



Genetic Biomarkers of Aging in *Drosophila Melanogaster*

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ABSTRACT

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Genetic Biomarkers of Aging in *Drosophila melanogaster*

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The thesis was done in the Mitochondrial Gerontology and Age-related Disease group at the University of Tampere. The subject concerns mitochondrial gerontology, and the aim of the thesis was to find the genetic biomarkers of aging in *Drosophila melanogaster* using several experimental models. The purpose of the work was to study the levels of expression of different genes which are related to the mitochondrial energy metabolism using qPCR technique.

To accomplish the purpose, the levels of expression of the chosen genes were studied in the following experiments: different ages in females of two wild-type strains Oregon R and Dahomey, Dahomey males, flies with overexpressed mitochondrial superoxide dismutase, Dahomey females grown at different temperatures, and Dahomey females with increased flying activity.

As a result, appreciable changes in the expression of the following genes were observed: Impl3, Hsp22 and Hsp70. Impl3 expression increased with age in all the conditions studied, so it can be used as a biomarker of aging in all experimental strains. The expression of Hsp22 and Hsp70 changed markedly only in some of the strains and did not change in others.

Key words: aging, mitochondria, *Drosophila melanogaster*, energy metabolism, qPCR, gene expression.

TIIVISTELMÄ

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Ikääntymisen Geneettiset Biomarkkerit *Drosophila melanogaster*issa

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Opinnäytetyö tehtiin Tampereen Yliopiston mitokondriaa ja ikääntymistä tutkivassa ryhmässä. Aihe koskee mitokondrion gerontologiaa, ja työn tavoitteena oli löytää vanhenemisen geneettisiä biomarkkereita *Drosophila melanogaster*:n useissa koemalleissa. Työn tarkoituksena oli tutkia erilaisten mitokondrion metaboliaan liittyvien geenien ekspressiotasoja qPCR-tekniikan avulla.

Tarkoituksen toteuttamiseksi valittujen geenien ekspressiotasot tutkittiin muutamissa kärpäslinjoissa. Näitä oli: eri-ikäiset villityypin Oregon R ja Dahomey naarat, Dahomey urokset, kärpäset, joiden mitokondriaalinen superoksidi dismutaasi oli yli-ilmennetty, erilaisissa lämpötiloissa kasvatetut Dahomey naarat ja Dahomey naarat, joiden lentoaktiivisuutta oli lisätty.

Tulokseksi saatiin, että seuraavien geenien ekspressiotasojen muutokset olivat olennaisia: Impl3, Hsp22 ja Hsp70. Impl3 geenin ekspressiotaso kasvoi vanhenemisen myötä jokaisessa tutkitussa linjassa, jonka vuoksi Impl3 voidaan käyttää ikääntymisen geneettisenä biomarkkerina kokeissa käytetyissä kärpäsihmissä. Muutokset Hsp22 ja Hsp70 ekspressioissa eivät tapahtuneet jokaisessa tutkitussa linjassa.

Asiasanat: vanheneminen, mitokondriot, *Drosophila melanogaster*, energiametabolismi, qPCR, geenin ekspressio.

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ABBREVIATIONS

Acetyl-CoA	Acetyl coenzyme A
ATP	Adenosine triphosphate
cDNA	Complementary deoxyribonucleic acid
daGAL4	Yeast transcription factor GAL4 under <i>Drosophila melanogaster</i> ubiquitously expressed promoter <i>daughterless</i>
DAH	Dahomey (wild-type strain of fruit fly)
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dUTP	Deoxyuridine triphosphate
FADH2	Reduced form of flavin adenine dinucleotide
GTP	Guanosine-5'-triphosphate
mRNA	Messenger ribonucleic acid
NADH	Reduced form of nicotinamide adenine dinucleotide
OR	Oregon R (wild-type strain of fruit fly)
PCR	Polymerase chain reaction
Rpl32	Ribosomal protein L32
rRNA	Ribosomal ribonucleic acid
SOD2>dG	Superoxide dismutase II daGAL4
SOD2>w	Superoxide dismutase II wild-type
tRNA	Transfer ribonucleic acid
UAS	Upstream Activated Sequence
wd>G	Wild-type daGAL4

1 INTRODUCTION

This work was done in the Mitochondrial Gerontology and Age-related Disease group at the University of Tampere. The main study objectives of this group are aging and age-related diseases. The key interests of the group are molecular changes which occur during aging and the emergence of most common age-related diseases, mitochondrial processes, and formation of free radicals. It is well known that changes in processes occurring in mitochondria are involved in aging, and free radicals which are formed during the transfer of electrons may damage different parts of the cells.

The main model organism used in the host laboratory is *Drosophila melanogaster*. Fruit flies are widely used in multiple studies due to the following advantages: fast reproduction, short generation time, and the simplicity of cultivation and genetic crossing. It has only four pairs of chromosomes and its complete genome has been sequenced a few years ago. Another beneficial feature of *Drosophila* as a model organism is that it is free from ethical issues which often hamper the research progress when working with mammals. (Ashburner 2008.)

Although *Drosophila melanogaster* is an insect, a great deal of similarities with human exists. 50% of fruit fly genes have homologs in mammals, and it has been shown that 77% of known human disease-related genes are also expressed in flies. (Reiter et al. 2000, 1114, 1118.)

The aim of the work was to find genes which can be used as reliable markers of aging in different strains of *Drosophila melanogaster*. Since aging is related to the changes in mitochondrial processes, mitochondrial genes or related genes involved in energy metabolism were chosen. The purpose of this work was to study the levels of gene expression using quantitative (real-time) PCR and to find one or more genetic markers that can predict biological age in fruit flies.

To accomplish the purpose of the thesis the following tasks have been proposed:

- 1) Estimation of expression levels of the selected genes in different chronological ages of fruit fly wild-type strains,

- 2) Estimation of expression levels of the selected genes in flies with overexpression of mitochondrial superoxide dismutase,
- 3) Estimation of expression levels of the selected genes in flies with increased flying activity or in flying flies (this means that flies were grown in bottles, not in vials and thus their flying activity was increased),
- 4) Estimation of expression levels of the selected genes in flies maintained at different temperatures.

Finding the genes whose levels of expression change with age can help people understand the reason of aging. When the expression of some gene changes during aging, it is possible to increase or decrease its expression using such genetic engineering technologies as the over-expression of target gene or RNA-interference and see how this will affect longevity.

2 THEORETICAL BACKGROUND

2.1. Aging and mitochondria

The search for the cause of aging is one of the most interesting tasks in biology. Processes occurring during the aging are studied in numerous laboratories. Nowadays there are many theories of aging. One of them is the mitochondrial free radical theory of aging. Mitochondria-related aging may be associated with the generation of reactive oxygen species, the alteration of redox homeostasis, and the changes that happen in mitochondrial DNA, such as different point mutations and large-scale deletions (Leeuwenburgh et al. 2011).

2.2. Processes occurred in mitochondria

Mitochondria are important parts of the cell since they produce most of the energy. Mitochondria have their own genome, and the division of mitochondria happens independently from the cell division (Taylor & Turnbull 2005, 390). However, the majority of proteins required for mitochondrial functions is encoded in the nucleus, translated in the cytosol and then transported into mitochondria (Dujon 2009, 505). The transport is mediated by translocases - special proteins that recognize and help move the precursor of protein across the mitochondrial membrane (Wiedemann et. al. 2004, 14473). Proteins that are encoded in the nucleus are, for example, ribosomal proteins, enzymes for the citric acid cycle, and proteins, which participate in the DNA replication and transcription. The mitochondrial genome is also functional, but it is very small. In spite of this, mutations in mitochondrial DNA appear as one of the most important causes for genetic diseases (Taylor & Turnbull 2005, 389). In the mitochondrial genome, rRNA, tRNA, and some proteins involved in the electron transport chain are encoded (Clary & Wolstenholme 1985, 252). The number of mitochondria per cell depends on the cell's energy needs (Newsholme & Leech 2009, 4).

It is thought that mitochondria were formed through the merge of an ancient aerobic prokaryote cell and a nucleated cell. Thus, the aerobic cell could receive protection from the environment, and the other could benefit from the ability of more effective aerobic

energy production. There were found some similarities between mitochondria and modern prokaryotic cells. For example, the genome of mitochondria is circular, as in prokaryotic cells, and both the inner membrane of mitochondria and plasma membrane of prokaryotes contain electron transport proteins. (Newsholme & Leech 2009, 183.) The structure of the mitochondrion is shown in figure 1.

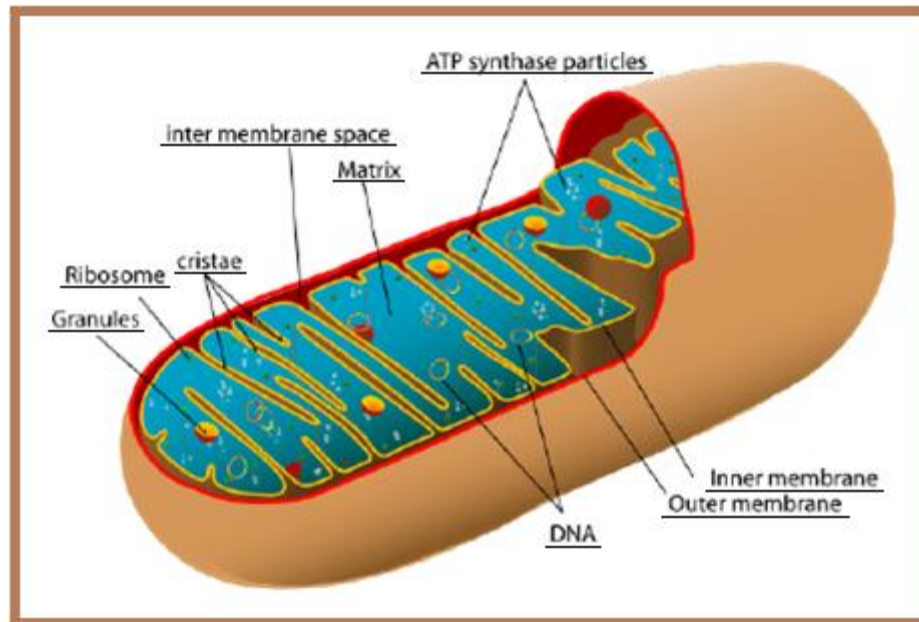


FIGURE 1. Mitochondrion. (Ruis 2006, modified)

The most important processes occurring in mitochondria are related to the cellular energy production, and these are citric acid cycle, or Krebs, or tricarboxylic acid cycle, β -oxidation of fatty acids, and oxidative phosphorylation, as a result of which ATP is produced. ATP is a molecule whose hydrolysis provides the energy required for the most cellular reactions. (Newsholme & Leech 2009, 183-184.) Glycolysis is also part of the cellular energy metabolism but unlike the above processes it takes place in the cytosol.

2.2.1 Krebs cycle

The citric acid cycle takes place in the mitochondrial matrix. When pyruvate derived from glycolysis is transferred to the mitochondrion, it is oxidized by pyruvate dehydrogenase. The result of this reaction is the formation of acetyl-CoA. (Koolman & Röhm 1996, 125-126.) After this reaction, acetyl-CoA enters the Krebs cycle, which is shown

in figure 3. During this cycle, 3 NADH molecules, 1 FADH₂ and 1 GTP molecule are formed. NADH and FADH₂ are used then as electron carriers to the inner membrane of mitochondria where oxidative phosphorylation takes place. (Newsholme & Leech 2009, 183.)

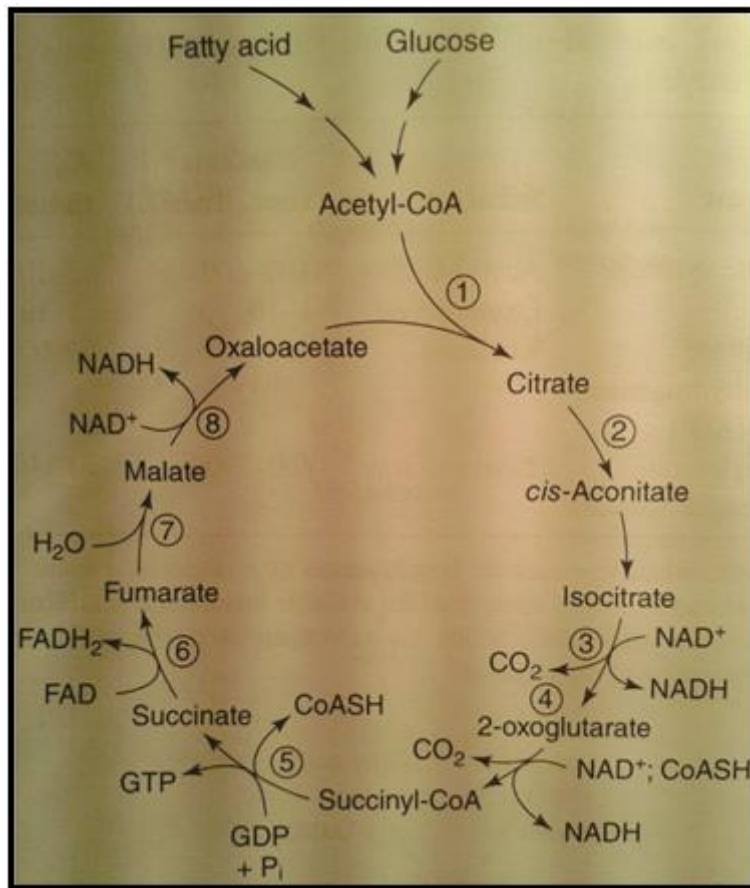


FIGURE 2. The scheme of the citric acid cycle. The enzymes which catalyze the reactions of the cycle are: 1) citrate synthase, 2) aconitase, 3) isocitrate dehydrogenase, 4) oxoglutarate dehydrogenase, 5) succinyl CoA synthetase, 6) succinate dehydrogenase, 7) fumarate hydratase, and 8) malate dehydrogenase. (Newsholme & Leech 2009, 183, modified)

Fatty acids and amino acids can also be used to produce energy in the case there are no carbohydrates available (Koolman & Röhm 1996, 126).

2.2.2 Oxidative phosphorylation and electron transport chain (ETC)

The most effective energy-producing process is the oxidative phosphorylation. Before it happens, electrons from the oxidation of electron carriers pass through the respiratory complex of ETC to the final electron acceptor (oxygen in aerobic organisms). NADH derived from glycolysis and the citric acid cycle is oxidized during this process by complex I to NAD^+ and donates an electron to the ETC. FADH_2 is oxidized by complex II (Cooper 2000). Electrons then flow through the remaining three protein complexes, which are situated in the inner mitochondrial membrane (figure 3). In the complex IV, or cytochrome c oxidase, electrons are transferred to the oxygen, which is then reduced to water. During the reduction and oxidation of complexes I, III and IV, protons are transferred from the matrix space to the intermembrane space. The exact number of protons passed through each complex is not clear, but the consensus is 4 protons for complexes I and III and 2 protons for complex IV. The movement of protons through the inner membrane results in the formation of an electrochemical gradient. (Koolman & Röhm 1996, 130-131.)

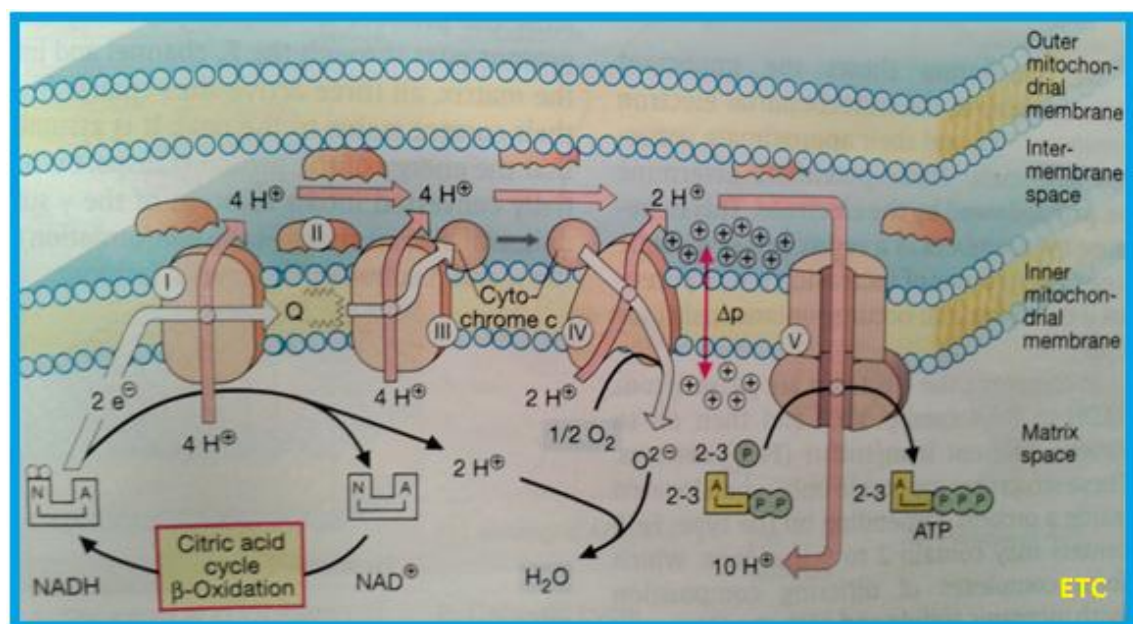


FIGURE 3. The simple scheme of ETC (Koolman & Röhm 1996, 131, modified)

The reaction of oxidative phosphorylation takes place in complex V. The difference between the charges of matrix and intermembrane space grows until a certain limit, after which protons flow back through complex V or ATP synthase. (Newsholme & Leech

2009, 186-187.) Complex V synthesizes ATP from ADP and the molecule of phosphate taken from the matrix space.

2.2.3 ROS

Reactive oxygen species (ROS) are chemical reactive molecules. This group includes superoxide anion, hydrogen peroxide, hydroxyl radical, and nitric oxide. Increased levels of ROS can cause damage to different parts of the cell, including nucleic acids, proteins and lipids. Oxidative stress or imbalance in the redox state of the cells is associated with some age-related diseases, such as Parkinson's disease, cancer or diabetes (Khansari et. al. 2009, 73). However, the role of ROS is not restricted only to the damage to cell components. In particular, they are a part of immune response to the invasion of external organisms or materials. ROS work also as signaling molecules in such processes as proliferation, antioxidant gene regulation, apoptosis, etc. (Ray et al. 2012, 981). The greatest part of the ROS is formed during the transport of electrons through ETC. (Held 2010, 1-11.)

Finally, ROS can increase the longevity of some animals as worms or flies. This is explained by increased adaptive response induced by stress which can protect cells further during chronological aging (mitohormesis). (Ristow & Zarse 2010, 410.)

2.3. References and candidates for biomarkers of aging

Since the research in the host laboratory is focused on the role of mitochondria in the process of aging, genes involved in the energy metabolism and maintenance of mitochondria were selected. The genes of interest and references used in this work are presented in table 1. All genes are protein-coding.

TABLE 1. Genes used in the experiment.

	Gene name	Description
References		
	Actin88F	Muscle isoform of actin
	GAPDH	Glyceraldehyde's 3-phosphate dehydrogenase
	Rpl32	Gene, which encodes ribosomal protein L32
	Actin5C	Cytoplasmic isoform of actin
	Tbp	TATA binding protein gene
	Hsp83	Heat shock protein gene with size 83 kDa
Genes of interest		
	Impl3	Lactate dehydrogenase, ecdysone-inducible gene L3
	39kDa (CG6020)	Mitochondrial NADH-ubiquinone dehydrogenase subunit 9
	COX I	Mitochondrial cytochrome C oxidase subunit I
	SOD2	Superoxide dismutase 2
	Aconitase	Aconitate hydratase
	Hsps-10,-22,-60,-70	Heat shock proteins with sizes 10, 22, 60, 70 kDa
	Pepck	Phosphoenolpyruvatecarboxykinase
	AcCoAs	Acetyl coenzyme A synthase
	Pgi	Phosphoglucoseisomerase
	Pgm	Phosphoglucose mutase
	Pfk	Phosphofructokinase
	Tpi	Triose phosphate isomerase
	whd(CG12891)	Withered (carnitine O-palmitoyltransferase)
	yip2	Gene, which encodes yippee interacting protein 2
	CG11198	Acetyl-CoA carboxylase

2.3.1 References

References (housekeeping genes) are genes which are used to exclude possible variations in gene expression resulting from differences in the sample preparation, like small mistakes in pipetting. The expression of a reference must be stable under the conditions of the experiment in every tissue throughout the development (Zhang et al. 2005). The references used in this work were chosen because, based on the observations in the host laboratory, they remained stable under oxidative stress.

Actin 88F encodes the muscle isoform of actin related to the formation of cytoskeleton microfilaments. Actin 88F is involved in skeletal myofibril assembly and phagocytosis. (Genes 2013.)

Actin 5C encodes the cytoplasmic isoform of the protein of the actin family. Biological processes in which it is involved are cytokinesis, phagocytosis, and the maintenance of protein location in cell. (Genes 2013.)

The molecular function of GAPDH is glyceraldehyde-3-phosphate dehydrogenase activity. The product of GAPDH catalyzes the sixth step of glycolysis. (Genes 2013.)

Rpl32 encodes a ribosomal protein that is a component of the 60S subunit of eukaryotic (80C) ribosome. It is also involved in the mitotic spindle elongation. (Genes 2013.)

Tbp encodes transcription factor - TATA binding protein. It is involved in transcription initiation from RNA polymerase II promoter, snRNA and tRNA transcription from RNA polymerase III promoter. (Genes 2013.)

Hsp83 is one of the heat shock proteins. It is involved in cellular component biogenesis, organelle organization, cell cycle, response to cold, and gamete generation. (Genes 2013.)

2.3.2 Genes of interest

Impl3 is a protein coding gene from *Drosophila melanogaster*. It encodes lactate dehydrogenase which catalyzes the conversion of pyruvate to lactate at the end of glycolysis. (Genes 2013.)

39 kDa encodes the subunit of mitochondrial complex I with molecular mass 39 kDa. It is involved in the transport of electrons from NADH to ubiquinone in ETC. (Genes 2013.)

COX I encodes the core subunit of mitochondrial complex IV of ETC encoded in mitochondrial DNA. It is involved in the terminal reduction of oxygen to water with four electrons and two protons. (Genes 2013.)

SOD2 encodes a mitochondrial matrix manganese superoxide dismutase protein that transforms toxic superoxide, a byproduct of the mitochondrial electron transport chain, into hydrogen peroxide and molecular oxygen (Held 2010, 2). Hydrogen peroxide can be then converted to water. SOD2 is involved in the determination of adult lifespan, regulation of metabolic process through manipulation of ROS level, response to oxidative stress, and removal of superoxide radicals. (Genes 2013.)

Aconitase encodes a protein which catalyzes the second step of Krebs cycle, and thus its molecular function is aconitase hydratase activity. It contains iron-sulfur clusters which are not stable and are readily disassembled under oxidative stress conditions. Iron from the cluster can react with hydrogen peroxide and convert it into hydroxyl radical, which is the most dangerous mitochondrial free radical. (Genes 2013.) Since aging is accompanied by the increased level of ROS, the level of aconitase expression is presumed to change as well.

Hsps are usually overexpressed when cells are exposed to stress, such as temperature rise, inflammation, or oxidative stress. These conditions can cause the unfolding of many proteins, which can result in the aggregation of proteins, loss of their functions, and cell death. During the unfolding, the protein exposes its hydrophobic domains. HSPs cover these domains protecting the protein from the after-effects of unfolding or bind to these domains providing refolding of these proteins and getting into the proper conformation. (Brock 2008, 36.)

Hsp22 encodes one of the chaperones widely used as oxidative stress marker. It is involved in the determination of adult lifespan, response to oxidative stress, and response to heat. Hsp60 encodes a chaperone which is involved in cellular response to heat, mitochondrion organization, protein targeting to mitochondrion, “de novo” protein folding, and protein refolding. Hsp70 encodes a chaperone which is involved in the following biological processes: response to hypoxia, heat shock-mediated polytene chromosome puffing, and response to heat. (Genes 2013.) Hsp10 encodes a chaperone involved

in protein folding and its expression is increased during cancer or autoimmune inhibition (Haibo et al. 2010, 47).

Pepck encodes the enzyme phosphoenolpyruvate carboxykinase which catalyzes the conversion of oxaloacetate to phosphoenolpyruvate in gluconeogenesis. Gluconeogenesis is a process of glucose formation from other compounds than carbohydrates. (Genes 2013.)

AcCoAs encodes a protein, which creates AcCoA from acetate and CoA and is involved in behavioral response to ethanol and acetyl-CoA biosynthetic process from acetate. It activates acetate so that it can be used for energy production or the synthesis of lipids. (Genes 2013.)

Pgi encodes a protein, which is involved in gluconeogenesis and glycolysis. Its molecular functions are glucose-6-phosphate isomerase activity in glycolysis and phosphogluconate dehydrogenase (decarboxylating) activity in pentose phosphate pathway. (Genes 2013.) Pentose phosphate pathway is a process of forming NADPH and the synthesis of five-carbon sugars (Berg, Tymoczko & Stryer 2002).

Pgm encodes an enzyme involved in the synthesis and breakdown of glucose, flight and lateral inhibition. Its molecular functions are phosphoglyceratemutase activity and phosphoglucomutase activity. (Genes 2013.)

Pfk encodes phosphofructokinase which catalyzes the conversion of fructose 6-phosphate to fructose 1,6-bisphosphate in glycolysis (Genes 2013). This reaction is irreversible and thus it is one of the main regulatory points for the glycolytic pathway.

The molecular function of Tpi is triose-phosphate isomerase activity. The protein encoded by this gene is involved in glycolysis (5th step), response to mechanical stimulus, the determination of adult lifespan, and neurological system process. (Genes 2013.)

Withered is involved in such biological processes as response to starvation, response to oxidative stress, and response to metal ions. (Genes 2013.)

Yip2 is involved in fatty acid beta-oxidation. Its molecular function is acetyl-CoA C-acyltransferase activity. (Genes 2013.)

The molecular function of CG11198 is biotin carboxylase activity, ATP and metal ion binding, and acetyl CoA carboxylase activity. The protein encoded by CG11198 is involved in fatty acid biosynthesis and cell morphogenesis. (Genes 2013.)

2.4. PCR

The levels of expression of genes were studied using the PCR, Reverse Transcriptase-PCR (RT-PCR) and quantitative-PCR (qPCR) techniques.

PCR is a process of amplification of DNA or cDNA fragments in order to estimate the level of gene expression or DNA copy number. PCR is carried out in a thermocycler and it is an automated process. The technique was discovered by Kary Mullis, a Nobel Prize laureate, while he was driving in the moonlight through the mountains of northern California.

RT-PCR and qPCR are the most used techniques to detect the levels of RNA expression. In this experiment, RT-PCR was used to synthesise cDNA from isolated total RNA. Common PCR was used to check the efficiency of cDNA synthesis and the presence of DNA in RNA samples. In RT-PCR, RNA is used as a template and there is just a sequence of temperatures instead of cycles. Reverse transcriptase is used for the extension of primers.

qPCR was used to determine the amount of transcripts in the sample. In qPCR, the detection of amplification is measured in real time, so that it can be seen after each cycle. To label PCR product, different fluorescent dyes are used. In this experiment, SYBR Green dye was used to detect the amplification. This dye intercalates between two strands of DNA helix and emits fluorescent light only if it is intercalated. Fluorescence increases with each cycle of PCR proportionally to the amount of product. SYBR Green has a maximum excitation of 495 nm and a maximum emission of 521 nm. (SYBR Green based qPCR 2013.) The detection takes place in the stage of extension. Also non-specific PCR products and primer-dimers can be labeled by SYBR Green, therefore, the

specificity of primers to target gene must be high. (Critical factors for successful Real-Time PCR 2009, 5.)

2.4.1 Steps of common PCR

Common PCR includes 3 steps: DNA denaturation, primer annealing and extension. All steps can be repeated 28-35 times, and the amount of the product increases exponentially. (McClellan 1997.)

In the denaturation step, double-stranded DNA is heated to around 95 °C for conversion to single-stranded DNA. (McClellan 1997.)

In the next step, specific primers bind to the complementary strands. The temperature of annealing can be at the range 55-65 °C and it depends on the properties of the primers. (McClellan 1997.)

In the last step, heat-stable DNA polymerase extends primers to obtain a double-stranded product (McClellan 1997). The temperature of the last step depends on the polymerase which is used in reaction; usually it is around 72 °C. High temperature in this step makes possible a high rate of the reaction. That is why polymerases are isolated from thermophilic bacteria, such as mostly used *Thermus aquaticus*. (Boyer 2012, 302.)

Reagents needed for PCR are DNA template, primers, DNA polymerase, and nucleotides. Template or target gene can be in a chromosome, plasmid or virus or it can be just a piece of DNA. Primers are around 20 base pairs long laboratory designed fragments of DNA, whose order of nucleotides is complementary to the 3' end of each strand in the amplified DNA fragment. DNA polymerase enzyme binds to the primers and adds nucleotides to the OH group in the 3' end of the strand. Nucleotides in the form of deoxynucleosides triphosphates (dATP, dGTP, dCTP and dUTP) are used for extension of the new strand. (Farrell & Taylor 2006, 359-361.) Also appropriate buffer is needed to maintain optimal pH for polymerase, and magnesium ions are used as its co-factor.

2.5. *Drosophila melanogaster* and genetics

Drosophila melanogaster is one of the most studied organisms in genetics. In this work, one of the study objects was a mutant line of fruit fly whose mitochondrial superoxide dismutase was overexpressed.

SOD2 overexpressor flies carry a UAS-SOD2 construct that allows the overexpression of the SOD2 gene when the protein GAL4 binds to the Upstream Activator Sequence. This is achieved by crossing the line carrying the UAS-construct (in this case SOD2) with another line that expresses the protein GAL4 and one specific promoter, in this case *daughterless* (that allows moderate but ubiquitous expression). Thus, the driver line (*daughterless*-GAL4) constantly stimulates the transcription of the gene of the expressor line (UAS-SOD2). These constructions are widely used in *Drosophila melanogaster* to find out how the upregulation of a certain gene can affect metabolic processes in the fly organism.

When overexpressed, SOD2 must decrease the amount of ROS in the cell. It has been shown in the previous section that SOD2 encodes for the mitochondrial superoxide dismutase which is involved in the elimination of superoxide anion, one of the by-products of mitochondrial respiratory chain function. However, the overexpression of SOD2 does not extend lifespan (Appendix 2, C).

In the experiment with SOD2 overexpression, there were 3 experimental groups: SOD2>dG (experimental flies carrying an UAS-SOD2 construct that expresses SOD2 in the presence of the protein GAL4), w>dG (control flies with the *daughterless* driver that expresses ubiquitously the GAL4 protein), and SOD2>w (control flies carrying an UAS-SOD2 construct but without the expression of the SOD2 protein since the GAL4 protein is absent).

3 MATERIALS AND METHODS

The levels of transcripts of the selected gene were studied using qPCR technique. Before this, the total RNA was extracted from frozen flies, and cDNA was made using RT-PCR.

3.1. Flies for the experiment

All flies were cultivated using standard cornmeal diet (Sanz et al. 2010², 136) at 25 °C, with the exception of group N 6, where temperatures 18 and 29 °C were used. Flies can survive in the temperature interval between 10 and 32 °C, but 25 °C is the optimal one.

Flies were grown until a specific age (5, 25, 45, 75 days old or when 100 (young), 80 (old), and 50% (very old) of the starting population were still alive), then frozen in liquid nitrogen and stored at -80 °C. Correlation between chronological (days) and biological (% of survival) aging is shown in table 2.

TABLE 2. Correlation between chronological and biological lifespan in *D. melanogaster*.

% alive		100	85	82	66	43
days old	DAH	3-5	56	63	74-77	79-81
	OR	3-5	36	51	79-81	84-86

Females were used as samples with the exception of one group N 3 where males were used. Samples are shown in table 3. All flies with the exception of group N 5 (SOD2 overexpressor flies) were wild-type strains.

Both the expressor (UAS-SOD2, Stock Number 24494) and the driver (*daughterless-GAL4*, Stock Number 27608) lines of SOD2 overexpressor flies were obtained from Bloomington stock center (<http://flystocks.bio.indiana.edu/>) and then backcrossed 6-7 times with DAH flies to exclude the possible influence of genetic background.

TABLE 3. Flies for the experiment.

	Flies
1	Dahomey ♀, 5, 25, 45, 75 days old
2	Oregon R ♀, 5, 25, 45, 75 days old
3	Dahomey ♂, 100, 80, 50 % alive
4	Dahomey ♀, 100, 80, 50 % alive, flying flies
5	SOD2 overexpressor ♀, 100, 80, 50 % alive
6	Dahomey ♀, 100, 80, 50 % alive, grown at 29 °C and 18 °C

Three last groups were chosen for the experiment to see how regulation of ROS production affects the levels of expression of selected genes. In Dahomey flying flies level of ROS is increased since flies are able to move more. In SOD2 overexpressor flies the level of ROS is presumed to be decreased by overexpression of SOD2, which decreases mitochondrial superoxide anion concentrations. In the last group flies were grown at different temperatures, and it is known, that at higher temperatures ROS production is increased while lower temperature reduces it.

3.2. RNA extraction

Total RNA extraction was made by homogenizing flies (10/vial) in TRI reagent (MRC). After the first step, a short incubation was made to provide complete lysis of cells (5 min). TRI reagent method is tailored to be performed on fruit flies. The solution contains guanidinium thiocyanate and phenol and is used also for the extraction of DNA and proteins. The solution inhibits RNase activity and facilitates the maintenance of RNA integrity. (Molecular Research Center 2012.) RNA is susceptible to enzymatic and non-specific degradation, so all the samples and reagents were kept on ice between the isolation steps.

After homogenization, chloroform was added to separate RNA in the water phase from the rest of the cell debris and samples were centrifuged at +4 °C. RNA stays in the aqueous phase, DNA in interphase, and proteins in organic phase. Supernatant with RNA was transferred to the new tube and isopropanol was added to precipitate RNA out of the solution. The pellet of RNA was washed with 75% ethanol. After removing the ethanol, pellets were dried at room temperature (RT) and dissolved in PCR-water

(DNase and RNase free water from Bioline). DNase (ThermoScientific) was added to the solution with RNA (40 units/probe) and the reaction was incubated 1 hour at 37 °C. After this step, ethanol precipitation was made with 300 mM of sodium acetate (pH 5,2) and 95% ethanol at -20 °C overnight (precipitation and RNA yield is increased at a low temperature). Sodium acetate contains ions of sodium needed for nucleic acid precipitation and it helps precipitation by making solution acidic. After this, three series of centrifugation were performed and the pellets were washed with 75% ethanol. The pellets were dried 5 minutes at RT and dissolved in PCR-water. The concentration of RNA was measured by NanoDrop2000 spectrophotometer and adjusted to 1ug/ul for further work. A few microliters of extracted RNA was used to make cDNA and the rest of RNA stored at -80 °C before the actual use.

3.3. cDNA synthesis

For each biological replicate, 3 separate cDNA synthesis reactions were carried out and then pooled together. Two master mixes were prepared (table 4). Random hexamer primers were used, so cDNA was synthesized from the total RNA. All reagents excluding PCR-water were obtained from ThermoScientific.

TABLE 4. Master mixes for cDNA synthesis.

MM I	
	PCR water
	10 mM dNTP in DEPC
	0,2 ug/ul random hexamer primers
MM II	Reverse transcriptase buffer
	40 u/ul RNase inhibitor

The reaction was carried in a thermal cycler with the program shown in table 5. Master mix I was mixed with RNA and put in the step 1: denaturation of RNA which provides unfolding of its secondary structure (Table 5). Master mix II was added after the first step. The second and third steps were needed for the attachment of primers to the RNA strand and for mixing the solution. After the second step, reverse transcriptase was added and the extension of primers took place in the next step. The last step was needed for the inactivation of the ferment.

TABLE 5. Program for cDNA synthesis.

steps	1	2	3	4
°C	90	25	37	70
time, min	3	10	60	10

After the cDNA synthesis, the product was checked with common PCR. Rpl32 primers (c=20uM/L) were used to check the efficiency of the cDNA synthesis and DNA contamination. Rpl32 primers are designed to amplify a portion of two different exons separated by an intron. The amplification of DNA with these primers produces a product of 184 base pair (bp). The amplification of cDNA produces a smaller product of 125 bp since it does not contain introns. Phusion polymerase (c=2u/ul) was used with a special buffer for it. The program for PCR is shown in table 6. Steps 2-4 were repeated 35 times.

TABLE 6. Program for PCR

steps	1	2	3	4	5
°C	98	98	60	72	72
time	1min	30sec	30sec	30sec	10min

After that, electrophoresis was made with the product from PCR. An example of the results of electrophoresis is shown in figure 4. Size of the product was defined by using GeneRuler DNA ladder (ThermoScientific). cDNA was stored at -20 °C.

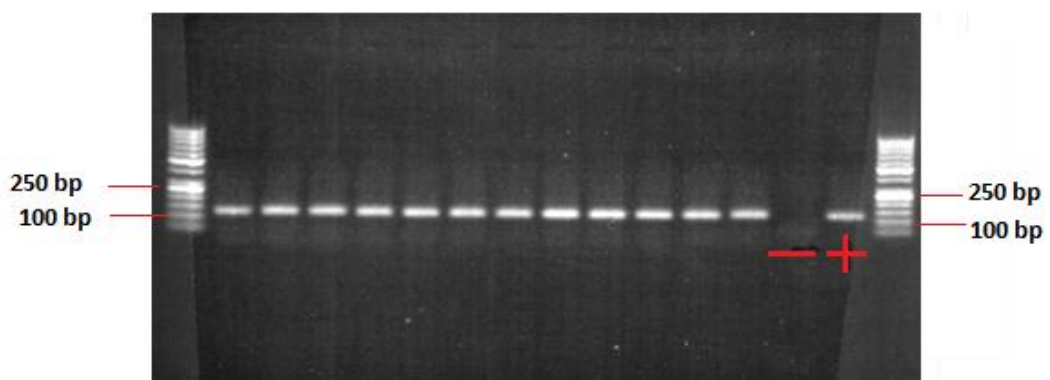


Figure 4. Electrophoresis of Rpl32 fragments. The size of amplicons is ~125 bp. 14 and 15 wells are negative and positive controls, respectively.

3.4. qPCR

To estimate the levels of gene expression, an absolute quantification was selected. A standard curve with four different points was prepared. The first point of the calibration curve was made by mixing equal amounts of each cDNA sample and then diluted 1:5. Next three points were prepared using serial 10-fold dilutions from the first point. For qPCR, a 1:20 dilution of the sample was made, and 3 replicates were done. With these dilutions, the samples fit within the calibration curve.

The amount of cDNA to prepare the calibration curve depends on the total number of samples and the expected amount of PCR runs. For one qPCR reaction, 4 ul of diluted sample is needed, so calculations were made according to this. qPCR was carried out on a special qPCR plate. Besides samples and calibration solution, negative control was needed to check the presence of DNA contamination in reagents. Samples, negative control and calibration points have been made in triplicates. The master mix was made with PCR-water, primers (c=20uM/L) complementary for the cDNA fragment of interest, and 2x SYBR Green SensiFast Hi-Rox buffer (Bioline), which includes all reagents required for PCR, with the exception of primers. Parameters for PCR were taken from the SYBR Green Sensi Fast Hi-ROX kit manufacturer's guide.

TABLE 7. Program for qPCR. The reading of fluorescence takes place at the last step of the cycling stage.

	Holding stage	Cycling stage, 40 cycles		
T, °C	95	95	60	72
Time	2 min.	5 sec.	10 sec.	10 sec.

Expression values for the reference genes and the genes of interest were obtained using StepOne software (Applied Biosystems). The mean of the replicates was calculated with Excel (Microsoft Office TM). Prism was used to calculate the mean from the samples, to build graphs, to calculate standard deviation (variability between samples in one group), and to analyze the statistical difference between the levels of gene expression (Prism 2013). Statistical significance was calculated by one-way ANOVA with Newman-Keuls test and was considered significant when P-value was less than 0,05.

Initial expression values for each gene of interest were normalized to the reference gene. Normalizing of the results by the reference means that the amount of target gene transcripts is divided by the amount of reference transcripts. References undergo the same procedure as the genes of interest, so dividing compensates mistakes which can occur during the processing of samples. After normalization by reference, the amount of transcripts was also normalized to a control group specific for each experiment. Normalization was made to the mean of replicates from the control groups. In the experiments with Dahomey and Oregon R aged to 5, 25, 45 and 75 days, the control group was 5 days old flies. In the rest of the strains, 100% alive flies were used as a control. In the case of references, the mean amount of transcripts in the control groups was taken as one.

4 RESULTS

The results are shown as graphs which represent the levels of gene expression. During the experiment, all references shown in table 1 were tested, and it was revealed that Act88F was the most stable in all the studied strains. That is why it was used in calculation to normalize all the results. Figure 5 shows the changes of expression in other references. The results for Actin88F are shown in figures 6-9.

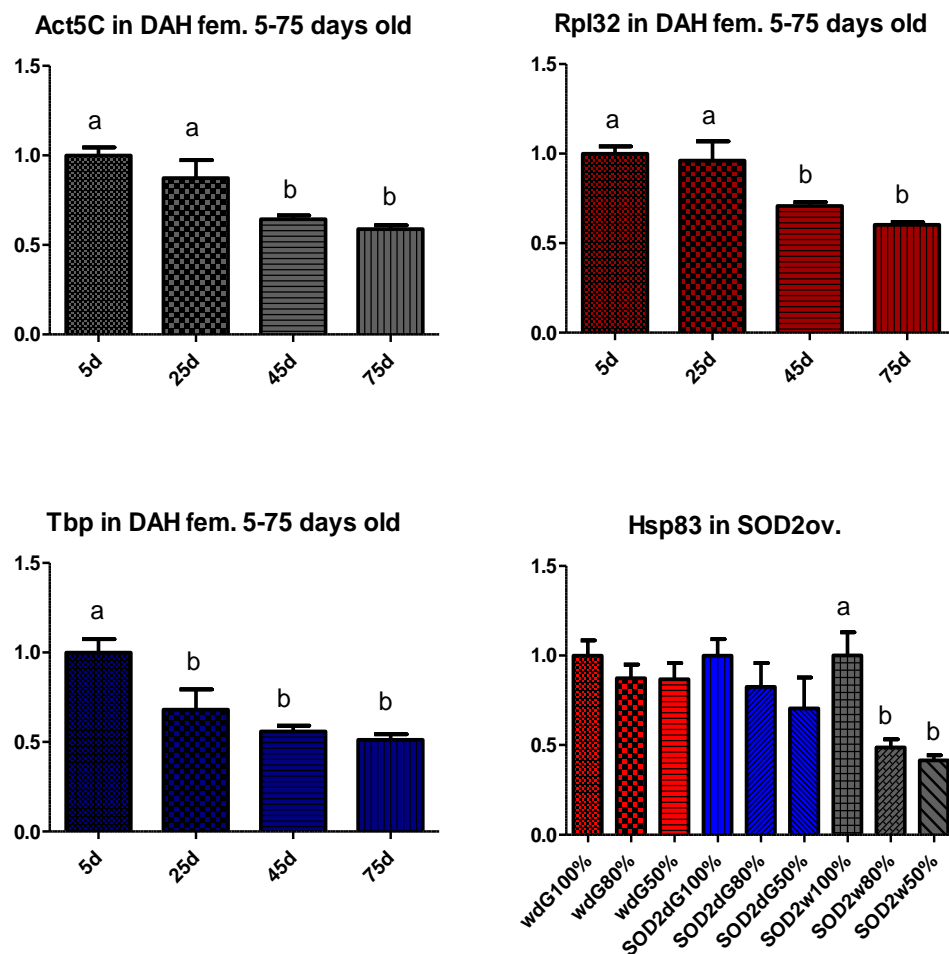


Figure 5. Levels of expression of the references analyzed by PRISM. The data are shown as mean and standard deviation.

X-Axis – the age of flies, y-axis – the amount of transcripts, normalized to the control within the group.

Different letters mean that there is a significant difference between the columns.

Table 8 shows which genes change with the age in different strains of fruit fly. First, all the genes were checked on Dahomey females aged to 5, 25, 45, and 75 days old and then in other experiments. If the expression of the gene did not change or changed insignificantly, it was not used in the following experiments. That is why in table 8 there are no results for all the genes in every group. Figures 5-9 show the data from Prism. Graphs for the genes whose levels of expression did not change or changed slightly or just in a few strains are not presented here.

TABLE 8. Changes in the levels of expression of different genes during aging. (+) means that there is significant correlation ($P < 0,05$) in the expression, (-) means that there is no correlation, and (~) means a non-significant trend in the expression. Blank means that these genes were not studied in that specific experiment.

Genes	♀Dah 5-25-45-75d	♀ Ore-gon R 5-25-45-75	♂ DAH 100-80-50% alive	♀ DAH flying100 -82-40% alive	♀ SOD2 OV 100-80-50% alive	DAH ♀, 29°/18 °C, 100, 80, 50% alive
GAPDH	-	+	+	+		
Rpl32	+					
Act88F	+	-	+	~+	-	-
Act5C	+					
Tbp	+					
Hsp83					+	
Impl3	++	+	~+	+	++	+
39kDa	~+	-				
COX1	~+	~+		-		
SOD2	-	+	~+			
Acon	-	+	-			
Hsp60	-	-				
Hsp22	~+	++	++	-	+	+
Hsp70	++	~+	+	-	+	+
Hsp10	-	~+				
Pepck	++	-	+	+	~+	
AcCoAS	+	-	+	++	-	
Pgi	+	-	+	~+	-	
Pgm	-					
Pfk	-					
Tpi	-					
whd	+	-	-	-	-	
yip2	+	-	~+	-	-	
CG11198	-					

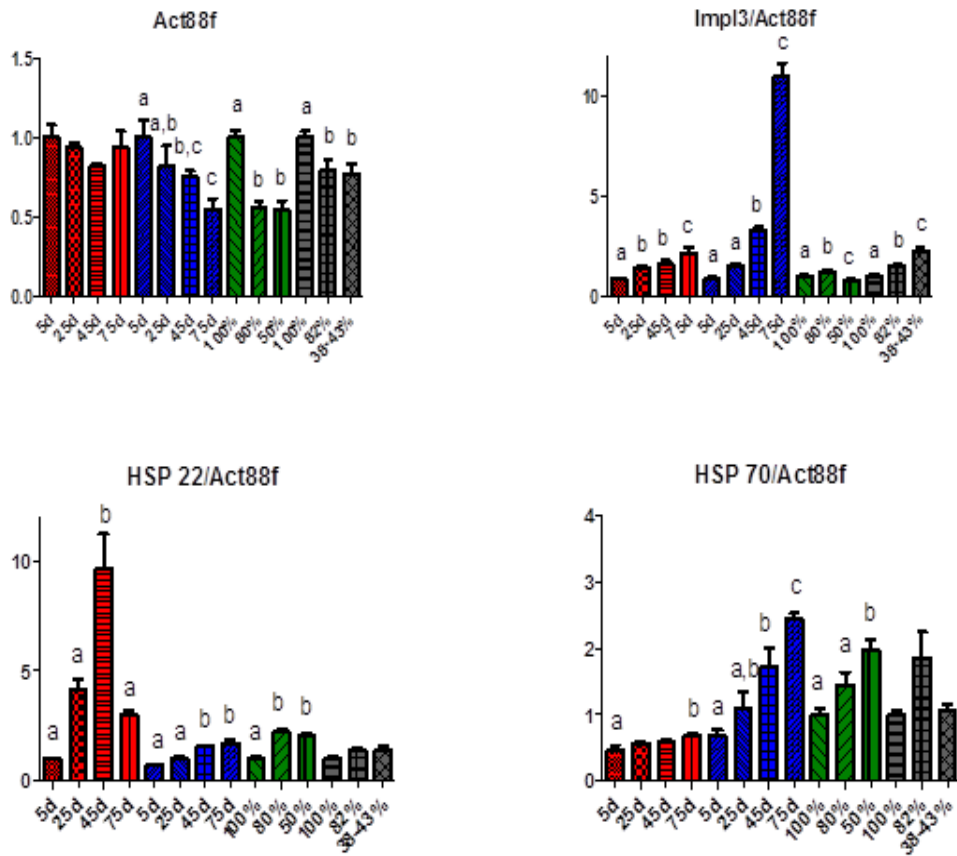


FIGURE 6. Levels of expression analyzed by PRISM. Red – OR, blue – DAH, green – DAH males, gray – DAH flying. The data are shown as mean and standard deviation. X-axis – the age of flies, y-axis – the amount of transcripts, normalized to the control within the group.

Different letters mean that there is a significant difference between the columns.

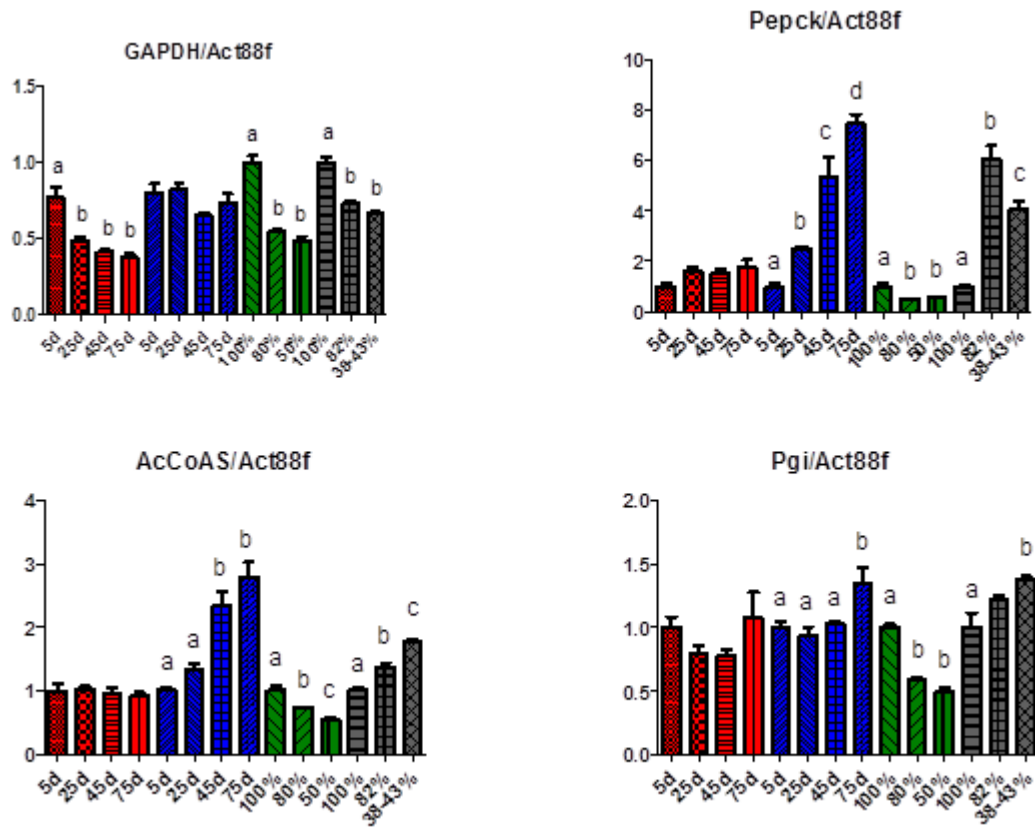


FIGURE 7. Levels of expression, analyzed by PRISM. Red – OR, blue – DAH, green – DAH males, gray – DAH flying. The data are shown as mean and standard deviation. X-Axis – the age of flies, y-axis – the amount of transcripts, normalized to the control within the group.

Different letters mean that there is a significant difference between the columns.

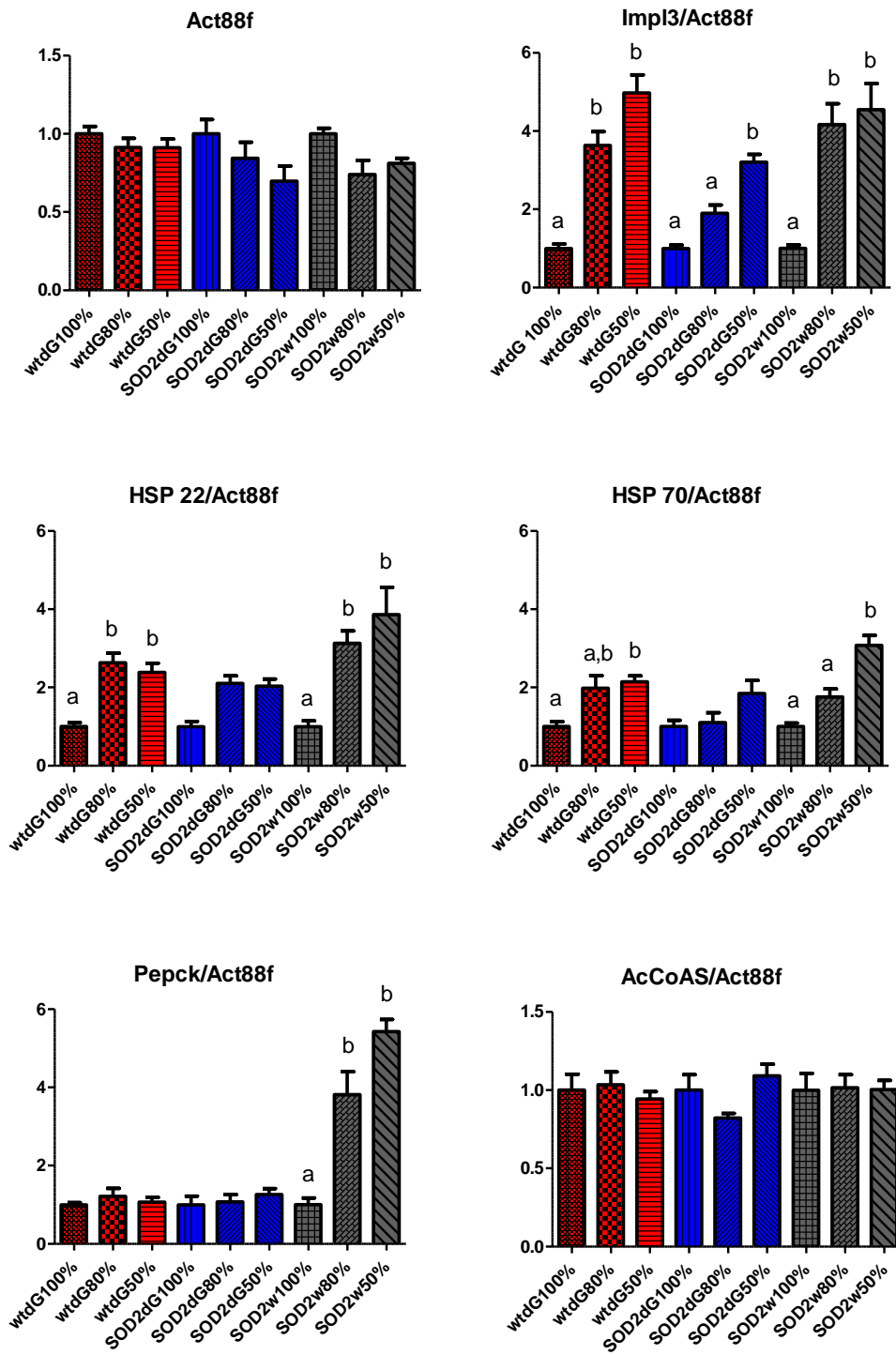


FIGURE 8. Changes in the levels of expression in SOD2 overexpressor flies and controls. The data are shown as mean and standard deviation.

X-Axis – the age of flies of overexpressors and controls, y-axis – the amount of transcripts, normalized to Actin88F and to the control within the group.

Different letters mean that there is a significant difference between the columns.

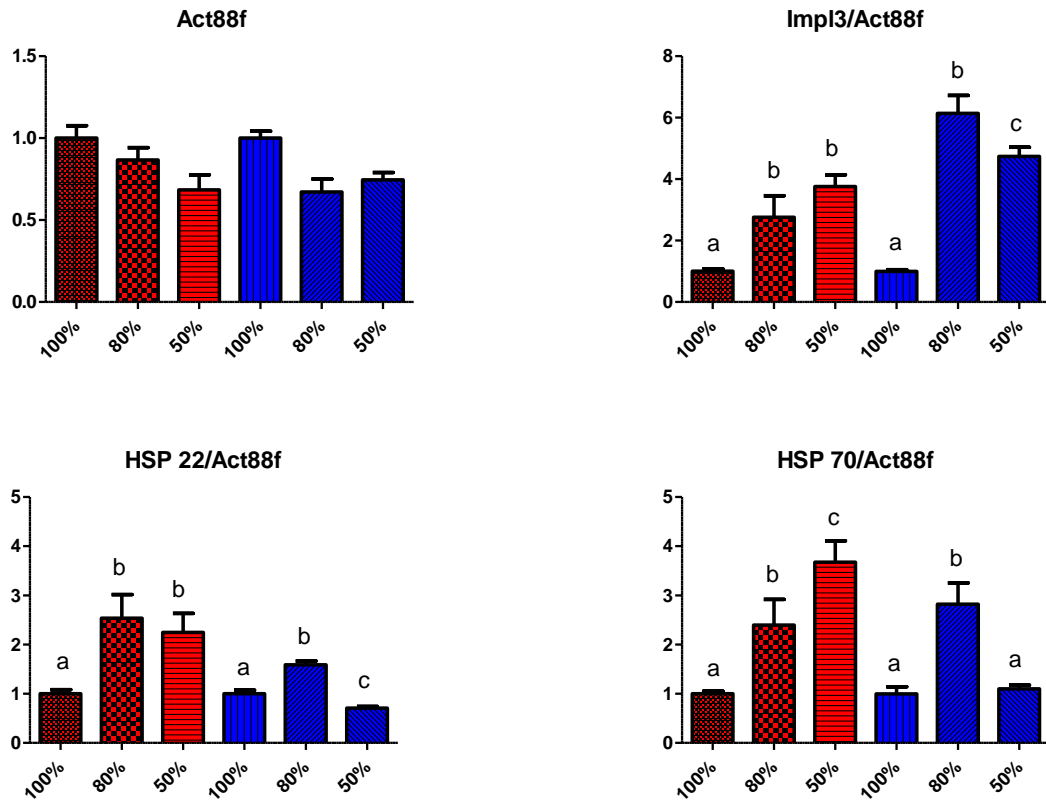


FIGURE 9. Changes in the levels of expression in DAH, grown at different temperatures. Red – 18 °C, blue – 29 °C. The data are shown as mean and standard deviation.

X-axis – the age of flies, y-axis – the amount of transcripts, normalized to the control within the group.

Different letters mean that there is a significant difference between the columns.

5 DISCUSSION

Only one gene (*Impl3*) whose expression changed in all the experiments done during aging was found. However, the expression of this gene was only slightly changed in Dahomey males. There were also a few other genes which can be used as biomarkers of aging at least in some experimental situations. They are *Hsp70* and *Hsp22*. The expression of these genes did not change in flies with increased flying activity and changed slightly in females of Dahomey (*Hsp70*) and Oregon R (*Hsp22*). The lifespans of Dahomey and Oregon R are provided in the Appendix 2, A. OR flies live noticeably longer than DAH. It can be seen from figures 6 and 7 that in DAH, the expression of most of the best candidates for aging changes distinctly during aging. In OR, only the change of expression in *Hsp22* is sharp. In DAH, the change in the expression of this gene is slight.

One of the main things in the estimation of gene expression levels is validation of the references. The references must be abundantly expressed and stay stable under the conditions of the experiment, so that the results of the calculation can be reliable. From table 8 and the graphs, it can be seen that in the experiments with some of the strains (1, 3 and 4, table 8), the expression of *Act88F* decreased. This can be a result of a common decrease of protein biosynthesis in the organism occurring with age. On the other hand, it could indicate damage in mRNA, since it has been described previously that mRNA in general is oxidized during aging (Hofer et al. 2008, 563). Oxidized mRNA might be a problem for reverse transcriptase, and less cDNA or with less quality would be synthesised. In the third group (DAH males), the decrease in the level of transcription for the references is even more obvious, and this correlates with the fact that the lifespan of males is much shorter than that of females (Appendix 2, A). Anyway, as one can see in the figures, the impact of the decrease of the reference gene expression on the changes in the target gene expression level is insignificant.

Now things can be considered in more detail starting from *Impl3*. This gene encodes protein that catalyzes the conversion of pyruvate to lactate at the end of glycolysis. Increase in the expression of *Impl3* can result in the increased formation of lactate. This may mean that the producing of pyruvate is also increased. However, it can be asked why this occurs with age. It is proved that the activity of mitochondrial ETC decreases

with age (Fergusson et al. 2005, 501). That is why the system tries to obtain more energy using alternative pathways. During glycolysis, 2 molecules of ATP are created, so increased glycolysis rate is one option if ETC does not function properly. However, this strategy will raise lactate concentration making the environment acidic, which can adversely affect the cell and the whole organism homeostasis, and possibly the lifespan.

Two other genes with increased levels of expression were Hsp70 and Hsp22. As it was discussed earlier, heat shock proteins can refold other proteins under stress conditions. This means that the amount of unfolded proteins increases with age which can be a result of oxidative damage to cell compartments, metabolic dysfunction, etc.

The expression of Pepck was increased dramatically in Dahomey females. Pepck encodes phosphoenolpyruvate carboxykinase which catalyzes the conversion of oxaloacetate to phosphoenolpyruvate in gluconeogenesis. This process is induced when there is no glucose to start glycolysis in tissues. In gluconeogenesis, glucose can be formed from pyruvate, lactate, glycerol, and amino acids, etc. (Newsholme & Leech 2009, 113). Increasing levels of Pepck expression can be explained by glucose starvation of tissues. It has been shown in a few experiments that the expenditure of glucose in organism slows down during aging (Davidson 1979, 688). Dahomey females become aged faster than Oregon R females in which expression of Pepck does not change during aging (figure 7). This difference in lifespans may be due to the altered glucose metabolism in Dahomey females.

From figure 9, one can see that at a higher temperature (29 °C), the expression of studied genes is increased in 80% but drops in 50% alive flies, unlike as in flies grown at a normal temperature. This can be a result of metabolism dysfunction related to the continuous stress induced by an adverse temperature. Indeed, the flies grown at a high temperature live at least 3 times shorter than the flies grown at a low temperature (Appendix 2, B).

The lifespans of SOD2 overexpressor flies and controls are provided in the Appendix 2, C. It should be noted that the overexpression of superoxide dismutase II does not affect the lifespan much, and there is no difference between the strains in gene expression levels with exception of Pepck, whose expression does not change with age in wtdG and

SOD2dG, but is significantly increased in SOD2wt. To find out the reason of such a difference, more experiments should be done.

The lifespans of flying and non-flying DAH are provided in the Appendix 2, D. Flying flies live significantly less than non-flying. It can be possible that changes in the expression of the following genes are related to the difference in their longevity: Impl3, HSP 70, and Pepck (figures 6 and 7).

From the results obtained in this work, following conclusions can be made:

1. Changes in the expression of Impl3, Hsp22 and -70, and Pepck are related to aging.
2. On the basis of this experiment, Impl3 can be used as a biomarker of aging in all the studied strains.
3. Hsp22 and -70 can be used as biomarkers of aging, although it is possible to find a situation where they are not good predictors of biological aging (i.e. Dahomey flying flies).
4. Obtained results suggest that a different rate of aging can be partially explained by the dysfunction of ETC, oxidative damage, and the dysfunction of glucose methabolism.

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Appendix 1. Sequences of primers

Genes		Sequences
GAPDH	F R	GAC GAA ATC AAG GCT AAG GTC G AAT GGG TGT CGC TGA AGA AGT C
Rpl32	F R	GTT CGA TCC GTA ACC GAT GTT CAC CAG TCG GAT CGA TAT GC
Act88f	F R	AGG GTG TGA TGG TGG GTA TG CTT CTC CAT GTC GTC CCA GT
Act5C	F R	GAG GAA GCA GCA GCG AAA GT TTT TGT TGT GCT GCA CTC CAA
Tbp	F R	GCA TCA TCC AAA AGC TCG GT AGC GTA TGG GGA ACT TGA CA
Hsp83	F R	ACC TGG TCA ACA ACT TGG GA GGA GTA GAA ACC CAC ACC GA
Impl3	F R	CGG GAT TGG ACA CCA TAA GC GCA ACA CCG ACA TCC TCA AG
39kDa	F R	CGC TTA ATC CAC CTG TCC TCT AGC TCA CCC TCG TAC TTG CTT
COX1	F R	CCT GAT ATA GCA TTC CCA CGA A TGT TCC AGC TCC ATT TTC AAC
SOD2	F R	GTC TGG TGG TGC TTC TGG TG GCC CGT AAA ATT TCG CAA AC
Acon	F R	CAA CAT GGG TGC TGA GAT CG TCT TGG CCT GGT ACT TCT GG
Hsp60	F R	ATG TTC CGT TTG CCA GTT TC GAT CAC ATT GCG ACC CTT G
Hsp22	F R	GCT AAA GGT CAA GGT GCT GG CTT GTC CGC CTC GTA TCC TT
Hsp70	F R	CCA ATG GAA TCC TGA ACG TC CCT TGT CGT TCT TGA TCG TG
Hsp10	F R	GCT GAC CAA GAC GAA AGG AG GTT GCC AGT GGA GGC ATT AC
Pepck	F R	GAC GAC ATT GCGTGG ATG AA CAT GGC AAT GGG ATT GGT CT
AcCoAS	F R	CAA CAA CCA TGA ACG CTTCG TCC ACA AAT AGC CGT CAG CA
Pgi	F R	GCC TTC TAC CAG CTC ATC CA GCC ATT AAA GCC TCC GTC TG
Pgm	F R	GGG CAA GGA AGT GTT TGA GG CCC AGA TGC CAT CCT TTT CG
Pfk	F R	GCG GAA TCT GGA GAC CTA CA CTT CGA TTC CCT TGC CAT CG

Appendix 1, continuation

Tpi	F	ATA GAT GGC TTC CTG GTC GG
	R	GCT TGA GAT CGC GTT GCT TA
whd	F	GTC TAT CCG GCT CAC ATC CA
	R	TAT CGT GTT GGA AGG CAG GT
yip2	F	AGG AGG CGC TTA AGG AGT AC
	R	GCC AAT GCC CAT GAT CTC AG
CG11198	F	CAC AAC AAG GAC GAC ATG CA
	R	AGC AGA GTG ACC AGC AGA TT

Appendix 2. Lifespans of the studied fly strains

