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# The circadian clock is required for rhythmic lipid transport in *Drosophila* in interaction with diet and photic condition

**Kelechi M. Amatobi[1,2], Ayten Gizem Ozbek-Unal[1], Stefan Schäbler[1], Peter Deppisch[2], Charlotte Helfrich-Förster[2], Martin J. Mueller[1], Christian Wegener[2]*, and Agnes Fekete[1]***

[1]Biocenter, Julius-von-Sachs-Institute, Pharmaceutical Biology, and [2]Biocenter, Theodor-Boveri-Institute, Würzburg Insect Research (WIR), Neurobiology and Genetics, Julius-Maximilians-Universität Würzburg, Würzburg, Germany

**Abstract Modern lifestyle is often at odds with endogenously driven rhythmicity, which can lead to circadian disruption and metabolic syndrome. One signature for circadian disruption is a reduced or altered metabolite cycling in the circulating tissue reflecting the current metabolic status. *Drosophila* is a well-established model in chronobiology, but daytime dependent variations of transport metabolites in the fly circulation are poorly characterized. Here, we sampled fly hemolymph throughout the day and analyzed diacylglycerols (DGs), phosphoethanolamines (PEs) and phosphocholines (PCs) using LC-MS. In wild-type flies kept on sugar-only medium under a light-dark cycle, all transport lipid species showed a synchronized bimodal oscillation pattern with maxima at the beginning and end of the light phase which were impaired in *period*$^{01}$ clock mutants. In wild-type flies under constant dark conditions, the oscillation became monophasic with a maximum in the middle of the subjective day. In strong support of clock-driven oscillations, levels of the targeted lipids peaked once in the middle of the light phase under time-restricted feeding independent of the time of food intake. When wild-type flies were reared on full standard medium, the rhythmic alterations of hemolymph lipid levels were greatly attenuated. Our data suggest that the circadian clock aligns daily oscillations of DGs, PEs, and PCs in the hemolymph to the anabolic siesta phase, with a strong influence of light on phase and modality.**

**Supplementary key words** hemolymph lipids • lipidomics • circadian rhythm • feeding • locomotor activity • light-driven metabolism

Physiological processes need to be temporally aligned with each other and with the environment to ensure the health and well-being of organisms. In animals, including humans, circadian clocks in the brain and peripheral organs are endogenous timekeepers that are essential for temporal alignment throughout the body even in the absence of environmental synchronizing cues (Zeitgeber such as light or feeding) (1–3). In addition, circadian clocks allow animals to adjust their physiology and behavior in anticipation of periodic biotic and abiotic changes in the surrounding environment (4). Circadian disruption and misalignment with daily environmental changes promotes diverse physiological pathologies, including metabolic disorders such as type-2 diabetes and obesity in shift workers or people with sleep disorders (5, 6).

Like other metabolic pathways, lipid metabolism shows daily rhythmicity and is under circadian regulation in mammals (see (7, 8)). In particular, there is substantial daily rhythmicity in plasma transport lipids, including diacylglycerols and triacylglycerols (DGs, TGs), free fatty acids, sphingolipids, and phospholipids (e.g., (9–12)). The fruit fly *Drosophila*, a long-standing model in circadian biology, is attracting increasing attention as a model to study lipid metabolism in health and disease (13), including circadian aspects (14). Global metabolic profiling in *Drosophila* identified a larger set of molecules including acyl carnitines, fatty acids, and other lipids that rhythmically oscillate in a light- and clock-dependent manner (15–17). In addition, transcripts for a large number of lipid-metabolizing enzymes are cycling in a circadian fashion in the gut and fat body (14, 18), major organs involved in the regulation of systemic lipid metabolism in the fly. These enzymes are responsible for different chemical steps in lipid metabolism, including long-chain fatty acid metabolism, fatty-acyl-CoA reduction, TG/DG breakdown, and synthesis as well as β-oxidation.

In contrast to mammals, the diel temporal patterns of lipids transported in the *Drosophila* hemolymph (the fly's "blood") remain largely uncharacterized, and their dependence on the circadian clock is unclear. To fill this gap, we profiled the diel and circadian temporal patterns of lipids in the hemolymph of *Drosophila* using ultra performance liquid chromatography coupled to time-of-flight mass spectrometry in Canton-S wild-type

*For correspondence: Christian Wegener, christian.wegener@biozentrum.uni-wuerzburg.de; Agnes Fekete, agnes.fekete@uni-wuerzburg.de.



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($WT_{CS}$) flies and *period*$^{01}$ mutants (*per*$^{01}$) with a defective molecular clockwork. We focused on the two major groups of transported lipids: glycerolipids (DGs) and phospholipids [phosphoethanolamines (PEs), phosphocholines (PCs)]. DGs were chosen because they represent the transport form of fatty acids used for cellular energy and lipid homeostasis in insects (19, 20) and because TG/DG breakdown and synthesis are under transcriptional control of the fat body clock (18). Phospholipids play a major role as components of biological membranes, in signal transduction and in membrane trafficking, and serve as building blocks for more complex cellular lipids. DGs, PEs, and PCs are major lipid constituents of lipophorins, the major hemolymph carrier vehicles for water-insoluble metabolites (20, 21).

Since the temporal profile of lipid metabolism is strongly influenced by time-restricted feeding (TRF) in flies and mammals (e.g., (22–25)), we performed hemolymph lipid profiling in flies kept on different diets or TRF regimes. Our findings show that the circadian clock, light, and feeding are important factors that shape the temporal profile of circulating lipids in flies. Our results further provide evidence for a role of de novo lipid synthesis in clock-dependent temporal changes of transport lipids in the hemolymph. Interestingly, the clock appears to align peak levels of transport lipids to the anabolic siesta time, which may have a systemic and health-relevant impact.

## MATERIALS AND METHODS

### Fly husbandry

$WT_{CS}$ and cantonized *per*$^{01}$ flies were kindly provided by Bambos Kyriacou (Leicester) and are described in (26). Flies were raised on standard *Drosophila* medium containing 0.8% agar, 2.2% sugar beet syrup, 8.0% malt extract, 1.8% yeast, 1.0% soybean meal, 8.0% maize meal and 0.3% hydroxybenzoic acid (per 100 g: 2.4 g protein, 13.03 g carbohydrates, 0.38 g lipids) under a 12:12 h light:dark (LD) regime at 25 ± 0.2°C and 60 ± 2% relative humidity. For the experiments, young males were collected within 24 h after eclosion and transferred to vials containing either standard medium or sugar-only medium consisting of 5% sucrose and 2% agar (per 100 g: 5 g carbohydrates, no proteins or lipids).

### Hemolymph sampling

Six-days-old male flies were transferred from their food vials to a clean vial and anesthetized on ice for 3 min. The males were then placed on a plastic Petri dish wrapped in parafilm to reduce water condensation under a stereomicroscope. A small incision was made in the thorax of the flies using a sharp tungsten needle. After incision, 20 male flies were pooled into a 0.5 ml Eppendorf tube with three small holes at the bottom, which was inserted into a 1.5 ml Eppendorf tube (supplemental Fig. S1A). The tubes containing the flies were then centrifuged for 5 min at 4°C at 3,075 rcf (5,000 rpm) in a benchtop centrifuge. After centrifugation, clear and light-yellowish hemolymph samples were typically obtained. Cloudy dark yellow samples were discarded. The hemolymph obtained in the 1.5 ml Eppendorf tube was then taken up using 0.5 or 1 µl microcapillaries. The length occupied by the hemolymph in the microcapillaries was measured under a stereomicroscope, and the volume was calculated. Afterward, the microcapillaries were each placed in an Eppendorf tube containing 25 µl of Millipore water and the entire contents ejected using a small rubber ejector. The test tubes containing the samples were stored at −80°C until the samples were prepared for lipid analysis.

### Sample preparation and lipid analysis

Prior to analysis, the diluted hemolymph samples were dried to complete dryness in a rotary evaporator at 50°C until complete dryness and reconstituted in 75 µl of cold isopropanol containing internal standards [1 ng/µl of DG(20:0), PE(34:0) and PC(34:0), and 0.1 ng/µl of TG(30:0)], followed by ultrasonication for 15 min. Analyses were performed on an Acquity Ultra Performance LC coupled to a Synapt G2 HDMS (Waters) as previously described (16). Briefly, a BEH C18 column (2.1 × 100 mm, 1.7 µm, Waters) at 60°C was used for chromatographic separation. A linear binary solvent gradient of 30–100% eluent B was applied over 10 min at a flow rate of 0.3 ml/min. Eluent A consisted of 10 mM ammonium acetate in 60/40% water/acetonitrile, and eluent B consisted of 10 mM ammonium acetate in 90/10% isopropanol/acetonitrile. After chromatographic separation, metabolites were ionized using an electrospray ionization source operated in positive mode. The quadrupole was operated in a broadband mode, and data were acquired in a centroid data mode over the mass range of 50–1,200 Da. Leucine-enkephalin (m/z 556.2771) was used for internal calibration every 0.3 min. MassLynx and QuanLynx (version 4.1; Waters) were used to acquire and process the chromatograms. For quantification, the peak areas of the analytes and internal standards in the extracted ion chromatogram were integrated. Concentrations were calculated using an internal standard technique (response factor of one) for each analyte/internal standards pair, and plots were generated using either R or MS-Excel. Retention times and mass-to-charge ratios of the lipids are given in supplemental Table S1.

### Locomotor activity recording and feeding assay

*Drosophila* activity monitors (DAM-2, Trikinetics, Waltham) were used to record locomotor activity. For both feeding conditions (standard medium or sugar-only medium), 32 male flies per genotype ($WT_{CS}$ and *per*$^{01}$) were individually placed in small glass tubes (5 mm diameter) containing food on one side closed with a rubber stopper. The other side was closed with a small foam plug to allow for air exchange. The standard medium consisted of 3.6% yeast extract, 5% sucrose, and 2% agar. Sugar-only medium consisted of 2% agar with 4% sucrose. Flies were monitored under LD12:12 conditions for 7 days, and under constant darkness (DD) for the rest of the experiments at constant humidity (60%) and temperature (25°C). Fly activity was measured by counting the interruptions of the infrared beam in the middle of the tubes.

The capillary feeding assay was performed as previously described (16). The standard medium contained 3.6% yeast extract and 5% sucrose, while the sugar-only medium consisted of 5% sucrose. Both media contained 0.3% FD&C Blue No 1 (E133) as a coloring agent.

### Statistical analysis

Time-dependent lipid oscillations and food consumption were analyzed using JTK_CYCLE in an R environment (27).

To identify oscillation profiles, period length was set to 6–24 h. Lipids and feeding were considered oscillatory if the adjusted *P* value (adj.p) was less than 0.05. JTK_CYCLE also calculated amplitude (AMP) and time of maximum also known as lag phase (LAG). The rhythmicity of locomotor activity was analyzed using the Lomb–Scargle and Chi-squared test in ActogramJ (28).

## RESULTS

### Diacylglycerol and phospholipid levels vary throughout the day in a clock- and light-dependent manner on sugar-only medium

Previous studies have reported daily oscillations of several lipids in fly bodies (thorax and abdomen) or in whole flies (15–17), but it remained unclear whether lipid levels in the fly hemolymph vary according to the time of day. To fill this gap, we analyzed lipids in extracted hemolymph every 3 h over the entire day in 6 days old male $WT_{CS}$ flies maintained under LD (supplemental Fig. S1B) and fed *ad-libitum* with sugar-only medium containing sugar as the sole carbon and energy source. We first profiled the major hemolymph lipid species (DGs, PEs, and PCs) using ultra performance liquid chromatography coupled to time-of-flight mass spectrometry and identified 20 lipid species [DG(26:0), DG(26:1), DG(28:0), DG(28:1), DG(30:1), DG(32:1), DG(32:2); PE(30:1), PE(32:1), PE(32:2), PE(34:1), PE(34:2), PE(34:3), PE(36:2), PE(36:3); PC(32:1), PC(32:2), PC(34:1), PC(34:2), PC(34:3) listed in supplemental Table S1]. Interestingly, all lipid species of the three different lipid classes showed very similar temporal oscillation patterns in phase with each other (**Fig. 1**A–C), indicating that the daily variation is independent of the lipid species. This allowed us to sum up the total levels for each lipid class. The experiments were independently repeated seven times over a period of 3 years. In six out of the seven experiments, the levels of total DG, PE, and PC species peaked at the beginning (ZT1) and end (ZT10-13) of the light phase with variable AMPs (supplemental Fig. S2). In addition to manual inspection, daily oscillations were analyzed using the nonparametric algorithm JTK_CYCLE (27) which revealed significant daily oscillations in five out of the seven individual experiments (adj.$P < 0.01$, supplemental Table S2). We then combined and averaged the normalized individual experiments which showed strong bimodal daily oscillations in hemolymph DGs, PEs, and PCs with peaks at ZT1 and 10 (**Fig. 2**A, A′′), as reflected by the low adj.*P* values ($4e^{-7}$-$3e^{-6}$) shown in supplemental Table S2.

To test whether the observed bimodal oscillation of hemolymph lipids is endogenously driven by the circadian clock, we next analyzed DGs, PEs, and PCs in the hemolymph of $WT_{CS}$ in DD. Flies were synchronized for 3 days in LD and switched to DD conditions before hemolymph was sampled every 3 h on the 6th day under red light to which the circadian clock of the fly is blind (supplemental Fig. S1C). In two out of three independent experiments, the total levels of DGs, PEs, and PCs showed a monomodal peak at subjective midday (supplemental Fig. S3). However, JTK_CYCLE identified significant oscillation in only one experiment (supplemental Table S2). The combined normalized and averaged lipid levels of all three experiments showed a monomodal peak at CT7 (Fig. 2B, B′′). Although the profiles of DGs, PEs and PCs are very similar, JTK_CYCLE revealed rhythmicity only for PCs (adj.p: 0.003 for PC, 0.2 for PE and 1 for DG, supplemental Table S2).

The difference in the temporal profile between LD and DD suggests that light has a significant effect on the daily fluctuations of hemolymph DGs, PEs, and PCs in *Drosophila*. In LD, lipid levels peak bimodally around the times of lights on and off, while in DD only a single peak occurs in the middle of the subjective day (CT7). Nevertheless, the monomodal oscillation in DD provides strong evidence for a circadian basis underlying the daily changes of hemolymph lipids. In addition, light appears to have a positive effect on total lipid levels in the hemolymph of $WT_{CS}$. Quantified lipid levels after averaging of all sampling times were considerably higher in LD than in DD in two out of three experiments (fold changes of averaged levels between DD and LD: 0.2, 0.3, and 1, supplemental Table S3).

To better disentangle the effect of light and the circadian clock on the daily fluctuations of circulating lipids in *Drosophila*, we determined the levels of DGs, PEs, and PCs in the hemolymph of $per^{01}$ clock mutants under LD (supplemental Fig. S2B). Since light has a significant effect on hemolymph lipid oscillations in wild-type flies, we expected a weak bimodal activity pattern in the clock-impaired flies. Yet, in contrast, daytime-dependent levels of DGs, PEs, and PCs in the hemolymph of $per^{01}$ mutants under LD varied greatly between the three independent experiments (supplemental Fig. S4), and the oscillations were significantly rhythmic in only one out of three experiments according to JTK_CYCLE (supplemental Table S2). After averaging the normalized data, hemolymph lipids levels showed no systematic daily variations (Fig. 2C, C′′), and rhythmicity was not detected (JTK_CYCLE adj.p: 0.6 and 1, supplemental Table S2). Interestingly, the averaged total lipid levels in the clock null mutant were wild type–like under LD conditions, with fold changes between $per^{01}$ and $WT_{CS}$ of 1.0–1.4 for DG, 1.1–2.1 for PEs, and 1.3–2.1 for PC (supplemental Table S3). These results suggest that a disrupted clock in $per^{01}$ mutants mainly affects the temporal profile of hemolymph lipid levels but not total levels. Importantly, the observed arrhythmicity of $per^{01}$ further demonstrates that light alone is not sufficient to drive the rhythmicity of hemolymph lipid levels but rather modulates the phase and shape of the clock-dependent rhythm.

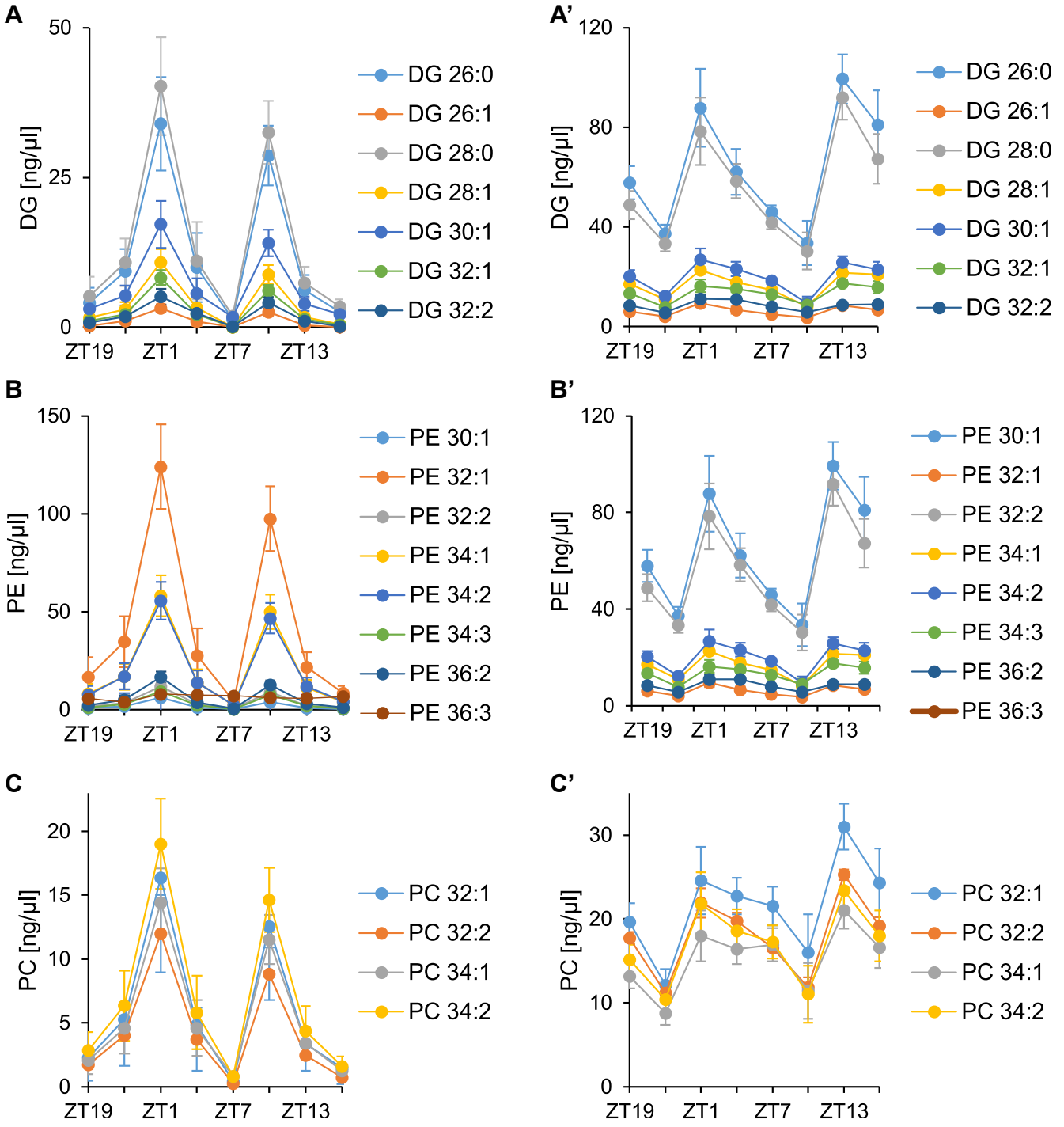


**Fig. 1.** Temporal pattern of hemolymph lipids in male $WT_{CS}$ flies is similar between individual lipid species. Levels of detected DG- (A, A'), PE- (B, B'), and PC-species (C, C') in the hemolymph of 6-day-old male $WT_{CS}$ flies fed ad libitum with sugar-only medium (A, B, C) or standard medium (A', B', C'). Six-day-old flies were sampled every 3 h over the course of 1 day. A lipid species is defined by the lipid class (first 2 letters), the number of acyl carbons (first 2 numbers), and the number of acyl double bonds (last number). The x-axis shows the Zeitgeber time in h (ZT0 = lights on, ZT12 = lights off). Data are presented as mean ± standard error (n=13).

### Access to a rich medium masks diel lipid oscillation patterns in the fly hemolymph

The above results show that hemolymph lipids oscillate in $WT_{CS}$ flies when the diet is restricted to sugar. We next asked whether daily rhythmicity of circulating lipids in *Drosophila* persists on a rich standard medium containing carbohydrates, proteins, and lipids. We determined the levels of DGs, PEs, and PCs in the hemolymph of $WT_{CS}$ male flies fed ad libitum on standard medium at 3-h intervals over a day in LD (supplemental Fig. S2B). On standard medium, DGs in the hemolymph can originate from the gut where they can be reassembled from dietary fatty acids or produced de novo from sugars or from TG mobilization

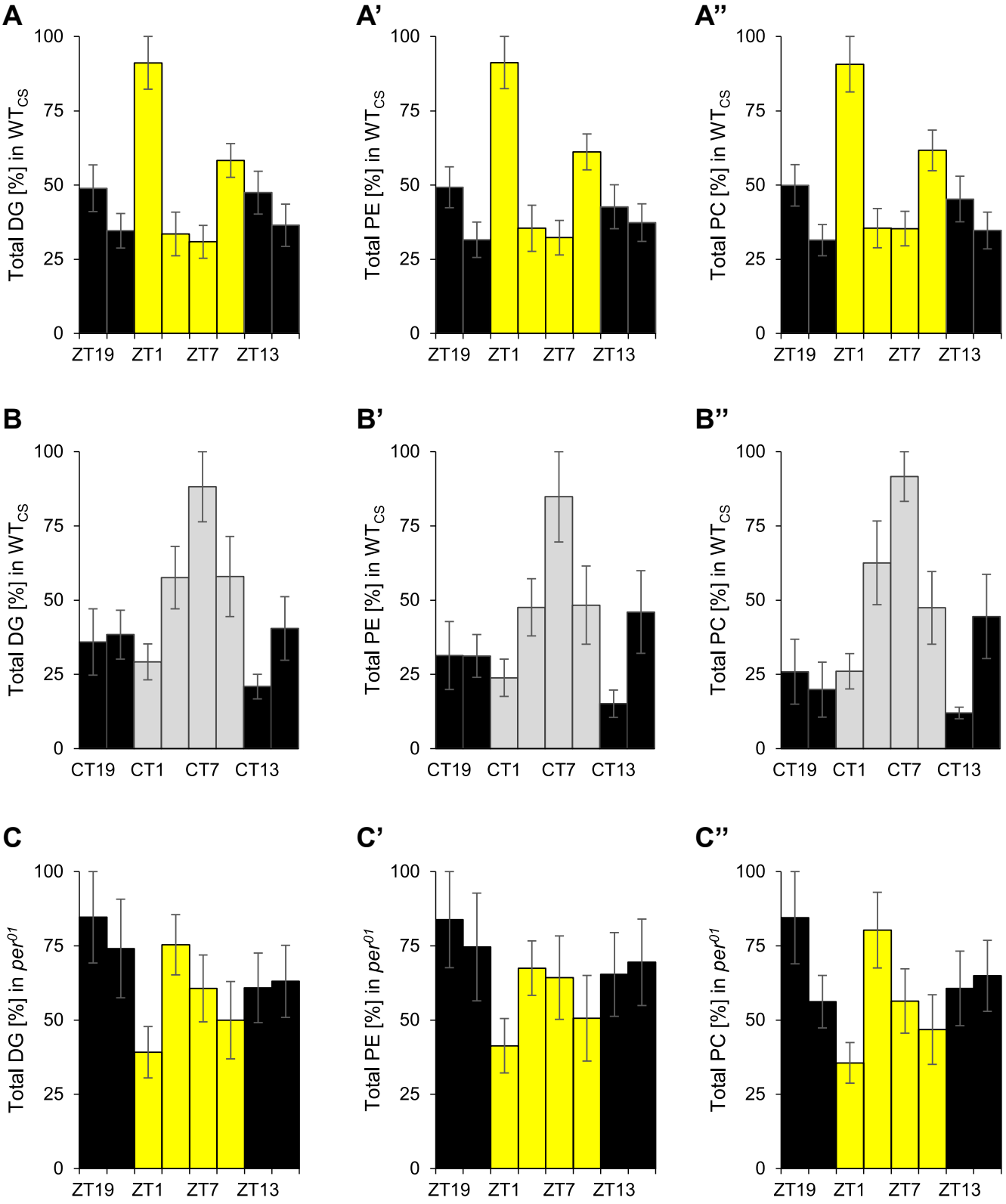


**Fig. 2.** Clock and light dependent diel oscillation of hemolymph lipids. Normalized levels of DGs (A, B, C), PEs (A', B', C), and PCs (A'', B'', C) in male flies reared on sugar-only medium. $WT_{CS}$ flies in LD (A, N = 7, n = 91) and DD (B, N = 3, n = 39) and $per^{01}$ flies in LD12:12 (C, N = 3, n = 39). The color of the bars indicates the light conditions. (LD: *yellow* = lights on, *black* = lights off; DD: *light gray* = subjective light phase, *dark gray* = subjective dark phase). Data represent mean ± standard error. The x-axis shows the Zeitgeber time (LD conditions) or circadian time (third day of DD conditions).

from the fat body (29). We found that the absolute levels in the hemolymph of flies reared on standard medium were approximately 5-fold higher for DGs, 7-fold higher for PEs, and 2-fold higher for PCs compared to sugar-only medium (supplemental Table S3), in agreement with a previous study (21). The relative amounts of hemolymph DGs were biased toward medium chain lengths (26–28) compared to a bias toward longer chain lengths (30–34) in whole bodies or heads containing the fat body (supplemental Fig. S5). Importantly, the DG composition differed considerably between the hemolymph, head and body, and the standard medium (supplemental Fig. S5, head and body data from a previous study (16)).

The temporal patterns of the individual DG, PC, and PE species on standard medium were largely similar (**Fig. 1**A′–C). This allowed us again to calculate their total levels which fluctuated only weakly with maxima between ZT22-ZT1 and at ZT13 in three out of six independently performed experiments (supplemental Fig. S6, supplemental Table S2). This weak bimodal peak pattern (**Fig. 3**) showed a relative increase of the evening peak compared to the situation on sugar-only medium (Fig. 2A). However, after normalization and averaging of all six experiments, JTK_CYCLE did not detect a significant daily rhythmicity (adj.*P* values = 0.6 and 1.0 shown in supplemental Table S2). One possible reason for the loss of hemolymph lipid oscillations on standard medium is masking by food-derived lipid uptake and production. Collectively, the results suggest that the daily rhythmicity in the hemolymph profiles of the analyzed lipids is mainly determined by mobilization from intracellular stores in the fat body or de novo synthesis from the gut, whereas flies stabilize hemolymph transport lipids at a constant high level when they have ad libitum access to a rich medium containing also proteins and lipids. These levels may mask or impair the effect of rhythmic lipid de novo synthesis or mobilization from intracellular stores.

### Feeding and locomotor activity rhythmicity are not phase-coupled to diel lipid oscillations under laboratory conditions

Feeding and locomotor activity are closely linked to metabolism and are modulated by the circadian clock in *Drosophila* (30–32). Therefore, we asked to what extent both behaviors contribute to the temporal profile of hemolymph lipids. We used a capillary feeding assay (33) to measure feeding, and TriKinetics activity monitors to record locomotor activity of $WT_{CS}$ and $per^{01}$ male flies. Consistent with our previous work (16), we observed a significant diel rhythmicity in locomotor activity (supplemental Fig. S7A) and food consumption (supplemental Fig. S8A) in $WT_{CS}$ on standard medium under LD (**Fig. 4**A, A′). In DD, circadian rhythmicity of locomotor activity (supplemental Fig. S7A) and food

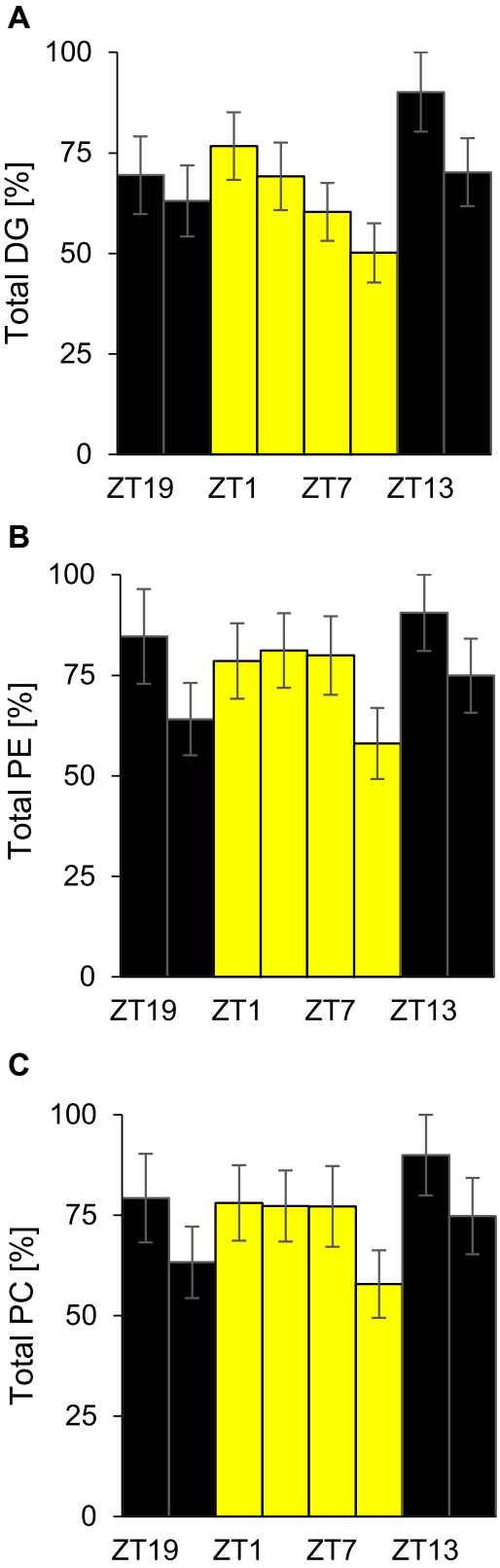


**Fig. 3.** Dietary lipids mask diel rhythmicity in hemolymph lipids. Normalized total levels of DGs (A), PEs (B) and PCs (C) in the hemolymph of $WT_{CS}$ male flies on standard medium. Color of the bars indicates light conditions (*yellow* = lights on and *black* = lights off). Data represent mean ± standard error (N = 6, n = 78).

consumption (supplemental Fig. S8B) persisted but with lower AMP (adj.p food consumption: $6e^{-18}$ in LD, $1e^{-6}$ in DD; % flies with rhythmic locomotor activity: 97% in LD, 97% in DD, supplemental Table S2). In contrast, the rhythmicity of $per^{01}$ mutants was already low in LD (adj.p food consumption: $4e^{-3}$; 81% flies with rhythmic locomotor activity, supplemental Tables S2 and S4) and became arrhythmic in DD (supplemental Figs. S7B and S8D).

We next asked whether a sugar-only diet alters the rhythmicity in feeding and locomotor activity. On sugar-only medium, the bimodal pattern of locomotor activity (supplemental Fig. S7A′) persisted in $WT_{CS}$ under LD (Fig. 4B) and DD conditions (Fig. 4C) in $WT_{CS}$ (91% rhythmic flies in LD and 78% rhythmic flies in DD, supplemental Table S4). This is not surprising as many *Drosophila* chronobiology labs routinely use sugar-only medium when recording locomotor activity. However, the diel oscillation of food consumption in $WT_{CS}$ was strongly reduced on sugar-only medium in LD (Fig. 4B′). JTK_CYCLE analysis of the normalized data of food consumption on each diet confirmed the reduced feeding rhythmicity on sugar-only medium compared to standard medium (adj.p in sugar-only medium: $1e^{-3}$, standard medium: $1e^{-18}$, supplemental Table S2) and revealed a phase shift of about 4 h (maximum (LAG) in sugar-only medium: ZT5, standard medium: ZT1, supplemental Table S2). Manual inspection of food consumption revealed damped oscillations in $WT_{CS}$ in DD (Fig. 4C′) compared to LD (Fig. 4B′), with similar oscillation parameters of the normalized data (adj.p: $1e^{-3}$ in LD, $2e^{-5}$ in DD; LAG in LD and DD: ZT5 and CT5; relative AMP: 13% in LD, 11% in DD, supplemental Table S2). The rhythmicity of feeding on sugar-only medium appears to be under the control of the circadian clock as feeding behavior in $per^{01}$ clock mutants was arrhythmic in both LD (Fig. 4D′, adj.p: 0.6–1, supplemental Table S2) and DD (supplemental Fig. S8B).

On standard medium, the peaks in food consumption and locomotor activity of $WT_{CS}$ flies (Fig. 4A, A′) did not correlate with peaks in hemolymph lipids (Fig. 3). However, on sugar-only medium in LD, the peaks of both behaviors (Fig. 4B, B′) temporally coincided with peaks in the circulating lipid titer (Fig. 2A, A′′), suggesting that light synchronizes locomotor activity, feeding, and circulating lipid levels in the absence of dietary lipids. This synchronization was lost in DD, as the phases of locomotor activity, feeding (peaks around dusk and dawn, Fig. 4C, C′). and hemolymph lipids (peak at midday siesta, Fig. 2B, B′′) separated. Specifically, locomotor activity peaked around CT0 and CT12, whereas the maximum of feeding behavior was CT5 (supplemental Table S2), and hemolymph lipid titers peaked between CT7 and CT9 (supplemental Table S2). This suggests that, under our conditions, the observed peaks in hemolymph lipids are not a consequence of activity-induced lipid mobilization.

### Time of restricted feeding does not alter the phase of hemolymph lipid oscillations

To test more directly whether lipid oscillations are a consequence of food consumption, we used a TRF paradigm. Flies were fed sugar-only medium only during the 12 h light phase or the 12 h dark phase. First, we monitored food consumption in $WT_{CS}$ flies over 6 days. We found no significant difference in total food consumption during this period between light- and dark phase-TRF flies (1.6 ± 0.4 vs. 2.0 ± 0.6 µl/fly).

During light phase-TRF, the flies showed a tendency to eat more between ZT3 and ZT6 (**Fig. 5**A), but consumption was more or less stable throughout the light phase. During dark phase-TRF, food consumption was more variable with no consistent pattern during the 12 h of darkness (Fig. 5B). Switching the flies back from TRF to ad libitum feeding resulted in a normal diel rhythmicity of food consumption on the 2nd day (Fig. 5C,D and supplemental Table S2), as previously reported (34).

Next, we analyzed the levels of DGs, PEs, and PCs in LD in the hemolymph of $WT_{CS}$ flies subjected to light- or dark-phase TRF for 3 consecutive days. Hemolymph was sampled every 3 hours on the last day of TRF (supplemental Fig. S1D, E). Irrespective of the TRF conditions, hemolymph lipids levels oscillated unimodally with a maximum at ZT6-ZT10 (**Fig. 6**). The main differences in the pattern pertained to the AMP at ZT7, which was significantly higher for DGs (Fig. 6A) and significantly lower for PEs (Fig. 6B) and PCs (Fig. 6C) in light-phase than in dark-phase TRF. Moreover, the levels of all three lipid classes were constantly higher from ZT10-ZT19 in dark-phase TRF flies compared to light-phase TRF flies. These differences correlated well with the time the flies had access to food. Time-dependent levels of DGs, PEs, and PCs in the hemolymph were not always classified as rhythmic by JTK_CYCLE possibly due to the high variance resulting in a high adj.*P* value (supplemental Table S2). Interestingly, the oscillation profiles of $WT_{CS}$ hemolymph lipids under TRF in LD were very similar to the profile observed under ad libitum feeding in DD, with a monomodal peak around midday. Furthermore, the finding of a monomodal peak in lipid levels around midday regardless of whether TRF was restricted to the photophase or the scotophase (Fig. 6) provides strong evidence that the temporal profile of hemolymph lipid levels is under the control of the circadian clock and is not a direct consequence of feeding.

## DISCUSSION

The fruit fly *Drosophila* is a prime model for circadian research (35) and provides ample opportunity to study the interplay between the circadian clock and metabolism. As a result, several metabolomic studies have characterized the global effects of an impaired clock on

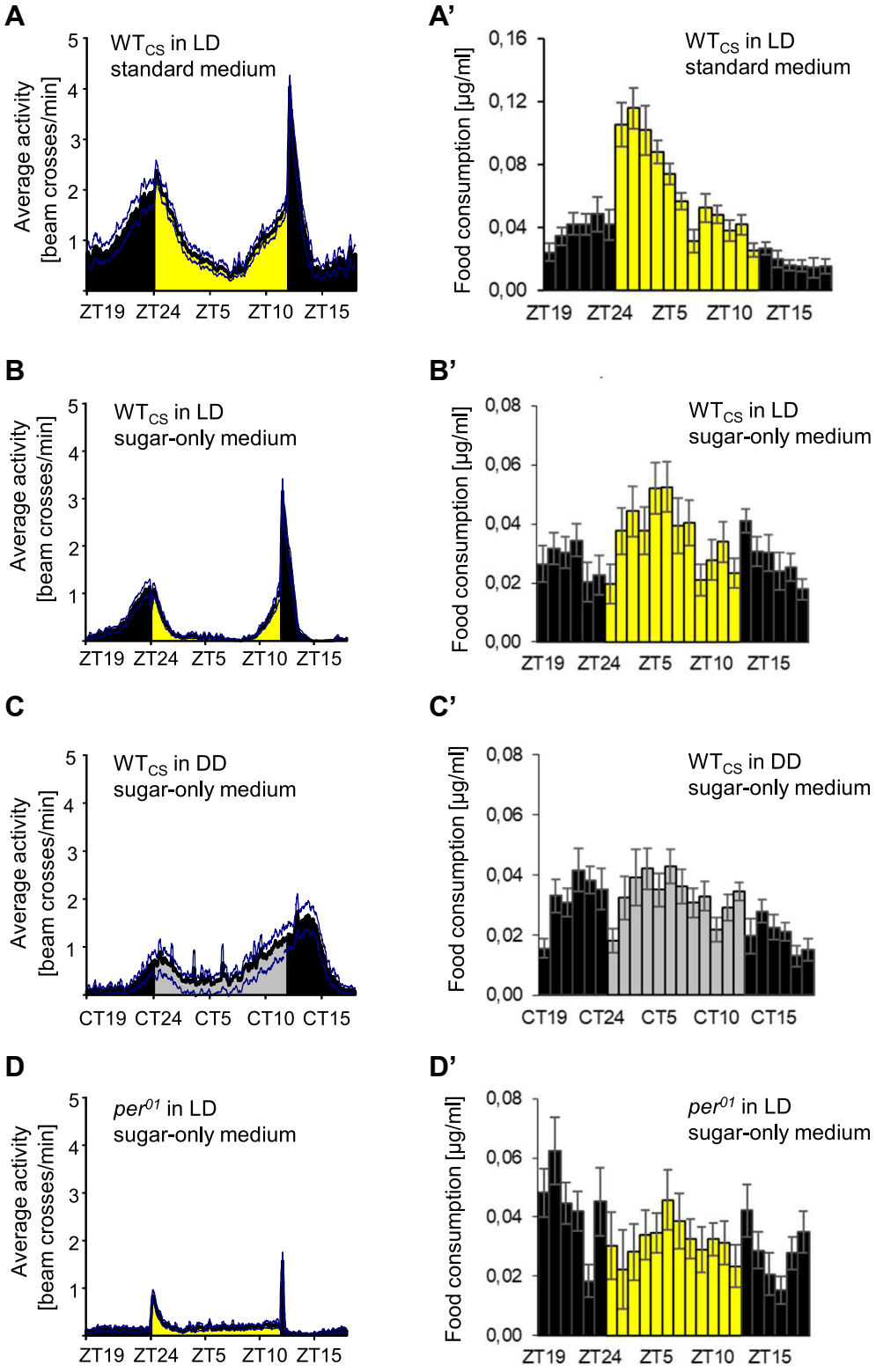


**Fig. 4.** Diel rhythmicity profiles of locomotor activity and food consumption differ in $WT_{CS}$ flies. Locomotor activity (A, B, C, D) and food consumption (A′, B′, C′, D′) were monitored in $WT_{CS}$ in LD on standard medium (A, A′) and sugar-only medium (B, B′). In addition, both behaviors were examined in $WT_{CS}$ in DD (C, C′) and $per^{01}$ in LD (D, D′) on sugar-only medium. Color of the bars indicates light conditions (LD: *yellow* = lights on and *black* = lights off; DD: *light gray* = subjective light phase; *dark gray* = subjective dark phase). Data represent mean ± standard error (N = 2, n = 10 for CAFÉ assays and n = 29–32 for locomotor activity). CAFÉ, capillary feeding assay.

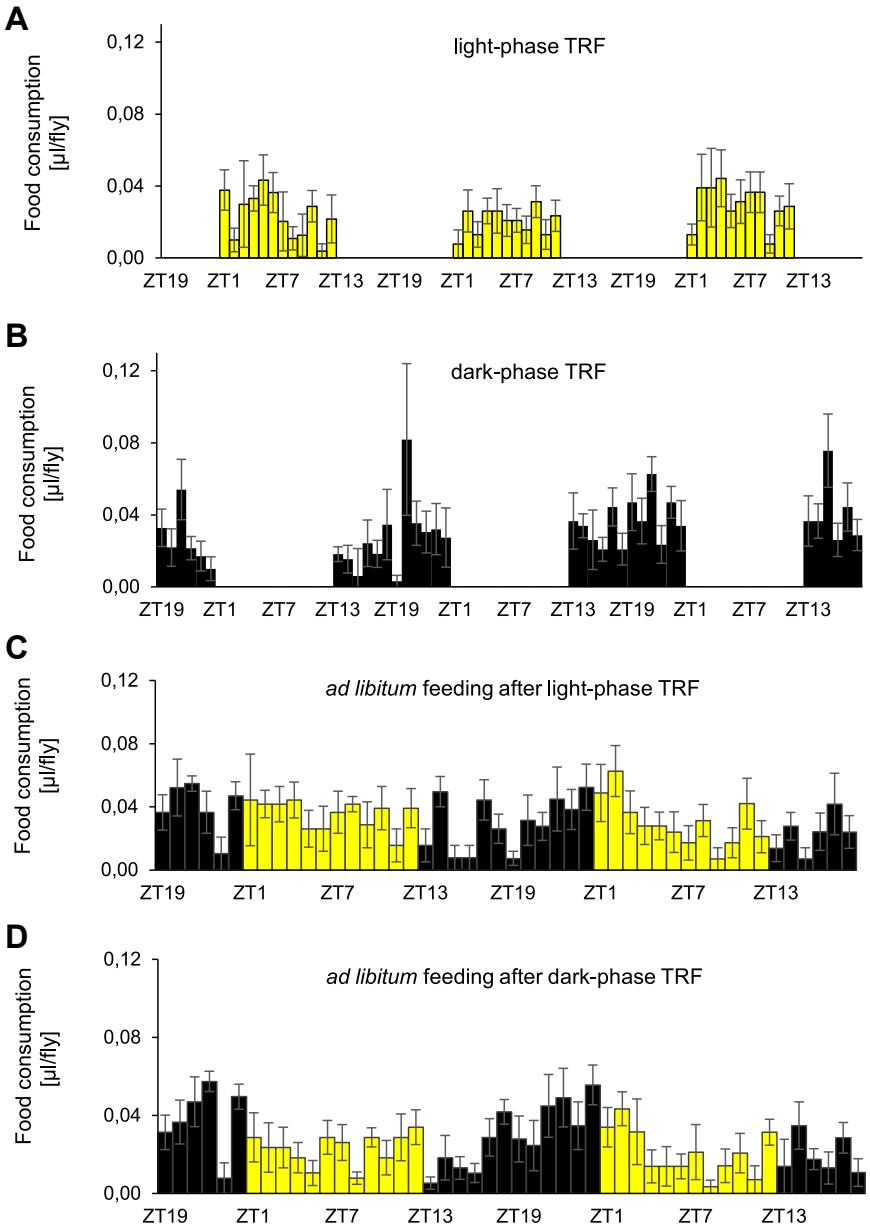


**Fig. 5.** Time-restricted feeding does not entrain the temporal pattern of food consumption. $WT_{CS}$ males were fed only during the light (A) or dark (B) phase of LD. Food consumption was monitored from day 4 to day 6 using a modified CAFÉ assay. After light-phase (C) or dark-phase TRF (D), flies were fed ad libitum with sugar-only medium and food consumption in LD was monitored for two further days. The color of the bars indicates the light conditions (*yellow* = lights on and *black* = lights off). Data are presented as mean ± standard error (n = 5). CAFÉ, capillary feeding assay.

metabolism in *Drosophila*, including lipids (15–17). However, the temporal profile and clock dependence of transport lipids in the hemolymph remained unknown. Transport lipids represent a hub for lipid mobilization and de novo synthesis and can serve as a proxy to assess overall lipid metabolism. Therefore, here we characterized the temporal profiles of the major lipid classes in the hemolymph of the fruit fly *Drosophila* and their dependence on the circadian clock, light, locomotor activity, and feeding.

### Lipids in the fly hemolymph show diel oscillations that are influenced by the clock and light and masked by access to a full diet

A major conclusion of our work is that hemolymph DG, PE, and PC levels show clock- and light-dependent oscillations in phase with each other in flies kept on sugar-only diet. Specifically, the levels of DGs, PEs, and PCs peaked in the early morning and late afternoon in the hemolymph of male $WT_{CS}$ in LD on sugar-only medium. This temporal profile was altered to a

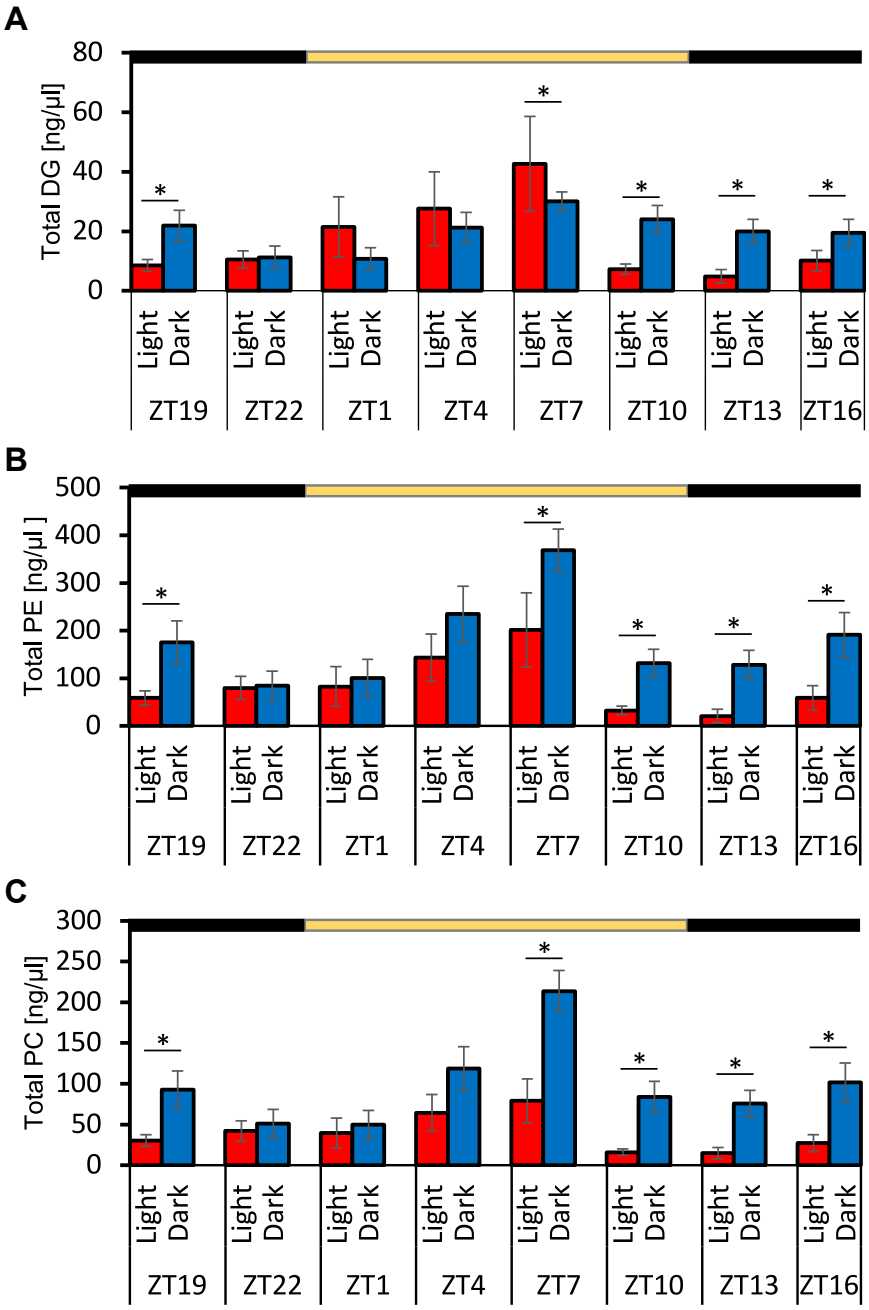


**Fig. 6.** Time-restricted feeding does not alter the phase of hemolymph lipid oscillations. $WT_{CS}$ males were fed for 3 days only during the light (red bars) or dark (blue bars) phase in LD, and the total levels of DGs (A), PEs (B), and PCs (C) in the hemolymph were measured. *Asterisks* indicate statistically significant differences between light- and dark phase-TRF ($P < 0.05$, *t* test). The top bar indicates lights-on (*yellow*) and lights-off (*black*). Data are presented as mean ± standard error (n = 11).

monomodal oscillation with a peak at the subjective midday in DD, whereas the oscillations were severely impaired in LD in $per^{01}$ clock mutants. Taken together, the results suggest that the circadian clock is the main driver of the oscillations, while light has a significant effect on the phase and temporal profile. These findings are consistent with metabolomic studies on *Drosophila* heads and bodies, which found an influence of both the circadian clock and the light phase on the oscillations of lipids and other metabolites (15, 16).

Notably, $per^{01}$ clock mutant flies in our study showed unaltered total levels of circulating lipids in LD. This is in contrast to the situation in rodents where disruption of the molecular clock leads to altered levels of circulating lipids (see (36)). For example, plasma TG levels were higher in *Bmal1* (37) and lower in *Per2* mutant mice (38).

Another interesting finding of our work is that the levels of DGs, PEs, and PCs in the hemolymph oscillate in phase with each other. This result is in contrast to the situation in humans, where the daily oscillation of blood glycerolipids and PCs are out of phase on a lipid-poor diet (9). This difference may be easily explained by differences in the mode of lipid transport between

insects and mammals. In mammals, the interorgan transport of lipids occurs by multiple and organ/lipid species-specific means, including chylomicrons and lipoproteins, and the timing of circulating TGs is dependent on the type of lipoprotein (high density lipoproteins or apoB-lipoprotein) (39). In the insect hemolymph, DGs are primarily transported by lipophorin particles that are surrounded by phospholipids including PEs and PCs derived from the fat body (20, 21). We propose that this co-shuttling of glycerolipids and phospholipids between organs in lipophorin particles underlies the observed phase synchronicity of circulating lipids in flies.

Diet affects the circadian clock machinery (40), and diet has been shown to affect metabolite oscillations as well (41–43). In mice, for example, most serum lipids lose diel rhythmicity after a high fat diet (41). Our study shows that hemolymph lipid oscillations were strongly dampened and disappeared when flies had ad libitum access to a rich standard medium containing lipids, sugars, proteins, and micronutrients. We conclude from this that continuous access to exogenous (dietary) supply of lipids, carbohydrates, and proteins masks the diel oscillations of glycerolipids and phospholipids in the hemolymph of *Drosophila*. Combined with the increased food intake on standard medium compared to sugar-only medium, the loss of circadian fluctuations may be interpreted as a sign of stability. The high levels of hemolymph lipids may serve as a buffer to compensate for short-term changes resulting from physical activity, feeding, or lipid synthesis/mobilization.

The overall higher levels of hemolymph lipids in flies on a full diet compared to sugar-only medium is consistent with previous data (21, 44) and might be attributed to increased lipid production and uptake.

Collectively, our data suggest an overall dominant influence of dietary lipid and protein intake on fly hemolymph lipid levels.

The composition of DGs in our standard medium (biased toward long chain fatty acids) was clearly different from the glycerolipid composition in the hemolymph (biased toward medium chain fatty acids). This suggests that dietary DGs are not directly transported into the hemolymph but are metabolized or modified in the insect digestive tract. This observation is consistent with previous data that suggest a difference in DG species due to shortening of dietary fatty acids prior to export to target organs via the hemolymph (44).

### Rhythmicity of DGs in the fly hemolymph on sugar-only medium likely involves de novo synthesis in the gut

The primary sources of hemolymph lipids on sugar-only medium are either mobilization from the fat body or de novo synthesis from the gut (see (19, 21, 45)). We have not investigated this further here, although it could be done by stable isotope-labeled feeding. Nevertheless, two findings suggest that lipid de novo synthesis is daytime-dependent at least on a nutrient-depleted diet. First, the peak times of hemolymph lipid levels and locomotor activity (the dominant energy-consuming behavior possible under our laboratory conditions) are not synchronized or in a fixed phase-relationship (as would be expected if mobilization is involved) but dissociate in DD. Second, we observed a bias toward medium-chain DGs (DG26:X, DG28:X) and a lower degree of desaturation for DGs in the hemolymph than in whole heads or bodies comprising the fat body. While the majority of DGs is found in the hemolymph, DGs occur in lower levels also in the fat body (44). In the hemolymph, medium-chain DGs are by far the dominant DG species, while long-chain DGs are a very small fraction. In contrast, the fat body contains approximately equal amounts of DGs with medium and long-chain fatty acyls (44). The medium-chain DGs are mostly produced in the gut, as their levels are massively increased in the gut upon genetic downregulation of lipophorin (*Lpp*) (21). This effect is also observed in lipid-depleted medium (21).

### Diel oscillations in hemolymph lipids are not driven by rhythmic locomotor activity or feeding

Metabolism is strongly influenced by physical activity and nutrient intake. To analyze their influence on diel oscillations in hemolymph lipids, we compared the temporal lipid profiles with those of the energy supplying behavior (feeding) and the most overt energy-consuming behavior (locomotor activity). On standard medium in LD, hemolymph lipid levels remained a relatively constant level, whereas both feeding behavior and locomotor activity oscillated with distinct morning and smaller evening peaks. This suggests that energy supply and expenditure are balanced under these conditions. In addition, both feeding behavior and locomotor activity obviously do not have a direct instantaneous influence on hemolymph lipid oscillations. This also appears to be the case on sugar-only medium, where lipid levels were out of phase with feeding (LD) or locomotor activity (DD). Further support comes from the finding that $per^{01}$ mutants in LD lost their rhythmic diel oscillations in hemolymph lipids and feeding behavior although locomotor activity maintained light-driven rhythmicity.

We note that our experiments were conducted with flies housed in small vials, which provide little opportunity and incentive for very intense and sustained physical exercise such as longer flight bouts requiring lipid mobilization. It would therefore be interesting to repeat similar experiments in a more natural environment that allows energy-demanding activities such as intensive foraging or flight.

In mammals as well as in *Drosophila*, TRF is a strong "Zeitgeber" capable of resetting the circadian clock (23, 46–50). Changes in feeding pattern or feeding time with different TRF feeding paradigms affect hepatic lipid homeostasis in mice (51, 52). Also, in lactating

Holstein cows, an 8–12 h shift in peak levels of metabolites, plasma hormones, and milk synthesis was observed between night-time and day-time restricted feeding (53). Here, we found that both light-phase TRF and dark-phase TRF shifted the hemolymph lipid peak to the middle of the light phase when compared to ad libitum feeding on sugar-only medium. Thus, TRF does not entrain but phase-shifts the rhythmicity of circulating glycero(phospho)lipids in *Drosophila*, an effect strikingly similar to the situation for hepatic TGs in mice (51).

In conclusion, our data show that DGs, PEs, and PCs in the fly hemolymph oscillate in a clock- and light-dependent manner in flies without access to dietary proteins and lipids. This cycling appears to be largely independent of the time of feeding. Based on the observed differences in lipid species composition between the hemolymph, fat body, and food source, we suggest that the observed lipid oscillations on lipid-free diet is primarily due to rhythmic de novo lipid synthesis in the gut, rather than rhythmic lipid mobilization. This hypothesis can be tested in the future, for example in flies with disrupted peripheral clocks specifically in the fat body or gut. Regardless whether hemolymph transport lipids originate from de novo synthesis in the gut or are mobilized from the fat body, the temporal profile under constant conditions suggests that the clock serves to align transport lipid levels with an optimal time window for anabolism. This hypothesis and possible health implications need to be tested with tissue-specific genetic manipulations of the clock and underlying metabolic pathways, for which *Drosophila* provides ample tools.

## Data availability

The data that support the findings of this study are available from the last author (Dr Agnes Fekete, agnes.fekete@uni-wueruburg.de) upon reasonable request.

*Supplemental data*

This article contains supplemental data.

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*Author contributions*

Kelechi Michael Amatobi: performing metabolite analysis including hemolymph extraction, monitoring of food consumption, fly rearing, writing. Ayten Gizem Ozbek-Unal: monitoring of locomotor activity, writing. Stefan Schäbler: performing metabolite analysis, analysis troubleshooting. Peter Deppisch: monitoring locomotor activity. Martin J Mueller: designing experiments, consultancy in metabolite analysis. Charlotte Förster: designing experiments, consultancy in chronobiology. Christian Wegener: designing experiments, writing, consultancy in monitoring of food consumption, supervision. Agnes Fekete: designing experiments, writing, consultancy in metabolite analysis, supervision.

*Author ORCIDs*

Ayten Gizem Ozbek-Unal https://orcid.org/0000-0002-0548-9568
Peter Deppisch https://orcid.org/0000-0002-7897-5348
Charlotte Helfrich-Förster https://orcid.org/0000-0002-0859-9092
Christian Wegener https://orcid.org/0000-0003-4481-3567
Agnes Fekete https://orcid.org/0000-0003-3058-7570

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*Conflict of interest*

The authors declare that they have no conflicts of interest with the contents of this article.

*Abbreviations*

AMP, amplitude; DD, constant darkness; DG, diacylglycerol; LAG, lag phase; LD, light:dark; PC, phosphocholine; PE, phosphoethanolamine; TG, triacylglycerol; TRF, time-restricted feeding.

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