Circadian clocks determine transport and membrane lipid oscillation in *Drosophila* hemolymph in complex interactions between nutrient-type, photic conditions and feeding behaviour

Die innere Uhr bestimmt den Transport und die Membranlipid-Oszillation in der *Drosophila* Hämolymphe in komplexen Interaktionen zwischen Nährstofftyp, photischen Bedingungen und Fressverhalten

Doctoral Thesis

for the conferral of the degree "Doctor rerum naturalium" (Dr. rer. nat.)

at the Graduate School of Life Sciences, Integrative Biology Section

Julius-Maximilians-Universität

Würzburg

Submitted by

Kelechi Michael Amatobi

Born in Gboko, Nigeria

Würzburg, 2021

Submitted on: ……...……...

Office stamp

Members of the Thesis Committee:

Chairperson: Prof Dr. Thomas Müller

Primary Supervisor: Dr. Agnes Fekete

Supervisor (Second): Prof. Christian Wegener

Supervisor (Third): Prof Charlotte Förster

Supervisor (Fourth): Prof. Dr. Dr. Martin J. Müller

Date of Public Defence: ……..……

Date of Receipt of Certificates: ……

Summary

The interaction between circadian clocks and metabolism is of increasing interest, since clock dysfunction often correlates with metabolic pathologies. Many research articles have been published analysing the impact of factors such as circadian clock, light, feeding time and diet-type on energy homeostasis in various tissues/organs of organisms with most of the findings done in mammals. Little is known about the impact of circadian clock and the above-mentioned factors on circulating lipids, especially the transport form of lipids - diacylglycerol (DG) and membrane lipids such as phosphatidylethanolamine (PE) and phosphatidylcholine (PC) in the *Drosophila* hemolymph. The fruit fly *Drosophila* is a prime model organism in circadian, behaviour and metabolism research.

To study the role of circadian clock and behaviour in metabolism, we performed an extensive comparative hemolymph lipid (diacylglycerol: DG, phosphatidylethanolamine: PE, phosphatidylcholine: PC) analysis using ultra performance liquid chromatography coupled to time-of-flight mass spectrometry (UPLC-MS) between wild-type flies (WT_{CS}) and clock disrupted mutants (per^{01}). In addition, clock controlled food intake– feeding behaviour was investigated. Time-dependent variation of transport (DG) and membrane lipids (PE and PC) were not rhythmic in WT_{CS} under constant darkness and in per^{01} under LD, suggesting an impact of light and clock genes on daily lipid oscillations. Day-time and night-time restriction of food led to comparable lipid profiles, suggesting that lipid oscillations are not exclusively entrained by feeding but rather are endogenously regulated. Ultradian oscillations in lipid levels in WT_{CS} under LD were masked by digested fatty acids since lipid levels peaked more robustly at the beginning and end of light phase when flies were fed a lipid- and protein-free diet. These results suggest that metabolite (DG, PE and PC) oscillation is influenced by complex interactions between nutrient-type, photic conditions, circadian clock and feeding time.

In conclusion, the results of this thesis suggest that circadian clocks determine transport and membrane lipid oscillation in *Drosophila* hemolymph in complex interactions between nutrient-type, photic conditions and feeding behaviour.

Zusammenfassung

Die Interaktion zwischen Innerer Uhr und Metabolismus ist von zunehmendem Interesse, weil Störungen der Inneren Uhr oft mit metabolischen Störungen assoziiert sind. Zahlreiche Untersuchungen zum Einfluss verschiedener Faktoren, u.a. der Inneren Uhr, Lichtregime, Zeitpunkt der Nahrungsaufnahme und Art der Diät, auf die Energiehomöostase in verschiedenen Geweben und Organen wurden vor allem in Säugetieren durchgeführt. Der Einfluss der Inneren Uhr und der oben genannten weiteren Faktoren auf zirkulierende Lipide in der Hämolymphe von *Drosophila*, insbesondere auf die Transportform Diacylglycerol (DG) und Membranlipide (wie z.B. Phosphatidylethanolamin (PE) und Phospathidylcholine (PC)), ist jedoch kaum untersucht. Die Taufliege *Drosophila* dient dabei als hervorragendes Modell in der circadianen Verhaltens- und Metabolismusforschung.

Um die Rolle der Inneren Uhr und circadianen Verhaltens auf den Metabolismus zu untersuchen, haben wir eine extensive und vergleichende Lipidanalyse (DG, PE, PC) in der Hämolymphe von Wildtyp-Fliegen (WT_{CS}) und Uhrmutanten (per^{01}) mittels Ultrahochleistungs-Flüssigkeits-chromatographie gekoppelt mit Flugzeit-Massenspektrometrie (UPLC-MS) durchgeführt. Gleichzeitig wurde auch die circadian gesteuerte Nahrungsaufnahme untersucht. Die zeitabhängigen Schwankungen der Transport- (DG) und Membranlipide (PE, PC) unterlagen keiner tageszeitlichen Rhythmik in konstanter Dunkelheit in Wildtypfliegen, und unter Licht-Dunkelwechsel (LD) in per^{01} Mutanten. Dies weist auf einen Einfluss der Inneren Uhr und des Lichts auf tägliche Lipidschwankungen hin. Restriktion der Futtergabe auf entweder Tag oder Nacht ergab ähnliche Lipidprofile, was darauf hinweist, daß Schwankungen in den Lipidkonzentrationen nicht ausschliesslich durch die Nahrungsaufnahme, sondern auch endogen geregelt werden. Ultradiane Oszillationen in der Lipidkonzentration in WT_{CS} unter LD-Bedingungen wurden durch mit der Nahrung aufgenommene Fettsäuren maskiert, zeigten sich aber deutlicher zu Beginn und Ende der Lichtphase wenn die Fliegen auf einer Lipid- und Protein-freien Diät gehalten wurden. Diese Ergebnisse weisen darauf hin, daß Oszillationen in Lipiden (DG, PE und PC) in der Hämolymphe durch eine komplexe Interaktion zwischen Diättyp, Lichtregime, Innerer Uhr und Zeitpunkt der Nahrungsaufnahme bestimmt wird.

Zusammengenommen zeigen die Resultate dieser Arbeit, dass die Innere Uhr in komplexer Interaktion mit Diättyp, Lichtregime und Freßverhalten das zeitliche Profil von Transport- und Membranlipiden in der *Drosophila*-Hämolymphe bestimmt.

Table of Contents

1 INTRODUCTION

1.1 Circadian clock

1.1.1 Circadian clock and general health

A very common phenomenon observed by organisms including humans is the sunrise and sunset which occurs due to the Earth's rotation on its own axis every 24hrs. This phenomenon is anticipated by organisms using circadian clocks or biological clocks. Circadian clock is a conserved molecular machinery consisting of a transcriptional/translational feedback loops that generates circadian rhythms. Furthermore, circadian rhythms regulate a variety of biological processes in many organisms from unicellular to multicellular organisms (Bell-Pedersen et al., 2005; Lowrey & Takahashi, 2004). These biological processes include, but are not limited to, metabolic and physiological processes. When these circadian controlled rhythms and processes are altered via genetic and/or environmental agitation, different metabolic and physiological associated diseases could ensue (Hastings et al., 2003; Lucassen et al., 2012; Zizi et al., 2010). Among these disease conditions developed by disruption of circadian rhythm, metabolism-related diseases seem to be on the rise especially in modern day society due to changes in lifestyle, nutrition, social and commercial pressures. Such perturbations result in the destabilization of internal timing order thereby leading to increase in circadian stress which ultimately progresses to illnesses such as cardiovascular diseases, diabetes and hypercholesterolemia (Ghiasvand et al., 2006; A. Pan et al., 2011; Rajaratnam & Arendt, 2001). Therefore, it is pertinent to understand the mechanisms by which the circadian clock influences physiological and metabolic processes in humans and model organisms such as *Drosophila*.

1.1.2 Similarities and differences between circadian clocks of mammals and *Drosophila*

Scientific methods such as genetics, recombinant DNA technology, genetic screens, DNA sequencing and PCR methods over the past years has given great insight into understanding circadian rhythms (Rosbash, 2009). Genetic screens and DNA sequencing of many living organisms such *Drosophila*, Neurospora, humans, mice, plants and cyanobacteria revealed multiple circadian genes and protein sequences. When the above-mentioned methods were coupled to PCR methods to bootstrap different living organisms (Sun et al., 1997; Tei et al., 1997), similarities in clock proteins were observed between the systems. For instance, *Drosophila* and mammals construct their molecular clocks using ortholog genes (Hamilton &

Kay, 2008; Young & Kay, 2001; Yu & Hardin, 2006). Clock-Cycle (CLK-CYC) heterodimer as well as the negative regulator period (PER) in fly have orthologs in mammals known as CLK-BMAL and PER1/PER2 indicating that the basic clock mechanism in animals emanated from a common ancestor which then diversified between insects and mammals 500 million years back (Rosbash, 2009). Apart from the similarities, there are obvious differences from an evolutionary point between the mammalian and fly clocks (Rosato et al., 2006). Mammals tend to possess clock genes with duplicated copies (paralogues) whereas *Drosophila* clock genes usually do not possess duplicated copies (Looby & Loudon, 2005; Tauber et al., 2004). For instance, cryptochrome (*cry*) genes have at least two copies in mammals whereas in *Drosophila* it exists in only one copy (Rosato et al., 2006). When duplication of genes occurs, it leads to less selective constraint on the new gene thereby leading to functional divergence (Susumu, 1970; Zhang, 2003). All in all, the first circadian gene (per^{01}) was discovered in *Drosophila* and has been well characterized as reviewed by Charlotte Förster (Helfrich-Forster, 2002). This has given *Drosophila* an advantageous position as one of the main model organisms to study circadian related processes.

1.1.3 Circadian clock in mammals

Mammalian circadian clock can be said to be divided into a central clock and peripheral clocks. These clocks work in synchrony with the environment and other factors to drive various physiological, metabolic and behavioural changes in animals (Kumar Jha et al., 2015). Initially, the interaction between the central and peripheral clocks was thought to be hierarchical whereby the central clock acts as the "master" regulator that controls the peripheral clocks (Albrecht & Eichele, 2003; Ralph et al., 1990). But current studies have shown that the peripheral clock could be using the central clock for better synchronization and this is supported by the fact that time restricted feeding disconnects the peripheral clock alignment from the central clock (Damiola, 2000; Stokkan et al., 2001). Furthermore, the mechanism of the central clock seems to be different from that of peripheral clocks (Glossop & Hardin, 2002a; Oishi et al., 2000).

1.1.3.1 Mammalian central clock

The central clock in mammals is situated in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus (Klein et al., 1990). The SCN is made up of a network of approximately 10,000

to 20,000 neurons with each neuron believed to contain an independent cell autonomous circadian oscillator capable to generate self-sustained circadian rhythms shown (Herzog, 2007; Lehane, & Billingsley, 1996; Lowrey & Takahashi, 2011). The population of SCN neurons couple together forming a network that generates synchronized rhythms (Herzog, 2007; Welsh et al., 2010). Molecularly, the central circadian clock composes of transcriptional/translational positive and negative interdependent feedback loops shown in Fig 1 (Ravi Allada et al., 2001; Meyer-Bernstein & Sehgal, 2001; Young & Kay, 2001).

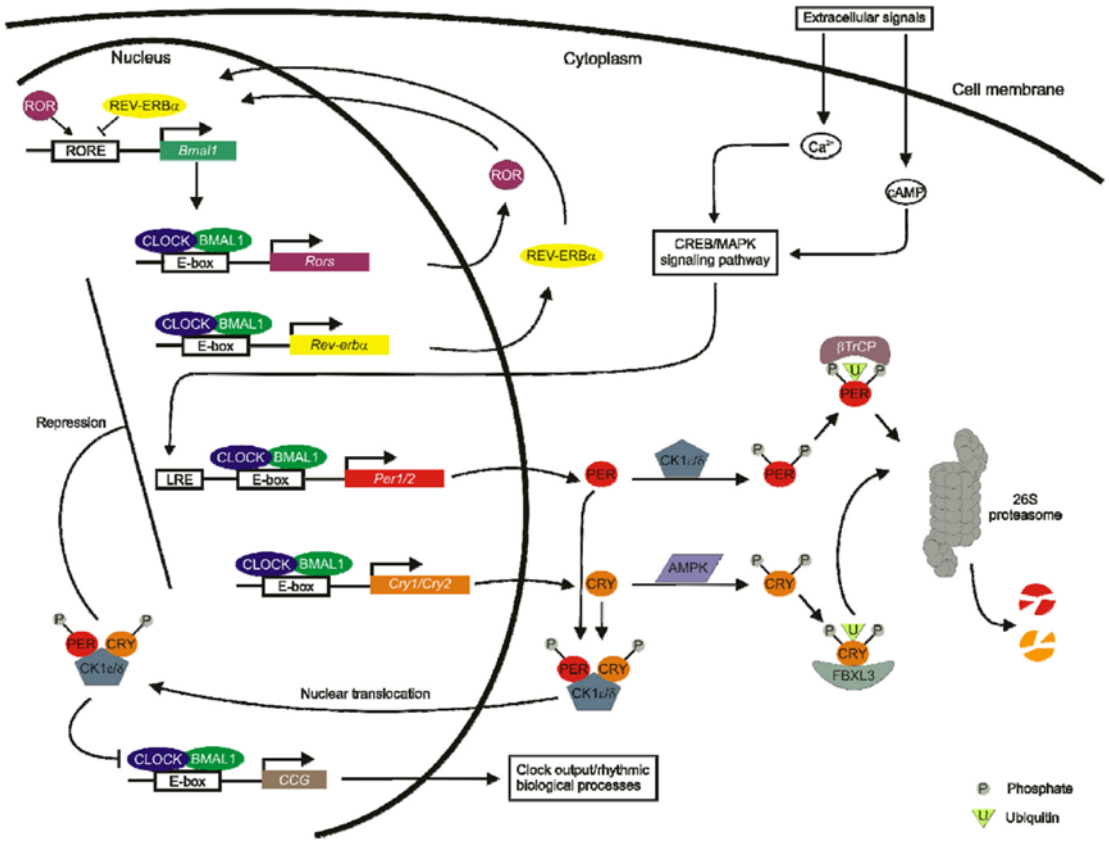


Figure 1. Schematics of mammalian circadian oscillator (Lowrey & Takahashi, 2011)

The positive feedback loop consists of CLK/BMAL1 heterodimers which are two basic helix-loop-helix transcriptional activators. They bind on the E-boxes that are situated on the regulatory region of period (*per*) and *cryptochrome* (*cry*) genes, thereby leading to their transcription (Albrecht & Eichele, 2003; Reppert & Weaver, 2002). PER and CRY which are translational products of *per* and *cry* form oligomers which accumulate in the cytoplasm and then translocate into the nucleus where they repress their own gene transcription by inhibiting the actions of CLK/BMAL1 thereby constituting a negative feedback loop (Mohawk et al.,

2012). Additionally, there is another negative feedback loop which consist of REB-ERBα and –β and they bind to ROR response element (RRE) of CLOCK/BMAL1 promoters to repress their own transcription. Conversely, RORα/β/γ binds on RRE of BMAL1 to induce its own transcription (Crumbley & Burris, 2011; Douglas, 2013; Preitner et al., 2002). The above regulations become more complex when the number of genes and paralogs are considered for example in mammals there are three per genes (per^1, per^2 and per^3) and two cry genes (cry^1 and cry^2) (Lowrey & Takahashi, 2004). Additionally, $BMAL^1$ has a paralog known as $BMAL^2$ which is functionally redundant while $NPAS^2$ is the paralog of CLK and it collaborates with $BMAL^1$ to sustain circadian clock function when CLK protein is absent (Bertolucci et al., 2008; DeBruyne et al., 2006, 2007; S. Shi et al., 2010).

1.1.3.2 Mammalian peripheral clocks

Peripheral clocks or oscillators exist throughout the cells and tissues of mammals. At first, it was thought that SCN neurons (central clock) were the only oscillators capable of self-sustained oscillations. But studies performed in culture have shown that non-SCN cells are capable of endogenous circadian oscillations (Aurélio Balsalobre et al., 1998; Yamazaki, 2000a; Yoo et al., 2004a). Although the molecular machinery of circadian clock is conserved in peripheral clocks just like in SCN (Yagita, 2001), there seems to be obvious differences in terms of resetting, output pathways and regulation (Glossop & Hardin, 2002a; Mohawk et al., 2012). Despite these differences, they both share the same core clock components based a transcriptional/translational feedback loop (Dibner et al., 2010).

Peripheral organs which showed self-sustaining oscillations as reflected by *ex vivo* cultures of liver, skeletal muscle tissue and lung showed dampened oscillation and eventually lost their synchrony after SCN lesion (Yamazaki, 2000b; Yoo et al., 2004b). However, there are studies that have shown that cell autonomous clocks of peripheral tissues can support self-sustaining rhythmic cell functions without dependence on SCN (M. Shi & Zheng, 2013). For example, a number of human metabolic pathways are controlled by circadian peripheral clocks without dependency on sleep and feeding (Dallmann et al., 2012a).

1.1.3.3 Interaction between the SCN and peripheral clocks in mammals

To address the interaction of the central clock with peripheral clocks, it is important to know how the different neurons in the SCN are synchronized to the environment and to one another before its synchronization with peripheral clocks. The SCN is synchronized to the environment by light which is the primary Zeitgeber signal. Light is recognized by the eye as photic signals which are then transmitted to the SCN through cyclic AMP response element–binding (CREB) protein allowing the synchronization of postsynaptic neurons (C. Liu et al., 2007). Rods and cones are photoreceptors found in the outer retina of the eye and they serve as important mediators transmitting light signals to the retina ganglion cells (RGCs) which are neurons occupying the inner part of the retina (C. Liu et al., 2007). A subset of RGCs known as ipRGCs (intrinsically photosensitive RGC) which expresses photosensitive melanopsin and can fire independently of rods and cones when exposed to light (Berson, 2002; Hattar, 2002). SCN is needed for the synchronization of peripheral clocks to environmental factors such as light entrainment (A. C. Liu et al., 2007). The synchronization of the SCN to peripheral tissues is achieved via neuronal transmission (neuronal projections and intermediate neurons) and neurotransmitters such as vasoactive intestinal polypeptide (VIP). Circadian timing information from SCN has been shown to be transmitted to other brain parts via neuronal projections to pineal gland and liver through intermediate neurons (Buijs & Kalsbeek, 2001; Kalsbeek et al., 2006). Moreover, loss of VIP has shown altered phase relationship between the peripheral organs and SCN (Loh et al., 2011). In addition, SCN was shown to influence peripheral clock rhythmicity in mammals by regulating systemic cues such as hormones, feeding behaviour and body temperature as shown in Fig 2 (A. Balsalobre, 2000; Brown et al., 2002; Damiola, 2000; Vujović et al., 2008).

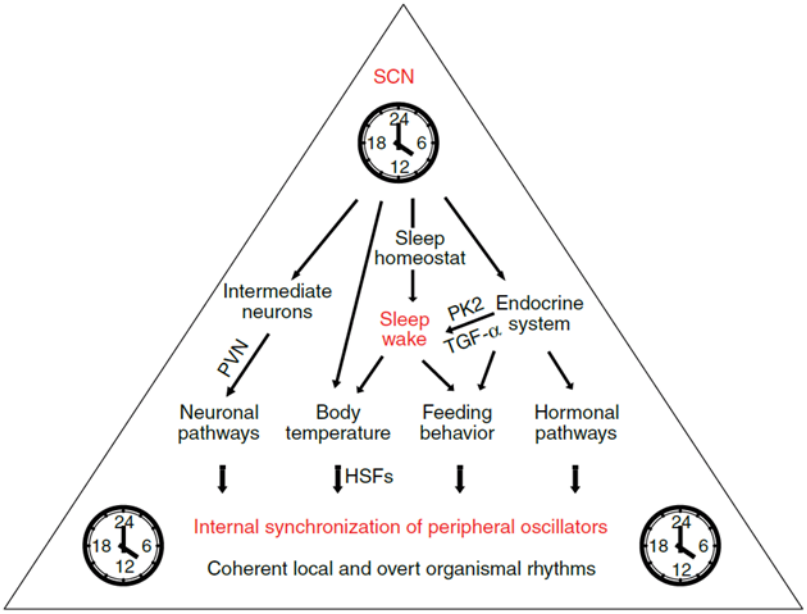


Figure 2. A classical organismal level internal synchronization adapted from (A. C. Liu et al., 2007).

1.1.3.4 Mammalian circadian clock influence physiological processes

The mammalian circadian system modulates many different physiological processes. These processes include – metabolism, cardiovascular system, renal function, endocrine system, reproductive system, immune system etc. (Gachon et al., 2004; Richards & Gumz, 2013). Mammalian SCN coordinates rhythms associated with metabolic processes via transmitters and hormones which orchestrates metabolic rates, feeding behaviour and appetite (Bechtold et al., 2008; Dudley, 2003; Turek, 2005). For example, feeding rhythmicity which is a behaviour closely linked to metabolism has been shown to be diminished in circadian clock mutants and lack of VIP or its receptor in mice led to advancement of feeding phase with lower metabolic rate (Bechtold et al., 2008; Turek, 2005; Yang et al., 2009). In addition, circadian clocks of organs such as liver, pancreas and gastrointestinal modulate tissue specific functions such as rate-limiting steps in metabolism, bile metabolism, gastric emptying etc. (Cagampang & Bruce, 2012). Circadian clock coordinates an important role in synchronizing the endocrine system which in turn regulates peripheral clocks (Richards & Gumz, 2013). For example, glucocorticoids and melatonin is involved in the circadian mechanism where they regulate peripheral clocks and endocrine systems (Richards & Gumz, 2013). In addition, both hormones have been implicated as modulators of immune system (Franchimont, 2004; Radogna et al.,

2010). For example toll-like receptor 9 (TLR9), which is an important receptor expressed in immune system cells has been shown to be a circadian target gene (Richards & Gumz, 2013). Influence of mammalian circadian clock on reproductive system especially female reproductive system has also been investigated. This investigation has been centred around gonadotropin-releasing peptide hormone (GnRH) neurons (Richards & Gumz, 2013). GnRH is a releasing hormone in charge for releasing follicle-stimulating hormone (FSH) and luteinizing hormone (LH) which are involved reproductive processes (such as pubertal maturation) and ovulation triggering respectively. The release of GnRH has been shown to emulate circadian rhythm (Mahoney et al., 2004; Oster et al., 2017).

1.1.4 *Drosophila;* a model organism for circadian, behaviour and metabolic studies

For centuries circadian rhythms have been observed (Marian, 1729) but the breakthrough came in the 1960s when *Drosophila* became unique in facilitating the understanding of the genetic and molecular nature of circadian clock (Dubowy & Sehgal, 2017; Pittendrigh, 1967). Konopka and Benzer in their pioneering work identified the first clock gene *per* through genetic screen (Konopka & Benzer, 1971) which was mapped and then cloned (Bargiello et al., 1984; Konopka & Benzer, 1971; Zehring et al., 1984). *Drosophila* has numerous advantages in biological research such as low genetic redundancy, simplicity in function, easy to propagate in a laboratory, possibility of large-scale genetic screens etc. (Dubowy & Sehgal, 2017). These above-mentioned characteristics has made *Drosophila* a valuable model organism in the study of circadian molecular mechanisms and also in the discovery of new regulators involving circadian rhythms (Dubowy & Sehgal, 2017; Ozgur & Tataroglu, 2014).

Apart from the use of *Drosophila* in circadian studies, it has also been used extensively in the field of metabolic studies (C. Helfrich-Förster, 1998; Seay & Thummel, 2011; Xu et al., 2011). Many metabolic pathways are conserved between flies and humans although these species diverged millions of years ago (Bharucha, 2009; Rajan & Perrimon, 2013). In addition, *Drosophila* presents numerous genetic manipulation possibilities to generate human-like phenotypes such as diabetes, obesity etc. (Graham & Pick, 2017; Musselman & Kühnlein, 2018).

The fruit fly has also proved to be indispensable when it comes to behavioural studies. Many behaviours such as eclosion, locomotor activity and feeding can be studied effectively in flies

(Chiu et al., 2010; Konopka & Benzer, 1971; Pittendrigh, 1967). Such behaviours have shown to be controlled by the circadian clock, making it possible to study circadian related behaviour and metabolism using one model organism.

1.1.5 *Drosophila* circadian clock

Drosophila shares fundamental clock mechanisms with mammals in the form of homologues (Rosato et al., 2006). This remarkable characteristic similarity has made the *Drosophila* circadian clock an important study area which helps in answering many questions about the mammalian circadian clock. One of the famous studies that comes to mind is the study by Konopka and Benzer in which the first circadian clock gene *per* was identified and was later discovered to occur also in mammals (Buhr & Takahashi, 2013; Konopka & Benzer, 1971). As observed in mammals, the central clock of *Drosophila* regulates rhythms of locomotor activity and sleep-wake cycles and it is situated in a small group of neurons residing in the lateral part of the brain (Hardin, 2005). However, in *Drosophila*, autonomous peripheral light-sensitive clocks are ubiquitous in the fly as demonstrated by reporter assays incorporating luciferase expression (Giebultowicz & Hege, 1997; Plautz et al., 1997). These peripheral clocks are found in various tissues and organs where they regulate different functions of the organs/tissues. For example, feeding behaviour has been found to be under circadian regulation with clocks in the fat body regulating the feeding rhythm phase (Xu et al., 2008).

1.1.6 Circadian clock architecture of *Drosophila*

The central clock consists of approximately 150 clock gene expressing neurons located in the brain (Shafer & Yao, 2014; Taghert & Shafer, 2006). It is located in two neural groups namely – lateral neurons (LNs) and dorsal neurons (DNs). LNs consist of three clusters - small ventral LNs (s-LNv), large ventral LNs (l-LNv), and dorsal LNs (LNd) while the DNs comprises of DN1, DN2, and DN3 (Helfrich-Förster, 2004; Hermann-Luibl & Helfrich-Förster, 2015). Furthermore, the s-LNv are regarded as the most important group in the central clock network (Grima et al., 2004; Stoleru et al., 2004) and they are important in driving locomotor activity under constant darkness also known as free running condition (Helfrich-Förster, 1998; Renn et al., 1999). They are also involved in light perception; s-LNv receive light input from retinal photoreceptors located within the compound eyes and extra-retinal photoreceptors within the brain (Glossop & Hardin, 2002b). Nevertheless, s-LNv can be entrained by light that pierce

through the cuticle (Helfrich-Förster et al., 2001; Stanewsky et al., 1998). The neuronal network of the central clock is regulated by pigment dispersing factor (PDF) expressed in the s- and l-LNvs and it is responsible for synchronizing circadian rhythms to environmental cycles (Hermann-Luibl & Helfrich-Förster, 2015; Miyasako et al., 2007; Shafer & Yao, 2014, 2014).

Peripheral clocks are the other part of the circadian clocks which are located in different tissues/organs and regulate the rhythms of the tissue and organ (Ito & Tomioka, 2016). These clocks are based on cell-autonomous molecular oscillation and they show diversity in their organization as shown by molecular oscillation and output rhythms (Ito & Tomioka, 2016; Krupp et al., 2013; Myers et al., 2003; Plautz et al., 1997).

1.1.7 Molecular mechanism of the *Drosophila* circadian clock

The molecular machinery of circadian clock is based on transcriptional/translational feedback loops (Fig 3&4). In Drosophila, it comprises of DNA-binding heterodimer CLK/CYC proteins which acts as transcriptional activators and forms the positive limb (Allada et al., 1998; Rutila et al., 1998). In the main feedback loop, CLK-CYC dimerizes and activates the transcription of repressor genes *per* and timeless (*tim*) genes which occurs during the daytime (Darlington, 1998; Myers et al., 1995; Sehgal et al., 1994, 1995). The PER protein which is newly synthesized is phosphorylated by Doubletime (DBT) (a homologue of casein kinase 1 in mammals) and further subjected to E3 ligase-mediated proteasomal degradation (Grima et al., 2002; Kloss et al., 1988; Ko et al., 2002; Price et al., 1998). On the other hand, TIM is degraded by light input via CRY, a blue light receptor which causes the clock to reset (Ceriani, 1999; Grima et al., 2002; Hunter-Ensor et al., 1996; Myers et al., 1996; Stanewsky et al., 1998; Zeng et al., 1996). During the night-time, the negative limb PER and TIM proteins accumulate and form a heterodimer which translocate to the nucleus to inhibit the transcriptional activation function of CLK-CYC (Darlington, 1998; Lee et al., 1999; Menet et al., 2010). During daybreak, PER and TIM are degraded and CLK-CYC starts a new transcriptional cycle leading to rhythmic expression of *per* and tim including other CLK-CYC controlled genes (Hardin, 2005; Williams & Sehgal, 2001). A second feedback loop involves the expression of Clk itself. In this loop, CLK-CYC activates the mRNA transcription of two regulators which include - vrille (vri) and Pdp1ε (PAR domain protein 1ε). The negative regulator *vri* inhibits the transcription of *Clk* while the positive regulator (Pdp1ε) activates Clk transcription (Cyran et al., 2003). Furthermore, CLK protein levels do not appear to oscillate, therefore the importance

of *Clk* mRNA oscillation is not clear (Shi & Zheng, 2013). Nonetheless, the phase of *Clk* mRNA oscillation align with CLK phosphorylation which advances degradation indicating that Clk mRNA seem to maintain total CLK levels (Kim & Edery, 2006; Shi & Zheng, 2013; Yu, 2006). The above mentioned regulation is vital for stable maintenance of circadian rhythms. Importantly, loss of Pdp1ε leads to decrease of CLK protein level in the central neurons resulting behavioural arrhythmic flies (Zheng et al., 2009).

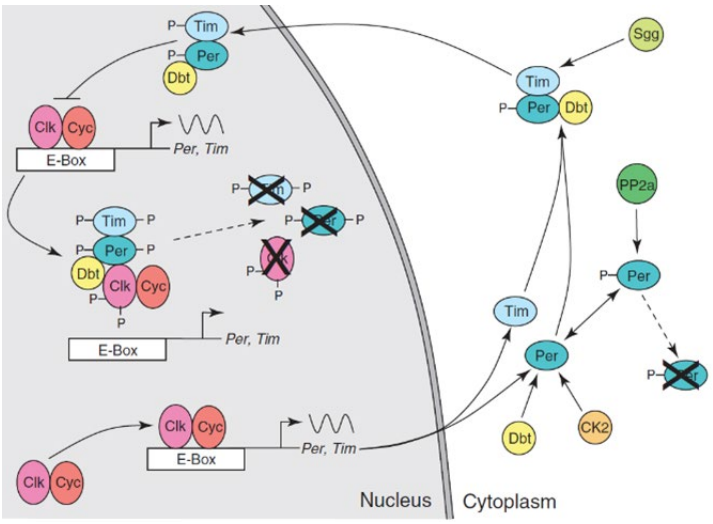


Figure 3. A schematic model of Per/Tim feedback loop (Hardin, 2005)

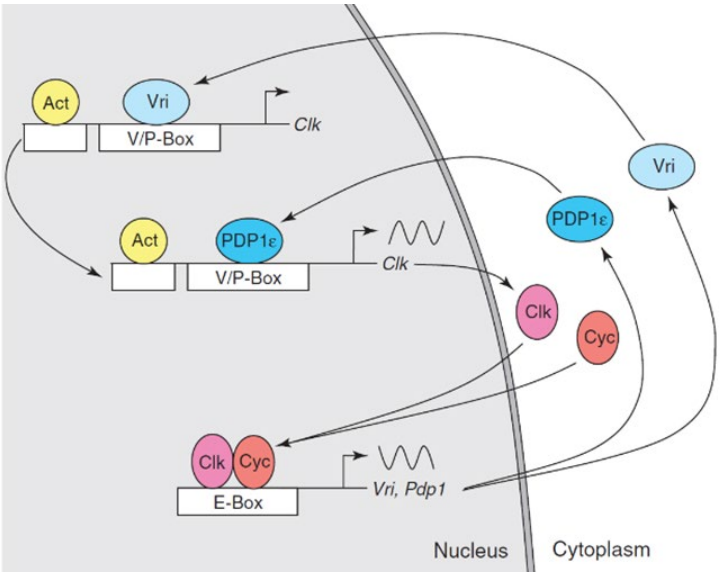


Figure 4. Model of the Clk feedback loop (Hardin, 2005)

The central and peripheral clocks share the above fundamental molecular mechanism of the circadian clock. In the central clock, CRY, compound eyes, ocelli and Hofbauer-Buchner eyelets are involved in light entrainment of the clock (Helfrich-Förster et al., 2001; Stanewsky et al., 1998). Nevertheless, among peripheral clocks, the function of CRY is varied with two different possible functions proposed – It may function as both photoreceptor and core clock component (Collins et al., 2006; Ivanchenko et al., 2001; Stanewsky et al., 1998) or as only a photoreceptor (Ito et al., 2008).

1.2 Feeding behaviour

1.2.1 Circadian clock and *Drosophila* feeding behaviour

Feeding is one of the most important activities performed by organisms and proper alignment of this activity is vital. Studies have shown that circadian clocks synchronize feeding and related physiological processes such as energy consumption and metabolism to daily environmental cycles in many organisms such as *Drosophila* (reviewed by (Allada & Chung, 2010). Feeding behaviour of organisms in nature tends to be maintained in controlled laboratory situations and this is the case with fruit flies (Xu et al., 2008). Rhythmic food consumption occurs during the daytime under light-dark conditions (Seay & Thummel, 2011; Xu et al., 2008). Flies prefer to feed in the beginning of the light phase but in some studies an additional peak was observed at the end of the light phase (Xu et al., 2008). Oscillation in food consumption persisted even under constant darkness. Feeding behaviour of *Drosophila* has been shown to be influenced by two major mechanisms - circadian clock and light. Furthermore, peripheral clocks in *Drosophila* play important roles in the feeding behaviour. These peripheral clocks include those found in olfactory receptor neurons which sense the smell of food and metabolic tissues like fat body that perceives the energy status of the fly (Colombani et al., 2003). Moreover, when the fat body clock is knocked down in fat body there is an increase in the amount of food consumed during the night phase (Xu et al., 2008). Apart from the effect of circadian clocks, other factors such as dietary composition and group size can play significant roles on feeding behaviour (Wong et al., 2009).

1.2.2 Consequences of restricted feeding in *Drosophila*

Most animals restrict their feeding to a specific period with short periods of fasting in-between which correspond to their sleeping time (Longo & Panda, 2016). This sequence of feeding and fasting is coordinated by the circadian clock and helps maintain homeostasis. Time restricted feeding (TRF) is a feeding program mimicking feeding/fasting sequences whereby food access is limited to a specific period of time, usually 12 hours during daytime (Gill et al., 2015). TRF is used in *Drosophila* extensively to study various behavioural and physiological parameters (Adamovich et al., 2014; Gill et al., 2015; Longo & Panda, 2016). For example, the effect of TRF on neural, peripheral and cardiovascular physiology was studied (Gill et al., 2015). The result showed improvement in sleep, slowing down of cardiac aging as well as weight gain prevention (Gill et al., 2015). In addition, it has been shown that circadian clock and time of feeding modulate hepatic triglyceride levels and oscillation in mice (Adamovich et al., 2014). Putting together the above information, it is clear that TRF can be a useful tool to address important questions related to circadian clock-controlled feeding behaviour, and related physiological processes such as metabolism in the fruit fly.

The concept of dietary restriction (DR) and its ability to extend life span was first tested in rodents (McCay et al., 1935). Also in *Drosophila*, dietary restriction which is the reduction of dietary intake leads to extended life span (Piper & Partridge, 2007). DR involves the limited availability of yeast or by dilution of whole food medium (Partridge et al., 2005; Tatar, 2006). It is important to know that *Drosophila* standard food differs from laboratory to laboratory and fly stocks may respond differently to dilutions (Partridge et al., 2005). Overall, the amount of food consumed increases when the food is diluted in order to make up for the low nutrient density (Driver et al., 1986). In addition, studies have shown increase in feeding rate especially among female flies when presented with different sucrose concentrations from 0.5 to 17% after 3 days of sugar-only diet (Edgecomb et al., 1994). However, flies do not change the time spent on feeding with regards to DR (dilution of whole food or restriction of yeast) (Partridge et al., 2005).

1.3 Digestive and metabolic system of *Drosophila*

1.3.1 Structure of the digestive system of *Drosophila*

In the body cavity of *Drosophila*, the gut is one of the largest organs and it plays a vital role such as absorption of nutrients, defence against pathogens and major origin of neuronal and endocrine signals (Lemaitre & Miguel-Aliaga, 2013). The adult fly gut is a tube with its interior lined by epithelia monolayer comprising of four different type of cells namely - intestinal stem cells, absorptive enterocyte, secretory enteroendocrine cells, and enteroblasts (Miguel-Aliaga et al., 2018). The adult *Drosophila* gut is divided into three regions and they are elastic (Miguel-Aliaga et al., 2018). These regions include - foregut, midgut and hindgut with different developmental origins (Fig 5).

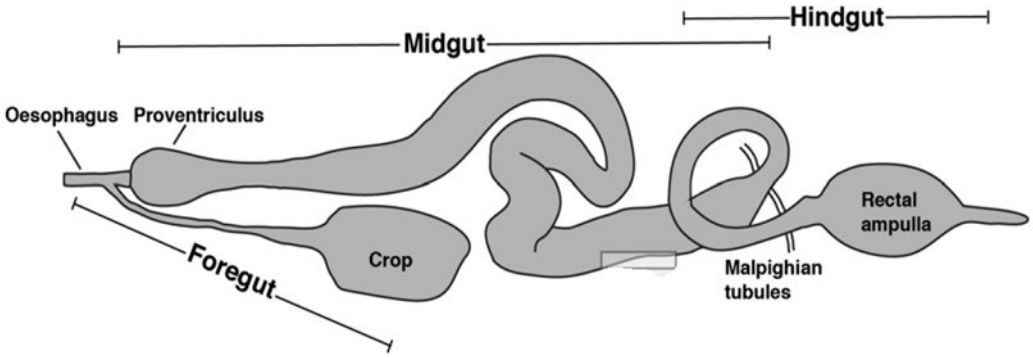


Figure 5. Anatomical structure of adult Drosophila gut (Miguel-Aliaga et al., 2018)

Importantly, the foregut and hindgut are of ectodermal origin while the midgut is of endodermal origin (Miguel-Aliaga et al., 2018). The adult foregut is subdivided into three main parts namely - oesophagus, crop, and cardia. The crop is a structure which comprise of sphincters and valves and serves to transit contents of intestine in and out of the alimentary canal. In addition, from other insect studies it seems to be involved in digestion, food storage, microbial regulation and detoxification (Stoffolano & Haselton, 2013). The cardia, a bulb-shaped structure produces the peritrophic matrix, antimicrobial peptides and regulates the movement of ingested food into the midgut (King, 1988; Tzou et al., 2000). The midgut on the other hand has an average length of 6 mm and is the main digestive portion of the gut and occupies a large swath of the abdomen (Demerec, 1950; Douglas, 2013). Furthermore, the hindgut is comprised of the pylorus, ileum and rectum and is the site of water/ion exchange (Demerec, 1950; Douglas, 2013). It is worthy of note that the physiology of *Drosophila* gut is modulated by hormones and autonomic innervation (Miguel-Aliaga et al., 2018).

When it comes to digestion and adsorption, the midgut of *Drosophila* is the main site although there is evidence of enzymatic conversion taking place in the foregut and/or crop and extra oral digestion (Lehane, & Billingsley, 1996). When the fly eats, the food enters into the digestive tracts where the complex macromolecules are broken down into simpler molecules and absorbed by the intestinal epithelium (Miguel-Aliaga et al., 2018). The digestion of food in the gut is influenced by many factors including – temperature, pH, redox potential, intestinal transit and gut bacteria (Douglas, 2013; Huang & Douglas, 2015; Lehane, & Billingsley, 1996).

1.3.2 Fat body

Feeding is a behaviour that is closely connected to metabolism and the fat body is the main metabolic organ in *Drosophila*. It serves similar function as liver and adipose tissues in mammals (Yongmei Xi, 2015). The fat body is an important metabolic tissue in flies because it serves not only as storage organ but also helps in detoxification and immune response (Musselman et al., 2013). In addition, it is an endocrine organ and it is able to synthesize triglyceride, diacylglycerol, glycogen and trehalose when there is energy demand (Yongmei Xi, 2015). However, lipids, especially diacylglycerol (DG) which is the major transport form of neutral lipid obtained from the digestive process in the midgut, is incorporated into lipoprotein complexes and transported to the fat body via the hemolymph (Palm et al., 2012). This makes the fat body a principal organ in metabolism (Canavoso et al., 2001; Palm et al., 2012). The fat body also serves as the site for lipophorin (Lpp) formation which are dense lipid carrier particles that are rich in phospholipid (Palm et al., 2012). The Lpp is needed for the acquisition of sterols and DG from the gut which it transfers via the hemolymph back to the fat body (Palm et al., 2012).

1.3.3 Hemolymph

The hemolymph is an extracellular fluid found in arthropods for example *Drosophila,* it flows in an open circulatory system soaking all internal organs (Handke et al., 2013). It is the only extracellular fluid found in insects unlike the extracellular fluids in animals which consist of blood plasma or interstitial fluid (Handke et al., 2013). Like blood in animals, hemolymph acts as transport system involved in the transportation of metabolites, nutrients and humoral factors between tissues (Handke et al., 2013; Heier & Kühnlein, 2018). The other important chemical communication occurring in the hemolymph involves the transportation of metabolic waste

products out of cells and tissues (Handke et al., 2013). Importantly, hemolymph plays an important role when it comes to lipid metabolism. DG is exported from the midgut via the hemolymph in the form of lipoprotein complexes to other metabolic tissues such as the fat body (Heier & Kühnlein, 2018).

1.3.4 Circadian clock in digestive/metabolic tissues of *Drosophila*

The *Drosophila* digestive system is made up of different parts of the gut such as- foregut, midgut, hindgut etc. (Miguel-Aliaga et al., 2018). The gut (intestine) is under constant environmental stress such as digestive proteins, ingested harmful pathogens or chemicals. Due to these constant stress and damages on *Drosophila* intestine, intestinal stem cells proliferate and renew the dead and damaged intestinal cells. Disruption of clock components results in arrhythmic intestinal stem cell division (Karpowicz et al., 2013). Interestingly, *Drosophila* intestinal cells are mostly synchronized by photoperiod, however intestinal stem cells are highly sensitive to homeostatic signalling pathways such as Wnt and Hippo (Parasram et al., 2018). Furthermore, the intestinal cell clocks can be regulated by time of food uptake. Moreover, a study showed that TRF modulates CLK/CYC activity in CRY mutants (insensitive to photoperiod) and wild-type flies in the absence of photoperiod. This suggest that food intake may be considered as a supporting synchronizing factor in metabolic tissues (Parasram et al., 2018). It is important to note that *Drosophila* cells such as retinal ganglion cells are responsive to light and photoperiod still remains the main entrainment factor as shown by earlier studies on feeding behaviour and metabolism (Albrecht & Eichele, 2003; Xu et al., 2011). Furthermore, microarray analysis has shown that the fat body contains a molecular clock which regulates genes involved in metabolism, steroid regulation, detoxification and immune response (Xu et al., 2008, 2011). Furthermore, when it comes to metabolism, neuronal clocks counter the effects of the fat body clock (Xu et al., 2008) and this shows that the circadian regulation of metabolism and energy homeostasis is complex.

1.4 Metabolism

1.4.1 Lipids as important group of metabolites in *Drosophila*

Lipids are vital metabolites in organisms and they are essential for life. They provide organisms with energy, precursors for structural components and serve as signalling molecules.

Structurally, lipids are diverse and they are composed of diverse moieties. This diversity emanates from combinations of different fatty acids and functional group heads combined together in multiple ways (Carvalho et al., 2012; Wenk, 2010). According to an on-line database called LIPID MAPS (https://www.lipidmaps.org/) and other publications , lipids are classified into eight (8) different categories based on condensation reactions involving carbanion of ketoacyl thioesters and/or carbocation of isoprene units (Fahy et al., 2009, 2011). Examples of the lipid categories include - glycerolipids (TAG, DG), phospholipids (PE or PC), sphingolipids, fatty acyls, saccharolipids, polyketides, prenol lipids, sterol lipids. Glycerolipids and phospholipids have been quantified in different organs and tissues of *Drosophila* larvae which shows the extent of lipid diversity in the fruit fly (Carvalho et al., 2012). Neutral lipids are known as hydrophobic molecules which do not possess charged groups. TAGs and DGs are one of the most abundant neutral lipids found in fat body and hemolymph respectively. In *Drosophila*, DGs consist of a glycerol backbone and two fatty acid moieties with total carbon number of 26-28 (Fig 6), which is made up of medium chain saturated and unsaturated fatty acids of length 12-14 (Carvalho et al., 2012).

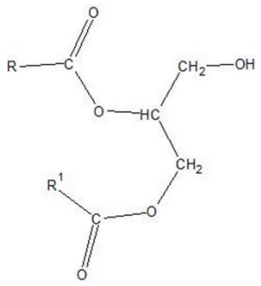


Figure 6. General diacylglycerol (DG) structure. R and R^1 representing same or different fatty acid side chains.

Another important group of lipids in *Drosophila* are the phospholipids (PLs). Biological membranes consist of PLs influencing biochemical and biophysical characteristics of cell barrier (Godzien et al., 2015). In addition, PLs regulate membrane trafficking of molecules and act as precursors for different molecules involved in cell functions (Godzien et al., 2015). Phospholipids can be divided into phosphoethanolamine(PE), phosphocholine(PC), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidylglycerol (PG) (Godzien et al., 2015). Glycerophospholipids comprises of a glycerol backbone with alkylation/acylation at sn-1 and/or sn-2 and a phosphate ester at sn-3 position (Godzien et al., 2015). The phosphate ester (Fig 7) defines the lipid subclass. Among the various phospholipids identified in *Drosophila*, PE is the most abundant phospholipid especially in lipoprotein (Carvalho et al.,

2012). Nevertheless, there is a small fraction of lipoproteins that is made up of PC (Carvalho et al., 2012).

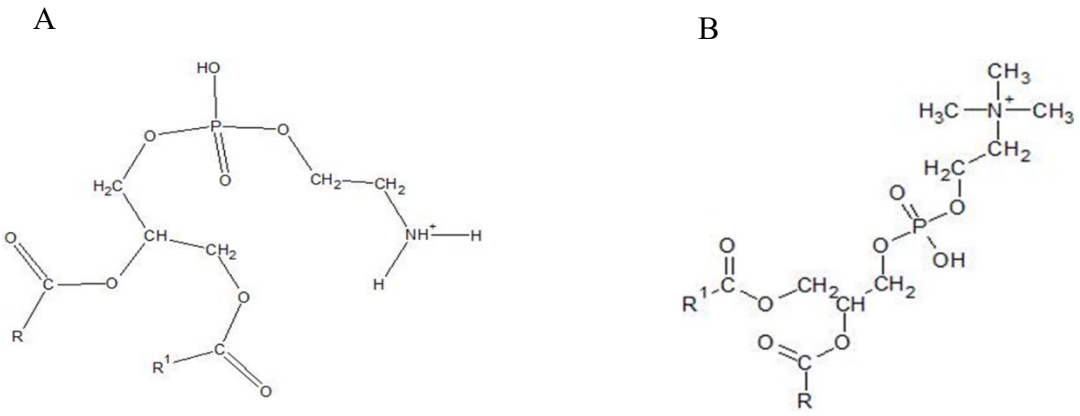


Figure 7. General glycerophosphoethanolamine (PE) and glycerophosphocholines (PC) structure. R and R^1 representing same or different fatty acid side chains.

1.4.2 Lipid metabolism in *Drosophila*

Glycerolipids are one of the most essential lipid groups when it comes to energy homeostasis across animal taxa. Triacylglycerol (TAGs) are important not only as a caloric source but as a vital source for building up structural and signalling molecules (Heier & Kühnlein, 2018). In addition, they are involved in many metabolic processes such as lipid anabolism, lipid catabolism and lipid transport. In *Drosophila* as well as other organisms, TAG and glycogen are the most important energy reservoirs (Heier & Kühnlein, 2018). TAG has some advantages over glycogen. For instance, the carbon atoms in TAG are more reduced compared to glycogen leading to higher caloric yield when completely oxidized (Heier & Kühnlein, 2018). Lipids are an essential components of *Drosophila* diet. In the midgut lumen of *Drosophila*, ingested glycerolipids are hydrolysed into free fatty acids, glycerol, and/or other acylglycerol intermediates by digestive lipases (reviewed in (Heier & Kühnlein, 2018). The metabolites derived from the breakdown of TAG are absorbed by enterocytes and repackaged into complex lipids (Canavoso et al., 2001; Sieber & Thummel, 2012). Enterocytes (intestinal absorptive cells) combine dietary fatty acids and glycerol groups into diacylglycerol (DG) which is the main transport form of lipid in the hemolymph (Palm et al., 2012).

The transportation of lipids is done primarily by Lipophorin (Lpp). As it is well known, DG is the major neutral lipid transported via the hemolymph in a complex with Lpp (Palm et al., 2012). However, TAG and fatty acids are transported in trace amounts (Heier & Kühnlein, 2018). Other lipids that are transported by Lpp includes phosphatidylethanolamine (PE), phosphatidylcholine (PC), free sterol, sphingolipids and hydrocarbons (Carvalho et al., 2012; Fernando-Warnakulasuriya & Wells, 1988). It is noteworthy to know that despite the midgut being the centre for lipid absorption, *de novo* synthesis and lipid export (to lesser extent when compared to fat body) to the circulatory system, it lacks the ability to synthesize lipophorin, instead, it depends on the fat body (Palm et al., 2012). Apart from dietary sources of TAG, there is *de novo* lipogenesis in midgut whereby acetyl-CoA units from carbohydrate are converted to fatty acids which are then incorporated into TAG for storage in different tissues (Song et al., 2014). The excess remaining of fatty acids from enterocytes are converted to TAG and stored in lipid droplets for short term storage (Carvalho et al., 2012; Palm et al., 2012). When there is shortage of food, the fat body releases TAG reserves in a process known as lipolysis (enzymatic hydrolysis) to other tissues (Arrese, 1997; Chino & Gilbert, 1964; Grönke et al., 2005, 2007). Notably, different tissues such adipose tissues also have their own TAG reserves and they express enzymes necessary for TAG synthesis although the fat body is the primary TAG storage tissue (Chintapalli et al., 2007; Kühnlein, 2011).

1.4.3 Circadian clock regulation of (lipid) metabolism in *Drosophila*

The circadian clock was shown to regulate expression of genes that are vital for metabolism and physiology in Drosophila (Xu et al., 2011). In addition, rhythmic feeding behaviour which is closely linked to metabolism is known to be regulated by the circadian clock (Seay & Thummel, 2011; Xu et al., 2008). Both the neuronal and peripheral clocks help in maintaining feeding rhythms and energy metabolism (Xu et al., 2008). The fat body clock is proposed to regulate metabolic processes through driving rhythmic gene expression (Shi & Zheng, 2013). This regulation is supported by genome wide transcriptional study of the fat body whereby many genes associated with metabolism, steroid hormone regulation, immune response and detoxification were expressed in a rhythmic fashion. In addition, disruption of CLK in the fat body is enough to change the feeding rhythm and eliminate the cyclic expression of clock-controlled genes (Xu et al., 2011).

Generally, proper balance between lipid synthesis and breakdown is necessary in all organisms. The mechanisms surrounding such proper homeostasis is complex and not fully understood,

however there has been increased number of studies in this direction. *Drosophila* has been a helpful model organism to continuously unravel the underlying mechanism of lipid metabolism. The central clock has been implicated in lipid metabolism for example when the circadian gene, disruption of the Clk in the neuronal clock cells, was shown to result in increased triglycerides in the fat body of the fly (DiAngelo et al., 2011). The peripheral clocks, especially the fat body, drive rhythmic metabolic genes associated with lipid metabolism for example GNS1/SUR4 membrane proteins, long-chain-fatty-acid-CoA ligase (CG9009), some MFS genes and ABC genes (Xu et al., 2011). A metabolomic study showed a strong effect of non-functional *per* in the head or body metabolome especially on DG (major transport form of lipids in *Drosophila*) and acyl carnitines (AC) (Schäbler et al., 2020a). Other evidences have also shown the relationship between circadian clock and lipid metabolism. For example, non-rhythmic levels of triacylglycerol (TAG) were lower in clock mutants such as tim^{01} and cry^{01} (Seay & Thummel, 2011). However, investigation into circadian influence on lipids in *Drosophila* is still at its nascent stage and more studies are required for deeper understanding of circadian influence on lipid metabolism in the fruit fly.

1.5 Aims and objectives

The aim of my thesis was to test whether lipids oscillate diurnally in the hemolymph of *Drosophila*, and to identify whether such lipid oscillations are under circadian influence, and whether there are other factors, such as rhythmic food consumption, that contribute to lipid oscillations. To achieve this, high abundant lipids in the hemolymph (DG, PE, PC) were analysed every three hours in the hemolymph of wild-type male *Drosophila* flies entrained to a 12hr/12hr light-dark cycle (LD) and fed *ad libitum* on standard and lipid-free medium. To interlink circadian clocks and circulating target lipids, levels of DGs, PEs and PCs were determined in the hemolymph of wild type flies under constant darkness (DD) and in the clock mutant per^{01} under LD. Finally, to determine if feeding behaviour contributes to the oscillation of lipids in *Drosophila* hemolymph, a CAFE assay (Ja et al., 2007)was used to monitor both wild-type and per^{01}. Subsequently, a time-restricted feeding (TRF) paradigm was used to ascertain if lipid oscillations were affected by time of feeding in lipid-free fed wild-type flies.

2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Consumables, devices and software

Name	**Manufacturer**
Cylindrical Bottles (43mm X 13mm)	Germany
30µl open ended capillary tube (drummond microcaps)	Merck, Germany
10µl open ended capillary tube (drummond microcaps)	Merck, Germany
Micro-capillary tubes (0.5 µl minincaps)	Hirschmann, Eberstadt
Eppendorf Tubes (1.5 ml & 0.5 ml)	Eppendorf, Hamburg
UPLC Vials	Chromacol, Herts (UK)
Pipettes Tips	Sarstedt, Nuernberg
Camera (DMK22BUP031)	Germany
IC capture	The Imaging Source Europe GmbH, Bremen
Microscope	Olympus SZ61, Germany
Analytical Balance (XPE 205 Delta Range)	Mettler Toledo, Columbus (USA)
Centrifuge (541712)	Eppendorf, Hamburg
Ultrasonic Bath	VWR, Darmstadt
UPLC (Acquity Ultra Performance LC)	Waters, Milford (USA)
Vacuum Rotary Evaporator (RVC 2-18)	Christ, Osterode
Vortex shaker (Vortex Genie 2) /FRESCO 21	Thermo scientific, Germany
Rios Water Purification System	Millipore, Billerica (USA)
Quadrupole Time-Of-Flight Mass Spectrometer	Synapt HDMS G2, Waters, Milford (USA)
Microsoft Office	Microsoft
Image J	National Institutes of Health
R Programming Language	R core team
METLIN	Scripps Research Institute
Lipidmaps	www.lipidmaps.org
Masslynx	Waters Corporation
JTK Cycle Algorithm	Hughes Lab
Chemsketch	ACD/Labs

2.1.2 Chemicals and standards

Name	**Manufacturer**
2 – Propanol (LC-Ms Grade)	Biosolve, Dieuze (FR)
Acetonitrile (Gradient Grade)	Biosolve, Dieuze (FR)
Acetonitrile (LC-Ms Grade)	Biosolve, Dieuze (FR)
Ammonium Acetate	Biosolve, Dieuze (FR)
Chloroform Normapur	VWR, Radnor (USA)
Methanol (Gradient Grade)	Merck, Darmstadt
Sucrose	Carl Roth GmbH & Co. KG
Agar	Carl Roth GmbH & Co. KG
Bromophenol Blue Sodium (Electrophoresis Grade)	AppliChem GmbH, Darmstadt
Yeast Extract Biochemica	AppliChem GmbH, Darmstadt
DG(20:0) (internal standard)	Avanti Polar Lipids, Alabster (USA)
PE 34:0 (internal standard)	Sigma Aldrich, St. Louis (USA)
PC 34:0 (internal standard)	Sigma Aldrich, St. Louis (USA)
TG 30:0 (internal standard)	Sigma Aldrich, St. Louis (USA)
PA 34:0 (internal standard)	Sigma Aldrich, St. Louis (USA)
PS 34:0 (internal standard)	Sigma Aldrich, St. Louis (USA)
PI 34:0(internal standard)	Sigma Aldrich, St. Louis (USA)
DG 36:2 (standard)	Sigma Aldrich, St. Louis (USA)
TG 51:0 (standard)	Sigma Aldrich, St. Louis (USA)
PE 36:2 (standard)	Larodan, Solna (Sweden)
PC 28:0 (standard)	Sigma Aldrich, St. Louis (USA)

2.2 METHODS

2.2.1 Fly lines

Canton-S (WTcs) were used as wildtypes, while *period*^{01} (*per*^{01}) was used as the clock disrupted mutant (Konopka & Benzer, 1971). Flies were kindly provided by Charlotte Foerster (Chair of Neurobiology and Genetics, University of Würzburg). All experiments were performed with male flies except otherwise stated.

2.2.2 Fly culture and rearing conditions

All flies were reared in small bottles (Fig 8) under a 12/12 h light/dark regime (LD) at 25±0.2 ºC and 60±2% relative humidity. They were fed standard food which consists of 0.8% agar, 2.2% sugar beet syrup, 8.0% malt extract, 1.8% yeast, 1.0% soy flour, 8.0% corn flour and 0.3% hydroxybenzoic acid. In some experiments, larvae were transferred from the standard medium to lipid-free medium which consists of 5% sucrose and 2% agar. For feeding assays, the standard food was liquid and consisted of 3.6% yeast extract, 5% sucrose and 0.3% FD&C Blue No 1 (E133) blue as colouring agent. While the liquid lipid-free medium consists of only 5% sucrose.

Figure 8. Schematic drawing of the cylindrical containers with food A) standard food and B) lipid-free food

2.2.3 Preparation of the CAFE assay

The model of capillary feeding (CAFE assay) used in the feeding experiment was a modified version from the original setup (Ja et al., 2007). The main chamber of the flies was made from a glass cylinder measuring 13 mm X 43 mm. A rectangular box was constructed out of a mesh and gummed to one side of the glass cylinder and this serves to protect the control capillary containing food from flies. 1 ml of 2% agar was added to each the chamber and serves as water source and maintain humidity within the chamber. Five air holes were made on the vial cap, two of these holes hold a calibrated feeding capillary tube (centre hole) and a calibrated control capillary tube (side hole) respectively (Fig 9). The capillary tubes were used to take up liquid medium via capillary action. Each capillary tube was able to hold 30 µl of liquid medium and it was changed every 2 days for first 4 days and subsequently every day. At the end of the experiment, the volume measurements from the control capillary is subtracted from the actual feeding measurement to account for evaporation. In addition, a mineral oil overlay (1 µl) was used to reduce evaporation rate in all experiments in both feeding capillary and control

capillary tubes. For each experimental condition, 10 CAFE vials were used and divided into 5 vials per genotype containing 10 flies per vial. A camera attached to a laptop and controlled by IC capture software was used to capture pictures of the capillary tubes every 1 hr. A timer was used to set 12/12 hr light/dark cycle and was put off totally when measuring in the constant darkness (DD).

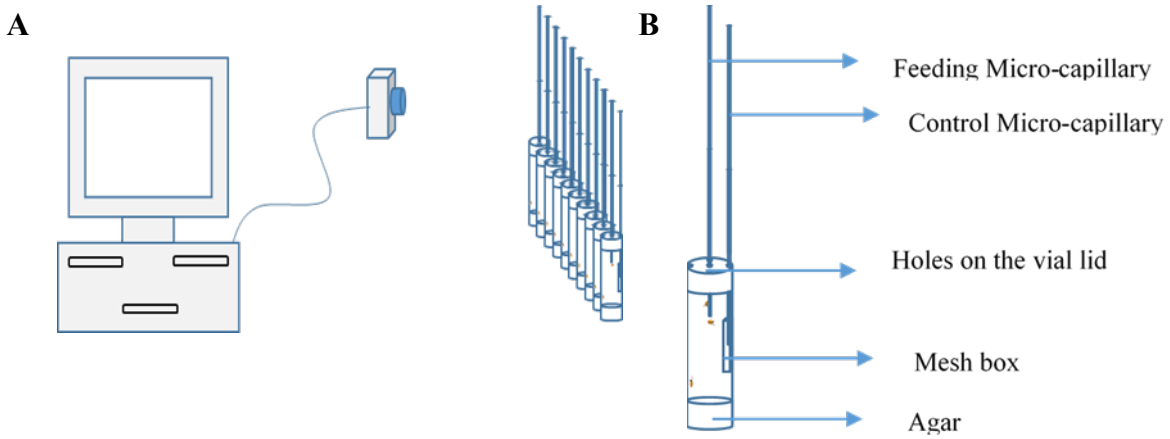


Figure 9. Schematics of the feeding assay set up. A) CAFE assay setup with camera connected to a computer and feeding vials B) a single vial with labelled parts

2.2.4 Determination of food volume from CAFE assay

The volume of food consumed by the flies using CAFE assay is determined using the formula below (Equation 1) after measuring the lengths of both the feeding capillary and control capillary tubes from the hourly pictures using Image J software.

$$V_h = T_h - E_h = \frac{(L_i \times -L_n) \times V_c}{Lc} - \frac{(L_i \times -L_n) \times V_c}{Lc}$$

Equation 1: Shows various equations to measure volume of food consumed. Abbreviations: V_h= food volume per hour, T_h= total food consumed including evaporated food volume (feeding capillary), E_h= evaporated volume (control capillary), L_d= capillary length difference between successive pictures, V_c= capillary volume of entire capillary used (e.g. 30 µl), L_c= capillary length of entire capillary used L_n= Next or following capillary length.

2.2.5 Light/dark, feeding and hemolymph sampling design

For all experiments LD was maintained at 12hr light and 12hr dark except for experiments involving constant darkness (DD). Under constant conditions, flies were entrained for 3 days under LD before switching them into constant darkness. During the experiment, the feeding regime was administered based as *ad libitum* in all experiments except during time restricted feeding when flies were either fed during the light phase only or dark phase only (Fig 10). Hemolymph sampling was performed on the 6th day at 8 different time points (ZT1, ZT4, ZT7, ZT10, ZT13, ZT16, ZT19, ZT22) within 24hrs.

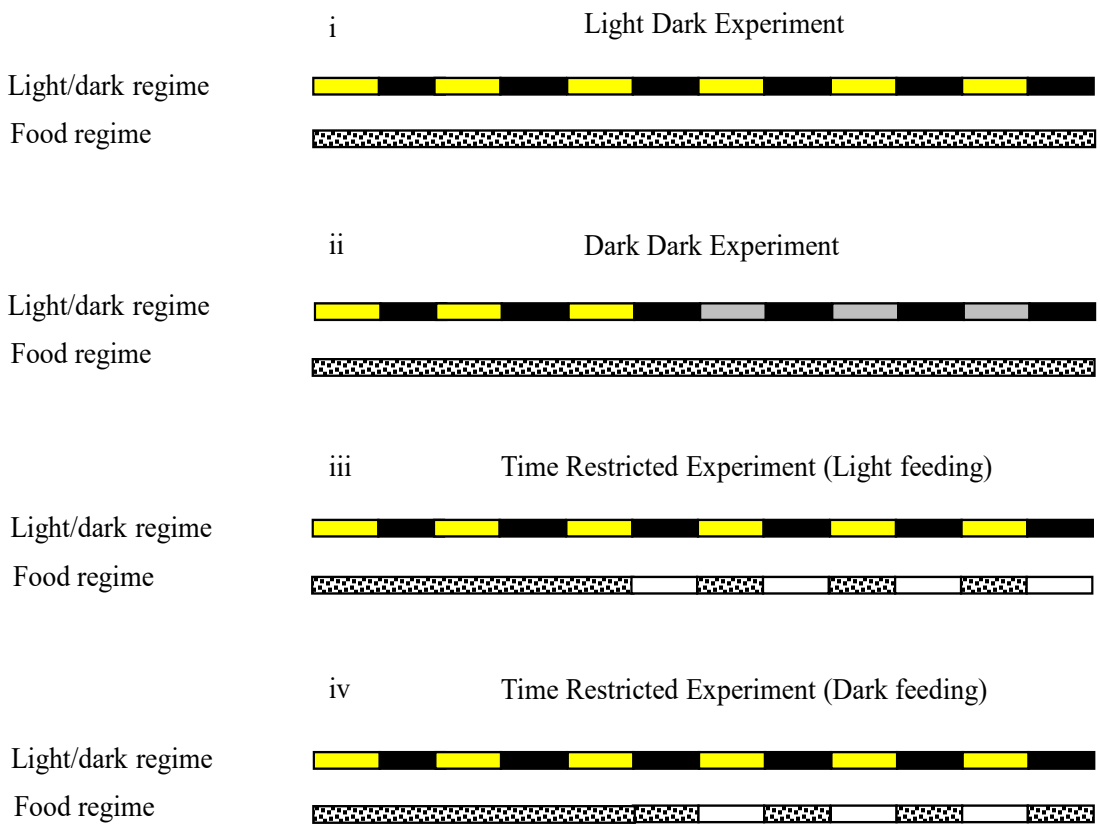


Figure 10. Experimental designs with the different light and feeding regimes. **i)** 12/12 h light-dark cycle and ad libitum feeding **ii)** 12/12 h light- dark cycle for 3 days before dark-dark cycle **iii)** 12/12 light-dark cycle with food limited to 12 hrs of light-on period **iv)** 12/12 light-dark cycle with food limited to 12 hrs of light-off period. All experiments were performed after 3 days of ad libitum feeding and 12/12 light-dark cycle entrainment unless otherwise stated. The lightening condition is designated by the colours of the bars such that yellow = light-on and black = light-off. The food conditions are the lower bars whereby white bars = presence of food and dotted bars = absence of food.

2.2.6 Hemolymph sampling

Drosophila flies were anesthetized by removing them from their food vials into a clean vial before placing them in an ice box for 3 mins post-extraction time points. Then they were placed on a plastic petri-dish wrapped with paraffin to reduce water condensation. The whole setup was placed under the microscope. Hemolymph was extracted from male *Drosophila* of both genotypes (WTcs and per^{01}) by making an incision on the thorax of the flies using a sharp tungsten needle. After the incision, 20 male flies were put into a 0.5 ml Eppendorf tube with 3 holes at the bottom which was inserted into a 1.5 ml Eppendorf tube. Subsequently, the flies contained in the tubes were centrifuged for 5 min at 4ºC and speed of 3075 rcf. After centrifugation, clear and yellowish hemolymph samples were used while cloudy deep yellow coloured samples were discarded. Micro-capillaries (0.5 or 1µl) were used to take up the hemolymph fluid from the 1.5 ml Eppendorf tube by capillary action. The length occupied by the hemolymph in the micro-capillaries was measured with a meter rule for subsequent volume calculation. Afterwards, the micro-capillaries containing the hemolymph were placed in Eppendorf tubes containing 25 µl of Millipore water each and all its content ejected using a small rubber ejector. The Eppendorf test tubes containing samples were stored at -80°C until lipid extraction.

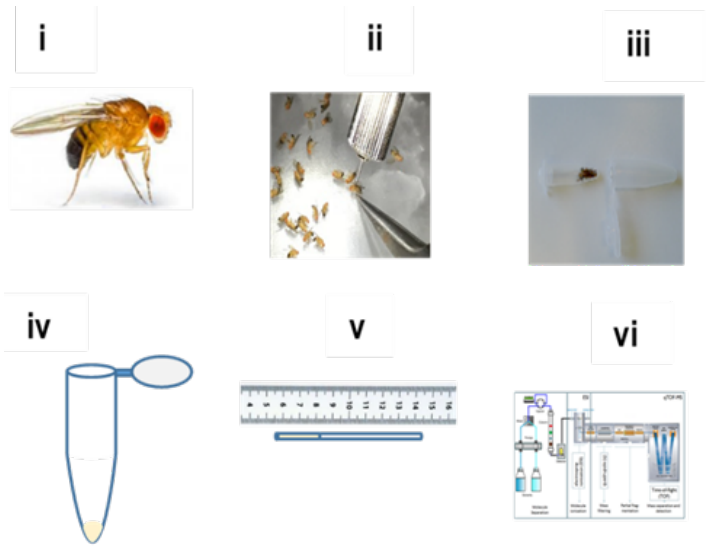


Figure 11. Drosophila hemolymph sampling and lipid measurement flow chart. i) Adult male fly (6 days old) (ii) Detailed view of the hemolypmh extraction. Flies are anesthetized on ice and a slight incision is made on the metathorax part of the fly with a tungsten needle (iii) Collection of 20 adult male flies in a 0.5 ml eppendorf tube (with 3 holes made under it) and insertion into 1.5 ml epperndorf tube. (iv) The collection of hemolymph (very clear light yellow

colour) after centrifugation in the 1.5 ml epperndorf tube (E) Measurement of tube length and length occupied the hemolymph (vi) Analysis of hemolymph lipid with liquid chromatography coupled to mass spectrometry.

2.2.7 Short validation

Calibration standards were prepared by spiking calibration solutions with lipid analytes (DG (36:2), TG(51:0), PE(36:2), PC(28:0)) with corresponding internal standards (DG(20:0), TG(30:0), PE(34:0), PC(34:0)). The lipid analytes had the following concentrations – 0.002, 0.01, 0.05, 0.25, 1.25, 6.25 and 31.25 ng/µl. The internal standards (IS) were spiked into the calibration solutions at 1 ng/µl of DG(20:0), PE(34:0) and PC(34:0) and at 0.1 ng/µl of TG(30:0).

The calibration curves were then constructed by plotting the peak area (analyte signal) against the concentration ranges. Linearity was determined by the values of coefficient of correlation (R^2) on the linear plots. The closer the value of R^2 to one signifies linearity of response. Sample-to-sample repeatability was determined by making 7 independent sample preparations of lipid ISs at concentrations of 1 ng/µl of DG(20:0), PE(34:0) and PC(34:0) and at 0.1 ng/µl of TG(30:0). Response factor (RF) was calculated from the calibration solutions containing the different lipid ISs and corresponding lipid analytes using the formula for response factor (Equation 2).

$$RF = \frac{P_A}{c_A} \times \frac{c_{IS}}{P_{IS}}$$

Equation 2: Shows the equation used in determining concentration of analyte or response factor. Abbreviaitons: RF= response factor, P_A= peak area of analyte, c_A = concentration of analyte, P_{IS}= peak area of internal standard, c_{IS} = concentration of internal standard.

2.2.8 Sample and internal standard preparation

Hemolymph samples dissolved in 25 µl of water was air dried in a speed vac at 50 ^{0}C until dryness. Subsequently, stock solutions (1 mg/ml) of the internal standards were prepared in a solution of chloroform/methanol/water (4:1:0.1). From the above stock solutions, the end concentration of the various internal standards was prepared as follows: 1 ng/µl of DG(20:0),

PE(34:0) and PC(34:0) and 0.1 ng/µl of TG(30:0). The above internal standards were mixed and air dried to evaporate the chloroform/methanol/water solution and then re-suspended in cold isopropanol. The required volume (75 µl) of internal standard mix was pipetted into each of the air dried hemolymph samples. Then the hemolymph samples are vortexed for 2 minutes and transferred to the ultrasonic bath for 15 minutes. Thereafter, the entire content of each hemolymph sample was pipetted into the MS vial for further measurements.

2.2.9 Lipidomics analyses

Mass spectrometric analyses were performed using Acquity Ultra Performance LC coupled to a Synapt G2 HDMS equipped with electrospray ionization source. For chromatographic separation a BEH C18 column (2.1 × 100 mm, 1.7 µm) at 60 ° C was used. A linear binary solvent gradient was applied using 30−100 % eluent B over 10 min at a flow rate of 0.3 ml/min. Eluent A consisted of 60:40 water/acetonitrile with 10 mM ammonium acetate and eluent B consisted of 90:10 isopropanol/acetonitrile with 10 mM ammonium acetate. After chromatographic separation, lipophilic metabolites were ionized using an electrospray ionization (ESI) source operated in positive mode and detected with a time-of-flight mass spectrometer. Ionization voltage was 1.8 kV in positive mode, source temperature of 120 ^{0}C and nitrogen was used as desolvation gas with desolvation temperature of 350 °C and flow rate of 800 L/hr. The quadrupole was operated in a wide-band mode, and data was acquired over the mass range of 50−1200 Da in a centroid data mode. Leucine-enkephaline ($[M+H]^+$: m/z 556.2771 and $[M-H]^-$: m/z 554.2615) was used as a reference compound in the lock spray. The lock spray had a scan time of 0.3 seconds which allowed for continuous mass correction and the data format was maintained as centroid with mass error of below 5 ppm. Data was acquired in low energy function (MS) and high energy function (MS/MS) using a collision energy ramp from 15 to 30 eV. MassLynx and QuanLynx (version 4.1; all Waters) were used to acquire and process chromatograms. For semi-quantitation, peak areas of the analytes and internal standards (IS) in the extracted ion chromatogram (XIC) were determined from extracted total ion chromatogram. Lipid concentrations were calculated using the formula below (Equation 2) with a response factor of approximately 1 for each analyte/IS pair (Table 1). The retention time (RT) and mass-to-charge ratios (m/z) of the lipids are included in supplementary Table 8,9 and 10.

3 RESULTS

3.1 Optimization of hemolymph extraction

To obtain hemolymph, a previously published incision-based extraction protocol was adapted and optimized (Haselton & Fridell, 2011; Naikkhwah & O'Donnell, 2011). The optimization process was necessary to avoid contamination from fat body. In addition, due to the open regulatory system of the fly, obtaining a high volume of hemolymph is challenging. During the optimization process, hemolymph extraction with a sharp tungsten needle was shown to make less contamination (Fig 12B) compared to normal needle by making pointed incisions. The incision point was made through the dorsal part between the thorax and scutellum (Fig 12A) to allow for exudation of a larger amount of hemolymph. In addition, the anesthetization process was done on ice instead of the common practice of using CO_2 which could lead to changes in some biochemical properties of hemolymph (MacMillan & Hughson, 2014). Furthermore, the centrifugation speed was maintained at 5000 rpm which produced hemolymph with high purity. The centrifugation speed is a critical point in the optimized extraction method because higher speed smashes the flies and releases fat body cells with high TAG content (Fig 12B).

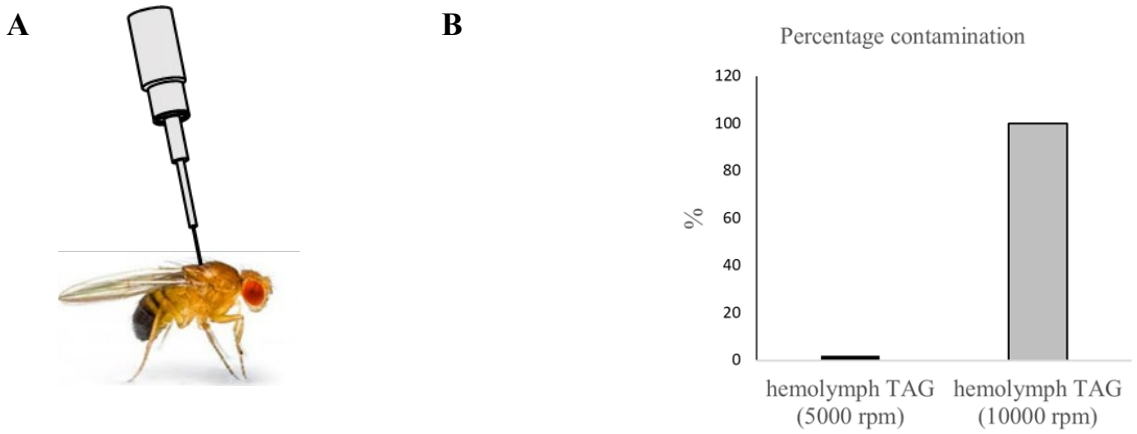


Figure 12. Purity and extraction of hemolymph. A) Incision point (between the thorax and scutellum) on the fruit fly for hemolymph extraction. B) Relative TAG levels indicating contamination of hemolymph during centrifugation.

3.2 Applied lipidomics approach for the determination of the hemolymph lipidome

3.2.1 LC-MS method

The hemolymph sample of the fruit flies were analysed for DGs, PEs and PCs followed a protocol that has already been developed in the laboratory and is described in the materials and method section. Shortly, a high resolution LC system coupled to a high accurate MS equipped with an ESI source operated at positive mode was used to determine our lipids of interest. Fig 13 represents a typical total ion current chromatograms (TIC) in which the most abundant lipid ions are detectable. DGs were detected between retention time of 6-9 minutes within a mass-to-charge (m/z) 383-580 (Fig 13, supplementary table 8). The targeted glycerophospholipids namely PEs and PCs had a close elution time since reversed phase LC was applied. They eluted between 7-10 mins with a m/z 660 – 750 (Fig 13, supplementary table 9 and 10).

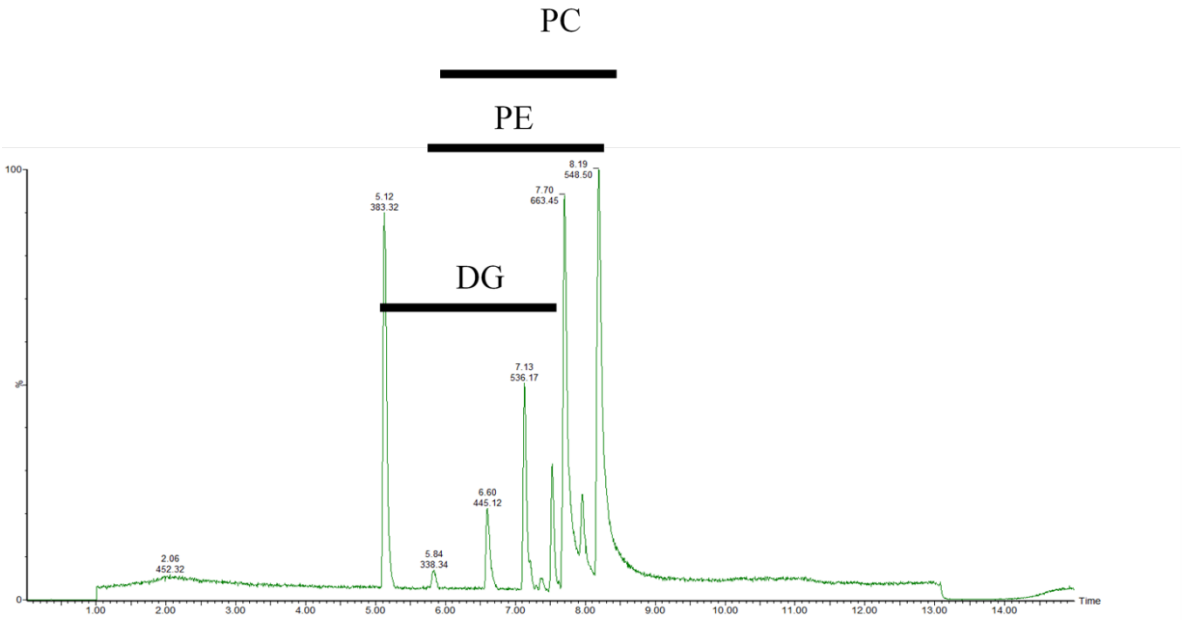


Figure 13. Typical total ion current chromatograms (TIC). Retention window of diacylglycerols (DG), phosphoethanolamines (PE) and phosphocholine (PC) are highlighted.

To determine specific lipids of interest, extracted ion chromatograms (XIC) were used where intensities of ions in function of retention time (rt) at defined mass-to-charge ratio (m/z) was plotted. Lipids of interest were defined as total number of carbons (first number) and double bonds (second number) of the fatty acyls (Fig 6). For example, DG(32:2) means that the DG-

species composes of two fatty acyls with a total sum of 32 carbon and 2 double bonds. After profiling, DG(26:0) at rt 6.94 min, DG(28:0) at rt 7.41 min, DG(30:1) at 7.46 min and DG(32:2) at rt of 7.79 min were always detectable (Fig 14) and, therefore, total levels of these four lipid-species was used to characterise daily variations of DGs in the hemolymph of *Drosophila*.

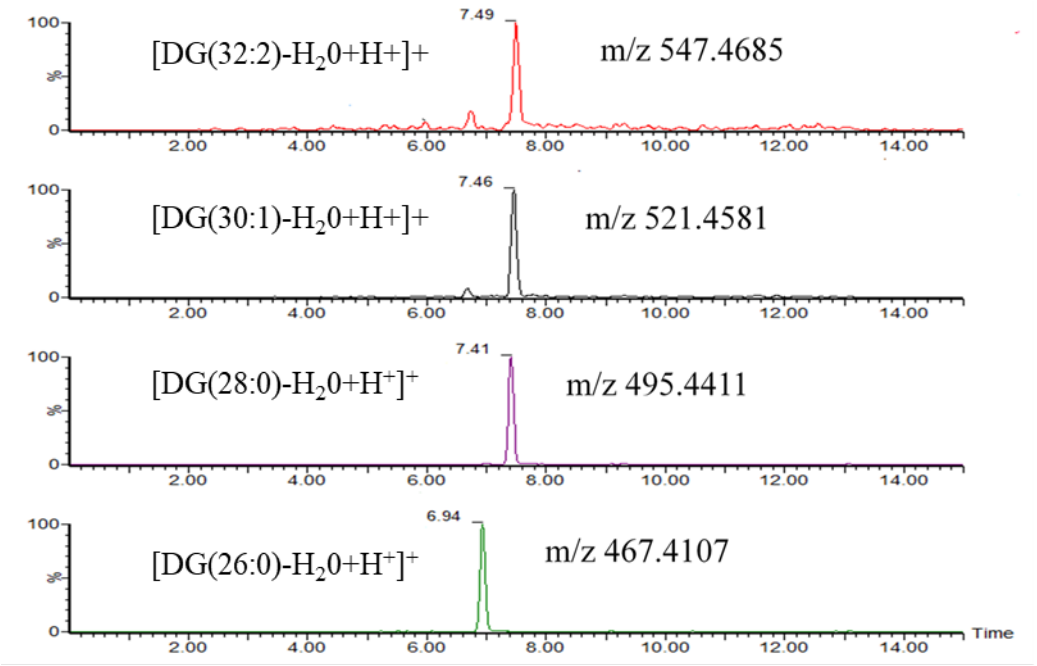


Figure 14. Extracted ion chromatograms of the most abundant DG species $[M\text{-}H_2O+H]^+$ in the hemolymph of WTcs. Abbreviation: DG(X:Y)= diacylglycerols defined as total number of acyl carbons (X) and total number of acyl double bonds (Y).

I also profiled phospholipid species in the hemolymph of 5-days old male flies. As expected, the most abundant phospholipids were PEs and PCs. Typical XICs of PE- and PC-species in WT_{CS} are presented in Fig 15 and Fig 16, respectively. Interestingly, total carbon number of fatty acyls in PEs and PCs were comparable (32-36), but they were longer as of DGs (26-32). For further examinations, I used total levels of PE(32:1), PE(34:1), PE(34:2) and PE(36:3) to follow PEs and total levels of PC(32:0), PC(34:1), PC(34:2) and PC(36:3) to determine PCs in the fly hemolymph.

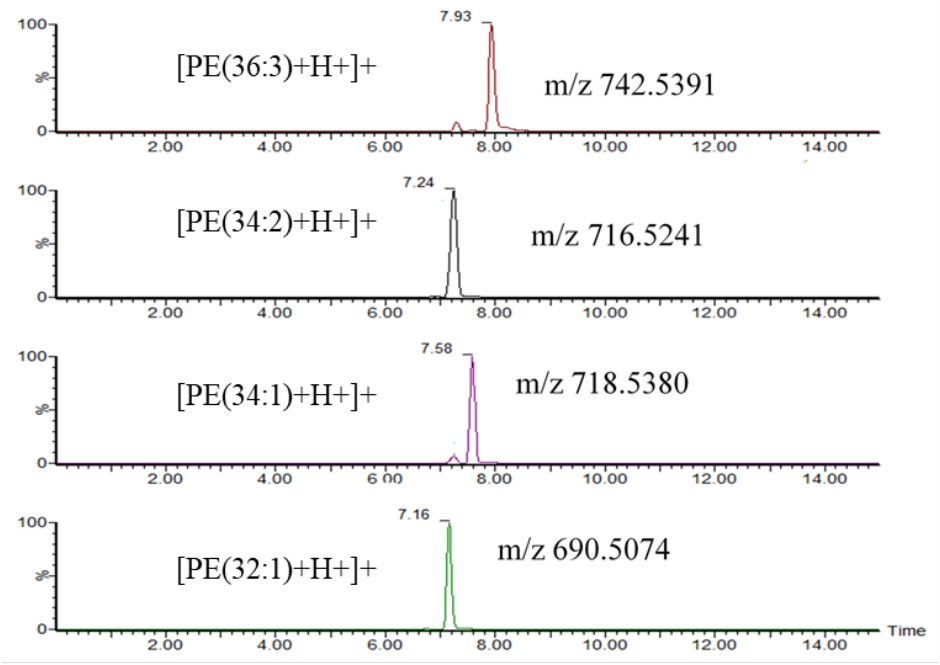


Figure 15. Extracted ion chromatograms of the most abundant PE species $[M+H]^+$ in the hemolymph of WTcs. Abbreviation: PE(X:Y)= phosphatidylethanolamine defined as total number of acyl carbons (X) and total number of acyl double bonds (Y).

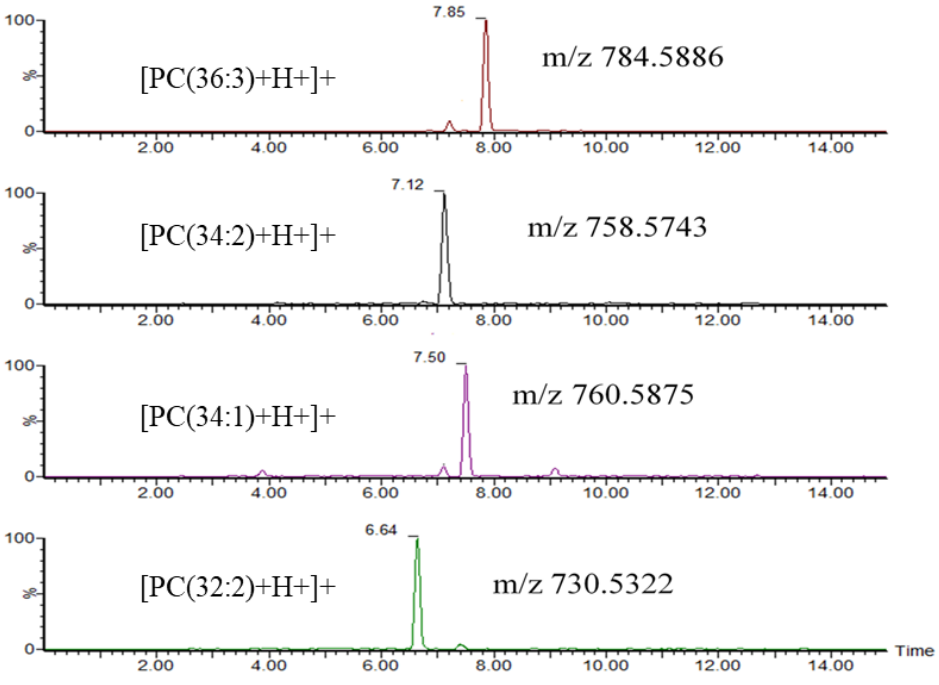


Figure 16. Extracted ion chromatograms (XIC). of the most abundant PC species [M + H] + in hemolymph of WTcs *Drosophila*. Abbreviation: PC(X:Y)= phosphatidylcholine defined as total number of acyl carbons (X) and total number of acyl double bonds (Y).

3.2.2 Mass spectrometry (MS) method

The targeted lipid classes – DG, PE and PC- were ionised in the positive ESI mode. The DG species were detected in the positive ESI mode with neutral loss of water $[M\text{-}H_20+H]^+$ as the most stable ion across experiments (Supplementary Table 1) although sodium adducts $[M+Na]^+$ and ammonium adducts $[M+NH4]^+$ were also detectable. Glycerophospholipids (PE and PC) were detected in protonated form $[M+H]^+$ as the most stable form (Supplementary Table 9 and 10). Fig 17-19 show typical time aligned mass spectra of each lipid class. The most intense peak is the monoisotopic peak and isotopic peaks could be separated from each other due to the high resolution characteristic of the MS instrument ($R_s \approx 13000$).

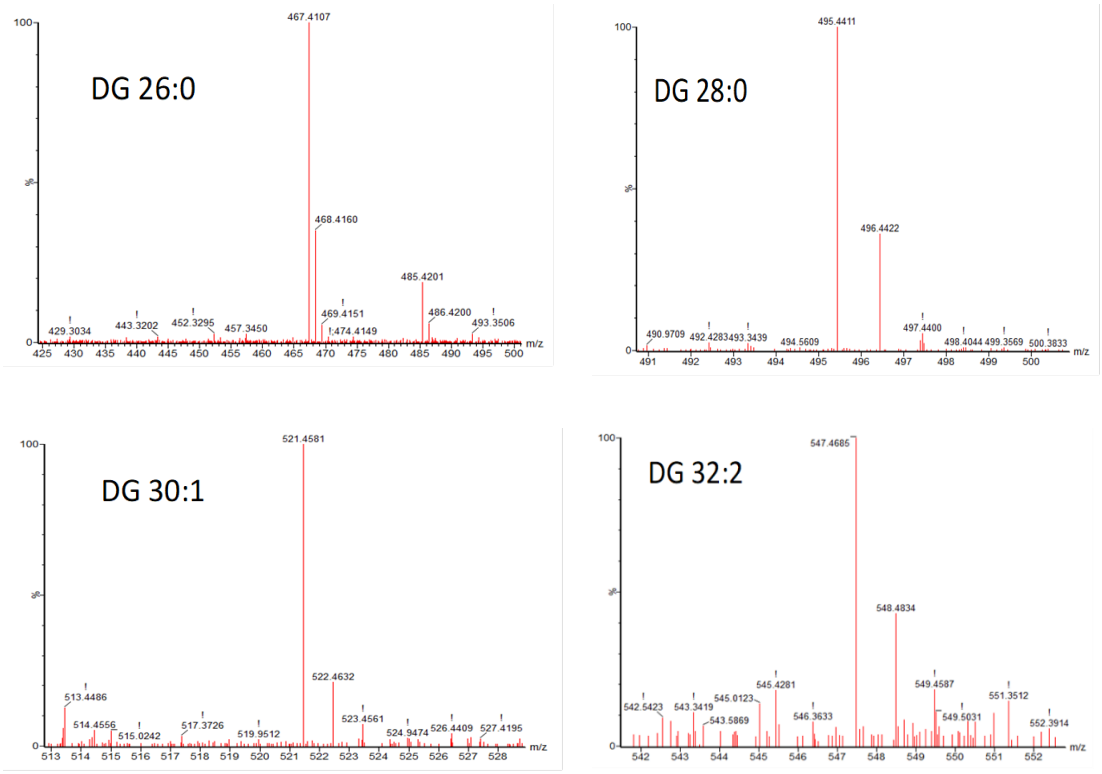


Figure 17. Time-aligned mass spectrum with positive ESI of the most abundant DG species (DG(26:0), DG(28:0), DG(30:1), DG(32:2)) ionised as neutral loss of water ($[M\text{-}H20+H]^+$) in hemolymph of WT_{CS}.

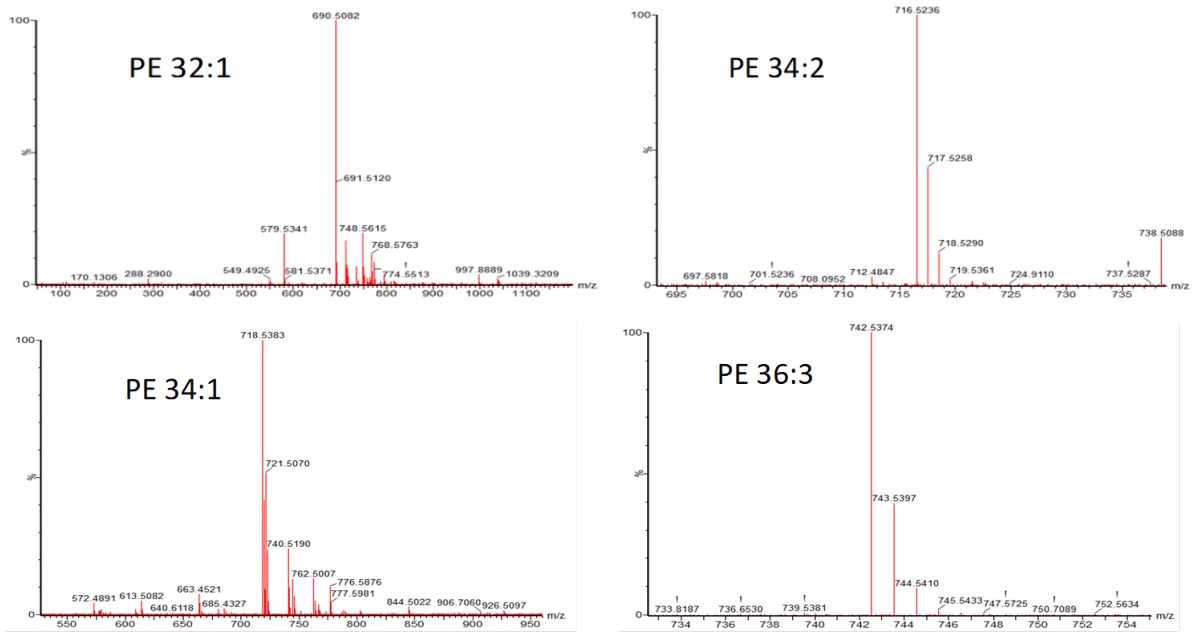


Figure 18. Time-aligned mass spectrum of the most abundant PE species (PE(32:1), PE(34:2), PE(34:1), PE(36:3)) protonised in positive ESI ($[M+H]^+$) in hemolymph of WT_{CS}.

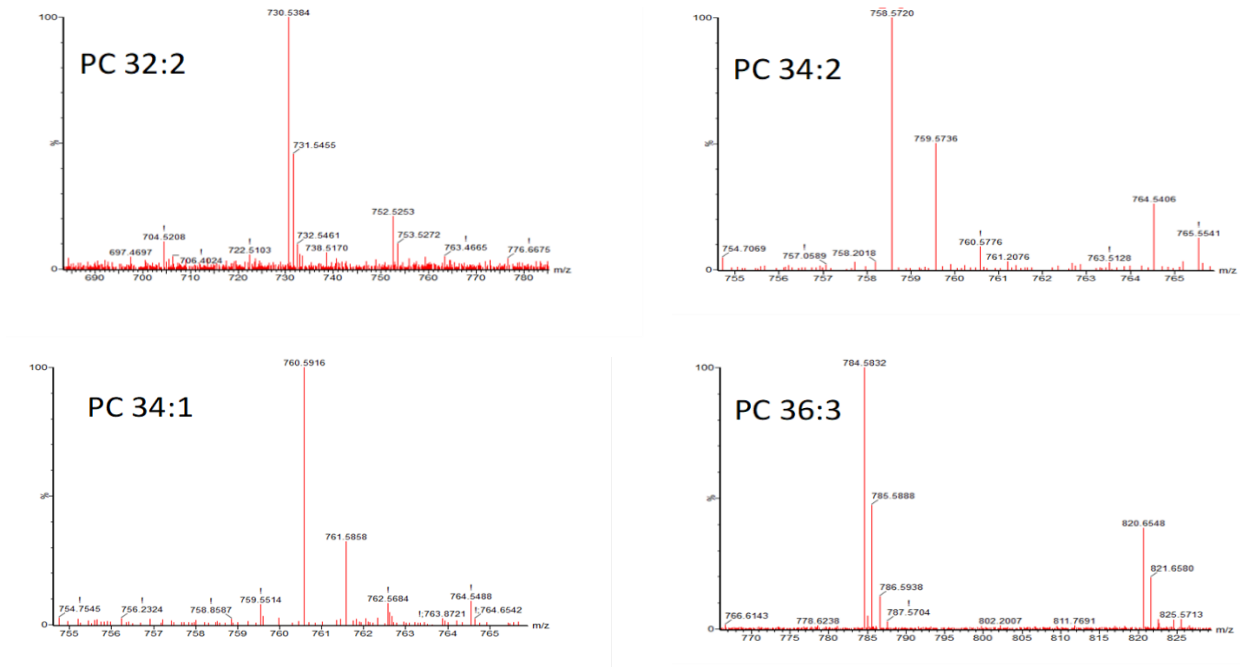


Figure 19. Time-aligned mass spectrum of the most abundant PC species (PC(32:2), PC(34:2), PE(34:1), PE(36:3)) protonised in positive ESI ($[M+H]^+$) in hemolymph of WT_{CS}.

3.2.3 MS/MS Method

Apart from detecting the MS of the precursor ions in full scan mode, product ion scan was performed to selectively detect characteristic fragments of DG, PE and PC in order to increase the confidence level in identifying the different lipid species (Knittelfelder et al., 2014; Pi et al., 2016; Sumner et al., 2007). In this case, precursor ions of DG 20:0, PE 34:0 and PC 34:0 (Fig 17-19) were selected and fragments formed in collision induced cell were. DG(20:0) after fragmentation showed the molecular ion of m/z 383 ($[M\text{-}H_20+H]^+$) and characteristic fragments were at m/z of 228, 85 and 71 at same rt (Fig 20). The fragment with m/z 228 corresponded to neutral loss of C10:0 and H_2O while elemental composition of m/z of 85 and 71 were C_6H_{12} and C_5H_{10} respectively. The predominant fragment ions formed when PE(34:0) was fragmented included ion such as m/z of 579 ($[M\text{-}141+H]^+$) which is the loss of the polar head group (Fig 21). In addition, PC 34:0 ($[M+H]^+$= 762) was subjected to fragmentation and readily formed molecular ion and m/z of 184 (elemental composition $C_5H_{15}O_4NP$) which is a characteristic fragment ion of PC (Fig 22).

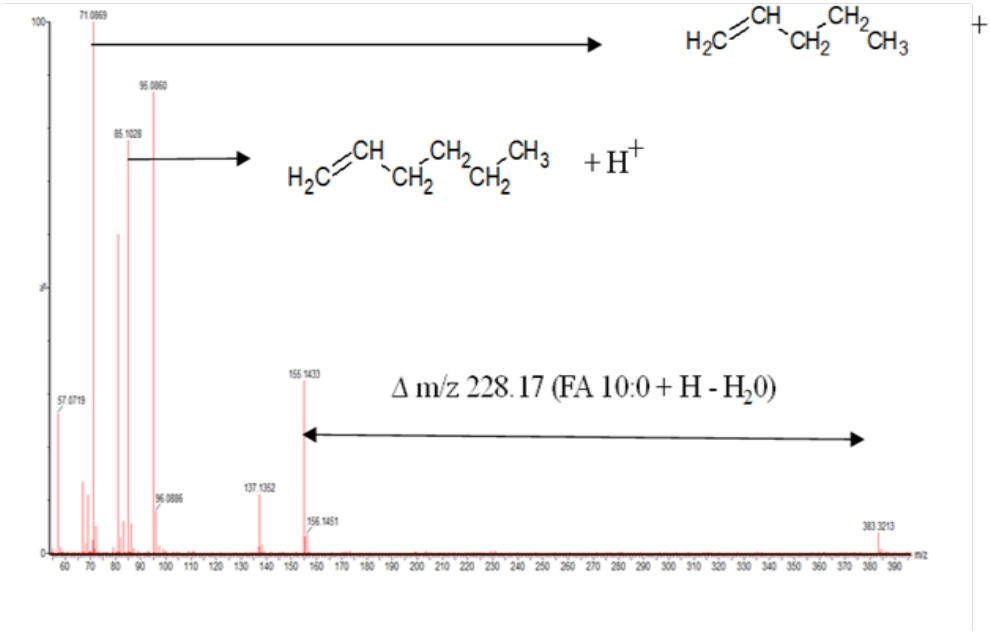


Figure 20. MS/MS spectra of DG(10:0/10:0) in ESI (+) mode with ramp collision energy (15 and 30 eV).

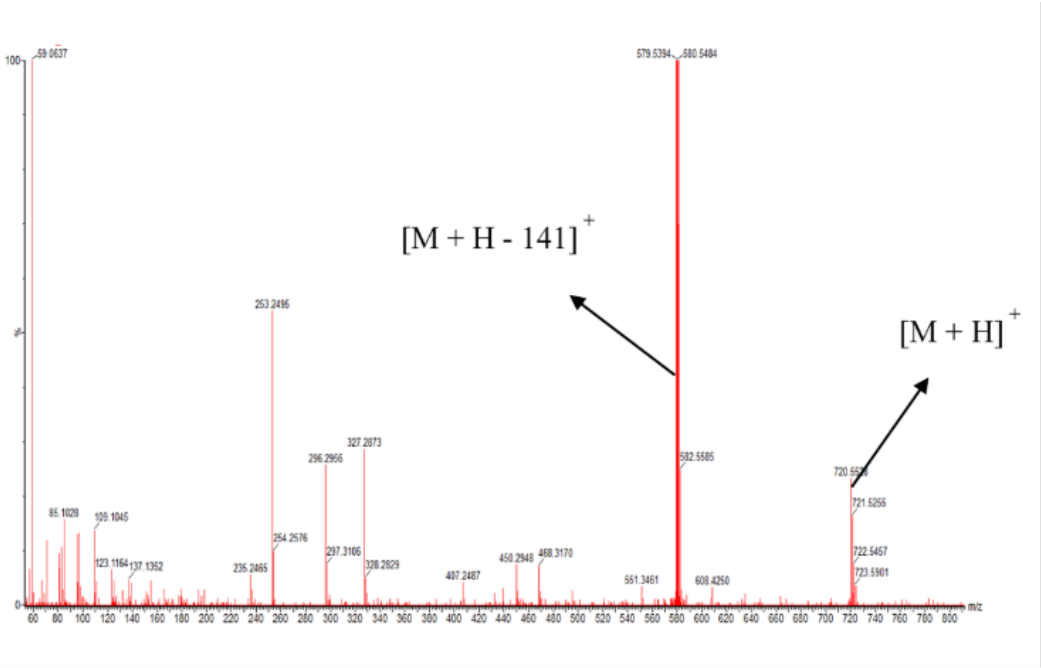


Figure 21. MS/MS spectra of PE(17:0/17:0) in ESI (+) mode with ramp collision energy (15 and 30 eV).

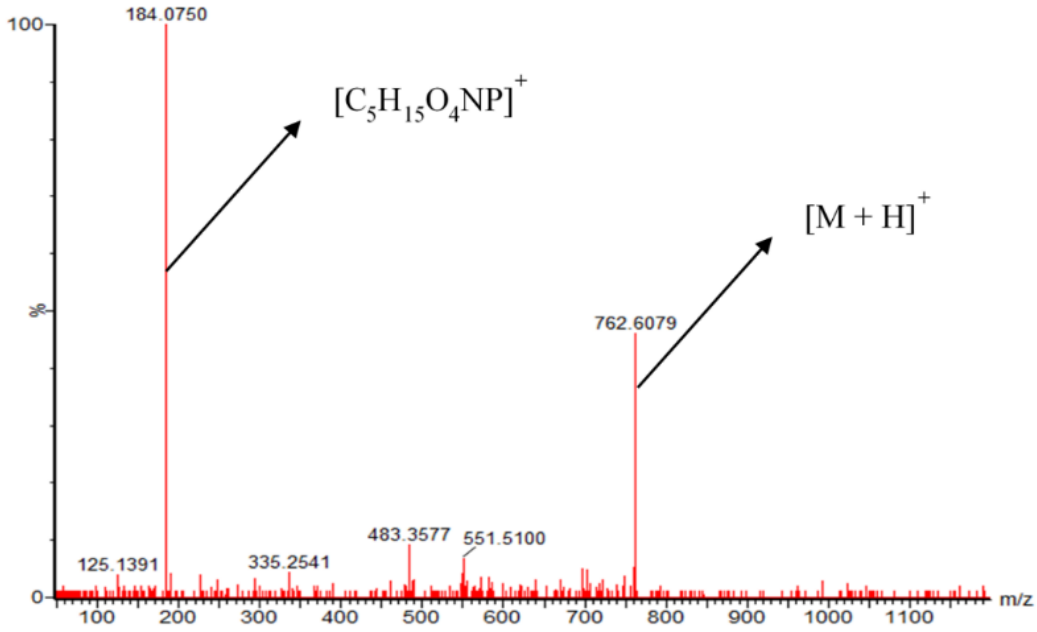


Figure 22. MS/MS spectra of PC(17:0/17:0) in ESI (+) mode with ramp collision energy (15 and 30 eV).

3.3.3 Method validation

3.3.3.1 Linearity

For reliable comparison of levels of analytes, the signal (in my case the peak area) has to be linearly dependent on the concentration. Therefore, a linear calibration curve was plotted using one lipid species within one lipid class (Fig 23). I also determined the linear range of TAGs since they have been used to check whether the hemolymph was contaminated by the fat body. Linear calibration curves with close to 0.99 were obtained over all lipid analyte concentration range between 0.01 and 31.25 ng/µl correspond to 0.001µg and 3.125µg in one sample. The lowest concentration, which peak was clearly distinguishable from the baseline was 0.01ng/µl and therefore this was used as limit of quantification.

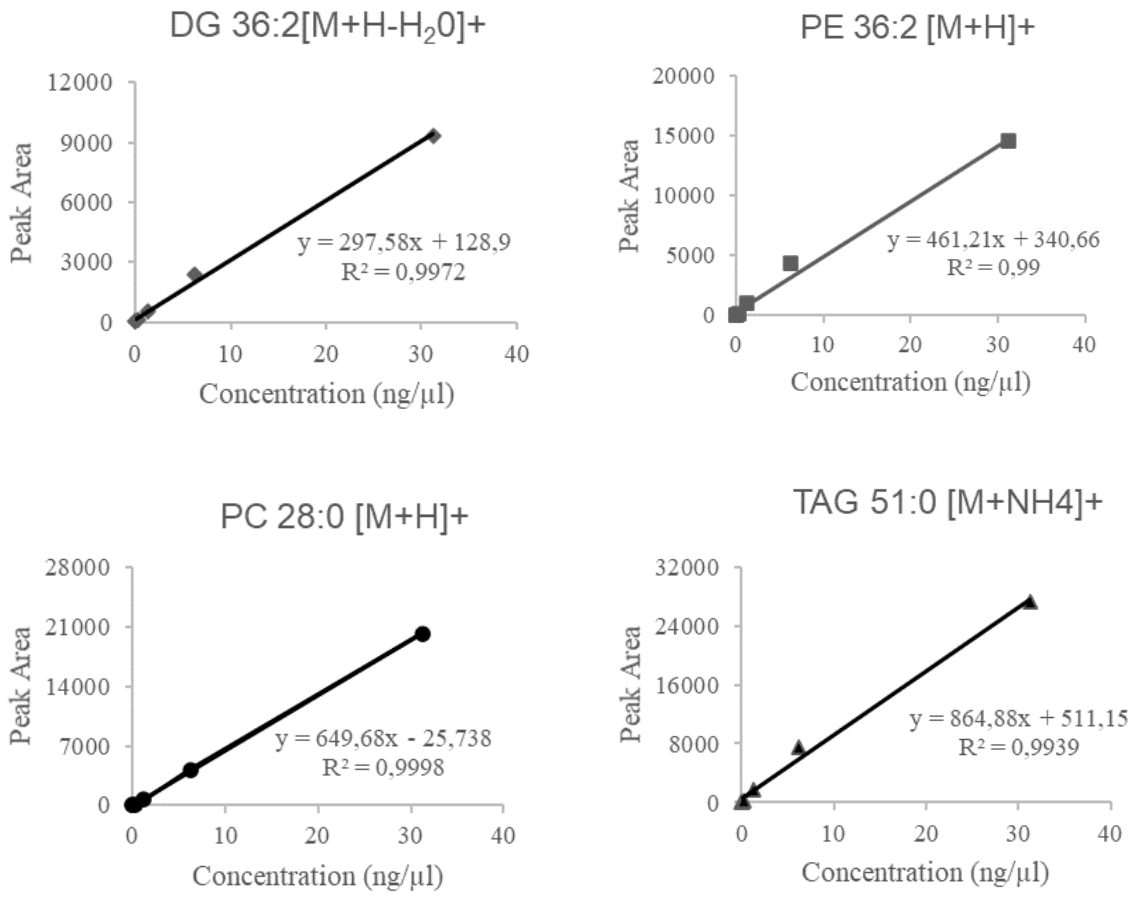


Figure 23. Linear curve plots of lipid analytes (Y-axis = Peak Area, X-axis = Analyte Concentrations). In all of the above plots , the coefficient of determination (R^2) ≥ 0.99. This indicates that variance proportion in the dependent variable (Peak Area) that can be predicted from independent variable (Concentration).

3.3.3.2 Sample-to-sample repeatability

To measure the ability of the method to generate similar result from independent preparations of calibration samples containing internal standards at same concentrations, plots were made of the peak area of six independent sample preparations (Fig 25). The outcome (Fig 24) shows low variations which can be deduced from the plots where the regression coefficient shows no linear correlation but a relative flat line. The relative standard deviations were between 14 -33 % suggesting that the protocol is repeatable and therefore can be used for real sample analysis.

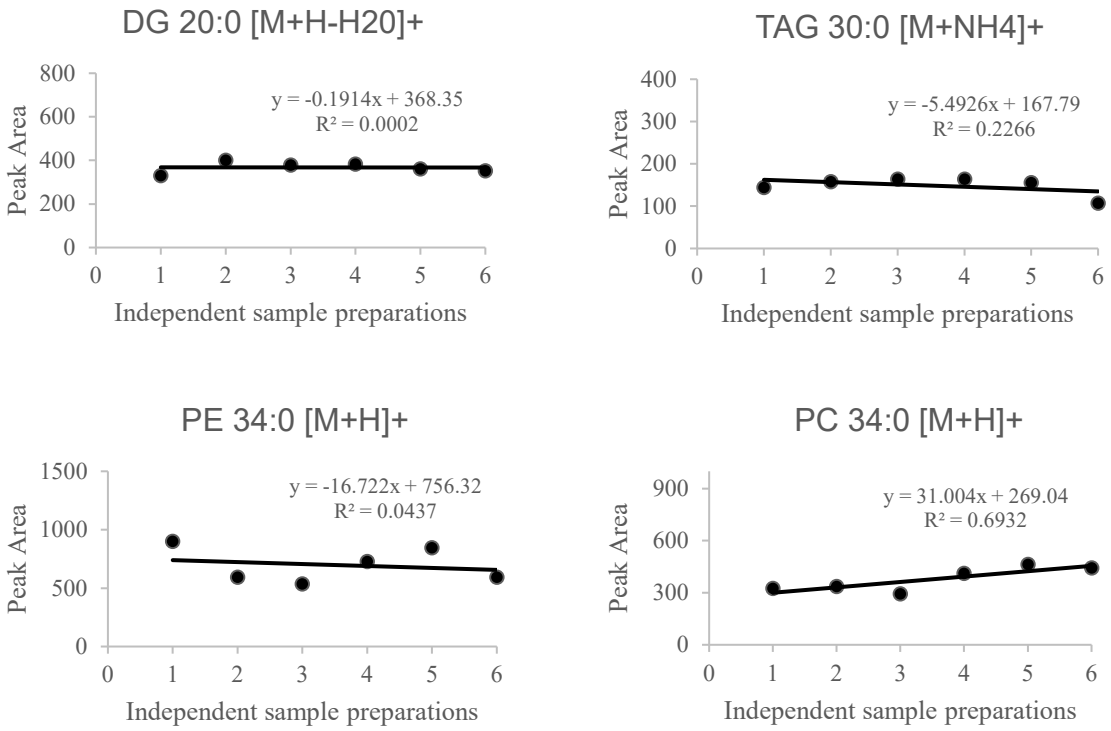


Figure 23. Shows the scatter plot of lipid internal standards from six (6) independently prepared samples.

3.3.3.3 Response factor

In order to evaluate the concentration of lipids in the hemolymph of fruit fly, the response factor (RF) of the various lipid classes was determined. RF was evaluated by injecting known amounts of both internal standards and lipid analytes and recording their peak areas. Using the formula in equation 2, the response factor was evaluated for each lipid class (Table 1). Results obtained shows that the response factor of all lipid classed was approximately one which suggest that lipid quantification can be carried out effectively.

Table 1. Shows the different lipid classes and response factor obtained.

Lipid species	Response factor
Diacyglycerol (DG)	1.0
Triacyglycerol (TAG)	0.8
Phosphoethanolamine (PE)	0.9
Phosphocholine (PC)	1.0

3.3 Transport lipids and phospholipids in the fly hemolymph remain its homeostasis under nutrition rich diet

To obtain a temporal picture of hemolymph lipids in *Drosophila*, I measured lipid levels of WT_{CS} wild-type male fruit flies at 8 different time points within 24hrs under 12hr/12hr light-dark cycle (LD). Wild-type flies were fed on standard medium for 5 days, and hemolymph samples collected on the 6^{th} day using the optimised protocol. Three lipid classes (DG, PE and PC) were determined in the raw extracts using UPLC-MS (Fig 25). To verify the veritable oscillation pattern, the experiment was independently repeated six times (Fig 25B, D and F) over two years at different seasons. Therefore, flies of different generations of the same stock were investigated. To evaluate if lipids levels in the fly hemolymph are rhythmic, I normalised all measurements to the highest concentration (n=10-13) of the six experiments and relative levels were averaged at each ZT. JTK cycle (Hughes et al., 2010) a non-parametric algorithm was used to identify rhythmicity of the detected lipids. The period length had to be set between 6-24hrs instead of 24hrs since samples were taken over the course of one day with 3hrs resolution. In addition, oscillation patterns other than unimodal can be also filtered out. Daily variations were identified as rhythmic if the adjusted p (ADJ.P) value was lower than 0.05. JTK- Cycle also calculates the ZTs in which the fitted waves has the highest value defined as lag phase (LAG) and the relative amplitudes.

As shown in Table 2, there were no oscillation in the levels of total DG as p-values were greater than 0.05 except for experiment 1. However, on close look into the data, there seem to be two peaks between ZT1-ZT4 and ZT13-ZT16 in all experiments (Fig 25B, supplementary fig 37). This daily fluctuation in the hemolymph DGs was clearly seen when all experiments were averaged (Fig 25A). Just like in total DG, JTK cycle did not reveal oscillations in total PE (Table 2). However, there seems to be a broad peak between ZT1 and ZT7 and then a trough

at ZT10 followed by a peak at ZT13 (Fig 25C and D, supplementary fig 38-39). A comparable pattern was obtained in total PC as in total PE. Although JTK cycle did not reveal daily rhythmicity in PCs, weak bimodal oscillation pattern was observed.

Putting these result together, it seems that hemolymph lipids including DG, PE and PC remain its homeostasis in male flies fed *ad libitum* with nutrition rich diet (standard medium). However, the observed weak time-dependent pattern can suggest suppressed or masked oscillation.

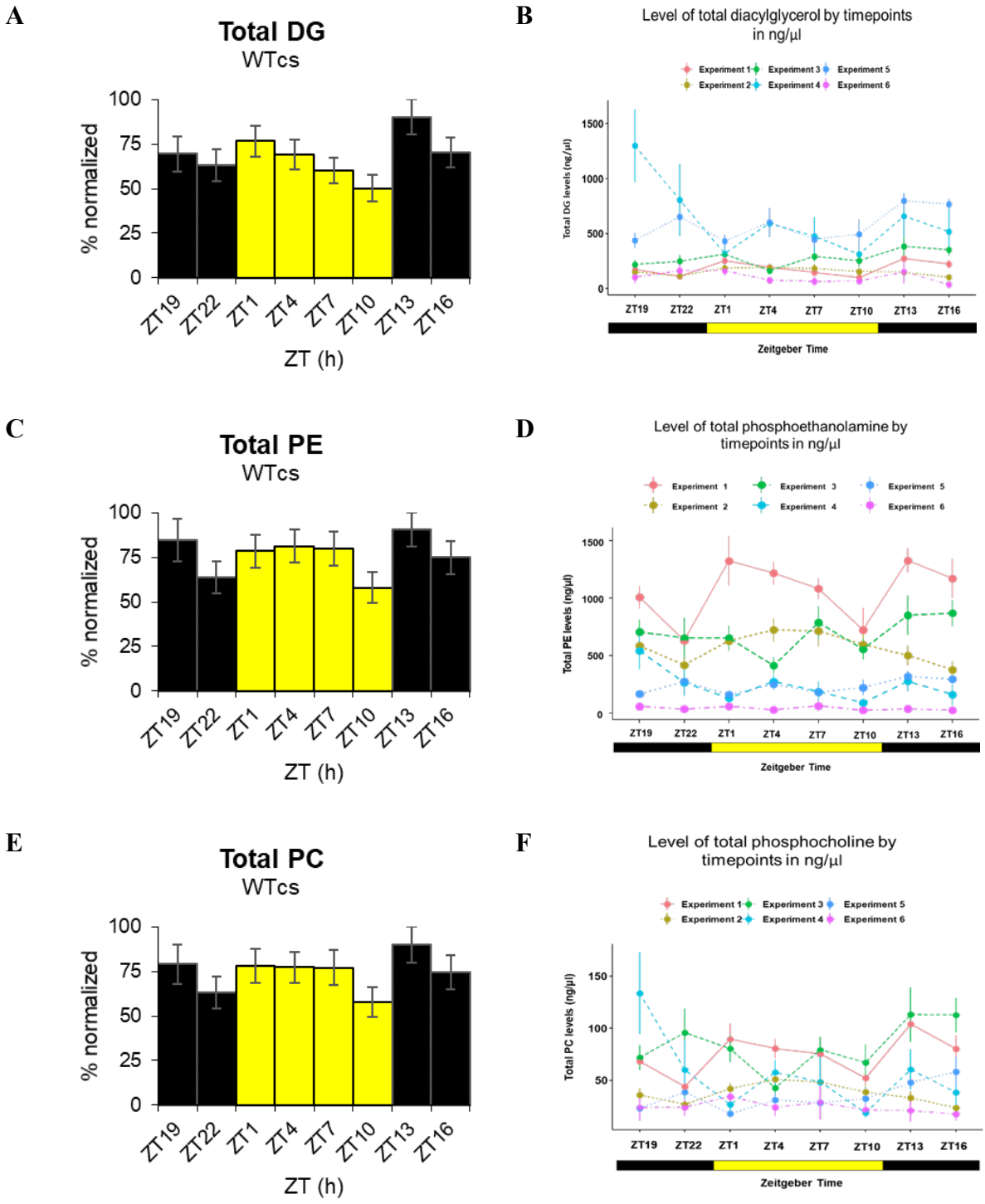


Figure 24. Shows oscillation profiles of DG, PE and PC in the hemolymph of WT_{CS} male flies fed on standard medium under LD at 8 different timepoints. Normalized levels of DG (A), PE (C) and PC (E) of all six experiments were averaged. Time dependent levels of DG (B), PE (D) and PC (F) in six independently performed experiments are also visualised. Data represents mean ± standard deviation (n=10-13 for one experiment). Rhythmicity is determined using JTK

cycle algorithm and adj.p < 0.05 shows oscillation (Table 2). The lightening condition is designated by the colours of the bars such that yellow = light-on and black = light-off.

Table 2. JTK cycle results of DG, PE and PC oscillation under LD when WT_{CS} flies were fed with standard medium (six independent experiments)**.** Abbreviations: BH.Q=Benjamin Hochberg coefficient, ADJ.P: adjusted p value. Green indicates oscillation ($p < 0.05$) while Red indicates no oscillation ($p > 0.05$).

Lipid class	Biological replicates	BH.Q	ADJ.P	PER	LAG	Relative Amplitude (%)
DG	Experiment 1	0.00	0.00	15	0	8.2
	Experiment 2	0.79	0.48	24	4.5	3.3
	Experiment 3	0.79	0.48	18	13.5	3.6
	Experiment 4	1.00	1.00	9	3	11.8
	Experiment 5	0.08	0.03	24	15	7.4
	Experiment 6	1.00	0.73	24	1.5	1.7
PE	Experiment 1	0.03	0.00	15	0	26.8
	Experiment 2	0.45	0.23	24	4.5	13.5
	Experiment 3	1.00	0.84	24	15	8.7
	Experiment 4	1.00	0.93	6	1.5	2.7
	Experiment 5	0.05	0.01	9	4.5	5.4
	Experiment 6	1.00	1.00	15	1.5	0.7
PC	Experiment 1	0.08	0.02	15	0	2.0
	Experiment 2	0.08	0.03	24	4.5	1.0
	Experiment 3	0.45	0.21	24	15	1.0
	Experiment 4	1.00	0.79	6	1.5	0.6
	Experiment 5	0.05	0.01	9	4.5	1.1
	Experiment 6	1.00	1.00	9	0	0.6
Lipid class	**Experiments**	**BH.Q**	**ADJ.P**	**PER**	**LAG**	**Relative Amplitude (%)**
DG	Normalized and averaged	1.0	1.0	15	13.5	45
PE	Normalized and averaged	1.0	0.6	6	1.5	27
PC	Normalized and averaged	1.0	1.0	15	0.0	28

3.4 *Drosophila* standard medium contains lipids found in hemolymph

One possible reason for the weak or absent oscillation of hemolymph lipids could be that their levels are compensated by dietary lipids leading to homeostasis. Therefore, I wanted to know

if standard medium contains similar lipids found in the hemolymph of *Drosophila*. As expected, the standard *Drosophila* food contained classes of lipids (DG, PE and PC) which were also detected in the hemolymph of the fruit fly (Fig 26). Furthermore, the result shows high abundance of polyunsaturated DGs in the standard food. Hence, I assumed that the lack of hemolymph lipid oscillation in wild-type flies may be due to masking of exogenous lipids from dietary lipids in the standard medium. This is supported by some studies showing that diet composition can influence Drosophila physiology, especially the lipidome (Bruce et al., 2013; Carvalho et al., 2012; Laye et al., 2015). To confirm the hypothesis, I decided to determine time-dependent levels of hemolymph lipids in flies which obtained a lipid-free diet. Therefore, I fed flies with agar containing sugar only, defined as sugar medium. As expected, no lipids were detectable in sugar medium. (Fig 26).

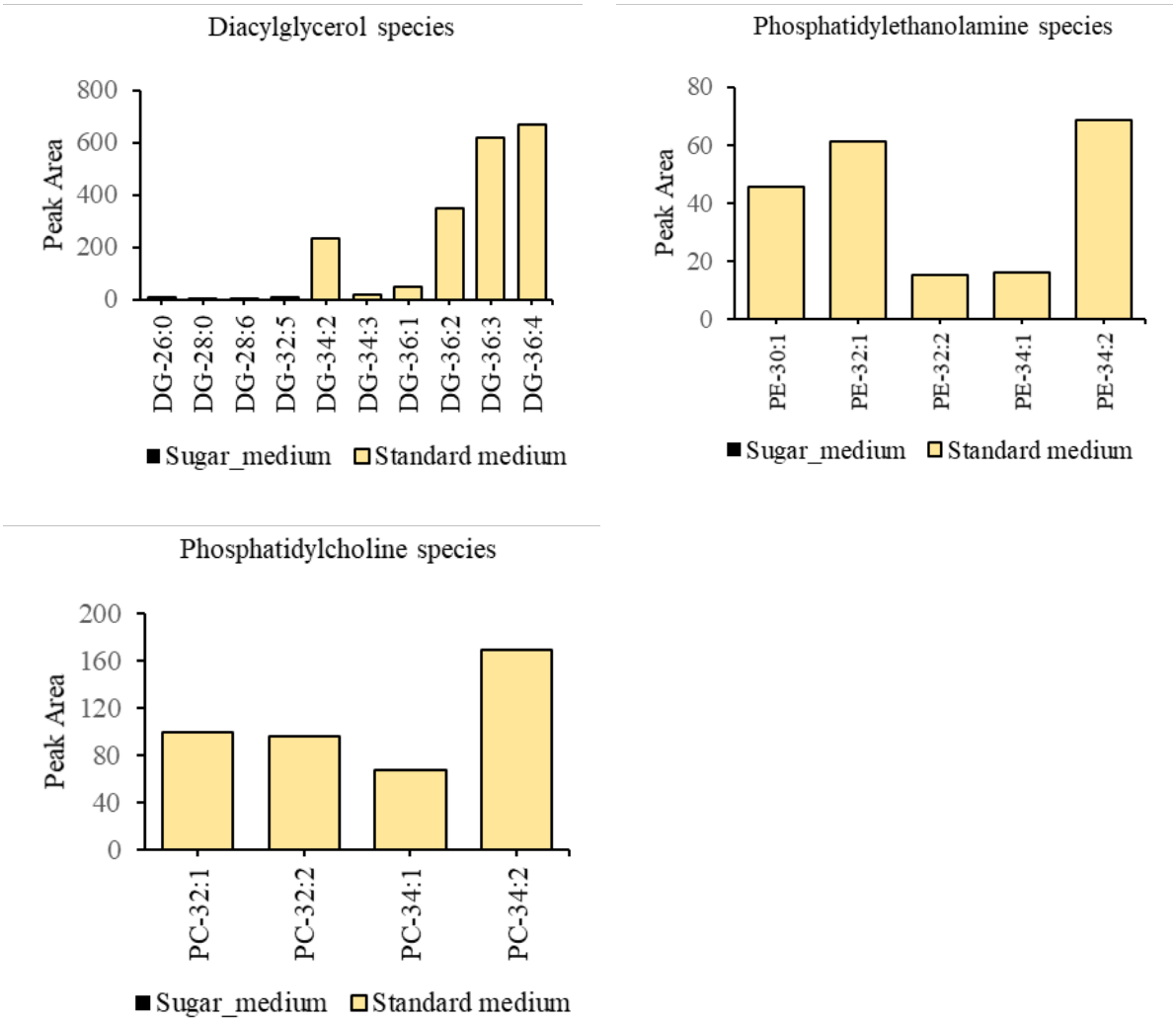


Figure 25. Shows the peak area of various lipid classes (DG, PE and PC) and sub-species detected in Drosophila standard food.

3.5 *Drosophila* hemolymph lipid exhibits oscillation under lipid-free medium

I hypothesised that daily rhythmicity in *Drosophila* hemolymph lipids was masked due to exogenous interference from dietary lipids. To investigate if hemolymph lipids oscillate in flies fed on lipid-free medium, levels of DG, PE and PC were measured in the hemolymph of WT_{CS} flies at 8 different time points. Flies were *ad libitum* fed with 5% sugar on agarose under LD (Fig 27).

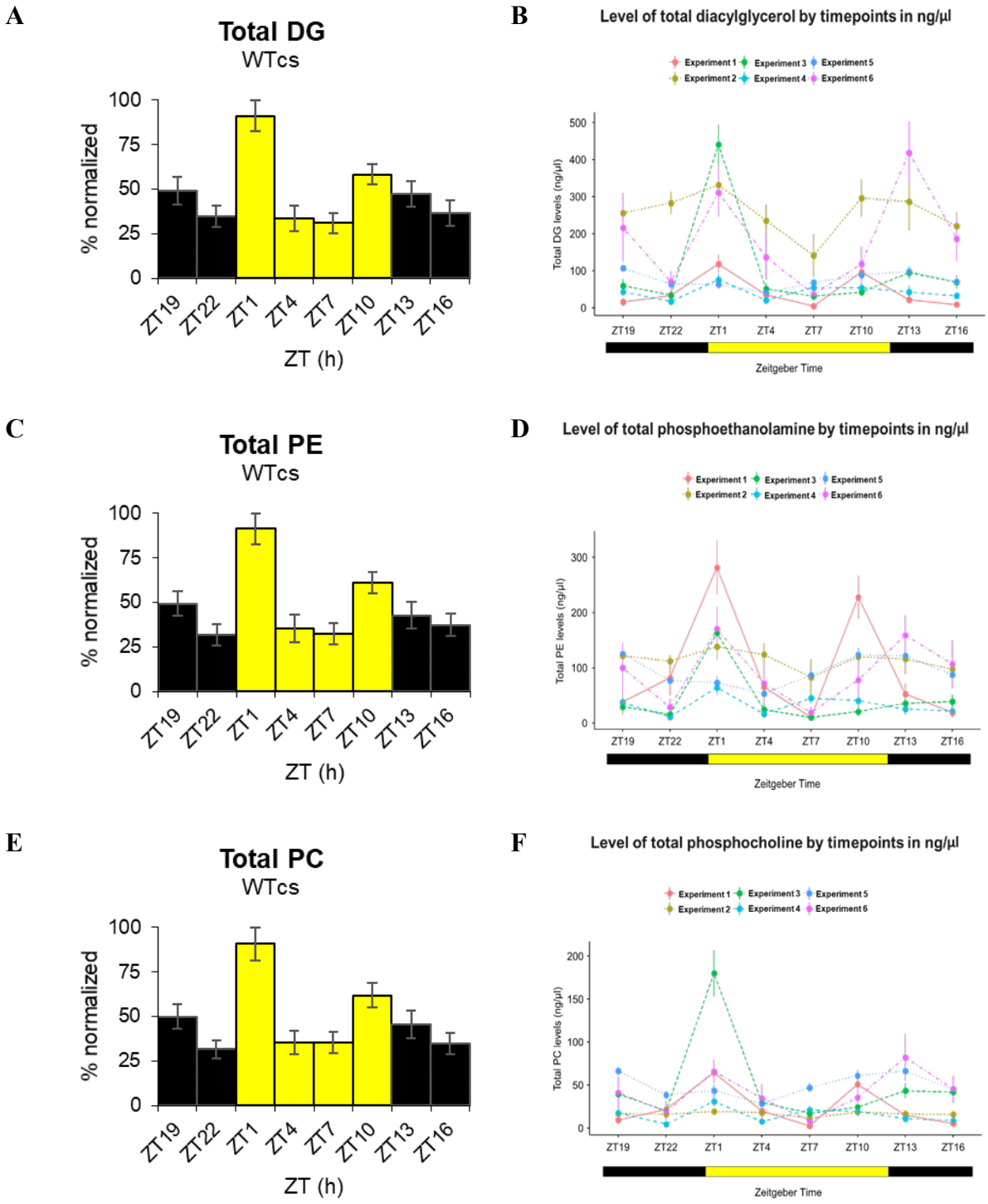


Figure 26. Oscillation profiles of DG, PE and PC in the hemolymph of WT_{CS} male flies fed on lipid-free medium under LD at 8 different timepoints. Normalized levels of DG (A), PE (C) and PC (E) of all six experiments were averaged. Time dependent levels of DG (B), PE (D) and PC (F) in six independently performed experiments are also visualised. Data represents mean ± standard deviation (n=10-13 for one experiment). Rhythmicity is determined using JTK

cycle algorithm and adj.p < 0.05 shows oscillation (Table 3). In the bar graphs, the lightening condition is designated by the colours of the bars (yellow = light-on and black = light-off).

Table 3. JTK cycle results of DG, PE and PC oscillation under LD when WT_{CS} flies were fed lipid-free medium (6 independent experiments) Abbreviations: BH.Q=Benjamin Hochberg coefficient, ADJ.P: adjusted p value. Green indicates oscillation ($p < 0.05$) while Red indicates no oscillation ($p > 0.05$).

Lipid class	Biological replicates	BH.Q	ADJ.P	PER	LAG	Relative Amplitude (%)
DG	Experiment 1	0.001	0.000	12	0.0	4.5
	Experiment 2	0.330	0.300	12	0.0	9.0
	Experiment 3	0.001	0.000	12	0.0	2.6
	Experiment 4	0.080	0.060	6	0.0	3.0
	Experiment 5	0.010	0.005	21	13.5	3.2
	Experiment 6	0.008	0.003	15	13.5	30.3
PE	Experiment 1	0.001	0.000	12	0.0	10.7
	Experiment 2	1.000	1.000	9	1.5	3.8
	Experiment 3	0.004	0.001	15	0.0	3.5
	Experiment 4	0.060	0.050	6	0.0	2.6
	Experiment 5	0.008	0.003	21	13.5	4.2
	Experiment 6	0.013	0.009	15	0.0	7.6
PC	Experiment 1	0.001	0.000	12	0.0	2.5
	Experiment 2	1.000	1.000	9	1.5	0.5
	Experiment 3	0.020	0.010	15	0.0	3.6
	Experiment 4	0.013	0.009	6	0.0	1.3
	Experiment 5	0.008	0.003	21	13.5	1.9
	Experiment 6	0.010	0.005	15	13.5	5.2
Lipid class	**Experiments**	**BH.Q**	**ADJ.P**	**PER**	**LAG**	**Relative Amplitude (%)**
DG	Normalized and averaged	1.10E-06	3.67E-07	12	0.0	25
PE	Normalized and averaged	1.66E-06	1.10E-06	9	0.0	39
PC	Normalized and averaged	2.67E-06	2.67E-06	9	0.0	36

Manual inspection as well as JTK cycle revealed a clear oscillation in the levels of hemolymph DG (ADJ.P=3.67E-07) when all six experiments were normalised and averaged (Fig 27, table 3). In the light phase, two peaks were observed with maxima that occur at ZT1 and ZT10 and

troughs between ZT4 and ZT7 (Fig 27A). In the dark phase, the total DG level was constant. This experiment was repeated in six times (Fig 27B, supplementary fig 40) and all experiments showed relatively similar pattern of total DG levels with only one experiment (experiment 2) showing no oscillation (Table 3).

Total PE oscillated with a similar profile as total DG with two conspicuous peaks at ZT1 and ZT10, a trough between ZT4 and ZT7 and in the dark phase the PE levels remained constant (Fig 27C). Daily rhythmicity was confirmed by JTK cycle as p-values were lower than 0.05 in five out of six experiments shown in Table 3. In the six independent experiments (Fig 27D, supplementary fig 41), rhythmic patterns of PE levels were comparable. Also as total DG and PE, total PC showed oscillation according to JTK cycle (Table 3) with similar oscillation profile (Fig 27E and F, supplementary fig 42) in all six independently performed experiments.

My result suggests that levels of DG, PE and PC in the hemolymph of wild-type flies fed *ad libitum* on lipid-free medium oscillate in daily manner. This observation seems to be due to the absence of exogenous dietary lipid. In addition, it is safe to say that the diet-type exerts a strong influence on rhythmicity of circulating lipids.

3.6 *Drosophila* hemolymph lipids has a circadian component and is affected by light

To determine if the observed oscillation is endogenously driven, I analysed lipids in the hemolymph of WT_{CS} wild-type flies fed on lipid-free medium under DD at 8 time points (Fig 28). JTK cycle did not indicate daily oscillation in the levels of total DG and PE as in the two independently performed experiments (Fig 28A, B, C and D, Table 4). Interestingly, on closer look the CT1 peak diminishes while CT10 peak is still present and an additional peak at CT22 appeared (Fig 28A,C). On the other hand, levels of PC showed oscillation as indicated by the JTK cycle results (Table 4) with peaks at CT10 and 22 but absence of peak at CT1 as observed in DG and PE (Fig 28E,F). This result indicates the importance of light in lipid oscillation in fly hemolymph.

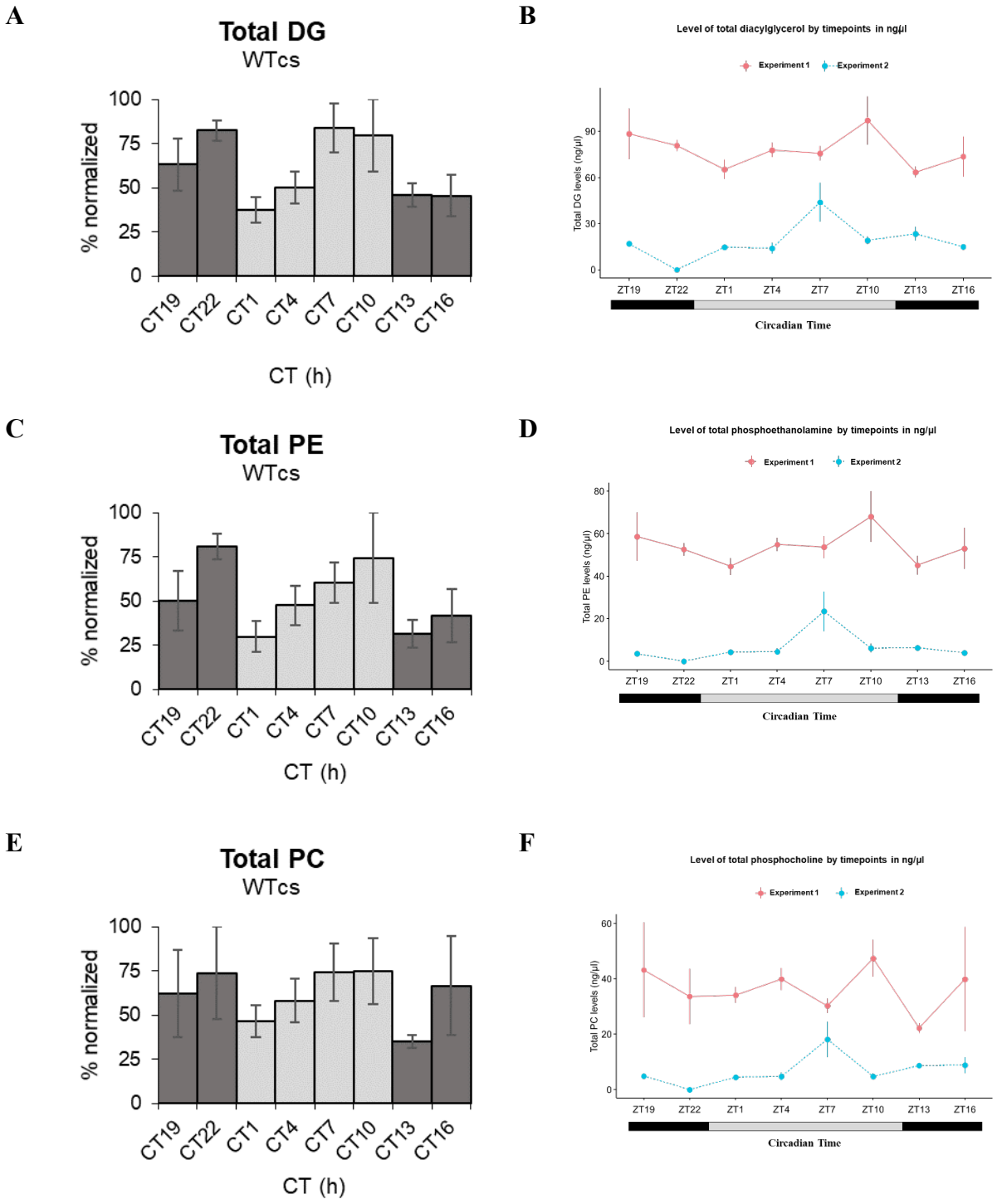


Figure 27. Oscillation profiles of DG, PE and PC in the hemolymph of WT_{CS} male flies fed on lipid-free medium under DD at 8 different timepoints. Normalized levels of DG (A), PE (C) and PC (E) of all six experiments were averaged. Time dependent levels of DG (B), PE (D) and PC (F) in two independently performed experiments are also visualised. Data represents mean ± standard deviation (n=10-13 for each experiment). Rhythmicity is determined using JTK cycle algorithm and adj.p < 0.05 shows oscillation (Table 4). In the bar graphs, the

lightening condition is designated by the colours of the bars (light grey = subjective light-on and dark grey = subjective light-off)

Table 4. JTK cycle results of DG, PE and PC oscillation under constant darkness when when WT_{CS} flies were fed lipid-free medium (two independent experiments)**.** Abbreviations: BH.Q=Benjamin Hochberg coefficient, ADJ.P: adjusted p value. Green indicates oscillation ($p < 0.05$) while Red indicates no oscillation ($p > 0.05$).

Lipid class	Biological replicates	BH.Q	ADJ.P	PER	LAG	Relative Amplitude (%)
DG	Experiment 1	0.162	0.135	15	7.5	18.6
	Experiment 2	0.160	0.107	15	9.0	20.9
PE	Experiment 1	0.999	0.999	18	6.0	24.2
	Experiment 2	0.325	0.325	21	7.5	4.0
PC	Experiment 1	0.013	0.009	24	4.5	26.6
	Experiment 2	0.039	0.013	9	6.0	5.7
Lipid class	**Experiments**	**BH.Q**	**ADJ.P**	**PER**	**LAG**	**Relative Amplitude (%)**
DG	Normalized and averaged	1.0	1.0	18	7.5	64
PE	Normalized and averaged	0.3	0.2	24	9.0	15
PC	Normalized and averaged	0.009	0.003	24	9.0	20

As rhythmicity of lipid levels dampened and changed in WT_{CS} flies under DD compared to LD, the question if circadian clocks modulates lipid levels remained unclear. Therefore, I analysed the targeted lipids in hemolymph of per^{01} clock mutant flies under LD (Fig 29). Oscillation in levels of normalized and averaged levels of DG, PE and PC were diminished as indicated by the JTK cycle results (Table 5). The oscillation patterns varied considerably from one experiment to another indicating complete arrhythmicity of hemolymph lipids in per^{01} flies (Fig 29B, D & F). This suggest that daily rhythmicity of the analysed lipids in the fly hemolymph is dependent on the *per* clock gene.

From all results above, it is clear that in the absence of light rhythmicity in hemolymph lipid levels is dampened and ZT1 peak diminished in WT_{CS} wild-type flies (Fig 29A,C,E). This indicates that the light-dark cycle is necessary for hemolymph lipid oscillation. Additionally,

hemolymph lipid oscillation has a circadian component due to lack of oscillation and arrhythmicity observed in *per*^{01} mutant flies.

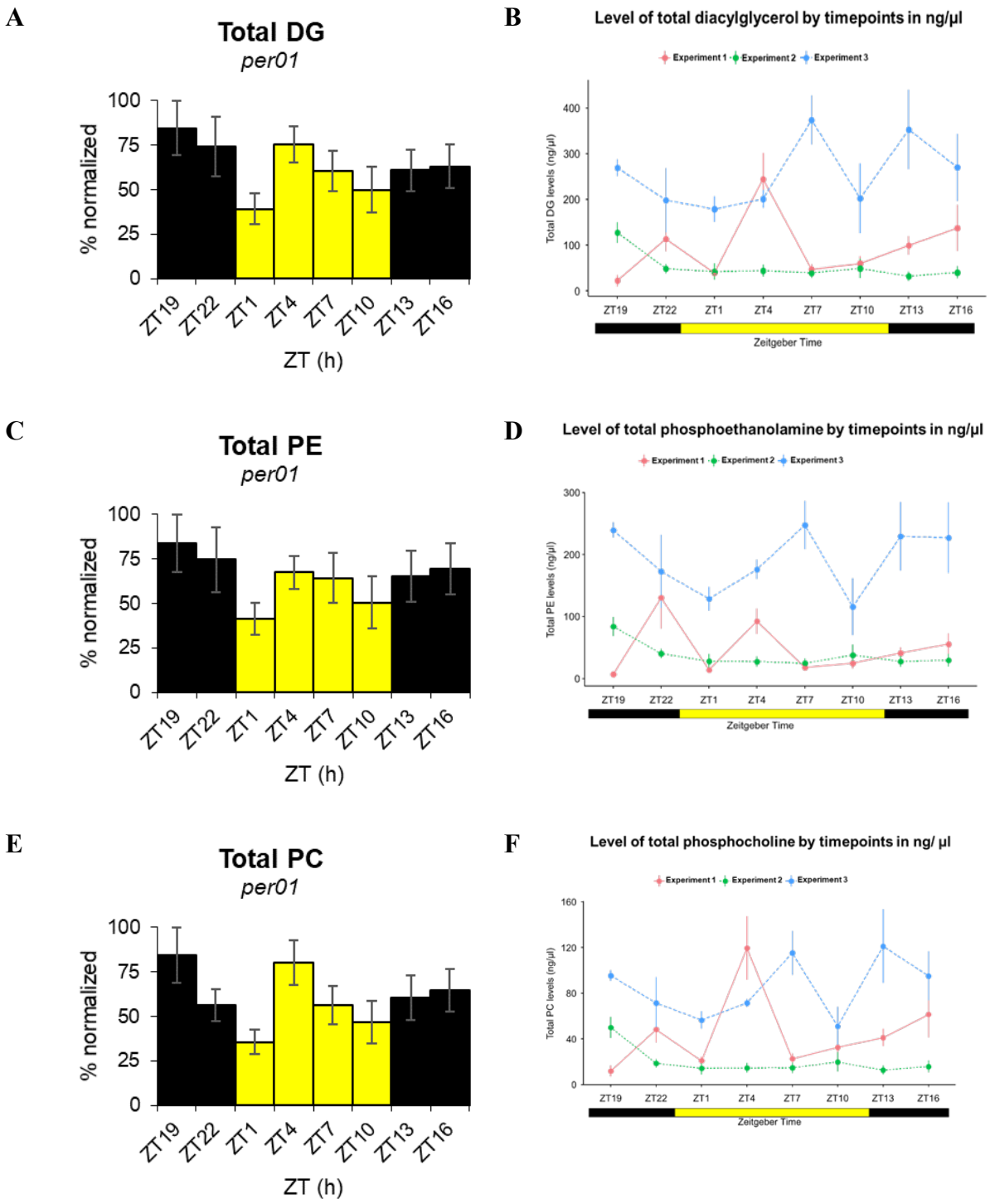


Figure 28. Oscillation profiles of DG, PE and PC in the hemolymph of *per*^{01} male flies fed on lipid-free medium under LD at 8 different timepoints. Normalized levels of DG (A), PE (C) and PC (E) of all three experiments were averaged. Time dependent levels of DG (B), PE (D)

and PC (F) in three independently performed experiments are also visualised. Data represents mean ± standard deviation (n=10-13 for each experiment). Rhythmicity is determined using JTK cycle algorithm and adj.p < 0.05 shows oscillation (Table 5). In the bar graphs, the lightening condition is designated by the colours of the bars (yellow = light-on and black = light-off).

Table 5. JTK cycle results of DG, PE and PC oscillation under LD when per^{01} flies were fed lipid-free medium (two independent experiments)**.** Abbreviations: BH.Q=Benjamin Hochberg coefficient, ADJ.P: adjusted p value. Green indicates oscillation ($p < 0.05$) while Red indicates no oscillation ($p > 0.05$).

Lipid class	Biological replicates	BH.Q	ADJ.P	PER	LAG	Relative Amplitude (%)
DG	Experiment 1	0.036	0.012	12	3.0	15.9
	Experiment 2	0.584	0.357	15	4.5	2.2
	Experiment 3	0.727	0.646	24	12.0	38.3
PE	Experiment 1	0.005	0.001	6	4.5	13.8
	Experiment 2	0.584	0.282	15	4.5	0.6
	Experiment 3	0.584	0.411	12	6.0	10.9
PC	Experiment 1	0.036	0.009	12	3.0	6.8
	Experiment 2	0.584	0.282	15	4.5	0.6
	Experiment 3	0.584	0.411	12	6.0	10.9
Lipid class	**Experiments**	**BH.Q**	**ADJ.P**	**PER**	**LAG**	**Relative Amplitude (%)**
DG	Normalized and averaged	1.0	1.0	9	4.5	34
PE	Normalized and averaged	1.0	0.6	9	4.5	47
PC	Normalized and averaged	1.0	1.0	12	6.0	19

3.7 *Drosophila* feeding behaviour on standard medium exhibits a clock-modulated rhythm

Since levels of DGs in the hemolymph of *Drosophila* fed on standard medium showed a tendency of an early light phase peak, I wanted to ensure that the used wild type stock exhibits a circadian controlled behaviour. Since energy levels is tightly connected to feeding (Xu et al.,

2008), I therefore tested for clock-controlled feeding behaviour and monitored food consumption of ten WT_{CS} wild-type male flies in a modified CAFE assay chamber (Fig 9).

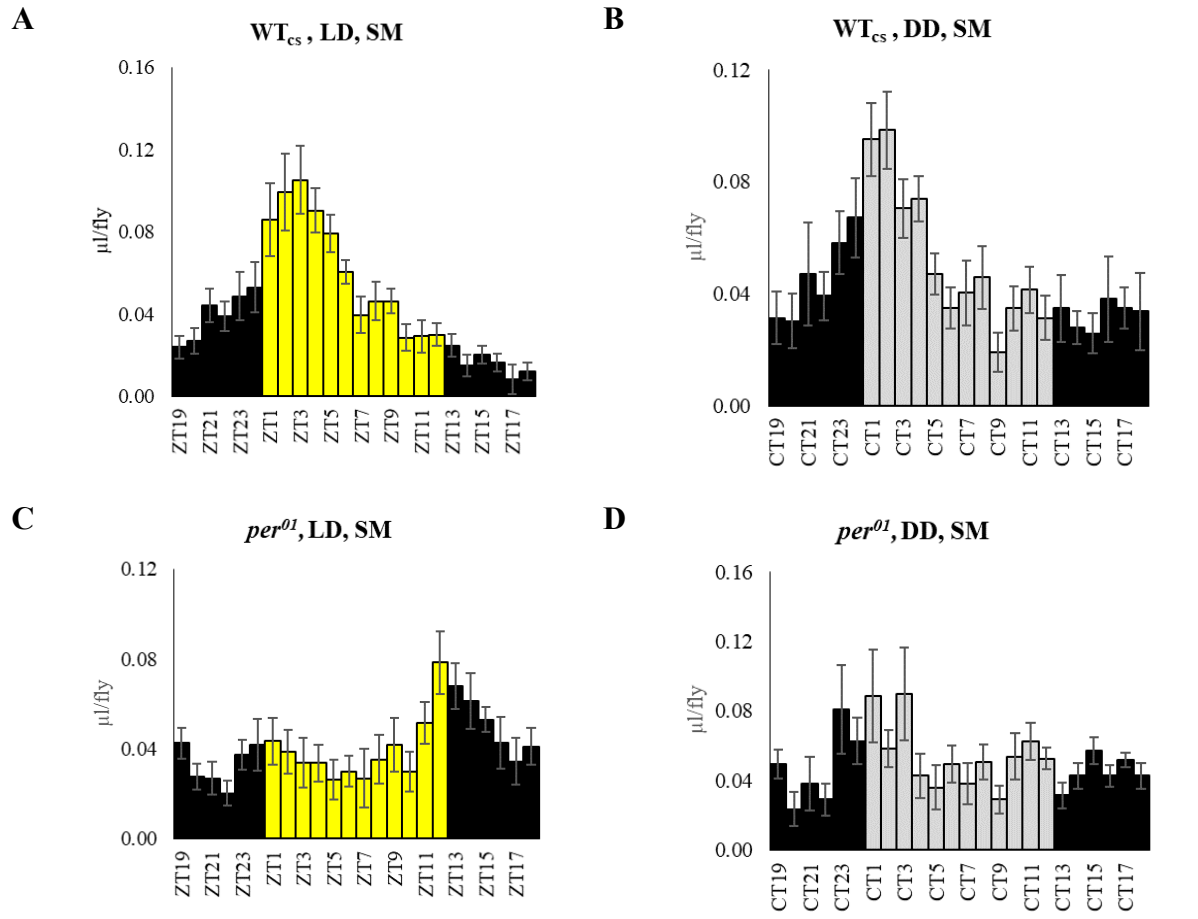


Figure 29. *Drosophila* shows circadian feeding behaviour when fed standard medium**.** Feeding rhythm of WTcs flies under light-dark cycle (A) and under constant darkness (B). Time-dependent food consumption of per^{01} flies under light-dark cycle (C) and under constant darkness (D). All flies were fed standard medium *ad libitum* and a modified CAFÉ assay was used to determine hourly food consumption of 10 male flies in a chamber. Abbreviations: SM=standard medium, LD=light-dark cycle, DD=constant darkness, WT_{CS}=Canton-S, per^{01}=$period^{01}$. All bar plots were plotted using standard error and n=10.). In the bar graphs, the lightening condition is designated by the colors of the bars (LD: yellow = light-on and black = light-off; DD: light grey = subjective light-on and dark grey = subjective light-off).

The results showed a robust rhythmic feeding pattern under LD with a large peak in the early light phase between ZT1 to ZT5 and a trough during the dark phase (Fig 30A, supplementary

fig 47A, B and C). JTK cycle confirmed a strong oscillation with an adjusted p-value= 6.37E-18 (Supplementary table 11) which is in line with the literature (Seay & Thummel, 2011; Xu et al., 2008).

To determine if the observed feeding rhythm is under circadian control, I monitored food consumption of wild-type flies maintained in constant darkness after 3 days of entrainment under light-dark condition (Fig 30B, supplementary fig 47A,C,D). As expected, WTcs flies maintained their feeding oscillation under DD with a peak between CT23 –CT5 and a trough in the dark phase (Fig 30B). Further, I characterised if the per^{01} feeding behaviour oscillates under LD when fed on standard medium. In line with the literature (Xu et al., 2008), I found that the clock mutant flies showed oscillation under light/dark condition with a minor early light phase peak between ZT24 –ZT3 and a major late light phase/early dark phase peak between ZT11-ZT15 (Fig 30C, supplementary fig 47). Under free running condition, per^{01} showed no discernible feeding oscillation as also reflected by the JTK result (p-value=0.39) (Supplementary Table 11).

These observations suggest that the feeding rhythm is under circadian clock control, and a functional *period* gene is needed to maintain oscillation under constant condition. In addition, there seems to be a light component which enables feeding oscillation in the absence of functional *per* gene addition such as has been also shown in mutant flies with non-functional clock (Seay & Thummel, 2011). Interestingly, my results suggest that daily rhythmicity of lipids and feeding behaviour are not synchronized since hemolymph lipids seem not to oscillate (but presence of observable early light phase peak) in flies fed on standard medium although their feeding behaviour show rhythmic daily pattern.

3.8 *Drosophila* feeding behaviour persists in lipid-free medium and seems to be under the control of circadian clock

My previous results indicated that time-dependent lipid levels in the fly hemolymph oscillate rhythmically when flies were fed lipid-free medium. To address the question whether the oscillation of circulating lipid and food consumption is synchronised under lipid-free medium, I characterised feeding behaviour under *ad-libitum* feeding on lipid-free medium (Fig 31A, Supplementary fig 48). Therefore, ten wild-type male flies were placed in a CAFE assay chamber under 12hr/12hr light/dark cycle. The results showed a rhythmic feeding pattern under

light/dark condition with a peak in early light phase between ZT1 to ZT5 and a trough in night phase (Fig 31A, supplementary fig 48A, B and C). The oscillation was determined using JTK cycle and it showed a significant oscillation with p-value =1.16E-06 (Supplementary table 11).

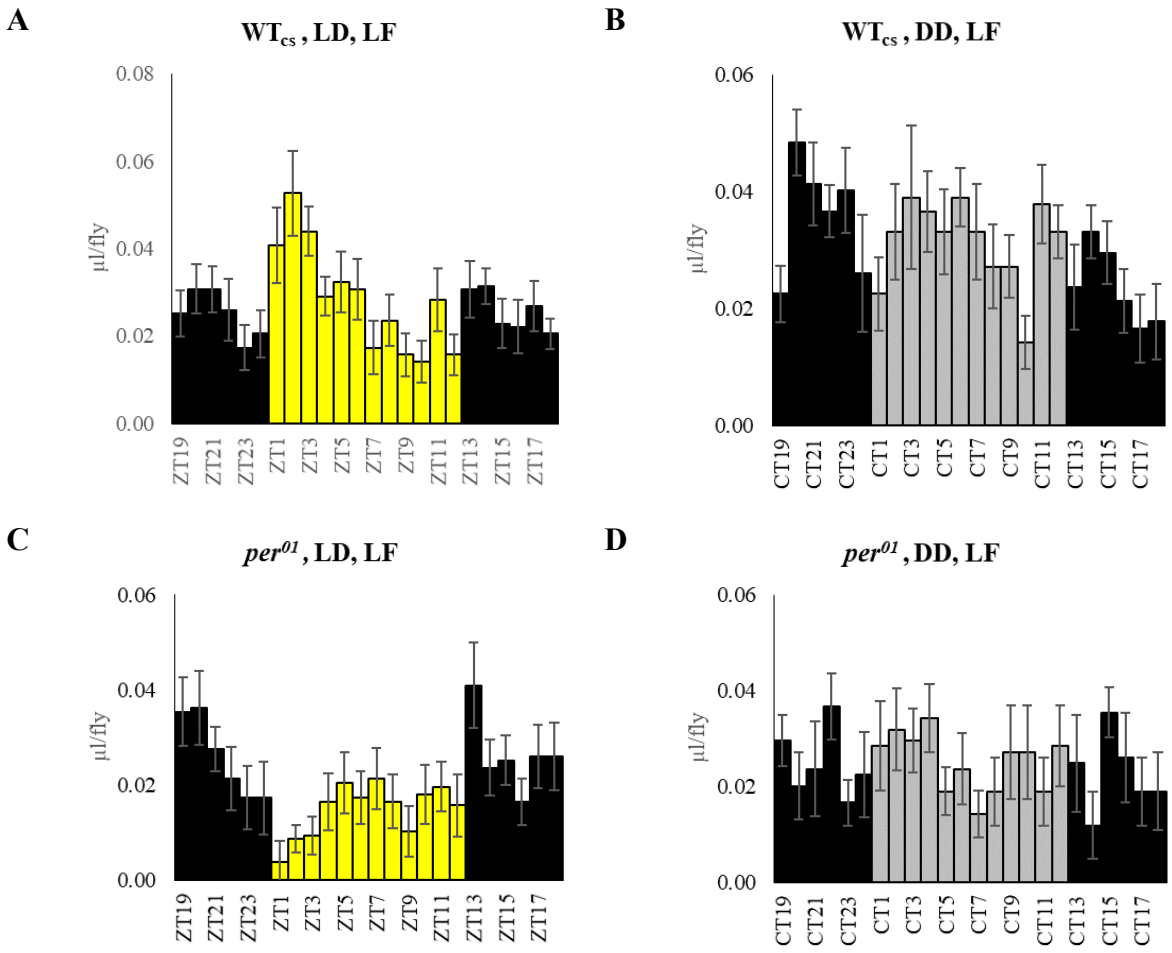


Figure 30. *Drosophila* shows circadian feeding behaviour when fed lipid-free medium. Feeding rhythm of WTcs flies under LD (A) and DD (B). Time-dependent food consumption of *per*^{01} flies under LD (C) and DD (D). All flies were fed lipid-free medium *ad libitum* and a modified CAFÉ assay was used to determine hourly food consumption of 10 male flies in a chamber. Abbreviations: LF=lipid-free medium, LD=light-dark cycle, DD=constant darkness, WT_{CS}=Canton-S, *per*^{01}=*period*^{01}. All bar plots were plotted using standard error and n=10. In the bar graphs, the lightening condition is designated by the colours of the bars (LD: yellow = light-on and black = light-off; DD: light grey = subjective light-on and dark grey = subjective light-off).

To determine if the observed feeding rhythm is under circadian control, I tested whether these oscillations persist in *ad-libitum* feeding under constant condition (dark/dark) after 3 days of light-dark entrainment (Fig 31B, supplementary fig 48A, C and D). Interestingly, the feeding behaviour oscillation persisted but weakened under DD (p-value<0.05) with early light phase peak between CT1 to CT7 and a dark phase peak between CT21 to CT23. Further, I characterised if the mutant flies *(per^{01})* feeding behaviour oscillates under light/dark condition when fed on lipid-free medium. I discovered that mutant flies seem to show no oscillation under LD according to JTK cycle (Supplementary table 4) although its feeding profile showed a main peak between ZT15 to ZT21. However, mutant flies *(per^{01})* fed on lipid-free medium under DD after 3 days of entrainment became completely arrhythmic as confirmed by the JTK results (p-value>0.05) (Supplementary Table 4).

3.9 The circadian clock does not affect total food consumption in wild-type flies fed on standard medium.

Considering the feeding rhythm oscillation of flies fed with standard medium, I noticed that the feeding peak was high in the early light phase in WT_{CS} (Fig 30A). On the other hand, the feeding peak in the clock mutant *per^{01}* seems to be high at the early night phase (Fig 30C). Hence, I quantified the total amount of food intake per fly for a period of 24 hrs (LD) in 1 day old male flies (Fig 32A) and in DD (Fig 32D). Subsequently, I determined the total food consumption in light phase (ZT0 – ZT12) and dark phase (ZT13 – ZT24) in both genotypes (Fig 32 B & C). Furthermore, I quantified the food consumption of the two genotypes under constant darkness in the subjective light phase (CT0 – CT12) and subjective dark phase (CT13 –CT24) (Fig 32D & F).

Interestingly, I found that both genotypes consume similar daily amounts of food for a period of six days except for the third day (Fig 32A). The results of the light phase showed that wild-type flies consumed more than *per* mutants (Fig 32B), while the mutants consumed more than WT_{CS} during the dark phase (Fig 32C). Similarly, the results of the total amount of daily food consumption under DD showed no significant difference between the genotypes (Fig 32D). In line, the two genotypes showed similar food consumption in the subjective light phase (Fig 32E) and subjective dark condition (Fig 32F). It is worth to mention that in the subjective light phase WTcs showed a tendency to eat more especially in the 2nd and 3rd day (Fig 32E). On the

contrary, during the subjective dark phase, the mutant showed a tendency to consume more food (Fig 32F). These results suggest that the circadian clock seems not to regulate daily total food consumption. However, the influence of circadian clock is clearly seen when comparing food intake during the light or dark phase.

On the other hand, daily, subjective light phase and subjective dark phase total food consumption between the wild-type and mutant in DD remains similar suggesting there may be compensatory mechanism in the mutant.

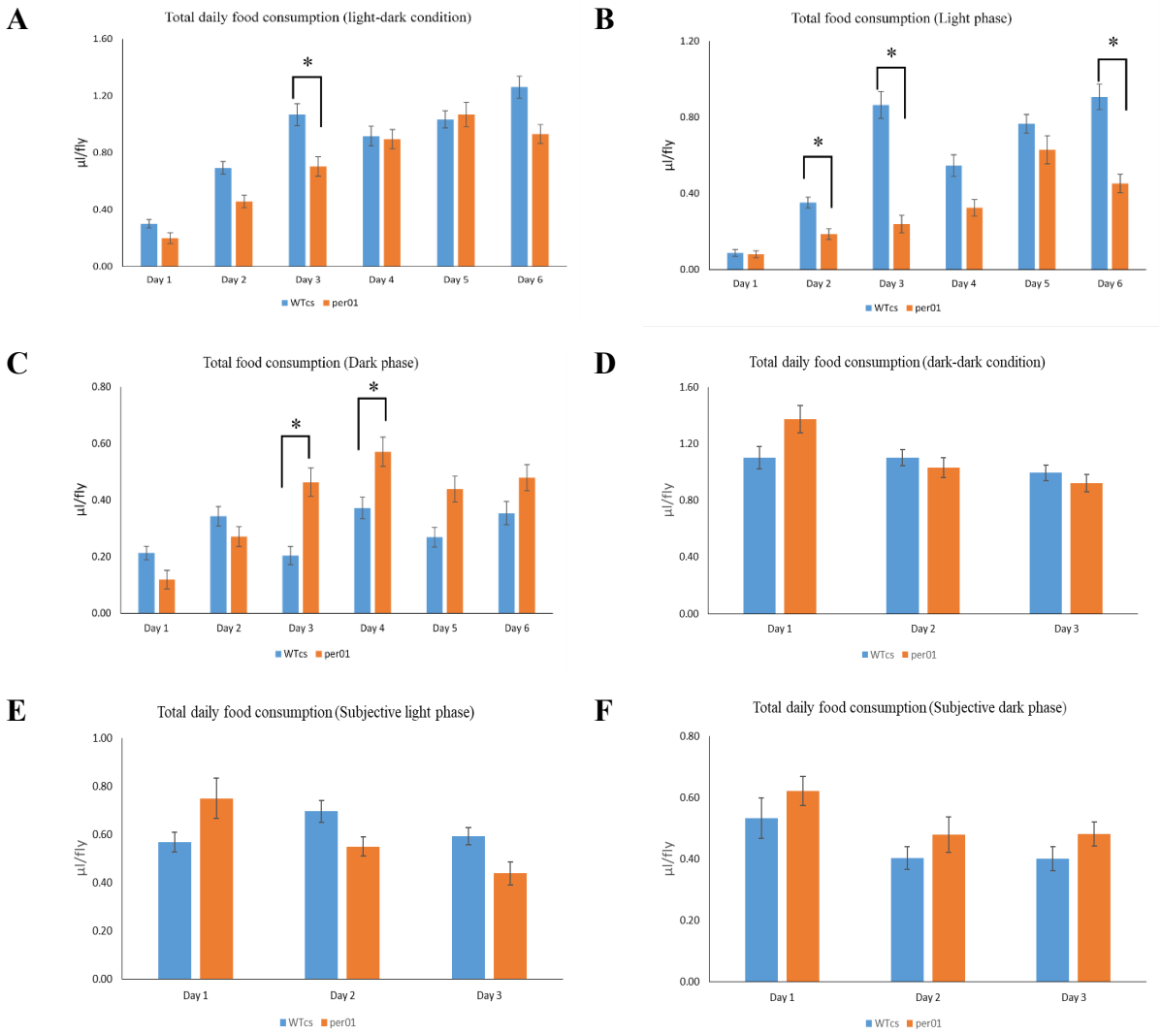


Figure 31. The circadian clock does not impact total food consumption in flies fed with standard medium**.** The total food consumed is the sum during 24h. **A)** Total daily food

consumption of WT_{CS} (blue) and *per*^{01} (orange) flies over 6 days of feeding in light-dark condition. **B)** Total food consumption during light phase of LD between WT_{CS} (blue) and *per*^{01} (orange) flies over 6 days of feeding. **C)** Total food consumption during dark phase of LD between WT_{CS} (blue) and *per*^{01} (orange) over 6 days of feeding. **D)** Total daily food consumption of WT_{CS} (blue) and *per*^{01} (orange) flies over 3 days of feeding under DD. **E)** Total food consumption during subjective light phase of DD between WT_{CS} (blue) and *per*^{01} (orange) over three days of feeding. **F)** Total food consumption during subjective dark phase of DD between WT_{CS} (blue) and *per*^{01} (orange) flies over 3 days of feeding. Asterisks indicate significant differences (Student-t test, $p<0.05$). All bar plots were plotted using standard error and n=10.

3.10 The circadian clock modulates total food consumption in wild-type flies fed on lipid-free medium.

The feeding oscillation of flies fed on lipid-free medium showed a peak in the early light phase in WT_{CS} wild-type flies (Fig 31A) and a peak in *per*^{01} clock mutant during the dark phase period (Fig 31C). I was interested if total food consumption is compensated in the clock mutant independently of the diet. Therefore, I quantified the total amount of food consumed per fly for a period of 24 hrs for the two genotypes under LD (Fig 33A) and DD conditions (Fig 33D) on lipid-free medium. Afterwards, I measured the total food consumption in the light phase (ZT0 – ZT12) and dark phase (ZT13 – ZT24) between the genotypes (Fig 33 B,C). Additionally, I quantified the total food consumed by the different genotypes under DD in the subjective light phase (CT0 – CT12) and subjective dark phase (CT13 –CT24) (Fig 33 E, F).

As under nutrition-rich diet (standard medium), both genotypes consume similar daily amount of food although the wild-type seems to show a tendency to consume more (Fig 33A). Furthermore, the results of the light phase experiments show that wild-type flies consumed more (Fig 33B) while in the dark phase both genotypes seem to consume similar amount of food (Fig 33C). On the other hand, the wild-type consumes significantly more than the mutant under DD (Fig 33D).The two genotypes showed similar food consumption during the subjective light phase with exception of the 3rd day (Fig 33E). Also in subjective dark phase, there was no significant difference between the wildype and mutants (Fig 33B). Taking the results together, it seems that a non-functional *per* gene leads to lower food consumption especially under nutrition-depleted diet.

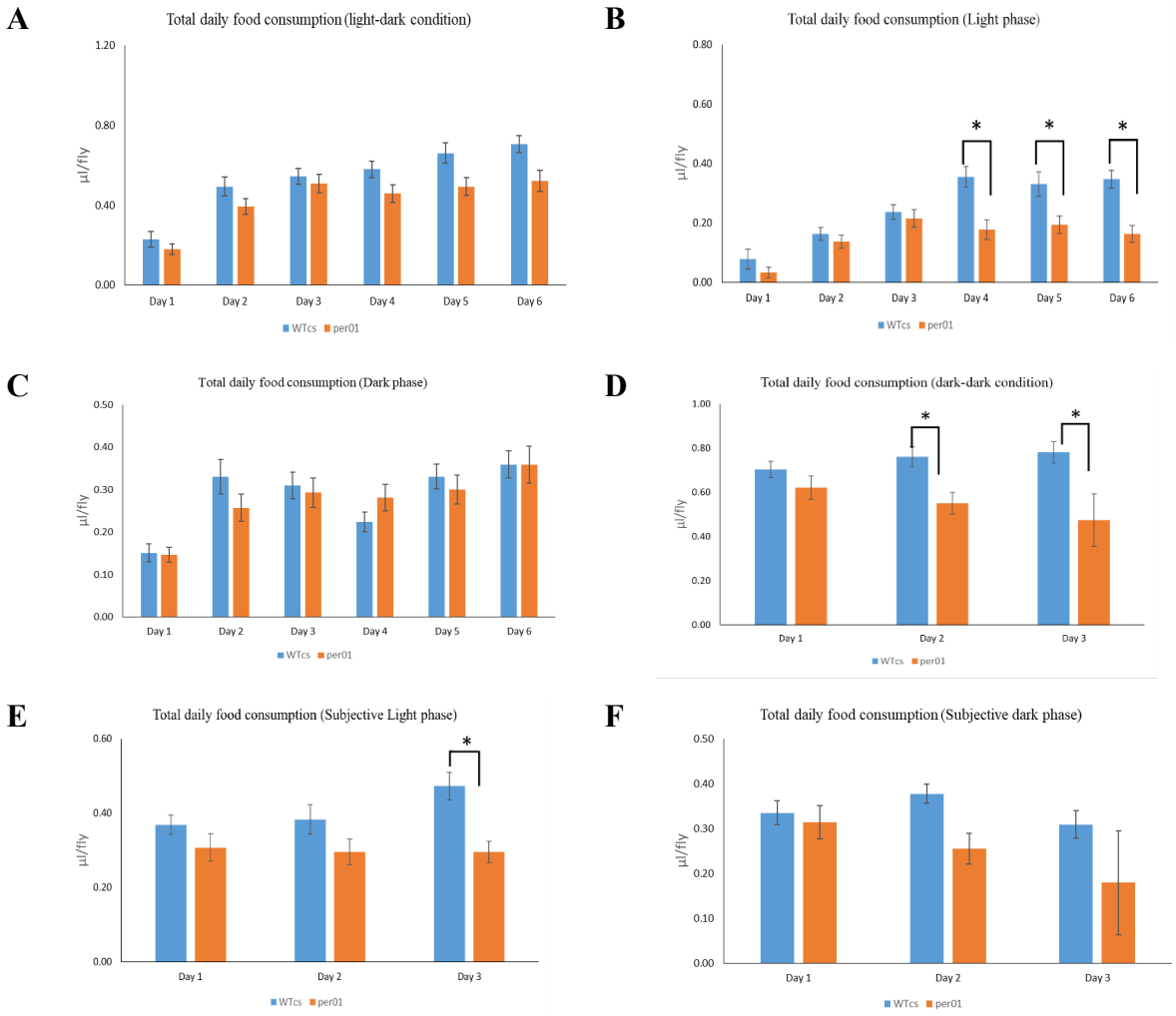


Figure 32. The circadian clock modulates total food consumption in wild-type flies fed with lipid-free medium. The total food consumed is the sum of 24h. **A)** Total daily food consumption of WT_{CS} (blue) and *per*^{01} (orange) flies over 6 days of feeding in LD. **B)** Total food consumption during dark phase of LD between WT_{CS} (blue) and *per*^{01} (orange) flies over 6 days of feeding. **C)** Total food consumption during dark phase of LD between WT_{CS} (blue) and *per*^{01} (orange) over 6 days of feeding. **D)** Total daily food consumption of WT_{CS} (blue) and *per*^{01} (orange) flies over 3 days of feeding under DD. **E)** Total food consumption during subjective light phase of DD between WT_{CS} (blue) and *per*^{01} (orange) over 3 days of feeding. **F)** Total food consumption during subjective dark phase of DD between WT_{CS} (blue) and *per*^{01} (orange) flies over 3 days of feeding. Asterisks indicate significant differences ($p<0.05$). All bar plots were plotted using standard error (SE) and n=10.

3.11 Time-restricted feeding modulates the timing of feeding behaviour and lipid oscillations in the hemolymph.

From previous results, I found out that wild-type feeding behaviour and lipid oscillations have a peak in the early light phase especially when they are fed with lipid-free medium. To understand if lipid oscillation is the direct consequence of feeding behaviour, I decided to use a time restricted feeding paradigm. WTcs flies were fed within a 12hr feeding window period during light phase (ZT0 – ZT12) or dark phase (ZT13 – ZT24) under LD (Fig 34A,B). Further, WTcs flies were subjected to a 12hr feeding window period during subjective light phase (CT0 – CT12) or subjective dark phase (CT13 – CT24) under DD (Fig 34C,D).

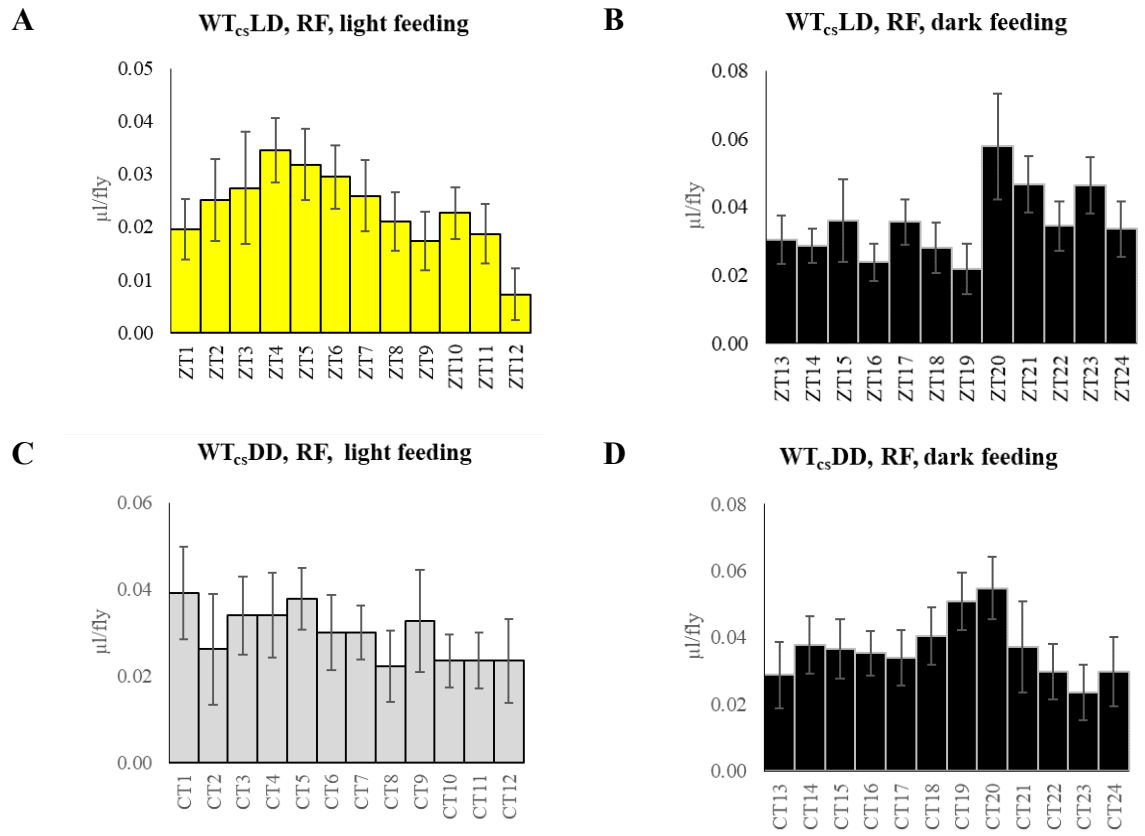


Figure 33. Time restricted feeding modulates feeding behaviour of WTcs flies under lipid-free diet**.** A) Feeding rhythm of flies which were fed for 12 hours during light phase (ZT0 – ZT12) of LD. B) Feeding rhythm of flies which were fed for 12 hours during dark phase (ZT13 – ZT24) of LD. C) Feeding rhythm of flies which obtained food for 12 hours during subjective light phase (CT0 – CT12) of DD. D) Feeding rhythm of flies which obtained food for 12 hours

during subjective dark phase (CT13 – CT24) of DD. Data represents mean ± standard error (n=5).). In the bar graphs, the lightening condition is designated by the colours of the bars (LD: yellow = light-on and black = light-off; DD: light grey = subjective light-on and dark grey = subjective light-off).

Interestingly, during the light phase period under LD cycle, the flies showed a tendency to feed more between ZT3 to ZT6 (Fig 34A) but it was not significant based on JTK analysis (Supplementary table 12). Similarly, in the dark phase (under 12hr/12hr light-dark cycle), JTK results showed no oscillation in wild-type feeding behaviour (Fig 34B, Supplementary table 12). Under constant condition (dark-dark), wild-type flies showed no oscillation in feeding behaviour during both the subjective light period (Fig 34C, supplementary table 12) and dark period (Fig 34D, Supplementary table 12).

To determine if the circadian clock is actually regulating the feeding rhythm and if the effect of TRF on feeding behaviour is temporal, I decided to return the flies to *ad libitum* feeding (Fig 35 A,B).

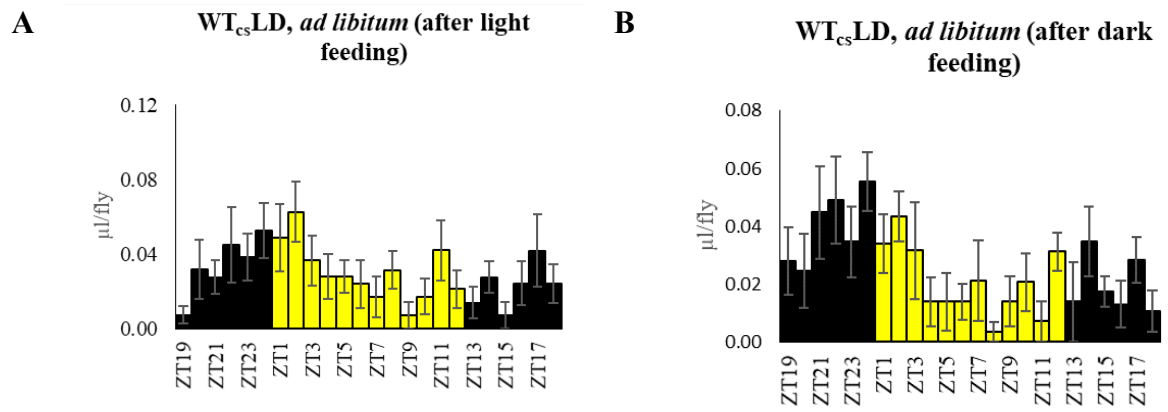


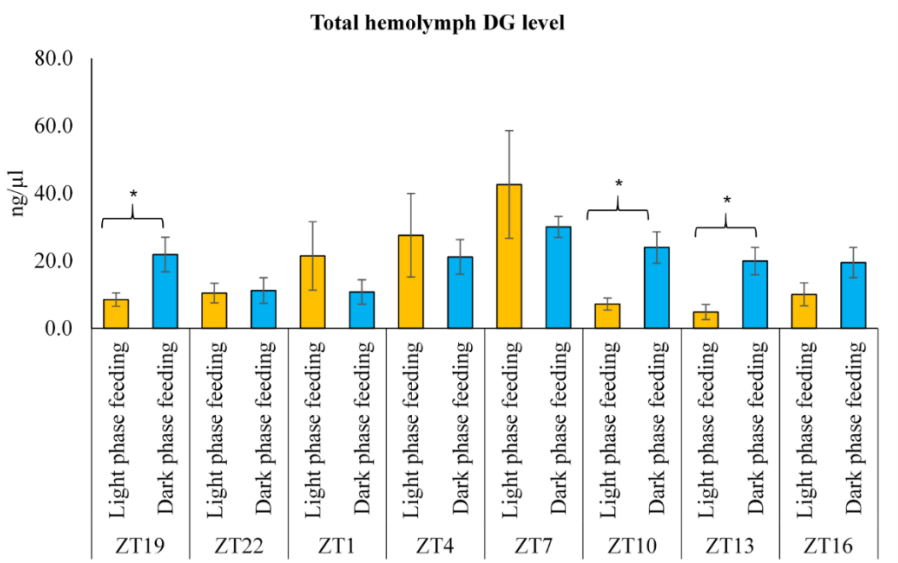
Figure 34. The circadian clock exerts control on the feeding behaviour of WTcs after TRF under LD. A) Feeding rhythm of flies fed lipid-free medium (LF) *ad libitum* after light phase TRF (ZT0 – ZT12). B) Feeding rhythm of flies which were fed lipid-free medium (LF) *ad libitum* after dark phase RTF (ZT13 – ZT24). Data represents mean ± standard error (n=5). In the bar graphs, the lightening condition is designated by the colours of the bars (LD: yellow = light-on and black = light-off).

In line with the literature (Xu et al., 2008), 24h after returning the flies to *ad libitum* feeding, wild-type flies that were fed only during light phase (ZT0 - ZT12) and those fed only during the dark phase (ZT13 - ZT24) under LD, daily rhythmicity in food consumption returned back. Wild-type flies fed only during the light phase after returning to *ad libitum* feeding showed a peak between ZT23 and ZT3 (Fig 35A) whereas the dark phase fed flies showed peak between ZT21 and ZT3 (Fig 35B). This was confirmed by JTK analysis (Fig 35A & B, Supplementary table 13).

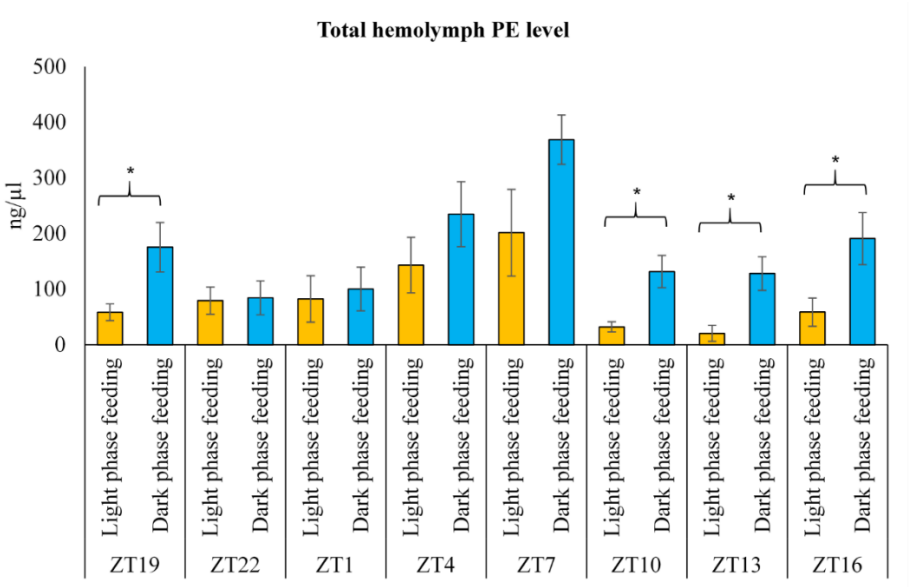
From the results above, it is seen that TRF modulates feeding behaviour but its modulation is temporary due to the strong influence of the circadian clock in the absence of TRF. This temporary effect can be exploited to study if lipid oscillations are a direct consequence of feeding behaviour. Hence, wild-type flies were subjected to either light phase TRF (ZT0 – ZT12) or dark phase TRF (ZT13 – ZT24) conditions. Afterwards, their lipid levels in the hemolymph were analysed with LC-MS at 8 different time points (Fig 36A,B,C). The result showed that in both TRF conditions, the lipid oscillation profiles were completely changed compared to previous results under *ad libitum* feeding. All the lipids measured (DG, PE and PC) showed similar oscillation profiles in both TRF conditions (Supplementary fig 54-56). However, in flies fed during the light phase (ZT0 – ZT12), only PE was oscillating according to JTK result (Supplementary table 14). The oscillation showed a peak between ZT4 and ZT7 while the early light phase peak present in the *ad libitum* feeding in previous result was absent (Supplementary fig 54-56). For the dark phase fed flies (ZT13 – ZT24), all lipid species measured oscillated according to JTK result (Supplementary table 14) and they have similar oscillation pattern with a peak maximum between ZT4 and ZT7 (Supplementary fig 54-56). Interestingly, when the different lipid species between light phase fed flies (ZT0 – ZT12) and dark phase fed flies (ZT13 – ZT24) are compared, differences were found. These differences occurred during the dark phase under light-dark condition, the lipid levels of PE, PC and DG are higher in dark phase fed flies (ZT13 – ZT24) compared to light phase fed flies (ZT0 – ZT12) (Fig 35A,B,C).

Taken together, the above results suggest that lipid oscillations may not be a direct consequence of feeding behaviour since the early light phase peak of lipids observed in *ad libitum* feeding was missing in light phase only fed flies (ZT0 – ZT12). However, there seems to be an indirect consequence because the lipid oscillation changed when the feeding time changed. In summary, TRF seems to modulate both feeding behaviour and hemolymph lipid oscillation which indicates that these two oscillations can be decoupled just by changing feeding time.

A


Total hemolymph DG level
80.0
60.0
40.0
20.0
0.0
ng/µl
*
*
*
Light phase feeding
Dark phase feeding
ZT19
ZT22
ZT1
ZT4
ZT7
ZT10
ZT13
ZT16

B


Total hemolymph PE level
500
400
300
200
100
0
ng/µl
*
*
*
*
Light phase feeding
Dark phase feeding
ZT19
ZT22
ZT1
ZT4
ZT7
ZT10
ZT13
ZT16

C

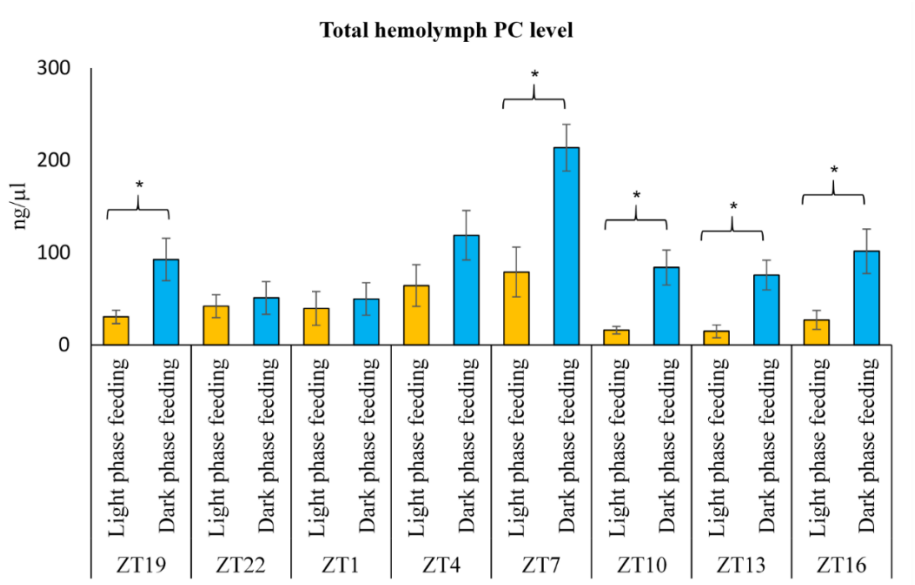


Figure 35. Time restricted feeding influences lipid oscillation in light phase fed flies (ZT0 – ZT12) and dark phase fed flies (ZT13 – ZT24)**.** Wild-type flies were maintained on the time restricted paradigm for 6 days before and during the sampling. Levels of DG (A), PE (B) and PC (C) were measured in hemolymph of both light phase fed flies (ZT0 – ZT12) (orange) and dark phase fed flies (ZT13 – ZT24) (blue). Asterisks indicate significant differences between the TRF paradigms ($p<0.05$). Data represents mean ± standard error (n=10-13).

4 DISCUSSION

4.1 Lipid levels show daily rhythmicity in the fly hemolymph

Only few studies so far have focused on deciphering oscillating metabolites from global metabolic profiles in whole bodies of *Drosophila* fed on standard cornmeal agar medium (Gogna et al., 2015; Rhoades et al., 2018; Schäbler et al., 2020a). Using mass spectrometry or nuclear magnetic resonance spectrometry, different molecules ranging from sugars, amino acids, acyl carnitines, fatty acids, lipids etc. were identified to be rhythmic. However, the question whether metabolite levels in the hemolymph are dependent on the time of the day in the circulating tissue remained to be answered. Main metabolites in the fly hemolymph are amino acids, sugars (including trehalose) and DGs (Herren et al., 2014; Piyankarage et al., 2008). A preliminary study indicated that levels of hydrophylic metabolites remain in homeostasis (Forster, 2017), whereas DG levels showed diurnal oscillation in WT_{CS} under LD and standard cornmeal agar medium (Amatobi, 2016). Therefore, I studied further daily variations of glycerol(phospho)lipids in the hemolymph of *Drosophila*.

Generally speaking, organisms including humans store lipids in lipid droplets and also in insects, lipids are an important source of energy (Chino & Gilbert, 1964; Van der Horst, 1990). Furthermore, vertebrates digest and absorb lipids in a similar manner as insects yet there are obvious differences between insect species (Canavoso et al., 2001). The major neutral lipids that are transported by lipophorin, a multimeric hemolymph protein that transports dietary lipids, pheromones and other lipids to utilization sites, are different among insect (Pennington & Wells, 2002; Soulages & Wells, 1994). For example, insects belonging to the Culicomorpha from the infraorder of Nematocera (e.g. Mosquitos) have TG as its major transport lipid (Pennington & Wells, 2002). The lipophorin of crane flies (*Nephrotoma abbreviate)* from the infraorder Tipulomorpha transports equivalent amount of DG and TG (Pennington & Wells, 2002). In *Drosophila melanogaster*, lipophorin transports DGs as the major transport lipid (Palm et al., 2012; Pennington & Wells, 2002). Phospholipids are also components of lipophorin forming phospholipid-protein surface (Canavoso et al., 2001; Pennington & Wells, 2002). These lipid molecules are composed of a hydrophilic head group and hydrophobic tails. The hydrophilic head group comprises of a phosphate group modified by organic molecules such as ethanolamine, choline or serine while the hydrophobic tail is derived from fatty acids linked by alcohol residues of glycerol. In *Drosophila*, lipophorin is the major hemolymph lipid

carrier and contains DG and PE as the most abundant glycerol- and phospholipids, respectively (Palm et al., 2012; Van der Horst, 1990). Additionally, it contains smaller amounts of TG, PC, ceramides and ceramide phosphorylethanolamine (Palm et al., 2012). The lipid composition of lipophorin present in the fly hemolymph may explain the observed oscillation pattern of DG, PE and PC which were similar in every experimental condition. For example, the oscillation profile of hemolymph DG was similar to both PE and PC oscillation when wild-type flies were fed on lipid-free medium. These oscillation profiles were bimodal with an early morning peak and late evening peak. Similarly, when mutant flies (per^{01}) were fed on lipid-free medium, hemolymph DG, PE and PC oscillation profiles were comparable.

Daily rhythmicity of glycero(phospho)lipids identified in the hemolymph differed from the oscillation profile measured in heads and bodies of WT_{CS} wild-type flies (Schäbler et al., 2020b). In the fly body parts, levels of PEs and PCs were constant in time whereas levels of DGs slightly varied in a daily manner (Schäbler et al., 2020b). Interestingly, DG levels showed an early morning peak in hemolymph as well as in body parts. This indicates that light is a contributing factor to the early light phase peak irrespective of organ or tissue sampled. Many other molecules such as sugars and fatty acids also showed early light phase peaks which were interpreted as responsiveness to light (Gogna et al., 2015; Rhoades et al., 2018). Additionally, levels of DGs peaked in the early night in the fly hemolymph., whereas a sharp maximum in the middle of the light phase was observed in heads and bodies (Schäbler et al., 2020b). These differences may be due to the analysed sample type. Heads and bodies contain multiple organs whose circadian clocks may tick differently leading to complex daily rhythmicity in lipid profile (Schäbler et al., 2020a). In contrary, the hemolymph is a specific tissue with composition reflecting the actual metabolic status. To know to what extent lipid oscillation in the hemolymph contributes to the oscillation in the whole body, absolute quantification of lipids in dissected metabolic organs such fat body, digestive tract and oenocytes will be required.

4.2 Rhythmicity of circulating transport and membrane lipids are under control of circadian clocks

Generally, metabolite oscillations have been shown to be disrupted in clock mutants of many organisms (Adamovich et al., 2014; Beaver et al., 2012). For example, hepatic lipid

accumulation showed differences in phase and composition of TG-species between wild-type and clock mutant mice (Adamovich et al., 2014). In *Drosophila melanogaster*,a null mutation in the *per* gene lead to different oscillation pattern flies not only in glutathione (Beaver et al., 2012) but also in acylcarnitines (Schäbler et al., 2020b). A similar observation was made in this study whereby the lipid oscillation in the hemolymph of WT_{CS} wild-type flies diminished in the per^{01} clock disrupted mutant when flies were reared under LD and obtained a lipid-free diet. Interestingly, levels of DGs in the hemolymph of the two genotypes were similar whereas lower levels were determined in the bodies of per^{01} compared to WT_{CS}. This observation clearly shows that circadian clock is necessary for proper whole-body oscillation of lipids but not for their levels in the fly hemolymph. One possibility for this phenomenon might be explained via the different feeding behaviour between the two genotypes. Clock disrupted mutant flies have been shown to be arrhythmic in feeding behaviour (Xu et al., 2008) and they constantly feed which suggests a constant supply of energy molecules like sugar or lipids which leads to dampening of hemolymph lipid oscillations.

In the hemolymph of *Drosophila* fed with standard medium, glycerophospholipids (PE and PC) oscillation profile showed weak bimodal oscillation profiles, However, amplitudes were higher in the absence of dietary lipid supply. In addition, oscillation profiles of lipids in WT_{CS} become altered in per^{01} clock mutant under LD which has also been shown in mice whereby disruption of circadian clock (Cry1-/-, Cry2-/-) led to alteration in lipids such as lyophosphatidylcholine (Minami et al., 2009).

In mammals, many studies have been carried out to find out how metabolite oscillations are regulated in different organs, including blood plasma, liver and others (Adamovich et al., 2014; Ang et al., 2012; Aviram et al., 2016; Chua et al., 2013; Dallmann et al., 2012b; Kasukawa et al., 2012; Maillot et al., 2005; Minami et al., 2009). For example, TGs and microsomal triglyceride transfer protein have been shown to be rhythmic in mouse plasma (Pan & Hussain, 2007). Furthermore, expression of genes associated with lipid biosynthesis revealed a circadian oscillation (Adamovich et al., 2014; Kohsaka et al., 2007; Kudo et al., 2007; Shostak et al., 2013). Enzymes involved in lipid biosynthesis including lipin, diacylglycerol acyltransferase, glycerol-3-phosphate acyltransferase and 1-acylglycerol-3-phosphate acyltransferase were expressed depending on time of day in liver of *ad libitum* fed wild-type mice (Adamovich et al., 2014; Kumar Jha et al., 2015).

Several studies have been performed in terms of oscillating metabolites in humans (Ang et al., 2012; Chua et al., 2013; Dallmann et al., 2012b; Eckel-Mahan et al., 2013; Kasukawa et al., 2012). In human plasma, TG, DG and PC exhibit rhythmicity, although there is variability in the occurrence of these peaks from one individual to another.(Chua et al., 2013). However, group-level analysis showed that TG and DG showed morning peaks whereas a small subset of PCs exhibited evening peaks (Chua et al., 2013). Daily rhythmicity of phospholipids in the circulating tissue of human and *Drosophila* differed from each other; PC oscillation in human plasma peaks in the evening (Chua et al., 2013) while in *Drosophila* hemolymph it peaks in the morning. Surprisingly, the TG and DG oscillation in human plasma and *Drosophila* hemolymph had a similar early morning peak, although there is a presence of a late minor evening peak in the case of the fruit fly. It seems that the increase in hemolymph DG in the morning hours may be attributed to circadian regulation of lipid hydrolysis and not absorption of dietary DG since the flies are fed on a lipid-free medium (Chua et al., 2013; Douris et al., 2011). Taken together, since rhythmicity of lipid levels in the hemolymph did not diminish in WT_{CS} flies under DD but changed in per^{01} mutants under LD, I surmise that circadian clocks are able to modulate circulating lipid levels in *Drosophila* such as in mammals.

4.3 Time-dependent variations of circulating glycero(phospho)lipids are masked by consumption of dietary lipids

Masking as an English word refers to the act of concealing or obscuring something. In the context of circadian regulation, there are many factors that can mask a circadian influrence on metabolite oscillations such as light (Koronowski et al., 2019; Plano et al., 2017a; Seay & Thummel, 2011), time restricted feeding (Satoh et al., 2006; Sherman et al., 2012) and dietary nutrition (Abbondante et al., 2016; Carvalho et al., 2012; Schäbler et al., 2020b; Triscari et al., 1985; Wahle & Radcliffe, 1977). In my study, influence of dietary nutrition on circulating lipid oscillation in wild type flies was apparent. Levels of DGs, PEs and PCs in the hemolymph of WT_{CS} fed on standard medium remain virtually constant over the time as their daily oscillation were not significant according to JTK cycle. However, when flies were fed with lipid-free medium, the oscillation profile of the hemolymph glycero(phospho) lipids became robust and significant. Under both diets, the oscillation profile was bimodal with maxima in the subjective morning and evening. Effects of diet on metabolite oscillation have also been observed in mammals (Abbondante et al., 2016; Eckel-Mahan et al., 2013). For example, the majority of

lipids in the serum of mice lost their oscillation after high fat diet which was suggested to be due to 1) constitutive breakdown of adipose tissue, 2) fatty acids absorbed from the intestine due to increase in dietary lipids, and 3) desynchronization of peripheral clocks from SCN (Abbondante et al., 2016). Another study revealed that high fat diet could mask or influence oscillation of lipids, amino acids, carbohydrates, peptides, nucleotides etc. in mice through the induction of transcriptional reprogramming within the clock (Eckel-Mahan et al., 2013).

Diet or type of nutrition has also been shown to influence not only daily rhythmicity but also general metabolite levels in many organisms (Carvalho et al., 2012; Eckel-Mahan et al., 2013; Fernando-Warnakulasuriya et al., 1988; Huang & Douglas, 2015; Laye et al., 2015; Palanker Musselman et al., 2011; Poesen et al., 2015; Sherman et al., 2012; Skorupa et al., 2008). This phenomenon has been particularly evident in early studies involving lipid metabolism of rodents (Triscari et al., 1985; Wahle & Radcliffe, 1977). A diet rich in sunflower oil fed to rats was shown to reduce *de novo* lipid synthesis and depress the Δ9-desaturase activity in liver microsomes of obese and lean rats (Wahle & Radcliffe, 1977). Additionally, the consumption of high sunflower oil diet as compared to low sunflower diet led to increased proportion of 18:2ω6 in liver lipids and TGs in adipose tissues in both obese and lean rats. Also, Sprague-Dawley rats fed on a diet containing corn oil and condensed milk exhibited high rates of fatty acid synthesis in isolated hepatocytes *in vitro* (Triscari et al., 1985). In mice, a high fat diet administered within a defined period of time led to elevation of hepatic long-chain polyunsaturated fatty acids and also influenced TG levels (Mehus et al., 2019). Effects of dietary composition on insect metabolism has also been studied (Carvalho et al., 2012; Fernando-Warnakulasuriya et al., 1988; Skorupa et al., 2008). TG accumulation was shown to be enhanced by dietary carbohydrates and dampened by dietary yeast (Skorupa et al., 2008). Further, diet composition has been shown to affect not only TG metabolism but also food consumption, lifespan and fat deposition (Skorupa et al., 2008). Apart from the effect of dietary composition on TG accumulation, other lipids like glycerophospholipids composition were also modulated by the type of fatty acids in the food (Carvalho et al., 2012). Therefore, I hypothesised that flies under standard medium show elevated lipid levels in the hemolymph which can mask their oscillation. Indeed, levels of circulating lipids on standard medium was higher than lipid levels under lipid-free medium. This has also been determined in other insects. For example, lipoproteins of high-fat fed hornworm (*Manduca sexta*) larvae contained more lipids in comparison to fat-free fed hornworm larvae (Fernando-Warnakulasuriya et al., 1988). Composition of glycerolipids (DGs and TGs containing polyunsaturated long chain fatty acid)

in the standard food differed from the composition found in the hemolymph of WT_{CS} flies (containing medium chain fatty acid) suggesting that dietary lipids are metabolised in the digestive tract of the insects. This observation is in agreement with a previous study which indicates that the difference in DG species may be a result of a shortening of dietary fatty acids prior to exportation via the hemolymph (Carvalho et al., 2012). Under dietary restriction, TG metabolism was shifted by elevating fatty acid synthesis and breakdown (Katewa et al., 2012). The composition of dietary fatty acid was observed to exert direct influence not only on storage lipids but also on membrane lipids (phospholipids such as PEs and PCs), in every organ of *Drosophila* larvae (Carvalho et al., 2012). Interestingly, similar PE and PC species were found in adult fly hemolymph. However, DGs present in the adult hemolymph are mostly composed of medium chain fatty acids shorter than the fatty acids found in the diet.

Diet can influence not only circulating lipids but also feeding. Therefore, a masking effect of diet on glycerol(phospho)lipid oscillation might be an indirect consequence of changed consumption. Flies (both genotypes) consumed more on standard medium relative to the lipid-free medium. This observation is surprising because previous studies showed that flies consumed more when fed with carbohydrate rich-food (Edgecomb et al., 1994; Skorupa et al., 2008). However, this idea is challenged by the "composition-central" hypothesis (Skorupa et al., 2008) whereby the standard medium may be more palatable which could drive increased food consumption relative to lipid-free medium. Interestingly, circadian clocks seem to contribute to food consumption (Xu et al., 2008), whereby clock disrupted flies consumed more during the night period compared to the wild type flies. A similar observation was made in this study whereby the wild-type flies fed more during the light phase relative to the clock disrupted mutant when flies were fed with standard medium (Fig 33B), while the clock disrupted mutant fed more during the dark phase. However, the total daily food consumption was similar between the two genotypes (Schäbler et al., 2020a), which I confirmed under LD as well as under DD. Importantly, under constant conditions there were no significant differences in the daily total food consumption between the two genotypes. In lipid-free medium, a similar trend was observed except in the dark phase where there was no significance difference in total food consumed between wild-type and mutant. Interestingly, unlike in the standard medium, in the lipid-free medium, there was a significant difference in the daily total food consumption under constant conditions. My results indicate that food consumption is clock-controlled with a stronger effect when flies are given lipid-free medium. Taken together, I surmise that dietary nutrition can mask oscillations of transport- and membrane lipids in the hemolymph of

Drosophila which is probably via incorporation of dietary lipids from the intestine when available in the diet. To confirm that exogenously applied glycerolipids mask daily rhythmicity in circulating DGs, their levels should be measured in flies which are fed with medium containing certain lipids in certain amounts.

4.3 Time restricted feeding impact lipid oscillations in the fly hemolymph

Time restricted feeding (TRF) is the limitation of food accessibility to a specific period of time and this period varies throughout different studies. In some studies, the time for food access is limited to 12 hours, in others to 10 hours, 8 hours or 4 hours during day time or night time depending on the research question (Adamovich et al., 2014; Chaix et al., 2019; Gill et al., 2015; Hatori et al., 2012; Lundell et al., 2020; Moro et al., 2016; Satoh et al., 2006). Importantly, TRF is all about restricting food access to a specific time window without changing the caloric input via different diets (Katewa et al., 2016; Katewa et al., 2012; Waldman et al., 2020). In humans, many studies have been carried out on the effect of TRF on lipid factors and it is clear that TRF modulates lipid factors (Fakhrzadeh et al., 2003; Lundell et al., 2020; Moro et al., 2016; Ongsara, 2017; Temizhan et al., 2000). Studies in other mammals have also revealed the effect of TRF on plasma metabolites; for example, night-time restricted feeding of dairy cows modified plasma glucose, non-esterified fatty acids and insulin in plasma (Salfer & Harvatine, 2020).

Many studies showed that TRF is a strong "Zeitgeber" in mammals and can reset the circadian clock (Cassone & Stephan, 2002; Froy, 2010; Hirota & Fukada, 2004; Schibler et al., 2003; Sherman et al., 2012; Stephan, 2002). For example, TRF shifted the daily oscillations of metabolite levels, plasma hormones and milk synthesis in lactating Holstein cows (Salfer & Harvatine, 2020). In mice liver, TRF influenced the oscillation profile of TGs by shifting the phase (Adamovich et al., 2014). Similar observation were made in blood plasma of mice where lipids as well as microsomal triglyceride transfer protein (MTP) oscillated with the same phase which was altered under TRF (X. Pan & Hussain, 2007). Not much results are available when it comes to effect of TRF on metabolites in insects. Villanueva and colleagues (Villanueva et al., 2019) used TRF intervention in countering metabolic dysfunction by minimizing intramuscular fat deposit, insulin markers and phospho-AKT levels in skeletal muscles of *Drosophila*. However, the difference between my work and the published study is that I

focused on the effect of TRF on lipid metabolites in a specific *Drosophila* tissue (hemolymph) which is so far unique when it comes to metabolism.

Generally, both day-time (DT-TRF) and night-time (NT-TRF) restricted feeding shifted the hemolymph lipid peaks to the middle of the light phase when compared to *ad libitum* feeding. One reason for the shift may be attributed to changes in the feeding behaviour. In *ad libitum* feeding, the feeding behaviour oscillates in a bimodal manner with its peak coinciding with the hemolymph lipid peaks whereas under DT-TRF as well as under NT-TRF, food consumption was constant during the feeding time. Interestingly, both TRF paradigms (DT-TRF and NT-TRF) lead to similar oscillation pattern of the hemolymph lipids. The only difference is that levels of the analysed lipids dropped in the middle of the light phase and remained constantly low during dark phase under DT-TRF, whereas a continuous drop was observed under NT-TRF. This could suggest that for DT-TRF flies, other sources of energy are enough when there is food availability but later in the day lipolysis starts taking place to supply energy for the night. On the other hand, NT-TRF flies hemolymph lipids increases due to lipolysis before the beginning of the night to supply energy for the fly's activity and remains stable when food becomes available. Putting these observations together suggest that TRF does not entrain but influences rhythmicity of circulating glycero(phospho)lipids in *Drosophila*, in line with excisting observations on hepatic TGs in mice (Adamovich et al., 2014).

4.4 Hemolymph lipid oscillations are controlled by light and clock-controlled feeding rhythm

Feeding behaviour is closely associated with energy metabolism and is under control of circadian clocks. The rhythmicity of food consumption in *Drosophila* under nutrition-rich diet has already been shown (Turek, 2005; Xu et al., 2008). However, I investigated whether circadian clocks regulate feeding specifically under a lipid- and protein-free diet. On both standard and lipid-free media, wild-type flies showed a clearly oscillating feeding pattern under LD cycles and the oscillation persisted under constant condition. The oscillation was dampened in clock disrupted mutant (per^{01}) under LD cycles and disappeared in constant darkness. Having established the fact that feeding rhythm is clock controlled also under lipid- and protein-free diet, the important question now is, whether the feeding behaviour pattern has any relationship with the observed hemolymph lipid oscillations? This thesis provides some

observations suggesting that clock regulation of feeding and circulating lipids are mutually interconnected. For example, feeding behaviour of wild-type flies on standard medium in LD cycle shows a broad morning peak which coincides with the morning peak in hemolymph lipid levels. A similar observation was made when flies were fed lipid-free medium under LD cycles. Yet, there were two peaks (in the morning and evening) in the feeding oscillation as well as in levels of lipids in the hemolymph. Strikingly, this relationship was lost under constant condition in which the feeding oscillation persisted while the morning peak of the hemolymph lipid strongly dampened. In other species such as mice, the role of feeding behaviour in TG oscillation is much more prominent (Adamovich et al., 2014). TG accumulation was affected by the time of feeding with an increase in TG accumulation experienced during night fed mice (Adamovich et al., 2014). These aforementioned studies support the idea that feeding behaviour has impact oscillation of energy lipids not only in vertebrates but also in insects. In line with the results of TRF, feeding behaviour cannot completely explain the circulating lipid oscillation prompting the possibility of additional factors.

My results indicate that lipids in the *Drosophila* hemolymph is circadian controlled. However, lipid oscillations in the fly hemolymph dampened and changed under free running condition with the absence of the early morning peak under DD compared to LD, indicating that the morning peak may be light sensitive. Light cycles are considered as an important Zeitgeber for circadian rhythms in numerous organisms (Elmore et al., 1994; Yoshii et al., 2009). Changes in light duration like non-24hrs light regimes can lead to misalignment of oscillators (Plano et al., 2017b). For example, the importance of light in mammals is reflected in the modulation of temporal homeostatic processes associated with feeding and sleeping via SCN clock entrainment (Plano et al., 2017b). More insight on how light synchronizes the SCN in mammals has been dealt with in many reviews (e.g. Husse et al., 2015; Lucas et al., 2001). Although mammals may be diurnal or nocturnal, the effect of light cycle on their metabolism is noticeable. For example, in both nocturnal and diurnal animals, the light cycle influences circadian rhythms which in turn modulate lipid metabolism (Kumar Jha et al., 2015). In mice which are nocturnal animal, plasma lipids and microsomal triglyceride transfer protein (MTP) oscillate in a diurnal manner (X. Pan & Hussain, 2007). However, when these mice were exposed to constant light or dark, the lipid and MTP oscillation were altered (X. Pan & Hussain, 2007). In fruit flies, the molecular mechanism of light interaction with circadian clock and the downstream effect have been intensely studied (Benito et al., 2008; Ceriani, 1999; Charlotte Helfrich-Förster et al., 2001; Hunter-Ensor et al., 1996; Ivanchenko et al., 2001; Mazzoni et

al., 2005; M. P. Myers et al., 1996; Schlichting & Helfrich-Förster, 2015; Suri et al., 1998; Zeng et al., 1996). Nevertheless, still very little is known in terms of light effects on fly metabolism and metabolite oscillation in conjunction with the circadian clock (J. M. Giebultowicz, 2018; Rhoades et al., 2018; Paolo Sassone-Corsi & Christen, 2016; Seay & Thummel, 2011; Sehgal, 2016). Light has been shown to suppress late day feeding via cryptochrome (CRY) and CRY has been shown to delay the accumulation of glycogen (Seay & Thummel, 2011). Interestingly, further studies have indicated that light can drive metabolic rhythms because the metabolome during the light phase differed between LD and DD (Rhoades et al., 2018; Schäbler et al., 2020b). My study expanded the idea of a light effect on to hemolymph lipid oscillations involving the main transport form of lipid (DG) and glycerophospholipids (PE & PC). The approach used shows that light is needed for hemolymph oscillations despite the presence of an intact clock. However, the regulatory effects of light on circulating lipids in *Drosophila* may be indirect. For example, photoreceptors such as cryptochrome were shown to influence metabolite oscillation via the suppression of feeding bouts in *Drosophila* (Seay & Thummel, 2011). It is likely that light is necessary for hemolymph lipid oscillation yet the mechanisms behind remain unclear.

In summary, this thesis shows that the circadian clock, feeding behaviour, light, diet-type and feeding time all interact via yet unknown complex mechanism to maintain oscillation and metabolism of circulating lipids. This complex interaction offers an insight into how insects are able to tightly regulate metabolism using both internal and external cues. Importantly, this study shows how daily rhythmicity of circulating lipids are conserved between fruit fly and mammals including humans, thereby providing a model system for better understanding of underlying mechanisms of lipid metabolism in human health and disease.

5 OUTLOOK

This body of scientific work has been able to look into the circadian influence on one of the major energy macromolecules – lipids. Furthermore, it unravelled other factors which could influence lipid oscillations in the circulating tissue of *Drosophila* such as light, feeding behaviour and diet. Though the thesis represents a first and important step towards fully understanding hemolymph lipid oscillation and mechanisms behind, it is more of a descriptive work. Therefore, the next crucial question is to ascertain if the profile of hemolymph lipid oscillation is also modulated by other circadian molecular components apart from the *period* gene using mutant flieswith other mutation on other genes.

The other pertinent question is the identification of candidate metabolic pathways that interact to influence behaviour and physiology in fruit flies. This will enable a deeper understanding of how and why metabolites such as lipids oscillate in the hemolymph and how such oscillations can be manipulated. With such knowledge, the health and physiological consequences will be better understood.

Lastly, manipulating peripheral clocks of fly metabolic organs such as fat body, gut and oenocytes through genetic alterations will give additional insights into the interactions between these organs and how the influence circadian controlled hemolymph lipid oscillations.

6 REFERENCES

Abbondante, S., Eckel-Mahan, K. L., Ceglia, N. J., Baldi, P., & Sassone-Corsi, P. (2016). Comparative Circadian Metabolomics Reveal Differential Effects of Nutritional Challenge in the Serum and Liver. *Journal of Biological Chemistry*, *291*(6), 2812–2828. https://doi.org/10.1074/jbc.M115.681130

Adamovich, Y., Rousso-Noori, L., Zwighaft, Z., Neufeld-Cohen, A., Golik, M., Kraut-Cohen, J., Wang, M., Han, X., & Asher, G. (2014). Circadian Clocks and Feeding Time Regulate the Oscillations and Levels of Hepatic Triglycerides. *Cell Metabolism*, *19*(2), 319–330. https://doi.org/10.1016/j.cmet.2013.12.016

Albrecht, U., & Eichele, G. (2003). The mammalian circadian clock. *Current Opinion in Genetics & Development*, *13*(3), 271–277. https://doi.org/10.1016/S0959-437X(03)00055-8

Allada, R., & Chung, B. Y. (2010). Circadian organization of behavior and physiology in Drosophila. *Annu Rev Physiol*, *72*, 605–624. https://doi.org/10.1146/annurev-physiol-021909-135815

Allada, Ravi, Emery, P., Takahashi, J. S., & Rosbash, M. (2001). Stopping Time: The Genetics of Fly and Mouse Circadian Clocks. *Annual Review of Neuroscience*, *24*(1), 1091–1119. https://doi.org/10.1146/annurev.neuro.24.1.1091

Allada, Ravi, White, N. E., So, W. V., Hall, J. C., & Rosbash, M. (1998). A Mutant Drosophila Homolog of Mammalian Clock Disrupts Circadian Rhythms and Transcription of period and timeless. *Cell*, *93*(5), 791–804. https://doi.org/10.1016/S0092-8674(00)81440-3

Amatobi, K. M. (2016). *Determination of circadian metabolites in the hemolymph of Drosophila. (Unpublished)*.

Ang, J. E., Revell, V., Mann, A., Mäntele, S., Otway, D. T., Johnston, J. D., Thumser, A. E., Skene, D. J., & Raynaud, F. (2012). Identification of Human Plasma Metabolites Exhibiting Time-of-Day Variation Using an Untargeted Liquid Chromatography–Mass Spectrometry Metabolomic Approach. *Chronobiology International*, *29*(7), 868–881. https://doi.org/10.3109/07420528.2012.699122

Arrese, E. L. (1997). Adipokinetic hormone-induced lipolysis in the fat body of an insect, Manduca sexta: Synthesis of sn-I,2-diacylglycerols. *Journal of Lipid Research*, 9.

Aviram, R., Manella, G., Kopelman, N., Neufeld-Cohen, A., Zwighaft, Z., Elimelech, M., Adamovich, Y., Golik, M., Wang, C., Han, X., & Asher, G. (2016). Lipidomics Analyses Reveal Temporal and Spatial Lipid Organization and Uncover Daily Oscillations in Intracellular Organelles. *Molecular Cell*, *62*(4), 636–648. https://doi.org/10.1016/j.molcel.2016.04.002

Balsalobre, A. (2000). Resetting of Circadian Time in Peripheral Tissues by Glucocorticoid Signaling. *Science*, *289*(5488), 2344–2347. https://doi.org/10.1126/science.289.5488.2344

Balsalobre, Aurélio, Damiola, F., & Schibler, U. (1998). A Serum Shock Induces Circadian Gene Expression in Mammalian Tissue Culture Cells. *Cell*, *93*(6), 929–937. https://doi.org/10.1016/S0092-8674(00)81199-X

Bargiello, T. A., Jackson, F. R., & Young, M. W. (1984). Restoration of circadian behavioural rhythms by gene transfer in Drosophila. *Nature*, *312*(5996), 752–754. https://doi.org/10.1038/312752a0

Beaver, L. M., Klichko, V. I., Chow, E. S., Kotwica-Rolinska, J., Williamson, M., Orr, W. C., Radyuk, S. N., & Giebultowicz, J. M. (2012). Circadian Regulation of Glutathione Levels and Biosynthesis in Drosophila melanogaster. *PLoS ONE*, *7*(11), e50454. https://doi.org/10.1371/journal.pone.0050454

Bechtold, D. A., Brown, T. M., Luckman, S. M., & Piggins, H. D. (2008). Metabolic rhythm abnormalities in mice lacking VIP-VPAC 2 signaling. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *294*(2), R344–R351. https://doi.org/10.1152/ajpregu.00667.2007

Bell-Pedersen, D., Cassone, V. M., Earnest, D. J., Golden, S. S., Hardin, P. E., Thomas, T. L., & Zoran, M. J. (2005). Circadian rhythms from multiple oscillators: Lessons from diverse organisms. *Nature Reviews Genetics*, *6*(7), 544–556. https://doi.org/10.1038/nrg1633

Benito, J., Houl, J. H., Roman, G. W., & Hardin, P. E. (2008). The Blue-Light Photoreceptor CRYPTOCHROME Is Expressed in a Subset of Circadian Oscillator Neurons in the *Drosophila* CNS. *Journal of Biological Rhythms*, *23*(4), 296–307. https://doi.org/10.1177/0748730408318588

Berson, D. M. (2002). Phototransduction by Retinal Ganglion Cells That Set the Circadian Clock. *Science*, *295*(5557), 1070–1073. https://doi.org/10.1126/science.1067262

Bertolucci, C., Cavallari, N., Colognesi, I., Aguzzi, J., Chen, Z., Caruso, P., Foa, A., Tosini, G., Bernardi, F., & Pinotti, M. (2008). Evidence for an Overlapping Role of CLOCK and NPAS2 Transcription Factors in Liver Circadian Oscillators. *Molecular and Cellular Biology*, *28*(9), 3070–3075. https://doi.org/10.1128/MCB.01931-07

Bharucha, K. N. (2009). The Epicurean Fly: Using Drosophila Melanogaster to Study Metabolism. *Pediatric Research*, *65*(2), 132–137. https://doi.org/10.1203/PDR.0b013e318191fc68

Brown, S. A., Zumbrunn, G., Fleury-Olela, F., Preitner, N., & Schibler, U. (2002). Rhythms of Mammalian Body Temperature Can Sustain Peripheral Circadian Clocks. *Current Biology*, *12*(18), 1574–1583. https://doi.org/10.1016/S0960-9822(02)01145-4

Bruce, K. D., Hoxha, S., Carvalho, G. B., Yamada, R., Wang, H.-D., Karayan, P., He, S., Brummel, T., Kapahi, P., & Ja, W. W. (2013). High carbohydrate–low protein consumption maximizes Drosophila lifespan. *Experimental Gerontology*, *48*(10), 1129–1135. https://doi.org/10.1016/j.exger.2013.02.003

Buhr, E. D., & Takahashi, J. S. (2013). *Molecular components of the mammalian circadian clock*. 22.

Buijs, R. M., & Kalsbeek, A. (2001). Hypothalamic integration of central and peripheral clocks. *Nature Reviews Neuroscience*, *2*(7), 521–526. https://doi.org/10.1038/35081582

Cagampang, F. R., & Bruce, K. D. (2012). The role of the circadian clock system in nutrition and metabolism. *British Journal of Nutrition*, *108*(3), 381–392. https://doi.org/10.1017/S0007114512002139

Canavoso, L. E., Jouni, Z. E., Karnas, K. J., Pennington, J. E., & Wells, M. A. (2001). Fat Metabolism in Insects. *Annual Review of Nutrition*, *21*(1), 23–46. https://doi.org/10.1146/annurev.nutr.21.1.23

Carvalho, M., Sampaio, J. L., Palm, W., Brankatschk, M., Eaton, S., & Shevchenko, A. (2012). Effects of diet and development on the Drosophila lipidome. *Mol Syst Biol*, *8*, 600. https://doi.org/10.1038/msb.2012.29

Cassone, V. M., & Stephan, F. K. (2002). *Central and Peripheral Regulation of Feeding and Nutrition by the Mammalian Circadian Clock: Implications for Nutrition During Manned Space Flight*. *18*(10), 6.

Ceriani, M. F. (1999). Light-Dependent Sequestration of TIMELESS by CRYPTOCHROME. *Science*, *285*(5427), 553–556. https://doi.org/10.1126/science.285.5427.553

Chaix, A., Lin, T., Le, H. D., Chang, M. W., & Panda, S. (2019). Time-Restricted Feeding Prevents Obesity and Metabolic Syndrome in Mice Lacking a Circadian Clock. *Cell Metabolism*, *29*(2), 303-319.e4. https://doi.org/10.1016/j.cmet.2018.08.004

Chino, H., & Gilbert, L. I. (1964). Diglyceride Release from Insect Fat Body: A Possible Means of Lipid Transport. *Science*, *143*(3604), 359–361. https://doi.org/10.1126/science.143.3604.359

Chintapalli, V. R., Wang, J., & Dow, J. A. T. (2007). Using FlyAtlas to identify better Drosophila melanogaster models of human disease. *Nature Genetics*, *39*(6), 715–720. https://doi.org/10.1038/ng2049

Chiu, J. C., Low, K. H., Pike, D. H., Yildirim, E., & Edery, I. (2010). Assaying Locomotor Activity to Study Circadian Rhythms and Sleep Parameters in Drosophila. *Journal of Visualized Experiments*, *43*. https://doi.org/10.3791/2157

Chua, E. C.-P., Shui, G., Lee, I. T.-G., Lau, P., Tan, L.-C., Yeo, S.-C., Lam, B. D., Bulchand, S., Summers, S. A., Puvanendran, K., Rozen, S. G., Wenk, M. R., & Gooley, J. J. (2013). Extensive diversity in circadian regulation of plasma lipids and evidence for different circadian metabolic phenotypes in humans. *Proceedings of the National Academy of Sciences*, *110*(35), 14468–14473. https://doi.org/10.1073/pnas.1222647110

Collins, B., Mazzoni, E. O., Stanewsky, R., & Blau, J. (2006). Drosophila CRYPTOCHROME Is a Circadian Transcriptional Repressor. *Current Biology*, *16*(5), 441–449. https://doi.org/10.1016/j.cub.2006.01.034

Colombani, J., Raisin, S., Pantalacci, S., Radimerski, T., Montagne, J., & Léopold, P. (2003). A Nutrient Sensor Mechanism Controls Drosophila Growth. *Cell*, *114*(6), 739–749. https://doi.org/10.1016/S0092-8674(03)00713-X

Crumbley, C., & Burris, T. P. (2011). Direct Regulation of CLOCK Expression by REV-ERB. *PLoS ONE*, *6*(3), e17290. https://doi.org/10.1371/journal.pone.0017290

Cyran, S. A., Buchsbaum, A. M., Reddy, K. L., Lin, M.-C., Glossop, N. R. J., Hardin, P. E., Young, M. W., Storti, R. V., & Blau, J. (2003). Vrille, Pdp1, and dClock Form a Second

Feedback Loop in the Drosophila Circadian Clock. *Cell*, *112*(3), 329–341. https://doi.org/10.1016/S0092-8674(03)00074-6

Dallmann, R., Viola, A. U., Tarokh, L., Cajochen, C., & Brown, S. A. (2012a). The human circadian metabolome. *Proc Natl Acad Sci U S A*, *109*(7), 2625–2629. https://doi.org/10.1073/pnas.1114410109

Damiola, F. (2000). Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes & Development*, *14*(23), 2950–2961. https://doi.org/10.1101/gad.183500

Darlington, T. K. (1998). Closing the Circadian Loop: CLOCK-Induced Transcription of Its Own Inhibitors per and tim. *Science*, *280*(5369), 1599–1603. https://doi.org/10.1126/science.280.5369.1599

DeBruyne, J. P., Noton, E., Lambert, C. M., Maywood, E. S., Weaver, D. R., & Reppert, S. M. (2006). A Clock Shock: Mouse CLOCK Is Not Required for Circadian Oscillator Function. *Neuron*, *50*(3), 465–477. https://doi.org/10.1016/j.neuron.2006.03.041

DeBruyne, J. P., Weaver, D. R., & Reppert, S. M. (2007). CLOCK and NPAS2 have overlapping roles in the suprachiasmatic circadian clock. *Nature Neuroscience*, *10*(5), 543–545. https://doi.org/10.1038/nn1884

Demerec, M. (1950). *Biology of Drosophila*. John Wiley & Sons, Inc,.

DiAngelo, J. R., Erion, R., Crocker, A., & Sehgal, A. (2011). The central clock neurons regulate lipid storage in Drosophila. *PLoS One*, *6*(5), e19921. https://doi.org/10.1371/journal.pone.0019921

Dibner, C., Schibler, U., & Albrecht, U. (2010). The Mammalian Circadian Timing System: Organization and Coordination of Central and Peripheral Clocks. *Annual Review of Physiology*, *72*(1), 517–549. https://doi.org/10.1146/annurev-physiol-021909-135821

Douglas, A. E. (2013). *The alimentary canal in The Insects: Structure and Function,*. Cambridge University Press.

Douris, N., Kojima, S., Pan, X., Lerch-Gaggl, A. F., Duong, S. Q., Hussain, M. M., & Green, C. B. (2011). Nocturnin Regulates Circadian Trafficking of Dietary Lipid in Intestinal Enterocytes. *Current Biology*, *21*(16), 1347–1355. https://doi.org/10.1016/j.cub.2011.07.018

Driver, C. J. I., Wallis, R., & Cosopodiotis, G. (1986). IS A FAT METABOLITE THE MAJOR DIET DEPENDENT ACCELERATOR OF AGING? *Exp Gerontol*, 11.

Dubowy, C., & Sehgal, A. (2017). Circadian Rhythms and Sleep in *Drosophila melanogaster*. *Genetics*, *205*(4), 1373–1397. https://doi.org/10.1534/genetics.115.185157

Dudley, C. A. (2003). Altered Patterns of Sleep and Behavioral Adaptability in NPAS2-Deficient Mice. *Science*, *301*(5631), 379–383. https://doi.org/10.1126/science.1082795

Eckel-Mahan, K. L., Patel, V. R., de Mateo, S., Orozco-Solis, R., Ceglia, N. J., Sahar, S., Dilag-Penilla, S. A., Dyar, K. A., Baldi, P., & Sassone-Corsi, P. (2013). Reprogramming of the Circadian Clock by Nutritional Challenge. *Cell*, *155*(7), 1464–1478. https://doi.org/10.1016/j.cell.2013.11.034

Edgecomb, R. S., Harth, C. E., & Schneiderman, A. M. (1994). *Regulation of feeding behavior in adult drosophila melanogaster varies with feeding regime and nutritional state*. 21.

Elmore, S. K., Betrus, P. A., & Burr, R. (1994). Light, social zeitgebers, and the sleep–wake cycle in the entrainment of human circadian rhythms. *Research in Nursing & Health*, *17*(6), 471–478. https://doi.org/10.1002/nur.4770170610

Fahy, E., Cotter, D., Sud, M., & Subramaniam, S. (2011). Lipid classification, structures and tools. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, *1811*(11), 637–647. https://doi.org/10.1016/j.bbalip.2011.06.009

Fahy, E., Subramaniam, S., Murphy, R. C., Nishijima, M., Raetz, C. R. H., Shimizu, T., Spener, F., van Meer, G., Wakelam, M. J. O., & Dennis, E. A. (2009). Update of the LIPID MAPS comprehensive classification system for lipids. *Journal of Lipid Research*, *50*, S9–S14. https://doi.org/10.1194/jlr.R800095-JLR200

Fakhrzadeh, H., Lariiani, B., Sanjari, M., Baradar-Jalili, R., & Amini, M. R. (2003). Effect of Ramadan Fasting on Clinical and Biochemical Parameters in Healthy Adults. *Annals of Saudi Medicine*, *23*(3–4), 223–226. https://doi.org/10.5144/0256-4947.2003.223

Fernando-Warnakulasuriya, G. J. P., Tsuchida, K., & Wells, M. A. (1988). Effect of dietary lipid content on lipid transport and storage during larval development of Manduca sexta. *Insect Biochemistry*, *18*(2), 211–214. https://doi.org/10.1016/0020-1790(88)90025-X

Fernando-Warnakulasuriya, G. J. P., & Wells, M. A. (1988). Isolation and characterization of lipophorin fromDrosophila melanogaster larvae. *Archives of Insect Biochemistry and Physiology*, *8*(4), 243–248. https://doi.org/10.1002/arch.940080405

Forster, F. (2017). *Determination of hydrophilic metabolites in the hemolymph of drosophila*.

Franchimont, D. (2004). Overview of the Actions of Glucocorticoids on the Immune Response: A Good Model to Characterize New Pathways of Immunosuppression for New Treatment Strategies. *Annals of the New York Academy of Sciences*, *1024*(1), 124–137. https://doi.org/10.1196/annals.1321.009

Froy, O. (2010). Metabolism and Circadian Rhythms—Implications for Obesity. *Endocrine Reviews*, *31*(1), 1–24. https://doi.org/10.1210/er.2009-0014

Gachon, F., Nagoshi, E., Brown, StevenA., Ripperger, J., & Schibler, U. (2004). The mammalian circadian timing system: From gene expression to physiology. *Chromosoma*, *113*(3). https://doi.org/10.1007/s00412-004-0296-2

Ghiasvand, M., Heshmat, R., Golpira, R., Haghpanah, V., Soleimani, A., Shoushtarizadeh, P., Tavangar, S. M., & Larijani, B. (2006). Shift working and risk of lipid disorders: A cross-sectional study. *Lipids in Health and Disease*, 5,9.

Giebultowicz, J. M. (2018). Circadian regulation of metabolism and healthspan in Drosophila. *Free Radic Biol Med*, *119*, 62–68. https://doi.org/10.1016/j.freeradbiomed.2017.12.025

Giebultowicz, Jadwiga M., & Hege, D. M. (1997). Circadian clock in Malpighian tubules. *Nature*, *386*, 664.

Gill, S., Le, H. D., Melkani, G. C., & Panda, S. (2015). Time-restricted feeding attenuates age-related cardiac decline in Drosophila. *Science*, *347*(6227), 1265–1269. https://doi.org/10.1126/science.1256682

Glossop, N. R. J., & Hardin, P. E. (2002a). *Circadian oscillators in flies and mice*. 9.

Glossop, N. R. J., & Hardin, P. E. (2002b). Central and peripheral circadian oscillator mechanisms in flies and mammals. *Journal of Cell Science*, *115*(17), 3369.

Godzien, J., Ciborowski, M., Martínez-Alcázar, M. P., Samczuk, P., Kretowski, A., & Barbas, C. (2015). Rapid and Reliable Identification of Phospholipids for Untargeted Metabolomics with LC–ESI–QTOF–MS/MS. *Journal of Proteome Research*, *14*(8), 3204–3216. https://doi.org/10.1021/acs.jproteome.5b00169

Gogna, N., Singh, V. J., Sheeba, V., & Dorai, K. (2015). NMR-based investigation of the Drosophila melanogaster metabolome under the influence of daily cycles of light and temperature. *Mol Biosyst*, *11*(12), 3305–3315. https://doi.org/10.1039/c5mb00386e

Graham, P., & Pick, L. (2017). Drosophila as a Model for Diabetes and Diseases of Insulin Resistance. In *Current Topics in Developmental Biology* (Vol. 121, pp. 397–419). Elsevier. https://doi.org/10.1016/bs.ctdb.2016.07.011

Grima, B., Chélot, E., Xia, R., & Rouyer, F. (2004). Morning and evening peaks of activity rely on different clock neurons of the Drosophila brain. *Nature*, *431*(7010), 869–873. https://doi.org/10.1038/nature02935

Grima, B., Lamouroux, A., Chélot, E., Papin, C., Limbourg-Bouchon, B., & Rouyer, F. (2002). The F-box protein Slimb controls the levels of clock proteins Period and Timeless. *Nature*, *420*(6912), 178–182. https://doi.org/10.1038/nature01122

Grönke, S., Mildner, A., Fellert, S., Tennagels, N., Petry, S., Müller, G., Jäckle, H., & Kühnlein, R. P. (2005). Brummer lipase is an evolutionary conserved fat storage regulator in Drosophila. *Cell Metabolism*, *1*(5), 323–330. https://doi.org/10.1016/j.cmet.2005.04.003

Grönke, S., Müller, G., Hirsch, J., Fellert, S., Andreou, A., Haase, T., Jäckle, H., & Kühnlein, R. P. (2007). Dual Lipolytic Control of Body Fat Storage and Mobilization in Drosophila. *PLoS Biology*, *5*(6), e137. https://doi.org/10.1371/journal.pbio.0050137

Hamilton, E. E., & Kay, S. A. (2008). SnapShot: Circadian Clock Proteins. *Cell*, *135*(2), 368-368.e1. https://doi.org/10.1016/j.cell.2008.09.042

Handke, B., Poernbacher, I., Goetze, S., Ahrens, C. H., Omasits, U., Marty, F., Simigdala, N., Meyer, I., Wollscheid, B., Brunner, E., Hafen, E., & Lehner, C. F. (2013). The Hemolymph Proteome of Fed and Starved Drosophila Larvae. *PLoS ONE*, *8*(6), e67208. https://doi.org/10.1371/journal.pone.0067208

Hardin, P. E. (2005). The circadian timekeeping system of Drosophila. *Curr Biol*, *15*(17), R714-22. https://doi.org/10.1016/j.cub.2005.08.019

Haselton, A. T., & Fridell, Y.-W. C. (2011). Insulin Injection and Hemolymph Extraction to Measure Insulin Sensitivity in Adult Drosophila melanogaster. *Journal of Visualized Experiments*, *52*. https://doi.org/10.3791/2722

Hastings, M. H., Reddy, A. B., & Maywood, E. S. (2003). A clockwork web: Circadian timing in brain and periphery, in health and disease. *Nature Reviews Neuroscience*, *4*(8), 649–661. https://doi.org/10.1038/nrn1177

Hatori, M., Vollmers, C., Zarrinpar, A., DiTacchio, L., Bushong, E. A., Gill, S., Leblanc, M., Chaix, A., Joens, M., Fitzpatrick, J. A., Ellisman, M. H., & Panda, S. (2012). Time-restricted feeding without reducing caloric intake prevents metabolic diseases in mice fed a high-fat diet. *Cell Metab*, *15*(6), 848–860. https://doi.org/10.1016/j.cmet.2012.04.019

Hattar, S. (2002). Melanopsin-Containing Retinal Ganglion Cells: Architecture, Projections, and Intrinsic Photosensitivity. *Science*, *295*(5557), 1065–1070. https://doi.org/10.1126/science.1069609

Heier, C., & Kühnlein, R. P. (2018). Triacylglycerol Metabolism in *Drosophila melanogaster*. *Genetics*, *210*(4), 1163–1184. https://doi.org/10.1534/genetics.118.301583

Helfrich-Förster, C. (1998). Robust circadian rhythmicity of Drosophila melanogaster requires the presence of lateral neurons: A brain-behavioral study of disconnected mutants. *Journal of Comparative Physiology A: Sensory, Neural, and Behavioral Physiology*, *182*(4), 435–453. https://doi.org/10.1007/s003590050192

Helfrich-Forster, C. (2002). The circadian system of Drosophila melanogaster and its light input pathways. *Zoology (Jena)*, *105*(4), 297–312. https://doi.org/10.1078/0944-2006-00074

Helfrich-Förster, C. (2004). Neurobiology of the fruit fly's circadian clock: Drosophila circadian clock. *Genes, Brain and Behavior*, *4*(2), 65–76. https://doi.org/10.1111/j.1601-183X.2004.00092.x

Helfrich-Förster, Charlotte, Winter, C., Hofbauer, A., Hall, J. C., & Stanewsky, R. (2001). The Circadian Clock of Fruit Flies Is Blind after Elimination of All Known Photoreceptors. *Neuron*, *30*(1), 249–261. https://doi.org/10.1016/S0896-6273(01)00277-X

Hermann-Luibl, C., & Helfrich-Förster, C. (2015). Clock network in Drosophila. *Current Opinion in Insect Science*, *7*, 65–70. https://doi.org/10.1016/j.cois.2014.11.003

Herren, J. K., Paredes, J. C., Schüpfer, F., Arafah, K., Bulet, P., & Lemaitre, B. (2014). Insect endosymbiont proliferation is limited by lipid availability. *ELife*, *3*, e02964. https://doi.org/10.7554/eLife.02964

Herzog, E. D. (2007). Neurons and networks in daily rhythms. *Nature Reviews Neuroscience*, *8*(10), 790–802. https://doi.org/10.1038/nrn2215

Hirota, T., & Fukada, Y. (2004). Resetting Mechanism of Central and Peripheral Circadian Clocks in Mammals. *Zoological Science*, *21*(4), 359–368. https://doi.org/10.2108/zsj.21.359

Huang, J.-H., & Douglas, A. E. (2015). Consumption of dietary sugar by gut bacteria determines *Drosophila* lipid content. *Biology Letters*, *11*(9), 20150469. https://doi.org/10.1098/rsbl.2015.0469

Hunter-Ensor, M., Ousley, A., & Sehgal, A. (1996). Regulation of the Drosophila Protein Timeless Suggests a Mechanism for Resetting the Circadian Clock by Light. *Cell*, *84*(5), 677–685. https://doi.org/10.1016/S0092-8674(00)81046-6

Husse, J., Eichele, G., & Oster, H. (2015). Synchronization of the mammalian circadian timing system: Light can control peripheral clocks independently of the SCN clock: Alternate routes of entrainment optimize the alignment of the body's circadian clock network with external time. *BioEssays*, *37*(10), 1119–1128. https://doi.org/10.1002/bies.201500026

Ito, C., Goto, S. G., Shiga, S., Tomioka, K., & Numata, H. (2008). Peripheral circadian clock for the cuticle deposition rhythm in Drosophila melanogaster. *Proceedings of the National Academy of Sciences*, *105*(24), 8446–8451. https://doi.org/10.1073/pnas.0800145105

Ito, C., & Tomioka, K. (2016). Heterogeneity of the Peripheral Circadian Systems in Drosophila melanogaster: A Review. *Frontiers in Physiology*, *7*. https://doi.org/10.3389/fphys.2016.00008

Ivanchenko, M., Stanewsky, R., & Giebultowicz, J. M. (2001). Circadian Photoreception in Drosophila: Functions of Cryptochrome in Peripheral and Central Clocks. *J.Biol.Rhythms*, *16*, 205–215. https://doi.org/10.1177/074873040101600303

Ja, W. W., Carvalho, G. B., Mak, E. M., de la Rosa, N. N., Fang, A. Y., Liong, J. C., Brummel, T., & Benzer, S. (2007). Prandiology of Drosophila and the CAFE assay. *Proceedings of the National Academy of Sciences*, *104*(20), 8253–8256. https://doi.org/10.1073/pnas.0702726104

Kalsbeek, A., Palm, I. F., La Fleur, S. E., Scheer, F. A. J. L., Perreau-Lenz, S., Ruiter, M., Kreier, F., Cailotto, C., & Buijs, R. M. (2006). SCN Outputs and the Hypothalamic Balance of Life. *Journal of Biological Rhythms*, *21*(6), 458–469. https://doi.org/10.1177/0748730406293854

Kasukawa, T., Sugimoto, M., Hida, A., Minami, Y., Mori, M., Honma, S., Honma, K. -i., Mishima, K., Soga, T., & Ueda, H. R. (2012). Human blood metabolite timetable indicates internal body time. *Proceedings of the National Academy of Sciences*, *109*(37), 15036–15041. https://doi.org/10.1073/pnas.1207768109

Katewa, S. D., Akagi, K., Bose, N., Rakshit, K., Camarella, T., Zheng, X., Hall, D., Davis, S., Nelson, C. S., Brem, R. B., Ramanathan, A., Sehgal, A., Giebultowicz, J. M., & Kapahi, P. (2016). Peripheral Circadian Clocks Mediate Dietary Restriction-Dependent

Changes in Lifespan and Fat Metabolism in Drosophila. *Cell Metab*, *23*(1), 143–154. https://doi.org/10.1016/j.cmet.2015.10.014

Katewa, Subhash D., Demontis, F., Kolipinski, M., Hubbard, A., Gill, M. S., Perrimon, N., Melov, S., & Kapahi, P. (2012). Intramyocellular Fatty-Acid Metabolism Plays a Critical Role in Mediating Responses to Dietary Restriction in Drosophila melanogaster. *Cell Metabolism*, *16*(1), 97–103. https://doi.org/10.1016/j.cmet.2012.06.005

Kim, E. Y., & Edery, I. (2006). Balance between DBT/CKI kinase and protein phosphatase activities regulate phosphorylation and stability of Drosophila CLOCK protein. *Proceedings of the National Academy of Sciences*, *103*(16), 6178–6183. https://doi.org/10.1073/pnas.0511215103

King, D. G. (1988). Cellular organization and peritrophic membrane formation in the cardia (Proventriculus) ofDrosophila melanogaster. *Journal of Morphology*, *196*(3), 253–282. https://doi.org/10.1002/jmor.1051960302

Klein, D., moore, R., & Reppert, S. (1990). The Suprachiasmatic Nucleus: The Mind's Clock. In *New York: Oxford University Press*.

Kloss, B., Price, J. L., Saez, L., Blau, J., Rothenfluh, A., Wesley, C. S., & Young, M. W. (1988). *The Drosophila Clock Gene double-time Encodes a Protein Closely Related to Human Casein Kinase I□*. 11.

Knittelfelder, O. L., Weberhofer, B. P., Eichmann, T. O., Kohlwein, S. D., & Rechberger, G. N. (2014). A versatile ultra-high performance LC-MS method for lipid profiling. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*, *951–952*(100), 119–128. https://doi.org/10.1016/j.jchromb.2014.01.011

Ko, H. W., Jiang, J., & Edery, I. (2002). Role for Slimb in the degradation of Drosophila Period protein phosphorylated by Doubletime. *Nature*, *420*(6916), 673–678. https://doi.org/10.1038/nature01272

Kohsaka, A., Laposky, A. D., Ramsey, K. M., Estrada, C., Joshu, C., Kobayashi, Y., Turek, F. W., & Bass, J. (2007). High-Fat Diet Disrupts Behavioral and Molecular Circadian Rhythms in Mice. *Cell Metabolism*, *6*(5), 414–421. https://doi.org/10.1016/j.cmet.2007.09.006

Konopka, R. J., & Benzer, S. (1971). Clock Mutants of Drosophila melanogaster. *Proceedings of the National Academy of Sciences*, *68*(9), 2112–2116. https://doi.org/10.1073/pnas.68.9.2112

Koronowski, K. B., Kinouchi, K., Welz, P.-S., Smith, J. G., Zinna, V. M., Shi, J., Samad, M., Chen, S., Magnan, C. N., Kinchen, J. M., Li, W., Baldi, P., Benitah, S. A., & Sassone-Corsi, P. (2019). Defining the Independence of the Liver Circadian Clock. *Cell*, *177*(6), 1448-1462.e14. https://doi.org/10.1016/j.cell.2019.04.025

Krupp, J. J., Billeter, J.-C., Wong, A., Choi, C., Nitabach, M. N., & Levine, J. D. (2013). Pigment-Dispersing Factor Modulates Pheromone Production in Clock Cells that Influence Mating in Drosophila. *Neuron*, *79*(1), 54–68. https://doi.org/10.1016/j.neuron.2013.05.019

Kudo, T., Horikawa, K., & Shibata, S. (2007). Circadian Rhythms in the CNS and Peripheral Clock Disorders: The Circadian Clock and Hyperlipidemia. *Journal of Pharmacological Sciences*, *103*(2), 139–143. https://doi.org/10.1254/jphs.FMJ06003X3

Kühnlein, R. P. (2011). The contribution of the Drosophila model to lipid droplet research. *Progress in Lipid Research*, *50*(4), 348–356. https://doi.org/10.1016/j.plipres.2011.04.001

Kumar Jha, P., Challet, E., & Kalsbeek, A. (2015). Circadian rhythms in glucose and lipid metabolism in nocturnal and diurnal mammals. *Molecular and Cellular Endocrinology*, *418*, 74–88. https://doi.org/10.1016/j.mce.2015.01.024

Laye, M. J., Tran, V., Jones, D. P., Kapahi, P., & Promislow, D. E. L. (2015). The effects of age and dietary restriction on the tissue-specific metabolome of *Drosophila*. *Aging Cell*, *14*(5), 797–808. https://doi.org/10.1111/acel.12358

Lee, C., Bae, K., & Edery, I. (1999). PER and TIM Inhibit the DNA Binding Activity of a *Drosophila* CLOCK-CYC/dBMAL1 Heterodimer without Disrupting Formation of the Heterodimer: A Basis for Circadian Transcription. *Molecular and Cellular Biology*, *19*(8), 5316–5325. https://doi.org/10.1128/MCB.19.8.5316

Lehane, M. J., & Billingsley, P. F. (1996). *Biology of the Insect Midgut*. Chapman & Hall, London. https://doi.org/10.1007/ 978-94-009-1519-0

Lemaitre, B., & Miguel-Aliaga, I. (2013). The digestive tract of Drosophila melanogaster. *Annu Rev Genet*, *47*, 377–404. https://doi.org/10.1146/annurev-genet-111212-133343

Liu, A. C., Lewis, W. G., & Kay, S. A. (2007). Mammalian circadian signaling networks and therapeutic targets. *Nature Chemical Biology*, *3*(10), 630–639. https://doi.org/10.1038/nchembio.2007.37

Liu, C., Li, S., Liu, T., Borjigin, J., & Lin, J. D. (2007). Transcriptional coactivator PGC-1α integrates the mammalian clock and energy metabolism. *Nature*, *447*(7143), 477–481. https://doi.org/10.1038/nature05767

Loh, D. H., Dragich, J. M., Kudo, T., Schroeder, A. M., Nakamura, T. J., Waschek, J. A., Block, G. D., & Colwell, C. S. (2011). Effects of Vasoactive Intestinal Peptide Genotype on Circadian Gene Expression in the Suprachiasmatic Nucleus and Peripheral Organs. *Journal of Biological Rhythms*, *26*(3), 200–209. https://doi.org/10.1177/0748730411401740

Longo, V. D., & Panda, S. (2016). Fasting, Circadian Rhythms, and Time-Restricted Feeding in Healthy Lifespan. *Cell Metabolism*, *23*(6), 1048–1059. https://doi.org/10.1016/j.cmet.2016.06.001

Looby, P., & Loudon, A. S. I. (2005). Gene duplication and complex circadian clocks in mammals. *Trends in Genetics*, *21*(1), 46–53. https://doi.org/10.1016/j.tig.2004.11.012

Lowrey, P. L., & Takahashi, J. S. (2004). MAMMALIAN CIRCADIAN BIOLOGY: Elucidating Genome-Wide Levels of Temporal Organization. *Annual Review of Genomics and Human Genetics*, *5*(1), 407–441. https://doi.org/10.1146/annurev.genom.5.061903.175925

Lowrey, P. L., & Takahashi, J. S. (2011). Genetics of Circadian Rhythms in Mammalian Model Organisms. In *Advances in Genetics* (Vol. 74, pp. 175–230). Elsevier. https://doi.org/10.1016/B978-0-12-387690-4.00006-4

Lucas, R. J., Freedman, M. S., Lupi, D., Munoz, M., David-Gray, Z. K., & Foster, R. G. (2001). Identifying the photoreceptive inputs to the mammalian circadian system using transgenic and retinally degenerate mice. *Behavioural Brain Research*, *125*(1–2), 97–102. https://doi.org/10.1016/S0166-4328(01)00274-1

Lucassen, E. A., Rother, K. I., & Cizza, G. (2012). Interacting epidemics? Sleep curtailment, insulin resistance, and obesity: Short sleep and metabolic dysfunction. *Annals of the New York Academy of Sciences*, *1264*(1), 110–134. https://doi.org/10.1111/j.1749-6632.2012.06655.x

Lundell, L. S., Parr, E. B., Devlin, B. L., Ingerslev, L. R., Altıntaş, A., Sato, S., Sassone-Corsi, P., Barrès, R., Zierath, J. R., & Hawley, J. A. (2020). Time-restricted feeding alters lipid and amino acid metabolite rhythmicity without perturbing clock gene expression. *Nature Communications*, *11*(1). https://doi.org/10.1038/s41467-020-18412-w

MacMillan, H. A., & Hughson, B. N. (2014). A high-throughput method of hemolymph extraction from adult Drosophila without anesthesia. *Journal of Insect Physiology*, *63*, 27–31. https://doi.org/10.1016/j.jinsphys.2014.02.005

Mahoney, M. M., Sisk, C., Ross, H. E., & Smale, L. (2004). Circadian Regulation of Gonadotropin-Releasing Hormone Neurons and the Preovulatory Surge in Luteinizing Hormone in the Diurnal Rodent, Arvicanthis niloticus, and in a Nocturnal Rodent, Rattus norvegicus1. *Biology of Reproduction*, *70*(4), 1049–1054. https://doi.org/10.1095/biolreprod.103.021360

Maillot, F., Garrigue, M., Pinault, M., Objois, M., Théret, V., Lamisse, F., Hoinard, C., Antoine, J., Lairon, D., & Couet, C. (2005). Changes in plasma triacylglycerol concentrations after sequential lunch and dinner in healthy subjects. *Diabetes & Metabolism*, *31*(1), 69–77. https://doi.org/10.1016/S1262-3636(07)70169-6

Marian, J. (1729). Observation botanique. *Hist Acad Roy*.

Mazzoni, E. O., Desplan, C., & Blau, J. (2005). Circadian Pacemaker Neurons Transmit and Modulate Visual Information to Control a Rapid Behavioral Response. *Neuron*, *45*(2), 293–300. https://doi.org/10.1016/j.neuron.2004.12.038

McCay, C. M., Crowell, M. F., & Maynard, L. A. (1935). The Effect of Retarded Growth Upon the Length of Life Span and Upon the Ultimate Body Size: One Figure. *The Journal of Nutrition*, *10*(1), 63–79. https://doi.org/10.1093/jn/10.1.63

Mehus, A., Idso, J., Bukowski, M., & Picklo, M. (2019). Time-Restricted Feeding a High-fat Diet in Mice Elevates Hepatic Long-Chain Polyunsaturated Fatty Acid Content and Modifies the Triacylglyceryl Lipidome (P21-013-19). *Current Developments in Nutrition*, *3*(Supplement_1). https://doi.org/10.1093/cdn/nzz041.P21-013-19

Menet, J. S., Abruzzi, K. C., Desrochers, J., Rodriguez, J., & Rosbash, M. (2010). Dynamic PER repression mechanisms in the Drosophila circadian clock: From on-DNA to off-DNA. *Genes & Development*, *24*(4), 358–367. https://doi.org/10.1101/gad.1883910

Meyer-Bernstein, E. L., & Sehgal, A. (2001). Book Review: Molecular Regulation of Circadian Rhythms in Drosophila and Mammals. *The Neuroscientist*, *7*(6), 496–505. https://doi.org/10.1177/107385840100700606

Miguel-Aliaga, I., Jasper, H., & Lemaitre, B. (2018). Anatomy and Physiology of the Digestive Tract of *Drosophila melanogaster*. *Genetics*, *210*(2), 357–396. https://doi.org/10.1534/genetics.118.300224

Minami, Y., Kasukawa, T., Kakazu, Y., Iigo, M., Sugimoto, M., Ikeda, S., Yasui, A., van der Horst, G. T. J., Soga, T., & Ueda, H. R. (2009). Measurement of internal body time by blood metabolomics. *Proceedings of the National Academy of Sciences*, *106*(24), 9890–9895. https://doi.org/10.1073/pnas.0900617106

Miyasako, Y., Umezaki, Y., & Tomioka, K. (2007). Separate Sets of Cerebral Clock Neurons Are Responsible for Light and Temperature Entrainment of *Drosophila* Circadian Locomotor Rhythms. *Journal of Biological Rhythms*, *22*(2), 115–126. https://doi.org/10.1177/0748730407299344

Mohawk, J. A., Green, C. B., & Takahashi, J. S. (2012). Central and Peripheral Circadian Clocks in Mammals. *Annual Review of Neuroscience*, *35*(1), 445–462. https://doi.org/10.1146/annurev-neuro-060909-153128

Moro, T., Tinsley, G., Bianco, A., Marcolin, G., Pacelli, Q. F., Battaglia, G., Palma, A., Gentil, P., Neri, M., & Paoli, A. (2016). Effects of eight weeks of time-restricted feeding (16/8) on basal metabolism, maximal strength, body composition, inflammation, and cardiovascular risk factors in resistance-trained males. *Journal of Translational Medicine*, *14*(1). https://doi.org/10.1186/s12967-016-1044-0

Musselman, L. P., & Kühnlein, R. P. (2018). *Drosophila* as a model to study obesity and metabolic disease. *The Journal of Experimental Biology*, *221*(Suppl 1), jeb163881. https://doi.org/10.1242/jeb.163881

Myers, E. M., Yu, J., & Sehgal, A. (2003). Circadian Control of Eclosion: Interaction between a Central and Peripheral Clock in Drosophila melanogaster. *Current Biology*, 8.

Myers, M. P., Wager-Smith, K., Rothenfluh-Hilfiker, A., & Young, M. W. (1996). Light-Induced Degradation of TIMELESS and Entrainment of the Drosophila Circadian Clock. *Science*, *271*(5256), 1736–1740. https://doi.org/10.1126/science.271.5256.1736

Myers, M. P., Wager-Smith, K., Wesley, C. S., Young, M. W., & Sehgal, A. (1995). Positional Cloning and Sequence Analysis of the Drosophila Clock Gene, timeless. *Science*, *270*(5237), 805–808. https://doi.org/10.1126/science.270.5237.805

Naikkhwah, W., & O'Donnell, M. J. (2011). Salt stress alters fluid and ion transport by Malpighian tubules of Drosophila melanogaster: Evidence for phenotypic plasticity. *Journal of Experimental Biology*, *214*(20), 3443–3454. https://doi.org/10.1242/jeb.057828

Oishi, K., Fukui, H., & Ishida, N. (2000). Rhythmic Expression of BMAL1 mRNA Is Altered in Clock Mutant Mice: Differential Regulation in the Suprachiasmatic Nucleus and Peripheral Tissues. *Biochemical and Biophysical Research Communications*, *268*(1), 164–171. https://doi.org/10.1006/bbrc.1999.2054

Ongsara, S. (2017). The Effect of Ramadan Fasting on Biochemical Parameters in Healthy Thai Subjects. *JOURNAL OF CLINICAL AND DIAGNOSTIC RESEARCH*. https://doi.org/10.7860/JCDR/2017/27294.10634

Oster, H., Challet, E., Ott, V., Arvat, E., de Kloet, E. R., Dijk, D.-J., Lightman, S., Vgontzas, A., & Van Cauter, E. (2017). The Functional and Clinical Significance of the 24-Hour

Rhythm of Circulating Glucocorticoids. *Endocrine Reviews*, *38*(1), 3–45. https://doi.org/10.1210/er.2015-1080

Ozgur, T., & Tataroglu, O. (2014). Studying circadian rhythms in Drosophila melanogaster. *Methods*, *68*(1), 140–150. http://dx.doi.org/10.1016/j.ymeth.2014.01.001

Palanker Musselman, L., Fink, J. L., Narzinski, K., Ramachandran, P. V., Sukumar Hathiramani, S., Cagan, R. L., & Baranski, T. J. (2011). A high-sugar diet produces obesity and insulin resistance in wild-type Drosophila. *Disease Models & Mechanisms*, *4*(6), 842–849. https://doi.org/10.1242/dmm.007948

Palm, W., Sampaio, J. L., Brankatschk, M., Carvalho, M., Mahmoud, A., Shevchenko, A., & Eaton, S. (2012). Lipoproteins in Drosophila melanogaster—Assembly, Function, and Influence on Tissue Lipid Composition. *PLOS Genetics*, *8*(7), e1002828. https://doi.org/10.1371/journal.pgen.1002828

Pan, A., Schernhammer, E. S., Sun, Q., & Hu, F. B. (2011). Rotating Night Shift Work and Risk of Type 2 Diabetes: Two Prospective Cohort Studies in Women. *PLoS Medicine*, *8*(12), e1001141. https://doi.org/10.1371/journal.pmed.1001141

Pan, X., & Hussain, M. M. (2007). Diurnal Regulation of Microsomal Triglyceride Transfer Protein and Plasma Lipid Levels. *Journal of Biological Chemistry*, *282*(34), 24707–24719. https://doi.org/10.1074/jbc.M701305200

Partridge, L., Piper, M. D. W., & Mair, W. (2005). Dietary restriction in Drosophila. *Mechanisms of Ageing and Development*, *126*(9), 938–950. https://doi.org/10.1016/j.mad.2005.03.023

Pennington, J. E., & Wells, M. A. (2002). Triacylglycerol-rich lipophorins are found in the dipteran infraorder Culicomorpha, not just in mosquitoes. *J Insect Sci*, *2*, 15.

Pi, J., Wu, X., & Feng, Y. (2016). Fragmentation patterns of five types of phospholipids by ultra-high-performance liquid chromatography electrospray ionization quadrupole

time-of-flight tandem mass spectrometry. *Analytical Methods*, *8*(6), 1319–1332. https://doi.org/10.1039/C5AY00776C

Piper, M. D. W., & Partridge, L. (2007). Dietary Restriction in Drosophila: Delayed Aging or Experimental Artefact? *PLoS Genetics*, *3*(4), e57. https://doi.org/10.1371/journal.pgen.0030057

Pittendrigh, C. (1967). Circadian systems, 1. The driving oscillation and its assay in Drosophila pseudoobscura. *Proc. Natl. Acad. Sci.*, *58*, 1762–1767.

Piyankarage, S. C., Augustin, H., Grosjean, Y., Featherstone, D. E., & Shippy, S. A. (2008). Hemolymph Amino Acid Analysis of Individual *Drosophila* Larvae. *Analytical Chemistry*, *80*(4), 1201–1207. https://doi.org/10.1021/ac701785z

Plano, S. A., Casiraghi, L. P., García Moro, P., Paladino, N., Golombek, D. A., & Chiesa, J. J. (2017a). Circadian and Metabolic Effects of Light: Implications in Weight Homeostasis and Health. *Frontiers in Neurology*, *8*. https://doi.org/10.3389/fneur.2017.00558

Plano, S. A., Casiraghi, L. P., García Moro, P., Paladino, N., Golombek, D. A., & Chiesa, J. J. (2017b). Circadian and Metabolic Effects of Light: Implications in Weight Homeostasis and Health. *Frontiers in Neurology*, *8*. https://doi.org/10.3389/fneur.2017.00558

Plautz, J. D., Kaneko, M., Hall, J. C., & Kay, S. A. (1997). Independent Photoreceptive Circadian Clocks Throughout *Drosophila*. *Science*, *278*(5343), 1632–1635. https://doi.org/10.1126/science.278.5343.1632

Poesen, R., Mutsaers, H. A. M., Windey, K., van den Broek, P. H., Verweij, V., Augustijns, P., Kuypers, D., Jansen, J., Evenepoel, P., Verbeke, K., Meijers, B., & Masereeuw, R. (2015). The Influence of Dietary Protein Intake on Mammalian Tryptophan and Phenolic Metabolites. *PLOS ONE*, *10*(10), e0140820. https://doi.org/10.1371/journal.pone.0140820

Preitner, N., Damiola, F., Zakany, J., Duboule, D., Albrecht, U., & Schibler, U. (2002). *The Orphan Nuclear Receptor REV-ERB␣ Controls Circadian Transcription within the Positive Limb of the Mammalian Circadian Oscillator*. 10.

Price, J. L., Blau, J., Rothenfluh, A., Abodeely, M., Kloss, B., & Young, M. W. (1998). Double-time Is a Novel Drosophila Clock Gene that Regulates PERIOD Protein Accumulation. *Cell*, *94*(1), 83–95. https://doi.org/10.1016/S0092-8674(00)81224-6

Radogna, F., Diederich, M., & Ghibelli, L. (2010). Melatonin: A pleiotropic molecule regulating inflammation. *Biochemical Pharmacology*, *80*(12), 1844–1852. https://doi.org/10.1016/j.bcp.2010.07.041

Rajan, A., & Perrimon, N. (2013). Of flies and men: Insights on organismal metabolism from fruit flies. *BMC Biology*, *11*(1). https://doi.org/10.1186/1741-7007-11-38

Rajaratnam, S. M., & Arendt, J. (2001). Health in a 24-h society. *The Lancet*, *358*(9286), 999–1005. https://doi.org/10.1016/S0140-6736(01)06108-6

Ralph, M., Foster, R., Davis, F., & Menaker, M. (1990). Transplanted suprachiasmatic nucleus determines circadian period. *Science*, *247*(4945), 975–978. https://doi.org/10.1126/science.2305266

Renn, S. C. P., Park, J. H., Rosbash, M., Hall, J. C., & Taghert, P. H. (1999). A pdf Neuropeptide Gene Mutation and Ablation of PDF Neurons Each Cause Severe Abnormalities of Behavioral Circadian Rhythms in Drosophila. *Cell*, *99*(7), 791–802. https://doi.org/10.1016/S0092-8674(00)81676-1

Reppert, S. M., & Weaver, D. R. (2002). Coordination of circadian timing in mammals. *Nature*, *418*(6901), 935–941. https://doi.org/10.1038/nature00965

Rhoades, S. D., Nayak, K., Zhang, S. L., Sehgal, A., & Weljie, A. M. (2018). Circadian- and Light-driven Metabolic Rhythms in *Drosophila melanogaster*. *Journal of Biological Rhythms*, *33*(2), 126–136. https://doi.org/10.1177/0748730417753003

Richards, J., & Gumz, M. L. (2013). Mechanism of the circadian clock in physiology. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *304*(12), R1053–R1064. https://doi.org/10.1152/ajpregu.00066.2013

Rosato, E., Tauber, E., & Kyriacou, C. P. (2006). Molecular genetics of the fruit-fly circadian clock. *European Journal of Human Genetics*, *14*(6), 729–738. https://doi.org/10.1038/sj.ejhg.5201547

Rosbash, M. (2009). The Implications of Multiple Circadian Clock Origins. *PLoS Biology*, *7*(3), e1000062. https://doi.org/10.1371/journal.pbio.1000062

Rutila, J. E., Suri, V., Le, M., So, W. V., Rosbash, M., & Hall, J. C. (1998). CYCLE Is a Second bHLH-PAS Clock Protein Essential for Circadian Rhythmicity and Transcription of Drosophila period and timeless. *Cell*, *93*(5), 805–814. https://doi.org/10.1016/S0092-8674(00)81441-5

Salfer, I. J., & Harvatine, K. J. (2020). Night-restricted feeding of dairy cows modifies daily rhythms of feed intake, milk synthesis and plasma metabolites compared with day-restricted feeding. *British Journal of Nutrition*, *123*(8), 849–858. https://doi.org/10.1017/S0007114520000057

Sassone-Corsi, Paolo, & Christen, Y. (Eds.). (2016). *A Time for Metabolism and Hormones*. Springer International Publishing. https://doi.org/10.1007/978-3-319-27069-2

Satoh, Y., Kawai, H., Kudo, N., Kawashima, Y., & Mitsumoto, A. (2006). Time-restricted feeding entrains daily rhythms of energy metabolism in mice. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *290*(5), R1276–R1283. https://doi.org/10.1152/ajpregu.00775.2005

Schäbler, S., Amatobi, K. M., Horn, M., Rieger, D., Helfrich-Förster, C., Mueller, M. J., Wegener, C., & Fekete, A. (2020b). Loss of function in the Drosophila clock gene period results in altered intermediary lipid metabolism and increased susceptibility to

starvation. *Cellular and Molecular Life Sciences*, *77*, 4939–4956. https://doi.org/10.1007/s00018-019-03441-6

Schibler, U., Ripperger, J., & Brown, S. A. (2003). Peripheral Circadian Oscillators in Mammals: Time and Food. *Journal of Biological Rhythms*, *18*(3), 250–260. https://doi.org/10.1177/0748730403018003007

Schlichting, M., & Helfrich-Förster, C. (2015). Photic Entrainment in Drosophila Assessed by Locomotor Activity Recordings. In *Methods in Enzymology* (Vol. 552, pp. 105–123). Elsevier. https://doi.org/10.1016/bs.mie.2014.10.017

Seay, D. J., & Thummel, C. S. (2011). The Circadian Clock, Light, and Cryptochrome Regulate Feeding and Metabolism in *Drosophila*. *Journal of Biological Rhythms*, *26*(6), 497–506. https://doi.org/10.1177/0748730411420080

Sehgal, A. (2016). Control of Metabolism by Central and Peripheral Clocks in Drosophila. In P. Sassone-Corsi & Y. Christen (Eds.), *A Time for Metabolism and Hormones* (28892334; pp. 33–40). https://doi.org/10.1007/978-3-319-27069-2_4

Sehgal, A., Price, J. L., Man, B., & Young, M. W. (1994). *Loss of Circadian Behavioral Rhythms and per RNA Oscillations in the Drosophila Mutant timeless*. *263*, 5.

Sehgal, A., Rothenfluh-Hilfiker, A., Hunter-Ensor, M., Chen, Y., Myers, M. P., & Young, M. W. (1995). Rhythmic Expression of timeless: A Basis for Promoting Circadian Cycles in period Gene Autoregulation. *Science*, *270*(5237), 808–810. https://doi.org/10.1126/science.270.5237.808

Shafer, O. T., & Yao, Z. (2014). Pigment-dispersing factor signaling and circadian rhythms in insect locomotor activity. *Current Opinion in Insect Science*, *1*, 73–80. https://doi.org/10.1016/j.cois.2014.05.002

Sherman, H., Genzer, Y., Cohen, R., Chapnik, N., Madar, Z., & Froy, O. (2012). Timed high-fat diet resets circadian metabolism and prevents obesity. *The FASEB Journal*, *26*(8), 3493–3502. https://doi.org/10.1096/fj.12-208868

Shi, M., & Zheng, X. (2013). Interactions between the circadian clock and metabolism: There are good times and bad times. *Acta Biochimica et Biophysica Sinica*, *45*(1), 61–69. https://doi.org/10.1093/abbs/gms110

Shi, S., Hida, A., McGuinness, O. P., Wasserman, D. H., Yamazaki, S., & Johnson, C. H. (2010). Circadian Clock Gene Bmal1 Is Not Essential; Functional Replacement with its Paralog, Bmal2. *Current Biology*, *20*(4), 316–321. https://doi.org/10.1016/j.cub.2009.12.034

Shostak, A., Meyer-Kovac, J., & Oster, H. (2013). Circadian Regulation of Lipid Mobilization in White Adipose Tissues. *Diabetes*, *62*(7), 2195–2203. https://doi.org/10.2337/db12-1449

Sieber, M. H., & Thummel, C. S. (2012). Coordination of Triacylglycerol and Cholesterol Homeostasis by DHR96 and the Drosophila LipA Homolog magro. *Cell Metabolism*, *15*(1), 122–127. https://doi.org/10.1016/j.cmet.2011.11.011

Skorupa, D. A., Dervisefendic, A., Zwiener, J., & Pletcher, S. D. (2008). Dietary composition specifies consumption, obesity, and lifespan in *Drosophila melanogaster*. *Aging Cell*, *7*(4), 478–490. https://doi.org/10.1111/j.1474-9726.2008.00400.x

Soulages, J. L., & Wells, M. A. (1994). Lipophorin: The Structure of an Insect Lipoprotein and Its Role in Lipid Transport in Insects. In C. B. Anfinsen, J. T. Edsall, F. M. Richards, & D. S. Eisenberg (Eds.), *Advances in Protein Chemistry* (Vol. 45, pp. 371–415). Academic Press. https://doi.org/10.1016/S0065-3233(08)60644-0

Stanewsky, R., Kaneko, M., Emery, P., Beretta, B., Wager-Smith, K., Kay, S. A., Rosbash, M., & Hall, J. C. (1998). The cryb Mutation Identifies Cryptochrome as a Circadian

Photoreceptor in Drosophila. *Cell*, *95*(5), 681–692. https://doi.org/10.1016/S0092-8674(00)81638-4

Stephan, F. K. (2002). *The "Other" Circadian System: Food as a Zeitgeber*. 9.

Stoffolano, J. G., & Haselton, A. T. (2013). The Adult Dipteran Crop: A Unique and Overlooked Organ. *Annual Review of Entomology*, *58*(1), 205–225. https://doi.org/10.1146/annurev-ento-120811-153653

Stokkan, K., A., Yamazaki, S, Tei, H, Sakaki, Y, & Menaker, M. (2001). Entrainment of the Circadian Clock in the Liver by Feeding. *Science*, *291*(5503), 490–493. https://doi.org/10.1126/science.291.5503.490

Stoleru, D., Peng, Y., Agosto, J., & Rosbash, M. (2004). Coupled oscillators control morning and evening locomotor behaviour of Drosophila. *Nature*, *431*(7010), 862–868. https://doi.org/10.1038/nature02926

Sumner, L. W., Amberg, A., Barrett, D., Beale, M. H., Beger, R., Daykin, C. A., Fan, T. W.-M., Fiehn, O., Goodacre, R., Griffin, J. L., Hankemeier, T., Hardy, N., Harnly, J., Higashi, R., Kopka, J., Lane, A. N., Lindon, J. C., Marriott, P., Nicholls, A. W., … Viant, M. R. (2007). Proposed minimum reporting standards for chemical analysis: Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). *Metabolomics*, *3*(3), 211–221. https://doi.org/10.1007/s11306-007-0082-2

Sun, Z. S., Albrecht, U., Zhuchenko, O., Bailey, J., Eichele, G., & Lee, C. C. (1997). RIGUI, a Putative Mammalian Ortholog of the Drosophila period Gene. *Cell*, *90*(6), 1003–1011. https://doi.org/10.1016/S0092-8674(00)80366-9

Suri, V., Qian, Z., Hall, J. C., & Rosbash, M. (1998). Evidence that the TIM Light Response Is Relevant to Light-Induced Phase Shifts in Drosophila melanogaster. *Neuron*, *21*(1), 225–234. https://doi.org/10.1016/S0896-6273(00)80529-2

Susumu, O. (1970). Evolution by Gene Duplication. *Allen& Unwin, Springer-Verlag*.

Taghert, P. H., & Shafer, O. T. (2006). Mechanisms of Clock Output in the Drosophila Circadian Pacemaker System. *Journal of Biological Rhythms*, *21*(6), 445–457. https://doi.org/10.1177/0748730406293910

Tatar, M. (2006). Diet Restriction in Drosophila melanogaster. In C. V. Mobbs, K. Yen, & P. R. Hof (Eds.), *Interdisciplinary Topics in Gerontology* (pp. 115–136). KARGER. https://doi.org/10.1159/000096559

Tauber, E., Last, K. S., Olive, P. J. W., & Kyriacou, C. P. (2004). Clock Gene Evolution and Functional Divergence. *Journal of Biological Rhythms*, *19*(5), 445–458. https://doi.org/10.1177/0748730404268775

Tei, H., Okamura, H., Shigeyoshi, Y., Fukuhara, C., Ozawa, R., Hirose, M., & Sakaki, Y. (1997). Circadian oscillation of a mammalian homologue of the Drosophila period gene. *Nature*, *389*(6650), 512–516. https://doi.org/10.1038/39086

Temizhan, A., Tandogan, I., Dönderici, O., & Demirbas, B. (2000). The effects of Ramadan fasting on blood lipid levels. *The American Journal of Medicine*, *109*(4), 341–342. https://doi.org/10.1016/s0002-9343(00)00498-8

Triscari, J., Nauss-Karol, C., Levin, B. E., & Sullivan, A. C. (1985). Changes in lipid metabolism in diet-induced obesity. *Metabolism*, *34*(6), 580–587. https://doi.org/10.1016/0026-0495(85)90198-2

Turek, F. W. (2005). Obesity and Metabolic Syndrome in Circadian Clock Mutant Mice. *Science*, *308*(5724), 1043–1045. https://doi.org/10.1126/science.1108750

Tzou, P., Ohresser, S., Ferrandon, D., Capovilla, M., Reichhart, J.-M., Lemaitre, B., Hoffmann, J. A., & Imler, J.-L. (2000). Tissue-Specific Inducible Expression of Antimicrobial Peptide Genes in Drosophila Surface Epithelia. *Immunity*, *13*(5), 737–748. https://doi.org/10.1016/S1074-7613(00)00072-8

Van der Horst, D. J. (1990). Lipid transport function of lipoproteins in flying insects. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism*, *1047*(3), 195–211. https://doi.org/10.1016/0005-2760(90)90518-3

Villanueva, J. E., Livelo, C., Trujillo, A. S., Chandran, S., Woodworth, B., Andrade, L., Le, H. D., Manor, U., Panda, S., & Melkani, G. C. (2019). Time-restricted feeding restores muscle function in Drosophila models of obesity and circadian-rhythm disruption. *Nature Communications*, *10*(1). https://doi.org/10.1038/s41467-019-10563-9

Vujović, N., Davidson, A. J., & Menaker, M. (2008). Sympathetic input modulates, but does not determine, phase of peripheral circadian oscillators. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *295*(1), R355–R360. https://doi.org/10.1152/ajpregu.00498.2007

Wahle, K. W. J., & Radcliffe, J. D. (1977). Effect of a diet rich in sunflower oil on aspects of lipid metabolism in the genetically-obese rat. *Lipids*, *12*(2), 135–139. https://doi.org/10.1007/BF02533282

Waldman, H. S., Renteria, L. I., & McAllister, M. J. (2020). Time-restricted feeding for the prevention of cardiometabolic diseases in high-stress occupations: A mechanistic review. *Nutrition Reviews*, *78*(6), 459–464. https://doi.org/10.1093/nutrit/nuz090

Welsh, D. K., Takahashi, J. S., & Kay, S. A. (2010). Suprachiasmatic Nucleus: Cell Autonomy and Network Properties. *Annual Review of Physiology*, *72*(1), 551–577. https://doi.org/10.1146/annurev-physiol-021909-135919

Wenk, M. R. (2010). Lipidomics: New Tools and Applications. *Cell*, *143*(6), 888–895. https://doi.org/10.1016/j.cell.2010.11.033

Williams, J. A., & Sehgal, A. (2001). Molecular Components of the Circadian System in Drosophila. *Annual Review of Physiology*, *63*(1), 729–755. https://doi.org/10.1146/annurev.physiol.63.1.729

Wong, R., Piper, M. D. W., Wertheim, B., & Partridge, L. (2009). Quantification of Food Intake in Drosophila. *PLoS ONE*, *4*(6), e6063. https://doi.org/10.1371/journal.pone.0006063

Xu, K., DiAngelo, J. R., Hughes, M. E., Hogenesch, J. B., & Sehgal, A. (2011). The Circadian Clock Interacts with Metabolic Physiology to Influence Reproductive Fitness. *Cell Metabolism*, *13*(6), 639–654. https://doi.org/10.1016/j.cmet.2011.05.001

Xu, K., Zheng, X., & Sehgal, A. (2008). Regulation of Feeding and Metabolism by Neuronal and Peripheral Clocks in Drosophila. *Cell Metabolism*, *8*(4), 289–300. https://doi.org/10.1016/j.cmet.2008.09.006

Yagita, K. (2001). Molecular Mechanisms of the Biological Clock in Cultured Fibroblasts. *Science*, *292*(5515), 278–281. https://doi.org/10.1126/science.1059542

Yamazaki, S. (2000a). Resetting Central and Peripheral Circadian Oscillators in Transgenic Rats. *Science*, *288*(5466), 682–685. https://doi.org/10.1126/science.288.5466.682

Yamazaki, S. (2000b). Resetting Central and Peripheral Circadian Oscillators in Transgenic Rats. *Science*, *288*(5466), 682–685. https://doi.org/10.1126/science.288.5466.682

Yang, S., Liu, A., Weidenhammer, A., Cooksey, R. C., McClain, D., Kim, M. K., Aguilera, G., Abel, E. D., & Chung, J. H. (2009). The Role of *mPer2* Clock Gene in Glucocorticoid and Feeding Rhythms. *Endocrinology*, *150*(5), 2153–2160. https://doi.org/10.1210/en.2008-0705

Yongmei Xi, Y. Z. (2015). Fat Body Development and its Function in Energy Storage and Nutrient Sensing in Drosophila melanogaster. *Journal of Tissue Science & Engineering*, *06*(01). https://doi.org/10.4172/2157-7552.1000141

Yoo, S.-H., Yamazaki, S., Lowrey, P. L., Shimomura, K., Ko, C. H., Buhr, E. D., Siepka, S. M., Hong, H.-K., Oh, W. J., Yoo, O. J., Menaker, M., & Takahashi, J. S. (2004a). PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent

circadian oscillations in mouse peripheral tissues. *Proceedings of the National Academy of Sciences*, *101*(15), 5339–5346. https://doi.org/10.1073/pnas.0308709101

Yoo, S.-H., Yamazaki, S., Lowrey, P. L., Shimomura, K., Ko, C. H., Buhr, E. D., Siepka, S. M., Hong, H.-K., Oh, W. J., Yoo, O. J., Menaker, M., & Takahashi, J. S. (2004b). PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. *Proceedings of the National Academy of Sciences*, *101*(15), 5339–5346. https://doi.org/10.1073/pnas.0308709101

Yoshii, T., Vanin, S., Costa, R., & Helfrich-Förster, C. (2009). Synergic Entrainment of *Drosophila's* Circadian Clock by Light and Temperature. *Journal of Biological Rhythms*, *24*(6), 452–464. https://doi.org/10.1177/0748730409348551

Young, M. W., & Kay, S. A. (2001). Time zones: A comparative genetics of circadian clocks. *Nature Reviews Genetics*, *2*(9), 702–715. https://doi.org/10.1038/35088576

Yu, W. (2006). PER-dependent rhythms in CLK phosphorylation and E-box binding regulate circadian transcription. *Genes & Development*, *20*(6), 723–733. https://doi.org/10.1101/gad.1404406

Yu, W., & Hardin, P. E. (2006). Circadian oscillators of Drosophila and mammals. *Journal of Cell Science*, *119*(23), 4793–4795. https://doi.org/10.1242/jcs.03174

Zehring, W. A., Wheeler, D. A., Reddy, P., Konopka, R. J., Kyriacou, C. P., Rosbash, M., & Hall, J. C. (1984). P-element transformation with period locus DNA restores rhythmicity to mutant, arrhythmic drosophila melanogaster. *Cell*, *39*(2), 369–376. https://doi.org/10.1016/0092-8674(84)90015-1

Zeng, H., Qian, Z., Myers, M. P., & Rosbash, M. (1996). A light-entrainment mechanism for the Drosophila circadian clock. *Nature*, *380*(6570), 129–135. https://doi.org/10.1038/380129a0

Zhang, J. (2003). Evolution by gene duplication: An update. *Trends in Ecology & Evolution*, *18*(6), 292–298. https://doi.org/10.1016/S0169-5347(03)00033-8

Zheng, X., Koh, K., Sowcik, M., Smith, C. J., Chen, D., Wu, M. N., & Sehgal, A. (2009). An Isoform-Specific Mutant Reveals a Role of PDP1 in the Circadian Oscillator. *Journal of Neuroscience*, *29*(35), 10920–10927. https://doi.org/10.1523/JNEUROSCI.2133-09.2009

Zizi, F., Jean-Louis, G., Brown, C. D., Ogedegbe, G., Boutin-Foster, C., & McFarlane, S. I. (2010). Sleep Duration and the Risk of Diabetes Mellitus: Epidemiologic Evidence and Pathophysiologic Insights. *Current Diabetes Reports*, *10*(1), 43–47. https://doi.org/10.1007/s11892-009-0082-x

7 SUPPLEMENTARY RESULTS

Supplementary table 6. Shows the mass-to-Charge (m/z) and retention time (RT) of different DG species.

Substance	mass-to-charge ratio	retention time [min]
$[DG(20:0)-H20+H^+]^+$ Internal standard	383.3155	5.17
$[DG(26:0)-H20+H^+]^+$	467.4051	7.02
$[DG(26:1)-H20+H^+]^+$	465.4166	6.59
$[DG(28:0)-H20+H^+]^+$	495.4415	7.52
$[DG(28:1)-H20+H^+]^+$	493.4213	7.12
$[DG(30:1)-H20+H^+]^+$	521.4573	7.55
$[DG(32:1)-H20+H^+]^+$	549.4893	7.26
$[DG(32:2)-H20+H^+]^+$	547.4725	7.65

Supplementary table 7. Shows the mass-to-Charge (m/z) and retention time (RT) of different PE species.

Substance	mass-to-charge ratio	retention time [min]
$[PE(34:0)+H^+]^+$ Internal standard	720.553	8.02
$[PE(30:1)+H^+]^+$	662.4763	6.70
$[PE(32:1)+H^+]^+$	690.5074	7.23
$[PE(32:2)+H^+]^+$	688.4919	6.87
$[PE(34:1)+H^+]^+$	718.5376	7.64
$[PE(34:2)+H^+]^+$	716.5241	7.30
$[PE(34:3)+H^+]^+$	714.5083	6.91
$[PE(36:2)+H^+]^+$	744.5531	7.66
$[PE(36:3)+H^+]^+$	742.5391	7.36

Supplementary table 8. Shows the mass-to-Charge (m/z) and retention time (RT) of different PC species.

Substance	mass-to-charge ratio	retention time [min]
$[PC(34:0)+H^+]^+$ Internal standard	762.6004	7.94
$[PC(32:1)+H^+]^+$	732.5575	7.19
$[PC(32:2)+H^+]^+$	730.5402	6.70
$[PC(34:1)+H^+]^+$	760.5851	7.60
$[PC(34:2)+H^+]^+$	758.5666	7.26
$[PC(34:3)+H^+]^+$	756.5567	6.89

Supplementary table 9. JTK feeding result of wild-type and mutant flies under different light/dark conditions and diet type (green = oscillating, red = Not oscillating)

Experiment	Fly strain	Type of Food	Feeding method	Light condition	BH.Q	ADJ.P	PER	LAG	Relative Amplitude (%)
Replicate 1	WTcsLD	Standard	ad libitum	LD	4.2E-18	1.0E-18	24	1.5	9.0
Replicate 2	WTcsLD	Standard	ad libitum	LD	1.2E-22	1.5E-23	24	2.5	15.9
Replicate 1	WTcsDD	Standard	ad libitum	DD	2.1E-04	1.1E-04	24	22.5	6.7
Replicate 2	WTcsDD	Standard	ad libitum	DD	5.1E-15	6.4E-16	24	1.5	15.9
Replicate 1	per01LD	Standard	ad libitum	LD	6.7E-06	3.4E-06	24	20	6.8
Replicate 2	per01LD	Standard	ad libitum	LD	6.7E-06	4.2E-06	24	14.5	7.6
Replicate 1	per01DD	Standard	ad libitum	DD	5.2E-05	2.0E-05	24	20.5	11.1
Replicate 2	per01DD	Standard	ad libitum	DD	1.0	1.0	24	6	0.0
Replicate 1	WTcsLD	Lipid-free	ad libitum	LD	2.8E-03	2.1E-03	24	6.5	8.3
Replicate 2	WTcsLD	Lipid-free	ad libitum	LD	5.7E-02	5.0E-02	24	19	0.0
Replicate 1	WTcsDD	Lipid-free	ad libitum	DD	3.2E-06	8.1E-07	24	5	8.3
Replicate 2	WTcsDD	Lipid-free	ad libitum	DD	0.2	0.1	24	4.5	0.0

Replicate 1	per01LD	Lipid-free	ad libitum	LD	1.0	1.0	24	18	0.0
Replicate 2	per01LD	Lipid-free	ad libitum	LD	1.1E-06	4.2E-07	24	14	7.6
Replicate 1	per01DD	Lipid-free	ad libitum	DD	0.4	0.3	24	7.5	2.8
Replicate 2	per01DD	Lipid-free	ad libitum	DD	1.0	1.0	24	7	0.0
Experiment	**Fly strain**	**Type of Food**	**Feeding method**	**Light condition**	**BH.Q**	**ADJ.P**	**PER**	**LAG**	**Relative Amplitude (%)**
Normalized experiment	WTcsLD	Standard	ad libitum	LD	2.5E-17	6.4E-18	24	2	26.2
Normalized experiment	WTcsDD	Standard	ad libitum	DD	4.6E-06	1.2E-06	24	1	13.6
Normalized experiment	per01LD	Standard	ad libitum	LD	5.2E-03	3.9E-03	24	18	12.8
Normalized experiment	per01DD	Standard	ad libitum	DD	0.52	0.39	24	21	10.2
Normalized experiment	WTcsLD	Lipid-free	ad libitum	LD	2.7E-03	1.4E-03	24	5	12.6
Normalized experiment	WTcsDD	Lipid-free	ad libitum	DD	3.4E-05	1.7E-05	24	5	11.1
Normalized experiment	per01LD	Lipid-free	ad libitum	LD	1.0	1.0	24	18	5.6
Normalized experiment	per01DD	Lipid-free	ad libitum	DD	1.0	1.0	24	6	8.0

Supplementary table 10. JTK time restricted feeding result of wild-type flies fed during light phase or dark phase under LD condition and DD condition (Green = oscillating, Red = Not oscillating)

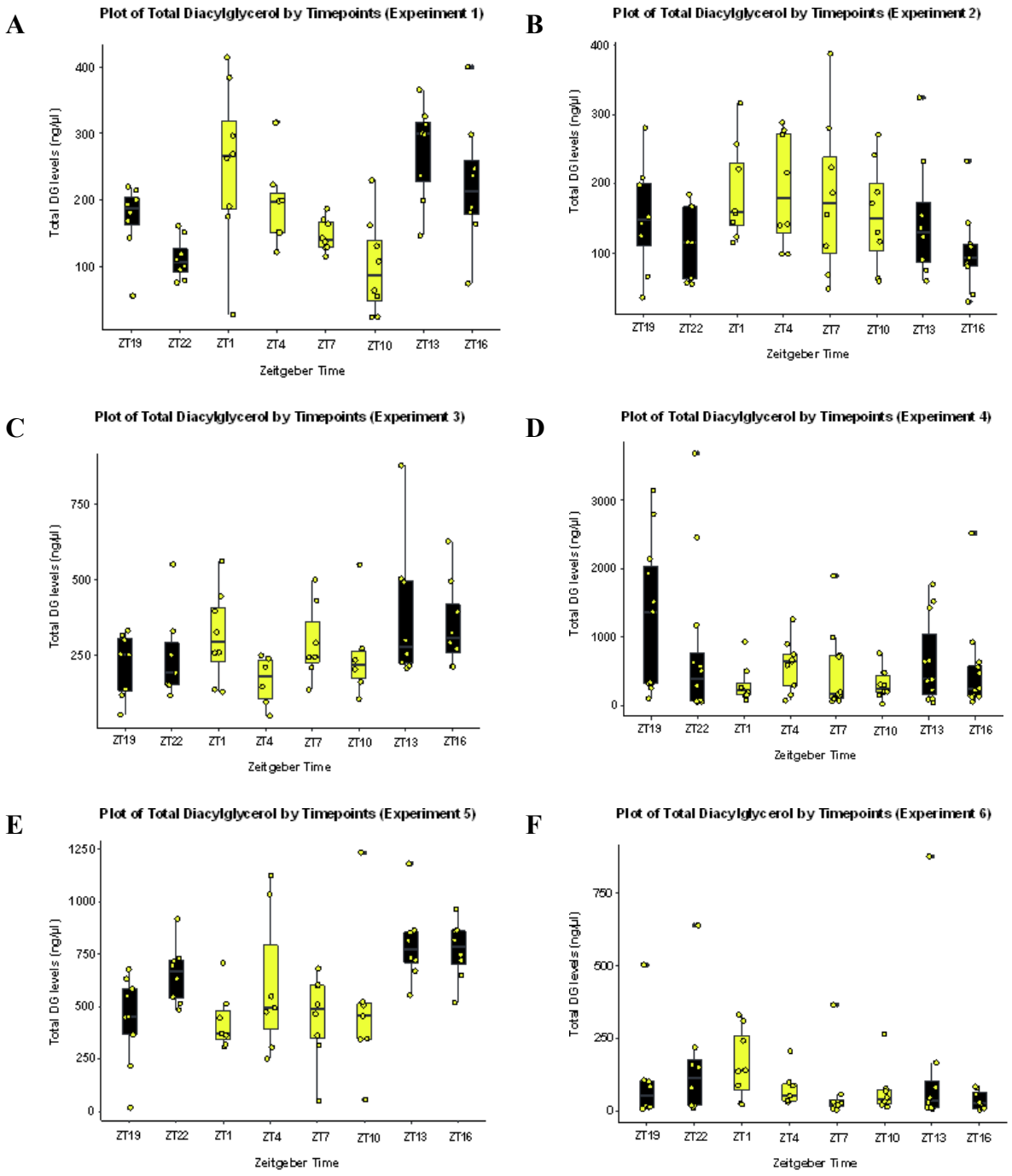
Experiment	**Fly strain**	**Type of Food**	**Feeding method**	**Light condition**	**BH.Q**	**ADJ.P**	**PER**	**LAG**	**Relative Amplitude (%)**
Time restricted	WTcsLD	Lipid-free	Day time restricted (DT-RF)	LD	0.3	0.1	12	9	20.8
Time restricted	WTcsLD	Lipid-free	Night time restricted (NT-RF)	LD	1.0	1.0	11	8	54.5
Time restricted	WTcsLD	Lipid-free	Subjective day-time restricted (SDT-RF)	DD	0.3	0.2	10	8	99.9
Time restricted	WTcsLD	Lipid-free	Subjective night-time restrcited (SNT-RF)	DD	1.0	1.0	6	5.5	0.1

Supplementary table 11. JTK *ad libitum* feeding result of wild-type flies after light phase feeding and dark phase feeding (Green = oscillating, Red = Not oscillating)

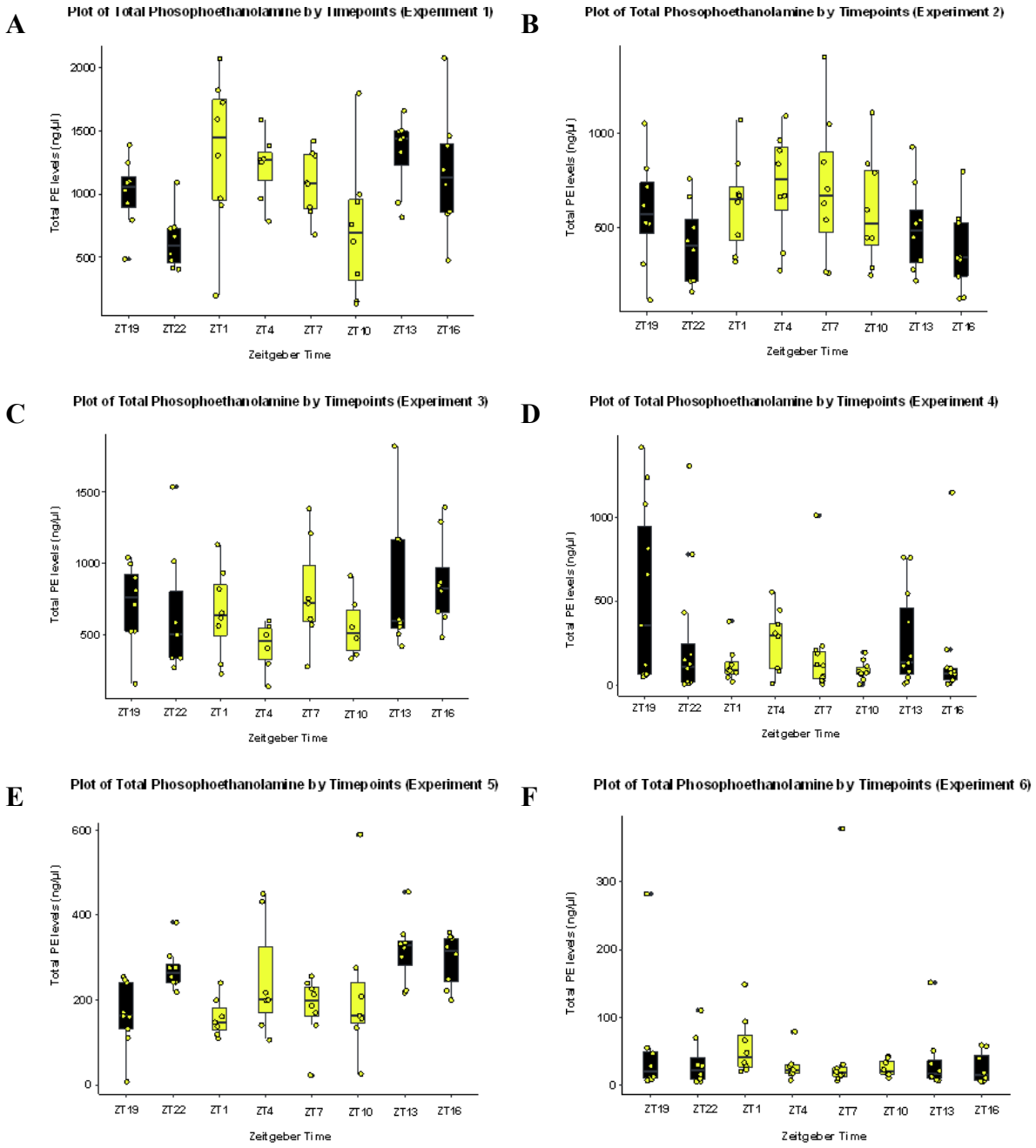
Experiment	Fly strain	Food	Feeding method	Light condition	BH.Q	ADJ.P	PER	LAG	Relative amplitude (%)
1st day after light time restriction feeding	WTcsLD	Lipid-free	ad libitum	LD	1.00	1.00	24	1.0	43.3
2nd day after light time restriction feeding	WTcsLD	Lipid-free	ad libitum	LD	0.05	0.02	24	0.5	56.7
1st day after dark time restriction feeding	WTcsLD	Lipid-free	ad libitum	LD	0.11	0.08	24	21.0	43.3
2nd day after dark time restriction feeding	WTcsLD	Lipid-free	ad libitum	LD	0.01	0.003	24	21.0	56.7

Supplementary table 12. Hemolymph lipid oscillation profile according to JTK cycle result of wild-type flies fed during light phase or dark phase under LD condition (Green = oscillating, Red = Not oscillating)

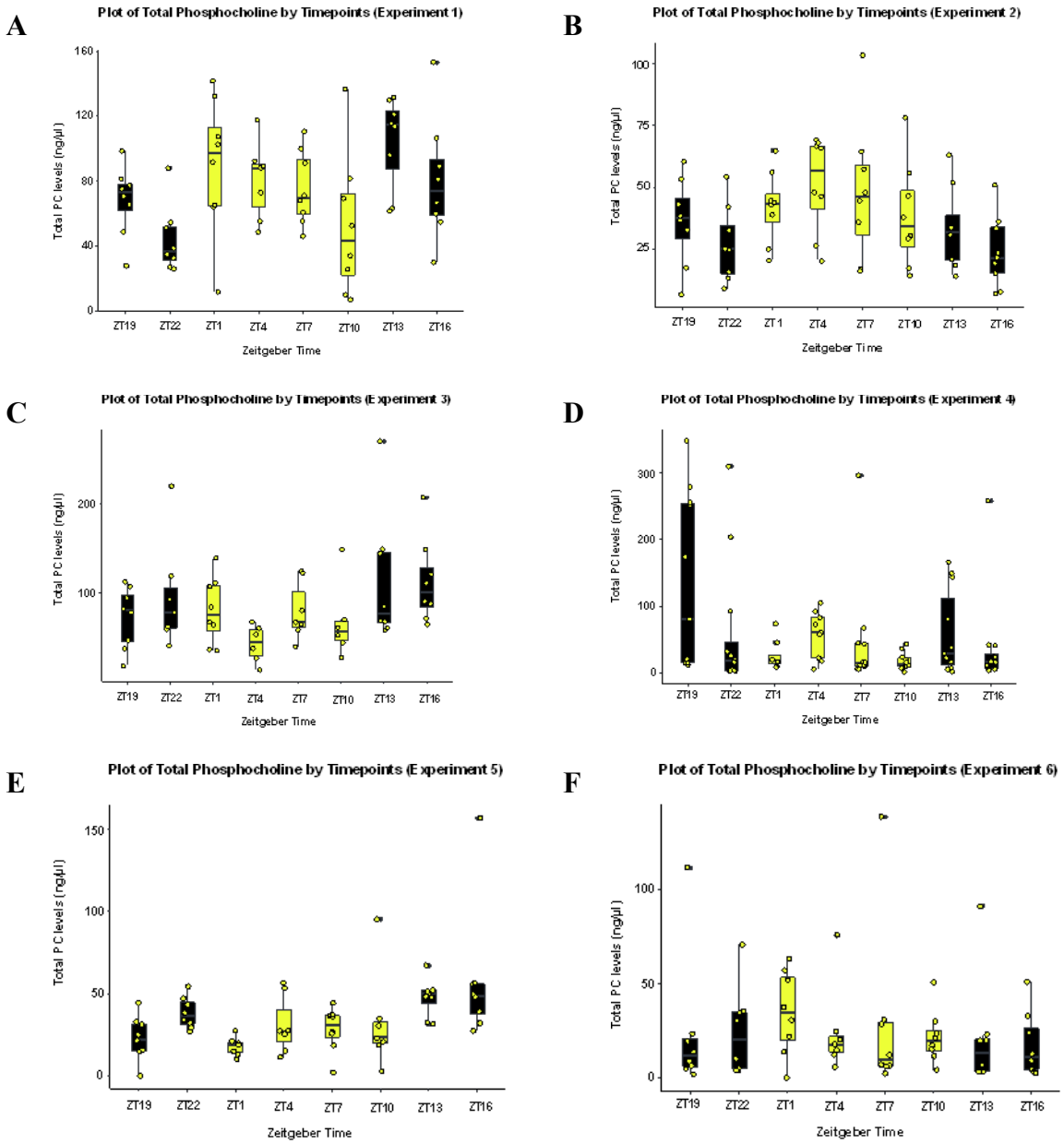
Metabolite	Fly strain	Food	Feeding method	Light condition	BH.Q	ADJ.P	PER	LAG	Relative amplitude (%)
DG	WTcsLD	Lipid-free	Light time restricted	LD	0.89	0.8912	21	4.5	12.2
DG	WTcsLD	Lipid-free	Dark time restricted	LD	0.05	0.0347	24	10.5	87.8
PE	WTcsLD	Lipid-free	Light time restricted	LD	0.03	0.0152	15	6.0	15.9
PE	WTcsLD	Lipid-free	Dark time restricted	LD	0.00	0.0004	12	6.0	84.1
PC	WTcsLD	Lipid-free	Light time restricted	LD	0.17	0.1416	18	4.5	20.2
PC	WTcsLD	Lipid-free	Dark time restricted	LD	0.00	0.0005	12	6.0	79.8



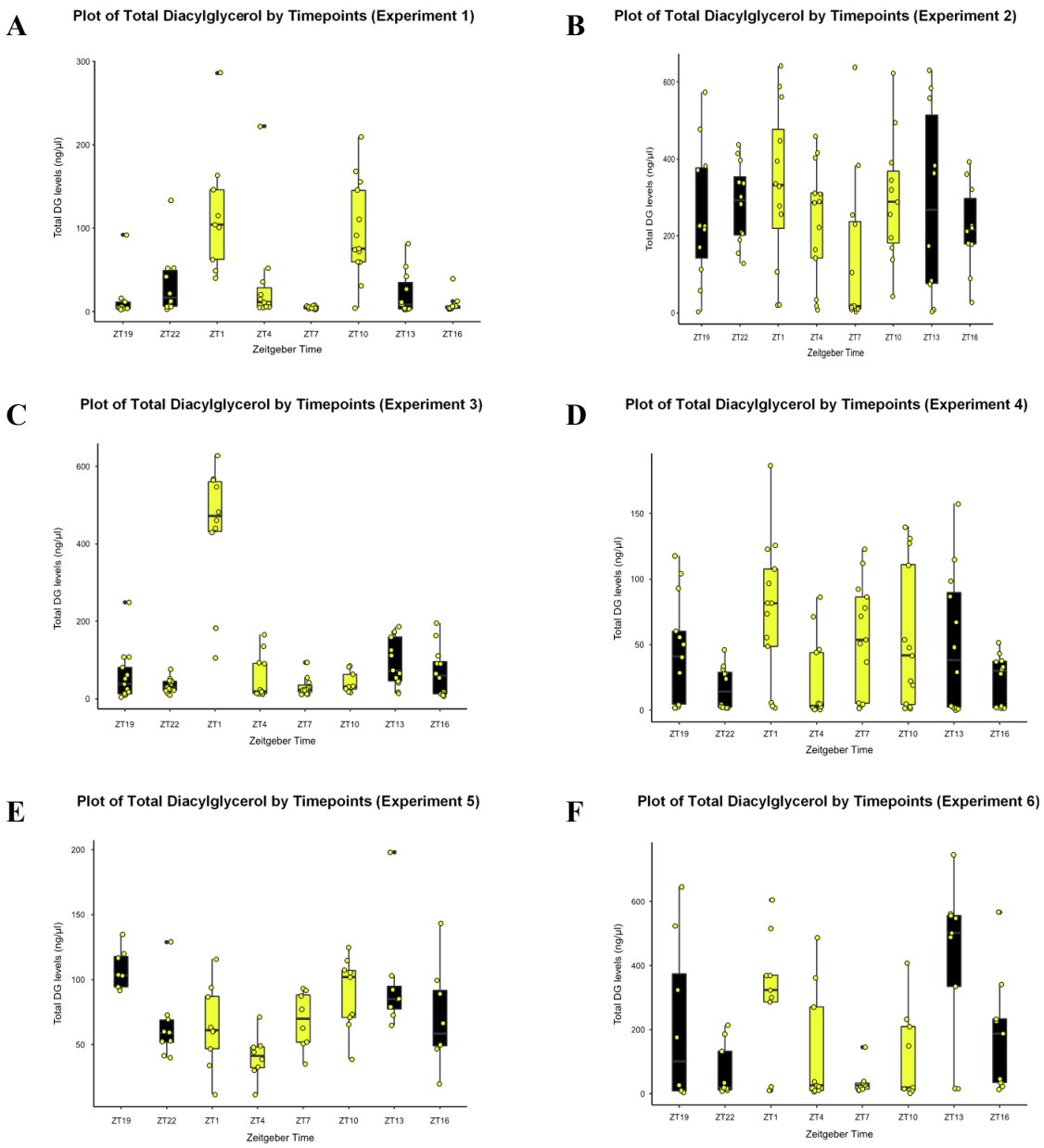
Supplementary Figure 36. Six (6) independent experiments (A, B, C, D, E, F) of hemolymph DG oscillation profile of *Drosophila* male flies fed on standard medium under 12hr/12hr light/dark cycle at 8 different timepoints (n= 10 - 13). In the box plot, the lightening condition is designated by the colours of the bars (yellow = light-on and black = light-off).



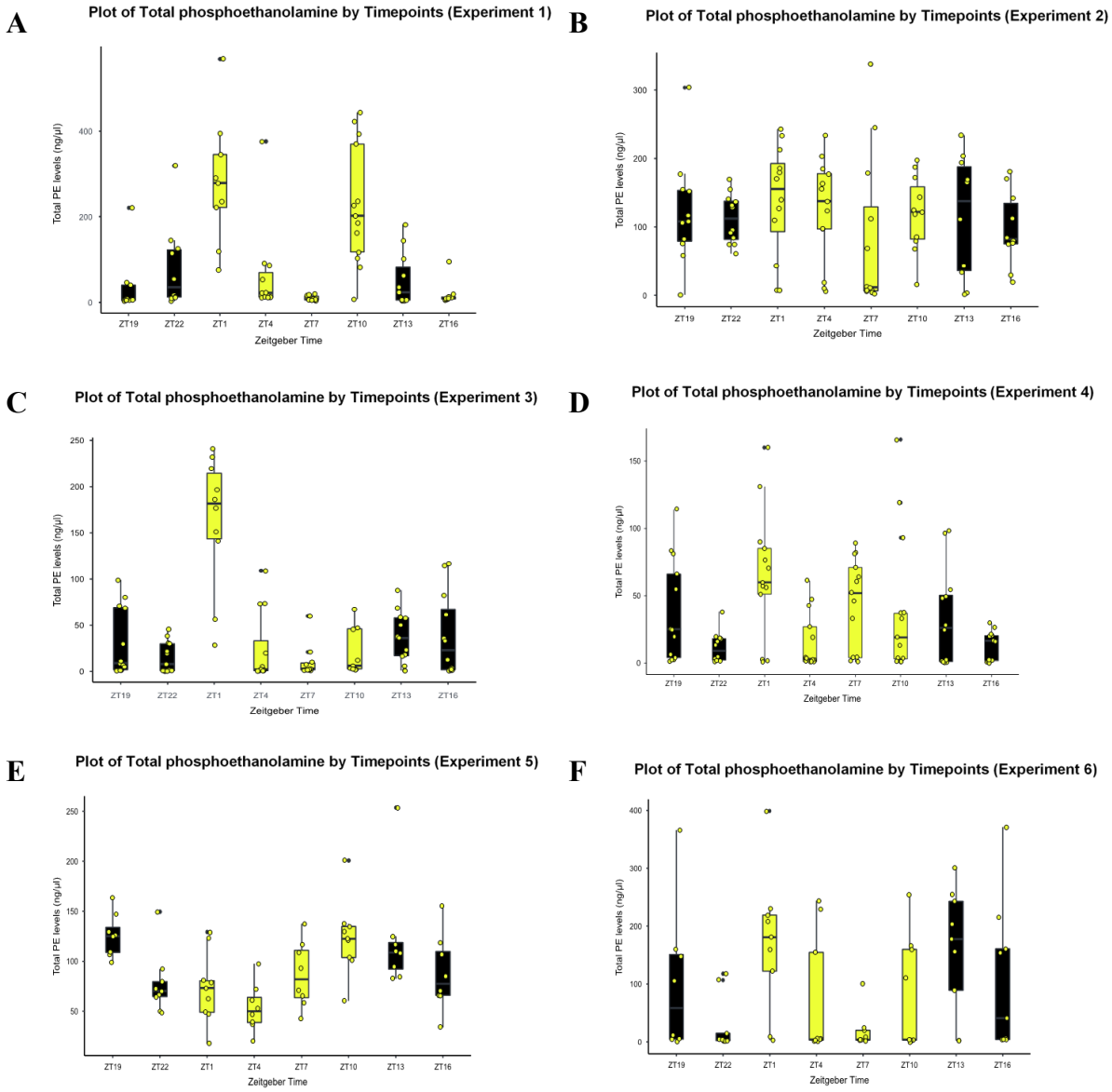
Supplementary Figure 37. Six (6) independent experiments (A, B, C, D, E, F) of hemolymph PE oscillation profile of Drosophila male flies fed on standard medium under 12hr/12hr light/dark cycle at 8 different timepoints (n= 10 - 13). In the box plot, the lightening condition is designated by the colours of the bars (yellow = light-on and black = light-off).



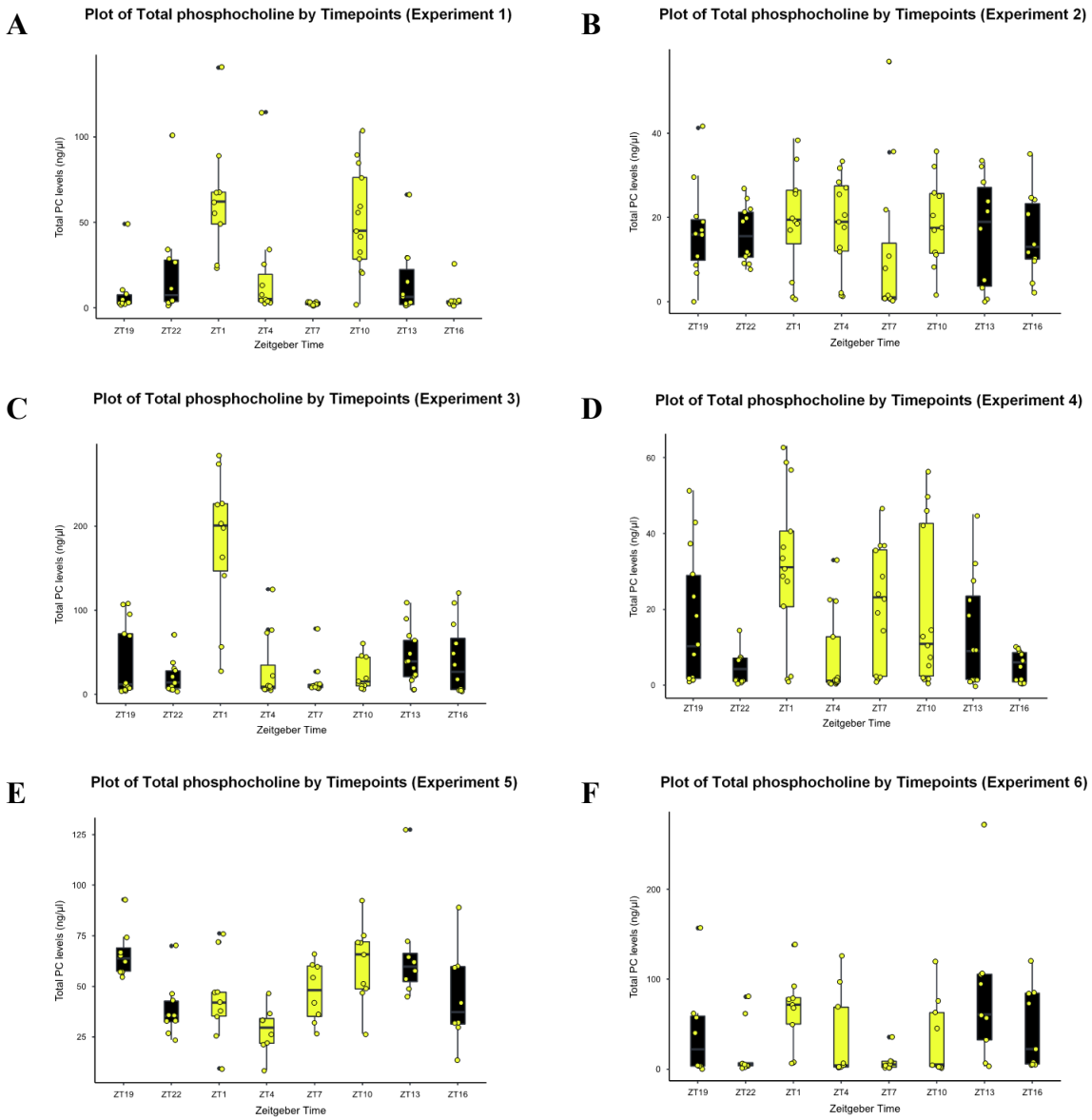
Supplementary Figure 38. Six (6) independent experiments (A, B, C, D, E, F) of hemolymph PC oscillation profile of Drosophila male flies fed on standard medium under 12hr/12hr light/dark cycle at 8 different timepoints (n= 10 - 13). In the box plot, the lightening condition is designated by the colours of the bars (yellow = light-on and black = light-off).



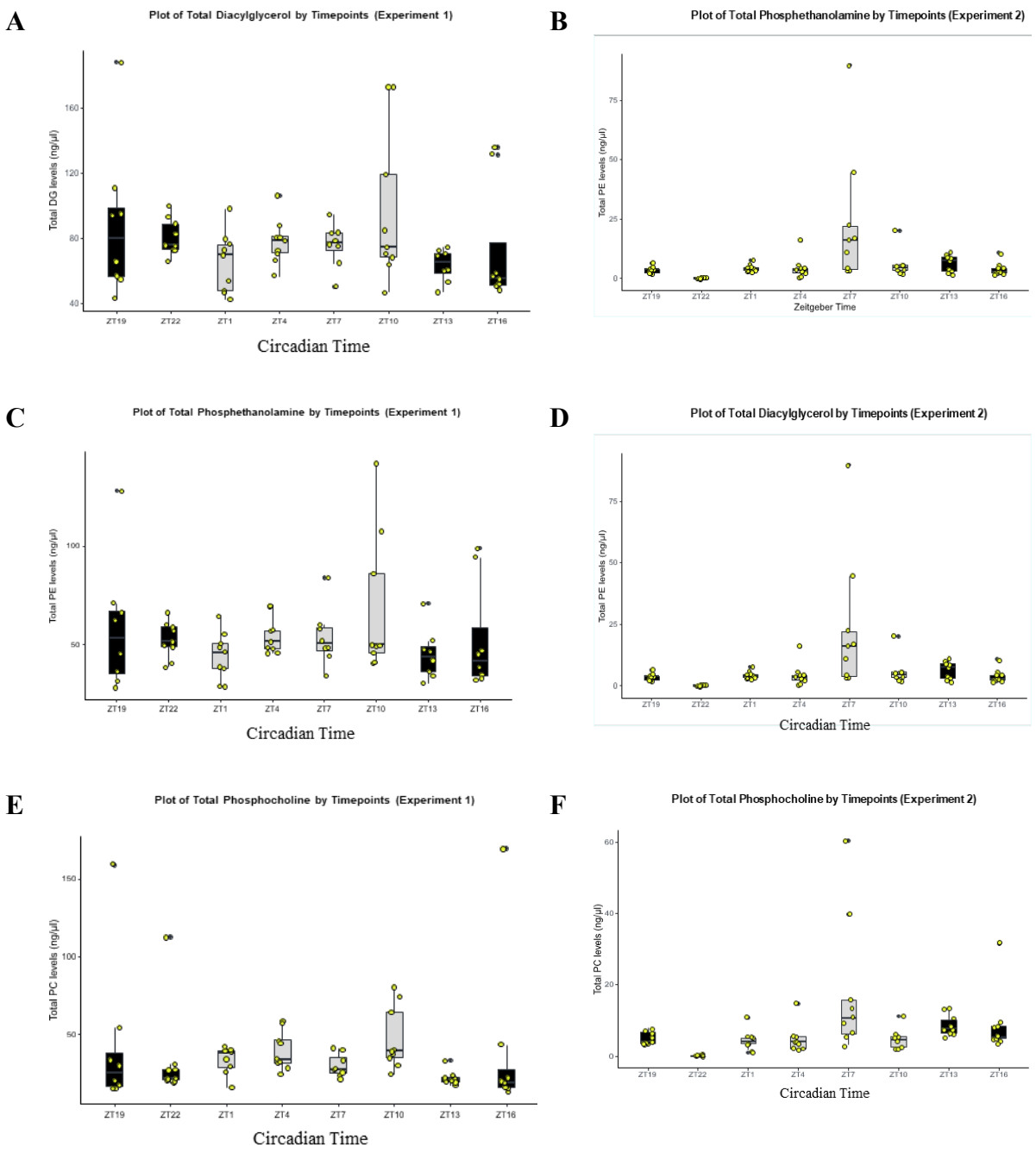
Supplementary Figure 39. Six (6) independent experiments (A, B, C, D, E, F) of hemolymph DG oscillation profile of *Drosophila* male flies fed on lipid free medium under 12hr/12hr light/dark cycle at 8 different timepoints (n= 10 - 13). In the box plot, the lightening condition is designated by the colours of the bars (yellow = light-on and black = light-off).



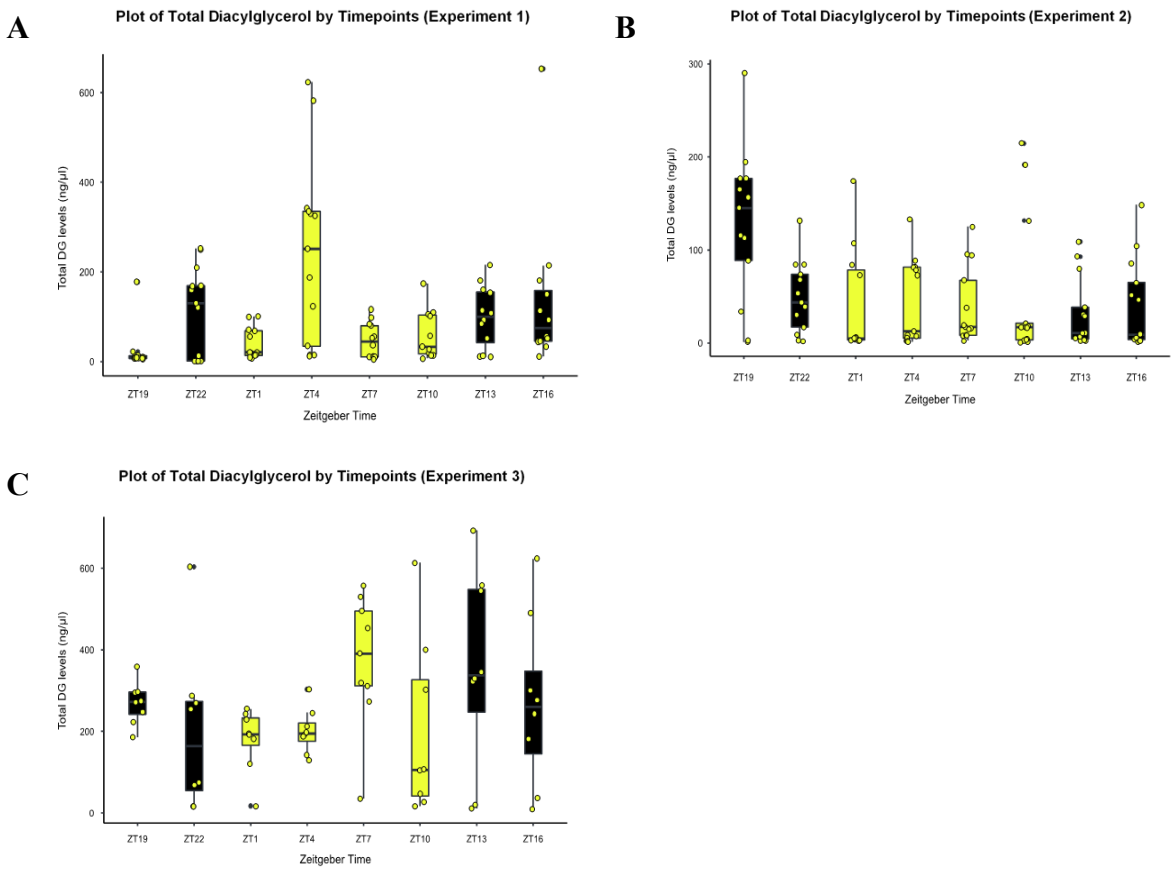
Supplementary Figure 40. Six (6) independent experiments (A, B, C, D, E, F) of hemolymph PE oscillation profile of Drosophila male flies fed on sugar medium under 12hr/12hr light/dark cycle at 8 different timepoints (n= 10 - 13). In the box plot, the lightening condition is designated by the colours of the bars (yellow = light-on and black = light-off).



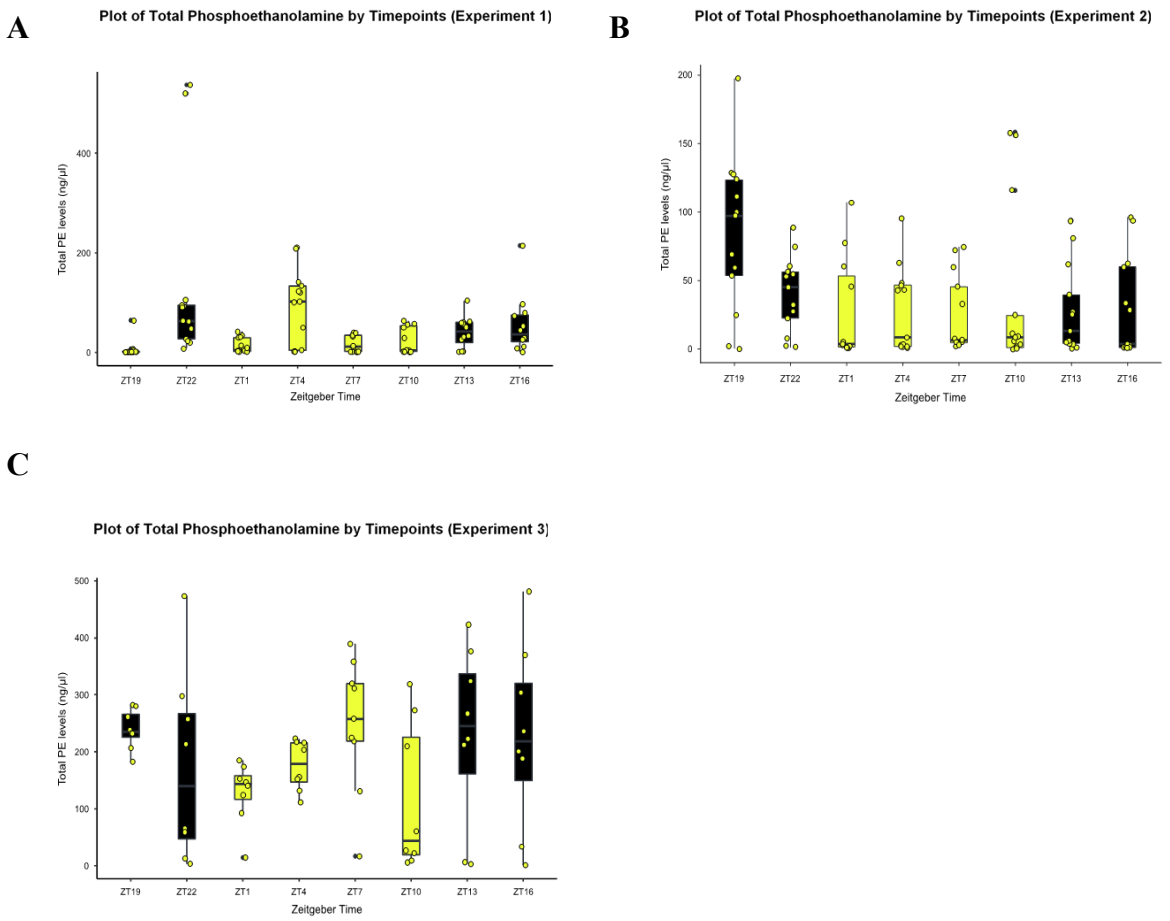
Supplementary Figure 41. Six (6) independent experiments (A, B, C, D, E, F) of hemolymph PC oscillation profile of Drosophila male flies fed on sugar medium under 12hr/12hr light/dark cycle at 8 different timepoints (n= 10 - 13). In the box plot, the lightening condition is designated by the colours of the bars (yellow = light-on and black = light-off).



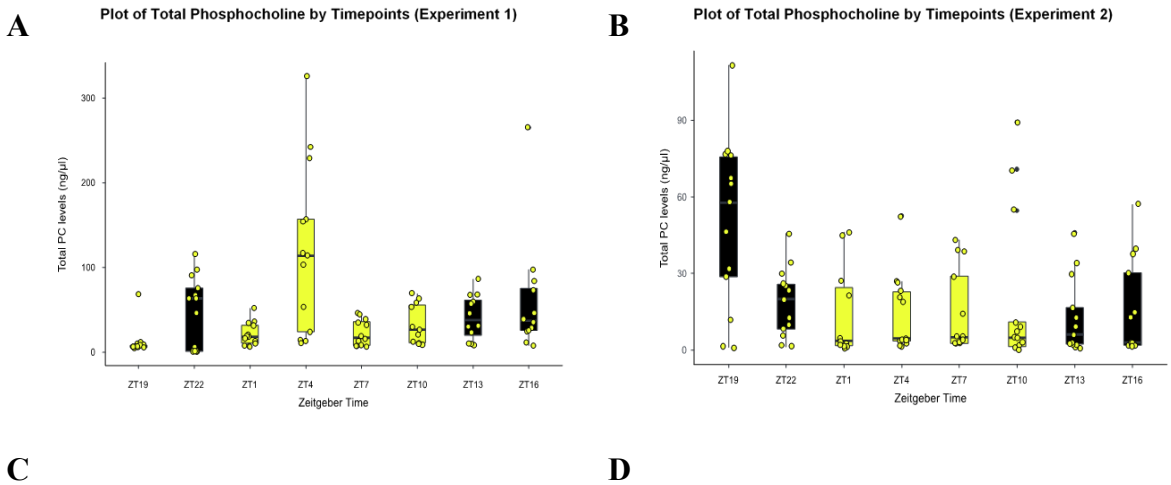
Supplementary Figure 42. Two (2) independent experiments (A, B, C, D, E, F) of hemolymph DG, PE and PC oscillation profile of Wild-Type *Drosophila* male flies fed on sugar medium under 12hr/12hr light/dark cycle at 8 different timepoints (n= 10 - 13). In the box plot, the lightening condition is designated by the colours of the bars (light grey = light-on and black = light-off).

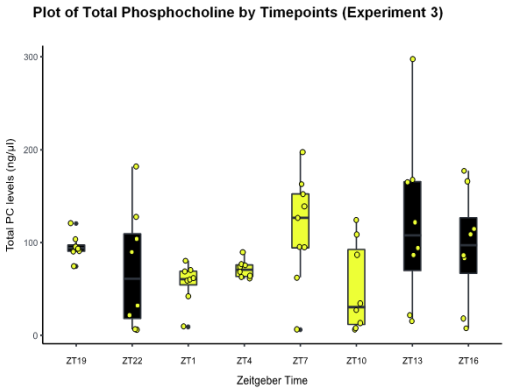


Supplementary Figure 43. Three (3) independent experiments (A, B, C) of hemolymph DG oscillation profile of mutant (*per*^{01}) *Drosophila* male flies fed on sugar medium under 12hr/12hr light/dark cycle at 8 different timepoints (n= 10 - 13). In the box plot, the lightening condition is designated by the colours of the bars (yellow = light-on and black = light-off).

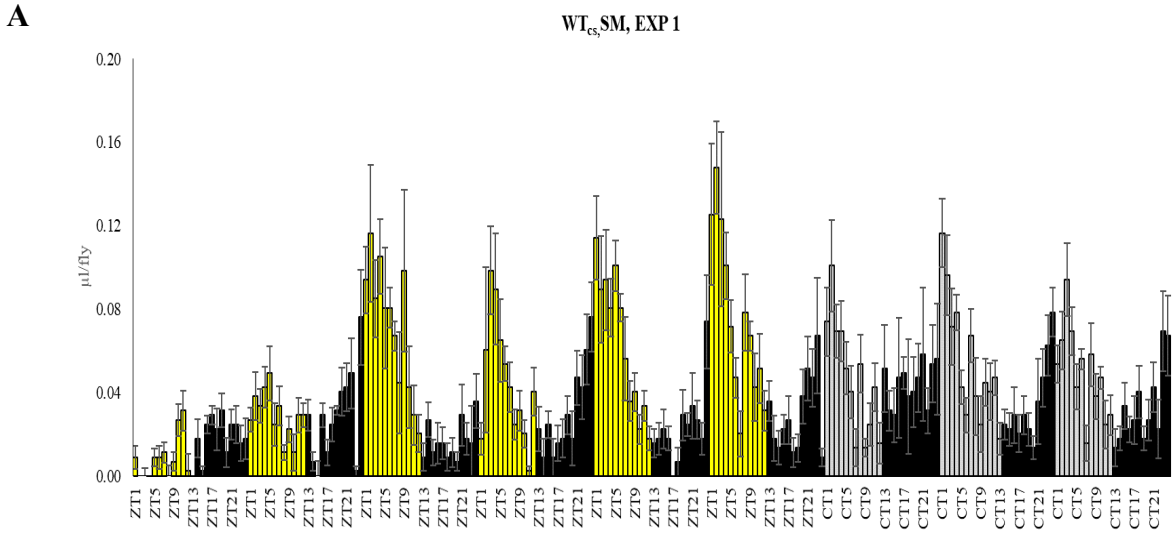


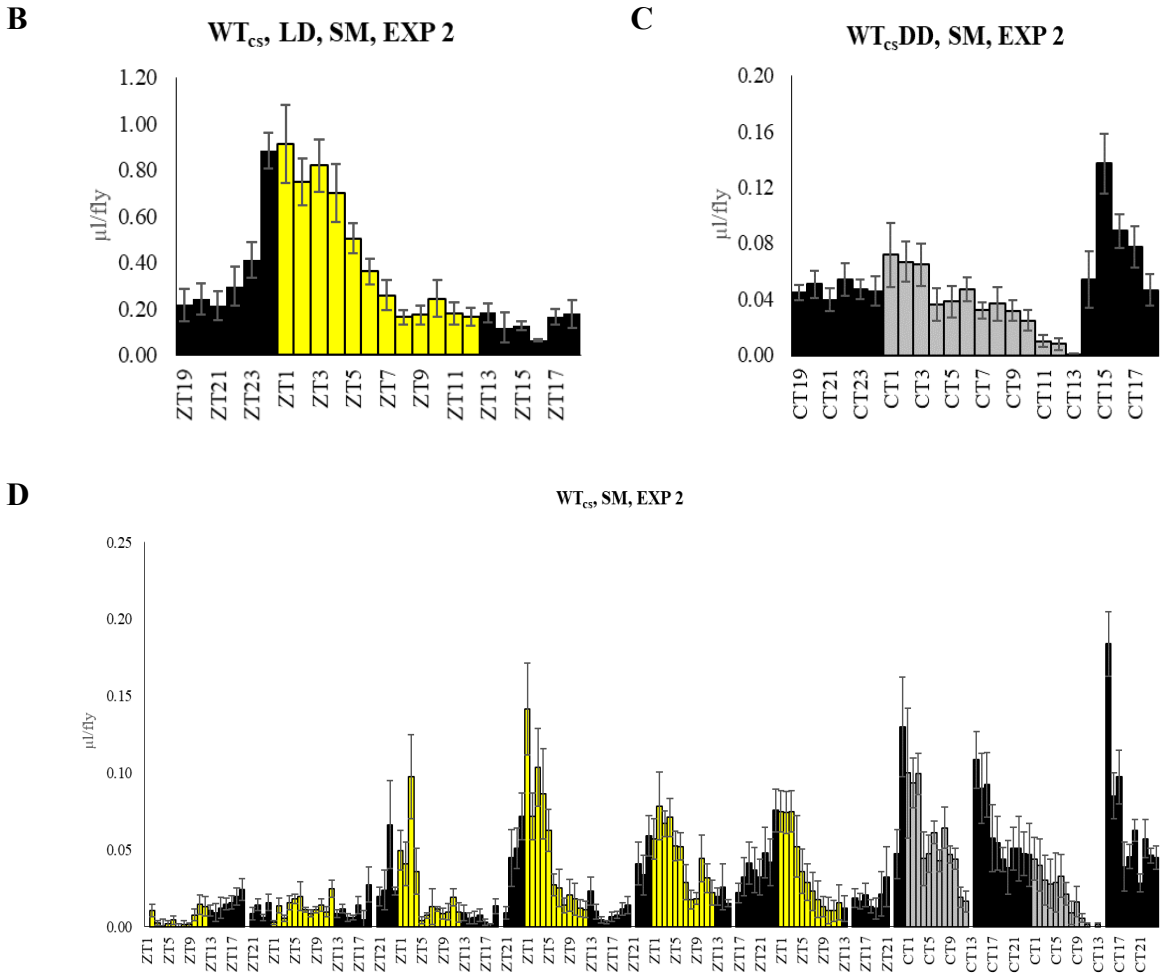
Supplementary Figure 44. Three (3) independent experiments (A, B, C) of hemolymph PE oscillation profile of mutant (*per*^{01}) *Drosophila* male flies fed on sugar medium under 12hr/12hr light/dark cycle at 8 different timepoints (n= 10 - 13). In the box plot, the lightening condition is designated by the colours of the bars (yellow = light-on and black = light-off).



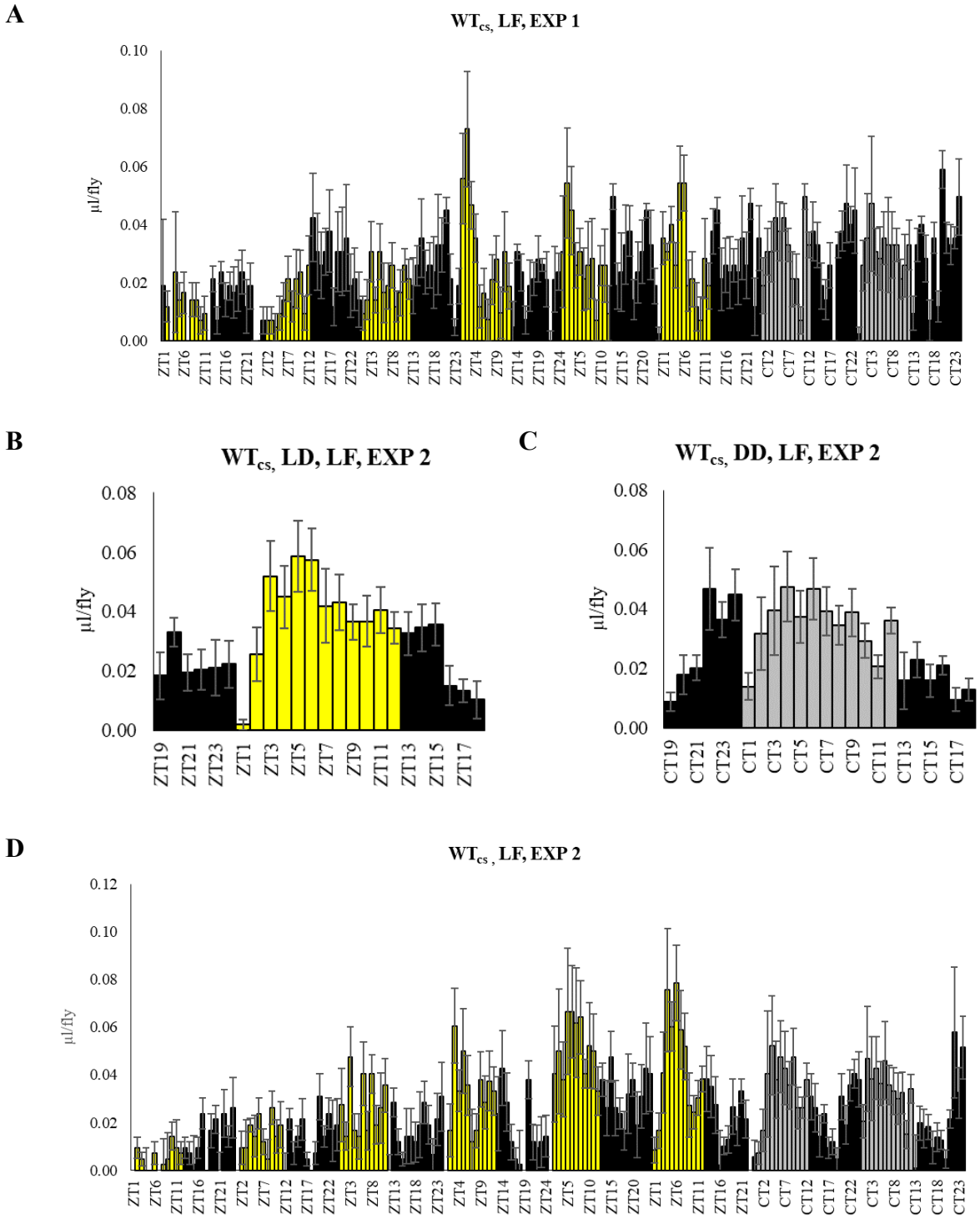


Supplementary Figure 45. Three (3) independent experiments (A, B, C) of hemolymph PC oscillation profile of mutant (*per*^{01}) *Drosophila* male flies fed on sugar medium under 12hr/12hr light/dark cycle at 8 different timepoints (n= 10 - 13). In the box plot, the lightening condition is designated by the colours of the bars (yellow = light-on and black = light-off).



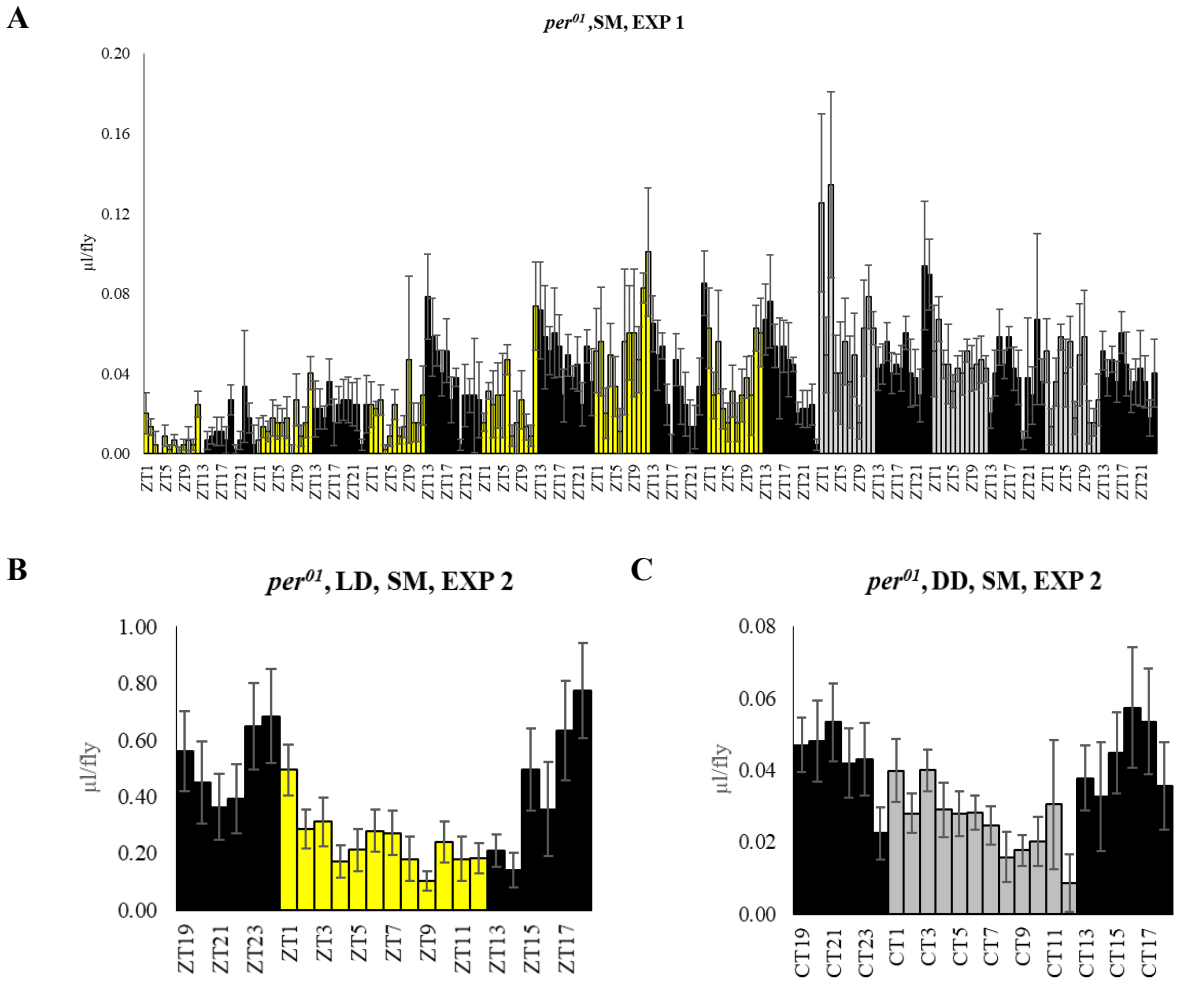


Supplementary Figure 46. Shows the feeding behaviour of wild-type fly fed on standard medium under 12hr/12hr light dark conditions and constant condition (dark/dark condition). A) 6 days light/ dark feeding and 3 days dark/dark feeding of wild-type fly. B) Averaged normalized feeding of 4th,5th and 6th day of wild-type feeding under light/dark condition. C) Averaged normalized feeding of 1st and 2nd day of wild-type feeding under dark/dark condition. D) 6 days light/ dark feeding and 3 days dark/dark feeding of wild-type fly (2nd independent experiment). In the bar graphs, the lightening condition is designated by the colors of the bars (LD: yellow = light-on and black = light-off; DD: light grey = subjective light-on and dark grey = subjective light-off)

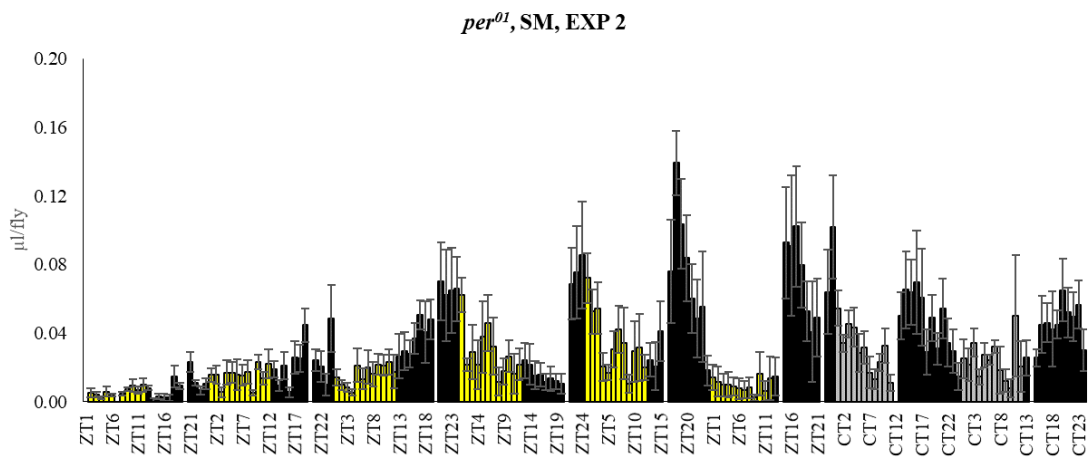


Supplementary Figure 47. Shows the feeding behaviour of wild-type fly fed on lipid-free medium under 12hr/12hr light dark conditions and constant condition (dark/dark condition). A) 6 days light/ dark feeding and 3 days dark/dark feeding of wild-type fly. B) Averaged normalized feeding of 4th,5th and 6th day of wild-type feeding under light/dark condition. C) Averaged normalized feeding of 1st and 2nd day of wild-type feeding under dark/dark condition.

D) 6 days light/ dark feeding and 3 days dark/dark feeding of wild-type fly (2nd independent experiment). In the bar graphs, the lightening condition is designated by the colors of the bars (LD: yellow = light-on and black = light-off; DD: light grey = subjective light-on and dark grey = subjective light-off)

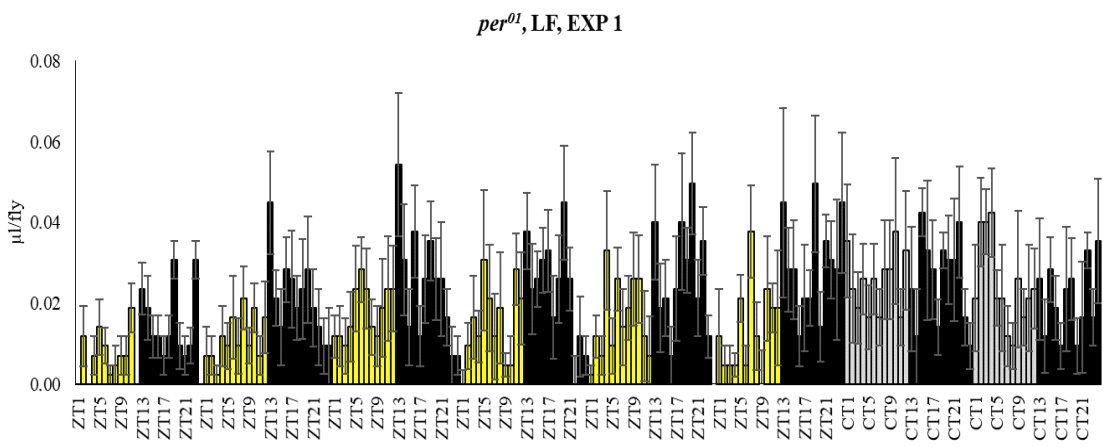


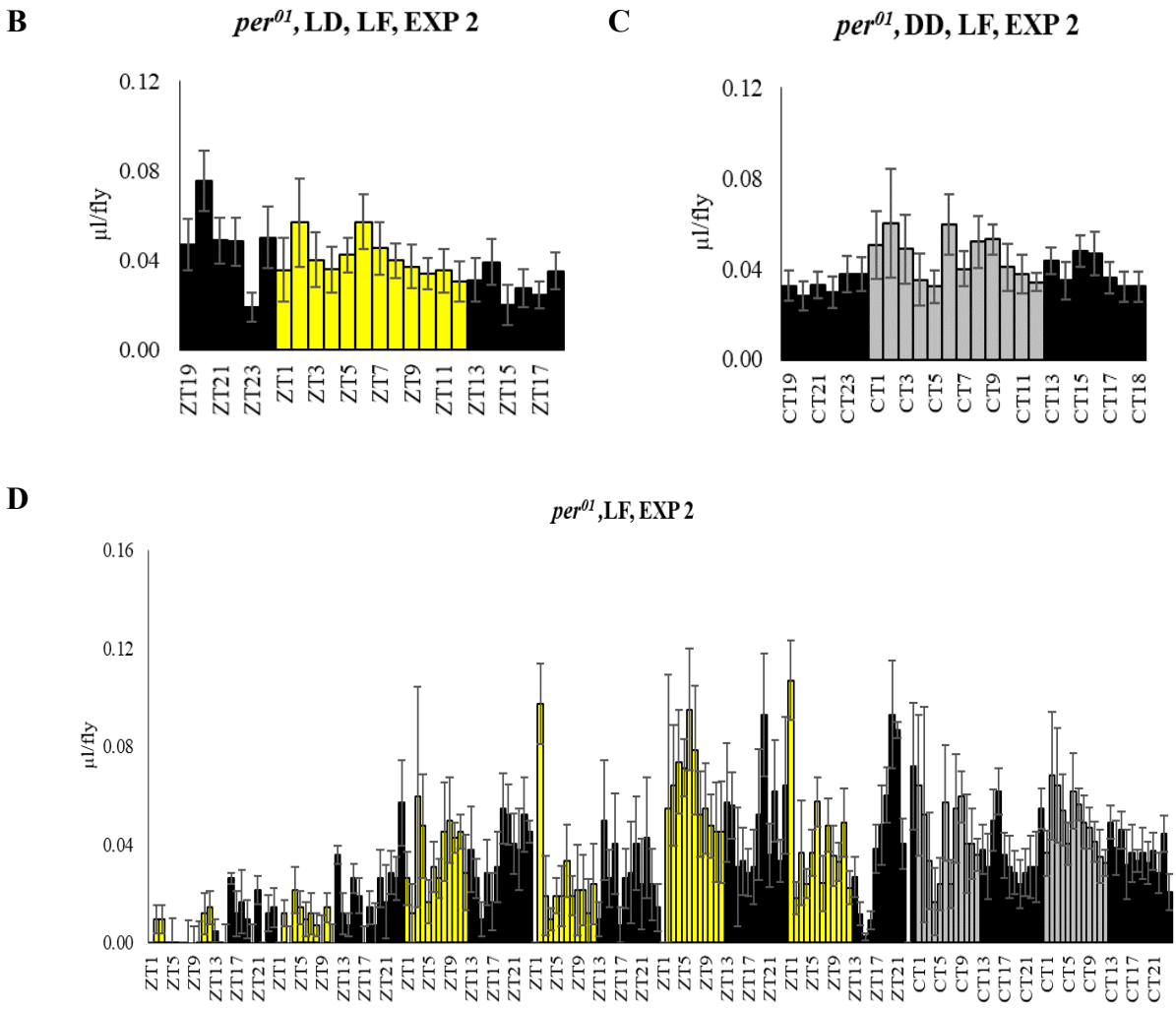
D



Supplementary Figure 48. Shows the feeding behaviour of mutant flies fed on standard medium under 12hr/12hr light dark conditions and constant condition (dark/dark condition). A) 6 days light/ dark feeding and 3 days dark/dark feeding of mutant flies. B) Averaged normalized feeding of 4th,5th and 6th day of mutant flies feeding under light/dark condition. C) Averaged normalized feeding of 1st and 2nd day of mutant flies feeding under dark/dark condition. D) 6 days light/ dark feeding and 2 days dark/dark feeding of mutant flies (2nd independent experiment). In the bar graphs, the lightening condition is designated by the colors of the bars (LD: yellow = light-on and black = light-off; DD: light grey = subjective light-on and dark grey = subjective light-off)

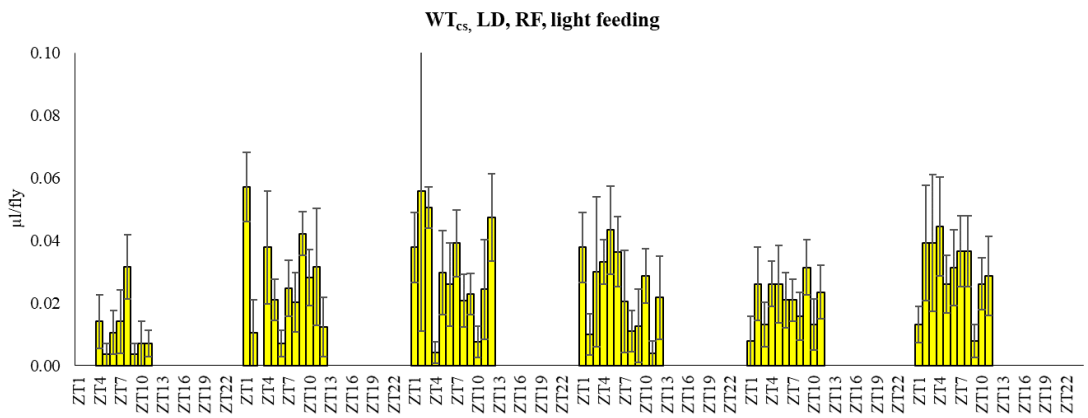
A



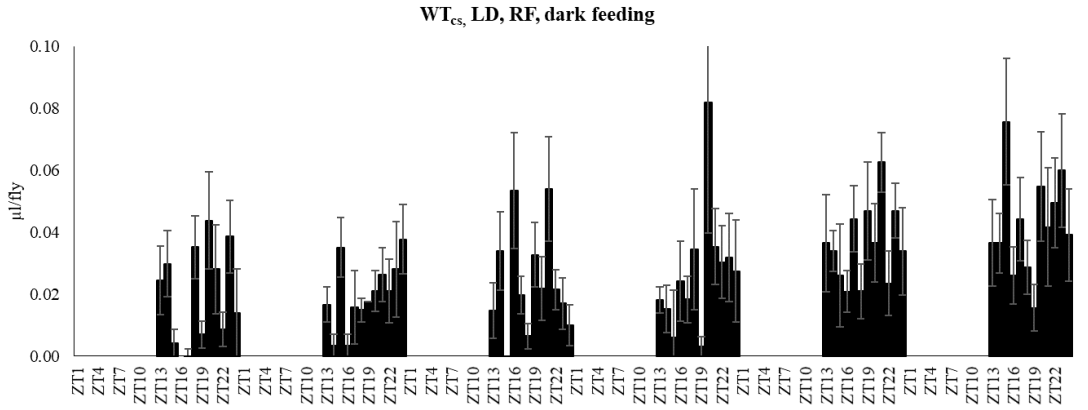


Supplementary Figure 49. Shows the feeding behaviour of mutant flies fed on lipid-free medium under 12hr/12hr light dark conditions and constant condition (dark/dark condition). A) 6 days light/ dark feeding and 3 days dark/dark feeding of mutant flies. B) Averaged normalized feeding of 4th,5th and 6th day of mutant flies feeding under light/dark condition. C) Averaged normalized feeding of 1st and 2nd day of mutant flies feeding under dark/dark condition. D) 6 days light/ dark feeding and 2 days dark/dark feeding of mutant flies (2nd independent experiment). In the bar graphs, the lightening condition is designated by the colors of the bars (LD: yellow = light-on and black = light-off; DD: light grey = subjective light-on and dark grey = subjective light-off).

A

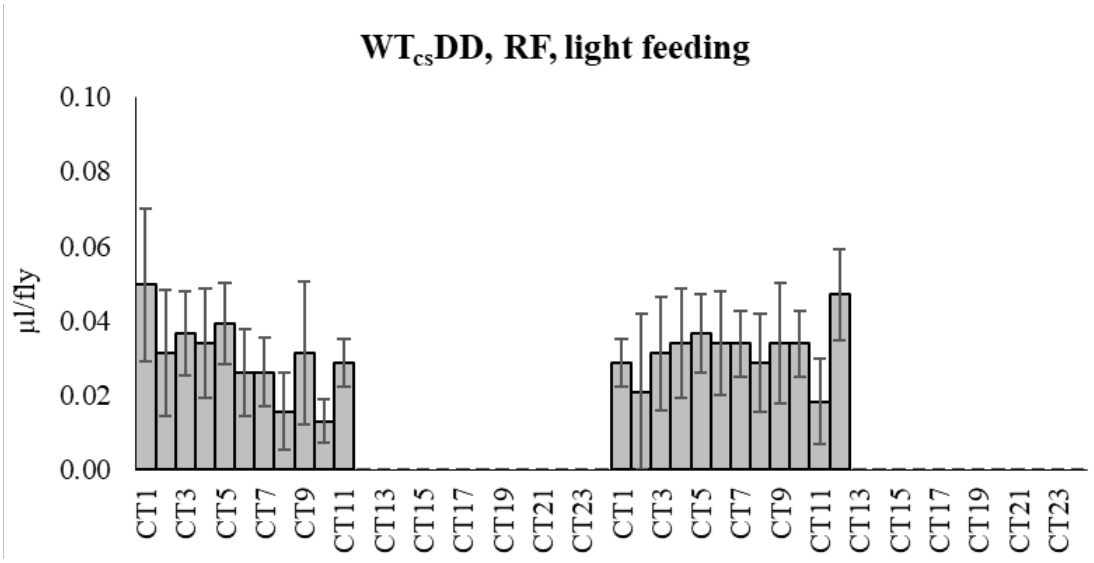


B

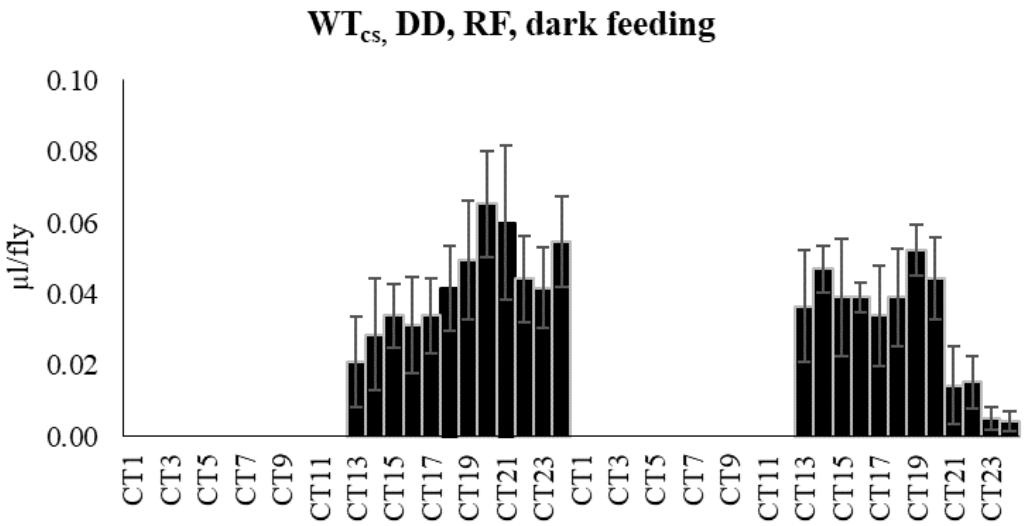


Supplementary Figure 50. Shows feeding behaviour of wild-type flies fed on lipid-free medium under time restricted feeding regime (light/dark condition) A) 6 days light phase restricted feeding. B) 6 days dark phase restricted feeding. In the bar graphs, the lightening condition is designated by the colors of the bars (yellow = light-on and black = light-off).

A

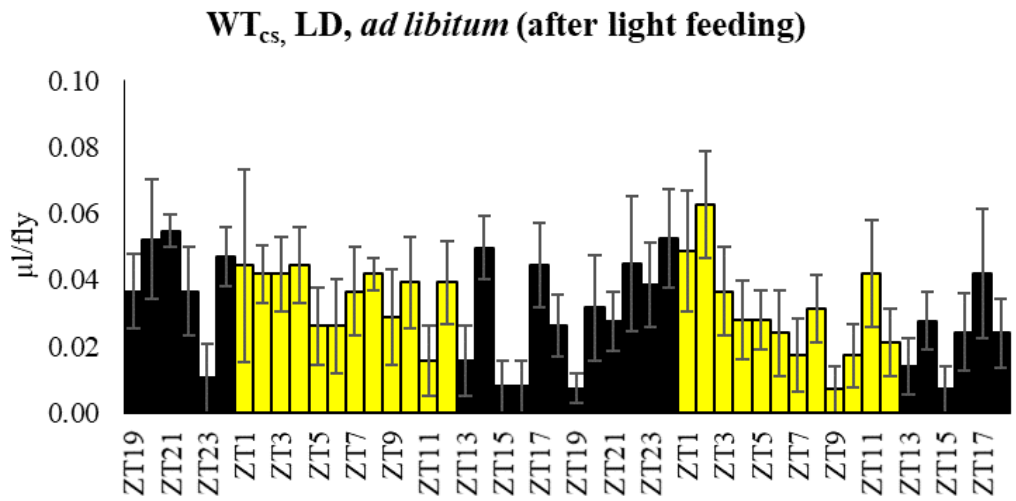


B

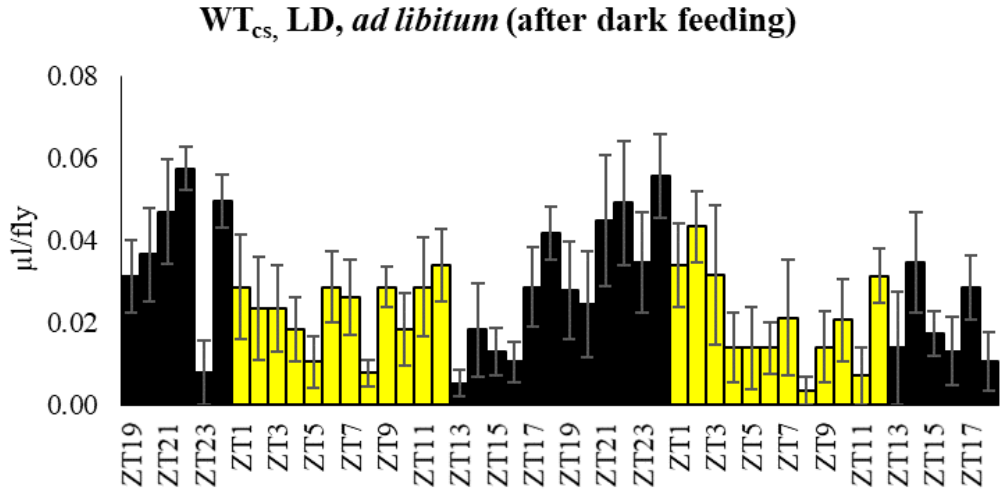


Supplementary Figure 51. Shows feeding behaviour of wild-type flies fed on lipid-free medium under time restricted feeding regime (constant condition) after 3 days on light/dark entrainment A) 2 days subjective light phase restricted feeding. B) 2 days subjective dark phase restricted feeding. In the bar graphs, the lightening condition is designated by the colors of the bars (light grey = subjective light-on and dark grey = subjective light-off)

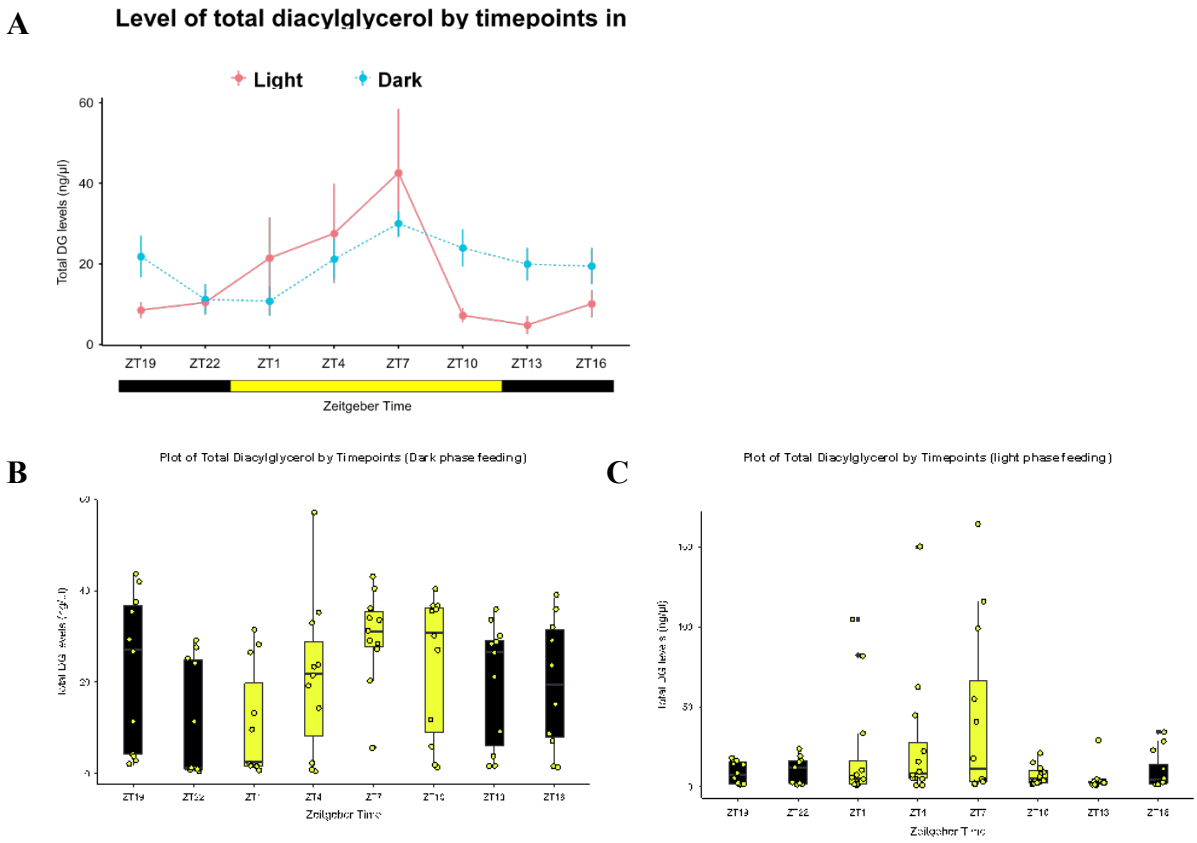
A



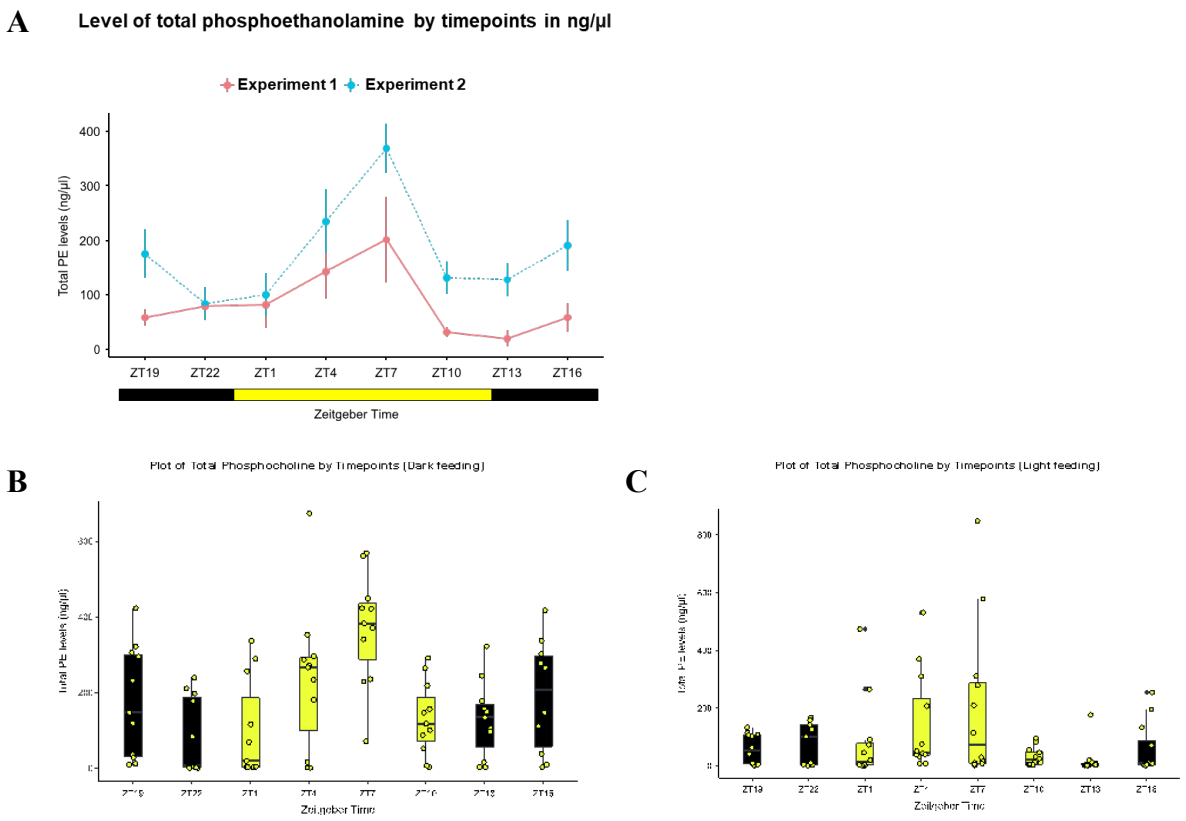
B



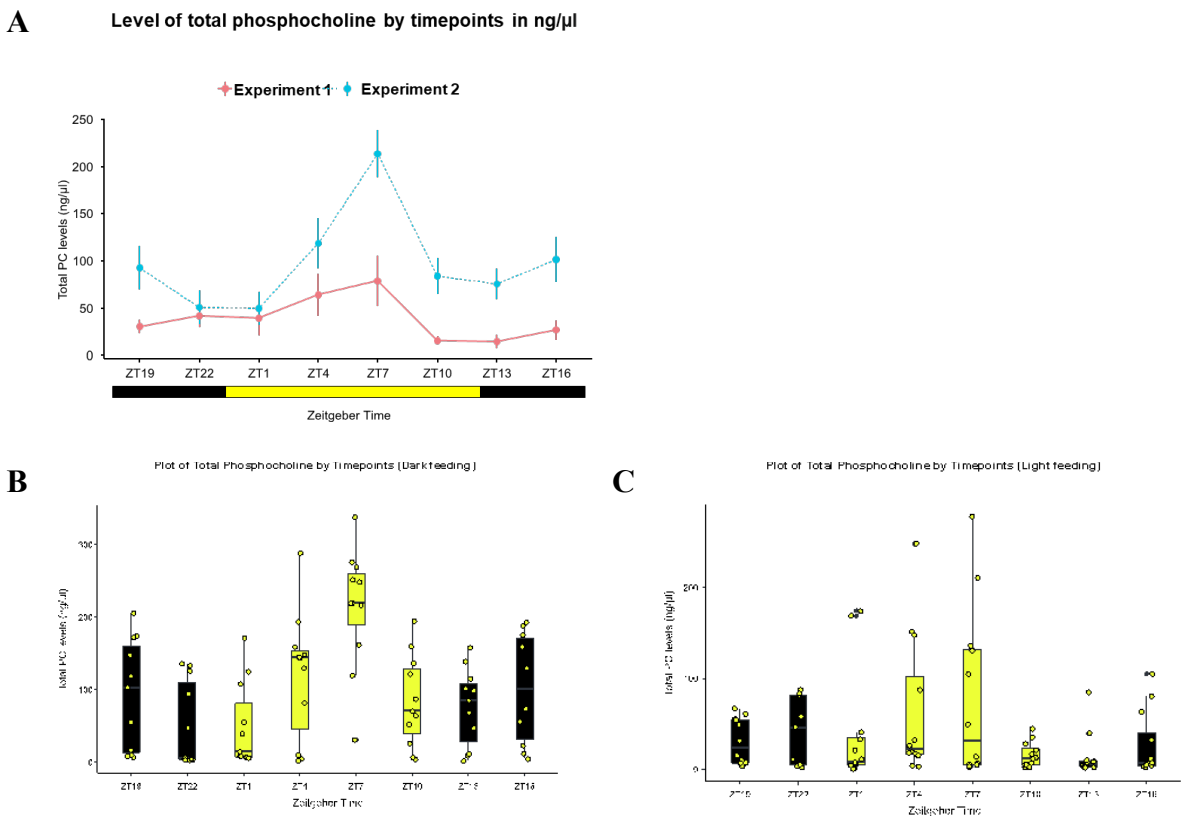
Supplementary Figure 52. Shows 2 days feeding behaviour of wild-type flies fed on lipid-free medium 3 days after time restricted feeding A) 2 days *ad libitum* feeding behaviour after 3 days of light time restricted feeding. B) 2 days *ad libitum* feeding behaviour after 3 days of dark time restricted feeding. In the bar graphs, the lightening condition is designated by the colors of the bars (yellow = light-on and black = light-off).



Supplementary Figure 53. Shows time restricted hemolymph DG oscillation profile of Wild-Type *Drosophila* male flies fed on lipid-free medium under 12hr/12hr light/dark cycle at 8 different timepoints. A) Line plot of superimposed DG profile of light phase and dark phase feeding B) Boxplot of DG profile of light phase and dark phase feeding respectively. In the graphs, the lightening condition is designated by the colors of the bars (yellow = light-on and black = light-off).



Supplementary Figure 54. Shows time restricted hemolymph PE oscillation profile of Wild-Type *Drosophila* male flies fed on lipid-free medium under 12hr/12hr light/dark cycle at 8 different timepoints. A) Line plot of superimposed PE profile of light phase and dark phase feeding B) Boxplot of PE profile of light phase and dark phase feeding respectively. In the graphs, the lightening condition is designated by the colors of the bars (yellow = light-on and black = light-off).



Supplementary Figure 55. Shows time restricted hemolymph PC oscillation profile of Wild-Type *Drosophila* male flies fed on lipid-free medium under 12hr/12hr light/dark cycle at 8 different timepoints. A) Line plot of superimposed PC profile of light phase and dark phase feeding B) Boxplot of PC profile of light phase and dark phase feeding respectively. In the graphs, the lightening condition is designated by the colors of the bars (yellow = light-on and black = light-off).

8 GENERAL ABBREVIATION

DG	Diacylglycerol
PE	Phosphatidylethanolamine
PC	Phosphatidylcholine
µ	Micro
TRF	Time restricted feeding
WTcs	Canton S
Per^{01}	period mutant
CAFE	Capillary feeder
LF	Lipid-free medium
SM	Standard Medium
DD	Dark-Dark (Constant condition)
LD	Light-Dark
R. F	Response Factor
P_A	Peak Area Analyte
$P_{i.s}$	Peak Area internal standard
C_A	Concentration of Analyte
$C_{i.s}$	Concentration of internal standard
TAG	Triacylglycerol
XIC	Extracted ion chromatogram
LC	Liquid chromatography
ZT	Zeitgeber Time
CT	Circadian Time
JTK	Jonckheere-Terpstra-Kendall
BH. Q	Benjamini-Hochberg critical value
ADJ.P	Adjusted P value
PER	Period
LAG	LAG phase

9 ACKNOWLEDGEMENTS

First and foremost, I sincerely and profoundly thank my primary supervisor Dr Agnes (Agi) Fekete (Manager metabolomics core facility) for teaching, guiding and correcting me throughout my PhD experience. I will not forget your support all through my studies at the University of Wuerzburg. Furthermore, I thank Prof. Dr. Dr. Martin Müller (Chair of Pharmaceutical Biology) who provided the enabling environment in his research group for me to carry out my project. I appreciate the offer you rendered to me. In addition, I deeply appreciate Prof Christian Wegener for supporting me in carrying out a crucial aspect of my project and giving me the first training in hemolymph extraction. Also, many thanks to Prof Charlotte Förster who provided additional support in materials and space I needed to run my experiments. Thanks to Dr Dirk Rieger who provided me with materials and space for my experiments.

I thank the graduate school of life sciences (GSLS) excellence initiative for providing the financial and academic support needed for me to complete my doctoral program. Specifically, I thank Dr. Gabriele Blum-Oehler (Manager) for her moral and academic support during this period.

My family during this period has been of immense support. Therefore, I thank my parents Mr and Mrs Amatobi who gave me constant encouragement. Additionally, I thank my siblings Mr Nnamdi Amatobi, Mr George Amatobi and Mrs Onyinyechi Otunneme who were supportive during this period. I wish to also acknowledge the support of my cousins and friends such as – Mr Chinedu Njokuoba, Kelenna Ogueri, Chimezie Amaefule, Ayoola Ogunleye, Atinuke, Evergreen, Chioma and Mojtaba Faryadras.

I wish to thank members of Prof. Martin Müllers lab who provided the enabling environment for my success such as – Dr Frank Waller, Dr Markus Kischke, Dr Gabriele Gresser, Dr Sussane Berger and Dr Daniel Maag. I want to specifically thank Maria for providing me with the assistance I needed during this period. In addition, I enjoyed the company of other students– Stefan, Michel, Steffie, Manuel, Elena, Mohamed, Jan, Ben and Atinuke. To my friends who I have lunch with such as Prathiba, Kushbu and Karimi, I say thank you.

Lastly, I wish to thank the organizers of the Fokus Life science program (master's program) that ushered me into the PhD program and all the friends I made in the program such as David, Simon, Maria, Ramon, Manju, Andrea, Gayatri, Aporva, Shruthi, Manu, Ruchi etc.

10 CURRICULUM VITAE

11 AFFIDAVIT

I hereby declare that my thesis entitled, "**Circadian clocks determine transport and phospholipid oscillation in complex interactions between nutrient-type, photic conditions, feeding behaviour and feeding time** "is the result of my own work. I did not receive any help or support from commercial consultants. All sources and/or materials applied are listed and specified in the thesis. Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Wurzburg, March 2021_____

EIDESSTATTLICHE ERKLARUNG

Hiermit erklare ich an Eides statt, die Dissertation „ **Zirkadiane Uhren bestimmen Transport und Phospholipid-Oszillation in komplexen Wechselwirkungen zwischen Nährstofftyp, photischen Bedingungen, Fressverhalten und Fütterungszeit** "eigenstandig, d.h. insbesondere selbststandig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben. Ich erklare auserdem, dass die Dissertation weder in gleicher noch in ahnlicher Form bereits in einem anderen Prufungsverfahren vorgelegen hat.

Wurzburg, March 2021_____