Characterization of Circadian Feeding Rhythms in *Drosophila* Using the Fly Liquid-Food Interaction Counter (FLIC) Assay

by

Qi Zhang

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Doctoral Committee:

Associate Professor Orie T. Shafer, Chair
Assistant Professor Laura Buttitta
Associate Professor Haoxing Xu
Associate Professor Scott Pletcher

Dedication

To all the researchers in biological science

It is never too late to pursue your dreams,

Whatever they may be.

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PREFACE

This work contains original work by Qi Zhang and members of the Shafer laboratory.

All of the content in Chapter 2 will be submitted to Journal of Biological Rhythms. Qi Zhang and Dr. Orie T. Shafer designed the experiments and Qi Zhang with the help from Jiang Zhu conducted the experiments, analyzed the data and prepared the figures. Dr. Orie T. Shafer and Qi Zhang prepared the manuscript.

Chapter 3 is the preliminary data intended to further our understanding of how the circadian clock regulates feeding rhythms and food consumption.

Chapter 4 is to examine how circadian clock regulate the circulating trehalose in *Drosophila*.

The content was generated by Qi Zhang and edited by Orie T. Shafer.

TABLE of CONTENTS

LIST of FIGURES

ABSTRACT

The circadian clock offers an internal estimate of the external time of day and is used to precisely time behavior and physiology to promote survival and fitness. In mammals, mounting evidence has established a correlation between a disrupted circadian clock and metabolic dysfunction, presumably due to the desynchronization of feeding with the optimal phase of endogenous metabolic rhythms. Although much is known about how feeding is positively and negatively regulated through endocrine and neuronal control, relatively little is known about how the central circadian clock regulates feeding. Thanks to its genetic tractability and relative simplicity of its central nervous system, *Drosophila melanogaster* is a powerful model organism for understanding the genetic and neural basis of circadian rhythms. Here we have used a sensitive and high through-put feeding assay, the Fly Liquid-food Interaction Counter (FLIC), to systematically characterize the circadian feeding behavior of flies in a manner that allows for the extended observation of feeding behavior in the absence of experimental perturbation, an approach that makes possible the recording of diurnal and circadian rhythms in feeding over many cycles. We found that wild-type and commonly used *Drosophila* strains display anticipatory morning and

evening peaks of feeding under a 12-hour-light/12-hour-dark cycle (LD) that resembles locomotor rhythms. Analysis of the feeding behavior of mutants lacking the central circadian rhythm gene clock revealed that morning and evening anticipation of feeding are dependent on the canonical molecular circadian clock. Moreover, flies lacking gene function in the positive limb of the molecular clock but not negative limb were characterized by increased food consumption, suggesting that feeding consumption is under the regulation of certain clock genes. We also found that the abundance of hemolymph (blood) trehalose (the predominant circulating sugar in flies) displayed circadian clock dependent regulation in a restricted feeding paradigm. We found that loss of molecular clock in the Dorsal and Lateral Dorsal clock neurons eliminates both the morning and evening anticipation of feeding behavior without changing the total amount of feeding. All of these findings lay a foundation for an understanding of circadian feeding behavior in flies and set the stage for further dissection of the neuronal circuitry underlying rhythmic feeding behavior.

CHAPTER 1

INTRODUCTION

The rotation of the earth around its own axis produces a predictable cycle of 24-hour changes in light and temperature. Evolution has shaped and tuned virtually all living organisms, from cyanobacteria to humans, to maintain circadian rhythms of around 24 hours. Circadian rhythms are programmed changes in physiology and behavior that persist with a period of about 24 hours in the absence of environmental cues. Such rhythms allow organisms to predict and anticipate environmental changes, both daily and seasonal, and are therefore thought to represent an important adaptation to a rhythmic and seasonal environment. Circadian rhythms are not simply direct responses to daily changes in temperature and light. Rather, they are driven by internal molecular clocks, as evidenced by the fact that circadian rhythms persist even when the organisms are kept under constant conditions (Moore-Ede et al., 1982). The circadian clock orchestrates a myriad of biological events including flower movements (e.g., sun flowers), locomotor behavior (e.g., fruit flies), feeding (e.g., mammals) etc. to optimally time behavior and physiological processes across

the day-night cycle. The focus of my work is to understand how the circadian system influences feeding behavior.

The following introduction describes circadian rhythms in general, particularly those of the fly *Drosophila melanogaster* and why this insect has become an important model organism to study the control of feeding by the circadian clock. The first section is an introduction of circadian rhythms in different organisms and why the circadian clock is relevant to human health. This section also introduces the molecular and neural basis of the circadian clock. The second section is focused on the circadian biology of *Drosophila melanogaster*, the model organism of my thesis research. This section will describe the fly's molecular circadian clock and the various outputs it controls. The final section in this chapter describes the tools and methods that make *Drosophila* a powerful model organism to study circadian clock.

1.1.1 Circadian rhythms

The word circadian is derived from the two Latin words "circa" and "diem" and therefore means "about a day". Circadian rhythms help the organisms living on the earth to anticipate predictable and often drastic daily environmental changes including changes in light and temperature. It is now widely accepted that circadian rhythms are orchestrated by circadian clocks in virtually all organisms, but the debate about whether circadian rhythms are endogenously regulated or just passive

responses to the changing environment lasted for more than two centuries (Moore-Ede et al., 1982).

In 1729, the observation by de Mairan of the rhythmic movement of the leaves of a "sensitive" heliotrope (probably *Minoso pudica*) persisted even though it had been isolated from daily changes in sunlight led him to question the assumption that biological rhythms are merely a consequence of a changing environment. Similar observations of heliotrope rhythms were made when light and temperature were kept constant thirty years later, suggesting the circadian time keeping is an endogenous property of plants (Duhamel DuMonceau, 1759). Since that time, more and more evidence has supported the concept that circadian rhythms are the outputs of an internal and endogenous time measuring system. For example, Beling (1929) and Renner carried out a series of sophisticated experiments to characterize the ability of honeybees to seeking food specifically at the time when food is provided. Moreover, honey bees transferred to a new time zone displayed food seeking activity corresponding to the original time zone rather than to local time (Renner, 1955), similar to the disorientation we often experience as "jet lag". Despite experiments like these, one can still argue that organisms are sensing some unnoticed time cues on the earth and simply responding to them. The final nail was placed in the coffin of this debate when the circadian rhythms of *Neurospora Crassa* were found to persist in space (Sulzman, 1984). The circadian rhythm of *Neurospora Crassa* is

evident in constant darkness through the observation of the daily shift between mycelia and aerial hyphase and conidia in a so-called racing tube. Dr. Sulzman found that this rhythm is sustained in space in absence of any daily cues from earth. The circadian clock is functions by receiving environmental time cues, entraining the central circadian clock, and modulating physiology and behavior to enable the biological events to happen at the optimal time of the day (Yu & Hardin, 2006).

In the field of chronobiology, it is now agreed that for a rhythm to be considered a circadian rhythm, it must satisfy three criteria: persistence, resetting, and temperature compensation. Persistence is the ability of the circadian clock to sustain an endogenous period of approximate 24 hours without any change of environmental cues. For example, the locomotor activity of wild type *Drosophila* displays a consistent period of ~23.5 hours in constant darkness and temperature (Fig. 1.2B). External stimuli can reset the circadian clock such that when we travel across time zones, a couple days later, our circadian clock will be synchronized to local time. Temperature compensation indicates a slight change of circadian period even given a temperature change, enabling the maintenance of circadian periodicity over a range of physiological temperatures (C. Johnson, 2004). As mentioned above, circadian rhythms exist in almost all organisms on the earth and allow them to cope with daily environmental changes. For example, nitrogen fixation of the cyanobacterium, *Synechococcus sp*, exhibits a peak activity at night while photosynthesis occurs

during the day (Huang et al., 1990); In bean plants (*phaseolus vulgaris L.*) as well as many other plants, leaf movement is regulated in a circadian fashion so that it is in a horizontal position at midday to receive the most sunshine (Hangarter et al., 2000), *Drosophila melanogaster,* are active around dawn and dusk when it is not too hot or dry (Fig. 1.2A). Mammals including humans contain circadian clocks that orchestrate daily outputs such as the sleep-active cycle, feeding rhythms, and hormonal cycles (C. Johnson, 2004).

A growing body of evidence suggests that the maintenance of a functional circadian clock is crucial for health, well-being and cognitive performance (Ramkisoensing & Meijer, 2015). Shift workers suffer from a higher risk of developing type 2 diabetes and other medical conditions due to the circadian disruption of nighttime sleep (Gallant et al., 2012; Knutsson 2003; Haus & Smolensky 2013; Rüger & Scheer 2009). Animal studies confirm the deleterious effects of the disruption of the circadian system due to the effects of irregular lighting conditions on sleep (Baron et al., 2011; Wulff et al., 2009), the cardiovascular system (Bray et al., 2008; Scheer et al., 2009; Oishi & Ohkura 2013), cognitive function (Gerstner et al., 2008), and metabolism (Marcheva et al., 2010; Turek et al., 2005; Landgraf et al., 2015). Thus, it is of great importance to understand the mechanisms underlying circadian timekeeping.

1.1.2 The Molecular Clock and Circadian Neural Networks in Different Organisms.

Underlying circadian rhythms in physiology and behavior sits a multifaceted timekeeping system that includes molecular clocks, neuronal networks and peripheral clocks depending on the complexity of the organism. Take cyanobacterium, *S. elongates*, for example, its core molecular clock consists of KaiA, KaiB, and KaiC, which form a posttranslational loop (C. H. Johnson, 2001). Plants have a spatial expression pattern of clock genes in different tissues with the core clock consisting of multi-feedback loops (Endo 2016; Jolma et al., 2010).

In mammals, the molecular circadian clock is expressed throughout the body. Conceptually, we divide the mammalian circadian system into the central clock, consisting of the suprachiasmatic nuclei (SCN) of the hypothalamus, and the peripheral clocks located in peripheral tissues such as the liver, kidney, pancreas, etc. (Kalsbeek et al., 2014). The SCN are located in the ventral periventricular zone of the hymothalamus and are recognized as a central clock that synchronizes the molecular circadian clocks in the peripheral tissues after receiving time cues via the retina (Jolma et al., 2010; Panda, 2002). The SCN orchestrates the peripheral clocks presumably via parasympathetic nervous system which innervates liver, pancreas, thyroid, and submandibular glands (Jolma et al., 2010). Furthermore, melatonin

from the pineal gland is also regulated by the SCN via nerve pulses to modulate sleep/wake rhythms, blood pressure, and other functions (Dunlap, J. C., 2004). It has also been shown that the SCN is resistant to temperature entrainment while peripheral clocks throughout the body are sensitive to the body temperature rhythm, suggesting a model where the SCN can drive the body temperature rhythm that serves as a universal entraining cue for peripheral clocks (Buhr et al., 2010). In addition to this central to peripheral regulation mechanism, it is also possible that peripheral organs receives time cues from daily metabolic or food-related changes. For example, liver transcription rhythms can be reset by oxyntomodulin secreted by the gut when food is present (Landgraf et al., 2015).

The mammalian molecular clock, at its core, is a transcriptional-translational feedback loop (TTFL). The genes making up this loop can be divided into positive limb genes, such as B*mal1* and *clock*, and negative limb genes, including the *period (per1-3)* genes, *the cryptochromes (cry1* and *cry2)* (Yu & Hardin, 2006). Heterodimers of BMAL1 and CLOCK activate the transcription of *per* and *cry*, thereby constituting a positive loop (Reppert & Weaver, 2002). PERs and CRYs accumulate in the cytoplasm, translocate into the nucleus and bind to BMAL1/CLOCK to inhibit their own transcription, thereby defining a negative loop (Mohawk et al., 2012) . On the other hand, heterodimers of BMAL1 and CLOCK will activate the transcription of many clock controlled genes such as retinoid-related

orphan receptors (*Ror*), reverse-Erb receptors*reb-erb*s (R*ev-erb*), and many other genes that could be important to cell activities (Reppert & Weaver, 2001). While REV-ERBs binds to the Rev-Erb/ROR response elements in the *Bmal1* promoter and inhibits its transcription, ROR binds the same element and positively regulate the transcription of *Bmal1*, forming the positive limb of the TTFL (Crumbley & Burris, 2011; Guillaumond, 2005).

1.1.3 Comparative Clocks: Flies and Mammals

The molecular mechanisms of the circadian clocks in *Drosophila* and mammals are highly conserved (Yu & Hardin, 2006). Both clocks are composed of TTFL featuring highly similar mechanisms. If we replace CLK-BMAL1 with CLK-CYC, mPER-mCRY with PER-TIM, REV-ERBα and RORα with VRI and PDP1ε, and RORε elements with V/P boxes, we will have converted the mammalian molecular clock to a *Drosophila* clock. One key difference between flies and mammals is the role of CRY. Mammalian CRY (mCRY) is a central component of the negative limb of the mammalian clock while *Drosophila* CRY (dCRY) functions as a cell autonomous light sensor in fly brain (Stanewsky et al., 1998). Interestingly, dCRY appears to serve an mCRY like function as a transcriptional repressor of the molecular clock in some peripheral tissues (Emery et al., 1998; Stanewsky et al., 1998).

1.2.1 Molecular Clocks in *Drosophila*

Similar to the molecular clock in mammals, the core of timekeeping in *Drosophila melanogaster* consists of interlocked transcriptional and translational feedback loops (TTFL). Following the discovery of the first clock gene, *period,* in the 1970s by Seymore Benzer and his post-doc Ronald Konopka via a chemical mutagenesis screen of flies, more clock genes were identified in the following decades. The characterization of these genes allowed a clear picture of the molecular clock in *Drosophila* to emerge. This section will focus on the molecular clock of *Drosophila* and highlight its similarities to the mammalian molecular clock.

Before we dive into the complex details of fly molecular clock, there are several important clock genes that must be introduced. These are *period* (*per*), *timeless* (*tim*), *clock* (*clk*), *cycle* (*cyc*), *double-time* (*dbt*), *shaggy* (*sgg*), *vrille* (*vri*) *and PAR domain protein1ε* (*pdp1ε*) (Yu & Hardin, 2006). At midday, heterodimers of helix-loop-helix transcription factors CLOCK and CYCLE bind to E-Box sequences (CACGTG) and activate the transcription of *per* and *tim* (Allada et al., 1998; Darlington et al., 1998; Rutila et al., 1998). This results in the accumulation of *per* and *tim* mRNA in evening and early night and this, with some delay, results in the accumulation and eventual nuclear translocation of PER and TIM before dawn (Curtin et al., 1995; Shafer et al., 2002; Peschel & Helfrich-Förster 2011). In the

nucleus PER and TIM bind to DBT to form a complex that inhibits CLK/CYC activity through the phosphorylation of CLK, thus causing CLK/CYC dimers to dissociate from the E-Boxes of *tim* and *per* (Lee et al., 1999; Yu et al., 2006). Under the control of attendant kinases, PER and TIM are degraded, thereby allowing the CLK and CYC to bind *per* and *tim* promoters and promote *per* and *tim* transcription.

The regulation of the transcription of *clk* is central to the positive limb of the molecular circadian clock. Two target genes, *vri* and *pdp1ε* are activated by E-box binding by CLK-CYC. The protein level of VRI rises first to bind the VRI/PDP1ε binding sites (V/P-boxes) of *clk* to repress clk transcription (Hardin, 2004). Then the DBT-PER repression of *vri* transcription lowers the level of VRI whereas the PDP1ε accumulates in the evening and outcompete VRI to bind the V/P box, activating the *clk* transcription(Hardin, 2004), thus forming a positive limb of the molecular circadian clock (Fig. 1.1).

1.2.2 Circadian Neural Networks in *Drosophila*

In this section, we will shift the focus to the circadian clock in the brain and peripheral tissues of the model organism, *Drosophila melanogaster*. As an adaptation to an ever-changing environment, *Drosophila*, like all animals, have evolved a central circadian clock in the brain as well as multiple peripheral clocks. For *Drosophila*, the central circadian clock consists of a group of about 150 clock

neurons residing in the brain. This central neuronal clock network consists of different classes of clock neurons that together orchestrate the circadian physiology and behavior of fruit flies via neural communication between clock neurons and between clock neurons and non-clock targets (Peschel & Helfrich-Förster, 2011).

The central clock coordinates circadian clocks located peripheral tissues, which contribute to specific rhythms in a tissue specific manner. Peripheral circadian clocks are found in sensory organs, digestive and reproductive systems, and other regions (Tomioka et al., 2012). For example, electroantennograms (EAG) revealed circadian rhythms in sensory transduction in the fly's antennae with a peak sensitivity at night (Krishnan et al., 1999). These rhythms are driven by molecular clocks in olfactory neurons (Tanoue et al., 2004). Though peripheral circadian clocks employ the same clock genes as the central clock, the mechanism of their molecular oscillations vary. The loss of cryptochrome, CRY, which was initially characterized as a cell autonomous blue light sensor in central clock neurons (Stanewsky et al., 1998), abolishes the rhythms in many peripheral clocks but not in the central clock, suggesting that CRY serves an important role in circadian timekeeping in peripheral tissues (Stanewsky et al., 1998). The extent of the dependence peripheral clocks on the central clock varies from tissue to tissue. For example, whereas the circadian rhythms of prothoracic gland are dependent on the central clock, the reproductive system can maintain its own rhythms without central clocks in *Drosophila* (Beaver

et al., 2002). The mechanisms through which the central clocks exert their effects on peripheral clocks remain elusive.

Although circadian clocks are expressed throughout the body, the central clock in the fly brain can orchestrate most behavioral and physiological rhythms (Tomioka et al., 2012). The adult fruit fly brain contains ~ 150 clock neurons which can be classified into 9 groups based on their anatomical positions (Shafer et al., 2006) (Fig. 1.3). In each hemisphere are five groups of laterally located neurons, referred to as lateral neurons. These consist of four large ventral lateral neurons (lLN_v), four small ventral lateral neurons (sLN_v), a single so-called "5^{th} sLNv," three or four lateral posterior neurons (LPNs), and six lateral dorsal neurons (LN_d). Each hemisphere also contains four groups of dorsally located neurons. These consist of two anterior DN1 neurons ($DN1_a$), approximately 16 posterior DN1 neurons ($DN1_p$), two DN2 neurons, and approximately 40 DN3 neurons (Almarestani et al., 2007; Helfrich-Förster et al., 2007; Taghert & Shafer 2006). It should also be noted that in addition to the clock neurons, there are a hundreds of glia cells that support clock gene cycling (Ewer et al., 1990). The function of these glial clocks is not currently understood.

How different classes of clock neurons coordinate with others to create a coherent circadian rhythm is a central question in the field. The fly's daily activity is characterized by two peaks of activity one around dawn and the other preceding

dusk by two or three hours (Aschoff, 1966). Current models of the clock neuron network in *Drosophila* identify subgroups of clock neurons as morning (M) oscillators and evening (E) oscillators, which are through to drive the two behavioral peaks, respectively. Two important studies provided the evidence for this model. Both Grima et al. (2004) and Stoleru et al. (2004) provided evidence that the sLN_v function as M-oscillators. Grima et al. (2004) E-oscillator through the rescued the loss of function *per* mutation in the LN_vs LN_d, and the 5^{th} sLN_v and found the rescue of both morning and evening anticipation of locomotor, suggesting the LN_d and the 5^{th} sLN_v may serve as E-oscillators. By changing the speed of molecular clock specifically in different subsets of clock neurons, Yao and Shafer (2014) further dissected the E-oscillator into E2, which included the 5^{th} s-LN_v and a single LN_d which are tightly coupled in their molecular timekeeping to the sLN_v, E1 as two LN_d that are receptive to sLN_v signals but are not tightly coupled to the sLN_v, and E3, which appear to be completely independent of the sLN_v (Yao & Shafer, 2014).

The sLN_v (the M oscillators) express the 18-amino acid neuropeptide pigment dispersing factor (PDF) (Renn et al., 1999). PDF is the most intensively studied neuropeptide in the circadian clock neuronal network of *Drosophila* since its discovery in 1999 (Renn et al., 1999). *pdf* null mutants (pdf^{01})and pdf receptor (*pdfR)* null mutants ($pdfr^{-}$) both fail to display anticipatory locomotor activity preceding dawn, have a significantly advanced evening peak of activity, and show severe

defects in circadian timekeeping under constant darkness and temperature (Renn et al., 1999; Hyun et al., 2005). The sLN_v of *pdf*^{01} flies fail to maintain synchrony in their molecular timekeeping, and molecular clock of the LN_ds fail to maintain high amplitude molecular rhythms in these mutants (Lin, 2004). This suggests that PDF and the LN_v that produce it are key mediators of molecular timekeeping throughout the clock neuron network of *Drosophila*.

1.2.3 Circadian Output in *Drosophila*

Ultimately, molecular and neuronal timekeeping must produce rhythms in physiology, metabolism, or behavior in order for flies to cope with a changing environment. In this section, I will focus on what is known about the outputs of the circadian system that produce daily rhythms in eclosion, locomotion, and feeding. In contrast to our understanding of timekeeping, very little is known about the output pathways that link the central circadian clock to specific outputs.

Eclosion is a key developmental event that consists of the fly's emergence from its pupal case following metamorphosis. The timing of eclosion is governed by a circadian clock (Konopka & Benzer, 1971). Eclosion rhythms are endogenous, entrainable and temperature compensated, meeting the three fundamental criteria of a circadian rhythm. The very first identified clock gene, *per,* was found in a forward

genetic screen for flies displaying abnormal eclosion timing (Konopka & Benzer, 1971). Two decades later, Sehgal and colleagues (1994) used eclosion rhythms as a behavioral readout in a forward genetic screen that identified the clock gene *tim*. Despite the importance of eclosion rhythms in the field of chronobiology, eclosion has now been less and less used primarily because it is a population rhythm and can be measured only once for a generation.

Locomotor activity is widely used as a circadian readout of activity and sleep in *Drosophila* thanks to the Trikinetics monitor. Most of the major discoveries regarding the genetics of central clocks and their organization is based on the monitoring of locomotor behavior. For example, the morning and evening anticipatory peaks of flies under a light/dark cycle were the basis of the discovery of M- and E- oscillators within the clock neuron network (D. Stoleru et al., 2004; Grima et al., 2004). Many studies have examined how central circadian neuronal network controls locomotor behavior. In 2014, Cavanaugh and colleagues conducted a screening of output neurons for circadian clock using GAL4-UAS system to express the heat-activated channel TrpA-1. The rational is that the constitutive activation of the output center should be deleterious to the locomotion rhythms of *Drosophila* but not affect the cycling of the molecular clock in the dominant pace makers. Indeed, they identified a cluster of corticotropin-releasing factor homolog, DH44, positive neurons in the pars intercerebralis region, which displayed a direct connection with

DN1s by GFP reconstitution across synaptic partners (GRASP) (Feinberg et al., 2008; Cavanaugh et al., 2014). Other potential locomotor output centers have also been identified by functional approaches. By expressing the mammalian ATP-gated cation channel P2X2 in the upstream neurons and the Ca2+ sensor, GCaMP6, in putative downstream neurons (Yao et al., 2012), Cavey and colleagues (2016) found that leucokinin neuropeptide (LK) and its receptor (LK-R) lie downstream of the central clock neurons, LN_vs. They also found that LK neurons' neuronal activity is rhythmically regulated by upstream clock centers (Cavey et al., 2016).

Bouts of inactivity of flies lasting five minutes or more are defined as sleep in *Drosophila*. Work done in the Greenspan and Sehgal labs established that this definition adheres to the classic definition of sleep: flies show increased arousal thresholds and increased need for sleep if they are deprived of such inactivity (Hendricks et al., 2000). Furthermore, sleep bouts in the fly are regulated by the circadian clock (i.e., the timing of sleep is controlled in a circadian manner), and mutations in the core circadian clock genes result in disrupted sleep patterns (M. N. Wu et al., 2008). The lLN_v clock neurons have been shown to play an important role in controlling sleep arousal and the light input into the clock neurons (Y. Wu et al., 2008; Parisky et al., 2008; Shang et al., 2008).

1.2.4 The Digestive System of *Drosophila*

In addition to its conserved molecular clock, there are several features of *Drosophila* that make it a great model for the study of the circadian control of feeding and metabolism. As in mammals, fruit flies have an anatomically and functionally segmented digestive system, of which anterior and posterior compartments are analogous to the stomach and intestines. The *Drosophila* adult midgut has 14 functional zones that cooperate to produce a sequential processing of food (Buchon et al., 2013). The copper cell region of midgut is a highly acidic compartment (pH<3) that supports a digestive function highly similar to that of the mammalian stomach (Filshie & Waterhouse 1955; Dubreuil et al., 2001; Shanbhag & Tripathi 2009). Due to the functional differences among different zones of the fly's digestive system, the cells of each zone have their own RNA expression profiles and populations of stem cells that can differentiate into different cell types, including enterocytes and enteroendocrine cells (Marianes & Spradling 2013; Biteau et al., 2011). After the absorption of nutrients, the enterocytes release the nutrients into an open circulatory system bathed in hemolymph, the fly's equivalent of blood. A tubular heart pumps hemolymph through the open circulatory system to ensure efficient metabolic exchange and hormonal dissemination.

1.2.5 Metabolism in *Drosophila*

Complex sugars are broken down by glucosidases into monosaccharides in the gut, absorbed, and then released by enterocytes (S. Turunen, 1996). Circulating monosaccharides are taken in by the fat body, the fly's functional equivalent of both the mammalian liver and adipose tissue, which catalyzes the formation of glycogen for energy storage or the glucose-glucose disaccharide, trehalose, which is subsequently released into the hemolymph where it is the fly's predominant form of circulating sugar (Reyes-DelaTorre et al., 2012). The synthesis of trehalose is through the trehalose phosphate synthase catalyzes and de-phosphorylation of trehalose phosphate phosphatase (Candy & Kilby, 1961). The use of trehalose allows passive influx of dietary sugars while maintaining a high level of circulating sugars (Klowden, 2013). It also stabilizes the high amounts of peptides, free amino acids, and proteins in the hemolymph because of the slow rate of hydrolysis of trehalose (Reyes-DelaTorre et al., 2012). As the fly's predominant circulating sugar, the level of trehalose within the hemolymph is a good indicator of the metabolic state of flies.

Dietary lipids are either transported from the gut as lipoprotein particles or stored as triacylglycerides (TAG) in lipid droplets, conversion of which mainly occurs in fat body with the help of lipid transfer particles (LTP) (Palm et al., 2012). The fat body serves as a primary regulator of energy metabolism. For example, the fat body converts glycogen into circulating sugars upon the activation of glycogen

phophorylase by insect adipokintic hormone (AKH), which is released exclusively from the corpora cardiaca (CC) of the ring gland, the fly equivalent of the vertebrate pituitary gland (Gade & Auerswald, 2003; Kim & Rulifson, 2004; Noyes, Katz, & Schaffer, 1995; Wu et al., 2003). The fat body also secretes humoral signals such as the leptin ortholog Unparied2 (Upd2) to balance carbohydrate, amino acid and fat levels through the regulation of the secretion of *Drosophila* Insulin Like Peptides (DILPs). Upd2 controls DILP release by relieving the inhibitory tone of the GABAergic neurons synapsing on the DILP Producing Cells (IPCs) (Rajan & Perrimon, 2012). The fat body supports molecular clock cycling, suggesting that it may play a role in the circadian regulation of feeding and metabolism (Xu et al., 2008).

1.2.6 Tools and Techniques in *Drosophila*

The GAL4-UAS system is currently the most widely used and well-developed binary expression system for transgene expression (Brand & Perrimon, 1993). This powerful tool has facilitated the analysis of clock mechanisms in *Drosophila*. GAL4 is a yeast transcriptional activator of an Upstream Activating Sequence (UAS) (Fischer et al., 1988). Neither GAL4 nor UAS is present in the genome of *Drosophila* (Brand & Perrimon, 1993). The coding sequence for GAL4 can be

placed under the control of selected fly enhancer/promoter sequences to produce cell or tissue specific expression of GAL4. Likewise, sequences-encoding transgenes can be placed under the control of UAS sequences. When a fly contains both an enhancer-GAL4 and a UAS-transgene element, the transgene will be expressed all cells expressing GAL4. Since its introduction into the fly system, thousands of GAL4 stocks have been created. In the field of circadian biology, many clock neuron specific drivers have been developed. For example *pdf-GAL4* is expressed only in the pigment dispersing factor positive s-LN_vs and and l-LN_vs (Renn et al., 1999), an element that has been used with great success to address the function of these neurons in the control of circadian rhythms (Renn et al., 1999; Grima et al., 2004; Shafer et al., 2008; Yao & Shafer 2014). It should be noted however that though *pdf-GAL4* directs GAL4 expression in only ~16 clock of the clock neurons in in the central brain, it also drives expression in PDF positive but CLOCK negative abdominal ganglion cells. Thus, when using the GAL4-UAS system, one should always be aware of possible expression outside of target regions. In my thesis work, I have used different GAL4 drivers to dissect the roles of certain clock neuron classes in feeding and metabolic rhythms or to manipulate the entire clock neuron network.

The promoter or enhancer fused with GAL4 determines the targeted cells while the sequence downstream of UAS achieves the purpose of manipulation. For example, *uas-reaper* can induce programmed cell death to ablate specific cells (Renn

et al., 1999), *uas-cycΔ* encodes a dominant negative form of *cyc* that lacks DNA binding regions and can abolish molecular clock cycling by binding CLK and preventing *per* and *tim* transcription. (Tanoue et al., 2004), and *uas-Kir2.1*, a potassium channel (Nitabach et al., 2002) can silence neuronal firing. These are only a few examples of many possible GAL4-UAS manipulations. The GAL4-UAS system allows for the directed expression of any sequence of interest in target cells to provide a remarkable number of genetic approaches for neuronal manipulation. At times, existing GAL4 elements may not provide sufficient specificity of transgene expression. For this reason, several approaches to limiting transgene expression to smaller and smaller subsets of cells has been important for progress in the field. For example, Gal80 blocks GAL4 transactivation (Suster et al., 2004). By placing Gal80 under the control of fly enhancers that produce expression that partially overlaps with GAL4 one can refine UAS-driven transgene expression patterns. Recently, similar but independent expression systems such as the LexA and QUAS systems have been developed to drive expression of genes independently of GAL4-UAS system (Shang et al., 2008; Lai & Lee 2006; Potter et al., 2010).

When experimentally perturbing circadian clocks, temporal control of the manipulation can be as important as spatial control of the manipulation. For example, one might like to excite or inhibit the firing of specific clock neurons at specific times of the day. TrpA, a temperature sensitive cation channel can be used

to activate circadian neurons using acute increases in ambient temperature (Hamada et al., 2008; Sivachenko et al., 2013). Temperature-sensitive Gal80 can also be used with GAL4 to enable a temperature dependent repression of UAS genes (McGuire et al., 2004). GAL4 has also been modified, by fusing to a fragment of the progesterone receptor, to make it dependent on the drug RU486, thereby making GAL4-UAS expression chemically inducible (Osterwalder et al., 2001; Roman et al., 2001). Of note, all of the above manipulations might affect circadian clock function independently and non-specifically, so proper and careful controls are needed to interpret the results.

In addition to making possible the manipulation of neuronal activity, the GAL4-UAS system has been used to measure activity in fly neurons. Electrophysiology is the most sensitive tool to study neuronal activity, but it is especially challenging to conduct in the circadian clock neurons of *Drosophila* because most clock neurons are quite small and reside deep within the brain, though there are some exceptions. Unlike the majority of clock neuron classes the membrane physiology of the lLN_vs and $DN1_ps$ has been measured using electrophysiology (Cao & Nitabach 2008; Seluzicki et al., 2014). The development of genetically-encoded sensors of cell signaling, for example sensors of Ca^{2+} and cAMP, allow for the measurement of neuronal activity in neurons that are inaccessible to electrophysiology. For example, the Ca^{2+} sensor GCamp is widely

used in the field to detect neural excitation (Akerboom et al., 2012). The FRET-based reporter for cAMP was also used to address the receptivity of the neuropeptide, Pigment Dispersing Factor (PDF) throughout the clock neuron network (Shafer et al., 2008). Both of the sensors of Ca^{2+} and cAMP have been fused with UAS and can be expressed via GAL4.

In addition to powerful genetic tools available in *Drosophila*, it is also possible to measure different physiological and behavioral rhythms of flies. Dr. Yoshiki Hotta leveraged the fact that the *Drosophila* circadian clock is not affected by infra-red light and designed a locomotor monitoring system based on registering the breaking of an infra-red beam by flies individually housed in a food-containing tube (Frank & Zimmerman, 1969). Such locomotor activity measurements are now routinely used via commercially available monitors.

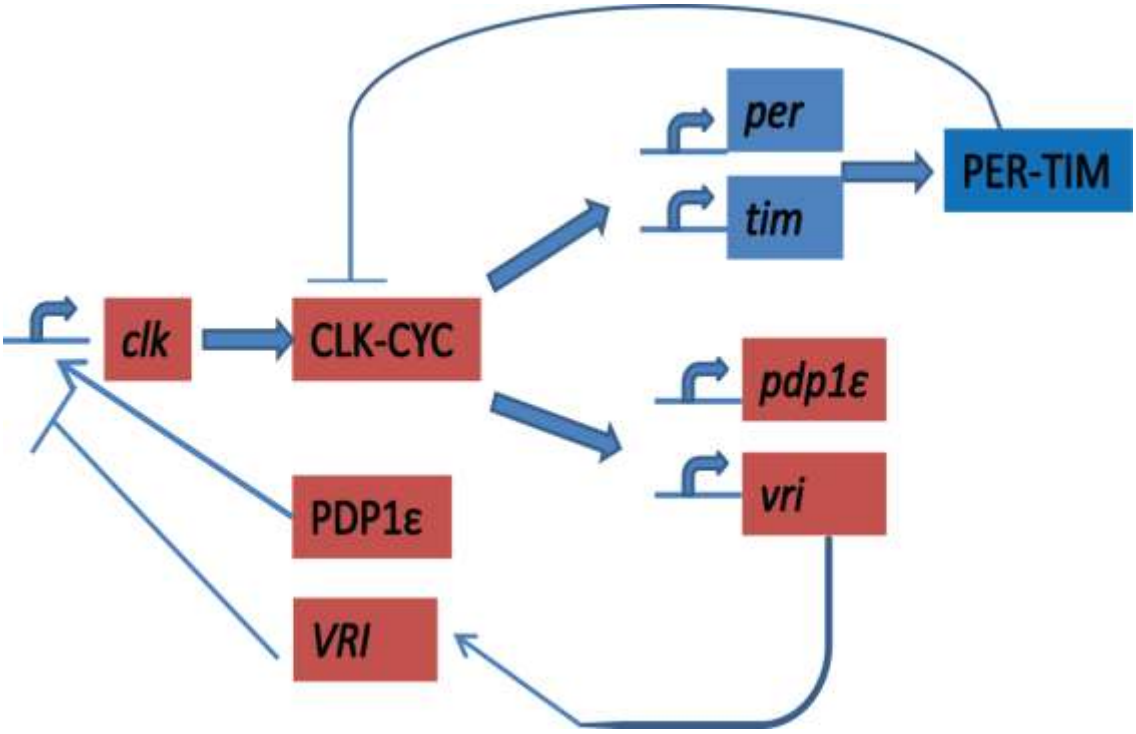
Recently new technologies have been developed for the measurement of other circadian outputs. Feeding is controlled by circadian clocks in the brain and digestive system (Xu et al., 2008). Traditionally, food intake in flies has been measured by loading food with vital dyes and measuring the amount of dye in the gut following food exposure (Edgecomb et al., 1994) or by observing the consumption of liquid food consumed by tracking the falling meniscus of a fluid-filled capillary tube (CAFE) (Xu et al., 2008; Ja et al., 2007). Both of these assays carry some caveats. The dye assay requires large number of flies and cannot measure the temporal pattern

of continuous feeding of the same fly over diurnal or circadian cycles. The CAFE assay makes it physically challenging for flies to feed and is subject to evaporation of the liquid food. In my thesis research, I have employed a newly developed apparatus for the measurement of feeding activity called Fly Liquid Interaction Counter (FLIC) to monitor the feeding behavior with high temporal resolution and for as long as seven days (Ro et al., 2014). I will discuss the rational and mechanism of the FLIC assay in Chapter Two in more detail.

Flies have also become an important model organism for the study of metabolism. As for circadian rhythms, this is due to the highly homologous nature of the genes governing metabolism and the power of the genetic tools available in *Drosophila* (Padmanabha & Baker, 2014). Previous work has investigated the circadian regulation of metabolites such as trehalose and triglyceride in whole body homogenates of flies (Seay & Thummel, 2011). In this thesis, I employ new, highly sensitive biochemical assays to specifically address the circadian control of blood sugar in the fly.

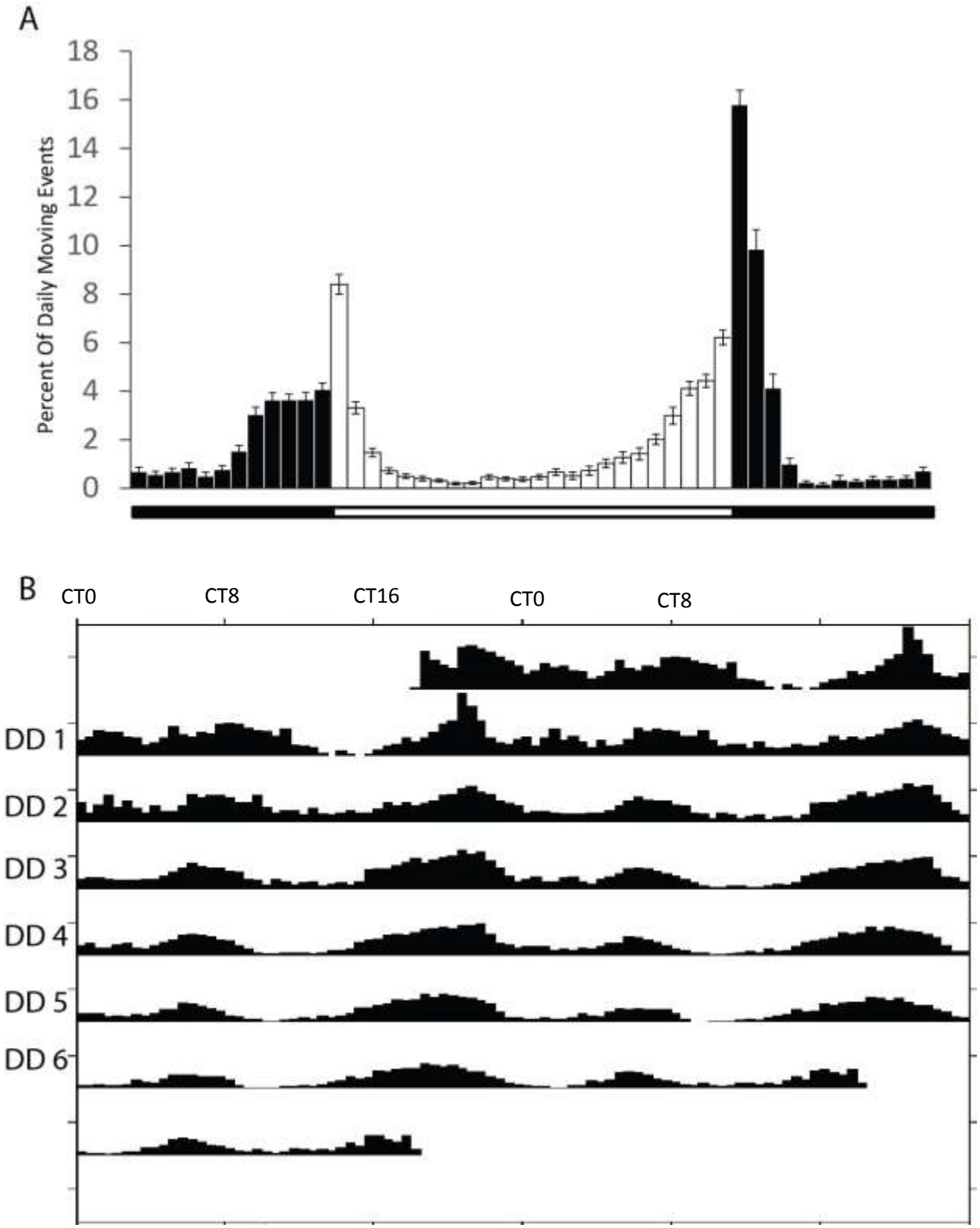
1.3 Figures

Fig. 1. 1. The molecular clock of *Drosophila*



The molecular of *Drosophila*. The components of the positive limb are colored red while those of the negative limb are colored blue.

Fig. 1. 2. Locomotion behavior of *Drosophila* in LD and DD



(A) Locomotion activity over a 12 h light and 12 h dark (LD) of CantonS flies. Each bar represents the percentage of daily moving events that occurred during the half hour bin. The bar below the graph indicates the light condition. The lights-on condition is indicated by white bars while the lights-off condition is indicated by black bars. (B) Rhythmic locomotion behavior of CantonS under constant darkness (DD).

Fig. 1. 3. Illustration of the circadian neuronal network of *Drosophila*

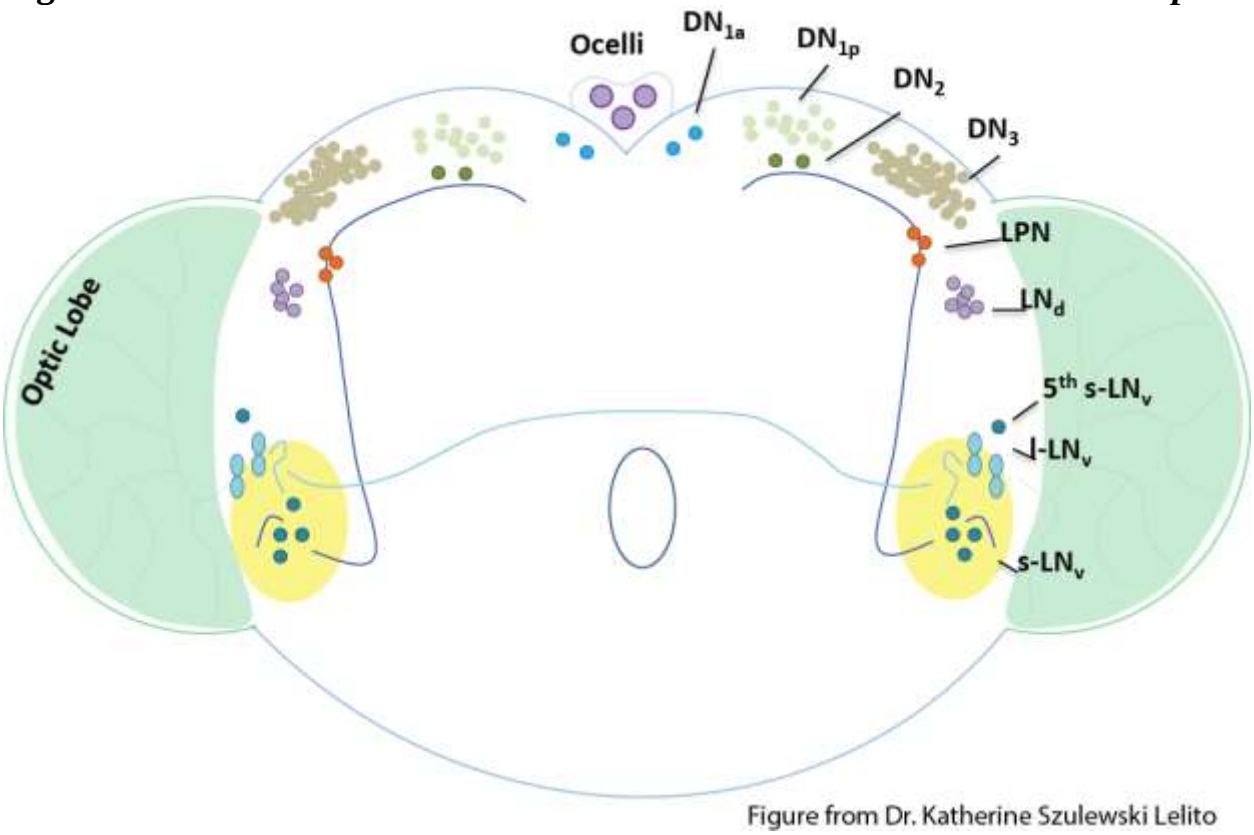


Illustration of different classes of clock neurons in circadian neuronal network of *Drosophila* from an anterior view. There are anterior dorsal neuron 1 (DN1a), posterior dorsal neuron 1(DN1p), dorsal neuron 2(DN2), dorsal neuron 3 (DN3), lateral posterior neuron (LPN), 5^{th} small lateral ventral neuron (5^{th} sLN_v), large lateral ventral neuron (lLN_v) and small lateral ventral neuron (sLN_v).

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CHAPTER 2

Clock genes regulate feeding behavior in *Drosophila*

2.1 Abstract

The circadian clock offers an internal estimate of the external time of day and is used to precisely time behavior and physiology to promote survival and fitness. In mammals, mounting evidence has established a correlation between a disrupted circadian clock and metabolic dysfunction, presumably due to the desynchronization of feeding with the optimal phase of endogenous metabolic rhythms (Froy, 2012). Although much is known about how feeding is positively and negatively regulated through endocrine and neuronal control, relatively little is known about how the central circadian clock regulates feeding. Thanks to its genetic tractability and the relative simplicity of its central nervous system, *Drosophila melanogaster* has become a powerful model organism for understanding the genetic and neural basis of circadian rhythms. Here we have used a sensitive and high through-put feeding assay, the Fly Liquid-food Interaction Counter (FLIC) (Ro et al., 2014), to systematically characterize the circadian feeding behavior of flies in a manner that allows for the extended observation of feeding behavior in the absence of perturbation, an approach that makes possible the recording of diurnal and circadian

rhythms in feeding over many cycles. We found that commonly used genetic fly strains display anticipatory morning and evening peaks of feeding under a 12-hour-light/12-hour-dark cycle (LD) that are highly reminiscent of those seen for locomotor rhythms. Analysis of the feeding behavior of loss of function clock gene mutants revealed that, as expected, morning and evening anticipation are dependent on the canonical molecular circadian clock. Moreover, flies lacking gene function in the positive limb of the molecular clock but not negative limb were characterized by increased food consumption, suggesting that feeding is promoted by the positive limb of the central clock and by as yet unidentified clock controlled genes. These findings establish a foundation for our understanding circadian feeding behavior in flies and set the stage for further dissection of the neuronal circuitry underlying rhythmic feeding behavior.

2.2 Introduction

The circadian clock offers an internal estimate of the external time of day and is used to precisely time behavior and physiology to promote survival and fitness (DeCoursey & Krulas, 1998). One key property of the circadian clock is that it can be entrained by light, temperature and food availability (Dunlap, J. C., 2004). In 1929, Ingeborg Beling discovered that bees can be trained to anticipate the presence

of food (sugar water) when it was made available at specific times of the day. In humans, mounting evidence suggests an important role of the timing of meals in human health. Baron and colleagues (2011) found that food intake in the late evening may increase the risk of obesity. Furthermore, an epidemiological study of 2, 372 nurses in Brazil suggested a strong association between shift work and Body Mass Index (Griep et al., 2014). The disruption of the circadian clock may affect metabolic state, thereby predisposing individuals to obesity and diabetes (Froy, 2012; Marcheva et al., 2010). Rats bearing mutations in *clock* genes display similar amounts of feeding between day and night whereas normal mice feed and drink at night (Stephan & Zucker, 1972). Furthermore, *clock* mutant mice are hyperphagic, suggesting an important role for the circadian clock in feeding and metabolism (Turek et al., 2005). Thus, the elucidation of the mechanisms through which the circadian clock regulates feeding behavior and metabolism is likely to shed light on the treatment of the metabolic disorders associated with the disruption of the circadian clock.

Thanks to its relatively simple clock neuron network and its genetic tractability, *Drosophila melanogaster* provides a powerful model system for the study of the neural mechanisms underlying circadian clock regulation (Weiner, 2000). The molecular clock of *Drosophila* displays striking homology to mammalian clocks, and consists of interlocked transcriptional/translational feedback

loops (Yu & Hardin, 2006). The negative loop starts with the transcription of the clock genes *period (per)* and *timeless (tim)* under the control of the positive transcription factors CLOCK (CLK) and CYCLE (CYC) in the late day (Peschel & Helfrich-Förster, 2011). The delayed accumulation of PER and TIM protein in the cytoplasm results in their translocation into the nucleus where they inhibit CLK-CYC, thereby repressing the transcription of *per* and *tim*. The positive limb of the clock is further regulated by two CLK-CYC target genes, *vrille* (*vri*) and *PAR domain protein 1ϵ* (*pdp1ϵ*), which negatively and positively regulate the transcription of CLOCK, respectively (Yu & Hardin, 2006).

Feeding is crucial for the maintenance of energy balance. Using tracer methods, previous research reported that *Drosophila* feeding under Light/Dark (LD) cycles display a single feeding peak around dawn (Seay & Thummel, 2011; Xu et al., 2008). The tracer method requires transfer of flies from normal food to food labeled with dye or radioactive chemicals for discrete windows of time and therefore the induction of mechanical perturbations. Such perturbations likely disrupt the fly's normal activity pattern thereby confounding the measurement normal feeding activity. Thus, a means to measure feeding behavior with high temporal resolution that does not require abrupt transfer of flies to and from labeled food is required for an accurate characterization of the circadian control feeding behavior.

The Fly Liquid-Food Interaction Counter (FLIC) system offers an excellent method for the measurement of feeding activity with high temporal resolution for extended periods of time without the need to interfere with normal feeding behavior (Ro et al., 2014). FLIC consists of a simple, low current electronic circuit that is closed only when the fly makes contact with a liquid food source. When the fly interacts with the liquid food, the completed electrical circuit is recorded as a small voltage change that is proportional to the contact the fly has made with the food, making it possible to distinguish between tasting and feeding (Ro et al., 2014). When fitted with a reservoir system, feeding behavior can be recorded for as long as seven days without disturbing the flies. This makes FLIC particularly useful for the chronobiological analysis of feeding behavior.

Using FLIC, we observed a bimodal feeding pattern of feeding in wild-type flies and established that it depends on *Drosophila*'s well-characterized molecular circadian clock. Flies bearing loss of function mutations in *clock* also display an increase in food consumption, suggesting that *clock* regulates food consumption.

2.3 Results

Drosophila displays a bimodal pattern of feeding behavior that requires the canonical molecular circadian clock.

Canton S (CS) flies, a commonly used wild-type *Drosophila* stock, were entrained to 12:12 light:dark (LD) cycle for five days, then loaded into FLIC arenas under the same LD cycle and monitored for feeding behavior for five days, with feeding events pooled into 30 min bins. CS flies exhibited morning and evening anticipatory bouts in LD (Fig. 2.1 A) that were highly reminiscent of the locomotor rhythms displayed in wild-type flies (Aschoff, 1966). Similar results were observed for *yw* flies, a second commonly used genetic background, though *yw* flies displayed a relatively larger morning peak and smaller evening peak than CS flies (Fig. 2.1 B). Based on results obtained using the tracer method, it was previously reported that flies display a single feeding peak in morning and a trough at night (Xu et al., 2008). We suggest that the strong bimodal pattern of feeding we observe may not be resolved by the tracer method, possibly due to the effects of physically transferring flies from labeled to unlabeled food. Thus, the FLIC method, which allows for the high resolution monitoring of feeding behavior in single flies, revealed a pattern of feeding behavior that could not be resolved by previous methods. These results are consistent with work done by Ro and colleagues on w^{1118} flies, another commonly used fly stock (Ro et al., 2014). A central characteristic of a circadian rhythm is that it is sustained under constant environmental conditions. To investigate if rhythmic feeding behavior persists under constant conditions, we monitored the feeding behavior of flies for six days under constant darkness and temperature with the help

of a liquid-food reservoir system (Fig. 2.2) that insured a constant supply of liquid food to FLIC arenas for the duration of the experiment. Wild-type CS flies displayed persistent circadian rhythms in feeding (Fig. 2.3A) with an average period of 23.7 hours and 91% rhythmicity (Table 1). *yw* and w^{1118} flies also displayed a rhythmic feeding behavior under DD (Fig. 2.3 C&E; Table 1).

To determine if the anticipatory peaks of feeding behavior are dependent on the canonical molecular circadian clock, we monitored the feeding behavior of three loss of function circadian rhythm gene mutants, including mutations in both positive and negative limb genes of the fly's molecular clock. We found that $period^{01}$ (per^{01}), $timeless^{01}$ (tim^{01}), $clock^{[jrk]}$(clk^{jrk}), and $cycle^{01}$ (cyc^{01}) display a complete absence of anticipatory feeding bouts under LD (Fig. 2.1C, D, E and F). These results reveal that an intact molecular clock is necessary for anticipatory bouts of morning and evening feeding under LD. Under DD, per^{01} (Fig. 2.3D), tim^{01} and clk^{jrk} mutants, failed to exhibit feeding rhythms under constant conditions revealing, as expected, that free running feeding rhythms require the canonical molecular circadian clock (Fig. 2.3 B,D, and F; Table 1).

Long- and short-period mutants of the *per* gene display shifted evening feeding peaks, and altered free running periods of feeding activity.

When Konopka and Dr. Benzer (1971) identified the *per* locus, they isolated a long period mutant (per^L) and a short period mutant (per^S), which are characterized by free-running locomotor rhythms with periods of ~28 hours and ~19 hours, respectively and altered phases of evening activity under LD cycles. To determine if these mutations have similar effects on affect diurnal and circadian feeding patterns, we monitored the feeding behavior of per^L and per^S mutants under LD and DD conditions. Under LD, per^L displayed an evening peak of feeding at ZT17.5±3.0, significantly delayed from the peak of control flies at ZT12±1.5 (calculated based on Butterworth filter; $p<0.05$ by T-test) (Fig. 2.4A). On the contrary, per^S was characterized by an advanced evening peak at ZT7.5±1.3, significantly advanced from the peak of control flies at ZT12.5±1.9 (calculated based on Butterworth filter; $p<0.05$ by T-test) (Fig. 2.4B).

per^L and per^S mutants displayed clear feeding rhythms under DD conditions (Fig. 2.4, Table 1), with per^L mutants displaying feeding rhythms with an average period of 28.3±0.8 hours (Fig. 2.4C; Table 1) and per^S mutants displaying an average period of 19.3±0.1 hours (Fig. 2.4D; Table 1). As for wild-type flies, the feeding rhythms of *per* period mutants were highly reminiscent of their well described locomotor rhythms. These results reveal that flies maintain daily crepuscular rhythms in feeding that mirror daily activity rhythms and that these rhythms require the canonical molecular circadian clock.

The positive limb clock genes, *clock*, regulates food consumption.

Feeding, both its timing and its absolute amount, is regulated by the molecular clock in both mammals and *Drosophila* (Turek et al., 2005; Xu et al., 2008). Furthermore, Turek and colleagues found that *clock* mutant mice developed a metabolic syndrome characterized by hyperleptinemia and hyperphagia on both a normal and high-fat diet (Turek et al., 2005). For the FLIC system the number of feeding events is highly correlated to total food consumption (Itskov et al., 2014; Ro et al., 2014). To investigate how the loss of circadian clock function affects food intake, we assayed food consumption in various clock mutants. We found that while per^{01} and tim^{01} mutants displayed normal levels of feeding compared to wild-type controls (Fig. 2.5A, B), the clk^{jrk} mutant, the most similar genotype to a *clk* null (Allada et al., 1998), were characterized by significantly higher levels of feeding, increases that were due to elevated nighttime feeding (Fig. 2.5C). That this increased feeding was due to the loss of *clk* function is supported by the failure of a third chromosome deficiency uncovering the *clk* locus to complement clk^{jrk} (Fig. 2.5D). We do note however that the per^{01} mutant, though it did not display significant

increases in total feeding, did show slightly elevated levels of feeding at night compared to controls (Fig. 2.5A). In addition to controlling the transcription of *per* and *tim*, *clk* functions to promote the rhythmic expression of many clock controlled genes (Abruzzi et al., 2011). Our results suggest that feeding is suppressed by the positive limb of the molecular circadian clock, likely through the transcription of one or more *clk* controlled genes.

Pigment Dispersing Factor (PDF) is required for normally phased feeding peaks.

PDFs are highly conserved 18-amino acid neuropeptides, which play important roles in the control of circadian rhythms in arthropods (Rao & Riehm 1989; Park & Hall 1998; Matsushima et al., 2004; Renn et al., 1999). In *Drosophila*, PDF is expressed by 16 ventral lateral neurons of the clock neuron network. Flies harboring a loss-of-function pdf^{01} mutation lack the anticipatory morning peak of locomotor activity and display an advanced evening peak under light/dark (LD) conditions. Pdf^{01} mutants also display weak or nonexistent circadian rhythms in locomotor activity under constant darkness and constant temperature (DD) (Renn et al., 1999), indicating that *pdf* is a critical neuronal output for the maintenance of locomotor rhythms. To investigate whether pdf^{01} mutants display similar changes in

the daily pattern of feeding behavior, we examined the feeding activity of pdf^{01} mutants and controls. We found that *pdf* mutant flies had a reduced morning peak of feeding activity and an advanced peak of evening feeding at ZT10±1.4, significantly advanced from the evening peak of control flies at ZT12±1.8 (calculated based on Butterworth filter (methods); $p<0.05$ by T-test) (Fig. 2.6A, B). We observied similar results with a chromosomal deficiency uncovering *pdf* over the pdf^{01} allele. The feeding behavior of pdf^{01}/df flies displayed a mutant-like loss of morning anticipation and an advanced evening peak of feeding behavior (ZT9.5±1.4 vs ZT12±2.4) (Fig. 2.6C, D). Under constant darkness and temperature, pdf^{01} mutants were characterized by significantly weaker feeding rhythms, indicating that, as for locomotor rhythms, PDF is a critical neuronal output for free-running rhythms in feeding behavior (Fig. 2.6G, H; Table 1).

The PDF has an identifed receptor, *pdfR* (also known as *han* (Hyun et al., 2005; Lear et al., 2005; Mertens et al., 2005; Shafer et al., 2008). Flies bearing a null mutation in *pdfR* display the same locomotor rhythm phenotypes as pdf^{01} mutants (Hyun et al., 2005). The null mutant of *pdfR* likewise displays an advanced evening peak of feeding at ZT10±1.5, which was significantly advanced from the feeding peak of control flies at ZT11.5 ± 1.6 (calculated based on Butter filter; T-Test is conducted, $p<0.05$) (Fig. 2.6E, F). Furthermore, the loss of PDFR results in increased arrhythmicity of the feeding rhythm under DD (Table 1).

Supplemented protein diet does not change the circadian feeding pattern but does affect feeding levels.

Like all animals, flies require nutrients to support normal development and metabolism. The nutritional make-up of the diet can affect feeding behavior via specific receptors in the fly's central nervous system. For example, a gustatory receptor, Gr43a, is present in both the brain and chemosensory organs where it detects fructose, promotes feeding in starved flies, and suppresses feeding in satiated flies (Miyamoto et al., 2012). In *Drosophila* larvae, a deficiency in essential amino acids in food can induce food rejection through General Control Nonderepressible 2 (GCN2) in dopaminergic (DA) neurons of the brain, where it functions in a manner highly similar to essential amino acid sensing in mammals (Bjordal et al., 2014; Hao et al., 2005).Thus, we hypothesized that the lack of protein in the liquid FLIC food might affect the circadian feeding pattern, perhaps inducing protein searching behavior that could have masked a more consolidated daily rhythm in feeding relative to the locomotor rhythm. To investigate how the protein in the diet might affect the pattern of diurnal feeding, we compared the feeding behavior of WT flies on a sugar (10% sucrose) only food source to flies on a diet containing both sugar (10% sucrose) and protein (1% yeast extract). Both morning and evening anticipatory feeding behavior was displayed by the flies on both diets with no obvious difference in the temporal organization of feeding activity compared to flies

feed on sugar only food, indicating that presence of protein in the diet did not alter the diurnal pattern of feeding behavior (Fig. 2.7A, B). Though the temporal patterns of feeding did not differ between diets, flies fed on sugar only food displayed higher levels of feeding caused by significantly higher levels of feeding during the day (Fig. 2.7C). Thus the amount of food consumed on the protein supplemented diet was significantly less than that of the sugar-only diet, which is consistent with unpublished work from Dr. Scott Pletcher and colleagues (S. Pletcher, Personal Communication).

2.4 Discussion

The use of the FLIC apparatus allowed us to make long-term recordings of feeding behavior in flies without perturbing the circadian system. Thus we were able to characterize the circadian control of feeding for up to seven circadian cycles. By carefully setting the threshold of the FLIC system, we were able to eliminate most of non-feeding or tasting interaction between flies and food (Ro et al., 2014). Thus, we are confident that our FLIC analysis represents feeding behavior and not simply the locomotor behavior of the flies within the arena. Our results reveal that in *Drosophila*, feeding rhythms essentially mirror locomotor rhythms, rely on the same molecular clock, and, more preliminarily, appear to rely on the same populations of neurons.

We have shown that WT and commonly used genetic backgrounds of *Drosophila* display two peaks of feeding behavior around dawn and dusk under light dark (Fig. 2.1). This daily pattern of feeding is different from the previous report of one primary feeding peak at ZT0, reported by Xu and colleagues (Xu et al., 2008). This may be because our protocol did not involve the transfer of flies from labeled to unlabeled food. Our data showed that loss of function mutations in key molecular circadian clock compenents are associated with the loss of both the morning and evening anticipatory feeding bouts, indicating an essential role of the canonical molecular circadian clock in regulation of daily feeding rhythms. This conclusion was further supported by the feeding behavior of the period mutants per^{L} and per^{S}, which displayed delayed and advanced feeding peaks respectively under LD conditions and slow and fast running free-running feeding rhythms under DD, respectively (Fig. 2.4).

Using Chromatin immunoprecipitation (ChIP) tiling array assays, Abruzzi and collegeus (2011) identified ~ 1500 direct transcriptional targets of CLOCK, including the known core clock genes *vri, rim, pdp1 and per*. Thus, CLOCK appears to be a master transcriptional regulator of the genome of *Drosophila*. Our results reveal that CLOCK normally acts to suppress feeding, as the loss of *clk* function results in increased feeding, particularly at night. Thus we propose that one or more targets of *clk* normally functions to decrease feeding. The circadian regulation may

emanate from the central brain, peripheral tissues, or both. Xu and colleagues have found increased food consumption in flies lacking functional clocks in the fatbody (Xu et al., 2008), suggesting the potential involvement of peripheral clocks.

PDF is a neuropeptide that is secreted by only 16 clock neurons in brain, the ablation of which abolishes the morning anticipation of locomotor behavior and advances the evening peak of locomotor activity under LD conditions while resulting in severely weakened circadian locomotor rhythms under constant conditions (Renn et al., 1999). Here we have confirmed a similar role for PDF in the circadian regulation of feeding behavior (Fig. 2.6).

Nutrients in the diet can affect the feeding behavior of *Drosophila* (Bjordal et al., 2014; Miyamoto et al., 2012). We found that feeding is significantly lower on protein containing food compared to a sugar only diet, particularly during the day, presumably because protein serves as a satiating factor and most feeding occurs during the day (Fig. 2.7). However, the diurnal feeding pattern of *Drosophila* on diets with and without protein was nearly identical (Fig. 2.7), suggesting at least in the short-term, that the absence of protein did not affect diurnal feeding patterns, suggesting that the feeding patterns we observed using FLIC were not caused by protein seeking behavior.

Taken together, our results comprise the first chronobiological analysis of feeding behavior in *Drosophila melanogaster*, a model system that continues to inform our understanding of the mechanisms underlying circadian timekeeping in all animals. This work lays the foundation for many future experiments aimed at understanding the molecular, genetic, and neuronal underpinnings of the circadian control of feeding and metabolism.

2.5 Methods

Fly strains

Flies were reared on cornmeal-yeast-sucrose medium and maintained at 25°C under a 12- light: 12-h dark cycle. All feeding and behavior assays were carried out on male flies that were 3-7 days old. All fly strains used in this study have been described previously. These were: *Canton-S*, *yw*, w^{1118}, per^{01}(Konopka & Benzer, 1971), tim^{01}(Sehgal et al., 1994), $clock^{jrk}$ (Allada et al., 1998), pdf^{01}(Renn et al., 1999), pdfr-mutant $pdfr^{5304}$(Hyun et al., 2005). The deficiency line used for *pdf* is Df(3R) /TM3, Ser1 (Bloomington Stock Number: 1909). The deficiency line used for *clk* is Df(3L)RM5-2/TM6B, Tb+ (Boomington Stock Number: 4502).

FLIC

Fly feeding behavior monitoring and data collection were done as previously described (Ro et al., 2014). For long-term FLIC assays, 53ml of 10% sucrose liquid food was added to a 300ml KIMAX 35 glass bottle. The bottle was then sealed with laboratory stopper (FISHER CODE# 14140C). A one way male lock (Cole-Parmer 30600-05), female luer thread style with 1/4" hex to 10-32 UNF thread (Cole-Parmer 45502-60), male luer integral lock ring to 200 series barb, 1/16" ID tubing (Cole-Parmer 45505-00), and 1/4" OD x 1/16" ID tubing (Saint-Gobain Tygon S3™ E-3003 NSF-5) were assembled to form a tubing system linking the *Drosophila* Feeding Monitor (DFM) and bottle. The connecting vessel formed between the bottle and DFM kept the liquid food at constant level allowing flies to feed without the disturbances associated with changing the food.

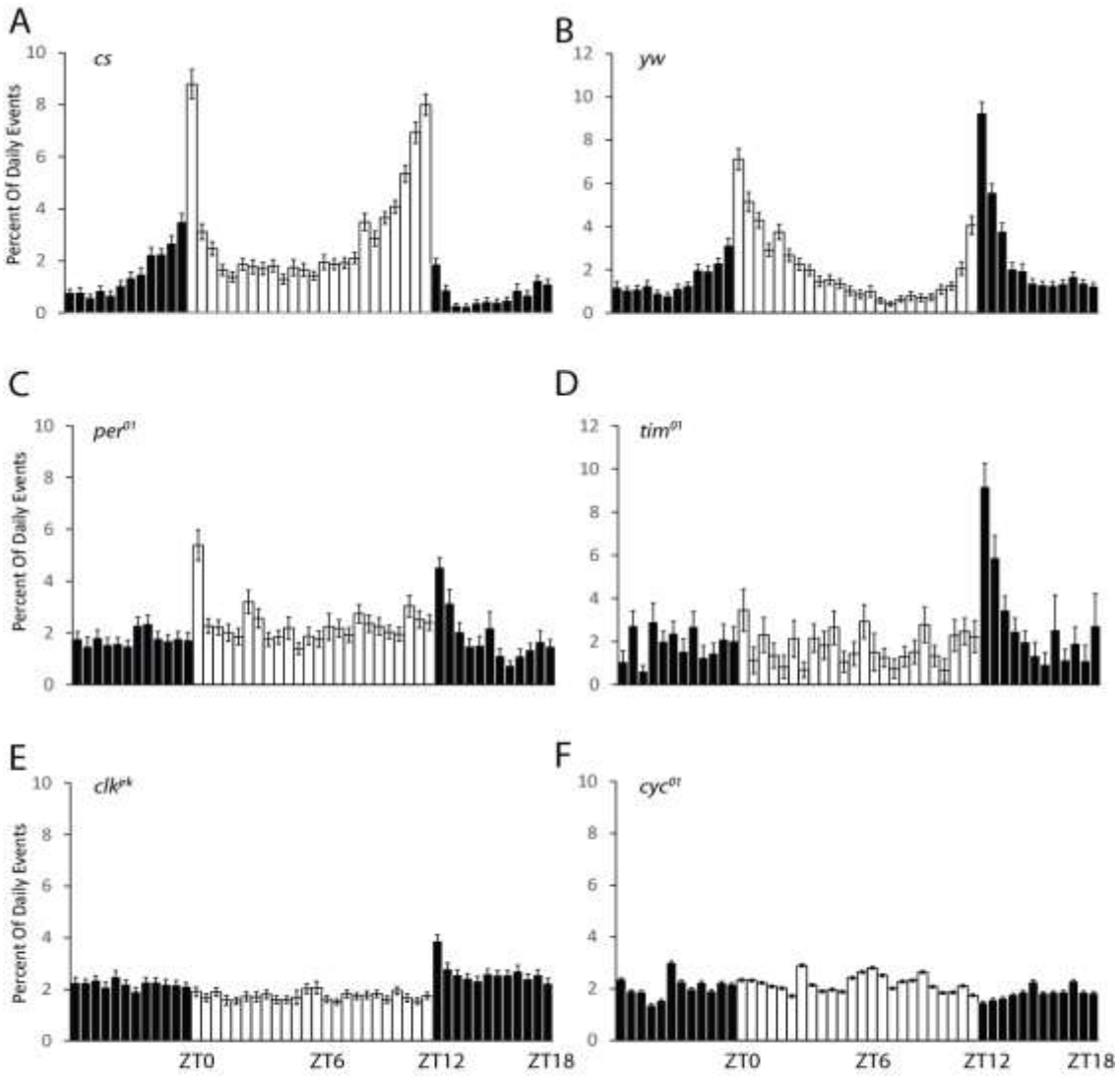
FLIC Data Analysis

When a fly extends its proboscis to feed or touches the liquid food with a leg, it completes an electrical circuit, which is registered as a voltage change which was recorded as an analog signal that ranging from 0-1023. In practice, almost all feeding events produce a signal between 0-700. We first corrected the signal by subtracting baseline signal, which was calculated by the median signal measured within five min

interval centered that time point. Because the high temporal resolution of the signal (five times in one second), feeding signals were rare in any given five min window, thus the median intensities within that five min interval can represent the background reasonably. We then compared our signal levels to the levels described for previous experiments and used their thresholds to calculate the threshold for feeding events in our setting (Ro et al., 2014). We compared the feeding signals from WT flies using different thresholds to an adaptive threshold (Ro et al., 2014) and concluded that any adjusted signal that surpassed a value of 50 should be identified as a feeding event. Feeding events were pooled into half-hour intervals. To determine if FLIC time series contained significant periodicities between 19 hours to 30 hours we employed χ-square periodogram analysis with a confidence level of 0.01 (Sokolove & Bushell, 1978). Eduction plots of feeding data in LD were smoothed by a Butterworth filter. The Butterworth filter was run in Matlab by the function code "BUTT_FILTER" attributed to Jeffery Hall Lab, Brandeis University. The peak time was calculated with the function, "findpeaks" from Signal Processing Toolbox with the facilitating code from Amy Bennet, Shafer lab.

2.6 Figures and Tables

Fig. 2. 1. *Drosophila* maintains a bimodal pattern of daily feeding that depends on the canonical circadian clock.



Average feeding activity over four days of 12 h light and 12 h dark (LD) of CantonS

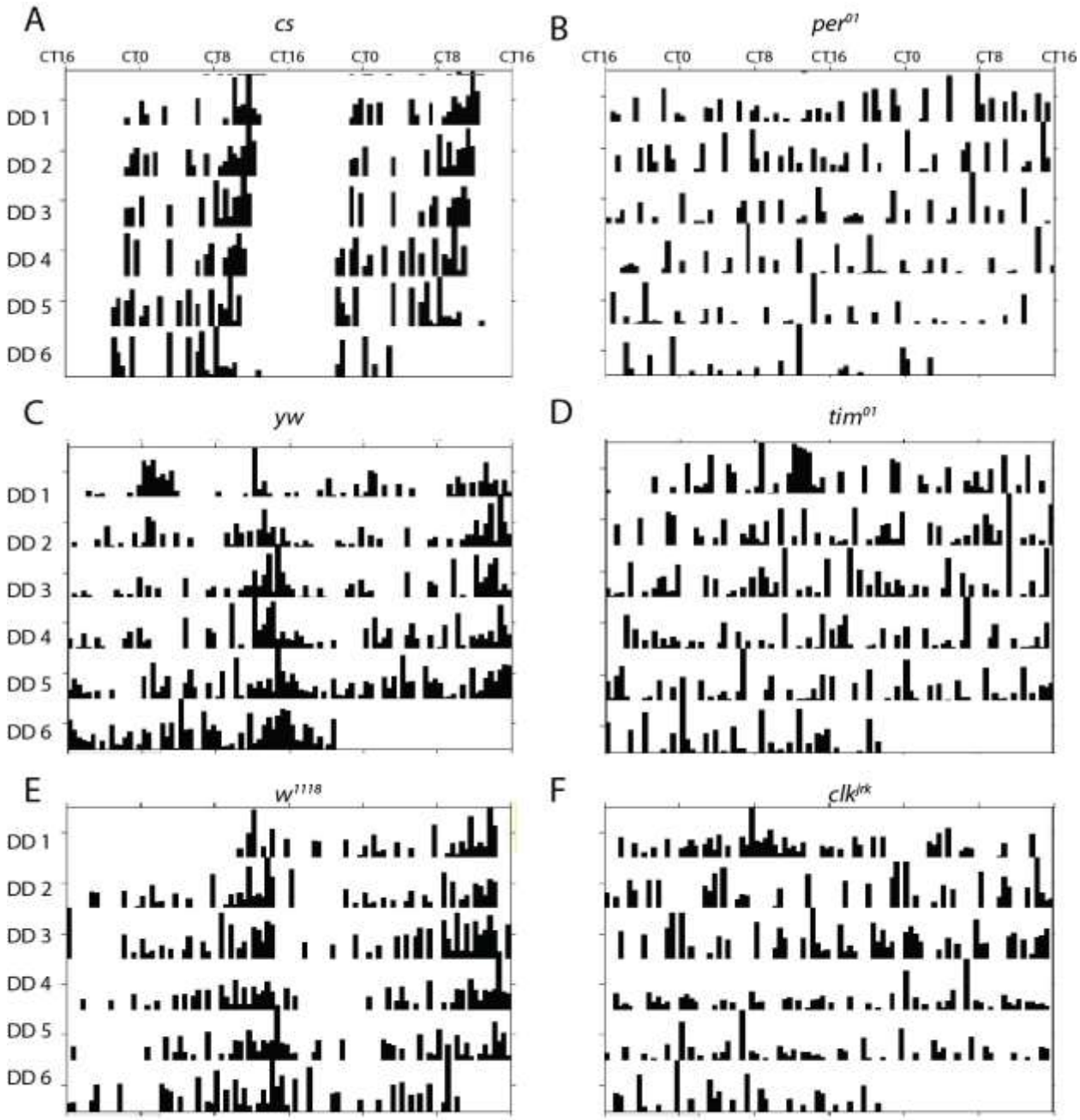
(N=59) flies (A), *per*^{01} (N=30) flies (B), *yw* (N=43) flies (C), *tim*^{01} (N=28) flies (D), *clk*^{jrk} (N=40) flies (E), and *cyc*^{01} (N=40) flies (F). For each plot, each bar represents the percentage of daily feeding events in that occurred during the half hour bin. The lights were turned on at ZT0 and off at ZT12. The lights-on condition is indicated by white bars while the lights-off condition is indicated by black bars.

Fig. 2. 2. Photo of the reservoir system for long term FLIC.

(A) *Drosophila* feeding monitor (DFM). Individual flies were kept in an arena containing a well of liquid food. Feeding behavior was registered whenever flies extended their proboscis onto the liquid food. (B) A two way valve to switch on and off the liquid food flow. (C) 1/4" OD x 1/16" ID tubing to connect the DFM and reservoir system. (D) A 300ml KIMAX 35 bottle with 53 ml of 10% sucrose water. The rubber stopper insures the seal. The tubing

between the bottle and DFM form a connecting vessel so that the liquid food can flow into the DFM from the bottle when the food level is low in the well.

Fig. 2. 3. *Drosophila* displays clock gene dependent rhythmic feeding activity under DD.



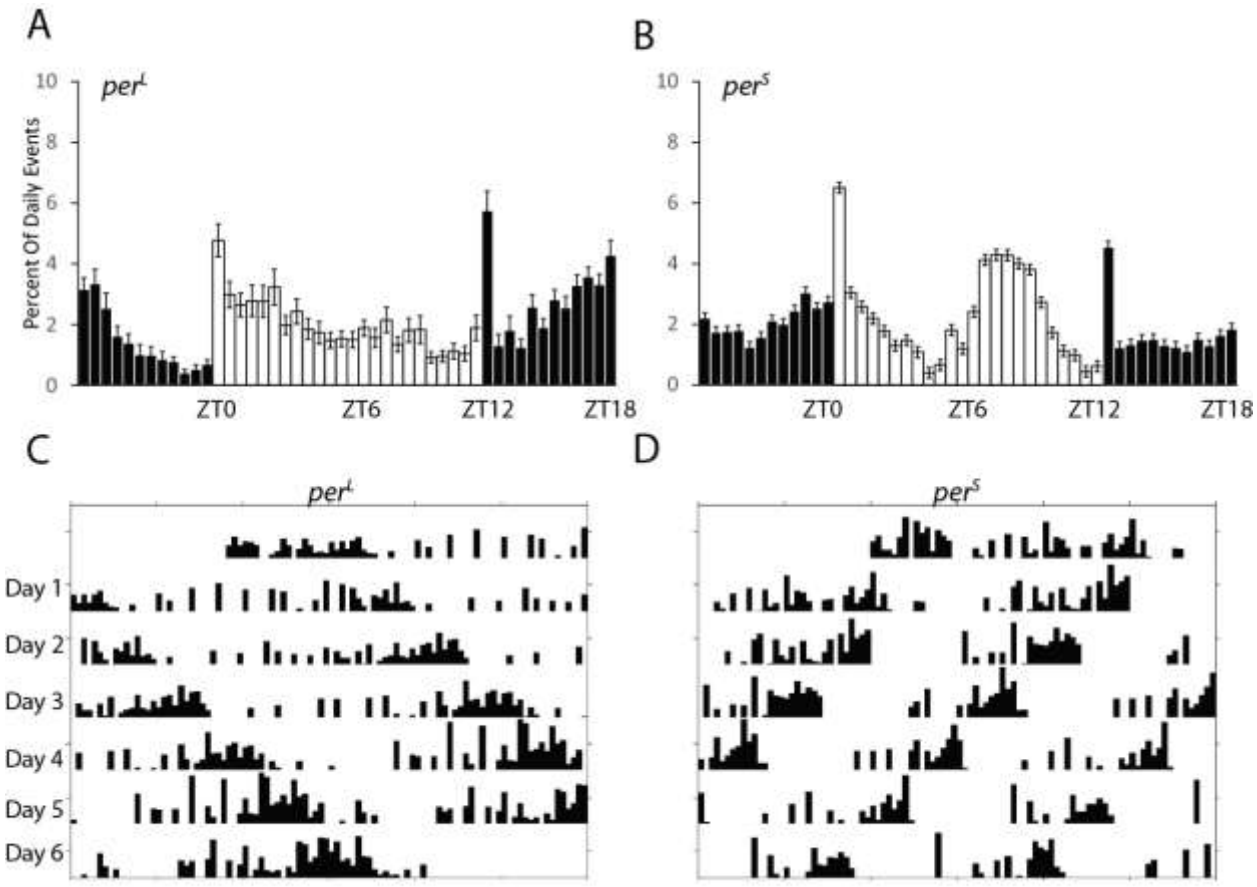
Representative feeding actograms of individual fly of CS (A), *per*^{01} (B), yw (C), *tim*^{01}(D), *w*^{1118} (E) and *clk*^{jrk} (F) flies under constant darkness are shown. For each

histogram, each bar represents the percentage of daily feeding events in that occurred during the half hour bin.

Table 1 Summary of the feeding activity under DD.

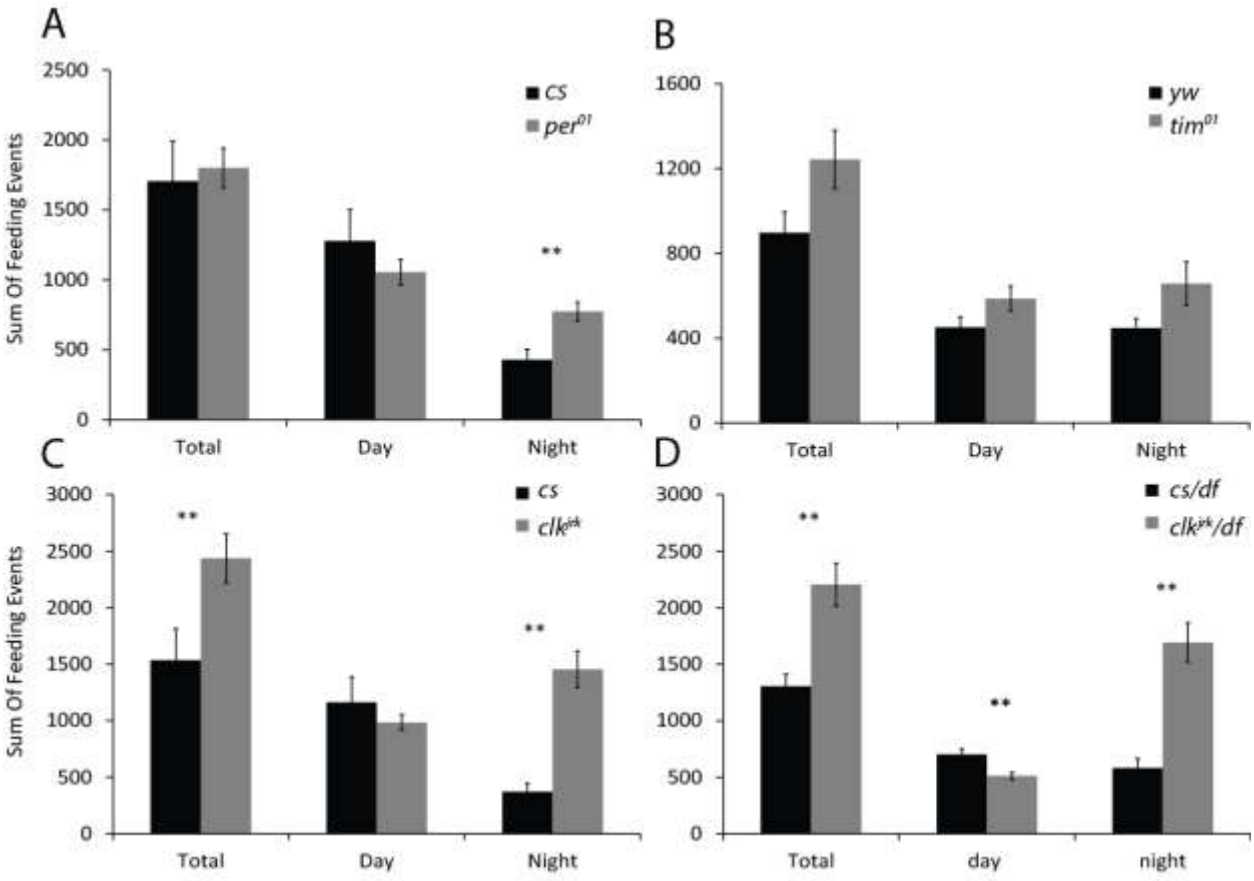
Genotype	Percent of Rhythmicity	Tau	Power	N
CS	91%	23.7±0.2	115.7±19.1	31
yw	67%	24.4±0.2	88.8±2.4	33
w^{1118}	74%	24.1±0.3	95.6±4.1	58
per^{01}	12%	22.1±0.2	78.5±8.5	31
tim^{01}	3%	22.5	80	31
clk^{jrk}	0%	//	//	29
per^{L}	58%	28.3±0.8	118.0±24.0	31
per^{S}	69%	19.3±0.1	94.1±5.3	39
pdf^{01}	24%	22.5±0.8	73.5±3.2	27
pdf^{+}	67%	23.8±0.1	97.5±5.5	29
han^{5304}	7%	23	74.8	29

Fig. 2. 4. The long- and short-period mutants display a delayed and advanced evening feeding peak, and long and short free running periods of feeding rhythms, respectively.



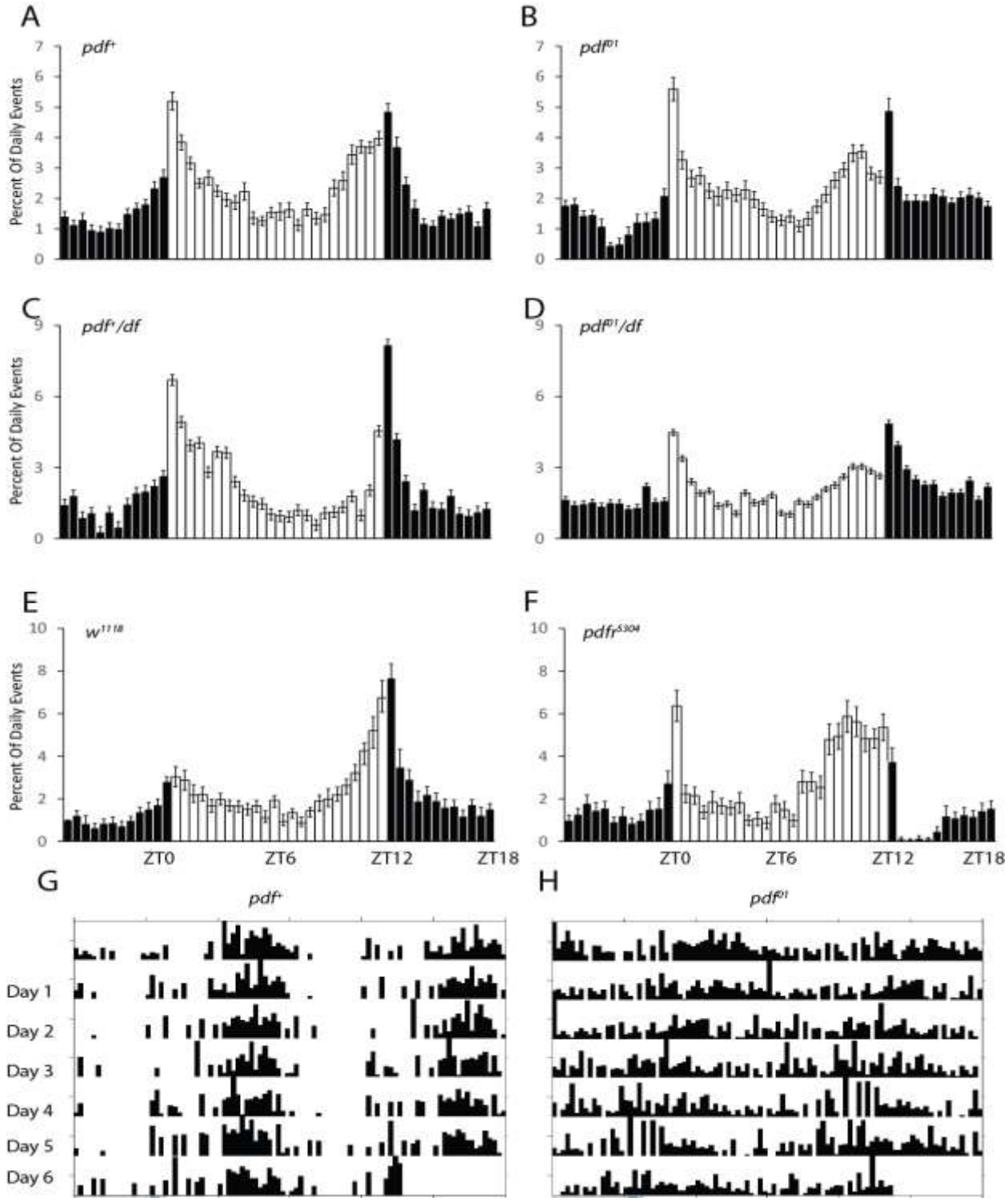
Average feeding activity under a 12:12 LD cycle of *per*^{L} (N=33) (A) and *per*^{S} (N=27) (B). Representative feeding actograms of individual fly of *per*^{L} (C) and *per*^{S} (D) flies under constant darkness are shown. For each histogram, each bar represents the percentage of daily feeding events in that occurred during the half hour bin.

Fig. 2. 5. The positive limb clock gene, *clock*, regulates food consumption.



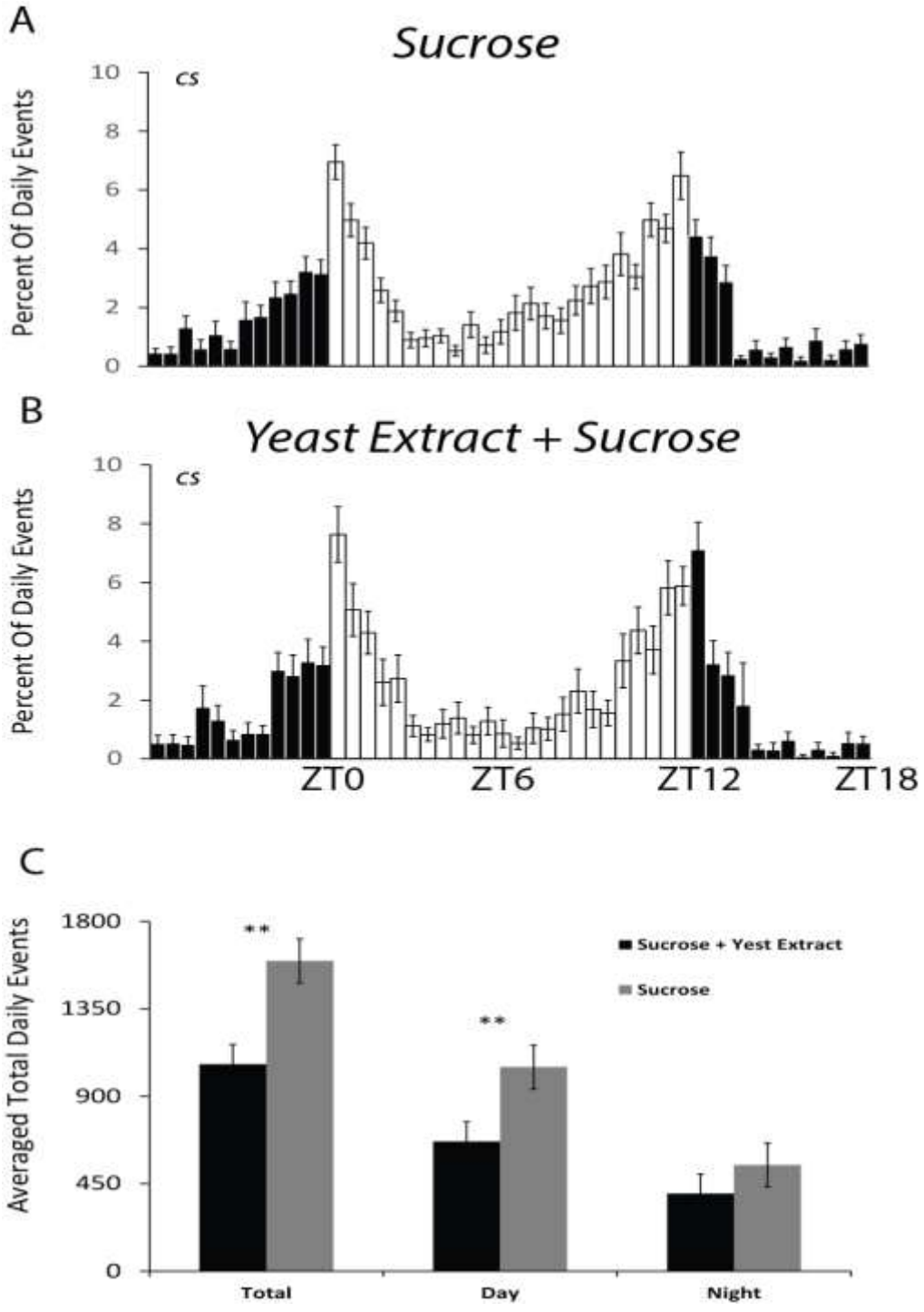
Average total feeding activity over a complete diurnal cycle (total), light phase (day) and dark phase (night) for *per*^{01} (N=30) and CantonS (N=22) flies (A), *tim*^{01} (N=26) and *yw* (N=24) flies (B), *clk*^{jrk} (N=40) and CS (N=43) flies (C), *+/df* (N=37) and *clk*^{jrk}*/df* (N=39) flies (D). In the histogram, each bar represents the number average feeding events during the indicated period of time. Error bars represent the pooled standard error. Each comparison was done by student's t test. One asterisk indicates a $P < 0.05$ while two represent a $P < 0.01$.

Fig. 2. 6. Pigment Dispersing Factor (PDF) is required for normally phased feeding peaks under LD and for strong feeding rhythms under DD.



Average feeding activity over five days of 12:12 LD) cycle of pdf^{+} (N=21) flies (A), pdf^{01} (N=24) flies (B), pdf^{+}/df (N=38) flies (C), pdf^{01}/df (N=38) flies (D), han^{5304} (N=32) flies (E), and W^{1118} (N=14) flies (F). For each histogram, each bar represents the percentage of daily feeding events in that occurred during the half hour bin. The lights are turned on at ZT0 and off at ZT12. The lights-on condition is indicated by white bars while the lights-off condition by black bars.

Fig. 2. 7. The diurnal pattern of feeding behavior is not a reflection of protein seeking behavior but protein supplementation decreases total feeding in the FLIC assay.



Average feeding activity of 12 h light and 12 h dark (LD) of wild-type CS flies on a diet of 10% liquid sucrose (N=44) (A) and on a diet of 10% sucrose plus 1% yeast extract (N=45) (B). (C) Average total feeding events and average daytime and nighttime feeding events are significantly different between sugar only diet (N=44) sugar plus yeast extract diet (N=45) in LD. (** indicates P<0.01, T- Test).

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CHAPTER 3

Exploring the roles of specific classes of clock neurons and the molecular clock in the fat body play in the regulation of feeding behavior

3.1 Abstract

The circadian clock offers an internal estimate of the external time of day and is used to precisely time behavior and physiology to promote survival and fitness. Feeding behavior is no exception. Here, using the highly sensitive and high throughput feeding monitoring system, Fly Liquid-food Interaction Counter (FLIC), we aim to further dissect the neural and cellular mechanisms by which the circadian clock regulates feeding behavior. We address whether specific subsets of circadian clocks, such as the $DN1_p$ neurons or fat body cells, are responsible for different aspects of the feeding rhythm. We find that *glass*^{60j}mutants, which lack external photoreceptors and DN1p clock neurons, lacks an evening anticipatory feeding peak while maintaining the anticipatory evening peak in locomotor activity. We found that the loss of the molecular clock in all neurons eliminates both the morning and evening anticipation of feeding behavior without changing the total amount of feeding. We also found that the elimination of molecular clock in a subset of the

$DN1_p$s does not have measurable effects on the feeding rhythm. We further investigated the role of the molecular clock in the fat body, the major glucose metabolism organ of *Drosophila*, and found no evidence that the molecular clock is required in this important metabolic tissue for normal feeding rhythms.

3.2 Introduction

The circadian clock orchestrates most daily physiological and behavioral processes including feeding time in animals. In mammals, feeding time is dependent at least partially on circadian clock (Turek et al., 2005). Though we know that feeding is regulated by a complex interplay between orexigenic and anorexigenic hormones and neuromodulators (Pool & Scott, 2014), it remains unclear that how the circadian clock regulates feeding behavior.

There is strong evolutionary conservation of many critical biological processes among flies and humans, making *Drosophila* an excellent model organism for the study of the control feeding, results from which have consistently informed our understanding of similar processes in humans. Underlying many of these conserved biological processes are homologous genes shared by humans and flies (Gustavson, Goldsborough, Ali, & Kornberg, 1996). For example, out of the pool,

287 genes are implicated in humans diseases, 178 (62%) have a homolog or functional homolog in flies (Rubin et al., 2000).

In the previous chapter, I used FLIC to systematically characterize the daily bimodal feeding pattern of wild type and commonly used laboratory strains of flies and showed that it is dependent on the core molecular clock under both a 12h:12h light/dark cycle and under constant conditions. Precisely how central circadian clocks in the brain and peripheral circadian clocks regulate circadian feeding behavior remains unknown. In this chapter, I address this question by addressing the role that different cell types and tissues play in the circadian regulation of feeding

One model of how the circadian neuronal network orchestrate distinct behavioral, endocrine, and physiological outputs predicts that different classes of clock neurons, and perhaps subsets of these classes, are capable of autonomous timekeeping and independently controlling various outputs via connections to different brain regions. Testing this hypothesis requires a reliable method to disrupt the circadian clock within specific subsets of clock neurons. As described in the previous chapter, the molecular clock of *Drosophila* consists of a negative limb including the transcriptional repressors PER and TIM, and a positive limb that includes CLK and CYC (Yu & Hardin, 2006). CLK and CYC activate the transcription of *per* and *tim* while accumulated PER and TIM translocate into nucleus to inhibit the transcriptional activity of CLK and CYC (Yu & Hardin, 2006).

In addition to the neurons of central clock neuron network, peripheral tissues also support molecular clock cycling. For example, the fat body, the fly's functional equivalent of both the liver and adipose tissue, possesses a molecular clock that is thought to control the feeding behavior and energy storage (Xu et al., 2008). We used the GAL4-UAS system to express a dominant negative form of CYC (CYCΔ) to cell autonomously abolish molecular clock cycling. This form of CYC is the result of 17 base pair deletion from the *cyc* gene that blocks the ability of CYC-CLK complexes to bind the E-box of *tim* and promote its transcritpion (Tanoue et al., 2004).

Using FLIC in combination with genetic tools, I show that the elimination of the molecular clock in neurons is sufficient to abolish feeding rhythms in LD, suggesting molecular timekeeping is required in the nervous system for normal feeding rhythms. Interestingly, we also found that the *glass*^{60j}mutant, which lacks external photoreceptors and the $DN1_p$ clock neurons, fails to display an evening anticipatory feeding peak while maintaining the anticipatory evening peak of locomotor activity. We found that the expression of CYCΔ in DNs and LN_d eliminates both the morning and evening anticipatory feeding behavior without changing the total amount of daily feeding. Furthermore, to determine if the loss of molecular timekeeping in the $DN1_p$s was responsible this phenotype, we attempted to abolish the molecular clock in a subset of DN1s and found that this manipulation

was not sufficient to produce measurable changes in the daily feeding rhythm. Finally, we investigated the role of the molecular clock of the fat body by expressing CYCΔ specifically in these cells. This manipulation did not produce any measurable change in the daily feeding rhythm or in total feeding. Taken together, our results indicate that molecular timekeeping in the PDF^{-} clock neurons are important for daily rhythms in feeding behavior, whereas circadian timekeeping is likely not required in the fat body for such rhythms.

3.3 Results

The bimodal pattern of feeding behavior requires molecular timekeeping in neurons.

In the previous chapter, we found that wild-type and commonly used genetic backgrounds exhibit anticipatory morning and evening bouts of feeding under LD cycles. To determine where molecular timekeeping is required for feeding rhythms, we examined the feeding pattern of flies in which the molecular clock was abolished in all neurons. CYCΔ, a dominant negative form CYC, lacks a DNA binding region while maintaining the ability to form a heterodimer with its binding partner CLK (Tanoue et al., 2004), thereby preventing rhythmic transcription of *per* and *tim* and stopping the molecular clock. When we used a pan-neuronal driver, *nSyb-GAL4* to

drive the expression of *uas- cycΔ*, the flies lost their morning and evening anticipatory feeding bouts in LD (Fig. 3.1B) while the control *uas-cycΔ*/+ without GAL4 flies exhibit normal anticipatory feeding peaks around dawn and dusk (Fig. 3.1A), suggesting that molecular clock cycling is required in neurons for the anticipatory feeding bouts. We also found that the flies without functional clocks in neurons were largely arrhythmic with respect to feeding under constant darkness and temperature (Fig. 3.1D) with only 9.1% of experimental flies displaying a significant periodicity in feeding behavior compared to 95.5% rhythmicity in *uas-cycΔ* control flies (Fig. 3.1C). To confirm that we did in fact abolish molecular clock cycling through CYCΔ expression, we also conducted immunocytochemistry (ICC) to examine the PER expression in a subset of central clock neurons, the sLN_vs. As shown in Figure 3.11E and F, the PER cycling was abolished in *nSyb-GAL4/uas-cycΔ* flies, confirming that our *uas-cycΔ* expression abolished molecular clock cycling.

Drosophila mutants (_galss^{60j}_) lacking external photoreceptors and DN1p clock neurons exhibit a loss of anticipatory evening feeding bouts but maintain the evening peak of anticipatory locomotion.

The *glass* gene encodes a transcription factor required for the development of external photoreceptor cells (Moses, Ellis, & Rubin, 1989). Flies deficient in *glass* (*glass*^{60j}) lack all retinal photoreceptor cells and all extra-retinal photoreceptors such as the ocelli and Hofbauer-Buchner eyelet. These mutants also lack the $DN1_p$ clock neurons (Lindsley, D.L, and Zimm, 1992; Peschel & Helfrich-Förster, 2011). The H-B eyelet is an important circadian photoreceptor located between the retina and the medulla in the fly optic lobes (Veleri, Rieger, Helfrich-Förster, & Stanewsky, 2007). Thus, *glass*^{60j} mutants lack the $DN1_p$ and all known photoreceptors except for the deep brain photoreceptor cryptochrome (CRY). To examine how the loss of the external phototreceptors and the $DN1_p$ might affect diurnal feeding patterns, we ran the FLIC assay on the null-mutant *glass*^{60j} flies under a 12:12 LD cycle. We found that the *glass*^{60j} flies exhibit a loss of the anticipatory evening feeding bouts but nevertheless maintained the evening anticipatory peak in locomotion (Helfrich-Förster et al., 2001) (Fig. 3.2A, B), demonstrating a potentially informative difference between the control of feeding behavior and locomotor behavior. Since the *glass*^{60J} mutant lacks the $DN1_p$ and the fact that the $DN1_p$ are not required for normal locomotor rhythms (Stoleru et al., 2004; Grima et al., 2004), we wondered if these neurons might be required specifically for the anticipatory evening bout of feeding.

Expression of CYCΔ in a subset of $DN1_p$s does not affect the diurnal pattern of feeding or total food consumption.

To test the hypothesis that circadian timekeeping is required in the $DN1_p$ for the anticipatory evening bout of feeding. We drove the expression of CYCΔ using the *clk4.1m-GAL4* driver, the expression of which is restricted to a subset of the $DN1_p$ (Zhang et al., 2010). *Clk4.1m-GAL4/uas-cycΔ* flies exhibit both morning and evening anticipatory bouts of feeding under LD (Fig. 3.3B) that were similar to those displayed by *uas-cycΔ* control flies (Fig. 3.3A), suggesting that the expression of CYCΔ in this subset of $DN1_p$ has no significant effects on the bimodal pattern of diurnal feeding behavior. The diurnal pattern of locomotor activity was also normal for both experimental and control flies (Fig. 3.3C and D). The total food consumption of *Clk4.1m-GAL4/uas-cycΔ* flies was not significantly different from control *uas-cycΔ/+* flies (Fig. 3.3E). These results suggest that molecular timekeeping within this subset of $DN1_p$ is not required for either the diurnal pattern of feeding behavior or the level of food consumption. However, it must be stated that in the absence of a confirmation of the successful suppression of the molecular clock in this subset of the $DN1_p$, another formal possibility that the *Clk4.1m-GAL4* element did not produce sufficiently strong CYCΔ expression to completely suppress molecular clock cycling. Furthermore, it is possible that the subset of

$DN1_p$ that do not express *Clk4.1m-GAL4* are by themselves sufficient to drive the normal diurnal pattern of feeding.

The expression of CYCΔ in the DNs and LN_d results in the loss of the normal bimodal pattern of feeding.

Based on the anatomy and function, the ~150 clock neurons are divided into nine groups. There are 4 large ventral lateral neurons (lLN_v), 4 small ventral lateral (sLN_v), the 5th sLN_v, lateral posterior neurons (LPN) and lateral dorsal neurons (LN_d); More dorsally there are three groups of neurons called dorsal neurons 1-3 (DN1-3) which can be further divided into $DN1_a$, $DN1_p$ (Almarestani et al., 2007; Helfrich-Förster et al., 2007; Taghert & Shafer, 2006). Among them, the PDF positive LN_v seem to be the critical pacemakers. Flies with LN_v ablated by apoptosis or electrically silenced display arrhythmic locomotion activity under DD (Blanchardon et al., 2001; Nitabach et al., 2002; Renn et al., 1999). Furthermore, multiple studies suggested a dual oscillator model where the LN_v serves as the morning oscillator driving the morning peak of activity, while the 5^{th} sLN_v and LN_d serves as the evening oscillator driving the evening pea of activity (D. Stoleru, 2004; Grima et al., 2004). Yao and Shafer (2014) found that the LN_d (PDF-negative clock neurons) exert measurable control over free-running activity rhythms that is

independent of PDF-positive clock neurons. Our results in the previous chapter indicated that the PDF-positive neurons are indeed important for normal feeding rhythms. However, the role the PDF-negative neurons play in regulating the daily bimodal pattern of feeding behavior has not been addressed. We therefore drove the expression of CYCΔ throughout the clock neuron network using the *Clk-GAL4* driver while silencing the expression of CYCΔ in the LN_v using *Pdf-GAL80*, which drives expression of the GAL4 inhibitor GAL80 only in PDF-expressing neurons (Gummadova et al. 2009; D. Stoleru et al., 2004). We found that flies expressing CYCΔ in PDF-negative (between *Clk856-GAL4, Pdf-GAL80*) neurons exhibit no morning or evening anticipatory bouts in either locomotor or feeding behavior compared to their controls (Fig. 3.4A-D). Interestingly, total activity and feeding were not significantly different between *Clk856-GAL4, Pdf-GAL80/uas-cycΔ* flies and the *uas-cycΔ* controls (Fig. 3.4E and F). Taken together, these results suggest that the normal bimodal pattern of feeding and locomotor behavior requires molecular clock cycling in PDF-negative neurons. These results are somewhat surprising given that the PDF negative neurons are thought to specifically govern the evening peak of locomotor activity while the PDF-positive LN_v are thought to govern morning peaks. However, given the absence of evidence that this genetic combination resulted in the loss of molecular clock cycling only in the PDF-negative

DNs and LN_d, it is also possible that the *Pdf-GAL80* element did not prevent CYCΔ expression in the LN_v thereby abolishing morning peaks in feeding and locomotion.

Expression of CYCΔ in the fat body does not affect the diurnal pattern of feeding or total food consumption.

In flies, peripheral tissues also support molecular clock cycling. The fat body, a tissue playing physiological roles equivalent of those of vertebrate fat and liver, in *Drosophila*, supports molecular clock cycling that appears to contribute to the control the feeding behavior and overall nutrition storage (Xu et al., 2008). To address the potential role of circadian timekeeping in the fat body in the regulation of the diurnal pattern of feeding behavior and total levels of food consumption, we drove the expression of CYCΔ specifically in the fat body using the *Takeout-GAL4* (*To-GAL4*) driver (Dauwalder et al., 2002). *To-GAL4/uas-cycΔ* flies exhibited normal morning and evening anticipatory feeding bouts and normal total food consumption relative to the *uas-cycΔ* control flies (Fig. 3.5A, B, C). These results suggest that molecular clock cycling might not be required in the fat body for the normal diurnal pattern of feeding. Though we examined expression pattern of GFP in *To-GAL4->uas-eGFP* flies to confirm the expression of to-GAL4 in fat body, we did not exclude the possibility that *To-GAL4/uas-cycΔ* combination failed abolish

the molecular clock completely in the fat body. Given the absence of evidence that this genetic combination resulted in the loss of molecular clock cycling in the fat body, it is therefore a formal possibility that this negative result simply reflects a failure to stop such cycling in the fat body.

To examine the potential role of molecular clock cycling in the fat body in the regulation of food consumption, we compared the level of feeding in *To-GAL4/uas-cycΔ* flies to that of *uas-cycΔ* control flies. These two genotypes exhibit similar levels of food consumption.

3.4 Discussion

Using the GAL4-UAS system, we found that the flies without a functional molecular clock in the nervous system fail to display the normal bimodal feeding pattern in LD and feeding rhythms under DD conditions (Fig. 3.1), suggesting the necessity of molecular clock cycling in the brain for normal diurnal and circadian feeding behavior. By combining the GAL4-UAS system and FLIC assay, we have established a method of interrogating the role that specific clock neurons and tissues play in the regulation of feeding over diurnal and circadian cycles.

Indeed, overexpression of CYCΔ in PDF-negative clock neurons (driven by *clk856-GAL4, pdf-GAL80*), the flies lost both their morning and evening anticipatory

feeding bouts, suggesting that the PDF-negative clock neurons play important roles in the orchestration of feeding behavior. However, the possibility cannot be excluded that the genetic manipulation also affected the molecular clock in PDF-positive clock neurons due to an incomplete silencing of CYCΔ expression. Further experiments on the PDF-positive neurons should be carried out the exclude this alternative explanation.

Our results, including those described in the previous chapter, suggest that the feeding rhythm of *Drosophila* is temporally indistinguishable from the activity rhythm, suggesting that activity rhythms and feeding rhythms may be governed by the same genetic and neural mechanisms. However, the finding that *glass*^{60j} mutant flies displayed a clear discrepancy between the diurnal pattern of locomotor and feeding behaviors suggest that some aspects of these two rhythms may depend on independent regulatory mechanisms. It is also possible that the differences we observed between locomotion and feeding behaviors in the *glass*^{60j} mutant might simply be a reflection of the different environments in which these two behaviors were recorded, the FLIC assay for feeding and *Drosophila* activity monitors for locomotion. A video system that independently tracks locomotor activity during FLIC assay experiments would address this possibility. Though the diurnal feeding pattern of the *glass*^{60j} mutant is interesting, it is difficult to interpret because of the syndrome of phenotypes displayed by this mutant. For example, the *glass* gene is

required for the normal function of the ring gland, which is a major neuroendocrine center controlling many physiological processes, including glucose sensing and hormonal regulation (De Velasco, Shen, Go, & Hartenstein, 2004; Kim & Rulifson, 2004).

We used the GAL4/UAS mediated disruption of molecular clock cycling to address where circadian timekeeping is required for normal patterns of daily feeding, I did not observe any significant effects of CYCΔ in a subset of DN1 (driven by *Clk4.1m-GAL4)* or the fat body (driven by *To-GAL4*). One formal possibility is that *Clk4.1m-GAL4* and *To-GAL4* were not sufficiently strong to stop the molecular clock. To address this possibility, we should use ICC of PER level at different time points through LD to determine if the cycling of the molecular clock was stopped when CYCΔ expression was driven using these drivers.

3.5 Methods

Fly strains

Flies were reared on cornmeal-yeast-sucrose medium and maintained at 25°C under a 12- light: 12-h dark cycle. All feeding and behavior assays were carried out on male flies that were 3-7 days old. All fly strains used in this study have been described previously. These were: *uas-cycΔ* (Tanoue et al. 2004), *nSyb-GAL4*

(generated by *Julie Simpson;* Bloomington Stock # 51635), *Clk4.1m-GAL4* (Zhang et al., 2010), *To-GAL4* (Dauwalder et al., 2002), *Clk856-GAL4* (Gummadova et al., 2009), *Pdf-Gal80* (D. Stoleru, 2004).

FLIC

Fly feeding behavior monitoring and data collection were done as previously described (Ro et al., 2014). For long-term FLIC assays, 53ml of 10% sucrose liquid food was added to a 300ml KIMAX 35 glass bottle. The bottle was then sealed with laboratory stopper (FISHER CODE# 14140C). A one way male lock (Cole-Parmer 30600-05), female luer thread style with 1/4" hex to 10-32 UNF thread (Cole-Parmer 45502-60), male luer integral lock ring to 200 series barb, 1/16" ID tubing (Cole-Parmer 45505-00), and 1/4" OD x 1/16" ID tubing (Saint-Gobain Tygon S3^{TM} E-3003 NSF-5) were assembled to form a tubing system linking the *Drosophila* Feeding Monitor (DFM) and bottle. The connecting vessel formed between the bottle and DFM kept the liquid food at constant level allowing flies to feed without the disturbances associated with changing the food.

FLIC Data Analysis

When a fly extends its proboscis to feed or touches the liquid food with a leg, it completes an electrical circuit, which is registered as a voltage change which was recorded as an analog signal that ranging from 0-1023. In practice, almost all feeding events produce a signal between 0-700 We first corrected the signal by subtracting baseline signal, which was calculated by the median signal measured within five min interval centered that time point. Because the high temporal resolution of the signal (five times in one second), feeding signals were rare in any given five min window, thus the median intensities within that five min interval can represent the background reasonably. We then compared our signal levels to the levels described for previous experiments and used their thresholds to calculate the threshold for feeding events in our setting (Ro et al., 2014). We therefore considered any adjusted signal that surpassed a value of 50 to be a feeding event. Feeding events were pooled into half-hour intervals. To determine if FLIC time series contained significant periodicities between 19 hours to 30 hours we employed χ-square periodogram analysis with a confidence level of 0.01 (Sokolove & Bushell, 1978).

Immunocytochemistry

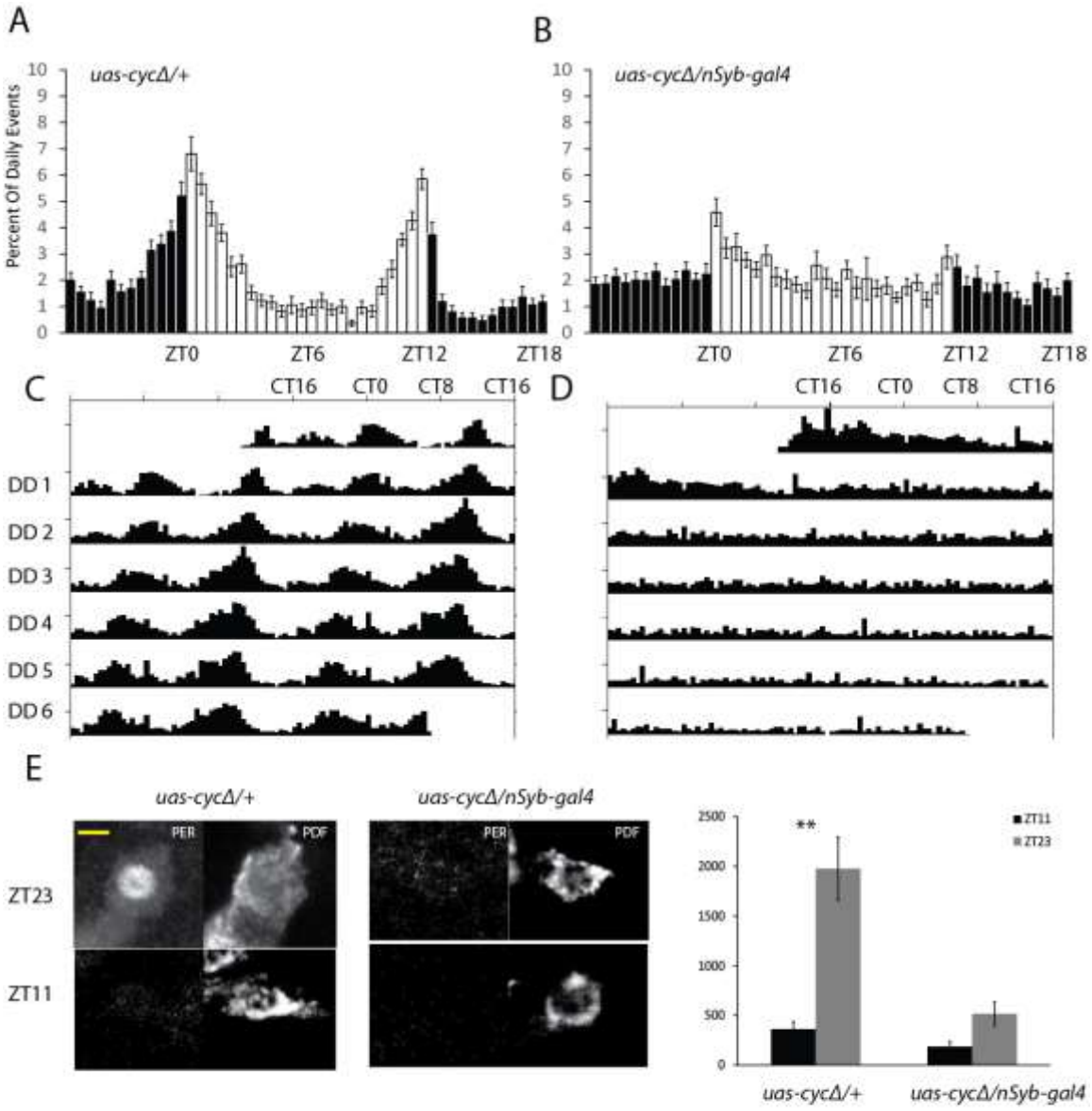
The immunocytochemistry methods used in this chapter have been previously described (Shafer et al. 2006). Fly brains were dissected in ice-cold Ca^{2+} - free

Drosophila Ringer's solution (182 mM KCl, 46 mM NaCl, 10mM Tris, pH 7.2) and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for one hour at room temperature. Brains were then rinsed with PBS with 0.3% Triton X-100 (PBS-TX), and blocked with 3% normal goat serum in PBS-TX for one hour at room temperature, After a brief rinse with PBS-TX, brains were incubated with primary antibodies for two nights at 4°C. Rat anti-PER antibody was used at a dilution of 1:500 (Liu et al., 1988) (Rat anti-PER was provided by Dr. Michael Rosbash, Brandeis University, Waltham, MA, USA). Mouse monoclonal anti-PDF was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa, and diluted at 1:200. After five 15-minute washes with PBS-TX, brains were incubated in 1:1000 dilutions of Alexa Fluor conjugated secondary antibodies (Invitrogen, Grand Island, NY, USA) at 4°C overnight. Brains were rinsed five times for 15-min followed by two washes of PBS. Brains were mounted on poly-L-lysine-coated cover slips with Fluoromount-G (Southern Biotech, CAT NO 0100-1). We imaged brains on an Olympus Fluoview 1000 confocal microscope with a 60X/1.10 NA water immersion objective (Olympus, Center Valley, PA, USA). Comparison of PER immune-signals between genotypes were based on images captured during the same imaging session with all settings held constant between genotypes for the same class of clock neurons. PER immune-signal intensity was

quantified using Imgage J software (National Institutes of Health, USA) as previously described (Shafer et al., 2002).

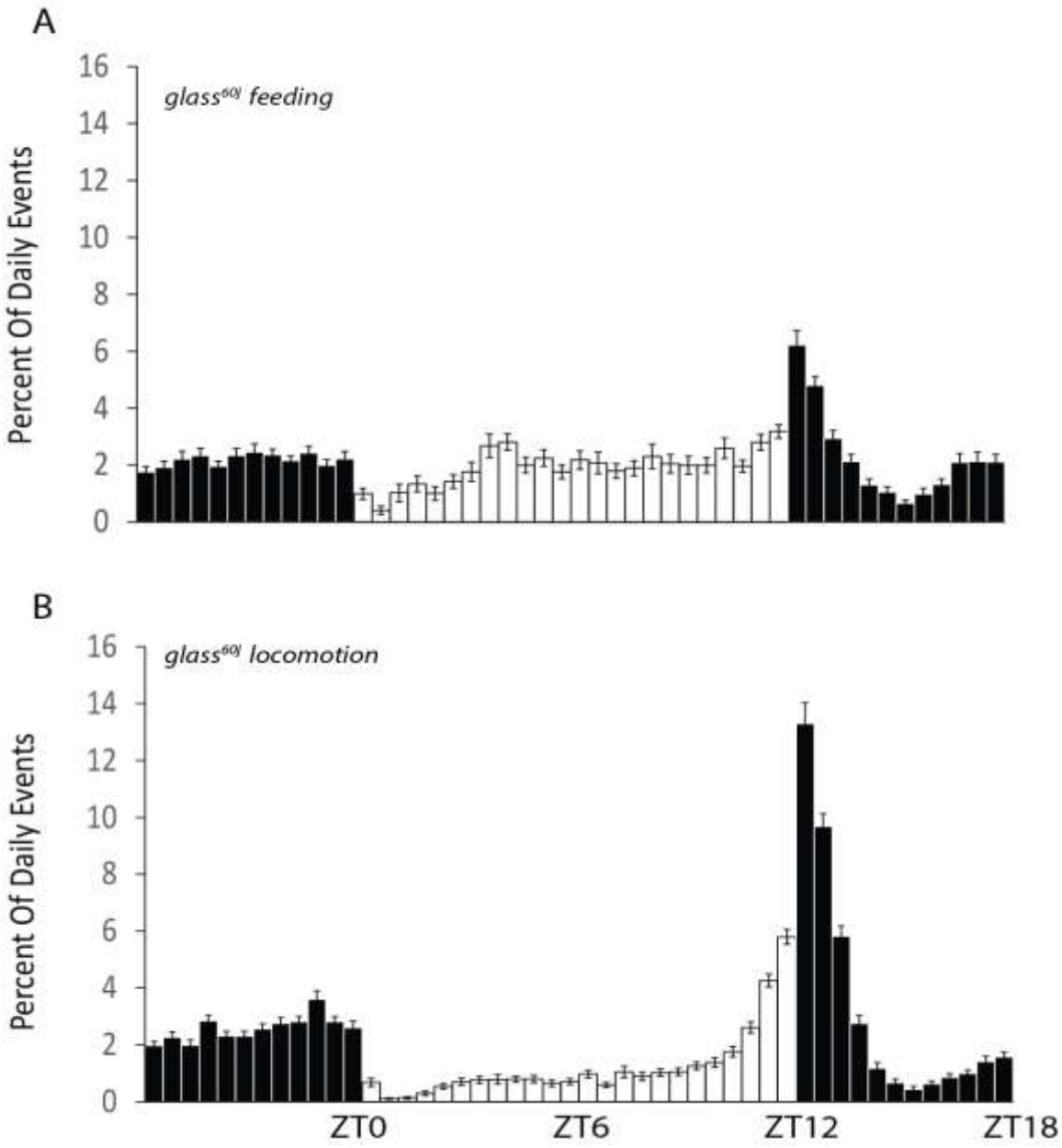
3.6 Figures

Fig. 3. 1. The bimodal pattern of feeding behavior requires molecular clock cycling in neurons.



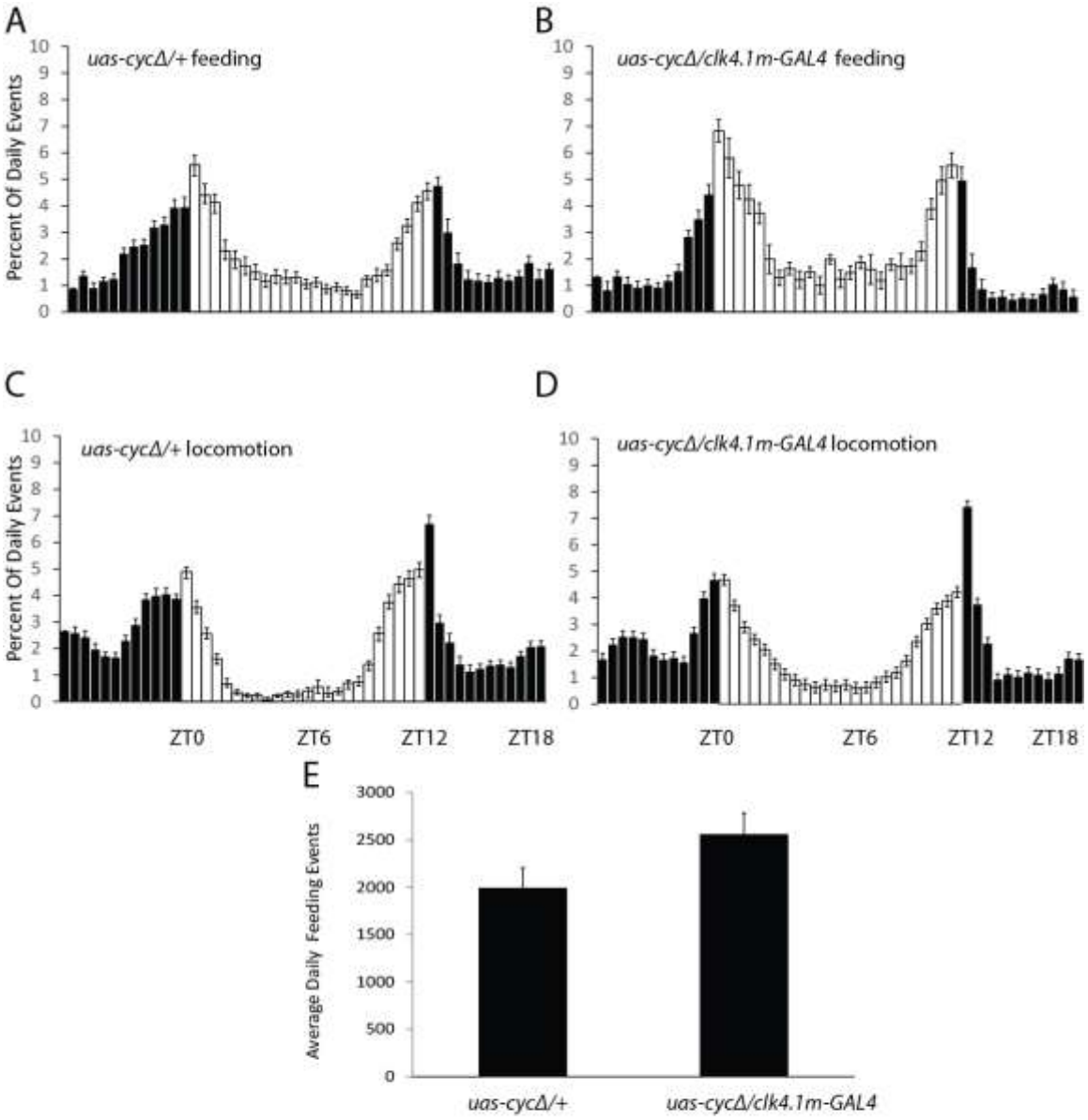
Average feeding activity over four days of 12 h light and 12 h dark (LD) for *uas-cycΔ/+* (N=36) flies (A) and uas-*cycΔ/nSyb-GAL4* (N=37) flies (B). For each plot in A and B, each bar represents the percentage of daily feeding events in that occurred during the half hour bin. The lights were turned on at ZT0 and off at ZT12. The lights-on condition is indicated by white bars while the lights-off condition is indicated by black bars. Representative feeding actogram under constant darkness and temperature for uas-*cycΔ/+* flies (C) and uas-*cycΔ/nSyb-GAL4* flies (D). (E) Representative micrographs of immunostaining of PDF and PER proteins expression in PDF-positive sLN_vs dissected at ZT11 and ZT23 of *uas-cycΔ/+* and *uas-cycΔ/nSyb-GAL4*. (F) Quantification of PER immunostaining intensity within sLN_vs of *uas-cycΔ/+* (ZT23, N=7; ZT11, N=10) and *uas-cycΔ/nSyb-GAL4* (ZT23, N=7; ZT11, N=7). The scale bar is 5 μm. Each comparison was done by student's t test. Two asterisks represent a $P < 0.01$.

Fig. 3. 2. *glass*^{60j} mutants exhibit a loss of evening feeding anticipatory bouts while maintaining the evening locomotor anticipatory peak.



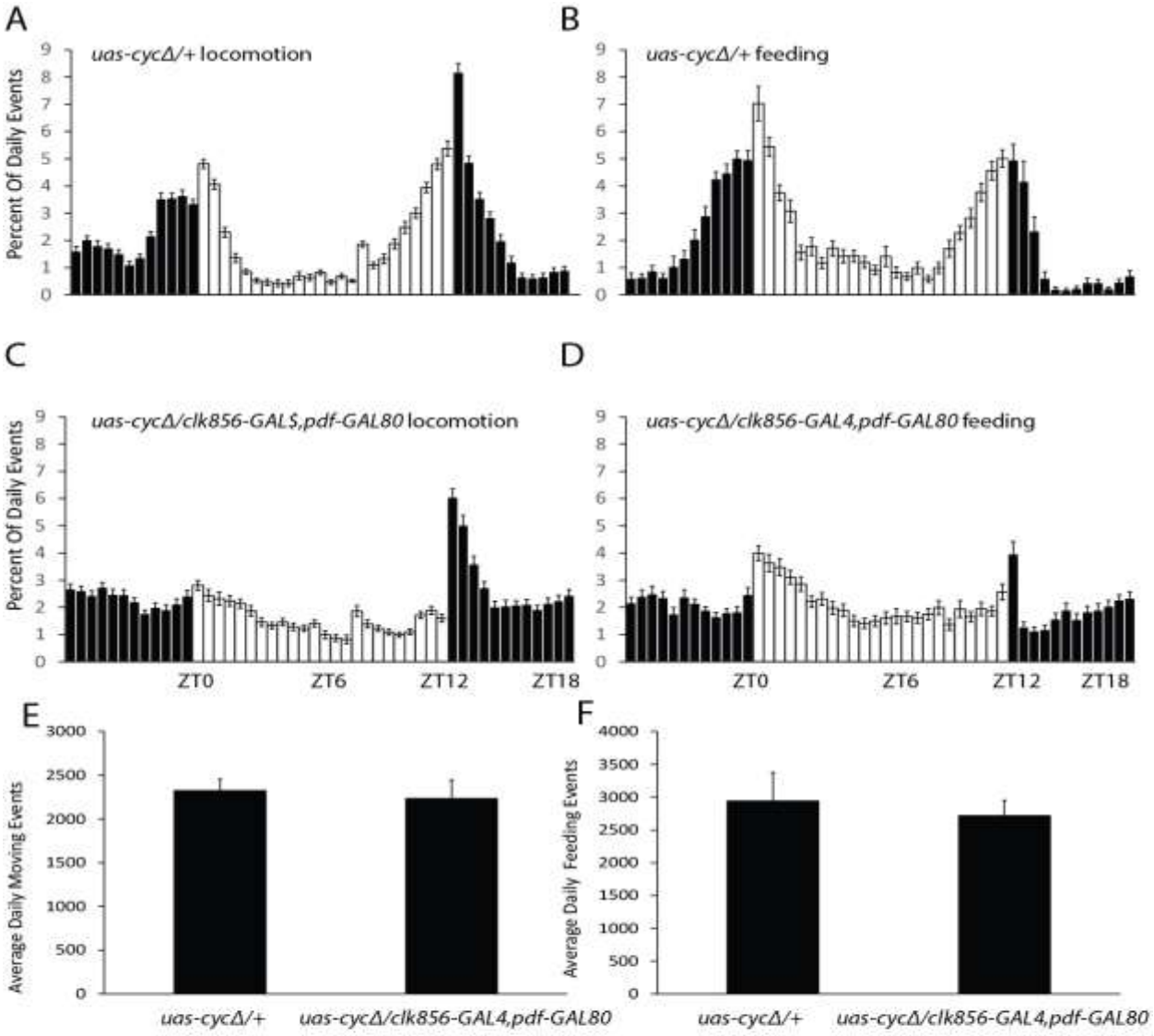
(A) Average feeding activity over four days of 12 h light and 12 h dark (LD) of *glass*^{60j} mutants (N= 49). (B) Average locomotion activity over four days of 12 h light and 12 h dark (LD) for *glass*^{60j} mutants (N=38) (Helfrich-Förster et al., 2001).

Fig. 3. 3. Expression of CYCΔ in a subset of the $DN1_p$ does not alter the diurnal pattern of feeding behavior or the levels of food consumption.



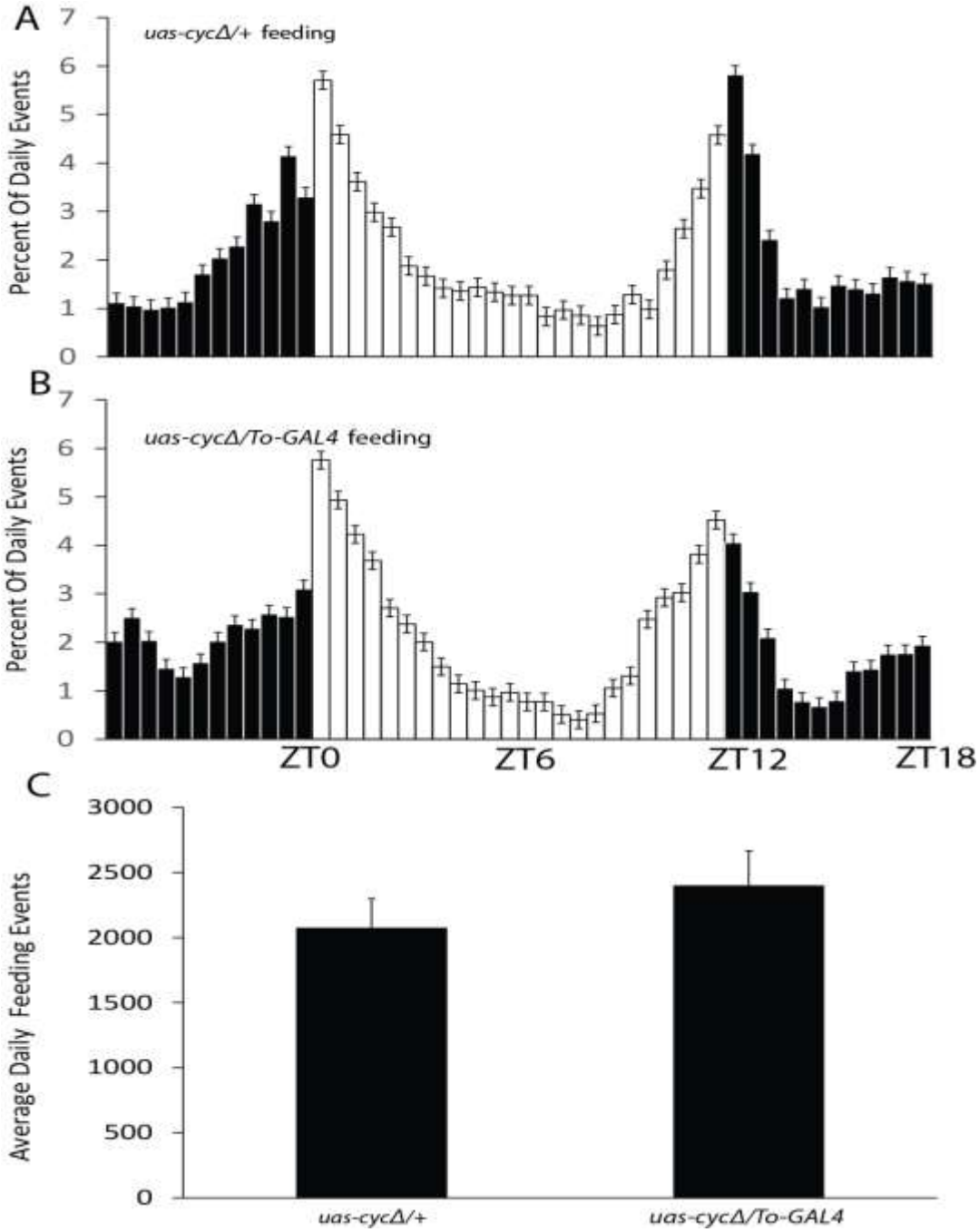
(A to D) Average feeding activity over four days of 12 h light and 12 h dark (LD) of *uas-cyc∆/+* flies (N=29) (A) *and uas-cyc∆/clk4.1m-GAL4* flies (N=30) (B). Average locomotor activity over four days of 12 h light and 12 h dark (LD) of *uas-cyc∆/+* flies (N=31) (C) and *uas-cyc∆/clk4.1m-GAL4* flies (N=31) (D). (E) Comparison of average total feeding events over a complete light dark day between *uas-cyc∆/+* flies (N=29) and *uas-cyc∆/clk4.1m-GAL4* flies (N=30). Each comparison was done by student's t test. No significant differences were found. No significant differences were found.

Fig. 3. 4. The expression of CYCΔ in PDF-negative neurons results in the loss of anticipatory morning and evening bouts of feeding activity under light dark cycles.



(A) Average locomotion activity over four days of 12 h light and 12 h dark (LD) of *uas-cyc∆/+* flies (N=28). (B) Average feeding activity over four days of 12 h light and 12 h dark (LD) of *uas-cyc∆/+* flies (N=23). (C) Average locomotion activity over four days of 12 h light and 12 h dark (LD) of *uas-cyc∆/clk856-GAL4, Pdf-GAL80* flies (N=28). (D) Average feeding activity over four days of 12 h light and 12 h dark (LD) of *uas-cyc∆/clk856-GAL4,Pdf-GAL80* flies (N=34). (E, F) Comparisons of average total locomotion (E) and feeding (F) activities over a complete light dark cycle between *uas-cyc∆/+* flies and *uas-cyc∆/clk856-GAL4,Pdf-GAL80* flies. Each comparison was done by student's t test. No significant differences were found.

Fig. 3. 5. The expression of CYCΔ in the fat body has no measurable effect on anticipatory morning and evening bouts of feeding activity under light dark cycles.



(A) Average feeding activity over four days of 12 h light and 12 h dark (LD) of *uas-cycΔ/+* flies (N=47). (B) Average feeding activity over four days of 12 h light and 12 h dark (LD) of *uas-cycΔ/To-GAL4* flies (N=45). (C) Comparisons of average total feeding events over a complete light dark cycle between *uas-cycΔ/+* flies (N=47) and *uas-cycΔ/To-GAL4* flies (N=45). Each comparison was done by student's t test. No significant differences were found.

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CHAPTER 4

The circadian clock regulates circulating trehalose in *Drosophila* under restricted feeding conditions

4.1 Abstract

In humans, daily feeding induces an increase of plasma glucose. However, how the bimodal feeding pattern affect the circulating trehalose in *Drosophila* is unknown. To answer this, we measured the level of circulating trehalose at different time points in LD. We found that the abundance of hemolymph (blood) trehalose (the predominant circulating sugar in flies) displays circadian clock dependent regulation in a restricted feeding paradigm, which might be under the regulation of clock gene, *per*.

4.2 Introduction

Monosaccharides are taken in by the fat body, the fly's functional equivalent of the mammalian liver and adipose tissue, which catalyzes the formation of glycogen for energy storage or the glucose-glucose disaccharide, trehalose, which is subsequently released into the hemolymph (Reyes-DelaTorre, Pena-Rangel, & Riesgo-Escovar, 2012). Trehalose is the main sugar form in hemolymph to allow

passive influx of dietary monosaccharides while maintaining a high level of circulating disaccharide (Klowden, 2013). It also stabilizes the high amounts of peptides, free amino acids, and proteins present in the hemolymph because of trehalose's slow rate of hydrolysis (Reyes-DelaTorre et al., 2012). Because it is the fly's predominant form circulating sugar, the level of trehalose within the hemolymph is a good indicator of the metabolic state of flies. Previous work has established the presence of postprandial changes in trehalose ~4 hour after the morning feeding peak when measured in whole fly extracts (Seay & Thummel, 2011). The measurement of trehalose from whole fly extracts includes the trehalose within the fat body which likely masked the contribution of hemolymph trehalose to post-prandial changes (Chang, 2012).

. To establish how bimodal feeding impacts fly physiology, we have employed a new method to draw hemolymph directly from a large number of flies (Tennessen, Barry, Cox, & Thummel, 2014) in order to directly measure circulating trehalose and compare the diurnal pattern of trehalose to the timing of feeding in the fly. We found that the hemolymph trehalose of wild-type flies display no obvious daily rhythms when flies are fed *ad libitum*. However, the use of a restricted feeding paradigm revealed a circadian clock dependent regulation of circulating trehalose, suggesting that the clock is required for the maintenance of steady blood sugar in the face of temporally restricted feeding.

4.3 Results

Levels of circulating trehalose level are regulated by the circadian clock gene *per* under a restricted feeding paradigm.

Feeding behavior leads to the uptake, metabolism, and storage of nutrients. In mammals, an increase in blood glucose is observed following a meal within two hours of eating (Cauter & Polonsky, 1997). Seay and Thummel (2004) examined trehalose levels of whole body extracts of flies and found that they increased significantly approximately 4 hours after what they considered the major feeding peak of the day: the morning peak of feeding determined by timed exposure to radioactively labeled food. However, as stated above, trehalose from whole body extracts may not be expected to faithfully reflect the level of circulating trehalose. Therefore, we extracted the hemolymph at various times of the day from flies which had been entrained to 12:12 LD cycle for three days and measured trehalose and glucose levels in the extracted hemolymph (see methods). Under *ad libitum* feeding conditions, we found that wild-type CS flies maintained steady tehalose and glucose levels in the hemolymph. The absence of daily peaks of circulating sugar suggested that flies maintain steady levels of circulating sugar despite maintaining two daily peaks of feeding at dawn and dusk (Fig. 2.8A). We observed a similar profile of hemolymph sugar in per^{01} mutant flies (Fig. 2.8A, B).

We speculate that postprandial hemolymph sugar changes during or after feeding peaks are buffered by the rapid conversion of circulating sugar into fat or glycogen for storage within fat-body or muscle cells. In fact, a significant increase of glycogen was observed after the morning feeding peak of feeding by Seay & Thummel (2011). We wondered if the wild-type flies would be capable of maintaining similarly constant levels of circulating sugar when challenged with time restricted feeding, which might lead to the daily depletion of calorie stores if feeding windows were sufficiently narrow. We introduced a feeding paradigm consisting of a one-hour feeding window during the last hour of a 12h day under LD 12:12 conditions. In these experiments, flies were kept on an agar substrate for twenty three hours a day and transferred onto cornmeal-yeast-sucrose food for one hour a day, from ZT 11 – ZT12, for 3 days. Hemolymph was extracted every six hours on the 4th day of restricted feeding. Under this restricted feeding paradigm, we found that wild-type flies were able to maintain constant levels of circulating sugar for at least 12 hours following the feeding window with a significant drop in circulating sugar five hours before the feeding window, i.e., 18 hours following the previous feed (Fig. 2.8C). These results suggested the homeostatic control of circulating sugar followed, 18 hours later, by the depletion of caloric stores. In contrast, per^{01} mutant flies displayed a single peak in circulating sugar immediately after the feeding window with relatively low trehalose levels comparable to the trough levels

of the wild-type control at the remaining time points. These results are consistent with the notion that the circadian clock is involved in the proper homeostatic control of circulating sugar in the context of restricted daily high amplitude daily feeding.

4.4 Discussion

In humans, the circadian clock regulates circulating glucose levels throughout the day, producing a regular rhythm in both blood sugar abundance and in post-prandial increase in circulating sugar (Saad et al., 2012). Furthermore, plasma glucose levels experience slight post-prandial increases following meals, falling back to baseline within two hours (Cauter & Polonsky, 1997). Similarly in *Drosophila*, Seay and Thummel (2011) found that following the four hours coinciding with the morning feeding peak (determined using the tracer method), whole body trehalose was increased significantly. However, their trehalose assay was likely not an accurate measurement of circulating trehalose (i.e., the equivalent of mammalian blood sugar) because it would have also sampled the trehalose present in fat body and other tissues in addition to that contained in the hemolymph. We therefore utilized a newly developed assay to directly extract the hemolymph from large amount of flies and biochemically assayed its sugar levels (Tennessen et al., 2014). Interestingly, we detected no significant rhythms in the abundance of

hemolymph trehalose in either the wild type flies or *per*^{01} mutants (Fig. 2.5A and B). We speculate that the absence of postprandial trehahlose peaks in wild-type flies is a reflection of the fact that trehalose level is tightly regulated by the fly. Dietary sugars can be converted to circulating trehalose, thereby causing increases in circulating sugar, or it can be converted for storage in the form of glycogen or triglycerides (Reyes-DelaTorre et al., 2012). It was not surprising to see a lack of sugar abundance in the hemolymph of *per*^{01} mutants, since these flies do not display rhythms in feeding (Fig. 2.1C).

In an attempt to overcome the potent homeostatic control of circulating sugar abundance in flies and to further gauge the influence of the circadian clock on metabolic state, we challenged wild-type flies with time restricted feeding. We placed flies under a restricted feeding paradigm where in flies were given access to food for only one hour between ZT 11 and ZT 12, in an effort to deplete stored carbohydrate daily without starving the flies. Under these conditions, wild type flies displayed relatively stable hemolymph trehalose levels following the feeding window, maintaining post-prandial sugar levels for around 18 hours before a significant fall was detected. In contrast, *per*^{01} mutants displayed significantly higher hemolypmh trehalose levels immediately following feeding compared with low hemolymph levels at all other time points. We hypothesize that the circadian clock plays an important role in regulating circulating sugar under conditions of time

restricted feeding, and perhaps normally in the presences of the normal bimodal pattern of feeding. It will be interesting to investigate if circadian timekeeping in peripheral organs such as fat body is responsible for such metabolic regulation (Xu et al., 2008; Erion et al., 2016).

4.5 Methods

Trehalose measurements

Our method was a modification of previous work from Tennessen and colleagues (2014). Flies were reared at 25 degrees Celsius under a 12-hour-light/12-hour-dark cycle. At the corresponding time points, 80 male flies were collected and placed in a DNA collecting tube. The DNA filtering layer was removed from DNA collecting tube (Zymo-Spin^{TM} IIIC) leaving only the glass wool. Then the tube was centrifuged at 9,000g for 5 min at 4 ° C to collect the hemolymph from 80 male flies. 0.5µl hemolymph was drawn into 99.5 µl ice cold Trehalase Buffer (TB) (5mM Tris PH6.6, 137 mM NaCl, 2.7 mM KCl). The buffer containing the hemolymph was then heated at 70 ° C for 5 min to denature the trehalase. We separated the 100µl of diluted hemolymph into two 50 µl aliquots in two 1.5 ml tubes. One aliquot was added to 50 µl trehalase (Sigma:T8778-5UN 3 µl to 1ml TB) to catalyze the trehalose into glucose for measurement. The other aliquot was added to 50 µl TB to determine free glucose. Glucose enzymatic assay described below determined the total glucose

concentration from trhalose and free glucose of the first aliquot and free glucose concentration of the other aliquot, thus enabling the calculation of the trehalose concentration in hemolymph by subtracting the subtracting the free glucose from the total glucose.

Glucose standards were made by diluting 16 µl of 1mg/ml glucose stock solution with 84 µl TB to generate a 0.16 mg/ml standard. A series of 2- fold serial dilutions of the 0.16 mg/ml standard using TB was conducted to generate 0.01, 0.02, 0.04, 0.08, and 0.16 mg/ml glucose standards. Trehalose standards were made by diluting 16 µl of 1 mg/ml trehalose (Sigma; 90208) with50 µl trehalase (TS) and 34 µl TB for 0.16 mg/ml. A series of 2-fold serial dilutions were conducted of the 0.16 mg/ml standard using 1:1 mix of TB and TS to generate 0.01, 0.02, 0.04, 0.08, and 0.16 mg/ml trehalose.

Both the glucose and trehalose standards were incubated with the samples at 37°C for 24 hours. After the incubation, we transfered 30 µl of each sample and each standard into an individual well of a 96-well plate. We added 100 µl glucose assay reagent to each well (Sigma-Aldrich G3293) and incubated it at room temperature for 15 min. Spectrometric readings at 340 nm were then used to assess the amount of glucose product.

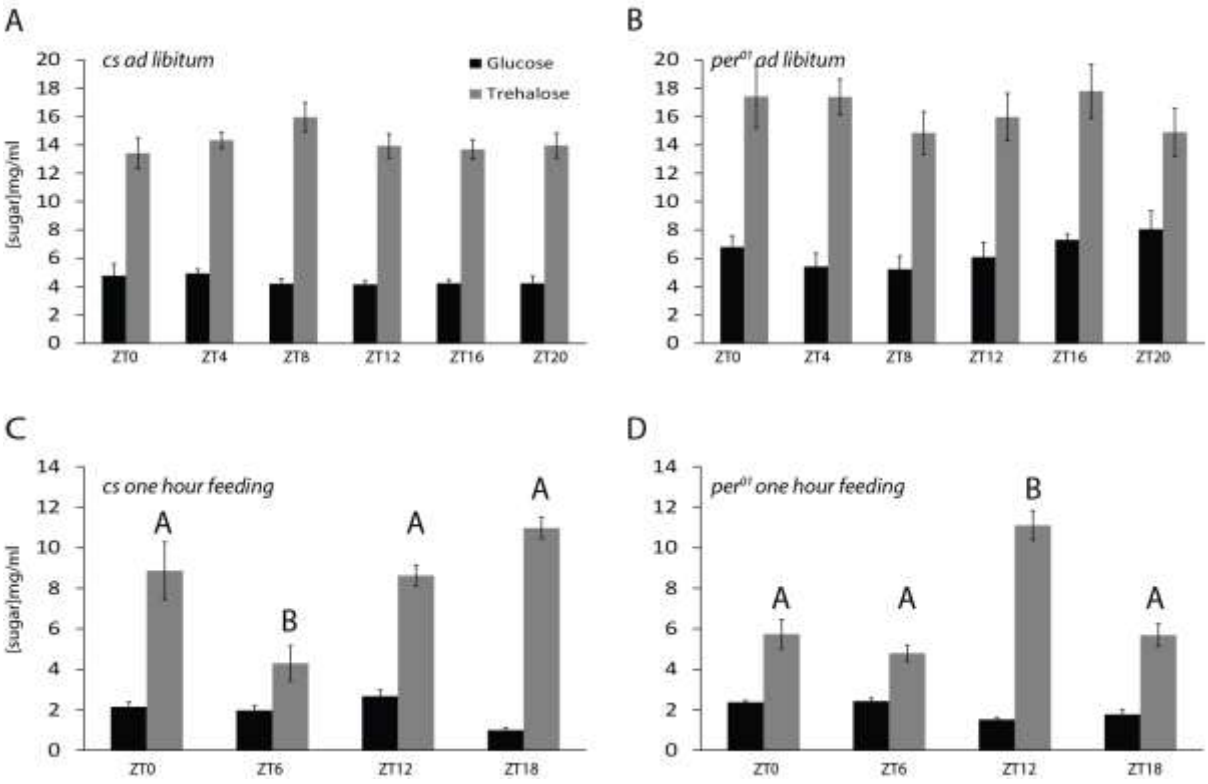
We collected and processed flies collected at night time time-points under a dark room red light.

Restricted Feeding Paradigm

Flies that were 3-7 days old were entrained on cornmeal-yeast-sucrose food (For 1.1L water, there are 12g Bacteriological Agar, 16.9 g Brewer's Yeast, 75 ml Corn Syrup, 71.2 g Cornmeal and 0.4 g Tegosept.) for 3 days under a 12:12 LD cycle. Flies were then transferred on to agar/water food (for 1.1 L water, there are 12 g Bacteriological Argar and 0.4 g Tegosept) for "restricted feeding conditions" at ZT 12 on day 4. For the next three days (days 5-7) flies were transferred at ZT11 into the cornmeal-yeast-sucrose food for feeding for an hour and then transferred back into agar food at ZT12. After three days of restricted 1-hour feeding on cornmeal-yeast-sucrose food, we collected hemolymph as described above at corresponding time points. For the ZT 12 time point, hemolymph was collected immediately after the one hour feed.

4.6 Figures

Fig. 4. 1. Wild-type flies maintain constant levels of circulating sugar but likely rely on the circadian clock to maintain sugar levels when challenged with timed restricted feeding.



Average concentrations of hemolymph glucose and trehalose from individual fly wild-type CS flies (A) and *per*^{01} mutant (B) throughout the diurnal cycle time points (CS: ZT0 N=480; ZT4 N=640; ZT8 N=720; ZT12 N=1040; ZT16 N=720; ZT20 N=640; *per*^{01}: ZT0 N=960; ZT4 N=720; ZT8 N=800; ZT12 N=640; ZT16 N=880; ZT20 N=960). Average concentrations of hemolymph glucose and trehalose from CS (C) and *per*^{01} (D) collected at four time points from flies challenged with time restricted feeding one hour feeding between ZT11 to ZT12 (CS: ZT0, N=640 ; ZT6 N=720; ZT12 N=800; ZT18 N=640; *per*^{01}: ZT0, N=640 ; ZT6 N=560; ZT12 N=640;

ZT18 N=640). The statistical test is one way ANOVA assay with significance set at P=0.05.

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CHAPTER 5

Concluding remarks and future directions

5.1 Concluding Remarks

In the field of chronobiology, locomotion of *Drosophila* has been used as the readout to study the mechanism of circadian clock for decades. There are a plethora of circadian physiological and behavioral outputs such as sleep and feeding that have recently gained the attention of the field. Though circadian feeding behavior has been studied by different groups (Seay & Thummel, 2011; Xu et al., 2008), the tracer methods they used to track feeding have likely interfered with the endogenous circadian clock and masked endogenous feeding behavior. Thanks to FLIC (Ro et al., 2014), I was able to systematically characterize the diurnal and circadian feeding behavior at a resolution of seconds and to measure their food consumption in real time. In my thesis work, I firstly investigated the diurnal and circadian feeding patterns of wild-type and commonly used genetic background (*yw* and w^{1118}) flies and found they exhibit a bimodal feeding pattern in LD and rhythmic feeding behavior in DD, which highly resembles the pattern of locomotion activity, as was previously described by Ro and colleagues (2014). Furthermore, I have shown that

the normal diurnal and circadian pattern of feeding requires the canonical molecular clock genes. By using GAL4-UAS mediated transgene expression and FLIC system, I established a way to examine whether different clock neurons or tissues regulate circadian feeding behavior. FLIC also offered a convenient way to calculate food consumption, which enabled me to find that the clock gene, *clk*, is involved in regulating the food consumption in *Drosophila*, which further the understanding from previous research done by Xu et al (2008). Lastly, I applied a recently developed hemolymph trehalose assay in my research and measured hemolymph trehalose levels at different time points, in an attempt to connect feeding behavior with metabolism. Using this method, I have shown that the circadian clock is involved in the homeostatic control of circulating sugar in the context of time restricted feeding.

5.2 Future Directions

In addition to accurately monitoring the feeding behavior of *Drosophila*, the FLIC assay provides the potential answers to other interesting questions regarding circadian feeding and metabolism. For example, since we know the shift work has a significant and detrimental effect on human metabolism and health (Cain, Filtness, Phillips, & Anderson, 2015; Haus & Smolensky, 2013; Knutsson, 2003), important

questions we can try to answer with FLIC are how altered feeding schedules analogous to those demanded by shift work might affect feeding and metabolism in *Drosophila* and what role the circadian clocks play in regulating feeding under shifting diurnal schedules.

In humans, the circadian clock regulates circulating glucose levels throughout the day, producing a regular rhythm in both blood sugar abundance and in post-prandial increase in circulating sugar (Saad et al., 2012). Our methods of measuring hemolymph trehalose provides a direct way to observe the downstream metabolites of feeding in the fly. In addition, we introduced a restricted feeding paradigm to further demonstrate the important role of clock gene, *per*, in regulating homeostatic control of circulating sugar. Using the same methods, it would be interesting to test if other clock genes such as *clk*, which controls the expression of hundreds of clock controlled genes (Abruzzi et al., 2011), are also involved in such regulation. With the GAL4-UAS system and trehalose assay, we can now determine whether it is the clock in the central nervous system and/or peripheral tissue such as fat body that are important in regulating the circadian homeostatic control of circulating sugar.

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