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Testing the Robustness of the Circadian Clock

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by
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Abstract

The surface of the Earth receives 24 hour cycles of light and darkness. Most living things have arisen under these conditions, and have evolved circadian clocks to keep track of time. In *Drosophila*, the circadian clock comprises the genes *Clock (CLK)* and *Cycle (CYC)* which are negatively regulated by the genes *Period (PER)* and *Timeless (TIM)*. *CLK/CYC* are transcription factors: their targets include *PER/TIM*, which accumulate as proteins to repress their own production. Light deactivates *PER/TIM* proteins, periodically activating *CLK/CYC*, and causing 24h rhythms of transcription activity.

Our lab has previously shown that intestinal stem cells are regulated by the clock during intestinal regeneration. *Drosophila*, maintained under constant 12h light/12h dark photoperiod, show a rhythmic intestinal stress response, and cell-specific disruption of the clock in different intestinal cell types causes arrhythmic repair and poor survival during damage. This suggests independent cell-autonomous clocks in the intestine coordinate their functions during stress. A central question is whether circadian clocks are coordinated in different tissues, and to what extent are their physiological outputs tissue or cell-autonomous.

Using a clock reporter, circadian clocks throughout *Drosophila* were found to be completely cell autonomous. However, both intestinal damage and aging were found to have attenuating effects on circadian clock function. Food intake was also shown to be able to affect the output of the circadian clock when restricted to a specific time of day. These findings demonstrate the robustness of the circadian system throughout the body.

Acknowledgments

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Chapter 1: Introduction

1.1 The Circadian Rhythm

Over billions of years life on earth has evolved in the presence of repeating 24 hour cycles of light and dark. Repeating cycles of light and dark create patterns of environmental favorability that has driven a planet wide evolutionary adaptation (Hut et al. 2013). The ability to innately measure these cycles and adapt behaviourally and physiologically has allowed organisms to better survive in their environment. For example, being able to anticipate dawn, helps nocturnal animals avoid diurnal predators, providing a more favourable environment for survival. This prediction ability is referred to as the circadian rhythm (Hardin 2011).

Circadian rhythms are entrainable biological processes which have a repeating 24 hour period, even in constant conditions (Bell-Pedersen 2005). In circadian biology these constant conditions are referred to as being on circadian time (CT) and are an important assay when determining if environmental input is effecting the circadian clock. Entrainment factors or zeitgebers (ZT), meaning time giver in German, are environmental cues capable of altering activity peaks and troughs in clock gene expression, also referred to as the rhythm's phase. Entrainment factors include external factors such as photoperiod (cycles of light and darkness), the timing of food intake, and changes in temperature all of which have been shown to effect circadian rhythms within the *Drosophila's* body (Zeng et al. 1996; Xu et al. 2011; Glaser and Stanewsky 2005). Although temperature is an entrainment factor, circadian clock processes are temperature compensated. This means that temperature can alter the phase of clock activity but not

the period, which stays around 24 hours. For example, when *Drosophila* were raised in constant light conditions (LL) and constant temperature conditions (25^{o}C) their clocks become arrhythmic due to the constant environmental light stimulus. Under these constant light conditions, flies were subjected to temperature cycles of 10hrs at 25^{o}C and 14hrs at 17^{o}C for 3 days. This shift from constant light and temperature, to constant light with temperature cycles was enough to entrain core clock transcripts, which became rhythmic over 24 hours peaking at ZT16 (Glaser and Stanewsky 2005). Finally, circadian rhythms are thought to be autonomous to each individual cell; however, this is a point of investigation since it has been found that this is not the case in all animal systems. Although self sustained rhythms are present in *Drosophila* malpighian tubules, proboscis and antennae (Plautz et al., 1997; Giebultowicz and Hege 1997), it has also been discovered that the fat body requires rhythmic input from the brain in order to maintain its rhythmicity (Erion et al. 2016). This also happens to be the case for maintaining rhythmicity in the murine liver (Kornmann et al. 2007).

Circadian disruption has been found to shorten the lifespan of various species of animal. Hamsters with circadian disruptions die pre-maturely with an increased incidence of renal and cardiovascular disease. (Tami et al. 2008). Studies in mice correlate circadian disruptions to an increased incidence of cancer and cancer progression (Savvidis and Koutsilieris 2012). And *Drosophila* with circadian disruptions show increased neurodegeneration in old age (Krishnan et al. 2009). With the abundance of evidence, it is clear that maintaining circadian rhythms is important for organismal longevity.

1.2 The Circadian Clock

The circadian clock is a molecular pacemaker that is responsible for all 24 hour rhythmic processes (Figure 1). Circadian rhythms are the consequence of the interactions between the circadian clock machinery and other components of the cellular machinery that are responsible for the circadian timing of many different physiological states (Sassone-Corsi 1994). In animals, the circadian clock involves an auto-regulatory feedback loop of transcription activators and deactivators (Sehgal et al. 1994) In *Drosophila*, dimerization of the positive transcription factors Clock (CLK) and Cycle (CYC) allows binding to the Ebox promoter regions of the genes *Timeless* (*TIM*) and *Period* (*PER*), just two of many possible genetic targets (Abruzzi et al. 2011). This CLK/CYC activity takes place from mid-day to early night. *PER* and *TIM* RNA peak early evening but do not start accumulating as protein until later in the evening. This is due to PER protein destabilization by Double Time (DBT) kinase phosphorylation. Stabilization occurs when TIM protein binds to phosphorylated PER protein creating a DBT-PER-TIM complex. This complex accumulates in the cytoplasm until phosphor-kinases Shaggy (Sgg) and CK2 phosphorylate TIM and PER respectively, causing the complex to become active. (Wang et al. 2001; Edery et al. 1994). The activated complex translocates into the nucleus where the PER component of the DBT-PER-TIM complex represses CLK/CYC activity by inhibiting its DNA binding potential. This causes uncoupling of CLK/CYC from both *TIM* and *PER* promoter regions. In order to reset this process, the photoreceptive protein cryptochrome (CRY) becomes activated by light (Stanewsky et al. 1998). Once activated CRY transforms to uncover a TIM binding site which binds to and phosphorylates TIM

marking it for degradation. Without TIM as a stabilizing factor, PER is degraded and the CLK/CYC dimer can reinitiate transcription of *PER* and *TIM*. This process is thought to take place in every cell in the *Drosophila* body.

FIGURE 2

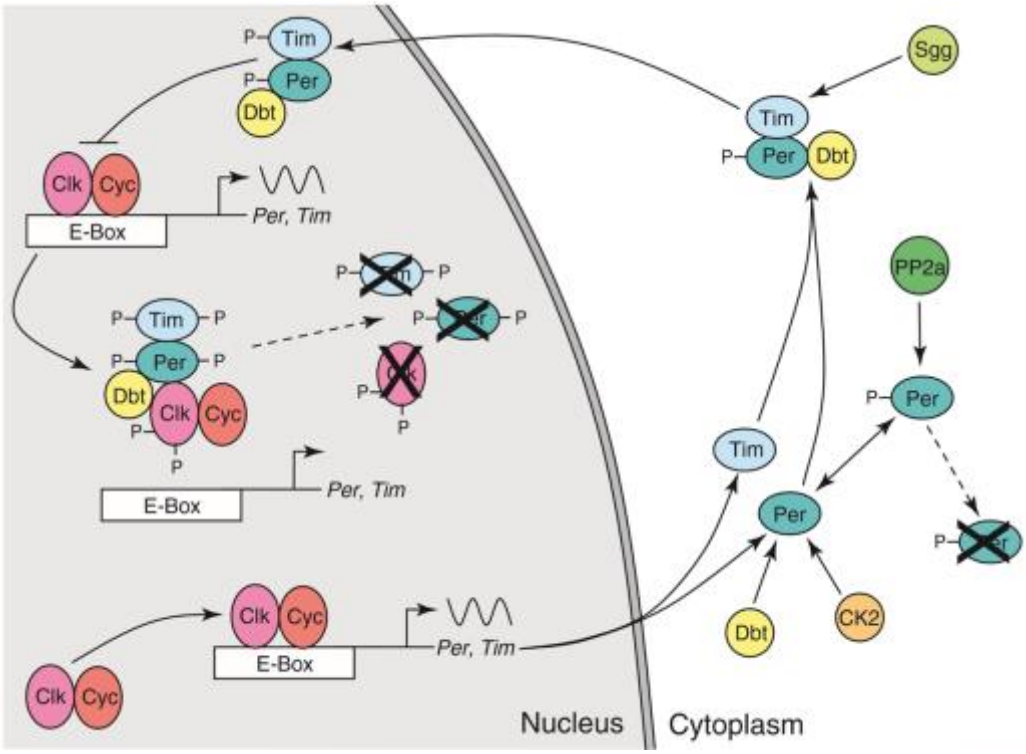


Figure 1: Drosophila circadian clock schematic. Hardin 2011

1.3 The Central Clock Vs Peripheral Clocks

The way that light affects clock systems of different organisms is restricted to the organisms' physiological capabilities for exposure to and detection of the stimulus. In mammals, the only way that light is detected is through retinal ganglion cells (Figure 2). These cells relay neuronal light information to the circadian clock by directly synapsing with the posterior hypothalamus in the brain (Berson et al. 2002). Within this region lies

the Suprachiasmatic Nucleus (SCN) This is considered the master or central clock in mammals (Weaver 1998). It has the responsibility of synchronizing its clock to external light signals and relaying it to the rest of the clocks throughout the body, referred to as peripheral clocks, by way of hormonal and neuronal signals (Buijs and Kalsbeek 2001).

Unlike mammals, *Drosophila* physiology permits light penetration to each individual cell (Figure 2). These cells contain the light sensing protein, CRY, which allows them to be directly synchronized by external light cues (Stanewsky et al. 1998). With a direct synchronization pathway for peripheral clocks this brings into question the need for a central pacemaker in this system, however, one does exist. This central pacemaker consists of a group of lateral ventral neurons (LNv) in the brain (Park et al. 1999). These cells are unique, in that the express Pigment dispersing factor (PDF). PDF is a neural hormone which is necessary for propagating behavioural rhythmicity of eclosion and sleep wake activity in *Drosophila* (Renn et al. 1999). PDF+ cells have also been found to be required to maintain metabolic rhythms of some clock related genes in the fat body (Erion et al. 2016). Although the central clock has been found to be necessary for synchronizing some peripheral clocks, others exist within other *Drosophila* anatomy that can function autonomously. Such clocks have been found in the wings, antennae, proboscis, and Malpighian tubules. (Plautz et al., 1997; Giebultowicz and Hege 1997). Clocks in these regions can be directly entrained to light cues and maintain their rhythmicity even in the absence of PDF+ cells. With the existence of multiple synchronization strategies in peripheral clocks, it is important that each individual clock be tested for any cell autonomous characteristics.

FIGURE 1

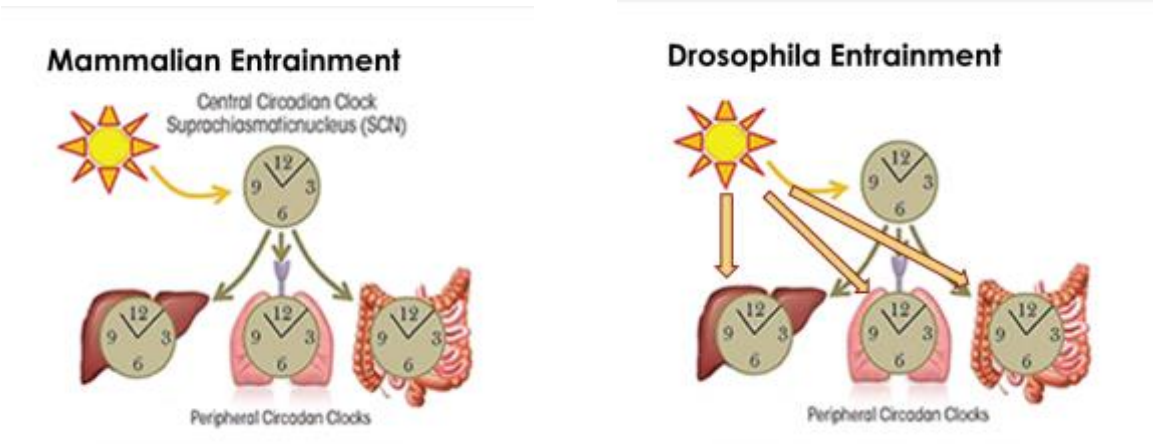


Figure 2: Schematics displaying systemic circadian light entrainment methods in Mammals and Drosophila. (Voigt R et al. 2013)
A: Mammalian peripheral clocks rely on a central pacemaker in the brain. The central pacemaker is entrained to light cues and then relays that information to other peripheral clocks by way of neuronal and hormonal signals
B: Drosophila peripheral clocks are able to be synchronized directly by light cues. This is because each individual cell contains a CRY light sensing protein which acts to directly entrain circadian clocks through light activation.

1.4 Time to Eat: Nutrition and the Circadian Clock

An interesting aspect of the circadian system is that the time of food intake can entrain circadian clocks as well. Evidence has been uncovered that suggest that food acts as secondary entrainment factor in metabolic systems, in both *Drosophila* and murine systems (Xu et al. 2011; Carneiro and Araujo 2012) (Figure 3). In these systems it has been shown that restricted feeding regimens have been able to differentially synchronize the phase of metabolism related genes in the fat body and liver of *Drosophila* and mice

respectively. For example, in the *Drosophila* fat body restricted feeding synchronizes the phase of PER/TIM transcript expression independently of the central clock. (Xu et al. 2011). *Drosophila* normally feed early in the morning (ZT0-3) and decrease this behaviour towards the evening (ZT9-12). Xu et al. found that when flies were allowed to feed when ever they wanted (*ad libitum*) PER and TIM transcripts found peaks around ZT12 in the fatbody. This rhythm was propagated in constant conditions with peaks in gene expression occurring at CT12. However, when food intake was restricted to a time of day in which feeding did not normally occur (ZT9-12), PER and TIM peaks were shifted to CT4 and CT8 respectively. When restricted feeding is done at a time when food would normally be eaten (ZT0-3) then the phase of TIM/PER gene expression was maintained with a stronger amplitude. This process occurs without an effect on the circadian clock in the brain (Xu et al. 2011). This means that clocks in two different tissues can be desynchronized because some tissues respond to time of feeding directly, while others do not.

FIGURE 3

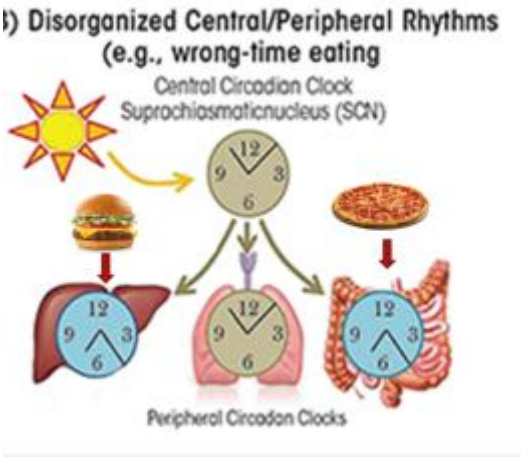


Figure 3: Schematics displaying systemic circadian nutritional entrainment in the Mammalian and Drosophila systems. Systems that are responsible for nutritional processing are able to be differentially synchronized from the brain when a restricted feeding regimen is implemented. (Voigt R et al 2013)

1.5 Anatomy of the *Drosophila* Intestine

The intestine is responsible for receiving and extracting nutrition from ingested items and as such is subjected to continuous external cues. The *Drosophila* intestine has become a prime model for investigation since its functional characteristics are comparable to the mammalian intestine (Casall and Batlle 2009). The *Drosophila* intestine is separated into 3 distinct regions. The foregut is the most proximal feature and includes the pharynx, esophagus, and crop. Food passes through the cardia, a sphincter for controlling food passage to the midgut. The midgut is the largest part of the intestine and is the primary site of digestion and absorption. The most distal region of the intestine is the hindgut, this is where water is reabsorbed by concentrating waste before evacuation (Cognigni et al., 2011).

The midgut can further be separated in to 5 distinct regions (R1-R5) with as many as 13 sub-regions delineated by intestinal morphology, cell morphology and gene expression. The midgut is comprised of four types of cells (Figure 4). Stem cells are the only dividing cell in the intestine, these cells divide and differentiate into an intermediate cell called an enteroblast (Patel and Edgar 2014). Depending on the needs of the system, the enteroblast differentiates into either an enterocyte or enteroendocrine cell which primarily function to absorb nutrients or secrete hormones, respectively (Biteau et al 2011). Since nutrient intake has been shown to effect clocks in the fat body of the *Drosophila*, we hypothesized that the clock in the intestine would also be affected by restricted feeding since it is the primary receptacle for environmental nutrition.

FIGURE 4

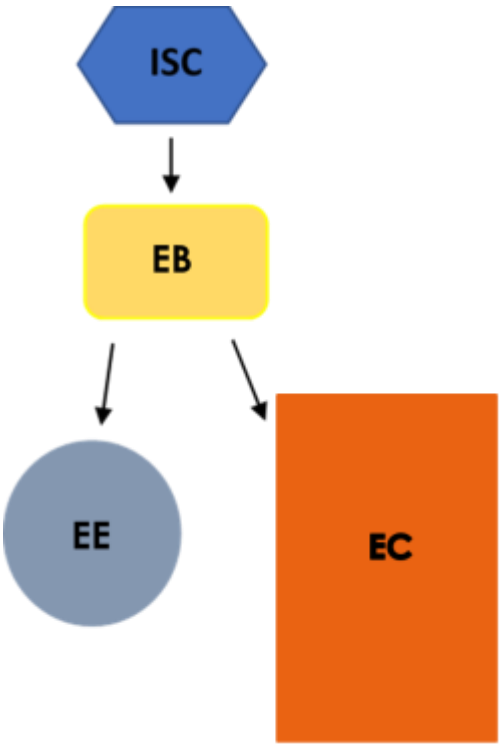


Figure 4: Schematic of Drosophila intestinal cell lineage. There are 4 different cell types in the Drosophila intestine. Intestinal stem cells (ISCs) are the only dividing cells. These divide and differentiate into a precursor cell called an enteroblast (EB). Depending on the needs of the system, the EB will differentiate into either an Enteroendocrine cell (EE) or an Enterocyte (EC).

1.6 Studying Regeneration in the Intestine

During times of high stress, the intestine follows a circadian rhythm in regeneration, displaying a peak in mitosis in the late night to early morning transition (Karpowicz et al 2013). Intestines become arrhythmic when the essential clock genes *PER* and *CYC* are mutated, highlighting the importance of the clock in regeneration. A similar non-rhythmic phenotype is produced when clock gene expression is knocked down specifically in intestinal stem cells. This makes sense since stem cells are the only dividing cell in the intestine, it would need a functional clock to do so rhythmically. However, the rhythmic regenerative phenotype is also lost when clocks are knocked down specifically in enterocytes. Since enterocytes do not divide in the intestine, it is curious that enterocyte clocks would be necessary for rhythmic mitoses in stem cells. These findings suggest the existence of a circadian communication pathway between enterocytes and stem cells. With the potential for elucidating non-autonomous circadian mediated cell processes and secondary entrainment pathways, the intestine becomes an exciting model for discovery.

1.7 Hypotheses

1. I hypothesize that Circadian clocks in *Drosophila* intestinal cells function autonomously from one another by responding directly to entrainment cues.

2. Due to the evidence of nutritional entrainment in the fat body, I hypothesize that restricted feeding can also synchronize the intestinal clock independent of photoperiod.

Chapter 2: Methods of Investigation

Due to the relative simplicity of the *Drosophila* genome and the extensive genetic work that has taken place, there are many transgenic tools that can be used not only to study the clock, but manipulate its output as well. *Drosophila*'s life cycle consists of four stages. Females lay eggs which hatch into larvae after two days, at 25oC. As they grow, larvae go through four molting stages (first, second and third instar), with the fourth molt resulting in a pupa. The immobile pupa provides the environment for complete tissue remodeling into the adult *Drosophila*. The adult *Drosophila* hatches (ecloses) roughly 10 days later, at 25oC. The transformation from egg to adult takes approximately 10 days and the average lifespan is about 40-50 days (Stocker and Gallant 2008).

2.1 Fly Husbandry

Flies were reared in bottles with food containing: 15% dry yeast, 10% soy flour, 65% cornmeal, 5% malt and 5% agar. Flies were housed at 24C under a 12:12 LD cycle (ZT0 = 7am) and synchronized for at least 5 days before the experiment. At the time of experimentation flies were no older than 10 – 12 days. Flies were dissected at 8 different time points (ZT/CT0, ZT/CT3, ZT/CT6, ZT/CT9, ZT/CT12, ZT/CT15, ZT/CT18,

ZT/CT21) over 24 hours. At each time point 10 – 12 intestines were harvested by first soaking flies in 70% EOH for 1 minute and then dissected in PBS (1mM Na2HPO4, 0.18mM KH2PO4, 15.5mM NaCl at a pH of 7.37). Once dissected, intestines were immediately transferred to Fix (4% PFA (Electron microscopy sciences) in PBS at a 1:4 ratio). Intestines were fixed for at least 40 minutes. Intestines were rinsed using cold PBS and then stained with Dapi (ThermoFisher Scientific, 1:5000) in PBS-T (PBS + 0.2% triton) for 5 mins. Intestines were then washed 3 times for 10 minutes each using PBS-T and mounted on slides with ProLong Gold antifade reagent (ThermoFisher Scentific)

2.2 Circadian Clock Reporters

In order to visualize and measure dynamic clock activity in the *Drosophila* intestine two novel clock reporters were produced by our research group (Phillip Karpowicz, Li He and Norbert Perrimon, unpublished). Each reporter contains minimal promoters from the clock targets: *TIM* or *PER*, arranged in a 4x tandem series to increase transcriptional activation. Each promoter is a minimal (122-174 bp) sequence containing E-box binding sites and other regulatory sites for the CLK/CYC dimer that have been previously shown to drive rhythmic expression of *LacZ* (Hao et al 1997; McDonald et al 2001). The minimal promoter array was introduced upstream from a superfolder destabilized-GFP, that lasts about an hour and then is degraded (Li He and Norbert Perrimon unpublished). This means that when the CLK/CYC dimer binds to intrinsic promoter regions of *PER* and *TIM* it will also bind to our extrinsic reporters' minimal promoter and drive the transcription

of a GFP molecule at the same time. Due to a designed PEST domain in the reporter, the GFP signal degrades shortly after being produced so clock activity could be measured dynamically over time.

2.3 Circadian Reporter Validation

To validate that these reporters are displaying circadian clock activity two methods were utilized, qPCR and fluorescence microscopy. To set up flies for dissection, Drosophila were synchronized to 12 hours of light and 12 hours of dark (12:12 LD) for at least 5 days. The initial onset of light, or ZT0, was at 7am and the onset of darkness, or ZT12, was at 7pm. Intestines were harvested from flies containing either the $Clock^{TIM}$ reporter or the $Clock^{PER}$ reporter every 3 hours over 24 hours starting at ZT0 (ZT0, ZT3, ZT6, ZT9, ZT12, ZT15, ZT18, ZT21). The negative control for these experiments was the *CYC*^{01} null mutant (flybase ID: FBal0195440), which were collected also collected across 8 timpoints. These flies do not produce functional CYC protein, disrupting clock function throughout the organism. When extracting RNA intestines were homogenized in RLT Buffer (Qiagen) using a Bullet blender as directed by the manufacturers protocol (Next Advance, Averill Park, NY). RNA was extracted following the RNeasy Mini RNA purification Kit instruction (Qiagen). RNA concentration was verified using Nano drop . cDNA was produced using the ISCRIPT RT Supermix (Bio-Rad, Mississauga, Ontario, Canada and associated protocol cDNA synthesis kit (BIO-RAD). Samples were placed in BIO-RAD PCR machine. From the undiluted sample, a serial dilution was performed using RNase free

H2O (1:4, 1:16, 1:64). Master mix for each sample contained 5microL 2X Quantitect SYBR Green PCR, 3.2microL of RNase free H2O, 0.4microL of forward primer and 0.4microL of reverse primer Master mix (9µL) and sample (1µL) were added to each well. Plate was spun down (4000rpm for 1 min). Quantification took place using the ViiA7 PCR plate reader.

To study the clock using GFP reporter fluorescence, *Drosophila* intestines were dissected and stained with DAPI, then visualized using the slide scanner (Zeiss Axio Scan), and Axio Scan.Z1 software. Entire intestines were scanned and examined for changes in the ratio of GFP:DAPI signal over time. Secondarily, at the end of the entrainment period flies were released into constant darkness (DD) to ensure that any observations were instances of the circadian clock and not just responses to light. Intestines were harvested from flies containing the $Clock^{PER}$ reporter every 3 hours over 24 hours starting at CT0 (CT0, CT3, CT6, CT9, CT12, CT15, CT18, CT21). The negative control for these experiments was the CYC^{01} null mutant (flybase ID: FBal0195440), which were collected at 4 time points CT0, CT6, CT15, CT21. When mutant flies are crossed to reporter flies, there should be no GFP expression since the reporter's driver (CLK/CYC) is no longer functional. If GFP is present in the mutants, then it is hard to conclude that GFP presence in the wild type flies is from the reporter. Anterior and posterior regions were also examined in this way.

2.4 Single Cell Reporter Expression

Due to noted complexity of the *Drosophila* intestine, confocal microscopy was utilized (Olympus confocal imager) to examine the reporter at a single cell resolution. Flies were synchronized as stated above, ISCs were stained with mouse anti-Delta stain (1:50), and EEs were stained with mouse anti-prospero (1:50). Secondary stain was performed with goat anti-rabbit (1:2000) and Dapi (1:5000). ECs and EBs were identified based on size and location. ECs are the largest cell in the intestine. Since EBs differentiate from ISCs they should be in the vicinity of delta-positive cells. High resolution single cell images from the intestine were taken with a 40x lens with water.

2.5 Testing Intestinal Circadian Autonomy

In order to test cellular autonomy in the *Drosophila* intestine clock function in one cell type must be knocked down, while leaving the rest of the cells with functional clocks. If clock function in other cells is perturbed by the disruption of clock function in another cell, then clocks within the intestine would have to be cellularly interdependent and not autonomous. To accomplish this the GAL4-UAS (Duffy 2002) system and RNAi (Mohr et al. 2014) were utilized in tandem. The GAL4-UAS system allows for targeted expression of genes in a wide variety of cell types. The transcriptional activator GAL4 is introduced into the genome downstream from a gene of interest, that is expressed specifically in a certain cell type. This is called a cell marker. The cell marker becomes a gene driver, capable of turning on gene expression in that cell. Elsewhere in the genome transgenes are inserted

downstream of an upstream activating sequence (UAS). The two flies are mated together, and their offspring contain both the GAL4 driver, and the UAS sequences upstream of a transgene. When GAL4 protein is produced as a by-product from normal cellular expression, it is able to locate and bind to any UAS to drive the production of its downstream gene. Because GAL4 is only being expressed in cells that contain a marker, it is only driving UAS expression specifically within that cell type (Brand and Perrimon 1993).

Interference RNA or RNAi is a biological process which can down regulate the concentration of specific RNA transcripts in the cytoplasm, pre-translation. When small interfering RNA (siRNA) is expressed in the cell, associated proteins come together and bind to the siRNA creating the RNA-induced silencing complex (RISC). Once activated RISC uses the siRNA sequence to identify homologues RNAs and mark them for degradation. (Agrawal et al 2003). So any time the cell produces RNA for a specific gene transcript, it will be degraded before it is translated into protein. This can effectively and selectively knock down gene expression in specific cells when driven by the GAL4-UAS system. If RNAi components are placed downstream from a UAS binding site, then any cells expressing GAL4 would also have a specific gene knockdown.

To test cellular clock autonomy within the intestine, I used the GAL4-UAS system to drive the production of CYC interference RNA (CYC-RNAi) specifically in enterocytes and then in intestinal stem cells. To knockdown clock function in intestinal stem cells, the *escargot* (*ESG*) driver was used and to knockdown clock function in EEs the MYO1A driver was used. This allowed me to knockdown the function of the circadian clock in one cell type and study how it effects other cells which will contain functional clocks. In control

samples the GAL4-UAS system will be driving RNAi for Luciferase transcripts (LUC-RNAi). This is a control because luciferase is not endogenously produced by the *Drosophila* and so knocking down its expression should produce no effect on the intestine. Using the clock reporters and assay described above, I predict that cell specific clock knockdowns will have no effect on $Clock^{PER}$ reporter signal from other cells with functional clocks. Flies were entrained as stated above. For the stress experiments, the same synchronization assay was utilized as described above, however; 2 days prior to the experiment, flies were put on food containing 25μg/mL bleomycin to cause intestinal damage. Aged flies were aged to 35-40 days prior to dissection. Synchronization methods as stated above.

To further test intestinal clock autonomy, I looked to see if the intestine is capable of propagating direct light signals in the absence of a functional central clock in the brain. Utilizing the GAL4-UAS system, CYC-RNAi was produced by the brain driver ELAV therefore, only cells expressing ELAV will have perturbations in clock function. The $Clock^{Per}$ reporter in the intestine was examined from flies containing an intact brain clock and a disrupted brain clock, and compared to see if a lack of circadian brain function has an effect on intestinal clock function. Once again control samples with intact brain clocks will be driving LUC-RNAi specifically in brain cells. Without luciferase transcripts, there should be no difference from a clock normal fly. I predict that in the absence of a brain clock, the intestinal clock will continue to produce the same characteristic signal in GFP expression, similar to wild type flies

2.6 Food Entrainment

To study the effects of restricted feeding on the entrainment of the intestine, flies were subjected to the same 12:12LD synchronization schedule as above. During 5 days of light entrainment, three separate groups of flies were placed on different feeding regimens. Control flies were given food at all times (ad libitum) and allowed to feed as normal. The first test group had their food restricted from ZT0-3, a time when flies normally consume lots of food. The second test group was restricted to later in the day, ZT9-12, when food is not usually consumed. When not on food, flies were given a hydration supplement consisting of 2.4g of agar in 500mL water. In an attempt to eliminate the effects of direct light entrainment on intestinal cells, *CRY*^{01} null mutant flies (Flybase ID: FBal0218575) were used. These flies do not produce a functional CRY protein, so they are not be able to be directly entrained by light, and feeding should become the dominant entrainment factor if possible. Positive control flies were wild type Clock^{PER} reporter flies. I predict that restricted feeding will become the primary entrainment factor in *CRY*^{01} intestines, and the phase of reporter expression will differ between flies fed *ad libitum* and those fed restrictively. I believe wild type Clock^{PER} reporter flies will continue to be entrained by light and produce its regular rhythm, irrespective of feeding time.

Chapter 3: Results

3.1 Reporters for Testing the Circadian Clock

Before the Clock^{PER} and Clock^{TIM} reporters could be used to test the circadian clock, they had to be tested to determine if clock activity was being accurately reported. To characterize reporter function over time, time series were performed where groups of *Drosophila* were synchronized to 12 hours of light and 12 hours of darkness (12:12 LD). After at least 5 days of synchronization intestines were harvested at 8 different time points over 24 hours (ZT0, ZT3, ZT6, ZT9, ZT12, ZT15, ZT18, ZT21). The tested groups included Clock^{PER} and Clock^{TIM} reporter flies with functional clocks (Wild Type, WT) and their counterpart reporter flies with non-functional clocks through *Cycle* (*CYC*) null mutation.

The first method used to test reporter function was Quantitative PCR (qPCR). This enabled the quantification of specific gene transcript levels (RNA) and allowed us to track the changes in target gene expression in the intestine over time. Targets of the qPCR were the transcripts for the genes *TIM*, *PER*, *GFP* and *GAPDH*. A mentioned above, *TIM* and *PER* are genes involved in the core feedback loop of the circadian clock in *Drosophila*. Different levels of these transcripts should be produced rhythmically over 24 hours when the clock is functional. *GFP* is not a native gene to the *Drosophila* and should only be present due to the activity of the Clock^{PER} and Clock^{TIM} reporters. *GAPDH* is a cellular house-keeping gene which is produced around a constant rate at all times (Barber et al. 2005). This meant that relative abundances of *PER*, *TIM* and *GFP* transcript could be measured over time using *GAPDH* levels as a constant. *PER* and *TIM* transcripts should be produced with a

circadian rhythm over time. These rhythms are used to predict how *GFP* transcript production should occur depending on whether it is being driven by the *TIM* or *PER* promoter. *GFP* transcripts produced with a circadian rhythm similar to their *PER* and *TIM* counter parts would indicate a functional circadian reporter. The negative control for the experiment were flies containing a copy of one of the clock reporters along with a null mutation in its *CYC* gene. *CYC* is part of the dimer transcription activator which drives circadian clock function. With this rendered inactive there should be no clock activity in these flies, with arrhythmic diminished expression of *TIM*, *PER* and *GFP* transcripts.

To further study clock reporter function, intestines were examined for GFP fluorescence over time. Intestines were stained with DAPI nuclear stain to visualize and verify that cells are present and the sources of GFP fluorescence were coming from the cells. GFP and DAPI fluorescence levels were quantified over time using the Zeiss Axio Scan slide scanner. GFP fluorescence for each intestine was normalized to its DAPI expression since DAPI fluorescence should stay consistent across time points and samples. This is because all intestines within an experiment are exposed to the same concentration of DAPI for the same amount of time. If functional, reporters should produce GFP with a circadian rhythm in WT flies while *CYC* mutants should produce small amounts of arrhythmic GFP transcript.

3.2 Testing the Clock^{TIM} Reporter

The first reporter to be examined was the *TIM* reporter, which contains a 174 bp minimal promoter of the *TIM* gene (McDonald et al. 2001) upstream of destabilized GFP. When WT Clock^{TIM} reporter intestines were analyzed using qPCR relative abundances of *TIM*, *PER* and *GFP* transcripts produced robust circadian rhythms over a 24 hour period in two experiments (Figure 5). In Experiment A: *PER*, *TIM* and *GFP* transcripts all peaked at ZT15. *CYC* mutants produced less PCR product than the WT with no discernible rhythm. In Experiment B: *PER* and *TIM* RNA peaked at ZT15, while *GFP* peaked later at ZT18. Similar to Experiment A, Experiment B's *CYC* mutants produced no rhythmic transcript signal. When GFP fluorescence was quantified in WT Clock^{TIM} reporter intestines, it produced a circadian rhythm which showed significant variation over 24 hours ($F(7,87) = 11.42$, $P<0.0001$). GFP fluorescence peaked at ZT0 and troughed at ZT19. *CYC* mutants produced greatly attenuated fluorescence compared to the WT with no discernible rhythm (Figure 6).

FIGURE 5

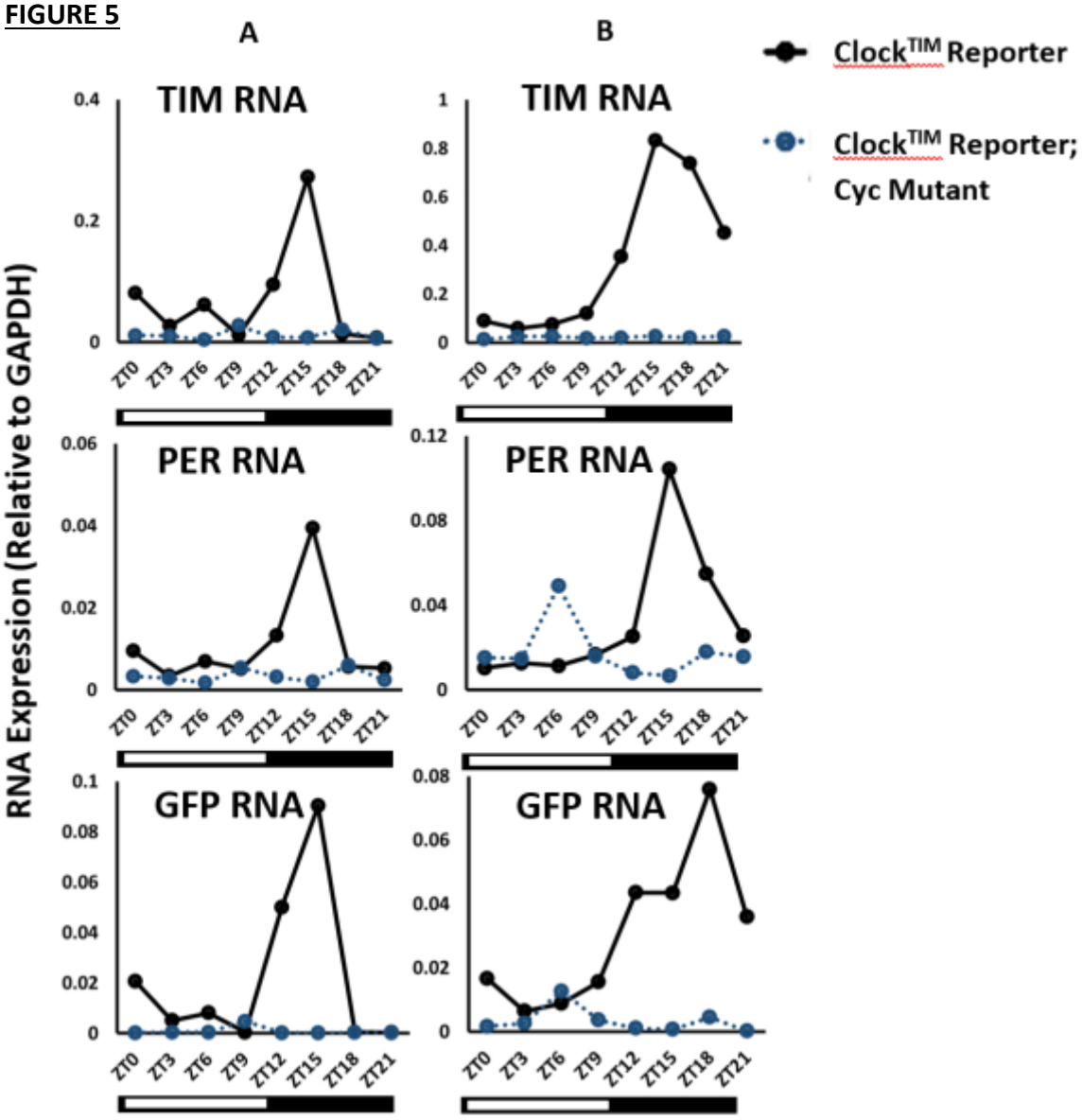


Figure 5: Two separate time series depicting the changes in the levels of *TIM*, *PER* and *GFP* transcript compared to *GAPDH* in the intestine over time. qPCR results from Clock^{TIM} Reporter flies, and control flies *CYC* mutant flies (n=10 intestines per timepoint)

A: When compared to *GAPDH*; *TIM*, *PER* and *GFP* transcripts in Clock^{TIM} reporter flies followed circadian rhythms, all peaking at ZT15. *CYC* mutants showed no discernible rhythms.

B: When compared to *GAPDH*; *TIM*, *PER* and *GFP* transcripts produced circadian rhythms. *TIM* and *PER* transcripts peaked at ZT15 and GFP peaked at ZT18, *CYC* mutants show no discernible rhythm.

FIGURE 6

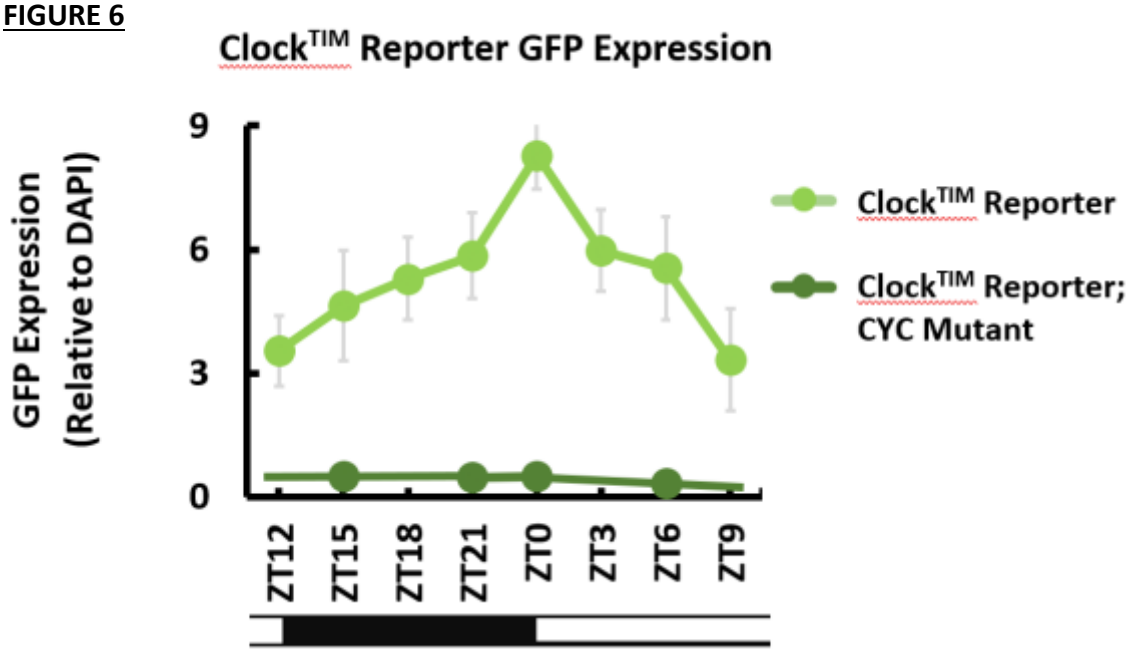


Figure 6: Time series of GFP fluorescence produced by the Clock^{TIM} reporter in WT flies and flies containing a *CYC* null mutation (n=12 intestines per time point). The Clock^{TIM} reporter in WT flies produced a robust circadian rhythm in LD, peaking at ZT0. *CYC* mutants showed no discernible signal rhythm in LD. This indicates that the reporter is producing GFP through the circadian clock mechanism.

3.3 Evaluating the Clock^{PER} Reporter

The PER reporter was next tested, which similarly contains a minimal 122 bp promoter region (Hao et al. 1997) upstream of destabilized GFP. When WT Clock^{PER} reporter intestines were analyzed using qPCR relative abundances of *TIM*, *PER* AND *GFP* transcripts produced robust circadian rhythms over a 24 hour period, as they had with the Clock^{TIM} reporter (Figure 7). In experiment A: *PER* and *TIM* transcripts peaked at ZT18 and troughed around ZT3. *GFP* transcripts differed slightly and peaked at ZT18, while maintaining a ZT3 trough. *CYC* mutants produced much less PCR product than the WT with no discernible rhythm. In Experiment B: all transcripts were once again rhythmic however, *PER* peaked at ZT15, *GFP* peaked at ZT18 and *TIM* seamed to maintain peak expression from ZT15-18. When *GFP* fluorescence was quantified in WT Clock^{PER} reporter intestines, it produced a circadian rhythm with significant variation over 24 hours ($F(7, 87) = 50.38$, $P<0.0001$)(Figure 8). *GFP* fluorescence peaked at ZT0 and troughed at ZT15. As with the TIM reporter experiments, the *CYC* mutants produced greatly attenuated fluorescence compared to the WT with no discernible rhythm.

FIGURE 7

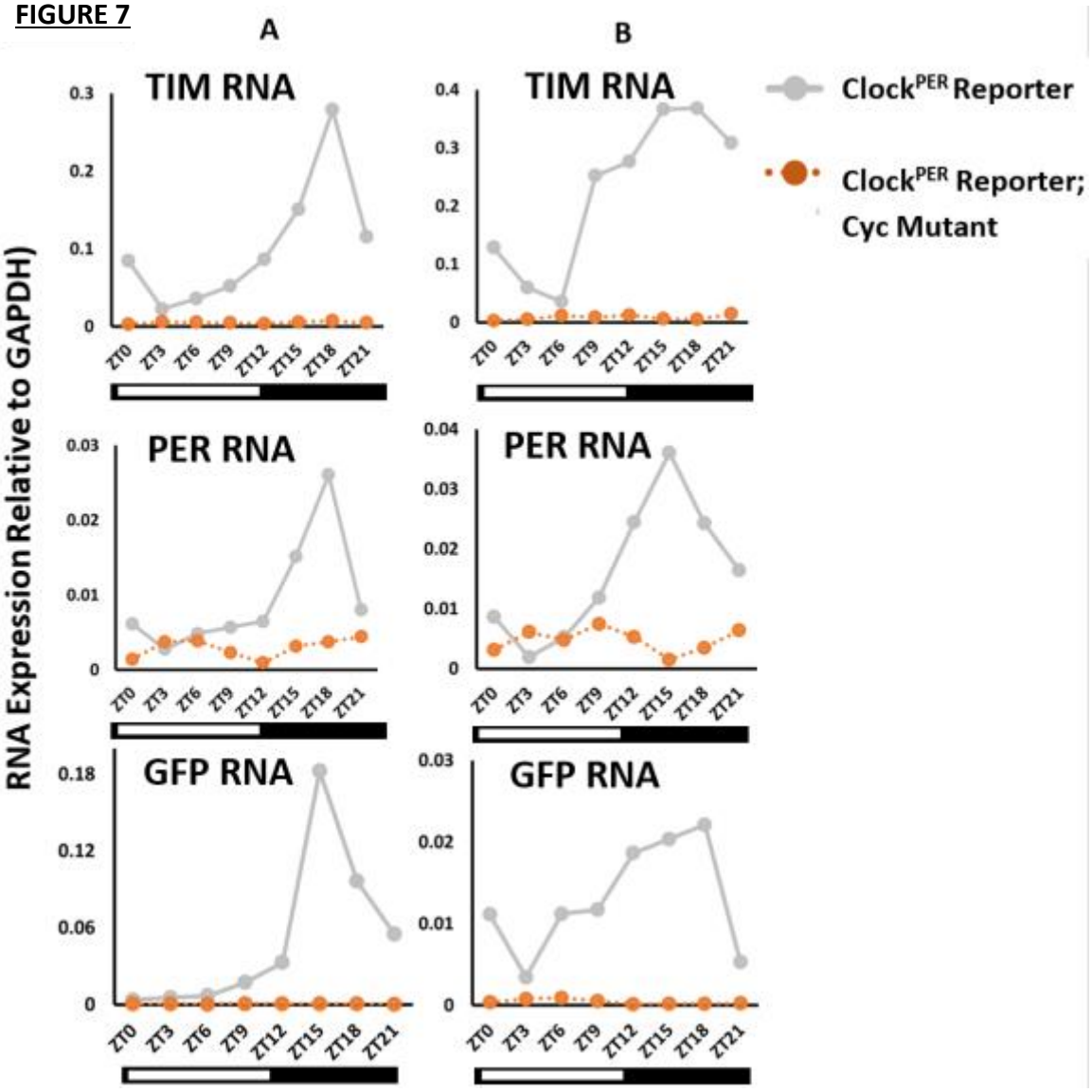


Figure 7: Two separate time series depicting the changes in the levels of *TIM*, *PER* and *GFP* transcript compared to *GAPDH* in the intestine over time. qPCR results from Clock^{PER} reporter flies, and control flies *CYC* mutant flies (n=10 intestines).

A: When compared to *GAPDH*; *TIM*, *PER* and *GFP* transcripts in Clock^{PER} reporter flies followed circadian rhythms, *PER* and *TIM* peaking at ZT18 and *GFP* peaking at ZT15. *CYC* mutants showed no discernible rhythms.

B: When compared to *GAPDH*; *TIM*, *PER* and *GFP* transcripts produced circadian rhythms. *PER* transcripts peaked at ZT15 and *GFP* peaked at ZT18. *TIM* transcripts peaked somewhere in between. *CYC* mutants show no discernible rhythm.

FIGURE 8

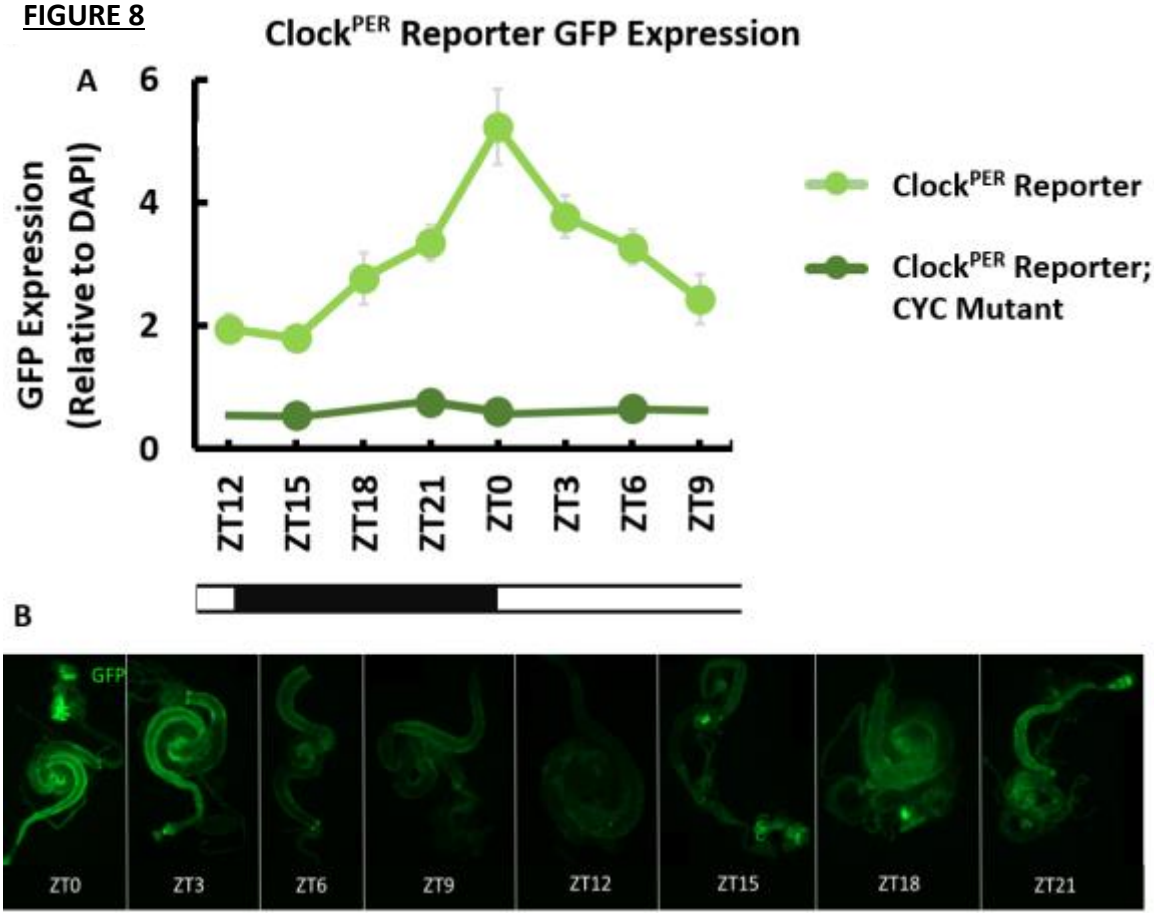


Figure 8: Time series of GFP fluorescence from Clock^{PER} reporter.
A: The Clock^{PER} reporter in WT flies produced a robust circadian rhythm in LD, peaking at ZT0 (n= 12 intestines per timepoint). The *CYC* mutant expressed no discernible GFP signal. This indicates that the reporter is producing GFP through the circadian clock mechanism.
B: Representative intestinal fluorescence from each dissection time point.

3.4 Clock^{PER} Reporter is Functional in Total Darkness

Since Clock^{PER} and Clock^{TIM} reporters produced similar rhythms in GFP expression, only the Clock^{PER} reporter was arbitrarily chosen to measure clock activity in subsequent experiments. An important characteristic of a circadian rhythm is that the rhythm persists in the absence of light cues. Clock^{PER} reporter flies and Cyc mutants were synchronized to a 12:12LD cycle. On the day of the experiment they were released into total darkness (DD) to see if reporter function was a simply a response to light presence or the actual circadian clock. WT Clock^{PER} reporter intestines DD (indicated by change in ZT to CT on x-axis in graphs) produced a circadian rhythm with significant variation over time ($F(7, 75) = 16.5$, $P<0.0001$). GFP reporter signal in constant conditions continued to peak at CT0 and trough around CT15. CYC mutants continued to produce no fluorescence (Figure 9). This confirmed that the Clock^{PER} reporter was detecting and reporting free-running circadian clock activity.

FIGURE 9

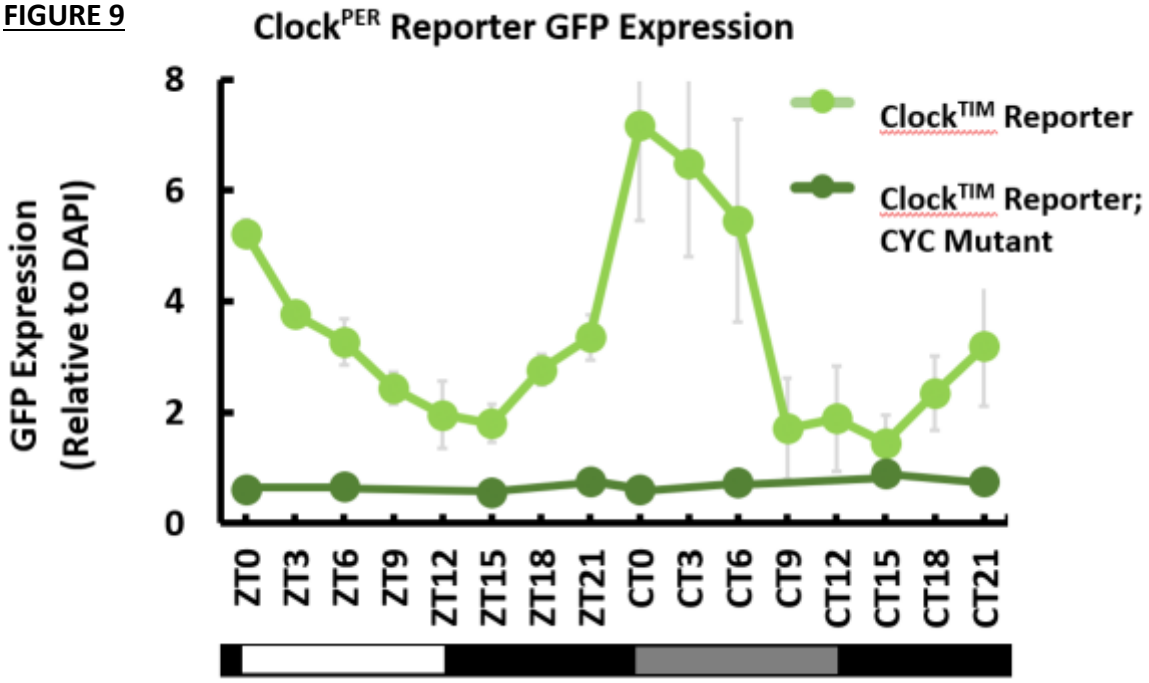


Figure 9: A 48 hour time series of GFP fluorescence from Clock^{PER} reporter in WT flies and flies containing a Cyc null mutation (n=12 intestines per timepoint). First 24 hours of reporter activity is under light entrainment cues (ZT) was tested (data same as in Figure 8), then flies are shifted to constant darkness to visualized reporter on circadian time (CT) The ClockPER reporter in WT flies produced a robust circadian rhythm in LD peaking at ZT0. This rhythm was propagated in constant conditions, peaking again at CT0. Clock mutant flies produced no GFP fluorescence. This indicates that the reporter displays an entrained rhythm validating the use of this reporter for further circadian experiments.

3.5 Single Cell Analysis of Clock^{PER} Reporter Function

With the Clock^{PER} reporter deemed functional, it was important to know if all 4 cell types in the intestine were expressing it. In order to differentiate between cell types, markers specific to those cells were used. Intestinal stem cells (ISCs) are identified by their relative size and positive Delta receptor staining. Delta receptors are unique to ISCs and allow for Notch mediated cell differentiation (Ohlstein and Spradling 2007). Once division completes and differentiation begins, the enteroblast (EB) can be identified by its small size and lack of delta stain as well as its vicinity to Delta positive cells. Since EBs are directly dividing from delta positive ISCs then EBs should be found close to Delta positive cells. Enteroendocrine (EE) cells are slightly larger than EBs and are identified by prospero stain, a well-known marker of these cells in the intestine. Enterocytes (ECs) are the largest cells in the intestine and identified solely by their size.

To test whether the clock is expressed in these different cell types high resolution confocal images were taken of different regions of the *Drosophila* midgut at the highest time of reporter activity, ZT0 (Figure 10). Fluorescence was quantified in each cell type using ImageJ software. ISCs, EBs and ECs, all produced similar fluorescence levels indicating the clock reporter functioned in these cells. However, EEs produced little to no detectable reporter signal ($F(3, 44) = 44.05$, $P<0.0001$). This leads me to conclude that either EEs lack a functional clock, or the reporter is present in an area of the genome that is silenced in mature EEs.

FIGURE 10

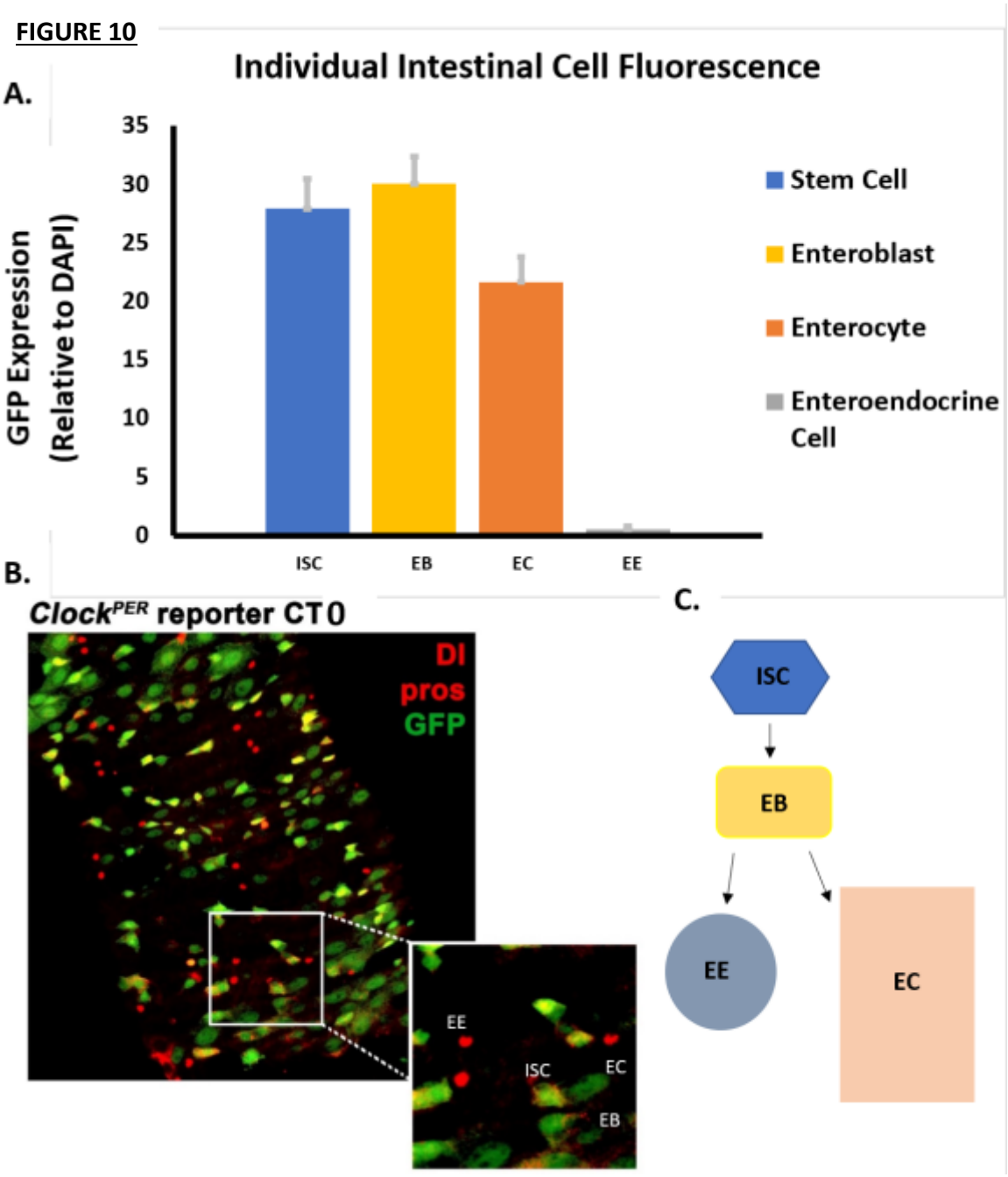


Figure 10: Single Cell analysis of the ClockPER reporter

A: Graph showing quantification of signals from each cell type (n=12 cells tested from 3 separate images). EEs show no GFP fluorescence.

B: High resolution confocal image of portion of *Drosophila* intestine with delta and Prospero staining. No reporter activity is visible in EEs.

C: Schematic of intestinal cell lineage, showing Intestinal stem cells (ISC), Enteroblasts (EB), Enterocytes (EC) and Enteroendocrine cells (EE).

3.6 Testing Regional Variation in Clock^{PER} Reporter Function

The mammalian intestine is a complex system with specialized regions for various processes, such as secretion, absorption, and digestion. Recently, the complexity of *Drosophila* intestine was revealed with two publications suggesting the fly intestine is compartmentalized into as many as 13 different sub regions based on, cell presence, morphology, and gene expression. Over half of the *Drosophila's* genome is expressed in the intestine (Fly atlas database Chintapalli et al 2007). This was confirmed when 210 randomly selected reporter transgenes were tested, and it was found that 151 were expressed in intestinal tissue (Buchon et al. 2013).

With this highlighted complexity in intestinal transgene expression, it was important to observe how the Clock^{PER} Reporter acts in these different regions. Using the Clock^{PER} reporter, the anterior (R2) and posterior (R5) midgut was analyzed and contrasted for any regional difference. Despite their many region-specific difference (Buchon et al. 2013), both regions exhibit similar reporter activity (Figure 11), and there was no significant regional variation when it came to the phase of the clock in the anterior vs posterior ($F(7, 114) = 0.3244$, $P=0.9416$).

FIGURE 11

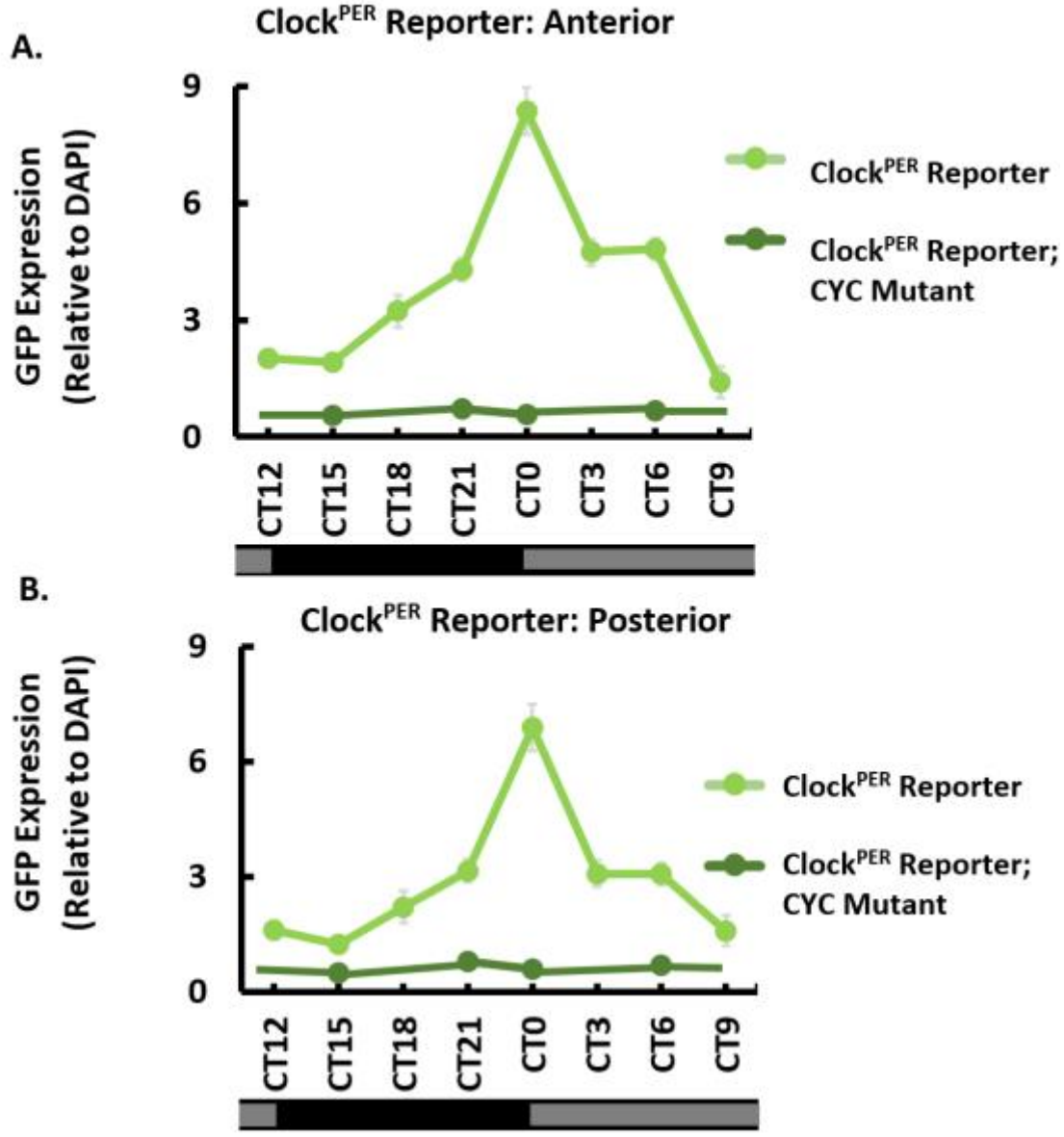


Figure 11: Time series of Clock^{PER} reporter GFP fluorescence in the anterior vs posterior midgut (n= 12 guts per timepoint).
A: The Clock^{PER} reporter in the anterior midgut of WT flies produced a robust circadian rhythm in constant conditions, peaking at ZT0. Clock mutants show no GFP fluorescence.
B: The Clock^{PER} reporter in the posterior midgut of WT flies produced a robust circadian rhythm in constant conditions, peaking at ZT0. Clock mutants show no GFP fluorescence.
There was no regional variation in reporter expression.

3.7 Testing Intra-Cellular Autonomy of the Intestine

Regeneration is a homeostatic process which facilitates the replacement of damaged cells through division and differentiation of ISCs. When damaged intestines were analyzed, it was found that the rate at which ISCs divided followed a circadian rhythm over time. Cell specific clock knock down performed in ISCs and then ECs, both negatively affected rhythmic regeneration (Karpowicz et al. 2013). The need for a functional clock in ISCs is reasonable, as ISCs are the only dividing cell in the intestine and would therefore need a functioning clock to divide with a rhythm. However, non-dividing, fully differentiated ECs were also required to generate rhythmic regeneration in ISCs. This finding raised the question of whether a non-autonomous clock communicates between ECs and ISCs.

To test intestinal cell autonomy in clock function, the GAL4-UAS system was utilized in tandem with RNAi to attenuate clock function in a specific cell types. This was accomplished by placing GAL4 down-stream from genes uniquely expressed in those cells. For example, ISCs uniquely express the gene *ESG*, while ECs are the only cells expressing *MYO1A*. When GAL4 is placed downstream from *ESG*, it is only expressed in ISCs and when placed downstream from *MYO1A* it is only expressed in ECs. Once activated, GAL4 seeks out and binds to UAS binding sites. Once bound, the UAS activates transcription of components necessary to form an RNAi complex. The RNAi complex is responsible for circadian clock attenuation through marking *CYC* RNA transcripts for degradation, thus not allowing active CYC protein to form. When this approach was used in tandem with the $Clock^{PER}$ reporter, it enabled tracking of any changes in the function of clock positive

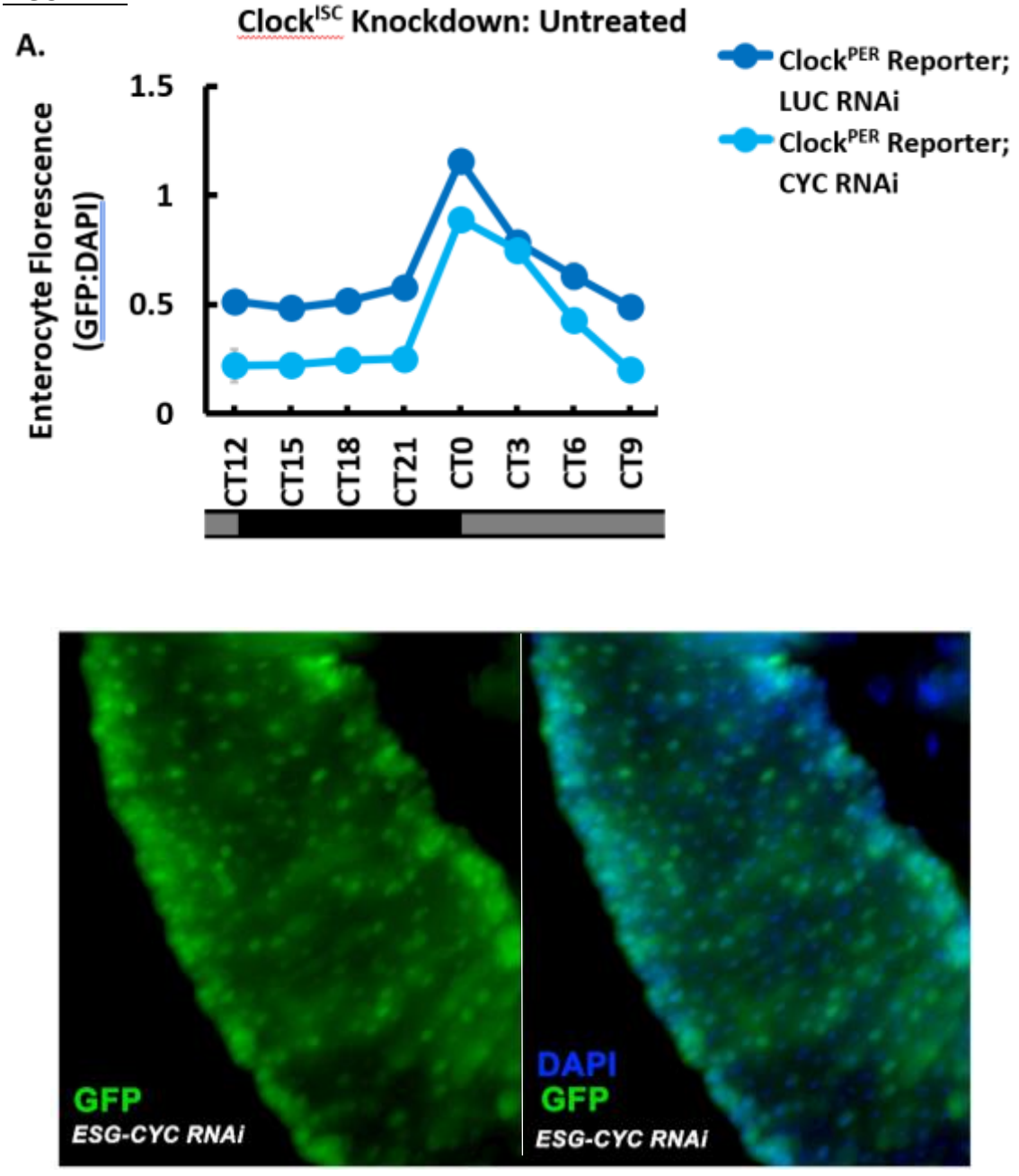
cells when a specific cell type had a clock that was disrupted. The groups of positive control flies (ISC^{Luc} KD/ EC^{Luc} KD) contained a KD for *Lucferase* (*LUC*) transcript driven by ISC and EC markers. This gene does not exist in the *Drosophila* genome so the KD should have no effect and reporter activity should occur as normal.

To determine if clock disruption is occurring in each cell type, high resolution photos of the intestine with a GFP and DAPI overlay were examined. ISCs and ECs were both identified by their relative sizes, ISCs being the smallest cells and ECs being the largest. Cells containing a clock disturbance should be stained with DAPI but produce no GFP signal, and this was confirmed when images were examined (Figure 12).

ISC^{Luc} KD control flies produced a robust circadian rhythm (Figure 12) with significant variance over time ($F(7, 77) = 41.01$, $P<0.0001$). This rhythm peaked at CT0 and troughed at CT9. ISC^{CYC} KD flies produced a significantly variant rhythm ($F(7, 73) = 49.72$, $P<0.0001$) with the same phase as its control but the amplitude of reporter signal was significantly lower (Figure 12) at each time point ($F(7, 168) = 3.603$, $P=0.0012$). This was due attenuated signal output from ISCs leaving only the signal produced by ECs and EBs. EC^{LUC} KD control flies also produced a circadian rhythm with significant variation over time (Figure 12) peaking at CT0 and a troughing around CT9 ($F(7, 88) = 24.95$, $P<0.0001$). EC^{CYC} KD flies also produced a significant circadian rhythm ($F(7, 84) = 39.36$, $P<0.0001$) with a peak at CT0 and a trough around CT12 but with a significantly lower amplitude (Figure 12) than its control ($F(7,168) = 21.17$, $P<0.0001$) Once again, differences in reporter expression amplitude is most likely due to the lack of reporter signal coming from ECs leaving signal only from ISCs and EBs to be measured. This data suggests that under

normal conditions the circadian clocks in the *Drosophila* ISCs/EBS versus ECs function completely autonomously from one another.

FIGURE 12


A.
Clock^ISC Knockdown: Untreated
Clock^PER Reporter; LUC RNAi
Clock^PER Reporter; CYC RNAi
Enterocyte Florescence (GFP:DAPI)
1.5
1
0.5
0
CT12
CT15
CT18
CT21
CT0
CT3
CT6
CT9
GFP
ESG-CYC RNAi
DAPI
GFP
ESG-CYC RNAi

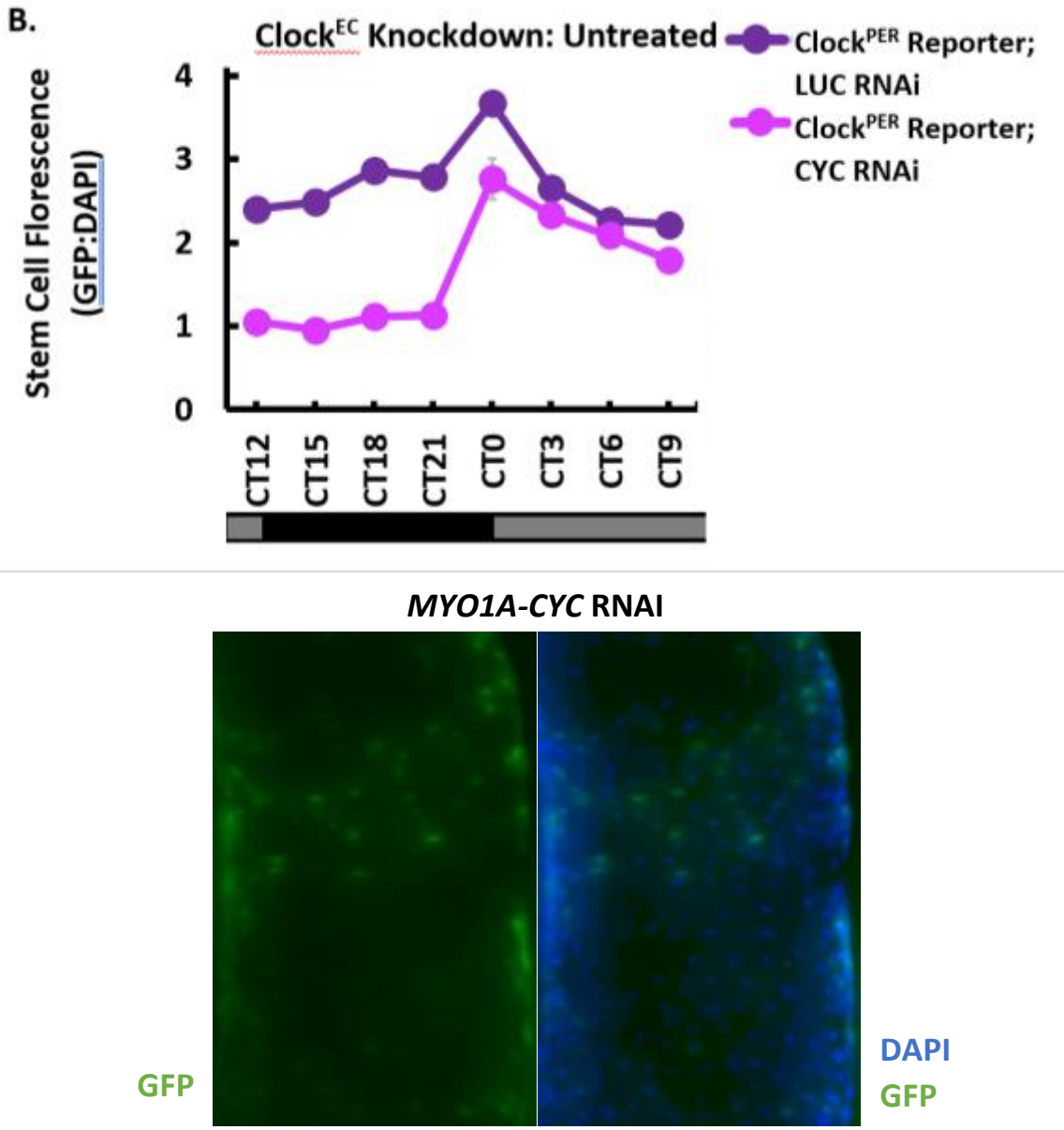


Figure 12: Time series of Clock^{PER} reporter GFP fluorescence when the clock is knocked down in ISCs, and ECs (n=12 intestines per timepoint)
A: Clock^{PER} reporter signal from *LUC* knockdown and a *CYC* knockdown in the ISCs. *CYC* knockdown in ISCs has no effect on clocks in the rest of the system. Images of intestine found below graph
B: Comparing Clock^{PER} reporter signal from intestines containing a *LUC* knockdown and a *CYC* knockdown in the ECs. *CYC* knockdown in ECs has no effect on clocks in the rest of the system. Images of intestine found below graph.

3.8 Circadian Autonomy in Regeneration

Since regeneration only takes place when the intestine is damaged and in need of repair, it is possible that the normally autonomous circadian clocks in ISCs/EBs and ECs become interlinked only under stressed conditions. To induce damage dependent processes in the intestine, fly food was infused with bleomycin. Bleomycin is a chemotherapeutic which is used to damage DNA in cells, leading to apoptosis (Amcheslavsky et al. 2009). Under damaged conditions both ISC^{LUC} control KD and ISC^{Cyc} KD flies maintained significant rhythms (Figure 13) in reporter expression over time ($F(7, 38) = 3.623$, $P=0.0044$; $F(7, 38) = 13.71$, $P<0.0001$). ISC^{CYC} KD flies produced an overall lower amplitude rhythm than its control ($F(7, 168) = 2.973$, $P=0.0058$), similar to previous findings (Figure 12A). EC^{LUC} KD and EC^{CYC} KD flies also maintained a significant circadian rhythm (Figure 13) in reporter expression under stress ($F(7, 40) = 6.667$, $P<0.0001$; $F(7, 37) = 4.575$, $P=0.0009$). An overall lower reporter expression was measured in EC^{CYC} KD compared to its control ($F(7, 168) = 4.871$, $P<0.001$). Under bleomycin stress ISC and EC control flies both produced extremely attenuated reporter fluoresce when compared to unstressed conditions ($F(7, 114) = 10.6$, $P<0.0001$; $F(7, 172) = 19.24$, $P<0.0001$). Although Bleomycin did not have an effect on the autonomous state of the intestine, it did have an overall attenuating effect on WT flies in both ISC and EC knockdown experiments when compared to undamaged conditions.

FIGURE 13

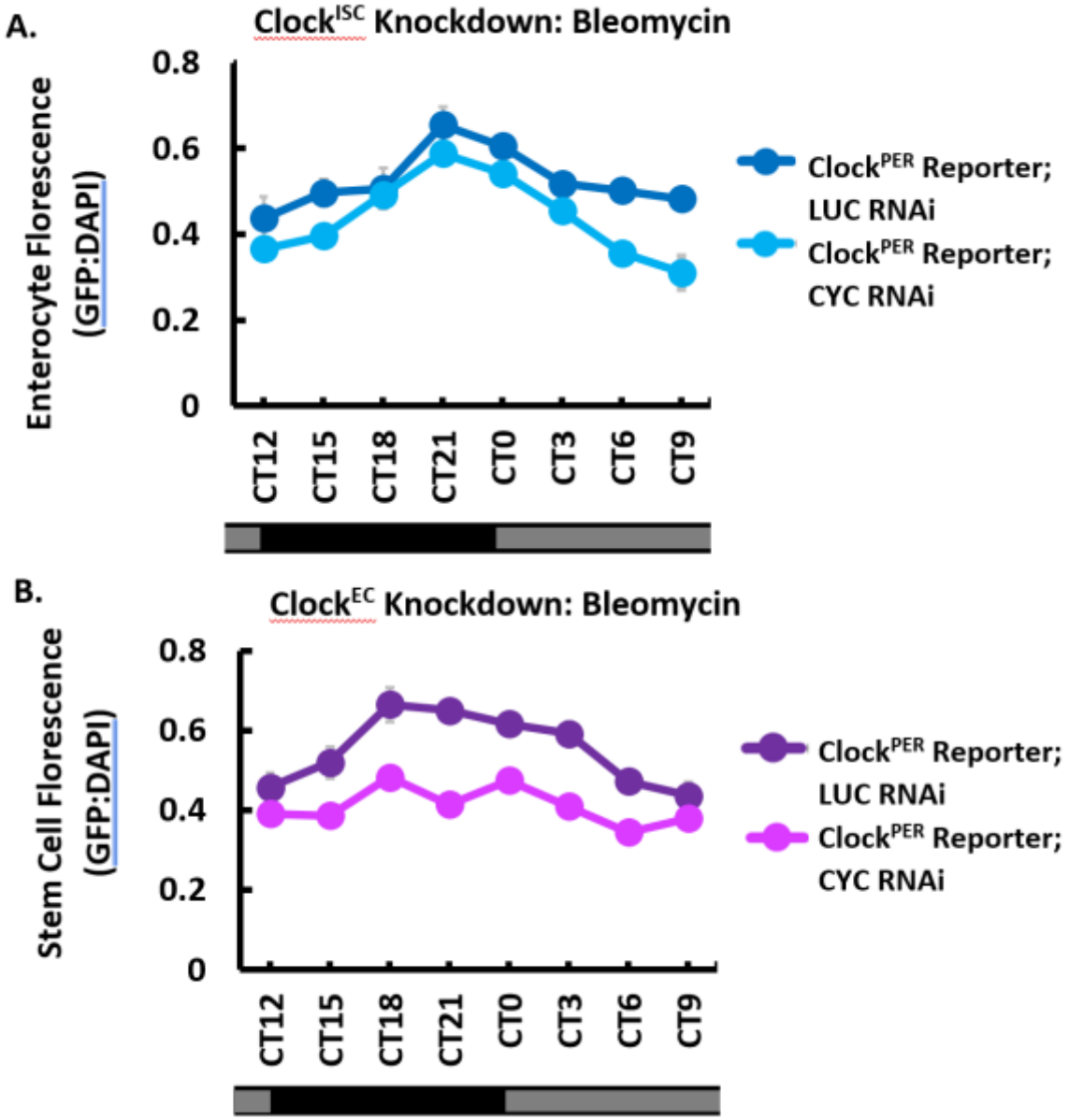


Figure 13: Time series of Clock^{PER} reporter GFP fluorescence when the clock is knocked down in ISCs, and ECs under acute damage conditions(n=12 intestines per timepoint)

A: Comparing Clock^{PER} reporter signal from intestines containing a *LUC* knockdown and a *CYC* knockdown in the ISCs. *CYC* knockdown in ISCs has no effect on clocks in the rest of the system. Both control and experimental knockdowns show a damage related disruption in clock function

B: Comparing Clock^{PER} reporter signal from intestines containing a *LUC* knockdown and a *CYC* knockdown in the ECs. *CYC* knockdown in ECs has no effect on clocks in the rest of the system. Both control and experimental knockdowns show a damage related disruption in clock function.

3.9 The Old Intestinal Circadian Clock

Aging is a natural chronic stress that is experienced by all living organisms. In *Drosophila* aging has been shown to lower the output of the critical clock genes *TIM* and *PER*, overall lowering the efficiency with which the clock functions (Rakshit 2012). This is not the case in aged mice which continue to produce strong central clock oscillations in the brain although evidence has been shown that there are age related peripheral clock changes in the murine liver (Hatanaka et al. 2017). In *Drosophila* the effects of aging on peripheral clocks has not been well developed.

When the $Clock^{PER}$ reporter was examined in aged ISC^{Luc} KD flies (Figure 14), a statistically significant rhythm was produced ($F(7, 37) = 13.32$, $P<0.0001$), with a primary peak at CT0 and smaller peak at CT15. ISC^{Cyc} KD flies also produced a significantly variable reporter signal (Figure 14), with a primary peak at CT0 and a smaller second peak at CT9 ($F(7, 38) = 26.43$, $P<0.0001$). The overall rhythm signal was lower when compared to the control ($F(7, 168) = 15.04$, $P<0.0001$). When the effects of aging were examined in EC^{Luc} KD flies and EC^{Cyc} KD flies, aberrant but significant rhythms were still produced ($F(7, 38) = 11.21$, $P<0.0001$; $F(7, 44) = 9.898$, $P<0.0001$). The signal attenuation between control and clock knockdown flies was significant ($F(7, 168) = 12.84$, $P<0.0001$). Although aging did not have an effect on the autonomous state of the intestine, it did have an overall attenuating effect on reporter expression similar to bleomycin treatment (Figure 12A/B). When compared to undamaged conditions (Figure 12A/B) aged ISC and EC knockdown controls (Figure 14A/B) showed significant attenuation from their undamaged counterparts ($F(7, 114) = 3.498$, $P=0.0020$; $F(7, 172) = 13.73$, $P<0.0001$).

FIGURE 14

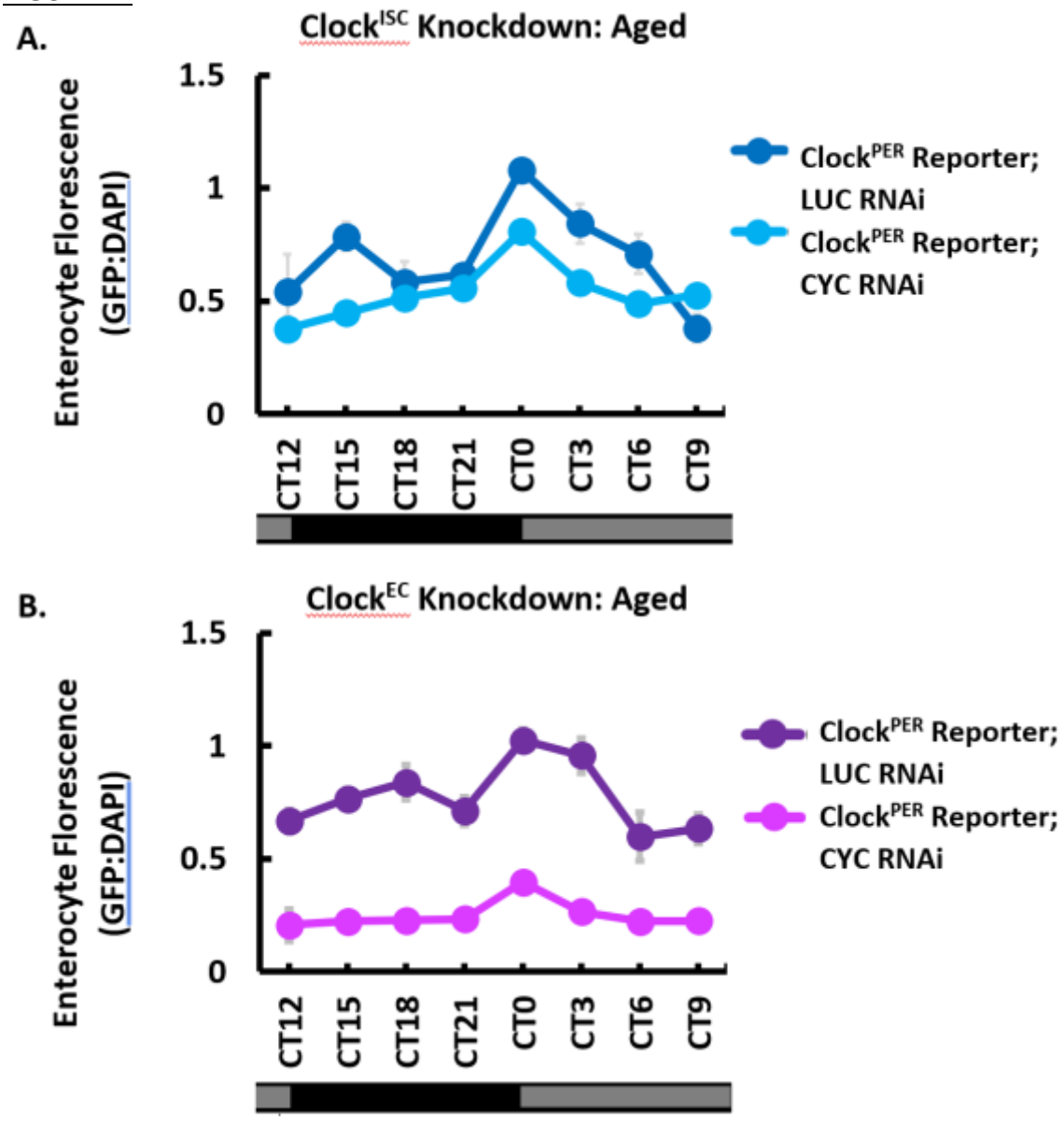


Figure 14: Time series when the clock is knocked down in ISCs, and ECs under chronic damage conditions (n= 12 intestines per timepoint)

A: Intestines containing a *LUC* knockdown and a *CYC* knockdown in the ISCs. *CYC* knockdown in ISCs has no effect on clocks in the rest of the system. Both control and experimental knockdowns show a damage related disruption in clock function

B: Intestines containing a *LUC* knockdown and a *CYC* knockdown in the ECs. *CYC* knockdown in ECs has no effect on clocks in the rest of the system. Both control and experimental knockdowns show a damage related disruption in clock function.

3.10 Does the *Drosophila* Intestine Rely on a Central Pacemaker?

Unlike *Drosophila*, mammalian cells have no way of detecting photoperiod to entrain circadian clocks and, the Suprachiasmatic nucleus (SCN) is required to convey light queues to peripheral clocks throughout the body (Weaver 1998). In *Drosophila*, light can directly affect each cell directly through the protein Cryptochrome (CRY) (Stanewsky et al 1998). When light activates CRY it is responsible for degrading TIM in the PER/TIM dimer complex that represses CLK/CYC dimer activity. With CLK/CYC no longer repressed, it reinitiates *PER* and *TIM* transcription resetting the circadian clock. The ability for light to directly effect each cell brings into question the need for a SCN-like synchronizing factor.

To test a possibleinfluence of the central pacemaker on the peripheral intestinal clock, clock function in the brain was knocked down and $Clock^{PER}$ reporter expression was observed in the intestine over time. To knockdown the clock specifically in the brain the GAL4-UAS/RNAi system was utilized, as previously explained. This time GAL4 was driven by the ELAV brain marker. This drove the production of LUC ($ELAV^{Luc}$ KD) and CYC ($ELAV^{Cyc}$ KD) RNAi, specifically in *Drosophila* brain cells effectively knocking down clock function in that organ. The control, $ELAV^{Luc}$ KD flies, produced a significant typical circadian rhythm (Figure 15) in intestinal reporter expression ($F(7, 78) = 28.91$, $P<0.0001$), with a peak at CT0 and a trough around CT12. $ELAV^{Cyc}$ KD flies, lacking a functional brain clock (Figure 15), also produced a significant rhythm ($F(7, 84) = 14.75$, $P<0.0001$). Since the intestinal rhythm during clock knockdown in the brain was not significantly different from its control ($F(7, 168) = 1.807$, $P=0.0890$), evidence suggests that the intestinal clock is autonomous from the central clock in the brain.

FIGURE 15

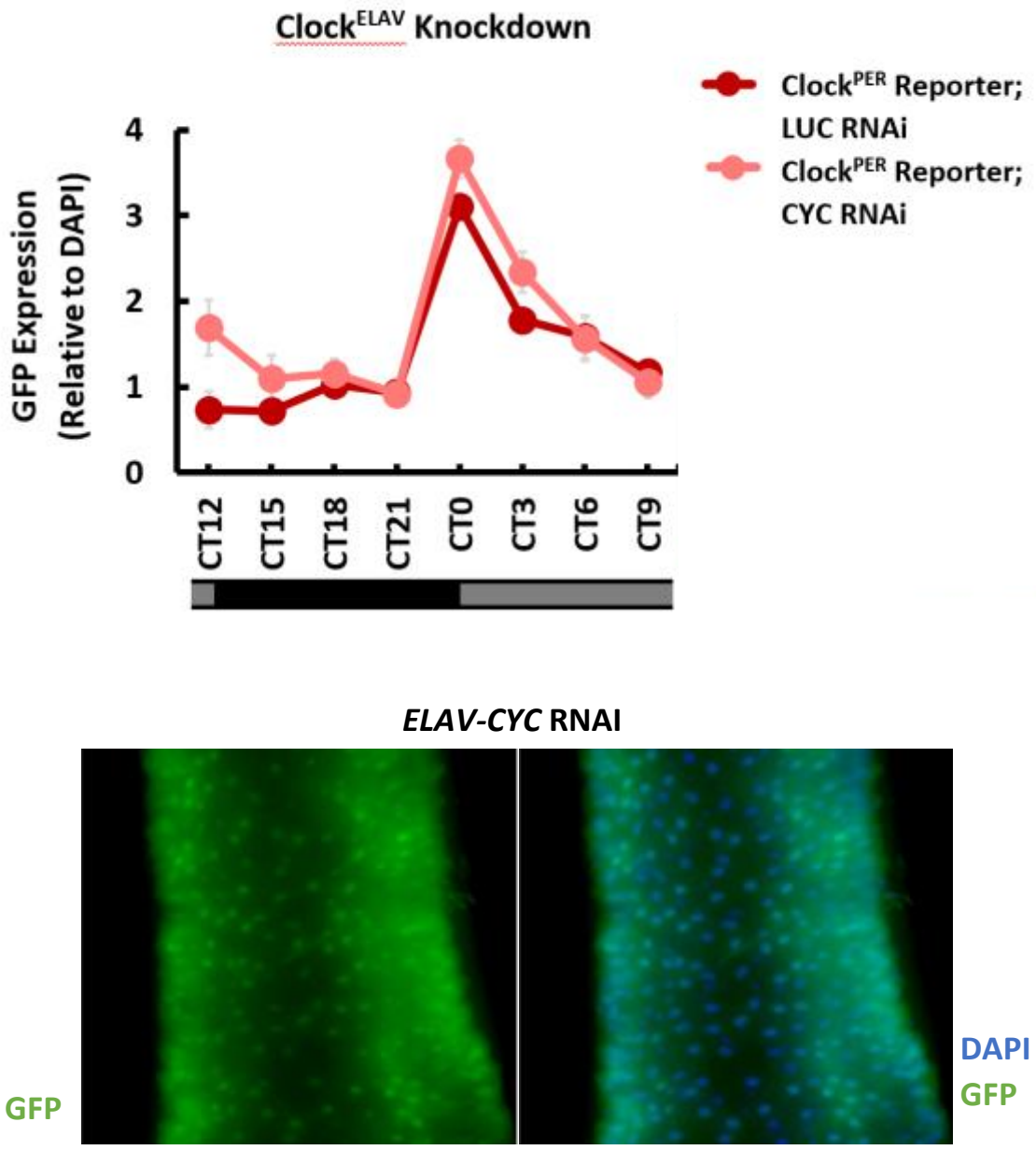


Figure 15: Time series of Clock^{PER} reporter GFP fluorescence from the intestine when the clock is knocked down in the brain (n=12 intestines per timepoint). *LUC* brain knockdowns had no effect on the rhythm in the intestine, as predicted. When the clock is knocked down in the brain, the intestinal clock remains rhythmic producing its primary peak at CT0, similar to the control. Intestinal clock function is autonomous from the brain clock. Image of intestine below graph.

3.11 Can Restricted Feeding Entrain the Intestinal Clock?

In circadian biology, light has been thought to be the primary zeitgeber in *Drosophila*; however, evidence of alternative synchronization methods, such as food and temperature have become recently established in the *Drosophila* system (Xu et al. 2011; Glaser and Stanewsky 2005). I predict that eliminating the ability for light to reset the clock would reveal a secondary light-independent synchronization pathway in *Drosophila*. To test this, flies containing a *Cryptochrome* null mutation (CRY^{01}), that no longer produce CRY protein and are not longer able to reset their clocks via light stimulation, were tested The $Clock^{PER}$ reporter in CRY^{01} flies fed *ad libitum* produced a significant rhythm (Figure 15) with a peak at ZT9 and a sharp drop to a trough at ZT12 ($F(7, 85) = 7.109$, $P<0.0001$). This differs from WT $Clock^{PER}$ reporter flies, whose peak is located at ZT0 and trough at ZT12 (Figure 16).

To further test the ability of food intake time to entrain clocks, I performed restricted feeding experiments, where flies were only allowed to eat at specific time of day. When flies were placed on restricted feeding from ZT0-3 CRY^{01} flies produced a significant rhythm (Figure 16) with a peak at ZT9, and a trough around ZT15 ($F(7,79) = 2.417$, $P=0.0270$), similar to flies fed *ad libitum*, WT flies containing the $Clock^{PER}$ reporter produced its characteristic significant rhythm with a peak at ZT0 and trough at ZT12($F(7, 81) = 8.886$, $P<0.0001$). Alternatively, when feeding was restricted to ZT9-12, rhythms in both CRY^{01} and WT $Clock^{PER}$ reporter flies remained significantly rhythmic($F(7, 87) = 6.083$. $P<0.0001$; $F(7, 85) = 38.69$, $P<0.0001$); however, signal expression was greatly attenuated when compared to ad libitum feeding ($F(7, 114) = 12.23$, $P<0.0001$). These

results suggest that *CRY* mutant flies are able to maintain different rhythms under LD, than WT controls.

FIGURE 16

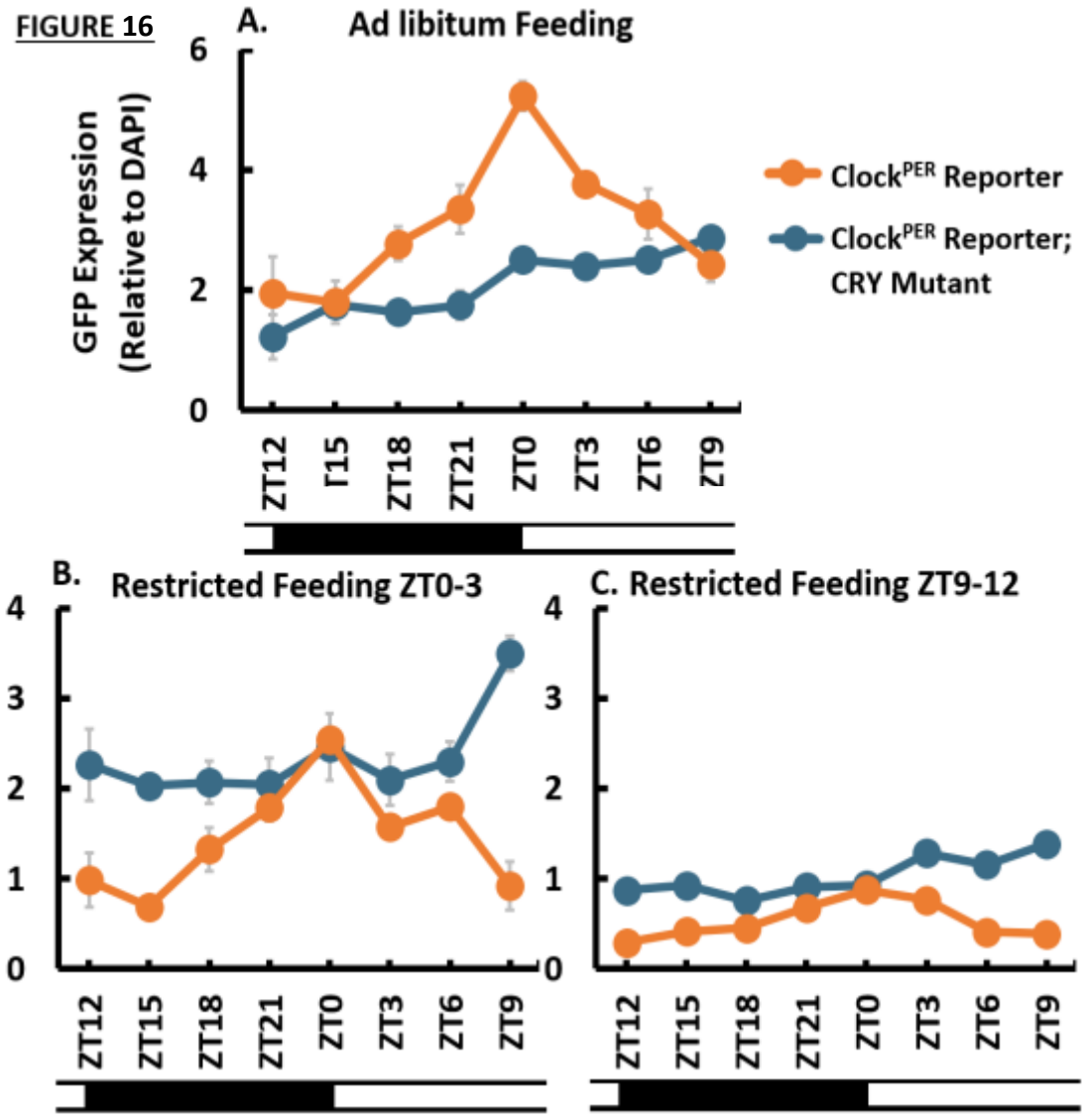


Figure 16: Time series of clock reporter due to restricted feeding regimens (n=12 intestines per time point). Test samples had a CRY mutation to eliminate direct light synchronization.

A: Under *adlib* feeding conditions, the WT reporter produced a rhythm with a peak at ZT0. CRY mutant flies also produced a rhythm, peaking at ZT9 and dropping quickly to ZT12

B: When food is restricted from ZT0-3, the WT produced a rhythm with a peak at ZT0, CRY mutants peak at ZT9 and sharp drop at ZT12.

C: When food is restricted from ZT9-12, the reporter in WT reporter retains its rhythmicity but the signal becomes attenuated. This also appears in CRY mutant flies indicating the restricted feeding is effecting the intestinal system.

Chapter 4: Discussion

As the earth rotates around its axis, 24-hour periods of light and dark are created. Light and dark bring with them different conditions that, if adapted to appropriately, increase fitness. (Hut et al. 2013). For example, terrestrial mammals that require light in order to visually perceive the world around them would be at an immediate disadvantage if their behaviour was nocturnal, their sense of sight would be perturbed, leading to an overall decrease in survival. Endogenous processes are also environmentally dependent. Some organisms have been shown to shift DNA replication to night to avoid exposure to deleterious UV radiations that are present during the day (Nikaido and Johnson 2000). This is considered energetically advantageous. With various environmental dependent processes throughout the body, it would be advantageous for an organism to measure and predict changes in the environment. This ability is referred to as the circadian clock (Hardin 2011). The clock is a genetically based system that allows for predictable physiological responses to environmental changes, enabling animals to better survive in their environment.

The circadian clock reporters described in this thesis contain a minimal promoter for either the *TIM* gene or *PER* gene, arranged in a 4x tandem series to increase transcriptional activation. Each promoter is a minimal (122-174 bp) sequence containing E-box binding sites and other regulatory sites for the CLK/CYC dimer that have been previously shown to drive rhythmic expression of *LacZ* (Hao et al. 1997. McDonald et al. 2001) The promoter array is placed upstream from a superfolder destabilized-GFP, that lasts about an hour and then is degraded (Norbert Perrimon lab unpublished results). So

when the CLK/CYC dimer binds to intrinsic promoter regions of *PER* and *TIM* it also binds to our extrinsic reporters' minimal promoter and drives the transcription of a GFP protein at the same time. Due to a designed PEST domain in the reporter, the GFP signal degrades shortly after being produced so clock activity could be measured dynamically over time

4.1 The Novel Clock Reporters Measure Time Accurately

To characterize how the $Clock^{TIM}$ and $Clock^{PER}$ reporters functioned in the intestine, qPCR and fluorescence time series' were performed. Quantitative polymerase chain reaction is a process that allows for the quantification of specific gene transcripts and tracking of changes in target gene expression over time. *PER* and *TIM* transcripts are known to be rhythmic, so if *GFP* transcripts are also rhythmic then clock reporters will be verified under circadian control. When this technique was applied to $Clock^{TIM}$ reporter intestines over 2 experiments, PER, TIM and GFP transcripts were all produced rhythmically over time, but peaks in expression at varied from ZT15-18 (Figure 5 A/B). When observed in $Clock^{PER}$ reporter intestines, the three transcripts were also rhythmic over time; and produced peaks varying from ZT15-18 (Figure 6 A/B). Previous analysis of the *Drosophila* intestine found that *PER* and *TIM* transcripts peaked at ZT15 (Karpowicz et al 2013). However, both experiments may have missed the true peak of reporter expression due to the chosen strategy of dissection. Since both experiments start at ZT0 and measure transcript activity in increments of 3 hours, the actual peak of activity may actually be between ZT15 and ZT18. For instance, when *TIM* and *PER* qPCR transcripts are

measured in the retina over time, peak expression of both genes occurs at ZT16 (Hardin et al. 1990). Although not completely precise, this level of analysis was suitable for verifying circadian functionality of the reporters at the level of transcription. Future studies could elucidate a more accurate peak of clock transcript activity in the intestine by harvesting samples from ZT14-19 every hour.

Once the peak of *GFP* transcription was established it was important to understand the kinetics of GFP translation. When intestinal fluorescence was observed, both $Clock^{TIM}$ (Figure 6) and $Clock^{PER}$ (Figure 8 A/B) reporters expressed GFP rhythmically over time, with the peak protein expression at ZT0 and a trough around ZT15. This means that there is approximately 5-8 hours between peak RNA expression and peak protein expression of our reporters. Flies containing a CYC null mutation should not produce any GFP signal, since part of its transcriptional activator is non-functional. CYC mutant flies were found to produce no GFP signal (Figure 6/8). Since $Clock^{PER}$ and $Clock^{TIM}$ reporters produced the same rhythm in GFP expression overtime, the $Clock^{PER}$ reporter was arbitrarily chosen for further validation and future experiments.

One important defining aspect of a circadian rhythm is that once a circadian clock is entrained to a rhythm, it will continue to be active under the influence of that rhythm even in the absence of an external stimulus (Bell-Pedersen 2005). To understand how the $Clock^{PER}$ reporter functions in constant conditions, flies were synchronized to a 12:12LD cycle for 5 days. On the day of the experiment flies were transferred to a 12:12 DD constant conditions and dissected across 8 different time points. Under constant conditions, the $Clock^{PER}$ reporter produced GFP following the same pattern of expression

as LD experiments, with a peak at CT0 (Figure 9). Once again, CYC mutant intestines did not produce any GFP signal. With these findings, along with qPCR data, it is clear these reporters can measure and report dynamic clock activity accurately.

4.2 Single Cell Analysis of Clock^{PER} Reporter

Depending on its location, physiology and actively transcribed genes, a cell can take on many identities throughout the body. The *Drosophila* midgut is comprised of 4 different cell types. Stem cells (ISCs) are the only dividing cell in the intestine and are identified by their delta receptors. Upon systemic signals, ISCs divide and differentiate into an intermediate cell called an enteroblast (EB) (Patel and Edgar 2014). The enteroblast no longer divides, however depending on the needs of the system, the enteroblasts differentiates into either an enterocyte (EC) or enteroendocrine cell (EE) (Biteau et al 2011). The enterocyte is the largest cell in the *Drosophila* intestine and has the primary function of nutrient absorption. Enteroendocrine cells primarily function to secrete hormones and are identified by prospero staining. Since each cell has its own genetic identity, physiology and activity, I asked how the Clock^{PER} reporter functioned in each cell type.

Single cell analysis of the intestine was performed at ZT0, the highest time point of reporter GFP expression (Figure 10A/B). At high resolution, it was found that all ECs, EBs, and ISCs produced high levels of circadian reporter GFP. However, GFP signal was not detected in majority of prospero stained EEs (Figure 10A/B). This indicates either EEs do

not have a functional clock, our reporter does not function in mature EEs or circadian clock function is context dependent in these cells. For cells to grow and differentiate, there are multitudes of processes that must occur in a harmonious succession. These processes are subject to activation and silencing through various molecular changes, including epigenetic changes that either open or restrict access to sections of DNA. The end result of differentiation is a new mature cell that contains all the DNA of its progenitor cell, but is differentiated from its progenitor cell in function and gene expression. In these samples the Clock^{PER} reporter was inserted into the second chromosome at an insertion point known as Attp40. This insertion site is utilized do to its well-established history of active transcription in various *Drosophila* tissues. (Markstein et al. 2008) To test if the reporter is not functional at this insertion site, another reliable insertion site, such as Attp2, could be utilized. If reporter expression is still absent in EEs then there is a good chance that the clock is either completely non-functional in EEs or only functional in EEs under the right physiological context.

4.3 *Drosophila* EBs and ISCs Have Cell-Autonomous Clock Function

In *Drosophila*, the clock has been shown to be a highly complex mechanism comprised of both autonomous and non-autonomous entrainment pathways depending on the tissue. Therefore, it is important to understand how the clock functions in each tissue. Previous research established in this lab has suggested that rhythms in intestinal regeneration are dependent on functional clocks in both ECs and ISCs, inferring a non-

autonomous circadian pathway (Karpowicz et al. 2013). When cellular clock autonomy in the intestine was tested by knocking down the clock in ISCs, the rest of the intestine was found to be autonomous continuing to produce peak reporter expression at CT0 (Figure 12A). Knocking out the clock in ECs also had no effect on the clocks in the rest of the intestine which continued to peak at CT0 similar to its control (Figure 12B). Functional cell types still perpetuated a circadian rhythm in DD conditions, leading me to conclude that under non-stressed conditions clocks in *Drosophila* intestinal cells are cell-autonomous. Since the intestine is responsible for handling direct external cues that are not guaranteed to be consistently present, cellular autonomy may be advantageous for faster homeostatic responses. This may be because autonomous cells eliminate the need for cellular communication decreasing the overall processes that need to occur for compensation and entrainment.

4.4 The Intestinal Clock is Cell-Autonomous Under Stress

To investigate the cell autonomy of intestinal clocks further, it was postulated that since the previous evidence of cellular dependency in clock function only occurs in a regenerative context then the intestine must be damaged in order to reveal clock interdependence. Under acute damaged conditions, when the clock is KD in ISCs the $Clock^{PER}$ reporter in the intestine remains rhythmic in all genotypes, however, the overall signal when compared to undamaged conditions seems attenuated. (Figure 13A). When the clock is KD in ECs, the rhythm is diminished. However, since the control also appeared

to show different rhythmicity from undamaged conditions it is hard to conclude that this change is due to clock KD. Since the clock is unaffected by the non-functional clocks around it, intestinal cells are autonomous under acute stress. Although clock knockdowns did not have an effect on the system, it appears as though bleomycin stress causes an overall attenuation in clock reporter signal, compared to their undamaged counterparts.

Aging is a different kind of chronic stress experienced by almost all living organisms. Aging has the ability to disrupt systemic homeostasis which leads to an overall decrease in energetic favourability. When enough of these systems become disrupted, death occurs. Aging has been shown to have abnormal effects on the clocks in various tissues. For example, old mice show a marked decrease in the amount of rhythmic gene expression in their liver when compared to a younger mouse reared under the same conditions (Sato et al 2017). In *Drosophila,* aged flies lose rhythms in their sleep/wake cycles (Koh et al. 2006) and head samples show dampened oscillations in PER and TIM transcripts (Rakshit et al 2012). Aging has also been shown to cause widespread deregulation of stem cell activity in the *Drosophila* intestine causing an increase in intestinal stress markers (Buchon et al. 2013).

When analyzing the Clock^{PER} reporter in control flies which contain LUC knockdown in their ISCs, the characteristic reporter rhythm maintained its central peak at CT0 (Figure 14A). Similarly, when the Clock^{PER} reporter was analyzed in flies containing a CYC knockdown in ISCs the functional cells produce rhythmic reporter expression, with a central peak at CT0 (Figure 14A). When the Clock^{PER} reporter was analyzed in control flies that contained a *LUC* knockdown in their ECs, the characteristic reporter rhythm was

still present (Figure 14B). Similarly, when clock function is knocked down in ECs, functional cells still produce a rhythm in GFP activity (Figure 14B). Similar to the bleomycin-induced stress, aging stress also greatly attenuates reporter signal when compared to younger tissues.

Although the intestine remains autonomous under aged and bleomycin treatments, stress itself seems to have a negative effect on reporter function, inferring that stress also has the potential to disrupt the functionality of core circadian processes. This may highlight an evolutionary flaw in the Drosophila circadian system. Without intracellular communication of circadian clock entrainment, each individual cell is responsible for its own synchrony. This is a problem, since stress induced clock disruptions cannot be overcome by compensatory signalling from other cells.

4.5 Intestinal Clock Synchrony Does Not Rely on a Functional Brain Clock

With no indication of interdependence between clocks in different intestinal cell types, I asked whether the whole intestine was autonomous from the brain, or if it relied on non-autonomous brain signals to remain entrained under constant conditions. To test if the intestinal clock was autonomous from the brain clock, brain clock functionality was knocked down and the reporter in the intestine was observed (Figure 15). Control flies containing a LUC knockdown in the brain, produced the characteristic Clock^{PER} reporter rhythm in their intestine, with a peak at CT0. When the clock was knocked down specifically in brain cells the intestinal clock continued to produce a circadian rhythm

similar to the control sample, with a primary peak at CT0. This evidence suggests that the intestinal clock is autonomous and self sustained by light cues.

4.6 Restricted Feeding Entrains the Intestine

Feeding has been thought to be a behaviour driven by light dark cues. Feeding timing can act as its own synchronization factor as demonstrated in restricted feeding experiments done on mice and flies. (Xu et al. 2011; Carneiro and Araujo 2012). In the *Drosophila* fat body restricted feeding has been shown to synchronize the phase of *PER*/*TIM* transcript expression independently of the central clock (Xu et al. 2008). Previous studies have shown that *Drosophila* normally feed early in the morning (ZT0-3) and *PER* and *TIM* transcripts peak at CT12. However, if food intake is restricted to a time of day in which feeding does not normally occur (ZT9-12), *PER* and *TIM* peaks are shifted to CT4 and CT8 respectively (Xu et al. 2008).

When the Clock^{PER} reporter is observed in the intestines of wild type flies fed ad libitum, the characteristic reporter rhythm is produced, peaking at ZT0 (Figure 16A). However, when observed in CRY^{01} mutants, the Clock^{PER} reporter peaked at ZT9 and sharply dropped to a trough at ZT12. When food intake is restricted to ZT0-3, a normal feeding time, WT and mutant flies remained rhythmic maintaining their peaks at ZT0 and ZT 9 respectively (Figure 16B). However, when food intake is restricted to a time of the day when they do not normally eat, ZT9-12, Clock^{PER} reporter expression changes in both WT and mutant flies (Figure 16C), by greatly attenuating reporter expression. In all cases,

the Cry^{01}mutant shows a different rhythm that the WT control, suggesting that without photoperiod entrainment, circadian clocks have an altered phase in the CRY mutant. These findings are coherent with previous data established in the fat body (Xu et al 2001). This new data suggests that restricted feeding affects the overall efficiency with which the intestinal clock coordinates food cues with light cues.

As mentioned previously, the intestine comes in direct contact with external cues that are not guaranteed to be consistently present. I would assume that being able to decouple circadian clock synchrony from the brain would be advantageous, since it allows quick and direct organ entrainment without the need for inputs from other clock systems, when a change in environmental nutrition occurs. Since the brain does not appear to be effected by nutritional restriction, it is important that the intestine be able to sense these changes and react accordingly to maintain circadian homeostasis.

Chapter 5: Conclusion

My research on circadian rhythms in the *Drosophila* intestine has provided insight into the field of food entrainment, clock autonomy, and circadian responses to stress. I conclude that intestinal cell clocks function extremely robustly in a cell-autonomous fashion and that both photoperiod and restricted feeding have the ability to directly influence the core circadian clock in the intestine. Although the circadian clock is a robust mechanism, new research findings indicate that homeostatic disruptive process' such as cancer and aging can have strong effects on circadian function. For example, lung adenocarcinoma is able to distally rewire which transcripts in the liver become rhythmic, as well as many different metabolites when compared to controls (Masri et al 2016). Aged mice also show a difference in their rhythmic transcriptome when compared to younger mice (Sato et al. 2017). When the rhythmic transcriptome of young mouse livers was compared to old mouse livers it was found that many of the once rhythmic transcripts were no longer rhythmic. However new transcripts, not rhythmic in young mice, were found to be rhythmic in old mice. With the boom of industrialism and home electricity, our connection between our circadian rhythms and the natural environment have become drastically decoupled. By developing the field of circadian biology, we are able to understand the damaging effects of a disrupted circadian clock and work towards strategies to prevent or mitigate its negative effects on our health.

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