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Increased *hsp22* RNA Levels in *Drosophila* Lines Genetically Selected for Increased Longevity

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RNAs for the small heat shock protein (hsp) genes *hsp22* and *hsp23* are induced during *Drosophila* aging, suggesting that these genes might have specific functions at late ages. To determine if *hsp22* and *hsp23* gene expression might correlate with life span, RNA levels for these and additional genes were analyzed throughout the adult life span in a set of five outbred "O" lines, which have been genetically selected for increased longevity, and in five matched control "B" lines. Control ribosomal protein genes *rp49* and *AP3/RpPO* RNA levels were similar in O and B lines. In contrast, *hsp22* RNA levels were twofold–tenfold higher in all five O lines relative to all five B lines, while *hsp23* exhibited a smaller but significant increase. Thus increased *hsp22* and *hsp23* RNA levels correlate with the increased life span and increased stress resistance of the genetically selected O lines.

AMONG the proteins induced in response to heat stress in eukaryotes are the "small" heat shock proteins (shsps), with molecular masses ranging from 15–30 kD (1). The shsps in eukaryotes are related by a conserved region of the protein called the α-crystallin domain. Mammalian shsps include αA-crystallin, αB-crystallin, and hsp27. There are four predominant shsps in *Drosophila* induced in response to heat stress, called hsp22, hsp23, hsp26, and hsp27. These shsps also have characteristic developmental expression patterns and are regulated by the hormone ecdysone. The function of the *Drosophila* hsp22 and hsp23 proteins has not been directly examined. However, *Drosophila* hsp27 and human hsp27 and αB-crystallin have each been found to confer increased resistance to heat and oxidative stress when expressed in cultured cells (1–5). Human hsp27 and bovine αA- and αB-crystallins have been shown to act in vitro as molecular chaperones, in that they can suppress thermally induced aggregation of other proteins and can facilitate refolding of denatured proteins (6–8). Taken together, the data suggest that one role of the shsp family may be the prevention of protein aggregation and denaturation during thermal and oxidative stress. The hsp70 heat shock protein is also induced in response to heat stress. The well-characterized hsp70 family proteins can prevent protein aggregation, facilitate protein refolding, and facilitate entry of damaged proteins into proteolytic pathways (9).

Drosophila exhibit a characteristic pattern of heat shock gene expression during aging, involving *hsp70*, *hsp22*, and *hsp23*. hsp70 protein is induced approximately 5-fold, specifically in thoracic muscle tissue, and may be a response to oxidative damage (10). Induction of *hsp70* during aging appears to involve both transcriptional and post-transcriptional regulation and requires functional heat shock response elements (HSEs) in the promoter region (11). *hsp22* RNA and, to a lesser extent, *hsp23* RNA were found to be induced during normal aging throughout the fly (10). *hsp22* expression during aging has been analyzed in detail (12). *hsp22* is induced in all tissues with regulation at both the transcriptional and post-transcriptional levels. *hsp22* RNA is induced up to 60-fold and hsp22 protein more than 150-fold. Like *hsp70*, transcriptional upregulation of *hsp22* during aging requires functional HSEs in the promoter. However, the greater magnitude of *hsp22* induction suggested the possible involvement of additional element(s). Aging also affects the expression of hsps in response to a heat stress, with old flies exhibiting a prolonged response and greater levels of protein turnover (13,14).

Aging is associated with the accumulation of abnormal and/or malfolded proteins and oxidatively damaged proteins in many organisms, including nematodes, flies, and mammals (15–17). This suggested the hypothesis that induction of hsp genes during *Drosophila* aging might have beneficial effects and help prolong the functional life span of the fly (10,12). At least two lines of evidence support this hypothesis and suggest a function for hsps in regulating *Drosophila* aging and life span. First, mild heat stress induces multiple hsps and increases resistance to subsequent stresses, an effect known as induced thermotolerance (18). Such mild heat stresses can also cause small increases in life span (19–21). Second, mild heat stress of *Drosphila* transgenic for extra copies of the *hsp70* gene exhibited increased hsp70 expression and an increase in life span several percent greater than controls (22,23).

The selection of genetically heterogeneous *Drosophila* lines over many generations for late-life fecundity results in increased longevity (24–27). The long-lived lines also exhibit a number of correlated phenotypes, including increased resistance to stresses such as starvation, desiccation, and oxidative stress (28–33). RNA levels for several antioxidant genes have been analyzed in a single long-lived line which exhibits increased oxidative stress resistance relative to its matched control line. Expression of Cu/Zn-superoxide dis-

mutase, catalase, and xanthine dehydrogenase were found to be increased (34).

The studies presented here utilize a specific set of five genetically selected long-lived (O) lines, which have been extensively characterized and exhibit up to twice the life span of their five paired control (B) lines (35). The analysis of five independent sets of lines allows the identification of characteristics that are consistently altered by selection. To determine if selection of the long-lived O lines affected the aging-related expression of the *hsp22* and *hsp23* genes, these genes' RNA levels were quantitated throughout the adult life span of the O and B lines. Although there was considerable variability in expression of these genes between lines and time points, *hsp22* expression was consistently found to be 2–10-fold higher in each of the five long-lived O lines relative to the B control lines. Smaller, but significant, increases in expression were also observed for the *hsp23* gene.

Methods

Drosophila Stocks and Culture

O and B lines were cultured as previously described (35). O lines were taken off of selection for three generations prior to analysis. To obtain flies of defined ages, newly eclosed males were maintained at 25°C at a density of 40 per vial and were transferred to fresh vials every 4 days to prevent growth of bacteria or mold.

Northern Blot Analysis

Northern blot analysis was performed using standard methods (36). Briefly, for each age time point, 25 males were dissected into head, thorax, and abdomen. Body segments were homogenized in guanidine hydrochloride extraction buffer and extracted with phenol/chloroform. Nucleic acids were precipitated with ethanol, and the pellet was resuspended directly into deionized formamide/formaldehyde gel loading buffer. The total RNA was fractionated using 1% agarose/formaldehyde gels and transferred to GeneScreen membranes (Dupont, Boston, MA/NEN). Membranes were hybridized successively with radiolabeled gene-specific probes: *hsp22*, *hsp23*, *hsp26*, and *hsp27* (10,37); ribosomal protein 49 gene *rp49* (38); ribosomal protein/apurinic endonuclease gene *AP3*, also called *RpPO* (39–40); and the myosin heavy chain gene *Mhc* (41). Northern blot results were quantitated by phosphoimager analysis and/or autoradiography and densitometry. Densitometry was performed on autoradiographs exposed within the linear range of the film. Darker exposures are presented in the figures to allow visualization of signal from B line samples. All data are presented in relative band intensity units normalized to the signal with the *rp49* probe, as a control for loading.

Statistical Analyses

Five long-lived (O) and 5 control (B) populations were used in this study. Each time point for each population represented the average of 50 flies, as RNA was isolated from 50 flies for each time point and approximately one fifth of this RNA was used for each Northern blot gel lane. Statistical analyses were then based on the averages of the five replicate O populations versus the averages of the five replicate B populations.

In the statistical analyses the dependent variable was always the character under study (e.g., *hsp22*, *hsp23*, *Ap3*), and the factors were selection treatment (B vs O), age, and region (head, thorax, abdomen). Results of the experiment shown in Table 1 and Figure 1, involving data from day 5 only, were analyzed using a two-way factorial analysis of variance (ANOVA), with selection treatment and region as factors. These same data were also analyzed by region using a one-way ANOVA with selection treatment as the only factor.

Results of the experiments shown in Figures 2 and 3, analysis of RNA levels throughout the adult life span, were analyzed using a three-way factorial ANOVA with selection treatment, age, and region as factors. All ANOVAs were performed using Statview statistical analysis software (Abacus Concepts, Berkeley, CA). Results are presented in the legends to Figures 2 and 3 and Table 1.

To address possible differences in variance between groups of data, all statistical analyses were repeated using log-transformed data, and the results are presented in brackets in the legends to Table 1 and Figure 3. The significance of the results was unchanged, with two minor exceptions that tend to further support the general conclusions of the study. In Table 1, the difference in Myosin expression between O and B lines in thorax becomes insignificant, and in Figure 3, the difference in *hsp23* expression between O and B lines becomes significant.

Results

Northern blot analysis was used to compare RNA expression in long-lived O lines and short-lived B control lines. Each of the five O lines and five B lines was taken off of selection for three generations to avoid any possible direct effects of the culture conditions on gene expression. Adult male flies of each line were maintained at 25°C until 5 days, and then 25 flies were dissected into head, thorax, and abdomen. RNA was isolated from the dissected body segments and analyzed by Northern blots. The Northern blot of thorax RNA is presented (Figure 1). Each Northern blot was hybridized with a probe specific for the constitutively expressed control gene ribosomal protein 49 (*rp49*) as a loading control. *rp49* RNA levels are constant with age relative to several other housekeeping-type gene RNAs (10) and rRNA (34). The blots were hybridized with a probe specific for *hsp22*, and as a control for the specificity of *hsp22* expression, expression of the non-hsp genes ribosomal protein/apurinic endonuclease *AP3* and the myosin heavy chain *Mhc* were also analyzed. Results were quantitated by densitometry, normalized to *rp49* expression, and are presented in Table 1. The largest differences in expression between the five O populations and the five B populations were for *hsp22*. *hsp22* expression was found to be increased by an average 3.7-fold in thorax of O lines relative to B line controls (Figure 1 and Table 1). *hsp22* was also increased in head in O lines by an average 4.14-fold (Table 1). *hsp22* RNA levels were similar in O and B line abdomen in this

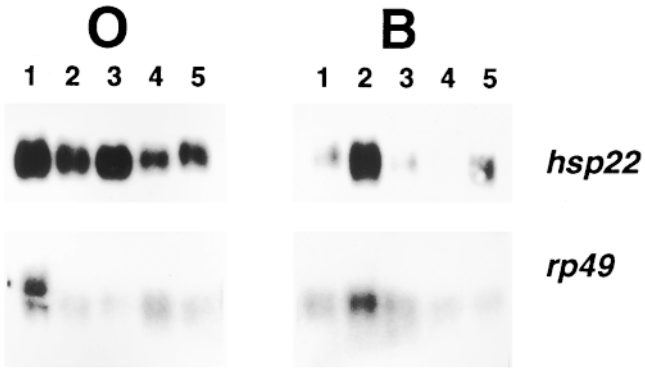

Figure 1. Northern blot analysis of 5-day-old O and B line fly thoraces. Total RNA was isolated from 25 thoraces at 5 days of age for each of the O1-5 and B1-5 lines, as indicated. RNA was transferred to Northern blot and hybridized successively with probes specific for *hsp22* and the *rp49* loading control, as indicated. Quantitation of data is presented in Table 1.

experiment. Smaller differences were observed for expression of the control genes. On average, *AP3* expression was increased by 85% in O line heads and decreased by 8% in O line abdomens. *Mhc* was increased by 57% in O line thorax. However, as shown below, the small differences in control gene expression were not reproduced in a larger experiment, whereas dramatically increased expression of *hsp22* in O line flies was consistently observed.

To confirm the upregulation of *hsp22* RNA expression in the O line flies, RNA expression was analyzed throughout the life span of independently cultured cohorts of the O and B lines. Each of the five O lines and five B lines was again taken off of selection for three generations, and adult male flies of each line were maintained at 25°C until defined ages. Then at various time points, 25 flies were dissected into head, thorax, and abdomen, and RNA was isolated from the dissected body segments and analyzed by Northern blot.

Blots for each O line and its matched B control line (O1 and B1, O2 and B2, etc.) were always hybridized together in the same container with a given probe so that the intensity of signal could be directly compared between the O and B lines of each set. Representative Northern blots are shown in Figure 2. Thorax RNA was isolated from flies of O and B lines 1–5. Each blot was hybridized successively with probes specific for *hsp22* and *rp49*. For each set of O and B lines, *rp49* expression was found to be approximately equal between the O and B lines (Figure 2). In O line flies, *hsp22* expression was 2–10-fold higher than in the B line flies throughout the period from about 2–40 days. The life span of the B line flies corresponds roughly to this period. *hsp22* RNA levels in the O line flies subsequently decreased between days 50–70.

Northern blot analysis was also performed for the head and abdomen body segments. The results for the head, thorax, and abdomen were quantitated by densitometry, normalized relative to *rp49* RNA levels, and are plotted in Figure 3. The greatest differences in expression were observed in the thorax, where *hsp22* RNA levels were 2–10-fold higher in 27 out of 30 (90%) of the time points for which both strains had detectable expression. A similar, but somewhat smaller difference was observed in the head. While at a subset of time points *hsp22* RNA levels were similar between O and B lines in the head, at the majority of time points *hsp22* RNA levels were 2–10-fold higher in O lines. Finally, the smallest difference was observed in the abdomen. Increased *hsp22* RNA levels in the O line flies' abdomens were observed at many time points for each set of lines except O2/B2. However, the increases were generally smaller and observed at fewer time points than for the thorax or head. A three-way factorial ANOVA demonstrates that *hsp22* expression is significantly increased in O lines

Table 1. Quantitation of RNA Levels in O and B Line Heads, Thoraces and Abdomens, at 5 Days of Age

	Population													
	O1	O2	O3	O4	O5	Oavg (*SD*)	B1	B2	B3	B4	B5	Bavg (*SD*)	O/B	*p*
Head														
AP3	236	227	231	215	189	219.6 (18.78)	145	90	130	126	102	118.6 (22.22)	1.85	<.0001 [.0002]
Mhc	45	42	39	72	45	48.6 (13.32)	60	17	34	75	71	51.4 (25.0)	0.94	.8306 [.8829]
hsp22	259	212	64	188	155	175.6 (73.0)	10	49	87	30	36	42.4 (28.6)	4.14	.0052 [.0072]
Thorax														
AP3	139	130	111	124	143	129.4 (12.7)	125	111	123	139	122	124.0 (10.0)	1.04	.4765 [.5237]
Mhc	170	155	176	162	126	157.8 (19.4)	126	102	25	102	148	100.6 (46.4)	1.57	.0346 [.1031]
hsp22	165	286	225	58	68	160.4 (98.7)	30	122	24	9	34	43.8 (44.8)	3.7	.0428 [.0241]
Abdomen														
AP3	103	92	93	98	98	96.8 (4.44)	100	99	111	106	112	105.6 (6.02)	0.92	.0302 [.0183]
Mhc	32	30	ND	60	38	40.0 (13.8)	41	37	36	39	34	37.4 (2.7)	1.07	.6873 [.6408]
hsp22	95	108	103	115	83	100.8 (12.3)	79	107	102	88	66	88.4 (16.7)	1.14	.2192 [.2186]

Notes: Northern blot data were quantitated for *hsp22*, *Mhc*, and *AP3* gene RNA levels for head, thorax and abdomen (data of Figure 1 and additional data), normalized to *rp49* levels as loading controls, and are presented as a table of band intensity values in relative units. Two-way factorial ANOVA was used to analyze the significance of the differences in RNA levels between O and B lines for each gene combining all data: *hsp22*, $p = .0002$ [.0001]; *Mhc*, $p = .055$ [.1499]; *AP3*, $p < .0001$ [<.0001]. One-way ANOVA was also used to analyze the significance of the differences in RNA levels between O and B lines for each gene in each body segment, and *p* values are presented above. When the significance of the differences in RNA levels between O and B lines for each gene in each body segment were analyzed using one-tailed paired *t* tests, the results were the same with the exception of *hsp22* in abdomen, which was then found to be significant ($p = .0348$). Bracketed numbers are results of statistical analyses using log-transformed data. Oavg = average value for O lines; Bavg = average value for B lines; O/B = Oavg divided by Bavg; ND = not done.

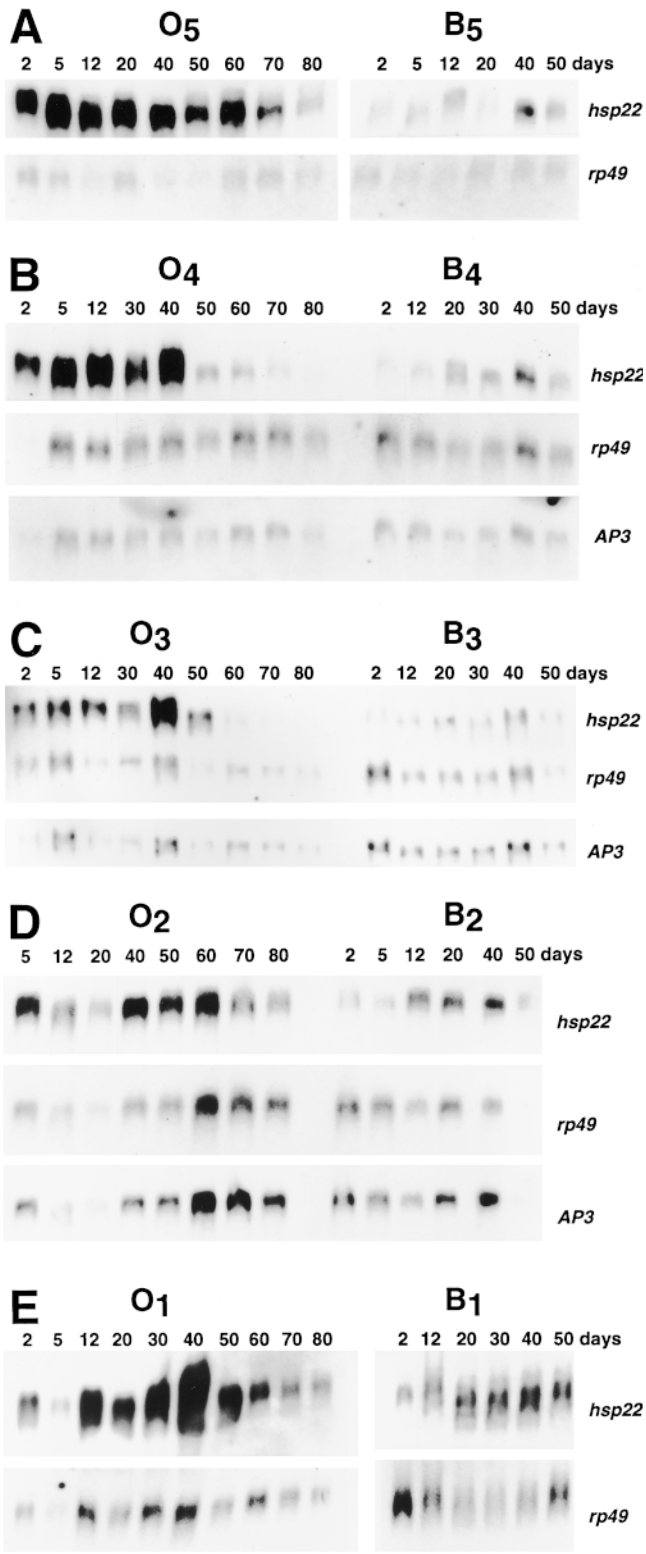

Figure 2. Northern blot analysis of O and B line thoraces throughout the adult life span. Total RNA was isolated from 25 thoraces at each of the indicated adult age time points for O and B line flies. Matched O and B samples were always hybridized together with the indicated probes so that band intensities could be directly compared between O and B. **A**, O5 and B5 thorax. **B**, O4 and B4 thorax. **C**, O3 and B3 thorax. **D**, O2 and B2 thorax. **E**, O1 and B1 thorax. The multiple *hsp22* bands in **E** were not reproducible, and appear to be an artifact of RNA isolation and/or southern conditions. Signal from the multiple bands was summed during quantitation. Quantitation of data is presented in Figure 3.

when the body segments are considered together or separately (Figure 3). Control *AP3* RNA levels were found to be equal between each of the five sets of O and B line flies, in the head, thorax and abdomen, at virtually every time point assayed. In the rare instances where *AP3* RNA levels differed between O and B line flies (c.f., Figure 3, A-04 and H-01) the differences were neither consistent across time points nor reproducible across the five lines. ANOVA demonstrates that *AP3* expression is not significantly different between the O and B lines when body segments are considered either together or separately (Figure 3).

Each Northern blot was also hybridized to a probe specific for *hsp23*. *hsp23* expression is somewhat more difficult to quantitate because its expression level in the adult fly is lower than that for *hsp22*. *hsp23* RNA expression was also found to be significantly greater in the O lines than in the B lines (Figure 3); however, the difference between O and B lines was generally smaller for *hsp23* than for *hsp22*. ANOVA demonstrates that *hsp23* expression is significantly increased in O lines when the body segments are considered together, and is significantly increased in the head and thorax when the body segments are analyzed separately (Figure 3). Finally, the Northern blots were also hybridized to probes specific for *hsp26* and *hsp27*. In general, the *hsp26* and *hsp27* RNAs were too rare to be quantitated in the O and B lines. In the cases where a Northern signal was observed, there was no reproducible difference in expression between the O and B lines (data not shown).

Discussion

The data of two independent Northern blot experiments demonstrates a consistent upregulation of *hsp22* RNA levels by 2–10-fold in five O lines relative to their B line controls. This increase was observed in the head and thorax, and in one of two experiments (the larger experiment) in the abdomen. A smaller, but significant, increase in expression was also observed for *hsp23* in head and thorax. In the O line samples, *hsp22* RNA levels are high throughout the first part of the life span, from 0 to ~50 days, which corresponds to the entire length of adult life span of the shorter-lived B lines. At around 50 days, *hsp22* expression in the O lines generally declines and remains lower for the rest of the O line life span. This dynamic pattern of expression may be occurring in each fly, with a high level of expression followed by a decrease. Alternatively, expression may be different in different subpopulations of the O line flies, and the decrease in RNA levels at later ages in the Northern blot analysis may represent a longer-lived subpopulation characterized by lower levels of *hsp22* RNA expression. Distinguishing between these two possibilities will require the development of a means to quantitate *hsp22* expression at the level of individual flies, such as by use of transgenic GFP reporter constructs (42,43).

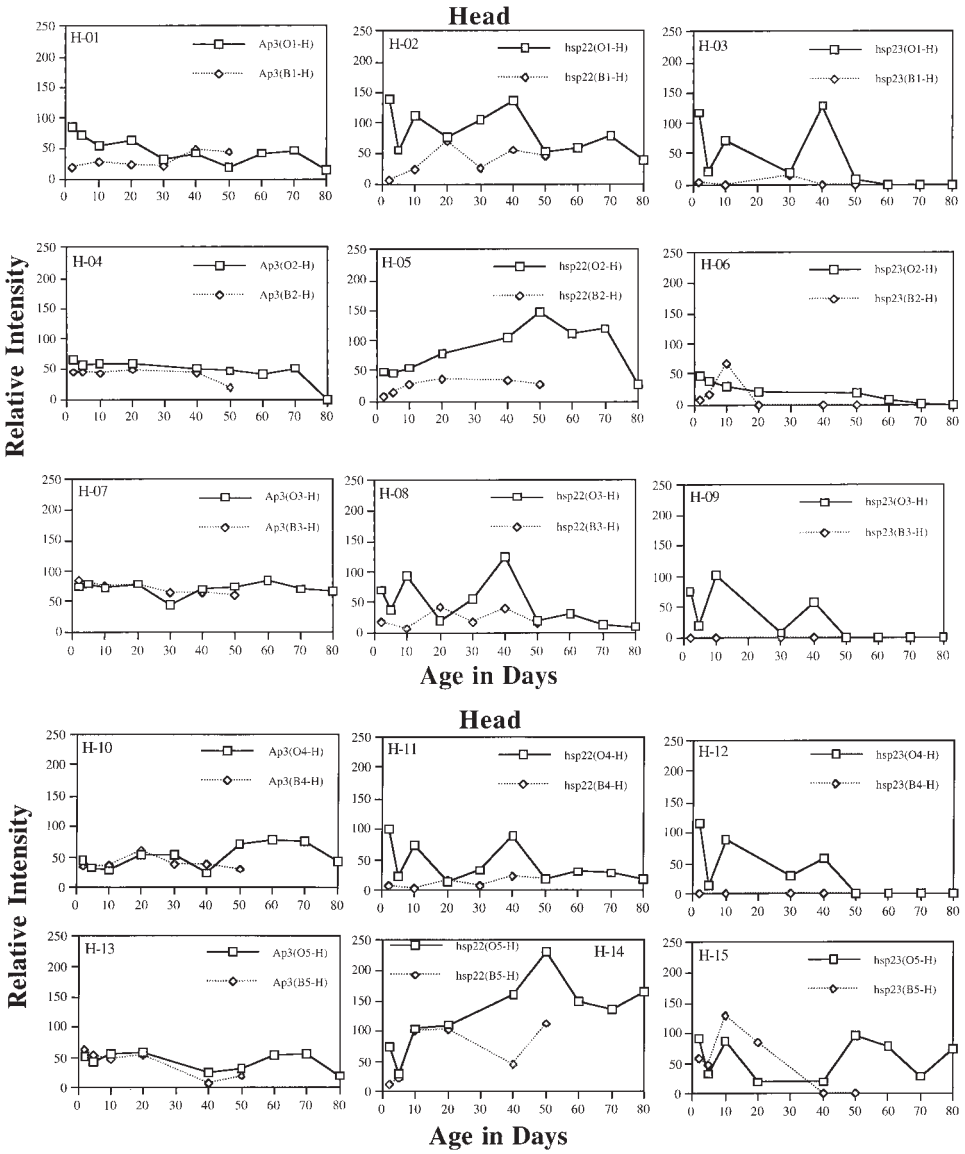

Figure 3. Quantitation of RNA levels in O and B line heads, thoraces, and abdomens, throughout the adult life span. Northern blot data were quantitated for *hsp22, hsp23,* and *AP3* RNA levels (data of Figure 2 and additional data), normalized to *rp49* levels as a loading control, and plotted. Plots are of band intensity in relative units versus adult age in days. Data plots are presented for all five O lines and all five B lines, for head (H-01 through H-15), thorax (T-01 through T-15), and abdomen (A-01 through A-15), as indicated. Three-way factorial ANOVA was used to analyze the significance of the differences in RNA levels between O and B lines for each gene combining all data: *hsp22*, $p < .0001$ [<.0001]; *hsp23*, $p = .0011$ [<.0001]; *AP3*, $p = .1616$ [.4249]. One-way ANOVA was also used to analyze the significance of the differences in RNA levels between O and B lines for each gene in each body segment. Head: *hsp22*, $p < .0001$ [<.0001]; *hsp23*, $p = .0046$ [<.0001]; *AP3*, $p = .0941$ [.055]. Thorax: *hsp22*, $p < .0001$ [.0001]; *hsp23*, $p = .0022$ [<.0001]; *AP3*, $p = .6405$ [.4984]. Abdomen: *hsp22*, $p = .0015$ [<.0001]; *hsp23*, $p = .4143$ [.0174]; *AP3*, $p = .6505$ [.7648]. Bracketed numbers are results of statistical analyses using log-transformed data.

In previous studies, two general patterns of *hsp22* RNA expression during aging have been observed (10,12). With Oregon-R wild type and *white*^{1118} strain flies cultured at low density in culture bottles, *hsp22* RNA levels were low or undetectable in young flies. Under these optimal conditions the increases in RNA levels observed during aging were large, ranging from 2.5–60-fold, depending on the body region assayed. In contrast, with the same strain of Oregon-R flies cultured at higher density using population cage protocols, the starting levels of *hsp22* RNA in young flies were higher, particularly in the head and abdomen, and the fold inductions observed during aging were small or undetectable (10). In the experiments presented in this article, the O and B line flies were cultured using higher density, population cage type protocols. Consistent with previous observations, *hsp22* RNA levels were relatively high in young animals and no significant induction with age was detectable. We conclude that these presumably more stressful culture conditions result in abundant *hsp22* RNA expression in young animals, which in turn masks the induction due to ag-

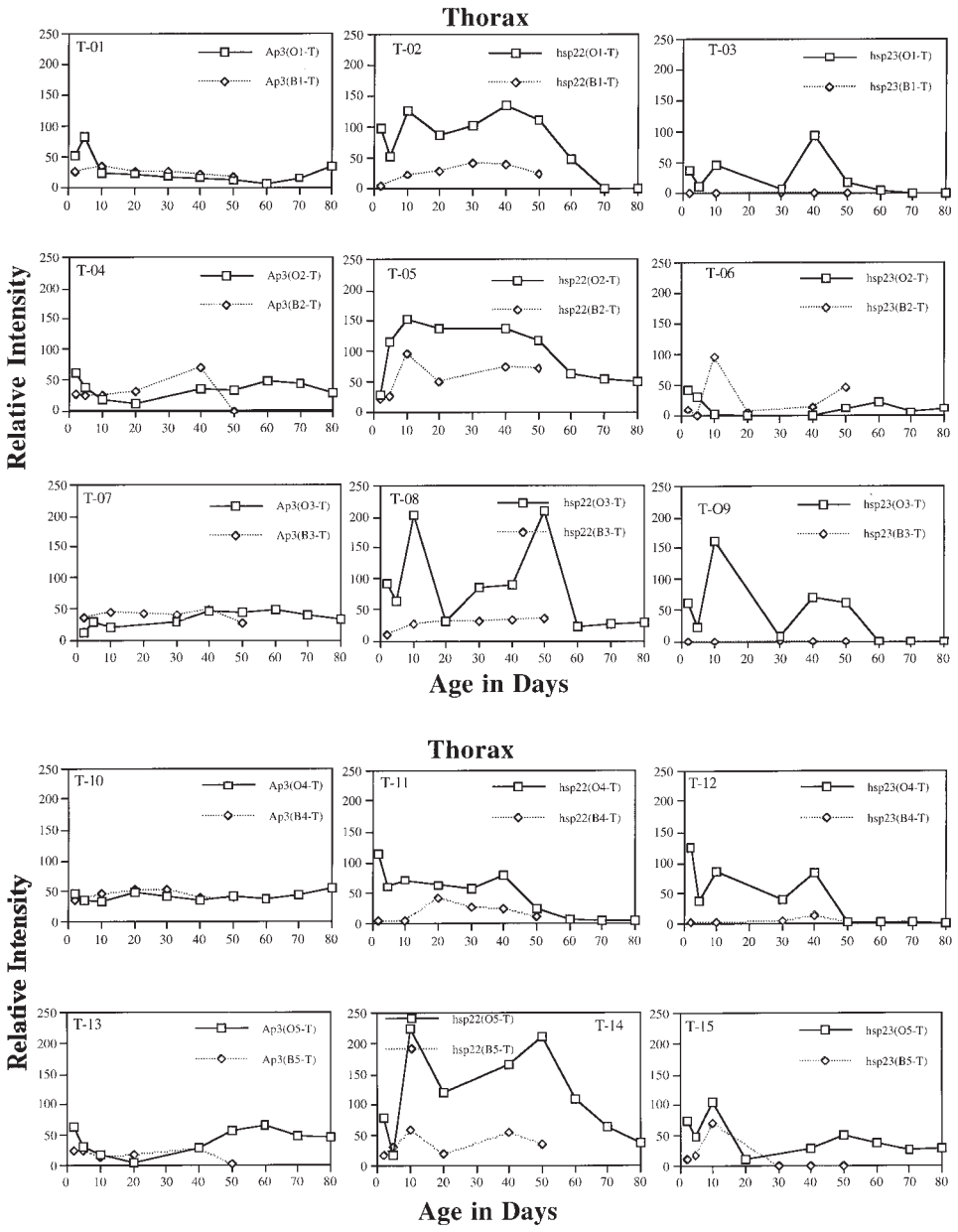

Figure 3. continued

ing. Further experiments will be required to test this idea conclusively. This situation does not alter the interpretation of the present result of increased *hsp22* RNA levels in O lines relative to B lines, because their culture conditions were identical. However, it is important to note that this difference is being observed under conditions that may be more stressful than the optimum.

hsp22 and, to a lesser extent, *hsp23* are induced at the RNA level during *Drosophila* aging under the appropriate conditions (10,12). Other hsps, such as *hsp26*, *hsp27*, and *hsp70* do not exhibit this induction at the RNA level or exhibit markedly smaller inductions. These results suggest a specialized mechanism or pathway for preferential induction of *hsp22* and *hsp23* RNAs during aging. The fact that it is *hsp22* and *hsp23* that are upregulated in the O line flies suggests that it may be this same aging-associated mechanism or pathway that is upregulated in the O line flies relative to the B lines.

Selection for increased life span in the O line flies is associated with a correlated phenotype of increased stress resistance. In particular, O line flies are more resistant to stresses such as desiccation, starvation and forced flight (28,32). Increased stress resistance is also associated with increased life span in other organisms. For example, specific mutations in genes of the dauer formation pathway in *C. elegans* increase both life span and resistance to stresses such as heat stress, oxidative stress, and ultraviolet irradiation (44–48). A problem afflicting all of these experiments, including

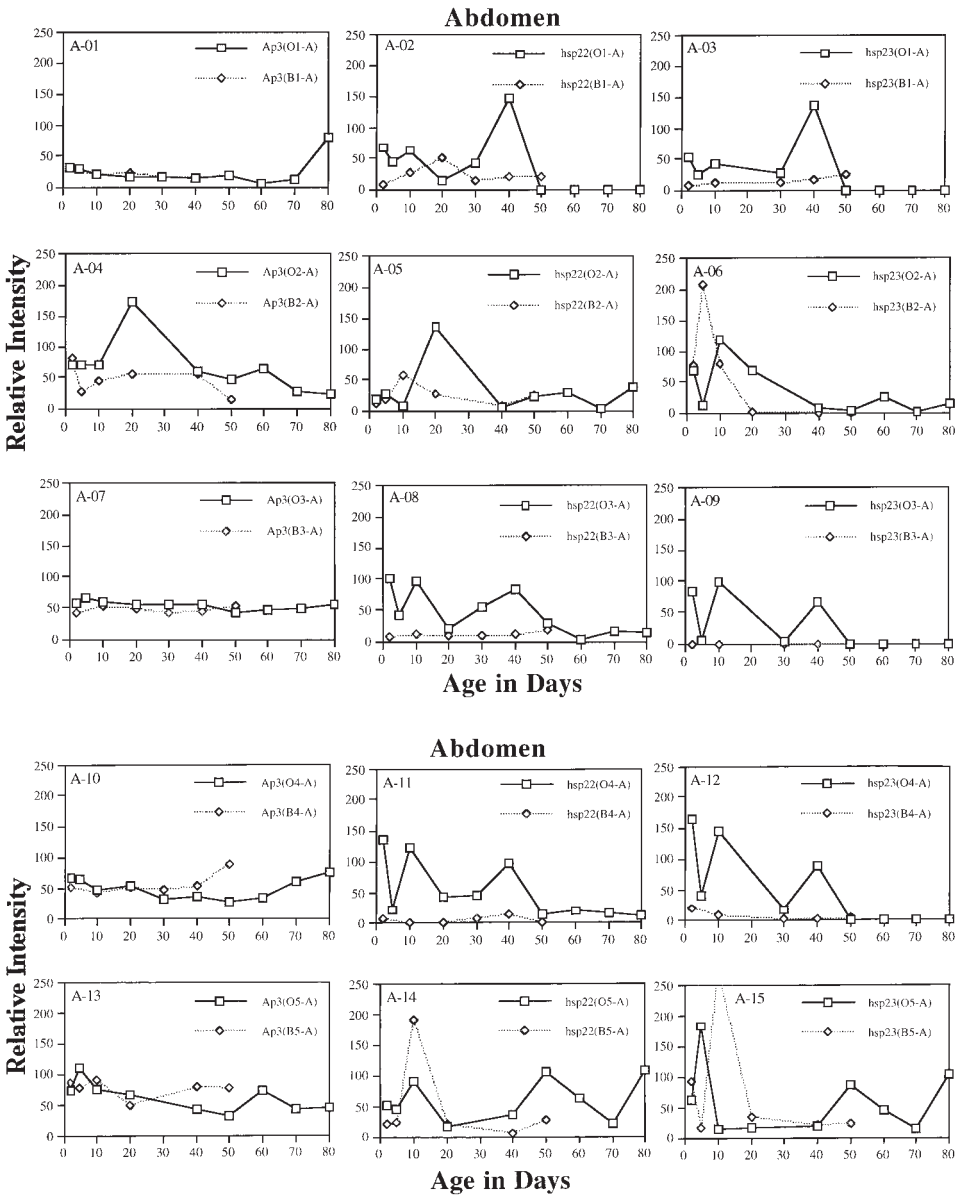

Figure 3. continued

the work presented here, is genotype by environment interaction. The effects of genetic differences can depend critically on assay environments. This has been shown to confuse inferences of character correlations in certain earlier studies of aging (24). The resolution of this difficulty requires additional experimentation, with a variety of genotypes and environments. However, initial significant correlations between specific characters such as stress resistance, hsp expression, and aging are not without interest because of this problem.

The correlation between increased stress resistance and increased life span suggests a testable model for the function of increased *hsp22* and *hsp23* expression in the O line flies. Other members of the shsp family have been demonstrated to be molecular chaperones, capable of conferring increased resistance to stresses such as heat and oxidative damage when overexpressed in cultured cells. We hypothesize that the upregulated expression of *hsp22* and *hsp23* in the O line flies contributes directly to their increased stress resistance and increased life span. A critical test of this hypothesis will require experimentally upregulating and/or downregulating the expression of *hsp22* and *hsp23* in transgenic flies, and assaying the effects of this altered expression on stress resistance and life span.

Acknowledgments

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