Characterizing the role of *vrille* and *par domain protein1* in the initiation of the Northern House Mosquito's diapause response

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Introduction

Many insects and arthropods go into diapause, arresting their development and becoming dormant. This seasonal response helps these organisms survive unfavorable developmental conditions such as cold winters or dry tropical summers that result in food scarcity, unfavorable temperatures, and increased susceptibility to desiccation (Danilevsky et al., 1970; Denlinger, 2002; Tauber and Tauber, 1976). To survive winters, temperate insects enter diapause including the bumble bee (Alford, 1969), stink bug (Saulich and Musolin, 2012), and mosquito (Denlinger and Armbruster, 2014). These insects initiate diapause in response to the short daylengths of late summer and early fall and as such, diapause is also a photoperiodic response.

The mechanism by which insects measure daylength is still not fully understood. However, many have hypothesized that a functional circadian clock helps these insects to measure daylength and appropriately regulate their seasonal responses (Bünning, 1936; Pittendrigh, 1981). The circadian clock and the underlying biochemical and molecular mechanisms of diapause have been extensively studied in the fruit fly, *Drosophila melanogaster* (Hardin, 2005, Sandrelli et. al., 2008). In the *Drosophila* clock, clock genes *cycle* (*cyc*) and *Clock* (*Clk*) code for two transcription factors which then form a heterodimer complex. The CLK/CYC protein complex is able to bind to an E box sequence found in the promoter region of the clock genes *period* (*per*) and *timeless* (*tim*) resulting in those genes' transcription. The protein products PER and TIM form another heterodimer that is able to translocate back into the nucleus and inhibit the activity of CYC and CLK. This results in a self-regulatory negative feedback loop. PER and TIM are eventually degraded allowing CLK and CYC to activate *per* and

tim's transcription. Although the clock is well-understood in *D. melanogaster*, studies have shown many insects have clocks that work differently than that of the fruit fly. For example, the circadian gene *Clk* mRNA cycle throughout the day whilst *cyc* mRNA expression is constant in *D. melanogaster*. However, *Clock* expression is constant while *cycle* mRNA oscillate in the honey bee (Rubin et al., 2006), monarch butterfly (Reppert, 2006), and Northern House Mosquito (Meuti et. al., 2015).

Our test organism, the Northern House Mosquito (*Culex pipiens*), is the major disease vector for West Nile Virus, Canine Heartworm, and St. Louis Encephalitis. The females of this species enter an adult reproductive diapause in which they arrest their egg follicle development and increase their lipid accumulation. Thus, small egg follicles are one of the many phenotypes associated with diapausing *Cx. pipiens.* Previous work has already identified the expression pattern of core circadian clock genes *Clk*, *cyc*, *per*, and *tim* in diapausing and non-diapausing mosquitoes (Meuti et. al., 2015). Clock genes *per*, *tim*, and *cyc* expression oscillate in non-diapausing females and continue to do so during diapause as well as post-diapause whereas *Clk* expression remains constant during all three phases. However, a secondary feedback loop consisting of circadian transcription factors *vrille* (*vri*) and *Par domain protein 1* (*Pdp1*) are also regulated by the CLK/CYC proteins and are responsible for cyclic expression of *Clk* mRNA in *D. melanogaster* where VRI represses *Clk* and PDP1 activates *Clk* (Cyran et. al., 2003). As *cyc* expression oscillates in *Culex* mosquitoes (Gentile et al. 2009; Meuti et al. 2015), we hypothesize that VRI and PDP1 act on *cyc* in our test organism (Fig. 1).

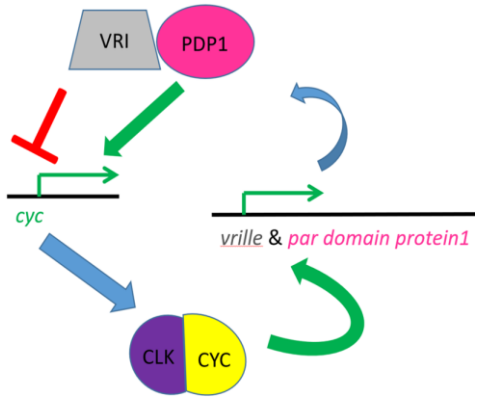

Figure 1 Insect circadian model focusing on *vri* and *pdp1* adapted from Meuti and Denlinger (2013).

The rhythmic daily changes in the levels of clock gene transcripts and their associated proteins are directly responsible for generating daily behavioral rhythms. When the levels of clock genes are disrupted, behavioral outputs are also disrupted. One method to study the clock genes' role in behavior utilizes RNA interference (RNAi) in which dsRNA of the gene of interest is injected into the organism, and a reduced level of the gene is observed. Ikeno et. al. (2010) showed the daily rhythm of cuticle deposition in bean bugs were disrupted when RNAi directed against *per* and *cyc* was performed. Furthermore, she observed ovarian development in *per* dsRNA injected bean bugs raised in diapause inducing conditions and a suppression of ovarian development in *cyc* and *Clk* dsRNA injected bean bugs raised in diapause averting conditions (Ikeno et. al., 2010;2013). Similarly, Meuti et al. (2015) observed that females of *Cx. pipiens* injected with *per* and *tim* dsRNA averted diapause even though they were raised in short day, diapause inducing conditions. This suggests that the clock may also be responsible for generating seasonal rhythms.

Materials and Methods

Insect Rearing

Cx. pipiens (Buckeye strain) was reared as previously reported (Robich and Denlinger 2005; Meuti et al. 2018). In brief, larvae from the main colony were reared at 25°C with a 16h L: 8h D. Approximately 10 days after adult emergence, adult mosquitoes were fed chicken blood (Pelfreez Biologicals) using an artificial blood feeding system (Hemotek; UK). First instar larvae were either placed in long day, diapause-averting conditions (16h L:8h D at 18°C), or short day, diapause-inducing conditions (8h L: 16h D at 18°C).

Expression Profiles of Genes Determined by qRT-PCR

Seven days after adult emergence, groups of ~65 female mosquitoes from long day (LD) conditions and short day (SD) conditions were killed at 4 h intervals over a 24h-period (Zeitgebers, or ZT1, 5, 9, 13, 17 and 21 where ZT0 indicates the time when lights turned on). The brain of each female was dissected on dry ice in 100% ethanol. RNA was extracted from the dissected brains (n = 10 brains/biological replicate; 5 biological replicates/ZT = 60 biological samples total) using the Ambion mirVana™ miRNA isolation kit (Ambion, USA), treated with TurboDNase (Invitrogen, Green Island, NY), and subsequently purified using a Zymo Clean and Concentrator Kit (Zymo Research, Orange, CA) according to the manufacturers' instructions. The quantity and quality of the RNA was assessed using a Nanodrop spectrophotometer (Nanodrop Products, Wilmington, DE, USA). cDNA was synthesized using 0.5µg of RNA and the miScript Reverse Transcription (RT) Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol using the HiFlex Buffer.

We measured the relative level of *vri*, *pdp1*, *Clock* (*Clk*), *Rp49* and *RpL19* using quantitative Real Time PCR (qRT-PCR) using previously described primer sequences (Gentile et al. 2009; Zhang and Denlinger 2010). qRT-PCR was performed in a 96 well plate using an iQ5

real-time PCR detection system (Bio-Rad, Hercules, CA, USA). All reactions were performed in triplicate in a total volume of 20µL containing 10µL iTaq Universal SYBR green PCR Master Mix (Bio-Rad, Hercules, CA, USA), 400 nmol of each primer, and 2 µl sample cDNA.

The qRT PCR data were analyzed as previously described (Meuti et al. 2015). In brief, the background subtracted values were smoothed and normalized according to Larionov et al. (2005). The relative cycle threshold (CT) of each was averaged across technical replicates. The resulting CT value for each gene of interest (*vri* and *pdp1*) was normalized to the geometric average of the CT values of three reference genes (*Rp49*, *RpL19*, and *Clk*) by subtracting the CT of reference genes from the gene of interest ($2^{-\Delta CT}$ method). The large number of samples (n = 30 LD and 30 SD biological replicates) prevented us from running LD and SD samples on the same plate. Therefore, a subset of LD and SD samples were run on both plates, to ensure that plate-to-plate variation was low. This allowed us to accurately compare expression of LD and SD samples run on separate plates. We determined whether a correction factor is necessary when the average difference CT of the same samples on both plates was ≥0.5, and when the standard deviation of the difference between all technical replicates was ≤0.1 CT's. However, none of the plates met that criteria. Thus, we did not apply any correction factors.

Synthesizing and Injecting dsRNA into Adult Females

dsRNA for *vri*, *pdp1*, and *β-galactosidase* (positive control) were synthesized using the T7 RiboMAX Express RNAi System (Promega, Madison, WI, USA), according to the manufacturer's instructions. *β-gal* dsRNA was made using previously published primer sequences (Meuti et al., 2015), while *vri* and *pdp1* dsRNA was synthesized using unique primer sequences (Table 1).

Primer Name	Sequence	Tm
Cp.vri.RNAi.Fw	GGGACGCGATCAAGAACAAAT	60.8
Cp.vri.RNAi.Rev	GGATGAGTGGACTTCGGTCGT	60.12
Cp.pdp1.RNAi.Fw	GGATGAGTGGACTTCGGTCGT	59.3
Cp.pdp1.RNAi.Rev	GCCGCCATGTTGTTCTTCC.	60.1

Table 1 Primer sequences used to make *vri* and *pdp1* dsRNA.

Approximately one day after adult emergence cold anesthetized female mosquitoes were injected in their thorax using a microinjector (Tritech Research, Los Angeles, CA, USA) with 2.2 µg of *vri* and *pdp1* dsRNA and 2.0-2.2 µg of *β-galactosidase* dsRNA (injection volume = 0.70-1µL).

Assessing Diapause Status of dsRNA-injected Females

The egg follicle length of each female was measured five days post-injection. Ten egg follicle lengths from fifteen different females in each dsRNA-treatment were measured using an inverted microscope (Nikon). We considered a female to be in diapause when her egg follicles are around 50 to 75µM, and we considered a female to not be in diapause if her egg follicles are bigger than 90µM.

Statistical Analyses

All statistical analyses were performed in R.3.32 (R Core Team, 2017). Changes in mRNA expression over time (from ZT1-ZT21) for each photoregime (either LD or SD) were evaluated using a one-way ANOVA followed by Tukey's post-hoc test. Similarly, differences in overall mRNA expression for each gene over time between the two photoregimes were evaluated using a two-way ANOVA followed by Tukey's post-hoc test. Egg follicle length were evaluated using Student's t-test.

Results

Expression profiles of clock gene mRNA

To determine whether *vri* and *pdp1* are differentially expressed between long-day reared and short-day reared female mosquitoes, and whether the expression of these genes oscillated throughout the day we characterized their expression profiles in long-day reared and short-day reared females using qRT-PCR. We characterized mRNAs as cycling if they had equivalent abundance near the beginning and end of the day (ZT1 and ZT21) and if their abundance significantly changed during the course of the day (reviewed by Dunlap, 1996). In short day females, only *vri* oscillated with peak expression occurring at ZT5 (Fig. 2A; p=0.0000036). In contrast, *pdp1* did not oscillate in short day females (Fig. 2B). In long day females, both genes oscillated with peak expression occurring at ZT9 for *vri* and ZT13 for *pdp1* (Fig. 2A, B; p=0.0000725, p=0.0161). The relative mRNA abundance of *pdp1* were higher in diapausing females compared to non-diapausing females (Fig. 2B; p< 0.0342).

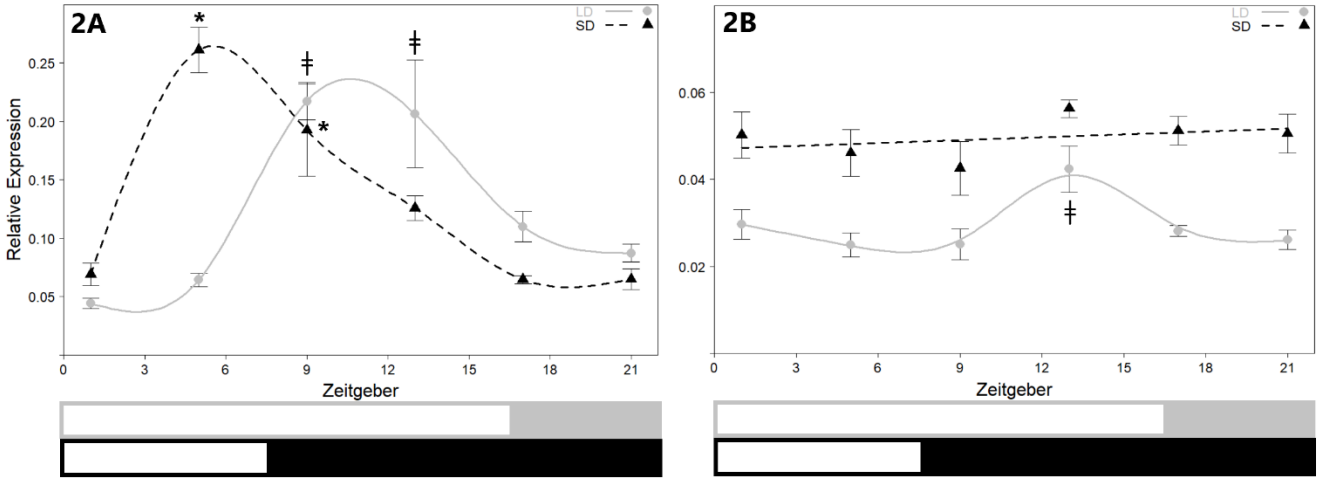

Figure 2 **The expression profile of clock genes differ under different photoperiodic conditions.** Relative mRNA abundance of (A) *vrille* (*vri*), (B) *Par domain protein1* (*pdp1*) in female brains were measured by quantitative real-time PCR and normalized to the geometric mean of three reference genes (*Rp49*, *RpL19*, and *Clk*). One-week old short day females (exposed to 8h L: 16h D at 18°C) are represented by

dashed, black lines with triangular points, and long day females (exposed to 16h L:8h D at 18°C) are represented by solid, grey lines with circular points. Each point represents the mean relative mRNA expression of 5 biological replicates each containing 10 brains, and bars represent the standard error. A spline curve was fitted to the expression data of all 5 biological replicates. Asterisks (*) represent significant differences in expression between ZT1 and other time points for SD females and ‡ for LD females (one way ANOVA followed by Tukey's post-hoc test; $p<0.05$)

Effects of RNAi on Diapause Initiation

We measured the egg follicle lengths of dsRNA-injected females to determine whether the females had appropriately responded to their rearing conditions. As expected, long day-reared females injected with *ßgal* dsRNA averted diapause and had large egg follicles, whereas short day-reared females that had been injected with *ßgal* dsRNA were in diapause and have small egg follicles (Fig 3). Injecting *vri* dsRNA in long day females had no effect, but injecting *vri* in short day females stimulated their ovarian development such that these females had larger egg follicles than the *ßgal*-injected control (Fig. 3A; $p=0.024$). Injecting *pdp1* dsRNA did not affect their egg follicle development in long or short day-reared females (Fig. 3B).

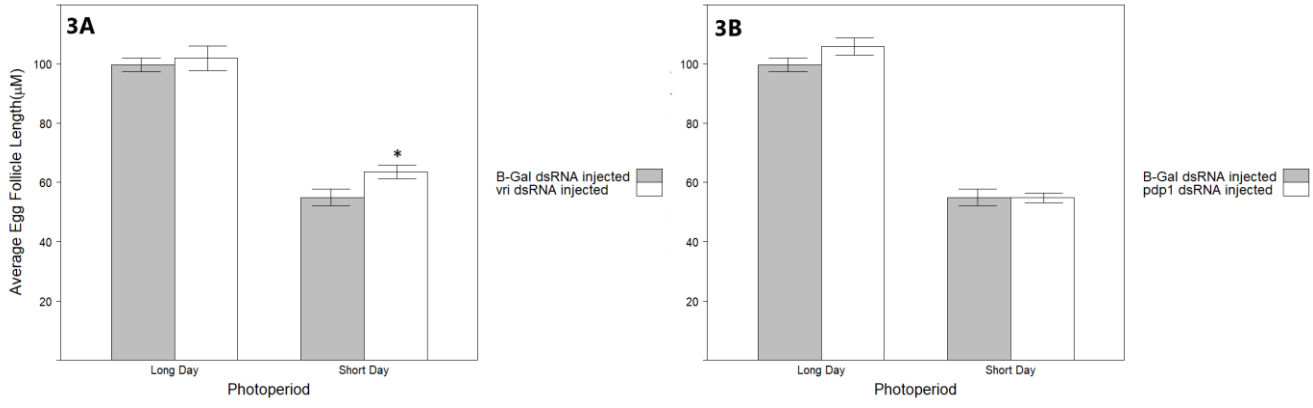

Figure 3 **SD females injected with *vri* dsRNA enhanced egg follicle development (A) while *pdp1* dsRNA had no effect (B).** Five days post-injection, the average egg follicle length of 10 eggs from 15 females

injected with *vri* dsRNA (A) and *pdp1* dsRNA (B) were measured. Asterisks (*) represent significant differences between the control and treatment (Student's t-test; $p<0.05$).

Discussion

The expression profile for *vri* and *pdp1* in long day-reared females agree are consistent with previously published literature which examined *vri* and *pdp1* expression in two other species of mosquitoes *Aedes aegypti* and *Culex quinquefasciatus* under a 12L:12D photoregime and constant darkness (Gentile et. al., 2009). Lowest expression in *cyc* mRNA occurs around ZT12 in long day females whereas *vri* and *pdp1* expression peaks around ZT9 and ZT13 respectively (Meuti et. al., 2015; Fig. 2A,B). This shows a negative correlation between the rise and fall of *vri* and *cyc* expression and a positive correlation between the rise of *pdp1* and *cyc* expression. Differential expression of *vri* and *pdp1* between long day and short day females hint at their importance in the initiation of diapause (Fig. 2A,B). The overall higher levels of *pdp1* constitutively expressed in SD females suggest that *pdp1* plays a role in driving changes in gene expression resulting in a diapause phenotype. More specifically, we think *pdp1* is linked to the around the clock feeding behavior of females preparing for diapause previously reported by Bowen (1992). This behavior then leads to higher lipid accumulation which is one of the key phenotypes of diapause. The link between *pdp1* and lipid accumulation was observed when RNAi directed against *pdp1* in short-day reared females caused less fat accumulation compared to the control (data not shown). Furthermore, previous studies on *Drosophila melanogaster* have shown that *pdp1* is a regulator of *takeout*, an output gene of the circadian oscillator (Benito et. al., 2010). Since *takeout* is preferentially expressed in fat body and *pdp1* is a

regulator of *takeout*, we concluded that PDP1 may very likely be a transcriptional regulator in fat body.

We performed RNAi directed against *vri* and *pdp1* and observed whether females are still able to appropriately respond to their living conditions. Although we have not been able to confirm that dsRNA injection significantly reduced the level of *pdp1* and *vri* mRNA, we observed a difference in phenotype in SD females injected with *vri* dsRNA (Fig. 3A). Previous attempts to knock down *cyc* in *Cx. pipiens* were met with limited success even though up to 6.0µg of dsRNA was injected (Meuti et. al., 2015). This leads us to believe that the circadian clock in *Cx. pipiens* is robust and recalcitrant to perturbations. Thus, we still need to confirm the knockdown efficiency of our RNAi experiments.

We were surprised to find that females injected with *pdp1* dsRNA did not deviate from the controls since *pdp1* is differentially expressed between long day and short day females (Fig 2B, 3B). However, it is also likely that *pdp1* levels were not reduced since we haven't been able to confirm RNAi knockdown. It is also possible that other parameters such as lipid accumulation needs to be tested. It was exciting to observe *vrille* dsRNA injected short day females developing larger egg follicles compared to the control (Fig. 3A). *vrille* plays an important role in embryogenesis in *Drosophila*, more specifically, it enhances the expression of a key morphogen in the fruit fly's development (George and Terracol, 1997). Altered *vrille* expression has also been shown to produce behavioral arrythmia in which the period of locomotor activity is altered as well as a disruption in the rhythmic expression of other clock genes such as *per* and *tim* (Blau and Young, 1999). Thus, we know *vrille* plays a role in development and the clock, but we do not know for a fact its role in diapause. This clock gene may possibly be pleiotropically

involved in egg follicle development. It could also be another form of evidence towards the clock's involvement in measuring daylength to help regulate diapause since knocking down other negative elements of the clock (*per*, *tim*, *cryptochrome2*) has induced ovarian development in diapausing females (Meuti et. al., 2015).

Ongoing work is attempting to confirm knock down efficiency of our RNAi experiments. Instead of injecting about one day after the females' emergence as adults, we are now injecting them within 24 hours of their eclosion to ensure the introduction of dsRNA occurs when they are most photoreceptive. Furthermore, our sampling time has moved from 5 days post injection to 2 days post injection to ensure that the measurement of mRNA levels occur before the organism is able to correct the disruption. Other future work involves studying circadian output genes such as *takeout* and *susi* and identifying how they are regulated and their roles in diapause. Unfortunately, the work reported here does not implicate whether *vri* and *pdp1* play an essential role in the initiation of diapause, but it does provide compelling support that *vri* plays a role in repressing egg follicle development and *pdp1* is necessary for lipid accumulation, both of which are essential phenotypes of diapause.

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