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Essential physiological differences characterise short- and long-lived strains of *Drosophila melanogaster*

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Abstract

Aging is a multifactorial process which affects all animals. Aging as a result of damage accumulation is the most accepted explanation but the proximal causes remain to be elucidated. There is also evidence indicating that aging has an important genetic component. Animal species age at different rates and specific signalling pathways, such as insulin/insulin-like growth factor, can regulate lifespan of individuals within a species by reprogramming cells in response to environmental changes. Here, we use an unbiased approach to identify novel factors that regulate lifespan in *Drosophila melanogaster*. We compare the transcriptome and metabolome of two wild-type strains used widely in aging research: short-lived *Dahomey* and long-lived *Oregon R* flies. We found that *Dahomey* flies carry several traits associated with short-lived individuals and species such as increased lipoxidative stress, decreased mitochondrial gene expression and increased Target of Rapamycin signalling. *Dahomey* flies also have upregulated octopamine signalling known to stimulate foraging behaviour. Accordingly, we present evidence that increased foraging behaviour, under laboratory conditions where nutrients are in excess increases damage generation and accelerates aging. In summary, we have identified several new pathways, which influence longevity highlighting the contribution and importance of the genetic component of aging.

Keywords: *RNA sequencing, metabolomics, lipidomics, Drosophila, aging*

Introduction

Aging is a multifactorial process that reduces an individual's ability to handle stress, increasing mortality over time. In the last century, there has been increasing interest in understanding and manipulating the rate of aging. Yet, we still ignore the proximal causes and the possibility that the same mechanisms drive aging in different animal species (1). However, two characteristics have been shown to be conserved across the evolutionary spectrum. Firstly, aged individuals generate and accumulate different types of damage (2) and strategies that reduce the accumulation of damage extend lifespan (3). Secondly, aging has a significant genetic component as demonstrated by the differential rate of aging between animal species or the extension of lifespan in several model organisms through manipulation of pathways such as insulin/insulin-like growth factor signalling (4).

Genetic manipulations that alter lifespan have greatly contributed to our understanding of the aging process. However, in order to control for genetic background effects experiments are performed in highly inbred strains. In some cases the experimental manipulation only restores lifespan to that of outbred animals (5). In fact, the differences observed in lifespan between long- and short-lived wild type populations are best explained by low levels of variation conferred by single nucleotide polymorphisms in several genes than by mutations in individual genes that severely disrupt specific pathways (6). In accordance with this long-lived mutants (e.g. *chico* mutants) are less fit than wild type animals and are more sensitive to natural environmental challenges such as a limited energy supply(7). Therefore, using wild type populations for aging studies offers a unique opportunity to isolate factors regulating aging rate and in some respects is more representative of the variation present in the human population, characterized by high levels of genetic variability and a continuous exposure to various stresses.

Here, we study the transcriptome and metabolome of two wild-type strains of *Drosophila melanogaster* that are widely used in aging studies: a short-lived strain, Dahomey (DAH) and a long-lived strain Oregon R. In our laboratory conditions, DAH flies live consistently (8-9%) shorter than OR flies (8, 9). However, lifespan of wild type strains can vary depending on rearing conditions and there are reports where DAH flies are long-lived compared to OR (10). We chose to analyse 10 day old flies in our study, reasoning that factors affecting aging rate are already present in young sexually active adult flies and precede any aging phenotypes. Using this approach, we found that short-lived DAH flies have several traits that have been previously described in short-lived species and individuals, including increased oxidative stress, reduced expression of mitochondrial genes and increased Target of rapamycin (TOR) signalling. In addition, DAH flies showed accumulation of triacylglycerides (TAG) and depleted carbohydrate stores that correlated with increased octopamine signalling, a well-known promotor of starvation-like foraging behaviour (11). Increased foraging behaviour under laboratory conditions where nutrients are in excess could result in increased generation of damage and accelerated aging. Our results indicate that short- and long-lived fly populations display several distinct physiological traits that can explain differences in lifespan. Studying only one of these traits is not sufficient if we want to understand why some individuals live longer than others. Unbiased approaches like those we have employed here will help to uncover the various physiological traits which may require simultaneous modification to significantly and consistently extend longevity.

Materials and methods

A more detailed version of materials and methods is included in supplementary information.

Fly husbandry

Wild-type short-lived Dahomey (DAH) and long-lived Oregon R (OR) strains including lifespan records have been described before (8, 9). In these studies, we found that DAH flies have a median lifespan of 74-79 days and a maximum lifespan of 79-84 *versus* OR flies with a median lifespan of 81-86 and a maximum lifespan of 86-91 days. Female flies 10 days old were used in all experiments unless otherwise stated.

RNA sequencing analysis

Four independent samples of 60 thoraxes-and-heads were prepared per background. Total RNA was isolated using RNeasy Microarray Tissue Mini Kit (Qiagen, Maryland, USA). Library preparation and indexing were performed using TruSeq RNA sample prep v2 kit (Illumina, California, USA). Libraries were sequenced by HiSeq 2500 instrument (Illumina, California, USA) with 2 x 100bp sequencing chemistry, 4 samples per lane, at the Finnish Functional Genomics Centre, Turku, Finland. Data analysis was performed using the following pipeline: FastQC to check the quality of reads, TopHat2 for the alignment, DESeq2 for the differential gene expression and DAVID for the pathway enrichment. Raw sequence reads were submitted to NCBI's Gene Expression Omnibus and are available through GEO Series accession number GSE101465.

Survival to dry starvation

20 flies were put into an empty vial and survival was scored at three different time points: 16, 20 and 24 h.

Metabolomics analysis

After snap-freezing, samples were homogenized in 1:20 w:v cold methanol (-20°C) with 1 mM butylated hydroxytoluene and centrifuged at 13,000g for 10 min at 4°C. The supernatant was stored at -80°C until Mass Spectrometry (MS) analysis. 2µL of extracted sample was injected into an Agilent 1290 LC system coupled to an ESI-Q-TOF MS/MS 6520 instrument (Agilent, California, USA). MassHunter Data Analysis Software (Agilent) was used to collect the results. MassHunter Mass Profiler Professional Software (Agilent) was used to perform a non-targeted metabolomics analysis of the samples.

Analysis of fatty acid composition

Fatty acyl groups of total lipids from *Drosophila* homogenates were analysed as methyl esters derivatives by GC/MS as previously described (12).

Determination of oxidation-derived markers of protein damage

We measured five different markers of protein damage: two protein carbonyls glutamic and aminoadipic semialdehydes, two markers of glycoxidation Nε-(carboxyethyl)-lysine, and Nε-(carboxymethyl)-lysine (CML), and one marker of lipoxidation Nε-(malondialdehyde)-lysine (MDAL) by gas chromatography/mass spectrometry (GC/MS) as described in (13).

Determination of TAG, glucose, trehalose and glycogen content

Measurements of trehalose, glucose, glycogen and triacylglycerides (TAG) were performed as described in (14).

Enzymatic activity assays

Activities of aconitase, superoxide dismutase, catalase, glutathione-S-transferase, aspartate aminotransferase malate, isocitrate, lactate and glucose-6-phosphate dehydrogenases were measured as described in (15).

Redox state of biological thiols

Total thiol content (sum of low- and high-molecular mass thiol-containing molecules) was measured spectrophotometrically in whole body extracts as described in (16).

Measurement of the ubiquinone content

Measurement of Coenzyme Q isoforms was performed as described in (17).

Measurement of NADPH/NADP⁺ and AMP/ADP/ATP levels

Levels of NADPH/NADP and AMP/ADP/ATP were detected as described in (18) adapted for *Drosophila*.

Western Blot analysis

Protein extracts were prepared from flies homogenized in 0.5 ml of PBS containing 1.5% Triton X-100 and Complete mini EDTA-free Protease Inhibitor Cocktail (Sigma, Missouri, USA). 50µg of protein was mixed with 4X SDS PAGE loading buffer and denatured at 95°C. Protein mixtures were loaded in Bio-Rad precast gels (Bio-Rad, California, USA) and run at 130 V. Separated proteins were transferred to a nitrocellulose membrane at 30V. Membranes were blocked in 5% BSA in PBS-Tween 1X. Antibodies used were anti-phospho-Drosophila p70 S6 Kinase (Thr398) (Cell Signalling, Massachusetts, USA), anti-p70 S6 kinase alpha (C-18) (Santa Cruz, USA), anti-beta Tubulin [EPR16774] (Abcam, Cambridge, UK), HRP goat anti-rabbit IgG antibody

(Vector Laboratories, Peterborough, UK). ImageJ was used for quantification of protein band intensities.

Statistical analysis

Data were analysed with Prism 6 (GraphPad) using unpaired two-tailed Student's *t*-test with Newman-Keuls adjustment for multiple testing where appropriate unless otherwise stated. Data on plots are displayed as means $\pm$ standard deviation (SD). *n* indicates the number of independent samples per experiment. $p < 0.05$ was taken as statistically significant. In the figures *, ** and *** denotes $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively.

Results

We have used transcriptomic and metabolomic analysis to identify novel factors that can explain differences in lifespan observed between short- and long-lived strains of *Drosophila melanogaster*. Comparative analysis of the transcriptome of these strains revealed significant differences between short-lived (DAH) and long-lived (OR) flies (Figure 1A), with differential expression of 3939 genes (~26% of the transcriptome); with 1970 genes upregulated and 1969 genes downregulated in DAH flies (Supplementary Table 1). The magnitude of changes in gene expression (on a logarithmical scale) ranged from 8.3 to -8.3, however, the majority of differences between genes were smaller (± 0.2 -0.4 by log2 fold change) (Figure 1B). The most highly upregulated and downregulated genes can be found in Supplementary Table 2.

We used the DAVID Bioinformatics Database to perform Gene Ontology term and KEGG Pathway enrichment analysis (19) on our data set (Supplementary Table 3). We found that pathways related to oxidation-reduction process (GO:0055114), oxidative phosphorylation (dme00190), NADH dehydrogenase activity (GO:0003954) and mitochondrial respiratory chain complex I (GO:0005747) were downregulated in short-lived flies. While carbohydrate metabolic process (GO:0005975, dme00520), fatty acid metabolism (dme01212) and protein translation (GO:0005840, GO:0022625, GO:0022627, dme03010) were upregulated. We completed our analysis by performing manual pathway enrichment as described in Supplementary File 1 and shown in Supplementary Table 4. This analysis revealed significant changes, associated with longevity, in genes involved in the synthesis of low molecular weight antioxidants, lipogenesis, sugar response, starvation and locomotor behaviour.

There were also significant differences between DAH and OR flies at the metabolic level (Figure 1C-D). We observed changes in 83 out of 1003 metabolites detected with upregulation of 46 and downregulation of 37 metabolites in DAH flies (Supplementary Tables 5 and 6). Pathway

analysis using MetaboAnalyst 3.0 (20) revealed only minor changes (Supplementary Table 7), so we performed manual annotation of metabolites significantly changed in DAH flies (Supplementary Table 8).

DAH flies membranes have higher levels of unsaturation and lipoxidative damage.

Increased production of oxidising agents such as Reactive Oxygen Species (ROS) is a universal hallmark of aging (2) which is paralleled in old flies (17, 21). Mitochondria isolated from whole DAH flies produce more H₂O₂ than those isolated from OR flies (8). However, this was not observed when reactive oxygen species (ROS) levels were measured in dissected brains (17). In this study, we did not detect differences in the expression (levels of mRNA) of key genes involved in antioxidant defence (e.g. superoxide dismutases, catalases, glutathione-S-transferases) (Supplementary Table S4a) or in the enzymatic activity of catalase and glutathione-S-transferase (Figure 2A). However, we did find upregulation of several parameters that indicate higher levels of oxidative stress in DAH.

Firstly, enzymatic activity of superoxide dismutase and levels of uric acid (a potent antioxidant) were both higher in DAH flies (Figure 2A, Supplementary Table 6). We propose that this is a response to the higher level of basal mitochondrial superoxide production observed in isolated mitochondria from DAH flies (9). Supporting this further, we found that levels of coenzyme-Q10 and Q9 increased and decreased respectively in DAH (Figure 2B). The former has been previously described as an adaptation to increased electron leak across mitochondrial membranes (22). Superoxide dismutase enzymes and uric acid are potent ROS scavengers in hydrophilic environments (22), but show poor efficiency in hydrophobic environments such as lipid membranes (23). Accordingly, we observed fewer protein carbonyls and a higher number of reduced thiols in proteins in DAH flies (see below).

Secondly, DAH flies had a lower ratio of GSH:GSSG (Supplementary Table 6), number of low molecular weight thiols (Figure 2C) and ratio of NADPH:NADP (Figure 2D). Since DAH flies have higher levels of lipid peroxidation (see below), we hypothesize that NADPH and GSH are being oxidized to protect lipid membranes. Accordingly, we observed a transcriptional upregulation of genes involved in their *de novo* synthesis, probably to compensate for their increased use to recycle oxidized antioxidants. In addition, we observed an increase in the activity of two enzymes which provide reductive power in the form of NADPH in response to oxidative stress - glucose-6-phosphate dehydrogenase and isocitrate dehydrogenase - in DAH flies (Figure 2E, F). In summary, these data suggest that higher levels of oxidative stress are generated in DAH flies.

To test if higher levels of oxidising agents led to increased molecular damage, we measured levels of protein damage in our short- and long-lived strains. We quantified five markers across three types of protein damage: glycoxidation, lipoxidation and protein carbonylation. We found higher levels of lipoxidation, N^ε-(carboxymethyl)lysine (CML) and N^ε-(malondialdehyde)lysine (MDAL), and lower levels of amino-adipic semialdehyde, a marker of protein carbonylation in DAH flies (Figure 3A, B). We also found that high molecular weight thiols (i.e. proteins with reduced –SH groups) were increased (Figure 2C), further supporting reduction of protein carbonyls in DAH flies. As mentioned above, this could be due to the higher activity of superoxide dismutases and uric acid levels found in DAH flies. In addition, it could also indicate that GSH (whose levels are lower in DAH) is used to maintain protein thiols in a reduced state. Interestingly, higher levels of protein lipoxidation, but fewer protein carbonyls is a profile similar to that of short-lived mammals when compared to long-lived mammals (24).

There is a strong negative correlation between lifespan and membrane unsaturation in animals both vertebrates and invertebrates (25). PUFAs (Polyunsaturated Fatty Acids) with four or more double bonds are replaced by PUFAs with three or less double bonds and MUFAs

(Monounsaturated Fatty Acids) in the membranes of long-lived individuals(26). This change in membrane composition has been hypothesised to be an adaptation to oxidative stress since peroxidation sensitivity is proportional to the number of double bonds (25). Here (Supplementary Table 9), we found a higher UFA content (13%) in short-living DAH flies and consequently a higher Peroxidation Index (66% more). These differences are caused mainly by differences in PUFA content (70% more in DAH) than in MUFA (only 5% higher). PUFAs are more sensitive to oxidative attack, this would explain the higher levels of lipoxidation observed in DAH flies (Figure 3A, B). Interestingly, Shmookler Reis et al. reported a similar negative correlation between lifespan and membrane unsaturation in different mutant strains of *Caenorhabditis elegans* (27). In this study, administration of eicosapentaenoic acid (a PUFA with five double bonds) shortened lifespan. More recently, Han et al. (28) have shown that increasing, using several genetic approaches, the proportion of MUFAs in the worm intestine or by supplementing their diet with MUFAs extends worm lifespan, while no or negative effects were observed when their diet was supplemented with PUFAs. Although, Han et al. (28) did not study the mechanism(s) by which MUFAs extend lifespan, it can be hypothesized that part of the effect is mediated through replacement of PUFAs by MUFAs within lipid membranes and the consequent reduction in sensitivity to lipid peroxidation (26).

Our results demonstrate that DAH flies have higher levels of oxidative stress, membrane unsaturation and upregulation of specific antioxidants. In the future, it would be interesting to determine the degree to which higher levels of lipoxidative damage contribute to shortened lifespan in flies as has been investigated in worms(27).

Transcriptome profiling shows more cataplerotic mitochondria in DAH flies.

Accumulation of damaged mitochondria that produce less ATP is a well-characterized hallmark of aging (2). Accordingly, the majority of genes encoding mitochondrial proteins are

downregulated during aging (29). It is currently unknown if this underlies the age associated reduction in mitochondrial function or is an adaptation which prevents the assembly of defective respiratory complexes. In a previous study (8) in isolated mitochondria we did not observe differences in oxygen consumption between DAH and OR flies. However, in the current study we found several signs of altered mitochondrial function in these strains. Firstly, in DAH flies mitochondrial genes including subunits of the respiratory complexes (particularly those encoding Complex I subunits), ATP synthase, Tricarboxylic Acid Cycle (TCA cycle) enzymes and components of the mitochondrial import system were downregulated (Supplementary Table 4b, g). Secondly, the transcriptomic signature of DAH flies revealed a reduction in anaplerotic reactions with decreased expression of genes that import glutamate to the mitochondrion and subsequently convert it into alpha-ketoglutarate or succinic semialdehyde (Supplementary Table 4b). Thirdly, cataplerotic reactions involving alpha-ketoglutarate export from the mitochondria to the cytosol and its subsequent conversion into glutamate were increased in DAH flies (Supplementary Table 4b). Consistently, we found that glutamate and succinic semialdehyde levels were increased and decreased respectively in DAH flies (Supplementary Tables 6 and 8), while the activity of aconitase and isocitrate dehydrogenase, responsible for alpha-ketoglutarate generation from acetyl-CoA, was upregulated with no change in malate dehydrogenase activity (Figure 2F). Similarly, both expression and activity of aspartate aminotransferase were upregulated in DAH flies indicating a conversion of oxaloacetate to aspartate and alpha-ketoglutarate to glutamate (*Got1* and *Got2* in Supplementary Table 4b, AST in Figure 2E). Thus, amino acid flux into the TCA cycle is probably limited by reduced proteolysis in DAH flies as indicated by changes in the transcriptome (Supplementary Table 4g). These results indicate that DAH flies, compared to OR flies, predominantly use their mitochondria to provide raw materials for biosynthesis than for energy production.

Export of alpha-ketoglutarate and aspartate from the TCA cycle can be used for protein and antioxidant biosynthesis. In DAH flies we found an upregulation of genes (*HEX-C* and *Pgi*), enzymatic activity (glucose-6-phosphate dehydrogenase) and metabolites (beta-d-fructose-6-P) involved in shuttling glucose to the pentose phosphate pathway, providing reductive power in the form of NADPH (Supplementary Table 4d, Figure 2E and Supplementary Table 6). This is probably required to neutralize lipid peroxides produced because of more unsaturated membranes (Supplementary Table 9) and to reduce oxidised glutathione (Supplementary Table 4a). Finally, underlining that mitochondria in DAH flies produce less energy, we found increased levels of expression and activity of lactate dehydrogenase (*Impl3* in Supplementary Table 4d and LDH in Figure 2E). This indicates an upregulation of glycolysis that will allow energy to be acquired quickly in DAH flies. However, glycolysis is less efficient, generates more toxic by-products (such as methylglyoxal) than oxidative phosphorylation and may cause lactic acidosis. Increased glycolysis with oxidation of pyruvate to lactate is characteristic of old individuals, where accumulation of dysfunctional mitochondria reduces mitochondrial ATP generation and forces the use of this alternative pathway (30). In DAH flies, we found decreased expression of *Mpc1* (the main mitochondrial pyruvate carrier) and four subunits of the pyruvate dehydrogenase complex. This suggests that less pyruvate is being imported into the mitochondria to be oxidized (Supplementary Table 4b) and provides evidence for increased anaerobic glycolysis in DAH flies. However, levels of ATP, ADP and AMP were higher in DAH flies with no differences in the ratio of ADP to ATP or AMP to ATP (Figure 3C). This means that either no decrease in mitochondrial production of ATP occurs *in vivo* or there is a compensation in ATP production via glycolysis or fatty acid beta-oxidation (see below). In summary, the data presented here support a less efficient energy metabolism, where pyruvate is oxidized primarily by fermentation to lactate than by oxidative phosphorylation in DAH flies. This could contribute to the higher levels of toxic by-products (e.g. see CML and MDAL levels in Figure 3A) observed in these flies, accelerating aging.

DAH flies use more fatty acids to obtain energy

Further supporting a preference for less efficient oxidative glycolysis in DAH flies, we found evidence suggesting an increase in the utilization of fatty acids to produce energy. Firstly, key genes involved in the synthesis of saturated (e.g. *FASN1* and *ACC*) and unsaturated (e.g. *desat1*) fatty acids were upregulated in DAH flies (Supplementary Table 4c). Upregulation of unsaturated fatty acid biosynthesis was confirmed by measuring the lipid membrane composition (Supplementary Table 9). DAH fly membranes were enriched with MUFAs and PUFAs which explains the higher levels of lipoxidation observed in these flies (see MDAL in Figure 3A). Secondly, we observed increased expression of several genes that participate in coenzyme A synthesis (*fbl*, *ppc* and *Ppat-Dpck*, Supplementary Table 4c) while the concentration of the coenzyme A precursor, pantothenic acid, was reduced (Supplementary Tables 6, 8). Thirdly, the concentration of 5-(Tetradecyloxy)-2-furoic acid, a potent inhibitor of fatty acid biosynthesis (31) was reduced in DAH flies (Supplementary Table 8). Fourthly, genes encoding enzymes involved in beta-oxidation of fatty acids were upregulated (Supplementary Table 4c). Accordingly, there was an increase in the levels of C18-C20 and a decrease in the levels of C14-C16 fatty acids (Supplementary Table 9), a profile characteristic of enhanced fatty acid degradation (32). Paradoxically, increased fatty acid oxidation occurred in parallel to a reduction in lipolysis (Supplementary Table 4c). The reduction in lipolysis can explain the higher levels of TAG observed in DAH flies (Figure 4A). Increased use of fatty acids has been proposed to be partially responsible for the positive effects of dietary restriction (33). However, the accumulation of fat has been related to aging and diabetes (34), associating yet another trait characteristic of accelerated aging with DAH flies.

DAH flies have increased protein synthesis and activation of growth signalling pathways

In addition to increased biosynthesis of NADPH and fatty acids, DAH flies have a significant upregulation of genes required for cytosolic translation (Supplementary Table 4g). We also found higher levels of phosphorylated S6K (Ribosomal protein S6 kinase, Figure 3D, E) that stimulates ribosomal protein synthesis. S6K is directly regulated by Target of rapamycin (TOR) (35). TOR is one of the most important regulators of cellular metabolism and growth. When resources are abundant, TOR is activated stimulating cellular growth (35). TOR is also a highly evolutionarily conserved regulator of the rate of aging (36). Pharmacological or genetic suppression of TOR activity boosts proteasomal activity, autophagy and extends lifespan in fruit flies and other animal models (37, 38) . Furthermore, we have recently shown that activation of TOR, measured as phosphorylated S6 kinase, negatively correlates with lifespan in flies cultured at three different temperatures (39) .

Our previous (39) and current results suggest a key role for TOR in explaining differences in lifespan between wild type flies in different environmental conditions. Accumulation of damage is the most supported hypothesis to explain ageing (40). However, there is an alternative model that proposes extended activation of pro-growth pathways (e.g. Target of Rapamycin (Tor)) beyond sexual maturity as the main cause of ageing (41). This theory has received experimental support from data obtained mainly in *C. elegans* (42). Accordingly, it could also be proposed that DAH flies age faster not because they generate more damage but because the basal activity of nutrient sensing pathways is higher in these flies. In fact, a different interpretation of data presented so far e.g. more cataplerotic mitochondria, upregulation of biosynthetic pathways and higher levels of TOR activation, would support this hypothesis. On the other hand, no differences in weight or developmental time (data not shown) between short and long-lived strains were observed. Furthermore, the only suggestion of an accelerated metabolism, which might explain the need for

elevated protein synthesis in DAH flies was their higher levels of activity, which we have previously reported (17). Therefore, proposing differences in growth signalling, as an alternative to differential damage generation, as the main responsible for the observed differences in lifespan will require further experimental evidence in the future.

Octopamine and dopamine signalling is altered in DAH flies

Finally, we found changes in the expression of two neurotransmitters that may explain many of the phenotypes we have presented here. Octopamine and dopamine regulate feeding behaviour circuits in the brain (11). Octopamine, the fly analogue of norepinephrine, is synthesized from tyrosine and stimulates foraging behaviour under starvation (43). Dopamine is also synthesized from tyrosine, but works to inhibit feeding (44). DAH flies upregulate and downregulate octopamine and dopamine signalling respectively. We found increased expression of genes which synthesize octopamine from tyrosine (*Tdc1* and *Tbh*) and of downstream targets of octopamine such as *Dsk* (Supplementary Table 4h) in DAH flies. Thus, the octopamine-mediated starvation response (45, 46) was activated in DAH flies. First, we found less trehalose and glycogen in tissues, but more trehalose in haemolymph in DAH flies (Figure 4A, B). Second, there was also an accumulation of TAG (Figure 4A) via upregulation of fatty acid biosynthesis and suppression of TAG catabolism in DAH flies (Supplementary Table 4c). Third, DAH flies were more resistant to starvation (Figure 4C). Finally, expression of genes associated with locomotion, muscle function and heart contraction were also upregulated in DAH flies (Supplementary Table 4i). Indeed, we have previously reported that DAH flies are more active than long-lived OR flies (17). Conversely genes involved in dopamine biosynthesis (*Ple*, *Ddc*, *PPO3*) were downregulated in DAH flies, whereas *Catsup*, an inhibitor of dopamine biosynthesis, was upregulated (Supplementary Table 4h). These phenotypes also add support indirectly for the presence of a higher metabolic rate in DAH flies. Metabolic rate correlates negatively with longevity in several species and model organisms including *Drosophila*

melanogaster (25, 39) and may be an important factor explaining the shorter lifespan of DAH flies by increased generation of damage.

Discussion

We have found many differences between the transcriptome and metabolome of short-lived DAH and long-lived OR flies. DAH flies display many traits associated with short-lived individuals and species including: (i) higher level of lipoxidative damage to proteins, (ii) increased content of PUFAs in membranes, (iii) less efficient oxidative phosphorylation and (iv) increased TOR activation. Many publications have reported correlations between these variables and lifespan (25, 39). Moreover, experimental manipulation of growth signalling, mitochondrial function or membrane unsaturation extends lifespan in model organisms (27, 38, 47). Our data reveal that these parameters vary significantly between wild type populations. Importantly, alterations in these parameters seem to be interconnected. For example, increasing the levels of PUFAs in membranes increases lipoxidative damage (39). Future research will delineate how each of these variables contribute to the rate of aging, alone or in synergy with described and yet to be described variables.

It is worth mentioning that DAH flies also display many traits associated with the so-called “rover” phenotype (48). A specific allelic dimorphism in the gene *foraging* (*for*) confers one of two phenotypes: (i) the “rover” or (ii) the “sitter”. The “rover” phenotype causes higher expression and activity of *for* (49) as well as increased fly activity, reduced expression of mitochondrial genes, TAG accumulation and reduction in carbohydrates storage (50, 51). All these behavioural and metabolic modifications promote foraging. Stimulation of foraging is selected for environments where nutrient resources are scarce (52). The different origins of DAH and OR flies may explain the existence of such important transcriptomic and metabolomic differences. DAH flies were originally collected in Western Africa, an environment characterized by food and water restrictions. Under these conditions, it seems logical that adaptations which promote foraging were selected. The intriguing question is why these differences have remained after so many years of laboratory culture

conditions where resources are not limited. One possible explanation is that the “rover” allele is dominant in our DAH flies. However, our transcriptomic analysis did not reveal any genetic evidence (SNP, insertions/deletions in coding sequences, splicing variants) that the "rover"-like phenotype in DAH flies is due to the presence of "rover" allele. Allelic dimorphism in *for* has recently been described to occur through epigenetic regulation of its expression (53). As our transcriptome contains only spliced mRNAs, it is not possible to confirm or to discard the presence of cis-elements regulating *for* expression in introns, promoters and adjacent sequences.

Octopamine signalling promotes foraging behaviour (11) and its increase could account for the “rover”-like phenotype displayed by DAH flies. Furthermore, increased octopamine signalling would have additional consequences that may affect lifespan (see Figure 5). Foraging behaviour stimulates fatty acid metabolism including the synthesis of unsaturated fatty acids that increase lipoxidative damage (Figure 3A). To compensate for increased damage, anabolic pathways which synthesize antioxidant molecules (GSH, NADPH) and new proteins are stimulated (Supplementary Table 4a). Again, foraging-related locomotion enhances muscle function intensifying the need for protein synthesis. This requires an increase in TOR signalling (54) to reprogram cellular metabolism and stimulate protein synthesis (Figure 3D, E). TOR activation will inhibit autophagy and protein turnover and accelerate the accumulation of damage (38, 55). The damage theory is the currently most supported explanation for aging (2, 40) and the increased generation and accumulation of damage found in DAH flies could account for their reduced longevity. Alternatively, increased activation of pro-growth pathways independently of damage generation could be responsible for accelerated aging in DAH flies (41). Finally, it is also plausible that damage generation and alterations in pro-growth (or other) pathways are connected by one leading to another or by contributing to different aspects of ageing in parallel. In future, it will be important

to understand the mechanisms that accelerate aging in DAH flies and the contribution of octopamine signalling.

In summary, our study highlights the importance of selecting an appropriate genetic background when studying complex phenotypes like aging. Our data emphasizes the importance of not over-stating conclusions based on analysis of only one or a few physiological variables. Differences in lifespan between DAH and OR flies are not explained by a single factor, but by a synergy of small physiological differences. Similarly, a recent report studying the transcriptome of 14 *Drosophila* species (differing six fold in lifespan) discarded that differences in lifespan were due to the expression of individual genes, alternatively supporting small contributions of many distinct genetic networks as the better explanation (56). Interestingly, DAH flies display traits found in short-lived individuals or species. In the future, strategies which act to modify several different pathways simultaneously may be the most effective way to extend lifespan. Studies using wild type populations with high levels of genetic and phenotypic variability, as is seen in the human population, together with unbiased approaches will be an essential. The data presented in this study suggests that experimental manipulation of octopamine signalling could be an effective method to change several pathways at once, modifying the rate of aging.

Supplementary Material

Please visit the article online at <http://gerontologist.oxfordjournals.org/> to view supplementary material containing extended materials and methods. Supplementary information referring to supplementary tables 1 to 9 has been deposited at Harvard Dataverse repository: <https://doi.org/10.7910/DVN/CHAOKB>

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Figure captions

Figure 1. Global analysis of the transcriptome and metabolome of short- and long-lived strains of *Drosophila*. (A) Principal component analysis (PCA) plot displays transcriptomic profiles of all samples. X axis – Principal component 1 (pca1), Y axis – Principal component 2 (pca2). DAH flies are represented by red dots and OR are represented by blue dots (n=4). (B) Histogram of Log2 fold change distribution for genes with an adjusted *p*-value <0.05. X axis – log2 fold change, Y axis – frequency. (C and D) PCA displays metabolomic profiles using both positive (C) and negative (D) ionization metabolites (n=4). X axis – Principal component 1, Y axis – Principal component 2, Z axis– Principal component 3. DAH flies are represented by red dots and OR are represented by blue dots (n=16-19).

Figure 2. Antioxidant defences and redox homeostasis are altered in short-lived flies. (A) Enzymatic activity of (total) superoxide dismutase (SOD), glutathione-S-transferase (GST) and catalase (CAT, right Y-axis) (n = 7). (B) Levels of three isoforms of ubiquinone–Q8, Q9, Q10, and ratio of Q9 to Q10 (right Y-axis) (n = 3). (C) Levels of high and low (right Y-axis) molecular weight thiols (n = 7). (D) Levels of NADPH, NADP⁺ and the ratio of NADPH to NADP⁺ (right Y-axis) (n = 4-11). (E) Enzymatic activities of glucose-6-phosphate dehydrogenase (G6PDH), lactate dehydrogenase (LDH) and aspartate aminotransferase (AST, right Y-axis) (n = 7). (F) Enzymatic activity of isocitrate dehydrogenase (Idh), aconitase (Acon) and malate dehydrogenase (Mdh) (n = 7).

Figure 3. High levels of protein damage, energy and Tor signalling in short-lived flies. (A) Levels of markers of protein lipoxidation [N^ε-(carboxymethyl)lysine (CML), N^ε-(malondialdehyde)lysine (MDAL)] and glycooxidation [N^ε-(carboxyethyl)lysine (CEL, right Y-axis)]; CML is also a product of protein glycooxidation (n = 5). (B) Levels of markers of protein carbonylation glutamic semialdehyde (GSA), amino-adipic semialdehyde (AASA) (n = 5). (C).

AMP, ADP, ATP content and AMP/ATP and ADP/ATP ratio (right Y-axis) (n=3-10). **(D)**. Representative blot of S6K and pS6K with tubulin as loading control. **(E)** Protein levels of S6 kinase normalized to loading control Tubulin (S6K/Tub), phosphorylated S6 kinase normalized to loading control Tubulin (pS6K/Tub) and ratio of pS6K to S6K (n = 3).

Figure 4. Higher levels of triacylglycerols, reduced storage of carbohydrates and increased starvation resistance in short-lived flies. **(A)** Total levels of trehalose, glycogen and triacylglycerols (TAG) in fly homogenates (n=5-6) **(B)** Levels of trehalose and glucose in fly haemolymph (n=6). **(C)** Survival under dry starvation (n = 8).

Figure 5. Model outlining how octopamine signalling regulates lifespan. Activation of octopamine signalling increases foraging behaviour promoting fly activity and feeding. Thus, it increases fatty acid biosynthesis causing an increase in total TAG content and in membrane unsaturation. In conditions where food supply is not limited, former physiological changes cause an increase in damage generation (particularly lipoxidative damage) as well as activation of anabolic pathways to sustain fly activity, to synthesize antioxidants and to replace damaged proteins. Metabolic reprogramming is executed by activation of TOR. The former can cause damage accumulation by inhibiting autophagy and proteolysis or by promoting aging independently of damage generation. On the other hand, increased feeding could stimulate glycolysis as a result of higher glucose intake. The end products of glycolysis are converted to lactate and dumped via the pentose pathway (promoting generation of glycoxidative damage), instead of being converted to acetyl-CoA by pyruvate dehydrogenase, contributing to reduced lifespan. Solid lines – confirmed interactions. Dotted lines – hypothetical interactions. Red – processes found to be upregulated in DAH flies. Blue – processes downregulated in DAH flies. Filled boxes – traits previously associated with reduced lifespan.

Essential physiological differences characterise short- and long-lived strains of *Drosophila melanogaster*

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Experimental procedures

Fly husbandry

Wild type short-lived *Dahomey* (DAH) and long-lived *Oregon R* (OR) strains have been previously described (1, 2). In the referred studies, we found that DAH flies have a median lifespan of 74-79 days and a maximum lifespan of 79-84 *versus* OR flies with a median lifespan of 81-86 and a maximum lifespan of 86-91 days. Unless otherwise stated 10 day old female flies were used in all experiments. Flies were maintained at 25°C on a standard oatmeal-molasses diet (1% agar, 1.5% sucrose, 3% glucose, 3.5% dried yeast, 1.5% maize, 1% wheat, 1% soya, 3% treacle, 0.5% propionic acid, 0.1% Nipagin) in a controlled 12:12 hour light:dark cycle. They were collected 24 hours after eclosion using CO₂ anaesthesia and kept at a density of 20 flies per vial. Flies were transferred to a fresh vial every 2-3 days.

RNA sequencing analysis

Fly dissection and RNA extraction

Flies were flash-frozen in liquid nitrogen and stored at -80°C prior to RNA extraction. Abdomens were removed before the RNA extraction. Four independent samples of 60 heads and thoraxes were prepared for each strain. Total RNA was isolated using an RNeasy Microarray Tissue Mini Kit (Qiagen, Maryland, USA) according to manufacturer's instructions. Eluted RNA was DNase I treated (RNase-free) (Thermo Fischer Scientific, Massachusetts, USA). RNA was quantified using NanoDrop 2000 (Thermo Fischer Scientific, Massachusetts, USA) and quality control performed using the Agilent 2100 Bioanalyser (Agilent, California, USA). All RNA samples were prepared simultaneously.

Library preparation and sequencing

Library preparation and indexing were performed using TruSeq RNA sample prep v2 kit (Illumina, California, USA), which provides poly(A)-mRNA enrichment of samples at the first step. Library quality was checked with the Agilent 2100 Bioanalyser (Agilent, California, USA) and the concentration was measured with Qubit dsDNA kit (Invitrogen, California,

USA) following manufacturer's instructions. Fragment sizes ranged from 200bp to 670bp with an average size of 313-350bp. Libraries were sequenced using a HiSeq 2500 instrument (Illumina, California, USA) with 2 x 100bp sequencing chemistry, 4 samples per lane, at the Finnish Functional Genomics Centre, Turku, Finland (<https://www.btk.fi/functional-genomics/>). The depth of sequencing was 90 million reads corresponding to 300x coverage of the whole *Drosophila* transcriptome.

Data analysis and statistical treatment.

The quality of reads was checked using FastQC tool (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>). Reads were aligned to the *Drosophila melanogaster* reference genome assembly BDGP5.25 (3) (http://www.ensembl.org/Drosophila_melanogaster/Info/Annotation) using Tophat2 version 2.0.7 with default parameters (4). Raw sequence data were submitted to the NCBI's Gene Expression Omnibus repository (5), GEO Series accession number GSE101465. Normalization of data, statistical treatment and differential gene expression analysis was carried out using DESeq2 package (6). Differential gene expression values were obtained for comparison DAH vs OR, where DAH flies are the “experimental group” and OR flies the “control group”. Differences in gene expression were expressed as 2-base logarithmic fold changes calculated as follows:

$$\log_2 \text{ fold change} = \log_2 \left(\frac{\text{read counts in DAH}}{\text{read counts in OR}} \right)$$

In this case, a positive value of $\log_2$ fold change means the gene is upregulated in DAH flies compared to OR flies. Conversely, a negative value means that the gene is downregulated. The statistical significance of each of $\log_2$ fold change value was estimated by adjusted p-values with Benjamini-Hochberg correction for multiple testing. Genes with adjusted p-values <0.05 were selected for further analysis.

R free software (<http://www.R-project.org/>) was used to generate principal component analysis plots of the normalized read counts and $\log_2$ fold change distribution diagram based on DESeq2 output. The plots showed that DAH flies clearly cluster away from OR flies (Figure 1A) but this is a result of small changes in gene expression: median $\log_2$ fold change values were 0.4043 for upregulated and -0.4289 for downregulated genes (Figure 1B). Four independent biological samples provide enough statistical power to make statistically

significant inferences about differences in gene expression, we chose not to use an arbitrary cut-off for log₂fold change to filter differentially regulated genes.

Functional annotation and pathway analysis of differentially expressed genes

Pathway analysis was performed using DAVID (7) (<https://david.ncifcrf.gov/>) with Gene Ontology (GO) and KEGG Pathway term enrichment with default parameters and an EASE score threshold of less than 0.05. Gene Ontology and KEGG Pathway terms with a p-value less than 0.05 adjusted by Benjamini-Hochberg correction were selected (Table S2). Additionally, we performed manual pathway analysis using current GO annotation downloaded from FlyBase (8), data of known enzymatic activity – EC numbers – downloaded via DAVID software, data from the KEGG compound and pathway database (9) and bibliographic search. Lists of up and downregulated genes were analysed using two criteria. Firstly, genes whose GO term was described as a “non-traceable author statement” were discarded. Secondly, if an equal number of genes were upregulated and downregulated in a specific pathway, we considered that pathway as unchanged. However, up and downregulation of well-established key or rate limiting enzymes was considered as indicative of that particular pathway. The results of manual pathway enrichment are summarized in Table S3. Figure 5 was drawn using PathVisio3(10).

Dry starvation

For dry starvation 20 flies were transferred to an empty vial. Survival was scored at three different time points: 16, 20 and 24 h at 25°C.

Metabolomics analysis

Sample Preparation

10 flies were starved for 2 h before snap-freezing in liquid nitrogen. Samples were then immediately homogenized in 1:20 w:v cold MetOH (-20°C) with 1mM butylated hydroxytoluene and centrifuged at 13,000g for 10 min at 4°C. The supernatant was transferred to a 1.5ml tube and stored at -80°C until Mass Spectrometry (MS) analysis.

Analysis of metabolites

For metabolomics analysis, an Agilent 1290 LC system coupled to an ESI-Q-TOF MS/MS 6520 instrument (Agilent, California, USA) was used. In all cases, 2 μ L of extracted sample (methanolic phase) was applied onto a reversed-phase column (Zorbax SB-Aq 1.8 μ m 2.1 x 50mm; Agilent) equipped with a precolumn (Zorba-SB-C8 Rapid Resolution Cartridge 2.1 x 30mm 3.5 μ m; Agilent) with a column temperature of 60°C. The flow rate was 0.6 mL/min. Solvent A was composed of water containing 0.2% acetic acid and solvent B was composed of methanol 0.2% acetic acid. The gradient started at 2% B and increased to 98% B in 13 min and held at 98% B for 6 min. Post-time was established in 5 min. Data were collected in positive electrospray mode time of flight operated in full-scan mode at 100–3000 m/z in an extended dynamic range (2 GHz), using N₂ as the nebulizer gas (5 L/min, 350°C). The capillary voltage was 3500 V with a scan rate of 1 scan/s. The ESI source used a separate nebulizer for the continuous, low-level (10 L/min) introduction of reference mass compounds: 121.050873, 922.009798 (positive ion mode) and 119.036320, 966.000725 (negative ion mode), which were used for continuous, online mass calibration. MassHunter Data Analysis Software (Agilent) was used for data collection and MassHunter Qualitative Analysis Software (Agilent) was used to determine molecular features, co-migrating ionic species of a given molecular entity were differentiated using the Molecular Feature Extractor algorithm (Agilent) as described in (11, 12). Finally, MassHunter Mass Profiler Professional Software (Agilent) was used to perform non-targeted metabolomics analysis of the extracted features. We selected samples with a minimum of 2 ions, with multiple charge states not considered. Compounds from different samples were aligned using a retention time window of 0.1% $\pm$ 0.25 minutes and a mass window of 10.0 ppm $\pm$ 2.0 mDa. Only common features found in at least 75% of the samples in the same group were analysed correcting for individual bias. Data for PCA analyses were obtained using this software.

Masses with significant differences (Student's *t* test, $p < 0.05$, Benjamini-Hochberg false discovery rate) were searched against the PCDL database from Agilent (Agilent), which uses retention times in a standardized chromatographic system as an orthogonal search parameter to complement accurate mass data (accurate mass retention time approach) according to previously published works (11).

Functional annotation and pathway enrichment of metabolites

Enrichment of metabolites was performed using the hypergeometric test and relative-betweenness centrality using MetaboAnalyst 3.0 (13). In addition, we performed manual functional annotation of metabolites using the KEGG compound and pathway database (9), the Human Metabolome Database (14), PubChem compound database (15) and Lipid MAPS (16). Only metabolites reported to be part of the *Drosophila* metabolome in one or more of these databases were considered for analysis.

Analysis of fatty acid composition

Fatty acyl groups of total lipids from *Drosophila* homogenates were analysed as methyl esters derivatives by GC/MS as previously described (17). Separation was performed in a SP2330 capillary column (30m x 0.25mm x 0.20µm) in a GC Hewlett Packard 6890 Series II gas chromatograph (Agilent, California, USA). A Hewlett Packard 5973A mass spectrometer was used as a detector in the electron-impact mode. Fatty acyl methyl esters were identified by comparison with authentic standards on the basis of mass spectra. Results are expressed as mol%. Indexes of the following fatty acyls were calculated: saturated fatty acids (SFA); unsaturated fatty acids (UFA); monounsaturated fatty acids (MUFA); polyunsaturated fatty acids (PUFA) from n-3 and n-6 series (PUFAn-3 and PUFAn-6); acyl chain length (ACL) = $[(\Sigma\%Total14 \times 14) + (\Sigma\%Total16 \times 16) + (\Sigma\%Total18 \times 18) + (\Sigma\%Total20 \times 20)]/100$; double bond index (DBI) = $[(1 \times \Sigma\text{mol\% monoenoic}) + (2 \times \Sigma\text{mol\% dienoic}) + (3 \times \Sigma\text{mol\% trienoic})]$, and peroxidizability index (PI) = $[(0.025 \times \Sigma\text{mol\% monoenoic}) + (1 \times \Sigma\text{mol\% dienoic}) + (2 \times \Sigma\text{mol\% trienoic})]$.

Determination of oxidation-derived markers of protein damage

We measured five different markers of protein damage: two protein carbonyls, glutamic (GSA) and aminoadipic (AASA) semialdehydes, two markers of glycooxidation, Nε-(carboxyethyl)-lysine (CEL) and Nε-(carboxymethyl)-lysine (CML) and one marker of lipoxidation Nε-(malondialdehyde)-lysine (MDAL) by gas chromatography/mass spectrometry (GC/MS) as described in (18). Briefly, protein non-enzymatic modification markers were determined as trifluoroacetic acid methyl ester (TFAME) derivatives in acid hydrolysed, delipidated and reduced protein samples by GC/MS using a HP6890 Series II gas chromatograph (Agilent, California, USA) with a MSD5973A Series detector and a 7683

Series automatic injector, a HP-5MS column (30-m x 0.25-mm x 0.25- μ m) and temperature program described in (18). Quantification was performed by internal and external standardization using standard curves constructed from mixtures of deuterated and non-deuterated standards. Analyses were carried out by selected ion-monitoring GC/MS (SIM-GC/MS). The amount of each product was expressed as μ moles of protein damage marker per mol of lysine.

Determination of TAG, glucose, trehalose and glycogen content

Measurements of trehalose, glucose, glycogen and triacylglycerides (TAG) were performed as described in (19, 20). Briefly, glucose was measured in haemolymph, trehalose was measured in haemolymph and whole body extracts and glycogen and TAG were measured in whole body extracts. Glucose, trehalose and glycogen were measured using a diagnostic kit, Licquick Cor-Glucose (PZ Cormay SA, Łomianki, Poland). TAG levels were measured using a diagnostic kit, Liquick Cor-TG (PZ Cormay SA, Łomianki, Poland). 5-6 independent samples were used for each experiment.

Enzymatic activity assays

Activities of aconitase, superoxide dismutase, catalase, glutathione-S-transferase, aspartate aminotransferase, malate, isocitrate, lactate and glucose-6-phosphate dehydrogenase were measured as described in (21). Briefly, flies were homogenized 1:10 w:v in cold 50mM potassium phosphate (KPi) buffer (pH 7.5) containing 0.5mM EDTA and 1mM phenylmethane sulfonyl fluoride. Homogenates were centrifuged (16,000g, 15min, 4 °C) and supernatants collected and used for the different assays and protein measurements. One unit of SOD activity was defined as the amount of enzyme required to inhibit the maximal oxidation of quercetin by 50% expressed per milligram of protein. For the rest of enzymes, one unit of activity was defined as the amount of the enzyme that consumed 1 μ mol of substrate or generated 1 μ mol of product per minute. Activities of the enzymes are expressed as international units (or milliunits) per milligram of soluble protein.

Redox state of biological thiols

Total thiol content (sum of low- and high-molecular mass thiol-containing molecules) was measured spectrophotometrically using 5, 5-dithiobis (2-nitrobenzoic acid) at 412 nm in whole body extracts as described in (22). Briefly after addition of 10% trichloroacetic acid fly homogenates were centrifuged (13,000g, 5 min, 4°C). Supernatants were used for measurement of low-molecular mass thiols as described in (22) and concentration of high-molecular weight thiols was calculated by subtracting low molecular weight thiols values from the total value. Thiol concentrations are expressed as micromoles of SH-groups per milligram of wet fly mass.

Measurement of the ubiquinone content

Measurement of specific Coenzyme Q isoforms was performed as described in (23) with minor modifications to adapt the method to *Drosophila* (24). Briefly, volumes were scaled down to extract and vortex samples in 2ml polypropylene tubes. Lipid extraction was done by adding SDS (1% final concentration) to 100µl of cuticle-free whole fly homogenate followed by 200µl of 2-propanol:ethanol (5:95 v/v) and 500µl of hexane. Hexane fractions were evaporated under vacuum and samples were dissolved in ethanol prior to HPLC analysis. Lipids were separated in C18 RP-HPLC columns (5µm, 150 × 4.6 mm) and analysed as described in (23). Levels of ubiquinone isoforms are expressed as % from total content of ubiquinone.

Measurement of NADPH/NADP⁺ and AMP/ADP/ATP levels

Levels of NADPH/NADP and AMP/ADP/ATP were detected as described in (25) adapted for *Drosophila*. 40 flies were homogenised in 200µL of 10 mM KH₂PO₄ (pH 7.5) and centrifuged (17000g 1min 4°C). Supernatants were filtered using 0.45µm Ultrafree® centrifugal filters (Millipore, Darmstadt, Germany) and 100µl of sample was injected into an Agilent 1100 LC system (Agilent, California, USA) coupled to an Agilent 1100 Series Variable Wavelength Detector (Agilent). Metabolites were separated using a ZORBAX Eclipse Plus C18 column (4.6.x150mm, 5 µm, Agilent). The mobile phase consisted of two eluents: buffer A (10 mM tetrabutylammonium hydroxide, 10 mM KH₂PO₄, 1% methanol, pH), and buffer B (2.8 mM tetrabutylammonium hydroxide, 100 mM KH₂PO₄ , 30%

methanol, pH 5.5). Flow rate was 1.2 mL/min and the gradient used for separation was: 12 min buffer A 100%, 2 min increase to 40% buffer B, 11 min increase to 44% buffer B, 10 min increase to 100% buffer B, 100% buffer B for 5 min and 10 min 100% buffer A. All metabolites were detected at 254 nm. HPLC grade standards were used to identify the elution time for each metabolite and to build calibration curves for quantification. Each sample was normalized to g of fly homogenate.

Western Blot analysis

Whole protein extracts were prepared from 20 flies homogenized in 0.5 ml of PBS containing 1.5% Triton X-100 and Complete mini EDTA-free Protease Inhibitor Cocktail (Sigma, Missouri, USA) at the manufacturer's recommended dilution. After 10 min of incubation on ice, samples were centrifuged (13,000g, 15 min, 4°C). Supernatants were transferred to a new tube and protein concentration was estimated by the Bradford protein assay. For SDS-PAGE prior to western-blotting, 50µg of protein was mixed with 4x SDS PAGE loading buffer (40% glycerol, 8% SDS, 25% 1 M Tris-HCl pH 6.8, 0.015% Bromophenol Blue Slurry) and denatured at 95°C for 5 min. Samples were loaded into Bio-Rad precast gels (Bio-Rad, California, USA) and run for 1 h at 130 V in a Bio-Rad Criterion-Cell electrophoresis tank (Bio-Rad). Separated proteins were transferred to a nitrocellulose membrane (Amersham Protran, Little Chalfont, UK) using a Bio-Rad Wet Transfer system (Bio-Rad) at 30V for 1 h. Membranes were blocked in 5% BSA in PBS-Tween 1X for 1 h at 25°C. Antibodies used: 1:500 anti-phospho-Drosophila p70 S6 Kinase (Thr398) (Cell Signalling, Massachusetts, USA), 1:500 anti-p70 S6 kinase alpha (C-18) (Santa Cruz, USA), 1:1000 anti-beta Tubulin [EPR16774] (Abcam, Cambridge, UK), 1:1000 HRP goat anti-rabbit IgG antibody (Vector Laboratories, Peterborough, UK). Primary antibodies were incubated over-night at 4°C and secondary 1h at room temperature. Membranes were incubated with Clarity™ Western ECL Blotting Substrate (Bio-Rad, California, USA) and membranes were imaged with a Fujifilm LAS4000 Luminescence Imager (GE Healthcare Life Sciences, Buckinghamshire, UK). ImageJ was used for quantification of protein band intensities. pS6K and S6K bands were quantified and normalized to the loading control tubulin, after normalization the ratio of pS6k to S6K was calculated.

Statistical analysis

Data were analysed with Prism 6 (GraphPad) using the unpaired two-tailed Student's *t*-test with Newman-Keuls adjustment for multiple testing where appropriate unless otherwise stated. Data on plots are shown as means $\pm$ standard deviation (SD). *n* indicates the number of independent samples per experiment. Test $p < 0.05$ was taken as statistically significant. In the figures *, ** and *** means $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively.

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A **B** **C** **D** **E** **F**

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