and $\beta$ are greater than zero) and increased robustness (parameter $r$ is less than one). Course treatment made the population slightly less heterogeneous (parameter

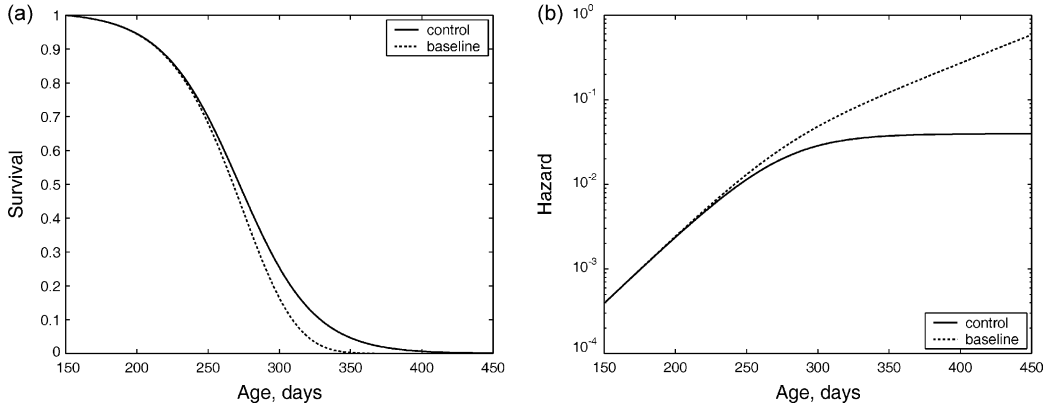


Fig. 3. Estimated conditional baseline hazard and survival function for female *HER-2/neu* transgenic mice, compared to the control group.

$\gamma$ is less than one), while constant treatment made it slightly more heterogeneous (parameter $\gamma$ is greater than one). Debilitation and its amplification are smaller in the group subjected to saline five times monthly than in the group subjected to saline constantly. Saline treatment made both groups less frail on average, compared to the control group. Mice who received the higher dose of saline became more robust on average than the mice who received lower dose treatment. Because of smaller debilitation and increased homogeneity, the mice subjected to the course saline injections demonstrated higher survival values than mice subjected to the constant treatment.

Different treatments with Epitalon produced different effects on survival. The population of mice subjected to the course injections experienced amplified debilitation, it became more frail in average and slightly more homogeneous. The population subjected to the constant treatment experienced vanishing adaptation, became more robust on average, and slightly more heterogeneous. Because of adaptation and increased robustness, the mice constantly treated with Epitalon had significantly higher chances of survival compared to the course treated and the control group.

Melatonin given five times monthly produced vanishing adaptation, slightly increased robustness, and made the population slightly more homogeneous; given five times weekly it produced vanishing debilitation, significantly increased the average frailty of the population, and made the population significantly more homogeneous. That explains why mice exposed to constant treatment with melatonin had lower chances of survival.

The hormetic effect of three regimen treatments (dark/dark, light/light with different illumination) consists of vanishing adaptation, a significant increase in average robustness, and a significant increase in population heterogeneity. The group exposed to a constant light regimen with an illumination of 300 lux became less robust on average than the light-deprived group, but it became more heterogeneous and this explains the lengthening of the tail of survival distribution in this group. Constant light regimen with illumination of 2500 lux produced a greater adaptation effect than any other treatment applied. This group became the most robust on average and the most heterogeneous of all the others. The combination of adaptation, increased robustness and heterogeneity ensured the highest survival values in mice subjected to this treatment.

Mixed treatment (constant light and melatonin) produced vanishing adaptation, increased robustness and increased population heterogeneity. Because of these effects, we can

Table 4
Parameter estimates of the heterogeneous mortality model for the groups of female *HER-2/neu* mice subjected to different treatments

| | $\alpha\times10^{-3}$ | $\beta\times10^{-3}$ | $r$ | $\gamma$ |
|---|---|---|---|---|
| Saline 5tm | 0.31(0.28, 0.32) | 1.4(1.3, 1.5) | 0.35(0.29, 0.41) | 0.934(0.93, 0.94) |
| Saline 5tw | 4.2(3.9, 4.6) | 31(28, 35) | 0.06(0.04, 0.08) | 1.0013(1.001, 1.002) |
| Epitalon 5tm | $2.9\times10^{-8}$($2.6\times10^{-8}$, 3. $2\times10^{-8}$) | 101(77, 121) | 1.14(1.12, 1.15) | 0.95(0.94, 0.96) |
| Epitalon 5tw | −34.5(−40, −30) | −1.6(−1.8, −1.4) | 0.4(0.3, 0.5) | 1.0011(1.001, 1.0012) |
| Melatonin 5tm | −70.1(−82, −57) | −63(−71, −54) | 0.958(0.95, 0.96) | 0.954(0.95, 0.96) |
| Melatonin 5tw | 1.5(1.4×10, 1.7) | −28(−30, −26) | 1.8(1.7, 2.1) | 0.56(0.53, 0.58) |
| light deprivation | −14(−15, −13) | −16(−18, −15) | 0.37(0.29, 0.49) | 1.65(1.58, 1.71) |
| 300 lux | −0.2(−0.23, −0.17) | −49(−54, −45) | 0.41(0.33, 0.51) | 2.2(1.91, 2.35) |
| 2500 lux | −480(−512, −455) | −30(−35, −31) | 0.023(0.022, 0.027) | 8.2(7.7, 8.5) |
| 300 lux and melatonin 5tw | −1.2(−1.3, −1.1) | −4529(−4892, −4217) | 0.83(0.81, 0.84) | 1.97(1.85, 2.17) |
| | $\sigma^2$=0.39 (0.37, 0.41) for all groups | | | |

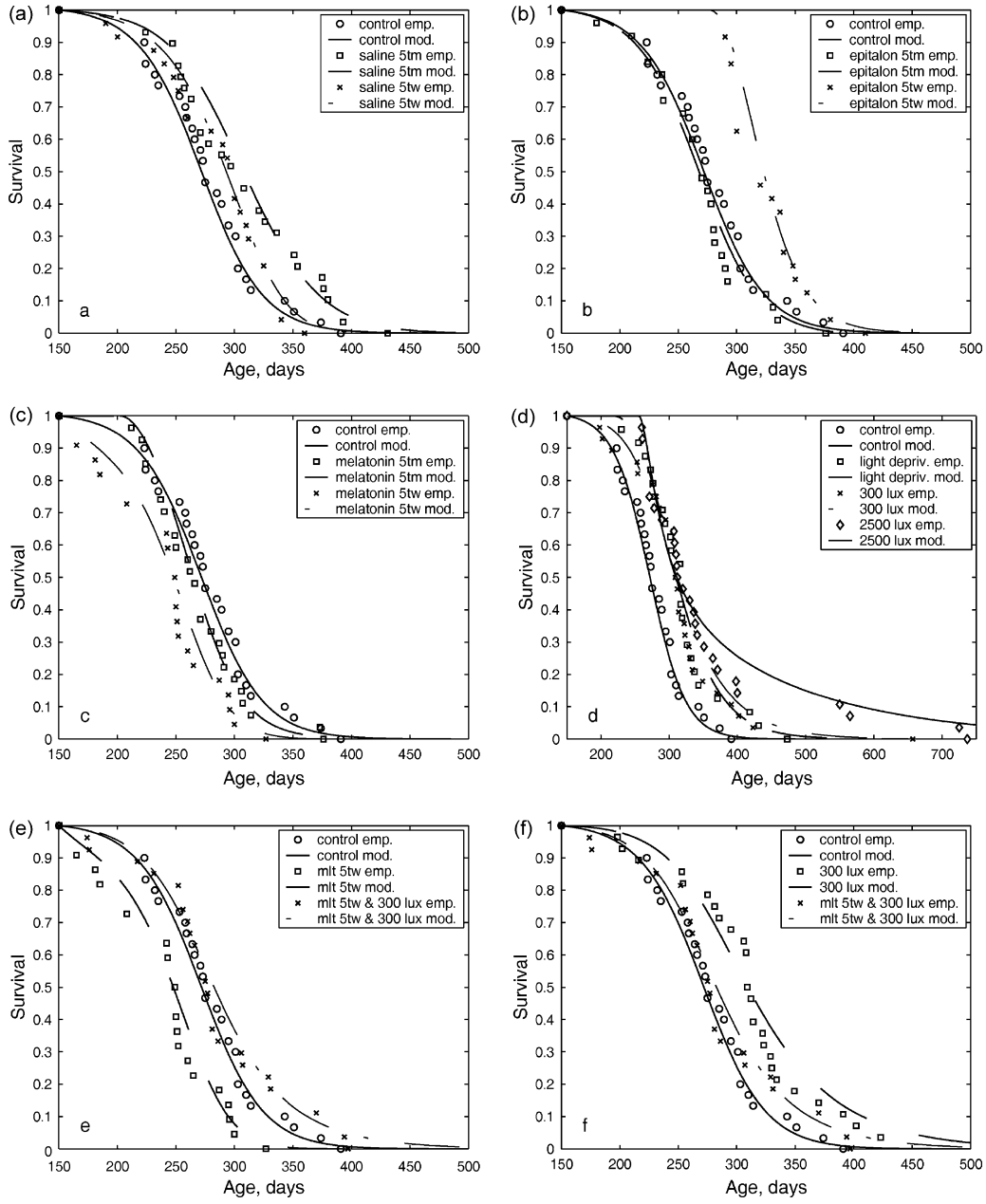


Fig. 4. Empirical and modeled survival functions of female *HER-2/neu* transgenic mice subjected to different treatments, grouped by type of treatment, and compared to the control population.

observe an intersection of survival curves for the experimental and control groups (incomplete hormesis).

Now let us take a look at the effects, which explain the significant increase in life span produced by the different treatments. Empirical analysis showed that course saline, constant Epitalon, light deprivation and constant light treatments all changed the survival distribution of transgenic *HER-2/neu* mice in a very similar way. Estimated parameter values for the first group indicate the presence of amplified debilitation, increased robustness, and increased homogeneity for the population of mice subjected to the course saline injections. To be precise, debilitation prevents the survival function from being rectangular. The model of heterogeneous mortality describes the effects in the last three groups as vanishing adaptation, increased robustness and increased heterogeneity. Greater heterogeneity led to the lengthening of the tail of the survival function. Greater adaptation made the survival curve more rectangular. Constant saline and mixed treatments affected the life span of the last 10% of survivors differently because the former treatment produced amplified debilitation, whereas the latter led to an adaptation, albeit a vanishing one. Survival values in the group exposed to constant saline treatment are greater than in the control group in the age

interval from 225 to 340 days of life (Fig. 2a) because of significantly increased average robustness. The survival function for this group failed to have a long tail because of insufficient heterogeneity. The survival curve in the group subjected to the mixed treatment comes very close to the survival curve in the control group between ages of 260 and 310 days of life (Fig. 2e and f) because of an insufficient increase in robustness. A significant increase in heterogeneity ensures a long tail for the survival function in the experimental group subjected to the constant light and melatonin treatments. Empirical analysis revealed no significant difference between the survivals in the groups subjected to course Epitalon and melatonin treatments. According to the model both groups became less heterogeneous. Exposure to Epitalon made mice slightly more frail on average in addition to leading to an amplified, though small, debilitation, whilst exposure to melatonin produced vanishing adaptation and made mice slightly more robust.

## 4. Discussion

According to the free radical theory of aging, free radicals are involved in the production of changes in cellular metabolism that lead to a time-dependent functional decline in all living beings. Consequently, antioxidants and/or free radical scavengers may retard the aging process.

Given the antioxidant and free radical scavenger properties of melatonin, it can be seen that this hormone prevents oxidative damage of tissues and slows down the process of aging. It was shown by Bonilla et al. (2002) that melatonin, added daily to the nutrition medium at a concentration of 100 μg/ml, significantly increased the life span of *Drosophila melanogaster* (Oregon wild strain). Furthermore, it increased the resistance of flies to paraquat and to an ambient temperature of 36 °C. It has also been shown that treatment with the pineal indole hormone melatonin inhibits the development of mammary gland tumorigenesis both in vitro and in vivo (Blask, 1993; Musatov et al., 1999; Cos and Sanchez-Barcelo, 2000; Bartsch et al., 2001). It has been shown that melatonin increases both life span and tumor incidence in female CBA mice (Anisimov et al., 2001b). In *HER-2/neu* transgenic mice, comparison with the control group showed that treatment with melatonin slowed down age-related disturbances in estrous function, decreased the incidence and size of mammary adenocarcinomas, and the incidence of lung metastases (Anisimov et al., 2003). Polycystic kidney disease is common in this transgenic line. The adverse effect of constant melatonin treatment on life span in these experiments (debilitation, increased average frailty and homogeneity) may be unique to the transgenic model used.

It was recently shown that Epitalon increased the life span in two strains of fruit flies and in female *CBA* mice, and inhibits the spontaneous tumorigenesis in mice (Khavinson et al., 2000; Mylnikov, 2000; Anisimov et al., 2001a). The inhibitory effect of Epitalon in the development of spontaneous mammary tumors in *HER-2/neu* mice was shown in Anisimov et al. (2002b). In the present study we showed that, depending on dosage, Epitalon can either decrease maximum life span of mice—by debilitation and accumulation of frail individuals in the population—or significantly increase chances of survival in transgenic mice—because of adaptation, increase of average robustness and heterogeneity.

An inhibition of the pineal function with the exposure to the constant light regimen stimulates mammary carcinogenesis, whereas the light deprivation inhibits the carcinogenesis (see Anisimov (2002, 2003), for the review). The influence of visible light and constant darkness on the life span of *D. melanogaster* (Oregon R) males was investigated by Massie and Whitney (1991) and Massie et al. (1993). It was shown that a reduction of illumination significantly increased survival. Even dim light (65 lux) affected life span in a negative manner. Fruit flies exposed to constant darkness lived 43.2% longer than those exposed to constant light at a light intensity of 2000 lux. In our study, we observed prolongation of life span in *HER-2/neu* mice subjected to light deprivation and those subjected to constant light treatments with both illuminations: this is probably due to the specificity of transgenic model used. We attributed the longevity hormesis in these cases to adaptation, increased robustness and increased variability with respect to the individuals' frailty.

Li and Xu (1997) studied the influence of light/dark shift manipulations and melatonin treatment on immune function, oncogenicity and the life span of rats, mice and fruit flies. They concluded that the alternating photoperiod is stressful for all species considered. Moreover, the life span of fruit flies was shortened by photoperiodic shifting. They also showed that melatonin treatment counteracted the deleterious influences of photoperiodic shifting in the above animals. Experiments performed by Natelson et al. (1996, 1997) and on cardiomyopathic hamsters (CMHs) showed that animals live longer if they spend their lives in an environment devoid of time cues (in constant light or other non-24-h light–dark cycles). Authors also suggested that inhibition, rather than stimulation, of pineal function might be beneficial for those with congestive heart failure. Since our observations with *HER-2/neu* mice were the detrimental effect on survival of constant melatonin treatment and the hormetic effect of constant light regimen, we conclude that in the mixed treatment the positive effect of the stressor counteracted the negative effect of geroprotector. The cumulative effect of both treatments appeared to be a slight increase in average robustness, a strong increase in population heterogeneity, and adaptation.

Since living organisms are exposed to stresses of different kind during their lives, they have developed various strategies to cope with them. Evolution has seen the development of a resistance to stress that is often related to

longevity (Parsons, 1996). This leads to the hypothesis that the stress response may also counteract the negative effects of aging, and that inducing a stress response by exposing organisms to mild stress may help them to live longer (Yashin et al., 2001a). Mild stress has been reported to increase longevity (Neafsey, 1990; Le Bourg and Minois, 1999; Minois, 2000; Verbeke et al., 2001; Hercus et al., 2003); iradiation, heat and cold shock, starvation, desiccation, hypergravity, and exercise are some examples of the stress studied. The present study included the first investigation of the influence of different saline treatments on survival of transgenic mice. We observed a significant increase in longevity among the mice subjected to course saline injections and a decrease in mean life span of the last 10% of survivors under constant treatment. According to our calculations, a greater dose of stressor produced greater debilitation.

It is now apparent that environmental stress does more than eliminate the weakest individuals from the population and thereby altering the mortality patterns of the surviving population (Yashin et al., 1996,2001b; Michalski and Yashin, 2002). The mechanism whereby stresses increase longevity has not yet been elucidated. However, the studies conducted so far do show that it may involve metabolic regulation and induction of stress proteins. We presume that the longevity hormesis observed in our experiments can be explained by the inhibiting effect of glucocorticoids on kidney pathology, which is common in the *Her-2/neu* transgenic line. But neither the data itself nor the modeling results give an exact answer to the question about survival mechanisms of individuals who live long even after a severe stress. More studies of the biological nature of stress response are needed to address this important question.

The Gompertz model (Gompertz, 1825) was previously used to analyze survival in the experiments discussed. Its parameters are associated with the rate of aging and initial mortality, but these associations are biologically unjustified. It is well documented that mortality rates for humans (Strehler and Mildvan, 1960; Vaupel et al., 1979; Manton and Stallard, 1984), as well as for laboratory animals (Finch et al., 1990; Curtsinger et al., 1992; Carey et al., 1992; Fukui et al., 1996), decelerate at advanced ages and deviate from the Gompertz law. The theoretical challenge is to understand how different effects combine to produce post-stress survival patterns (Boxenbaum, 1991; Yakovlev et al., 1993; Lithgow et al., 1994, 1995). The application of sophisticated mathematical models advances our understanding of biological phenomena as they appear at both individual and population levels. The Cox's regression model (Cox, 1972) is a method of choice in the case of observed covariates. When it is impossible to observe covariates, the specification for two-sample problem allows to estimate relative risk of death in the treatment group compared to the control population. Application of a frailty model in this case is appropriate (Vaupel et al., 1979). Our specification of model allows to estimate baseline survival function, heterogeneity of the control group and possible influences of the treatment on the frailty distribution and baseline hazard.

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## Appendix A

### *A1. Heterogeneous mortality model*

Let $T$ and $Z$ be the life span and the heterogeneity (frailty) variable such that the conditional hazard of death given $Z$ is $Zh_0(x)$, where $h_0(x)$ is the underlying hazard (Vaupel et al., 1979). Let us assume that frailty $Z$ is gamma $(k, \lambda)$ distributed with mean 1 and variance $\sigma^2$, i.e. $k=\lambda$, and $\sigma^2=(1/\lambda)$. Let $H(x)=\int_0^x h_0(u)\mathrm{d}u$ be the cumulative underlying hazard. Then the observed mortality $\bar{h}(x)$ is:

$$\bar{h}(x)=\frac{h_0(x)}{1+\sigma^2 H(x)}. \tag{A1}$$

The marginal survival function $S(x)$ is:

$$S(x)=\left(1+\frac{1}{\lambda}H(x)\right)^{-k}=(1+\sigma^2 H(x))^{-1/\sigma^2}. \tag{A2}$$

In the case of homogeneous population $\sigma^2=0$, expression (A1) transforms into $\bar{h}(x)=h_0(x)$. Using the L'Hospital's rule, it is easy to show that $S(x)\to\exp(-H(x))=S_0(x)$, when $\sigma^2\to 0$ in Eq. (A2).

In our further calculations, we will follow the methodology for the analysis of data from the stress experiment suggested by Yashin et al. (1996). The application of this model to the analysis of post-stress survival of *D. melanogaster* flies is described in Semenchenko et al. (2004).

Let us consider two identical heterogeneous populations whose chances of survival correspond to the proportional hazards model and assume that the initial frailties are gamma-distributed with means 1 and variances $\sigma_1^2, \sigma_2^2$. The first population—the control group—experiences standard living conditions without any interventions and the second is subjected to some treatment at the age interval $[x_0,x^*]$. To compare the survival functions after age $x^*$ in the experimental and in the control group let us assume that in the control group the underlying hazard $h_{01}(x)$ does not change and in the experimental cohort the underlying hazard $h_{02}(x)$ increases at the interval $[x_0, x^*]$ and that after age $x^*$ it is $h_{02}(x)=h_0(x)+f(x)$. Note that if $f(x)\equiv 0$ the underlying hazard returns to its standard level, a negative $f(x)$ manifests the presence of adaptive effect, and a positive represents debilitative effects. It follows from Eq. (A2) that the marginal survival functions $S_i(x)$, $i=1, 2$ for those who

survived age $x^*$ are:

$$S_i(x) = \left(1 + \frac{1}{\lambda_i^*} H_i^*(x)\right)^{-k_i}, \quad i = 1, 2, \tag{A3}$$

where

$$\lambda_i^* = 1/\sigma_i^2 + H_i(x^*), H_i(x^*) = \int_0^{x^*} h_{0i}(u)\, \mathrm{d}u,$$

$$H_1^*(x) = \int_{x^*}^{x} h_0(u)\, \mathrm{d}u,$$

$$H_2^*(x) = \int_{x^*}^{x} h_0(u)\, \mathrm{d}u + F(x), \quad F(x) = \int_{x^*}^{x} f(u)\, \mathrm{d}u,$$

and

$$k_i = 1/\sigma_i^2,\ i = 1, 2.$$

Let us assume that under normal living conditions an individual's susceptibility to death does not change during its life and that any exogenous intervention can increase or decrease an individual's frailty.

Note that in the control population even the 'natural' selection process does not change the shape parameter $k_1 = 1/\sigma_1^2$ of the frailty distribution. If the application of treatment does not influence an individual's frailty, this parameter also does not change in the experimental cohort, i.e. $k_2 = 1/\sigma_2^2$.

Let us assume that $\sigma_1^2 = \sigma_2^2 = \sigma^2$ and that the application of a treatment can also change the shape parameter of the frailty distribution by a factor $\gamma$, i.e. $k_2 = 1/\gamma\sigma^2$. Changes in frailty variance reflect non-linear changes in population heterogeneity: this can occur, for example, when weak individuals become weaker, robust individuals increase their robustness, and so on. So, for the survival in the control cohort after age $x^*$, one can write:

$$S_1(x) = \left(1 + \frac{k_1^*}{l_1^*}\sigma^2 H_1^*(x)\right)^{-k_1^*}$$

$$= (1 + m_1^* \sigma^2 H_1^*(x))^{-1/\sigma^2} \tag{A4}$$

and for the survival in the experimental cohort ($x > x^*$)

$$S_2(x) = (1 + m_2^* \gamma\sigma^2 H_2^*(x))^{-1/\gamma\sigma^2} \tag{A5}$$

where $m_1^*$ and $m_2^*$ are the mean values of the frailty distribution in the control and in the experimental populations at age $x^*$, respectively, and $\gamma$ is the factor which shows the presence of changes in the frailty distribution that are not associated with changes of average frailty in the population during the treatment.

Note further that it follows from Eq. (A4) and from the definition of $F(x)$ that

$$H_2^*(x) = \frac{S_1(x)^{-\sigma^2} - 1}{\sigma^2 m_1^*} + F(x). \tag{A6}$$

Replacing $H_2^*(x)$ in Eqs. (A5) with (A6) we obtain the following equation for the survival $S_2(x)$, ($x > x^*$) in the experimental group

$$S_2(x) = (1 + r\gamma(S_1(x)^{-\sigma^2} - 1) + m_1^* r\gamma\sigma^2 F(x))^{-1/\gamma\sigma^2} \tag{A7}$$

with $r = m_2^*/m_1^*$. In our calculations we use $f(x) = a\, \mathrm{e}^{\beta(x-x^*)}$. Denoting $\alpha = a m_1^*$, Eq. (A7) can be rewritten as:

$$S_2(x) = \left(1 + r\gamma(S_1(x)^{-\sigma^2} - 1) + \gamma r\sigma^2 \frac{\alpha}{\beta}(\mathrm{e}^{\beta(x-x^*)} - 1)\right)^{-1/\gamma\sigma^2} \tag{A8}$$

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