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Supplementary Information

NMR-based investigation of the *Drosophila melanogaster* metabolome under the influence of daily cycles of light and temperature

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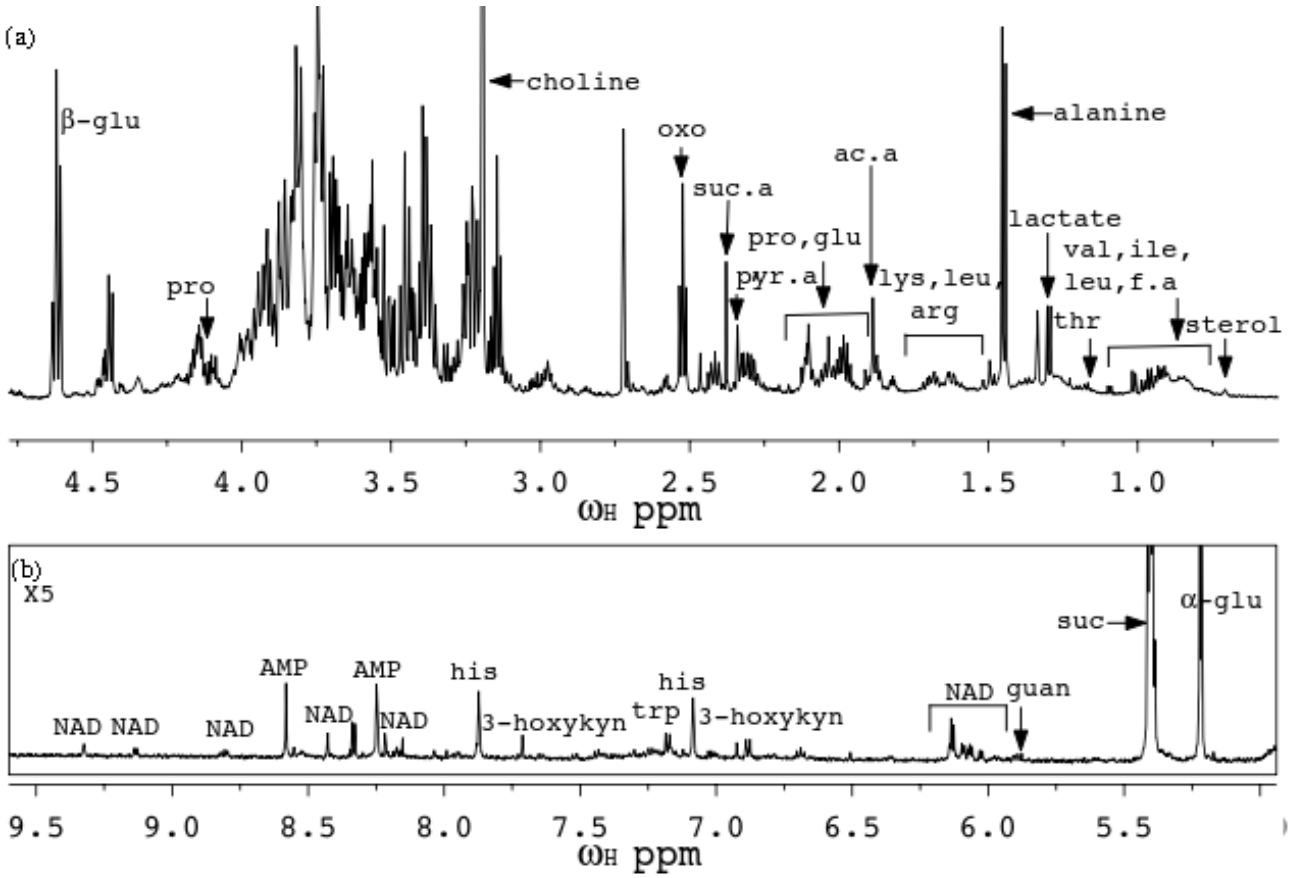

Figure S1: 1D ^{1}H NMR spectrum of *D. melanogaster*, recorded at 600 MHz, showing specific resonances of metabolites identified. Metabolites marked (a) in spectral region 0.0 - 4.6 ppm: sterols, valine (val), leucine (leu), isoleucine (ile), fatty acids (f.a), threonine (thr), lactic acid, alanine, lysine (lys), arginine (arg), acetic acid (ac.a), proline (pro), glutamic acid (glu), pyruvic acid (pyr.a), succinic acid (suc.a), oxo fatty acids (oxo), choline, beta-glucose (β-glu), and (b) in spectral region 5.0 – 9.5 ppm alpha-glucose (α-glu), suc (sucrose), guanosine (guan), histidine (his), tryptophan (trp), 3-hoxykyn (3-hydroxykynurenine), nicotinamide adenine dinucleotide (NAD) and adenosine monophosphate (AMP).

Metabolite	**Chemical shift in ppm (multiplicity, J in Hz)**	**Metabolite**	**Chemical shift in ppm (multiplicity, J in Hz)**
Sterols	0.74 (s)	**Carbohydrates**	
Lipids		Ribose	2.21 (s)
terminal methyl group	0.89 (t,6.9)	Sucrose	4.2 (d,8.7), 5.4 (d,3.8)
-(CH_2)n	1.28 (m)	Erythrose	4.4 (m)
-CH_2-CH_2-COOH (C3)	1.64 (m)	β-glucose	4.63 (d,7.9)
-CH_2-COOH (C2)	2.36 (t,7.5)	α-glucose	5.22 (d,3.7)
Amino acids		**Organic acids**	
Leucine	0.94 (t,5.9), 1.70 (m)	Propionic acid	2.16 (q,7.5)
Valine	0.97 (d,7.0), 1.02 (d,7.1), 2.29 (m)	Lactic acid	1.32 (d,6.9), 4.04 (q,6.9)
Isoleucine	0.99 (d, 6.9)	Acetic acid	1.91 (s)
Alanine	1.47 (d,7.2)	Succinic acid	2.41 (s)
Arginine	1.68 (m), 1.90 (m)	Citric acid	2.53 (d), 2.65 (d)
Lysine	1.71 (m), 1.87 (m)	Fumaric acid	6.51 (s)
Serine	3.84 (dd,5.6,3.8), 3.96 (m)	**Others**	
Proline	1.99 (m), 2.06 (m), 4.12 (dd,8.6,6.4)	Choline Creatine	3.22 (s) 3.92 (s)
Glutamic acid	2.04 (m), 2.34 (m)	Myoinositol	4.05 (t,2.8)
Glutamine	2.44 (m)	NAD	6.03 (d,5.8), 6.08 (d,5.7), 6.12 (d,5.8), 8.16 (s), 8.20 (m), 8.44 (s), 8.83 (d,8.0), 9.15 (d,6.3), 9.33 (s)
Histidine	7.09 (d,0.6), 7.90 (d,1.1)	3-hydroxykynurenine	6.7 (t), 7.43 (d)
Tryptophan	7.31 (s), 7.53 (d,8.1), 7.68 (d,8.0)	AMP	8.26 (s), 8.59 (s)

Table ST1: List of metabolites identified from 1D ^{1}H and 2D NMR experiments, with chemical shift given in ppm and the corresponding multiplicity and scalar coupling J values (in Hz)

Figure S1 shows 1D ^{1}H NMR spectra of *Drosophila*, recorded at 600MHz. The 1D ^{1}H NMR spectrum was crowded with many overlapping peaks. A high- field (low frequency) region from 0.8 to 3.5 ppm had peaks mainly consisting of amino acids and acyl peaks of lipids. The mid low-field region from 3.5 to 5.5 ppm showed peaks mainly from carbohydrates. Further, low-field region beyond 6.0 ppm showed peaks mainly from aromatic compounds. The assignments of the ^{1}H NMR spectrum are summarized in Table ST1.

The presence of sterols was confirmed by the singlet peak identified at 0.74 ppm. The region from 0.8 to 1 ppm was crowded with peaks from lipid terminal methyl and several amino acids. The metabolites were then identified by their multiplicity and J values and found to include amino acids such as leucine, isoleucine and valine. Other amino acids identified included glycine, alanine, arginine, lysine, serine, proline, glutamic acid, glutamine,histidine, threonine and tryptophan. Several organic acids were identified, including propionic acid, lactic acid, acetic acid, succinic acid, fumaric acid and citric acid.

Lipids were identified by the presence of a triplet at 0.89 (J=6.9 Hz), multiplets at 1.28 ppm and 1.64 ppm, and a triplet at 2.36 ppm (J=7.5 Hz). The presence of oxo fatty acids was confirmed by the presence of a triplet peak at 2.55 ppm (J=6.7 Hz). Among sugars, both alpha and beta glucose peaks were identified by the presence of doublets at 5.22 (J=3.7 Hz) and 4.63 ppm (J=7.9 Hz) respectively. Sucrose was identified by a peak at 5.4 ppm (d, J=3.8 Hz). Other sugars identified included erythrose and ribose. Presence of the glucose moiety of aliphatic and aromatic glucosinolates was confirmed by the assignment of peaks with large coupling constants (J=9.5- 10 Hz). Other metabolites identified included peaks for choline at 3.22 (s), creatinine at 3.92 (s) ppm, myoinositol at 4.05 ppm (t, J=2.8) and 3- hydroxykynurenine at 6.7 (t) ppm and 7.43 (d) ppm. The aromatic region beyond 6 ppm showed peaks mainly for NAD and AMP, and also peaks for some of the aromatic amino acids mentioned above. We verified all inferences about metabolite identification by cross-validation with the 2D NMR spectra and spectral database matching. For instance, while in the 1D NMR spectrum, the peak at 5.4 ppm overlaps for both maltose and sucrose, in the corresponding 2D NMR spectra, the peak at 4.2 ppm (d,8.58) is present for sucrose but absent for maltose and we hence used this fact to infer that in our spectra the maltose peak is missing and the peak at 5.4 ppm belongs to sucrose.

Metabolite	**p.value**	**-log10(p)**	**FDR**	**Fisher's LSD**
Fatty acid CH_3	0.000070037	4.1547	0.0083435	ZT 0 - ZT 10; ZT 22 - ZT 0; ZT 0 - ZT 4; ZT 18 - ZT 10; ZT 2 - ZT 10; ZT 20 - ZT 10; ZT 22 - ZT 10; ZT 18 - ZT 12; ZT 20 - ZT 12; ZT 22 - ZT 12; ZT 22 – ZT 14; ZT 14 - ZT 4; ZT 20 - ZT 16; ZT 22 - ZT 16; ZT 22 - ZT 18; ZT 18 - ZT 4; ZT 18 - ZT 8; ZT 22 - ZT 2; ZT 2 - ZT 4; ZT 22 - ZT 20; ZT 20 - ZT 4; ZT 20 - ZT 6; ZT 20 - ZT 8; ZT 22 - ZT 4; ZT 22 - ZT 6; ZT 22 - ZT 8
NAD	0.000071009	4.1487	0.0083435	ZT 20 - ZT 0; ZT 22 - ZT 0; ZT 20 - ZT 10; ZT 22 - ZT 10; ZT 20 - ZT 12; ZT 22 - ZT 12; ZT 22 - ZT 14; ZT 20 - ZT 16; ZT 22 - ZT 16; ZT 22 - ZT 18; ZT 18 - ZT 4; ZT 18 - ZT 6; ZT 18 - ZT 8; ZT 20 - ZT 2; ZT 22 - ZT 2; ZT 20 - ZT 4; ZT 20 - ZT 6; ZT 20 - ZT 8; ZT 22 - ZT 4; ZT 22 - ZT 6; ZT 22 - ZT 8
Choline	0.00027004	3.5686	0.021153	ZT 0 - ZT 10; ZT 0 - ZT 12; ZT 0 - ZT 16; ZT 0 - ZT 4; ZT 0 - ZT 6; ZT 0 - ZT 8; ZT 18 - ZT 10; ZT 22 - ZT 10; ZT 18 - ZT 12; ZT 22 - ZT 12; ZT 22 - ZT 14; ZT 14 - ZT 4; ZT 22 - ZT 16; ZT 18 - ZT 4; ZT 18 - ZT 8; ZT 22 - ZT 2; ZT 2 - ZT 4; ZT 22 - ZT 20; ZT 22 - ZT 4; ZT 22 - ZT 6; ZT 22 - ZT 8
Glucose	0.00072222	3.1413	0.042431	ZT 0 - ZT 10; ZT 22 - ZT 0; ZT 0 - ZT 4; ZT 18 - ZT 10; ZT 22 - ZT 10; ZT 22 - ZT 12; ZT 22 - ZT 14; ZT 14 - ZT 4; ZT 18 - ZT 16; ZT 22 - ZT 16; ZT 18 - ZT 4; ZT 18 - ZT 6; ZT 22 - ZT 2; ZT 2 - ZT 4; ZT 22 - ZT 20; ZT 22 - ZT 4; ZT 22 - ZT 6; ZT 22 - ZT 8
Valine	0.0019417	2.7118	0.077665	ZT 0 - ZT 4; ZT 0 - ZT 8; ZT 14 - ZT 10; ZT 18 - ZT 10; ZT 22 - ZT 10; ZT 22 - ZT 12; ZT 14 - ZT 4; ZT 14 - ZT 6; ZT 14 - ZT 8; ZT 18 - ZT 16; ZT 22 - ZT 16; ZT 18 - ZT 2; ZT 18 - ZT 4; ZT 18 - ZT 6; ZT 18 - ZT 8; ZT 22 - ZT 2; ZT 22 - ZT 20; ZT 22 - ZT 4; ZT 22 - ZT 6; ZT 22 - ZT 8
Sucrose	0.0020945	2.6789	0.077665	ZT 2 - ZT 0; ZT 22 - ZT 0; ZT 4 - ZT 0; ZT 8 - ZT 0; ZT 2 - ZT 10; ZT 22 - ZT 10; ZT 2 - ZT 12; ZT 22 - ZT 12; ZT 4 - ZT 12; ZT 8 - ZT 12; ZT 2 - ZT 14; ZT 22 - ZT 14; ZT 4 - ZT 14; ZT 6 - ZT 14; ZT 8 - ZT 14; ZT 2 - ZT 16; ZT 2 - ZT 18; ZT 2 - ZT 20; ZT 2 - ZT 4; ZT 2 - ZT 6; ZT 2 - ZT 8
Proline	0.0044418	2.3524	0.11598	ZT 0 - ZT 10; ZT 0 - ZT 8; ZT 14 - ZT 10; ZT 18 - ZT 10; ZT 22 - ZT 10; ZT 22 - ZT 12; ZT 14 - ZT 4; ZT 14 - ZT 6; ZT 14 - ZT 8; ZT 22 - ZT 16; ZT 18 - ZT 4; ZT 18 - ZT 6; ZT 18 - ZT 8; ZT 22 - ZT 2; ZT 22 - ZT 20; ZT 22 - ZT 4; ZT 22 - ZT 6; ZT 22 - ZT 8
Lysine	0.0049408	2.3062	0.11611	ZT 0 - ZT 10; ZT 0 - ZT 16; ZT 0 - ZT 4; ZT 22 - ZT 10; ZT 22 - ZT 12; ZT 22 - ZT 14; ZT 18 - ZT 16; ZT 22 - ZT 16; ZT 22 - ZT 18; ZT 22 - ZT 2; ZT 22 - ZT 20; ZT 22 - ZT 4; ZT 22 - ZT 6; ZT 22 - ZT 8
Serine	0.0073728	2.1324	0.13619	ZT 12 - ZT 0; ZT 12 - ZT 10; ZT 12 - ZT 18; ZT 12 - ZT 2; ZT 12 - ZT 20; ZT 12 - ZT 22; ZT 14 - ZT 22; ZT 16 - ZT 22; ZT 4 - ZT 18; ZT 4 - ZT 2; ZT 4 - ZT 20; ZT 4 - ZT 22; ZT 6 - ZT 22; ZT 8 - ZT 22

Table ST2: ANOVA and Post hoc analysis showing metabolites which are responsible for differentiation between all 12 time points given the p value threshold of 0.01.

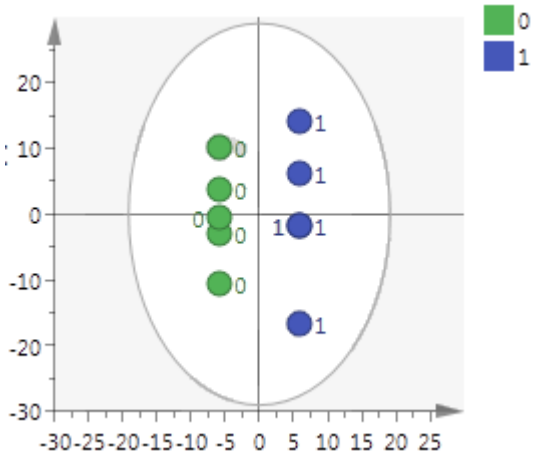

Figure S2: Score plot from ^{1}H NMR spectra of ZT8 and ZT20 time points. 0 denotes ZT20 and 1 denotes ZT8.

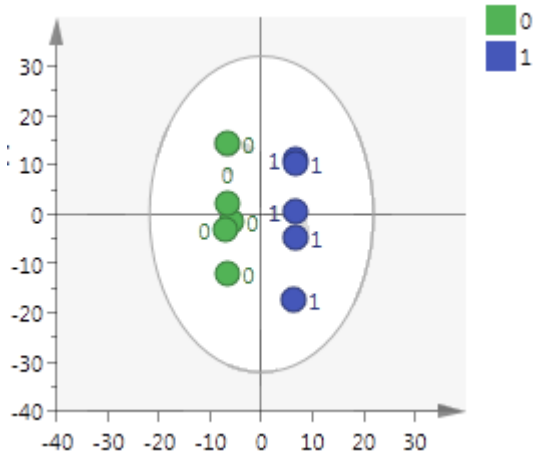

Figure S3: Score plot from ^{1}H NMR spectra of ZT6 and ZT18 time points. 0 denotes ZT18 and 1 denotes ZT6.

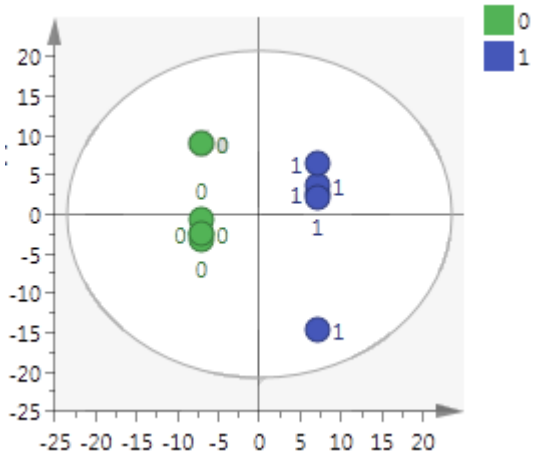

Figure S4: Score plot from ^{1}H NMR spectra of ZT4 and ZT16 time points. 0 denotes ZT16 and 1 denotes ZT4.

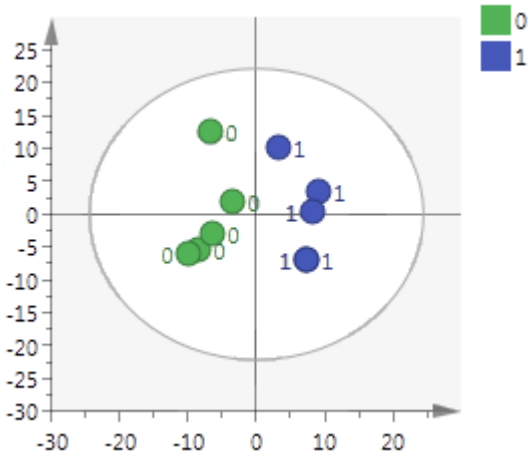

Figure S5: Score plot from ^{1}H NMR spectra of ZT0 and ZT22 time points. 0 denotes ZT22 and 1 denotes ZT0.

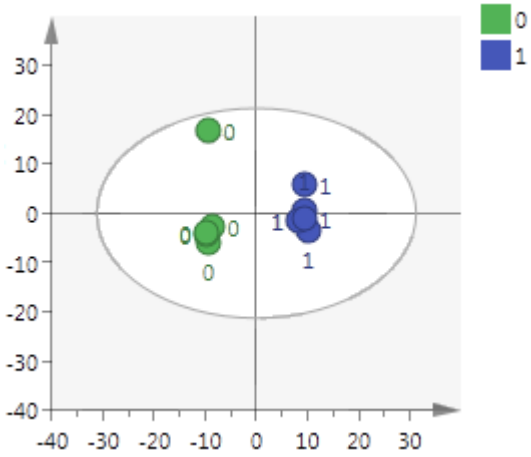

Figure S6: Score plot from ^{1}H NMR spectra of ZT2 and ZT22 time points. 0 denotes ZT22 and 1 denotes ZT2.

Metabolite (ZT6 vs ZT18)	**Metabolite (ZT4 vs ZT16)**
NAD	lipid
valine	sucrose
erythrose	valine
isoleucine	ribose
histidine	
Metabolite (ZT0 vs ZT22)	**Metabolite (ZT2 vs ZT22)**
leucine	alanine
valine	NAD
isoleucine	lipid
NAD	lysine

Table ST3: Metabolites identified to be responsible for separation between the two time points considered, using t-test ($p < 0.01$).