Characterizing the relative abundance of circadian transcription factors in diapausing and nondiapausing Northern house mosquitoes

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Abstract:

The Northern house mosquito (*Culex pipiens*) is a major vector of West Nile virus. To survive harsh conditions in winter adult females of *Cx. pipiens* enter a state of arrested reproductive development called diapause. Diapause is triggered by the short daylengths of late summer and early fall. The methods by which *Cx. pipiens* measures daylength are still unknown. However, it is suspected that clock genes, which provide information on daylength, may also regulate diapause. The proteins produced by these genes often cycle in abundance throughout the day in diapausing and nondiapausing insects. Two clock genes suspected to control diapause are *cycle (cyc)* and *Par domain protein1 (Pdp1)* as they encode circadian transcription factors that may regulate genes that are involved in diapause. Using Western blotting we measured the relative protein abundance of CYC and PDP1 throughout the day in the whole bodies and the heads of *Cx. pipiens* reared under either long-day, diapause-averting conditions or short-day, diapause-inducing conditions. We found that in whole bodies there was no significant oscillation of CYC or PDP1 abundance in both long day and short day-reared mosquitoes. In the heads of long day-reared mosquitoes both CYC and PDP1 cycled. In contrast, only PDP1 abundance showed diel differences in abundance in the heads of short day-reared mosquitoes. These data bring us one step closer to understanding the role that CYC and PDP1 may play in regulating diapause and other biological processes.

Key words: Clock proteins, Western blotting, *Culex pipiens*, Cycle (CYC), Par Domain Protein1 (PDP1), seasonal responses

1.0 Introduction

Diapause is a state of arrested development that is used by arthropods to survive prolonged periods of unfavorable conditions. Facultative diapause is triggered by external cues (reviewed by Denlinger et al., 2017), as opposed to obligatory diapause that occurs automatically at a specific life stage (Frohne, 1954; West and Black IV, 1998). In temperate environments insects often enter facultative diapause in response to short daylengths in the late summer and early fall (Gill et al., 2017; Tauber and Kyriacou, 2001). Adult females of the Northern house mosquito, *Culex pipiens*, enter a facultative, reproductive diapause before winter's onset (reviewed by Gill et al., 2017; Spielman and Wong, 1973). To prepare for diapause, females of *Cx. pipiens* seek out protective refuges (Eldridge, 1987) and feed on sucrose-rich nectar (Bowen, 1992). While in diapause females are characterized by having low levels of juvenile hormone (JH; Spielman, 1974) and therefore do not develop egg follicles or lay eggs (Spielman and Wong, 1973). Instead, diapausing females of *Cx. pipiens* divert energy gained from nectar-feeding to build fat stores (Robich and Denlinger, 2005). Diapausing female mosquitoes also halt host-seeking-behavior (Bowen et al., 1988), meaning they do not take a bloodmeal and therefore do not transmit disease. The molecular mechanisms that insects such as *Cx. pipiens* use to distinguish long, summer days from short winter days and thereby initiate diapause are still unknown, but evidence suggests that the circadian clock may be involved (Meuti et al., 2015; Chang and Meuti, 2020).

The circadian clock consists of a network of genes and provides information on the time of day (Schotland and Sehgal, 2001). Bünning (1936) hypothesized that the circadian clock might also measure daylength and therefore be involved in photoperiodic or seasonal responses. The circadian clock has been extensively studied in *Drosophila melanogaster* (Cyran, 2005;

Cyran et al., 2003; Sehgal et al., 1994, 1992) where clock proteins CLOCK (CLK) and CYCLE (CYC) form a heterodimer that binds to E-box promoters in the *period* (*per*) and *timeless* (*tim*) genes (Rutila et al., 1998), thereby increasing the transcription of *per* and *tim* until their mRNA levels peak during the early scotophase (Darlington, 1998; Hardin, 2005). PER and TIM protein abundance peaks late in scotophase, allowing TIM to facilitate PER's entry into the nucleus (Hardin, 2005). PER then represses the activity of CLK:CYC and thereby inhibits its own transcription which completes the core negative feedback loop of the clock (Allada et al., 2001; Hardin, 2005). In *D. melanogaster* CYC:CLK also upregulate transcription of *vrille* (*vri*) and *Par domain protein1* (*Pdp1*), two components of a secondary feedback loop (Hardin, 2005). While *vri* mRNA peaks at the onset of scotophase and its protein peaks ~3 hrs later, *Pdp1* mRNA and PDP1 protein show peak abundance in the mid-scotophase in the heads of *Drosophila* (Cyran et al. 2003). However, Bu et al. (2020) found a slightly different pattern where *Pdp1* mRNA peaked at the onset of scotophase (ZT12), whereas the abundance of PDP1 protein peaked occurred 4 hours later. Although both studies report peak PDP1 protein levels occur in the mid-scotophase, it is surprising that they describe different peaks in *Pdp1* mRNA levels as both studies characterized clock transcript and protein abundance the heads of *D. melanogaster* that were exposed to the same light regime (Light:Dark 12:12). In contrast to VRI, which acts as a transcriptional repressor, PDP1 activates the transcription of *Clk* (Cyran et al., 2003). Due to the opposing actions of VRI and PDP1 on *Clk* transcription, *Clk* mRNA oscillates throughout the day in *D. melanogaster* (Bae et al. 2000; reviewed in Hardin 2005).

Clock genes are highly conserved among insects and other animals (Helfrich-Förster, 2004; Reitzel et al., 2010) and regulate many physiological responses (Meireles-Filho and Kyriacou, 2013). Expression of *Pdp1* mRNA oscillates in the mosquitoes *Aedes aegypti* and *Cx.*

quinquefasciatus with peak transcript abundance occurring at mid-scotophase and the lowest levels of mRNA in the middle of the photophase (Gentile et al., 2009), matching the expression profile seen in *Drosophila* (Cyran et al., 2003). Similarly, *Pdp1* transcripts were most abundant at mid-scotophase and were lowest in mid-photophase in the heads of the mosquito *Anopheles gambiae* (Rund et al., 2011). In the brains of *Cx. pipiens*, *Pdp1* mRNA cycled under long day conditions with peak expression occurring at late scotophase and lowest abundance at mid-photophase, whereas *Pdp1* transcripts did not cycle in the brains of short-day reared females but were more abundant than in the brains of long day-reared females (Chang and Meuti, 2020). Daily changes in PDP1 abundance have not been measured in any insect apart from *D. melanogaster* where researchers have observed a peak protein abundance in the middle of the scotophase (ZT18 by Cyran et al. 2003; ZT16 by Bu et al. 2020).

Although the profile of *Pdp1* mRNA transcripts is relatively consistent across all insects where it has been measured, *cyc* mRNA and protein levels are more variable. For example, CYC protein abundance is constant in the cricket *Dianemobius nigrofasciatus* (Shao et al., 2008), and neither *cyc* mRNA nor CYC protein abundance oscillate in higher dipterans including *D. melanogaster* (Bu et al., 2020) and the flesh fly *Sarcophaga crassipalpis* (Goto and Denlinger, 2002), as well as the hymenopteran sawfly *Athalia rosae* (Bembenek et al., 2007). However, long day-reared parasitoid wasps of *Nasonia vitripennis*, another hymenopteran, *cyc* transcripts peaked during mid-photophase and were at their lowest levels at mid-scotophase (Benetta et al., 2019). Notably, *cyc* mRNA does not oscillate in the silkworm *Bombyx mori* (Markova et al., 2003). However, *cyc* mRNA oscillates in another lepidopteran, *Sesamia nonagrioides*, such that *cyc* mRNA transcripts peak in the mid-scotophase and are lowest at mid-photophase (Kontogiannatos et al., 2017). Unlike the higher dipterans where *cyc* expression remains constant

(Bu et al., 2020; Goto and Denlinger, 2002), *cyc* mRNA oscillates with peak abundance at lights-on and lowest at lights-off in mosquitoes such as *An. gambiae*, *Ae. aegypti*, *Cx. quinquefasciatus* and *Cx. pipiens* (Rund et al., 2011; Gentile et al., 2009; Meuti et al., 2015). This demonstrates that although the sequences of clock genes are conserved, the daily profiles of transcript abundance can vary across different insects and even among insects within the same order.

Although the mRNA expression profiles of *cyc* and *Pdp1* have been characterized under different seasonal conditions in *Cx. pipiens* (Meuti et al., 2015; Chang and Meuti, 2020), we still do not know if these translate to changes in their protein levels. Moreover, we do not fully understand the role that these circadian transcription factors play in initiating seasonal responses. Therefore, to clarify the role of CYC and PDP1 in the circadian clock of *Cx. pipiens* and whether their daily patterns of protein abundance change under different seasonal conditions, we measured the relative protein abundance of CYC and PDP1 in the whole bodies and heads of female *Cx. pipiens* that were reared under long- and short-day conditions. We expected that the relative protein abundance would track daily changes in mRNA transcripts with a 4-hour delay as was seen in clock genes and proteins in *D. melanogaster* (Bu et al., 2020). Specifically, we hypothesized that relative CYC protein abundance would peak at mid-photophase and be lowest at mid/late-scotophase in both nondiapausing and diapausing females of *Cx. pipiens*, corresponding with previously observed trends in its mRNA expression (Meuti et al., 2015). In contrast, we predicted that the relative abundance of PDP1 would peak at mid-scotophase and be lowest during the late photophase in nondiapausing females and not cycle in diapausing females, as again *Pdp1* transcripts oscillated in nondiapausing mosquitoes but were constitutively upregulated and did not oscillate in diapausing females of *Cx. pipiens* (Chang and Meuti, 2020). As CYC and PDP1 are transcription factors that regulate the expression of genes both within and

outside of the circadian clock (Rutila et al., 1998; Cyran et al., 2003; Benito et al., 2010), knowing when CYC and PDP1 proteins are most abundant and whether this changes in response to different seasonal conditions could explain how *Cx. pipiens* distinguish long, summer days from short winter days, and to what extent these circadian transcription factors may regulate the diapause phenotype.

2.0 Materials and Methods

2.1 Mosquito rearing

Larvae of *Cx. pipiens* (Buckeye strain, established in 2013) were reared in environmental chambers set to 18°C and either short day, diapause-inducing conditions (8 hours of light and 16 hours of dark or L:D 8:16) or long day, diapause-averting conditions (L:D 16:8). Larvae were fed a diet of ground Tetramin fish food (Melle, Germany) until pupation. Plastic cups containing pupae were placed into mesh cages and continuously exposed to either long-day or short-day conditions. As adults emerged, pupal dishes were moved to new cages every 24 hours to ensure that adults were the same age. Adults were provided constant access to 10% sucrose solution and water. Four days after adult emergence, long and short-day reared females were divided equally among seven small plastic containers (one for each sampling timepoint) that were placed within the same incubator and given constant access to 10% sucrose solution and water. Mosquitoes were kept in these containers for three days and then were euthanized at specified times (7 days after adult emergence).

2.2 Sample collection and preparation

Protein isolation, separation and Western blotting methods were adapted from Piermarini et al. (2009, 2010). Protein was isolated from 5-8 biological replicates each containing either 15

whole bodies or 45 heads of long or short day-reared females collected one week after adult emergence (n = 5-8 biological replicates per sampling time per photoperiod). Collections began one hour after the lights turned on (Zeitgeber 1 or ZT1), and subsequent samples were collected every 4 hrs (ZT 5, 9, 13, 17 and 21; Fig. 1) by freezing groups of mosquitoes in a -70° C freezer. Tissues were homogenized in 500µl Urea lysis buffer (for 1L: 1.8 g Urea, 1.25g NaCl, 1.5 ml of 10% Sodium dodecyl sulfate, 1.50µl Trition x 100, topped off to 50ml water) with 2µl EDTA and 2µl protease inhibitor (Thermo Scientific) until a uniform consistency was reached. Tubes containing homogenized females were centrifuged at 750 g for 15 minutes and the supernatant was transferred to a clean 1.5 ml tube and frozen at -80° C until analysis. After isolation, the protein concentration of each sample was measured using a NanoDrop One Spectrophotometer (Thermo Scientific). Proteins were then diluted and 60 µg of each protein sample and 10 µl LSB (600 ul Tris HCl, 5 ml 50% glycerol, 2 ml SDS, 1 ml 1% bromophenol blue, 900 ul deionized water, 1.055 ml 2-Mercaptoethanol) was incubated at 95°C for 5 minutes. Each sample was then loaded into wells of a 4% stacking and 10% separating polyacrylamide gel, and 6 µl PageRuler Plus Pre-Stained Protein ladder (Thermo Scientific) was loaded into the first lane. The gel was run for 1 hour at 200V in a Mini Blot Module (Invitrogen) with running buffer (3 g Tris Base, 14.4 g Glycine, 1 ml 10% SDS, brought up to 1 L with DI water [pH 8.3-8.48]). After electrophoresis, the stacking gel was discarded, and the separated proteins were transferred to a PDVF membrane (0.45 pore size; Thermo Scientific) in transfer buffer (1.48 g Tris Base, 7.2 g Glycine, 800 ml DI water, and 200 ml MeOH [pH 8.1-8.4]) using electrophoresis (15V applied overnight).

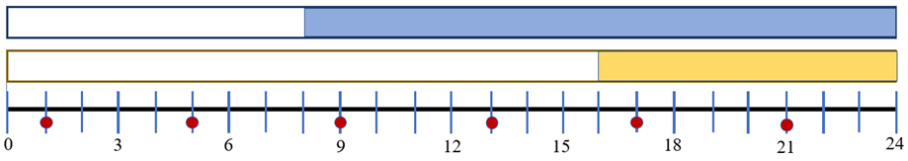

Figure 1: Photoperiodic regimes and sample times

Photoperiodic regimes for short-day (blue) and long-day (yellow) conditions. Open bars represent when the lights were on, while solid bars indicate when the lights off. Zeitgeber time points are given along the bottom, while red dots that signify when mosquitoes were sampled.

2.3 Western blotting

Ponceau stain (100 mg Ponceau Dye power, 5 ml acetic acid, and 95 ml water) was used to confirm a successful transfer of protein to the PDVF membrane. The membrane was incubated in blocking solution containing 5% milk in TBST (8.006g NaCl, 10 ml Tris HCl, DI water to 1L, brought to a pH of 7.5 with NaOH) for 2 hours at room temperature on a rocking platform. Custom, primary monospecific antibodies were made against *Cx. pipiens* CYC [amino acid sequence: KRKFSYNDNSDIEDDTGDDAKSVR] and PDP1 [amino acids sequence RIFDAGKSSNNNKDIC] in rabbit by Pacific Immunology. Primary anti-CYC and anti-PDP1 antibodies were applied to the membrane at a concentration of 1:5,000. To ensure that equal amounts of protein were loaded in each sample, a primary antibody specific to glyceraldehyde 3-phosphate dehydrogenase (GAPDH; GeneTex) isolated in rabbit was applied to the membrane at a concentration of 1:5,000 in 5% milk in TBST. All primary antibodies were incubated overnight on a rocking platform with ice. The membrane was washed three times for 5 minutes each in TBST to remove excess primary antibodies. A secondary antibody (goat anti-rabbit conjugated with HRP; Invitrogen) at a concentration of 1:20,000 in 5% milk in TBST was added to the membrane and incubated for 2 hours. Immunoreactive protein bands were visualized by applying 300 ul SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Scientific) to the

membranes which were then covered with cling film and incubated for 15 minutes in darkness. The cling film-wrapped membrane was scanned in a digital imager (BioRad) first under the chemiluminescent setting without light and then with UV light to visualize protein bands.

2.5 Data analysis

To analyze relative immunoreactive protein abundance the average band thickness and intensity of each sampling timepoint was measured using ImageJ (1.52a). The relative protein abundance of CYC and PDP1 was normalized by dividing the CYC and PDP1 band by the size and intensity of GAPDH's band within the same protein sample, and then further normalized by dividing the band size in each sample by the lowest point in expression for each blot. The 1.5*IQR method in R was used to locate and remove outliers (R version 4.0.2), and the relative protein levels were graphed using ggplot2 and ggpubr (R version 4.0.2). A Kruskal-Wallis test was performed in R (version 4.0.2) to determine if there was a significant difference in relative abundance between sampling timepoints and the fold difference was calculated between the lowest and highest abundance. When significant changes in daily abundance were detected, a Mann-Whitney U test with normal approximation was performed *post hoc* to determine which Zeitgeber times were significantly different from one another. We considered a protein to be cycling if there were significant differences in its abundance throughout the 24-hr sampling period and if the relative abundance at the beginning of the day (ZT1) and near the end of the day (ZT21) were not statistically different.

3.0 Results

3.1 Relative abundance of CYC and PDP1 in whole bodies

By comparing the normalized relative protein abundance (Fig. 2A, B) across timepoints we were able to determine whether CYC and PDP1 cycled over the 24-hour sampling period in the whole bodies and heads of long and short-day-reared female mosquitoes. Specifically, there was no significant difference in the relative abundance of CYC in the whole bodies of nondiapausing mosquitoes (Fig. 3A). Although CYC abundance decreased 163% from ZT1 (maximum) to ZT21 (minimum) this change was not statistically significant (Kruskal-Wallis, $p = 0.18$). Additionally, in the whole bodies of diapausing mosquitoes, CYC abundance decreased 39% from its highest point at ZT9 to its lowest point at ZT13, but because of the high degree of variation in CYC protein abundance at ZT9, this change was also not statistically significant (Fig. 3B; Kruskal-Wallis, $p = 0.447$).

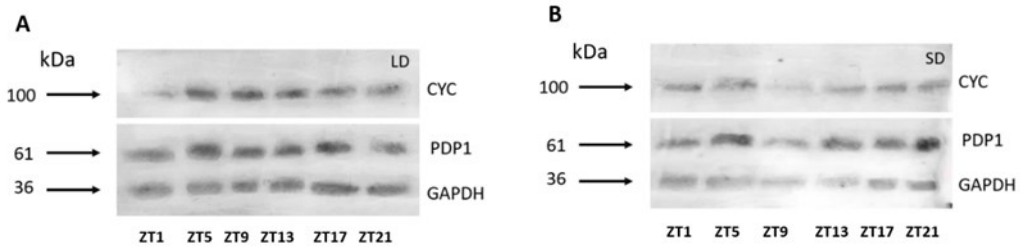

Figure 2: Representative Western blots showing the abundance of CYC, PDP1, and GAPDH proteins extracted from the whole bodies of mosquitoes reared under long-day (A) and short-day (B) conditions. The size of the protein in each band in kDa is listed to the left and the name of the corresponding protein is on the right.

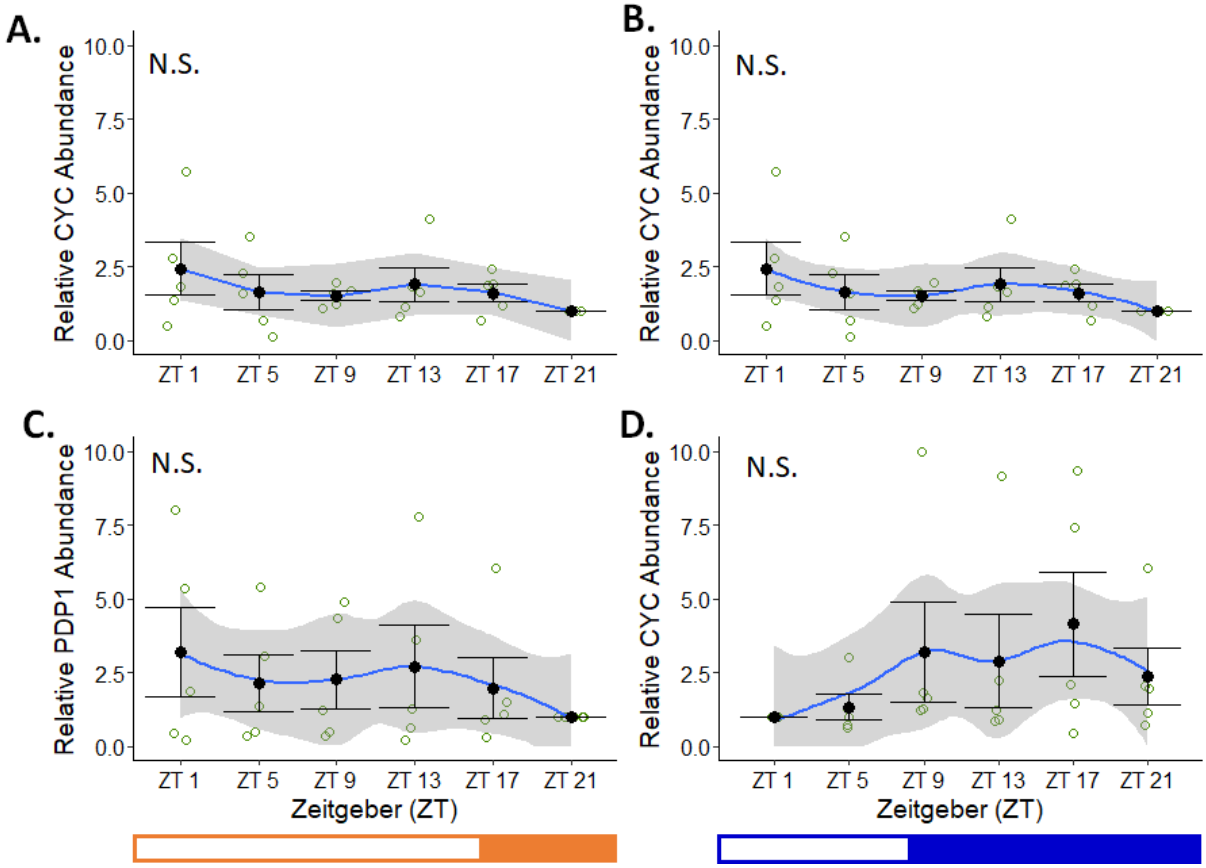

Figure 3: Relative abundance of CYC and PDP1 in whole bodies of nondiapausing (A, C) and diapausing (B, D) female mosquitoes obtained through Western blotting. Mean protein abundance at each ZT timepoint is represented by black dots (± SE). The relative abundance of individual samples is shown as open circles. The photoperiod regimes are represented by the bar below the graph where the open bar indicates when lights were on, and the solid bar indicates when lights were off. There was no significant difference of CYC or PDP1 relative abundance between the timepoints in whole bodies of either nondiapausing or diapausing mosquitoes (p = 0.18, 0.447, 0.053 and 0.49 respectively).

The relative abundance of PDP1 in whole bodies of female *Cx. pipiens* also did not show significant differences between timepoints in nondiapausing (Fig. 3C; Kruskal-Wallis, p = 0.053) and diapausing females (Fig. 3D; Kruskal-Wallis, p = 0.49). Relative PDP1 abundance decreased 69% from its maximum level at ZT1 to its lowest level at ZT9 in nondiapausing mosquitoes, which again was not statistically significant. In diapausing mosquitoes, changes in relative PDP1

abundance were less dramatic such that PDP1 abundance increased 12% from its minimum at ZT1 to its maximum at ZT9. Therefore, neither CYC nor PDP1 met our criteria for showing cyclic patterns of abundance in the whole bodies of *Cx. pipiens* that were reared under diapause-averting, long-day conditions or diapause-inducing, short-day conditions.

3.2 Relative abundance CYC and PDP1 in heads

In contrast to the whole-body data, we did see that CYC and PDP1 showed daily changes in relative abundance within female heads under specific photoperiodic conditions. In the heads of nondiapausing females reared under long-day conditions CYC protein abundance significantly differed between the timepoints (Fig. 4A; Kruskal-Wallis, $p = 0.0415$). Surprisingly, however, CYC protein abundance had a bimodal distribution with 2 peaks in abundance occurring at ZT9 (middle of photophase) and ZT17 (middle of scotophase) and two troughs occurring at ZT1 (early photophase) and ZT13 (late photophase). Relative CYC abundance decreased 76% from its highest peak at ZT13 to its minimum at ZT17, and this change was statistically significant (Mann-Whitney U Test, $p = 0.0176$). Additionally, CYC abundance significantly increased from ZT1 to ZT17 (Mann-Whitney U Test, $p = 0.0270$) while CYC levels at ZT1 and ZT21 were not statistically different (Mann-Whitney U Test, $p = 0.250$), and therefore met our criteria for cycling. In contrast, under short-day, diapause-inducing conditions CYC protein abundance did not significantly change throughout the day (Fig. 4B; Kruskal-Wallis, $p = 0.294$) even though CYC abundance decreased 152% from peak abundance at ZT17 to minimum abundance at ZT21.

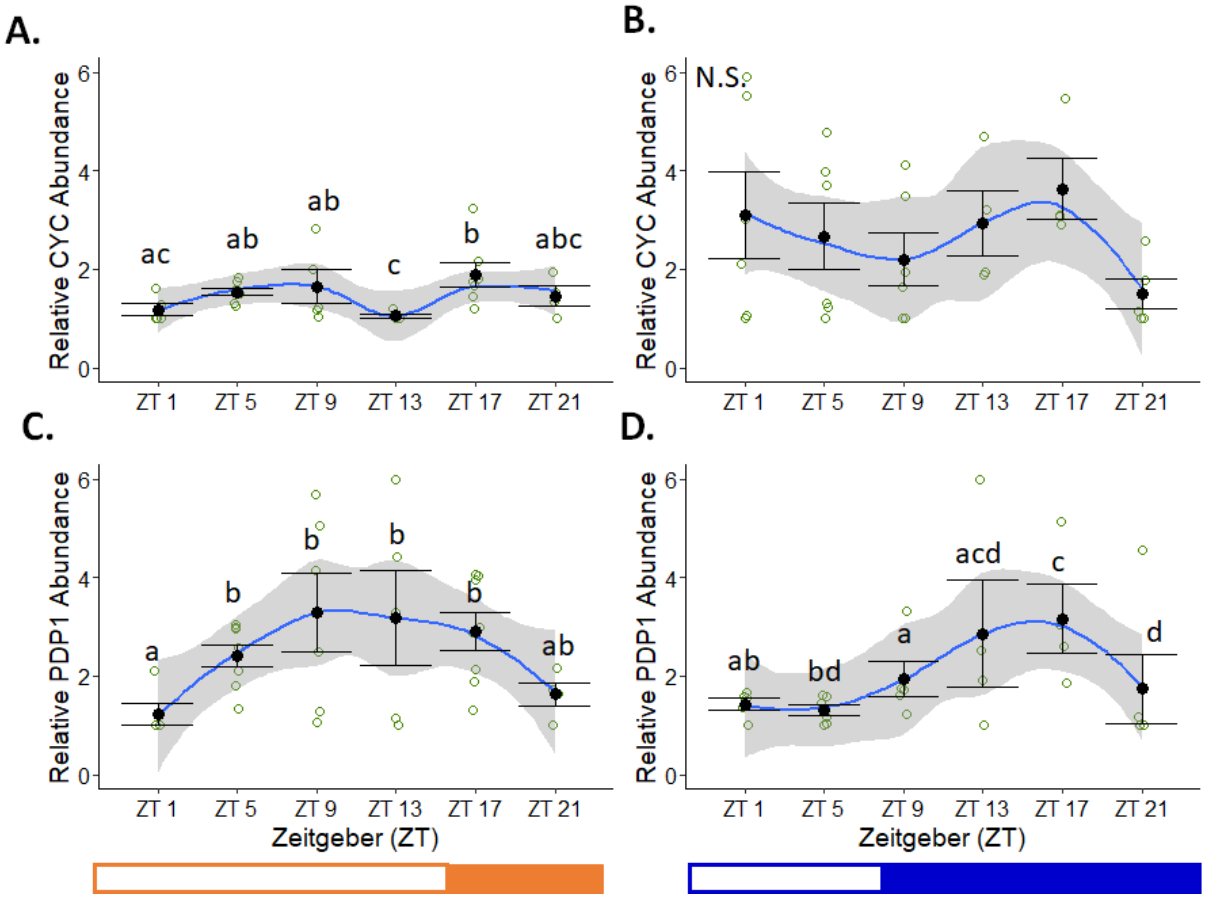

Figure 4: Relative abundance of CYC and PDP1 protein in the heads of nondiapausing (A, C) and diapausing (B, D) female mosquitoes obtained through Western blotting. Mean protein abundance at each ZT timepoint is represented by black dots. The relative abundance of individual samples is shown as open circles. The photoperiod regimes are represented by the bar below the graph where the open bar indicates when lights were on and the solid bar indicates when lights were off. There was significant cycling of CYC in the heads of nondiapausing females (A; Kruskal-Wallis test, $p = 0.0415$) but no significant cycling in the heads of diapausing females (B; Kruskal-Wallis test, $p = 0.294$). PDP1 relative abundance cycled in both nondiapausing (Kruskal-Wallis test, $p = 0.0290$) and diapausing ($p = 0.00681$) mosquito heads. Different letters indicate significant differences in average protein abundance between timepoints (Mann Whitney U test with normal approximation; $p < 0.05$).

PDP1 protein levels significantly changed in the heads of long day-reared, nondiapausing females (Fig. 4C; Kruskal-Wallis, $p = 0.0290$). Specifically, PDP1 increased 129% from its minimum abundance at ZT1 to its single peak at ZT13 (Mann-Whitney, $p = 0.0318$).

Additionally, the relative abundance of PDP1 at ZT1 and ZT21 was not significantly different in the heads of nondiapausing mosquitoes (Mann-Whitney, $p = 0.0572$), and therefore PDP1 met our criteria for cycling. Overall PDP1 protein abundance also significantly changed in the heads of short-day-reared, diapausing mosquitoes (Fig. 4D; Kruskal-Wallis, $p = 0.00681$), such that PDP1 increased 130% from its minimum in the middle of photophase at ZT5 to its maximum at ZT17 (Mann-Whitney, $p = 0.0105$). Although the average abundance of PDP1 at ZT21 was significantly higher than at ZT1 (Mann-Whitney, $p = 0.0178$), this PDP1 abundance only increased by 22%, and shows an overall expression profile that suggests it is oscillating in the heads of diapausing females of *Cx. pipiens*.

4.0 Discussion

We measured the abundance of CYC and PDP1 in both the whole bodies and heads of *Cx. pipiens* reared under diapause-averting, long day and diapause-inducing short-day conditions. No significant cycling of either protein was found in the whole bodies of diapausing and nondiapausing females (Fig. 3). This is likely because these proteins are produced in peripheral circadian clock cells throughout the body that are out of sync with one another, as well as with the central circadian clock in the brain, therefore diluting overall expression profiles (reviewed by Glossop and Hardin, 2002). Therefore, we decided to measure the protein abundance in only the heads of the females to characterize daily changes in the relative abundance of these proteins within the central circadian clock, as this is most likely regulating diapause. By doing this we found significant differences in the abundance of CYC and PDP1 in the heads mosquitoes reared under long-day conditions (Figs 4A, C) while only PDP1 protein levels cycled in the heads of short day-reared females (Fig. 4D).

In *D. melanogaster* and in the house fly *Musca domestica* levels of some circadian clock proteins show daily oscillations that lag 4 hours behind their mRNA expression profiles (Bu et al., 2020; Codd et al., 2007). Therefore, we predicted that CYC protein abundance in *Cx. pipiens* would show a similar pattern. However, we were unable to detect oscillations in CYC abundance in the heads of diapausing females of *Cx. pipiens* (Fig. 4B) although our previous work shows that *cyc* mRNA transcripts do oscillate in these mosquitoes (Meuti et al., 2015). Additionally, the relative abundance of CYC also differed from the patterns seen in its mRNA in the heads of non-diapausing mosquitoes; we observed 2 peaks in CYC abundance (Fig. 4A) whereas only 1 peak was measured in mRNA transcripts (Meuti et al., 2015). CYC's first peak at ZT9 under long-day conditions was close to the single predicted peak, while its second larger peak at ZT17 was unexpected and does not correspond with its mRNA abundance. Although the bimodal pattern in circadian protein abundance has not been reported in other insects, differences between mRNA expression and protein abundance of circadian clock genes are not unprecedented. For example, *Clk* mRNA levels oscillate throughout the day, while CLK protein is constant in the heads of *D. melanogaster* (Bae et al., 2000; Richier et al., 2008), demonstrating that protein abundance does not always correlate with the expression levels of its mRNA. Neither *cyc* mRNA nor CYC protein abundance oscillate in *D. melanogaster* (Bu et al., 2020), the flesh fly *Sarcophaga crassipalpis* (Goto and Denlinger, 2002), or the sawfly *Athalia rosae* (Bembenek et al., 2007), and CYC protein abundance is constant in the cricket *Dianemobius nigrofasciatus* (Shao et al., 2008). Therefore, our data represent the first time that anyone has shown significant daily changes in CYC protein, although it is interesting that this only occurred within the heads of nondiapausing females of *Cx. pipiens*.

We found that PDP1 protein abundance peaked in late photophase at ZT13 in the heads of non-diapausing female mosquitoes (Fig. 4C), which is similar to the peak in expression seen in its mRNA (Chang and Meuti, 2020). This is not consistent with the 4-hour delay between mRNA and protein levels that are seen with clock proteins in other insects (Codd et al., 2007; Bu et al. 2020), but is consistent with the patterns seen in the heads of *Drosophila* found by Cyran et al. (2003) where there was no difference between peak *Pdp1* transcript and PDP1 protein levels. As we previously noted, however, Bu et al. (2020) found that *Pdp1* mRNA peaked at the onset of the scotophase whereas PDP1 protein peaked 4 hours later in the heads of *D. melanogaster*. It is unclear why two different studies that examined *Pdp1* mRNA levels within the heads of *D. melanogaster* that were reared under the same photoperiodic conditions obtained slightly different results, yet both found that PDP1 protein was most abundant in the mid-scotophase. However, our results and those of Cyran et al. (2003) suggest that in certain contexts PDP1 may be more rapidly translated than other circadian clock proteins such that peaks in its transcripts and protein levels may coincide.

As *Pdp1* mRNA did not significantly change in the brains of diapausing females of *Cx. pipiens* (Chang and Meuti, 2020), we expected that PDP1 abundance would also be constant in the heads of diapausing mosquitoes. However, we observed that PDP1 did oscillate with peak abundance occurring at mid-scotophase (ZT9; Fig. 4D). It is unclear what might be driving this trend, but perhaps there are seasonal differences in how PDP1 is translated and/or degraded which merits further investigation. Additionally, there are clear differences in the circadian profile of PDP1 abundance between long day (peak at ZT13; 3 hrs before lights off) and short-day reared mosquitoes (peak at ZT9; 1 hr after lights off), suggesting that PDP1 is responsive to changes in daylength in *Cx. pipiens*.

CYC is an important circadian transcription factors that has the potential to regulate genes involved in JH synthesis and the insulin signaling pathway that are essential for regulating diapause (Fig. 5; Spielman, 1974; Sim and Denlinger, 2008, 2013). Notably, there are several CYC binding sites within the promoter of *allatotropin* (KF781633), a key component of the JH signaling pathway. Moreover, Kang et al. (2014) previously demonstrated that *allatotropin* is downregulated during diapause of *Cx. pipiens*, and this is likely one mechanism that leads to JH suppression and egg follicle arrest. Moreover, CYC binding sites are also present within the promoter of *pigment dispersing factor* (XP_001846575). Previous studies show that knocking down *pdf* caused long-day reared females of *Cx. pipiens* to enter a diapause-like state with small egg follicles and high levels of fat (Meuti et al. 2015), and levels of *Ilp1* and *InR* transcripts were also lower in *pdf* dsRNA-injected females (Supplemental Figure 1). Therefore, CYC may facilitate JH synthesis and insulin signaling by modulating the expression of *allatotropin* and *pdf*, allowing high levels of these transcripts to be produced in long day-reared, reproductively active females of *Cx. pipiens*.

In contrast, PDP1 may be involved in regulating genes and proteins involved in short day, diapause responses (Fig. 5). Knocking down *pdp1* in short day-reared females of *Cx. pipiens* prevented them from accumulating high levels of fat associated with diapause (Meuti and Chang, 2020). This affect may have been mediated through *takeout* as this gene is involved in involved in feeding behaviors, triglyceride accumulation, JH signaling and longevity (Sarov-Blat et al. 2000; Meunier et al. 2009; Chamseddin et al. 2012), which are all processes relevant to diapause. Furthermore, Benito et al. (2010) demonstrate that PDP1 indirectly regulates the *takeout* gene in *D. melanogaster* and *takeout* is also upregulated by the Forkhead transcription factor, FOXO, during diapause in *Cx. pipiens* (Sim et al., 2015). More recently, Jarwar et al. (2019)

demonstrated that *takeout* is upregulated in long-day reared migratory locusts and that knocking it down increased diapause incidence of short-day reared locusts. Surprisingly, however, knocking down *pdp1* led to an increased level of *takeout* expression in *Cx. pipiens* (Meuti & Chang, 2020), suggesting that other targets of PDP1 likely play a more important role in suppressing insulin signaling and acquiring fat. One of these potential PDP1 targets is *Susi*, a gene that strongly suppresses insulin signaling in *D. melanogaster* (Wittwer et al. 2005), but to date, the connection between PDP1 and *Susi* has not been demonstrated in any insect.

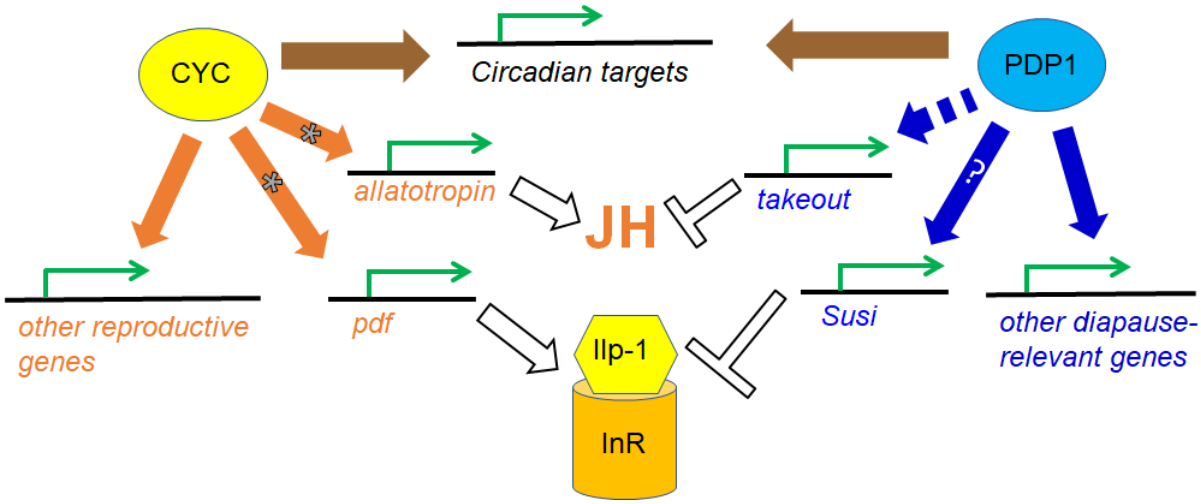

Figure 5: Possible mechanism by which CYC and PDP1 regulate diapause initiation. Under both long and short days, CYC and PDP1 regulate targets within the circadian clock (brown arrows). Under long day conditions (orange arrows), CYC likely binds to E-box promoters (gray asterisks) to increase the production of *allatotropin* and *pigment dispersing factor* (*pdf*) to stimulate JH production and insulin signaling (Ilp1 and InR), respectively. CYC may also regulate other genes that are involved in reproductive responses. In contrast, under short day conditions PDP1 may indirectly regulate (dashed blue arrows) the production of *takeout*, a known suppressor of JH signaling, and possibly regulate expression of *Susi*, a suppressor of insulin signaling, as well as other genes that are relevant to generating the diapause phenotype.

Future studies are needed to confirm the role of CYC and PDP1 and the diapause response within *Cx. pipiens*. Specifically, immunohistochemistry would reveal where CYC and PDP1 are produced within the brains of *Cx. pipiens* and thereby likely uncover neural connections between the circadian clock and hormonal regulators of diapause (e.g. allatotropin, Ilps, JH).

Additionally, Chromatin Immunoprecipitation followed by deep sequencing (ChIP-seq) should be employed to identify the genetic targets of CYC and PDP1 and how these change in response to seasonal conditions. The differences in the daily abundance of CYC and PDP1 that we have uncovered in the heads of diapausing and nondiapausing mosquitoes lays the foundation for this future work and suggests that these circadian transcription factors may detect seasonal changes in daylength and appropriately coordinate photoperiodic responses.

This is one of the few studies to characterize daily changes in the relative abundance of circadian transcription factors in insects that were exposed to different seasonal conditions. Therefore, these results are important in providing more information on the role of PDP1 and CYC play both within the clock and regulating seasonal responses in *Cx. pipiens*. Our data again affirm that the abundance of circadian clock proteins in the head does not always correlate with their mRNA levels, and that there is a great deal of variation in the expression and protein abundance of circadian proteins among insects. Additional studies that characterize daily and seasonal changes in the abundance of CYC and PDP1 and their mRNA targets in other insects will further elucidate the relationship between the circadian clock and seasonal responses, and how this might vary due to both the complexity and variation in clock gene expression and the independent evolution of diapause across insect species.

5.0 Acknowledgements

We thank Dr. Pete Piermarini and Dr. Megha Kalsi for assistance in conducting and interpreting the Western blots, as well as members of the Meuti lab for their assistance with mosquito rearing and collection. We also thank Alden Siperstein for creating the line images of *Cx. pipiens* used in the graphical abstract, and Lydia Fyie for assistance with statistical assays and making graphs in

R. This work was supported by a Graduate Student SEEDS Grant from the Ohio Agricultural Development Center awarded to Caitlin Peffers as well as an Interdisciplinary Seeds Grant from the Infectious Disease Institute at The Ohio State University and the National Science Foundation [IOS Grant 1944324] awarded to Megan Meuti.

6.0 Author Contributions

Caitlin Peffers: Methodology, Investigation, Data Curation, Visualization, Formal Analysis, Writing - Original draft Preparation; Funding Acquisition. Megan Meuti: Conceptualization, Resources, Supervision, Formal Analysis, Visualization, Writing - Review & Editing; Funding Acquisition.

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