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**Abstract**
Methionine sulfoxide reductases (Msrs) are enzymes that repair oxidized methionine residues in proteins. This function implicated Msrs in antioxidant defense and the regulation of aging. There are two known Msr types in animals: MsrA specific for the reduction of methionine-*S*-sulfoxide, and MsrB that catalyzes the reduction of methionine-*R*-sulfoxide. In a previous study, overexpression of MsrA in the nervous system of *Drosophila* was found to extend lifespan by 70%. Overexpression of MsrA in yeast also extended lifespan, whereas MsrB overexpression did so only under calorie restriction conditions. The effect of MsrB overexpression on lifespan has not yet been characterized in animal model systems. Here, the *GAL4-UAS* binary system was used to drive overexpression of cytosolic *Drosophila* MsrB and mitochondrial mouse MsrB2 in whole body, fatbody, and the nervous system of flies. In contrast to MsrA, MsrB overexpression had no consistent effect on the lifespan of fruit flies on either corn meal or sugar yeast diets. Physical activity, fecundity, and stress resistance were also similar in MsrB-overexpressing and control flies. Thus, MsrA and MsrB, the two proteins with similar function in antioxidant protein repair, have different effects on aging in fruit flies.

**Keywords:** aging, lifespan, methionine sulfoxide reductase, *Drosophila melanogaster,* protein repair

**Abbreviations:** Msr, methionine sulfoxide reductase; GSH, glutathione; GSSG, oxidized glutathione; MetO, methionine sulfoxide; Met-SO, methionine-*S*-sulfoxide; Met-RO, methionine-*R*-sulfoxide; ROS, reactive oxygen species; SY, sugar yeast food; DR, dietary restriction; DR SY, dietary restriction sugar yeast food; N SY, normal sugar yeast food; ORF, open reading frame; PBS, phosphate buffer saline; HPLC, high performance liquid chromatography.

## 1. Introduction

One of the prominent theories of aging is known as the free radical theory (Harman, 1956; Beckman and Ames, 1998). It postulates that an age-associated accumulation of macromolecules (e.g., proteins, nucleic acids, lipids) oxidatively damaged by reactive oxygen species (ROS) results in senescence. Oxidative damage appears from an imbalance among oxidant production, antioxidant defense, and repair processes. A logical prediction based on this theory is that the overexpression of antioxidant/repair enzymes should increase lifespan. *Drosophila melanogaster* is a short-lived model-organism that has been widely used to examine the correlation between antioxidant defense and aging; however, a number of conflicting data were obtained.

Strong evidence of lifespan extension in flies overexpressing Cu,Zn-superoxide dismutase (SOD) and Mn-SOD (Parkes *et al.*, 1998; Sun and Tower, 1999; Sun *et al.*, 2002, 2004) or neutral effect of catalase overexpression (Sun *et al.*, 2002; Orr and Sohal, 1992)

were found. The simultaneous overexpression of these enzymes in different combinations resulted in life-prolonging effects (Orr and Sohal, 1994; Sun *et al.*, 2004). Additionally, flies lacking either SODs (Kirby *et al.*, 2002; Phillips *et al.*, 1989) or catalase (Orr *et al.*, 1992) had reduced lifespan. However, other studies have shown that overexpression of these antioxidant enzymes in long-lived genetic background had no effect (Orr and Sohal, 2003; Seto *et al.*, 1990)on survivorship. The simultaneous overexpression of Cu,Zn-SOD, Mn-SOD and catalase in different combinations in long-lived flies also did not result in lifespan extension (Orr *et al.*, 2003) and even led to a decrease in lifespan by 43% for flies overexpressing Mn-SOD and catalase (Bayne *et al.*, 2005). As the correlation between these enzymes and lifespan was not direct and the observed differences in lifespan of transgenic flies were the function of (1) genetic background, (2) tissue/cell distribution of antioxidants, (3) temporal pattern of expression, and (4) source of transgene and type of expression system, it was suggested that antioxidant defense resulting from antioxidant enzymes is not a

limiting factor for *Drosophila* aging (Helfand and Inouye, 2003; Mockett *et al.*, 2001; Sohal *et al.*, 2002). Recent studies showed that overexpression of two enzymes, glucose-6-phosphate dehydrogenase (Legan *et al.*, 2008) and glutamate-cysteine ligase (Orr *et al.*, 2005), that increase low molecular weight antioxidants in cells (NADPH and glutathione (GSH), respectively), increases mean and maximum lifespan of long-lived *Drosophila* strain up to 50%. These studies, performed in one genetic background and with different transgene expression patterns, provide insights for understanding the processes that favor long lifespan.

In *Drosophila*, concentrations of low molecular weight antioxidants (and their precursors) decline in senescence-associated manner (Mockett *et al.*, 1999; Rebrin *et al.*, 2004). GSH as the most abundant low molecular weight antioxidant is considered as a major determinant of the cellular redox state. The reduced GSH/oxidized GSH (GSSG) ratio was decreased in exponential manner in the whole body of *Drosophila* (by over 70% from 10 to 60 days of age) (Rebrin *et al.*, 2004). In the same time scale, the concentration of free methionine, which also serves as the source for cysteine production via the transsulfuration pathway, was decreased by 50% (Rebrin *et al.*, 2004). Cysteine is a precursor for the GSH biosynthesis (Stipanuk, 2004). Various studies (reviewed in Stadtman *et al.*, 2005) revealed an age-related increase in methionine sulfoxide (MetO) levels in different tissues and organisms.

The repair of age-related protein damage has, to some extent, been studied in *Drosophila* (Chavous *et al.*, 2001; Ruan *et al.*, 2002). A balance between oxidative modifications of proteins and their repair was proposed to play an important role in senescence. Almost all amino acids are susceptible to oxidation by radicals that cause damage to amino acid side chains as well as protein backbones (Levine and Stadtman, 1996; Sohal, 2002; Stadtman, 2008). Thus, *Drosophila* shows an age-related increase in modified and oxidized proteins (Chavous *et al.*, 2001; Orr and Sohal, 1994; Sohal *et al.*, 1993). To repair oxidized side chains of sulfur-containing amino acids, cysteine and methionine, cells have evolved enzymes specific for oxidized forms of these amino acids. For example, two conserved redox systems, the thioredoxin and glutathione systems, maintain intracellular thiol groups of proteins and low molecular weight thiols in the reduced state in organisms from bacteria to humans (Holmgren, 2008; Lou, 2008).

Methionine sulfoxide reductases (Msrs) occur in most organisms and catalyze the thioredoxin-dependent reduction of free and protein-bound MetO to methionine (Brot and Weissbach, 2000; Kim and Gladyshev, 2007; Moskovitz, 2005). Msrs as well as glutathione and thioredoxin reductases are ubiquitously expressed in cells (Holmgren, 2008; Kim and Gladyshev, 2007; Lou, 2008) and together with their substrates and cofactors form repair systems that protect cells from oxidative stress, and maintain cellular redox homeostasis (Holmgren, 2008; Moskovitz, 2005; Kim and Gladyshev, 2007; Levine *et al.*, 2000).

Msrs are classified with respect to their substrate specificity into two types: MsrA that catalyzes the reduction of methionine-*S*-sulfoxide (Met-SO) in both protein-bound and free forms, and MsrB that is specific for the reduction of methionine-*R*-sulfoxide (Met-RO) primarily in proteins. *Msr* genes are found in most organisms except some parasites and hyperthermophiles (Kim and Gladyshev, 2007). The number of *MsrA* and *MsrB* genes varies in different organisms. One MsrA and one MsrB are present in *Escherichia coli, Saccharomyces cerevisiae, Caenorhabditis elegans,* and *D. melanogaster*. Mammals have one MsrA and three MsrBs (MsrB1, MsrB2, and MsrB3). Although MsrA and MsrB enzymes have the same function of MetO reduction, they are different not only in substrate specificity, but also in active site composition, protein fold, subcellular localization and evolution (Kim and Gladyshev, 2005, 2006, 2007; Kryukov *et al.*, 2002).

Several studies have provided evidence for an important role of Msrs in aging. An age-related decrease in total Msr activity and MsrA levels was observed in rat liver and kidney (Petropoulos *et al.*, 2001). Expression of MsrA and MsrB2 was decreased in senescent human WI-38 fibroblasts (Picot *et al.*, 2004). MsrA null mutants of yeast (Kryukov *et al.*, 2002; Moskovitz *et al.*, 1997) and mice (Moskovitz *et al.*, 2001) were more sensitive to oxidative stress than wild type organisms and had a shortened lifespan (decreased by ~26% in yeast (Koc *et al.*, 2004) and ~40% in mice (Moskovitz *et al.*, 1997)). Moreover, overexpression of MsrA in yeast (Moskovitz *et al.*, 1998) and fruit flies (Ruan *et al.*, 2002) led to protection against oxidative stress. In *S. cerevisiae,* overexpression of host MsrA extended lifespan by 25% (Koc *et al.*, 2004). Most remarkably, expression of GFP-fused bovine MsrA in the nervous system of *Drosophila* increased the median lifespan of fruit flies by 70% (Ruan *et al.*, 2002). Moreover, the authors observed that the MsrA-overexpressing transgenic flies had better physical activity, reproduction, and resistance to oxidative stress at the old age. Since MsrB is responsible for the reduction of one diastereomer of MetO (i.e., half of total MetO reduction), this enzyme is expected to have the same role in aging as MsrA. However, thus far, the effects of MsrB overexpression and knockout on aging were studied only in yeast (Koc *et al.*, 2004). The knockout of *MsrB* gene had little effect on the lifespan. Moreover, the overexpression of MsrB in yeast had no effect on the lifespan under standard growth conditions, but prolonged the lifespan under caloric restriction conditions (Koc *et al.*, 2004).

Since the most dramatic effect of MsrA overexpression on lifespan was observed in *Drosophila* (Ruan *et al.*, 2002), it was of interest to examine whether MsrB overexpression affects lifespan in this organism. Further considering the findings on the roles of MsrA and MsrB on aging in yeast, it was important to determine the effects of MsrB overexpression under both regular and calorie restriction conditions. In this study, we developed transgenic fruit flies overexpressing mouse mitochondrial MsrB2 or *Drosophila* cytosolic MsrB, and used them to address these questions.

## 2. Materials and methods

### 2.1. Constructs

The ORF of mouse MsrB2 was PCR-amplified from a pET28a-based construct (Kim and Gladyshev, 2004) with 5'-AAAAGAATTCATGGCGCGACTGCTGC-GAG-3'and 5'-ACACCTCGAGTTAGGGCTTGCTGGGTTTGAACT-3' primers and cloned into EcoRI/XhoI sites of pUAST vector (Brand and Perrimon, 1993) to yield pUAST-mMsrB2. The ORF of *Drosophila* MsrB was subcloned into EcoRI/XhoI sites of pUAST vector from pET28_MsrB construct (Kumar *et al.*, 2002) by using 5'-ACCGGAATTCATGGATAACAAGAGCGAGAAG-3' and 5'-AAAACTCGAGTCACTGCTGGGCAATG-3' primers to yield pUAST-dMsrB. Constructs were verified by DNA sequencing.

### 2.2. Transgenic Drosophila lines

Transgenic flies were obtained using standard techniques for germline cell injection (Rubin and Spradling, 1982) and subsequent crosses. Briefly, *white* (*w^1118^*) embryos were co-injected with pUAST-mMsrB2 or pUAST-dMsrB constructs and the P{Δ2-3} plasmid carrying transposase (Robertson *et al.*, 1988). Microinjection of the recombinant P-element plasmids yielded 8 independent transformants carrying *UAS-mMsrB2* and 5 transformants carrying *UAS-dMsrB* transgenes. P-element insertion was mapped to specific chromosome by genetic segregation analysis. A transgenic male was crossed with virgin females carrying the second-chromosome balancer [*w^1118^*; *amos^Tft^/CyO*] and third-chromosome balancer [*w^1118^*; *croc^5F59^e*/TM3, Sb^1^*]. Phenotypic analysis of the offsprings allowed for generation of the homozygous lines for each MsrB with P-element insertion on X, second or third chromosomes. Two independent homozygous *UAS-MsrB*-responder lines for each MsrB were used in the study. These MsrB lines were designated as *mMsrB2^2A^* , *mMsrB2^3A^* (mouse MsrB2 lines) and *dMsrB^2A^* , *dMsrB^2B^* (*Drosophila* MsrB lines). The designations of transgenic lines display the transgene (i.e., mouse MsrB2, mMsrB2) followed by the chromosome of insertion (i.e., 2 or 3), followed by a letter (i.e., A or B lines) indicating the independent insertion on that chromosome. None of these insertions had influence on viability or development of homozygous *UAS-MsrBs* lines.

Two GAL4-activator lines used in this study, *da-GAL4* [*w*; ; P{w^+mW.hs^ = GAL4-da.G32}UH1*] (stock #5460), *elav-GAL4* [*w*, P{w^+mW.hs^ = GawB}elav^c155^*]

(stock #458) and second-chromosome balancer [$w^{1118}$; $amos^{Tft}/CyO$] (stock #424) were obtained from the Bloomington *Drosophila* Stock Center at Indiana University. The *tubP-GAL4* [$w^*$; $P\{w^{+mC} = tubP\text{-}GAL4\}/CyO$] was kindly provided by J. P. Vincent (National Institute for Medical Research, London, U.K.). The third chromosome balancer [$w^{1118}$; $croc^{5F59}e^*/TM3$, $Sb^1$](Häcker *et al.*, 1995) and the *fat-body-GAL4* driver [$y^*w^*$; $P(w^{+mW.hs} = GawB)FB$ $P(w^{+m^*}$ *UAS-GFP 1010T2*)](Grönke *et al.*, 2003) was a kind gift from H. Jäckle and R. P. Kühnlein respectively (Max-Planck-Institut für Biophysikalische Chemie, Göttingen, Germany).

### 2.3. Fly culture media

Two food types were used in the lifespan experiments. The first was corn meal food (85.7 g corn meal ''Aunt Jemina''; The Quaker Oats Company, Chicago, IL), 50 ml golden A unsulfored molasses (Groeb Farms Inc., Onsted, MI), 71.4 g Torula yeast (MP Biomedicals, Solon, OH), 2.86 g *p*-hydroxybenzoic acid methyl ester (Sigma), 6.4 g agar (MoorAgar Inc., Loomis, CA) and 5.7 ml propionic acid (Sigma) per liter water. The second type was sugar yeast (SY) food. It included 20 g agar, 3 g *p*-hydroxybenzoic acid methyl ester, 3 ml propionic acid, and variable amounts of sugar and yeast per liter of water. 50 g/l dextrose was present in the dietary restriction sugar yeast food (DR SY) and 100 g in normal food (N SY). Autolysed yeast powder (T.P. Drewitt, London, UK) was added in quantity 50 g per liter of water in DR SY food and 100 g in N SY food (Chapman and Partridge, 1996).

### 2.4. Genetic crosses and Drosophila husbandry

The *GAL4-UAS* binary system (Brand and Perrimon, 1993) was used to drive overexpression of mouse mitochondrial MsrB2 and fruit fly cytosolic MsrB in *Drosophila*. To obtain heterozygous flies expressing mouse MsrB2 or *Drosophila* MsrB, homozygous $mMsrB2^{2A}$, $mMsrB2^{3A}$, $dMsrB^{2A}$, and $dMsrB^{2B}$ virgin males were crossed to the corresponding virgin females containing the *GAL4*-driver. F1 offspring from crosses between GAL4 drivers and *white* flies (*GAL4-activator/* $w^{1118}$) and between *white* flies and homozygous MsrB lines ($w^{1118}$/*UAS-MsrBs*) were obtained for control concurrently. Experimental flies were reared on corn meal food from vials seeded with less than 70 eggs per vial. Newly emerged flies were transferred to fresh corn meal food and allowed to mate for 1–2 days. 3-day-old mated flies were collected using ether, sorted by sex, and used for lifespan studies, paraquat resistance test, and starvation test. Unless otherwise stated, flies used in the experiment were held on the corn meal food and transferred to fresh vials without anesthesia every three days. Embryonic, larval, pupal, and adult animals were kept in temperature-controlled chamber at 25 °C with 12 h light/dark cycle and approximately 60% humidity.

### 2.5. Lifespan study

Newly eclosed adult animals were collected within 4 h and kept on corn meal food at density of 50 animals per vial. In a typical lifespan trial, 3-day-old mated flies were placed in cages. The cage was made from 1 L plastic container with a grommet for changing of fresh food vials and a slit for removal of dead flies by aspiration. 35 males and 35 females were kept in each cage for testing lifespan on sugar yeast or corn meal food. In one independent experiment, 70 mated males per cage were kept on dietary restriction SY food. Three replica cages were used for each lifespan simultaneously; survivor curves are presented the average of those independent replicas. Fresh food was supplied into cages and dead flies were removed by aspiration and counted every 3 days. Animals were maintained in temperature-controlled incubator at 25 °C with 12 h light/dark cycle and 50–60% humidity. The control and experimental group trials were always performed concurrently.

### 2.6. Paraquat resistance test

Male flies overexpressing mouse MsrB2 or *Drosophila* MsrB in nervous system were prepared as described above (Section 2.4) and cultured in half pint bottles on corn meal food at a density of 150 animals per bottle. 17 days old flies were sorted in groups of 10 or 20 animals with etherization 3 days before the test. The test vials were set up with sponge soaked with 2 ml of 20 mM or 30 mM freshly prepared paraquat (Sigma) and 5% sucrose (Sigma) solution and covered with a 3 MM filter paper circle. On the day of the test, flies were transferred without etherization to the test vials (for the experiment with 20 mM paraquat) or to vials with 1% agar and kept starved for 6 h (for the experiment with 30 mM paraquat). The starved flies were transferred to the test vials without etherization and the number of dead animals was counted at indicated time points. For the test with 20 mM paraquat, 8 replicates of 10 animals were used. For the test with 30 mM paraquat, 18 replicates of 20 animals were performed for each cross except for control cross *elav-GAL4*/$w^{1118}$ where 9 replicates were examined. All data are reported as the means ± S.D.

### 2.7. Starvation resistance test

Male flies were prepared by standard techniques (Section 2.4) and maintained on corn meal food for 20 days prior to the test. Groups of 20 flies were transferred without etherization to vials with 6 ml 1% agar and kept in 12 h light/dark cycle at 25 °C. Total number of flies tested for each genotype was 140 animals. Dead animals were counted every 8 h. All data are reported as the means ± S.D.

### 2.8. Reproduction

Age-specific changes in pupa production were determined from counts of pupal progeny produced by 5 females in 24 h. Females were placed in vials along with 5 males. 10 replicates of each vial were tested. Flies were transferred to vials with fresh food without anesthetization every 2–3 days. Females laid eggs for 24 h, then animals were removed and the number of pupa was counted after eclosion. All data are reported as the means ± S.D.

### 2.9. Enzyme activity assays

Homogenates of flies were prepared using the same procedure for enzyme activity assays, HPLC-based assay and Western blot analyses. Flies were homogenized in liquid nitrogen, resuspended in PBS buffer containing protease inhibitor mixture (Roche), incubated on ice for 20 min and centrifuged at 16,000 ×*g* for 20 min at 4 °C. Total protein concentration in supernatant was measured by the Bradford method using bovine serum albumin as a standard. 35 six-day-old males were resuspended in 350 µl of PBS buffer for activity measurements. MsrB activity was assayed in the supernatant by a standard procedure (Kumar *et al.*, 2002). Briefly, the reaction mixture (100 µl) contained 20 mM dithiothreitol, 200 µM dabsyl-Met-RO, and 200 µg of total protein in PBS, pH 7.4. The reaction was carried out at 37 °C for 30 min and stopped by adding 200 µl of acetonitrile. The reaction product, dabsyl-Met, was analyzed by an HPLC procedure using a $C_{18}$ column (Zorbax Eclipse XDB, USA). Measurements for each sample were performed in triplicate. All data are reported as the means ± S.D.

### 2.10. Analysis of Met/MetO content in proteins and free amino acid forms

Homogenates of 70 female flies in 800 µl of PBS buffer containing protease inhibitors were prepared as in Section 2.9. For each type of MsrB-overexpressing fly (mouse MsrB2 or *Drosophila* MsrB) two independent preparations were made. Proteins (2 mg) were precipitated from the homogenates by 5% trichloroacetic acid (Sigma) on ice for 10 min. Samples were centrifuged at 16,000 ×*g* for 20 min at 4 °C, supernatants were used for measurements of Met content immediately (or stored at –80 °C), and pellets were hydrolyzed with *p*-toluenesulfonic acid. *p*-Toluenensulfonic acid (Sigma, 98.5% purity) was prepared as described (Bayer *et al.*, 1985). Pellets were treated with 0.5 ml of 3 M *p*-toluenesulfonic acid under anaerobic conditions at 110 °C for 22 h, acidic samples were neutralized with equal volumes of 2 M NaOH and stored at –20 °C. The analysis of Met/MetO content in protein-free and hydrolyzed samples by HPLC was carried out as described (Lee *et al.*, 2008). *o*-Phthalaldehyde derivatization of amino acids were detected using a Zorbax Eclipse XDB-$C_8$ column (for Met content) or Zorbax Eclipse XDB-$C_{18}$ column (for MetO content). Measurements for each sample were performed in triplicate. All data are reported as the means ± S.D.

### 2.11. Western blotting

6-day-old flies were prepared as in Section 2.9. 100 µg of total protein was electrophoresed on NuPAGE® Novex 10% Bis–Tris gels, transferred onto PVDF membranes, and immunoblotted with antibodies specific for mouse MsrB2 (Kim and Gladyshev, 2005), *Drosophila* MsrB (Kumar *et al.*, 2002)or *Drosophila* β-actin (ab8224, Abcam). Dilution 1:1000 was used for anti-mouse MsrB2 antibodies, 1:2000 for anti-*Drosophila* MsrB antibodies and 1:1000 for anti-β-actin. Immunoblot signals were visualized using ECL detection system (Sigma).

### 2.12. Statistic analysis

The significance of the difference between the survivor curves was determined separately for each 3 replicates of transgenic flies and their corresponding control (*GAL4-activator*/$w^{1118}$, *UAS-MsrB*/$w^{1118}$) using SAS software v. 9.1 (SAS Institute Inc., Cary, NC). To compare the survivor curves of the MsrB-expressing and control flies the non-parametrical estimates of the survivor functions by Kaplan–Meier method were applied using the procedure LIFETEST provided by SAS. The PROC LIFETEST calculated statistics for testing the null hypothesis that the survivor functions are the same for testing and control flies; statistical parameters ($\chi^2$ and *p*-value) of log rank test were used for estimation of accuracy of the null hypothesis. To get the mean and maximum (90% mortality) lifespans for each set of replicates (transgenic and control flies) the procedure MEANS provided by SAS software was used.

## 3. Results

### 3.1. Overexpression of fruit fly MsrB and mouse MsrB2 in Drosophila

We used the *GAL4-UAS* binary system to drive overexpression of mouse mitochondrial MsrB2 and fruit fly cytosolic MsrB in *Drosophila* as described in Section 2.4. Two independent homozygous UAS-MsrB-responder lines for each MsrB were used, designated $mMsrB2^{2A}$ and $mMsrB2^{3A}$ for mouse MsrB2 and $dMsrB^{2A}$ and *dMs-*
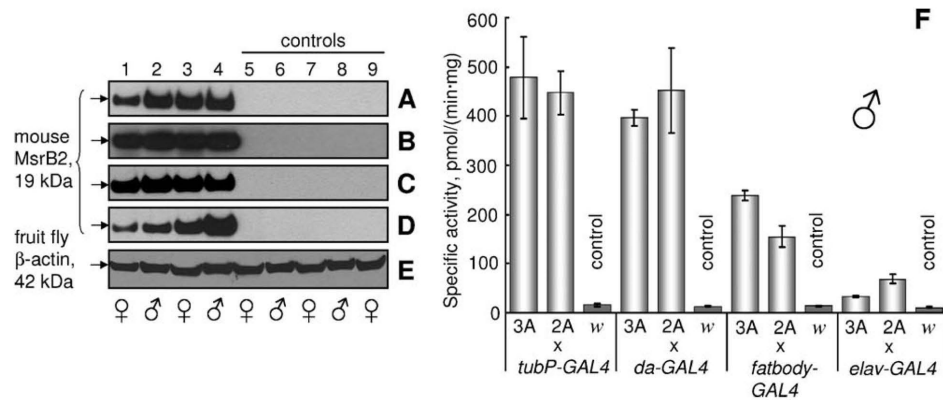
**Figure 1.** Expression of mouse MsrB2 in fruit flies. (**A–D**) Western blot analyses of *Drosophila* total homogenates with anti-mouse MsrB2 antibodies. *mMsrB2³ᴬ* (lanes 1, 2) and *mMsrB2²ᴬ* (lanes 3, 4) lines were crossed to *GAL4*-activator lines to drive mouse MsrB2 expression. Control flies were obtained by crossing *white* flies (*w¹¹¹⁸*) with *mMsrB2³ᴬ* (lanes 5, 6), *mMsrB2²ᴬ* (lanes 7, 8) and *GAL4*-activator (lane 9) lines. Expression of mouse MsrB2 (lanes 1–4) in whole body was achieved by crossing *UAS-mMsrB2* lines and *tubP-GAL4* (**A**) and *da-GAL4* (**B**) drivers, in fatbody by crossing *UAS-mMsrB2* with *fatbody-GAL4* driver (**C**), and in the nervous system by crossing *UAS-mMsrB2* with *elav-GAL4* driver (**D**). Migration of the 19 kDa band corresponding to mouse MsrB2 is indicated on the left in panels A–D. (**E**) Protein loading control (42 kDa *Drosophila* β-actin) for the Western blotting experiment shown in A. Gender of 6-day-old females and males analyzed is shown by the corresponding symbol below panel E. (**F**) MsrB activity of total homogenates of male flies used for Western blot analyses. Designations 3A, 2A, and letter *w* refer to *mMsrB2³ᴬ*, *mMsrB2²ᴬ*, and *w¹¹¹⁸* lines that were crossed with the *GAL4*-driver. Measurements of each sample were performed in triplicates. All data are reported as the means ± S.D.

*rB²ᴮ* for *Drosophila* MsrB. To test the efficacy of *UAS-MsrB* transgenes for whole body/ectopic expression, the responder lines were mated to four different *GAL4*-activator lines (drivers). Two drivers with constitutive broad range GAL4 expression, *tubP-GAL4* and *da-GAL4*, of different strength were selected because (1) flies express endogenous MsrB ubiquitously; and (2) we were interested in examining how MsrB expression level influences lifespan. The *GAL4-fatbody* driver was chosen as *Drosophila* fatbody includes many metabolic activities of the vertebrate liver such as storage and metabolism of fat, glycogen and proteins (Söndergaard, 1993). Mammalian liver shows high MsrB expression and activity (Kim and Gladyshev, 2007). The *elav-GAL4* activator line that mediates GAL4 expression in embryo, larvae and adult neurons was selected as the expression of bovine MsrA in *Drosophila* nervous system markedly extended lifespan of fruit flies in a previous study (Ruan *et al.*, 2002). We used *elav-GAL4* driver with the same genotype as that in the study that examined MsrA overexpression (Ruan *et al.*, 2002 and Section 2.2).

After crossing *GAL4*-activator lines with *UAS-MsrB*-responder lines, the overexpression of MsrBs in total body homogenate of

resulting progeny was verified by Western blot analyses and MsrB activity assays (Figures 1 and 2). The bands corresponding to mouse MsrB2 (19 kDa) (Figure 1A–C, lanes 1–4) and fruit fly MsrB (17 kDa) (Figure 2A–C, lanes 1–4) were detected in all analyzed flies carrying both *GAL4* and *UAS* transgenes. No bands with the same size were detected in control flies (Figures 1, 2A–C, lanes 5–9). As control, we used F1 progeny of crosses between corresponding *GAL4*-activator line and *white* flies (*GAL4-activator/w¹¹¹⁸*) as well as crosses between flies carrying *MsrB* transgene and *white* flies (*w¹¹¹⁸/UAS-MsrB*). Endogenous *Drosophila* MsrB was present at undetectable level in comparison with MsrB expressed from the transgene (Figure 2A–D, control lanes 5–9). The lowest level of MsrB expression was observed in the samples prepared from flies expressing *Drosophila* MsrB in the nervous system (Figure 2D), and this finding corresponded to only a small increase in MsrB activity driven by the *elav-GAL4* driver in these samples (compare cross *elav-GAL4/UAS-MsrBs* with other crosses on Figure 2F and Figure 1F). The low MsrB activity in flies with the transgene expressed in the nervous system reflects the fact that neuronal tissues contribute a smaller part by weight, and
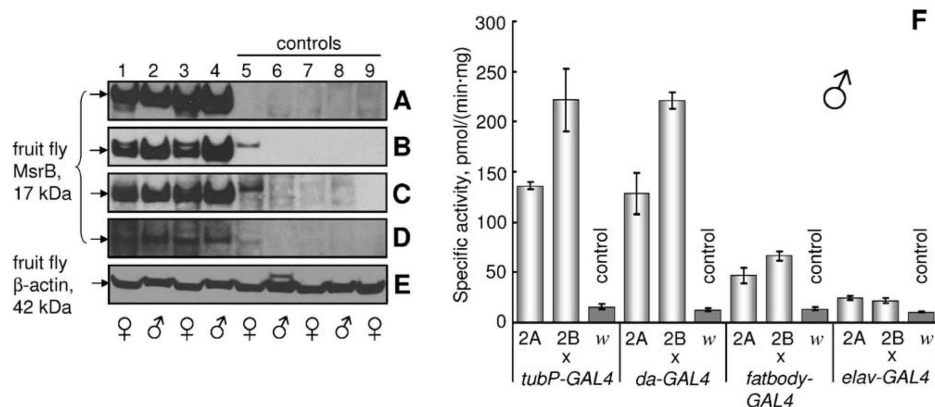


**Figure 2.** Expression of *Drosophila* MsrB in fruit flies. (**A–D**) Western blot analyses of fruit fly total homogenates with anti-*Drosophila* MsrB antibodies. *dMsrB²ᴬ* (lanes 1, 2) and *dMsrB²ᴮ* (lanes 3, 4) flies were crossed to *GAL4*-activator lines to overexpress *Drosophila* MsrB. Controls were obtained by crossing *white* flies with *dMsrB²ᴬ* (lanes 5, 6), *dMsrB²ᴮ* (lanes 7, 8), and *GAL4*-activator (lane 9) lines. F1 progeny of crosses between *tubP-GAL4* (**A**), *da-GAL4* (**B**), *fatbody-GAL4* (**C**), and *elav-GAL4* (**D**) drivers and *UAS-dMsrB* (lanes 1–4) and *white* flies *w¹¹¹⁸* (lane 9) were analyzed. Adult animals used were 6 days old. Migration of the 17 kDa band corresponding to fruit fly MsrB is shown by arrows on the left in panels A–D. (**E**) Protein loading control (42 kDa *Drosophila* β-actin) for the Western blotting experiment presented in A. Gender of 6-day-old females and males analyzed is shown by the corresponding symbols below panel E. (**F**) MsrB activity of total homogenate of fruit flies used for Western blot analyses. Designations 2A, 2B, and letter *w* refer to *mMsrB2³ᴬ*, *mMsrB2²ᴬ*, and *w¹¹¹⁸* lines that were crossed with the indicated *GAL4*-driver. Measurements were performed in triplicate. All data are reported as the means ± S.D.

the homogenates of flies expressing MsrB in the nervous system show lower levels of this enzyme in comparison, for example, with the homogenates of fruit flies expressing MsrB in the whole body. The highest MsrB activity was found in both ubiquitous *GAL4*-activator lines. Total body homogenates prepared from flies ubiquitously expressing mouse MsrB2 had almost 40-fold higher MsrB activity than the background activity due to endogenous fruit fly MsrB (Figure 1F). Homogenates prepared from fruit flies overexpressing *Drosophila* MsrB in whole body had almost 20-fold increase in activity (Figure 2F). No difference between two ubiquitous *GAL4*-activator lines (*tubP-GAL4* and *da-GAL4*) was found with regard to MsrB activity or expression (Figure 1A, B, and F), although the *da-GAL4* line is considered as a weaker driver. Fruit flies expressing MsrBs in the nervous system had statistically significant elevation in total MsrB activity (up to 6 times for mouse MsrB2 and up to 2 times for fruit fly MsrB overexpression) in comparison with controls. Clearly, efficient expression of active enzymes was achieved with both mouse MsrB2 and *Drosophila MsrB* transgenes. Background activity due to endogenous *Drosophila* MsrB was $13.2 \pm 1.9$ pmol/(min mg).

### 3.2. Overexpression of mouse MsrB2 has no effect on lifespan on corn meal food

Expression of MsrB2 in whole body had no life-prolonging effect; in contrast, a small reduction in the mean lifespans (Table 1; Figure 3B, E and F) or no effect (Table 1; Figure 3A) in MsrB2-expressing flies was observed. The longevity of females in which the expression of mouse MsrB2 was driven by *tubP-GAL4* activator line (*tubP-GAL4/mMsrB2$^{3A}$* and *tubP-GAL4/mMsrB2$^{2A}$*) was

not significantly different compared to driver line control *tubP-GAL4/w$^{1118}$* (Figure 3A; Table 1, column 5). However, male progeny of a cross *tubP-GAL4/mMsrB2$^{2A}$* showed a reduction (13%) in the mean lifespan in comparison with *tubP-GAL4/w$^{1118}$* control ($\chi^2 = 68.73$, $p < 0.0001$; Table 1, column 5 and Figure 3E). These three lines also demonstrated 10–20% reduction ($p < 0.0001$; Table 1, column 8) in the mean lifespan in comparison with the corresponding responder line controls (females *tubP-GAL4/mMsrB2$^{3A}$* vs. *w$^{1118}$/mMsrB2$^{3A}$* , females *tubP-GAL4/mMsrB2$^{2A}$* vs. *w$^{1118}$/mMsrB2$^{2A}$*, and males *tubP-GAL4/mMsrB2$^{3A}$* vs. *w$^{1118}$/mMsrB2$^{3A}$*). One male line *tubP-GAL4/mMsrB2$^{3A}$* displayed an 8% increase ($\chi^2 =10.76$, $p = 0.001$) and 30% increase ($\chi^2 =68.73$, $p < 0.0001$) in mean lifespan in comparison with driver line control and responder line control, respectively (Figure 3A; Table 1, columns 5 and 8). This was the only line in this study that showed lifespan extension with both controls.

When *tubP-GAL4* activator line was replaced with *da-GAL4* line, females expressing mouse MsrB2 lived significantly shorter ($p < 0.0001$) than driver control *da-GAL4/w$^{1118}$*. Experimental flies had 24% decrease in mean lifespan for cross *da-GAL4/mMsrB2$^{3A}$* vs. *da-GAL4/w$^{1118}$* ($\chi^2 = 39.42$, $p < 0.0001$) and 19% decrease for cross *da-GAL4/mMsrB2$^{2A}$* vs. *da-GAL4/w$^{1118}$* ($\chi^2 = 51.78$, $p < 0.0001$) (Figure 3B and Table 1, column 5). In males, respective 3% and 12% reductions in mean lifespan were observed (Figure 3F and Table 1, column 5). However, when a larger group was used (i.e., 210 males instead of 105) in the experiment with using sugar yeast (DR SY) food, no significant decrease in the mean lifespan for males with the same genotypes was observed (Figure 5B; Table 3).

**Table 1.** Statistical analysis of survivor curves presented in Figure 3 for mouse MsrB2-expressing flies and their controls.

| 1. Panel in Figure 3 | 2. Genotype | 3. Sex | 4. Mean (days) | 5. % vs. driver/$w^{1118}$ | 6. $\chi^2$ | 7. $p > \chi^2$ | 8. % vs. responder/$w^{1118\,op}$ | 9. $\chi^2$ | 10. $p > \chi^2$ |
|---|---|---|---|---|---|---|---|---|---|
| | $w^{1118}/mMsrB2^{3A}$ | F | 64.4 | | | | | | |
| | $w^{1118}/mMsrB2^{2A}$ | F | 65.0 | | | | +0.01* | 0.23* | 0.62* |
| A | $tubP\text{-}GAL4/mMsrB2^{3A}$ | F | 58.5 | +1 | 1.69 | 0.19 | –10 | 25.91 | <0.0001 |
| A | $tubP\text{-}GAL4/mMsrB2^{2A}$ | F | 52.5 | –1 | 3.89 | 0.05 | –19 | 68.51 | <0.0001 |
| A | $tubP\text{-}GAL4/w^{1118}$ | F | 54.1 | | | | | | |
| B | $da\text{-}GAL4/mMsrB2^{3A}$ | F | 49.2 | –24 | 39.42 | <0.0001 | –24 | 28.25 | <0.0001 |
| B | $da\text{-}GAL4/mMsrB2^{2A}$ | F | 53.2 | –19 | 51.78 | <0.0001 | –18 | 48.17 | <0.0001 |
| B | $da\text{-}GAL4/w^{1118}$ | F | 65.5 | | | | | | |
| C | $fatb\text{-}GAL4/mMsrB2^{3A}$ | F | 73.7 | –1 | 2.47 | 0.12 | +14 | 6.66 | 0.0099 |
| C | $fatb\text{-}GAL4/mMsrB2^{2A}$ | F | 72.8 | –4 | 3.21 | 0.06 | +12 | 7.28 | 0.0012 |
| C | $w^{1118}/mMsrB2^{2A}$ | F | | | | | | | |
| D | $elav\text{-}GAL4/mMsrB2^{3A}$ | F | 66.1 | –1 | 2.86 | 0.09 | +3 | 0.19 | 0.66 |
| D | $elav\text{-}GAL4/mMsrB2^{2A}$ | F | 64.9 | –1 | 4.26 | 0.04 | 0.01 | 0.21 | 0.95 |
| D | $elav\text{-}GAL4/w^{1118}$ | F | 67.1 | | | | | | |
| | $w^{1118}/mMsrB2^{3A}$ | M | 57.7 | | | | | | |
| | $w^{1118}/mMsrB2^{2A}$ | M | 61.4 | | | | +6* | 6.66* | 0.0099* |
| E | $tubP\text{-}GAL4/mMsrB2^{3A}$ | M | 75.8 | +8 | 10.76 | 0.001 | +30 | 66.71 | <0.0001 |
| E | $tubP\text{-}GAL4/mMsrB2^{2A}$ | M | 54.4 | –13 | 68.73 | <0.0001 | 11 | 16.69 | <0.0001 |
| E | $tubP\text{-}GAL4/w^{1118}$ | M | 70.5 | | | | | | |
| F | $da\text{-}GAL4/mMsrB2^{3A}$ | M | 76.5 | –3 | 1.09 | 0.29 | +32 | 67.56 | <0.0001 |
| F | $da\text{-}GAL4/mMsrB2^{2A}$ | M | 69.3 | –12 | 7.52 | 0.0061 | +13 | 28.89 | <0.0001 |
| F | $da\text{-}GAL4/w^{1118}$ | M | 78.4 | | | | | | |
| G | $fatb\text{-}GAL4/mMsrB2^{3A}$ | M | 79.6 | –6 | 2.31 | 0.11 | +38 | 114.72 | <0.0001 |
| G | $fatb\text{-}GAL4/mMsrB2^{2A}$ | M | 83.4 | –2 | 0.25 | 0.62 | +36 | 107.56 | <0.0001 |
| G | $fatb\text{-}GAL4/w^{1118}$ | M | 84.7 | | | | | | |
| H | $elav\text{-}GAL4/mMsrB2^{3A}$ | M | 73.7 | 0 | 4.44 | 0.04 | +28 | 58.10 | <0.0001 |
| H | $elav\text{-}GAL4/mMsrB2^{2A}$ | M | 69.3 | –6 | 0.42 | 0.52 | +13 | 30.38 | <0.0001 |
| H | $elav\text{-}GAL4/w^{1118}$ | M | 73.9 | | | | | | |

Column 1 indicates the letter of the corresponding panel in Figure 3. The genotypes and gender (female, F; males, M) are shown in columns 2 and 3, respectively. The mean lifespan is shown in column 4. The percent change in the mean lifespan compared with corresponding driver controls (*GAL4-driver/w$^{1118}$*) is displayed in column 5 and compared with corresponding responder control in column 8. Comparison of two types (transgenic and control) of survivor curves (three curves in each group, 105 flies total) was performed with SAS software (see Section 2.12). Statistics of non-parametrical log rank test ($\chi^2$) for comparison with driver control lines is shown in column 6; *p*-value in column 7 and for comparison with responder control line in columns 9 and 10. Star marks a comparison of two responder lines.
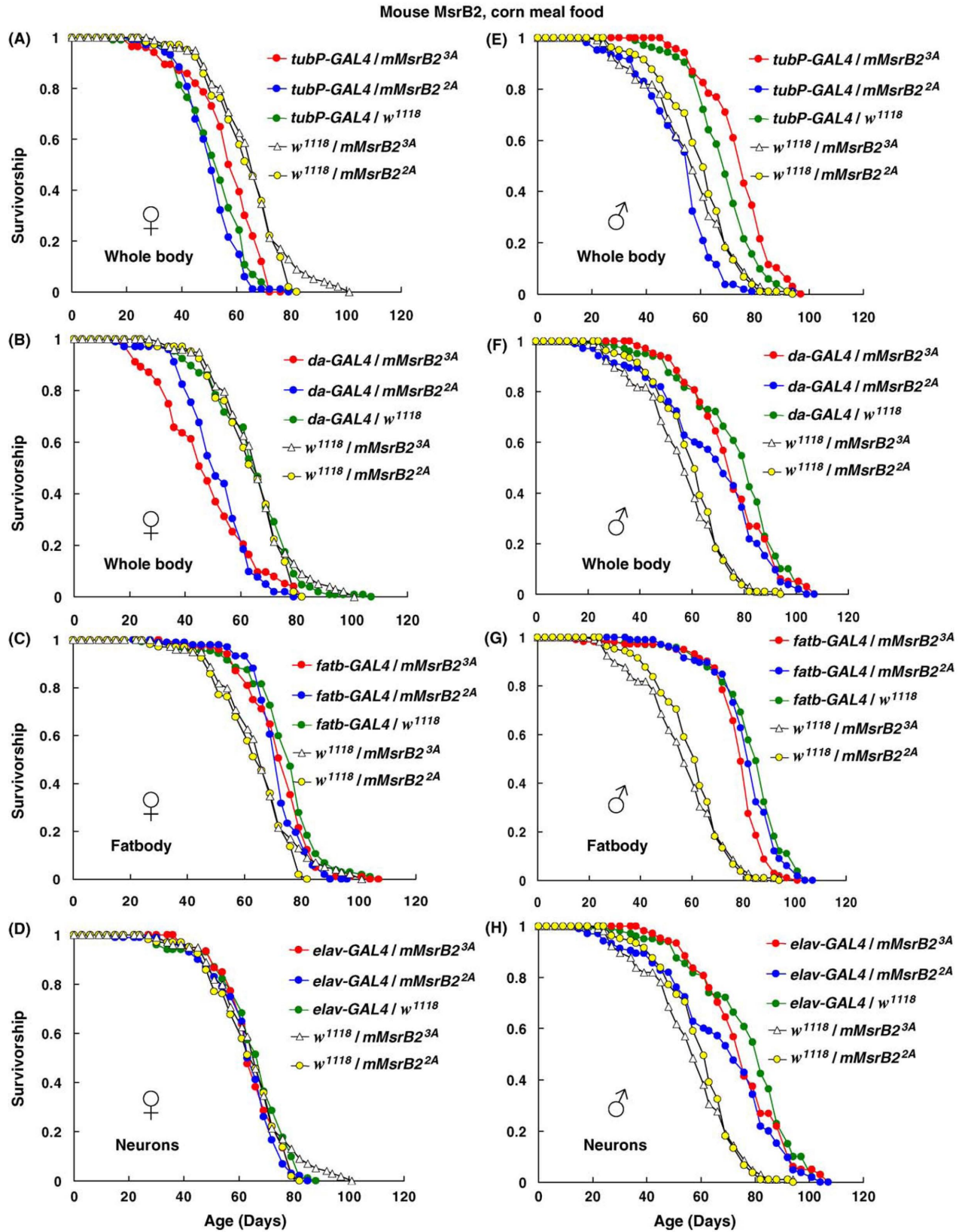
**Figure 3.** Survivorship curves of mouse MsrB2 expressing flies on corn meal food. 35 female and 35 male animals were housed together in plastic cages; three replica cages were used for each lifespan curve. Survivorship distribution presented shows the average of those independent replicas (105 females and 105 males for each curve). Fresh corn meal food was supplied and surviving animals were counted every 3–4 days. Expression of mouse MsrB2 in whole body was activated by using *tubP-GAL4* (**A** and **E**) or *da-GAL4* (**B** and **F**) activator lines; in fatbody by using *fatbody-GAL4* driver (**C** and **G**); in the nervous system by using *elav-GAL4* line (**D** and **H**). Genotypes and sexes are indicated on the plots. All trials were performed concurrently.
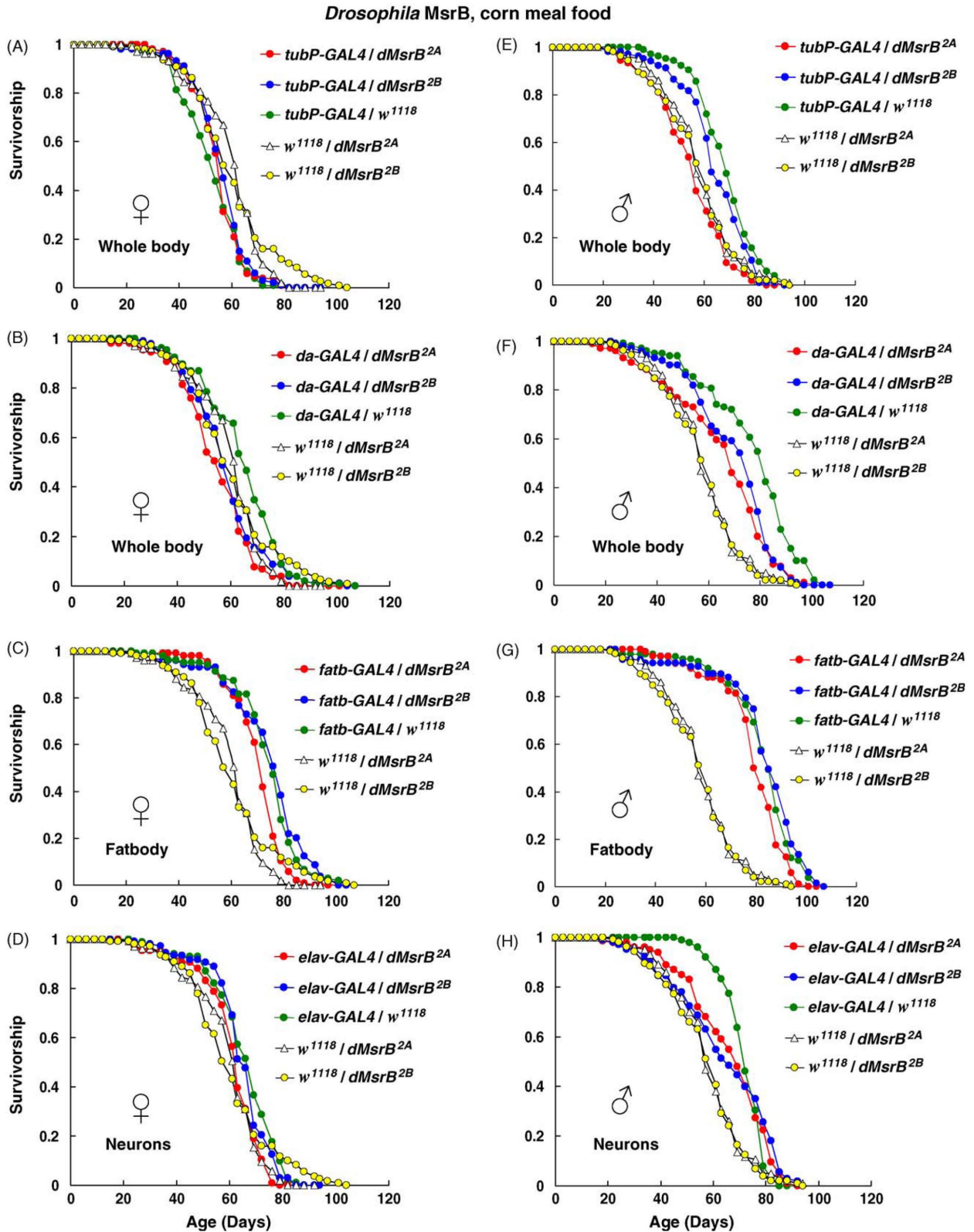
**Drosophila MsrB, corn meal food**



**Figure 4.** Survivorship curves of *D. melanogaster* MsrB-overexpressing flies on corn meal food. Males and females were kept together during lifespan study. Each survivorship curve represents 105 flies. Expression of *Drosophila* MsrB in whole body was driven by using *tubP-GAL4* (**A** and **E**) or *da-GAL4* (**B** and **F**) activator lines; in fatbody by using *fatbody-GAL4* driver (**C** and **G**); in the nervous system by using *elav-GAL4* line (**D** and **H**). Genotypes and sexes are shown on the plots. All trials were performed concurrently.

Although two ubiquitous *GAL4*-activator lines were used, conflicting data obtained does not allow making definitive conclusions with regard to negative influence of mouse MsrB2 overexpression in whole body on longevity of fruit flies. One of the possible reasons of such variations in lifespan could be the hybrid effect of two genetic backgrounds. Although both *UAS-mMsr2* flies and *GAL4*-drivers had *white* genetic background, they were not isogenic with experimental flies. The substantial effect of variations in genetic background was observed on responder line controls (Table 1, column 8). Although responder line controls had similar mean lifespan (≈ 65 days for females, $\chi^2$ = 0.23, $p$ = 0.62; 58 and 61 days for males, $\chi^2$ = 6.66, $p$ = 0.0099; Table 1), they showed statistically significant difference ($\chi^2 > 20$, $p$ < 0.0001) in mean lifespan in comparison with the GAL4-activator line control and experimental flies. The responder line controls $w^{1118}/UAS$-$mMsrB2$ had *white* genetic background. However, both the *GAL4*-activator line control and experimental flies had the same mixture (50:50) of *GAL4*-driver and *white* line chromatids. Therefore, an identical genetic background was achieved only in the driver line control, which should be viewed as the best control for the analysis of the effect of mMsrB2 expression on longevity. It was shown previously that genetic background itself could influence lifespan (Toivonen *et al.*, 2007); our data obtained for ubiquitous drivers are consistent with this idea. It should be noted that life-prolonging effect of MsrA overexpression in whole body (40% increase in median lifespan in compar-

ison with homozygous *GAL4*-activator line *Ubi-GAL4* and 80% increase in comparison with homozygous *UAS-MsrA* responder line) was obtained on non-backcrossed animals. Also, no responder line and driver line controls were analyzed in comparison with the flies overexpressing MsrA in the whole body (Ruan *et al.*, 2002).

Mouse MsrB2 ectopic expression in fatbody mediated by *fatbody-GAL4* activator line had no effect on lifespan in comparison with GAL4-activator line control (Figure 3C and G; Table 1, column 5). Male and female progeny of MsrB2-expressing flies had the similar mean lifespan as *GAL4-fatbody/w^{1118}* controls and their survivor curves were undistinguishable by statistical analysis (Table 1, column 5). The *fatbody-GAL4* flies (Grönke *et al.*, 2003) were generated on the basis of long-lived flies with *yellow* and *white* genetic background, *y w*. Long-lived flies expressing transgenes usually show more reproducible and reliable data than their short-lived counterparts (Orr *et al.*, 2003). Two heterozygous controls ($w^{1118}/mMsrB2^{3A}$ and $w^{1118}/mMsrB2^{2A}$) were tested concurrently with the experimental flies; no lifespan extension for MsrB2-expressing flies compared with their genetically matched $w^{1118}/mMsrB2^{3A}$ and $w^{1118}/mMsrB2^{2A}$ controls was found (Table 1, column 8). The positive effect of combination of two different genetic backgrounds ($w^{1118}$ and *y w*) on lifespan was observed. The heterozygotes combined $w^{1118}$ and *y w* chromosomes lived for approximately 13% (females) and 37% (males) longer than the responder line control with only *white* genetic background.

**Table 2.** Statistical analysis of survivor curves presented in Figure 4 for flies overexpressing *Drosophila* MsrB and their controls.

| 1. Panel in Figure 4 | 2. Genotype | 3. Sex | 4. Mean (days) | 5. % vs. driver/$w^{1118}$ | 6. $\chi^2$ | 7. $p > \chi^2$ | 8. % vs. responder/$w^{1118\,op}$ | 9. $\chi^2$ | 10. $p > \chi^2$ |
|---|---|---|---|---|---|---|---|---|---|
| | $w^{1118}/dMsrB^{2A}$ | F | 60.9 | | | | | | |
| | $w^{1118}/dMsrB^{2B}$ | F | 58.2 | | | | −5* | 3.17* | 0.12* |
| A | $tubP\text{-}GAL4/dMsrB^{2A}$ | F | 56.7 | +5 | 0.75 | 0.39 | −7 | 11.75 | 0.0006 |
| A | $tubP\text{-}GAL4/dMsrB^{2B}$ | F | 57.6 | +6 | 2.84 | 0.09 | −2 | 45.37 | 0.0003 |
| A | $tubP\text{-}GAL4/w^{1118}$ | F | 54.1 | | | | | | |
| B | $da\text{-}GAL4/dMsrB^{2A}$ | F | 56.2 | −14 | 25.9 | <0.0001 | −8 | 9.88 | 0.0017 |
| B | $da\text{-}GAL4/dMsrB^{2B}$ | F | 60.1 | −8 | 7.08 | 0.01 | +3 | 11.74 | 0.0006 |
| B | $da\text{-}GAL4/w^{1118}$ | F | 65.5 | | | | | | |
| C | $fatb\text{-}GAL4/dMsrB^{2A}$ | F | 71.2 | −6 | 14.2 | 0.0002 | +17 | 21.41 | <0.0001 |
| C | $fatb\text{-}GAL4/dMsrB^{2B}$ | F | 76.2 | +1 | 1.74 | 0.19 | +30 | 79.68 | <0.0001 |
| C | $fatb\text{-}GAL4/w^{1118}$ | F | 75.5 | | | | | | |
| D | $elav\text{-}GAL4/dMsrB^{2A}$ | F | 62.6 | −7 | 2.86 | 0.08 | +3 | 1.49 | 0.22 |
| D | $elav\text{-}GAL4/dMsrB^{2B}$ | F | 66.2 | −1 | 1.51 | 0.22 | +0.1 | 1.51 | 0.22 |
| D | $elav\text{-}GAL4/w^{1118}$ | F | 67.1 | | | | | | |
| | $w^{1118}/dMsrB^{2A}$ | M | 60.2 | | | | | | |
| | $w^{1118}/dMsrB^{3A}$ | M | 58.6 | | | | −3* | 0.03* | 0.86* |
| E | $tubP\text{-}GAL4/dMsrB^{2A}$ | M | 56.4 | −20 | 49.7 | <0.0001 | −7 | 1.56 | 0.2114 |
| E | $tubP\text{-}GAL4/dMsrB^{2B}$ | M | 66.1 | −6 | 4.7 | 0.03 | +9 | 17.47 | <0.0001 |
| E | $tubP\text{-}GAL4/w^{1118}$ | M | 70.5 | | | | | | |
| F | $da\text{-}GAL4/dMsrB^{2A}$ | M | 66.8 | −15 | 22.89 | <0.0001 | +11 | 16.8 | <0.0001 |
| F | $da\text{-}GAL4/dMsrB^{2B}$ | M | 71.5 | −9 | 16.85 | <0.0001 | +22 | 39.74 | <0.0001 |
| F | $da\text{-}GAL4/w^{1118}$ | M | 78.4 | | | | | | |
| G | $fatb\text{-}GAL4/dMsrB^{2A}$ | M | 80.8 | −5 | 2.31 | 0.11 | +34 | 82.4 | <0.0001 |
| G | $fatb\text{-}GAL4/dMsrB^{2B}$ | M | 77.2 | −9 | 0.25 | 0.62 | +31 | 73.4 | <0.0001 |
| G | $fatb\text{-}GAL4/w^{1118}$ | M | 84.7 | | | | | | |
| H | $elav\text{-}GAL4/dMsrB^{2A}$ | M | 56.6 | −23 | 4.44 | 0.04 | −6 | 1.18 | 0.28 |
| H | $elav\text{-}GAL4/dMsrB^{2B}$ | M | 65.5 | −11 | 0.42 | 0.52 | +11 | 20.67 | <0.0001 |
| H | $elav\text{-}GAL4/w^{1118}$ | M | 73.9 | | | | | | |

Column 1 indicates the letter of the corresponding panel in Figure 4. The genotypes and gender (female, F; males, M) are shown in columns 2 and 3, respectively. The mean lifespan is shown in column 4. The percent change in the mean lifespan compared with corresponding driver controls (*GAL4-driver*/$w^{1118}$) is displayed in column 5 and compared with corresponding responder control in column 8. Comparison of two types (transgenic and control) of survivor curves (three curves in each group, 105 flies total) was performed with SAS software (see Section 2.12). Statistics of non-parametrical log rank test ($\chi^2$) for comparison with driver control lines is shown in column 6; *p*-value in column 7 and for comparison with responder control line in columns 9, 10. Stars mark a comparison of two responder lines.
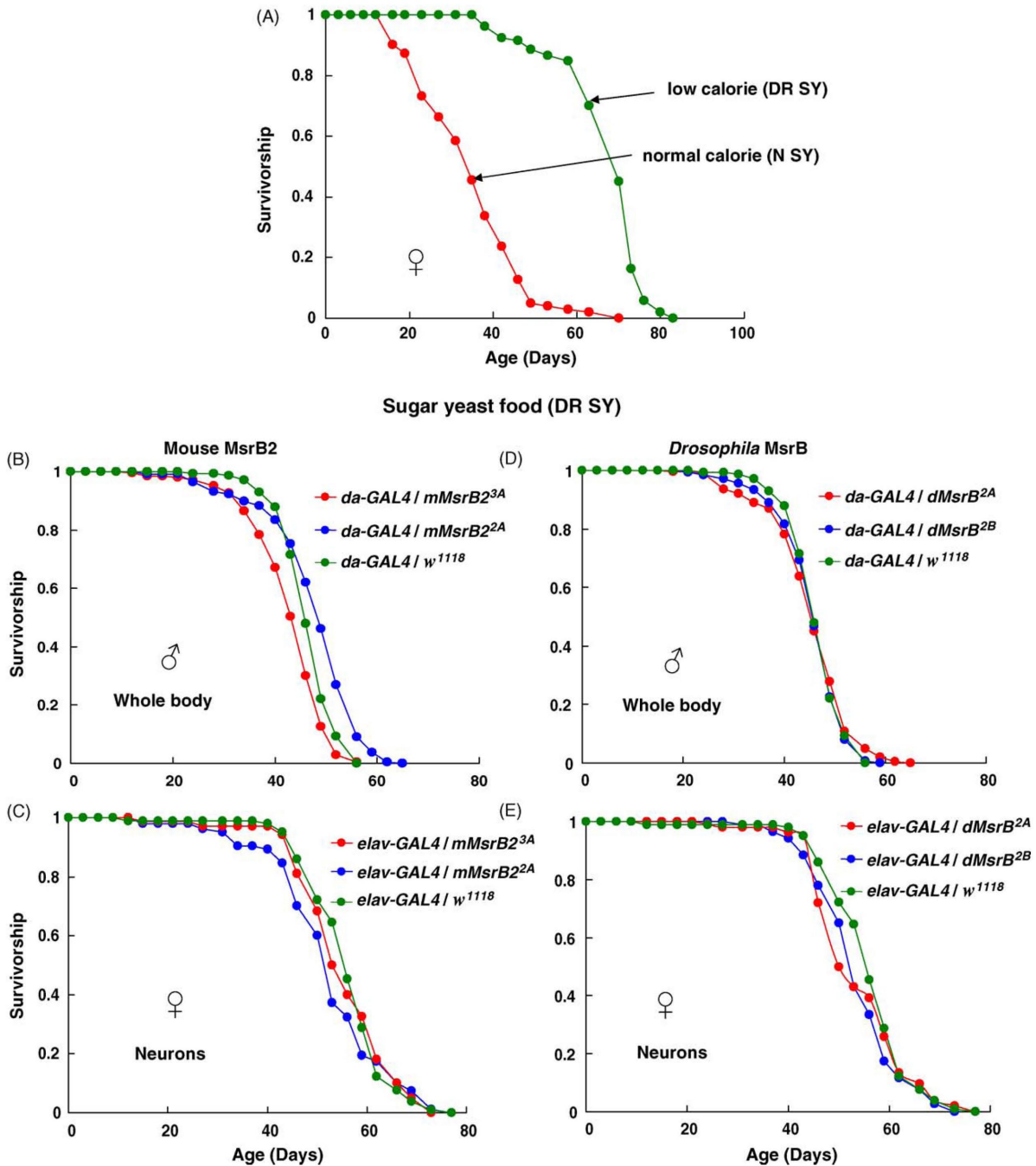
**Figure 5.** Survivorship curves of mouse MsrB2- and *Drosophila* MsrB-overexpressing flies on sugar yeast food. (**A**) Survivorship curves of control flies (*da-GAL4/w^{1118}*) on normal and dietary restriction sugar yeast food (N SY and DR SY, respectively). (**B–E**) Survivorship of flies overexpressing mouse MsrB2 (**B** and **C**) and *Drosophila* MsrB (**D** and **E**) on DR SY food. Overexpression of MsrBs in whole body (**B** and **D**) and nervous system (**C** and **E**) was driven by *da-GAL4* and *elav-GAL4* activator lines, respectively. Males and females were kept together (**A**, **C**, and **E**) or separate (**B** and **D**) during the lifespan study. 210 males were tested for each survivorship curve in panels B and D, and 105 animals for all other curves. Genotypes and sexes are marked on the plots. All trials were performed concurrently.

Ectopic expression of mouse MsrB2 in neurons by *elav-GAL4* driver had not revealed any effect of MsrB2 expression on lifespan in comparison with the *GAL4*-activator line control, *elav-GAL4/w^{1118}* (Figure 3D and H; Table 1, column 5). No statistically significant difference in survivor curves was observed (Table 1, column 5). Survivor curves of two other control lines, which do not express MsrB2 (*w^{1118}/mMsrB2^{3A}* and *w^{1118}/mMsrB2^{2A}*), were also obtained in a parallel experiment. Female responder line controls had the mean lifespans that were similar to those of the tested lines (Table 1, column 8). Consequently, we conclude

that neither ubiquitous nor ectopic expression of mitochondrial mouse MsrB2 in flies had the dramatic and consistent effect on lifespan on corn meal food.

*3.3. Overexpression of Drosophila MsrB has no effect on lifespan on corn meal food*

Similar results were obtained with flies overexpressing *Drosophila* MsrB (Figure 4 and Table 2, columns 5 and 8). Variations in mean lifespan (reduction up to 20%) were observed between

MsrB-overexpressing flies and their controls for both ubiquitous drivers (Figure 4A, B, E, F and Table 2). The most significant decrease in the mean lifespan in comparison with *GAL4*-activator line control was found for crosses *da-GAL4*/dMsrB2A and *da-GAL4*/*dMsrB^{2B}*. Females with respective genotype had 14% and 8% reduction in the mean lifespan, and males had 20% and 6% reduction in the mean lifespan (Figure 4B and F and Table 2, column 5). However, this effect was not seen with female progeny of a cross between *UAS-dMsrBs* lines and another ubiquitous driver *tubP-GAL4* (compare Figure 4A and B; Table 2, column 5). In addition, when more *da-GAL4*/*dMsrBs* males were studied on DR SY food (210 males instead of 105), no changes in mean lifespan of the experimental and the driver line control flies were found (Figure 5D; Table 3). The survivor curves of animals overexpressing *Drosophila* MsrB in fatbody as well as the mean lifespans were not changed significantly when compared with *fatbody-GAL4*/*w^{1118}* control (Figure 4C and G; Table 2, column 5). As in the case with expression of mouse MsrB2, responder line controls for *fatbody-GAL4*/*dMsrB* lines showed statistically significant (*p* < 0.0001) difference with experimental flies (Table 2, column 8). No dramatic effect of MsrB expression in the nervous system in comparison with driver line and responder line controls was detected (Figure 4D and H, Table 2, columns 5 and 8). In conclusion, strong MsrB overexpression in the nervous system, fatbody, or whole body of flies had no dramatic and consistent lifespan-prolonging effect on corn meal food.

### 3.4. Overexpression of MsrBs has no effect on lifespan on sugar yeast food

A link between overexpression of host MsrB in yeast and extended lifespan under caloric restriction was previously reported (Koc *et al.*, 2004). Under calorie restriction conditions, MsrB-overexpressing cells divided twice as many times (lifespan was assessed by replicative assays of yeast aging) (Koc *et al.*, 2004). Our initial purpose was to examine whether or not dietary restriction (DR) influences the lifespan of MsrB transgenic flies. It was demonstrated (Chapman and Partridge, 1996; Mair *et al.*, 2005; Kabil *et al.*, 2007) that sugar yeast diet (SY) regulated lifespan of flies in response to dietary dilution of sugar, but yeast dilution had a stronger effect on lifespan (Mair *et al.*, 2005). Dietary dilution in two fold provided up to 50% increase in the median lifespan for Da-

homey females (the effect was less pronounced for males) (Mair *et al.*, 2005; Chapman and Partridge, 1996). To confirm that dietary restriction works for our flies, the diet containing twice less sugar and yeast (DR SY food, Section 2.3)in comparison with normal sugar yeast food (N SY) was tested for *da-GAL4*/*w^{1118}* female flies. As expected, these females had 52% increase in the mean lifespan ($\chi^2$ = 187.7, *p* < 0.0001; Table 1, column 5 and Figure 5A). However, the mean lifespan of these flies on N SY food was significantly lower than that on corn meal food tested previously (36.3 days on N SY diet against 65.5 days on corn meal diet). The mean lifespan of tested females on DR SY diet (69.2 days) was comparable to that on corn meal food. We have not compared the nutritional composition and caloric content of corn meal diet and DR SY diet by analytical methods, but the lifespan showed that both foods had approximately the same calorie content. This finding led us to an additional test of MsrB-expressing flies on the other diet; however, even if we kept designation of this diet (DR SY) as accepted in the literature, this diet could not be considered as dietary restriction diet in comparison with corn meal food.

Overexpression of mouse MsrB2 or host MsrB in the nervous system (Figure 5C and E; Table 3) or the whole body (Figure 5B and D; Table 3) had no significant effect on lifespan on DR SY food. Slight reduction in the mean lifespan of *elav-GAL4*/*mMsrB2^{2A}* females (11%, $\chi^2$ = 41.25, *p* < 0.0001; Table 3, column 5) was not observed on previously tested corn meal food (compare Figure 3D and Figure 5C) and therefore appeared not to be the result of mouse MsrB2 expression. Again, the responder control lines showed a statistically significant difference (*p* < 0.0001) in mean lifespan (data not shown). Differences in metabolic pathways, MsrB expression levels, and MsrB localization between flies and yeast could possibly explain different outcomes of MsrB expression in these organisms.

### 3.5. Methionine/methionine sulfoxide content of MsrBs-overexpressing flies

Methionine sulfoxide content in proteins is considered as a measure of oxidative damage in cell (Stadtman *et al.*, 2005), but for *Drosophila* there are no direct experimental data confirming this observation. To compare experimental and control flies, we prepared 6-and 17-day-old *da-GAL4*/*dMsrB* and *da-GAL4*/*mMsrB2*

**Table 3.** Statistical analysis of survivor curves presented in Figure 5 for flies overexpressing mouse MsrB2 and *Drosophila* MsrB and their controls.

| 1. Panel in Figure 5 | 2. Genotype | 3. Sex | 4. Mean (days) | 5. % vs. driver/*w^{1118}* | 6. Max (days) | 7. % vs. driver/*w^{1118}* | 8. $\chi^2$ | 9. $p > \chi^2$ |
|---|---|---|---|---|---|---|---|---|
| A | *da-GAL4*/*w^{1118}* N SY | F | 36.3 | −52 | 64.4 | −15 | 187.7 | <0.0001 |
| A | *da-GAL4*/*w^{1118}* DR SY | F | 69.2 | | 76.1 | | | |
| B | *da-GAL4*/*mMsrB2^{3A}* | M | 44.7 | −5 | 51.8 | 0 | 12.7 | 0.0004 |
| B | *da-GAL4*/*mMsrB2^{2A}* | M | 49.7 | +6 | 59.9 | +16 | 35.99 | <0.0001 |
| B | *da-GAL4*/*w^{1118}* | M | 46.9 | | 51.8 | | | |
| D | *da-GAL4*/*dMsrB^{2A}* | M | 47.5 | −1 | 59.9 | +16 | 3.12 | 0.08 |
| D | *da-GAL4*/*dMsrB^{2B}* | M | 46.2 | −1 | 54.5 | +5 | 0.75 | 0.39 |
| D | *da-GAL4*/*w^{1118}* | M | 46.9 | | 51.8 | | | |
| C | *elav-GAL4*/*mMsrB2^{3A}* | F | 52.6 | −9 | 70.7 | 0 | 3.64 | 0.06 |
| C | *elav-GAL4*/*mMsrB2^{2A}* | F | 51.4 | −11 | 67.9 | −4 | 41.25 | <0.0001 |
| C | *elav-GAL4*/*w^{1118}* | F | 57.8 | | 70.7 | | | |
| E | *elav-GAL4*/*dMsrB^{2A}* | F | 54.2 | −6 | 67.1 | −5 | 49.7 | 0.02 |
| E | *elav-GAL4*/*dMsrB^{2B}* | F | 55.0 | −5 | 70.7 | 0 | 4.7 | 0.26 |
| E | *elav-GAL4*/*w^{1118}* | F | 57.8 | | 70.7 | | | |

Column 1 indicates panel in Figure 5. The genotypes and gender (female, F; males, M) of flies are shown in columns 2 and 3, respectively. The mean lifespan is indicated in column 4 and percent change in the mean lifespan compared with their genetically matched controls (*GAL4-driver*/*w^{1118}*) is displayed in column 5. Maximum lifespan and percent change of the maximum lifespan are shown in columns 6 and 7, respectively. Comparison of two types (transgenic and control) of survivor curves (three curves in each group, 210 flies for panels B and D and 105 flies for other panels) was performed with SAS software (see Section 2.12). Statistics of non-parametrical log rank test ($\chi^2$) is shown in column 8; *p*-value in column 9.

flies, their corresponding driver line control *da-GAL4/w[1118]*, and responder line controls *w[1118]/dMsrB* and *w[1118]/mMsrB2*. Proteins in homogenate were precipitated by trichloroacetic acid and methionine content in a protein-free form was measured (Figure 6A). The pellet was subjected to hydrolysis with ptoluenesulfonic acid followed by analysis of Met and MetO content (Figure 6B). Significant reduction of free Met during aging reduction of free methionine during aging (up to 35% decline, Figure 6A) was observed for both experimental and control flies. Our data are in agreement with the previously reported age-related decline of Met content (Rebrin *et al.*, 2004). Remarkably, 17-day-old flies expressing *Drosophila* MsrB showed approximately 30% increase in Met content in comparison with both control flies. Mouse MsrB2 expressing flies had the same content of free Met as control flies (within error).

Analysis of methionine sulfoxide content in hydrolyzed proteins revealed the presence of both diastereoisomers in equimolar ratio ([Met-RO]/[Met-SO] = 1.11 ± 0.04). We explain this finding by racemization of methionine sulfoxide during hydrolysis that was performed at 110 °C for 22 h. However, our experimental flies had a decreased methionine sulfoxide/methionine ratio in comparison with both types of controls (Figure 6B) suggesting the expression of functional MsrB. We observed an almost 40% increase in the MetO/Met ratio during aging for the flies with the same genotype. However, it should be noted that MetO in proteins still stays at low levels even in 17-day-old flies. The MetO/Met ratio in hydrolyzed protein samples was almost 50% higher in 17-day-old flies than that in 6-day-old flies with the same genotype (Figure 6B). This finding is consistent with the idea that Met residues in proteins become more oxidized during aging.

### 3.6. MsrB-expressing flies have the same stress resistance as controls

*Drosophila* longevity, when influenced by genetic interventions, DR, and selection for lines with postponed senescence, often positively correlates with enhanced resistance of animals to different stresses (e.g., oxidative stress, starvation, desiccation, heat) (Parkes *et al.*, 1998; Ruan *et al.*, 2002; Lin *et al.*, 1998; Orr *et al.*, 2005; Mockett *et al.*, 2001; Arking *et al.*, 1991; Vermeulen and Loeschcke, 2007). However, molecular mechanisms for such correlation remain elusive and there were several examples when oxidative stress response was not involved directly in aging (Bayne *et al.*, 2005; Vermeulen and Loeschcke, 2007). For example, the lifespan of mitochondrial Mn-SOD/catalase transgenic flies was decreased up to 43%, but their resistance to oxidative stress caused by $H_2O_2$ treatment or exposure to 100% oxygen was elevated (Bayne *et al.*, 2005). Similarly, although MsrB expression had no influence on lifespan, this is an important antioxidant enzyme and its protein repair function could presumably be uncoupled from its influence on lifespan. Thus, we examined resistance of transgenic MsrB flies to oxidative stress induced by dietary paraquat, which upon intake generates superoxide radicals (Arking *et al.*, 1991) that oxidize Met to MetO (Ruan *et al.*, 2002).

17-day-old males overexpressing MsrBs in the nervous system were fed with 5% sucrose and 20 mM paraquat (Figure 7A) or were starved for 6 h to minimize the variations in paraquat intake and then fed with 5% sucrose and 30 mM paraquat (Figure 7B). No statistically significant difference in survivorship between experimental (*elav-GAL4/mMsrB2[2A]* and *elav-GAL4/dMsrB[2A]*) and control (*elav-GAL4/w[1118]*) flies were observed on 20 mM paraquat (Figure 7A). The 17 h exposure to 30 mM paraquat killed 80% of experimental (*mMsrB2/elav-GAL4* and *dMsrB/elav-GAL4*) and control males (*w[1118]/UAS-MsrBs*); only 50% of other control flies (*elav-GAL4/w[1118]*) were dead at these conditions (Figure 7B). These data demonstrate no protective effect of MsrB overexpression against oxidative stress induced by paraquat. All

control animals (elavGAL4/*w[1118]*) fed with the diet containing 5% sucrose in the absence of paraquat were alive after 6 days.

Although we have shown that both types of MsrB-expressing flies have low concentration of MetO in protein-bound form and *Drosophila* MsrB-expressing flies have a lower decline of free Met, the protective affect of Met was not detected in assay with paraquat. The exact mechanism of paraquat *in vivo* toxicity is not known, even if it is evident that paraquat generates superoxide radicals. Probably, maintaining higher Met level in MsrB-overexpressing flies is not sufficient to overcome consequences of superoxide radical formation upon paraquat-induced oxidative stress.

As in the case of MsrA-overexpressing flies, MsrB overexpression did not protect against starvation (Figure 7C and D). The median lifespan was approximately 60 h for MsrBs-expressing and control flies. The observation of no influence of MsrA and MsrB overexpression on starvation seems logical, as both types of enzymes are unlikely to have direct influence on metabolic pathways linked to resistance against starvation (*e.g.,* increase in lipid content).

### 3.7. MsrB-expressing flies show no changes in reproduction and physical activity compared to controls

Aging in many species is associated with decline in physical activity and fertility. The long-lived MsrA-overexpressing flies with transgene expression predominantly in the nervous system
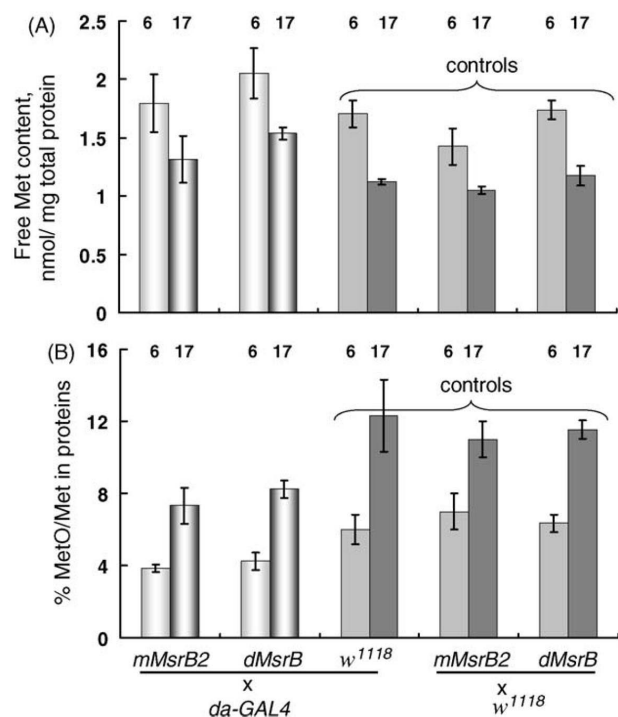


**Figure 6.** Analysis of Met and MetO content in 6- and 17-day-old flies. (A) Met content of the protein-free fraction of fruit fly homogenates. Met content is given in nmoles/mg of protein in homogenates. (B) Ratio of MetO to Met in a protein fraction. Flies (6- and 17-day-old) overexpressing mouse MsrB2 or *Drosophila* MsrB in whole body (*da-GAL4/dMsrB* and *da-GAL4/ mMsrB2*), their corresponding driver line control *daGAL4/w[1118]*, and responder line controls *w[1118]/dMsrB* and *w[1118]/mMsrB* were homogenized and proteins were precipitated. The supernatant was used to determine protein-free Met (A), and the pellet was used for protein hydrolysis and analysis of MetO and Met content (B). 70 females were used for each sample. Two independent preparations of each transgenic fly line as well as control flies were analyzed. Measurements for each sample were done in triplicate. All data are reported as means ± S.D. The age of flies (in days) is shown for each column. Genotypes are indicated.

had a markedly delayed age-related decline in general activity and reproduction (Ruan *et al.*, 2002). In contrast, we found that MsrBexpressing flies had similar physical characteristics and showed no changes in the number of developed pupa.

Timing of development of transgenic flies expressing MsrB from eggs to hatching was the same as in the parental and heterozygous control flies that did not express *MsrB* transgenes (9–10 days at 25 °C). Qualitative observation of flight, courtship, and feeding behavior revealed no significant differences between transgenic MsrB flies and their controls in early life. The average body weight of 10-day-old males expressing MsrBs in the nervous system (*elav-GAL4/mMsrB2$^{3A}$* and *elav-GAL4/dMsrB$^{2A}$*)was indistinguishable from the body weight of controls (*elav-GAL4/ w$^{1118}$* and *w$^{1118}$/UAS-MsrBs*); animals weighted 0.89 ± 0.02 mg (Supplementary Table S1). The average locomotor activity (mean ± S.E.) of 8-day-old males expressing MsrBs in whole body was similar with controls (Supplementary Figure S1).

In a further experiment characterizing overall reproductive vigor, we counted the number of pupa that developed from eggs laid by one female over 24 h (Figure 8). We used female animals expressing MsrBs in the nervous system (*elav-GAL/mMsrB2$^{3A}$* and *elav-GAL/dMsrB$^{2A}$*) and the offspring of a cross between *elav-GAL4* and white flies (*elav-GAL4/w$^{1118}$*) as a control. Pupal progeny developed into adult flies in more than 95% cases. No changes in the number of developed pupa were observed (Figure 8).

## 4. Discussion

Expression of GFP-fused bovine MsrA in the adult nervous system of fruit flies resulted in a greatly increased median lifespan (~70%) (Ruan *et al.*, 2002). To explain this finding, it was proposed that *Drosophila* has high levels of ROS and/or low levels of endogenous MsrA in the adult neurons, and that MsrA function might be a factor that limits the lifespan of flies (Ruan *et al.*, 2002). Other studies reported the low level of Cu,Zn-SOD in adult nervous system of fruit flies (Klichko *et al.*, 1999); and overexpression of human Cu,Zn-SOD in adult motorneurons using *GAL4-UAS* system extended the mean lifespan of fruit flies up to 40% (Parkes *et al.*, 1998). An attempt to reproduce this result using *elav-GAL4* driver failed (Ruan *et al.*, 2002). Based on this observation and on a greater life-prolonging effect of MsrA overexpression (70% against published 40% for Cu,Zn-SOD), it was suggested that the antioxidant mechanism involving MsrA-dependent reduction of oxidized Met residues in cellular proteins may be more robust than the SOD/catalase system with regard to lifespan (Ruan *et al.*, 2002). Since MsrA is specific for Met-SO, whereas Met-RO is reduced by MsrB, the analysis of fruit flies overexpressing MsrB provided an opportunity to better understand the unique role of MsrA and/or MetO reduction in influencing animal lifespan. In addition, since MsrA and MsrB catalyze the same reaction (but with different diastereomers of MetO), one would expect that the lifespan extension
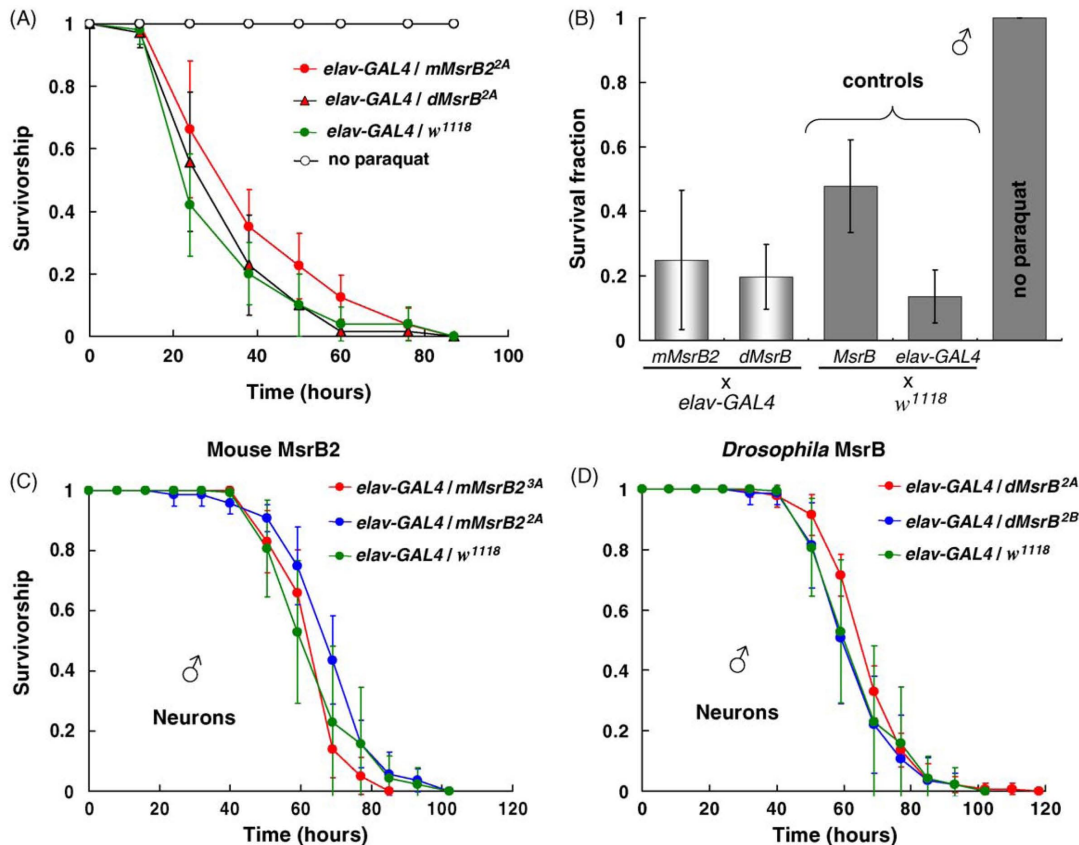


**Figure 7.** Stress resistance of mouse MsrB2- and *Drosophila* MsrB-overexpressing flies. 20-day-old male flies overexpressing MsrBs in the nervous system were examined for resistance to oxidative stress and starvation. Newly eclosed flies were mated, sex-segregated and maintained on corn meal food before the day of the tests. (**A** and **B**) Oxidative stress was induced by 20 mM (**A**) or 30 mM (**B**) dietary paraquat. (**A**) 80 animals were tested for each genotype (shown in the legend). Flies were transferred to test vials (5% sucrose, 20 mM paraquat) without preliminary starvation and dead animals were counted at fixed time points. 80 control flies (*elav-GAL4/w$^{1118}$*) were kept on 5% sucrose without paraquat (marked as ''no paraquat'' in the legend). (**B**) 360 animals were tested for flies overexpressing mouse MsrB2 (*elav-GAL4/mMsrB2*), *Drosophila* MsrB (*elav-GAL4/dMsrB*), and control flies (*MsrBs/w$^{1118}$*), except for control *elav-GAL4/w$^{1118}$* where 180 males were studied. Genotypes are shown in the *Y*-axis legend; 180 flies with genotype *elav-GAL4/w$^{1118}$* were kept on food without paraquat and noted in Figure 7B as ''no paraquat.'' All flies were starved for 6 h before the test and then fed with or without 30 mM paraquat and 5% sucrose for 17 h; animals alive were counted and normalized to the total number of animals used for each genotype. (**C** and **D**) Resistance to starvation was studied with flies expressing mouse MsrB2 (**C**) and *Drosophila* MsrB (**D**) in the nervous system. Flies were maintained in vials with 1% agar; dead animals were counted every 8 h. Total number of males tested for each genotype was 140. Genotypes are shown in the legends.
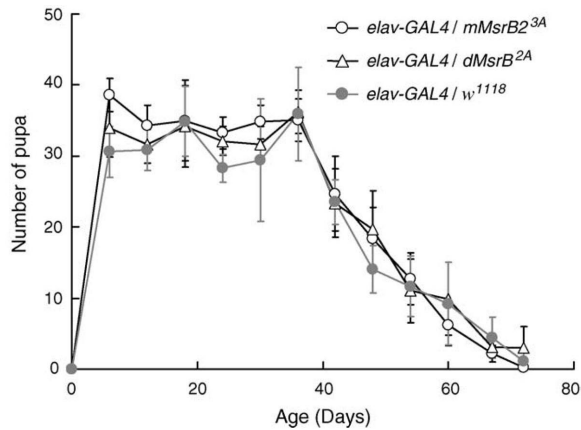
**Figure 8.** Age-associated changes in pupa production. Five females were kept with five males in a vial with corn meal food. Changes in pupa production were determined from counts of pupa developed from eggs laid by five female in 24 h; the developed pupa was counted after eclosion. The $Y$-axis represents the number of pupa produced by one female in one vial. 10 replica of each vial were tested. Offsprings of crosses used (*elav-GAL4/mMsrB2³ᴬ*, *elav-GAL4/dMsrB²ᴬ*, or *elav-GAL4/w¹¹¹⁸*) are shown in the legend.

due to MsrA overexpression would be reproduced in flies over-expressing MsrB.

However, we found that overexpression of either mouse mitochondrial MsrB2 or *Drosophila* cytosolic MsrB in neurons of fruit flies had no dramatic and consistent lifespan-prolonging influence in these animals. Our transgenic flies expressed high levels of catalytically active enzymes, and these animals were examined with four different *GAL4*-activator lines and on two different diets. None of the conditions showed clear lifespan extension. Since both MsrA and MsrB catalyze the reduction of MetO residues in proteins, it is puzzling how one enzyme could provide such a dramatic effect on lifespan by reducing half of MetO residues, and the other enzyme that reduces the other half of MetO residues could be ineffective under all conditions tested.

Apparently, MsrA has broader substrate specificity than MsrB. Besides Met-SO residues in proteins, this enzyme has activity for free Met-SO (Lee *et al.*, 2008). In yeast, MsrA transcriptional regulation clusters it with genes involved in methionine metabolism and sulfur acquisition, whereas MsrB does not show this expression pattern (Koc *et al.*, 2004). Moreover, MsrA deletion has a more significant effect on the growth of cells on free MetO than the deletion of MsrB (Kryukov *et al.*, 2002). We hypothesize that the role of MsrA in sulfur or methylation pathways is responsible for the observed role of this enzyme on lifespan extension and methionine metabolism.

Methionine serves as a source of sulfur for cysteine synthesis via the transsulfuration pathway (Stipanuk, 2004). Cysteine may influence cellular redox homeostasis and serves as an intermediate in GSH biosynthesis. The GSH/GSSG ratio determines an intracellular redox potential as GSH is a major antioxidant in the cell. In *Drosophila*, the GSH/GSSG ratio is decreased during aging (Rebrin *et al.*, 2004; Mockett *et al.*, 1999). Addition of GSH to food significantly increased resistance of fruit flies to oxidative stress induced by paraquat (Bonilla *et al.*, 2006). The importance of glutathione balance for *Drosophila* aging was previously shown in flies overexpressing glutamate-cysteine ligase, which catalyzes a rate-limiting reaction in GSH biosynthesis. These flies, expressing transgene in the nervous system, had extended mean and maximum lifespans up to 50% (Orr *et al.*, 2005).

Overexpression of MsrA in flies may influence GSH synthesis or methylation pathways by providing cells with an additional source of free Met. Additionally, *Drosophila* lacks glutathione re-

ductase and GSSG reduction is carried out by the thioredoxin reductase/thioredoxin system (Kanzok *et al.*, 2001)thatmay be affected during aging. Thus, it is possible that an altered metabolism of sulfur-containing compounds in MsrA-overexpressing flies may be one of the reasons for their delayed aging. Recent data on the role of MsrA in the aging process appear to support this idea. It was found that MsrA and a dietary supplementation of S-methyl-L-cysteine prevent Parkinson's like symptoms in a fruit fly model (Wassef *et al.*, 2007). In this model, S-methyl-L-cysteine could enhance MsrA function, presumably through its cyclic oxidation by ROS and reduction by MsrA. MsrB is not an efficient reductant for this oxidized substrate. Importance of free Met-RO reduction was demonstrated in human liver carcinoma SK-Hep1 cells expressing yeast free Met-RO reductase (Lee *et al.*, 2008). These cells had an increased resistance against oxidative stress induced by hydrogen peroxide as they had an additional source of Met derived from Met-RO.

An indirect proof of an important role of MsrA in processes other than reduction of Met-SO residues in proteins came from our experiment on the resistance of MsrB-overexpressing flies to oxidative stress induced by paraquat. It was previously shown (Ruan *et al.*, 2002) that long-lived flies expressing MsrA in the nervous system had an enhanced resistance to oxidative stress caused by dietary paraquat. Based on these data, the authors suggested that the lifespan extension is offered by the antioxidant action of MsrA (Ruan *et al.*, 2002). We used the same system for MsrB overexpression and the GAL4-driver with the same genotype as that in the study that examined MsrA overexpression. Our data demonstrated no protective effect of MsrBs overexpression in the fruit fly nervous system against paraquat-generated oxidative stress. We propose that an altered metabolism of sulfur-containing compounds in MsrA-overexpressing flies could provide an additional protection to oxidative stress in comparison with metabolism of MsrBs-overexpressing flies. In any case, our data suggest that further studies are needed to explain how MsrA increases resistance to oxidative stress.

MsrA expression in whole body (Ruan *et al.*, 2002) offered an approximately 40% increase in median lifespan in comparison with homozygous *GAL4*-activator line *Ubi-GAL4* and 80% increase in comparison with homozygous *UAS-MsrA* responder line. It is possible that the extension effect was caused by a combination of different genetic backgrounds from *UAS-MsrA* line and *Ubi-GAL4*. In our study, we used heterozygous lines as controls that combined genetic backgrounds of lines carrying *GAL4* and *UAS* transgenes (*GAL4-activator/w¹¹¹⁸* and *UAS-MsrBs/w¹¹¹⁸*). In the experiments with whole body MsrBs expression, two different drivers (*tubP-* and *da-GAL4*) and two different enzymes (mouse MsrB2 and *Drosophila* MsrB) were studied. No lifespan extension was observed at any conditions tested.

Overall, our study highlights an important difference in the aging process between two enzymes that catalyze the same reaction, MetO reduction, with different stereospecificity. Apparently, MsrA is unique in providing a remarkable lifespan extension, whereas MsrB is more in line with most other antioxidant enzymes in that its overexpression does not significantly influence lifespan in the fruit fly model. Since MsrA can also reduce free Met-SO, it would be important to examine the contribution of this process to *Drosophila* aging in future studies.

### Acknowledgments

## Appendix A. Supplementary data

Supplementary data can be found online (for subscribers to *Mechanisms of Ageing and Development*) at doi:10.1016/j.mad.2009.04.003.

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