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ORIGINAL PAPER

Age-dependent expression profiles of two adaptogenic systems and thermotolerance in *Drosophila melanogaster*

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Abstract

Here, we monitored the expression of three genes (*hsp70*, *hsp22*, and *hsf1*) involved in heat shock response in *Drosophila melanogaster* in males and females of different age. Also, we investigated age- and sex-dependent expression of three major genes participating in the production of hydrogen sulfide (H_2S) (*cse*, *cbs*, and *mst*), implicated in stress resistance and aging. In addition to the control strain, we monitored the expression of all of these genes in a *cbs* knockout strain (*cbs*−/−) generated using the CRISPR technique. The tested strains differ in the induction capacities of the studied genes. Relative to the control strain, under normal conditions, the *cbs*−/− strain expresses all of the studied genes more abundantly, especially *hsp22*. In the control strain, aging leads to a dramatic increase in *hsp22* synthesis, whereas in the *cbs*−/− strain, *hsp22* induction is not pronounced. Furthermore, in 30-day-old *cbs*−/− flies, the constitutive expression of *hsp70* and *mst* is decreased. Surprisingly, in the *cbs*−/− strain, we detected an upregulation of *hsf1* transcription in the 30-day-old females. After heat shock in the control strain, *hsp*70 and *hsp22* induction decreased with age in males and *hsp22* decreased in females, while in the *cbs*−/− strain, a pronounced drop in the induction capacity of both *hsp* genes was seen in 30-day-old males and females. However, in most cases, the expression levels of *hsf1* and H_2S-producing genes do not exhibit pronounced changes depending on sex, age, or heat shock. Flies of control and *cbs*−/− strain exhibited strong reduction in basal thermotolerance with age. Our data suggest a cross-talk between the two studied ancient and universal adaptive systems.

Keywords *D. melanogaster* · *hsp* genes · Hydrogen sulfide · *cbs* deletion · Aging · Heat shock

Introduction

Multiple studies have reported a decrease in the adaptive response with age in various organisms (Pomatto and Davies 2017). In the process of normal aging, various degenerative changes occur at the molecular and tissue levels in all organisms ranging from flies to humans (Iliadi et al. 2012). These changes are mostly due to the accumulation of inactive or denatured proteins, including enzymes and structural proteins (Lithgow 2006; Morimoto 2008). The accumulation of abnormal or denatured proteins in the cells of aged organisms results in the induction of the heat shock proteins (HSPs), including the major stress protein Hsp70 (Tower 2009). Due to its chaperone properties, Hsp70 binds to partially unfolded or misfolded proteins and either assists in their refolding or directs them to a safe disposal site (Mayer and Bukau 2005; Duncan et al. 2015). However, the activity of endogenous Hsp70 appears to be diminished and insufficient in various neurodegenerative disorders and in aged individuals (Guzhova and Margulis 2006; Pratt et al. 2015). It is also known that in humans, Hsp70 levels and production in response to stress declines in neurons in the course of aging (Latchman 2004; Calderwood et al. 2009; Leak 2014), likely with negative implications for the ability of brains in aged individuals to retain normal function. It is known that HSPs are required for longevity and many long-lived species (among mammals and birds) have a higher constitutive expression of Hsp70 (Singh et al. 2007; Salway et al. 2011). Furthermore, overexpression of the genes encoding HSPs

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increases longevity in Drosophila, nematodes, and vertebrates (Morrow et al. 2004; Kurapati et al. 2000; Walker et al. 2001; Tower 2011). Hsp70 has been shown to diminish oxidative stress damage, prevent denatured or damaged proteins aggregation, and inhibit pro-inflammatory signaling in a wide spectrum of organisms (Broome et al. 2006; Kalmar and Greensmith 2009; Yenari et al. 2005; Liberek et al. 2008).

Among low molecular weight Hsps, Hsp22 plays a special role in the aging process. Hsp22 was shown to be the most upregulated protein especially in the fly heads during aging (King and Tower 1999; Landis et al. 2012). In the genetically developed fly strains with increased longevity, young flies exhibited significantly higher levels of Hsp22 expression compared to short-lived flies (Kurapati et al. 2000). Also, these flies are more resistant to heat stress (Kurapati et al. 2000). In the absence of Hsp22 expression, the lifespan and resistance to stress declines (Morrow et al. 2004).

Herein, we report the results of our studies using *Drosophila melanogaster*, describing how major genes of the heat shock system, including HSP-encoding genes and *hsf1*, are expressed under normal condition and after heat shock challenge in young and old flies, as well as monitor the thermotolerance of flies of different age and sex.

Research on the aging process of distantly related model organisms such as yeast, nematodes, mice, and *Drosophila* has led to the idea that regulation of lifespan and the process of aging are modulated by several common and often interacting ancient adaptogenic systems (Calderwood et al. 2009). Thus, the discovery that exogenous hydrogen sulfide (H_2S) might prolong the lifespan of nematodes, and other model organisms suggested its important role in health and disease, as well as in the aging process (Qabazard et al. 2014). It was also demonstrated that in 50- to 80-year-old human subjects, an age-dependent decline of H_2S level in plasma usually takes place (Perridon et al. 2016). It is known that in higher organisms, H_2S is generated by the three major H_2S-producing genes: cytoplasmic cystathionine β-synthase (*cbs*), cystathionine γ-lyase (*cse*), and mitochondrial gene 3-mercaptopyruvate sulfurtransferase (*mst*) (Kimura 2014). Hydrogen sulfide is now recognized as a biological mediator with various roles, including cytoprotection, anti-inflammation, and oxygen sensing (Paul and Snyder 2015; Stein and Bailey 2013), which in several important aspects coincides or overlap with the described above activities of the HSP system (Calderwood et al. 2009). However, the precise relationship and possible interaction between these two vital adaptogenic systems in the process of normal aging and after heat shock challenge are still largely unknown. Notably, the scattered data obtained mostly in plants suggest the interaction of these two very ancient and universal systems and support their adaptive homeostasis capacity (Li et al. 2013; Min et al. 2016). Here, our data support the notion of an interaction between these two systems in the course of aging and in response to heat shock in *D. melanogaster*.

Materials and methods

Drosophila stocks and maintenance

In our study, we worked with flies of the w^{1118} strain obtained from the Bloomington *Drosophila* Stock Center (stock number #6326) and transgenic *cbs*−/− strains developed in our laboratory. Flies (separately males and females) were maintained at 22 °C on standard yeast, sugar, and agar medium in 40-ml vials at a density of 20 flies per vial throughout the experiment. All flies were synchronized by age: enclosing individuals were collected daily, transferred to new vials with medium, and then aged appropriately; we considered 2-day-old flies as "young" and 30-day-old flies as "old."

The development of flies with deletion of *cbs* gene using CRISPR/CAS9 technique

cbs gene deletion, two plasmids were generated: the pAc-dual-sgRNA-cbs (carrying dual spacers against *cbs*) and pSK-mCherry-cbs integration plasmids. For the generation of the pAc-dual-sgRNA plasmid, the *cbs* gene sequence (CG1753) was obtained from Flybase (Thurmond et al. 2019). Target regions in the *cbs* gene were chosen as having low nucleosome occupancy according to the data described in Mieczkowski et al. (2016). Sequences of the target regions were amplified with the pairs of primers cbs_CG1753-5′-flank-genome-check-F with cbs_CG1753-5′-flank-XhoI-R and cbs_CG1753-3′-flank-NotI-F with cbs_CG1753-5′-flank-genome-check-R, followed by Sanger sequencing. The resulting sequences were used to design spacers for the CRISPR/SpyCas9 system using CRISPOR (http://crispor.tefor.net/ (Haeussler et al. 2016). High-ranked spacers having the least possible off-targets were chosen for further cloning into the pAc-dual-sgRNA plasmid described in Zolotarev et al. (2019). A fragment including the full first sgRNA, U6-1 terminator, U6-2 promoter, and spacer for the second sgRNA was amplified using a pair of primers (cbs_CG1753-5′-flank-sgRNA-F and cbs_CG1753-3′-flank-sgRNA-R) using the pAc-dual-sgRNA plasmid as a template. The PCR product was cloned into the BbsI-cut pAc-dual-sgRNA plasmid using Gibbson assembly (Gibson et al. 2009). The correctness of the dual-sgRNA construct was verified by Sanger sequencing. An integration plasmid for the *cbs* gene deletion carrying mCherry as a reporter was constructed as follows. The upstream *cbs* fragment was amplified by overlap PCR with XbaI site mutated using primers cbs_CG1753-5′-flank-XbaI-F, cbs_CG1753-5′-flank-XbaI-mut-F, cbs_CG1753-5′-

flank-XbaI-mut-R, and cbs_CG1753-5′-flank-XhoI-R, and cloned into a pSK-mCherry integration vector (Zolotarev et al. 2019) at the XbaI and XhoI sites. The downstream section of the *cbs* gene was amplified using primers cbs_CG1753-3′-flank-NotI-F and cbs_CG1753-3′-flank-SacI-R and cloned into the pSK-mCherry integration vector at the NotI and SacI sites. The correctness of the inserts was verified by Sanger sequencing. The primers used in CRISPR experiments are listed in Table S1.

Embryo injection

Preblastoderm embryos of *Drosophila melanogaster* line 58492 with genotype y1 M{Act5C-Cas9.P.RFP-}ZH-2A w1118 DNAlig4169 from the Bloomington center were used for injection; as described in Zhang et al. (2014), we used a 500 ng/μl mixture of two plasmids, SgRNA coding plasmid pAc-dual-sgRNA-cbs and homologous pSK-mCherry-cbs integration plasmid (1:5). A total of 220 embryos were injected. One hundred twenty adults that developed from injected embryos were outcrossed to laboratory strain *yw* (df (1)w, yw67c23 (2)) and flies carrying the gene deletion were selected based on the expression of the mCherry gene under control of the Actin 5C promoter. Three independent homozygous viable lines were obtained. Deletion of the *cbs* gene was checked by PCR using primers cbs_CG1753-deletion-check-F and cbs_CG1753-deletion-check-R (Table S1) using genomic DNA, and qRT PCR studies were performed using RNA from the mutant lines with deletions.

Thermotolerance

To study the thermotolerance of young and old flies (2 or 30 days old, respectively), the standard protocol from our laboratory was used (Garbuz et al. 2002): appropriately aged male and female flies are separated and transferred into 50-ml polypropylene vials, and then placed into a preheated water bath. To study basal thermotolerance, the flies of the compared strains were incubated at 38 or 39 °C for 30 min; the proportion of flies that were able to walk 24 h after heat shock treatment was used as a measure of thermotolerance. Between 50 and 100 flies were used in each experiment, which was repeated at least three times.

Protein extraction and Western blot analysis

To measure Hsp70 levels, flies were frozen in liquid nitrogen, then briefly grinned with an ice-cold pestle and lysed in Laemmli buffer (Laemmli 1970). The concentration of proteins was normalized after their separation by SDS-PAGE and staining with Coomassie brilliant blue. Equal quantities of the material were applied to the gel. After sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, proteins were transferred to a nitrocellulose blotting membrane (Amersham Protran Supported 0.45-μm membrane) by a semi-dry technique, following the manufacturer's instructions. Hsp70 was detected using monoclonal antibody 7FB (dilution 1:500), which is specific for the inducible Hsp70 of Drosophila. Actin was detected with the mab 1501 anti-Actin antibody, clone 4C (Sigma-Aldrich). After incubation with secondary antibodies conjugated with horseradish peroxidase, immune complexes were detected on a ChemiDoc MP system (Bio-Rad, USA) using a reagent for chemiluminescent detection (Thermo Scientific SuperSignal West Pico Plus Chemiluminescent substrate) of Hsp70 after heat shock and actin as internal control. For detection Hsp70 in control conditions, we used the Thermo Scientific Super Signal West Femto Maximum Sensitivity substrate. The results were processed using ImageJ. The measured levels of Hsp70 in each sample were normalized to the amount of actin.

RNA extraction and quantitative real-time PCR

Procedures were identical to those described in Shilova et al. (2017). Briefly, total RNA was extracted from adult flies using guanidine isothiocyanate (RNAzol RT, Sigma-Aldrich, cat.# R4533) following the manufacture's protocol. One microgram of total RNA was used for cDNA synthesis with an MMLV RT kit (Evrogen, Moscow, Russia, cat.# SK021). All qRT-PCR reactions were conducted using the SYBR Green fluorescent dye (Evrogen, Russia, cat.# PK156S) in an ABI PRISM VR 7500 device (Applied Biosystems). The relative expression of studied genes was calculated based on the $\Delta\Delta Ct$ method (Schefe et al. 2006). Quantifications were normalized to the housekeeping gene rp49 (Ponton et al. 2011). All experiments were performed with three to five biological replicates and three experimental replicates. The primer sequences are given in Supplemental Table S2.

Statistical analysis

Thermotolerance experiments were analyzed using the V^2 test (χ^2 adjusted for sample size) and the Benjamini and Hochberg method adjusted for multiple comparisons (Benjamini and Hochberg 1995). Factorial ANOVA and Tukey's HSD test for post hoc correction of multiple comparisons (STATISTICA 10, StatSoft, Inc. 2011) was used to compare mRNA levels between the studied groups. P values ≤ 0.05 were considered statistically significant. The statistical method used in the analyses was the ANOVA test with multiple comparisons of Tukey or Games-Howell. When the parametric model (ANOVA) was not adequate, we used the Mann-Whitney test. All tests were performed with a reliability level of 95% ($\alpha = 0.05$). The statistical analyses were conducted using GraphPad Prism (version 5).

Results

Constitutive expression of adaptogenes in young and old w^{1118} strain flies

We have focused our attention on the study of age-associated changes of two ancient adaptive systems: heat shock response (HSR) and hydrogen sulfide (H_2S) production. Both of these systems were shown to exhibit clear roles in lifespan extension and stress resistance (Calderwood et al. 2009; Miller and Roth 2007). Therefore, by qRT-PCR and Western blotting, we investigated the transcription levels of three major genes responsible for H_2S production in Drosophila (*cbs*, *cse*, *mst*), as well as the major genes involved in HSR, including *hsp70*, *hsp22*, and the heat shock transcriptional factor *hsf1*. It is evident from Fig. 1a that in 2-day-old males of the w^{1118} strain, the expression level of mRNA of *hsp70* and *hsp22* is slightly higher than in same-aged females, while the expression levels of *hsf1* and *cbs* are relatively higher in females.

Interestingly, while the expression level of *hsp70* mRNA does not change with age, the level of small mitochondrial *hsp22* expression increases dramatically, especially in males (Fig. 1b). The expression of *hsf1* and major H_2S-producing genes (*cbs* and *cse*) was enhanced in the old flies of both sexes, while in the case of the *mst* gene expressed in mitochondria, we detected only a tendency for an increase in the course of aging (Fig. 1b). To analyze the content of Hsp70, we performed Western blotting analyses using total protein extracts from the whole flies (young and old, male and female). Surprisingly, in contrast to the qRT-PCR findings, under normal physiological temperature, Western blotting analyses detected a significant increase in Hsp70 level with increasing age in males and females, and this was more pronounced in females (Fig. 2). Therefore, since *hsp70* RNA levels were not enhanced with age in males and females, the observed age-dependent increase in Hsp70 protein levels is apparently due to post-transcriptional regulation (e.g., differences in Hsp70 stability).

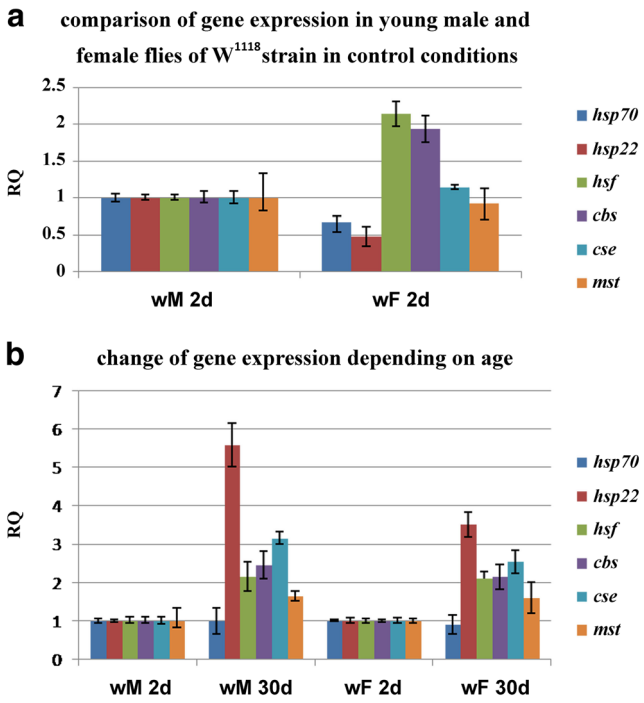

Fig. 1 **a** Comparative analysis of basal level expression of *hsp70*, *hsp22*, *hsf1*, *cbs*, *cse*, and *mst* genes in young (2-day-old) male and female w^{1118} *Drosophila* under normal conditions (22 °C). **b** Expression of the *hsp70*, *hsp22*, *hsf1*, *cbs*, *cse*, and *mst* genes under normal conditions (22 °C) in 30-day-old male and female w^{1118} *Drosophila* relative to 2-day-old flies. The relative quantity of transcripts was measured using qRT-PCR. The thin vertical lines show the average error

The induction of adaptogenes by stress in flies is age- and sex-dependent

We investigated the induction levels of major genes involved in HSR of *D. melanogaster* flies of the w^{1118} strain (*hsps 22* and *70*) and found that in the young males, both *hsp* genes exhibited higher levels of transcription after heat shock when comparing to same-aged females (Fig. 3a). Regarding old flies, males and females exhibited similar levels of investigated *hsp* gene induction after heat shock challenge (Fig. 3a, b). *Hsp70* induction in 30-day-old females is similar to 2-day-old flies. As expected, we failed to detect pronounced differences in the expression of *hsf1* in the flies after heat shock. However, *hsf1* was modestly induced in young but not in the old males after temperature elevation (Fig. 3c). Similar studies monitoring the expression of H_2S-producing genes after temperature elevation demonstrated that all three genes under investigation produce the same level of transcription after heat shock (Fig. 3d). After temperature elevation, however, in young males, we detected a slight elevation of *cse* and *mst* expression (Fig. 3d).

Western blot analysis exploring 7FB antibodies recognizing only inducible Hsp70 family members confirmed the results of qRT-PCR studies and demonstrated that with increasing age, the level of Hsp70 induction is significantly decreased both in males and females (Fig. 4). After heat shock, the accumulation of Hsp70 in females exceeded that accumulated in males and the decrease in Hsp70 accumulations after HS is observed only in 60-day-old females (Fig. 4).

The thermotolerance of flies is age- and sex-dependent

Young and old flies of both sexes of the w^{1118} and *cbs*−/− strain were used to investigate the basal tolerance after HS treatment of different strength (38 and 39 °C). We found that 2-day-old flies are significantly more thermotolerant than 30-day-old flies in both strains (Fig. 5 a and b). This difference is particularly pronounced after heavy HS (39 °C). Furthermore, the experiments demonstrated that

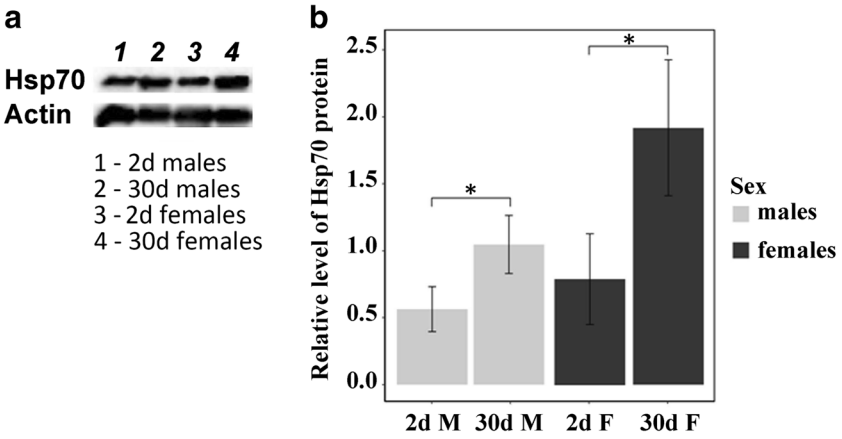

Fig. 2 Western blot analysis of proteins from 2- and 30-day-old male (M) and female (F) w^{1118} *Drosophila* grown under normal conditions (22 °C). Upper panel, HSP70 detection using monoclonal antibody 7FB; lower panel, the same blot rehybridized with anti-actin antibodies. The bars represent HSP70 expression changes. For quantification, the optical density of HSP70 was normalized to actin. Each value represents the mean ± SD of at least three independent experiments. *$p < 0.02$

in all cases, females exhibited a tendency for higher thermotolerance, which became highly significant after heavy HS (39 °C) (Fig. 5 a and b). These results correlate with a higher level of Hsp70 accumulation after HS in old females when compared to same-aged males (Fig. 4). Flies of *cbs*−/− strain exhibited similar decrease in thermotolerance in aged flies for both sexes although the flies with deletion are characterized by slightly higher basal thermotolerance in the case of heavy heat shock (39 °C) in comparison with the control strain (Fig. 5 a and b).

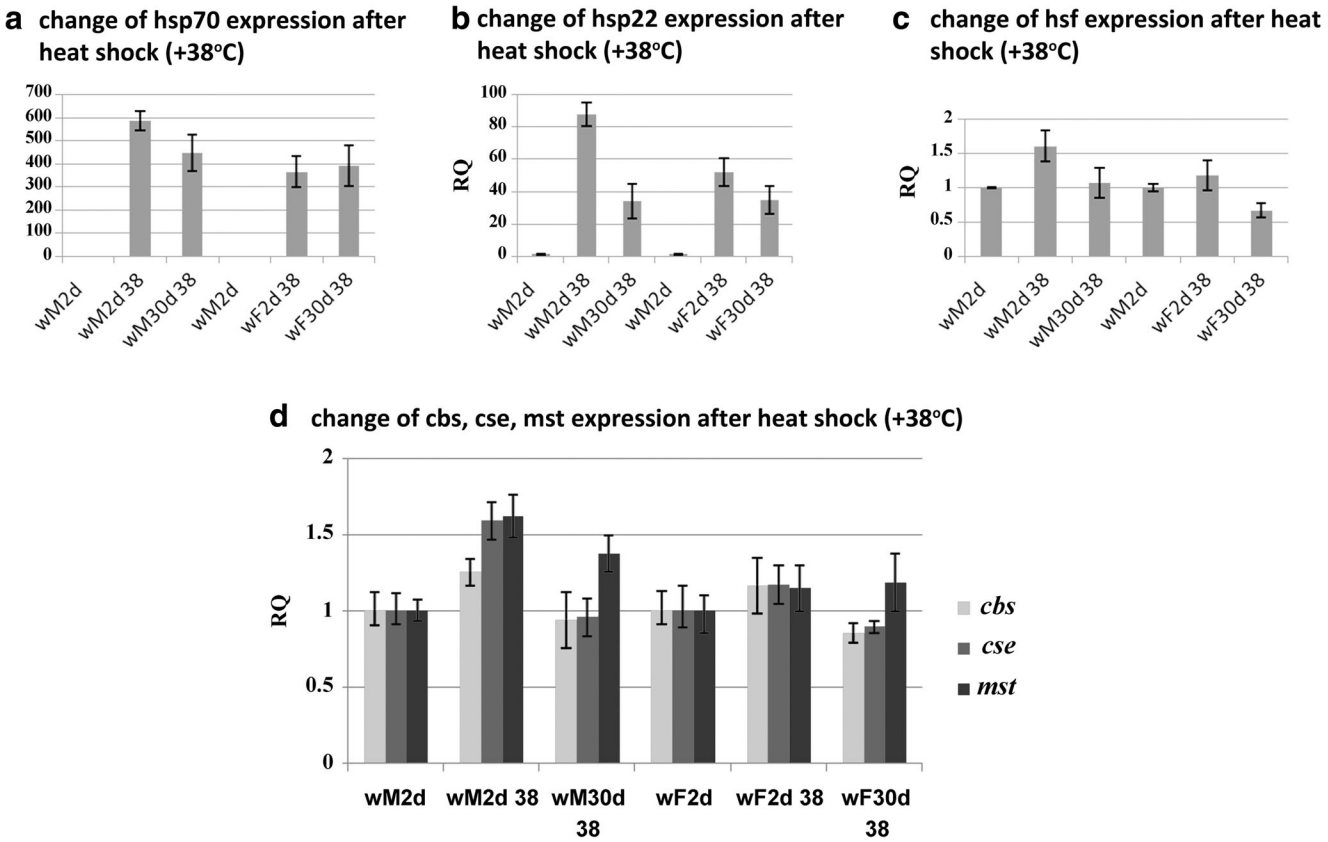

Fig. 3 The changes in *hsp70* (**a**), *hsp22* (**b**), and *hsf1* (**c**) expression in 2- and 30-day-old male and female w^{1118} *Drosophila* after heat shock (38 °C for 30 min) relative to 2-day-old flies grown under normal conditions (22 °C). The relative quantity of transcripts was measured using RTPCR. The thin vertical lines show the average error

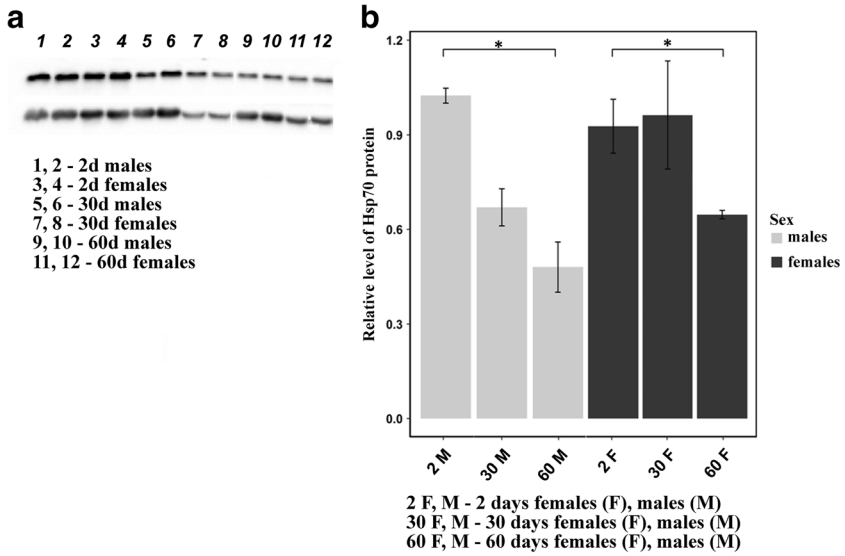

Fig. 4 Western blot analysis of proteins from 2-, (30- and 60-day-old male (M) and female (F) w^{1118} *Drosophila* after HS (38 °C for 30 min) and recovery (30 min at 22 °C). Upper panel, HSP70 detection using monoclonal antibody 7FB; lower panel, the same blot rehybridized with anti-actin antibodies. The bars represent HSP70 expression changes. For quantification, the optical density of HSP70 was normalized to actin. Each value represents the mean ± SD of at least three independent experiments. $*p < 0.02$

Cross-talk between HSR genes and the genes responsible for hydrogen sulfide production

To investigate the possible interaction between heat shock genes and major genes responsible for the production of H_2S, we deleted one of the major genes belonging to this system (*cbs*) using CRISPR technology and monitored the response to heat shock in the resulting transgenic strain. The deletion of *cbs* was confirmed by qRT-PCR (Supplemental Fig. S1) and Southern blot analysis (data not shown). The performed experiments demonstrated that 2-day-old males with a deleted *cbs* gene (*cbs*−/− strain) are characterized by a significantly higher level of *hsp22* expression (10 times) and a modest elevation of *hsp70*, *hsf1*, *cse*, and *mst* (2–3 times) mRNA under normal temperature than in the w^{1118} strain (Fig. 6a). Furthermore, 2-day-old females had significantly increased expression of the *hsp22* (6-times) and *cse* (5-times) genes (Fig. 6b). During aging in *cbs*−/− males, we observed pronounced elevation of the *hsp22* and *cse* gene expressions (Fig. 6a and Fig. 7a). However, in the w^{1118} strain, elevation of *hsp22* during aging is more significant than in the *cbs*−/− strain. In old females carrying the *cbs* deletion, we detected a significant increase in the levels of *hsp22*, *hsf1*, and *cse* expression (Fig. 6b and Fig. 7b). It is important that

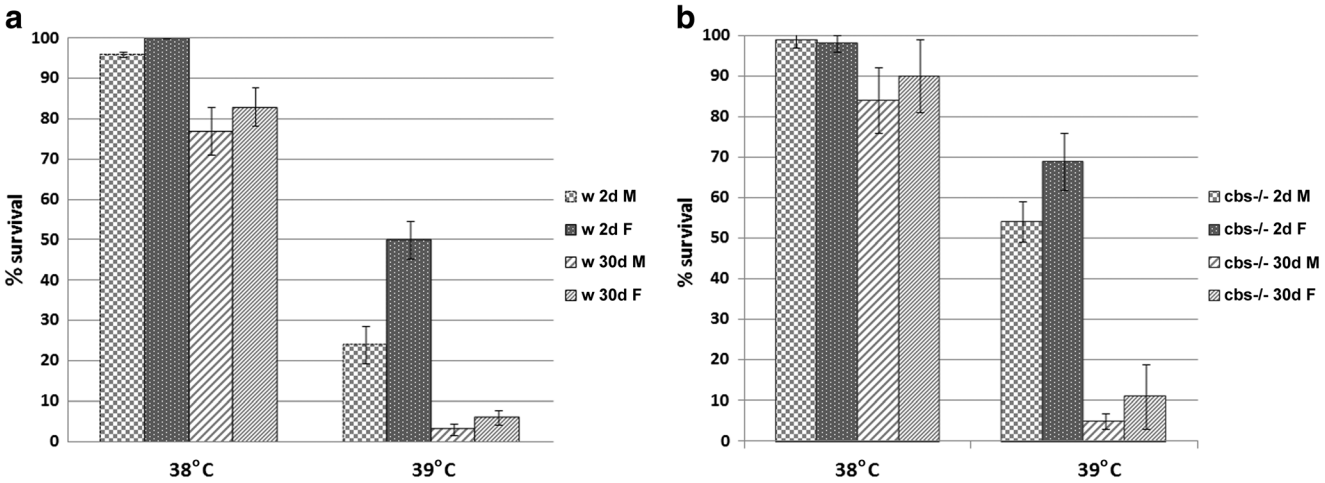

Fig. 5 Basal thermotolerance of 2- and 30-day-old male and female flies of strain w^{1118} (**a**) and strain cbs−/− flies (**b**) after exposure to different strengths of heat treatment (38 °C for 30 min or 39 °C for 30 min). The vertical *y*-axis indicates the proportion of survived flies (in %). The 95% confidence intervals for each sample are shown as error bars. A more detailed description of the statistics is given in the Supplementary Materials, which includes analysis of variance and post hoc tests

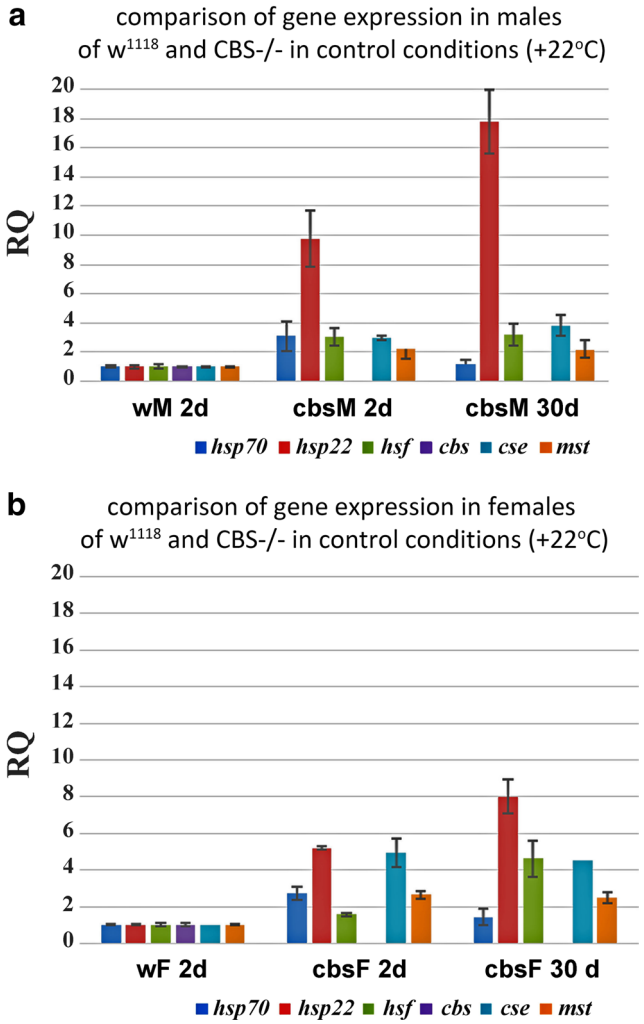

Fig. 6 Expression of the *hsp70*, *hsp22*, *hsf1*, *cbs*, *cse*, and *mst* genes in 2- and 30-day-old male (**a**) and female (**b**) cbs−/− *Drosophila* relative to 2-day-old w^{1118} flies grown control conditions. The relative quantity of transcripts was measured using the qRT-PCR. The thin vertical lines show the average error

during aging of the w^{1118} strain, the level of increase in the expression of all adaptogens is higher than during aging of *cbs*−/− flies. It is noteworthy that the level of expression of all studied genes (except *hsf1*) was lower in 2-day-old females than in same-aged males (Fig. 7c).

Because we failed to detect significant changes in the expression of the studied sulfur metabolism genes after heat shock in the w^{1118} strain, in the *cbs*−/− strain, we monitored only the level of *hsps* induction (Fig. 8). In the deletion strain, we observed the same trend as in the w^{1118} strain (i.e., the decline of *hsps* induction with age) (Fig. 8a, b). On the other hand, in the strain with *cbs* deletion, we did not observe a significant difference between males and females in the level of *hsp22* induction after heat shock. Furthermore, in contrast to the w^{1118} strain, where we detected a slight decrease in *hsf1* expression in 30-day-old females after heat shock (38 °C), in the *cbs*−/− strain, *hsf1* expression increased by 2.5 times in 30-day-old females (Fig. 8c).

Discussion

There is now substantial literature detailing a decline in adaptive homeostasis that manifests with aging in various model organisms and humans (Pomatto et al. 2017a, b). Multiple studies have implicated Hsps synthesis in the regulation of the aging process (Tower 2009, 2011). Hsp70 and Hsp22 attracted special attention and were studied in detail regarding their role in lifespan extension and stress resistance (Tower 2009, 2011; King and Tower 1999; Morrow and Tanguay 2015). Generally speaking, the existing literature suggests that an evolutionarily conserved feature of aging is the insufficient induction of *hsp* genes—often referred to as "adaptogenes"—at advanced age in response to oxidative and other forms of stress, ultimately resulting in protein damage. In the last decade, hydrogen sulfide (H_2S)—a pungent gas formed by many tissues—has emerged as an important transmitter belonging to the adaptogene category and involved in the control of stress resistance in various organisms, ranging from flies to humans (Shaposhnikov et al. 2018; Kimura et al. 2010; Kimura 2014). Recent experimental studies have highlighted the role of H_2S in aging and longevity (Qabazard et al. 2014; Shaposhnikov et al. 2018; Perridon et al. 2016; Paul and Snyder 2015). Increasing evidence suggests beneficial effects of H_2S in several disease models, including age-related pathologies (Predmore et al. 2012; Hu et al. 2010). The age-dependent decline in plasma H_2S levels in aged humans supports the link between H2S and the aging process (Perridon et al. 2016).

Many groups have investigated the mechanism of heat shock gene induction by heat stress in flies, including ourselves (Evgen'ev et al. 2014; Lis and Wu 1993). Thus, it was demonstrated that Drosophila males and females differ significantly in terms of stress resistance and kinetics of Hsps synthesis and induction after temperature elevation (Pomatto et al. 2017a, b; Shilova et al. 2017). Thus, *Drosophila* females are usually more stress-resistant than males. This applies to heat stress (Tower 2011), oxidative stress (Pomatto and Davies 2017), and starvation stress (Jang and Lee 2015). It is believed that the increased resistance in females is due to the double dose of the X chromosome, which carries important genes for homeostasis. Also, the higher stress-resistance often exhibited by females might be associated with larger body size and maternal inheritance of mitochondria. The role of specific hsp proteins in stress resistance and longevity in *Drosophila* has also been demonstrated (Tower 2009, 2011; Shaposhnikov et al. 2018; Morrow and Tanguay 2015).

It is noteworthy that while a cross-talk between H_2S and NO was shown to play an important role in the regulation of

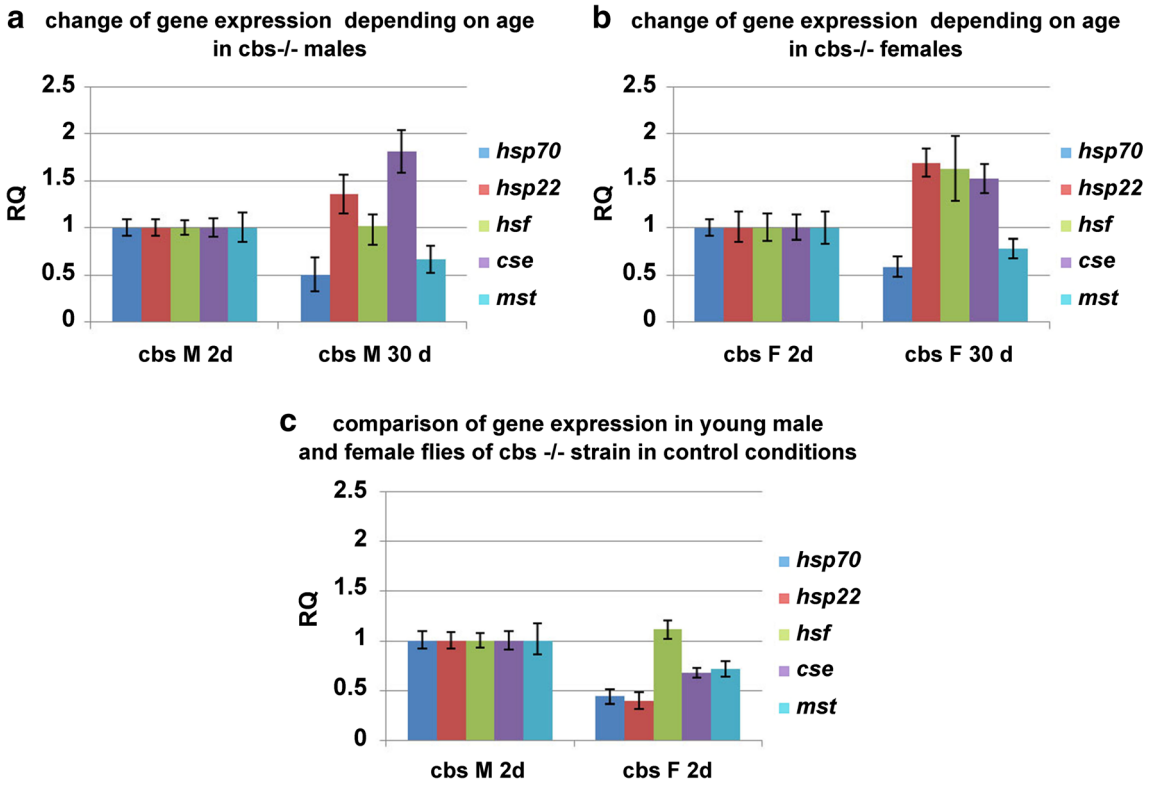

Fig. 7 The changes in expression *hsp70*, *hsp22*, *hsf1*, *cbs*, *cse*, and *mst* genes during aging. 30-day-old male (**a**) and female (**b**) cbs−/− flies compared to 2-day-old males and females of cbs−/− flies. **c** Comparative analysis of the basal expression levels of *hsp70*, *hsp22*, *hsf1*, *cbs*, *cse*, and *mst* at normal temperature (22 °C) in 2-day-old male and female of cbs−/− flies

various physiological processes (Kolluru et al. 2013; Bianco and Fukuto 2015), a possible interaction between H_2S levels and heat shock system functioning was not fully elucidated in animal models.

Therefore, in our investigation, we monitored the age-dependent expression of Hsp70 representing a major stress gene implicated by numerous studies in stress resistance and longevity and another well-characterized Drosophila *hsp22* gene (Morrow and Tanguay 2015), separately in males and females known for its preferential upregulation during aging and in oxidative stress conditions (King and Tower 1999; Landis et al. 2004). Hsp22 is a beneficial protein because its overexpression increases lifespan and resistance to stress, while its downregulation is detrimental (Morrow et al. 2004; Kurapati et al. 2000). We also monitored the sex- and age-dependent expression of the conserved heat shock transcription factor-1 (*hsf 1*), which is essential to cellular stress resistance and contributes to life span. HSF binding to the HSEs results in high-level transcription of all heat shock genes. The major function of HSF-1 is to regulate a network of *hsps* genes that protect proteins from damage caused by extrinsic environmental stress or age-related deterioration (Morimoto 1998; Nielsen et al. 2005).

In addition to the above genes involved in HSR, we monitored the expression of all three genes participating in H_2S production in Drosophila to reveal possible cross-talk between these presumably adaptive systems. We also explored a strain with a deletion of the *cbs* gene. The overexpression of this gene was implicated in the control of stress resistance and life extension in *D. melanogaster* and other organisms (Kabil et al. 2011; Hine et al. 2015; Shaposhnikov et al. 2018).

Our analysis demonstrated a higher level of most H_2S producing genes and lower level of hsps in 2-day-old females relative to males in the control w^{1118} strain and significant upregulation of transcription of all studied genes, except *hsp70* in the old flies of both sexes (Fig. 1). Interestingly, in the *cbs*−/− strain in 2-day-old females, we observed lower expression of all genes, except the *hsf1* gene. On the other hand, in the *cbs*−/− strain, the *hsps* induction level after heat shock in 2-day-old females is equal to or even higher than in 2-day-old males. The *cbs*−/− strain exhibited significantly higher levels of all studied genes, especially when comparing the *hsp22* gene against the control strain under normal temperature (Fig. 6). However, we observed a less pronounced increase in the expression of the studied genes in the old flies, *hsp22* being an exception (Fig. 6). This suggests that in the young flies, the absence of the *cbs* gene leads to oxidative stress, which induces

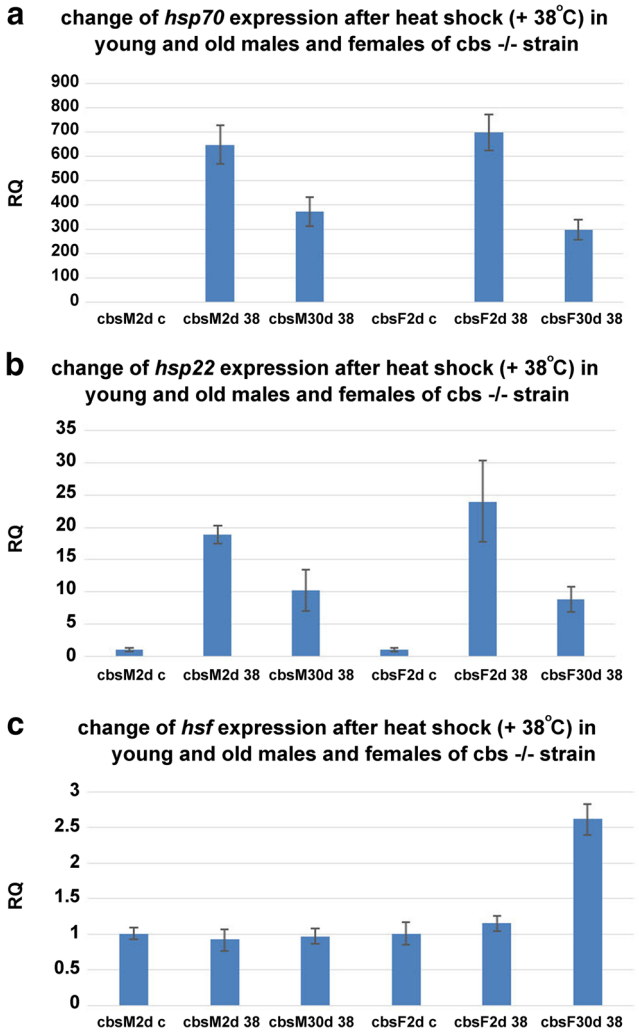

Fig. 8 The changes in *hsp70* (**a**), *hsp22* (**b**), and *hsf1* (**c**) expression levels after heat shock (38 °C for 30 min) in 2- and 30-day-old cbs−/− *Drosophila* relative to 2-day-old flies kept under normal temperature conditions (22 °C). The relative quantity of transcripts was measured using RTPCR. The thin vertical lines show the average error

the expression of other studied adaptogens. Since the adaptogens are already expressed at a high level in young flies, aging resulted in a less pronounced increase in their expression than in the w^{1118} control strain. Notably, age-dependent induction of small hsps, including *hsp22*, was previously has been reported by other authors (King and Tower 1999; Morrow and Tanguay 2015). However, in the case of the *cbs*−/− strain, the transcription level of *hsp22* in 2-day-old flies is higher than in 30-day-old flies of w^{1118} strain. Notably, it was shown that hsp22 mRNA is post-transcriptionally regulated. Thus, during aging hsp22 mRNA reaches a high level by 30 days old, and the protein was detected only at 40 days old in the flies (King and Tower 1999).

A significantly higher level of *hsf1* expression represents another interesting feature of the strain with *cbs* deletion (Fig. 6). On the other hand, a significant age-dependent decrease of hsp70 expression is observed in this strain (Fig. 6). It was shown that while *hsf1* is activated and undergoes trimerization after heat shock, the level of its RNA is not changed (Lis and Wu 1993; Morimoto 1993). A lower level of *hsp* gene induction in the old flies—as demonstrated in our studies—is not associated with a lower level of *hsf1* transcription and is likely due to malfunction of Hsf or its posttranslational modification. It is noteworthy that results from cultured cells suggest that the age-related decline in Hsp70 induction is constitutive and due to decreased binding of the heat shock factor 1 (Hsf-1) to the heat shock elements (HSEs) after stress and, hence, diminished *hsp70* transcription. These changes might explain the decreased thermotolerance upon aging observed in our studies in the strains studied including *cbs*−/− flies (Heydari et al. 1994).

Notably, the levels of *hsp70* expression revealed by qRT-PCR in the studied strains do not correlate with Hsp70 protein content estimated by Western blotting (Figs. 2 and 4). Such analysis demonstrated a clear increase of Hsp70 level with more pronounced age in females (Fig. 2). Previously, we described a similar phenomenon when studying different Stratiomydae species (Garbuz et al. 2009). These data suggest that the post-transcriptional upregulation of HSP levels during aging are due to an increase in protein or RNA stability, but this has not yet been directly tested. Higher constitutive level of Hsp70 in the females correlates with a significantly higher level of thermotolerance demonstrated for old females, particularly after "heavy" heat shock (Fig. 5).

The strains used in the investigation differ in the induction capacities of the studied genes. Thus, while in the control strain *hsp*70 induction was significantly decreased with age only in males, in the *cbs* deletion strain, we detected a pronounced drop in the induction of both hsps in both old males and females (Fig. 8). Furthermore, while in the control strain, we observed some decrease in *hsf1* expression in the old females after HS, surprisingly, in the *cbs*−/− strain, we detected a clear upregulation of *hsf1* transcript levels in females of the same age after HS (Fig. 8). However, in our experiments, in most cases, the level of *hsf1* expression does not exhibit pronounced changes depending on sex or temperature elevation. Similarly, the expression of all three studied genes involved in H_2S production did not fluctuate significantly after HS treatment.

Here we have described the fluctuations in the expression of genes involved in HSR and three major genes responsible for H_2S production in *D. melanogaster* during the aging process. Relative to the control strain, we detected significant differences in the expression of these genes in a *cbs* deletion strain. These data suggest a cross-talk between the two studied ancient and universal adaptive systems. However, we are well aware that the differences observed in the cbs−/− may be due to general changes in cell physiology of this mutant strain that only indirectly bring about changes in heat shock gene expression that may have little or no useful effect on thermotolerance.

The demonstrated sex- and age-dependent variations in adaptive homeostasis, as well as the adaptive stress responses, including the expression of major hsps and H_2S synthesis genes, offer insight into the underlying mechanisms for the male vs. female survival differences that have been reported in many organisms.

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