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RESEARCH ARTICLE

Mild heat treatments induce long-term changes in metabolites associated with energy metabolism in *Drosophila melanogaster*

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Abstract Heat-induced hormesis, the beneficial effect of mild heat-induced stress, increases the average lifespan of many organisms. Yet little is known about the mechanisms underlying this effect. We used nuclear magnetic resonance spectroscopy to investigate the long-term effects of repeated mild heat treatments on the metabolome of male *Drosophila melanogaster*. 10 days after the heat treatment, metabolic aging appears to be slowed down, and a treatment response with 40 % higher levels of alanine and lactate and lower levels of aspartate and glutamate were measured. All treatment effects had disappeared 16 days later. Metabolic reprogramming has been associated with the life extending effects of dietary restriction. The metabolite changes induced by the hormetic treatment suggest that the positive effects might not be limited to the repair pathways induced, but that there also is a change in energy metabolism. A possible direct link between changes in energy metabolism and heat induced increase in *Hsp70* expression is discussed.

Keywords Energy metabolism · NMR · Heat stress · *Hsp70* · Hormesis · Longevity · Metabolomics

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Introduction

Short bouts of mild stress early in life can affect many life-history-traits at later life-stages (Sørensen et al. 2009). Depending on the severity of the stress and the state of the individual experiencing the stress, the late-acting effects might be beneficial or detrimental (Kuether and Arking 1999; Gómez et al. 2009; Sarup and Loeschcke 2011). The beneficial effects occur when stressful conditions induce maintenance and repair pathways to an extent that exceeds the damage that the stress has caused. This is termed hormesis and among other effects it can result in increased general stress resistance and lifespan. Hormesis has been reported in a diverse set of species and resulting from a large number of stressors such as heat, hyper gravity and cold (Rattan 1998; Verbeke et al. 2000; Le Bourg et al. 2004; Vermeulen and Loeschcke 2007; Gems and Partridge 2008; Le Bourg 2010). In human skin fibroblasts, multiple heat shocks can delay the onset of aging as measured by growth parameters and various biochemical characteristics in vitro (Rattan 1998; Verbeke et al. 2000; Beedholm et al. 2004). The effect of a mild heat treatment early in life is thus apparently long lasting after the stress has ceased. A brief heat shock 4 days after eclosion can decrease mortality rate during a period up to 5 weeks in *Drosophila* (Khazaeli et al. 1997). Both the initial mortality rate and the Gompertz rate of increase in mortality are decreased by multiple bouts of mild heat stress in *Caenorhabditis elegans* (Wu et al. 2009). These results indicate that the progress of aging can be slowed down by hormesis. Hormesis induced increases in stress resistance have also been found to last long after the stress (Hercus et al. 2003; Le Bourg 2015).

Although it is clear that episodes of stress early in life can alter later life history traits, the mechanisms behind are more elusive, and not much is currently known of the late acting physiological effects of mild stresses. One of the defence systems that are potentially involved in hormesis is the heat shock response including the heat shock proteins. Flies that have an unstable heat shock factor, and therefore are unable to up-regulate their heat shock response during stress, do not have beneficial effects of an otherwise hormetic treatment (Sørensen et al. 2007). In addition, Hsp70 remains up-regulated several days after a series of hormesis inducing mild heat treatments (Sarup et al. 2014). However, we know that the organism's immediate response to stress includes changes in the expression of many other groups of genes, alterations in membrane lipid composition and the concentration of several metabolites within the first 8 h after a mild stress (Sørensen et al. 2005; Malmendal et al. 2006), all of these could contribute to the hormetic effect. To further understand how a short pulse of heat early in life decreases mortality in the following weeks, we investigate if and how the metabolome changes at later ages after exposure to a mild stress. Is the late acting effect of mild stress due to short-term changes in the flies' homeodynamics during or immediately after the stress, or is there a lasting effect that can be detected in the metabolome in ageing flies?

Nuclear magnetic resonance (NMR) metabolomics has been applied to characterize stress responses in *Drosophila* (Malmendal et al. 2006; Overgaard et al. 2007), and in a number of other organisms, including plants (Arbona et al. 2013) and a number of different metazoans (Michaud et al. 2008; Hawes et al. 2008; Colinet et al. 2012; Butler et al. 2013). This method may provide a comprehensive impression of the relative abundance of a large number of metabolites simultaneously. It should be noted that this method does not measure the metabolite flux, but provides snapshots of their concentrations. It is not possible to identify all the detected compounds yet, however, the behaviour of the combined metabolome data set, as analysed by principal component analysis, elucidates patterns that cannot be detected by analysis of individual metabolites (Sarup et al. 2012).

Materials and methods

Fly lines, maintenance and experimental procedures

We used three *Drosophila melanogaster* lines that were selected for increased longevity independently for 33 generations as previously described (Bubliy and Loeschcke 2005; Sarup and Loeschcke 2011). This selection did not affect the heat resistance of the lines (Wit et al. 2013). Flies were set-up in 200 mL bottles under uncrowded conditions (allowing ten pairs to lay eggs for 24 h). Upon the start of emergence the bottles were emptied and flies were collected less than 24 h old under light CO_2 anaesthesia. We collected 15 males and 15 females per vial. As flies died the number of vials was gradually reduced keeping density in the

vials close to 30. Within each line, half of the replicates was subjected to a hardening treatment previously shown to induce hormesis eight generations prior to the sampling for the current experiment (Sarup and Loeschcke 2011), the remaining replicates were kept at constant 25 °C. Heat hardening took place three times during the experiments, when the flies were 3, 6, and 9 days post eclosure, at 34 °C for 2 h in pre-heated water baths and subsequently the flies were returned to 25 °C.

Sampling

Five samples of 50 males per line were sampled from three vials at day 3 post eclosure, just before the heat treatments began, and for both treated and non-treated flies at day 19 and 35, 10 and 26 days after the heat treatment ended. Female longevity (and hormetic response to mild stress) is highly variable depending on their mating frequency (Sarup and Loeschcke 2011), which changes e.g. according to the sex ratio in the vials. The sex ratio changes due to different mortality rate in the different sexes and lines. Pilot studies showed a more robust response to hormetic treatment in males. Therefore only males were used for metabolomic studies and related transcriptomic studies (Sarup et al. 2011, 2012, 2014).

All samples were frozen in liquid nitrogen at the same time of the day (2 p.m.) to minimize the influence of daily rhythm on the metabolome.

Preparation of samples and NMR spectroscopy

Flies from each sample were mechanically homogenized with a Kinematica, Pt 1200 (Buch & Holm A/S) in 1 mL of ice-cold acetonitril (50 %) and centrifuged for 30 min (4 °C). The supernatant was transferred to new tubes, lyophilized and stored at −80 °C. Immediately before the NMR measurements, samples were rehydrated in 650 µL of 50 mM phosphate buffer (pH 7.4) in D_2O, and 600 µL was transferred to a 5 mm NMR tube. The buffer contained 50 mg/L of the chemical shift reference 3-(trimethylsilyl)-propionic acid-D4, sodium salt (TSP). The NMR measurements were performed at 25 °C on a Bruker Avance-2 700 MHz spectrometer (Bruker Biospin, Rheinstetten, Germany), operating at a ^{1}H frequency of 700.09 MHz, equipped with a 5 mm HCN triple-resonance probe. ^{1}H NMR spectra were acquired using a single −90°-pulse experiment with a Carr–Purcell–Meiboom–Gill (CPMG) delay added in order to attenuate broad signals from high-molecular-weight components. The total CPMG delay was 40 ms and the spin-echo delay was 200 µs. The water signal was suppressed by presaturation of the water peak during the relaxation delay of 1.5 s. A total of 256 transients of 16 K data points spanning a spectral width of 24 ppm were collected, corresponding to a total experimental time of 10 min. For assignment purposes two-dimensional ^{1}H–^{1}H TOCSY and ^{1}H–^{13}C HSQC spectra were acquired. Metabolites were identified from ^{1}H and ^{13}C chemical shifts only.

Data reduction and statistics

The spectra were processed using iNMR (www.inmr.net). An exponential line-broadening of 0.5 Hz was applied to the free-induction decay prior to Fourier transformation. All spectra were referenced to the TSP signal at −0.017 ppm, automatically phased and baseline corrected. The spectra were aligned using icoshift (Savorani et al. 2010). The region around the residual water signal (4.85–4.67 ppm) was removed in order not to compromise the analysis. The high- and low-field ends of the spectrum, where no signals except the reference signal from TSP appear, were also removed (i.e., leaving data between 9.5 and 0.5 ppm). The integrals were normalized to total intensity in order to suppress trivial separation based on variations in amount of sample.

Principal component analysis (PCA) was carried out on pareto scaled data (Craig et al. 2006). Initial PCA identified one 35 day old heat-treated replicate as a significant outlier. This replicate was excluded. Significant effects were assessed by applying MANOVA on all PCA scores. Orthogonal projection to latent structures discriminant analysis (O2PLS-DA) was used to illustrate the overall variation between flies at different ages without or with heat treatment and displaying scores calculated using leave one out cross validation (Wold 1978). O2PLS-DA partitions the metabolomic variation into a part that is correlated to the studied phenotype and the remaining orthogonal part. This approach results in fewer significant principal components, more robust models, and clearer interpretation of the results (Trygg and Wold 2002). To avoid problems with overfitting it is crucial to cross validate the model. This can be done by leaving out

randomly chosen groups of samples one at a time, calculate scores, and predict group membership for the left out samples. The scores displayed in Fig. 2 are calculated in this way. Metabolites affected by the mild heat treatment were identified using two-sample t-tests between H and L spectra at 19 or 35 days of age and using paired t-tests to account for baseline variations between lines. The spectral peaks identified in this manner were integrated and tested by applying sequential Bonferroni correction for a total number of 100 metabolites. All multivariate analysis was performed using the Simca-P software (Umetrics, Sweden).

Results

Overall effects

NMR spectra of 19 days old *D. melanogaster* metabolites are shown in Fig. 1. In order to characterize the overall metabolite response to the hormetic treatment metabolite NMR spectra were submitted to PCA, resulting in 11 principal components that explain 89 % of the variation. These were tested for significant effects of age, line, treatment and their interactions by three-way-MANOVA (Table 1), showing significant effects for all terms. An O2PLS-DA model was created to describe the separation between samples from flies of different ages subjected to different treatments. The scores of the two predictive components (PCs; Fig. 2) clearly show how the metabolome changes with age and heat treatment, with aging along PC1, the effect of heat treatment at day 19 along PC2, and no effect of heat treatment at day 35. Notably, the scores along PC1 of the heat-treated flies from day 19 were indistinguishable from those sampled at day 3 (Fig. 2) indicating delayed ageing of the metabolome. The metabolite changes associated with PC1 include increases in maltose (5.40 ppm) and fatty acid (0.88 ppm) and decreases in AMP (8.60, 6.13, 4.35 ppm), 3-hydroxykynurenine (7.44, 6.98, 6.69 ppm), glucose (4.62 ppm), glutamine (2.44 ppm), alanine (1.47 ppm) and lactate (1.31 ppm), while the metabolite changes associated with PC2 include increases in alanine (1.47 ppm) and lactate (1.31 ppm) and decreases in methionine sulfoxide (3.01, 2.74, 2.32 ppm), glutamine (2.44 ppm) and glutamate (2.34, 2.05 ppm).

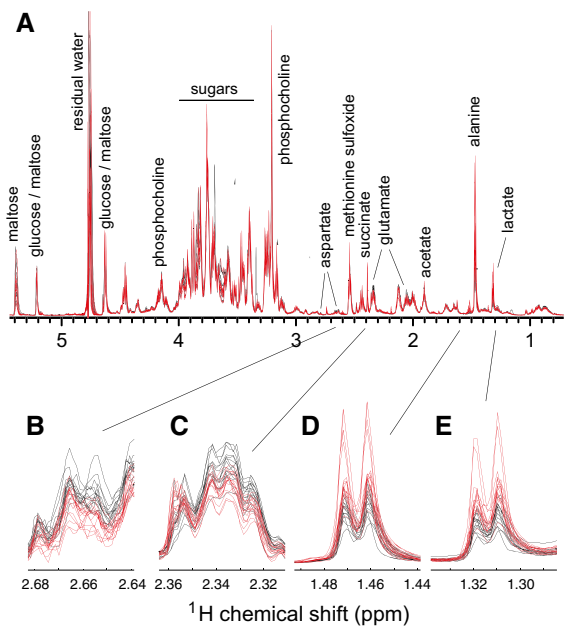

Fig. 1 ^{1}H NMR spectra of *D. melanogaster* metabolites. CPMG spectrum of 19-day-old flies acquired at 25 °C and pH 7.4. Spectra from flies submitted to heat treatment are shown in *red* and spectra from untreated flies in *black*. The upfield region where the significant metabolite signals are found is shown in (**a**). Signals from the significantly altered and other major metabolites are assigned, and the signals used for quantification are shown in (**b**–**e**), with **b** corresponding to aspartate, **c** to glutamate, **d** to alanine and **e** to lactate. (Color figure online)

Specific metabolite responses

In order to further characterize the metabolite response to the hormetic treatment, t-tests were performed on the NMR spectral intensities to identify the metabolites that were affected 10 and 26 days after the hormetic treatment, using Bonferroni correction

Table 1 MANOVA of PCA scores

Term	*P*-value [a]
Type	$5.5 \times 10^{-11***}$
Time	$<1.5 \times 10^{-15***}$
Line	$<1.5 \times 10^{-15***}$
Type × Time	$1.9 \times 10^{-6***}$
Type × Line	$2.2 \times 10^{-2*}$
Time × Line	$8.0 \times 10^{-3**}$
Type × Time × Line	$1.1 \times 10^{-5***}$

[a] Corrected for multiple comparisons using sequential Bonferroni correction for seven terms

* $P = 0.05$; ** $P = 0.01$; *** $P = 0.001$

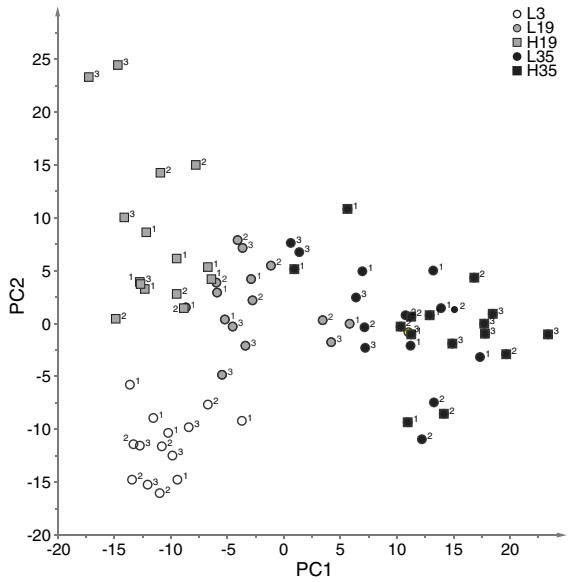

Fig. 2 O2PLS-DA scoresplot showing cross-validated scores separating age (3, 19, and 35 days) and treatment (untreated / heat). Day 3 flies are marked with open symbols, and day 19 and 35 flies with *grey* and *black* symbols, respectively. Untreated flies are marked with *circles* and heat treated flies with *squares*. *Numbers* indicate line number. The O2PLS-DA model was composed of 2 predictive and 3 orthogonal components. Predictive component 1 and 2 describe 11 % and 6 % of the variation, respectively

assuming a total number of 100 metabolites in the samples. 10 days after the hormetic treatment alanine (1.46, (3.76) ppm) and lactate (1.31, (4.10) ppm) showed 40 % higher levels (Figs. 1, 3, Table 2), and aspartate ((2.80), 2.65 ppm) showed 23 % lower levels as identified by two-sample t-tests. The chemical shift of each significant metabolite is given after each metabolite. (The chemical shifts for NMR signals from significant metabolites that did not change significantly due to overlap with signals from other metabolites are given in parenthesis.) Additional changes in unidentified aromatic substances were also detected. When using paired t-tests (accounting for the effect of selection line) also glutamate (2.34, 2.08 ppm) was lower by around 10 %. All these differences had disappeared 26 days after the heat treatment (Fig. 3). Metabolites that are detected and did not show significant changes include sugars, nucleotides, amino acids and other organic acids. It should be noted that there are additional treatment effects in the individual lines that will not be addressed here.

Discussion

The heat treatment used here has been shown to increase lifespan in the *D. melanogaster* lines used for this study eight generations prior to the sampling for metabolomics (Sarup and Loeschcke 2011). Here we show that the metabolome of the heat-treated flies is clearly distinguishable from control flies 10 days after the last bout of stress and that the result of the hormetic treatment appears to slow down metabolic aging (Fig. 2) in line with earlier transcriptomic studies (Sarup et al. 2011). When the effect of genetic differences among lines was removed from the data, four metabolites were significantly altered by the heat treatment (Table 2; Fig. 3). However, 26 days after the last bout of mild stress, the metabolite effect of the heat treatments had disappeared so that the fly metabolome was identical to that of untreated flies (Fig. 2).

Lactate

The metabolite that showed the largest effect of heat treatment was lactate, which increased by 43 % in relative abundance. In addition, lactate was the only metabolite in the current study which showed significant different abundance 19 days after heat treatment that was not also affected on the short term by heat treatment at 36 °C (Malmendal et al. 2006; Wu et al. 2009). Lactate does not respond following a heat treatment in *Escherichia coli* either (Ye et al. 2012).

Lactate abundance increases with age but is not affected by selection for longevity in *D. melanogaster* (Sarup et al. 2012). However, increased lactate levels have been linked to longevity as keeping adult male flies on medium containing lactate increases medium lifespan with 12 % (Massie and Williams 1979) and fibroblast cell cultures from offspring of human centenarians have higher production of lactate than their partners (Dekker et al. 2012), possibly due to a higher metabolism and cell proliferation.

Alanine

The heat-treated flies showed 40 % increased relative abundance of alanine 10 days after the stress treatment. This metabolite also showed a 20 % increase eight hours after a mild heat stress (Malmendal et al. 2006). The plastic response to increased temperature

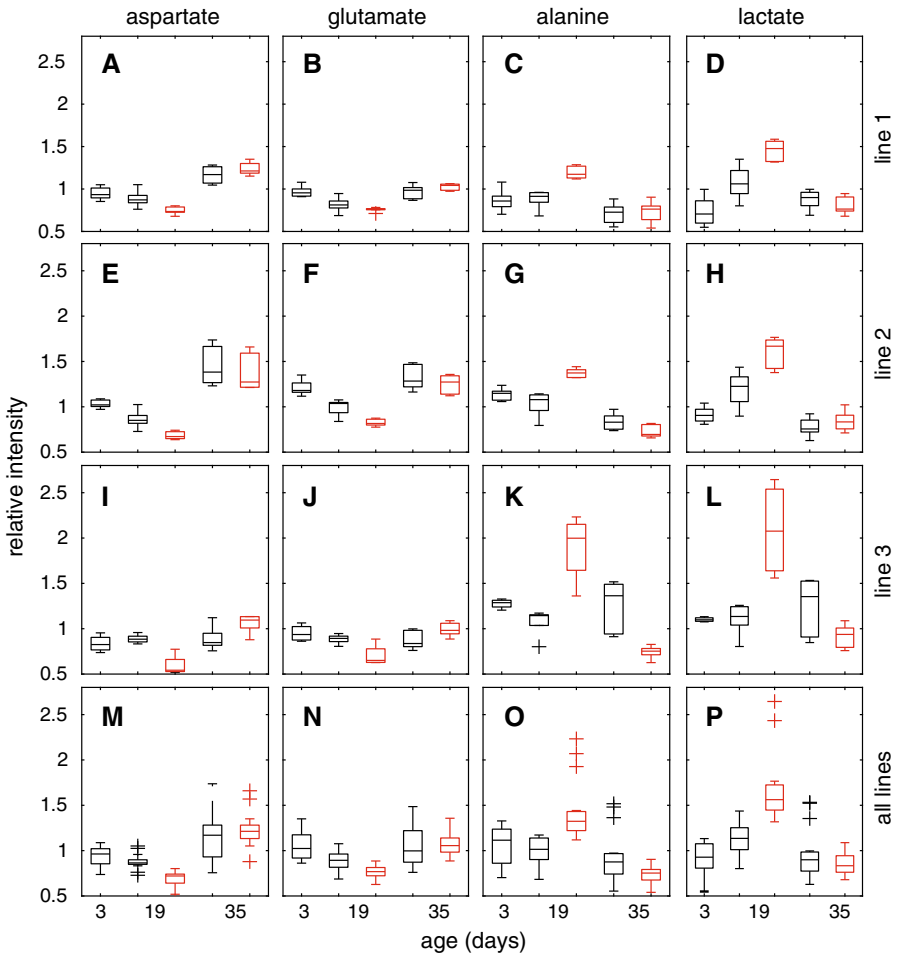

Fig. 3 Whisker box plots of relative concentrations in aspartate, glutamate, alanine, and lactate in individual lines and overall. *Red* symbols denote flies submitted to heat treatment. **a–d** Show results for *line 1*, **e–h** for *line 2*, **i–l** for *line 3* and **m–p** for all lines together. The *y*-axis describes the concentration relative to the average concentration for all untreated flies. The *central mark* is the median. The edges of the box are the 25th and 75th percentiles. The *whiskers* extend to the most extreme data points not considered outliers. Outliers are plotted individually. (Color figure online)

Table 2 Long-term effects of heat treatment on metabolites

Metabolite	Chemical shift range (ppm)	Change in % day 19[b]	P-values[a]			
			Two-sample t-test[c]		Paired t-test[d]	
			19 days	35 days	19 days	35 days
Aspartate	2.669, 2.651	−23	$1.4 \times 10^{-4***}$	5.2×10^{1}	$8.9 \times 10^{-5***}$	3.2×10^{1}
Glutamate	2.345, 2.330	−10	6.6×10^{-2}	6.4×10^{1}	$1.0 \times 10^{-2*}$	4.0×10^{1}
Alanine	1.475, 1.456	+40	$1.1 \times 10^{-2*}$	2.1	$1.1 \times 10^{-4***}$	8.8×10^{-1}
Lactate	1.321, 1.305	+43	$3.1 \times 10^{-3**}$	1.8×10^{1}	$8.5 \times 10^{-4***}$	1.2×10^{1}

[a] Corrected for multiple comparisons, using Bonferroni correction for 100 metabolites

[b] Difference in relative abundance between treated and control flies at day 19 relative to abundance in control samples

[c] Not accounting for between line variations

[d] Accounting for between line variations

* $P = 0.05$; ** $P = 0.01$; *** $P = 0.001$ respectively

with regards to alanine abundance seems evolutionary conserved. It also increases in *Arabidopsis thaliana, E. coli,* and in some grasses in response to heat stress (Kaplan and Guy 2004; Du et al. 2011; Ye et al. 2012).

Interestingly, increased alanine production was also found in fibroblast cell cultures from offspring of human centenarians (Dekker et al. 2012). This effect could be the result of a higher metabolic activity compared to that in fibroblast cell cultures originating from the partners of the offspring.

While increased availability of alanine in the medium did not alter lifespan in a study on *D. melanogaster* (Yuneva et al. 2002), it can increase lifespan in *C. elegans* (Edwards et al. 2015). However, we should be careful when comparing changes in steady state abundances of a metabolite with supplying increased amounts of this metabolite in the diet. Change in steady state abundancies is the result of changed metabolic pathways; Changes in diet composition will most likely change metabolic pathways. However, which pathways that change and in what direction is not clear though and the overall impact on the steady state metabolite abundances were not measured in either study.

Aspartate

We observed a decrease in the relative abundance of aspartate by 23 % in the heat-treated flies (Table 2). Aspartate homeostasis has been linked to the hormetic effects of calorie restriction (Easlon et al. 2008) and to neurodegenerative diseases (Verdin 2015). High concentrations of aspartate in the growth medium decreased the lifespan of *C. elegans* (Edwards et al. 2015).

Glutamate

Heat treatment decreased the relative abundance of glutamate by 10 %. The short-term effect of heat stress in *Drosophila* and *E. coli* is in the opposite direction. This effect has disappeared within 8 h after hardening though (Malmendal et al. 2006; Ye et al. 2012). However, low relative abundance of glutamate is also seen in cold acclimated flies (Overgaard et al. 2007).

In yeast lifespan can be prolonged by both increasing and decreasing concentration of glutamate in the substrate (Wu et al. 2013). Adding 1 or 5 mg of glutamate to the standard growth medium of *C. elegans* increased lifespan while adding 10 mg decreased it (Edwards et al. 2015).

Overall pattern in metabolomic effects of repeated mild heat shock

The metabolite changes induced at day 19 by the mild heat treatment are indicative of a shift in the energy metabolism. Glucose is metabolized to pyruvate, which in turn can be transformed to acetyl CoA, lactate and alanine. Lactate is synthesized by lactate dehydrogenase, and alanine by alanine transaminase using glutamate for the transamination. Glucose is regenerated from alanine and lactate in the fat body. Thus, to obtain the observed changes either the glucose intake has been increased, the need for glucose generated acetyl-CoA is smaller, or there is a change in regulation of the energy metabolism.

Metabolic reprogramming has been associated with the life extending effects of dietary restriction (Masoro 2005). Dietary restriction has been shown to increase mitochondrial efficiency allowing maintenance of cellular metabolism with reduced accumulation of oxidative stress damage, resulting in slower aging (Martin-Montalvo and de Cabo 2013).

Interestingly, lactate can function as an alternative electron transporter across the mitochondrial membrane (Kane 2014) so when aspartate and glutamate levels are lower this can be compensated by increased lactate.

Irrespective of the mechanism, a shift in the energy metabolism induced by the heat treatment opens up for the possibility of favourable effects comparable to those of dietary restriction in addition to those of the repair pathways induced by the treatment.

Sarup et al. (2014) found an over-expression of Hsp70 both 10 and 26 days after the last stress, which could potentially have a direct effect on the metabolism. The over-expression of Hsp70 is so small (1.8-fold) (Sarup et al. 2014) that the energetic costs of this expression are expected to be limited, and considering the efficient oxygen transport in insects not likely to result in increased anaerobic metabolism. Several studies have shown increased lifespan resulting from elevated levels of Hsp70 expression (see reviews Vermeulen and Loeschcke 2007; Tower 2011), and this effect has been interpreted as a direct benefit from the molecular chaperone activity of the stress inducible protein. However, evidence that long-term

elevated levels of Hsp70 expression might induce changes in the regulation of energy metabolism is accumulating.

Data from rodent models and humans indicate that Hsp72, the inducible isoform of mammal Hsp70, stimulates fat oxidation in skeletal muscle with a consequent reduction in fat storage and adiposity (Chung et al. 2008; Henstridge et al. 2010, 2014a) and that the effect is achieved through both increases in mitochondrial number and oxidative capacity (Henstridge et al. 2014b). Indeed heat therapy increases both mitochondrial enzyme activity and exercise endurance capacity in rats (Chung et al. 2008), and such an increase in fat oxidation would agree with the metabolite changes observed here.

A shift in energy metabolism could be an adaptive plastic response to repeated mild heat stress. While investigation of whole body gene expression can detect general changes in gene expression it is not unlikely to miss changes in genes that are only expressed in certain organs or tissues, or whose expression is only differentially regulated in specific organs or tissues. As the main site for storage and release of glucose to the haemolymph is the fat body, it is quite possible that a tissue specific change in gene expression regarding glucose metabolism in the fat body was not picked up by the prior study of whole fly gene expression, but still could have had effects on glucose levels at the whole body level in the flies. In addition, other types of regulation, e.g. on the post-translational and posttranscriptional or epigenetic levels (Vaiserman 2011; Keating and El-Osta 2015) might give rise to the observed effect.

Finally, an indirect effect, in terms of signal enhancement/attenuation or behavioural changes such as elevated activity or feeding behaviour, could link heat induced gene expression and metabolite changes as well.

Conclusion

In conclusion, the hormetic heat treatment appeared to slow down metabolic aging 10 days after the heat treatment. The treatment induced metabolite changes are indicative of a change in energy metabolism. Metabolic reprogramming has been associated with the life extending effects of dietary restriction. Thus, the favourable effects of the hormetic treatment might not be limited to those of the repair pathways induced. Furthermore, the effects of changes in Hsp70 expression might be more diverse than previously thought (Sørensen et al. 2009).

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Authors' contribution PS: planned the experiment, cultured and sampled the flies, contributed to statistical analysis, discussed the results and wrote the paper. SMMP: performed NMR experiments, initial statistical analysis and discussion of results, contributed to the paper. NCN: funding of instrument, planed the experiment, contributed to the paper. VL: funding of fly work, initiation and maintenance of selection lines, planned the experiment, discussed the results and contributed to the manuscript. AM: planed the experiment performed NMR experiments, statistical analysis, discussed the results and wrote the paper.

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