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Multiple Mild Heat-Shocks Decrease The Gompertz Component of Mortality in *C. elegans*

Deqing Wu^{*,#}, James R. Cypser^{*}, Anatoli I. Yashin, and Thomas E. Johnson^{*,†}

^{*} Institute for Behavioral Genetics, University of Colorado at Boulder, Box 447, Boulder, CO 80309, USA

[†] Department of Integrative Physiology, University of Colorado at Boulder, Box 354, Boulder, CO 80309, USA

Abstract

Exposure to mild heat-stress (heat shock) can significantly increase the life expectancy of the nematode *Caenorhabditis elegans*. A single heat-shock early in life extends longevity by 20% or more and affects life-long mortality by decreasing initial mortality only; the rate of increase in subsequent mortality (Gompertz component) is unchanged. Repeated mild heat-shocks throughout life have a larger effect on life span than does a single heat-shock early in life. Here we ask how multiple heat-shocks affect the mortality trajectory in nematodes and find increases of life expectancy of close to 50% and of maximum longevity as well. We examined mortality using large numbers of animals and found that multiple heat-shocks not only decrease initial mortality, but also slow the Gompertz rate of increase in mortality. Thus, multiple heat-shocks have anti-aging hormetic effects and represent an effective approach for modulating aging.

Keywords

heat-shock; Gompertz; mortality; *C. elegans*; hormesis; caloric restriction; stress; stressors; initial mortality; demography; aging; longevity; life span; anti-aging

1. INTRODUCTION

A mild heat shock is an effective way to induce an increase in longevity in a widely-recognized environmental intervention that we have termed heat-induced hormesis (Cypser et al, 2006). In early studies, we measured the effect of single heat shocks on life expectancy. In 1995, Lithgow and colleagues reported that exposures of a few hours to elevated levels of heat resulted in about a 15% increase in the mean life span of the nematode *Caenorhabditis elegans*, compared to non-heat-shocked controls (Lithgow et al, 1995). Subsequently, a single mild heat shock was observed to extend longevity in several species, including *C. elegans* (Butov et al., 2001; Michalski et al., 2001; Yashin et al., 2001; Yashin et al., 2002, Cypser and Johnson, 2002; Cypser and Johnson, 2003; Rea et al, 2005; Wu et al, 2008), several species of flies: *Drosophila melanogaster* (Khazaeli et al, 1997; Le Bourg et al, 2001; Hercus et al,

#To whom correspondence should be addressed: Deqing.wu@gmail.com, phone: (303) 960-3916, fax: (919) 684-3861.

Current address of Deqing Wu: Center for Population Health and Aging, Duke University, Box 90408, 002 Trent Hall, Durham, NC 27708

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2003), *D. buzzatii* and *D. koepferae* (Scannapieco et al, 2007), as well as in the yeast *Saccharomyces cerevisiae* (Shama et al, 1998; Swiecilo et al, 2000). In *C. elegans*, and *D. melanogaster*, multiple heat-shocks throughout life extend longevity even more than does a single mild heat treatment early in life (Hercus et al, 2003; Olsen et al, 2006). Multiple heat-shocks have been reported to delay the onset of several age-related changes and to have anti-aging hormetic effects on growth and various cellular and biochemical characteristics of human skin fibroblasts *in vitro* (Rattan 1998; Verbeke et al, 2000; 2001; 2002; Rattan et al, 2004; Beedholm et al, 2004; Rattan, 2005; Rattan, 2008).

Here we assess age-specific mortality, which has major advantages over simple assessment of life expectancy as a measure of aging: 1) there is an age interval where mortality increases exponentially with chronological age in most species, including humans, 2) mortality captures information about the entire life span distribution, 3) mortality responds instantaneously to interventions (Mair et al, 2003; Partridge et al, 2005), 4) it is central to many definitions of aging (Finch, 1990; Vaupel et al, 1998; Carey, 2003) and 5) it is altered by many interventions that extend life, especially by genetic alterations in *C. elegans* (Johnson, 1987; 1990; Vaupel et al, 1998; Johnson et al, 2001).

A few studies of the effect of single heat shock have used demographic approaches, in that the populations were large enough to measure age-specific mortality, rather than merely life expectancy. Khazaeli et al (1997) investigated age-specific mortality of 28,000 *Drosophila melanogaster* adults after a mild heat stress, and found that life expectancy was extended as a demographic consequence of reduced age-specific mortality over a period of several weeks after the heat treatment. In *C. elegans*, both hormesis after short exposure to heat shock and debilitation after long exposure were observed. In order to describe these survival patterns, Michalski et al (2001) constructed a biphasic survival model with Gamma-distributed frailty; Butov et al (2001) proposed a stochastic model of stress which describes the accumulation of damage in the cells of the worm as the worm ages; and Yashin et al (2001) developed an adaptation model of stress. Later, Yashin et al (2002) proposed a discrete heterogeneity model in which isogenic populations of *C. elegans* consist of three subpopulations with distinct Gompertz mortality patterns (Gompertz, 1825). They concluded that the hormetic effect of a single mild heat shock could be explained by the transfer of worms from one subpopulation to another. We investigated age-specific mortality following a single mild heat shock in a large population of worms, and found that the hormetic effect on longevity is driven by a decrease of initial mortality only (Wu et al, 2008).

Although multiple heat-shocks were reported to extend longevity (Hercus et al, 2003; Olsen et al, 2006), no demographic studies of the effects of multiple heat shocks on mortality have been reported. We previously demonstrated that a single heat-shock early in life decreased *only* initial mortality without changing the subsequent rate of increase in mortality (Wu et al, 2008). Because of the close association between aging and the rate of increase in mortality (Finch, 1990; Mair et al, 2003; Wu et al, 2009, submitted), our earlier observations suggest that a single hormetic treatment early in life does not alter the rate of aging. We therefore questioned whether multiple heat-shocks administered during adulthood influenced the rate of increase in mortality in *C. elegans*.

2. MATERIALS AND METHODS

2.1 Strain and age-synchronization of populations

We used TJ375 (*gplIs1 [hsp-16.2::GFP]*), which carries the *hsp-16.2* promoter, driving expression of GFP (Green Fluorescent Protein), integrated into the genome of the N2CGCb background strain, which has been employed to predict longevity (Rea et al, 2005) and to examine mortality of worms following single mild heat-shock early in life (Wu et al, 2008).

Animals were maintained as frozen stocks as described (Johnson and Wood, 1982) until needed. To establish age-synchronous populations, worms were grown at 20 °C on NGM agar plates seeded with live *Escherichia coli* (OP50). Gravid adult worms were put onto fresh NGM plates for 5 hours and then removed by washing with S Basal solution. The eggs remaining on the plates were permitted to develop into young adults at 20 °C.

2.2 Heat-shock

Young adults were divided into six populations: control (no heat-shock), heat-shocked only on day 1 of adulthood within 24 hours after the onset of fertility, heat-shocked on days 1 and 4, on days 1, 4 and 7, on days 1, 4, 7 and 10, and those heat-shocked on days 1, 4, 7, 10, and 13. Adults were maintained in fresh liquid medium at 20 °C for longevity assessments, which are described below. On the day of heat-shock, populations were transferred to 10 cm NGM/OP50 plates and heated at 35 °C for 1 hour on day 1, 55 minutes on day 4, 50 minutes on day 7, 40 minutes on day 10, and/or 30 minutes on day 13. Preliminary Results suggested that these times would yield hormetic effects on longevity at these ages.

2.3 Assessment of longevity

After heat-shock, worms were transferred to fresh liquid medium for longevity assessment, as described previously (Johnson and Wood, 1982). About eighty worms were cultured in plastic Petri plates containing 4 ml of liquid medium (1×10^9 cells/ml OP50) but with the addition of 5-fluoro-2'-deoxyuridine (FUdR, Sigma) at a final concentration of 25 μ M/ml to prevent development of progeny. Previous experiments (Mitchell et al, 1979; Apfeld, 1999) and our own unpublished data indicate that FUdR does not significantly affect life span under these conditions. Worms were transferred to fresh medium every day and the numbers of worms alive, dead, or censored (lost or accidentally killed) was recorded using the criteria of death described in Johnson and Wood (1982).

2.4 Statistical Analyses

We assigned the life span of worms on the first day of adulthood as zero. The calculation of mean life span and statistical tests were described in Wu et al (2006). Age-specific probability of death was estimated and plotted to reflect mortality trajectory with age. It was estimated by the formula

$$q_x = \frac{d_x}{n_x}$$

where q_x is the probability of death at age (day) x ; d_x is the number of worms that died in the age interval $(x, x+1)$ and n_x is the number of worms at risk at the beginning of age x . Age-specific force of mortality was fitted with the following Gompertz model to reflect the initial mortality and the rate of increase in mortality with age. Gompertz parameters were estimated by using the Maximum likelihood method (Wu et al, 2008). The Likelihood Ratio Test was employed for the comparison of the rates of increase in mortality between two populations.

3. RESULTS

3.1 Mean life span

Worms were subjected to six sets of conditions: control (not heated), and experimental groups heat-shocked 1, 2, 3, 4, or 5 times. The first heat shock was always administered on day 1 of adulthood within 24 hours after the onset of fertility and subsequent shocks were administered in consecutive fashion on days 4, 7, 10 and 13. Two independent experiments were conducted.

In the first experiment, the mean life spans of animals heat-shocked, 1, 2, 3, 4, and 5-times are 19.0 days, 21.9 days, 23.7 days, 24.9 days, 25.8 days, and 26.4 days, respectively (Figure 1, Table 1). When worms were exposed to mild heat stress on day 1 of adulthood, the mean life span increased from 19.0 days to 21.9 days, and the increase of 15.3% compared to controls was highly significant ($p < 0.0001$). When two mild heat-shocks were applied, the mean life span of worms was extended to 23.7 days, an increase of 24.7% as compared to control. When worms received 3, 4 or 5 heat-shocks, they displayed increasing extensions of mean life span: 31.1%, 35.8% and 38.9% as compared to control, respectively. The second experiment produced similar results. A single heat-shock on day 1 of adulthood significantly increased longevity (a 16.7% increase compared to controls, $p < 0.0001$). Multiple heat shocks of 2, 3, 4 or 5 times extended mean life span 24.2%, 29.3%, 34.3% or 38.4% as compared to control animals, respectively. Therefore, repeated exposure to mild heat-stress can significantly extend longevity; each single heat-shock has a hormetic effect on life span, but the magnitude of the hormetic effect of additional single heat-shocks decreases as the age of heat shock increases.

3.2 Age-specific mortality

We used demographic analysis to examine the effect of multiple heat shocks on mortality at specific ages. Fig. 2 illustrates age-specific probability of death of worms under 6 sets of conditions. In both experiments, worms heat-shocked one or more times display lower mortality than controls at all ages. Although daily mortality fluctuated in all groups, there was a strong association between the number of heat-shocks and the daily mortality at most ages.

We fit our age-specific data to a Gompertz model to examine how fast mortality increase with age (Table 2). In the first experiment, the rates of increase in mortality of worms heat-shocked 0, 1, 2, 3, 4 or 5 times were 0.2368, 0.2352, 0.2119, 0.1901, 0.1454, and 0.1344, respectively; each additional heat shock further decelerated the rate of increase in mortality. We used the likelihood ratio test and found that the difference in the rates of increase in mortality between control worms and worms heat-shocked only one time was not significant ($p = 0.9173$). Therefore, the 2.9 additional days of longevity displayed by the once-shocked group compared to control (Table 1) was driven by the decrease of initial mortality of the shocked group from 0.0016 to 0.0008 (Table 2). Again using the likelihood ratio test, we also found that the difference in the rate of increase in mortality between controls and twice-heated animals was not significant ($p = 0.0948$). However, the differences between controls and each group heat-shocked more than twice is significant: for animals heated 3, 4, or 5 times, the likelihood ratio test produced p values of 0.001, $2.9\text{E-}12$ and $4.6\text{E-}14$, respectively. In summary, three or more heat-shocks can significantly decrease the rate of increase in mortality.

In the second experiment, the rates of increase in mortality under the six conditions were 0.2194, 0.2025, 0.1816, 0.1790, 0.1462, and 0.1329, respectively (Table 2). Again, the number of heat-shocks predicted monotonic lowering of mortality. As in the first experiment, the differences between controls and animals heat-shocked either once or twice were not significant ($p = 0.7355$ and 0.4521 , respectively). However, the rates of increase in mortality between controls and the group heat-shocked three times was significant ($p = 0.0209$, Table 2). And both remaining groups (heat-shocked four times and five times) displayed rates of increase in mortality that were each much lower than that of the control ($p = 4.7\text{E-}08$ and $3.7\text{E-}14$). The second experiment also showed that multiple heat-shocks (three times or more) significantly reduced the rate of mortality increase with age.

4. Discussion

The data presented here demonstrates the effects of multiple heat shocks on mean life span and age-specific mortality of *C. elegans*. Multiple mild heat-shocks extended mean lifespan of worms more than a single heat-shock; each individual heat-shock causes a hormetic increase

in life span, but the magnitude of each successive hormetic benefit decreases as the animals age. By decomposing the effect on mean lifespan into the effect on mortality at specific ages, we found that worms heat-shocked one or more times display lower mortality than controls at all ages. However, worms heat-shocked three or more times display a different pattern of age-specific mortality as compared to worms heat-shocked only one or two times. Worms that were heat-shocked one or two times, displayed a significant decrease in initial mortality, but the subsequent rate of increase in mortality remained unchanged; thus, life extension was driven solely by the decrease in initial mortality. When worms were heat-shocked three times or more, *both* initial mortality *and* the subsequent rate of increase in mortality decreased significantly. Therefore, hormesis can extend lifespan via multiple heat-shocks (numbering three or more) by decreasing both initial mortality and the rate of increase in mortality.

Initial mortality and the rate of increase in mortality are two parameters of the Gompertz model, which can reflect accurately the exponential increase of age-specific mortality of *C. elegans* with chronological age (Johnson, 1987; 1990). Interventions, either genetic or environmental, could extend the longevity of *C. elegans* by lowering initial mortality, or by reducing the rate of increase in mortality with age, or by combined effects (Johnson, 1987; 1990). There are many studies where initial mortality alone was altered. For example, the mutant *C. elegans* strain HG23 [*age-2(yw23)*] extends life span by reducing the initial mortality rate (Yang and Wilson, 1999). Exposure to lithium at clinically relevant concentrations throughout adulthood decreases initial mortality only and thus increases longevity of worms by as much as 46% (McColl et al, 2008). A single mild heat-shock of *C. elegans* also demonstrated that the hormetic effect on longevity was driven by the decrease of initial mortality (Wu et al, 2008). In another group of studies, longevity is extended only via reduction of the rate of increase in mortality, without affecting initial mortality. For example, the increased longevity of recombinant inbred lines resulted from a lower rate of increase in mortality (Johnson, 1987). Also, *age-1* mutants displayed a decrease in the rate of increase in mortality by 30%–40% with no change in initial mortality as compared to wild-type controls and thus extended longevity (Johnson, 1990; Johnson et al, 2001). *daf-2(e1370)* had a longer mortality rate doubling time (3.25 d at 25°C) than wild-type animals (1.11 d at 25°C), implying that the rate of increase in mortality in *daf-2(e1370)* animals is lower than in wild-type *C. elegans* (Samuelson et al, 2007). After short-term exposure to hyperoxia, wild-type worms increased their longevity through a lower rate of increase in mortality than control worms (Honda and Matsuo, 1992; Honda et al, 1993; Matsuo, 1993; Honda and Honda, 2002). Life span extension induced by CR in *C. elegans*, is also caused by a decreasing of the rate of increase in mortality (Lenaerts et al, 2007). Two genetic interventions have demonstrated increased longevity caused by the combination of decreasing initial mortality and slowing the rate of increase in mortality. These are the strain HG284 [*age-1(hx546) II; age-2(yw23)*] and the recombinant inbred strain TJ143 of *C. elegans*, both of which provide strong evidence that the extended longevity results from both decreasing initial mortality and the rate of increase in mortality (Yang and Wilson, 1999; Johnson, 1987). Here we report that anti-aging hormetic effects by lowering both initial mortality and the rate of increase in mortality can also be achieved via an environmental intervention: multiple mild heat-shocks.

The hormetic effect of multiple heat-shocks on longevity of *C. elegans* has been observed in other studies as well. Olsen et al (2006) reported that repeated mild heat treatments for 2 or 4 hours at 30 °C throughout life had a larger effect on lifespan compared to a single mild heat treatment early in life. Galbadage and Hartman (2008) showed that wild-type worms incubated at 25 °C lived almost as long as those that were incubated constantly at 12 °C, when they were “thermocycled” between 25 °C and 12 °C at 10-min intervals. Multiple heat-shocks also exerted hormetic effect in other species and human cell culture. Wild-type *D. melanogaster* exposed 5 min per day, 5 days per week for one week to 37 °C live on average 2 days longer than the control ies (Le Bourg et al., 2001). In another study on *D. melanogaster*, exposure of

young female ies to four rounds of mild heat-shocks at 34 °C significantly increased average and maximum lifespan and increased their resistance to potentially lethal heat stress (Hercus et al., 2003). In human cell culture, anti-aging hormetic effects of multiple heat-shocks have been reported in a series of experiments (Rattan, 1998; Verbeke et al, 2000, 2001, 2002; Fonager et al, 2002; Rattan et al, 2004; Rattan, 2005, 2008). These effects include the maintenance of youthful morphology, reduced accumulation of oxidatively- and glycoxidatively-damaged proteins, as well as increased resistance to ethanol, hydrogen peroxide and UV-A irradiation. Therefore, multiple heat-shocks have anti-aging hormetic effects and represent an effective approach for modulating aging.

We cultured worms in liquid medium (1×10^9 OP50/ml and 10 µg/ml cholesterol) at 20 °C, and performed multiple heat-shocks on 10 cm solid NGM/OP50 plates at 35 °C. Our method is different from the multiple heat-shock method of Olsen et al (2006), wherein worms were cultured at 20 °C and heated at 30 °C or 33 °C for 4 hours on solid NGM plates. It is also different from the single heat-shock method employed in Cypser and Johnson (2002; 2003) where worms were both cultured (at 20 °C) and heated (at 35 °C) on solid NGM plates, and different from the single heat-shock method employed in Rea et al (2005) wherein worms were both cultured and heated in liquid medium. The reasons we used a different method are as follows: (1) To maximize hormetic response; worms live longer in liquid medium than on solid NGM plates, thus at a given chronological age, they are healthier and more responsive to heat-shock. (2) To avoid damage to a frail subpopulation; heat-shock at 35 °C for 1 hour in liquid medium extends longevity of a majority of worms but shortens the life span of a minority (Wu et al, 2008). However, heat-shock of 1 hour at 35 °C on solid NGM plates increases longevity of 100% of the population (as indicted in Yashin et al, 2002).

Induced as part of the response of an organism to heat shock, heat shock proteins (HSPs) form the front line of a cell's defenses against protein damage, functioning to refold, sequester, or aggregate damaged proteins, or to target proteins for degradation when damaged past repair (Jolly et al, 2000; Verbeke et al, 2001; Morrow et al, 2003; Rattan, 2005). The nematode *C. elegans* exhibits a typical response to heat shock and the most prominent HSPs in *C. elegans* include polypeptides of masses 16,000 and 18,000 Da (Russnak et al, 1983). Link et al (1999) constructed a novel *hsp-16.2::GFP* reporter transgene to monitor HSP16.2 expression in response to heat shock and found that HSP16.2 levels increased and reached a maximum 24 hours after heat shock, and then decreased by 25% 48 hours after heat-shock as compared to the maximum. Because HSP levels decrease quickly over time, we repeated heat-shocks every 3 days beginning at the first day of adulthood. This is different from the multiple heat-shock method Olsen et al (2006) used, wherein worms were heated every 4 days.

By using the discrete heterogeneity model, Yashin et al (2002) proposed that heat-shock at 35 °C on solid NGM plates for 1 hour extended longevity of all individuals, but heat-shock for 2 hours caused 4.8% of worms to become frail. Therefore, in order not to damage worms, we employed the method of multiple heat-shocks, heating worms on the first day of adulthood for 1 hour, and performing repeated heat-shocks on day 4, day 7, day 10, and day 13 for 55 minutes, 50 minutes, 40 minutes and 30 minutes, respectively. Before these two independent experiments, pilot experiments revealed that multiple heat-shocks for the durations indicted above extended longevity and showed no excess mortality during the stress in either of the two experiments.

Originally proposed in 1825 as an empirical model for human aging, the Gompertz model suggests that as populations increase in age, the force of mortality increases exponentially (Gompertz, 1825; Finch, 1990). This model has been the paradigm of mortality studies in gerontology for many years (Finch et al, 1990; Carey and Liedo, 1995; Carey, 1997). The use of this model has been questioned because doubt has been cast on the assumption that the force

of mortality increases consistently with age. In fact, mortality has been shown to decelerate at advanced ages in a variety of organisms. For example, in the medfly, the rate of increase in mortality was found to generally slow at advanced ages, and also actually decreased with age (Carey et al, 1992). In *Drosophila* (Curtsinger et al, 1992) and *C. elegans* (Johnson et al, 2001), mortality was found to level off at advanced ages and is better fit by a two-stage Gompertz model. However these latter studies used hundreds of thousands of individuals while the studies reported here are not feasible with those population sizes. Our data on multiple heat-shocks also indicate that the force of mortality does not consistently increase with age. We have tried a two-stage Gompertz model, but it does not have an easily interpretable biological meaning in the case of multiple heat-shocks. Our aim is not to find the best-fitting model, but rather to determine whether the rate of increase in mortality is affected by multiple heat-shocks. As has been argued by others (Carey and Liedo, 1995), the Gompertz model is useful as a starting point in comparing differences in rates of aging between populations. Therefore, our use of the Gompertz model to study the effect of multiple heat-shocks is appropriate.

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Fig. 1.

Survival of worms following heat-shock treatment. The solid line (—) represents control (no heat-shock). Solid lines with filled circles (—●—) and open circles (—○—) represent worms heat-shocked only on day 1 of adulthood, and repeatedly heat-shocked on day 1 and day 4, respectively. Dashed lines with filled up-triangle (—▲—), open down-triangle (—▼—), and cross (—+—) represent worms repeatedly heat-shocked on day 1, 4, and 7, on day 1, 4, 7, and 10, on day 1, 4, 7, 10 and day 13 of adulthood, respectively. (A) the first experiment. (B) the second experiment.

Fig. 2.

Age-specific probability of death following heat-shock treatment plotted using a log scale. Solid line (—) represents control (no heat-shock). Solid lines with filled circles (—●—) and open circles (—○—) represent worms heat-shocked only on day 1 of adulthood, and repeatedly heat-shocked on day 1 and day 4, respectively. Dashed lines with filled up-triangle (---▲---), open down-triangle (---▽---), and cross (---+---) represent worms repeatedly heat-shocked on day 1, 4, and 7, on day 1, 4, 7, and 10, on day 1, 4, 7, 10 and day 13 of adulthood, respectively. (A) the first experiment. (B) the second experiment.

Table 1

Mean Life-span and Descriptive Statistics

Exp. ID	Populations	Sample size	Mean life span	Standard Error	Percent of control
I	Control	305	19.0	0.3	100.0
	Day 1	286	21.9*	0.3	115.3
	Day 1, 4	292	23.7*	0.3	124.7
	Day 1, 4, 7	307	24.9*	0.3	131.1
	Day 1, 4, 7, 10	348	25.8*	0.3	135.8
	Day 1, 4, 7, 10, 13	309	26.4*	0.4	138.9
II	Control	203	19.8	0.3	100.0
	Day 1	211	23.1*	0.4	116.7
	Day 1, 4	240	24.6*	0.3	124.2
	Day 1, 4, 7	206	25.6*	0.4	129.3
	Day 1, 4, 7, 10	220	26.6*	0.4	134.3
	Day 1, 4, 7, 10, 13	247	27.4*	0.4	138.4

* p<0.0001 in comparison to control

Table 2

Maximum likelihood estimates of Gompertz parameters and likelihood ratio tests of the rate of increase in mortality

Exp. ID	Populations	Gompertz parameters		<i>p</i> for comparison in <i>b</i> with control
		<i>a</i>	<i>b</i>	
I	Control	0.0016 ± 0.0003 [*]	0.2368 ± 0.0108	
	Day 1	0.0008 ± 0.0002 [*]	0.2352 ± 0.0109	0.9173
	Day 1, 4	0.0016 ± 0.0003 [†]	0.2119 ± 0.0102	0.0948
	Day 1, 4, 7	0.0032 ± 0.0006 [‡]	0.1901 ± 0.0093	0.0010
	Day 1, 4, 7, 10	0.0088 ± 0.0012 [§]	0.1454 ± 0.0077	2.9E-12
	Day 1, 4, 7, 10, 13	0.0148 ± 0.0020 [¶]	0.1344 ± 0.0086	4.6E-14
II	Control	0.0019 ± 0.0004 [*]	0.2194 ± 0.0035	
	Day 1	0.0011 ± 0.0003 [*]	0.2025 ± 0.0111	0.7355
	Day 1, 4	0.0023 ± 0.0005 [†]	0.1816 ± 0.0095	0.4521
	Day 1, 4, 7	0.0034 ± 0.0007 [‡]	0.1790 ± 0.0107	0.0209
	Day 1, 4, 7, 10	0.0077 ± 0.0014 [§]	0.1462 ± 0.0094	4.7E-08
	Day 1, 4, 7, 10, 13	0.0128 ± 0.0020 [¶]	0.1329 ± 0.0092	3.7E-14

* mortality at day 1 of adulthood.

† mortality at day 4.

‡ mortality at day 7.

§ mortality at day 10.

¶ mortality at day 13.