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Identification of CYCLE targets that contribute diverse features of circadian rhythms in the mosquito *Culex pipiens*☆

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ABSTRACT

Culex pipiens demonstrates robust circadian rhythms in adult eclosion, flight activity, mating, and development. These rhythmic patterns are believed to be controlled by the endogenous light-entrainable circadian clock that consists of positive and negative regulators working in a transcription-translation feedback loop. Moreover, these mosquitoes undergo seasonal diapause in exposure to the short photoperiod of late summer or early fall. However, the exact genetic and cellular mechanism behind the clock gene-mediated activity pattern, seasonal time measurement, and subsequent diapause initiation still need to be unraveled. To determine the possible linkage between clock genes and downstream processes, here we employed ChIP-sequencing to identify the direct targets of one of the core clock proteins, Cycle (CYC). The nearest genes with peaks mapping to their 1Kb upstream region of the transcription start site were extracted and scanned for consensus E box sequences, resulting in a dataset comprising the target genes possibly regulated by CYC. Based on the highest fold enrichment and functional relevance, we identified genes relating to five gene categories of potential interest, including peptide/receptors, neurotransmission, olfaction, immunity, and reproductive growth. Of these, we validated fourteen genes with ChIP-qPCR and qRT-PCR. These genes showed a significantly high expression in dusk compared to dawn in concert with the activity level of the CYC transcription factor and are thus strong candidates for mediating circadian rhythmicity and possibly regulating seasonal shifts in mosquito reproductive activity.

1. Introduction

The circadian clock is present across all domains of life, from ancient Archaea (Maniscalco et al., 2014) to photosynthetic cyanobacteria (Kondo and Ishiura, 2000) to eukaryotic plants (Sanchez and Kay, 2016) and animals (Bell-Pedersen et al., 2005), where it aids in shaping organismal behavior and physiology according to daily changes in external environmental factors such as light, temperature, and resource availability (Bell-Pedersen et al., 2005). This is true for the Northern house mosquito, *Culex pipiens*, the primary vector of pathogens like West Nile virus, where the circadian clock anticipates changes in the light-dark cycle and regulates peak locomotor activity to occur around dawn and dusk (Hubálek and Halouzka, 1999; Rivas et al., 2018). Moreover, rhythmic patterns in many other behaviors such as mating, host-seeking, blood feeding, and oviposition are precisely coordinated to occur at the correct time of day in disease vector mosquitoes, including *Cx. pipiens* (Meireles-Filho and Kyriacou, 2013; Rund et al., 2016). Additionally, previous studies have also demonstrated the rhythmic expression of genes attributable to periodic behaviors in mosquitoes (Beehler et al., 1993; Savage et al., 2008; Fritz et al., 2014; Rivas et al., 2018; Liu et al., 2022), but the regulation of these genes by the circadian clock has not been specifically evaluated.

The mosquito circadian clock comprises component genes that operate in two light-entrainable transcriptional-translational feedback loops which complete the cycle every 24 h. The core component genes at the center of this feedback loop are *cycle* (*cyc*), *clock* (*Clk*), *period* (*per*), *timeless* (*tim*), *par-domain protein 1* (*pdp1*), and *cryptochrome-2* (Gentile et al., 2009; Meireles-Filho and Kyriacou, 2013; Meuti et al., 2015). The

Abbreviations: qRT-PCR, quantitative real-time PCR; ChIP-qPCR, Chromatin immunoprecipitation quantitative real-time PCR; ZT, Zeitgeber Time.

☆ Data deposition: The sequences reported in this paper have been deposited in the Genbank database (accession no. **PRJNA957333**).

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working mechanism of these genes is mostly similar to the *Drosophila melanogaster* model where CLK/CYC heterocomplex binds to E box element (CACGTG) and upregulates the transcription of *per* and *tim* until they reach their highest peak at early scotophase (Rutila et al., 1998). PER and TIM when translated translocate into the nucleus bind to CLK/CYC heterodimer and repress their transcription. In the next loop, the heterodimer regulates the transcription of *pdp1* and *vri* which binds to the promoter of *clk* and enhances or suppresses the expression and abundance of CLK and contributes to the clock (reviewed in Hardin, 2005). But in contrast to *D. melanogaster,* insects like *Cx. pipiens*, and *Bombyx mori* lack the oscillating expression of *clk* and show the rhythmic expression of *cyc* (Markova et al., 2003; Meuti et al., 2015). Moreover, unlike *Drosophila*, the CYC protein retains the c-terminal transactivation domain resembling the mammalian orthologue BMAL and binds to the E box element in the promoter region of the downstream genes. These target genes include the clock component genes such as *period* and other downstream genes responsible for various physiological and behavioral changes (Rutila et al., 1998; Chang et al., 2003). However, very few target genes of CYC have been identified, especially in mosquito species, leaving a gap in the complete understanding of how the circadian clock mediates differences in daily behavior.

In addition, since the hypothesis put forward by Bünning in 1936,

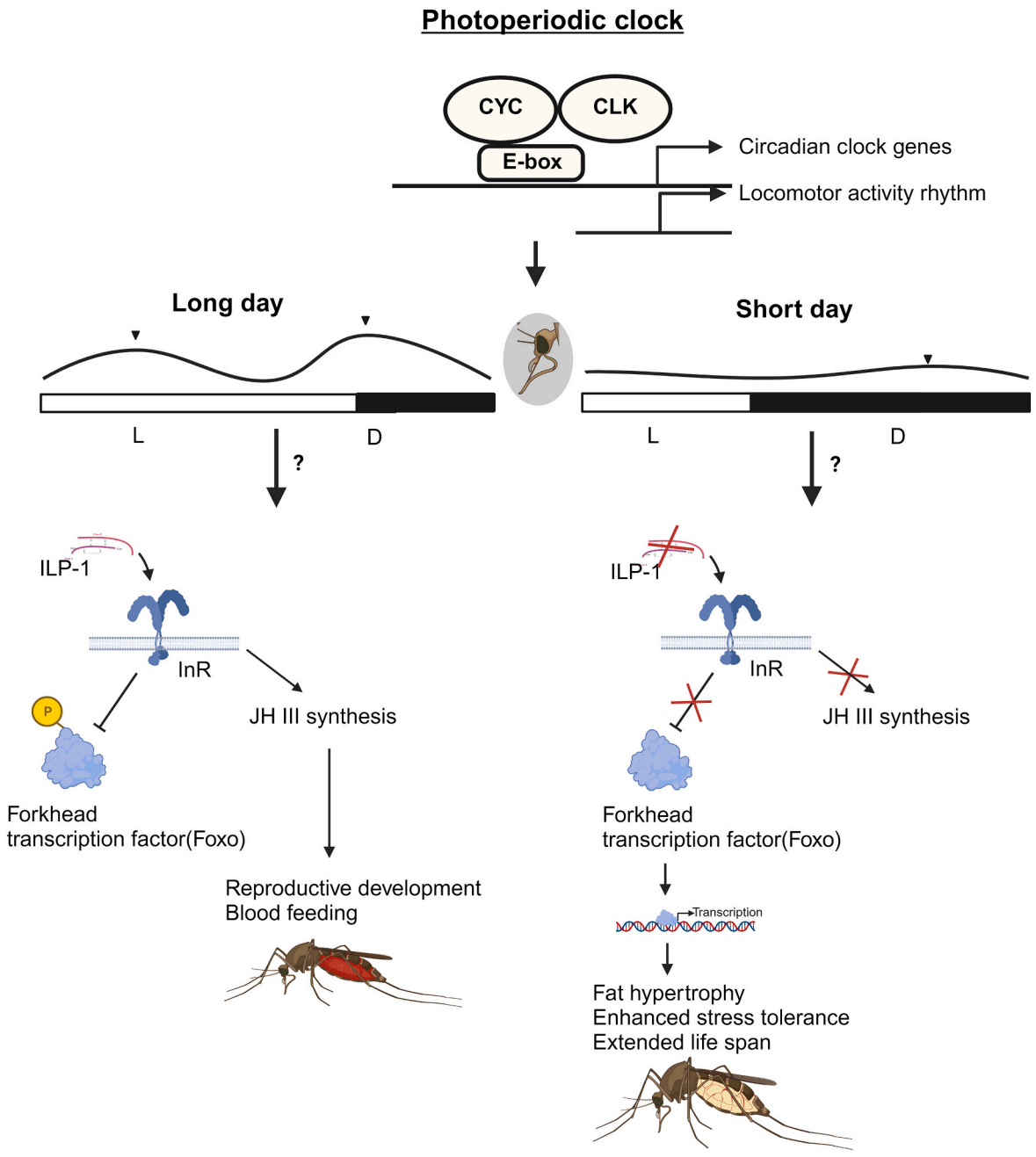

Fig. 1. A model for the role of CYCLE (CYC) in measuring the photoperiodic time in *Culex pipiens*. CYC differentially oscillates in the female heads under the different light regimes (the line graph on top of the box shows photoperiod with an arrowhead pointing to the time point with peak abundance/activity) and binds to the E-box element in the promoter of genes related to the circadian clock and downstream genes, thereby transmitting photoperiodic information to the *pars intercerebralis* (PI) within the insect brain to produce insulin-like peptides. In *Cx. pipiens*, long day lengths induce the elevated production of ILP-1, deactivating the Foxo and stimulating the production of JH III, and leads to reproductive growth and blood feeding. In contrast, short days induce suppressed levels of ILP-1 leading to the activation of the Foxo transcription factor and upregulation of downstream diapause-related genes. For further explanation see the main text.

many studies have reported that insects use the circadian clock to anticipate seasonal changes and undergo diapause to evade an inimical environment (Bünning, 1936; Meuti et al., 2015; Des Marteaux et al., 2022). This is conserved in several species of temperate *Culex* mosquitoes in which adult females show a strong photoperiodic effect and undergo seasonal diapause in anticipation of the short-day length of late summer and early fall to overwinter the inimical cold condition (Eldridge, 1987). Diapause syndrome in the Culex mosquito manifests as a halting of reproductive maturation, a shift from blood feeding to sugar consumption, migration to sheltered overwintering areas, reduction of metabolic activity, and heightened stress responses to cope with cold temperatures, dry conditions, and pathogens (Robich and Denlinger, 2005). Under the diapause-inducing conditions, the complex interplay between the hormonal and endocrinal pathways leads to the repression of insulin-like peptide 1 and subsequently to the decreased production of juvenile hormone III (JH) by the *corpora allata*. In the absence of insulin signaling (IS) and JH signaling, the FOXO transcription factor is activated and promotes the transcription of downstream genes involved in diapause-specific metabolic, physiological, and behavioral changes in *Cx. pipiens* as depicted in Fig. 1 (Sim and Denlinger, 2013; Sim et al., 2015). The involvement of the clock components in the regulation of diapause induction has been well-established by previous studies that used RNA interference (RNAi) to temporarily knock down clock genes expression, buttressing the supposition of the link between the endogenous clock and photoperiodic diapause response (Meuti et al., 2015; Chang and Meuti, 2020). Nevertheless, we still lack a mechanistic understanding of how the circadian clock regulates the genes and hormones involved in photoperiodic responses.

We propose that CYC acts as a key transcriptional regulator of downstream genes and mediates circadian behaviors and determines photoperiodic responses in *Cx. pipiens*. To decipher the genetic mechanism in the circadian regulation of physiology and behaviors, we exploited ChIP-sequencing to evaluate the hypothesis that differential expression of the target genes induced by CYC-mediated regulation results in a multitude of circadian behaviors and photoperiodic responses. Our results demonstrated that CYC binds to and regulates the expression of several peptide hormones and genes involved in neurotransmission, reproductive development, immunity, and olfaction finally revealing how the circadian clock regulates rhythmic behaviors and photoperiodic effects.

2. Materials and methods

2.1. Insect rearing

The laboratory colony *Cx. pipiens pipiens* was started in September 2000 from field-collected larvae in Columbus, OH, and invigorated later in 2009 and 2021 by adding field-trapped females of natural populations (Meuti et al., 2015). The colony was reared under a 12 h light:12 h dark (L:D) daily light cycle at 25 °C and 75 % relative humidity and adequately provided with honey water-soaked paper cords and water. Adult females were induced to produce eggs by feeding with chicken blood (Pel-Freez Biologicals, Rogers, AK) using a membrane feeding system. The eggs were put in a container filled with deionized water and allowed to hatch and fed with ground TetraMin fish food (Tetra Holding Inc., Blacksburg, VA). After the larvae molt into the second-instar stage larvae were placed under long-day rearing conditions consisting of 15 h light: 9 h dark, 18 °C, and 75 % relative humidity. Adults were provided with 10 % honey water-soaked wicks and kept in screened cages of 33.5 cm × 30 cm × 30 cm dimensions.

2.2. Preparation of ChIP samples

ChIP samples were prepared from whole bodies of long-day reared females collected at ZT16 (one hour after the lights had turned off), 1 week of adult eclosion. In the *Culex* mosquito, CYC oscillates in the head of females under LD conditions with the highest peak at ZT16 (Peffers and Meuti, 2022), and the chromatin sample from this time point will likely represent the maximum targets bound to CYC. Frozen whole bodies were crosslinked with 1 % formaldehyde and lysed to extract the nuclei using a Magna ChIP Kit (Protein G; Millipore) as per the manufacturer's instructions. Extracted chromatin was sonicated for fragmentation and immunoprecipitated with a custom-made monospecific antibody raised against *Cx pipiens* CYC peptide (amino acid sequence: KRKFSYNDNSDIEDDTGDDAKSVR) in rabbits by Pacific Immunology as described previously (Sim et al., 2015). Three biological replicates were prepared from fifty mosquitoes per sample and pooled together to increase the sequencing depth. After size fractionating the ChIPed DNAs, a ChIP library was constructed and sequenced at the PSOMAGEN genomics service (https://www.psomagen.com).

2.3. ChIP seq data analysis and prioritization

Sequence reads were trimmed and mapped to the *Culex quinquefasciatus* genome (JHB 2020; VectorBase) using Bowtie-2 (Langmead and Salzberg, 2012). Uniquely mapped reads were selected using Picard Mark Duplicates (V 2.18.2.3) (https://broadinstitute.github.io/picard/) and filtered to a Phred scale of at least thirty. Peak calling was carried out with MACS2 (Feng et al., 2012), and the resulting peaks were annotated with the ChIPseeker package in R (Yu et al., 2015). Peaks mapping to the 10 kb promoter region of nearest genes were selected and the genomic sequence of the peaks was extracted and scanned for the E box element (CACGTG) using a FIMO scan (Grant et al., 2011). The nearest gene to the predicted binding site was compiled into a dataset containing genes potentially regulated by CYC. The peak position in the promoter region was further confirmed by visualizing the ChIP-seq signal using the Integrated Genomics Viewer software (Robinson et al., 2011). Functional annotation of the candidate genes was performed using DAVID analysis, v 6.8 (Dennis et al., 2003), and clusters were made using UniProt IDs (https://www.uniprot.org/).

2.4. Validation of chromatin enrichment by ChIP-qPCR

Independent ChIP samples were prepared from the whole bodies of long-day reared females collected at ZT16 one week post eclosion. Anti-CYC antibodies or pre-immune serum (control) were used to immunoprecipitate samples from twenty-five whole bodies per biological replicate, and DNA samples were then analyzed using ChIP-qPCR. DNA copy numbers between the ChIPed DNA samples were determined and compared using qPCR in an iQ5 real-time PCR detection system (Bio-Rad). The sequences of gene-specific qPCR primer sets are listed in Table S1. All reactions were performed in at least three technical replicates in a total volume of 10 µl containing 5 µl SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA), 400 nmol of each primer, and 2 µl of ChIPed DNA per replicate at the following conditions: initial heating at 95 °C for 3 min followed by 40 cycles of denaturation at 95 °C for 15 s, followed by annealing at 55 °C for 30 s, and finally extension at 72 °C for 30 s. All Ct values were normalized to the mean Ct value of 1 % volume of sheared chromatin without immunoprecipitation as input. The statistical significance of differences in copy numbers of candidate sites between test and control ChIPed DNA was determined by using the Student's *t*-test considering a P-value of <0.05. All the statistical tests were done in GraphPad Prism 9 (GraphPad Software, USA) software.

2.5. Transcript levels measurement of candidate genes from ChIP-seq in ZT0 and ZT16

Gene expression was measured at ZT16 when the CYC transcription factor is reported to be most active in expressing the target genes, and ZT0 when it is least active. After 7 days post-eclosion, TRIzol (Invitrogen) was used to extract each RNA sample from the heads of female adults (3 biological replicates from 10 heads each replicate per time

point summing to 60 adult female heads). cDNA was synthesized using one μg of total RNA with SuperScript IV-reverse transcriptase (Invitrogen) and used to measure the relative transcript level of genes of interest using qRT-PCR. All qRT-PCR reactions were performed in a total volume of 10 μl containing 5 μl SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA), 400 nmol of each primer, and 2 μl of cDNA, and were conducted in iQ5 real-time PCR detection system (Bio-Rad) using the gene-specific primers listed in Table S2. The abundance of each transcript of interest was standardized by comparing it with the abundance of *Ribosomal protein L19* (*RpL19*; reference gene) using the $2^{-\Delta CT}$ method and compared between time points by $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). For each gene evaluated, statistical significance was determined using a Student's *t*-test between the relative expression values at two different time points. A P-value <0.05 was considered a significant difference between the transcript levels in GraphPad Prism 9 (GraphPad Software, USA) software.

3. Results

3.1. Genome-wide mapping of CYC binding sites

To identify the genes regulated by CYC, we utilized the ChIP-seq technology combining the deep Illumina sequencing of immunoprecipitated DNA fragments. The chromatin sample was prepared from the whole-body sample of long-day reared (15 h Light:9 h Dark) 7-day-old adult female mosquitoes collected at ZT16. Under these circumstances, the CYC protein level oscillates in the head of female *Cx. pipiens* with the highest expression at ZT16 and thus provided a potentially enriched source of CYC targets (Peffers and Meuti, 2022). The ChIPed DNA fragments were size-fractionated and amplified for the library preparation following the standard protocol and subjected to paired-end sequencing. Total reads of 78,476,068 DNA fragments were generated from Illumina sequencing and were processed through a bioinformatic pipeline (Fig. S2). The reads were quality assessed, trimmed, and then mapped to the *Cx. quinquefasciatus* genome (JHB 2020; VectorBase). Of them, 8,615,023 reads were mapped uniquely corresponding to the 44,439 binding sites across the genome (Fig. 2 A and B). We then scanned the genomic sequences corresponding to the peaks for the E box element (CACGTG), consensus CLK/CYC binding sites (Fig. 2C), with the exception for allatotropin based on the functional importance to photoperiodism, and our initial analysis yielded 1245 peaks that mapped to <10 kb promoter region comprising E box element. Of these, we narrowed down our selection to 462 genes with the peaks mapping to <1 kb promoter region. The selection was further narrowed down by selecting genes based on the fold enrichment of at least five-fold and annotation to uniprot ID. The peaks were visualized using Integrated Genome Viewer (Robinson et al., 2011) to locate the E-box in the upstream region of transcription start site (TSS) of the nearest gene (Fig. S1) and a total of 336 genes were selected as potential targets (Supplementary file 1).

The selected genes were then analyzed for GO enrichment and molecular function. They represented various molecular functions involved in different biological processes such as intra- and extracellular signaling, transcription, development, metabolism, and transport. We then grouped the genes based on their functional roles and selected 14 candidate genes belonging to five categories particularly relevant to neuropeptides and their receptors, neurotransmission, reproductive growth, immunity, and olfaction (Table. 1) for further analysis.

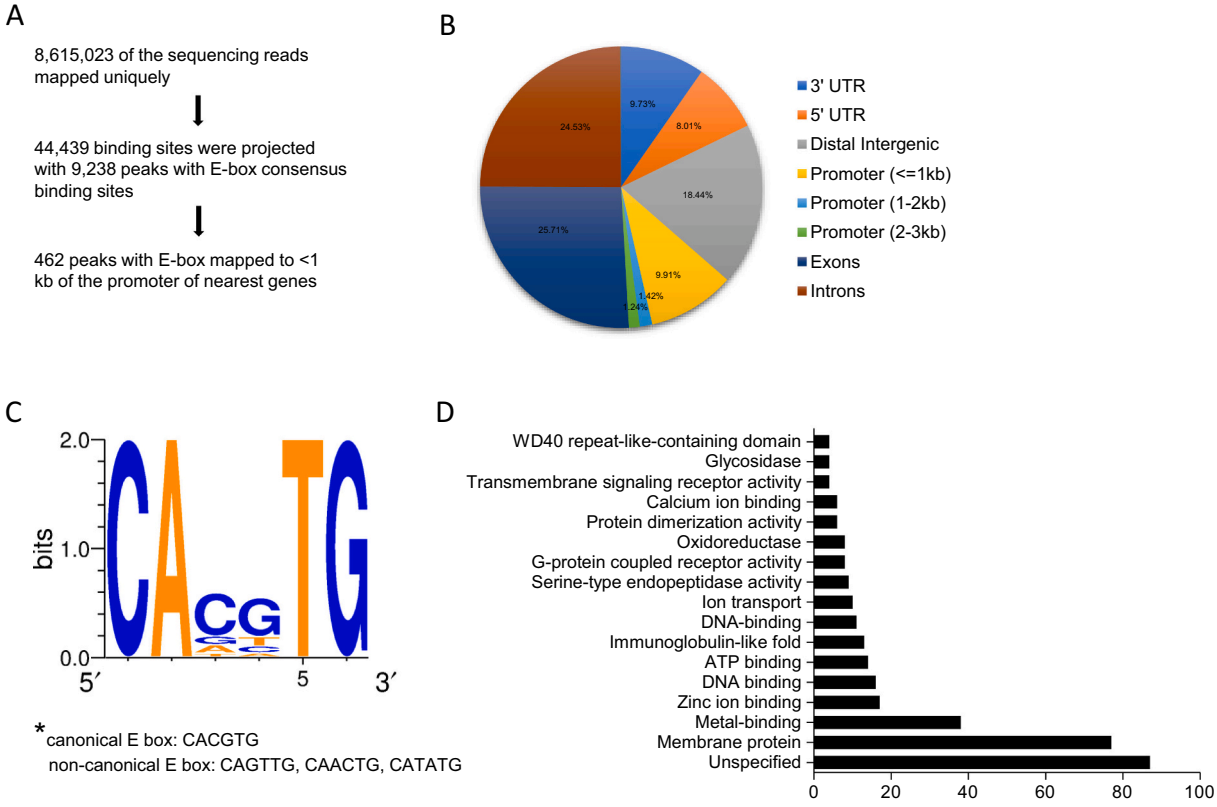

Fig. 2. ChIP-seq analysis using anti-CYC antibody revealed 462 genes with E box located within the <1 kb promoter region, and these putative CYC targets have diverse molecular functions. (A) A summary of the analysis of the peaks (B) Distribution of 44,439 peaks yielded by MACS2 in different genomic regions. (C) The motif logo of the E-box showing the canonical and uncanonical E-box elements. (D) Functional categories of putative genes identified by ChIP-seq analysis. 336 genes were clustered based on GO analysis in DAVID.

Table 1
Potential targets of CYC identified by ChIP assay.

VectorBase accession no.	Gene ID	Description	UniProt ID	E-box binding site
Peptide/receptor				
CQUJHB017763	Allatotropin	Allatotropin	B0WLE6	CM027411.1:78086540-78086545 (+), 78086559-78086564 (+)[a]
CQUJHB015305	CCKAR	Cholecystokinin receptor type A	B0XAR6	CM027410.1:131716084-131716089 (-)
Neurotransmission				
CQUJHB001203	Neuroligin	Neuroligin-1, transcript variant X2	B0WSL2	CM027411.1:114611626-114611631 (+) 114611657-114611662 (+)
CQUJHB012771	nAChRa	Acetylcholine receptor subunit alpha-like	B0W7P0	CM027410.1:70470841-70470846 (+)
CQUJHB011350	nAChRa7	Neuronal acetylcholine receptor subunit alpha 7	B0XLU4	CM027411.1:200130343-200130348 (-), 200130134-200130139 (-)
CQUJHB007660	Oct-Tyr R	Tyramine/octopamine receptor	B0WGI0	CM027410.1:96367351-96367356 (-)
CQUJHB000643	OctB2R	Octopamine beta-2 receptor	B0X4Z5	CM027410.1:49790034-49790039 (+)
CQUJHB003034	Stum	Protein stum	B0WS83	CM027411.1:76961431-76961436 (+)
Reproductive growth				
CQUJHB001065	Groucho	Groucho, transcript variant X10	B0WU77	CM027412.1:32359725-32359730 (+)
CQUJHB015490	Kr-h1	Krüppel homolog 1, transcript variant X2	B0X1K7	CM027411.1:126385900-126385905 (+)
Immunity				
CQUJHB002678	Takeout	Takeout	B0X3U4	CM027411.1:212021133-212021138 (+)
CQUJHB006434	Eip-74EF	Ecdysone-induced protein 74EF, transcript variant X3	B0W6X3	CM027412.1:189472324-189472329 (+)
Olfaction				
CQUJHB007574	Ir75a	Ionotropic receptor 75a-like, transcript variant X2	B0WNS8	CM027412.1:187141344-187141349 (-), 187141290-187141295 (-)
CQUJHB017442	Orco	Odorant receptor co-receptor	B0WR66	CM027412.1:46949894-46949899 (-)

[a] Uncanonical E box element (CACTTG).

3.2. ChIP-qPCR analysis confirms the binding of CYC to the promoter of target genes

To validate the in vivo specificity of CYC binding to the E box in the promoter of candidate targets, we tested the selective enrichment of DNA fragments using ChIP-qPCR. We found the high fold enrichment of targeted fragments in DNA immunoprecipitated with anti-CYC antibody compared to the pre-immune serum (Fig. 3). Only the base level binding of the target genes promoter was found in the control DNA sample obtained with the preimmune serum. This base level binding in control and significantly high fold enrichment in the test DNA sample confirmed the specificity of the genes we identified as potential targets of the CYC transcription factor.

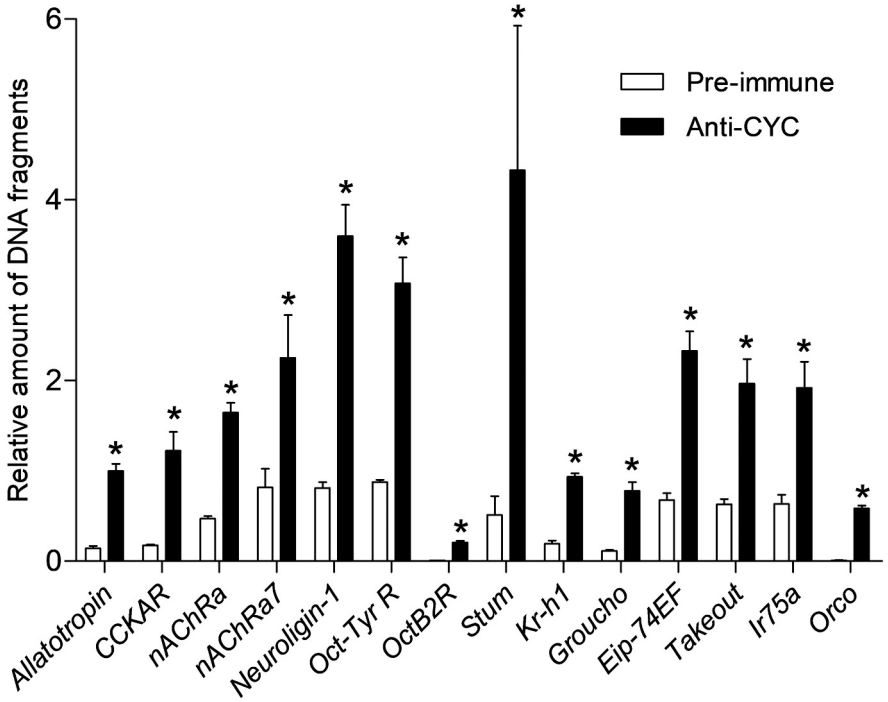

Fig. 3. ChIP-qPCR analysis shows the high enrichment of DNA fragments with motifs from the promoter of candidate genes of CYC. The Relative enrichment by pre-immune serum (control) to input is presented as white bars whereas the black bars show the relative enrichment with anti-CYC antibody (test). Sheared chromatin without immunoprecipitation was used as input control. Both groups were maintained under long day length at 18 °C and a whole-body mosquito sample was collected at ZT16. (*Significant difference at P = 0.05 by *t*-test). Data are expressed as mean DNA fragment level ± SD; *n* = 3 groups of 25 individuals each, each fragment level is normalized to fragment level present in 1 % input chromatin.

3.3. qRT-PCR shows the circadian regulation of candidate gene transcription

We then asked whether the expression of these candidate genes is in line with the level of CYC and limited to the central clock in the insect brain. qRT-PCR was used to test the relative abundance of transcripts in cDNA prepared from the head-only samples collected at ZT0 and ZT16. All the target genes previously validated for specific target binding by CYC showed daily differences in transcript abundance with a significantly higher level at ZT16 compared to ZT0 ($P < 0.05$, Student's *t*-test; Fig.4). The differential expression of these genes at tested two-time points correspond to the peak and trough of CYC protein abundance in the head of the female *Cx. pipiens* mosquitoes (Peffers and Meuti, 2022). These results imply that CYC acts as a key regulator to control the target genes that are involved in generating rhythmic behaviors and developmental changes in females of *Cx. pipiens*.

4. Discussion

Our study utilized the Illumina sequencing of ChIPed DNA obtained with the highly specific antibody to map the targets of CYC in females of mosquito *Cx. pipiens*. We predict that the CYC transcription factor affects the transcript abundance of target genes resulting in circadian patterns of behavior and physiological functions. Moreover, we also envision that by controlling the downstream target genes, CYC regulates photoperiodic responses and influences seasonal changes in development like diapause. The genes we have confirmed in this study represent genes involved in many functional categories pertaining to the major behaviors with circadian patterns one might think about the mosquito and reproductive development exhibited by the *Culex* mosquitoes under long-day, summer-like conditions, as outlined later. Besides these, our ChIP-seq analysis has identified many more target genes involved in several other signaling pathways that also likely contribute to the rhythmic behavior exhibited by mosquitoes and other insects.

4.1. Neurotransmitters

Circadian behavior in insects is the result of time-related memory and is governed by many factors including neurotransmitters such as acetylcholine, dopamine, octopamine, and glutamate (Xia et al., 2005; Raza et al., 2022). Our ChIP analysis identified that CYC regulates the transcription of the nicotinic acetylcholine receptor genes *nAChRa* and *nAChRa7*. Acetylcholine, found predominantly in the insect brain, serves as the primary excitatory neurotransmitter, mainly in sensory pathways and notably in the olfactory system. Blocking acetylcholine receptors leads to disturbances in memory, circadian rhythm, and sleep (Gauthier, 2010; Tasman et al., 2021). The circadian regulation of receptors to such excitatory neurotransmitters by CYC might thus control the periodic locomotory behavior to dusk in *Cx. pipiens*. Moreover, our analysis identified two genes encoding the receptor for the monoamine neurotransmitter octopamine, *Oct/TyrR*, and *Octβ2R*. In invertebrates, octopamine along with its derivative, tyramine, acts as a neurohormone and neurotransmitter and thus binds to the G-protein coupled receptors present in many tissues such as presynaptic and postsynaptic neurons and brain mushroom body, and oversees crucial functions such as memory and learning, locomotion, feeding behaviors, response to pheromones, and cardiac activity (Roeder, 1999; Farooqui, 2007). For example, octB2R is essential for female fertility and deficiency of such receptors prevents females from laying eggs despite their ability to engage in typical courtship, mating, copulation, sperm storage, and behaviors related to post-mating rejection (Lim et al., 2014). Another target gene identified from our study, *neuroligin1* is involved in synapsis (the fusion of chromosome pairs at the start of meiosis) and is expressed in mushroom bodies of the brain that process short-term memory (Krzeptowski et al., 2018; Xing et al., 2018). Moreover, a previous study demonstrated that neuroligin1 is linked to behavioral and neuronal plasticity in response to olfactory stimulation in insects (Durand et al., 2021); this combined with our results suggests that circadian behaviors such as blood feeding could be attributed to increased sensing of host odor. However, demonstrating the connection between CYC, neuroligin1, and host-seeking demands functional validation. In addition, *stum*, a gene known to be essential for coordinated locomotion has also been identified as a target of CYC. Previous research in *Drosophila* demonstrated that *stum* plays an important role in mechanosensation and reported its abundant presence in neuronal termini in leg joints (Desai et al., 2014). Our finding contrasts with these results, as we found that *stum* was abundantly expressed in mosquito heads at dusk when the mosquitoes were more mobile showing a correlation with the circadian locomotor activity.

4.2. Olfaction

Olfaction is one of the key features of mosquitoes and is essential for blood and sugar feeding, mating, and oviposition; therefore, appropriate

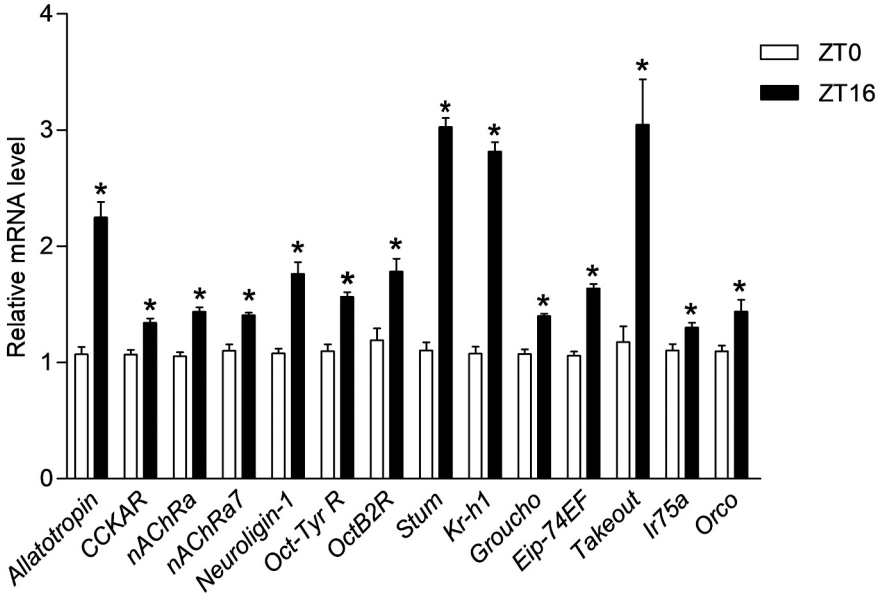

Fig. 4. qRT-PCR showing the relative abundance of candidate genes regulated by CYC in the head of 7–10 days old females of *Cx. pipiens* collected at two time points, ZT0 (lights on) and ZT16 (1 h after lights had turned off; peak CYC activity). The relative expression level at ZT0 is presented as white bars whereas black bars show the relative expression at ZT16. Both groups were maintained at 18 °C under long-day conditions. The relative abundance of each gene of interest was normalized to the abundance of *ribosomal protein large subunit* (*RpL19*). (*Significant difference at $P = 0.05$ by *t*-test). Data are expressed as mean transcript level ± SD; $n = 3$ groups of 10 individuals each.

circadian regulation of olfactory systems has a profound effect on mosquito fitness and survival. These responses are mediated by different receptors and are crucial for detecting a wide range of environmental stimuli such as pheromones, odor, bitterness, sweetness, carbon dioxide, humidity, and carbonated water (Wheelwright et al., 2021). The daily changes in olfactory response occur in several insects, including mosquitoes, and these changes continue even without any external light-dark cues indicating that they are governed by the endogenous circadian clock present mainly in antennae (Zhou et al., 2005; Eilerts et al., 2018). *Ionotropic receptor 75a-like* (*Ir-75*) is another gene that our study indicates is potentially regulated by the CYC transcription factor. *Ir-75a* is an ionotropic glutamate receptor and is tuned to sense volatile acidic compounds, particularly acetic acid, and propionic acid (Benton et al., 2009). Similarly, another putative CYC target gene, *Orco*, is involved in sensing the blood meal host and mating partner which is important in mosquitoes for blood feeding and mating success (DeGennaro et al., 2013). Recently, mutants of *Aedes aegypti* that lacked a functional CYC protein were reported to show low mating success and disrupted blood-feeding patterns (Shetty et al., 2022). In another study, knocking out *Orco* in the malaria vector *Anopheles sinesis* resulted in a similar loss of sensitivity to the host (Wang et al., 2022). This suggests the potential link between CYC and *Orco* regulating the behaviors rhythmically and is governed by an endogenous circadian clock.

4.3. Immune function

Immune functions in mosquitoes are not only crucial in combating infections, enhancing survival and maintaining reproductive fitness but are also in their vectorial capacity (King, 2020). The circadian rhythm in the immune system enables the host to better anticipate and manage microbial threats, leading to improved efficiency in dealing with them. Additionally, it aids in promoting tissue recovery and eliminating potentially harmful cellular components from the bloodstream (Scheiermann et al., 2013). Interestingly, our analyses indicate that CYC regulates *Eip74EF* (*E74*) and *takeout/JHBP* genes, which have previously been reported to mediate immunity in insects. The *takeout* gene family comprises a diverse suite of genes sharing similarities with juvenile hormone-binding proteins (JHBPs) (Saito et al., 2006). The *takeout*, identified in our analysis, is like JHBP and thus possesses an interesting functional relevance for rhythmic activation of the immune system. JHBPs bind to JH (Juvenile Hormone) before transporting to the target tissue and serve the crucial function of safeguarding the JH against the hydrolytic action of JH esterase and thus ensures the maintained titer of JH in hemolymph (de Kort and Granger, 1996). In addition, mosquito JHBP controls hemocyte numbers and the structure of hemocyte populations thereby regulating the antibacterial response (Kim et al., 2020). Our finding that CYC regulates *takeout/JHBP* expression suggests a mechanistic detail of the circadian control of immune functions. Moreover, the insect immune system requires ecdysone signaling for innate immune response and involves ecdysone-regulated transcription factors including *E74* (*ecdysone-inducible early gene 74*) (Rus et al., 2013). The *E74* encodes a transcription factor ETS (erythroblast transformation specific), that plays a crucial role in mediating the response to 20-hydroxyecdysone (20E) during gene regulation. It exists in two isoforms, E74A and E74B, with distinct patterns of expression: E74A activates 20E-responsive genes, while E74B represses them (Fletcher et al., 1997). Previously the members of the ETS family have been reported to be involved in immune response in a diverse range of insect species including *B. mori* and *D. melanogaster* where they directly govern the expression of antimicrobial peptide genes and affect immune responses (Tanaka et al., 2012; Rus et al., 2013).

4.4. Neuropeptides

Insects have a multitude of neuropeptides and are described to act as co-transmitters, neuromodulators, and hormones by interacting with different receptors, primarily G protein-coupled receptors (GPCRs) (Schoofs et al., 2017). Our analysis suggests that CYC likely enhances the transcription of the neuropeptides including *allatotropin*, and *cholecystokinin receptor type A* (*CCKAR*). Allatotropin (AT) is a pleiotropic neuropeptide with diverse effects on various tissues in insects. Initially known for its stimulatory activity for the synthesis of Juvenile Hormone (JH), AT also influences numerous other processes. These include regulation of cardiac rhythm, oviduct and hindgut contractions, nutrient absorption, and even impacting the circadian cycle (Kataoka et al., 1989; Elekonich and Horodyski, 2003). *Cx. pipiens* shows facultative reproductive diapause in response to short photoperiod and is characterized by the reduced abundance of *allatotropin* mRNA transcripts and suppressed JH III production (Denlinger and Armbruster, 2014; Kang et al., 2014). This points towards the potential link between circadian clock genes and JH III synthesis and therefore holds an important mechanism for reproductive success under a long-day photoperiod. An additional target gene, *CCKAR*, is a G-protein coupled receptor that binds to the cholecystokinin (CCK)/sulfakinin and is distributed in the insect brain and ventral nerve cord (Słocińska et al., 2020). CCK/Sulfakinin, originally identified in cockroach heads, is produced mainly by neurons and is colocalized in the brain with cells that produce insulin-like peptides (Nichols et al., 1988; Słocińska et al., 2020). CCK/sulfakinin with its pleiotropic regulatory activity signals many functions relating to diverse behaviors including gustatory sensitivity, satiety and feeding, locomotion, aggression, and reproductive behavior (Nässel and Wu, 2022). Our finding indicates that CYC regulates higher expression of *CCKAR* transcripts and likely leads to increased behaviors during dusk and nighttime. Such pattern in behavior is of utmost importance to the crepuscular host-seeking and blood-feeding of *Culex* mosquitoes which contributes to their reproductive success and survival.

4.5. Reproductive development

Cx. pipiens show strong photoperiodic responses, continuing with reproductive development during the long-day lengths and entering adult diapause in response to short-day lengths. The molecular mechanisms that female mosquitoes use to acquire and process photoperiodic information are still unclear, but previous reports documented the indispensability of the circadian clock for the induction, maintenance, and termination of such seasonal responses (Meuti et al., 2015; Chang and Meuti, 2020). Our ChIP analysis identified that CYC regulates the *Krüppel homolog 1* (*Kr-h1*) gene. Previous studies demonstrate that the expression of *Kr-h1* is also regulated by photoperiod and controls the expression of downstream target genes by binding to the promoter region and thereby inducing reproduction in many arthropods (Ojani et al., 2018; Dong et al., 2021; Guo et al., 2021). Although the transcriptional change and importance of this gene in seasonal diapause have been reported by previous studies, the exact genetic mechanism for such abundance remained unexplored to date. Our discovery of the transcriptional regulation of *Kr-h1* by CYC is thus an important finding in the regulation of seasonal adaptation by the internal circadian clock. Notably, another target of CYC that we identified, the gene encoding for the Groucho (GRO) protein, acts as a cofactor of the HAIRY to repress critical downstream genes in the JH signaling pathway and is necessary for the reproductive development (Saha et al., 2016). Additionally, GRO is involved in ovarian development and oviposition and plays a pivotal role in embryonic development, and neurogenesis (Orian et al., 2007; Gao et al., 2022). This points towards the putative role of the endogenous clock in regulating reproductive development in response to long-day photoperiods.

5. Conclusions

Our study is the first to identify the targets of the CYC transcription factor in insects. It helps fill the existing knowledge gap in the underlying mechanisms by which the circadian clock regulates daily

behaviors and photoperiodic responses in *Cx. pipiens*. Our findings are consistent with the crucial role of transcription factor CYC in governing important circadian behaviors, including neurotransmission, immunity, and olfaction, as well as the seasonal induction of insulin signaling and reproductive processes. Our study suggests that the oscillating abundance of CYC across a day regulates the expression of downstream target genes activating diverse gene networks and leading to behavioral changes in a time-of-day-dependent fashion. The control of CYC is not limited to circadian behavior but extends beyond that to translating photoperiodic information to developmental pathways such as diapause. Future studies that confirm the function of the CYC targets we've identified will allow us to determine how mosquitoes and other insects use shifts in the daily and seasonal light and dark cycles to regulate their behavior and physiology.

CRediT authorship contribution statement

C.S., M.M. and P.D. designed the research; P.D. performed research and analyzed data; XW assisted in sample preparation and C.S., M.M. and P.D. drafted the paper.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

The NCBI SRA accession number has been provided in manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbd.2023.101140.

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