CHANGES IN SUPEROXIDE DISMUTASE AND CATALASE IN AGING HEAT-SHOCKED DROSOPHILA

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We have examined the age-dependent expression of CuZn superoxide dismutase (SOD) and catalase (CAT) in Drosophila melanogaster following heat shock. Quantitative northern blot analysis was performed after heat shock on 2-, 23- and 49-day-old flies, using a 0.48 kb SalI EcoR1 fragment of the Drosophila SOD cDNA and a 1.4 kb fragment of the human catalase cDNA. Heat shock induction was monitored with a 5.4 kb DNA fragment of the Drosophila hsp70 gene. After exposure to 37˚C for 30 min and 60 min, the level of SOD RNA in young flies was elevated above that of nonstressed conditions. Changes in the transcription of SOD gene with age paralleled the expression of hsp70 RNA. The SOD RNA was elevated in heat-shocked middle aged (23–25 days old) flies compared to young Drosophila (2 days old), then it decreased in 49–50-day-old flies. The relative expression of CAT RNA did not change with age or after heat shock. Enzymatic activities of these two antioxidant enzymes were evaluated in nondenaturing polyacrylamide electrophoresis gels. SOD migrates on this gel as three different electromorphs. These were designated as fast, intermediate and slow migrating bands. The most intense activity was associated with the fast band in these flies. In the absence of heat shock, there was an age-dependent decrease in the intermediate, but not in the slow or fast bands. Heat shock does not affect the intensity of the fast band in young or old flies, however; in middle aged flies, there is a shift in this band toward the slow position. No change was detected in the activity of catalase with age or heat shock, although flies of all ages exhibited a shift toward a faster-migrating electromorph with increasing time of heat shock. This effect was also observed in flies fed H2O2 and is more pronounced in insects fed higher concentrations. These results are discussed in relation to the role of these antioxidant enzymes in protecting against age-induced oxidative stresses.

KEY WORDS: Superoxide dismutase, catalase, hsp70, heat shock, aging, Drosophila melanogaster

INTRODUCTION

Cells continuously exposed to either exogenous or endogenous stress such as heat, oxygen radicals or radiation have developed a variety of protective mechanisms, such as the synthesis of stress proteins, antioxidant defense or DNA repair enzymes. The similarity of responses to elevated temperatures, and various oxidative stresses in bacteria,1–4 Drosophila5 and even in plants6 suggest the possibility that increased temperature and oxidative stress may be related. Accordingly, cells or organisms exposed to heat or oxidative stress synthesize a similar subset of “heat shock proteins”.

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The induction of these proteins is necessary to survive a given stress and recover to normal metabolic conditions.

Exposure to heat is accompanied by an elevation in oxygen consumption. Thus, exposure to high temperatures most likely results in an increased production of free radicals which would generate the need for an elevated level of scavenging enzymes to remove these toxic species. Consequently, enzymes such as superoxide dismutase (SOD) and catalase (CAT) which defend cellular components against oxidative damage may contribute to the cellular defense system during heat stress. SOD, which is the primary defense against intracellular superoxide, exists in two forms in eukaryotic cells: as a Cu/Zn SOD which resides in the cytosol and also may reside in the peroxisomes and a Mn SOD which is localized in the mitochondria. The cytoplasmic form of SOD is a 32 kDa dimer with two Cu and two Zn atoms and catalyzes the dismutation of the superoxide anion ($O_2^-$) to hydrogen peroxide and molecular oxygen.\textsuperscript{7,8} The product of the dismutation reaction, $H_2O_2$, is removed by catalase, a 240 kDa tetramer, in peroxisomes and by glutathione peroxidase in other cell compartments.

Though an effective antioxidant-scavenging system is necessary during normal metabolic conditions to protect cellular components against oxidative damage, the need for this protection increases during periods of stress, such as exposure to heat. Experimental evidence suggests that if heat-induced oxidative stress exceeds the antioxidant defense capability of the cell, this can result in a heat-induced injury and lead to cell death. Accordingly, it has been shown that normal mouse embryo cells with 2–5 times higher level of SOD, CAT and glutathione peroxidase than SV-40 transformed cells are more resistant to hyperthermia (cell survival 75% vs. 5%).\textsuperscript{9} Pretreatment of normal cells with diethylthiocarbamate (the inhibitor of SOD activity) significantly reduced this resistance and in the case of transformed cells made them extremely thermosensitive.\textsuperscript{9} Also, in several other systems, an increased antioxidant level has been correlated with thermal tolerance.\textsuperscript{10,11}

The extent of oxidative damage to biological molecules has been suggested to play a major role in the aging process.\textsuperscript{12} Numerous studies have shown that during normal cellular aging the level of oxidative stress (i.e. balance between prooxidants and antioxidants) shifts toward a relatively more prooxidizing status.\textsuperscript{13} This may result in the increase in oxidative damage to cellular components with age\textsuperscript{14} and consequently reduce the metabolic efficiency of a cell. Accordingly, a decrease in enzymatic activity of the scavenging enzymes SOD, CAT, and also glutathione was observed in house flies with age.\textsuperscript{15} Also SOD and CAT expression was lower in the brain and liver of aging rats.\textsuperscript{16} However, the literature contains conflicting data in this regard and the analysis of antioxidant enzymes in some systems indicates that there is no significant decrease in antioxidant defense with age.\textsuperscript{17–19}

The extent of cellular damage may be especially relevant in senescent organisms during adaptation to environmental stresses such as heat shock. We have previously shown that old Drosophila are less resistant to heat than young flies, and we have postulated that this may be related to an increase in age-dependent protein damage.\textsuperscript{20} There are no data describing the heat-dependent regulation of antioxidant genes in aging organisms. In this study we have investigated the expression of SOD and CAT in aging Drosophila during exposure to heat shock. These results have important implications for evaluating mechanisms that protect organisms against heat stress during aging.
MATERIALS AND METHODS

_Drosophila Cultures and Heat Shock_

_Drosophila melanogaster_ of the Oregon R strain, obtained in 1981 from NASA Ames Research Center, were maintained at 24°C in half-pint bottles containing Formula 4-24 instant _Drosophila_ medium (Carolina Biological Supply Co.). Heat shock was applied by transferring 20 male flies of given age group to 35 ml thin-walled glass vials (containing filter paper moistened with 25% sucrose) and placing them in a 37°C incubator for different periods of time.

_Isolation of RNA_

Total RNA was prepared by placing 20 flies in a 1.5 ml microcentrifuge tube and homogenizing in a solution containing equal volumes of phenol and buffer (10 mM Tris-HCl pH 7.5, 20 mM EDTA, 1% SDS) as described by Xiao and Lis.21 The RNA was sequentially extracted by phenol, phenol/chloroform, and chloroform; collected by ethanol precipitation, and suspended in H2O. Samples were kept frozen at −70°C until use. The concentration of RNA was determined by taking an absorbance reading at 260 and 280 nm, assuming that 1 mg of RNA/ml of solution gives 25 A units at 260 nm.22

_Analysis of RNA by Northern Blotting_

Total RNA (4 ug/lane) was size fractionated on 0.9% formaldehyde-agarose gels according to the method described by Maniatis.22 RNA was transferred to a piece of Biotrans nylon membrane. The membrane was baked for 2 h at 80°C in vacuo and prehybridized for 3 h at 45°C in a prehybridization mixture containing 50% (v/v) formamide, 5 × SSC, 5 × Denhardt’s solution, 0.5% SDS, 25 ug/ml sonicated sperm DNA. The membrane was probed with denatured 32P-labeled 0.48 kb _SalI/EcoR1_ fragment of the pGem2 containing the _Drosophila_ Cu/Zn SOD (gift from J. Kwiatoowski, UC Irvine, CA) gene or with 1.4 kb fragment of _EcoR1/SalI_ human catalase gene (obtained from R. Hallewell, Chiron Corp., Emeryville, CA).23 Hybridization was performed at 45°C overnight. The membrane was washed for 1 h at room temp, in 2 × SSC, 0.1% SDS and then for 1 h at 50°C in 0.1 × SSC, 0.5% SDS and finally rinsed in 0.1 × SSC. The membrane was exposed to Kodak XRP-1 x-ray film with an intensifying screen at −70°C for different times. The filters were stripped of the label and rehybridized under the same conditions (except the wash in 0.1 × SSC, 0.5% SDS was at 58°C) with a 5.4 kb _SalI_ fragment of the p132E3 plasmid of the _Drosophila_ hsp70 gene (gift from S. Munroe, Stanford University, CA) and 0.8 kb _SalI_ fragment of _Drosophila_ actin 42A gene (gift from B.B. Mathews, UCLA, CA).

_SOD Activity_

Extraction of Cu/Zn SOD from flies was performed by the method of Bannister and Bannister,24 modified as follows: 25 flies were homogenized in 150 ul of 20 mM Tris-acetate buffer pH 7.8 containing 0.2% Triton X-100 and 1 mM phenylmethyl-
sulfonyle fluoride. The homogenate was centrifuged at 11,000 \times g for 15 min at 4°C. The resulting supernatant was collected, heated rapidly to 60°C for 10 min, acidified to pH 5.5 with 1 M acetic acid, and centrifuged at 11,000 \times g for 15 min at 4°C. Proteins were separated on a 10% non-denaturing polyacrylamide gel for 18 h in 35 V. After electrophoresis, SODs were identified in the gel by the Nitro Blue Tetrazolium method.\textsuperscript{25} The gels were soaked in 2.5 mM Nitro Blue Tetrazolium for 30 min with shaking. They were rinsed and immersed in a solution of 0.028 M tetramethylene diamine, 2.8 \times 10^{-5} \text{M riboflavin and 0.036 M potassium phosphate at pH 7.8.} The gels were then illuminated on a fluorescent light box for 5 to 15 min. During that time, gels gradually become uniformly blue (reduced Nitro Blue Tetrazolium) except at the location of SOD.

**Catalase Activity**

Protein crude extracts were prepared from *Drosophila* of different age groups. 25 males were homogenized in 250 ul of 20 mM Tris-Acetate buffer pH 7.8, 0.1% Triton X-100, 1 mM phenylmethylsulfonyle fluoride. The homogenate was centrifuged at 11,000 \times g for 15 min at 4°C and the collected supernatant was used for protein analysis. Crude extracts were loaded on a 8% polyacrylamide nondenaturing gel and the proteins were separated overnight at 55 V. After electrophoresis, the CAT bands were identified in the gel using a modification of the method of Gregory and Fridovich.\textsuperscript{26} Gels were soaked for 15 min in a 1 to 1000 dilution of 3% H\textsubscript{2}O\textsubscript{2}. Then gels were immersed in freshly prepared solution of 0.2% potassium ferricyanide, 0.2% ferric chloride and mixed gently for a few minutes. Catalase activity appears as yellow zones on a blue-green background.

**RESULTS**

We have analysed the expression of SOD and CAT RNA in male *Drosophila* 2 days, 23–24 days and 49–50 days old. Northern blots were performed from total RNA isolated from these flies kept at normal environmental temperature (25°C) and heat shocked at 37°C for 30 min and 60 min. Blots were probed with 0.48 kb fragment of *Drosophila* SOD cDNA and 1.4 kb fragment of human catalase cDNA. Blots were also probed with 5.4 kb DNA fragment containing *Drosophila* hsp70 gene which served to monitor the induction of the heat shock response (Figure 1). To correct for differences in gel-loading transfer or RNA degradation, the amount of hsp70, CAT and Cu/Zn SOD was determined as a ratio to actin (Table I). This was accomplished by rehybridization of filters with 0.8 kb *Sal I* fragment of *Drosophila* actin 42A gene. Blots for each particular probe were exposed for different times to obtain the optimal density of bands for densitometric scanning of autoradiographs. As can be seen in both Figure 1 and Table I, the exposure of flies of different ages to 37°C results in the induction of 2.3 kb RNA corresponding to the full size hsp70 RNA transcript. In RNA samples isolated from flies kept at 25°C, no hybridization signal with hsp70 probe was detected. Data in Table I show that hsp70 RNA synthesis was higher after 60 min than after 30 min of heat treatment. This is in agreement with the results reported from Craig’s laboratory on adult *Drosophila*\textsuperscript{27} as well as with reports showing that an increase in hsp70 is correlated with the severity of heat
stress in cultured Schneider cells.\textsuperscript{28} According to our earlier report,\textsuperscript{20} hsp70 synthesis was highest in middle aged flies compared to young and old \textit{Drosophila}.

Total RNA isolated from control and heat-shocked flies of different ages was rehybridized with 0.48 kb fragment of \textit{Drosophila} Cu/Zn SOD cDNA. As can be seen on Figure 1, a band corresponding the \textit{Drosophila} Cu/Zn SOD transcript hybridizes to the probe in flies of all ages kept both at 37°C and at their normal environmental temperature of 25°C. Following exposure to 37°C for 30 min and 60 min, the level of SOD RNA was elevated above that observed under non-stressed conditions in 2-day-old insects (Table I). However, it did not increase further with time of heat shock. We did not observe significant changes in SOD expression by heat shock in flies of other ages. No significant differences in the level of SOD RNA
FIGURE 2  Polyacrylamide gel analysis of SOD activity in heat-shocked aging flies. The positions of MnSOD and three different Cu/Zn SOD electromorphs (A, B, C) are shown in young (4 days old), middle aged (23–24 days old) and old (50 days old) control flies and flies heat-shocked for 30 and 60 min. Purified bovine Cu/Zn SOD was coelectrophoresed as a standard.

were detected between 30 and 60 min of heat shock for flies that are young (2 days old) or middle aged (23–24 days old). In the case of 49–50-day-old insects, a decrease in SOD expression was noted after 60 min (compared to 30 min) of heat treatment. The SOD RNA level in flies kept at normal environmental temperature was 3.5 times elevated in 23–24-day-old flies compared to 2-day-old insects, then decreased in old flies. However, old flies still expressed 2 times more SOD than the young ones in the absence of heat shock. The same age-dependent pattern was also observed in heat-stressed flies and parallels the changes for hsp70 RNA expression with age (Table I).

Figure 1 also shows a northern blot analysis of CAT RNA in flies of different ages exposed for 30 min and 60 min and kept at 25°C. Calculated CAT/actin ratios show that CAT RNA level does not significantly change in flies both with age and after heat treatment for either 30 min and 60 min (Table I).

Changes in enzymatic activity of SOD and CAT in flies of different ages exposed to heat stress were analyzed by polyacrylamide gel analysis. SOD polyacrylamide activity gel is shown in Figure 2 and densitometric profiles from this gel are presented in Figure 3, for young, middle aged and old flies kept at 25°C and heat-shocked for 30 min and 60 min at 37°C. The active Cu/Zn SOD enzyme is a homodimer composed of two 16-kDa subunits. On the activity gel, 3 bands corresponding to different electromorphs of Drosophila Cu/Zn SOD were detected (A, B, C). Among these electromorphs the strongest activity was associated with the fastest migrating SOD band on this gel. The upper, slowest-migrating band on the gel corresponds to the position of Drosophila Mn SOD. We observed an age-dependent small decrease in the intensity of the intermediate Cu/Zn SOD electromorph band (B) in flies not
exposed to heat stress (see Figure 3). The intensity of the fast electromorph (C) was not affected by age. Exposure of flies to 37°C for 30 min and 60 min does not significantly change the intensity of the faster-migrating band (C) in young and old *Drosophila* on the activity gel. However in middle aged flies, there is a shift in the position of this SOD band toward slowest-migrating electromorph (A) at the expense of the fastest one (C). This pattern in the shift in Cu/Zn SOD activity is very clear in middle aged flies after 60 min of heat exposure (Figure 3). The intensity of the Mn SOD band on the activity gel shows a tendency to decrease slightly during heat-shock treatment compared to nonstressed conditions in flies of all ages studied.

Polyacrylamide gel analysis and densitometric profiles of CAT activity in flies of different ages exposed to 30 min and 60 min of heat stress is presented on Figures 4 and 5, respectively. There is no evident change in CAT activity in flies with age both during normal and heat-shock conditions. However, flies of all ages exhibit a shift toward fastest-migrating electromorph with the increasing time of heat shock (Figure 5). The tendency to this change is observed earlier during heat stress in old than in young flies. For instance, the activity shift can be seen in old flies after 30 min of heat exposure, but not in young and middle aged organisms, where it is evident after 60 min of heat stress.

It has been reported that aging flies have increased level of H₂O₂ released from mitochondria as a result of mitochondrial membrane damage and/or increased intensity of oxidative stress with age. Thus, we addressed the question of how the exposure of *Drosophila* to an increased level of H₂O₂ affects CAT activity. We fed young *Drosophila* on 1% and 3% H₂O₂ for 48 hours and analyzed CAT activity by polyacrylamide gel electrophoresis. The results of this assay are shown on Figure 6. Flies fed on sucrose display one band of CAT activity on the gel. In flies exposed to an increasing concentration of H₂O₂ for 24 hrs, there is a shift in the CAT activity band toward faster-migrating position. The shift toward faster-migrating band is more pronounced in CAT isolated from flies exposed to a H₂O₂ concentration of 3% than of 1%.
FIGURE 4  Polyacrylamide gel analysis of catalase activity in aging heat-shocked flies. Catalase activity was determined as described in Materials and Methods. Purified bovine catalase was used as a standard.

FIGURE 5  Densitometric profiles of catalase activity from polyacrylamide gels. The migration position of catalase activity is shown in young (4 days old) middle aged (23-24 days old) and old (50 days old) flies non-stressed and exposed to 30 and 60 min of heat stress. The direction of band migration is left to right.
DISCUSSION

Because SOD and CAT play an important role in free radical detoxification, the age-related changes in the expression of these two enzymes during exposure to stress may predispose organisms to free-radical damage.

In our studies we analyzed how exposure to heat shock affects the transcription and enzymatic activity of Cu/Zn SOD and CAT and whether these changes are age-related.

We observed that the expression of Cu/Zn SOD and CAT were differently affected by elevated temperature in aging Drosophila. The level of Drosophila Cu/Zn SOD RNA changed with both age and heat stress. Exposure of young flies to 30 min and 60 min of heat stimulated expression of Drosophila SOD, however, in middle aged and old flies SOD RNA level was not much affected compared to nonstressed conditions. The induction of Cu/Zn SOD by heat shock was observed in mammalian cells, also bacterial SOD was elevated by heat exposure.

It is interesting that Cu/Zn SOD RNA synthesis in aging heat stressed flies parallels the age related changes in hsp70 expression under these conditions. The maximum expression for both Cu/Zn SOD and hsp70 was observed during heat-stress in middle aged (23–24-day-old) flies. For instance, old flies (50 days old) exposed to 30 min of heat treatment had about 22% less RNA for hsp70 and 26% for SOD compared to middle aged insects. However, old flies still had about 1.2 times more hsp70 RNA and 2.3 times more Cu/Zn SOD RNA compared to 2-day-old Drosophila. On the other hand, while hsp70 RNA synthesis increased with time of heat shock, the Cu/Zn SOD RNA level in flies of different ages did not change significantly between 30 and 60 min of heat exposure.

Thermodynamic analysis shows that the primary targets of heat-induced damage are proteins. Moreover, oxygen radicals have been shown to induce posttranslational damage to polypeptides in vivo. The synthesis of hsp70 is necessary for the protection of cellular proteins against heat-induced denaturation, and the extent of its expression has been correlated with the severity of cellular damage. According to this, an elevated level of hsp70 was observed in heat-stressed middle aged flies relative to young flies. However, in old Drosophila, where even higher accumulation of abnormal proteins is expected, the hsp70 synthesis is lower.
than in middle aged organisms. The decrease in hsp70 synthesis in heat-stressed old compared to middle aged flies is also accompanied by lower Cu/Zn SOD expression. This may result in more cellular protein damage generated during heat exposure in flies of this age. Thus, the coordinate expression of Cu/Zn SOD and hsp70 during heat stress with age suggest that these proteins may be a part of a protective system that is induced by heat stress and similarly affected by age. The indirect protective effect of Cu/Zn SOD against protein oxidation may be involved in other cellular events in addition to heat stress, such as the resistance to TNF,\textsuperscript{35} and to viral infection.\textsuperscript{36} In all of these cases, both the increase in the level of abnormal cellular proteins and oxidative damage seem to play a role.

Induction of Cu/Zn SOD by heat in young flies and its age related coordinate expression with hsp70 would also suggest that Cu/Zn SOD may play a function of stress protein. However, the published data do not show the presence of the Heat Shock consensus sequence in the promoter region of the Drosophila Cu/Zn SOD gene which is necessary for heat induction. It is likely that different types of regulation may also be involved in the transcriptional stimulation of Drosophila Cu/Zn SOD gene during heat stress and they may undergo age related changes. Examples of such regulation have been found in yeast CAT\textsuperscript{37} and also in S. cerevisiae DDR2 gene, which can be activated by both heat and DNA damage without Heat Shock Element involved.\textsuperscript{40}

Heat-induced transcription of Drosophila Cu/Zn SOD does not directly correlate with the changes in Cu/Zn SOD enzymatic activity in aging flies during heat shock. The enzymatic activity of Cu/Zn SOD is affected by heat shock only in middle aged flies. In young and old heat stressed flies, the relative intensities of the Cu/Zn SOD electromorphs are not substantially different from nonstressed Drosophila of corresponding ages. This lack of correlation between heat-induced increase in transcription of Cu/Zn SOD and lack of changes in its enzymatic activity may be related to the findings reported for Cu/Zn SOD in lungs of aging rats. Despite an age-related increase in Cu/Zn SOD transcript, total Cu/Zn SOD enzymatic activity remained constant due to an increase in the relative proportion of inactive Cu/Zn SOD in this aging species.\textsuperscript{39} In middle aged flies, we observed maximum expression of Cu/Zn SOD RNA during heat stress and it is interesting that only at this age Cu/Zn SOD activity was affected by heat. This change in activity was manifested by a shift in the position of the fast and slow electromorph on the nondenaturing gel. Our activity gel shows that the various Cu/Zn SOD electromorphs have different scavenging activities. It has been also observed that there is a selection for one of these electromorphs in long-lived Drosophila strain (M. Rose, personal communication).

In flies of all ages, CAT RNA level was not changed during heat treatment compared to nonstressed conditions. The similar lack of induction of CAT was also observed for rat hearts during heat shock.\textsuperscript{40} However, it has been reported that heat stress induced CAT RNA in yeast\textsuperscript{37} and Neurospora.\textsuperscript{41}

It is interesting that the expression of catalase RNA is not affected by heat stress, whereas the expression of the Cu/Zn SOD transcript increases following heat shock. Cu/Zn SOD is an exclusive O$_2^-$ scavenger, while H$_2$O$_2$, the product of the Cu/Zn SOD dismutation reaction, is converted to H$_2$O and O$_2$ not only by CAT but also other scavengers such as GSH, ascorbate and glutathione peroxidase.\textsuperscript{42} The efficiency of H$_2$O$_2$ scavengers other than CAT may partially explain the lack of change in the transcription rate of the CAT gene during heat stress. This is supported by the
observation that changes in cellular GSH level following heat stress in CHO cells are correlated with the thermal sensitivity. Our results show that despite lack of induction of transcription heat shock alters CAT enzymatic activity. We observed a shift in the migration of CAT activity band on nondenaturing polyacrylamide gel toward a faster migrating position. The shift in CAT activity band is related to the intensity of the heat stress (it is more pronounced after 60 min than 30 min of heat treatment) (Figures 4 and 5).

We have observed that exposure of young flies at normal physiological temperature to increased concentration of H₂O₂ (1% to 3%) resulted in a shift in CAT activity similar to the one observed in Drosophila exposed to heat stress. Sohal reported an increasing release of H₂O₂ from mitochondria with age, but there are no data regarding any concomitant physicochemical change of the CAT protein. In aging Drosophila maintained at physiological temperature, we did not observe any change in the position of the CAT band on non-denaturing activity gel. However, during heat stress, the shift of CAT activity toward faster-migrating position was noted and it was observed earlier in senescence than in young Drosophila (Figure 5, compare 30 min heat shock for young, middle aged and old flies). This would suggest the existence of a threshold in the cellular H₂O₂ level affecting CAT protein resulting in a change in the migration of this protein on non-denaturing gel.

Our results conclude that in aging Drosophila, heat stress affects both transcription of Cu/Zn SOD and CAT and their enzymatic activity but in a different, not necessarily coordinate, manner. In young flies Cu/Zn SOD transcription was stimulated by heat treatment. Changes in transcription of the Cu/Zn SOD gene with age paralleled the expression of hsp70 RNA. After heat shock, the level of RNA for these two proteins increases in middle aged flies. The maximum expression of Cu/Zn SOD in middle aged flies correlated with changes in the distribution of different Cu/Zn SOD electromorphs at this age.

The expression of CAT was not affected by either heat and age. However, during heat exposure, CAT showed a shift toward the fastest electromorph, which may be related to the accumulation of H₂O₂ in cells and may reflect differential effects of oxidative stress in flies of different age.

References