Metabolomic profiling of heat stress: hardening and recovery of homeostasis in *Drosophila*

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ECTOTHERMIC ANIMALS EXPERIENCE fluctuating temperatures on both a daily and seasonal scale. In some cases, these fluctuations may become severely stressful or even lethal, and so these animals possess a number of physiological, biochemical, or behavioral adaptations that allow them to overcome such stressful situations. For instance, the fruit fly (*Drosophila melanogaster*) has the capacity to survive transient exposures to temperatures ranging from less than −10°C to above 40°C, and hence this species has been widely used as a model to investigate different aspects of the temperature stress response (21).

Physiological stress can have effects on many biological levels. Because metabolites are downstream of both gene transcripts and proteins, changes in metabolite levels can provide an indication of the overall integrated response of an organism. To obtain a better understanding of the effects of heat stress on metabolite concentrations, it is relevant to adopt an integrative approach, such as simultaneous measurement of the response to stress situations of all metabolites present (above a concentration threshold) in the organism, e.g., by NMR spectroscopy. This approach, termed metabolomics, is a holistic approach complementary to genomics and proteomics for studying the complex biological system response to chemical, physical, and genetic factors on the metabolite level (15–17, 28, 29, 34). The simultaneous measurement of a large number of metabolites may, in combination with the use of multivariate pattern recognition techniques, allow for an assessment of the overall effects of a stress or biological perturbation.

The present study investigates the effects of different degrees of heat stress and how a heat-hardening treatment affects the metabolomic stress response in *D. melanogaster*. Without previous exposure to elevated temperatures, even short exposures to temperatures above 38°C cause mortality in *D. melanogaster*. Survival is, however, increased considerably if flies have previously been exposed to a milder, hardening, heat stress (21, 25). The hardening activates a heat shock response that alters both transcription and translations of a large number of genes (11, 12, 39). In particular, it is known to massively increase the concentration of stress or heat shock proteins (HSPs) as these genes are triggered by non-native/denatured proteins in the cell (1). The role of HSPs is primarily to counteract the effects of stress by stabilizing, refolding or degrading denatured proteins (14, 31–33). However, there seems to be a temporal mismatch of the heat shock response in terms of hsp expression and the heat-hardening effects: the actual hardening effect is at maximum some time after HSP expression and the heat-hardening effects: the actual hardening effect is at maximum some time after HSP expression.
thermore, we compared the temporal development in gene transcription with changes in the metabolome after heat hardening using previously published data (39) on the same lines, and we, therefore, gained novel insight into the nature of the heat shock response. Our main hypothesis was that heat stress will disrupt cellular homeostasis and that the dynamic progress of this disruption can be seen as significant changes in metabolite concentrations. This can be thought of as a “metabolic trajectory,” i.e., a perturbation in multivariate metabolic space following heat stress (28). The specific hypotheses that we made were as follows: 1) that the metabolic trajectory would be a sensitive monitor of departure and return from biological homeostasis; 2) that a severe heat stress would cause similar metabolic effects as a milder heat-hardening stress, but that the overall physiological disruption will be greater; and 3) that the protective effect conferred by heat-hardening would reduce the metabolic perturbations of a subsequent severe heat stress. To test these hypotheses, we exposed *D. melanogaster* to three thermal stress treatments and compared the temporal changes in metabolic profile to an untreated control using high-resolution 1H NMR spectroscopy.

**Materials and Methods**

Maintenance and collection of flies. *D. melanogaster* (Meigen, 1830) were derived from a mass population on the basis of four preexisting laboratory stocks originating from different geographical regions (4). The mass population was created in September 2002 and was kept at the University of Aarhus in high numbers. The flies were held under well-controlled laboratory conditions (25°C and 12:12-h light-dark cycle) on a standard agar-sugar-yeast-oatmeal medium (see Ref. 4 for further details). Newly hatched (< 1 day old) female flies were collected under light CO2 anesthesia and distributed in 100 batches of 50 flies. These samples were transferred to food vials where they were maintained at standard conditions for 2 days before starting the experiments.

**Experimental protocol.** The flies were randomly divided and submitted to four treatments: 1) untreated control (C flies), 2) exposure to heat-hardening stress (36°C for 1 h, no mortality observed; H flies), 3) exposure to severe heat stress (38°C for 1 h, ~ 20% mortality observed after 8 h; S flies, and 4) exposure to severe heat stress after the flies had been heat hardened (36°C for 1 h followed by 1 h at 38°C 4 h later, no mortality observed; HS flies). Before heat exposure, the flies were transferred to glass vials containing agar to prevent them from getting stuck in the medium. The vials were then immediately immersed in water at the desired temperature. After the treatment, the flies were immediately returned to their food vials. The experiment started shortly after the onset of the 12-h light cycle at which point the HS flies were exposed to the heat-hardening treatment (1 h at 36°C). Five hours later H, S, and HS flies were exposed to 1 h at 36 or 38°C. Five replicates of 50 live flies were sampled for each treatment group immediately following the stress period, and at 1, 2, 4, and 8 h after the exposure to heat stress. The entire experimental protocol lasted 14 h but in order not to get artifacts from changing the light regimen, the lights were not turned off before the protocol had ended. Mortality was not observed immediately among S flies (no dead flies at the 0- and 1-h samplings), whereas dead flies could be removed from the 2-, 4-, and 8-h samplings. Thus some moribund flies are likely to have been included in the samples of the S flies. Quickly after sampling, the flies were transferred to microcentrifuge tubes and snap frozen in liquid nitrogen. Samples were stored at −80°C until extraction of metabolites.

**Sample preparation.** The frozen samples were homogenized in 400 μl ice-cold acetonitrile (50%) and centrifuged at 17,000 g for 10 min (at 5°C), and the supernatant was passed through a 10-kDa microfilter (Ultrafree-MC; Millipore, Molsheim, France). The microfilters were washed four times in distilled water before use to remove glicerol used to preserve the microfilters. The samples were spun for ~ 45 min at 6,000 g, and the filtered supernatant was then lyophilized and stored at −80°C until NMR analysis. Immediately before the NMR measurements, the samples were rehydrated in 650 μl of 50 mM phosphate buffer made up in D2O (pH 7.4), and 600 μl was transferred to a 5-mm NMR tube. The buffer contained 50 mg/l of the chemical shift reference dimethylsialpentesulfonic acid (DSS).

**NMR spectroscopy.** The NMR measurements were performed at 25°C on a Bruker Avance 400 spectrometer, operating at a 1H frequency of 400.13 MHz, and equipped with a 5-mm HX inverse probe. 1H NMR spectra were acquired using a single-90°- pulse experiment, with water suppression by presaturation of the water peak during the relaxation delay of 1.5 s. A total of 128 transients of 8-K data points spanning a spectral width of 12 ppm were collected, corresponding to a total experimental time of 6 min. For assignment purposes, a two-dimensional 1H-1H TOCSY (2, 3) spectrum with 80 ms DIPSI2 mixing (5), and a 13C-1H HSQC spectrum (30) were acquired on a sample containing 500 flies.

**Data reduction/treatment.** The spectra were processed using 1D NMR processor (Advanced Chemistry Development, Toronto, ON). An exponential line broadening of 1 Hz was applied to the free induction decay before Fourier transformation. All spectra were referenced to the DSS signal at 0 ppm. Data reduction was accomplished by dividing the spectrum into 0.01-ppm regions (bins) over which the signal was integrated to obtain the signal intensity. Resonances from DSS, residual water, and residual glicerol from the filters were removed so as to not compromise the analysis (regions 5.00–4.70, 3.75–3.60, 2.95–2.87, and 1.80–1.65 ppm). The high- and low-field ends of the spectrum where no signals appear were also removed (i.e., leaving data between 9.5 and 0.7 ppm). The integrals were normalized to a total intensity of 1,000 to suppress trivial separation based solely on variations in amount of sample. The variation of the concentration of individual metabolites was measured as a function of time and treatment by summation over the regions containing only signals from this metabolite.

For the initial principal component (PC) analysis (PCA) (36), the signals were scaled to obtain variance within the control set (i.e., each region was divided by the standard deviation of the integral of that region within the control set) and then centered. This scaling reduces the weight of variations that are not related to the treatment, i.e., random variations between identical samples in the control set or variations with time (circadian) within the control set are reduced, and the analysis is not biased toward metabolites present in high concentrations. The explained variation for each bin was used to identify the metabolites having a large contribution from PC1. The first four principal components were subjected to two-way ANOVA (10) for treatment and time. These tests were made at the 0.05 significance level using ranked Bonferroni correction for multiple treatments. The PCA was performed using Simca-P (Umetrics, Umeå, Sweden). Partial least squares discriminant analysis (PLS-DA) (41), was used to evaluate how similar the different treatments were to each other at different time points. In contrast to PCA, PLS-DA takes the treatment into account in the analysis and tries to separate the different treatments. It therefore sharpens separation between groups of variables relative to PCA. The data were scaled so that each time point in the control set on average has unit variance (i.e., each region was divided by its average standard deviation at the different time points in the control set) and then centered. Dendrograms were created for the data at each individual time point using hierarchical cluster analysis (HCA) with PLS-DA scores as input. Fitting two axes for each PLS-DA model was judged optimal in all cases, on the basis of cross-validation. HCA was then carried out using average (centroid) linkages in SPPLUS 2000 (Insightful, Seattle, WA) of the averaged PLS scores for each treatment. PLS-DA was also done on the same data to compare the C and H flies data sets after 4 and 8 h and the S and HS flies data.
sets at time 0 to identify effects of heat-hardening. One-way ANOVA (10) was used to identify the metabolites that were most affected by the stress treatment. For each bin at each time point it was tested if at least one of the stress treatments or untreated control was different from the rest. The most responsive bins were found by probing significant differences at the (uncorrected) 0.001 significance level. The stress-affected metabolite signals were identified from two-dimensional $^{1}$H-$^{1}$H TOCSY and $^{13}$C-$^{1}$H HSQC spectra by comparison with known metabolite chemical shifts (13, 23). Intensities of the signals from individual metabolites were measured for all time points of all treatments to follow their temporal changes. These metabolites were then subjected to two-way ANOVA (10) for treatment and time. These tests were made at the 0.05 significance level using ranked Bonferroni correction for multiple treatments. Other discussed changes/differences were tested at the 0.05 significance level.

The metabolite data were compared with gene expression data from Sørensen et al. (39) who examined the overall gene expression pattern of D. melanogaster 0, 1, 2, 4, 8, 16, 32, and 64 h after treatments identical to our heat-hardening treatment using flies from the same original population. Here, 1,222 significant, differentially expressed genes were found. Of these, 999 genes were divided into three main stress-responsive clusters: early up, late up, and early down on the basis of their expression pattern (further details are found in Ref. 11). To compare the effect of the heat-hardening treatment on metabolite concentrations and gene expression (39), both types of data were $\log(x + a)$ transformed, where $a$ is a constant chosen to make the standard deviation independent on the amplitude ($a = 0.16$ and 1.8 for metabolite and gene data, respectively) and then centered to treat the two data types as similarly as possible. Both data sets were then independently analyzed by PCA. For the metabolite data, only C and H flies were included in the PCA. A measure of the hardening effect was then obtained by subtracting the average control PC1 scores for the C flies from the H flies at each time point. For gene expression, all time points were included in the PCA, and the hardening effect was obtained by subtracting the average PC1 scores of the samples 16 to 64 h after stress treatment at all time points. This approach was chosen because there was no parallel untreated control for the gene data, and the last time points (16, 32, and 64 h) showed little variation in PC1. Furthermore, only data for the early-up, late-up, and early-down stress-responsive gene clusters (39) were included in the PCA of the gene data, because the other groups of genes may include genes that show an expression pattern that varies because of circadian rhythm (39). Finally, the metabolite and gene data were normalized to a maximum amplitude of one.

**RESULTS**

In this paper, we present results from a NMR spectroscopic study of metabolomic changes following heat stress in D. melanogaster. A typical D. melanogaster metabolite $^{1}$H NMR spectrum is shown in Fig. 1 where well-resolved signals from the metabolites discussed in this study have been assigned.

**Multivariate analysis.** PCA was performed to explore the possibility to characterize different levels of heat stress, and the effect of heat-hardening in D. melanogaster. This was done by finding correlated changes at the metabolite level. A plot of the scores of the different treatments along the first principal component, PC1, is shown in Fig. 2. PC1, by definition, accounts for a greater part of the variance in the data set than any other single PC. In this case, 21% ($R^2$) of the scaled data set used for PCA was accounted for by PC1. The major contributions to the PC1 loadings do not come from a few highly abundant metabolites, but from a range of metabolites at different concentrations. However, if the loadings are scaled back to original signal intensities, PC1 accounts for 39% of the original intensities, which clearly shows that although the intense peaks were scaled down for the PCA, a large part of the high amplitude changes were picked up by PC1. We also examined further PCs (down to PC4) for evidence of metabolic effects. As shown in Table 1, PC1 was by far the component that best discriminated between the four treatments. PC1 can, therefore, be interpreted as a “stress” component describing much of the variation related to heat stress. From Fig. 2, it is evident that each of the heat stress treatments resulted in metabolite profiles that were markedly different from the untreated control. Variations in PC1 scores (Fig. 2) accounted for more than 50% of the variation in the concentration of many major metabolites, including amino acids (leucine, isoleucine, valine, β-alanine, glutamate, tyrosine, and proline), ATP, NAD, glucose, and glycogen. Accordingly, many metabolites showed correlated changes, e.g., initial increases of tyrosine, valine/isoleucine, and leucine/isoleucine correlated with initial decrease of glucose. However, few metabolites showed strong correlations throughout the experiment.

**Variation of individual components.** The most responsive spectral regions were identified as those having the most significant differences between treatments at one or more time points. From 51 different spectral regions (most of which span several contiguous bins, and some of which contain signals from the same metabolites), 16 metabolites were identified: alanine, β-alanine, glutamate, glutamine, isoleucine, leucine, proline, tyrosine, valine, acetate, choline, glucose, glycogen, ATP, NAD, and a fatty acid-like metabolite. The metabolites were followed by looking at regions unique for the following metabolites: fatty acid-like metabolite, 0.83–0.88 ppm; leucine/isoleucine, 0.92–0.97 ppm; valine/isoleucine, 0.97–1.05 ppm (the signals in the two latter regions could not be safely separated); alanine, 1.44–1.49 ppm; acetate, 1.88–1.92 ppm; proline, 1.97–2.04 ppm; glutamate, 2.30–2.37 ppm; glutamine, 2.40–2.47 ppm; β-alanine, 2.50–2.57 ppm; choline, 3.19–3.23 ppm; trehalose, 5.15–5.20 ppm; glucose, 5.20–5.25 ppm; glycogen, 5.35–5.45 ppm; tyrosine, 6.86–6.90, 7.15–7.20 ppm; ATP, 6.11–6.17, 8.48–8.57, 8.23–8.29 ppm; and NAD, 8.80–8.84, 9.11–9.16, 9.31–9.35 ppm. The temporal
The levels of glutamate and choline (Fig. 3, C and J) were still elevated after 8 h in the S flies. In contrast, these metabolites had returned to control levels in the H and HS flies.

The heat-hardening stress had a more subtle effect. As seen in Fig. 3, the metabolite changes were not merely milder versions of those of the more severe stress treatment, and although there was an overall recovery after 4 h (Fig. 2), there were some effects on metabolite levels, which persisted throughout the experiment. A prior heat-hardening stress also resulted in observable differences between the S and HS flies immediately after the heat stress, primarily a reduction in the increase of leucine/isoleucine, valine/isoleucine, and tyrosine concentrations caused by the stress (Fig. 3, E, F, and H).

Severity of heat stress and the effect of heat-hardening. The overall pattern of stress effects and recovery is very clear on PC1 (Fig. 2). Here the untreated flies exhibited an overall decrease in PC1 scores over the 8-h period. The flies exposed to heat-hardening stress had a delayed response; they were similar to the controls immediately after the treatment, but PC1 then increased until 2 h after the treatment. By the 4- and 8-h time points, the PC1 scores were back at control levels. Both the S and HS flies showed an immediate difference in metabolite levels and had higher PC1 scores than the C and H flies at 0 h. The magnitude of this immediate response was the same for both the S and HS flies. However, the S and HS flies exhibited different recovery phases so that the metabolite perturbation of the S flies continued for at least the entire 8-h period, whereas the HS flies had largely recovered already 4 h after the stress treatment.

Although PC1 accounts for a larger part of the variation than the other PCs, these also contain information of biological relevance. We therefore also analyzed data from each time point individually by using PLS-DA and hierarchical cluster analysis. By doing the analysis separately at each time point changes in the relative concentrations of these metabolites were followed for the different treatments, and significant effects were identified by two-way ANOVA (Table 1). Trehalose was also included because inspection of the spectra showed that the variance of the concentration of this metabolite was affected by heat stress, a feature that is not picked up by regular ANOVA. Plots of changes in concentration for the most responsive metabolites are shown in Fig. 3. In general, the magnitude of the metabolite responses to the different stress treatments was quite similar. The main differences between treatments were in recovery: how long it took for the perturbed metabolites to return to control levels. For example, Table 1. Two-way ANOVA for time and treatment of the first four principal components and the most affected metabolites

<table>
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<tr>
<th>Principal Component/ Metabolite</th>
<th>Treatment</th>
<th>F</th>
<th>P†</th>
<th>Time</th>
<th>F</th>
<th>P†</th>
<th>Treatment × Time</th>
<th>F</th>
<th>P†</th>
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<td></td>
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<td>PC1</td>
<td></td>
<td>71.7</td>
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<td></td>
<td>25.4</td>
<td>&lt;0.001*</td>
<td></td>
<td>4.42</td>
<td>&lt;0.001*</td>
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<td></td>
<td>0.877</td>
<td>0.48</td>
<td></td>
<td>1.21</td>
<td>0.29</td>
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<td>0.018</td>
<td></td>
<td>0.767</td>
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<td></td>
<td>1.04</td>
<td>0.42</td>
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<td>3.51</td>
<td>0.019</td>
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<td>0.800</td>
<td>0.53</td>
<td></td>
<td>1.26</td>
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<td></td>
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<tr>
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<td>15.6</td>
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<td></td>
<td>7.95</td>
<td>&lt;0.001*</td>
<td></td>
<td>3.74</td>
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<td>&lt;0.001*</td>
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<td></td>
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<td></td>
<td>20.2</td>
<td>&lt;0.001*</td>
<td></td>
<td>11.6</td>
<td>&lt;0.001*</td>
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<td>3.41</td>
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<td>&lt;0.001*</td>
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<td>2.70</td>
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<td>&lt;0.001*</td>
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<td>&lt;0.001*</td>
<td></td>
<td>1.59</td>
<td>0.19</td>
<td></td>
<td>2.48</td>
<td>0.008</td>
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</table>

For the principal components (PC), n = 4 and for the metabolites, n = 16. *Significance using ranked Bonferroni correction, †P values are not corrected for multiple treatments.
the relative metabolic differences at different times after heat stress were much more clearly revealed. Dendrograms showing the relative similarity of the treatments for early, middle, and late times (0, 2, and 8 h) are given in Fig. 4, A–C. (Clusters observed after 1 and 4 h were very similar to those observed after 0 h and 2 h, respectively.) PLS-DA plots gave a measure of the relative metabolic effect of each treatment (Fig. 4, D–F).

At time 0, the untreated controls were the most distinct with all of the heat-stressed flies falling into a single cluster; within this cluster, the two groups of flies that had been exposed to severe heat stress treatments were the most similar of all, and not significantly different from each other (ellipses overlap in Fig. 4D). After 2 h, the S flies were found in a distinct cluster, whereas C, H, and HS flies at this time were in a single cluster. Here, H and HS flies were not significantly different from each other (Fig. 4E). At the end of the incubation, after 8 h, the S flies were still found in a distinct cluster, but the most similar treatments within the remaining group were at this time H and C flies, which were not significantly different (Fig. 4F).

For hardened flies, separate PCA of metabolite and gene transcription data were made to get a general and unbiased comparison of the two types of responses. As seen in Fig. 5A, their timeframes of recovery are strikingly similar. It should be noted that the absence of the more severe treatments in this analysis and the different scaling of the data slightly changes the weight of the different metabolites contributing to PC1 compared with the PC1 described in Fig. 2. However, much of the apparent difference in the time course of the two metabolite PC1s can be explained by the fact that the analysis in Fig. 5A is on the basis of log-transformed data, whereas that in Fig. 2 is not. Fig. 5B shows the average relative expression of the early-up (hsp genes excluded) and late-up and early-down gene clusters, and for the hsp (23, 26, 27, 67Ba, 67Bc, 68, 70, and 83) genes. The hsp genes are of particular interest because they were at maximum immediately after stress and stayed upregulated throughout the experiment, whereas the changes in expression of the larger gene clusters were comparatively smaller and did not maximize until 1 h after heat stress. Hsp70 gene expression was 6–9 times more upregulated than the average hsp genes, but showed the same time dependence (8).

DISCUSSION

The present study demonstrates the strength of 1H NMR-based metabolomics as a tool to assess the overall physiological phenotype of heat-stressed flies. Together with sample-
Fig. 5. PC analysis (PCA) of gene transcription and metabolite concentrations and relative gene transcription induced by heat hardening. A: normalized PC1 of stress-induced changes in metabolite concentrations (filled squares, solid line) and in gene transcription (open triangles, dashed line) after heat-hardening stress. B: geometric average of the gene transcription relative to 32–64 h after stress treatment for 257 early-up gene clusters (hsp genes were excluded; filled squares), 734 late-up and early-down gene clusters (filled triangles), and differentially expressed hsp 23, 26, 27, 67Ba, 67Bc, 68, 70, and 83 genes (open squares) obtained from Ref. 39. Late up and early down are shown together because their time dependence is essentially similar within this time frame. Error bars represent 1 SE.

Based multivariate analysis (pattern recognition), this allows one to visualize the overall metabolic effects of stress by comparison to the untreated control, and forms a quantitative and functional basis for a discussion of the overall progression of the effects of heat stress that would have been difficult to establish by other means. We were thus able to confirm our hypothesis that the metabolic trajectory was a sensitive monitor of departure and return from biological homeostasis and that this method was able to clearly separate different intensities of stressful treatments.

Metabolic responses to heat stress. Although the experimental design of the present study provides information about changes in metabolite concentrations rather than the pathways involved, it can provide important information about the mechanisms of heat stress response. If a metabolite plays a specific role in physiological adaptation to an abiotic stressor, this often implies the occurrence of extremely high concentrations of a certain metabolite, e.g., sorbitol providing resistance to high temperature in whiteflies (42) or glucose protecting against freezing damages in frogs (7). Although we found no evidence of any major metabolite-level adaptation to heat stress in D. melanogaster, it is still possible to generate new hypotheses about biological function on the basis of the present data.

An important feature of metabolomic data is that it simultaneously measures a large number of metabolites in a quantitatively reliable manner and therefore allows for discovery of correlations between pairs of metabolites, even when the biological connections between them are at present not obvious. In this study, which involves metabolites collected from whole animals, few strong correlations were found. However, we observed that several of the responsive metabolites had substantial positive or negative contributions from the stress component PC1. Accordingly, the abundance of several of these metabolites also varied in a concerted fashion. For example, the metabolites that were decreased relative to the controls following heat stress are involved in energy metabolism. Energy storage in the form of glycogen and presumed fatty acid-like or lipid-like molecules (Fig. 3, K and P) were decreased after heat stress, as is also the case for glucose levels (Fig. 3G). Indeed, comparison of Figs. 2 and 3L shows that there is a very good agreement between glucose concentrations and PC1 scores. The immediate reductions in metabolites involved in the energy metabolism may be caused by a general increase in metabolic rate because of the elevated temperature.

Heat stress was also associated with a general increase in the levels of free amino acids. This increase is likely to be associated with the breakdown of proteins because of the stress treatment, but it is possible that some amino acids changed for other reasons. In relation to energy metabolism, we noted that the concentration of alanine, which is one of the major end products of anaerobic metabolism in insects (20) was markedly increased after heat stress. Although tracheal respiration is an efficient mean for oxygen transport (18, 22), we cannot exclude the possibility that flies partially used anaerobic respiration during heat stress. Indeed, a partial reliance on anaerobiosis would intensify the depletion of glucose and glycogen, because this type of respiration is less efficient in generating ATP. The increased alanine levels could, however, also originate from glutamine, which has been found to be metabolized to alanine and/or glutamate during heat stress in isolated cell cultures from Drosophila (35). In fact, glutamine was slightly decreased immediately after severe heat stress, while both alanine and glutamate were increased. However, the glutamine levels quickly recovered and increased above control levels after 2 h. This latter increase may be important for the general stress response, as the presence of glutamine during heat stress has been found to enhance the expression levels and reduce the turnover rate of HSPs (35, 40).

Another amino acid that plays an important role in the Drosophila stress response is tyrosine. Tyrosine acts as a precursor of several stress hormones in insects, including dopamine, octopamine, and tyramine (19, 37). In the present study, we found that tyrosine was markedly elevated in the S flies, whereas the levels were intermediate in the H flies and close to control in the HS flies. Changes in tyrosine levels have been shown to correlate with the changes in the levels of stress hormones during heat stress (19). From this point of view, our results suggest that the flies that were hardened and subse-
quent received a severe heat stress were in fact less stressed after the severe stress than they were after the (initial) harden-
ing (36°C) heat stress. In contrast, tyrosine levels remained elevated throughout the experiment in the flies that were directly exposed to a severe stress.

Trehalose is of interest because it has been shown to play an important role in protecting protein and membrane integrity during exposure to various stressors, such as desiccation, cold, anoxia, and heat in invertebrates and fungi (6, 8, 27). Compared with the metabolites discussed so far, trehalose showed a somewhat different behavior. Although there were no clear tendencies in the mean values, the variance was markedly increased in flies subjected to both heat-hardening stress and severe heat stress treatments. It seems, therefore, that the control of trehalose levels was reduced in these treatments (Fig. 3M). The increased variance stems from a few samples with elevated trehalose concentrations, indicating that in a few of the animals, trehalose is less regulated under stressful conditions. Again, our results for this metabolite indicate that the HS flies were actually less affected than the H flies although they had received a more dramatic stress treatment.

Effects of heat hardening on homeostatic control. In contrast to our hypothesis that heat-hardening would decrease the metabolic perturbations following heat stress, we found that the acute effect was similar in flies that were heat-hardened and those that were not. Instead, we found that heat-hardened flies showed a much faster recovery to normal metabolite levels. The flies that were heat-hardened before severe stress recovered and reached their normal metabolic homeostasis after 4 to 8 h, whereas those that were not hardened were pushed over a homeostasis boundary and were unable to recover fully within a reasonable period of time or suffered from permanent damage. In this context, it is interesting to note the increasing levels of acetate at the later time points for the severely stressed flies (Fig. 3I). It is possible that this is an indicator of a damaged state. For all other treatments acetate levels were fairly con-
stant.

The increased heat stress resistance brought about by heat-hardening has been demonstrated repeatedly, and the effect has mainly been linked to induction of HSPs (9, 38). HSP levels maximize immediately after a heat-hardening treatment (1). However, as mentioned earlier, the maximal heat-hardening effect does not occur until after 4 to 32 h after the heat-
hardening (9). Studies have shown that costs associated with heat-hardening (e.g., in terms of reduced fecundity) in general are alleviated within a few hours after a moderate heat-hardening stress has been applied (41, 42), lending support to a time separation of costs and benefits of the heat shock response. This temporal mismatch has been attributed to the homeostatic perturbation resulting from heat-hardening itself, which is “paid” primarily in the initial phase after heat-hardening (24, 26, 38). Indeed, along this line of reasoning, our results demonstrate that a moderate heat-hardening stress does lead to metabolic perturbation, but that homeostasis is recovered between 4 and 8 h after the heat-hardening stress.

Comparison with transcription data. The temporal perturba-
tion and return of homeostasis found in this work agreed well with previous observations of gene transcription in Drosophila. DiDomenico et al. (11, 12) showed that a heat-hardening treatment similar to the one used in the present study caused transcription and translation of stress proteins in Drosophila cells to be elevated for the first 4 h, whereas translation of normal proteins was reduced during this period. However, after 4 h of recovery, both transcriptional and translational levels had largely returned to normal. These observations were sub-
stantiated by Sørensen et al. (39) who examined the overall gene expression pattern of D. melanogaster following a 1-h heat-hardening stress at 36°C. Figure 5A shows a direct comparison of PC1 from transcriptional and metabolomic data derived from two separate data sets using the same stock of D. melanogaster. Following a heat-hardening treatment, the metabolomic perturbations reached a maximum a little later than the transcriptional changes, possibly because these changes were downstream of gene transcription. However, the similar temporal development of these responses indicates a strong coupling between the degrees of disturbance at two different biological levels.

As can be seen from Fig. 5B, the majority of the differenti-
ally expressed genes has recovered within the first 8 h. However, despite the fact that hsp expression declined faster than the rest of the genes in the early-up cluster, hsp transcription was still fourfold upregulated after an 8 h recovery period (Fig. 5B). This transcriptional activity was also observed at the protein level as, for example, Hsp70 was nearly fourfold above control levels 8 h after a similar heat-hardening stress (17). This demonstrates the essential role of HSPs for the ability of cells and organisms to harden before a subsequent stress exposure (14). Furthermore, this shows that a large proportion of the costs (perturbations) related to heat-hardening, were paid both on the transcriptional and metabolomic levels within 4 to 8 h (Fig. 5), after which only the beneficial effects (HSP upregulation) remain. This may explain why maximal heat-hardening effects (heat shock survival) occur several hours later than the peak of hsp transcription and HSP protein levels.

In conclusion, we confirmed that NMR metabolomic profil-
ing could be used to visualize the perturbation of and return to cellular homeostasis caused by heat stress in D. melanogaster. With this, we have demonstrated that analysis of the metabolic trajectory caused by a biological stress is a powerful way of monitoring effects on homeostasis, which could be widely applied within cellular/molecular physiology and biochemistry. The data revealed changes in several specific metabolites that are likely to be related to the stress, and both the overall metabolomic response and the changes in the specific metabolites varied with the harshness of the heat stress applied to the flies. Contrary to our original hypothesis, we showed that the protective effect of heat hardening did not reduce the acute metabolic perturbation, but instead resulted in a quicker return to homeostasis. The present study corroborated that HSPs play an essential role in heat hardening. Finally, there is a striking similarity in the temporal development of metabolite concentrations and transcriptional levels suggesting a strong coupling between these two levels.

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