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Expression of *hsp22* and *hsp70* Transgenes Is Partially Predictive of Drosophila Survival Under Normal and Stress Conditions

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Drosophila Hsp70 is a highly conserved molecular chaperone with numerous cytoplasmic targets. Hsp22 is an alpha-crystallin–related chaperone (small hsp) that localizes to the mitochondrial matrix. The *hsp70* and *hsp22* genes are induced in response to acute heat and oxidative stress and are also upregulated during normal aging. Here the *hsp22* promoter (−314 to +10) and the *hsp70* promoter (−194 to +10) were used to drive expression of the fluorescent reporter proteins green fluorescent protein (GFP) and Discosoma sp. red fluorescent protein (DsRED) in transgenic flies. Multiple transgenic lines were analyzed under normal culture conditions and under oxidative stress and heat stress conditions that significantly shorten life span. Flies were individually housed, and GFP (or DsRED) was quantified at young-age time points using the fluorescence stereomicroscope and image analysis software. Expression of the *hsp* reporters in young flies was partially predictive of remaining life span: Young flies with high expression tended to die sooner under both control and stress conditions.

Key Words: Biomarker—Aging—Oxidative stress—GFP—hsp.

THE reasons for individual variability in life span are not understood and are a topic of active research (1). Only about 30% of life span appears to be "heritable" in humans (and flies) based on standard Mendelian genetic models. Because of the enormous individual variability in aging and life span, chronological age usually does not reflect the physiological age of humans and model organisms. A biomarker of aging, therefore, would be a powerful tool in aging studies. By definition, a biomarker of aging is "a biological parameter of an organism that either alone or in some multivariate composite will, in the absence of disease, better predict functional capability at some late age than will chronological age" (2). Thus, genes whose expression changes during aging are good candidates.

The 14,000 or so genes in Drosophila have been assayed in cross-sectional studies of aging, and only a few hundred change in expression, and they do so in a characteristic and relatively predictable manner (3–5). Some of these changes appear to be conserved in human aging and are therefore of particular interest, including the downregulation of electron transport chain and energy metabolism genes and the tissue-specific upregulation of oxidative stress response genes (6).

The current evolutionary theory of aging suggests that aging in flies and humans results from the decreasing force of natural selection with age (7). This predicts that genes with expression exclusively in very old animals should not exist, and so far there is no data to contradict this idea. Much of the aging-specific gene expression pattern observed in flies and humans should therefore result from normal homeostatic and stress response pathways responding to the particular types of disorganization and damage incurred during aging.

Aging in Drosophila is associated with changes in gene expression similar to an oxidative stress response, including the tissue-specific upregulation of *hsp22* and *hsp70* gene expression (4,8–10). During normal aging, *hsp70* is upregulated preferentially in flight and leg muscle, whereas *hsp22* is upregulated in all tissues, especially nervous tissue and retina tissue. Both genes respond to acute heat and oxidative stress; however, *hsp22* is relatively more responsive to oxygen stress whereas *hsp70* is relatively more responsive to heat stress (4), for as-yet-unknown reasons. Both the *hsp70* and *hsp22* aging expression patterns require functional heat shock response elements (HSEs) in the promoter (9,10). HSEs are the binding sites for the conserved heat shock factor (HSF; 11,12), and HSF has been shown to be a regular of life span in *Caenorhabditis elegans* (13–15).

Tissue-specific induction of *hsp70* family and *hsp22* family genes is also observed in mammals, both during normal aging and in neurodegenerative diseases (6,16). This indicates that *hsp* gene induction during aging is evolutionarily conserved across a broad range of taxa. Mammalian *hsp27* is a critical regulator of apoptosis in neurons and is regulated at both the transcriptional and posttranscriptional levels (17,18). Tissues from old mammals, including skeletal muscle and T cells, exhibit both increased "basal" levels of Hsp70, as well as a reduced accumulation of Hsp70 in response to acute stresses (12,19,20). Strikingly, *hsp70* gene alleles and blood Hsp70 levels have been found to correlate with longevity in human studies (21,22), making *hsp70* a promising biomarker for human aging. Taken together, the current data suggest that studying *hsp* gene expression in Drosophila may ultimately inform on human aging mechanisms and health interventions.

In Drosophila, experimental manipulation of *hsp* gene expression is associated with increased life span under certain conditions and decreased life span under other conditions

(23–27). In mammals, both *hsp22* family and *hsp70* gene expressions are observed in apoptotic cells; however, their function can be proapoptotic or antiapoptotic depending on the tissue and age of the animal (20). Taken together, the data suggest a model in which robust and regulated expression of *hsp* genes (on and off) favors longevity, whereas accumulation of Hsps to high levels in old animals may be toxic (28–30). Consistent with this idea, it was recently reported that in *C. elegans* the life span of genetically identical individuals correlates with ability to induce expression of heat shock genes in response to heat stress when they are young (31).

Here *Drosophila melanogaster* was transformed with constructs containing *hsp* gene promoter sequences fused to enhanced green fluorescent protein (eGFP), or to DsRED, to test for aging biomarker activity of both *Drosophila hsp22* and *hsp70*. GFP and DsRED expression level was quantified using multiple images of individual flies generated with a fluorescence stereomicroscope. Although there was considerable variability across lines and experiments, both *hsp22* and *hsp70* transgene expression at early age time points was partially predictive of remaining life span of adult flies during normal aging and when flies were subjected to heat stress and oxidative stress.

Materials and Methods

DNA Constructs

Plasmids for making transgenic GFP reporter flies were derived from pGreen pelican (32). phsp22p-Gpel was a derivative of pGreen pelican in which the sequence between the *Kpn*I and *Bgl*II sites was replaced by the sequences from −312 to +10 of the *hsp22* gene of *D. melanogaster*, and in phsp70-Gpel, these sequences were replaced by the sequences from −194 to +10 of the *hsp70* gene. The *hsp* gene sequences used in cloning were generated using the following oligonucleotides containing engineered *Bgl*II and *Kpn*I sites (underlined): *hsp22* forward primer CC AGA TCT TCA ATG TGT CTC TCT GCG, *hsp22* reverse primer CC GGT ACC TTT GAA CTG AGA GCG TAG, *hsp70* forward primer CC AGA TCT CTC GAG AAA TTT CTC TGG, and *hsp70* reverse primer CC GGT ACC GAA TTG AAT TGT CGC TCC. The template for amplification of *hsp70* sequences was plasmid pBS70Z (9), and the template for amplification of *hsp22* sequences was plasmid "hsp22 5′Δ(−314)" (10). Plasmids for making transgenic DsRED reporter flies were derived from Plasmid pRHP (hsp70core-DsRed-Pelican), which was obtained from James Posakony (University of California, San Diego). The pRHP plasmid was first modified by removing the *hsp70* core promoter sequences. This was done using *Bam*HI plus *Age*I restriction digestion, followed by treatment with Klenow fragment to fill in 5′ overhangs and generate blunt ends. The linear blunt-ended plasmid was then ligated using DNA ligase to create circular plasmid pRpel. The hsp-DsRED reporter plasmids were then constructed analogous to the construction of the GFP reporters described earlier: phsp22p-Rpel was a derivative of pRpel in which the sequence between the *Kpn*I and *Bgl*II sites were replaced by the sequences from −312 to +10 of the *hsp22* gene, and in phsp70-Rpel, these sequences were replaced by the sequences from −194 to +10 of the *hsp70* gene.

Drosophila P Element–Mediated Transformation

Transgenic fly strains were generated by microinjection of the *P*-element constructs into fly embryos along with delta2–3 "turbo" helper plasmid (pUChspD2-3wc) as a source of transposase, using standard methods (33). Strains with multiple inserts were generated in order to increase reporter expression levels, as follows: To generate strains with multiple inserts, strains with single inserts were crossed to the delta2-3 transposase source (34) to mobilize the inserts, and chromosomes bearing multiple inserts were first identified by increased expression of the *mini-white+* marker gene, and then insert copy number was confirmed using genomic Southern blotting.

Generation of PEPCK-GFP Reporter Flies

A strain of flies was generated where GFP expression is under the control of a promoter that is not induced by heat or oxidative stress, in this case the *PEPCK* gene promoter. A gene-trap line of genotype *w[1118]; P{w[+mGT] = GT1}BG02569* was obtained from Bloomington Drosophila stock center and produces GAL4 protein under the control of *PEPCK* gene regulatory sequences (35); this strain is hereafter referred to as *PEPCK-GAL4*. A strain was constructed containing multiple copies of a construct with a upstream activating sequence (UAS) promoter driving expression of eGFP as follows: starting strains were obtained from Ron Davis (Baylor College of Medicine) that contained the UAS-2XeGFP construct (36) inserted on the second chromosome (line U-202-3) and on the third chromosome (line U-307-1). These lines were crossed to a strain bearing the delta2-3 transposase source (34) to mobilize the inserts, and chromosomes were derived that contained multiple copies of the insert on the second and third chromosomes, named *UAS-2xEGFP[m5B29]* and *UAS-2xEGFP[m6B1]*, respectively, where the "*m*" stands for multimer derivative chromosome. These second and third chromosomes bearing the multiple inserts were then placed into the same genetic background and made homozygous by appropriate crosses to double-balancer strains, to generate strain *w*; *UAS-2-xEGFP[m5B29]*; *UAS-2xEGFP[m6B1]*. This strain was crossed to the *PEPCK-GAL4* strain described earlier to generate progeny of genotype *w; UAS-2xEGFP[m5B29]/PEPCK-GAL4; UAS-2xEGFP[m6B1]*. In these progeny flies, the *PEPCK* gene regulatory sequences drive expression of GAL4 transcription factor, which in turn binds to the multiple UAS promoter constructs to drive expression of eGFP. Therefore, in these flies the GFP expression is ultimately

driven by the regulatory sequences of *PEPCK*, and they are hereafter referred to as "*PEPCK-GFP*" reporter flies.

Drosophila Culture

Flies were cultured as previously described (4). Newly eclosed male flies were collected over a period of 24 hours, maintained at 25°C at 10 per vial in culture vials with food. All flies were transferred every other day into fresh media. At 4 days of age, flies were transferred to fresh media at one fly per vial. Each fly was given a specific number for the purpose of measuring its life span and reporter expression. The time of death of the flies was recorded at every transfer. For normal aging assays, flies were kept in an incubator at 25°C on a 12-h:12-h light–dark cycle.

Oxygen Stress Assay

Flies were treated in an enclosed chamber with 100% oxygen gas flow and transferred to fresh media every day, as previously described (4). GFP or DsRED expression level for each fly was assayed every other day (days 2, 4, and 6).

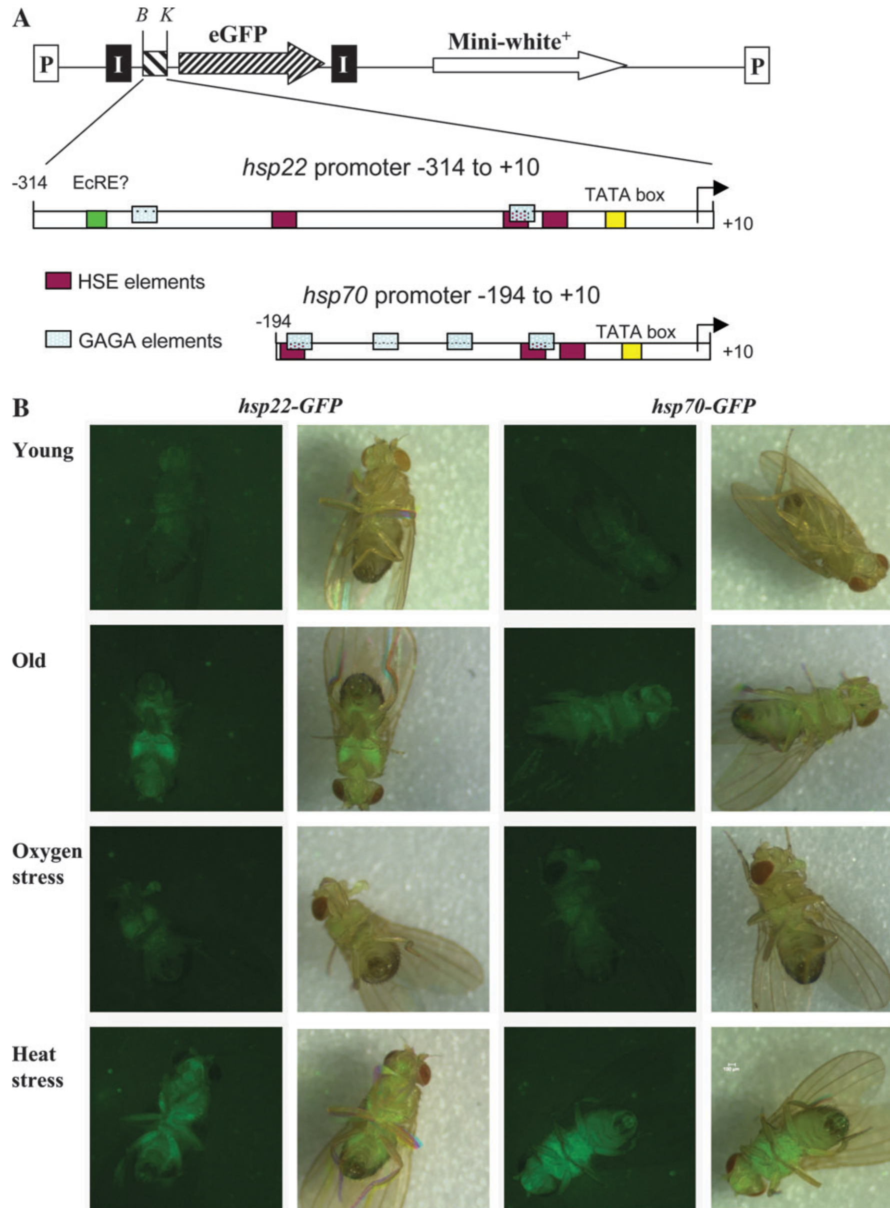


Figure 1. Transgenic *hsp22* and *hsp70* reporters. (**A**), Construct maps. The vector region between the P element inverted repeats (P) is diagrammed at the top, and the *hsp* gene sequences are diagrammed below. Solid boxes (I) indicate the insulator elements. (**B**) Image capture assay. Representative images are presented for male flies of the *hsp22-GFP* and *hsp70-GFP* transgenic strains under normal aging conditions (young = 4-d old; old = 70-d old) and heat stress (3 d at 34°C) and oxidative stress conditions (4 d in oxygen stress), as indicated. For each fly, the GFP fluorescence image is presented to the left and an overlay of the GFP and visible images is presented to the right. The young fly pictures are from line *hsp22-GFP(3)1/TM3 Sb e* and *hsp70-GFP(3)2/TM3 Sb e*. All other pictures are from lines *hsp22-GFP(3)1 MI1/TM3 Sb e* and *hsp70-GFP(3)2 MI4/TM3 Sb e*.

Table 1. Transgenic Reporter Strains and Summary of Results

Strain Name	Inserts	Predictivity		
		Aging	Oxygen	Heat
hsp22-GFP(2)1	2	nc [67]	Neg (1) [10]	Neg (1)* [5]
hsp22-GFP(3)1/TM3	1	Neg (2) [74]	Neg (1,2,3) [13]	Nc* [10]
hsp22-GFP(3)1MI1/TM3	2	Neg (3) [56]	Neg (2)* [10]	Nc† [6]
hsp22-DsRed(3)1/TM3	1	ND	Nc† [7]	nc† [4]
hsp70-GFP(3)1 MI2/TM3	2	Neg (2,3) [52]	Nc* [15]	Nc† [10]
hsp70-GFP(3)2/TM3	1	nc [80]	Neg (1) [10]	ND
hsp70-GFP(3)2 MI4/TM3	2	Neg (2,3) [60]	Nc* [8]	Nc† [13]
hsp70-DsRed(3)1 MI2/TM3	2	ND	Nc† [14]	Neg (1)† [12]
PEPCK-GFP	1, >4‡	nc [77]	nc [11]	ND

Notes: The *hsp22GFP(2)1* insert is on the second chromosome and is homozygous. The remaining inserts are on the third chromosome and are balanced over *TM3*, *Sb*, *e*. Numbers in parentheses indicate at which of the three time points (TP1, TP2, TP3) a significant negative (Neg) correlation between GFP intensity and subsequent life span was detected by regression analysis ($p < .05$). ND = assay not done; nc = no significant correlation detected. Certain experiments included less than three time points due to the limited survival of the flies.

*Experiments that included only two time points (TP1 and TP2).

†Experiments that included only one time point (TP1).

‡The *PEPCK-GFP* reporter flies contain one insert of the *PEPCK-GAL4* gene trap and ≥4 inserts of the *UAS-GFP* construct (Materials and Methods). The median life span of each cohort is indicated in brackets.

Heat Stress Assay

Flies were maintained in a 34°C incubation chamber and transferred to fresh media every day. GFP or DsRED expression level for each fly was assayed every other day (days 2, 4, and 6).

Quantification of GFP Expression in Live Flies

GFP fluorescence levels for each fly at each time point were determined from triplicate fluorescence images generated with the Leica MZFLIII fluorescence stereomicroscope (Leica, Inc., Deerfield, IL) and SPOT and Image-plus software, as previously described (4). This method yields GFP density, which is the average of green pixel numbers in the digital pictures, which is a measure of how green the picture is in red/green/blue (RGB) space. For clarity of presentation, the images of flies presented in Figure 1B were adjusted by increasing the contrast enhancement setting gamma from 1 to 1.6. Each data set was also analyzed using a custom Matlab script to quantify the GFP intensity of the fly, where GFP intensity is the hue value of a specific green wavelength (510 nm) with fixed pixel number, which indicates how bright the color is in HSL (hue/saturation/lightness) space. Similar results were obtained with both quantification methods (data not shown): Greater GFP expression should produce more emission of green light, thereby making the picture both greener and brighter, and therefore values calculated from both methods are reflective of GFP levels in the flies. The DsRED expression levels were quantified using analogous RGB methods.

Statistical Analysis of GFP and DsRED Expression

Linear regression analysis was conducted to determine the significance of the correlation between GFP expression and life span (if any), using SPSS statistical software. Both the *hsp-GFP* strain and the *PEPCK-GFP* strain were analyzed. First, each data set was tested to confirm that the data conformed to a normal distribution using Shapiro–Wilks test, and each data set had a normal distribution except for one. The one nonnormal data set could not be corrected by transforming the data and was therefore discarded. For the regression analyses, the predictor variables were always GFP levels, whereas the outcome variable was always life span. Adjusted R^2, significance of the Predictor, and standardized beta are presented for each data set (Table 2).

Results

Longitudinal Assay of GFP and DsRED Reporter Expression

Transgenic fly strains were generated where GFP or DsRED expression was under the control of the regulatory regions of either the *hsp70* gene or the *hsp22* gene (Figure 1A), whereas in control flies GFP expression was driven by the regulatory region of the *PEPCK* gene (Materials and Methods). *PEPCK* was chosen because it is not induced by heat or oxidative stress, and in this way serves as one control to demonstrate that any relationship between transgene expression and life span that might be observed with the *hsp* reporters is not simply due to a correlation between life span and global levels of transcription, translation, or GFP turnover. The *hsp* transgenic strains generated and analyzed in this study are listed along with a summary of results (Table 1). Age-synchronized cohorts of approximately 50 male flies were generated for each strain. Reporter expression was assayed at three young-age time points: 10, 20, and 30 days for normal aging conditions and at 2, 4, and 6 days for oxygen stress and heat stress conditions where life span was reduced. This allowed for assay of most flies before significant mortality of the cohort had begun (Figure 2), which is the time period at which a predictive biomarker would be most

Table 2. Regression Analyses

	GFP at TP1	GFP at TP2	GFP at TP3
Normal aging			
hsp22GFP(3)1/TM3 Sb e			
N	43	41	40
Adjusted R^2	.017	.141	.000
Significance of predictor	.198	.015	.960
Standardized beta	−.200	−.376	−.008
hsp22GFP(2)1			
N	38	38	35
Adjusted R^2	.069	.012	.007
Significance of predictor	.061	.234	.271
Standardized beta	−.307	−.198	−.191
hsp22 GFP (3)1MI1/TM3 Sb e			
N	45	45	43
Adjusted R^2	−.022	−.023	.237
Significance of predictor	.850	.918	.001
Standardized beta	−.029	.016	−.505
hsp70 GFP (3)2/TM3 Sb e			
N	45	45	45
Adjusted R^2	−.019	−.007	−.019
Significance of predictor	.672	.408	.666
Standardized beta	.065	.126	.066
hsp70 GFP (3)2MI4/TM3 Sb e			
N	48	47	47
Adjusted R^2	.008	.116	.064
Significance of predictor	.246	.011	.047
Standardized beta	−.171	−.368	−.291
hsp70 GFP (3)1MI2/TM3 Sb e			
N	47	46	44
Adjusted R^2	.000	.064	.120
Significance of predictor	.320	.049	.012
Standardized beta	−.148	−.292	−.374
PEPCK-GFP			
N	46	46	45
Adjusted R^2	−.006	−.023	.002
Significance of predictor	.402	.952	.304
Standardized beta	.127	.009	−.157
Oxygen stress			
hsp22 GFP (3)1/TM3 Sb e			
N	29	29	29
Adjusted R^2	.234	.151	.193
Significance of predictor	.005	.021	.010
Standardized beta	−.511	−.425	−.471
hsp22 GFP (2)1			
N	28	27	27
Adjusted R^2	.123	.051	.054
Significance of predictor	.038	.135	.128
Standardized beta	−.394	−.295	−.301
hsp22 GFP (3)1MI1/TM3 Sb e			
N	29	29	
Adjusted R^2	.084	.285	
Significance of predictor	.070	.002	
Standardized beta	−.341	−.557	
hsp70 GFP (3)2/TM3 Sb e			
N	30	30	29
Adjusted R^2	.113	−.034	−.029
Significance of predictor	.039	.831	.649
Standardized beta	−.379	.041	−.088
hsp70 GFP (3)2MI4/TM3 Sb e			
N	30	30	
Adjusted R^2	.031	−.036	
Significance of predictor	.175	.969	
Standardized beta	−.254	−.007	
hsp70 GFP (3)1MI2/TM3 Sb e			
N	30	30	
Adjusted R^2	−.033	.041	
Significance of predictor	.780	.146	
Standardized beta	−.053	−.272	
PEPCK-GFP			
N	30	30	30
Adjusted R^2	−.030	−.020	.002
Significance of predictor	.709	.517	.315
Standardized beta	−.071	−.123	−.190
Heat stress			
hsp22 GFP (3)1/TM3 Sb e			
N	48	47	
Adjusted R^2	−.021	−.007	
Significance of predictor	.860	.421	
Standardized beta	−.026	.120	
hsp22 GFP (2)1			
N	49	32	
Adjusted R^2	.088	−.021	
Significance of predictor	.022	.545	
Standardized beta	−.328	−.111	
hsp22 GFP (3)1MI1/TM3 Sb e			
N	50		
Adjusted R^2	−.020		
Significance of predictor	.816		
Standardized beta	−.034		
hsp70 GFP (3)2MI4/TM3 Sb e			
N	39		
Adjusted R^2	.020		
Significance of predictor	.191		
Standardized beta	−.214		
hsp70 GFP (3)1MI2/TM3 Sb e			
N	47		
Adjusted R^2	.004		
Significance of predictor	.281		
Standardized beta	−.161		
	DsRed at TP1		
Oxygen stress			
hsp22 DsRed (3)1/TM3 Sb e			
N	26		
Adjusted R^2	−.022		
Significance of predictor	.507		
Standardized beta	.136		
hsp70 DsRed (3)1MI2/TM3 Sb e			
N	30		
Adjusted R^2	−.033		
Significance of predictor	.808		
Standardized beta	−.046		
Heat stress			
hsp22 DsRed (3)1/TM3 Sb e			
N	22		
Adjusted R^2	.058		
Significance of predictor	.146		
Standardized beta	−.320		
hsp70 DsRed (3)1MI2/TM3 Sb e			
N	33		
Adjusted R^2	.200		
Significance of predictor	.005		
Standardized beta	−.474		

(*Table 2 Continues*)

Table 2. Regression Analyses (*Continued*)

TP1 = time point 1; TP2 = time point 2; TP3 = time point 3.

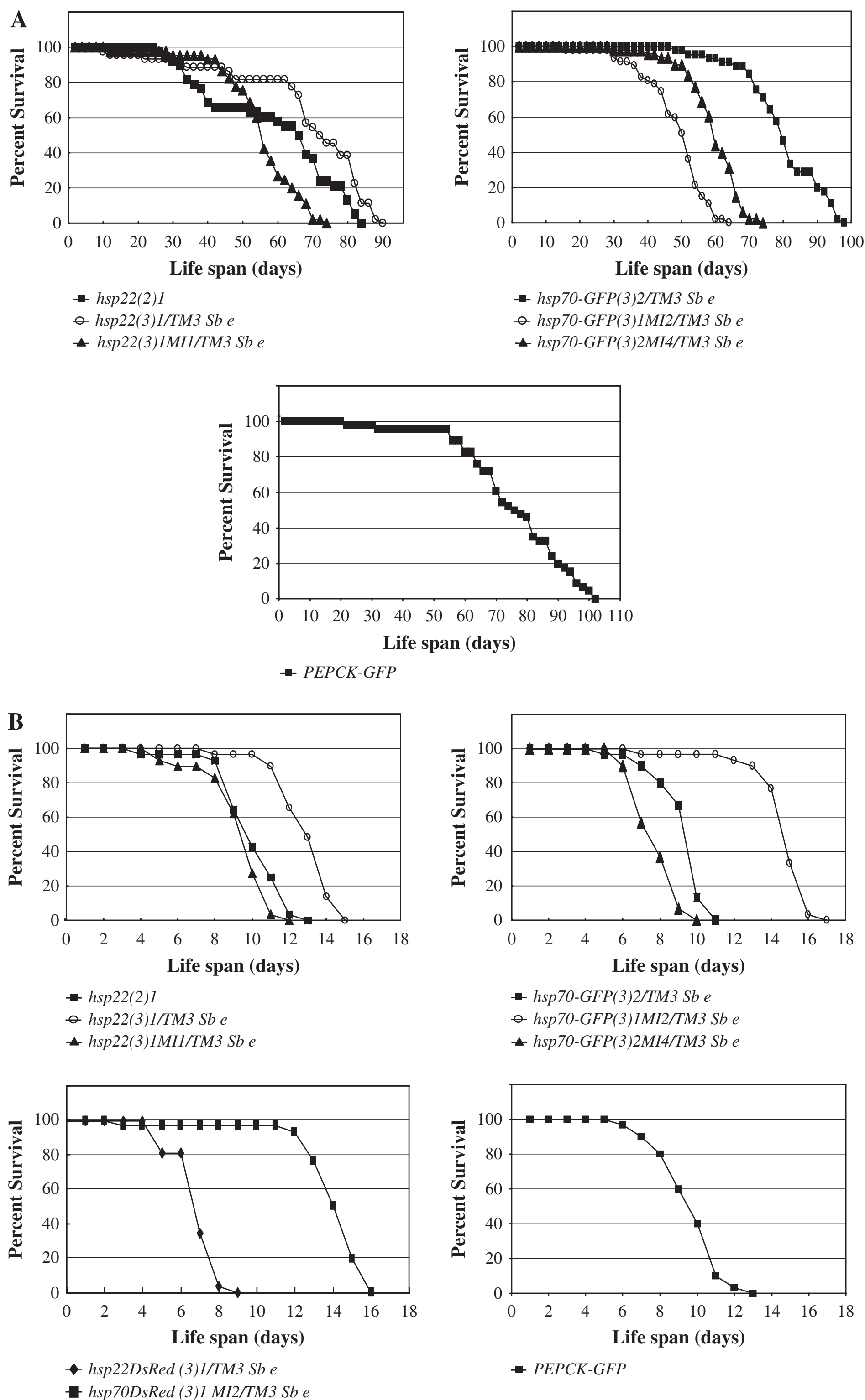
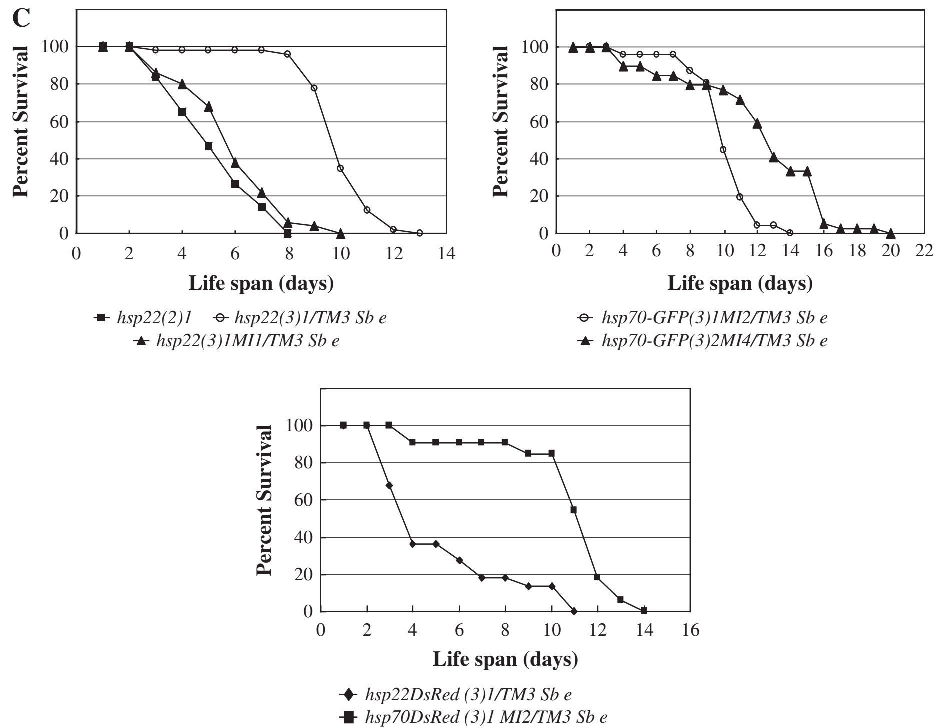


Figure 2. Survival curves for transgenic fly cohorts. (**A**) Survival curves for flies under normal aging conditions. Time points for fluorescence assay were 10 d (time point 1), 20 d (time point 2), and 30 d (time point 3), respectively. (**B**) Survival curves for flies under oxygen stress conditions. Time points for fluorescence assay were 2 d (time point 1), 4 d (time point 2), and 6 d (time point 3), respectively. (**C**) Survival curves for flies under heat stress conditions. Time points for fluorescence assay were 2 d (time point 1), 4 d (time point 2), and 6 d (time point 3), respectively. Median life spans for each cohort are summarized in Table 1.



(*Figure 2 Continued*)

useful. The flies were maintained individually and transferred to fresh culture vials every other day, so that GFP or DsRED intensity could be correlated with individual life spans. GFP and DsRED intensity was quantified at each time point by briefly anesthetizing the fly with CO_2 gas and then taking three photographs of the fluorescence of the fly using the Leica MZFLIII fluorescence stereomicroscope; and this assay has little to no effect on fly life span (4). Representative images of GFP fluorescence from transgenic reporter flies are presented (Figure 1B), and as expected, the expression of both *hsp22* and *hsp70* transgenic reporters increased during aging and in response to heat and oxygen stress. Image analysis software was used to outline the part of the image representing the flies' body, and the pixel intensity in that region of the three pictures was averaged. The GFP or DsRED intensity measured for each fly at each age time point was then correlated with the subsequent life span of the flies. Experiments were conducted under normal culture conditions, as well as under heat stress conditions and with exposure to 100% oxygen atmosphere. Statistical significance of the correlations of GFP or DsRED expression and life span was determined using linear regression to compare life span and fluorescence for each cohort in each experiment (Figure 3A; Table 2; data summarized in Table 1). For ease of presentation and comparison of certain aspects of the data, the life span for flies was separated in to three groups containing equal numbers of flies, short lived, medium lived, and long lived, and the average plus or minus standard deviation of GFP or DsRED intensity in each group at each time point is presented in bar graph form (Figures 3 and 4).

Previously, the expression pattern of *hsp22* and *hsp70* during aging has been characterized using Northern blots, Western blots, and transgenic reporter constructs where the *hsp22* gene region from −314 to +275 and the *hsp70* gene region from −194 to +276 were fused to *LacZ*. In those studies, the expression of *hsp22* during aging was tissue general (10), whereas *hsp70* expression was preferentially observed in flight muscle and leg muscle (8,9). In the present experiments, the *hsp70-GFP* reporter was expressed in other tissues in addition to flight and leg muscle, including the head and abdomen (Figure 1B). The reason for this apparently broader distribution of the *hsp70-GFP* reporter relative to the *hsp70-LacZ* reporters may be the smaller amount of *hsp70* gene sequences present in the *hsp70-GFP* construct (−194 to +10) and/or differences in the stability and resolution afforded by GFP relative to LacZ.

hsp Expression and Drosophila Life Span Under Normal Aging Conditions

When adult flies were cultured under normal aging conditions, GFP levels were negatively correlated with remaining life span for two out of three *hsp22-GFP* lines assayed, at a subset of the young time points (Table 1). For example, flies of line *hsp22-GFP(3)1MI1* that had lower expression of GFP at 30 days of age (time point 3) tended to be longer lived than their siblings (Figure 3A and B). Similarly, GFP levels were negatively correlated with remaining life span for two out of three *hsp70-GFP* lines assayed, at a subset of the young time points (Figure 3C; Table 1). No correlation

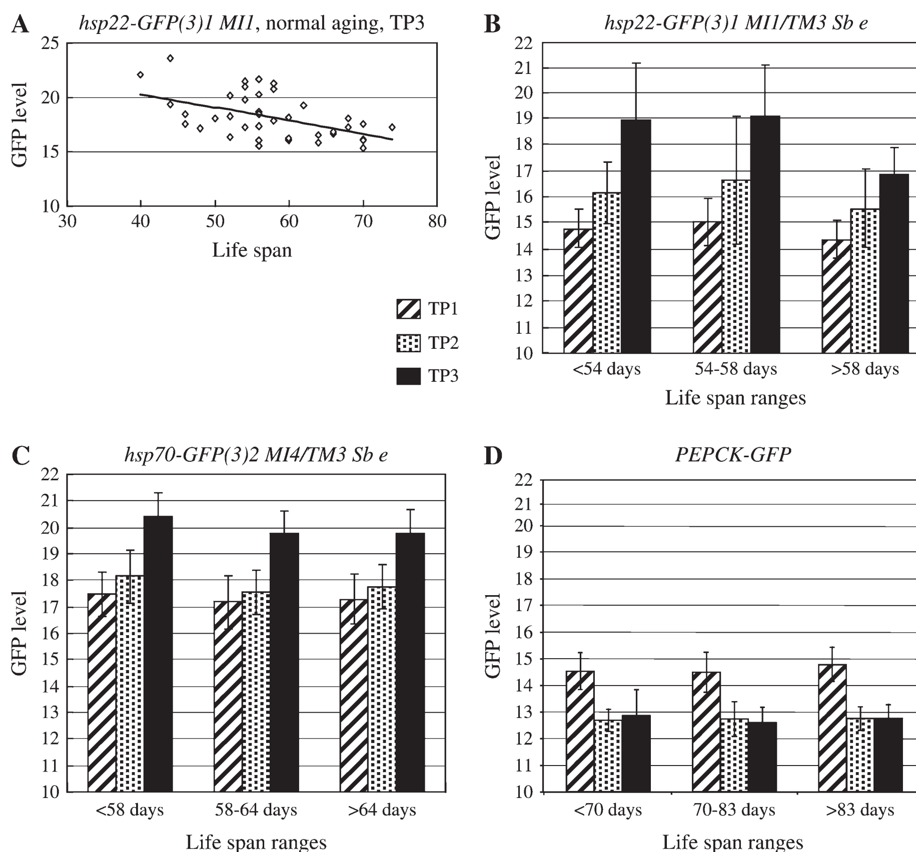


Figure 3. GFP levels of flies with different life spans under normal aging conditions. Image capture was performed in triplicate for each fly at time point 1 (TP1 = Day 10), time point 2 (TP2 = Day 20), and time point 3 (TP3 = Day 30). (**A**) Scatter plot, line *hsp22-GFP (3)1/TM3 Sb e*, data for time point 3. (**B–D**) The data for the flies were divided into three equal groups based on their life spans. (**B**) *hsp22-GFP(3)1MI1/TM3 Sb e*. (**C**) *hsp70-GFP(3)2MI4/TM3 Sb e*. (**D**) *PEPCK-GFP*.

was observed between GFP expression and life span with the *PEPCK-GFP* control at any time points in any experiment (Figure 3D; Table 1).

hsp Expression and Drosophila Life Span Under Oxidative Stress Conditions

When adult flies were cultured in 100% oxygen atmosphere, life spans were dramatically reduced, as expected (Figure 1; Table 1). Under these oxidative stress conditions, expression levels were negatively correlated with remaining life spans for three out of four *hsp22* reporter lines assayed and for one out of four *hsp70* reporter lines, at a subset of young time points (Figure 4 and Table 2), whereas again no correlation was observed for the *PEPCK* control strain.

hsp Expression and Drosophila Life Span Under Heat Stress Conditions

When flies were cultured at an elevated temperature (34°C), life spans were again significantly reduced relative to normal culture conditions, as expected (Figure 1; Table 1). Under these heat stress conditions, expression levels were negatively correlated with remaining life spans for one out of four *hsp22* reporter lines and for one out of three *hsp70* reporter lines (Table 2). No correlation was observed for the *PEPCK* control strain.

Discussion

In transgenic lines where *hsp22* and *hsp70* gene promoters were used to drive expression of eGFP or DsRED, the fluorescence intensity could be assayed longitudinally and correlated with individual life span. The *hsp22* and *hsp70* genes are known to be induced in response to heat and oxidative stress and to be induced during normal aging. Consistent with this, in the longitudinal assays of individual flies, the expression of the *hsp22* and *hsp70* reporters was generally observed to increase with age and duration of stress treatment. However, at the level of cohorts of flies, the changes in average expression with age and duration of stress treatment were more complex. For most cohorts, the average level of expression increased across the three time points in concordance with the increase in expression level in the individual flies (e.g., see Figure 3B and C; Figure 4B). In contrast, for certain cohorts, the average expression level remained relatively constant with time, or actually went down, for example the line *hsp70-GFP(3)1MI2* with oxygen stress (Figure 4C). We conclude that this decrease in average

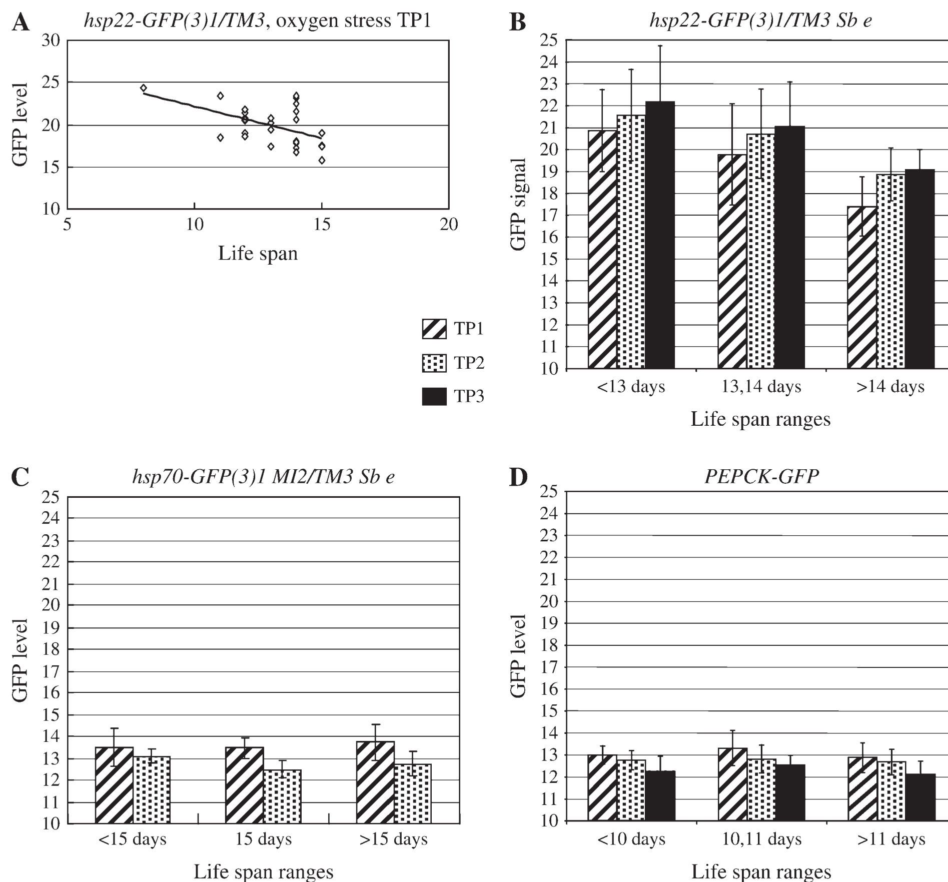


Figure 4. GFP level of flies with different life span ranges under oxygen stress. Image capture was performed in triplicate for each fly at time point 1 (TP1 = Day 2), time point 2 (TP2 = Day 4), and time point 3 (TP3 = Day 6). (**A**) Scatter plot, line *hsp22-GFP (3)1/TM3 Sb e*, data for time point 1. (**B–D**) The data for the flies were divided into three equal groups based on their life spans. (**B**) *hsp22-GFP(3)/TM3 Sb e.* (**C**) *hsp70-GFP(3)1MI2/TM3 Sb e.* (**D**) *PEPCK-GFP.*

expression in certain cohorts is due to "cohort effects": because the most highly expressing flies die earlier, this can sometimes produce a decrease in the average expression of the cohort over certain time intervals.

In the experiments where a significant correlation was observed, the expression of the *hsp* reporters was always negatively correlated with life span under both normal and stress conditions. The negative correlation between *hsp* reporter expression and life span is likely related to the fact that these genes are induced in response to stress; flies experiencing a relatively larger degree of physiological stress are expected to exhibit a larger induction of the transgene and may survive for a shorter period due to the life-shortening effects of that stress. This result is somewhat counterintuitive in relation to studies where *hsp* gene expression has been examined in animals that have been genetically selected for increased life span: In cross-sectional assays of control and long-lived fly strains, the long-lived strains exhibited higher levels of *hsp22* RNA (37). Moreover, in *C. elegans* strains that are long lived due to reduced insulin/insulin like growth factor 1-like signaling, the long-lived strains exhibit higher levels of expression of certain small *hsp* genes in response to heat stress (38). We conclude that the correlation between longevity and robust *hsp* expression in such cross-sectional assays indicates a more effective stress response, likely involving the ability to efficiently turn the genes off as well as on. Consistent with this idea, in young *C. elegans,* the level of induction of a *shsp* reporter in response to a mild and transient heat stress is positively correlated with remaining life span (31), consistent with the idea that animals with a more robust stress response live longer. In contrast, in longitudinal assays of individual animals under normal conditions or constant stress, the level of *hsp* reporter expression may be indicative of that particular individuals' susceptibility to stress, failing homeostasis and imminent mortality. In other words, in cross-sectional comparisons of strains with different life spans, the induction of *hsp* genes positively correlates with life span, whereas within a given strain, the longitudinal time course of *hsp* gene expression in an individual animal is negatively correlated with remaining life span. In the future, the longitudinal assay of the Drosophila *hsp* reporters in control and long-lived genetic backgrounds should allow for definitive tests of this model.

One possible way that the correlation between life span and *hsp-GFP* reporter expression could be an artifact would

be if GFP expression itself were having a significant negative effect on life span. For example, the *PEPCK-GFP* control flies had lower expression of GFP at late ages than did the *hsp-GFP* flies, and conceivably the GFP itself could have been toxic to the flies. However, the *PEPCK-GFP* flies had GFP expression at time point 1 that was equal to that of several *hsp-GFP* lines (compare Figure 3D with 3B), yet these *PEPCK-GFP* flies had normal life span and no correlation between *GFP* expression and life span. Moreover, the *hsp70-GFP(3)2* strain had robust GFP expression at late ages and the longest life span of all the strains analyzed (Table 1). Therefore, there was no evidence for a negative effect of GFP itself on life span.

The Drosophila *Drosomycin* gene encodes a small peptide that is induced in response to microbial (fungal) infections and has antimicrobial properties (39). Previously, a transgenic reporter construct consisting of the *Drosomycin* gene promoter fused to GFP was analyzed in young adult flies and shown to be partially predictive of remaining life span (4). Similar to the *hsp* gene reporters, the *Drosomycin-GFP* reporter was negatively correlated with life span, in that young flies with high expression tended to die sooner. Flies cultured in the absence of detectable microbes and in the presence of antimicrobial drugs had unchanged life spans (40), indicating that microbes do not typically limit life span in the laboratory, and making it unlikely that the predictive power of the *Drosomycin* reporter was due to a response to some life span–limiting infection. However, antimicrobial peptides can sometimes have additional functions, for example the human Hepcidin antimicrobial peptide is also a critical regulator of iron homeostasis (41). Like *hsp70* and *hsp22*, the Drosophila *Drosomycin* gene is induced in response to oxidative stress and during normal aging (4). Because oxidative stress appears to increase during aging, it may be the ability of the *hsp70*, *hsp22*, and *Drosomycin* genes to respond to oxidative stress that marks a physiologically older fly.

The ability of the Drosophila *hsp22* and *hsp70* reporters to act as predictors of remaining life span is promising; however, the predictive power is partial. One way, we are attempting to improve the predictive power is to further increase the copy number of the reporter constructs in the same fly strain, in an attempt to produce higher levels of expression. Similarly, we are developing more sensitive video-based methods for continuous and longitudinal assay of GFP fluorescence levels during fly aging (D. Grover, J. Yang, S. Tavaré, and J. Tower, 2009, unpublished data). Another approach will be to combine two different gene reporters in the same strain, for example *hsp22-GFP* along with *hsp70-DsRED*, and such strains are under construction. Even the partial predictive power of the present strains and assays should be useful in experiments such as in genetic screens designed to identify life span regulators, by screening for increased or decreased reporter expression in young- or middle-aged animals.

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