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RESEARCH ARTICLE

Age-related and sex-specific differences in proteasome activity in individual *Drosophila* flies from wild type, longevity-selected and stress resistant strains

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Abstract We have measured the caspase-like proteasome activity in individual male and female *Drosophila* flies by using a non-denaturing lysing technique that consistently extracts total protein. The mean proteasome activity in control C1 females was more than two times higher as compared with that in C1 males. However, in longevity-selected LS1 flies the proteasome activity was significantly lower compared to C1 flies, but the sex differences were maintained to some extent. Five other stress resistant lines also had significantly reduced proteasome activity in both sexes. During ageing, there was a progressive decrease in proteasome activity in C1 females, but not in C1 males. This age-related decline in proteasome activity observed in C1 females was not observed in LS1 flies. We conclude that the proteasome activity in control male and female flies is significantly different from each other and that increased lifespan and stress resistance lead to a reduction in proteasome activity and recession of the age-related decline observed in control females.

Keywords Longevity selection · Hormesis · Stress resistance · Variance · Ageing · *Drosophila melanogaster*

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Introduction

The ageing process at the molecular level is characterized by a progressive accumulation of damage in macromolecules, including DNA, RNA and proteins (Rattan 2006, 2008a, 2012). The evolutionary reason for the accumulation of molecular damage is the imperfect nature of maintenance, repair and removal systems. For example, lysosomal and proteasomal pathways of degradation are the main mechanisms for the removal of damaged and otherwise abnormal proteins from within the cells, but these are not perfect systems and some damaged proteins are always left behind (Shang and Taylor 2011; Markaki and Tavernarakis 2011). The proteasome is a proteolytic multisubunit complex and is the main player in non-lysosomal protein degradation. During ageing, the proteasome itself is prone to modifications like oxidation, glycosylation, glyoxidation and formation of conjugates of lipid peroxidation products. Different

subunits are prone to different kinds of modifications, and a change in proteasome structure leads to decreased proteasome activity (Carrard et al. 2003). An age-related decline in proteasomal activity has been reported in a variety of systems, including human cells, yeast, rodent organs and flies (Grune et al. 2005; Bulteau et al. 2000; Anselmi et al. 1998; Vernace et al. 2007; Sitte et al. 2000; da Cunha et al. 2011; Chondrogianni and Gonos 2004). The consequence of reduced proteasome activity is an accumulation of abnormal proteins and their aggregates, which can have severe consequences on the cellular physiology and survival.

In the case of *Drosophila melanogaster* an age-related decline of proteasomal activity was reported in lysates from a mix of 30 male and female flies at young (1–2 days) and old age (43–47 days) (Vernace et al. 2007). All three proteasomal peptidase activities displayed a significant age-related decline. Furthermore, old flies had less 26S proteasome available compared to the young flies, indicating that the age-related decline in proteasome activity was due to a shift in the ratio between the active 26S and inactive 20S proteasome (Vernace et al. 2007). The assemblance of the active 26S proteasome is dependent on ATP and the decrease in 26S and increase in 20S proteasome in old flies was further correlated with a drop in ATP levels by about 50 %.

However, within a *Drosophila* population, natural as well as laboratory strains, there is a certain level of genetic variation among individual flies. In fact this variation is what makes it possible to select for traits such as longevity and stress resistance (Fallis et al. 2011; Sarup et al. 2011; Mueller et al. 1985; Bubliy and Loeschcke 2005; Tatarenkov and Ayala 2007; Le Bourg 1996). In order to account for genetic variance within the *Drosophila* strains we measured the proteasome activity in individual female and male flies from a longevity-selected line and the corresponding wild type control line at different points in their life time, and in several lines selected for increased stress resistance. The reason for choosing stress resistant lines is to explore the relationship between stress resistance, longevity and proteasomal activity, and the fact that exposing cells and organisms to mild stress can increase proteasomal activity, slow down ageing and extend lifespan, a phenomenon known as hormesis (Hercus et al. 2003; Beedholm et al. 2004; Olsen et al. 2006; Rattan 2008b; Le Bourg and Rattan 2008; 2009).

Here we present our data on the age-related change in proteasome activity in individual male and female flies. Our observations show that the proteasome activity in control male and female flies is significantly different from each other and that increased longevity and stress resistance lead to a reduction in proteasome activity and recession of the age-related decline observed in control females.

Materials and methods

Drosophila melanogaster culture

Flies were raised in sex-mixed cultures on standard cornmeal-molasses-agar-yeast medium with propionic acid added to the food as an anti-fungal agent. All mating, egg laying and hatching were done in a 25 °C ± 1 °C temperature-controlled room with 12:12 h light/dark cycle and 50–60 % humidity. Food was changed every third day throughout their lifespan. Control flies, C1, were derived from a mass laboratory population of *D. melanogaster* (Bubliy and Loeschcke 2002). The longevity strain, designated LS1, was generated from this mass population based on selection for fecundity at old age for 17 generations (for details see Bubliy and Loeschcke (2005)). The stress resistant strains used were from already established selection lines, and had been selected for their abilities to endure cold-shock (CS), heat-shock (HS), heat-knockdown (KS), desiccation (DS) and starvation (SS) (Bubliy and Loeschcke 2005).

Survival curves

Female flies (less than 24 h old) were set up in five replicates for both C1 and LS1 with 10 flies per vial. The flies were transferred to fresh food every third day. The number of dead flies was counted at each transfer throughout the survival study. Survival was estimated by Kaplan–Meier methods and compared among strains by log rank tests implemented in Prism (Graphpad Software, San Diego, CA, USA). Figure 1 is a representative curve from one experiment and the survival data for all 5 experiments are given in Table 1.

Sample preparation

Fourteen flies from each sex were collected every third day from C1 and every seventh day from LS1 until day 27 and 57, respectively. Since LS1 flies live 40 % as long as C1, we collected their samples once a week instead of every third day. In the case of the stress resistant strains, flies were collected only at day 15. Flies were sorted into males and females under light CO_2 anesthesia. During sample preparation everything was kept on ice. Each sample contained one fly which initially was crushed using liquid nitrogen and a disposable pistil. The ruptured tissues were dissolved in 500 µl buffer containing 50 mM NaCl, 10 mM HEPES pH 8, 250 mM sucrose, 1 mM EDTA and 0.2 % TritonX100. Further tissue separation and lysis of cells were established by extensive vortexing of the samples. The samples were stored at −80 °C until use.

Total protein estimation

Protein concentration was estimated based on a BSA standard curve spanning from 0 to 1.4 mg/ml, using Bradford reagent (Sigma-Aldrich) in a 96-well microtiter plate. The OD595 was measured in a BioRad plate reader. Each sample was measured in triplicate. The total protein concentration per sample was used for normalizing the kinetic data output from the FluoStar Optima, to account for differences in weight and protein content in individual flies.

Proteasome activity measurement

The synthetic fluorogenic peptide substrate, Z-Leu-Leu-Glu-7-amido-4-methylcoumarin (Sigma-Aldrich), was used for measuring the caspase-like activity of the proteasome (Lima and Rattan 2010). The peptide was diluted to a final concentration of 25 µM in assay buffer 50 mM NaCl, 10 mM HEPES pH 8, 250 mM sucrose, 1 mM EDTA and 0.2 % TritonX100 per reaction. Samples were loaded into a black 96 MicroWell Nunclon Δ Optical Bottom Plate and fluorescence/min was measured on an automated microplate based multi-detection reader at ex355 nm and em460. Flourescence intensity was converted to FI/min (rate of proteolytic cleavage). Background protease activity was measured by adding the proteasome inhibitor epoxomycin (Sigma-Aldrich) to a final concentration of 2 µM. This background activity was subtracted from the sample wells. Slope/min was normalized against total protein concentration per sample. Conversion into pmol AMC/min/µg protein was achieved according to following equation.

$$\text{Mol AMC/min/g protein} = \frac{\text{FI/min} \times \text{M}_{\text{AMC standard}} \times \text{V}_{\text{Assay}}}{\text{FI}_{\text{AMC standard}} \times \text{m}_{\text{protein}}}$$

The AMC standard is given at 5 µM AMC 300 FI. All proteasome activity data are presented as absolute numbers with the unit pmol AMC/min/µg protein.

Statistical analysis

Statistical analysis was executed in both GraphPad Prism 4.0 and the statistical software program Stata/IC 11.2 for Windows (32-bit). Test of variance in protein extraction procedure was evaluated using a 99 % confidence interval, and the data are presented with ±standard deviation (SD). ANOVA was used for analysing the effect of sex and strain on proteasome activity in the first study and for the effect of age and sex on proteasome activity in the second study. Sexes were further compared by a non-parametric Mann–Whitney test in individual strains. In addition to ANOVA, we performed linear regression analysis for testing the regression over time.

Results

The maximum life expectancy of C1 female flies at 25 °C and 50–60 % humidity was about 50 days, whereas in LS1 flies the maximum life expectancy had increased to around 75 days and the mean survival increased from 38.4 to 52.8 days (Fig. 1; Table 1). Survival data for male flies are not shown, but in

mixed cultures males from both C1 and LS1 had a 10 day longer mean survival compared to females (Sarup and Loeschcke 2011).

We have standardized the method for handling individual flies for the extraction of proteins and for the measurement of the proteasome activity. Comparing the protein yield from the lysis of 14 individual C1 males and females showed a fairly low and insignificant variation. The mean protein concentration for C1 females and males was 440 ± 59 (SD) and 356 ± 42 (SD) μg/fly, respectively. Further, samples prepared from C1 flies harvested at different time points showed that protein concentration did not change notably in extracts prepared on different days. Protein yield from males was 14–19 % lower than that from females. The sample variance indicates that this difference in protein yield was likely to be due to size difference between the sexes (females are larger than males) and not due to differences in the extent of lysis and extraction. The low variance among samples further validates that the lysis technique applied in this study extracted the proteins in a consistent manner. Variation in protein concentration among samples generated on different days was also not significant, thus single-fly sample preparation was not prone to day to day variation, supporting that the method was consistent and suitable for studies on individual flies. The protein concentration was later used for normalizing the proteasome activity measurements.

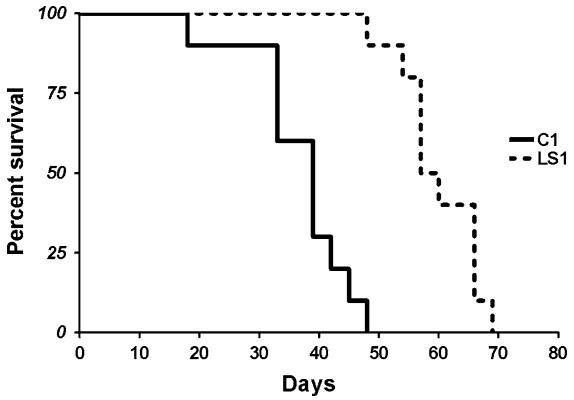

Fig. 1 Survival curves for female C1 and LS1 flies. The presented graph is a representative of one of five replicates comprised of ten flies per replicate. Selecting for longevity significantly increased both the mean lifespan of all replicates on average from 38.4 to 52.8 days and the maximum lifespan from approximately 50 to 75 days

Table 1 Median survival of control C1 and longevity selected LS1 female flies in five independent experiments

Flies	Median survival (days)	*n*	Log rank *p* value
Expt #1			
C1	42	10	0.001
LS1	51	10	
Expt #2			
C1	39	10	0.06
LS1	46.5	10	
Expt #3			
C1	36	10	<0.0001
LS1	54	9	
Expt #4			
C1	39	10	<0.0001
LS1	58.5	10	
Expt #5			
C1	36	10	<0.0001
LS1	54	10	
Mean			
C1	38.4	50	<0.0001
LS1	52.8	49	

Sex-differences in proteasome activity levels

There was a significant interaction between the effect of sex and strain on proteasome activity in 15 day old flies (14 day old LS1s). A joint factor test revealed significant effects of both sex and proteasome activity (Table 2). By analyzing each strain individually by a non-parametric Mann–Whitney test, we found a significant difference in proteasome activity between sex in C1, LS1, KS1, HS1 and DS1 ($P < 0.05$), but not in CS1 and SS1 flies ($P > 0.05$) (Fig. 2).

Table 2 Two-way ANOVA of the effect of sex and strain on proteasome activity at day 15 (day 14 for LS1s)

Source	Degrees of freedom	Sum of squares	F ratio	Prob > F
Strain	6	17	13	<0.0001
Sex	1	13	62	<0.0001
Strain * sex	6	14	11	<0.0001
Joint factor test				
Strain	12	31	12	<0.0001
Sex	7	28	18	<0.0001

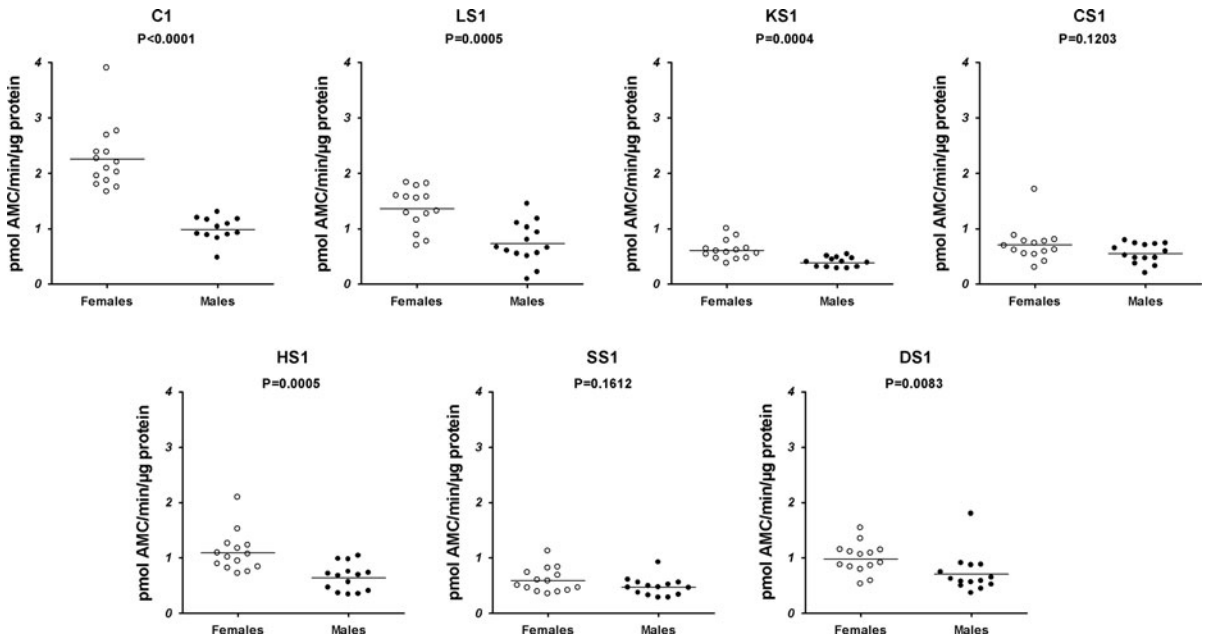

Fig. 2 Proteasome activity (PA) in individual male and female flies at day 15 (day 14 for LS1). A difference between sexes was observed in C1, LS1, KS1, DS1 and HS1, while male and female CS1 and SS1 selected lines had no difference in PA

Level of proteasome activity in selected lines

There was a significantly reduced proteasome activity in both LS1 and stress resistance-selected male and female flies as compared with C1 female flies (Table 2). Further analysis using separate paired *t* tests between the individual selected strains and C1 flies revealed that C1 females had a significantly higher proteasome activity compared to all selected lines (LS1, HS1, DS1, CS1, KS1 and SS1) ($P < 0.05$) (Fig. 3). However, comparing the individual stress resistant lines showed that there was no significant difference in proteasome activity between HS1 and DS1 ($P = 0.5658$) females and both lines had a significantly higher proteasome activity ($P < 0.05$) compared to CS1, KS1 and SS1, which all displayed significantly similar proteasome activities ($P > 0.05$) (Fig. 3). In males, all but LS1 had a significantly lower proteasome activity compared to C1 males ($P < 0.05$). LS1, HS1 and DS1 males had significantly similar proteasome activities ($P > 0.05$) and all three lines had significantly higher proteasome activity compared to CS1, KS1 and SS1 ($P < 0.05$). These results suggest that proteasome activity is affected differently depending on the trait selected for.

Proteasome activity during ageing

The control line C1 used in this study had maximum lifespan expectancy close to 50 days (Fig. 1). We measured proteasome activity in 14 female and 14 male individual flies every third day until day 30. C1 females displayed a significantly higher proteasome activity (Table 3), and large inter-individual variation early in life as compared to males; and as ageing progressed proteasome activity regressed from 4.77 pmol AMC/min/µg protein at day 6 to 3.27 pmol AMC/min/µg protein at day 30 ($P = 0.038$) (Fig. 4). In C1 males the proteasome activity remained low at 1.16 pmol AMC/min/µg protein at day 6 and declined to 0.82 pmol AMC/min/µg protein at day 30. The regression analysis of the mean proteasome activity to the various time points showed no evidence of an age-related decline in proteasome activity in C1 males ($P = 0.933$). At 27 days the mean proteasome activity was no longer different between the sexes ($P = 0.8296$) with males having a mean activity of 1.7 pmol AMC/min/µg protein and females a mean activity of 1.82 pmol AMC/min/µg protein. This activity level may indicate a lower threshold for proteasome activity for C1 flies.

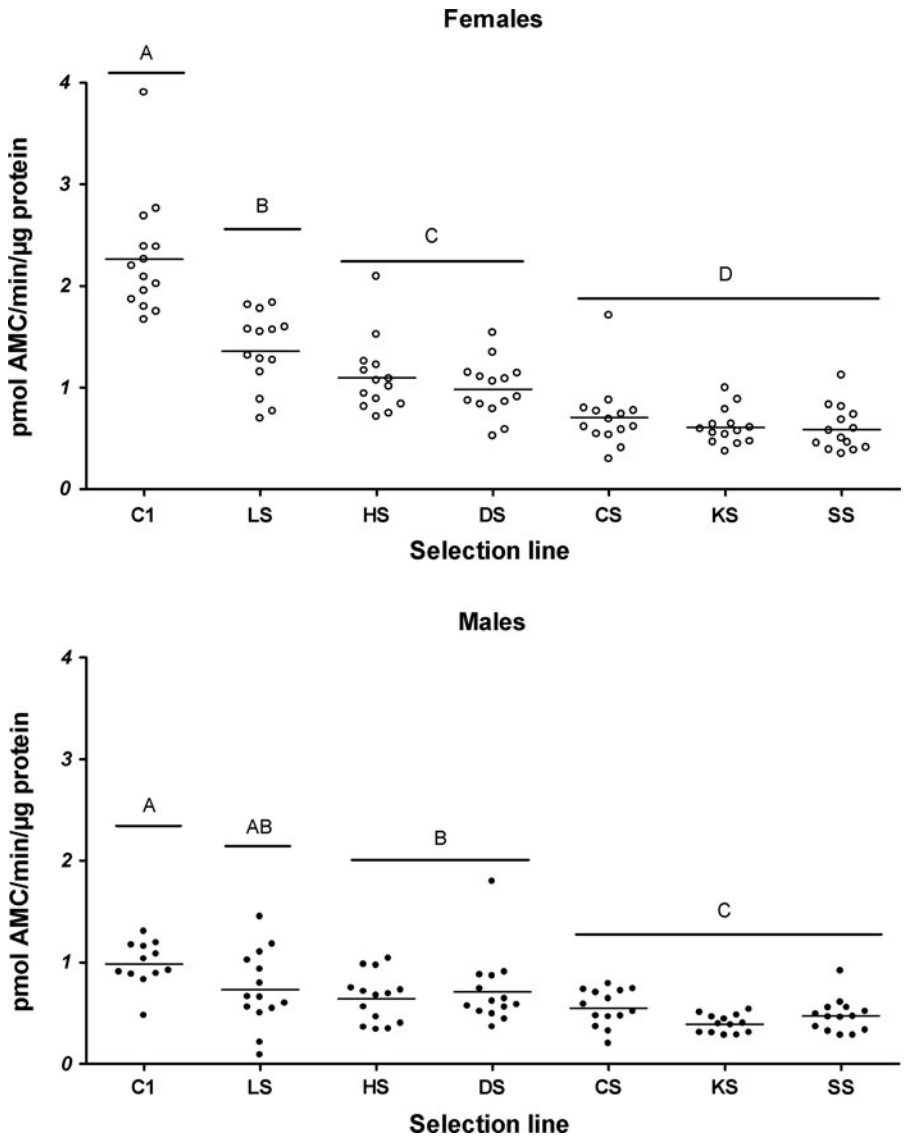

Fig. 3 Proteasome activity (PA) in stress resistance selection lines in individual females and males. PA in 15 day old flies (14 day old LS1) from various strains showed that C1 females had a significantly higher activity as compared to all selection lines ($P < 0.05$). However, C1 males did not have a significantly higher PA compared to LS1 males ($P = 0.076$) but they did have a significantly higher PA compared to the stress resistant lines ($P < 0.05$). CS1, KS1 and SS1 were the strains with the significantly lowest PA compared to all strains in both males and females ($P < 0.05$). *Letters A*, *B*, *C* and *D* divide the strains into groups that are statistically identical

In the longevity selected LS1 strain, samples were collected until day 56 and it was observed that while the proteasome activity was reduced in these flies as compared with C1 flies, there was no further significant regression of proteasome activity with age in either males ($P = 0.431$) or females ($P = 0.412$) (Fig. 5; Table 4). However, the mean proteasome activity was significantly higher in LS1 females (1.70 pmol AMC/min/µg protein) compared to males (1.04 pmol AMC/min/µg protein) within the time span measured ($P = 0.007$).

Table 3 Two-way ANOVA of the effect of age and sex on proteasome activity in C1 flies

Source	Degrees of freedom	Sum of squares	F ratio	Prob > F
Days	1	34	22	<0.0001
Sex	1	111	73	<0.0001
Days * sex	1	37	24	<0.0001
Joint factor test				
Days	2	72	24	<0.0001
Sex	2	148	49	<0.0001

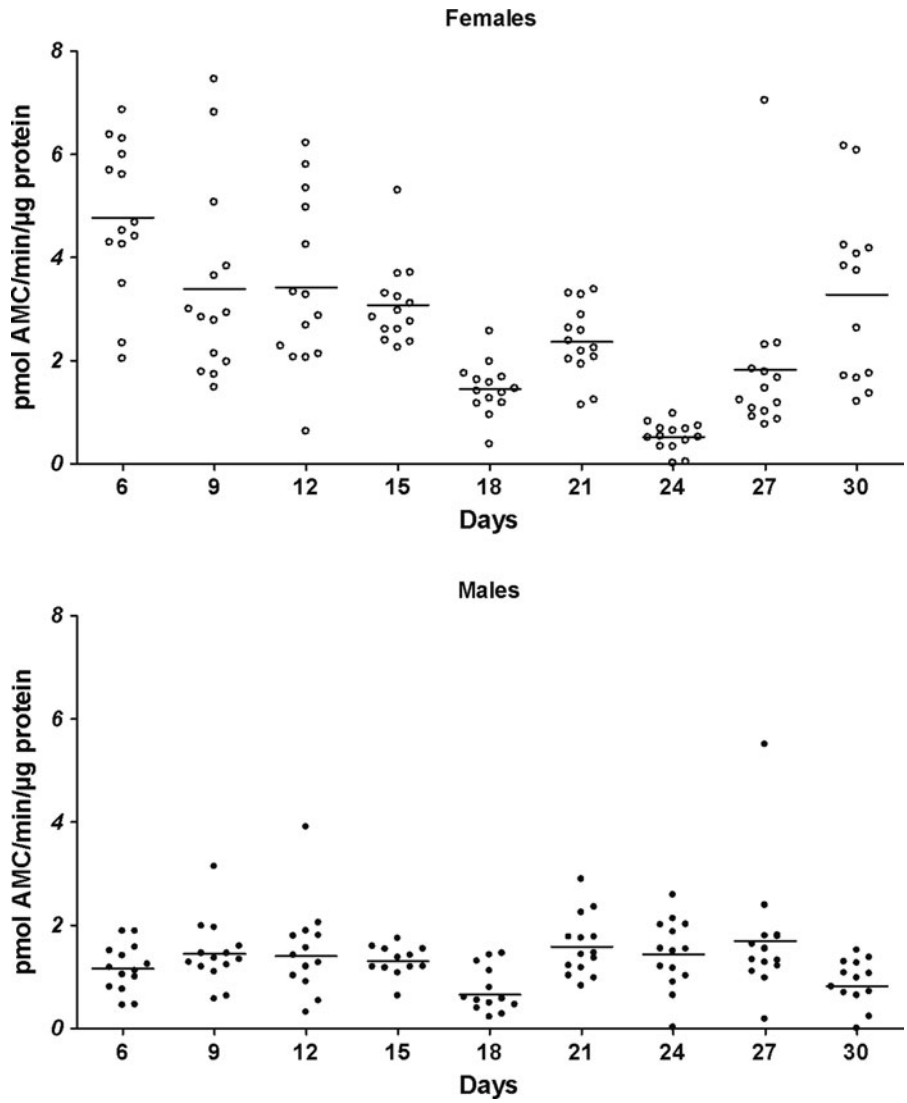

Fig. 4 Proteasome activity (PA) in individual C1 males and females measured at different time points during ageing. PA significantly decreased with age in females ($P = 0.038$), whereas it remained constant in males throughout the time period measured ($P = 0.933$)

Discussion

In this study we have measured proteasome activity in individual flies by using a non-denaturing lysing technique that consistently extracts total protein. We measured proteasome activity in males and females separately for both C1 and LS1 strains at day 15 and 14, respectively. We saw that the mean proteasome activity in 15 day old C1 females was more than two times higher than C1 males. In LS1 the proteasome activity had significantly decreased compared with control flies, but the sex differences were maintained to some extent. We further harvested flies from both strains and sexes at increasing age and measured proteasome activity in individual females and males. We saw an overall significant higher level of proteasome activity in females compared to males. Furthermore, in C1 females we saw an age-related decrease in proteasome activity that was not obvious in C1 males.

The age-related decline in proteasome activity observed in C1 females had disappeared during longevity selection. We further investigated the proteasome activity level in five fly strains that each had been selected for resistance towards five different types of environmental stress. At day 15 the proteasome activity was significantly lower in all stress resistant lines compared to C1 in both females and males. Previous studies have shown that in addition to lifespan extension LS1 flies have an increased tolerance to starvation, desiccation, heat-knockdown heat-

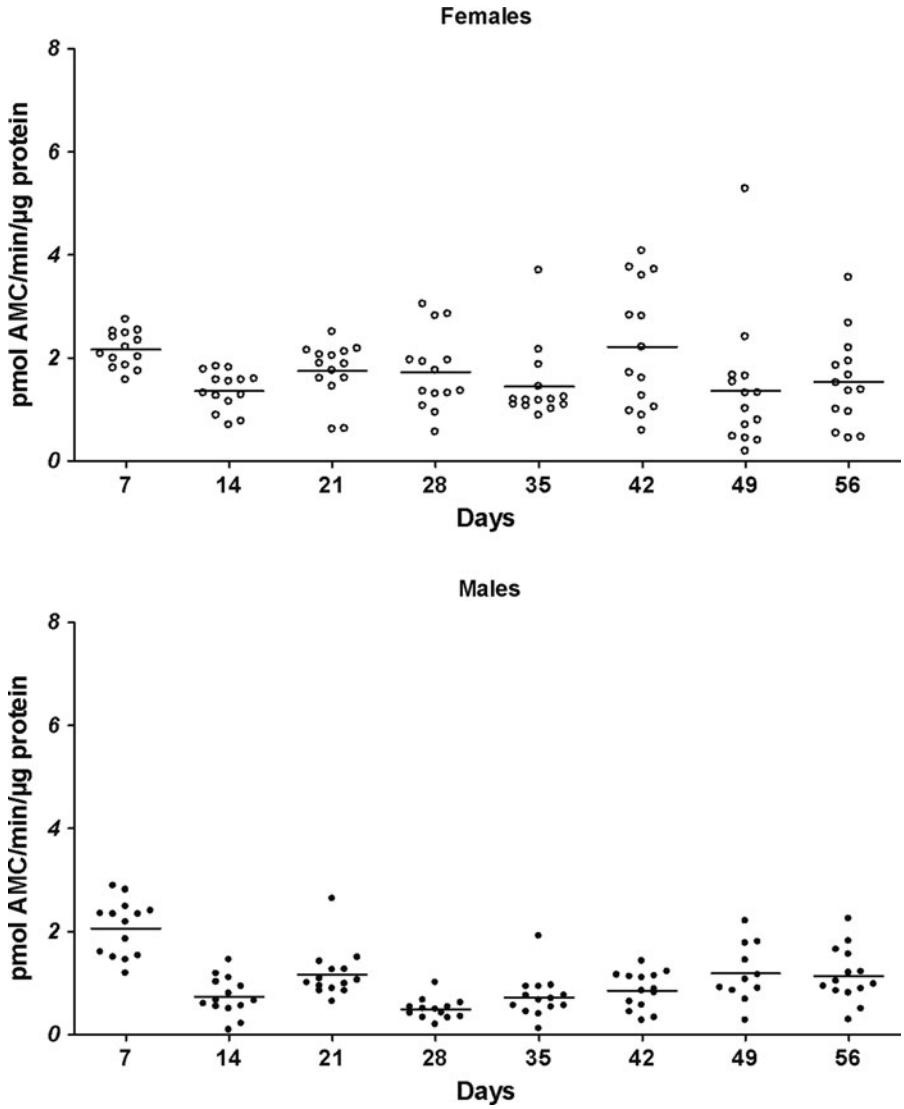

Fig. 5 Proteasome activity (PA) in individual LS1 males and females measured at different time points during ageing. There was no significant decline in PA with age in LS1 females and males ($P = 0.431$ and $P = 0.412$, respectively). PA was in general higher in LS1 females compared to LS1 males ($P < 0.0001$, see Table 4)

Table 4 Two-way ANOVA of the effect of age and sex on proteasome activity in LS1 flies

Source	Degrees of freedom	Sum of squares	F ratio	Prob > F
Days	1	3	5	0.02
Sex	1	24	41	<0.0001
Days * sex	1	0.17	0.30	0.58

and cold-shock compared to C1, while other traits, such as developmental time and productivity at early age remain unchanged after selection (Bubliy and Loeschcke 2005). One could speculate whether the difference in proteasome activity observed between LS1 and C1 flies is related to fertility. The disposable soma theory suggests that evolution favors spending energy on reproduction rather than granting longevity to the individual (Kirkwood 1977). This means that there is a trade-off between long life and reproduction. According to this theory LS1 flies should therefore be less productive. However, in a previous study (Bubliy and Loeschcke 2005), it was demonstrated that the productivity at early age in the LS1 lines was unaffected compared to C1. (C1 progeny 271.92 ± 13.13 and LS1 progeny 285.28 ± 7.93). It can thus be concluded that the higher proteasome activity observed in C1 females compared to LS1 females cannot be explained by higher fertility.

Previous studies of *D. melanogaster* have supported the currently accepted hypothesis that age-

related decline in proteasome activity is a crucial factor responsible for ageing at the cellular level (Vernace et al. 2007). However, these studies have looked at the activity of combined samples of several flies of both sexes. When individual flies from several genetically independent lines were investigated we could not confirm this relationship. On the contrary, flies with increased lifespan and stress resistance had a lower level of proteasome activity.

There is no straight forward explanation for this except for the possibility that reduced proteasome activity is an indicator of reduced occurrence of molecular damage in long lived and stress resistant flies. Some other studies on longevity and stress resistance in nematodes have shown that an increase in the expression of the enzymatic antioxidant superoxide dismutase may account for a decrease in molecular damage (Yanase et al. 2002). Furthermore, a recent study made on *Drosophila* has demonstrated the ability of blueberry extract to up-regulate antioxidant gene expression along with a 10 % increase in lifespan (Peng et al. 2012). However, no up-regulation of SOD was observed in the longevity selected LS1 strain used in our study (Sarup et al. 2011). Additionally, Sørensen et al. (2007) had analyzed the gene expression profile in all selection lines and in general had found only a minor overlap in gene expression among the lines, mostly in the genes involved in the immediate heat shock response. These results indicate that an increase in the efficiency of damage-repair may accordingly reduce the requirement for protein degradation by the proteasome and thus be an explanation for the reduced proteasome activity observed in the longevity and stress resistant selection lines in our study. Investigating other elements within the protein turnover cycle will give further biochemical explanation as to why an increase in lifespan and stress resistance leads to a reduced proteasome activity in *D. melanogaster*.

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