The microbiome interacts with the circadian clock and dietary composition to regulate metabolite cycling in the gut

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The gut microbiome plays a key role in the maintenance of host metabolic homeostasis and health. Most metabolic processes cycle with a 24 hour rhythm, but the extent to which the microbiome influences metabolite cycling under different conditions, such as variations in dietary composition, is largely unknown. In this study, we utilized high temporal resolution metabolite profiling of the *Drosophila* gut to investigate the role of the microbiome in metabolite cycling. Although the microbiome was previously shown to dampen transcript cycling in the gut, we find that in contrast it increases the number of oscillating metabolites. Interestingly, effects of microbiome loss on metabolite cycling are reduced in a time restricted feeding (TF) paradigm. Conversely, promotion of cycling by TF is reduced in microbiome-containing flies, suggesting that TF compensates for deficits in the microbiome to some extent. In a clock mutant background, loss of the microbiome increases cycling of some classes of metabolites but profoundly affects phase of all of them, indicating the host clock modulates effects of the microbiome on cycling and maintains phase in the face of microbial changes. Lastly, a high protein diet increases microbiome-dependent metabolite cycling but a high sugar diet suppresses such cycling while altering phase. Indeed, we observe that amino acid metabolism is the metabolic pathway most affected by changes in the gut microbiome, the circadian clock and timed feeding. Collectively, our observations highlight a key role of the gut microbiome in contributing to host metabolite cycling, and reveal a complex interaction with internal and external factors.

Introduction

To adapt to the 24-hour light and temperature cycle caused by the Earth's rotation, organisms have evolved endogenous circadian clocks comprised of auto-regulating clock genes (1-3). Circadian clocks can be in the brain (i.e. central) or in peripheral tissues, and show differential responses to external entraining cues. In general, central clocks are mainly regulated by light signals, while dietary signals affect the rhythm of several peripheral circadian clocks(4, 5). Central and peripheral clocks coordinate with

each other to achieve synchronization of physiological and behavioral circadian rhythms. Disrupting circadian rhythms can result in a range of metabolic and neurological disorders (6-8), underscoring the importance of a well-synchronized circadian system.

The gut microbiome consists of microorganisms, including bacteria, viruses, protists, archaea and fungi, that coexist symbiotically with hosts. Indeed, compelling evidence shows that gut microbiomes play a vital role in host nutrition, immunity, and neurodevelopment (9-11). About 10% to 15% of the microbe species in the mammalian gut exhibit rhythms of abundance, and that is considered to be a key determinant of host health and fitness (12-14). Time restricted feeding (TF), which restricts food intake to a specific daily interval, enhances oscillations of the microbiome and improves health conditions in animals with obesity or metabolic syndrome (15-17). On the other hand, western diets (high fat, high protein, and high sugar), which significantly increase the occurrence of metabolic diseases, can disrupt microbiome rhythms (18-20). In addition, gut microbiomes regulate circadian rhythms of gene expression in metabolic tissues (21), however their impact on host metabolite cycling remains largely unknown.

We previously reported that the gut microbiome in *Drosophila* does not cycle, but it regulates cycling of the host transcriptome. Specifically, it suppresses transcript rhythms in the gut while TF enhances these rhythms (22). Here we sought to test whether the microbiome regulates metabolite oscillations and how the host clock and dietary manipulations interact with those cycles. Thus, we assayed metabolite rhythms in wild type flies with and without a microbiome in different dietary paradigms and in clock mutant flies. Overall, we observed that the microbiome increases metabolite cycling. However, in the absence of a host clock, the microbiome decreases cycling of metabolites in total while dramatically affecting the phase of all. A TF feeding paradigm increases metabolite cycling, although less so in animals that contain a microbiome. On the other hand, a protein-rich diet enhances microbiome-dependent cycling, but this is not the case in sugar-rich diet. Overall, our results suggest the gut microbiome plays a critical role in maintaining cycles of metabolic activity in the gut, in a manner that depends on the host circadian clock, diet composition and feeding behavior.

Methods

Generation and maintenance of fly lines

The w^{118} *iso31,* per^{01} fly lines present in the study were maintained on standard cornmeal/molasses medium at 25°C under 12:12 LD conditions. The sterile flies used in this study are the same as in the previous report (22). The Newborn fly embryos within 12 hours were rinsed in 100% ethanol, dechorionated in 10% bleach for 2 minutes, then immediately rinsed three times in sterile PBS. The sterile embryos were transferred to autoclaved standard sterile molasses-cornmeal-yeast medium containing 1 mM kanamycin, 650 μM ampicillin (61-238-RH, MediaTech), and 650 μM doxycycline (D9891, Sigma-Aldrich). Germ-free flies were maintained on sterile mediums with three antibiotics for four generations and then maintained *on* the same *medium without*

antibiotics for the next four generations. Bacterial contamination of flies was monitored by homogenizing larvae in sterile PBS. Aspirate supernatant after brief centrifugation and monitoring the bacterial growth on DeMan, Rogosa and Sharpe (MRS)-agar plates.

Diets Preparation and Sample collection

Diets preparation followed the protocols below. Normal chow diets: 64.67g of corn meal, 27.1g of dry yeast, 8g of agar, and 61.6ml of molasses in 1L ddH_2O. For high protein diets: 64.67g of corn meal, 150g of dry yeast, 8g of agar, and 61.6ml of molasses in 1L of ddH_2O. For high sugar diets: 64.67g of corn meal, 27.1g of dry yeast, 8g of agar, 61.6ml of molasses, and 243g of sucrose in 1L of ddH_2O. After autoclaving the diets at high temperature, temperature was allowed to drop to 40°C and then 10.17ml of 20% Tegosept was added to each 1L diet and mixed thoroughly. Sterile conditions were maintained throughout. Adult flies were provided standard sterile medium in groups of 40 for 3 days. 5-7 day old flies were separated by gender and subjected to the feeding paradigm in the presence of light:dark cycles starting on day 5. Ad libitum (AF) and flies subjected to timed feeding (TF) were shifted to sterile medium vials at zeitgeber time 0 (ZT0), (which corresponded to actual time of 9:00 am) and then were transferred to either a new sterile medium or a 1.1% agar vial at ZT10 (7pm) for 14h fast. Fly guts were dissected on the fifth days at ZT0, ZT6, ZT12, and ZT18 time points in a 12:12 LD cycle, after being continuously fed for 4 days under both ad lib and timed feeding conditions, with 4 repeat samples collected for microbiome-containing and sterile female flies at each time point (>200 guts per sample, which corresponds to at least 10 mg for each sample). All experimental processes were carried out on ice, and samples quickly transferred to dry ice for storage after each sample was collected.

Metabolite profiling

Primary metabolites: Samples were extracted using the Matyash extraction buffer which includes MTBE, MeOH, and H_2O. The organic (upper) phase was dried down and submitted for resuspension and injection onto the LC while the aqueous (bottom) phase was dried down and submitted to derivatization for GC. 10 ul of Methoxamine in pyridine was added to the aqueous phase, after which it was shaken at 30 C for 1.5 hours. Then 91 uL of MSTFA + FAMEs was added to each sample and shaking was continued at 37 C for 0.5 hours to finish derivatization. Samples were then vialed, capped, and injected onto the instrument. We use a 7890A GC coupled with a LECO TOF. 0.5 uL of derivatized sample is injected using a splitless method onto a RESTEK RTX-5SIL MS column with an Intergra-Guard at 275C with a helium flow of 1 mL/min. The GC oven is set to hold at 50C for 1 min then ramped at 20C/min to 330C and held for 5 min. The transferline is set to 280C while the EI ion source is set to 250C. The Mass spec parameters collect data from 85m/z to 500m/z at an acquisition rate of 17 spectra/sec.

Lipids: Samples were extracted using the Matyash extraction procedure, which includes MTBE, MeOH, and H_2O. The organic (upper) phase was dried down and submitted for resuspension and injection onto the LC while the aqueous (bottom) phase was dried down and submitted to derivatization for GC. It was resuspended with 110 uL

of a solution of 9:1 methanol: toluene and 50 ng/mL CUDA. This was then shaken for 20 seconds, sonicated for 5 minutes at room temperature, and then centrifuged for 2 minutes at 16100 rcf. The samples were then aliquoted into three parts. 33 uL were aliquoted into each of two vials with a 50 uL glass insert for positive and negative mode lipidomics. The last part was aliquoted into an eppendorf tube to be used as a pool. The samples are then loaded up on an Agilent 1290 Infinity LC stack. The positive mode was run on an Agilent 6530 with a scan range of m/z 120-1200 Da with an acquisition speed of 2 spectra/s. Positive mode has between 0.5 and 2 uL injected onto an Acquity Premier BEH C18 1.7 µm, 2.1 x 50 mm Column. The gradient consists of two mobile phases, of which only B is controlled by the software as follows: 0 min 15% (B), 0.75 min 30% (B), 0.98 min 48% (B), 4.00 min 82% (B), 4.13-4.50 min 99% (B), 4.58-5.50 min 15% (B) with a flow rate of 0.8 mL/min. The other sample aliquot was run in negative mode, which was run on Agilent 1290 Infinity LC stack, and injected on the same column, with the same gradient and using an Agilent 6546 QTOF mass spec. The acquisition rate was 2 spectra/s with a scan range of m/z 60-1200 Da. The mass resolution for the Agilent 6530 is 10,000 for ESI (+) and 30,000 for ESI (-) for the Agilent 6546.

Biogenic Amines: Sample extraction for biogenic amines is a Liquid-Liquid extraction using the Matyash extraction containing MTBE, Methanol and Water and creating a biphasic partition. The polar phase is then dried down to completeness and run on a Waters Premier Acquity BEH Amide column. A short 4-minute Liquid Chromatography method is used for separation of polar metabolites from a starting condition of 100% LCMS H_2O with 10 mM ammonium formate and 0.125% formic acid to an end condition of 100% ACN: H_2O 95:5 (v/v) with 10 mM ammonium formate and 0.125% formic acid. A Sciex Triple-ToF scans from 50-1500 m/z with MS/MS collection from 40-1000 selecting from the top 5 ions per cycle.

Data processing and enrichment analysis

After acquisition primary metabolomics data is converted from LECO peg files to NetCDF files for processing. Files are submitted to in-house data processor BinBase for annotation and a report is exported into an excel file. In excel data is curated by blank reduction with a fold change of 3 or lower by using the max peak height from samples/ average blank peak height. After reduction peak heights are normalized by sum normalization. This is done using the un-normalized peak height of the feature divided by the mTIC (sum of the annotated metabolites) of the respective sample multiplied by the average mTIC of the samples. Normalized peak heights are then reported in an excel document. Chromatograms first undergo a quality control check in which internal standards are examined for consistency of peak height and retention time. Raw data files are then processed using an updated version of MS-DIAL software which identifies and aligns peaks and then annotates peaks using both an in-house mzRT library and MS/MS spectral matching with NIST/MoNA libraries. All MS/MS annotations are then manually curated by a lab member to ensure that only high-quality compound identifications are included in the final report. Enrichment analysis was conducted through the online software MetaboAnalyst 5.0.

Statistical analysis

Statistical details of experiments can be found in the figure legends. Circadian statistical analysis was performed in R using JTK_CYCLEv3.1. JTK_P value < 0.05 and JTK BH.Q < 0.2 deem as cycling metabolites.

Results

The microbiome enhances gut metabolite cycling

We showed previously that the *Drosophila* gut microbiome does not exhibit obvious oscillations in a 12:12 h LD cycle, but it significantly reduces the number and strength of host transcript cycling (22). To investigate the role of the microbiome in gut metabolite cycling, we assayed metabolite cycling at different times of day in microbiome-containing (AM) and sterile (AS) fed *ad libitum* (Figure. 1A). Metabolic profiling was performed separately to characterize primary metabolites, lipids and biogenic amines. Principal component analysis (PCA) of the total expressed primary metabolites did not show significant separation between AM and AS flies at any time of day, indicating that this class of molecules is not changed by loss of the microbiome. However, a clear separation between the two groups was observed for lipids and biogenic amines (Figure. S1A-C).

Given our transcriptomic results we were expecting that the gut microbiome would reduce the number of cycling metabolites, however, we observed the opposite. JTK cycling analysis of the three different types of metabolites revealed that the likelihood of oscillations increased in AM flies as compared to AS flies. 26.9% (227 of 843) primary metabolites were rhythmic in AM flies as compared to 20.3% (171 of 843) in AS flies. A further reduction in AS was observed for lipids whose rhythms dropped from 29.5%(1329 of 4510) in AM to 14.4% (648 of 4510) in AS. Similarly, the proportion of biogenic amines cycling decreased from 39.0% (1689 of 4330) to 24.7% (1068 of 4330) (JTK_P value < 0.05) (Figure. 1B, D, F, H, J and L). Consistent with the role of the microbiome in favoring metabolic rhythms, lipids and biogenic amines that cycle in both AM and AS flies have a higher amplitude in AM flies (Figure S2 A-C). Although the microbiome did not significantly change the overall distribution of phases for the cycling primary metabolites and lipids, it had a substantial effect on the phase of biogenic amine oscillations that shifted from peaking in the middle of the day (ZT6) in the AM flies to a peak in the early morning (ZT0) in AS flies (Figure. 1B, D, F, H, J and L).

Analysis of the abundance of cycling metabolites in AM, compared to their non-cycling counterparts in AS, also revealed that lipids and biogenic amines had higher expression levels in AM flies compared to AS flies. However, there was no significant difference between AM and AS flies for primary metabolites (Figure. S3A, E and I). Likewise, the overall abundance, regardless of cycling, of lipids and biogenic amines was higher in AM flies but primary metabolites were equivalent in AS and AM (Figure. S4A-C).

Overall, this indicates that the microbiome increases the cycling of all metabolites in the gut but has more dramatic effects on lipids and biogenic amines.

The microbiome has less effect on metabolite cycling under timed feeding conditions

Timed feeding affects host metabolism and benefits host health, according to several studies (16, 17, 23). In this study, we aimed to determine how microbiome-mediated metabolite cycling interacts with a timed feeding paradigm in which flies are provided with food only from ZT0 to ZT10. Similarly to *ad libitum* conditions, PCA of the total expressed primary metabolites showed only minor separation between the four groups (AM, AS, TM, TS), but a clear separation was observed for lipids and biogenic amines just as seen for AM and AS flies (Figure S1A-C). Consistent with the results of our previous study on transcript cycling, we found that timed feeding significantly increased the number of metabolites cycling in sterile and microbiome-containing flies (Figure 1B-M).

Time restricted feeding coordinated the phase of all three-metabolite classes with TM and TS flies displaying very similar phase distributions. Interestingly, primary metabolites peaked around a single time of day whilst lipids and biogenic amines displayed two distinct phases in timed feeding conditions (Figure 1B-M). Moreover, the observation that TM and TS flies display similar metabolite phases differs from what happens in ad lib conditions in which the phase of biogenic amines and lipids changes in the absence of a microbiome. More interestingly, metabolites peaking at ZT9 increased with TF in all metabolite groups. This could suggest an anticipation of sleep at the end of the feeding window.

Abundance analysis of cycling metabolites under ad lib feeding and timed feeding conditions revealed that, in general, rhythmic metabolites tend to have higher overall expression levels compared to non-rhythmic metabolites, and this phenomenon is more prominent in the case of cycling biogenic amines (Figure. S3A-L). Additionally, we also looked at the amplitude of metabolites that cycle in both microbiome containing and microbiome free flies under the two feeding paradigms. In this case, the three metabolite groups did not always show a larger amplitude in the timed fed groups, which slightly contradicts the potential benefits of this type of intervention (Figure. S2D-I).

To further determine whether timed feeding modulates the effect of the microbiome, we compared the number of metabolites cycling with TF in AS and AM flies and found that loss of the microbiome had a larger effect in ad lib fed flies. This was reflected in the ratios depicting metabolite cycling in microbiome-containing versus sterile flies, such that the AM/AS ratio was higher than the TM/TS for lipids and biogenic amines, although not for primary metabolites (Figure 1N-P). Moreover, the TS/AS ratio for lipids and biogenic amines was also higher than TM/AM (Figure, S1D-F). These data suggest that loss of the microbiome has less of an effect on timed fed flies than on ad lib fed,

and, conversely, timed feeding has less effect on microbiome-containing flies. Thus, time restricted feeding improves cycling and it may compensate for the lack of a microbiome.

Effects of the microbiome on the cycling metabolome are modulated by the circadian clock.

To determine whether circadian clocks play a role in the enhanced metabolite cycling in microbiome-containing flies, we next subjected *per*^{01} flies with (*per*^{01}-AM) and without (*per*^{01}-AS) a microbiome to ad lib feeding conditions and examined the cycling of metabolites in their guts (Figure 2A). The results showed that the PCA for the three metabolite classes differentiated *per*^{01}-AM and *per*^{01}-AS flies (Figure S5A-C). Surprisingly, loss of the microbiome increased metabolite cycling in *per*^{01} flies, not for primary metabolites, but increasing the proportion of lipids cycling from 17.8% to 22.2% and almost duplicating the proportion of biogenic amines cycling from 18% in *per*^{01}-AM to 30.5% cycling features in *per*^{01}-AS flies (Figure 2B, D, F). Moreover, all cycling metabolites exhibited completely opposite phases in *per*^{01}-AM relative to *per*^{01}-AS flies (Figure 2C, E and G). Phases also changed for cycling metabolites shared between AM and AS (Figure S6A-C).

Analysis of the abundance of cycling metabolites in *per*^{01} flies revealed that, in general, cycling lipids and biogenic amines have higher expression in AM compared with their non-cycling equivalents in AS, whereas primary metabolites show minor difference between the two groups (Figure S7A-C). This result is consistent with the comparison of abundance in microbiome-containing and sterile iso31 flies.

In addition, *per*^{01} flies showed different phases of metabolite expression from wild type flies. The average phase of primary metabolites in wild type flies falls around ZT6 while *per*^{01} flies mainly have a peak phase near ZT3. Lipids lose a morning peak in the clock mutant and most dramatically, biogenic amines go from peaking in the middle of the day to the middle of the night (Figure S8A, B, left panels). This effect of the host clock on the phase of cycling metabolites was even stronger in microbiome free flies (FigureS8A, B, right panels).

These datasets suggest a complex interaction between the microbiome and the host clock. It was noticeable that lack of a host clock resulted in reduced metabolite rhythms in microbiome containing flies (Figure S9A, E) but enhanced cycling in microbiome-free flies (Figure S9B, F). And also caused profound changes in phase. Overall, it appears that the host clock stabilizes metabolite rhythms against changes in the microbiome.

The microbiome enhances metabolite cycling in the presence of high protein diets and decreases cycling with high sugar diets

Changes in diet composition can significantly affect gut microbiome rhythms (11, 24). Therefore, we also explored the cyclic metabolome of flies with or without a microbiome and fed on two different diets; a high sugar and a high protein diet (Figure 3A and Figure 4A). From the PCA analysis we noticed that high sugar diets trigger metabolic differences between flies that contain a microbiome and microbiome-free flies, however, high protein diets do not prompt such difference (Figure S5D-F and G-I).

On high protein diets, the lack of a microbiome reduced the number of rhythmic metabolites (Figure 3B, D, F). This was consistent with the role of the microbiome in promoting metabolic rhythms. However, the effects of a high-sugar diet diverged substantially from those of a high-protein diet. With the high sugar diet, rhythmic lipids and biogenic amines increased in sterile flies, similar to what we observed in a clock mutant background (Figure 4B, D, F). Indeed, when the three metabolite groups were analyzed together, flies on a high sugar diet showed enhanced cycling of the metabolome when the microbiome was absent (Figure S9I and J).

We also looked at phases of cycling metabolites. In flies fed a high protein diet, a predominant peak around ZT15 was observed for all metabolites regardless of the presence or absence of a microbiome (Figure 3C, E, G). This suggests that protein metabolism imposes a specific metabolic rhythm that is not dependent on the gut microbiome. In case of the high sugar diet, we noticed that the absence of microbes changed the phase distribution of both lipid and biogenic amines but not of primary metabolites (Figure 4C, E and G).

We also compared the dataset generated from flies fed a standard diet versus these nutrient specific paradigms. For flies with a normal microbiome, the fraction of cycling metabolites decreased from 33.5% on standard diet to 22.2% and 24.5% with a high protein and high sugar diet respectively (Figure S9A, G and I). Thus, overall metabolite cycling decreases on high protein and high sugar diets. But while high protein potentiates the effect of the microbiome in promoting cycling, high sugar suppresses it. Phases were also different on the different diets, with just primary metabolites conserving a peak at ZT6 in both the standard and high sugar diet (Figure S8A, C, D left panels).

Overall, the results suggest that nutrient specific diets interact in different ways with the microbiome to regulate the cycling of gut metabolites.

Amino acid metabolism is modulated by the microbiome, timed feeding and the circadian clock.

The extensive metabolic profiling performed here indicates that the gut microbiome is a central piece in generating daily cycles of metabolites and that depending on the host circadian clock and the composition of the diet, the extent and directionality of that modulation varies.

To get a further understanding of the biological implications of this cycling metabolic datasets we performed pathway enrichment analysis. First, we explored the ad libitum dataset in which we found that absence of a microbiome reduced metabolic rhythms (Figure 1). To our surprise, despite the reduction in cycling metabolite number in flies lacking a microbiome, the pathway enrichment identified numerous pathways as significantly enriched in the AS only cycling group. These included biosynthesis of branched (Valine, leucine, isoleucine) and aromatic (Phenylalanine, tyrosine and tryptophan) amino acids as well as the aminoacyl-tRNA biosynthesis pathway that is relevant for protein synthesis (Figure 5A). Another amino acid pathway, involving alanine, aspartate and glutamate, was found significant in both the AS only and AS and AM cycling sets, with different specific metabolites cycling in these datasets. No pathway related to amino acids or protein metabolism was found specific to the AM cycling metabolites suggesting that, in general, the microbiome abrogates daily oscillations of protein anabolism.

We found that similar pathways were significant in the cycling dataset from microbiome-containing flies subjected to a daytime restricted feeding paradigm (TM) versus ad libitum feeding (AM) (Figure 5B). Timed feeding also affects valine, leucine and isoleucine metabolism in sterile flies but to a lesser extent than in flies with a microbiome (Figure 5C). Additionally, we determined whether pathways affected by timed feeding were different in flies with and without a microbiome and found that amino acid pathways cycle significantly in both groups (Figure 5D). All these data suggest that amino acid and protein biosynthesis cycles are dampened by the gut microbiome but potentiated by time restricted feeding and the latter has a larger effect in microbiome containing flies, indicating a microbiome and TF interaction. In addition, the TCA cycle appeared as a shared cycling category in all comparisons (AM vs AS, Figure 5A; AM vs TM, Figure 5B, AM vs *per*^{01} AM, Figure 5E), suggesting consistency in the rhythmicity of this pathway.

Lastly, we aimed to identify pathways whose cycles were exclusive to microbiome containing flies. We found that alpha-linoleic metabolism cycled significantly in AM flies as compared to AS flies (Figure 5A). In addition, this pathway cycled significantly in AM and TM flies (Figure 5B), indicating that its cycling is not dramatically influenced by timed feeding and may be largely dependent on the microbiome. However, in the absence of a microbiome, timed feeding seems to drive cycling of this pathway (see comparison of AS and TS in Figure 5C). In addition, cycling of this pathway requires a functional circadian clock, as it is lost in *per*^{01}-AM flies. Thus cycling of alpha-linoleic metabolism depends upon the microbiome and the clock and can also be driven by timed feeding.

Consistent with the results from flies fed a normal chow diet, we observed that sterile flies fed a high-sugar diet showed an enrichment of amino acid-related metabolism in the absence of a microbiome (Figure 5F), which indicated sugar content did not alter the functional category of cycling metabolites by KEGG analysis. However, pathway analysis of cycling metabolites under high protein conditions was not durable due to the reduced number of features.

Discussion

Our previous study reported that the microbiome dampens transcript cycling in the gut (22). By contrast, we now show that the microbiome increases the number of cycling metabolites. At first glance, the two results appear contradictory because enhanced cycling gene expression would appear to be a prerequisite for producing large amounts of metabolites in an oscillatory manner. However, gut microbiomes can effectively provide the host with nutrients and promote metabolite absorption and they may do so in a rhythmic fashion (11, 25). Thus, in the presence of a microbiome, the gut may not need to drive cycling of its own genes to promote metabolic oscillations since that might be facilitated by a healthy microbiome. In contrast, in sterile flies the host might need rhythmic expression of metabolism-related genes to achieve nutritional homeostasis. We report that the cycling metabolites in sterile flies are extensively enriched in amino acid metabolism. This may compensate for the loss of metabolic properties that would normally be conferred by the microbiome. Interestingly, this pathway is also a major target of timed feeding.

In this study, we first explored the cycling of metabolites in wild flies (iso31) under ad lib and timed feeding conditions, both in sterile and microbiome-containing flies (22). To better understand the role of the circadian clock and the effect of different diet compositions on metabolite cycling, we further investigated the microbiome-dependence of metabolite oscillations in *per*^{01} flies fed on normal chow diets and iso31 wild type flies fed on high protein and high sugar diets, respectively. Although these two sets of studies revealed similar numbers of named metabolites, the total numbers of metabolites exhibited some differences. For instance, the first study had 193/843, 456/4510 and 621/4330 named/total primary metabolites, lipids, and biogenic amines, respectively; in the second study, these numbers were 144/623, 513/2245, and 530/2791 respectively. We acknowledge that due to this fact, direct comparison of some of the datasets is not possible. As a strategy to eliminate the influence of different run conditions on the results, we also investigated metabolite cycling profiles using the shared named metabolites across the two batches and found that the results were highly consistent (data not shown). Although not ideal, this suggest that an overall comparison of all the samples included here is appropriate.

Some empirical evidence has found that timed feeding has a beneficial influence on metabolic health (15, 17, 23). As reported here for metabolite cycling, our previous study found that timed feeding not only enhances gene cycling but also changes the phase of cycling (22). Metabolite cycling was further measured under ad lib and timed feeding conditions in microbiome-containing and sterile flies. In line with the transcript results, timed feeding significantly enhanced metabolite cycling and changed the phase regardless of the presence of a microbiome. However, the microbiome has less effect in timed fed flies, and the reverse was also true, timed feeding had less effect on flies containing a microbiome relative to sterile flies. It is possible that the microbiome buffers the cyclic metabolome against strong external zeitgebers such as the time of feeding. Indeed, we previously showed that the gut microbiome stabilizes transcript cycling in the

gut such that the phase is not rapidly shifted by acute changes in the light: dark cycle (22). Alternatively, given that the presence of a microbiome also has less effect in a timed feeding paradigm, it is possible that TF and the microbiome have overlapping impact on metabolite cycling. On the other hand, oscillations in amino acid metabolism are characteristic of a timed feeding paradigm, as reported here and by others (17), and are enhanced in sterile flies as opposed to microbiome-containing flies. Perhaps these oscillations help the host cope with the lack of microbial driven cycles.

Keeping metabolic rhythms synchronized with the external environment is an important way to maintain optimal fitness for the host. The synchronization is mainly achieved through the circadian clock (2, 7, 26), and so mutations in core clock genes can lead to profound metabolic abnormalities (27, 28). To investigate whether the microbiome-dependent increase in the number of cycling metabolites depends on the endogenous circadian clock, metabolite cycling was further investigated in microbiome-containing and sterile *per*^{01} flies. Interestingly, the number of cycling metabolites was significantly decreased in *per*^{01} AM flies compared with the *per*^{01} AS flies. Also, unexpectedly, compared with *per*^{01} AS flies, cycling metabolites exhibit an opposite cycling phase in *per*^{01} AM flies. On the other hand, wild type flies show only a minor change in the phase of cycling primary metabolites with the loss of a microbiome. The cycling phase of biogenic amines shows a significant difference between AM and AS wild type flies but the difference is larger between AM and AS *per*^{01} flies. We speculate that the host circadian clock suppresses the effect of the microbiome in determining the phase of metabolite cycling. Thus, in clock mutant flies, the effect of the microbiome on phase is revealed. Cycling of metabolites involved in amino acid metabolism occurs in *per*^{01} AM flies just as it does in wild type sterile flies. This agrees with the idea that these oscillations represent some type of compensation, for lack of a clock or a microbiome.

Mice fed on western diets which contain high sugar amounts, show a significant reduction in the cycling of gut microbiomes, and it has been argued that the changed feeding rhythm may be the reason for this phenomenon (24, 29). In the current study, we found that the microbiome suppresses the number of cycling metabolites and substantially affects the phase when flies are fed high sugar diet. Conversely, the microbiome increases the number of cycling metabolites but does not change the cycling phase when flies are fed high protein diets. High sugar diet also resulted in phenotypic differences in the guts of AM and AS flies. In general, on normal chow or high protein diets, AM fly guts have good plasticity and are larger in size than those from AS flies. However, flies fed a high-sugar diet had smaller gut sizes than flies fed a high-protein or normal chow diets, and the microbiome-mediated gut plasticity difference between AM and AS conditions disappeared (data not shown). Moreover, metabolite abundance analysis showed higher expression of lipids and biogenic amines in AM flies than AS flies when flies were fed on normal chow or high protein diets, but on a high sugar diet AS flies had higher expression of lipids and biogenic amines, although the difference in biogenic amines was not significant (Figure S4). All these findings suggest that high-sugar diets disrupt microbiome function, and this is likely the cause of the increased number of cycling metabolites in AS flies compared to AM flies. Moreover, potential changes in the feeding rhythm in response to different diet compositions could

contribute to the observed variations in metabolite cycling phenotypes, as seen in mice fed different diets (29), but whether this phenomenon occurs in flies requires further research.

In summary, these extensive metabolic profiling data suggest that the gut microbiome maintains metabolic homeostasis in the gut. This effect is influenced by the host circadian clock and is sensitive to the influence of feeding behavior and diet composition (Figure 6). Interestingly, the microbiome and time restricted feeding paradigms can compensate for each other, suggesting that different strategies can be leveraged to serve organismal health.

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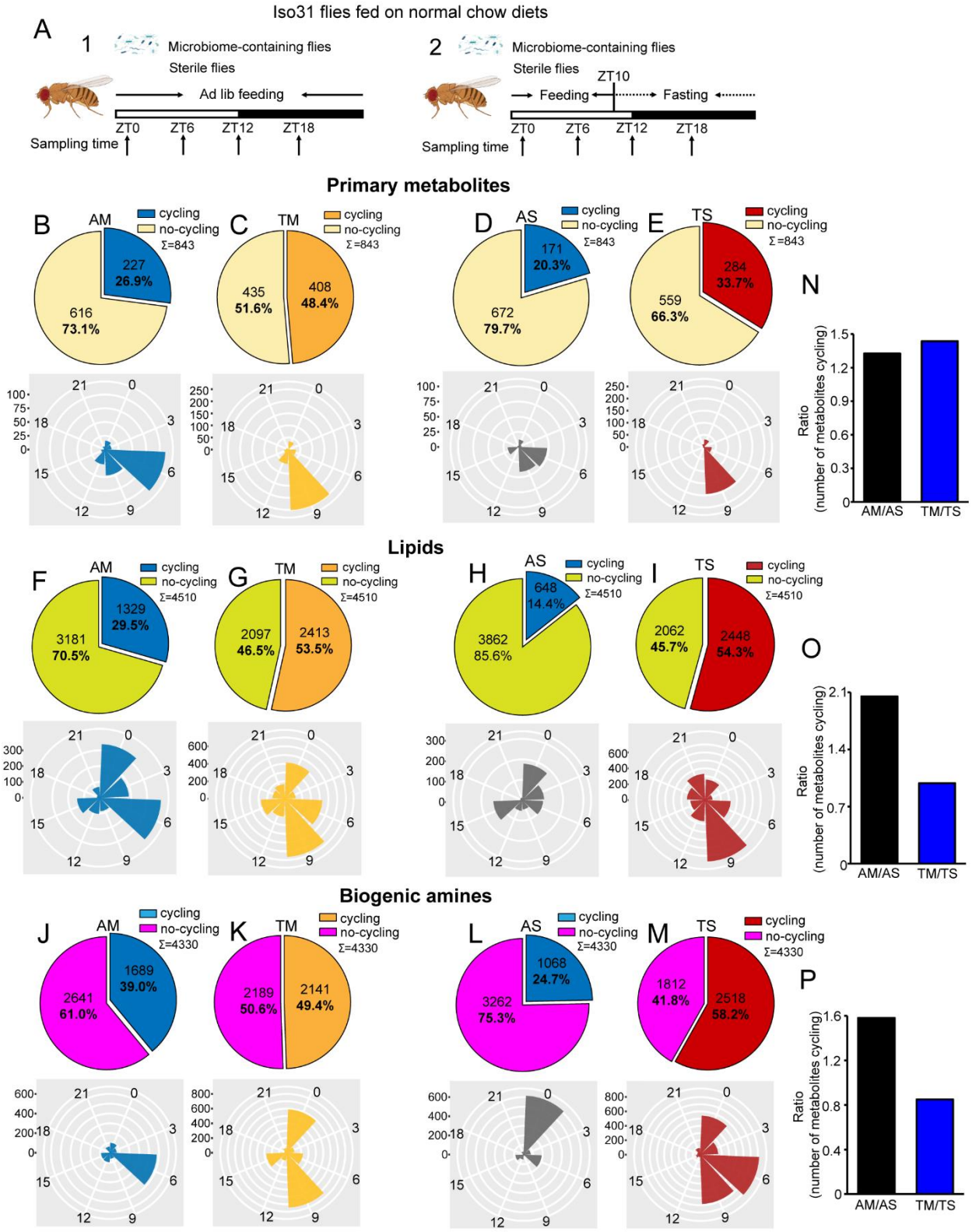
Iso31 flies fed on normal chow diets
A
1
Microbiome-containing flies
Sterile flies
Ad lib feeding
ZT0
ZT6
ZT12
ZT18
Sampling time
2
Microbiome-containing flies
Sterile flies
ZT10
Feeding
Fasting
ZT0
ZT6
ZT12
ZT18
Sampling time
Primary metabolites
B
AM
cycling
no-cycling
Σ=843
227
26.9%
616
73.1%
C
TM
cycling
no-cycling
Σ=843
435
51.6%
408
48.4%
D
AS
cycling
no-cycling
Σ=843
171
20.3%
672
79.7%
E
TS
cycling
no-cycling
Σ=843
284
33.7%
559
66.3%
N
Ratio
(number of metabolites cycling)
AM/AS
TM/TS
Lipids
F
AM
cycling
no-cycling
Σ=4510
1329
29.5%
3181
70.5%
G
TM
cycling
no-cycling
Σ=4510
2097
46.5%
2413
53.5%
H
AS
cycling
no-cycling
Σ=4510
648
14.4%
3862
85.6%
I
TS
cycling
no-cycling
Σ=4510
2062
45.7%
2448
54.3%
O
Ratio
(number of metabolites cycling)
AM/AS
TM/TS
Biogenic amines
J
AM
cycling
no-cycling
Σ=4330
1689
39.0%
2641
61.0%
K
TM
cycling
no-cycling
Σ=4330
2189
50.6%
2141
49.4%
L
AS
cycling
no-cycling
Σ=4330
1068
24.7%
3262
75.3%
M
TS
cycling
no-cycling
Σ=4330
1812
41.8%
2518
58.2%
P
Ratio
(number of metabolites cycling)
AM/AS
TM/TS

Figure 1. The microbiome reduces the effect of timed feeding on the cycling of lipids and biogenic amines.

(A) Schematic showing the experimental protocol for 1) analysis of gut metabolite cycling in microbiome-containing (AM) and sterile (AS) iso31 flies fed on normal chow diets under ad lib and 2) timed feeding (TF) conditions, respectively.
(B-E) Pie chart depicting the fraction of cycling primary metabolites and polar histogram plots of the peak phase for cycling primary metabolites in microbiome-containing and sterile flies under ad lib and timed feeding conditions (AM, TM, AS and TS), respectively, using a JTK_cycle cutoff of $P < 0.05$.
(F-I) Pie chart depicting the fraction of cycling lipids and polar histogram plots of the peak phase for cycling lipids in AM, TM, AS and TS flies, respectively, using a JTK_cycle cutoff of $P < 0.05$.
(J-M) Pie chart depicting the fraction of cycling biogenic amines and polar histogram plots of the peak phase for cycling biogenic amines in AM, TM, AS and TS flies, respectively, using a JTK_cycle cutoff of $P < 0.05$.
(N-P) The ratio of primary metabolites, lipids and biogenic amines that cycle in microbiome-containing versus sterile flies under ad lib feeding (AM/AS) and timed feeding (TM/TS) conditions, respectively.

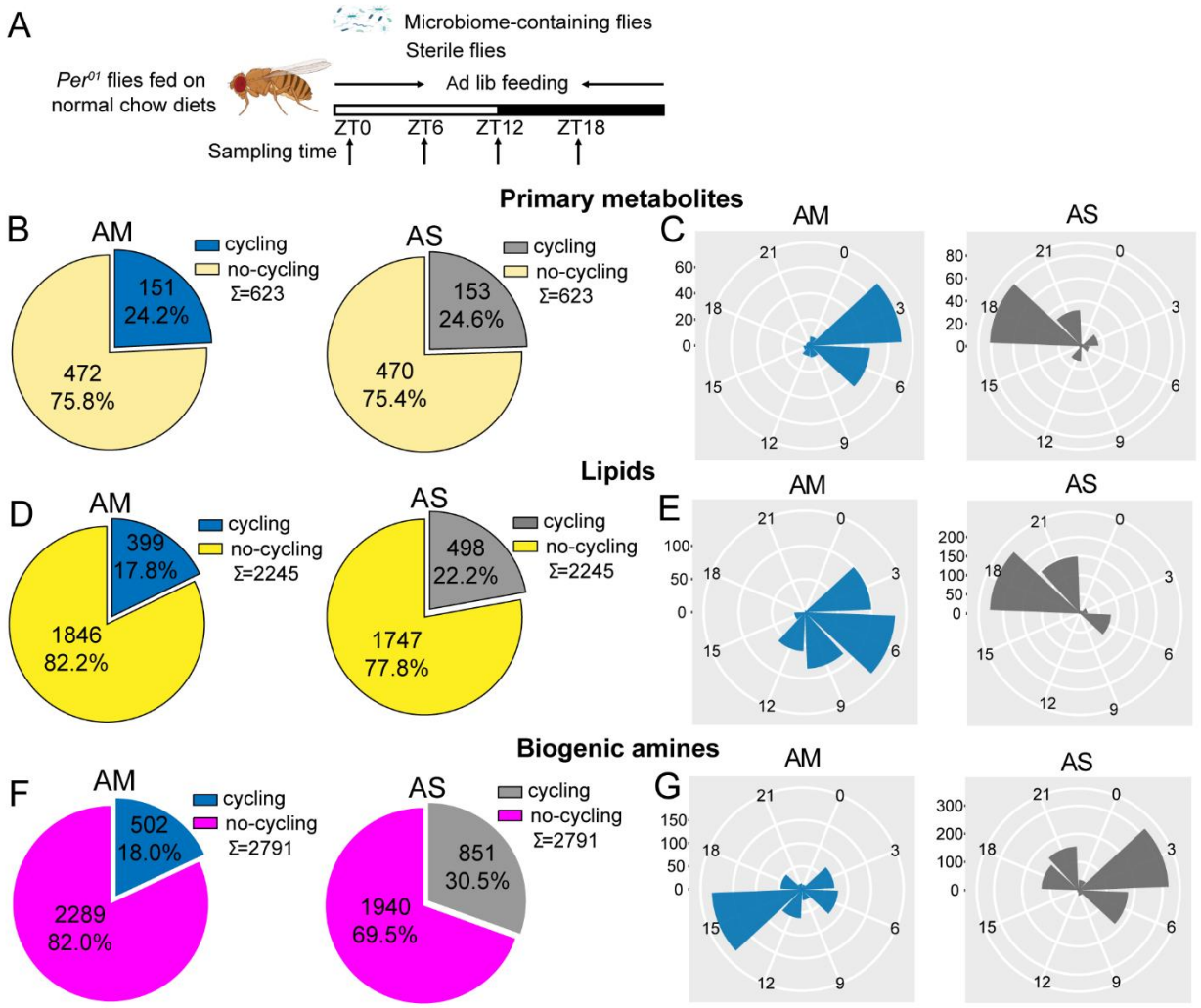

Figure 2. The effect of the microbiome on metabolite cycling depends on a functional circadian clock.

(A) Schematic showing the experimental protocol for analysis of gut metabolite cycling in microbiome-containing (AM) and sterile (AS) *per*^{01} flies fed on normal chow diets.
(B) Pie chart depicting the fraction of cycling primary metabolites, and (C) polar histogram plots of the peak phase for cycling primary metabolites in AM and AS *per*^{01} flies fed on normal chow diets, using a JTK_cycle cutoff of $P < 0.05$.
(D) Pie chart depicting the fraction of cycling lipids and (E) polar histogram plots of the peak phase for cycling lipids in AM and AS *per*^{01} flies fed on normal chow diets, using a JTK_cycle cutoff of $P < 0.05$.
(F) Pie chart depicting the fraction of cycling biogenic amines and (G) polar histogram plots of the peak phase for cycling biogenic amines in AM and AS *per*^{01} flies fed on normal chow diets, using a JTK_cycle cutoff of $P < 0.05$.

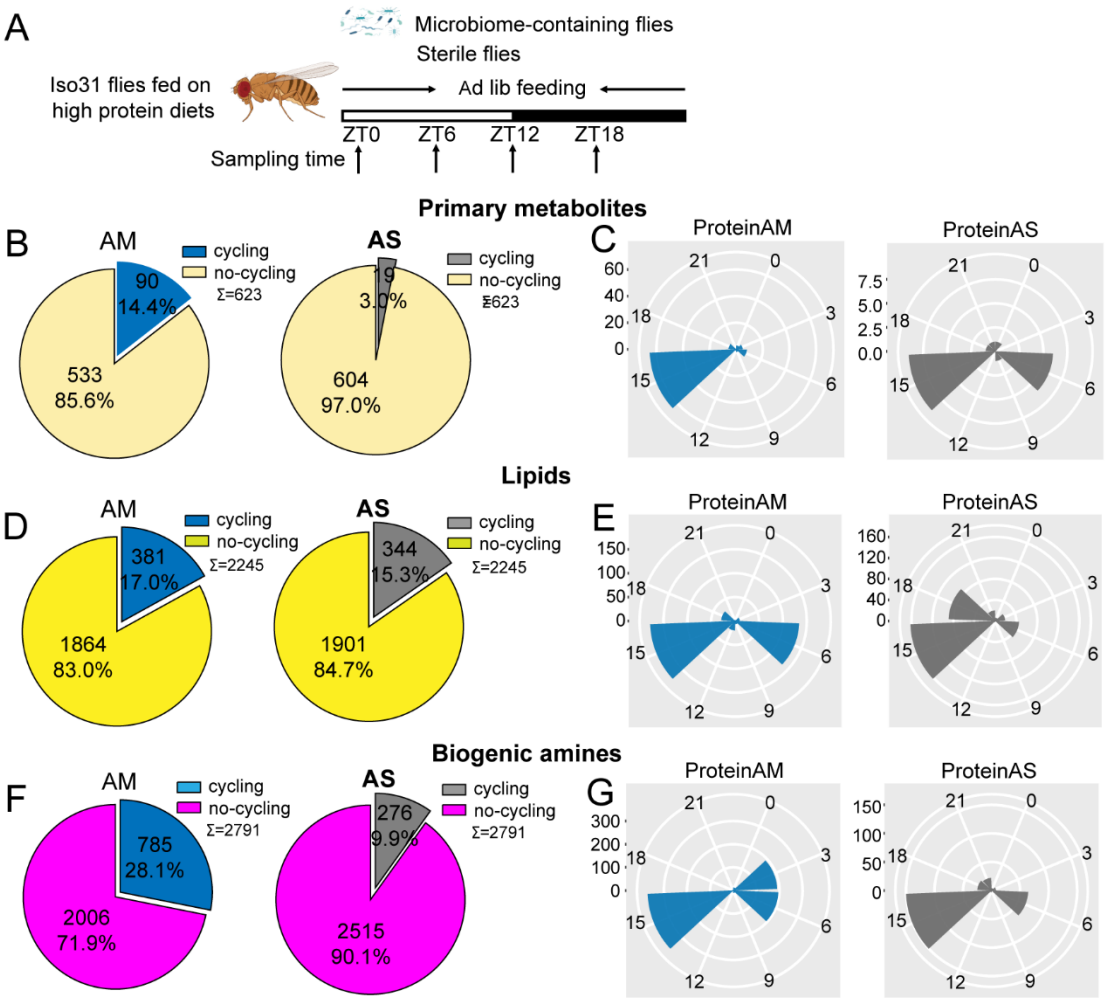

Figure 3. The microbiome increases the number of cycling of metabolites in flies fed on high protein diets.

(A) Schematic showing the experimental protocol for analysis of gut metabolite cycling in microbiome-containing (AM) and sterile (AS) Iso31 flies fed on high protein diets.
(B) Pie chart depicting the fraction of cycling primary metabolites, and (C) polar histogram plots of the peak phase for oscillating primary metabolites in AM and AS iso31 flies fed on high protein diets, using a JTK_cycle cutoff of $P < 0.05$.
(D) Pie chart depicting the fraction of cycling lipids, and (E) polar histogram plots of the peak phase for oscillating lipids in AM and AS iso31 flies fed on high protein diets, using a JTK_cycle cutoff of $P < 0.05$.
(F) Pie chart depicting the fraction of cycling biogenic amines, and (G) polar histogram plots of the peak phase for oscillating biogenic amines in AM and AS iso31 flies fed on high protein diets, using a JTK_cycle cutoff of $P < 0.05$.

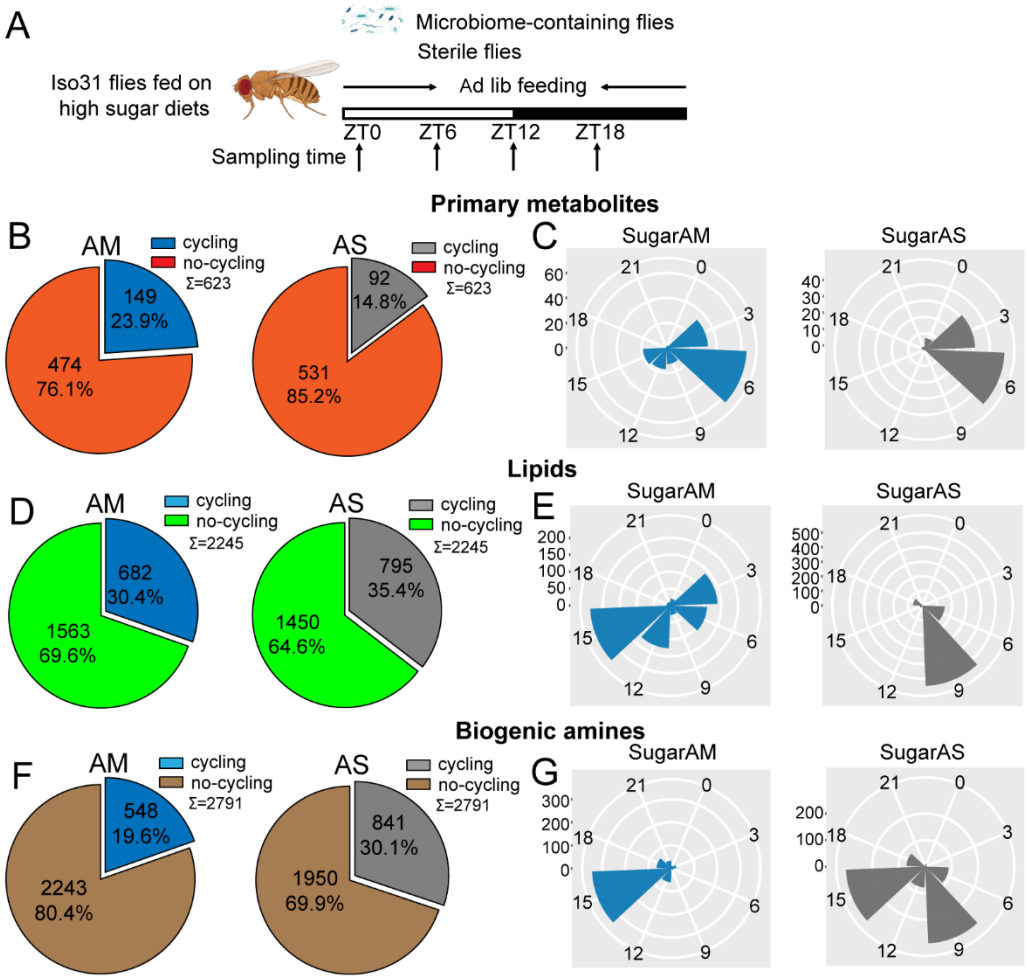

Figure 4. The microbiome decreases the number of cycling metabolites in iso31 flies fed on high sugar diets.

(A) Schematic showing the experimental protocol for analysis of gut metabolite cycling in microbiome-containing (AM) and sterile (AS) Iso31 flies fed on high sugar diets.
(B) Pie chart depicting the fraction of cycling primary metabolites, and (C) polar histogram plots of the peak phase for cycling primary metabolites in AM and AS iso31 flies fed on high sugar diets, using a JTK_cycle cutoff of $P < 0.05$.
(D) Pie chart depicting the fraction of cycling lipids, and (E) polar histogram plots of the peak phase for cycling lipids in AM and AS iso31 flies fed on high sugar diets, using a JTK_cycle cutoff of $P < 0.05$.
(F) Pie chart depicting the fraction of cycling biogenic amines and (G) polar histogram plots of the peak phase for cycling biogenic amines in AM and AS iso31 flies fed on high sugar diets, using a JTK_cycle cutoff of $P < 0.05$.

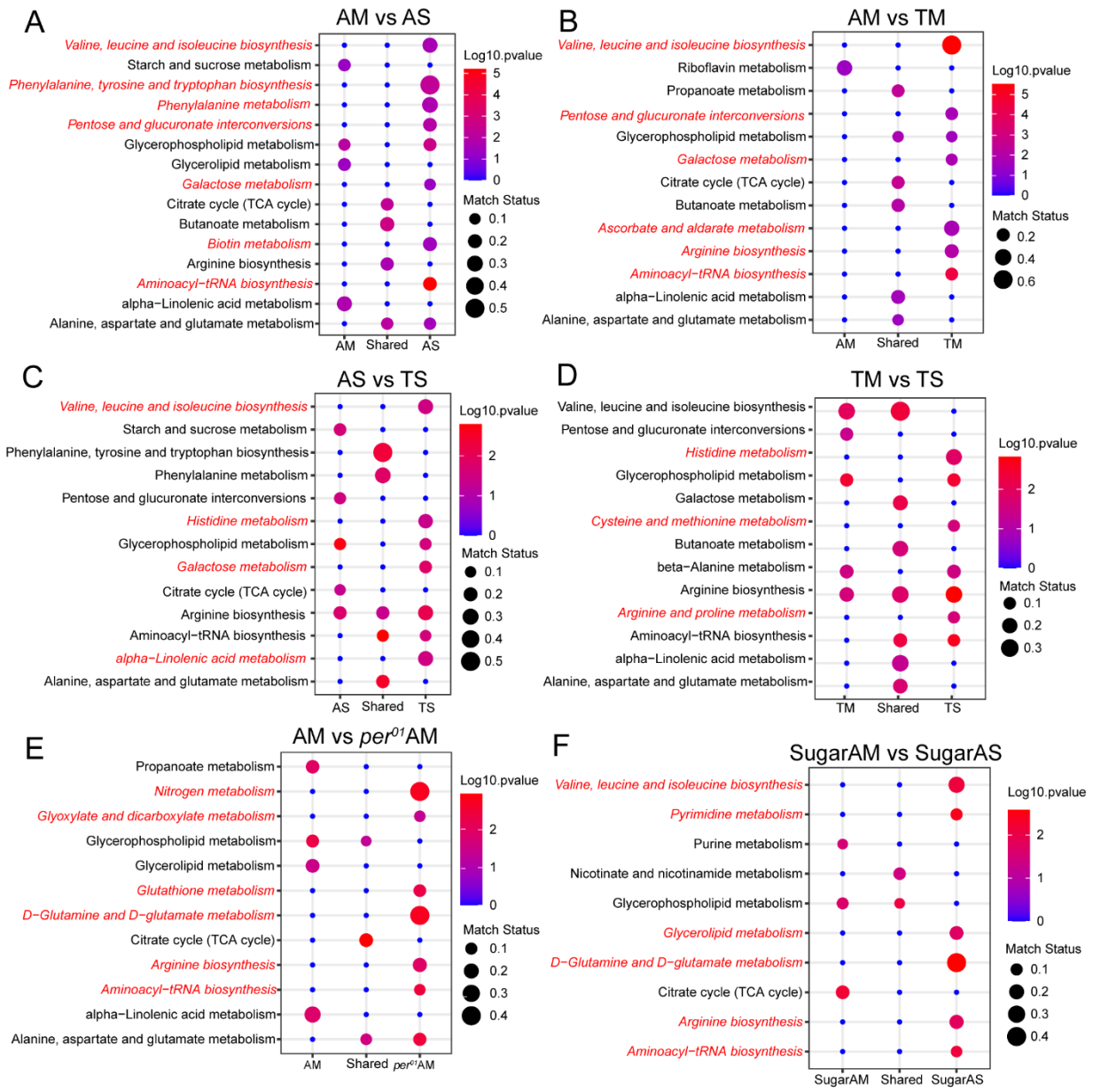

Figure 5. Amino acid metabolism is modulated by the microbiome, timed feeding and the circadian clock.

(A) KEGG enrichment analysis of oscillating metabolites unique to AM flies, shared in AM and AS flies and unique to AS flies. Data only show the $P < 0.05$ results.
(B) KEGG enrichment analysis of oscillating metabolites unique to AM flies, shared in AM and TM flies and unique to TM flies. Data only show the $P < 0.05$ results.
(C) KEGG enrichment analysis of oscillating metabolites unique to AS flies, shared in AS and TS flies and unique to TS flies. Data only show the $P < 0.05$ results.
(D) KEGG enrichment analysis of oscillating metabolites unique to TM flies, shared in TM and TS flies and unique to TS flies. Data only show the $P < 0.05$ results.

(E) KEGG enrichment analysis of oscillating metabolites unique to *per*^{01} AM flies, shared in AM and AS of *per*^{01} flies and unique in *per*^{01} AS flies. Data only show the P < 0.05 results.
(F) KEGG enrichment analysis of oscillating metabolites unique to high sugar fed AM flies, shared in AM and AS of *iso31* flies fed on high sugar diets and unique in high sugar fed AS flies. Data only show the P < 0.05 results.

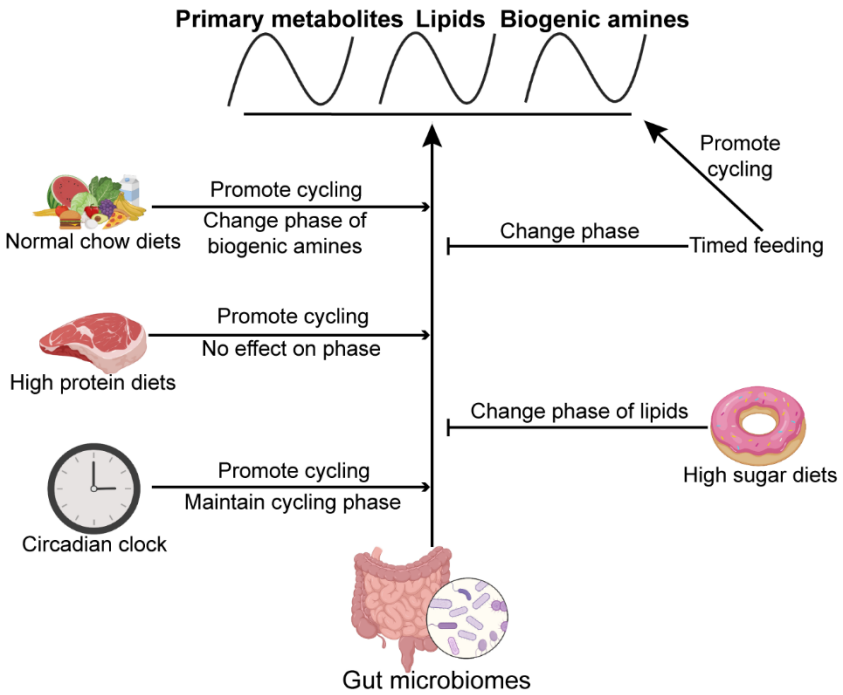

Figure 6. Model showing effects of the microbiome, timed feeding, the circadian clock and diet composition on metabolite rhythms in the gut.

The microbiome broadly increases metabolite rhythms on a normal chow diet and its effects are enhanced by high protein diet and the circadian clock. Timed feeding promotes cycling itself, but attenuates the effect of the microbiome on cycling, as does a high sugar diet. Effects of each of these manipulations on phase are also indicated.

Supplement Figures

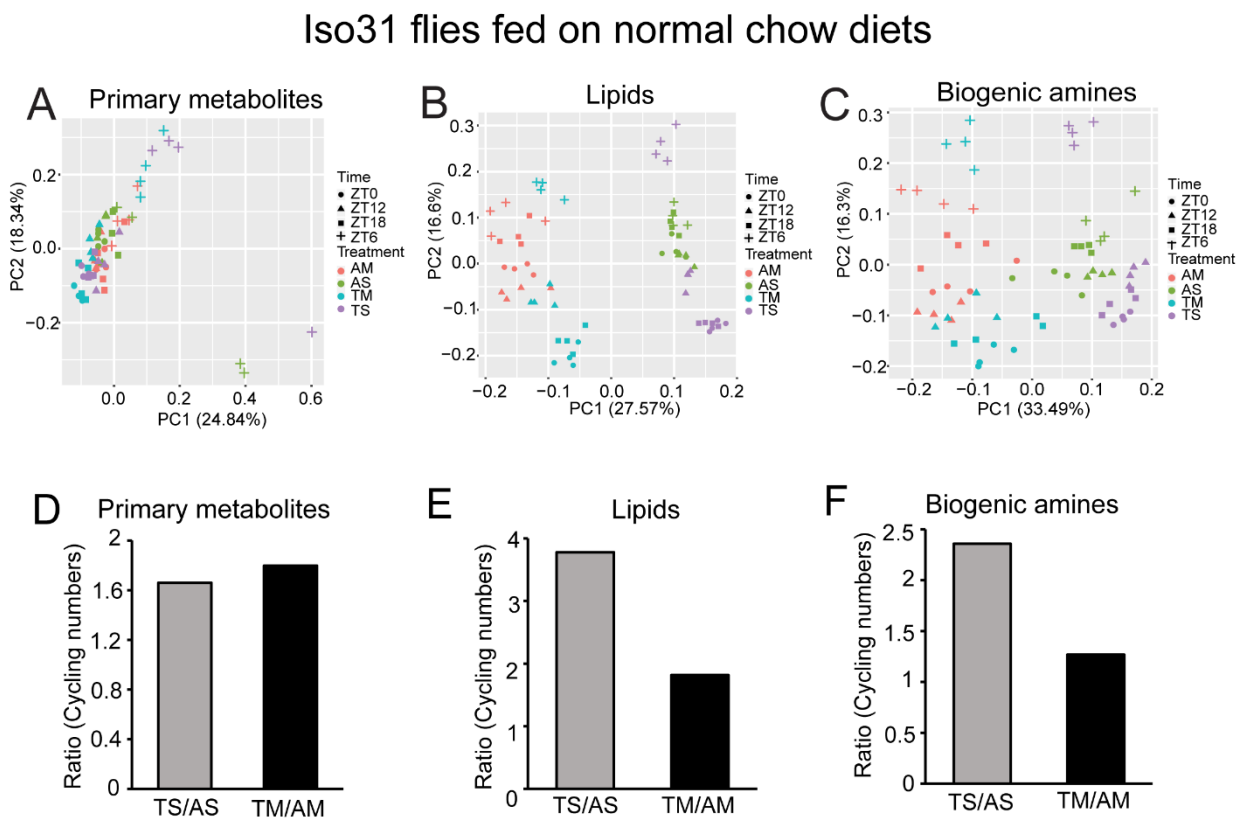

Figure S1. Timed feeding has less effect on cycling metabolites in microbiome-containing flies.

(A-C) A: Principal component analysis (PCA) of primary metabolites, lipids and biogenic amine abundance, respectively, at each time-point in a 12:12 LD cycle for AM, TM, AS, and TS flies.

(D-F) The ratio of primary metabolites, lipids and biogenic amines that cycle in microbiome-containing versus sterile flies under ad lib feeding (TS/AS) and timed feeding (TM/AM) conditions, respectively.

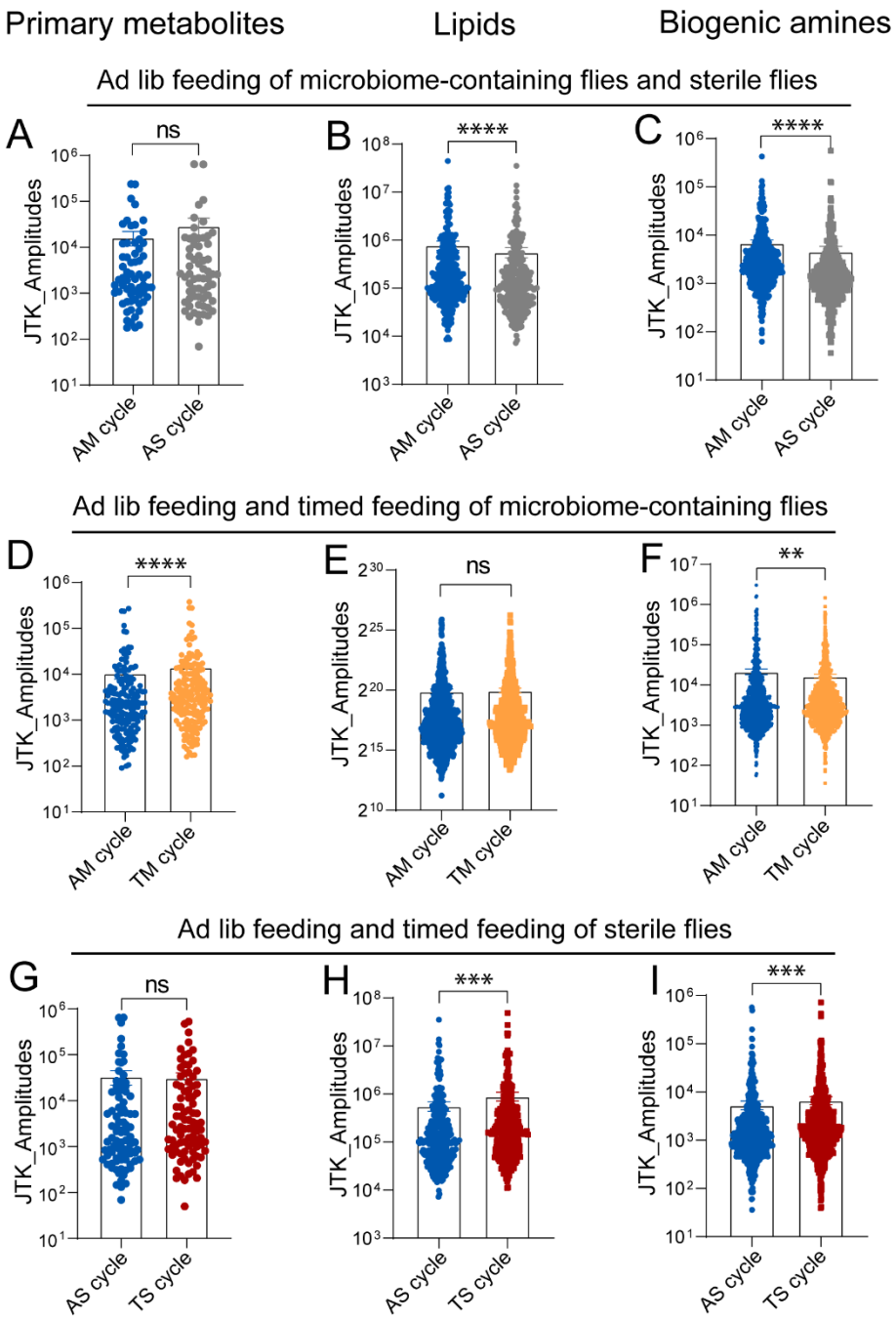

Figure S2. The amplitude of metabolite cycling is sometimes, but not always increased by the presence of a microbiome or by timed feeding.

(A-C) The comparison of amplitudes for cycling primary metabolites, lipids and biogenic amines, respectively, that overlap in AM and AS flies. Data are mean ± SEM, ****$p < 0.0001$ shown by Student's t test.
(D-F) The comparison of amplitudes for cycling primary metabolites, lipids and biogenic amines, respectively, that overlap in AM and TM flies. Data are mean ± SEM, **$p < 0.01$ and ****$p < 0.0001$ shown by Student's t test.

(G-I) The comparison of amplitudes for cycling primary metabolites, lipids and biogenic amines, respectively, that overlap in AS and TS flies. Data are mean ± SEM, ***$p < 0.001$ shown by Student's t test.

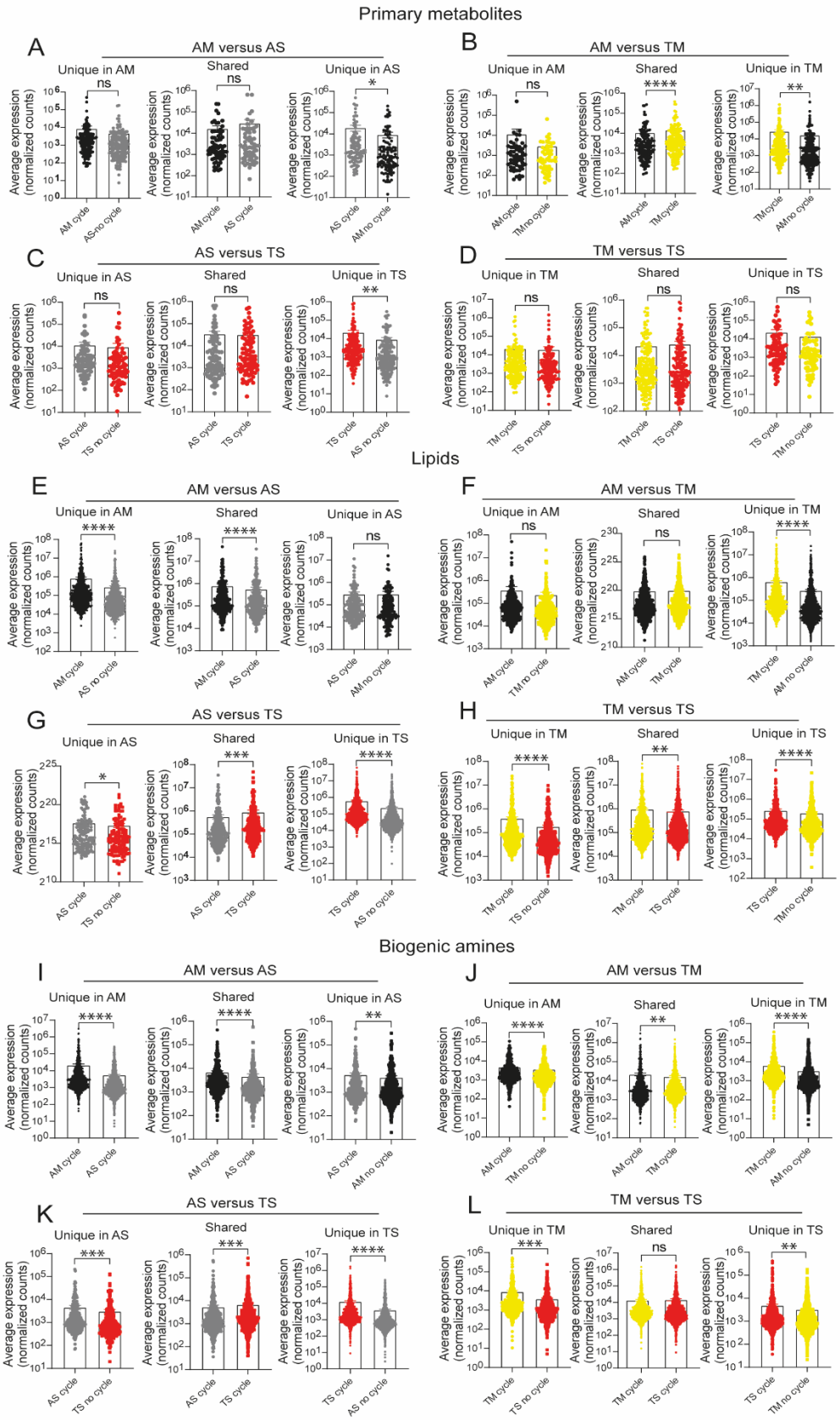
Primary metabolites
A
AM versus AS
Unique in AM
Shared
Unique in AS
ns
ns
*
B
AM versus TM
Unique in AM
Shared
Unique in TM
ns
****
**
C
AS versus TS
Unique in AS
Shared
Unique in TS
ns
ns
**
D
TM versus TS
Unique in TM
Shared
Unique in TS
ns
ns
ns
Lipids
E
AM versus AS
Unique in AM
Shared
Unique in AS
****
****
ns
F
AM versus TM
Unique in AM
Shared
Unique in TM
ns
ns
****
G
AS versus TS
Unique in AS
Shared
Unique in TS
*
***
****
H
TM versus TS
Unique in TM
Shared
Unique in TS
****
**
****
Biogenic amines
I
AM versus AS
Unique in AM
Shared
Unique in AS
****
****
**
J
AM versus TM
Unique in AM
Shared
Unique in TM
****
**
****
K
AS versus TS
Unique in AS
Shared
Unique in TS
***
***
****
L
TM versus TS
Unique in TM
Shared
Unique in TS
***
ns
**
Average expression (normalized counts)
AM cycle
AS no cycle
AS cycle
AM no cycle
TM no cycle
TM cycle
TS no cycle
TS cycle

Figure S3. Lipids and biogenic amines show higher abundance in microbiome-containing flies.

A-D: Average expression levels of gut primary metabolites that cycle in either microbiome-containing or sterile flies or both conditions (lost, shared and gained). The number indicates the average normalized counts in each condition for primary metabolites that cycle with $p < 0.05$, JTK_cutoff. Data are mean ± SEM, comparisons were with Student's t test. A: AS versus AM; B: TM versus AM; C: TS versus AS; D**:** TS versus TM.

E-H: Average expression levels of gut lipids that cycle in either microbiome-containing or sterile flies or both conditions (lost, shared and gained). The number indicates the average normalized counts in each condition for lipids that cycle with $p < 0.05$, JTK_cutoff. Data are mean ± SEM, comparisons were with Student's t test. E: AS versus AM; F: TM versus AM; G: TS versus AS; H**:** TS versus TM.

I-L: Average expression levels of gut biogenic amines that cycle in either microbiome-containing or sterile flies or both conditions (lost, shared and gained). The number indicates the average normalized counts in each condition for biogenic amines that cycle with $p < 0.05$, JTK_cutoff. Data are mean ± SEM, comparisons were with Student's t test. I: AS versus AM; J: TM versus AM; K: TS versus AS; L**:** TS versus TM.

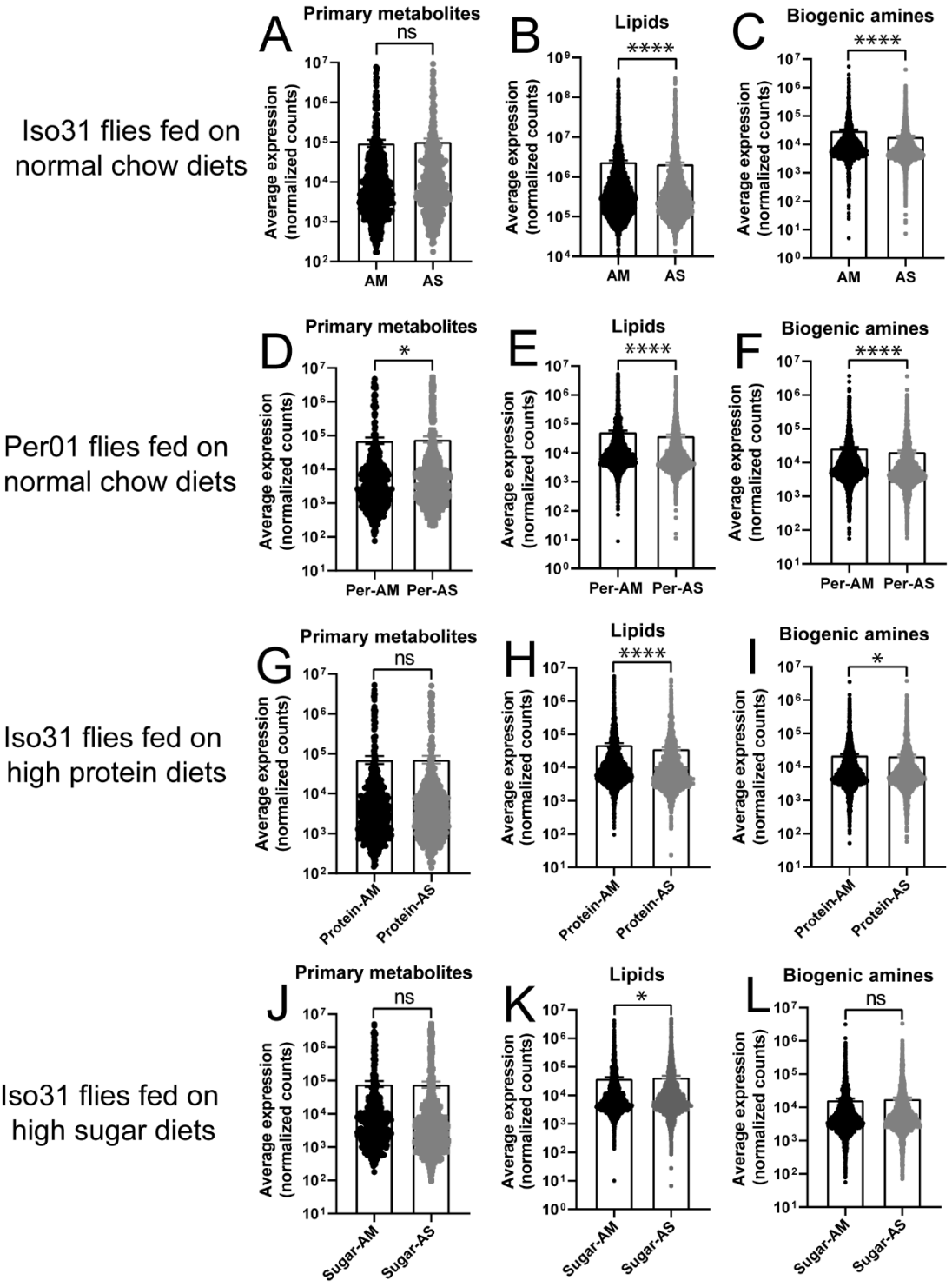

Figure S4. Abundance analysis of all metabolites in microbiome containing and sterile flies.

(A-C) Average expression levels of gut metabolites in microbiome-containing and sterile flies. iso31 flies are fed on normal chow diets. A: AS versus AM for primary metabolites; B: AS versus AM for lipids; C: AS versus AM for biogenic amines.
(D-F) Average expression levels of gut metabolites between microbiome-containing and sterile flies. *per*^{01} flies are fed on normal chow diets. D: AS versus AM for primary metabolites; E: AS versus AM for lipids; F: AS versus AM for biogenic amines.
(G-I) Average expression levels of gut metabolites between microbiome-containing and sterile flies. iso31 flies are fed on high protein diets. G: AS versus AM for primary metabolites; H: AS versus AM for lipids; I: AS versus AM for biogenic amines.
(J-L) Average expression levels of gut metabolites between microbiome-containing and sterile flies iso31 flies are fed on high sugar diets. J: AS versus AM for primary metabolites; K: AS versus AM for lipids; L: AS versus AM for biogenic amines.

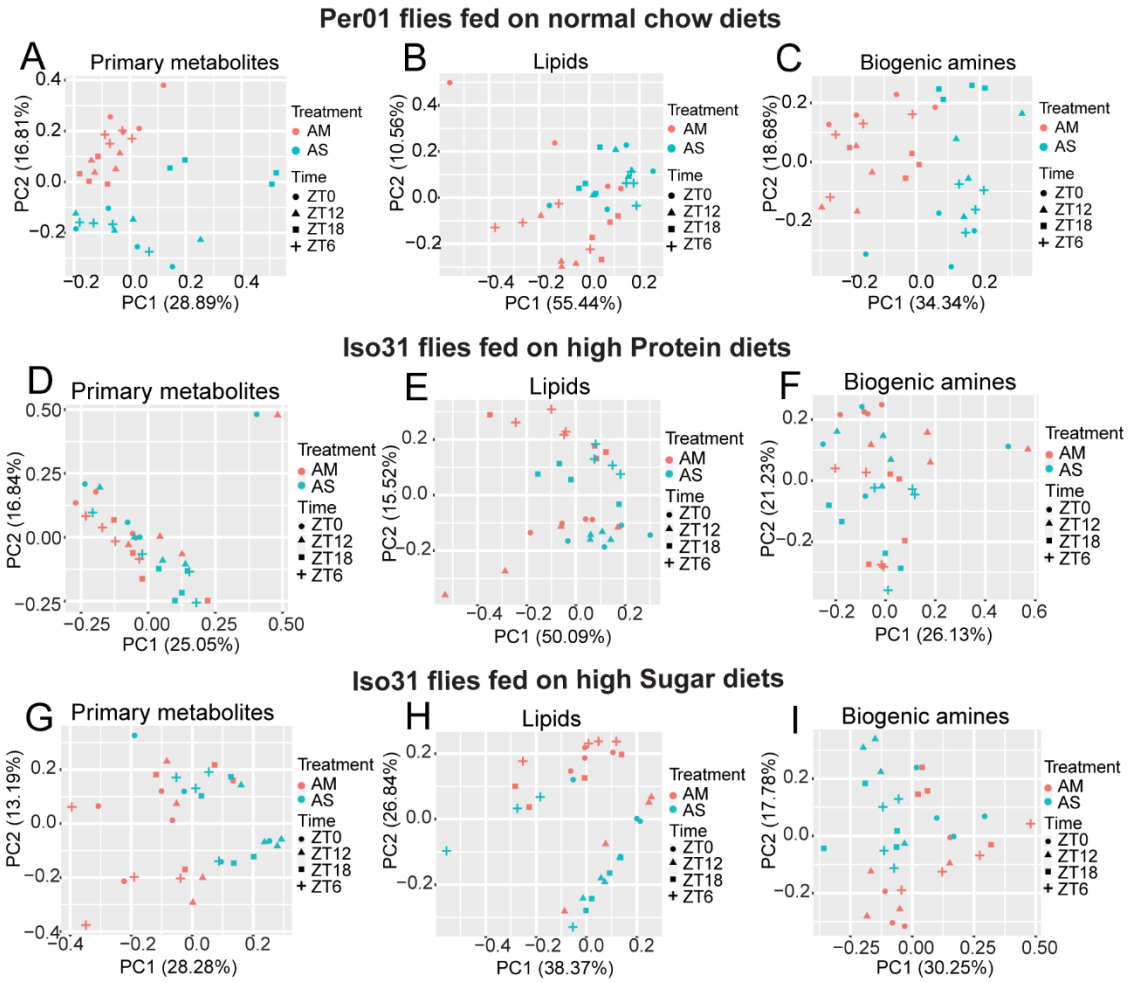

Figure S5. Principal component analysis (PCA) of total expressed metabolites in *per*^{01} flies fed on normal chow diets, iso31 flies fed on high protein and high sugar diets.

(A-C) Principal component analysis (PCA) of metabolite abundance at each time-point in a 12:12 LD cycle for *per*^{01} AM and AS flies fed on normal chow diets.
(D-F) Principal component analysis (PCA) of metabolite abundance at each time-point in a 12:12 LD cycle for iso31 AM and AS flies fed on high protein diets
(G-I) Principal component analysis (PCA) of metabolite abundance at each time-point in a 12:12 LD cycle iso31 AM and AS flies fed on high sugar diets.

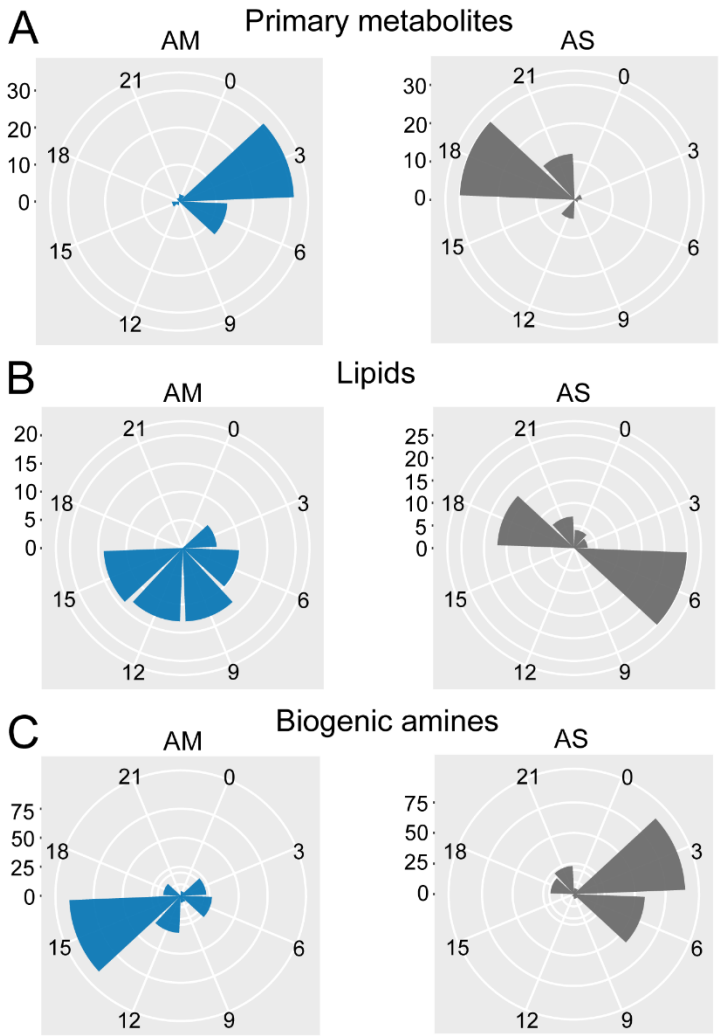

Figure S6. Phases also change for cycling metabolites shared between *per*^{01}-AM and *per*^{01}-AS.

(A-C) Polar histogram plots of the peak phase for shared oscillating primary, lipids, and biogenic amines, respectively, in *per*^{01} microbiome-containing and *per*^{01} sterile flies, using a JTK_cycle value of P < 0.05.

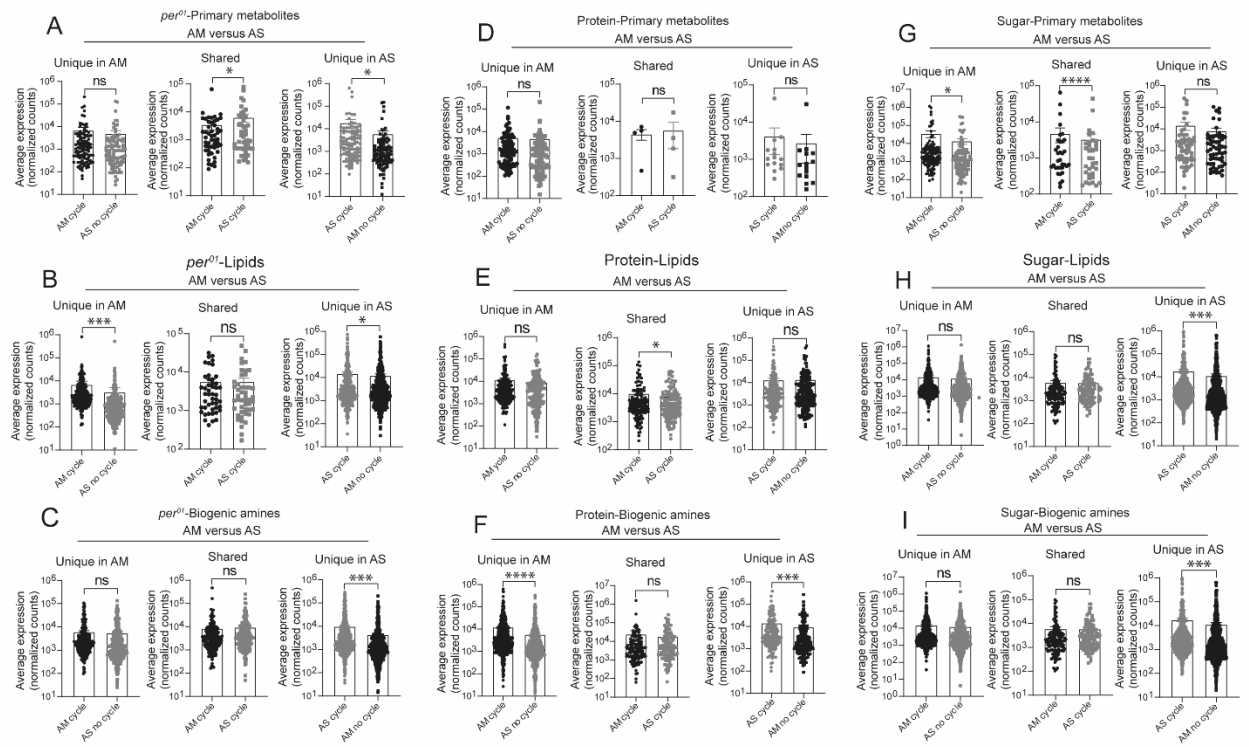

Figure S7. Effect of the microbiome on expression levels of cycling metabolites in clock mutants and under different dietary conditions.

A-C: Average expression levels of gut metabolites that cycle in microbiome-containing versus sterile *per*^{01} flies fed on normal chow diets. The middle group in each panel shows metabolites that cycle under both conditions, while those on either side indicate metabolites that cycle in only one condition. The number indicates the average normalized counts in each condition for metabolites that cycle with p < 0.05, JTK_cycle. Data are mean ± SEM, comparisons were with Student's t test. A: AS versus AM for primary metabolites; B: AS versus AM for lipids; C: AS versus AM for biogenic amines.
D-F: Average expression levels of gut metabolites that cycle in microbiome-containing versus sterile iso31 flies fed on high protein diets. The middle group in each panel shows metabolites that cycle under both conditions, while those on either side indicate metabolites that cycle in only one condition. The number indicates the average normalized counts in each condition for metabolites that cycle with p < 0.05, JTK_cycle. Data are mean ± SEM, comparisons were with student's t test. D: AS versus AM for primary metabolites; E: AS versus AM for lipids; F: AS versus AM for biogenic amines.

G-I: Average expression levels of gut metabolites that cycle in microbiome-containing versus sterile flies fed on high sugar diets. The middle group in each panel shows metabolites that cycle under both conditions, while those on either side indicate metabolites that cycle in only one condition. The number indicates the average normalized counts in each condition for metabolites that cycle with $p < 0.05$, JTK_cycle. Data are mean ± SEM, comparisons were with Student's t test. G: AS versus AM for primary metabolites; H: AS versus AM for lipids; I: AS versus AM for biogenic amines.

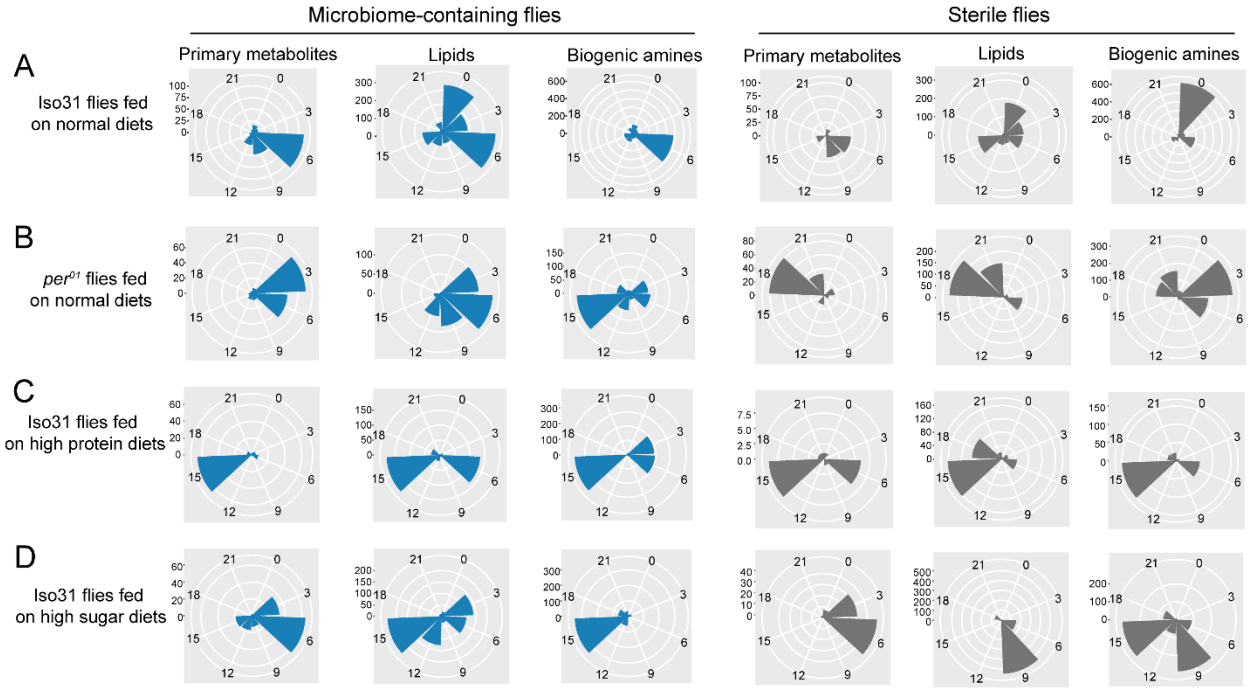

Figure S8. Effects of the circadian clock and dietary composition on the phase of metabolite cycling in microbiome-containing and sterile flies (these data are the same as Figure1-4).

(A-D) Polar histogram plots of the peak phase for oscillating primary, lipids, and biogenic amines, respectively, in microbiome-containing and sterile flies, using a JTK_cycle value of $P < 0.05$. A: iso31 flies fed on normal chow diets; B: *per*^{01} flies fed on normal chow diets; C: iso31 flies fed on high protein diets; D: iso31 flies fed on high sugar diets.

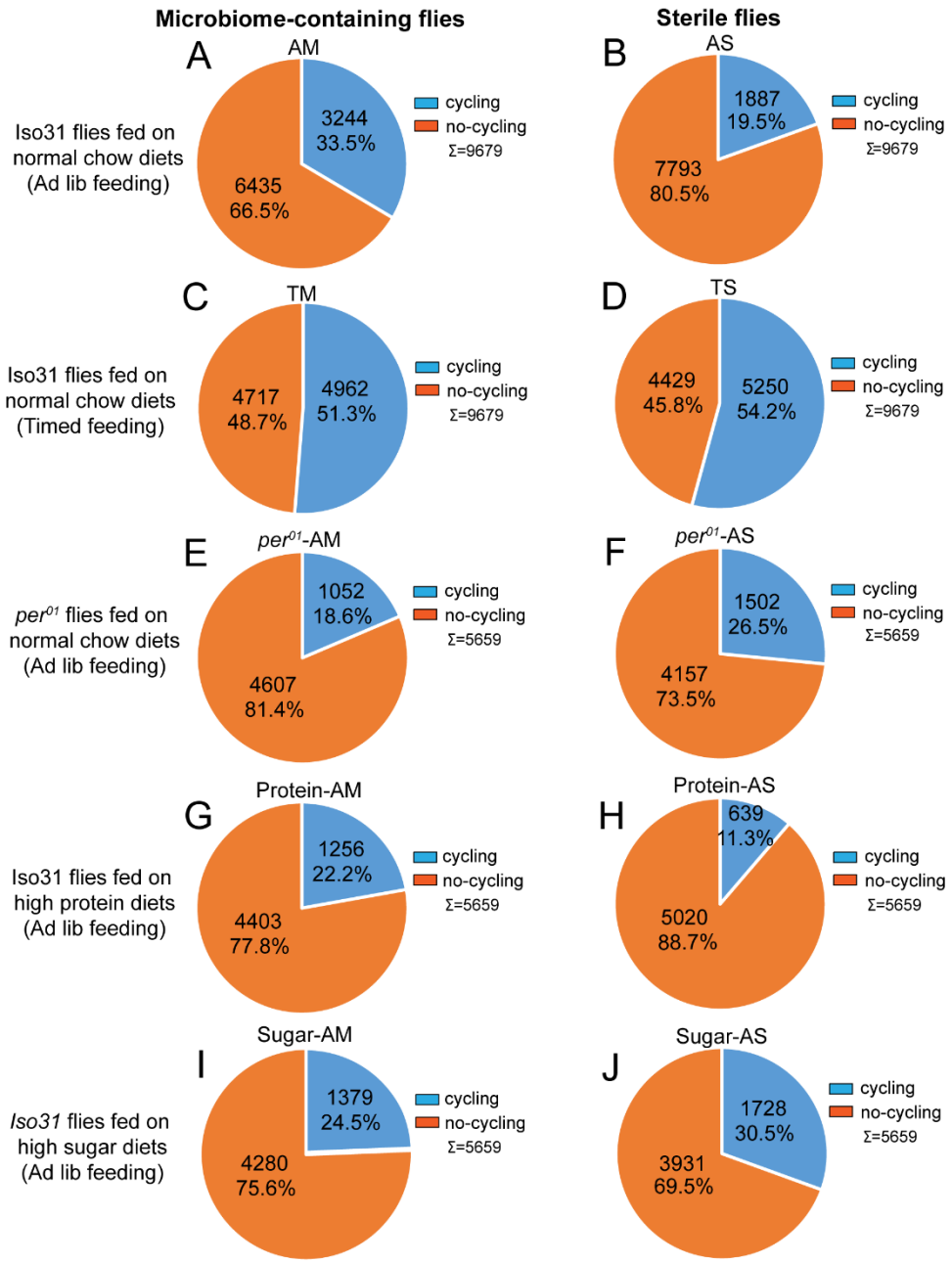

Figure S9. Loss of the circadian clock, high protein or high sugar diets decrease metabolite cycling in microbiome-containing flies.

(A,B) Pie charts depicting the fraction of metabolites cycling in AM and AS iso31 flies fed on normal chow diets; (C,D) Pie chart depicting the fraction of metabolites cycling in TM and TS iso31 flies fed on normal chow diets; (E,F) Pie charts depicting the fraction of metabolites cycling in AM and AS *per*^{01} flies fed on normal chow diets; (G,H) Pie chart depicting the fraction of metabolites cycling in AM and AS iso31 flies fed on high protein

diets; (I,J) Pie chart depicting the fraction of metabolites cycling in AM and AS iso31 flies fed on high sugar diets, using a JTK_cycle cutoff of $P < 0.05$.