

Visualizing hidden heterogeneity in isogenic populations of *C. elegans*

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Abstract

Age-specific mortality levels off at advanced ages in many species; one explanation for this phenomenon is provided by the population heterogeneity theory. Although mortality at advanced ages can be well fit by heterogeneity models, population heterogeneity remains theoretical, lacking much direct evidence to support the existence of unobserved heterogeneity. Here, we provide direct evidence to support the heterogeneity theory by using isogenic population of worms of *Caenorhabditis elegans*. We measure the ability of individual worms to respond to a heat stress using an HSP-16.2 promoter that has been attached to GFP, a fluorescent marker that can be assessed in living animals. Worms differ substantially in their response; worms with high response have a long lifespan, and worms with low response to stress have a short life. Each of these classes results from a mix of two distinct, heterogeneous classes of worms and the addition of more classes does not result in a better fit.

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1. Introduction

At advanced ages, mortality tends to plateau (Vaupel et al., 1998). Such plateaus have been observed in large isogenic populations of the nematode *Caenorhabditis elegans* (Johnson et al., 2001a,b; Vaupel et al., 1998) and in other species, including the medfly, *Ceratitis capitata* (Carey et al., 1992), *Drosophila melanogaster* (Curtsinger et al., 1992), three species of true fruit flies, *Anastrepha serpentina*, *Anastrepha obliqua*, and *Anastrepha ludens* (Vaupel et al., 1998); the bean beetle, *Collosobruchus maculatus* (Tatar et al., 1993), yeast, *Anastrepha* and wasps (Vaupel et al., 1998). In a cohort of 180,000 isogenic nematodes, mortality increased rapidly at young ages and more slowly at older ages (Brooks et al., 1994). Johnson et al. (2001a,b) also demonstrated that mortality at older ages levels off in wild type ($N=500,000$) and three long-lived (age) mutants of *C. elegans*: [*age-1(hx546)*, $N=300,000$; *clk-1*, ($N=300,000$) and *spe-26*, ($N=200,000$)]. Finally, the highest quality data on human centenarians hint at a similar phenomenon of mortality leveling off (Vaupel et al., 1998).

Two primary hypotheses have been put forward to explain this late-life mortality plateau: a slowing of individual aging

processes (Curtsinger et al., 1992; Curtsinger and Khazaeli, 1997), and population heterogeneity (Brooks et al., 1994; Vaupel et al., 1979, 1998; Vaupel and Yashin, 1985; Yashin et al., 1994, 1996). The first hypothesizes that mortality increases more rapidly at younger ages and more slowly at older ages, in every individual in a population. In contrast, population heterogeneity, suggests that individuals are heterogeneous, differing substantially in unobserved susceptibility to death; some individuals are frailer than others because of random developmental and environmental variation and these frail individuals have higher mortality than the more robust. For frail and robust individuals alike, the risk of death per unit time increases at a constant rate of acceleration throughout their lifespan. This compositional heterogeneity causes age-specific mortality at the population level to decelerate at advanced ages, even if at the individual level mortality continues to increase exponentially with age.

Although first noticed more than 150 years ago (Higham, 1851), only since Beard (1959) introduced the gamma distribution has unobserved heterogeneity become intensively studied (Yashin, 2002b). In 1979, Vaupel and colleagues formulated the continuous heterogeneity model that proved a turning point in heterogeneity analysis. Using this model Vaupel and Yashin (1985) investigated the effects of hidden heterogeneity on the age pattern of mortality curves. Their study, along with a later one (Vaupel and Carey, 1993), showed that mortality at advanced ages could be best fit by heterogeneity models. Yashin et al. (1996) extended the

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continuous hidden heterogeneity model to evaluate the effects of stress on mortality in heterogeneous populations. The properties of this revised model were later analyzed in depth by Michalski and Yashin (2001). Semenchenko et al. (2003) used this model to establish that resistance to stress in *Drosophila* declines with age. Finally, Michalski et al. (2001) and Yashin et al. (2001, 2002a,b) developed an alternative series of approaches to the analysis of experimental stress data collected using *C. elegans*. Despite this profusion of theoretical work, hidden heterogeneity has remained a mathematical model, lacking of direct experimental evidence.

A large body of evidence indicates that there is an intimate association between the increased longevity of long-lived mutants and increased resistance to various stressors in the nematode *C. elegans* (Johnson et al., 1996, 2000, 2001a,b; Henderson et al., 2005). Indeed, increased longevity appears to be associated with stress resistance in every species (Martin et al., 1996; Finkel and Holbrook, 2000). We ask here whether this association is maintained within species, at the individual level; is there variation in stress resistance? If so, is such variation correlated significantly with longevity? To address these questions, we have employed isogenic population of *C. elegans*. Instead of testing the heterogeneity hypothesis indirectly as in previous papers (Khazaeli et al., 1995, 1998; Drapeau et al., 2000), we now measure hidden heterogeneity directly using isogenic strains carrying the green fluorescent protein (GFP) as a reporter.

2. Materials and methods

2.1. Nematode strains, maintenance and mass culture

TJ375 (*gplIs1 [hsp-16.2::GFP]*) was previously described (Link et al., 1999; Rea et al., 2005). It is an in-frame fusion of GFP with the HSP-16.2 promoter. Standard techniques were used for maintenance of nematode strains (Brenner, 1974). Worms were maintained at 20 °C on NGM agar plates seeded with live *Escherichia coli* (OP50). For short periods (<1 month), when not in use, they were allowed to form dauers at 16 °C.

To avoid possible confounding effects of exposure to bleach, we established the following protocol for large-scale production of synchronized worm cultures (> 120,000 worms). For each experiment, 16 10 cm NGM/OP50 plates were each seeded with 1200 first stage larvae (L1, prepared as described below) and incubated at 20 °C. Animals were allowed to develop to 1-day-old (76 h) gravid adults and then suspended by washing the plates twice with 4 ml of S-Basal at room temperature (RT) and concentrated into a single 50 ml Falcon tube and allowed to pellet at 1 g (~5 min). (Note that in all procedures pelleting under gravity was used in place of centrifugation). Gravid adults were then washed five times with S-Basal (50 ml each), by which time the population was devoid of any previously laid eggs or hatched L1 progeny (visually confirmed via a dissecting microscope). Gravid adults were then re-suspended into ~1 ml S-Basal and spread evenly onto four 10 cm NGM/OP50 plates. Sufficient food was present on these plates so that the cultures became starved after 4 h and

hence stopped laying eggs (amounting to a 4-h limited lay). After ~24 h, hatched L1s were washed from these plates using a total volume of 8 ml of S-Basal (RT). An L1 aliquot was immobilized with 1 M sodium azide and the concentration determined using a dissecting microscope. Untreated L1s were used to seed new carry plates (1200 L1/plate) and/or several 10 cm NGM plates supplemented with 2% peptone (RNGM plates) which had been previously spread with a heavy lawn of RW2 bacteria (a wild-type *E. coli* strain). In the latter case, worms were seeded at 12,000 L1/plate, incubated at 20 °C, allowed to reach adulthood, harvested by washing each plate twice with 4 ml S-Basal (RT), pooled and washed twice with 50 ml S-Basal, then finally re-suspended in ~3 ml S-Basal, ready for assaying. We also typically fed the RNGM plates at +52 h with 1 ml of a 1×10^{11} OP50/ml *E. coli* stock solution (concentrated from an overnight-grown LB broth inoculum), to prevent the cultures from becoming starved.

2.2. Heat-shock

In each experiment a sub-sample of worms was not exposed to heat but instead transferred immediately to a 6 cm dish containing 4 ml of liquid food (20 °C). In order to facilitate even and reproducible heat-pulses to our mass cultures, young adults were transferred into a 2 l flask containing S-Basal, 1×10^9 OP50/ml and 10 µg cholesterol/ml (liquid food), that had been pre-heated to 35 °C. Worms were added to a final concentration of 300/ml. After 1 or 2 h at 35 °C with rotation (100 rpm in a New Brunswick Scientific Co., NJ, Series 25D incubator), worms were transferred to 50-ml Falcon tubes, pelleted under gravity (~5 min), and then quickly transferred to fresh media that had been pre-chilled to 20 °C. Recovering cultures were incubated at 20 °C with rotation (120 rpm in a New Brunswick Scientific Co., Innova 4230 incubator), and fed every 12–16 h with fresh bacteria in liquid. At the desired time, aliquots were withdrawn and processed for sorting.

2.3. Sorting procedure

Worms harvested during the recovery period were allowed first to pellet in 50 ml falcon tubes (RT). They were then pooled, washed twice with 50 ml S-Basal, and finally re-suspended to a final concentration of 0.5–1 worm/uL in S-Basal + 1×10^9 OP50. A COPAS Biosort 250 Worm Sorter, (Union Biometrica, Inc., Harvard Biosciences), was then employed to sort the worms into various fractions (at RT). Worms were selected on the basis of simultaneously fulfilling three user-defined criteria—time of flight (TOF, length), optical absorbance (extinction, EXT) and integrated fluorescence intensity at 488 nm (GFP). Values for TOF and EXT (gating) were kept constant during each run and typically 60–75% of the population was selected using these two parameters alone. From this sub-population two groups of worms were chosen, each expressing differing amounts of GFP—highest 2% (high), and lowest 2% (low). Percentages each represent 2% of the total starting population. An unsorted sample was also always taken as a control for sorter-induced alterations in our samples

but we found negligible changes in this population when compared to the flow-through (ungated) group (data not shown). Worms were not recycled through the sorter so a population comprised of about 30,000 individuals yielded ~150 worms per group. Worms were sorted directly into 6 cm dishes containing 4 ml of liquid food, after which they were incubated at 20 °C until further use.

2.4. Longevity assay

All lifespan analyses were performed in liquid food as previously described (Johnson and Wood, 1982). Briefly, worms per selected sample (sub-population) were grown in 4 ml liquid food at 20 °C. During the egg-laying period animals were transferred every day to fresh food, after this time they were transferred every second day until none remained alive. The number of animals surviving immediately prior to each transfer was recorded and all carcasses were removed.

2.5. Statistical analyses

2.5.1. Mean lifespan

C. elegans develops through four larval stages before reaching adulthood. All sorting experiments were undertaken on animals that had just moulted into adults. We assign the lifespan of worms on the first day of longevity assessment as zero. The mean lifespan is estimated by the formula

$$MLS = \frac{1}{N} \sum_j \frac{x_j + x_{j+1}}{2} d_j$$

where d_j is the number of worms that died in the age interval (x_j, x_{j+1}) and N is total number of worms. The standard error of the mean life span estimate was calculated using the equation

$$SE = \sqrt{\frac{1}{N(N-1)} \sum_j \left(\frac{x_j + x_{j+1}}{2} - MLS \right)^2 d_j}$$

2.5.2. Test for significance of the mean lifespan difference

Comparison of mean lifespan estimates between two populations was made by calculating the normalized statistics

$$z = (MLS_1 - MLS_0) / \sqrt{SE_1^2 + SE_0^2}$$

where MLS_0 , MLS_1 , SE_0 and SE_1 are mean lifespan estimates and standard error estimates in two populations.

2.5.3. Gompertz model and discrete heterogeneity model

Mortality of control worms that had been heat shocked but not sorted, (pre-sort), was analyzed using both the Gompertz model and the discrete heterogeneity model with sub-populations. The Gompertz model proposes an exponential increase of mortality with age and in the discrete heterogeneity model, there are two or more sub-populations, which differ significantly from each other in mortality.

Formula (1) is the hazard function (the force of mortality) in the Gompertz model. For the discrete heterogeneity model we

assume that the force of mortality in each sub-population follows a Gompertz distribution. For a discrete heterogeneity model with N sub-populations, the average survival function is given by Formula (2).

$$\mu(x) = ae^{bx} \quad (1)$$

$$\bar{S}(x) = \sum_{i=1}^N p_i e^{-(a_i/b_i)(e^{b_i x} - 1)} \quad (2)$$

where x is an individuals age, and i refers to sub-population. And p_i is the initial proportion of the i th sub-population in the whole population. To obtain estimates for model parameters, curve-fitting, using a maximum-likelihood estimation procedure was implemented. The Log Likelihood function can be expressed in the form

$$\text{Log Lik} = \sum_x (d_x \ln q_x + (n_x - d_x) \ln(1 - q_x))$$

where d_x is the number of deaths on day x , n_x is the number of worms at risk at the beginning of day x , and q_x is the probability of death on day x and is described by the equation $q_x = 1 - (\bar{S}(x+1)/\bar{S}(x))$.

2.5.4. The cox proportional hazard model

In the nematode lifecycle, eggs hatch into the first larval stage (L1), and after three more molts become adults. We use arrested L1s to obtain large synchronous populations. Several parameters differed slightly among replicate experiments; these include: the length of time from egg isolation to the time when arrested L1s were used (age of L1), and the length of time from egg-laying to the time when we began to measure longevity (time of longevity assay). To control for possible effects of these factors on longevity, we applied the Cox Proportional Hazard model as following

$$\mu(t) = \mu_0(t) e^{\beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_5 x_5}$$

where x_1 is individual GFP expression level (low=0, and high=1), x_2 the time of longevity assay, x_3 age of L1, x_4 the interaction between GFP level and the time of longevity assay, x_5 the interaction between GFP level and the age of L1.

All above statistical analyses were undertaken using SAS software (SAS Institute, Inc., NC), S-Plus 6.2 (Insightful Corporation) and Gauss (Aptech Systems, Inc.).

3. Results

3.1. Experimental design and overview

We have taken advantage of the optical transparency of *C. elegans* to design an in vivo method for assessing response to stress. This method has allowed us to monitor individual variation in response to stress and then use the living worms for further assessments. HSP-16.2 is a member of the family of small heat shock proteins (HSPs), which are a collection of low molecular weight polypeptides (15–30 kD), found in all eukaryotes; they have sequence similarity to the α B crystallins

(Arrigo and Landry, 1994). In *C. elegans*, the major small HSPs are a collection of 16 kD species encoded by six closely related genes (Candido et al., 1989). When *C. elegans* are stressed, especially by exposure to high temperature, worms activate HSP-16.2 expression, among other genes (GuhaThakurta et al., 2002). Therefore, the level of expression of HSP-16 is one assessment of the worm's ability to respond to stress.

Under normal circumstances HSP-16.2 is not directly detectable without killing the animal; so we utilized a transgenic strain carrying a chromosomally-integrated HSP-16.2::GFP expression vector (TJ375; Link et al., 1999; Rea et al., 2005). Green fluorescent protein (GFP) is observable using fluorescence optics, and is quantifiable. Our HSP-16.2::GFP reporter carries 400 bp, immediately 5' to the initiation site, of the HSP-16.2 promoter and functions as a reliable promoter when coupled to GFP (Link et al., 1999). The reporter encodes no HSP-16.2 product itself. When expressed via this construct, GFP protein has the same expression pattern as HSP-16.2 (Link et al., 1999). Therefore, the level of GFP fluorescence in an individual is a reflection of its level of HSP-16.2 expression and the mathematical relationship between individual GFP expression level and longevity should be the same as that between HSP-16.2 expression level and the individual's longevity.

In a typical experiment, about 120,000 age-synchronous *hsp-16.2::GFP* (TJ375) worms were obtained using a short period of egg lay and grown at 20 °C, until they were young adults. They were then shifted to 35 °C, by mixing in pre-warmed medium and maintained at this temperature for 1 or 2 h, and then returned to 20 °C, using pre-cooled medium, to recover. At 8–10 h post-heat-shock, young adults were suspended in running buffer and sorted using the COPAS BIOSORT 250 Worm Sorter (Union Biometrica, Inc., Harvard Biosciences) to assess GFP expression level of individual worms. We selected two samples of worms: those with GFP expression levels within the highest one to two percentiles and those with GFP expression levels in the lowest 1–2 percentile. Selected samples subsequently were measured for longevity. Controls included non-heat-shocked worms (pre-heat), worms that were heat shocked but not sorted (pre-sort), and worms that were sorted but were not selected (flow-through). We have repeated the 1- and 2-h heat-shock treatments multiple times (Rea et al., 2005) and here analyze data in which GFP levels

were measured 9 h after heat shock when GFP has the same level as HSP-16.2 and both approach their highest levels of expression within the population (Link et al., 1999).

3.2. Heterogeneity in individual GFP expression level

The three 1-h heat-shock experiments and the three 2-h heat-shock experiments utilized in this study were all conducted within a 3-month period (Table 1). For each experiment, we used 120,000 worms or more. All experiments showed significant inter-individual variation in GFP expression level, even though populations were comprised of isogenic worms (Fig. 1). The black lines in Fig. 1 are density estimations based on Gaussian Kernal with S-Plus 6.2. Mean GFP expression levels averaged about 256–290 units after a 1-h heat shock but were lower (about 152–154 arbitrary units) after 2-h heat shock. Even after correcting for mean:variance confounds, individual variation in GFP levels was higher after a 2-h treatment than after a 1-h treatment, as shown by examining the coefficient of variation (CV): For the three 1-h treatments, CV values were 22.1, 19.8 and 24.3%, respectively (Table 1), whereas for the three 2 h heat shock treatments, CV values were 26.1, 25.8, and 27.4%, respectively.

To test the normality of each GFP expression distribution captured for the six experiments (Fig. 1), we use Kolmogorov–Smirnov–Lillifors test ($N > 2000$). Table 1 shows that neither GFP expression after 1-h of heat shock nor GFP expression after 2-h of heat shock is normally distributed ($p < 0.01$). GFP distributions are skewed to the left (experiment B1) or to the right (experiment A1, A2, A3, and B2). If we look at Kurtosis, we can see that all Kurtosis values are positive which mean that GFP distributions are peaked distributions relative to a normal distribution.

3.3. Heterogeneity in individual frailty after heat-shock—theoretical support

Individual frailty is individual susceptibility to death (Vaupel et al., 1979). Is there heterogeneity in individual frailty? Under normal circumstances individual frailty, and its distribution within a population, cannot be observed directly in populations of randomly selected, heat-shocked animals. Theoretical support can, however, be provided if models that

Table 1
Values for GFP distributions in population

| Exp. ID | Date when measured (2002) | Duration after induction (h) | Moments | | | | | KSL test ^a | | |
|---------------------------------|---------------------------|------------------------------|----------|-------|------|--------|----------|-----------------------|----------------|----------------|
| | | | <i>N</i> | Mean | SD | CV (%) | Skewness | Kurtosis | <i>D</i> value | <i>P</i> value |
| <i>1-h heat-shock treatment</i> | | | | | | | | | | |
| A1 | Oct. 31 | 9 | 40,501 | 289.1 | 64.0 | 22.1 | 0.39 | 7.54 | 0.07 | <0.01 |
| A2 | Nov. 4 | 9 | 49,083 | 289.3 | 57.3 | 19.8 | 0.82 | 10.2 | 0.08 | <0.01 |
| A3 | Dec. 10 | 9 | 38,238 | 256.3 | 62.4 | 24.3 | 1.50 | 10.2 | 0.07 | <0.01 |
| <i>2-h heat-shock treatment</i> | | | | | | | | | | |
| B1 | Sept. 23 | 10 | 25,893 | 153.6 | 40.1 | 26.1 | −0.24 | 5.1 | 0.08 | <0.01 |
| B2 | Oct. 11 | 8 | 37,532 | 152.5 | 39.4 | 25.8 | 0.13 | 7.7 | 0.10 | <0.01 |
| B3 | Oct. 31 | 9 | 28,181 | 153.4 | 42.1 | 27.4 | 0.02 | 6.1 | 0.09 | <0.01 |

^a Kolmogorov–Smirnov–Lillifors test for normal distribution ($N > 2000$).

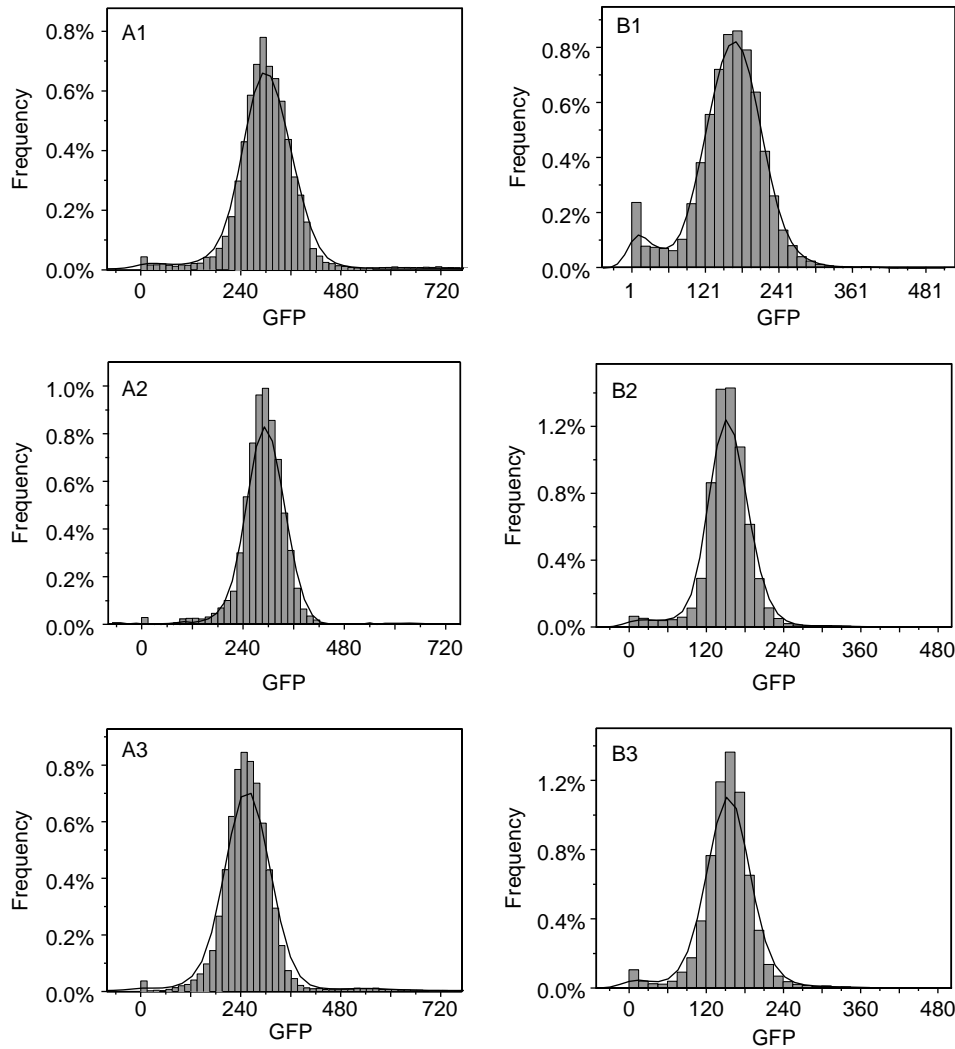


Fig. 1. Population distributions of GFP expression level in individual worms after a 1-h heat shock (A1, A2, and A3) or a 2-h heat shock (B1, B2, and B3). Density lines are estimated based on Gaussian Kernel with S-Plus 6.2.

fit longevity data for such populations show strong statistical support for the existence of sub-populations with differing mortality kinetics. To test this idea we fitted the longevity data collected from samples of each heat-shocked population using either a discrete heterogeneity model with different sub-populations, or a simple one population Gompertz model. Our results, summarized in Table 2, show that the discrete heterogeneity model with two-subpopulations fits the data significantly better than the Gompertz model, in all six heat-

shock experiments. For example, in the first experiment with 1-h heat-shock, the log maximum likelihoods for the Gompertz model and discrete heterogeneity model with two sub-populations are -241.1 and -232.1 , respectively, and the difference between the fits of the two models is significant ($p = 0.0004$). The fit by the discrete heterogeneity model with two sub-populations for each experiment are given in Fig. 2 where the observed survival function, the fitted survival function, and the survival functions of robust sub-population and frail

Table 2
Comparison between Gompertz and discrete heterogeneity models

| Heat-shock treatment (h) | Exp ID | Sample size (N) | Log maximum likelihood | | P values for likelihood ratio test |
|--------------------------|--------|-----------------|------------------------|------------------------------|------------------------------------|
| | | | Gompertz model | Discrete heterogeneity model | |
| 1 | A1 | 68 | -241.1 | -232.1 | 0.0004 |
| | A2 | 60 | -234.6 | -225.7 | 0.0005 |
| | A3 | 40 | -161.0 | -146.7 | 2.5×10^{-6} |
| 2 | B1 | 70 | -253.3 | -232.8 | 6.9×10^{-9} |
| | B2 | 91 | -360.4 | -337.8 | 1.9×10^{-11} |
| | B3 | 60 | -179.8 | -164.9 | 1.7×10^{-6} |

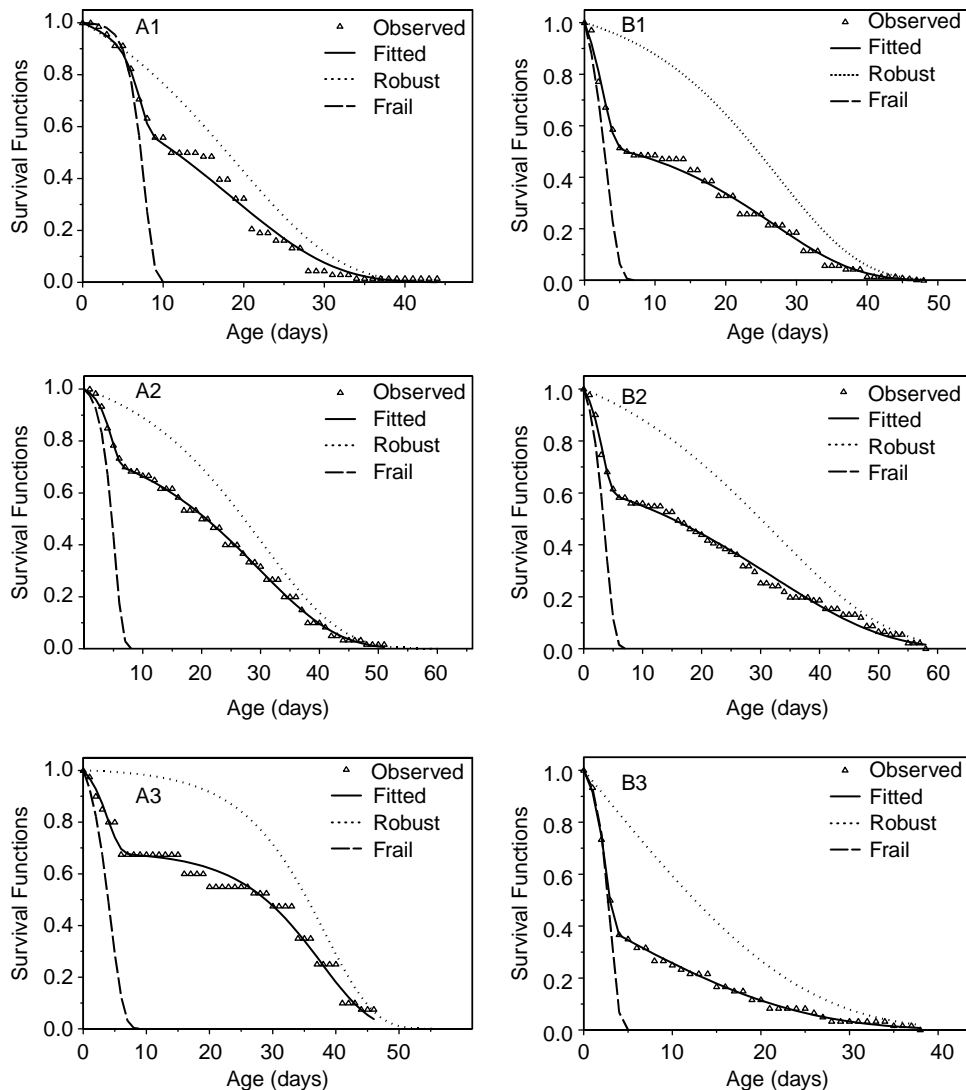


Fig. 2. Survival functions of worms following heat shock and before sorting (A1–3: 1-h heat shock; B1–B3: 2-h heat shock). Triangles represent observed survival function, whereas the solid line is the survival function fitted by the discrete heterogeneity model with two subpopulations: robust (dotted line) and frail (dashed line).

sub-population are shown. From Fig. 2 we can see that a discrete heterogeneity model with two sub-populations fits the observed longevity data very well, and the difference in mortality between the two sub-populations is very significant ($p < 0.0001$). We also fit the longevity data using a discrete heterogeneity model with three sub-populations and find that fittings fail to converge. We conclude that the mathematical existence of two sub-populations with significantly different mortality demonstrate the existence of heterogeneity in individual frailty and that individual frailty follows a two-point distribution (i.e. two sub-populations) in these isogenic worm populations after heat-shock.

3.4. The relationship between individual GFP level and frailty—direct observation of hidden heterogeneity

In order to test the relationship between individual GFP level and individual frailty, we employed a COPAS worm sorter to select worms displaying either high or low GFP expression after a

1- or 2-h heat shock and we then assessed their subsequent longevity. Fig. 3 reveals the relationship between GFP expression and longevity. In the three experiments with 1 h heat-shock (Fig. 3A1–3), the mean lifespan for high GFP and low GFP were 14.4 (SE 0.8) days and 10.6 (SE 1.0) days; 25.4 (SE 1.7) and 17.1 (SE 1.6) days; and 20.9 (SE 2.3) and 12.7 (SE 2.1) days, respectively. The p values for the difference in mean lifespan between high and low were 0.005, 0.0003 and 0.008, respectively. For worms exposed to 2 h of heat-shock (Fig. 3B1–3), the mean lifespan of high GFP expressing worms and low GFP expressing worms were 16.5 (SE 1.6) days and 3.0 (SE 0.5) days; 24.4 (SE 2.2) days and 16.3 (SE 2.1) days; and 8.6 (SE 1.0) days and 4.4 (SE 0.7) days, respectively. And the p values were 8.8×10^{-16} , 0.007 and 0.0005, respectively. These data show that worms which have high GFP expression live significantly longer than worms with low GFP expression, and the differences are consistent among replicates.

If we pool all three replicates, we can calculate the average risk ratios in mortality (Table 3). According to the Cox hazard

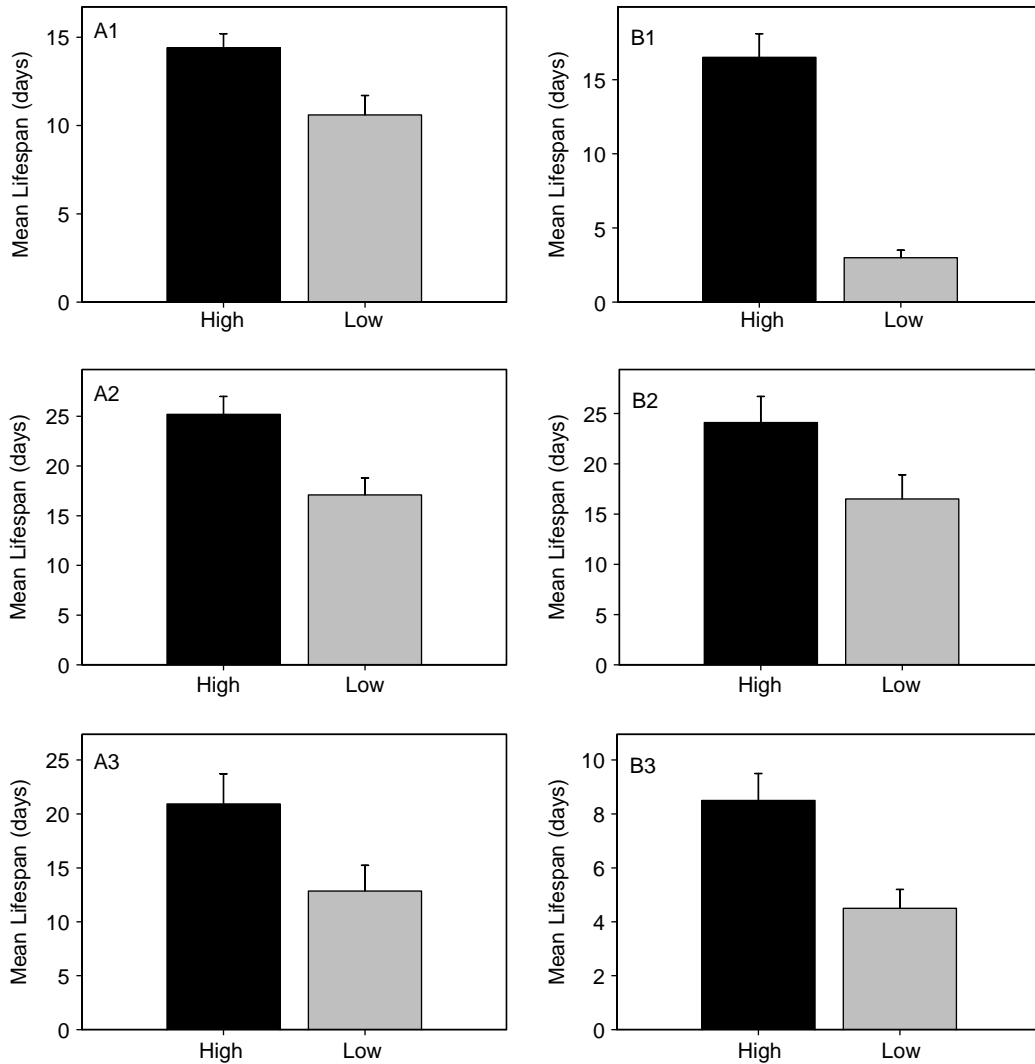


Fig. 3. Mean lifespan of worms expressing high and low levels of GFP after a 1-h heat shock (A1, A2, A3) or a 2-h heat-shock (B1, B2, B3). The sample sizes are as follows: A1: high (N=70), low (69); A2: high (70), low (70); A3: high (40), low (40); B1: high (70), low (80); B2: high (60), low (60); B3: high (100), low (103). All *p* values for the difference between mean survival of worms expressing high and low levels of GFP are <0.01.

model, controlling the effects of other factors such as age of L1, the time of longevity assay post-heat-shock, and the interactions between GFP and age of L1 and between GFP and the time of longevity assay, the mortality of worms with

high GFP expression is 0.67 ($p=0.0001$) times lower than that of worms with low GFP expression for 1-h heat-shock treatment, and 0.48 ($p=1.5 \times 10^{-14}$) times lower than that of worms with low GFP expression for 2-h heat-shock treatment.

Table 3
Estimates of the coefficients and the risk ratios based on the cox model

| Factors | Estimate | Risk ratio | χ^2 | Prob > χ^2 |
|--|------------|------------|------------|-----------------------|
| <i>1-h heat-shock treatment</i> | | | | |
| GFP (low=0, high=1) | -0.4069573 | 0.665673 | 14.5223205 | 0.0001 |
| Time of longevity assay | -0.3001711 | 0.740691 | 29.99696 | 0.0000 |
| Age of L1 | -0.0314866 | 0.969004 | 16.3411986 | 0.0001 |
| GFP ^a time of longevity assay | -0.0733322 | 0.929292 | 0.53101468 | 0.4662 |
| GFP ^a age of L1 | -0.0164014 | 0.983732 | 1.28306505 | 0.2573 |
| <i>2-h heat-shock treatment</i> | | | | |
| GFP (low=0, high=1) | -0.7353245 | 0.47935 | 59.0554156 | 1.5×10^{-14} |
| Time of longevity assay | -0.0878326 | 0.915914 | 3.38375161 | 0.0658 |
| Age of L1 | -0.0188548 | 0.981322 | 0.59202431 | 0.4416 |
| GFP ^a time of longevity assay | -0.2483215 | 0.780109 | 6.53737848 | 0.0106 |
| GFP ^a age of L1 | 0.14346822 | 1.15427 | 8.57755873 | 0.0034 |

^a Other interactions fail to converge.

Therefore, we experimentally confirm the existence of the two sub-populations detected in each heat-shocked population and the lower the GFP level, the frailer the worms.

4. Discussion

Until now, hidden heterogeneity has remained a theoretical concept with no direct experimental support. In the present study, we found significant variation in age at death within a 100% genetically identical (isogenic) population of the nematode *C. elegans*. Similar patterns of variation were seen in each population studied, consistent with the notion that the largest component of lifespan variance is non-genetic.

In human studies, only about 20% of the variation in length of life is heritable (Herskind et al., 1996) while in the nematode the estimate is about twice that much (Johnson and Wood, 1982). Worms (TJ375) that carry the *hsp-16.2::GFP* reporter transgene respond to heat shock by producing endogenously encoded heat shock protein (HSP-16.2) as well as transgenically encoded green fluorescent protein (GFP). Because GFP expression is driven by an *hsp-16.2* promoter, those worms with high GFP expression have high HSP-16 expression, and worms with low GFP expression have low HSP-16 expression. In this study, we analyzed the non-genetic component of variation in individual TJ375 life by utilizing variation in level of individual GFP expression. We demonstrate that the variation in GFP expression is predictive of subsequent length of life (see Rea et al., 2005). We show that worms, after heat-shock, consist of two sub-populations, which differ significantly in their mortality. The subpopulation with high GFP expression has a significantly longer lifespan than the subpopulation showing low GFP levels. We thus provide the first direct evidence of hidden heterogeneity in a population.

Heat shock proteins play a well established role in protecting cells from the many mis-folded proteins that commonly arise following heat shock and other types of stressors (Strayer et al., 2003). If left unattended, such wayward proteins can lead to cell death. In our current studies, we utilized a transgenic GFP reporter construct linked to the promoter of *hsp-16.2* to measure hidden heterogeneity. Since GFP levels and HSP-16.2 levels are strongly correlated in TJ375 (Link et al., 1999), one possibility is that HSP-16.2 levels directly determine the long life span of the high GFP-expressing worms. On the other hand, additional proteins were no doubt also induced following heat shock and it may have been one or more of these that were determining the observed lifespan responses. We favor this last hypothesis because over-expression of HSP-16 alone leads to only modest levels of increase in longevity (Walker et al., 2001; Walker and Lithgow, 2003). By this model, then, the greater the ability of individual worms to respond to stress, the longer the life, and increased response to stress would in turn be responsible for higher HSP-16.2 expression.

Is the ability to respond to stress the only hidden factor for which worms are heterogeneous? Our data suggests not. Comparing the observed and predicted lifespans of each sorted subpopulation in Fig. 3, we find that in some experiments, the

mean lifespan observed for sorted populations expressing low levels of GFP was higher than that predicted for corresponding frail populations; similarly the mean lifespan observed for some of the populations with high GFP was lower than that predicted for corresponding robust populations. This difference must be due to the presence of hidden factors other than those predicted by HSP-16. Clearly, the ability of worms to induce HSP-16 in response to heat stress is only one factor contributing to population heterogeneity, albeit a major one.

Several earlier studies attempted to detect hidden heterogeneity using indirect means but obtained conflicting results. Khazaeli et al. (1995) applied a brief, intense environmental stress to a cohort of genetically homogeneous population of *Drosophila* to kill frail individuals and then made a comparison of mortality at older ages between stressed populations and non-stressed controls. They reasoned that if their starting population was heterogeneous, post-stress mortality rates should drop below those of non-stressed controls, because weaker individuals will have been removed from the stressed population. If the population was not heterogeneous, post-stress mortality would not have differed. They found reduced mortality, following stress, suggesting that the population was heterogeneous for frailty. Importantly, however, these authors failed to observe heterogeneity directly and the environmental stress may have done more than just eliminate the weakest flies. Such stressors are known to alter the mortality trajectory of the stressed population even when no animals die; thus, the decrease in mortality following stress may result from a hormetic effect of the stress rather than by eliminating frail animals (Curtsinger and Khazaeli, 1997, Johnson and Bruunsgaard, 1998; Cypser and Johnson, 2002, 2003). In a later study, Khazaeli et al. (1998) attempted to reduce as much as possible the degree of pre-adult, environmentally induced heterogeneity among individuals of a genetically identical cohort through a careful process of selection of *Drosophila* eggs, larvae, pupae and adults. They then asked whether such cohorts, when compared to non-fractionated populations, exhibited less mortality deceleration at advanced ages. Finding no difference, they concluded that developmental/environmental heterogeneity is not a major factor contributing to mortality deceleration at older ages, but again, these authors did not determine whether individual heterogeneity was a factor or not. Finally, Drapeau et al. (2000) employed 61,404 flies from three lines of *D. melanogaster* that differ substantially in their resistance to starvation to demonstrate that heterogeneity is not a major contributor to the phenomenon of late-life mortality plateaus. However, they did not measure the difference in resistance to starvation among individuals within each line and so failed to measure hidden heterogeneity directly.

Our use of a non-invasive method to directly detect hidden heterogeneity in isogenic populations of *C. elegans* has provided the first concrete evidence of a phenomenon predicted by mathematics some 40 years ago to explain the leveling off of mortality at advanced ages. We conclude from our data that: (1) the ability to respond to stress is not the same from individual to individual; rather, worms differ substantially in their ability to respond to stress. (2) Individual ability to

respond to stress is not normally distributed. (3) There is a significantly positive association between individual ability to respond to stress and its longevity. Worms with high ability to respond to stress have long lifespan, and worms with low ability to respond to stress have short lifespan. (4) Among factors that affect longevity, the ability to respond to stress is a hidden and significant factor and is present on the first day of adult life.

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