A gut microbial factor modulates locomotor behaviour in *Drosophila*

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Sample Size and Statistics

Figure 1b – i
Analysis – (1b, 1i): Average speed calculated over the 10 min. testing period. (1d): Average bout length calculated over the 10 min. testing period. (1e): Average pause length calculated over the 10 min. testing period. (1f): Number of bouts over 10 min. testing period. (1g): Daily crossings sampled every min. per day. (1h): Stance linearity index, see ref. 64 for calculations
All are biological replicates assessed with a two-tailed test and data are representative of at least 3 independent trials for each experiment.

| Figure | Condition | # | Sample Size | Statistical Test | Comparison | P-value |
|--------|-----------|---|-------------|------------------|------------|---------|
| 1b     | Conv      | 1 | 36          | Kruskal-Wallis test | 1 v 2      | 0.0070  |
|        | Ax        | 2 | 36          | (P < 0.0001)      | 1 v 3      | 0.0024  |
|        | L.p       | 3 | 35          |                  | 1 v 4      | 0.9999  |
|        | L.b       | 4 | 36          |                  | 2 v 3      | 0.9999  |
|        |           |   |             |                  | 2 v 4      | 0.0112  |
|        |           |   |             |                  | 3 v 4      | 0.9999  |
| 1d     | Conv      | 1 | 32          | Kruskal-Wallis test | 1 v 2      | 0.0014  |
|        | Ax        | 2 | 36          | (P = 0.0026)     | 1 v 3      | 0.2111  |
|        | L.p       | 3 | 22          |                  | 1 v 4      | 0.9999  |
|        | L.b       | 4 | 20          |                  | 2 v 3      | 0.9999  |
|        |           |   |             |                  | 2 v 4      | 0.2814  |
|        |           |   |             |                  | 3 v 4      | 0.9999  |
| 1e     | Conv      | 1 | 32          | Kruskal-Wallis test | 1 v 2      | < 0.0001|
|        | Ax        | 2 | 36          | (P < 0.0001)     | 1 v 3      | 0.0034  |
|        | L.p       | 3 | 22          |                  | 1 v 4      | 0.9999  |
|        | L.b       | 4 | 20          |                  | 2 v 3      | 0.9999  |
|        |           |   |             |                  | 2 v 4      | 0.0335  |
|        |           |   |             |                  | 3 v 4      | 0.3321  |
| 1f     | Conv      | 1 | 32          | Kruskal-Wallis test | 1 v 2      | < 0.0001|
|        | Ax        | 2 | 36          | (P = 0.6685)     | 1 v 3      | 0.1980  |
|        | L.p       | 3 | 22          |                  | 1 v 4      | 0.9999  |
|        | L.b       | 4 | 20          |                  | 2 v 3      | 0.9999  |
|        |           |   |             |                  | 2 v 4      | 0.0335  |
|        |           |   |             |                  | 3 v 4      | 0.4716  |
| 1g     | Conv      | 1 | 8           | Kruskal-Wallis test | 1 v 2      | 0.0100  |
|        | Ax        | 2 | 8           | (P = 0.0042)     | 1 v 3      | 0.1980  |
|        | L.p       | 3 | 8           |                  | 1 v 4      | 0.9999  |
|        | L.b       | 4 | 8           |                  | 2 v 3      | 0.9999  |
|        |           |   |             |                  | 2 v 4      | 0.0335  |
|        |           |   |             |                  | 3 v 4      | 0.4716  |
| 1h     | Conv      | 1 | 6           | Kruskal-Wallis test | 1 v 2      | 0.0063  |
|        | Ax        | 2 | 7           | (P = 0.0057)     | 1 v 3      | 0.8248  |
|        | L.p       | 3 | 5           |                  | 1 v 4      | 0.9999  |
|        | L.b       | 4 | 5           |                  | 2 v 3      | 0.6907  |
|        |           |   |             |                  | 2 v 4      | 0.0508  |
|        |           |   |             |                  | 3 v 4      | 0.9999  |
| Figure | Condition | #  | Sample Size | Statistical Test     | Comparison | P-value |
|--------|-----------|----|-------------|----------------------|------------|---------|
| 1i     | Conv      | 1  | 25          | Kruskal-Wallis test  | 1 v 2      | 0.2846  |
|        | Ax        | 2  | 29          | (P = 0.0016)         | 1 v 3      | 0.0470  |
| 1i     | L.p       | 3  | 24          |                      | 1 v 4      | 0.9999  |
| 1i     | L.b       | 4  | 35          |                      | 2 v 3      | 0.9999  |
|        |           |    |             |                      | 2 v 4      | 0.0453  |
|        |           |    |             |                      | 3 v 4      | 0.0047  |

**Figure 2a – f**

Analysis – (2a – d, 2f): Average speed. (2e): Carbohydrate analysis of whole fly homogenates measured using HPAEC-PAD. Each sample contains 5 flies.

All are biological replicates assessed with a two-tailed test and data are representative of at least 2 independent trials for each experiment, except for Figure 2e in which one trial was performed.

| Figure | Condition | Measurement | #  | Sample Size | Statistical Test     | Comparison | P-value |
|--------|-----------|-------------|----|-------------|----------------------|------------|---------|
| 2a     | Ax        |             | 1  | 57          | Kruskal-Wallis test  | 1 v 2      | 0.0004  |
| 2a     | L.b       |             | 2  | 42          | test                 | 1 v 3      | 0.0294  |
| 2a     | L.b CFS   | 3           | 36 | (P = 0.0004) |                      | 1 v 4      | 0.9999  |
| 2a     | L.b HK    | 4           | 24 |             |                      | 2 v 3      | 0.9999  |
|        |           |             |    |             |                      | 2 v 4      | 0.1188  |
|        |           |             |    |             |                      | 3 v 4      | 0.8799  |
| 2b     | Conv      |             | 1  | 17          | Kruskal-Wallis test  | 1 v 2      | < 0.0001 |
| 2b     | Ax        |             | 2  | 45          | test                 | 1 v 3      | 0.9999  |
| 2b     | Xi*       | 3           | 29 | (P < 0.0001) |                      | 2 v 3      | < 0.0001 |
| 2c     | Ax        |             | 1  | 31          | Kruskal-Wallis test  | 1 v 2      | 0.0253  |
| 2c     | L.b CFS   | 2           | 12 | test        |                      | 1 v 3      | 0.0048  |
| 2c     | Xi*       | 3           | 28 | (P = 0.0014) |                      | 1 v 4      | 0.9999  |
| 2c     | Ai*       | 4           | 13 |             |                      | 2 v 3      | 0.9999  |
|        |           |             |    |             |                      | 2 v 4      | 0.2720  |
|        |           |             |    |             |                      | 3 v 4      | 0.2177  |
| 2d     | Ax        |             | 1  | 28          | Kruskal-Wallis test  | 1 v 2      | 0.0002  |
| 2d     | L.b       |             | 2  | 29          | test                 | 1 v 3      | 0.9056  |
| 2d     | L.b\(\Delta y/A\) | 3      | 18 | (P = 0.0003) |                      | 2 v 3      | 0.0424  |
| 2e     | Ax        | Total       | 5  |             | Mann-Whitney test    |            | 0.0079  |
| 2e     | Xi*       |             | 5  |             | test                 |            | 0.5000  |
| 2e     | Ax        | Gluc        | 5  |             | Mann-Whitney test    |            | 0.2222  |
| 2e     | Xi*       |             | 5  |             | test                 |            | 0.0079  |
| 2e     | Ax        | Fruc        | 5  |             | Mann-Whitney test    |            | 0.0079  |
| 2e     | Xi*       |             | 5  |             | test                 |            | 0.0079  |
| 2e     | Ax        | Ribo        | 5  |             | Mann-Whitney test    |            | 0.0079  |
| 2e     | Xi*       |             | 5  |             | test                 |            | 0.0079  |
| 2e     | Ax        | Treh        | 5  |             | Mann-Whitney test    |            | 0.0079  |
| 2e     | Xi*       |             | 5  |             | test                 |            | 0.0079  |
### Table 1: Statistical Analysis of Average Speed

| Figure | Condition | #  | Sample Size | Statistical Test          | Comparison | P-value  |
|--------|-----------|----|-------------|----------------------------|------------|----------|
| 2f     | Ax        | 1  | 16          | Kruskal-Wallis test        | 1 v 2      | 0.0465   |
|        | Xi*       | 2  | 18          | (P = 0.0057)               | 1 v 3      | 0.8352   |
|        | Ax+Treh   | 3  | 16          |                             | 1 v 4      | 0.9999   |
|        | Xi*+Treh  | 4  | 17          |                             | 2 v 3      | 0.9999   |
|        |           |    |             |                             | 2 v 4      | 0.0090   |
|        |           |    |             |                             | 3 v 4      | 0.3024   |

### Figure 3a – g

Analysis – (3a): Difference in average speed between flies calculated for each GAL4 line crossed with UAS-dTRPA1 tested at 27°C. Each point represents an independent trial, containing between 10 - 15 flies per group. Tdc2/Ddc/Th/ChAT, n = 5; Tβh, n = 6; Gad1, n = 3. For further details and graphs of each GAL4 line, see Extended Data Fig. 8. (3b – g): Average speed. For 3b and c, statistical significance was found for both variables (microbial status x genotype) calculated by Two-way ANOVA. In 3c, there is a significant interaction between microbial status and genotype (P = 0.0437). When examined separately, the Kruskal-Wallis test on only Conv flies across genotypes was significant (P = 0.0030) and the Kruskal-Wallis test on only ABX flies was not significant (P = 0.8426). All are biological replicates assessed with a two-tailed test and data are representative of at least 2 independent trials for each experiment.

| Figure | Condition | Genotype | Sample Size | Statistical Test       | P-value  |
|--------|-----------|-----------|-------------|------------------------|----------|
| 3b     | ABX       | UAS-TrpA1 | 15          | Mann-Whitney test      | 0.0139   |
|        |           |           | 12          |                         |          |
|        | ABX       | Tdc2-GAL4 | 23          | Mann-Whitney test      | 0.0177   |
|        |           |           | 23          |                         |          |
|        | ABX       | GAL4>UAS | (27°C)      | Mann-Whitney test      | 0.6308   |
|        |           |           | 14          |                         |          |
|        | ABX       | GAL4>UAS | (20°C)      | Mann-Whitney test      | 0.0238   |
|        |           |           | 15          |                         |          |
| 3c     | Conv      | UAS-TrpA1 | 45          | Mann-Whitney test      | 0.0033   |
|        |           |           | 30          |                         |          |
|        | Conv      | Tdc2-GAL4 | 48          | Mann-Whitney test      | 0.0263   |
|        |           |           | 39          |                         |          |
|        | Conv      | GAL4>UAS | (27°C)      | Mann-Whitney test      | 0.4929   |
|        |           |           | 49          |                         |          |
|        | Conv      | GAL4>UAS | (20°C)      | Mann-Whitney test      | 0.0143   |
|        |           |           | 31          |                         |          |
Extended Data Figure 1a – h
Analysis – (1a): Colony forming units. (1b – e, h): Average speed. (1f): Average speed calculated only within walking bouts. (1g): Tripod index: see ref. 64 for calculations. All are biological replicates assessed with a two-tailed test.
| Figure | Condition | # | Sample Size | Statistical Test | Comparison | P-value |
|--------|-----------|---|-------------|------------------|------------|---------|
| 1c     | Ax        | 1 | 58          | Kruskal-Wallis   | 1 v 2      | 0.0008  |
|        | L.b EW    | 2 | 57          | test            | 1 v 3      | < 0.0001|
|        | L.b Bb14  | 3 | 57          | (P < 0.0001)    | 2 v 3      | 0.9999  |
| 1d     | Ax        | 1 | 45          | Kruskal-Wallis   | 1 v 2      | 0.0465  |
|        | L.b EW    | 2 | 28          | test            | 1 v 3      | < 0.0001|
|        | L.b P-2   | 3 | 42          | (P < 0.0001)    | 2 v 3      | 0.0348  |
| 1e     | Diet 1 Ax | 1 | 20          | Mann-Whitney    | 1 v 2      | 0.0006  |
|        | Diet 1 L.b| 2 | 21          | test            |            |         |
|        | Diet 2 Ax | 3 | 18          | Mann-Whitney    | 3 v 4      | 0.0004  |
|        | Diet 2 L.b| 2 | 16          | test            |            |         |
|        | Diet 3 Ax | 5 | 6           | Mann-Whitney    | 5 v 6      | 0.0411  |
|        | Diet 3 L.b| 6 | 6           | test            |            |         |
| 1f     | Conv      | 1 | 23          | Kruskal-Wallis   | 1 v 4      | 0.9999  |
|        | Ax        | 2 | 35          | test            |            |         |
|        | L.p       | 3 | 22          | (P = 0.1731)    |            |         |
|        | L.b       | 4 | 22          |                 |            |         |
| 1g     | Conv      | 1 | 6           | Kruskal-Wallis   | 1 v 4      | 0.9999  |
|        | Ax        | 2 | 7           | test            |            |         |
|        | L.p       | 3 | 5           | (P = 0.1358)    |            |         |
|        | L.b       | 4 | 5           |                 |            |         |
| 1h     | Ax        | 1 | 18          | Kruskal-Wallis   | 1 v 2      | 0.9999  |
|        | L.p       | 2 | 24          | test            | 1 v 3      | 0.0090  |
|        | L.b       | 3 | 24          | (P < 0.0001)    | 1 v 4      | < 0.0001|
|        | L.p+L.b   | 4 | 24          |                 | 2 v 3      | 0.2590  |
|        |           |   |             |                 | 2 v 4      | 0.0037  |
|        |           |   |             |                 | 4 v 5      | 0.9631  |

**Extended Data Figure 2b – 1**

Analysis – (2b, e – g, i): Average speed. (2c, j): Average bout length. (2d, k): Average speed within walking bouts. (2l): Daily crossings.

All are biological replicates assessed with a two-tailed test.
| Figure | Condition | # | Sample Size | Statistical Test          | Comparison | P-value  |
|--------|-----------|---|-------------|--------------------------|------------|----------|
| 2e     | Conv      | 1 | 11          | Kruskal-Wallis test      | 1 v 2      | 0.0127   |
|        | Ax        | 2 | 53          |                          | 1 v 3      | 0.9999   |
|        | L.b 0d    | 3 | 53          | (P = 0.0004)             | 1 v 4      | 0.9999   |
|        | L.b 3-5d  | 4 | 52          |                          | 2 v 3      | 0.0065   |
|        |           |   |             |                          | 2 v 4      | 0.0056   |
|        |           |   |             |                          | 3 v 4      | 0.9999   |
| 2f     | Conv      | 1 | 32          | Kruskal-Wallis test      | 1 v 2      | 0.0122   |
|        | Ax        | 2 | 36          |                          | 1 v 3      | 0.0091   |
|        | ABX       | 3 | 36          | (P = 0.0038)             | 2 v 3      | 0.9999   |
| 2g     | Conv (OR) | 1 | 20          | Mann-Whitney test        | 1 v 2      | 0.0265   |
|        | ABX (OR)  | 2 | 22          |                          |            |          |
|        | Conv (CS) | 3 | 12          | Mann-Whitney test        | 3 v 4      | 0.0093   |
|        | ABX (CS)  | 4 | 17          |                          |            |          |
| 2i     | ABX       | 1 | 29          | Kruskal-Wallis test      | 1 v 2      | 0.9999   |
|        | L.p       | 2 | 24          |                          | 1 v 3      | 0.0203   |
|        | L.b       | 3 | 35          | (P = 0.0011)             | 2 v 3      | 0.0019   |
| 2j     | ABX       | 1 | 36          | Kruskal-Wallis test      | 1 v 2      | 0.9999   |
|        | L.p       | 2 | 30          |                          | 1 v 3      | 0.0698   |
|        | L.b       | 3 | 35          | (P = 0.0203)             | 2 v 3      | 0.0350   |
| 2k     | ABX       | 1 | 42          | Kruskal-Wallis test      | 1 v 2      | 0.9999   |
|        | L.p       | 2 | 30          |                          | 1 v 3      | 0.0607   |
|        | L.b       | 3 | 35          | (P = 0.0079)             | 2 v 3      | 0.0094   |
| 2l     | ABX       | 1 | 6           | Kruskal-Wallis test      | 1 v 2      | 0.8385   |
|        | L.p       | 2 | 6           |                          | 1 v 3      | 0.0148   |
|        | L.b       | 3 | 6           | (P = 0.0109)             | 2 v 3      | 0.2507   |

Extended Data Figure 3a–f

Analysis – (3a, b, f): Average speed. (3c): Average bout length. (3d): Average speed within walking bouts. (3e): Daily crossings.

All are biological replicates assessed with a two-tailed test.

| Figure | Condition | # | Sample Size | Statistical Test          | Comparison | P-value  |
|--------|-----------|---|-------------|--------------------------|------------|----------|
| 3a     | Ax        | 1 | 45          | Kruskal-Wallis test      | 1 v 2      | 0.9999   |
|        | L.p       | 2 | 17          | test                     | 1 v 3      | 0.3301   |
|        | L.b       | 3 | 42          | (P = 0.01)               | 1 v 4      | 0.9999   |
|        | L.p CFS   | 4 | 17          |                          | 1 v 5      | 0.0445   |
|        | L.b CFS   | 5 | 16          |                          | 3 v 5      | 0.9999   |
|        |           |   |             |                          | 4 v 5      | 0.0906   |
| 3b     | Ax        | 1 | 23          | Kruskal-Wallis test      | 1 v 2      | 0.1287   |
|        | L.p CFS   | 2 | 20          | test                     | 1 v 3      | < 0.0001 |
|        | L.b CFS   | 3 | 20          | (P < 0.0001)             | 2 v 3      | 0.0025   |
### Extended Data Figure 4a – e

#### Analysis – (4a, b): Average speed. (4c): Expression of gene transcripts, samples from whole body homogenates. (4d): Amount ingested. (4e): Gut content.

All are biological replicates assessed with a two-tailed test.

| Figure | Condition | Measurement | # | Sample Size | Statistical Test | Comparison | P-value |
|--------|-----------|-------------|---|-------------|------------------|------------|---------|
| 4a     | Wt Conv   |             | 1 | 16          | Mann-Whitney test| 1 v 2      | 0.0032  |
|        | Wt ABX    |             | 2 | 17          | Mann-Whitney test|            |         |
|        | IMD Conv  |             | 3 | 24          | Mann-Whitney test| 3 v 4      | 0.0168  |
|        | IMD ABX   |             | 4 | 25          | Mann-Whitney test|            |         |
| 4b     | Wt Conv   |             | 1 | 15          | Mann-Whitney test| 1 v 2      | 0.0299  |
|        | Wt ABX    |             | 2 | 17          | Mann-Whitney test| 3 v 4      | < 0.0001|
|        | Ti Conv   |             | 3 | 10          | Mann-Whitney test| 1 v 2      | 0.0004  |
|        | Ti ABX    |             | 4 | 11          | Mann-Whitney test| 1 v 4      | 0.4509  |
| 4c     | Ax        | Dpt         | 1 | 8           | One-way          |            |         |
|        | L.p CFS   |             | 2 | 10          | ANOVA test       |            |         |
|        | L.b CFS   |             | 3 | 10          | ANOVA test       |            |         |
|        | Ax        | Drs         | 4 | 10          | One-way          |            |         |
|        | L.p CFS   |             | 5 | 10          | ANOVA test       |            |         |
|        | L.b CFS   |             | 6 | 10          | ANOVA test       |            |         |
|        | Ax        | Cec         | 7 | 8           | One-way          |            |         |
|        | L.p CFS   |             | 8 | 10          | ANOVA test       |            |         |
|        | L.b CFS   |             | 9 | 10          | ANOVA test       |            |         |
|        | Ax        | AttA        | 10| 5           | One-way          |            |         |
|        | L.p CFS   |             | 11| 5           | ANOVA test       |            |         |
|        | L.b CFS   |             | 12| 5           | ANOVA test       |            |         |
|        | Ax        | Duox        | 13| 3           | One-way          |            |         |
|        | L.p CFS   |             | 14| 5           | ANOVA test       |            |         |
|        | L.b CFS   |             | 15| 5           | ANOVA test       |            |         |
### Extended Data Figure 5a – j

Analysis – (5a – f, h): Average speed. (5g): Daily crossings. (5i): Percent survival. Each sample consists of 15 – 25 flies. (5j): Percentage of apoptotic cells.

All are biological replicates assessed with a two-tailed test.

| Figure | Condition | # | Sample Size | Statistical Test | Comparison | P-value |
|--------|-----------|---|-------------|------------------|------------|---------|
| 5a     | Ax        | 1 | 18          | Kruskal-Wallis   | 1 v 2      | 0.0566  |
|        | L.b CFS   | 2 | 18          | test             | 1 v 3      | 0.9999  |
|        | + Typ     | 3 | 17          | (P = 0.0008)     | 1 v 4      | 0.0021  |
|        | - Typ     | 4 | 17          |                  | 3 v 4      | 0.0181  |
| 5b     | Ax        | 1 | 23          | Kruskal-Wallis   | 1 v 2      | 0.0081  |
|        | L.b CFS   | 2 | 18          | test             | 1 v 3      | 0.5168  |
|        | +PK       | 3 | 23          | (P = 0.0037)     | 1 v 4      | 0.0139  |
|        | - PK      | 4 | 23          |                  | 3 v 4      | 0.9999  |
| 5c     | Ax        | 1 | 18          | Kruskal-Wallis   | 1 v 2      | 0.0081  |
|        | L.b CFS   | 2 | 18          | test             | 1 v 3      | 0.1867  |
|        | +100°C    | 3 | 18          | (P = 0.0102)     | 2 v 3      | 0.7709  |
| 5d     | Ax        | 1 | 30          | Kruskal-Wallis   | 1 v 2      | 0.0170  |
|        | Am         | 2 | 17          | test             | 1 v 3      | 0.0036  |
|        | -amy      | 3 | 30          | (P = 0.0016)     | 2 v 3      | 0.9999  |
| 5e     | Ax        | 1 | 30          | Kruskal-Wallis   | 1 v 2      | < 0.0001|
|        | L.b       | 2 | 30          | test             | 1 v 3      | 0.2185  |
|        | L.p       | 3 | 29          | (P < 0.0001)     | 1 v 4      | 0.9140  |
|        | A.p       | 4 | 30          |                  | 1 v 5      | < 0.0001|
|        | E.c       | 5 | 18          |                  | 2 v 3      | 0.0074  |
| 5f     | Ax        | 1 | 65          | Kruskal-Wallis   | 1 v 2      | 0.0183  |
|        | E.c       | 2 | 52          | test             | 1 v 3      | 0.0003  |
|        | ΔtyrA     | 3 | 18          | (P < 0.0001)     | 1 v 4      | 0.0300  |
|        | ΔtrpC     | 4 | 17          |                  | 1 v 5      | < 0.0001|
|        | ΔmanX     | 5 | 45          |                  | 1 v 6      | < 0.0001|
|        | ΔtreA     | 6 | 46          |                  | 1 v 7      | 0.9999  |
|        | ΔxylA     | 7 | 20          |                  |            |         |
| Figure | Condition | # | Sample Size | Statistical Test | Comparison | P-value |
|--------|-----------|---|-------------|------------------|------------|---------|
| 5g     | Conv      | 1 | 16          | Kruskal-Wallis   | 1 v 2      | 0.0432  |
|        | Ax        | 2 | 24          | test             | 2 v 3      | 0.0640  |
|        | L.b CFS   | 3 | 19          | (P = 0.0027)     | 2 v 4      | 0.9999  |
|        | ΔxyA CFS  | 4 | 20          | Kruskal-Wallis   | 2 v 5      | 0.0134  |
|        | Xi        | 5 | 8           |                  |            |         |
| 5h     | Ax        | 1 | 16          | Kruskal-Wallis   | 1 v 2      | 0.0274  |
|        | L.b CFS   | 2 | 11          | test             | 1 v 3      | 0.9999  |
|        | 10 Xi     | 3 | 12          | (P = 0.0167)     | 1 v 4      | 0.0263  |
|        | 100 Xi    | 4 | 14          |                  |            |         |
| 5i     | Ax        | 1 | 4           | Kruskal-Wallis   |            |         |
|        | L.p CFS   | 2 | 5           | test             |            |         |
|        | L.b CFS   | 3 | 5           | (P = 0.4117)     |            |         |
|        | Xi        | 4 | 4           |                  |            |         |
| 5j     | Conv      | 1 | 7           | Kruskal-Wallis   | 1 v 2      | 0.9999  |
|        | Ax        | 2 | 5           | test             | 2 v 3      | 0.9999  |
|        | L.p CFS   | 3 | 4           | (P = 0.0383)     | 2 v 4      | 0.0343  |
|        | L.b CFS   | 4 | 6           |                  | 2 v 5      | 0.9999  |
|        | Xi        | 5 | 6           |                  |            |         |

**Extended Data Figure 6a – b**

Analysis – (6a, b): Sleep analysis.

All are biological replicates assessed with a two-tailed test.

| Figure | Condition | # | Sample Size | Statistical Test | Comparison | P-value |
|--------|-----------|---|-------------|------------------|------------|---------|
| 6a     | Conv      | 1 | 8           | Kruskal-Wallis   | 1 v 2      | 0.0193  |
|        | Light     | Ax| 2           | test             | 2 v 3      | 0.9999  |
|        | Phase     | L.p| 3          | (P = 0.0036)     | 2 v 4      | 0.0193  |
|        |           | L.b| 4          |                  |            |         |
| 6a     | Conv      | 1 | 8           | Kruskal-Wallis   |            |         |
|        | Dark      | Ax| 2           | test             |            |         |
|        | Phase     | L.p| 3          | (P = 0.0585)     |            |         |
|        |           | L.b| 4          |                  |            |         |
| 6b     | Conv      | 1 | 17          | Kruskal-Wallis   | 1 v 2      | 0.0911  |
|        | Light     | Ax| 2           | test             | 2 v 3      | 0.4850  |
|        | Phase     | L.p| 3          | (P = 0.0314)     | 2 v 4      | 0.9999  |
|        |           | L.b| 4          |                  | 2 v 5      | 0.0932  |
|        | Xi        | 5 | 8           |                  |            |         |
| 6b     | Conv      | 1 | 17          | Kruskal-Wallis   |            |         |
|        | Dark      | Ax| 2           | test             |            |         |
|        | Phase     | L.p| 3          | (P = 0.1477)     |            |         |
|        |           | L.b| 4          |                  |            |         |
|        | Xi        | 5 | 8           |                  |            |         |
Extended Data Figure 7a–k

Analysis – (7a–c, h–k): Average speed. (7d–e): Carbohydrate analysis measured using HPAEC-PAD. (7f–g): Trehalose levels in whole fly homogenate measured using a Megazyme Kit.

All are biological replicates assessed with a two-tailed test.

| Figure | Condition | Measurement | # | Sample Size | Statistical Test | Comparison | P-value |
|--------|-----------|-------------|---|-------------|------------------|------------|---------|
| 7a     | Ax        |             | 1 | 16          | Kruskal-         | 1 v 2      | 0.0021  |
|        | Xi        |             | 2 | 13          | Wallis test      | 1 v 3      | 0.1866  |
|        | +Fruc     |             | 3 | 13          | (P = 0.0041)     | 1 v 4      | 0.1992  |
|        | +Gluc     |             | 4 | 15          |                  |            |         |
| 7b     | Ax        |             | 1 | 26          | Kruskal-         | 1 v 2      | 0.0026  |
|        | Xi        |             | 2 | 21          | Wallis test      | 1 v 3      | 0.3686  |
|        | +Xylose   |             | 3 | 22          | (P = 0.0048)     | 1 v 4      | 0.1780  |
|        | +Xylulose |             | 4 | 18          |                  |            |         |
| 7c     | Ax        |             | 1 | 21          | Kruskal-         | 1 v 2      | 0.0003  |
|        | Xi        |             | 2 | 16          | Wallis test      | 1 v 3      | 0.2565  |
|        | Xi+EDTA   |             | 3 | 18          | (P = 0.0005)     | 2 v 3      | 0.0959  |
| 7d     | Ax        | Gluc        | 1 | 3           | Mann-            | 1 v 2      | 0.1000  |
|        | Xi        |             | 2 | 3           | Whitney test     |            |         |
|        | Ax        | Fruc        | 3 | 3           | Mann-            | 3 v 4      | 0.4000  |
|        | Xi        |             | 4 | 3           | Whitney test     |            |         |
|        | Ax        | Mann        | 5 | 3           | Mann-            | 5 v 6      | 0.7000  |
|        | Xi        |             | 6 | 3           | Whitney test     |            |         |
|        | Ax        | Xylu        | 7 | 3           | Mann-            | 7 v 8      | 0.4000  |
|        | Xi        |             | 8 | 3           | Whitney test     |            |         |
|        | Ax        | Treh        | 9 | 3           | Mann-            | 9 v 10     | 0.1000  |
|        | Xi        |             | 10| 3          | Whitney test     |            |         |
| 7e     | Ax        | Ribo        | 1 | 5           | Kruskal-         | 1 v 2      | 0.0908  |
|        | Xi        |             | 2 | 5           | Wallis test      | 1 v 3      | 0.0047  |
|        | Xi+EDTA   |             | 3 | 5           | (P = 0.0004)     | 2 v 3      | 0.9601  |
|        | Ax        | Treh        | 4 | 5           | Kruskal-         | 4 v 5      | 0.0027  |
|        | Xi        |             | 5 | 5           | Wallis test      | 4 v 6      | 0.1431  |
|        | Xi+EDTA   |             | 6 | 5           | (P = 0.0001)     | 5 v 6      | 0.5373  |
| 7f     | Conv      | Treh        | 1 | 9           | Kruskal-         | 1 v 2      | 0.0161  |
|        | Ax        |             | 2 | 6           | Wallis test      | 1 v 3      | 0.9999  |
|        | Xi        |             | 3 | 3           | (P = 0.0083)     |            |         |
| 7g     | Ax        | Treh        | 1 | 15          | Mann-            | 1 v 2      | 0.0059  |
|        | L.b       |             | 2 | 15          | Whitney test     |            |         |
| 7h     | Ax        |             | 1 | 40          | Kruskal-         | 1 v 2      | 0.0284  |
|        | Xi        |             | 2 | 40          | Wallis test      | 1 v 3      | 0.9999  |
|        | Xi+Treh   |             | 3 | 39          | (P = 0.0004)     | 1 v 4      | 0.0825  |
|        | Xi+Ara    |             | 4 | 18          |                  | 2 v 3      | 0.0028  |
|        |           |             |   |             |                  | 3 v 4      | 0.0160  |
| Figure | Condition | # | Sample Size | Statistical Test | Comparison | P-value |
|--------|-----------|---|-------------|------------------|------------|---------|
| 7i     | Ax        | 1 | 29          | Kruskal-Wallis test | 1 v 2      | 0.0350  |
|        | Xi        | 2 | 25          |                  | 1 v 3      | 0.9999  |
|        | +Ribo    | 3 | 12          | (P = 0.0286)     |            |         |
| 7j     | Conv      | 1 | 15          | Kruskal-Wallis test | 1 v 2      | 0.0022  |
|        | Ax        | 2 | 22          |                  | 1 v 3      | 0.0914  |
|        | Conv+Treh | 3 | 18          | (P = 0.0045)     | 3 v 4      | 0.9999  |
|        | Ax+Treh   | 4 | 15          |                  |            |         |
| 7k     | Ax        | 1 | 27          | Kruskal-Wallis test | 1 v 2      | 0.0361  |
|        | Xi        | 2 | 19          |                  | 1 v 3      | 0.8516  |
|        | Xi+EDTA   | 3 | 24          | (P = 0.0499)     | 1 v 4      | 0.9999  |
|        | Xi+Treh   | 4 | 19          |                  | 1 v 5      | 0.9999  |

Extended Data Figure 8b–h

Analysis – (8b–h): Average speed.
All are biological replicates assessed with a two-tailed test.

| Figure | Condition | Genotype | Sample Size | Statistical Test | P-value |
|--------|-----------|----------|-------------|------------------|---------|
| 8b     | ABX       | UAS-TrpA1 | 15          | Mann-Whitney test | 0.0027  |
|        | L.b CFS   |          | 14          |                  |         |
|        | ABX       | pBDPG4U-GAL4 | 24      | Mann-Whitney test | 0.0127  |
|        | L.b CFS   |          | 20          |                  |         |
|        | ABX       | GAL4>UAS (27°C) | 14     | Mann-Whitney test | 0.0456  |
|        | L.b CFS   |          | 9           |                  |         |
|        | ABX       | GAL4>UAS (20°C) | 16     | Mann-Whitney test | 0.0390  |
|        | L.b CFS   |          | 11          |                  |         |
| 8c     | ABX       | UAS-TrpA1 | 24          | Mann-Whitney test | 0.0008  |
|        | L.b CFS   |          | 24          |                  |         |
|        | ABX       | Tdc2-GAL4 | 24          | Mann-Whitney test | 0.0252  |
|        | L.b CFS   |          | 23          |                  |         |
|        | ABX       | GAL4>UAS (27°C) | 25    | Mann-Whitney test | 0.4265  |
|        | L.b CFS   |          | 26          |                  |         |
|        | ABX       | GAL4>UAS (20°C) | 19    | Mann-Whitney test | 0.0233  |
|        | L.b CFS   |          | 19          |                  |         |
| 8d     | ABX       | UAS-TrpA1 | 26          | Mann-Whitney test | 0.0012  |
|        | L.b CFS   |          | 18          |                  |         |
|        | ABX       | Tβh-GAL4 | 36          | Mann-Whitney test | 0.0003  |
|        | L.b CFS   |          | 24          |                  |         |
|        | ABX       | GAL4>UAS (27°C) | 53   | Mann-Whitney test | 0.5668  |
|        | L.b CFS   |          | 23          |                  |         |
|        | ABX       | GAL4>UAS (20°C) | 21   | Mann-Whitney test | 0.0006  |
|        | L.b CFS   |          | 7           |                  |         |
| Figure | Condition | Genotype         | Sample Size | Statistical Test | P-value |
|--------|-----------|------------------|-------------|------------------|---------|
| 8e     | ABX       | UAS-TrpA1        | 34          | Mann-Whitney     | 0.0089  |
|        | L.b CFS   |                  | 26          |                  |         |
|        | ABX       | Ddc-GAL4         | 34          | Mann-Whitney     | 0.0157  |
|        | L.b CFS   |                  | 28          |                  |         |
|        | ABX       | GAL4>UAS         | 10          | Mann-            | < 0.0001|
|        | L.b CFS   | (27°C)           | 17          | Whitney test     |         |
|        | ABX       | GAL4>UAS         | 17          | Mann-            | 0.0004  |
|        | L.b CFS   | (20°C)           | 13          | Whitney test     |         |
| 8f     | ABX       | UAS-TrpA1        | 36          | Mann-            | 0.0016  |
|        | L.b CFS   |                  | 30          | Whitney test     |         |
|        | ABX       | Th-GAL4          | 40          | Mann-            | 0.0041  |
|        | L.b CFS   |                  | 31          | Whitney test     |         |
|        | ABX       | GAL4>UAS         | 19          | Mann-            | < 0.0001|
|        | L.b CFS   | (27°C)           | 17          | Whitney test     |         |
|        | ABX       | GAL4>UAS         | 14          | Mann-            | 0.0103  |
|        | L.b CFS   | (20°C)           | 8           | Whitney test     |         |
| 8g     | ABX       | UAS-TrpA1        | 21          | Mann-            | 0.0330  |
|        | L.b CFS   |                  | 12          | Whitney test     |         |
|        | ABX       | Gad1-GAL4        | 28          | Mann-            | 0.0120  |
|        | L.b CFS   |                  | 24          | Whitney test     |         |
|        | ABX       | GAL4>UAS         | 24          | Mann-            | 0.0001  |
|        | L.b CFS   | (27°C)           | 20          | Whitney test     |         |
|        | ABX       | GAL4>UAS         | 16          | Mann-            | 0.0135  |
|        | L.b CFS   | (20°C)           | 15          | Whitney test     |         |
| 8h     | ABX       | UAS-TrpA1        | 31          | Mann-            | 0.0153  |
|        | L.b CFS   |                  | 20          | Whitney test     |         |
|        | ABX       | ChAT-GAL4        | 31          | Mann-            | 0.0179  |
|        | L.b CFS   |                  | 29          | Whitney test     |         |
|        | ABX       | GAL4>UAS         | 16          | Mann-            | 0.0207  |
|        | L.b CFS   | (27°C)           | 17          | Whitney test     |         |
|        | ABX       | GAL4>UAS         | 18          | Mann-            | < 0.0001|
### Extended Data Figure 9

Analysis – Average speed.

All are biological replicates assessed with a two-tailed test.

| Figure | Condition | Genotype | Sample Size | Statistical Test       | P-value |
|--------|-----------|-----------|-------------|------------------------|---------|
| 9      | ABX       | UAS-TrpA1 | 15          | Mann-Whitney test      | 0.3314  |
|        | L.b CFS   | Tβh<sup>M18</sup> | 14          | Mann-Whitney test      |         |
|        | ABX       | Tβh-GAL4  | 28          | Mann-Whitney test      | 0.2235  |
|        | L.b CFS   | Tβh<sup>M18</sup> | 20          | Mann-Whitney test      |         |
|        | ABX       | GAL4>UAS  | 11          | Mann-Whitney test      | 0.2284  |
|        | L.b CFS   | Tβh<sup>M18</sup> (27°C) | 13          | Mann-Whitney test      |         |
|        | ABX       | L.b CFS   | 9           | Mann-Whitney test      | 0.0745  |

### Extended Data Figure 10a – k

Analysis – (10a, d, e, f, h – k): Average speed. (10b – c): Expression of gene transcripts, samples from head homogenates.

All are biological replicates assessed with a two-tailed test.

| Figure | Condition | Measurement | # | Sample Size | Statistical Test       | Comparison | P-value |
|--------|-----------|-------------|---|-------------|------------------------|------------|---------|
| 10a    | Ax        | Tdc         | 1 | 26          | Kruskal-Wallis test    | 1 v 2      | 0.9999  |
|        | Ax+OA     |             | 2 | 27          | Unpaired t-test        | 1 v 3      | 0.9999  |
|        | Ax+L-dopa |             | 3 | 6           | (P = 0.0004)          | 1 v 4      | 0.0012  |
|        | L.b CFS   |             | 4 | 35          | Unpaired t-test        | 1 v 5      | 0.9965  |
|        | L.b CFS+OA|             | 5 | 26          | Unpaired t-test        | 1 v 6      | 0.0405  |
|        | L.b CFS+L-dopa |         | 6 | 6           |                        |            |         |
| 10b    | Ax        | Tdc         | 1 | 5           | Unpaired t-test        | 1 v 2      | 0.0754  |
|        | L.b CFS   |             | 2 | 5           | Unpaired t-test        | 1 v 3      | 0.0426  |
|        | Ax        | Tβh         | 3 | 5           | Unpaired t-test        | 3 v 4      | 0.6179  |
|        | L.b CFS   |             | 4 | 5           | Unpaired t-test        | 5 v 6      | 0.8425  |
|        | Ax        | Ddc         | 5 | 3           | Unpaired t-test        | 7 v 8      | 0.0030  |
|        | L.b CFS   |             | 6 | 5           | Unpaired t-test        | 2 v 4      | 0.0019  |
| 10c    | Ax        | Tdc         | 1 | 5           | Unpaired t-test        | 1 v 2      | 0.0122  |
|        | Xi        |             | 2 | 6           | Unpaired t-test        | 1 v 3      | 0.0036  |
|        | Ax        | Tβh         | 3 | 5           | Unpaired t-test        | 3 v 4      | 0.8425  |
|        | Xi        |             | 4 | 6           | Unpaired t-test        | 4 v 4      | 0.0296  |
| 10d    | Ax        | Tdc         | 1 | 21          | Kruskal-Wallis test    | 1 v 2      | 0.9999  |
|        | Ax+TA     |             | 2 | 10          | Unpaired t-test        | 1 v 3      | 0.0030  |
|        | L.b CFS   |             | 3 | 10          | (P < 0.0001)          | 1 v 4      | 0.0019  |
|        | L.b CFS+TA|             | 4 | 9           |                        | 2 v 4      | 0.0296  |
| Figure | Condition | Genotype | # | Sample Size | Statistical Test | Comparison | P-value |
|--------|-----------|----------|---|-------------|------------------|------------|---------|
| 10e    | Ax        | Tdc2-GAL4; Tsh-GAL80 | 1  | 25          | Mann-Whitney test | 1 v 2      | 0.0216  |
|        | Xi        |          | 2  | 18          |                  |            |         |
|        |           | UAS-DTI  | 3  | 26          | Mann-Whitney test | 3 v 4      | 0.0151  |
|        | Ax        |          | 4  | 21          | Whitney test     |            |         |
|        | Xi        | GAL4;GAL80 > UAS | 5  | 39          | Mann-Whitney test | 5 v 6      | 0.3173  |
| 10f    | Ax        | UAS-TβhRNAi | 1  | 9           | Mann-Whitney test | 1 v 2      | 0.0106  |
|        | L.b CFS   |          | 2  | 9           | Whitney test     |            |         |
|        | Ax        | Elav-GAL4 | 3  | 24          | Mann-Whitney test | 3 v 4      | 0.0040  |
|        | L.b CFS   |          | 4  | 19          | Whitney test     |            |         |
|        | Ax        | GAL4>UAS | 5  | 24          | Mann-Whitney test | 5 v 6      | 0.1703  |
|        | L.b CFS   |          | 6  | 21          | Whitney test     |            |         |
| 10h    | Ax        |          | 1  | 14          | Kruskal-Wallis test | 1 v 2      | 0.0007  |
|        | Xi        |          | 2  | 15          | Wallis test      | 1 v 3      | 0.5040  |
|        | Xi+Mianserin |          | 3  | 15          | (P = 0.0010)     | 2 v 3      | 0.0593  |
| 10i    | Conv      |          | 1  | 13          | Kruskal-Wallis test | 1 v 2      | 0.0010  |
|        | Ax        |          | 2  | 28          | Wallis test      | 1 v 3      | 0.9999  |
|        | Xi        |          | 3  | 24          | (P = 0.0015)     | 2 v 3      | 0.0234  |
|        | Conv+Mian |          | 4  | 27          |                  | 4 v 5      | 0.9999  |
|        | Ax+Mian   |          | 5  | 22          |                  | 4 v 6      | 0.9999  |
|        | Xi+Mian   |          | 6  | 22          |                  | 5 v 6      | 0.9999  |
| 10j    | Conv      | Wt (w+)  | 1  | 13          | Mann-Whitney test | 1 v 2      | 0.0093  |
|        | ABX       |          | 2  | 21          |                  |            |         |
|        |Conv       | Tdc<sup>R023</sup> | 3  | 28          | Mann-Whitney test | 3 v 4      | 0.6889  |
|        | ABX       |          | 4  | 34          |                  |            |         |
| 10k    | Conv      | Wt (CS)  | 1  | 38          | Mann-Whitney test | 1 v 2      | 0.0100  |
|        | ABX       |          | 2  | 42          |                  |            |         |
|        | Conv      | Tβh<sup>M18</sup> | 3  | 25          | Mann-Whitney test | 3 v 4      | 0.7435  |
|        | ABX       |          | 4  | 33          |                  |            |         |