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Integrated Respirometry and Metabolomics Unveil Circadian Metabolic Dynamics in Drosophila

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eLife Assessment

This **important** study investigates how sleep loss and circadian disruption affect whole-organ metabolism in flies (Drosophila melanogaster) and reports that wild-type flies align metabolism in anticipation of diurnal rhythm, while mutant flies with impaired sleep or circadian function shift to reactive or misaligned metabolism. The integration of chamber-based flow-through respirometry with LC-MS metabolomics is innovative, and the significance of the findings is significant. The strength of evidence needed to support the conclusions is generally **convincing**, although the absence of direct measures of locomotor activity makes it difficult to separate intrinsic metabolic changes from potential behavioral differences in mutants.

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Abstract

Sleep and circadian rhythms shape organismal energy patterns, but how this timing connects to oxygen use and carbon dioxide production remains incompletely understood. We combined high-resolution respirometry with LC-MS-based metabolomics to characterize respiratory dynamics and metabolic states in *Drosophila melanogaster*, resolving genotype-specific impacts of sleep disruption and circadian regulation. Wild-type flies under light-dark cycles (WT-LD) showed rhythmic respiratory patterns reflective of anticipatory coordination of mitochondrial energy metabolism, amino acid turnover, and redox cycling. Short-sleep mutants (*fmn*, *sss*) exhibited elevated metabolic rates with reactive shifts of fuel preferences toward lipid and amino acid catabolism and altered mitochondrial respiration. The clock-mutant (per^{01}) and flies under constant darkness (WT-DD) showed reactive and widespread metabolic dysregulation and impaired redox homeostasis. These findings demonstrate that both sleep and circadian systems contribute to aligning metabolic substrate selection with energy demands, offering mechanistic insights into how disruptions in behavioral states compromise metabolic health.

Introduction

Metabolism is a fundamental physiological process responsible for coordinating energy production and substrate utilization in response to changing physiological demands. This process is dynamically regulated by behavioral states, such as sleep and wakefulness, as well as directly by internal circadian clocks that align physiology with the external environment [1-3]. Disruptions in

these temporal processes are strongly associated with metabolic disorders, including obesity and type 2 diabetes [4-6] as well as other metabolic diseases such as cardiovascular dysfunction and cancer [7].

With conserved and well-characterized sleep and circadian mechanisms [8, 9], as well as metabolic pathways also found in mammals, *Drosophila* offers a genetically tractable model for dissecting interactions between these different systems. In particular, sleep mutants with robust phenotypes can be employed to determine how loss of sleep impacts metabolic parameters, including rhythms of metabolic activity. At the same time, additional tools are now available for metabolic measurements previously not possible. Multiple approaches can be combined for a comprehensive understanding of metabolism under precisely controlled environmental conditions and at high temporal resolution [10].

Indirect calorimetry is a non-invasive form of respirometry that enables real-time quantification of whole-organism metabolic rate by measuring oxygen consumption (VO_2) and carbon dioxide production (VCO_2)[11, 12]. These measurements allow for the estimation of energy expenditure and respiratory quotient (RQ), which can provide an index of relative substrate utilization, with appropriate caveats [13, 14]. Although widely applied in mammalian systems [15], indirect calorimetry in small model organisms such as *Drosophila melanogaster* has only recently become feasible with advances in high-resolution, small-volume flow-through systems [16-18]. This is a critical advancement, as dissecting time-of-day-dependent metabolic regulation in mammals is often confounded by overlapping effects of sleep, activity, and feeding rhythms [19, 20]. While this can also be true in flies, the genetic tools available allow distinction between these processes to a greater extent.

This study focuses on wild-type isogenic control flies alongside three genetically characterized mutants: two short-sleep mutants: *fumin* (*fmn*), which displays impaired dopamine transporter function and defective dopamine reuptake [21] and *sleepless* (*sss*), which lacks the Sleepless protein, resulting in altered membrane excitability and reduced GABA signaling [22-24]; and the circadian mutant *period*^{01} (*per*^{01}), which lacks a functional molecular clock [25]. To investigate how sleep and circadian disruption independently and interactively influence metabolic regulation, we utilized a small-animal respirometry platform built on the MAVEn-FT system, coupled with a Licor 7000 CO_2 analyzer and an Oxzilla differential O_2 analyzer. This system builds upon the Sleep and Activity Metabolic Monitor (SAMM), which first demonstrated the feasibility of simultaneous CO_2 and O_2 measurements in *Drosophila* [26]. This MAVEn-based platform enhances temporal resolution and throughput, enabling continuous measurement of respiratory parameters across multiple groups of flies over daily cycles, and allowing fine-scale profiling of metabolic rhythms and their disruption across sleep-wake cycles. In this study, we interpret RQ conservatively as reflecting relative shifts in substrate utilization over time and between genotypes, rather than as a precise quantitative measure of absolute carbohydrate versus lipid oxidation.

To provide a more comprehensive perspective on metabolic regulation, we complemented newly generated respiratory measurements with steady-state metabolomic profiling using liquid chromatography-mass spectrometry (LC-MS) data previously published from our group [27]. This integrative framework enabled time-matched and lag-aware analysis of respiratory output and metabolite profiles across Zeitgeber time in the LD cycle, providing detailed insights into how sleep and circadian mutations influence metabolic homeostasis [27-29]. By employing this framework, we sought to uncover conserved mechanisms linking temporal biological processes, such as sleep and circadian rhythms, to metabolic homeostasis and to elucidate how genetic and environmental perturbations in these systems influence energy balance and metabolic regulation.

Methods

Drosophila Strains, Entrainment and Collection

Drosophila melanogaster isogenic control (*iso*), two short-sleep mutants, fumin (*fmn*) [21] and sleepless (*sss*) [22-24], as well as a circadian clock mutant (per^{01}) [25]. were used for this study. Male flies were collected shortly after eclosion and entrained in light-dark (LD) incubators for a minimum of three days before diurnal (LD) time-course collection across Zeitgeber time (ZT). At the time of collection, flies were between five and seven days old. Wild-type (WT) isogenic control flies were either maintained under 12 hrs:12 hrs light versus dark conditions (WT-LD) or placed in constant darkness (WT-DD) for at least 24 hours prior to collection to examine light-independent rhythms. Mutant strains (*fmn, sss, and* per^{01}) were maintained under LD cycles. All flies were reared on a standard cornmeal/molasses medium at 25°C. Zeitgeber time (ZT) 0 was designated as lights-on, with lights-off occurring at ZT12 (12/12 LD).

Only male flies were used for all respirometry experiments and for integration with the metabolomics dataset. This was done to reduce variability introduced by female reproductive status (e.g., mating/egg production) and associated metabolic differences, enabling a clearer comparison across genotypes and lighting conditions.

Respirometry Setup

All experiments were performed using groups of 25 flies per chamber. To account for genotype-specific differences in body size, each group of 25 flies (*WT, fmn, sss, and* per^{01}) was weighed prior to respirometry, and these weights were used to normalize VCO_2 and VO_2 values for accurate comparison. Each chamber was provisioned with 2 mL of fly food (2% agar, 5% sucrose) to sustain the flies during the 24-hour continuous recording period. Control chambers containing only food, without flies, were included to account for background microbial metabolism. To simulate natural microbial inoculation, flies were briefly introduced into these control chambers and then removed before data acquisition began. Each genotype measurement represents an average of ~300 flies (25 flies/chamber × 4 chambers/experiment × 3 experimental days), with the chamber as the experimental unit.

The respirometry system continuously measured CO_2 production and O_2 consumption using a flow-through MAVEn system. It consisted of five main components: (1) a zero-grade air source and scrubbing column to remove residual CO_2, (2) mass-flow controllers to regulate airflow, (3) Sable Systems RC chambers to house the flies, (4) the MAVEn system to direct air sequentially from each chamber, and (5) gas analyzers for measuring CO_2 and O_2 concentrations. The system was calibrated every 3-5 trials using 100% N_2 (CO_2 = 0 ppm) and a known CO_2 standard to ensure measurement accuracy.

Zero-Grade Air Supply and Scrubbing System

Zero-grade compressed air (<0.1 ppm hydrocarbons) was first passed through a custom-built scrubbing column (#26800, Drierite) to remove residual CO_2. The column was packed with an inner layer of Ascarite (#81133–20–2, Acros Organics) flanked by two outer layers of Drierite (#7779–18–9, Drierite), separated by glass wool (#11–388, Fisher Scientific).

To re-humidify the air to ~9 parts per thousand (ppt) water vapor content, it was then passed through Nafion tubing (#TT-070, Perma Pure, LLC) submerged in deionized water. Bev-A-line nonpermeable tubing (#56280, United States Plastic Corp.) was used to connect all system components.

Airflow Regulation

Re-humidified, CO_2-free air was split into two streams, each regulated by mass flow controllers to maintain a constant flow rate of 25 mL/min. One stream provided reference air to the CO_2 and O_2 analyzers (controlled by a Side-Trak 840 Series, Sierra Instruments, Inc. MFCV), while the second stream provided flow to the fly chambers (controlled by the MAVEn's internal mass flow-controlled system).

Respirometry Chambers and MAVEn System's Airflow Management

Flies were housed in Sable Systems RC chambers (70 mm × 20 mm borosilicate glass tubes), integrated into a continuous flow through MAVEn system. The MAVEn splits airflow into 16 channels for fly chambers and one dedicated baseline channel. Airflow is constantly and continuously maintained through all fly chambers while the MAVEn's multiplexing system will switch the 'active' chamber's airflow toward the analyzer chain for a preset dwell time of 120 seconds per chamber, and 60 seconds for baseline, with a baseline interleave ratio of 4 to ensure accuracy in O_2 measurement (i.e a baseline measurement after every four chambers). In this system the MAVEn manages air flow to allow for the sequential measurements of individual chamber gas parameters and air flow rates. Experimental respirometry recording were conducted for 24-hour periods. System control and data acquisition were handled via Sable Systems MAVEn Controller software.

CO_2 Production Measurement

Baseline CO_2 levels were recorded from the reference air prior to entering the chambers. As flies respired, the CO_2 released in each chamber was subsequently flushed to the CO_2 analyzer (Li-7000, LI-COR Biosciences) via the MAVEn system.

O_2 Consumption Measurement

Similarly, O_2 consumed by flies was measured immediately using the Oxzilla differential O_2 analyzer (Oxzilla II Oxygen Analyzer, Sable Systems International) placed downstream from the CO_2 analyzer in the analyzer chain. In this setup-controlled flow reference air exiting the CO_2 analyzer's reference cell was routed into the Oxzilla's reference channel, while animal air exiting the CO_2 analyzer's sample cell was routed to the Oxzilla's sample channel. To prevent dilution effects, water vapor was removed prior to O_2 analysis using small scrubbing columns.

Water Vapor Scrubbing Columns

Separate scrubbing columns were used for reference and animal airflows and placed in-line directly prior to the Oxzilla differential O_2 analyzer's reference and sample channel inlet ports. Each column was made from a 10 mL syringe body (#14955459, Fisher Scientific) filled with an inner layer of Ascarite and two outer layers of magnesium perchlorate (M54, Fisher Scientific), separated by glass wool. Bev-A-line tubing was secured with rubber stoppers (#14–135E, Fisher Scientific). Columns were replaced twice during each 24-hour experiment to maintain performance.

Carbon Dioxide and Oxygen Analysis and Calculations

O_2 consumption was quantified by measuring the decrease in O_2 concentration as air passed through chambers containing flies. To ensure accuracy, a Savitzky-Golay filter (25-second window) was applied to smooth the raw signal, followed by a lag correction of 127 seconds accounted for delay between chamber exit and O_2 analyzer input. To match dry atmospheric air, the reference air was baseline corrected to 20.95% O_2. For both reference and animal air the $\%O_2$ values were converted to O_2 fractional contents by dividing percentage values by 100 (thus baseline air has a fractional content of 0.2095). The VO_2 value from an empty chamber was subtracted from experimental values [30]:

$$VO_2 = (\text{Animal } O_2 - \text{Reference } O_2) \times \text{Flow rate} / (1 - \text{Reference } O_2)$$

VO_2 values were expressed in μL/hr by summing all 5-minute bins into hourly averages.

CO_2 production was quantified by measuring the increase in CO_2 concentration as air passed through chambers containing flies. To ensure accuracy, a Savitzky-Golay filter (25-second window) was applied to smooth the raw signal, followed by a lag correction of 22 seconds with Z correction and 28 seconds without Z correction accounted for delay between chamber exit and O_2 analyzer input. To match dry atmospheric air, the reference air was baseline corrected to 0 parts per

million (ppm) CO_2. For both reference and animal air the ppm CO_2 values were converted to CO_2 fractional contents by dividing percentage values by 1000000. The VCO_2 value from an empty chamber was subtracted from experimental values [30]:

$$VCO_2 = (\text{Reference } CO_2 + \text{Animal } CO_2) \times \text{Flow rate} / \text{l-Reference } CO_2$$

VCO_2 values were expressed in µL/hr by summing all 5-minute bins into hourly averages.

Respiratory quotient (RQ) was calculated as:

RQ= VCO_2 / VO_2. RQ was used as an index of relative shifts in substrate utilization over time and between genotypes, interpreted conservatively rather than as a precise quantitative measure of absolute carbohydrate versus lipid oxidation as this has not been directly characterized in flies.

Gut Tissue Respirometry

Oxygen consumption rate (OCR) was measured in dissected gut tissue using the Resipher System (Lucid Scientific, GA, USA). Male flies were briefly anesthetized on ice and whole guts were dissected in Schneider's Media. 96-well flat-bottom plates were coated with 50 µL of Poly-D-lysine for 30 min to promote tissue adhesion; each well was then filled with 300 µL of Schneider's Media, a single gut was placed in a fixed position within each well, and the plate was fitted with the Resipher sensor lid. The Resipher hub recorded OCR (fmol/mm²/s) continuously for 72 h in a humidified incubator at 27 °C. OCR was extracted in 4-h bins and averaged over the 24-h window beginning 12 h after loading (the 12-36 h interval, once tissue respiration had stabilized). Experiments were performed in three independent runs; values were pooled, median-normalized to the iso^{31} controls within each run, and log2(x + 10)-transformed. A single iso^{31} gut in the third run of the per^{01} experiment was identified as an outlier (Prism "Identify Outliers") and excluded; no other values were removed. Normalized OCR was compared between each mutant and iso^{31} using the Mann-Whitney test in GraphPad Prism 10, with significance at $p < 0.05$ (*fmn* vs iso^{31}, n = 12 vs 12 guts; per^{01} vs iso^{31}, n = 12 vs 11 guts after the single iso^{31} exclusion).

Metabolite Extraction and LCMS Measurements

The metabolomics dataset analyzed in this study was previously published and is publicly available, as described in detail in [27, 31]. In the present study, these data were integrated with respirometry measurements to assess temporal relationships between metabolite abundance and respiratory output. Briefly, polar metabolites were extracted from fly bodies sampled every 2 hours from ZT0 to ZT22 using a modified Bligh-Dyer extraction method, as previously reported [31, 32]. The polar fraction of each extract was dried under vacuum and reconstituted in 100 µL of acetonitrile:Milli-Q water, followed by vortexing for 20 seconds. Samples were analyzed using an ion-switching LC-MS method, and peak integration and data processing were performed according to previously published procedures [27].

Statistical Analysis

All statistical analyses were conducted using GraphPad Prism. Non-parametric Spearman correlation was used to evaluate relationships among metabolic parameters. Metabolites with *p*-values below 0.05 were considered statistically significant and selected for further investigation. To assess genotype-specific differences, one-way ANOVA was performed followed by a pairwise unpaired two-tailed Student's *t*-tests comparing the control group (WT-LD) with each genotype (*fmn*, *sss*, per^{01}, and WT-DD).

Metabolomics-respirometry integration and lag analysis

To integrate respirometry with metabolomics, RQ was recorded continuously at 1-second resolution and averaged into 5-minute bins. Because steady-state metabolite measurements were acquired at 2-hour intervals, we extracted the RQ values corresponding to each 2-hour Zeitgeber Time (ZT) sampling point from the 5-minute-binned dataset to generate time-matched RQ-metabolite pairs. We additionally evaluated temporal relationships using a lag analysis by systematically shifting the RQ time series relative to the metabolite timepoints (−120, −60, −30, −15,

−5, +5, +15, +30, +60, and +120 minutes). Metabolite abundances were normalized as described above, and associations between RQ and individual metabolites were quantified using Spearman rank correlations (ρ) at each lag. Metabolites showing strong associations (e.g., $|\rho| > 0.7$ with nominal $p < 0.05$) were carried forward for visualization and summary, and lag direction was interpreted as metabolites preceding (negative lag) or following (positive lag) changes in RQ.

Rhythmicity analysis

Rhythmicity analysis was performed using Nitecap, a tool for circadian and rhythmic analyses [33]. Time-series metabolic data were analyzed to compute rhythmic parameters using the RAIN algorithm [34]. Rhythms were assessed for statistical significance using false discovery rate (FDR)-adjusted p-values, ensuring robust detection while minimizing false positives. The lag parameters of each rhythm were computed using JTK cycle algorithm [35].

Pathway Analysis

Significant metabolites identified from univariate analyses were used for metabolic pathway analysis via MetaboAnalyst 5.0. Metabolites were uploaded using HMDB identifiers and analyzed using the hypergeometric enrichment method, with relative-betweenness centrality applied for topology analysis based on the *Drosophila melanogaster* (KEGG) pathway library. Pathways with a p-value less than 0.05 were considered statistically significant.

Results

Metabolic Fuel Utilization Across Genotypes Based on Respiratory Quotient (RQ)

To assess whole-body metabolic activity, we performed respirometry on groups of 25 flies per genotype, measuring oxygen consumption (VO_2) and carbon dioxide production (VCO_2) across the 24-hour cycle. The respiratory quotient (RQ), calculated as the ratio of VCO_2 to VO_2, provides insight into the predominant metabolic substrate being utilized: an RQ of 1.0 reflects pure carbohydrate metabolism, values below 1.0 indicate lipid and protein oxidation, and values above 1.0 suggest lipogenesis (the conversion of carbohydrates into fats) [14]. RQ, VCO_2, and VO_2 were continuously recorded from Zeitgeber Time (ZT) 0 to 24 at one-second intervals and subsequently averaged into 5-minute bins for analysis. Figures 1a-c shows the 5-minute binned VCO_2, VO_2 and RQ profiles across the 24-hour cycle for all genotypes (separate individual plots for VCO_2, VO_2, and RQ for each genotype are provided in Figure S1. a-e, S2. a-e and S3. a-e respectively. Replicate information for respirometry provided in Supplementary Table S3). The average values of VCO_2, VO_2 and RQ across the full 24-hour cycle are summarized in Figure 1d-f.

Among the genotypes tested, WT-LD, WT-DD, and per^{01}flies exhibited similarly elevated RQ values (1.20, 1.19, and 1.20, respectively), consistent with active lipogenesis. Group differences were assessed using the Kruskal-Wallis test, followed by Dunn's multiple comparisons post hoc test, which revealed no statistically significant differences in RQ among these three genotypes (Figure 1f, ns), suggesting that neither free-running conditions in constant darkness (WT-DD) nor loss of clock function in the per^{01}mutant alters the dominant fuel utilization strategy under basal conditions.

In contrast, short-sleeping mutants displayed lower RQ values. The *fmn* mutant exhibited a moderate reduction (RQ = 1.09), reflecting increased reliance on carbohydrate metabolism. The *sss* mutant showed the lowest RQ (0.94), indicating a shift toward lipid and protein catabolism. Both mutants were significantly different from WT-LD ($p < 0.001$), highlighting genotype-specific alterations in fuel utilization associated with sleep loss (Figure 1f).

These results demonstrate that WT-LD, WT-DD, and per^{01} flies maintain a lipogenic profile, whereas sleep-deficient mutants exhibit altered substrate utilization, favoring catabolism of carbohydrates or lipids depending on the severity of sleep disruption.

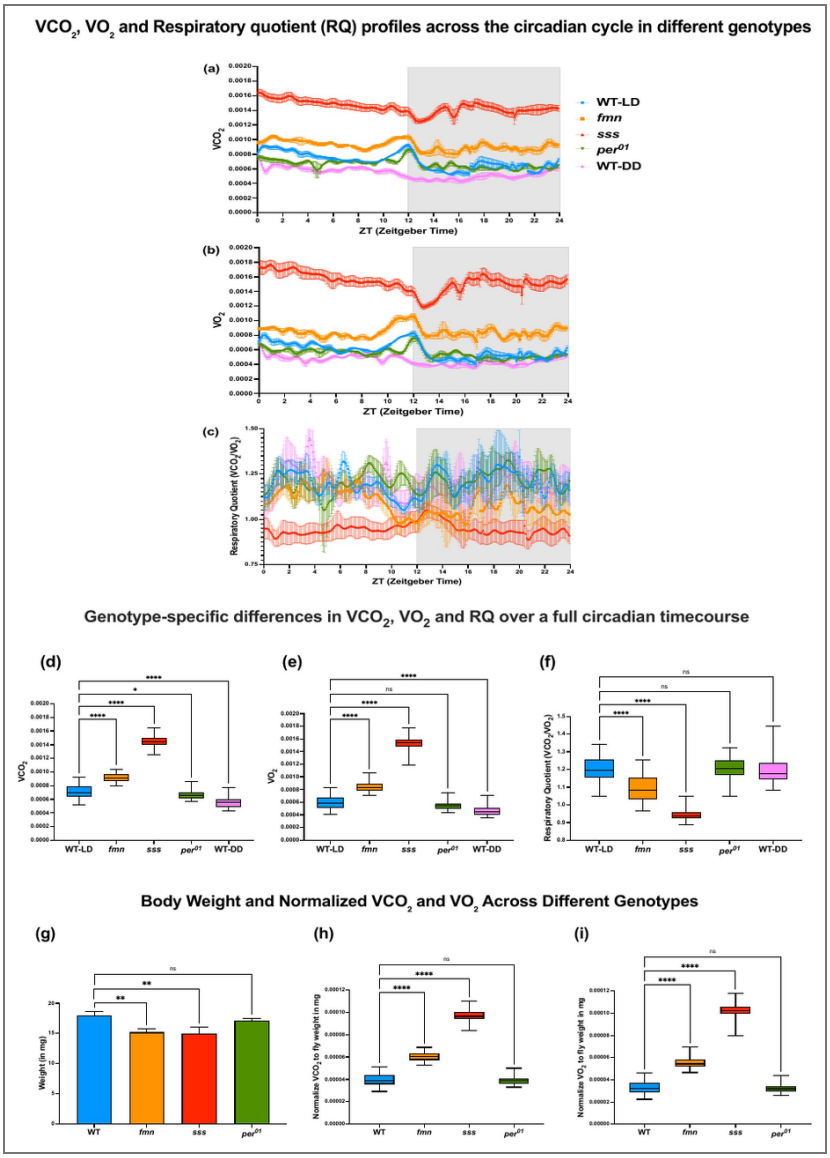

Figure 1.

a-c. *VCO_2, VO_2, and respiratory quotient (RQ) profiles across the 24-hour cycle in different genotypes*. VCO_2, VO_2 and RQ (VCO_2/VO_2) was continuously recorded at one-second intervals from Zeitgeber Time (ZT) 0 to 24 and subsequently averaged into 5-minute bins for analysis. Traces represent mean VCO_2, VO_2, and RQ values across the 24-hour recording period for wild-type flies under light-dark conditions (WT-LD), short-sleep mutants *fumin* (*fmn*) and *sleepless* (*sss*), the circadian clock mutant *period*^{01} (*per*^{01}), and wild-type flies maintained in constant darkness (WT-DD). Source data are the continuous respirometry recordings. **d-f. *Genotype-specific differences in VCO_2, VO_2, and RQ measured over a full 24-hour recording period*.** Boxplots show average values of (left to right) respiratory quotient (RQ), carbon dioxide production (VCO_2), and oxygen consumption (VO_2) across genotypes and lighting conditions. Measurements were taken continuously over a 24-hour period using a flow-through MAVEn system. Genotypes include wild-type under light-dark (WT-LD) and constant darkness (WT-DD), short-sleep mutants *fumin* (*fmn*) and *sleepless* (*sss*), and circadian mutant *per*^{01}. Group differences were assessed using the **Kruskal-Wallis test**, followed by **Dunn's multiple comparisons post hoc test**. Significance is denoted as: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***); ns = not significant. **g-i. *Body Weight and Normalized VCO_2 and VO_2 Across Different Genotypes*.** Body weight (mg) and respiratory parameters (VCO_2 and VO_2) normalized to body weight (mg) were measured in wild type (WT), *fmn*, *sss*, and *per*^{01} mutant flies. Statistical significance was assessed using one-way ANOVA followed by Dunnett's multiple comparisons test against WT. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***); ns = not significant. For all panels with error bars or shaded error bands, values are presented as mean ± SEM. Data represent approximately 300 flies per genotype across three experimental days (25 flies/chamber, four chambers/experiment). The chamber was used as the experimental unit; *n* denotes the number of chambers.

Furthermore, to account for potential differences in body size that could confound VCO_2 and VO_2 measurements, fly weights were recorded for each genotype before respirometry. Notably, *fmn* and *sss* mutants weighed significantly less than WT controls, whereas per^{01} flies had comparable weights (Figure 1g). Accordingly, VCO_2 and VO_2 values were normalized to fly weight, and the genotype-specific differences remained robust after normalization (Figure 1h-i). These data confirm that the observed metabolic differences are not attributable to body size but reflect inherent alterations in metabolic fuel utilization. Figures 1, 2, 3, and Figure S4 are derived from the same underlying continuous respirometry recordings but present different analytical summaries: Figure 1 shows 5-minute-binned time-course traces and 24-hour averages, Figures 2-3 present rhythmicity and phase analyses, and Figure S4 presents day (ZT0-12) versus night (ZT12-24) averages.

Diurnal Variation in CO_2 Production, O_2 Consumption, and Respiratory Quotient Across Genotypes

To investigate how sleep and diurnal regulation influence temporal patterns of metabolism, we measured carbon dioxide production (VCO_2), oxygen consumption (VO_2), and respiratory quotient (RQ) across day and night phases in wild-type flies maintained under light-dark cycles (WT-LD), wild-type flies kept in constant darkness (WT-DD) to assess free-running circadian regulation in the absence of external light-dark cues, short-sleep mutants (*fumin* [*fmn*] and *sleepless* [*sss*]), and the circadian clock mutant *period*^{01} (per^{01}). The average values of VCO_2, VO_2 and RQ during the day (ZT0-12) and night (ZT12-24) are presented in Figure S4.

The *sss* mutant exhibited the highest metabolic rates among all genotypes, with significantly elevated VCO_2 and VO_2 during both day and night relative to WT-LD ($p < 0.001$). A pronounced day-night difference in *sss* further indicated a strong phase-dependent increase in respiratory activity (Figure S4). The *fmn* mutant also showed significantly elevated VCO_2 and VO_2 compared to WT-LD ($p < 0.001$), with a detectable but less marked diurnal variation than in *sss* (Figure S4). WT-LD flies exhibited a robust diurnal pattern, with significantly higher respiratory rates during the day ($p < 0.001$), consistent with LD-associated temporal regulation of energy expenditure (Figure S4).

The circadian mutant per^{01}, lacking a functional molecular clock, displayed an attenuated and phase-altered respiratory pattern. Both VCO_2 and VO_2 were significantly reduced during the day ($p < 0.05$), with no significant changes at night, indicative of disrupted or misaligned metabolic patterning (Figure S4). WT-DD flies, maintained in constant darkness, exhibited the lowest overall respiratory activity. Despite the absence of environmental light cues, VCO_2 and VO_2 retained significant day-night differences (Figure S4), consistent with persistent free-running circadian control in constant darkness (with altered amplitude and/or phase relative to LD).

Diurnal and Circadian Variations in CO_2 Production, O_2 Consumption, and Respiratory Quotient Across Genotypes

To evaluate genotype-dependent 24-h rhythmic regulation of metabolism, we assessed rhythmicity in carbon dioxide production (VCO_2), oxygen consumption (VO_2), and respiratory quotient (RQ) in wild-type flies under light-dark conditions (WT-LD), wild-type flies in constant darkness (WT-DD), short-sleep mutants (*fumin* [*fmn*] and *sleepless* [*sss*]), and the circadian clock mutant *period*^{01} (per^{01}). All mutant strains were assayed under LD. Time-course data were analyzed using JTK CYCLE and RAIN algorithms, with rhythmicity classified as statistically significant ($p \leq 0.05$), trending ($0.05 < p < 0.1$), or not significant ($p \geq 0.1$). Rhythmic phase was estimated using JTK lag values (Figures 2-3, Table 1).

VCO_2 rhythms were statistically significant in WT-LD, WT-DD, *fmn*, *sss* and per^{01}(Figure 2, Table 1). These findings demonstrate that VCO_2 exhibits robust rhythmic oscillations under both light-dark and constant darkness conditions, and that rhythmicity persists even in the absence of a

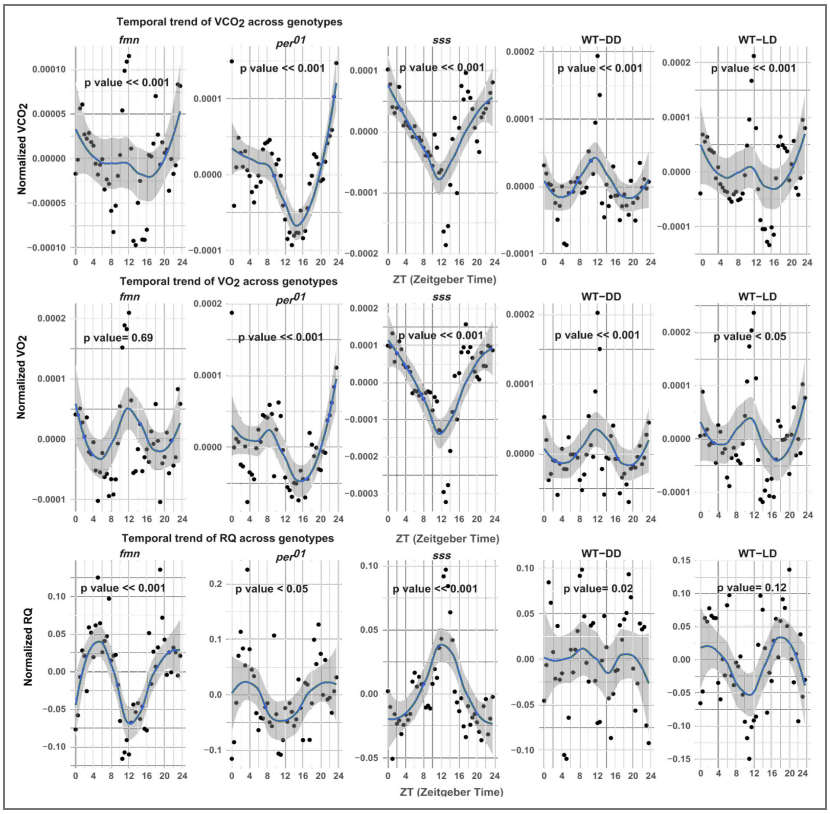

Figure 2. Twenty-four-hour time course of respiratory parameters across genotypes.

Normalized temporal profiles of carbon dioxide production (VCO_2) (top), oxygen consumption (VO_2) (middle), and respiratory quotient (RQ) (bottom) plotted over a 24-hour cycle (ZT0–24) for genotypes: wild-type in light-dark (WT-LD) and constant darkness (WT-DD), short-sleep mutants *fumin* (*fmn*) and *sleepless* (*sss*), and circadian mutant per^{01}. Curves reveal genotype-specific rhythmicity and metabolic dynamics across the 24-hour recording period. Significant genotype × time interactions were observed for all variables (VCO_2, VO_2, RQ), indicating genotype-dependent alterations in respiratory rhythmicity. $p < 0.05$ considered significant. For all panels with error bars or shaded error bands, values are presented as mean ± SEM. Source data are the same continuous respirometry recordings shown in Figure 1; Figure 2 presents rhythmicity analysis of VCO_2, VO_2, and RQ across the 24-hour cycle. Data represent approximately 300 flies per genotype across three experimental days (25 flies/chamber, four chambers/experiment). The chamber was used as the experimental unit; *n* denotes the number of chambers.

Figure 3. Phase distribution of respiratory rhythms across genotypes.

Polar plots depicting the phase (peak timing) of rhythmic expression for carbon dioxide production (VCO_2), oxygen consumption (VO_2), and respiratory quotient (RQ) over a 24-hour cycle (ZT0-24) for each genotype. Genotypes include wild-type in light-dark (WT-LD) and constant darkness (WT-DD), short-sleep mutants *fumin* (*fmn*) and *sleepless* (*sss*), and circadian mutant per^{01}. Statistical significance of rhythmicity was determined using the RAIN algorithm: darker-colored bars indicate significant rhythms ($p < 0.05$), and lighter-colored bars indicate non-significant rhythms ($p > 0.05$). Where applicable, plotted values are presented as mean ± SEM. Source data are the same continuous respirometry recordings shown in Figure 1; Figure 3 presents phase/peak-timing analysis derived from the rhythmicity analysis

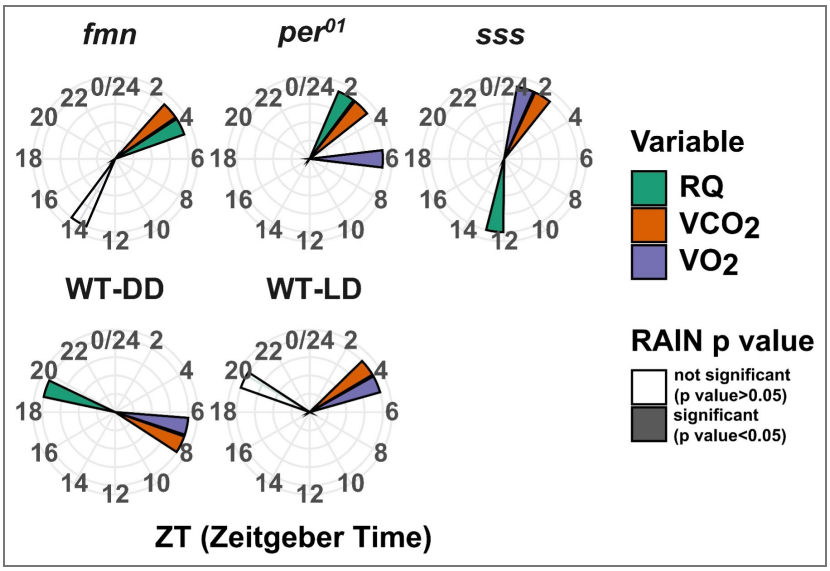

Table 1. Rhythmicity metrics for respiratory parameters across genotypes

Genotype	RQ JTK Lag	RQ JTK Period	RQ RAIN p-value	VCO_2 JTK Lag	VCO_2 JTK Period	VCO_2 RAIN p-value	VO_2 JTK Lag	VO_2 JTK Period	VO_2 RAIN p-value
WT-LD	19.75	20	0.12	4	24	1.1143e-05	4	24	0.001
Fumin	4.25	22.5	9.54e-08	3.25	24	0.0002	14	20	0.69
Sss	12.5	20	1.41e-07	2	24	1.33e-09	1.25	24	1.14e-10
per^{01}	2	20	0.006	3	24	1.05e-13	6	21.5	1.82e-07
WT-DD	19.25	23	0.02	7.5	24	0.0002	7	24	9.4145e-06

functional *period* gene. VCO_2 peak occurred near ZT ~4 in WT-LD, ZT ~7.5 in WT-DD, ZT ~3.25 in *fmn*, ZT ~3 in per^{01}, and ZT ~2 in *sss*, indicating genotype-specific shifts in respiratory phase (Figure 3, Table 1).

VO_2 rhythmicity was statistically significant in WT-LD, WT-DD, *sss* and per^{01} while not significant in *fmn* (Figure 2, Table 1). These findings indicate that rhythmic regulation of oxygen consumption is evident in constant darkness and in the absence of a functional *period* gene but is reduced in a sleep mutant. VO_2 peaked near ZT ~4 in WT-LD, ZT ~7 in WT-DD, ZT ~1.25 in *sss* and ZT ~6 in per^{01}, suggesting conserved phase alignment with all phases within ±3 h of WT-LD (Figure 3, Table 1).

RQ also showed genotype-dependent rhythmicity, with significant diurnal oscillations observed in *fmn*, *sss* and per^{01}, while no significant rhythms were observed in WT-LD or WT-DD (Figure 2, Table 1). In the rhythmic genotypes, RQ peaked at ZT ~4.25 in *fmn*, ZT ~2 in per^{01} and ZT ~12.5 in *sss*, indicating distinct RQ phase alignment across the sleep mutants (spread ~8 h apart), in contrast to the conserved phase alignment of respiratory output (VO_2) (Figure 3, Table 1).

Temporal Profiling of Respiratory Quotient in Wild-Type Flies Under Light-Dark Conditions

RQ values corresponding to each 2-hour Zeitgeber Time (ZT) point were extracted from the 5-minute-binned dataset. Building on this alignment, we explored temporal relationships by systematically shifting the RQ time series by −120, −60, −30, −15, −5, +5, +15, +30, +60, and +120 minutes relative to the metabolite dataset. The continuous respirometry time series showed an oscillatory day-night pattern in RQ; metabolomics was then used to relate time-matched and lagged metabolite dynamics to RQ patterns (Figure 4).

Correlation of Respiratory Quotient with Metabolite Profiles and Temporal Lag Analysis in WT-LD Flies

To investigate the association between respiratory quotient (RQ) and metabolite in WT-LD flies, we initially performed Spearman correlation analysis (ρ) across a range of temporal lags (−120 to +120 minutes). Several metabolites demonstrated strong correlations with RQ ($|\rho| > 0.7$, $p < 0.05$), suggesting tight coupling between metabolic fluctuations and respiratory output. The full set of metabolites meeting this threshold across WT-LD, *fmn*, *sss*, per^{01}, and WT-DD conditions, along with best lag direction, lag time, and Spearman ρ value and BH FDR, is provided in Supplementary Table 1. To minimize the influence of outliers and enhance data visualization, selected correlation patterns were subsequently presented as rank-based scatter plots. Representative examples are shown in Figure 5a to illustrate the metabolite-RQ correlation and lag-analysis workflow. Hydroxyhexadecenoylcarnitine and quinolinate are among the strongest positively- and negatively lagged RQ-correlated metabolites in WT-LD (ρ = +0.78 at +120 min lag and ρ = −0.77 at −120 min lag; Supplementary Table 1), illustrating the two opposite lag directions of the workflow. The full set of metabolites showing strong RQ-associated correlations across WT-LD, *fmn*, *sss*, per^{01}, and WT-DD conditions is provided in Supplementary Table 1. We define 'anticipatory' as metabolite changes that precede the associated respiratory shift (negative lag) and 'reactive' as changes that follow or coincide with the respiratory shift (positive lag), and we use these terms consistently throughout. To further characterize these associations, metabolites were categorized based on the timing of their peak changes relative to RQ fluctuations, exhibiting either positive or negative lag. To enable direct comparison of temporal dynamics between RQ and metabolite profiles across ZT 0 to ZT 24, z-scoring was performed to normalize differences in absolute magnitude and measurement units. Within this framework, a positive lag indicates that metabolite changes occur after shifts in RQ, requiring the metabolite profile to be shifted forward (rightward) along the time axis for optimal alignment. Conversely, a negative lag signifies that metabolite changes precede RQ fluctuations, necessitating a backward (leftward) shift of the metabolite profile to achieve alignment (Figure 5b).

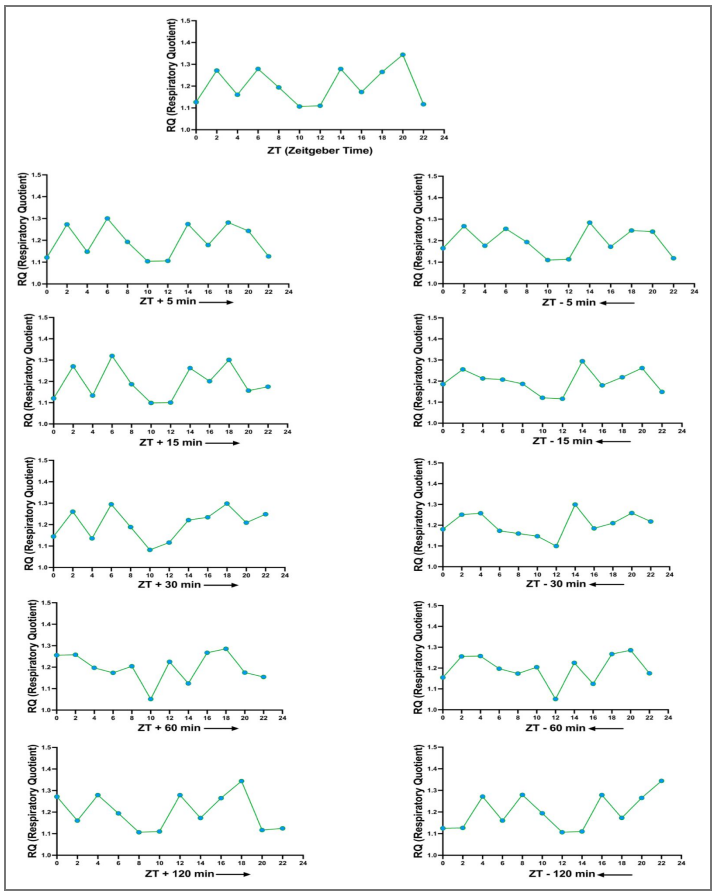

Figure 4. Temporal Profiling of Respiratory Quotient in Wild-Type Flies Under Light-Dark Conditions.

Respiratory quotient (RQ) was extracted every 2 h across a 24-hour light-dark (LD) cycle in WT flies. Lag analyses were performed by advancing or delaying the RQ data by −120, −60, −30, −15, −5, +5, +15, +30, +60-, and +120-minutes relative to Zeitgeber Time (ZT). Each panel represents the RQ profile corresponding to a specific time shift, illustrating phase-dependent respiratory dynamics across the 24-hour LD cycle. Data represent approximately 300 flies per genotype across three experimental days (25 flies/chamber, four chambers/experiment). The chamber was used as the experimental unit; *n* denotes the number of chambers. RQ rhythmicity itself was assessed from the continuous respirometry time series (Table 1); Figure 4 shows the day-night RQ pattern used as the reference for the lag-based metabolite correlations, to which the metabolomics data contribute.

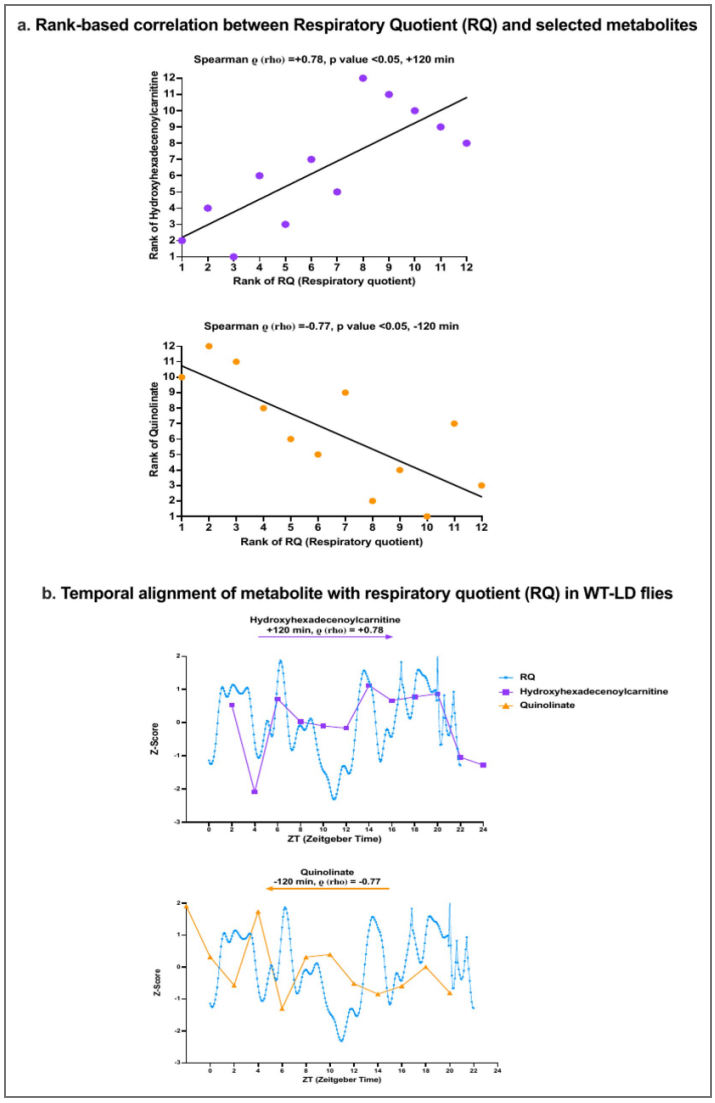

Figure 5.

a. Rank-based correlation between Respiratory Quotient (RQ) and selected metabolites. Scatter plots depict the rank-order relationships between RQ and (A) Hydroxyhexadecenoylcarnitine and (B) Quinolinate. Each point represents a timepoint after aligning data with respective time shifts. Spearman's rank correlation coefficient (ρ) and corresponding p-values are indicated on each panel. A positive lag (+120 minutes for Hydroxyhexadecenoylcarnitine) or negative lag (−120 minutes for Quinolinate) denotes the temporal shift in RQ relative to the metabolite dataset. Statistical significance was defined as $p < 0.05$. ***b. Temporal alignment of metabolite with respiratory quotient (RQ) in WT-LD flies.*** Z-scored time series of RQ, Hydroxyhexadecenoylcarnitine, and Quinolinate across a 24-hour light-dark cycle. **Top Panel:** Hydroxyhexadecenoylcarnitine shows a positive lag of +120 minutes and strong positive correlation with RQ (ρ=+0.78), suggesting its rise after changes in RQ. **Bottom Panel:** Quinolinate shows a negative lag of −120 minutes and strong negative correlation with RQ (ρ=-0.77), indicating it precedes changes in RQ.

Metabolite Response Dynamics Relative to RQ and Functional Pathway Enrichment of RQ-Associated Metabolites in WT-LD Flies

To further explore the temporal relationship between the metabolites and respiratory quotient (RQ), we generated a clustered heatmap of metabolites significantly correlated with RQ. Metabolites were filtered based on statistical significance ($|\rho| > 0.7$, $p < 0.05$ at any timepoint), and their temporal profiles were realigned relative to respiration, anchoring RQ at t = 0 (Figure 6, Supplementary Table 2). This flipping step was essential to standardize the visualization of metabolites either preceding or following respiratory changes, facilitating clearer interpretation of biological timing relationships.

To identify key metabolic pathways associated with diurnal regulation of respiration, we next performed pathway enrichment analysis on the RQ-correlated metabolites using MetaboAnalyst. Enriched pathways were identified at a significance threshold of $p < 0.05$. WT-LD-specific pathway enrichment results are presented in Figure 6. Consistent with these findings, WT flies under LD conditions exhibited diurnal-regulated metabolic rhythms, characterized by coordinated activity in amino acid metabolism, redox balance, and energy-generating pathways such as the TCA cycle and glyoxylate metabolism.

RQ-Linked Metabolic Shifts and Pathway Enrichment in Sleep Mutants

We applied the same analysis method to sleep mutant flies (*fmn* and *sss*) to identify metabolic pathways associated with altered respiratory rhythms. Metabolites significantly correlated with RQ ($|\rho| > 0.7$, $p < 0.05$) were identified, and their temporal profiles were realigned relative to respiration by anchoring RQ at $t = 0$. Clustered heatmaps were generated to visualize metabolite response dynamics, and pathway enrichment analysis of RQ-associated metabolites was conducted using MetaboAnalyst ($p < 0.05$) (Figure 7). This analysis revealed that sleep mutants exhibit genotype-specific metabolic disruptions implicating mitochondrial and energy-related pathways. In *fmn* flies, alterations were observed in arginine biosynthesis, purine and nitrogen metabolism, and butanoate pathways, suggestive of dysregulated nitrogen balance and mitochondrial dysfunction. In contrast, *sss* mutants showed perturbations in glycine, serine, and threonine metabolism, as well as carbohydrate and antibiotic biosynthetic pathways, reflecting altered carbon utilization and mitochondrial-associated metabolic stress.

RQ-Linked Metabolic Shifts in Circadian Mutants and WT Flies in Constant Darkness

Circadian clock mutant flies (per^{01}) and wild-type flies maintained under constant darkness (WT-DD) were analyzed using the same approach. We used constant darkness (DD) to assess free-running circadian regulation in the absence of external light–dark cues. Metabolites exhibiting strong correlations with RQ ($|\rho| > 0.7$, $p < 0.05$) were identified and temporally aligned by anchoring RQ at t = 0. Clustered heatmaps were constructed to visualize the dynamic responses of these metabolites, and pathway enrichment analysis of RQ-associated metabolites was performed using MetaboAnalyst ($p < 0.05$) (Figure 8). Both per^{01} and WT-DD flies exhibited disrupted temporal coordination of mitochondrial metabolism. per^{01} mutants showed dysregulation in amino acid metabolism, purine turnover, and redox-associated pathways such as glutathione and nicotinate metabolism, consistent with impaired redox buffering and energy imbalance. In contrast, WT-DD flies exhibited alterations in sulfur- and nitrogen-containing amino acid pathways, indicative of desynchronized but intact metabolic cycling in the absence of external light cues.

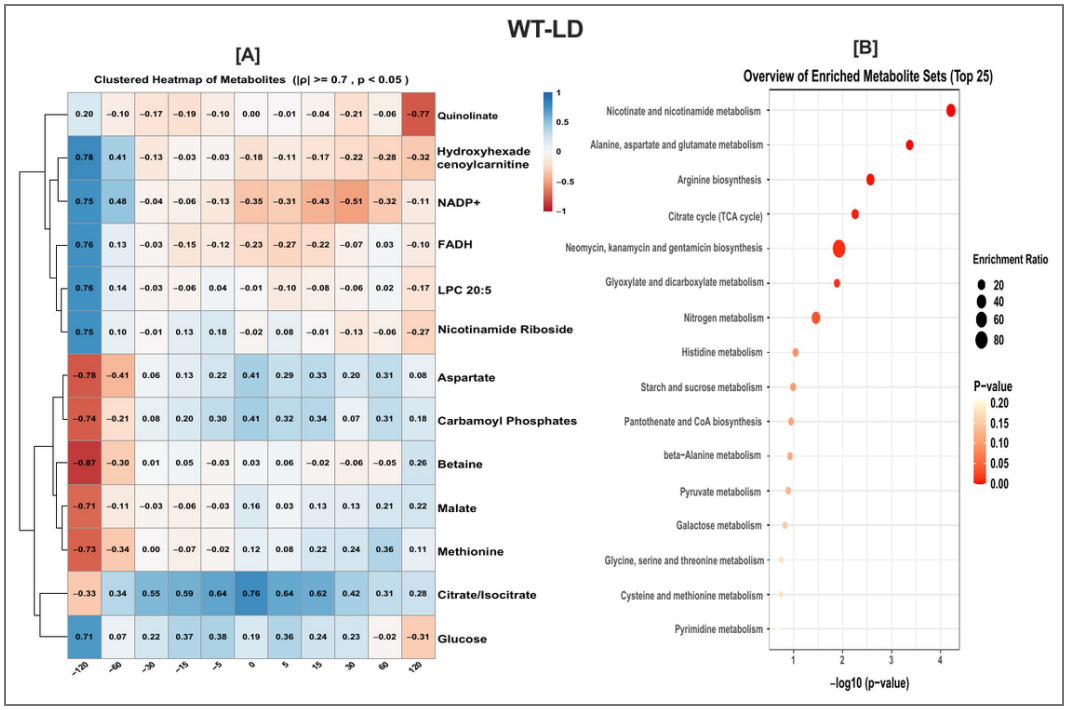

Figure 6. Metabolite-Respiratory Quotient Correlations and Pathway Enrichment in WT-LD Flies.

(A) Heatmap depicting Spearman correlations (|ñ| > 0.7, p < 0.05) between respiratory quotient (RQ) and metabolite across the 24-hour LD cycle in wild-type flies maintained under light-dark (LD) conditions. Metabolites were clustered based on correlation patterns, highlighting groups with similar temporal associations with respiratory activity. (B) Pathway enrichment analysis of significantly correlated metabolites, illustrating metabolic pathways most closely linked to respiratory dynamics. Pathways with a p-value less than 0.05 were considered statistically significant.

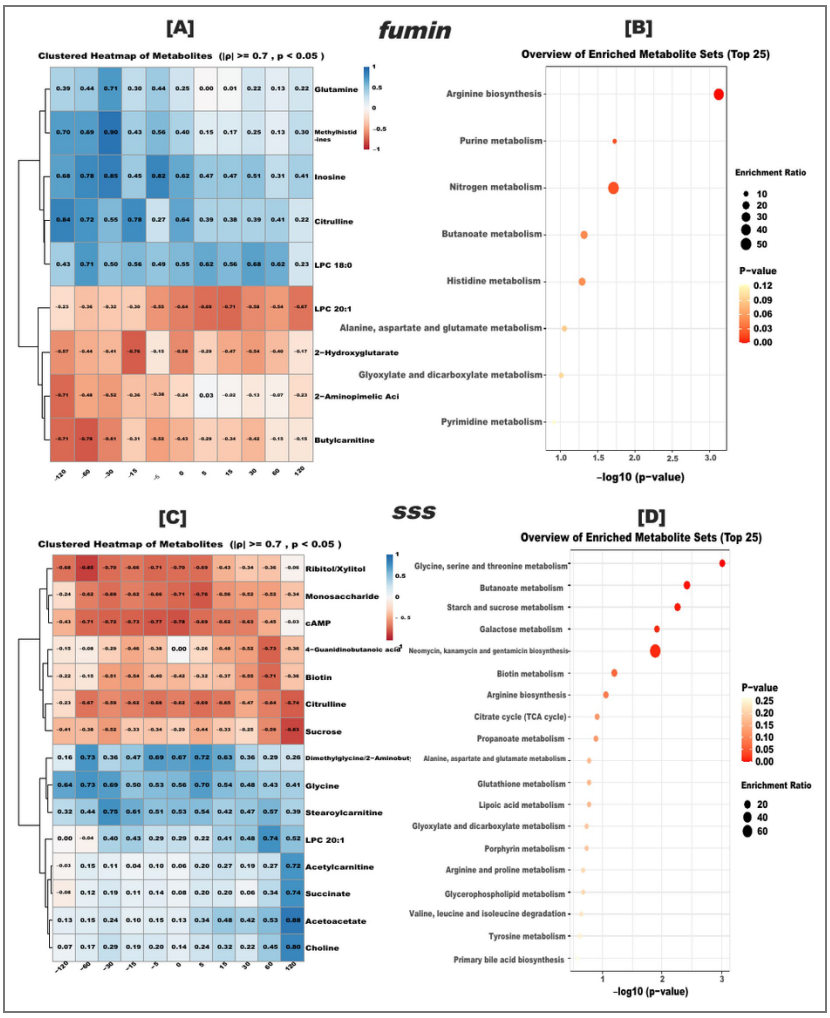

Figure 7. Correlation of Metabolites with Respiratory Quotient and Pathway Enrichment in Short-Sleep Mutants.

(A, C) Heatmaps showing Spearman correlations ($|\rho| > 0.7$, $p < 0.05$) between respiratory quotient (RQ) and metabolites in the short-sleep mutants *fmn* (A) and *sss* (C). (B, D) Pathway enrichment analyses of metabolites significantly correlated with RQ in *fmn* (B) and *sss* (D). Pathways with p-values less than 0.05 were considered statistically significant.

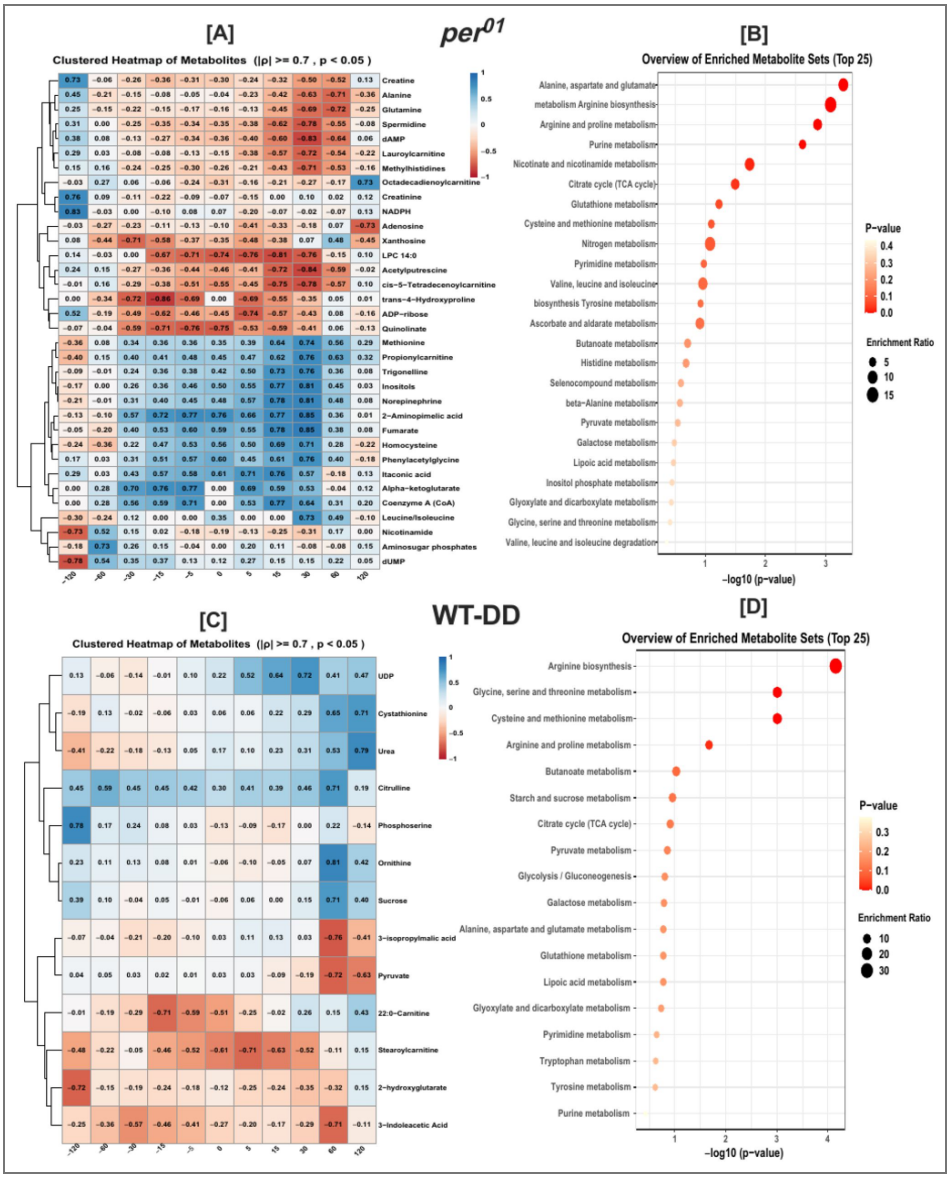

Figure 8. Correlation of Metabolites with Respiratory Quotient and Pathway Enrichment in Circadian Mutant and Wild-Type Flies in Constant Darkness.

(A, C) Heatmaps displaying Spearman correlations ($|\rho| > 0.7$, $p < 0.05$) between respiratory quotient (RQ) and metabolites in the circadian mutant *per*^{01} (A) and wild-type flies maintained in constant darkness (WT-DD) (C). (B, D) Pathway enrichment analyses of metabolites significantly correlated with RQ in *per*^{01} (B) and WT-DD (D). Pathways with p-values less than 0.05 were considered statistically significant.

Gut Tissue Respirometry Reveals Elevated Baseline Mitochondrial Respiration in fmn and per^{01} Mutants

Because pathway-level metabolomics implicated mitochondrial pathways in sleep and circadian mutants, we directly assayed mitochondrial respiration by measuring baseline oxygen consumption rate (OCR) in dissected gut tissue from *fmn* and per^{01} flies relative to iso controls. Both *fmn* and per^{01} guts showed significantly elevated baseline OCR (*fmn* vs iso^{31}, n = 12 vs 12; per^{01}vs iso^{31}, n = 12 vs 11 across 3 runs; Mann-Whitney test, $p < 0.05$; Figure 9A,B). Because only baseline OCR was measured, we interpret this as altered (elevated) baseline mitochondrial respiration rather than reduced capacity or a specific coupling defect (Figure 9).

Discussion

Temporal Misalignment Alters Fuel Utilization and Respiratory Rhythms

Our integrative approach, combining whole-organism respirometry [18, 26] with LC-MS-based metabolomics [27, 36-40], reveals how sleep and circadian disruptions distinctly alter energy metabolism in *Drosophila melanogaster*. By comparing wild-type flies with mutants affecting sleep (*fumin* and *sleepless*) [21, 22, 41, 42] and circadian clock function (per^{01}) [24], we have captured genotype-specific differences in respiratory dynamics and substrate usage across daily cycles under tightly controlled environmental conditions. These findings establish a functional framework for understanding how behavioral state and clock integrity shape metabolic homeostasis.

Across the manuscript, the central takeaway is that wild-type flies under LD exhibit anticipatory alignment of fuel selection with time of day, whereas short-sleep mutants (*fmn, sss*) and clock-disrupted flies (per^{01}) show reactive or misaligned metabolism under our conditions. We therefore focus the Discussion on loss of temporal coordination between respiratory output and pathway-level metabolism, rather than reiterating rate changes alone.

Metabolites in wild-type LD typically showed peak correlations preceding respiratory changes (negative lag), reflecting anticipatory diurnal metabolic regulation under LD conditions. In contrast, mutants displayed a shift to reactive correlations (positive lag), indicating loss of temporal synchronization and proactive metabolic adjustments. These phenotypes parallel mammalian systems, where sleep loss and circadian misalignment are linked to elevated basal metabolic rate, a shift toward carbohydrate oxidation and lipid/protein catabolism, and blunted, phase-shifted respiratory oscillations [3, 43, 44]. In our flies, the short-sleep mutants show the analogous catabolic shift (*fmn* RQ 1.09; *sss* RQ 0.94), and per^{01} and WT-DD show dampened, phase-shifted respiratory rhythms [45, 46]. These conserved responses underscore the translational value of *Drosophila* for dissecting the interplay between sleep, circadian timing, and metabolic regulation, while physiological differences between flies and mammals caution against direct mechanistic extrapolation.

Wild-Type Flies Exhibit Diurnal Regulation of Biosynthesis and Redox Balance Under LD Cycles

In wild-type flies maintained under a 12:12 light-dark (LD) cycle, RQ-correlated metabolites revealed tightly coordinated diurnal regulation of biosynthetic and redox pathways. An increase in nicotinate and nicotinamide metabolism-highlighted by changes in quinolinate, nicotinamide riboside, and L-aspartic acid-suggests a direct link between mitochondrial respiration and NAD^+ biosynthesis [47, 48]. These NAD^+ precursor changes occur before respiration increases (negative lag), highlighting anticipatory NAD^+ regulation. Concurrent alterations in alanine, aspartate, and glutamate metabolism (via citric acid and carbamoyl phosphate) and arginine biosynthesis indicate dynamic alignment of amino acid turnover with energy demand and nitrogen disposal [49, 50]. RQ-associated enrichment of citrate cycle intermediates such as malate and citric acid

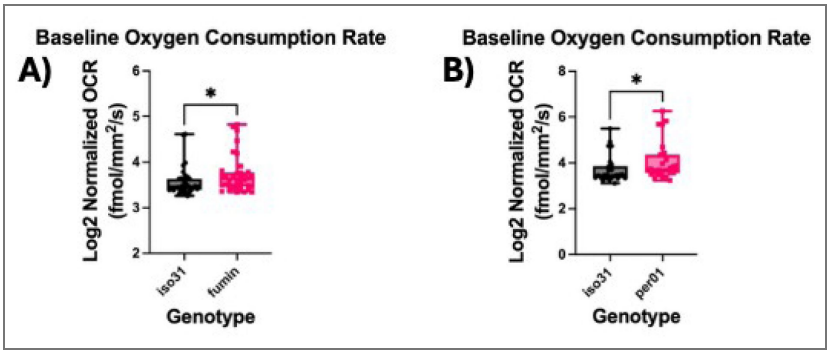

Figure 9. Baseline oxygen consumption in isolated gut tissue from fmn and per^{01} mutants.

Baseline oxygen consumption rate (OCR, fmol/mm²/s) measured by tissue respirometry in individual dissected guts from the short-sleep mutant *fmn* (A) and the circadian-clock mutant *per^{01}* (B) relative to *iso^{31}* genetic-background controls. For each gut, OCR was averaged over a 24-hour window beginning ~12 h after loading, once tissue respiration had stabilized (12–36 h). To combine independent runs, OCR was normalized to the median of the *iso^{31}* controls within each run and log2(x+10)-transformed. One *iso^{31}* gut in the third run of the *per^{01}* experiment was excluded as an outlier; no other values were removed. Baseline OCR differed significantly between *iso^{31}* and each mutant (Mann-Whitney test, $p < 0.05$; pooled across 3 independent runs; *fmn* vs *iso^{31}*, n = 12 vs 12; *per^{01}* vs *iso^{31}*, n = 12 vs 11 after the *iso^{31}* outlier exclusion).

further supports diurnal gating of oxidative phosphorylation [51]. Elevated levels of D-glucose were mapped to the KEGG pathway for neomycin, kanamycin, and gentamicin biosynthesis. While *Drosophila* does not synthesize these antibiotics, the enrichment likely reflects the presence of shared carbohydrate intermediates (e.g., glucose, glucose-6-phosphate, UDP-glucose) common to multiple biosynthetic processes. This mapping is more indicative of altered carbohydrate and amino sugar metabolism, or potentially reflects microbial contributions, rather than actual antibiotic production by the host [52, 53]. Additional contributions from glyoxylate and dicarboxylate metabolism reinforce the role of anaplerotic flux and redox cycling [54]. Notably, persistent correlations between RQ and pantothenate precursors suggest synchronized CoA biosynthesis, potentially coordinating fatty acid oxidation and TCA cycle input [55]. Together, these results demonstrate that under LD conditions, wild-type flies sustain temporal coordination between respiration and mitochondrial metabolism, integrating redox homeostasis, nitrogen metabolism, and substrate availability in an LD-associated diurnal manner.

Altered Amino Acid Metabolism and Mitochondrial Dysregulation in fmn Mutants

The *fumin* (*fmn*) mutant, characterized by chronic sleep loss, exhibited genotype-specific disruptions in metabolic coordination, as revealed by metabolites whose temporal dynamics were strongly correlated with respiratory quotient (RQ). In *fmn* flies, significant RQ correlations ($p < 0.05$) were identified in pathways including arginine biosynthesis (citrulline, glutamine), purine metabolism (inosine, glutamine), and nitrogen metabolism-suggesting a decoupling of amino acid turnover and nucleotide cycling from respiratory demand [56, 57]. Glutamine's strong association with RQ highlights its central role in buffering nitrogen flux and supporting biosynthetic processes under energetic stress [58]. Correlated dynamics of 2-hydroxyglutarate, a key intermediate in butanoate metabolism, point to impaired TCA cycle flux and redox imbalance [59]. Likewise, RQ-associated shifts in methylhistidine suggest altered histidine metabolism, potentially impacting mitochondrial protein turnover and methylation processes. Acylcarnitine, indicators of lipid oxidation, showed consistently strong RQ correlations across genotypes, most pronounced in *fmn*, underscoring lipid metabolism's role in respiratory coupling. Particularly in mutants, increased reliance on fatty acid oxidation may reflect compensatory responses to disrupted carbohydrate metabolism and sustained energetic demands. Together, these findings indicate a failure in aligning substrate oxidation with mitochondrial respiratory output in *fmn* mutants. Building on prior evidence of mitochondrial stress in sleep-deprived *fmn* flies [60], our integrative respirometry-metabolomics analysis reveals disrupted coupling between energy metabolism and amino acid pathways, underscoring the physiological cost of chronic sleep loss on mitochondrial homeostasis. These interpretations are further supported by gut-tissue respirometry showing elevated baseline mitochondrial respiration (OCR) in *fmn* relative to iso31 controls. Together with prior evidence of ROS accumulation (oxidative stress) in *fmn* [60], these functional data indicate that chronic sleep loss in *fmn* is associated with altered mitochondrial respiration.

Disrupted Carbon and Amino Acid Metabolism in sss Mutants

The *sleepless* (*sss*) mutant displayed significant disruptions in carbon and amino acid metabolism, as indicated by strong correlations between respiratory quotient (RQ) and metabolites involved in glycine, serine, and threonine metabolism (choline, dimethylglycine, glycine), butanoate metabolism (acetoacetate, succinate), and carbohydrate pathways [22]. Notably, RQ-associated shifts in choline and dimethylglycine suggest dysregulation of one-carbon metabolism and methyl group transfer, which can impair mitochondrial function and epigenetic stability [61, 62]. Correlated dynamics of acetoacetate, a ketone body often found in starvation, and succinate (both key intermediates of butanoate metabolism) are consistent with altered TCA cycle input and redox balance [63, 64]. In parallel, strong RQ correlations with sucrose and D-glucose (from both starch/sucrose and galactose metabolism) indicate disrupted carbohydrate processing and inefficient substrate utilization [65]. cAMP and biotin were also found to associate with respiration, both unique in metabolic regulation through altered neuromodulatory signaling and

cofactor metabolism. These findings suggest that *sss* mutants exhibit altered metabolic routing in response to respiratory demand, shifting substrate utilization toward carbohydrate and amino acid pathways under sleep-deprived conditions, consistent with metabolic stress responses observed in other models of sleep loss [66].

Loss of Temporal Coupling of Mitochondrial Metabolism in Clock Mutants

In *period* (per^{01}) mutants lacking a functional circadian clock, the temporal coordination between metabolism and respiration was disrupted across nearly every measured metabolite through intermittent strong correlations with respiration, reflecting broad metabolic chaos [1, 67]. Strong correlations with RQ were observed in alanine, aspartate, and glutamate metabolism (L-alanine, glutamine, fumarate, oxoglutarate) and arginine biosynthesis, suggesting inefficient routing of amino acid-derived substrates into mitochondrial energy production [50]. Delayed RQ-associated dynamics of glutamine, oxoglutarate, and fumarate indicate a mismatch between substrate availability and respiratory output [2]. Additionally, metabolites from arginine and proline metabolism (creatine, spermidine, N-acetylputrescine, 4-hydroxyproline) exhibited altered RQ correlations, suggesting impaired mitochondrial redox buffering and compromised integrity [68]. Perturbations in purine metabolism-including adenosine, deoxyadenosine monophosphate, xanthosine, and ADP-ribose-further point to dysregulated ATP turnover and nucleotide imbalance. Correlations with quinolinic acid and niacinamide reflect altered nicotinate and nicotinamide metabolism, implicating disrupted NAD^+ biosynthesis and redox state [48]. Additional RQ-associated changes in NADPH and TCA intermediates (fumarate, oxoglutarate) align with impaired glutathione metabolism and altered mitochondrial metabolism. Gut-tissue respirometry in per^{01} showed elevated baseline OCR. Because only baseline respiration was measured, we interpret this as altered (elevated) baseline mitochondrial respiration — which can reflect inefficient or uncoupled respiration, or compensatory upregulation, rather than increased functional capacity — consistent with the metabolomic signatures of disrupted redox balance and mitochondrial metabolism. Microbial-derived metabolites (trigonelline, phenylacetylglycine) were also identified in the correlation analysis, suggesting potential gut microbiome interactions that become evident in circadian disruption. Together, these findings suggest that the loss of circadian timing in per^{01} mutants lead to widespread uncoupling of substrate utilization and mitochondrial respiration. This breakdown in temporal regulation disrupts energy homeostasis, highlighting the essential role of the circadian clock in coordinating metabolic flux with respiratory demand [1, 51, 69].

Metabolic Desynchrony and Redox Imbalance in Wild-Type Flies Under Constant Darkness

In wild-type flies maintained under constant darkness (DD), the absence of environmental light cues led to disrupted temporal alignment between mitochondrial respiration and metabolic pathways [70, 71]. Generally, we note a shift towards reactive metabolic adjustments (positive lags) compared to WT-LD instead of anticipatory preparation, indicating reduced metabolic efficiency. RQ-correlated metabolites showed significant enrichment in arginine biosynthesis (citrulline, ornithine, urea), glycine, serine, and threonine metabolism (L-cystathionine, phosphoserine, pyruvic acid), and cysteine and methionine metabolism-indicating altered integration of nitrogen and sulfur amino acid metabolism with respiratory activity [49]. Notably, the convergence of pyruvic acid across multiple pathways suggests dysregulated entry points into the TCA cycle, while the consistent association of L-cystathionine highlights impaired redox buffering and methylation capacity [72, 73]. Altered correlations in arginine and proline metabolism (ornithine, pyruvic acid) further underscore compromised coupling between amino acid turnover and mitochondrial energy production [29]. Altered lipid metabolism and increased nitrogen catabolism are likely compensatory mechanisms in the absence of environmental cues. These findings build on previous reports of circadian desynchrony by providing functional evidence that environmental light cues contribute to coherent coordination between respiration and key metabolic circuits [50, 74]. The resulting metabolic misalignment under constant darkness emphasizes the circadian

clock's dependence on external entrainment to regulate energy homeostasis at the organismal level [54]. Our DD data show that endogenous free-running circadian regulation persists but that removing external light–dark cues degrade the temporal coordination between respiration and metabolism, indicating that external entrainment normally strengthens this coordination under our conditions; however, we acknowledge that the coupling may be different in mammals. We also restrict our conclusions to the genotypes and conditions tested (*fmn, sss, and per*^{01}) and do not generalize these effects to acute sleep deprivation, which was not performed in this study. Accordingly, the 'metabolic misalignment' observed in constant darkness (DD) likely results from a decrease in synchrony due to the removal of external light:dark cues. We also acknowledge the limits of this replication: with n = 12 chambers per genotype, statistical power to detect small-magnitude differences and subtle phase shifts is limited, and negative calls (e.g., arrhythmicity) should be interpreted with this caveat.

Locomotor activity and feeding could not be measured during the respirometry recordings, so genotype differences in respiratory parameters should be interpreted with caution. To assess whether behavioral timing could account for these differences, we compared our respiratory rhythms with the feeding and activity rhythms reported for these genotypes in our previous work [75]: wild-type feeding peaked at ZT ~3.25 and *fmn* feeding was phase-delayed to ZT ~4.5, while *fmn* also showed elevated locomotor activity, particularly during the dark period. In our data the *fmn* VCO_2 rhythm peaks at ZT ~3.25 (RQ at ZT ~4.25), so the respiratory peak slightly precedes the feeding peak; the phase of the *fmn* respiratory rhythm is therefore not driven by feeding, although the elevated activity of *fmn* may contribute to its higher overall metabolic rate. Feeding and activity were not measured for *sss*.

Because only males were analyzed, our conclusions may not generalize to females, which can show sex-specific metabolic physiology. Inclusion of female flies and direct sex comparisons across LD and DD conditions will be an important future direction. More specifically, females carry a higher reproductive and biosynthetic load (egg production) that typically raises metabolic rate and can shift RQ toward lipogenesis and alter the amplitude and phase of diurnal respiratory rhythms; females might therefore show larger or differently timed effects than the males studied here.

Conclusions

This study demonstrates that sleep loss and circadian disruption impair metabolic homeostasis in *Drosophila melanogaster* via distinct yet converging mechanisms. Wild-type flies under light-dark cycles (WT-LD) maintained coordinated anticipatory respiratory and metabolic rhythms, while sleep mutants (*fmn*, *sss*) and circadian-disrupted flies (*per*^{01}) showed altered substrate utilization, redox imbalance, and uncoupling of mitochondrial pathways, likely reactive to respiration. These findings highlight the essential role of both sleep and circadian timing in regulating energy metabolism. The presence of neuromodulatory metabolites (e.g., cAMP in *sleepless* mutants) and microbial-derived compounds (trigonelline, phenylacetylglycine in *per*^{01} mutants) points toward additional layers of metabolic regulation influenced by neuronal activity and gut microbiota interactions, revealing more complex metabolic dysregulation in sleep-deprived and circadian-disrupted states. By combining high-resolution respirometry with targeted metabolomics, we establish *Drosophila* as a powerful model for investigating the molecular basis of sleep- and clock-related metabolic dysfunction and potential therapeutic interventions.

Supplementary materials

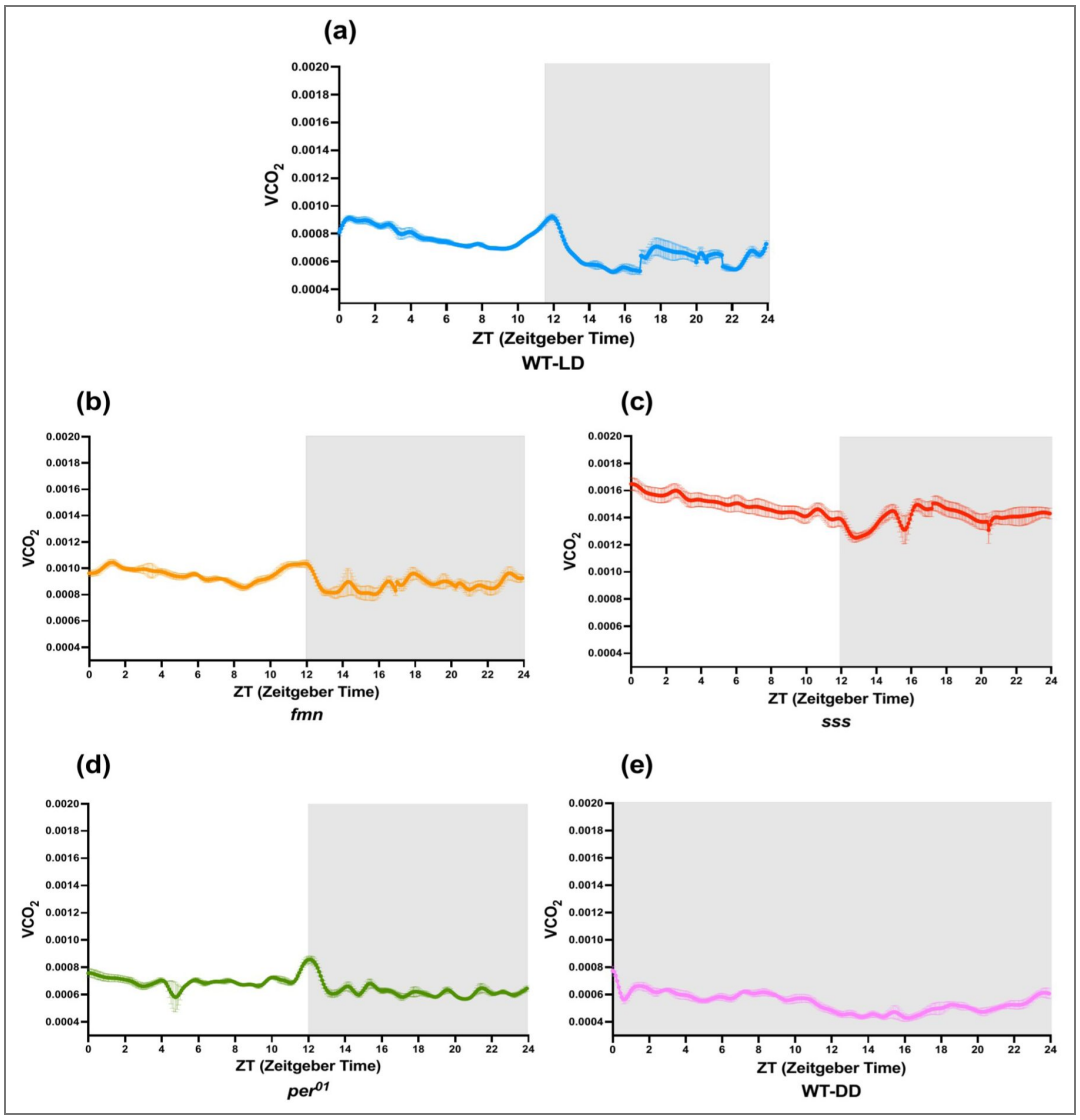

Figure S1. a-e. VCO_2 profiles across the 24-hour recording period in different genotypes. VCO_2 was continuously measured at one-second intervals from Zeitgeber Time (ZT) 0 to 24 and averaged into 5-minute bins for analysis. The traces display mean VCO_2 values across the 24-hour recording period for the following genotypes: wild-type flies under light-dark conditions (WT-LD), short-sleep mutants *fumin* (*fmn*) and *sleepless* (*sss*), the circadian clock mutant *period*^{01} (*per*^{01}), and wild-type flies in constant darkness (WT-DD). For all panels, traces are presented as mean ± SEM.. Data represent approximately 300 flies per genotype across three experimental days (25 flies/chamber, four chambers/experiment). The chamber was used as the experimental unit; *n* denotes the number of chambers.

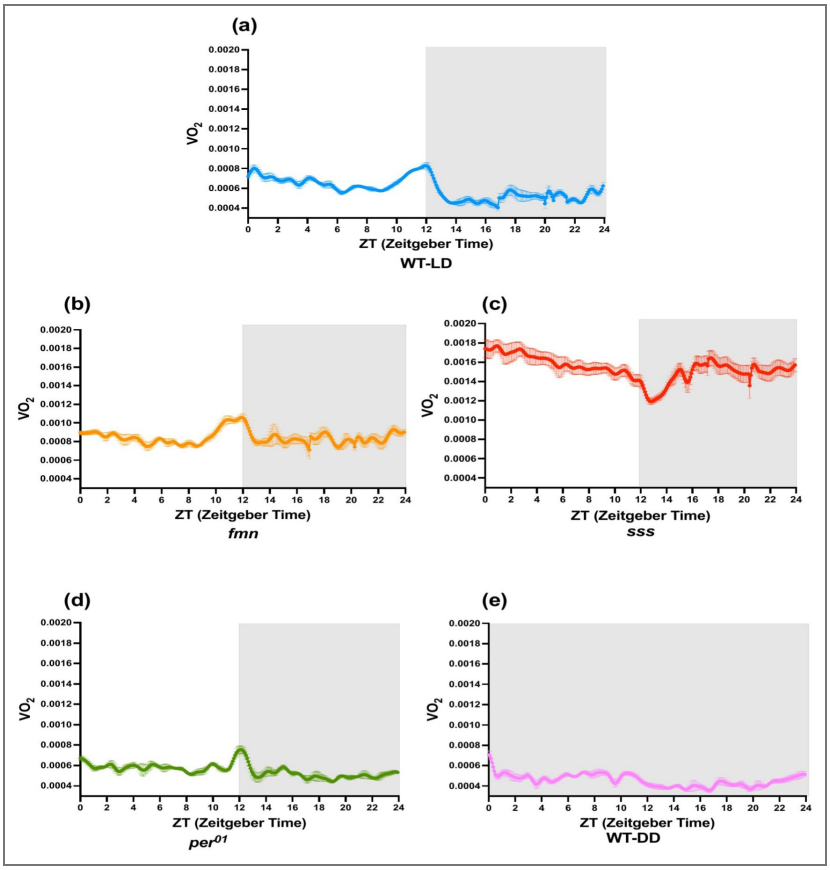

Figure S2.

a-e. VO_2 profiles across the 24-hour recording period in different genotypes. VO_2 was continuously measured at one-second intervals from Zeitgeber Time (ZT) 0 to 24 and averaged into 5-minute bins for analysis. The traces display mean VO_2 values across the 24-hour recording period for the following genotypes: wild-type flies under light-dark conditions (WT-LD), short-sleep mutants *fumin* (*fmn*) and *sleepless* (*sss*), the circadian clock mutant *period*^{01} (*per*^{01}), and wild-type flies in constant darkness (WT-DD). For all panels, traces are presented as mean ± SEM. Data represent approximately 300 flies per genotype across three experimental days (25 flies/chamber, four chambers/experiment). The chamber was used as the experimental unit; *n* denotes the number of chambers.

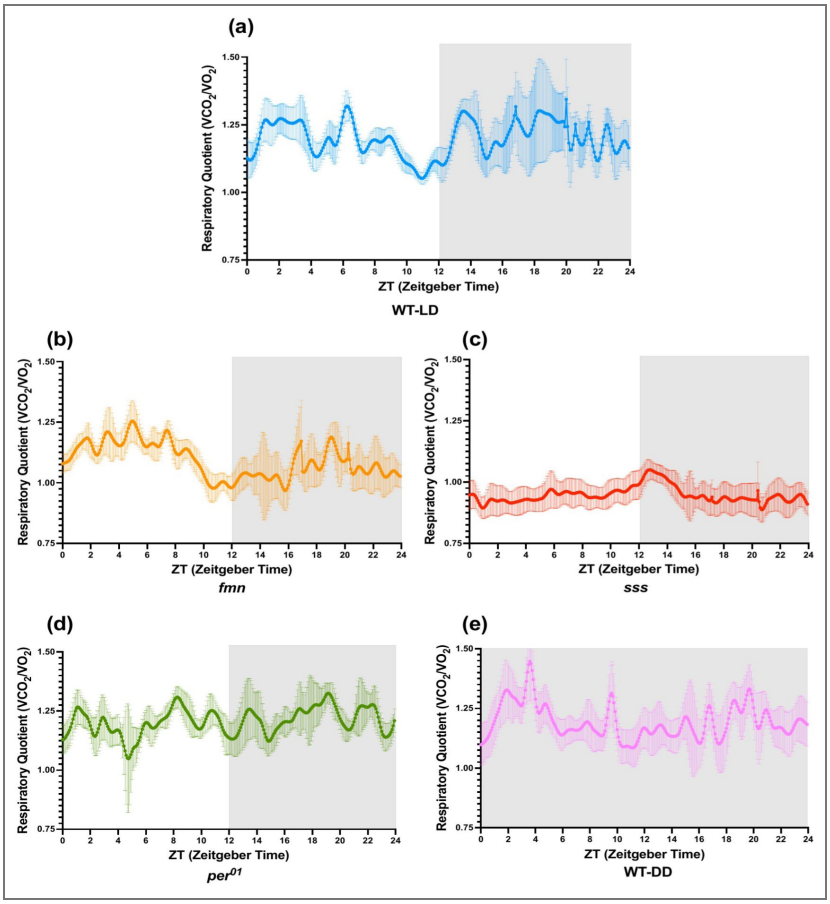

Figure S3.

a-e. Respiratory quotient (RQ) profiles across the 24-hour recording period in different genotypes. RQ (VCO_2/VO_2) was continuously recorded at one-second intervals from Zeitgeber Time (ZT) 0 to 24 and subsequently averaged into 5-minute bins for analysis. Traces represent mean RQ values across the 24-hour recording period for wild-type flies under light-dark conditions (WT-LD), short-sleep mutants *fumin* (*fmn*) and *sleepless* (*sss*), the circadian clock mutant *period*^{01} (*per*^{01}), and wild-type flies maintained in constant darkness (WT-DD). For all panels, traces are presented as mean ± SEM. Data represent approximately 300 flies per genotype across three experimental days (25 flies/chamber, four chambers/experiment). The chamber was used as the experimental unit; *n* denotes the number of chambers.

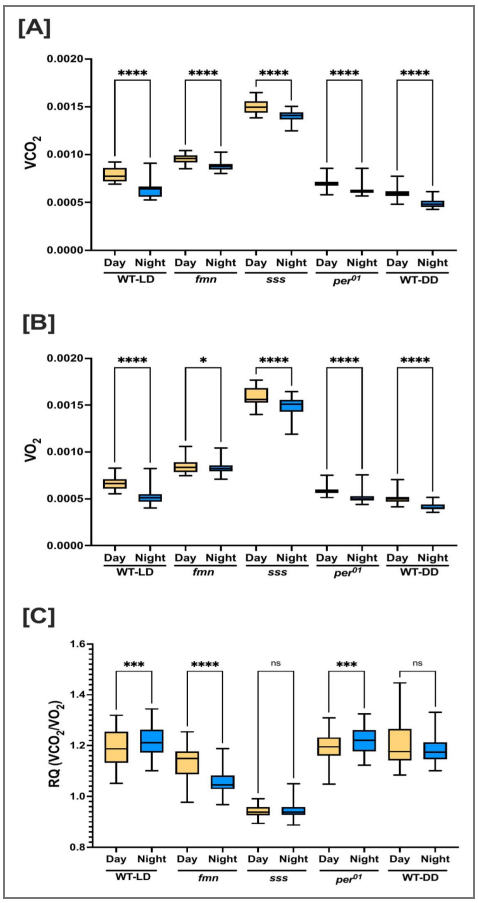

Figure S4. Day-night differences in genotype-specific metabolic rates.

Boxplots depict day (ZT0-12) and night (ZT12-24) averages plotted side-by-side for each genotype to facilitate direct day-night comparison. Panels show genotype-specific differences in (A) carbon dioxide production (VCO_2), (B) oxygen consumption (VO_2), and (C) respiratory quotient (RQ). Genotypes include wild-type flies under light-dark conditions (WT-LD), wild-type flies maintained in constant darkness (WT-DD), short-sleep mutants *fumin* (*fmn*) and *sleepless* (*sss*), and the circadian mutant *period*^{01} (*per*^{01}). Measurements were acquired continuously using a flow-through MAVEn system and binned into day and night intervals. Group differences were assessed using the Kruskal-Wallis test followed by Dunn's multiple comparisons post hoc test. Significance is denoted as: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***); ns = not significant. Source data are the same continuous respirometry recordings shown in Figure 1. Data represent approximately 300 flies per genotype across three experimental days (25 flies/chamber, four chambers/experiment). The chamber was used as the experimental unit; *n* denotes the number of chambers.

Supplementary Table 1. Metabolites showing strong lag-dependent correlations with respiratory quotient (RQ) across genotypes and lighting conditions.

Note: Metabolites were included if they showed a strong metabolite–RQ association at one or more temporal lags, defined as $|\rho| \geq 0.7$ with nominal $p < 0.05$. Nominal p-values are reported at the strongest lag for each metabolite. Benjamini–Hochberg false discovery rate (BH-FDR) adjusted p-values were calculated from the full metabolite × lag nominal p-value set within each genotype/condition, including all metabolites and all lag time points tested, and are reported at the strongest lag. BH-FDR adjusted $p < 0.05$ was considered significant after multiple-testing correction.

Genotype/ condition	Figure	Metabolite	Best lag direction	Best lag time	Spearman ρ at strongest lag	Nominal p-value
WT-LD	Fig. 6	Quinolinate	Negative lag	−120 min	−0.77	0.0049
WT-LD	Fig. 6	Hydroxyhexadecenoylcarnitine	Positive lag	+120 min	+0.78	0.0038
WT-LD	Fig. 6	$NADP^+$	Positive lag	+120 min	+0.75	0.007
WT-LD	Fig. 6	FADH	Positive lag	+120 min	+0.76	0.0062
WT-LD	Fig. 6	LPC 20:5	Positive lag	+120 min	+0.76	0.0062
WT-LD	Fig. 6	Nicotinamide riboside	Positive lag	+120 min	+0.75	0.007
WT-LD	Fig. 6	Glucose	Positive lag	+120 min	+0.71	0.0129
fmn	Fig. 7	Glutamine	Positive lag	+30 min	+0.71	0.0129
fmn	Fig. 7	Methylhistidines	Positive lag	+30 min	+0.90	0.0002
fmn	Fig. 7	Inosine	Positive lag	+30 min	+0.85	0.0009
fmn	Fig. 7	Citrulline	Positive lag	+120 min	+0.84	0.0011
fmn	Fig. 7	LPC 18:0	Positive lag	+60 min	+0.71	0.0129
fmn	Fig. 7	LPC 20:1	Negative lag	−15 min	−0.71	0.0129
sss	Fig. 7	Monosaccharide	Negative lag	−5 min	−0.76	0.0055
sss	Fig. 7	4-Guanidinobutanoic acid	Negative lag	−60 min	−0.73	0.0087
sss	Fig. 7	Biotin	Negative lag	−60 min	−0.71	0.0118
sss	Fig. 7	Citrulline	Negative lag	−120 min	−0.74	0.0078
sss	Fig. 7	Sucrose	Negative lag	−120 min	−0.83	0.0013
sss	Fig. 7	Dimethylglycine /2Aminobutyrate	Positive lag	+60 min	+0.73	0.0096
sss	Fig. 7	Glycine	Positive lag	+60 min	+0.73	0.0087
sss	Fig. 7	Stearoylcarnitine	Positive lag	+30 min	+0.75	0.007
per^{01}	Fig. 8	Creatine	Positive lag	+120 min	+0.73	0.0096
per^{01}	Fig. 8	Glutamine	Negative lag	−60 min	−0.72	0.0106
per^{01}	Fig. 8	Spermidine	Negative lag	−30 min	−0.78	0.0043
per^{01}	Fig. 8	dAMP	Negative lag	−30 min	−0.83	0.0016
per^{01}	Fig. 8	Lauroylcarnitine	Negative lag	−30 min	−0.72	0.0106
per^{01}	Fig. 8	Methylhistidines	Negative lag	−30 min	−0.71	0.0129
per^{01}	Fig. 8	Creatinine	Positive lag	+120 min	+0.76	0.0055
per^{01}	Fig. 8	NADPH	Positive lag	+120 min	+0.83	0.0013
per^{01}	Fig. 8	Adenosine	Negative lag	−120 min	−0.73	0.0096
per^{01}	Fig. 8	LPC 14:0	Negative lag	−15 min	−0.81	0.0022
per^{01}	Fig. 8	Acetylputrescine	Negative lag	−30 min	−0.84	0.0011
per^{01}	Fig. 8	Cis-5-Tetradecenoylcarnitine	Negative lag	−30 min	−0.78	0.0038
per^{01}	Fig. 8	ADP-ribose	Negative lag	−5 min	−0.74	0.0078
per^{01}	Fig. 8	Alpha-ketoglutarate	Positive lag	+5 min	+0.77	0.0049
WT-DD	Fig. 8	Phosphoserine	Positive lag	+120 min	+0.78	0.0038
WT-DD	Fig. 8	Pyruvate	Negative lag	−60 min	−0.72	0.0106
WT-DD	Fig. 8	Stearoylcarnitine	Negative lag	−5 min	−0.71	0.0118
WT-DD	Fig. 8	3-Indoleacetic acid	Negative lag	−60 min	−0.71	0.0129

Lag interpretation: Negative lag indicates that metabolite changes precede RQ changes and positive lag indicates that metabolite changes follow RQ changes. Metabolites correspond to those shown in the metabolite-RQ correlation heatmaps in Figures 6-8, with nominal p-values and BH-FDR values calculated from the full correlation output.

Genotype / condition	Figure	KEGG pathway	Total metabolites in set	Expected hits	Observed hits	Enrichment ratio	Raw p-value	Holm-adjusted p-value	FDR	Contributing metabolites
WT-LD	**6**	**Nicotinate and nicotinamide metabolism**	**15**	**0.088**	**3**	**34.21**	**6.08e-05**	**0.0049**	**0.0049**	**L-Aspartic acid, Quinolinic acid, Nicotinamide riboside**
WT-LD	**6**	**Alanine, aspartate and glutamate metabolism**	**28**	**0.164**	**3**	**18.29**	**0.0004**	**0.0333**	**0.0169**	**L-Aspartic acid, Citric acid, Carbamoyl phosphate**
WT-LD	**6**	**Arginine biosynthesis**	**14**	**0.082**	**2**	**24.42**	**0.0027**	**0.208**	**0.0712**	**L-Aspartic acid, Carbamoyl phosphate**
WT-LD	**6**	**Citrate cycle (TCA cycle)**	**20**	**0.117**	**2**	**17.09**	**0.0055**	**0.421**	**0.109**	**Malic acid, Citric acid**
WT-LD	**6**	**Neomycin, kanamycin and gentamicin biosynthesis**	**2**	**0.012**	**1**	**85.47**	**0.0117**	**0.887**	**0.173**	**D-Glucose**
WT-LD	**6**	**Glyoxylate and dicarboxylate metabolism**	**31**	**0.181**	**2**	**11.05**	**0.0129**	**0.971**	**0.173**	**Citric acid, Malic acid**
WT-LD	**6**	**Nitrogen metabolism**	**6**	**0.035**	**1**	**28.49**	**0.0346**	**1**	**0.396**	**Carbamoyl phosphate**
WT-LD	6	Histidine metabolism	16	0.094	1	10.68	0.09	1	0.847	
WT-LD	6	Starch and sucrose metabolism	18	0.105	1	9.52	0.101	1	0.847	
WT-LD	6	Pantothenate and CoA biosynthesis	20	0.117	1	8.55	0.111	1	0.847	
WT-LD	6	beta-Alanine metabolism	21	0.123	1	8.13	0.117	1	0.847	
WT-LD	6	Pyruvate metabolism	23	0.135	1	7.41	0.127	1	0.847	
WT-LD	6	Galactose metabolism	27	0.158	1	6.33	0.148	1	0.908	
WT-LD	6	Glycine, serine and threonine metabolism	33	0.193	1	5.18	0.178	1	0.947	
WT-LD	6	Cysteine and methionine metabolism	33	0.193	1	5.18	0.178	1	0.947	
WT-LD	6	Pyrimidine metabolism	39	0.228	1	4.39	0.207	1	1	
fmn	**7**	**Arginine biosynthesis**	**14**	**0.046**	**2**	**43.96**	**0.0008**	**0.0606**	**0.0606**	**Citrulline, Glutamine**
fmn	**7**	**Purine metabolism**	**70**	**0.227**	**2**	**8.81**	**0.0187**	**1**	**0.516**	**Glutamine, Inosine**
fmn	**7**	**Nitrogen**	**6**	**0.02**	**1**	**51.28**	**0.0194**	**1**	**0.516**	**Glutamine**

Supplementary Table 2. Metabolite Set Enrichment Analysis (MSEA ORA)

		metabolism								
fmn	**7**	**Butanoate metabolism**	**15**	**0.049**	**1**	**20.53**	**0.0479**	**1**	**0.816**	**2-Hydroxyglutarate**
fmn	**7**	**Histidine metabolism**	**16**	**0.052**	**1**	**19.23**	**0.051**	**1**	**0.816**	**Methylhistidine**
fmn	7	Alanine, aspartate and glutamate metabolism	28	0.091	1	10.99	0.0878	1	1	
fmn	7	Glyoxylate and dicarboxylate metabolism	31	0.101	1	9.9	0.0969	1	1	
fmn	7	Pyrimidine metabolism	39	0.127	1	7.87	0.121	1	1	
sss	**7**	**Glycine, serine and threonine metabolism**	**33**	**0.214**	**3**	**14.02**	**0.001**	**0.0779**	**0.0779**	**Choline, Dimethylglycine, Glycine**
sss	**7**	**Butanoate metabolism**	**15**	**0.098**	**2**	**20.51**	**0.0038**	**0.301**	**0.147**	**Acetoacetic acid, Succinic acid**
sss	**7**	**Starch and sucrose metabolism**	**18**	**0.117**	**2**	**17.09**	**0.0055**	**0.429**	**0.147**	**Sucrose, D-Glucose**
sss	**7**	**Galactose metabolism**	**27**	**0.175**	**2**	**11.43**	**0.0122**	**0.942**	**0.207**	**Sucrose, D-Glucose**
sss	**7**	**Neomycin, kanamycin and gentamicin biosynthesis**	**2**	**0.013**	**1**	**76.92**	**0.013**	**0.985**	**0.207**	**D-Glucose**
sss	7	Biotin metabolism	10	0.065	1	15.38	0.0633	1	0.844	
sss	7	Arginine biosynthesis	14	0.091	1	10.99	0.0876	1	1	
sss	7	Citrate cycle (TCA cycle)	20	0.13	1	7.69	0.123	1	1	
sss	7	Propanoate metabolism	21	0.136	1	7.35	0.129	1	1	
sss	7	Alanine, aspartate and glutamate metabolism	28	0.182	1	5.49	0.168	1	1	
sss	7	Glutathione metabolism	28	0.182	1	5.49	0.168	1	1	
sss	7	Lipoic acid metabolism	28	0.182	1	5.49	0.168	1	1	
sss	7	Glyoxylate and dicarboxylate metabolism	31	0.201	1	4.98	0.185	1	1	
sss	7	Porphyrin metabolism	31	0.201	1	4.98	0.185	1	1	
sss	7	Arginine and proline metabolism	36	0.234	1	4.27	0.211	1	1	
sss	7	Glycerophospholipid metabolism	36	0.234	1	4.27	0.211	1	1	
sss	7	Valine, leucine and isoleucine	39	0.253	1	3.95	0.227	1	1	

Supplementary Table 2. (continued)

		degradation								
sss	7	Tyrosine metabolism	42	0.273	1	3.66	0.242	1	1	
sss	7	Primary bile acid biosynthesis	46	0.299	1	3.34	0.262	1	1	
***per*^{01}**	**8**	**Alanine, aspartate and glutamate metabolism**	**28**	**0.4**	**4**	**10.0**	**0.0005**	**0.041**	**0.0334**	**L-Alanine, Glutamine, Fumaric acid, Oxoglutaric acid**
***per*^{01}**	**8**	**Arginine biosynthesis**	**14**	**0.2**	**3**	**15.0**	**0.0008**	**0.0659**	**0.0334**	**Glutamine, Oxoglutaric acid, Fumaric acid**
***per*^{01}**	**8**	**Arginine and proline metabolism**	**36**	**0.515**	**4**	**7.77**	**0.0014**	**0.107**	**0.0365**	**Creatine, Spermidine, N-Acetylputrescine, 4-Hydroxyproline**
***per*^{01}**	**8**	**Purine metabolism**	**70**	**1.0**	**5**	**5.0**	**0.0024**	**0.187**	**0.0485**	**Glutamine, Adenosine, Deoxyadenosine monophosphate, Xanthosine, Adenosine diphosphate ribose**
***per*^{01}**	**8**	**Nicotinate and nicotinamide metabolism**	**15**	**0.214**	**2**	**9.35**	**0.0183**	**1**	**0.293**	**Quinolinic acid, Niacinamide**
***per*^{01}**	**8**	**Citrate cycle (TCA cycle)**	**20**	**0.286**	**2**	**6.99**	**0.0317**	**1**	**0.423**	**Oxoglutaric acid, Fumaric acid**
***per*^{01}**	**8**	**Glutathione metabolism**	**28**	**0.4**	**2**	**5.0**	**0.0589**	**1**	**0.674**	**NADPH, Spermidine**
per^{01}	8	Cysteine and methionine metabolism	33	0.472	2	4.24	0.0789	1	0.737	
per^{01}	8	Nitrogen metabolism	6	0.086	1	11.66	0.0829	1	0.737	
per^{01}	8	Pyrimidine metabolism	39	0.558	2	3.58	0.105	1	0.75	
per^{01}	8	Valine, leucine and isoleucine biosynthesis	8	0.114	1	8.77	0.109	1	0.75	
per^{01}	8	Tyrosine metabolism	42	0.6	2	3.33	0.119	1	0.75	
per^{01}	8	Ascorbate and aldarate metabolism	9	0.129	1	7.75	0.122	1	0.75	

Supplementary Table 2. (continued)

per^{01}	8	Butanoate metabolism	15	0.214	1	4.67	0.195	1	1	
per^{01}	8	Histidine metabolism	16	0.229	1	4.37	0.207	1	1	
per^{01}	8	Selenocompound metabolism	20	0.286	1	3.5	0.252	1	1	
per^{01}	8	beta-Alanine metabolism	21	0.3	1	3.33	0.262	1	1	
per^{01}	8	Pyruvate metabolism	23	0.329	1	3.04	0.284	1	1	
per^{01}	8	Galactose metabolism	27	0.386	1	2.59	0.324	1	1	
per^{01}	8	Lipoic acid metabolism	28	0.4	1	2.5	0.334	1	1	
per^{01}	8	Inositol phosphate metabolism	30	0.429	1	2.33	0.353	1	1	
per^{01}	8	Glyoxylate and dicarboxylate metabolism	31	0.443	1	2.26	0.363	1	1	
per^{01}	8	Glycine, serine and threonine metabolism	33	0.472	1	2.12	0.381	1	1	
per^{01}	8	Valine, leucine and isoleucine degradation	39	0.558	1	1.79	0.434	1	1	
WT-DD	**8**	**Arginine biosynthesis**	**14**	**0.091**	**3**	**32.97**	**6.94e-05**	**0.0056**	**0.0056**	**Citrulline, Ornithine, Urea**
WT-DD	**8**	**Glycine, serine and threonine metabolism**	**33**	**0.214**	**3**	**14.02**	**0.001**	**0.0769**	**0.026**	**L-Cystathionine, Phosphoserine, Pyruvic acid**
WT-DD	**8**	**Cysteine and methionine metabolism**	**33**	**0.214**	**3**	**14.02**	**0.001**	**0.0769**	**0.026**	**L-Cystathionine, Phosphoserine, Pyruvic acid**
WT-DD	**8**	**Arginine and proline metabolism**	**36**	**0.234**	**2**	**8.55**	**0.0213**	**1**	**0.426**	**Ornithine, Pyruvic acid**
WT-DD	8	Butanoate metabolism	15	0.098	1	10.26	0.0936	1	1	
WT-DD	8	Starch and sucrose metabolism	18	0.117	1	8.55	0.111	1	1	
WT-DD	8	Citrate cycle (TCA cycle)	20	0.13	1	7.69	0.123	1	1	
WT-DD	8	Pyruvate	23	0.149	1	6.71	0.14	1	1	

Supplementary Table 2. (continued)

		metabolism								
WT-DD	8	Glycolysis / Gluconeogenesis	26	0.169	1	5.92	0.157	1	1	
WT-DD	8	Galactose metabolism	27	0.175	1	5.71	0.163	1	1	
WT-DD	8	Alanine, aspartate and glutamate metabolism	28	0.182	1	5.49	0.168	1	1	
WT-DD	8	Glutathione metabolism	28	0.182	1	5.49	0.168	1	1	
WT-DD	8	Lipoic acid metabolism	28	0.182	1	5.49	0.168	1	1	
WT-DD	8	Glyoxylate and dicarboxylate metabolism	31	0.201	1	4.98	0.185	1	1	
WT-DD	8	Pyrimidine metabolism	39	0.253	1	3.95	0.227	1	1	
WT-DD	8	Tryptophan metabolism	41	0.266	1	3.76	0.237	1	1	
WT-DD	8	Tyrosine metabolism	42	0.273	1	3.66	0.242	1	1	
WT-DD	8	Purine metabolism	70	0.455	1	2.2	0.373	1	1	

Note: MSEA over-representation analysis (ORA) was performed against KEGG pathways for each genotype/condition. Pathways listed correspond to the enrichment panels shown in Figures 6-8. Raw p-values, Holm-adjusted p-values, and FDR values are reported for each pathway. Contributing metabolites are shown for pathways/metabolite sets meeting the nominal significance threshold of $p < 0.05$. FDR-adjusted significance should be interpreted separately from the nominal p-value threshold.

Supplementary Table 2. (continued)

Figure/panel	Data shown	Genotypes /Conditions	Experimental unit	n per genotype/condition	Error bars/shaded bands
Fig. 1a-c	5-min VCO_2, VO_2, RQ traces	WT-LD, *fmn*, *sss*, per^{01}, WT-DD	Chamber	n = 12 chambers per genotype	Mean ± SEM
Fig. 1d-f	24-h average VCO_2, VO_2, RQ	WT-LD, *fmn*, *sss*, per^{01}, WT-DD	Chamber	n = 12 chambers per genotype	Mean ± SEM or boxplot details
Fig. 1g-i	Body weight / normalized VCO_2, VO_2	WT, *fmn*, *sss*, per^{01}	Chamber	n = 12 chambers per genotype	Mean ± SEM
Fig. 2	Rhythmicity time course	WT-LD, *fmn*, *sss*, per^{01}, WT-DD	Chamber	n = 12 chambers per genotype	Mean ± SEM
Fig. 3	Phase/peak timing	WT-LD, *fmn*, *sss*, per^{01}, WT-DD	Chamber	n = 12 chambers per genotype	Not applicable / phase estimate
Fig. S1-S3	Individual VCO_2, VO_2, RQ traces	WT-LD, *fmn*, *sss*, per^{01}, WT-DD	Chamber	12 chambers per genotype	Mean ± SEM
Fig. S4	Day vs night averages	WT-LD, *fmn*, *sss*, per^{01}, WT-DD	Chamber	12 chambers per genotype	Mean ± SEM or boxplot details

Note: Chamber was used as the experimental unit for all respirometry analyses. Each chamber contained 25 flies, and each genotype/condition was measured across four chambers per experiment over three experimental days, corresponding to n = 12 chambers per genotype/condition unless otherwise indicated. Error bars or shaded bands represent SEM. For boxplots, the plotted distribution/error representation is indicated in the corresponding figure legend.

Supplementary Table 3. Experimental unit, sample size, and error representation for respirometry-based figures.

This table summarizes the experimental unit, chamber-level sample size, and error representation for all respirometry-based analyses shown in Figures 1-3 and Figures S1-S4. For all respirometry analyses, the chamber was used as the experimental unit. Each chamber contained 25 flies, and each genotype/condition was measured across four chambers per experiment over three experimental days, corresponding to *n* = 12 chambers per genotype/condition unless otherwise indicated.

Data availability

All data used to generate figures in this study has been made available as supplemental tables.

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Additional information

Author Contributions

Conceptualization, F.A., A.S. and A.M.W.; methodology, F.A., D.M.M., P.H., J.K. A.N. and A.M.W.; formal analysis, F.A., A.S.G., A.N. and A.M.W.; investigation, F.A. A.N. and D.M.M.; resources, A.S. and A.M.W.; writing-original F.A.; writing-review and editing, F.A., A.S.G., J.K., A.S., and A.M.W.; visualization, F.A., A.S.G, and A.M.W.; supervision, A.M.W.; and funding acquisition, A.S. and A.M.W.

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Peer reviews

Reviewer #1 (Public review):

Summary:

This study by Akhtar et al. aims to investigate the link between systemic metabolism and respiratory demands, and how sleep and circadian clock regulate metabolic states and respiratory dynamics. The authors leverage genetic mutants that are defective in sleep and circadian behavior in combination with indirect respirometry and steady-state LC-MS-based metabolomics to address this question in the Drosophila model.

First, the authors performed respirometry (on groups of 25 flies) to measure oxygen consumption (VO2) and carbon dioxide production (VCO2) to calculate the respiratory quotient (RQ) across the 24-hour day (12h:12h light-dark cycle) and assess metabolic fuel utilization. They observed that among all the genotypes tested, wild type (WT) flies and per0 flies in LD and WT flies in DD exhibit RQ >1. They concluded the >1 RQ is consistent with active lipogenesis. In contrast, the short-sleep mutants fumin (fmn) and sleepless (sss) showed significantly different RQ; the fmn exhibits a slight reduction in RQ values, suggesting increased reliance on carbohydrate metabolism, while sss exhibit even lower RQ (0.94), consistent with a shift toward lipid and protein catabolism.

The authors then proceeded to bin these measurements in 12-hour partitions, ZT0-12 and ZT12-24, to assess diurnal differences in average values of VO2, VCO2, and RQ. They observed significant day-night differences in metabolic rates in WT-LD flies, with higher rates during the day. The diurnal differences remain in the short-sleep mutants, but the overall metabolic rates are higher. WT-DD flies exhibit the lowest respiratory activity although the day-night differences remain in free-running conditions. Finally, per01 mutants exhibit no significant change in day-night respiratory rates, suggesting that a functional circadian clock is necessary for diurnal differences in metabolic rates.

They then performed finer resolution 24-hours rhythmic analysis (RAIN and JTK) to determine if VO2, VCO2, and RQ exhibit 24-hour rhythmic and if there are genotype-specific differences. Based on their criteria, VCO2 is rhythmic in all conditions tested while VO2 is rhythmic in all conditions except in fmn-LD. Finally, RQ is rhythmic in all 3 mutants but not in WT-LD and WT-DD. Peak phases for the rhythms were deduced using JTK lag values.

The authors proceeded to leverage a previously published steady-state metabolite datasets to investigate potential association of RQ with metabolite profiles. Spearman correlation was performed to identify metabolites that exhibit coupling to respiratory output. Positive and

negative lag analysis were subsequently performed to further characterize these associations based on the timing of the metabolite peak changes relative to RQ fluctuations. The authors suggest that a positive lag indicates that metabolite changes occur after shifts in RQ, and a negative lag signifies that metabolite changes precede RQ changes. To visualize metabolic pathways that exhibit these temporal relationships, clustered heatmap and enrichment analysis were performed. Through these analyses, they concluded that both sleep and circadian systems are essential for aligning metabolic substrate selection with energy demands, and different metabolic pathways are misregulated in the different mutants with sleep and circadian defects.

Strength:

The research questions this study explore are significant given metabolism and respiratory demand are central to animal biology. The experimental methods used, including the well characterized fly genetic mutants, the newly developed method for indirect calorimetry measurements, and LC-MS based metabolomics, are all appropriate. This study provides insights into the impact of sleep and circadian rhythm disruption on metabolism and respiratory demand and serves as a foundation for future mechanistic investigations.

Comments on revised version.

The authors have thoughtfully revised the manuscript. They have now provided clarifications regarding perceived conceptual flaws in the original version. They have also performed additional data analysis to support their conclusions and provide clarifications on statistical methods when appropriate. Overall, the revised manuscript is much improved and the conclusions are generally well supported by their results.

https://doi.org/10.7554/eLife.108681.2.sa3

Reviewer #2 (Public review):

This is an innovative and technically strong study that integrates dual-gas respirometry with LC-MS metabolomics to examine how sleep and circadian disruption shape metabolism in Drosophila. The combination of continuous O_2/CO_2 measurements with high-temporal-resolution metabolite profiling is novel and provides fresh insight into how wild-type flies maintain anticipatory fuel alignment, while mutants shift to reactive or misaligned metabolism. The use of lag-shift correlation analysis is particularly clever, as it highlights temporal coordination rather than static associations. Together, the findings advance our understanding of how circadian clocks and sleep contribute to metabolic efficiency and redox balance.

However, there are several areas where the manuscript could be strengthened. The authors should acknowledge that their findings may be gene-specific. Because sleep deprivation was not performed, it remains uncertain whether the observed metabolic shifts generalize to sleep loss broadly or are restricted to the fmn and sss mutants. This concern also connects to the finding of metabolic misalignment under constant darkness despite an intact clock. The conclusion that external entrainment is essential for maintaining energy homeostasis in flies may not translate to mammals. It would help to reference supporting data for the finding and discuss differences across species. Ideally, complementary circadian (light-dark cycle disruption) or sleep deprivation (for several hours) experiments, or citation of comparable studies, would strengthen the generality of the findings. Figures 1-4 are straightforward and clear, but when the manuscript transitions to the metabolite-respiration correlations, there is little description of the metabolomics methods or datasets, which should be clarified. The Discussion is at times repetitive and could be tightened, with the main message (i.e., wild-type flies align metabolism in advance, while mutants do not) kept front and center. Terms such as "anticipatory" and "reactive" should be defined early and used consistently throughout.

Overall, this is a strong and novel contribution. With clarification of scope, refinement of presentation, and a more focused Discussion, the paper will make a significant impact.

Comments on revised version.

The authors have satisfactorily addressed my concerns in the revised manuscript

https://doi.org/10.7554/eLife.108681.2.sa2

Reviewer #3 (Public review):

Summary:

The authors investigate how sleep loss and circadian disruption affect whole-organism metabolism in Drosophila melanogaster. They used chamber-based flow-through respirometry to measure oxygen consumption, carbon dioxide production, in wild-type flies and in mutants with impaired sleep or circadian function. These measurements were then integrated with a previously published metabolomics dataset to explore how respiratory dynamics align with metabolic pathways. The central claim is that wild-type flies display anticipatory coordination of metabolic processes with circadian time, while mutants exhibit reactive shifts in substrate use, redox imbalance, and signs of mitochondrial stress.

Strengths:

The study has several strengths. Continuous high-resolution respirometry in flies is challenging, and its application across multiple genotypes provides good comparative insight. The conceptual framework distinguishing anticipatory from reactive metabolic regulation is interesting. The translational framing helps place the work in a broader context of sleep, circadian biology, and metabolic health.

Weaknesses:

At the same time, the evidence supporting the conclusions is somewhat limited. The metabolomics data were not newly generated but repurposed from prior work, reducing novelty. The biological replication in the respirometry assays is low, with only a small number of chambers per genotype. Importantly, respiratory parameters in flies are strongly influenced by locomotor activity, yet no direct measurements of activity were included, making it difficult to separate intrinsic metabolic changes from behavioral differences in mutants. In addition, repeated claims of "mitochondrial stress" are not directly substantiated by assays of mitochondrial function. The study also excluded female flies entirely, despite well-documented sex differences in metabolism, which narrows the generality of the findings.

https://doi.org/10.7554/eLife.108681.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *This study by Akhtar et al. aims to investigate the link between systemic metabolism and respiratory demands, and how sleep and the circadian clock regulate metabolic states*

and respiratory dynamics. The authors leverage genetic mutants that are defective in sleep and circadian behavior in combination with indirect respirometry and steady-state LC-MS-based metabolomics to address this question in the Drosophila model.

First, the authors performed respirometry (on groups of 25 flies) to measure oxygen consumption (VO2) and carbon dioxide production (VCO2) to calculate the respiratory quotient (RQ) across the 24-hour day (12h:12h light-dark cycle) and assess metabolic fuel utilization. They observed that among all the genotypes tested, wild type (WT) flies and per0 flies in LD and WT flies in DD exhibit RQ >1. They concluded the >1 RQ is consistent with active lipogenesis. In contrast, the short-sleep mutants fumin (fmn) and sleepless (sss) showed significantly different RQ; the fmn exhibits a slight reduction in RQ values, suggesting increased reliance on carbohydrate metabolism, while sss exhibits even lower RQ (0.94), consistent with a shift toward lipid and protein catabolism.

The authors then proceeded to bin these measurements in 12-hour partitions, ZT0-12 and ZT12-24, to assess diurnal differences in average values of VO2, VCO2, and RQ. They observed significant day-night differences in metabolic rates in WT-LD flies, with higher rates during the day. The diurnal differences remain in the short-sleep mutants, but the overall metabolic rates are higher. WT-DD flies exhibit the lowest respiratory activity, although the day-night differences remain in free-running conditions. Finally, per01 mutants exhibit no significant change in day-night respiratory rates, suggesting that a functional circadian clock is necessary for diurnal differences in metabolic rates.

They then performed finer-resolution 24-hour rhythmic analysis (RAIN and JTK) to determine if VO2, VCO2, and RQ exhibit 24-hour rhythmic and if there are genotypespecific differences. Based on their criteria, VCO2 is rhythmic in all conditions tested, while VO2 is rhythmic in all conditions except in fmn-LD. Finally, RQ is rhythmic in all 3 mutants but not in WT-LD and WT-DD. Peak phases for the rhythms were deduced using JTK lag values.

The authors proceeded to leverage a previously published steady-state metabolite dataset to investigate the potential association of RQ with metabolite profiles. Spearman correlation was performed to identify metabolites that exhibit coupling to respiratory output. Positive and negative lag analysis were subsequently performed to further characterize these associations based on the timing of the metabolite peak changes relative to RQ fluctuations. The authors suggest that a positive lag indicates that metabolite changes occur after shifts in RQ, and a negative lag signifies that metabolite changes precede RQ changes. To visualize metabolic pathways that exhibit these temporal relationships, a clustered heatmap and enrichment analysis were performed. Through these analyses, they concluded that both sleep and circadian systems are essential for aligning metabolic substrate selection with energy demands, and different metabolic pathways are mis regulated in the different mutants with sleep and circadian defects.

We thank the reviewer for summarizing the contributions made by this manuscript.

Strength:

The research questions this study explores are significant, given that metabolism and respiratory demand are central to animal biology. The experimental methods used, including the well-characterized fly genetic mutants, the newly developed method for indirect calorimetry measurements, and LC-MS-based metabolomics, are all appropriate. This study provides insights into the impact of sleep and circadian rhythm disruption on metabolism and respiratory demand and serves as a foundation for future mechanistic investigations.

We thank the reviewer for the positive comments.

> *Weaknesses:*
>
> *There are some conceptual flaws that the authors need to address regarding circadian biology, and some of the conclusions can be better supported by additional analysis to provide a stronger foundation for future functional investigation.*
>
> *At times, the methods, especially the statistical analysis, are not well articulated; they need to be better explained.*

Thank you for this suggestion and have revised and we expanded the Methods and figure legends to improve transparency and reproducibility.

Specifically, we have:

(i) Strengthened the rhythmicity description by specifying that rhythmicity was assessed in Nitecap using the RAIN algorithm with FDR-adjusted p-values (significant $p \leq 0.05$; trending 0.05–0.1), and that period and peak phase were estimated with JTK_CYCLE (JTK lag = peak phase), with per-genotype period, phase, and p-values reported in Table 1;

(ii) Clarified sample size and replication by adding these lines to the methods section and indicating sample sizes in figure legends. Figure legends now report n (chambers) and SEM.

"Each genotype measurement represents an average of ~300 flies (25 flies per chamber × 4 chambers per experiment × 3 experimental days). The chamber was treated as the experimental unit for all analyses."

In addition, we have expanded the description of metabolomics-respirometry correlation analyses to include dataset structure, time-matching across ZT, normalization steps, the use of Spearman correlations, and interpretation of lagged associations.

(iii) We have added these lines to the methods section:

"To integrate respirometry with metabolomics, RQ was recorded continuously at 1-second resolution and averaged into 5-minute bins. Because steady-state metabolite measurements were acquired at 2-hour intervals, we extracted the RQ values corresponding to each 2-hour Zeitgeber Time (ZT) sampling point from the 5-minutebinned dataset to generate time-matched RQ-metabolite pairs. We additionally evaluated temporal relationships using a lag analysis by systematically shifting the RQ time series relative to the metabolite time points (−120, −60, −30, −15, −5, +5, +15, +30, +60, and +120 minutes). Metabolite abundances were normalized as described above, and associations between RQ and individual metabolites were quantified using Spearman rank correlations (ρ) at each lag. Metabolites showing strong associations (e.g., $|\rho| > 0.7$ with nominal $p < 0.05$) were carried forward for visualization and summary, and lag direction was interpreted as metabolites preceding (negative lag) or following (positive lag) changes in RQ."

> ***Reviewer #2 (Public review):***
>
> *This is an innovative and technically strong study that integrates dual-gas respirometry with LC-MS metabolomics to examine how sleep and circadian disruption shape metabolism in Drosophila. The combination of continuous O_2/CO_2 measurements with high-temporal-resolution metabolite profiling is novel and provides fresh insight into how wild-type flies maintain anticipatory fuel alignment, while mutants shift to reactive or misaligned metabolism. The use of lag-shift correlation analysis is particularly clever, as it highlights temporal coordination rather than static associations. Together, the*

> *findings advance our understanding of how circadian clocks and sleep contribute to metabolic efficiency and redox balance.*

We thank the reviewer for the positive comments.

> *However, there are several areas where the manuscript could be strengthened.*
>
> *The authors should acknowledge that their findings may be gene specific. Because sleep deprivation was not performed, it remains uncertain whether the observed metabolic shifts generalize to sleep loss broadly or are restricted to the fmn and sss mutants. This concern also connects to the finding of metabolic misalignment under constant darkness despite an intact clock.*

We agree that our findings should be framed as genotype- and condition-specific. The phenotypes we report arise from chronic, genetically encoded sleep loss (fmn, sss) and clock loss (per01); because acute sleep deprivation was not performed, we do not claim these effects generalize to sleep loss broadly. This also bears on the reviewer's point about constant darkness: the metabolic misalignment we observe in WT-DD occurs despite an intact clock, and we therefore interpret it as a consequence of removing external light-dark cues under our conditions. We have scoped the claims accordingly in the Abstract, Results, and Discussion (subsection “Metabolic Desynchrony and Redox Imbalance in Wild-Type Flies Under Constant Darkness, DD”).

The text now reads as follows:

“We restrict our conclusions to the genotypes and conditions tested (*fmn*, *sss*, and per^{01}), and we do not generalize these effects to acute sleep deprivation because sleep deprivation was not performed in this study. Accordingly, the ‘metabolic misalignment’ observed in constant darkness (DD) likely results from a decrease in synchrony due to the removal of external light:dark cues.”

> *The conclusion that external entrainment is essential for maintaining energy homeostasis in flies may not translate to mammals. It would help to reference supporting data for the finding and discuss differences across species. Ideally, complementary circadian (lightdark cycle disruption) or sleep deprivation (for several hours) experiments, or citation of comparable studies, would strengthen the generality of the findings.*

Thank you. We have tempered the interpretation and expanded both the discussion and its citations. We now (i) avoid stating that external entrainment is universally “essential” for energy homeostasis, (ii) explicitly discuss fly-mammal differences (sleep architecture, thermoregulation, feeding control, and entrainment mechanisms), and (iii) anchor the translational comparison to the mammalian circadian-misalignment and sleep-loss literature already integrated in our Discussion (refs [3, 43-46]), noting that establishing cross-species generality will require additional paradigms (constant light, acute sleep deprivation).

The text now reads as follows:

“These phenotypes parallel mammalian systems, where sleep loss and circadian misalignment are linked to elevated basal metabolic rate, a shift toward carbohydrate oxidation and lipid/protein catabolism, and blunted, phase-shifted respiratory oscillations [3, 43-46]; physiological differences between flies and mammals nonetheless caution against direct mechanistic extrapolation. In constant darkness, our DD data show that endogenous free-running regulation persists but that removing external light-dark cues degrades temporal coordination between respiration and metabolism; however, we acknowledge that the coupling may be different in mammals.”

> *Figures 1-4 are straightforward and clear, but when the manuscript transitions to the metabolite-respiration correlations, there is little description of the metabolomics methods or datasets, which should be clarified.*

Thank you for noting this. We agree that the transition to the metabolite–respiration correlation analyses required clearer description of the metabolomics datasets and processing. We have revised the Methods and the corresponding Results text to briefly summarize the metabolomics dataset parameters and workflow, including how metabolomics and respirometry measurements were time-matched across ZT, the normalization procedures applied prior to analysis, the use of Spearman rank correlations, and how we interpret lagged relationships between metabolite abundance and respiratory outputs.

The text now reads as follows:

Methods:

“Metabolomics-respirometry integration and lag analysis

To integrate respirometry with metabolomics, RQ was recorded continuously at 1-second resolution and averaged into 5-minute bins. Because steady-state metabolite measurements were acquired at 2-hour intervals, we extracted the RQ values corresponding to each 2-hour Zeitgeber Time (ZT) sampling point from the 5-minutebinned dataset to generate time-matched RQ-metabolite pairs. We additionally evaluated temporal relationships using a lag analysis by systematically shifting the RQ time series relative to the metabolite timepoints (−120, −60, −30, −15, −5, +5, +15, +30, +60, and +120 minutes). Metabolite abundances were normalized as described above, and associations between RQ and individual metabolites were quantified using Spearman rank correlations (ρ) at each lag. Metabolites showing strong associations (e.g., $|\rho| > 0.7$ with nominal $p < 0.05$) were carried forward for visualization and summary, and lag direction was interpreted as metabolites preceding (negative lag) or following (positive lag) changes in RQ.”

Results:

“Temporal Profiling of Respiratory Quotient in Wild-Type Flies Under Light-Dark Conditions

RQ values corresponding to each 2-hour Zeitgeber Time (ZT) point were extracted from the 5-minute-binned dataset. Building on this alignment, we explored temporal relationships by systematically shifting the RQ time series by −120, −60, −30, −15, −5, +5, +15, +30, +60, and +120 minutes relative to the metabolite dataset. The continuous respirometry time series showed an oscillatory day-night pattern in RQ; metabolomics was then used to relate time-matched and lagged metabolite dynamics to RQ patterns (Figure 4).”

> *The Discussion is at times repetitive and could be tightened, with the main message (i.e., wild-type flies align metabolism in advance, while mutants do not) kept front and center.*

Thank you for this helpful suggestion. We have revised the Discussion to reduce repetition and improve focus by keeping the central takeaway explicit throughout, and by consolidating overlapping paragraphs into a more streamlined narrative.

We added this revision at the start of the Discussion, in the opening subsection “Temporal Misalignment Alters Fuel Utilization and Respiratory Rhythms.”

The Discussion now reads as follows:

“Across the manuscript, the central takeaway is that wild-type flies under LD exhibit anticipatory alignment of fuel selection with time of day, whereas short-sleep mutants (fmn,

sss) and clock-disrupted flies (per01) show reactive or misaligned metabolism under our conditions. We therefore focus the Discussion on loss of temporal coordination between respiratory output and pathway-level metabolism, rather than reiterating rate changes alone."

> *Terms such as "anticipatory" and "reactive" should be defined early and used consistently throughout.*

Thank you for this suggestion. We agree and have revised the manuscript to define these terms early (at first use) and apply them consistently throughout. We added this definition in two places:

(i) In the Results, at the start of the metabolomics-respirometry integration section where we first introduce the lag analysis, and

(ii) In the Methods, within the paragraph describing the lag analysis workflow, using identical wording.

The text now reads as follows:

"We define 'anticipatory' as metabolite changes that precede the associated respiratory shift (negative lag) and 'reactive' as changes that follow or coincide with the respiratory shift (positive lag), and we use these terms consistently throughout."

> *Overall, this is a strong and novel contribution. With clarification of scope, refinement of presentation, and a more focused Discussion, the paper will make a significant impact.*

We again thank the reviewer for the positive comments.

> ***Reviewer #3 (Public review):***
>
> *Summary:*
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> *The authors investigate how sleep loss and circadian disruption affect whole-organism metabolism in Drosophila melanogaster. They used chamber-based flow-through respirometry to measure oxygen consumption and carbon dioxide production in wild-type flies and in mutants with impaired sleep or circadian function. These measurements were then integrated with a previously published metabolomics dataset to explore how respiratory dynamics align with metabolic pathways. The central claim is that wild-type flies display anticipatory coordination of metabolic processes with circadian time, while mutants exhibit reactive shifts in substrate use, redox imbalance, and signs of mitochondrial stress.*

We thank the reviewer for summarizing the contributions made by this manuscript.

> *Strengths:*
>
> *The study has several strengths. Continuous high-resolution respirometry in flies is challenging, and its application across multiple genotypes provides good comparative insight. The conceptual framework distinguishing anticipatory from reactive metabolic regulation is interesting. The translational framing helps place the work in a broader context of sleep, circadian biology, and metabolic health.*

We thank the reviewer for the positive comments.

> *Weaknesses:*
>
> *At the same time, the evidence supporting the conclusions is somewhat limited. The metabolomics data were not newly generated but repurposed from prior work, reducing*

> *novelty.*

Thank you for raising this point. We now make the provenance of the metabolomics dataset explicit in the manuscript. Importantly, the current study uses this dataset in a new analytical context by integrating it with continuous VCO_2/VO_2 respirometry through timematched and lag-aware analyses. This approach allows us to evaluate dynamic relationships between respiratory output and metabolite profiles across circadian time, which was not addressed in the original metabolomics study. We have clarified this point in the Introduction and Methods.

The text now reads as follows:

In the Introduction:

"To provide a more comprehensive perspective on metabolic regulation, we complemented newly generated respiratory measurements with steady-state metabolomic profiling using liquid chromatography-mass spectrometry (LC-MS) data previously published from our group [27]. This integrative framework enabled timematched and lag-aware analysis of respiratory output and metabolite profiles across Zeitgeber time in the LD cycle…."

In the Methods:

"The metabolomics dataset analyzed in this study was previously published and is publicly available, as described in detail in [27, 31]. In the present study, these data were integrated with respirometry measurements to assess temporal relationships between metabolite abundance and respiratory output."

> *The biological replication in the respirometry assays is low, with only a small number of chambers per genotype.*

Thank you for highlighting this concern. We suggest that this is a lack of clarity in our initial description of the design and that the replication structure should be stated more explicitly. We have revised the Methods and all relevant figure legends to clearly report biological replication using the chamber as the experimental unit, including n (number of chambers) per genotype and the associated error structure. We also clarify sampling depth by stating that each genotype measurement reflects an average of ~300 flies (25 flies per chamber × 4 chambers per experiment × 3 experimental days). This information is now reported consistently to make the unit of analysis transparent.

We added this clarification in the Methods under "Respirometry Setup" (where chamber loading and experimental design are described) and ensured that each relevant figure legend explicitly reports n (chambers) and SEM.

The text now reads as follows:

"Each genotype measurement represents an average of ~300 flies (25 flies/chamber × 4 chambers/experiment × 3 experimental days), with the chamber as the experimental unit; n (chambers) and SEM are reported in each figure legend."

> *Importantly, respiratory parameters in flies are strongly influenced by locomotor activity, yet no direct measurements of activity were included, making it difficult to separate intrinsic metabolic changes from behavioral differences in mutants.*

A detailed timing comparison between behavior (feeding and locomotion) compared to respirometry is given in our master response to Reviewer 1, Major comment 4.

> *In addition, repeated claims of "mitochondrial stress" are not directly substantiated by assays of mitochondrial function.*

Thank you. We agree that “mitochondrial stress” requires direct functional evidence. We therefore directly assayed mitochondrial respiration (baseline gut-tissue OCR in fmn and per01 versus iso31 controls), added a new Methods subsection and Figure 9, and reframed our wording from “mitochondrial stress/impairment” to altered (elevated) baseline mitochondrial respiration. The experimental details are now in the Methods and the result in the Results (both quoted below). sss was not assayed, so we removed the functional mitochondrial-stress claim for sss; we retain Vaccaro et al. (2020) as prior support for fmn.

Methods — new subsection “Gut Tissue Respirometry” now reads: “Oxygen consumption rate (OCR) was measured in dissected gut tissue from iso31, fmn, and per01 flies using the Resipher System (Lucid Scientific, GA, USA). Baseline OCR (fmol/mm^2/s) was averaged over a 24-hour window following a 12-hour acclimation; values from 3 independent runs were pooled, median-normalized to iso31 within each run, and log2(x+10)-transformed. A single iso31 outlier (third per01 run) was excluded; no other values were removed. Each mutant was compared with iso31 using the MannWhitney test (GraphPad Prism 10), with significance at $p < 0.05$ (fmn vs iso31, n = 12 vs 12; per01 vs iso31, n = 12 vs 11 after excluding one iso31 outlier).”

The text has been added to Results:

“Because pathway-level metabolomics implicated mitochondrial pathways in the sleep and circadian mutants, we directly assayed mitochondrial respiration by measuring baseline oxygen consumption rate (OCR) in dissected gut tissue from fmn and per01 relative to iso31 controls. Both fmn and per01 guts showed significantly elevated baseline OCR (fmn vs iso31, n = 12 vs 12; per01 vs iso31, n = 12 vs 11 across 3 runs; Mann-Whitney test, $p<0.05$; Figure 9A,B). Because only baseline OCR was measured, we interpret this as altered (elevated) baseline mitochondrial respiration rather than reduced capacity or a specific coupling defect (Figure 9).”

Discussion- *fmn*:

“These interpretations are further supported by gut-tissue respirometry showing elevated baseline mitochondrial respiration in fmn relative to iso31 controls. Together with prior evidence of ROS accumulation (oxidative stress) in fmn (Vaccaro et al., Cell, 2020), these functional data indicate that chronic sleep loss in fmn is associated with altered mitochondrial respiration.”

Discussion- *per01*:

“Gut-tissue respirometry in per01 likewise showed elevated baseline mitochondrial respiration, providing functional evidence consistent with the metabolomic signatures of disrupted redox balance and mitochondrial metabolism.”

> *The study also excluded female flies entirely, despite well-documented sex differences in metabolism, which narrows the generality of the findings.*

Thank you for raising this point. We agree that sex is an important biological variable in metabolic regulation. While our Methods state that male flies were collected, we have now made this explicit and unambiguous by stating that only males were used for respirometry and metabolomics integration, and we have added this as a limitation in the Discussion, noting that sex-specific physiology could influence the magnitude and/or timing of the effects we report. We also highlight inclusion of females as an important future direction.

The text now reads as follows (Methods):

“Only male flies were used for all respirometry experiments and for integration with the metabolomics dataset. This was done to reduce variability introduced by female reproductive

status (e.g., mating/egg production) and associated metabolic differences, enabling a clearer comparison across genotypes and lighting conditions."

The text now reads as follows (Discussion):

"Because only males were analyzed, our conclusions may not generalize to females, which can show sex-specific metabolic physiology. Inclusion of female flies and direct sex comparisons across LD and DD conditions will be an important future direction. More specifically, females carry a higher reproductive and biosynthetic load (egg production) that typically raises metabolic rate and can shift RQ toward lipogenesis and alter the amplitude and phase of diurnal respiratory rhythms; females might therefore show larger or differently-timed effects than the males studied here."

> ***Recommendations for the authors:***
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> ***Reviewer #1 (Recommendations for the authors):***
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> *Major comments:*
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> *(1) The authors appear to be using WT-DD as a condition to disrupt circadian rhythm (line 216). Although rhythmicity is often dampened in DD compared to in LD, circadian rhythm is defined as rhythm in a constant condition (e.g., DD) after entrainment. WT-DD is not a condition that the authors should use if they want to disrupt circadian rhythms in flies. WTLL would be a better condition to use since flies become arrhythmic in LL, not DD.*

Thank you for this clarification. We agree that DD is not a circadian-disrupting condition; circadian rhythmicity is defined by rhythms that persist under constant conditions after entrainment. Our intent was not to treat WT-DD as arrhythmic, but to use DD to assess free-running circadian regulation in the absence of external light–dark cues. We have revised the manuscript to clearly distinguish diurnal rhythms under LD from free-running circadian rhythms under DD, and to avoid implying that DD abolishes rhythmicity.

The Results text now reads as follows:

"We used constant darkness (DD) to assess free-running circadian regulation in the absence of external light–dark cues."

In the Discussion, in the subsection "Metabolic Desynchrony and Redox Imbalance in Wild-Type Flies Under Constant Darkness," we revised the interpretation of WT-DD to clarify that the observed metabolic effects reflect removal of external light–dark cues under our experimental conditions, while avoiding overgeneralization.

The Discussion text now reads as follows:

Our DD data show that endogenous free-running circadian regulation persists but that removing external light–dark cues degrades the temporal coordination between respiration and metabolism, indicating that external entrainment normally strengthens this coordination under our conditions; however, we acknowledge that the coupling may be different in mammals.

> *(2) The authors need to be more precise in the use of the term "circadian" throughout the manuscript. When describing 24h rhythmicity in the LD condition in flies, they can use the diurnal rhythm. Circadian rhythm is an endogenous rhythm without external time cues (e.g., DD rhythm).*

Thank you for this point. We agree and have revised the manuscript to use terminology consistently: rhythms measured under LD are now referred to as diurnal (LD) rhythms/patterns, and the term circadian is reserved for endogenous free-running rhythms

in DD. We updated the Methods, Results, Discussion and figure legends throughout to correct instances where LD rhythmicity was previously labeled as “circadian.”

We added this clarification in the Methods (“*Drosophila* Strains, Entrainment and Collection”) and in the Results/figure legends where LD time courses are described.

The text now reads as follows (Methods):

“Male flies were collected shortly after eclosion and entrained in light-dark (LD) incubators for a minimum of three days before diurnal (LD) time-course collection across Zeitgeber time (ZT).”

We revised the Results text where WT-DD and LD time courses are described, replacing imprecise references to ‘circadian disruption’ or ‘circadian cycle’ with ‘free-running conditions in DD,’ ‘24-hour cycle,’ or ‘diurnal pattern under LD,’ as appropriate. We also revised the Discussion to avoid describing LD patterns as circadian and to avoid implying that DD disrupts circadian rhythmicity.

> *(3) Lines 253-256: The authors' interpretation of this data is not accurate. The authors observed significant day-night differences in VO2 and VCO2 in WT-DD. This suggests there is circadian control over metabolism rhythms. The authors noted there is "limited circadian control".*

Thank you for pointing this out. We agree that the significant day-night differences in VO_2 and VCO_2 in WT-DD support persistent endogenous (circadian) control of respiratory rhythms under constant darkness. We have revised the Results text (lines 253-256) to remove the statement implying “limited circadian control” and instead describe the WTDD effect as maintained rhythmicity with altered amplitude and/or phase relative to LD, rather than loss of rhythmic regulation.

We added this revision in the Results section under “Diurnal Variation in CO_2 Production, O_2 Consumption, and Respiratory Quotient Across Genotypes” (WT-DD description; lines 253-256).

The text now reads as follows:

WT-DD flies, maintained in constant darkness, exhibited the lowest overall respiratory activity. Despite the absence of environmental light cues, VCO_2 and VO_2 retained significant day-night differences (Figure S4), consistent with persistent free-running circadian control in constant darkness (with altered amplitude and/or phase relative to LD).

> *(4) Besides sleep, metabolic rates are known to be affected by food consumption. Measuring food consumption of the sleep and circadian mutants might provide insights into whether the metabolic rates are more affected by changes in sleep profile or food consumption. This might also be important given fumin displays impaired dopamine transport function and defective dopamine reuptake, and dopamine is known to affect eating behavior. This was not considered and/or discussed.*

It is technically challenging to measure feeding during respirometry, so we acknowledge it as a limitation. To test whether behavioral timing could explain our results, we compared our respiratory rhythms with the feeding and activity rhythms reported in Malik et al. (2026); the comparison and its interpretation are now in the Discussion (quoted below). This is our master response to the activity/feeding concern and is crossreferenced from Reviewer 3’s public review and Recommendation 1; feeding and activity were not measured for sss.

We added this clarification in the Discussion (Limitations/confounds) where we address potential behavioral contributors (activity/feeding) to respirometry outcomes.

The text now reads as follows:

"Locomotor activity and feeding could not be measured during the respirometry recordings, so genotype differences in respiratory parameters should be interpreted with caution. To assess whether behavioral timing could account for these differences, we compared our respiratory rhythms with the feeding and activity rhythms reported for these genotypes in Malik et al. (2026): wild-type feeding peaked at ZT ~3.25 and fmn feeding was phase-delayed to ZT ~4.5, while fmn also showed elevated locomotor activity, particularly during the dark period. In our data the fmn VCO2 rhythm peaks at ZT ~3.25 (RQ at ZT ~4.25), so the respiratory peak slightly precedes the feeding peak; the phase of the fmn respiratory rhythm is therefore not driven by feeding, although the elevated activity of fmn may contribute to its higher overall metabolic rate. Feeding and activity were not measured for sss."

> *(5) It is not clear whether the authors are simply analyzing the SAME dataset in Figure 1, Figure S4, and Figure 2-3, but just with different resolutions. They need to better articulate this point.*

We thank the reviewer for pointing this out. While the source data for Figures 1, 2-3, and Figure S4 use the same underlying respirometry dataset, they present different analyses to address specific questions. Figure 1 shows the full time-course traces (fine time bins), Figures 2-3 extract rhythmicity metrics (e.g., period/phase) from those same time-series, and Figure S4 collapses the same data into simple day (ZT0-12) vs night (ZT12-24) averages.

We added this clarification in the Figure legends for Figure 1, Figures 2-3, and Figure S4, and also noted it in the Methods where the respirometry analysis outputs (time-series binning, rhythmicity analysis, and day/night averaging) are described.

> *(6) Figure 3 and page 14: What are their criteria for differentiating "conserved" vs "distinct" phase alignment? It is not clear whether this conclusion is supported by any statistical analysis.*

We now specify that the "conserved" vs "distinct" phase descriptions refer to early- vs late-peaking rhythms, and we state the criterion explicitly: a phase was called "conserved" when it fell within ±3 h of the WT-LD peak. The revised text reads: "VO_2 peaked … suggesting conserved phase alignment, with all phases within ±3 h of WT-LD (Figure 3, Table 1)"; "RQ peaked … indicating distinct phase alignment, with the sleep-mutant phases ~8 h apart (Figure 3, Table 1)."

The text now reads as follows:

"VO_2 peaked …suggesting conserved phase alignment, with all phases within ±3 h of WT-LD (Figure 3, Table 1)"

"RQ peaked … indicating distinct phase alignment compared to respiratory output, with phases of the sleep mutants 8 h apart (Figure 3, Table 1)."

> *(7) Figure 4: The authors need to provide more details as to how the metabolite dataset was utilized to generate this figure and how they made the conclusion that their analysis "revealed a distinct circadian rhythmicity in RQ, characterized by oscillatory patterns indicative of coordinated substrate utilization across the day-night cycle".*

We have clarified how the respirometry and metabolomics data are used for Figure 4 and corrected the overstated rhythmicity claim:

(1) The RQ patterning in Figure 4 is derived from the continuous respirometry time series, not from the metabolomics dataset, which is used only for the time-matched and lagged correlation analyses that relate metabolite dynamics to RQ. (2) We removed the statement

that the analysis “revealed a distinct circadian rhythmicity in RQ”: RQ was not statistically rhythmic in WT-LD or WT-DD (Table 1), and Figure 4 instead shows the day-night RQ pattern that serves as the reference for the lag-based metabolite correlations. We revised the Methods, the Results paragraph introducing Figure 4, and the Figure 4 legend accordingly.

The text now reads as follows:

“Respiratory quotient (RQ) was recorded continuously and averaged into 5-minute bins. To integrate with metabolomics collected every 2 hours, we extracted the corresponding 2-hour ZT RQ values and performed a lag analysis (−120 to +120 min) to relate metabolite dynamics to RQ patterns; rhythmicity of RQ itself was assessed from the respirometry time series.”

> *(8) It is unclear why the examples of hydroxyhexadecenoylcarnitine and quinolinate were chosen to be presented in Figure 5a. The authors should clarify their choice of these two examples. Also, the authors should generate a supplemental table with the "several metabolites demonstrating strong correlations (line 294).*

Thank you for this suggestion. Hydroxyhexadecenoylcarnitine and quinolinate were selected as representative examples, and we agree that the metabolites supporting the strong RQ-associated correlations should be provided more explicitly. We have now added a Supplementary Table listing the metabolites demonstrating strong correlations with RQ across WT-LD, *fmn*, *sss*, per^{01}, and WT-DD conditions, using the same selection criterion applied in the heatmap analyses ($|\rho| \geq 0.7$, $p < 0.05$).

We also revised the Results text near the statement describing strong metabolite-RQ correlations to direct readers to this new table.

The text now reads as follows:

Hydroxyhexadecenoylcarnitine and quinolinate are among the strongest positively- and negatively-lagged RQ-correlated metabolites in WT-LD ($\rho = +0.78$ at +120 min and $\rho = -0.77$ at −120 min; Supplementary Table 1), illustrating the two opposite lag directions of the workflow. The full set of metabolites showing strong RQ-associated correlations across WT-LD, *fmn*, *sss*, per^{01}, and WT-DD conditions is provided in Supplementary Table 1.

> *(9) Although Spearman correlation analysis suggests some correlation between RQ and the two metabolites shown in Figure 5, the correlation shown in Figure 5b does not appear to be compelling. Results shown in Figure 5b do not provide confidence that conclusions based on clustered heatmap analysis shown in Figures 6 to 8 are meaningful. In addition to Spearman correlation, the authors might consider performing additional statistical methods to provide further support.*

Thank you for this comment. We suggest that Fig. 5b was not explained clearly and have clarified. Figure 5b is a lag analysis, not a separate correlation result: it shows how the Spearman correlation changes when the RQ time series is shifted forward or backward in time relative to the metabolite timepoints. The goal is to illustrate lead-lag timing (which shift gives the strongest association), rather than to present a single “strong” correlation as standalone proof.

To address the concern about confidence in the heatmap-based results (Figs. 6-8), we have strengthened the reporting by providing effect sizes (Spearman ρ) and lag for the metabolite-respirometry associations (now included as Supplementary Table 1). This allows readers to evaluate the statistical support underlying the clustering, beyond the visual patterns in the heatmaps.

We additionally report multiple-testing–corrected significance for the metabolite–RQ correlations (Benjamini–Hochberg FDR) alongside nominal p in Supplementary Table 1, using

the same correction already applied to the pathway enrichment in Supplementary Table 2.

> *Minor comments:*
>
> *(1) Line 82: The authors should clarify what they mean by "circadian collection". Except for WT-DD, my interpretation is that they collected their samples in LD, so that would not be "circadian collection".*

Thank you for catching this. We agree that “circadian collection” was imprecise. We have revised line 82 to clarify that samples collected under LD were collected across diurnal (LD) time (ZT), and we now reserve “circadian” specifically for collections under constant conditions (DD). We also updated the wording throughout the manuscript to maintain this distinction consistently. We added this clarification in the Methods section “*Drosophila* Strains, Entrainment and Collection” (line 82).

The text now reads as follows:

“Male flies were collected shortly after eclosion and entrained in light-dark (LD) incubators for a minimum of three days before diurnal (LD) time-course collection across Zeitgeber time (ZT).

> *(2) The authors cited Frayn 1983 to indicate how the RQ value can be used to reflect metabolic fuel utilization. Is this interpretation accepted for all animals, including flies?*

RQ is widely used in indirect calorimetry as an index of relative substrate utilization, including in small model organisms, but we agree that it should be interpreted with appropriate caveats. We have revised the manuscript to clarify that we interpret RQ conservatively as reflecting relative shifts in substrate utilization over time and between genotypes, rather than as a precise quantitative measure of absolute carbohydrate versus lipid oxidation.

We made this change in two places: in the Introduction, where RQ is introduced and Frayn is cited, and in the Methods, under “Carbon Dioxide and Oxygen Analysis and Calculations,” where RQ is defined.

The text now reads as follows:

Introduction: “These measurements allow for the estimation of energy expenditure and respiratory quotient (RQ), which can provide an index of relative substrate utilization, with appropriate caveats[13, 14]. In this study, we interpret RQ conservatively as reflecting relative shifts in substrate utilization over time and between genotypes, rather than as a precise quantitative measure of absolute carbohydrate versus lipid oxidation.”

Methods: “RQ was used as an index of relative shifts in substrate utilization over time and between genotypes, interpreted conservatively rather than as a precise measure of absolute carbohydrate versus lipid oxidation as this has not been directly characterized in flies.

> *(3) Figure S4: Since the authors are comparing day-night differences, they should plot them in the same graph to make it easier to compare.*

We have revised Figure S4 to plot day and night within the same graph/panel for each metric (VCO_2, VO_2, RQ), using side-by-side day vs night groupings per genotype to facilitate direct visual comparison, and we updated the legend accordingly.

> *(4) Line 252: When comparing diurnal differences, the authors should not use the word "rhythm". Pattern or profile might be a better word to use.*

We appreciate the suggestion. Where we use “rhythm” we refer specifically to 24-hour oscillations established statistically by JTK_CYCLE and RAIN (Figures 2-3, Table 1); for the

coarser day-versus-night comparisons we agree “pattern” or “profile” is preferable and have adopted it there. We have gone through the manuscript to apply this distinction consistently.

> *(5) Figure 6a: larger font labels are necessary for the metabolites.*

Thank you. We have revised Figure 6a to increase the metabolite label font size for improved readability.

> ***Reviewer #3 (Recommendations for the authors):***
>
> *(1) Activity controls: To strengthen the paper, include or reference direct measures of locomotor activity (e.g., DAM system). This would allow the separation of metabolic changes from behavioral differences and would enable better analysis of circadian patterns.*

This activity/feeding confound is addressed in full in our response to Reviewer 1, Major comment 4; we cross-reference it here to avoid repetition.

> *(2) Mitochondrial function: "mitochondrial stress" should be supported by additional assays such as mitochondrial enzyme activities, high-resolution respirometry, or reactive oxygen species measurements.*

See our full response to the mitochondrial point in Reviewer #3's public review above, including new Figure 9 and the Gut Tissue Respirometry Methods.

> *(3) Sex differences: Provide a clear justification for excluding female flies. If feasible, incorporate female data or explicitly discuss how sex differences could alter metabolic outcomes.*

This is addressed in the public review for reviewer 3.

> *(4) Statistical presentation: Increase n. Clarify in figure legends whether error bars represent SD or SEM and ensure consistency across all figures (Figure legends).*

We have revised all figure legends to clearly state that error bars represent SEM and ensured this is applied consistently across all figures. We also explicitly report the n for each genotype (with chambers as the experimental unit) in the relevant legends.

The text now reads as follows:

“Error bars represent SEM, and n denotes the number of chambers (experimental units) per genotype.”

> *(5) Sample size and replication: Indicate more clearly that the chamber, not the individual fly, is the experimental unit. Discuss limitations of replication and statistical power in the text.*

Thank you for this comment. We have clarified throughout the Methods and figure legends that the chamber (not the individual fly) is the experimental unit. We now state that each genotype measurement reflects an average of 300 flies (25 flies/chamber × 4 chambers/experiment × 3 experimental days), and we report n (number of chambers) and the error structure (SEM) for each genotype.

Added to Discussion:

“We also acknowledge the limits of this replication: with n = 12 chambers per genotype, statistical power to detect small-magnitude differences and subtle phase shifts is limited, and negative calls (e.g., arrhythmicity) should be interpreted with this caveat.”

> *(6) Writing and clarity: (a) Streamline the Discussion to focus on mechanistic themes (anticipatory vs reactive alignment, substrate shifts, redox imbalance).*
>
> *(b) The opening phrase ("Precise temporal regulation of metabolism by sleep and circadian rhythms is essential for dynamic energy homeostasis") is dense, vague, and difficult to interpret. Consider rephrasing to something more concrete, for example: "Sleep and circadian rhythms tightly control when and how the body uses energy, but we do not yet know exactly how this timing connects to oxygen use and breathing needs."*

Thank you for these helpful suggestions. We have revised the Discussion to reduce repetition and improve focus by organizing it around the key mechanistic themes raised by our data, including anticipatory vs reactive metabolic alignment, substrate-use shifts, and redox/mitochondrial imbalance. We revised the opening sentence of the Abstract to make the biological question more concrete and accessible, following the reviewer's suggestion.

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