Article

Diel rhythm of feeding and mating behaviors of the western flower thrips, *Frankliniella occidentalis*

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Simple Summary: Most insect behaviors exhibit the fixed action patterns based on innate programs. Underlying genetic factors drive the specific neuronal and muscular combinations to exhibit the stereotyped behaviors. The western flower thrips, *Frankliniella occidentalis*, is invasive and polyphagous insect pest. Identification and alteration of the genetic components controlling feeding and mating behaviors would provide novel pest management techniques against the thrips. This study showed the diurnal peaks of the feeding and mating behaviors of thrips. Most feeding occurred during photophase in both larvae and adults. Matings occurred mostly at 2~3 days-old young adults, in which peak callings for copulation were biased toward photophase. Four genes associated with the circadian clock were predicted in the *F. occidentalis* genome: period (*PER*), Timeless (*TIM*), Doubletime (*DBT*), and clock (*CLK*). Expressions of these genes were fluctuated in a 24 h-period, at which their high expression peaks were observed during photophase except for *DBT*. Interestingly, when the gene expressions except *DBT* were altered by RNA interference, the feeding and mating behaviors were altered by shifting or suppressing their active peaks. These results suggest that the circadian clock genes mediate the diurnal feeding and mating behaviors of *F. occidentalis*.

Abstract: The western flower thrips, *Frankliniella occidentalis*, is invasive and polyphagous. Its visual signal plays a crucial role in host location, suggesting that thrips has diurnal behavior. However, any diel rhythmicity of the thrips behavior is not yet well understood. This study analyzed the diurnal rhythmicity of the feeding and mating behaviors of thrips, and the patterns were explained in terms of diel rhythmicity by assessing the expression of genes associated with the circadian clock. Most feeding behaviors were observed during photophase in both larvae and adults. In mating behavior, which is mainly exhibited in 2~3 days-old young adults after emergence, most adults copulated at photophase. This diurnal pattern of the mating behavior was further supported by the analysis of a temporal calling behavior for mating, which was biased toward photophase. Four genes associated with the circadian clock were predicted in the *F. occidentalis* genome: period (*PER*), Timeless (*TIM*), Doubletime (*DBT*), and clock (*CLK*). All these genes exhibited a characteristic diel pattern in their gene expressions in a 24 h-period. The high expression peaks of these genes except for *DBT* were observed at photophase. To confirm the functional association of these genes with the feeding and mating behaviors, individual RNA interference specific to each gene was executed by feeding the gene-specific double-stranded RNA. Aside from *DBT*, suppression of these gene expressions resulted in significant deviations of the diel rhythms of the behaviors. These results suggest that the genetic components of the diel rhythmicity mediate the diurnal feeding and mating behaviors of *F. occidentalis*.

Keywords: Circadian clock; *Frankliniella occidentalis*; Feeding; Mating; Behavior

1. Introduction

The western flower thrips, *Frankliniella occidentalis*, has been seriously considered as an insect pest in Korea since its invasion in 1993 and its subsequent dispersion to the entire country [1,2]. Its polyphagous feeding behavior underscores its pest severity, causing serious economic damage to

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high-value crops such as hot pepper [3]. In particular, *F. occidentalis* is the major thrips in hot pepper that is cultivated in greenhouses, and it transmits a tospovirus called TSWV (tomato spotted wilt virus) [4]. Compared to its related native species (= *Frankliniella intonsa*), *F. occidentalis* exhibits a high tolerance in survival and longevity under a high CO_2 level (800 ppm), which can sometimes be the case in greenhouses [5]. This suggests that *F. occidentalis* would be a major thrips infesting various crops that are cultivated in greenhouses. Moreover, it has become more difficult to effectively control the insect pest due to its development of resistance to chemical insecticides [6].

Visual cues and olfactory signals are part of a major sensing system to locate hosts in *F. occidentalis*, wherein the visual recognition includes specific colors and shapes of objects [7]. Using an LED light source, its color discrimination was further analyzed, and the results suggested that it achieved color detection of three types of photoreceptors specific to blue, green, and UV [8]. In addition, short daylength reduces immature development and adult fecundity of *F. occidentalis* [9]. These suggest that *F. occidentalis* exhibits host-searching behavior in the daytime. However, its diel rhythmicity of the behaviors was still not well understood in genetic component(s).

The circadian clock represents a 24-h cyclic rhythmicity of various physiological processes in biological organisms including insects [10]. Moreover, entrainment to the daily environmental cycle such as the light-dark cycle is a fundamental property of the circadian clock. To adjust the rhythmicity to the light cycles, cryptochrome (CRY), which is a blue light receptor whose sequence is similar to that of DNA photolyases [11], plays a crucial role in the perception of light signal and transmitting the light signal to the other circadian clock components [12]. The circadian clock consists of two oscillatory loops: Period (PER)/Timeless (TIM) oscillatory loop and the CRY loop [13,14]. In each of these loops, Clock (CLK)/Cycle (CYC) acts as a transcriptional activator that promotes PER and TIM, or CRY transcription. The product proteins PER and TIM, or CRY are thought to provide negative feedback to inhibit the transcriptional activator. In the fruit fly, Drosophila melanogaster, CRY is expressed in specific clock neurons in the brain [15]. CRY is then activated by blue light and catalyzes TIM degradation through its protease activity, at which point the circadian clock is reset [16,17]. This resetting mechanism has been also demonstrated in monarch butterflies, which are known to navigate long distances during migration via time-compensated sun compass orientation [18]. Despite the well-known genetic components controlling circadian rhythm, little was known in the genetic factors modulating the behaviors such as feeding and mating in *F. occidentalis*.

The genome of *F. occidentalis* was first found in Thysanoptera, and it has 415.8 Mb encoding 16,859 genes [19]. The genome information allows us to investigate genes associated with circadian rhythm. This study demonstrated the diel rhythmicity of feeding and mating behaviors of *F. occidentalis*. To clarify their diel rhythmicity, circadian clock-associated genetic components were obtained from the annotated genome of the species and assessed in terms of their expression patterns. Moreover, the RNA interference (RNAi) of the circadian components followed by behavioral analysis supported the circadian rhythmicity of the feeding and mating behaviors.

2. Materials and Methods

2.1. Insect rearing

Both larvae and adults of *F. occidentalis* were obtained from the Department of Crop Protection, National Institute of Agricultural Sciences (Jeonju, Korea), and maintained at conditions of 25 ± 1°C temperature, 60 ± 5 % relative humidity, and a 14:10 h (L:D) light cycle without dawn and dusk transitions. Newly germinated beans (*Phaseolus coccineus* L.) were supplied *ad libitum* for immature growth and adult reproduction. Eggs that were newly laid on the beans in adult colonies were transferred to the breeding dish (10 cm diameter × 10 cm height, SPL Life Science, Seoul, Korea). After 3 days, at which point most larvae hatched, new beans were supplied every day. Under the laboratory conditions, larvae underwent two instars (L1 - L2) and were distinct from prepupae or pupae that developed wing pads.

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2.2. Feeding behavior assay

To determine the feeding behavior of larvae and adults, beans were supplied as a diet in the breeding dish covered with a wet filter paper (5 cm diameter, Whatman, Maidstone, UK). After the test thrips were introduced, they exhibited feeding behavior by inserting the stylets into the diet and keeping stand-still position which was observed under a stereomicroscope (EZ4, Leica, Wetzlar, Germany). This behavior was regarded as feeding and counted at 1-hour intervals. Each experimental unit consisted of 20 test insects. During each time interval, the positive insects exhibiting feeding behavior were counted and used to calculate the feeding frequency in percentage. Each experimental unit was independently replicated three times.

2.3. Mating behavior assay

Before measuring the mating behavior, pupae were separated from the colony and individually transferred to a 1.5 mL microtube (Axygen, Union City, CA, USA) to avoid mating. After measuring the emergence time, a diet of bean (0.2 × 0.2 cm) was provided in the tube. A pair of male and female unmated adults at 48-72 h after emergence was placed on a cap of 1.5 mL microtube, which served as an arena, and covered with cover glass (18 × 18 mm, Marienfeld, Königshofen, Germany) for observation under a stereomicroscope. Each mating behavior was examined for 10 min after the introduction of both males and females under the conditions of 25 ± 1°C temperature and 60 ± 5% relative humidity in different light-dark regimes. Mating behavior under the dark regime was observed using an infrared light (Infrared light 150W, Couyor, China). Copulation behavior within 10 min in the arena was recorded as a successful mating. Each observation was replicated 10 times with every fresh adult.

2.4. Y-tube olfactometer assay

This experiment used a Y-tube olfactometer containing the main length (10 cm) with two arms (9 cm each) separated by a 45° angle. The Y-tube was placed in a dark room with infrared light (SE601, Qingdao Hanil, Pingdu, China) at room temperature. During this experiment, the air was filtered by a charcoal filter at a rate of 0.6 L/min (Power Air Pump, Seoul, Korea) and passed through the two glass vessels, then entered each of the arms. A test chamber was connected to a vessel containing the test thrips (N = 50, 2-3 day-old adults). The other control had an empty chamber. A run (= an experimental unit) used 50 thrips (the same age of adults) and was replicated three times. The positive responses of thrips were recorded by having them pass through the arms and reach over 5 cm from the crossroad. The scoring was made at 10 min. After each run, the Y-tube was cleaned with 70% ethanol.

2.5. Bioinformatic analysis

DNA and amino acid sequences were obtained from NCBI (National Center for Biotechnology Information: https://blast.ncbi.nlm.nih.gov) with accession numbers. MEGA6.0 was used to construct a phylogenetic tree through clustering using Maximum likelihood, where the evolutionary distances were computed using the Poisson correction method. Bootstrapping values were obtained with 1,000 replications to support branching and clustering. Protein domains were predicted using a program searching the conserved domain (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), and InterPro (https://www.ebi.ac.uk/interpro/). The N-terminal signal peptide was determined using SignalP 5.0 server (https://services.healthtech.dtu.dk/ service.SignalP-5.0/). The resulting domains were drawn by Biorender (https://biorender.com/).

2.6. RNA extraction and cDNA preparation

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Total RNAs were extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. For each RNA extraction, 25 individuals were macerated using 500 μL of Trizol reagent. Following the RNA extraction, RNA was resuspended in 30 μL of diethyl pyrocarbonate (DEPC) and quantified using a spectrophotometer (Nanodrop, Thermo Fisher Scientific, Wilmington, DE, USA). For cDNA synthesis, 400 ng of RNA was used in each sample with RT oligo dT premix (Intron Biotechnology, Seoul, Korea) containing oligo dT primer. A reaction mixture consisted of 2 μL of RNA extract and 18 μL of DEPC-treated water and was run according to the manufacturer's instructions. The resulting cDNA samples were kept at -20°C before being used for experimentation.

2.7. RT-PCR and RT-qPCR

RT-PCR used the cDNA and amplified the target genes with a Taq polymerase (GeneALL, Seoul, Kore). A reaction mixture (25 μL) for PCR consisted of 2.5 μL of dNTP (each 10 pmol), 2.5 μL of 10× Taq buffer, 2 μL of forward and reverse primers (10 pmol/μL, Table S1), 0.5 μL of Taq polymerase, 1 μL of cDNA, and 16.5 μL of distilled deionized water. The PCR conditions began with an initial denaturation at 95°C for 5 min, which was followed by 35 amplification cycles consisting of 95°C for 1 min, 50 ~ 55°C for 30 sec, and 72°C for 1 min. At the end of the amplification cycle, an additional extension was performed at 72°C for 5 min. The PCR product was confirmed by 1% agarose gel electrophoresis. The qPCR used a Step One Plus Real-Time PCR System (Applied Biosystem) under the guidelines of Bustin et al. [20]. A sample of qPCR reaction (20 μL) contained 10 μL of Power SYBR Green PCR Master Mix (Toyobo, Osaka, Japan), 3 μL of cDNA template (100 ng), and 1 μL (10 pmol) each of the forward and reverse primers (Table S1). After an initial heat treatment at 95°C for 2 min, qPCR was performed with 40 cycles of denaturation at 95°C for 30 s, annealing at 50–55°C for 30 s, and extension at 72°C for 30 s. The transcript levels of elongation factor 1 (EF1) were used as a reference for the normalization of each test sample. Quantitative analysis was conducted using the comparative CT ($2^{-\Delta\Delta CT}$) method [21]. All experiments were independently replicated three times.

2.8. Measuring expression levels of circadian genes

For test thrips, L2 larvae and three-day-old adults were used. Each RNA extraction at every 2-h interval used 25 individuals and followed the cDNA synthesis and qPCR as described above. Each treatment was independently replicated three times.

2.9. RNA interference (RNAi) treatment and subsequent behavior assays

Template DNA was amplified with gene-specific primers (Table S1) containing T7 promoter sequence (5′-TAATACGACTCACTATAGGGAGA-3′) at the 5′ end. The PCR conditions were as described above. After confirming the PCR product, the resulting PCR product was used to in vitro synthesize double-stranded RNA (dsRNA) encoding *F. occidentalis* genes using T7 RNA polymerase with NTP mixture at 37°C for 3 h (MEGA script RNAi kit, Ambion, Austin, TX, USA). dsRNA (2,000 ng/10 μL) was mixed with a transfection reagent Metafectene PRO (Biontex, Plannegg, Germany) at a 1:1 (v/v) ratio and incubated at 25°C for 30 min to form liposomes, and it was then supplied with bean to *F. occidentalis*. Bean was coated with a mixture of 10 μL per 20 thrips and fed for 12 h. A control dsRNA ('dsCON') was prepared according to the method outlined by Vatanparast et al. [22]. Feeding and mating behaviors were assessed using the method described above.

2.10. Statistical analysis

Percent data were arcsine-transformed and the subsequent transformed data were confirmed to follow a normal distribution using PROC UNIVARIATE of SAS program [23]. Data obtained from the feeding or mating test were subjected to a one-way analysis of variance (ANOVA) using PROC

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GLM of the SAS program. The means were compared using the least significant difference (LSD) test at a Type I error of 0.05. Frequency data were analyzed by the mean difference test using PROC FREQ of the SAS program. Circadian rhythmicity of the gene expression was assessed using JTK_CYCLE [24].

3. Results

3.1. Diurnal feeding behavior of F. occidentalis

The feeding behavior of *F. occidentalis* was characterized by the insertion of their stylets to diet bean in both larvae and adults. Using this characteristic behavior, their diel rhythms were assessed in both developmental stages (Figure 1). The feeding behaviors were more active at photophase than they were at scotophase. This diurnal behavior was significant in larvae (F = 9. 45; df = 1, 22; P = 0.0050) and adults (F = 42.00; df = 1, 22; P < 0.0001).

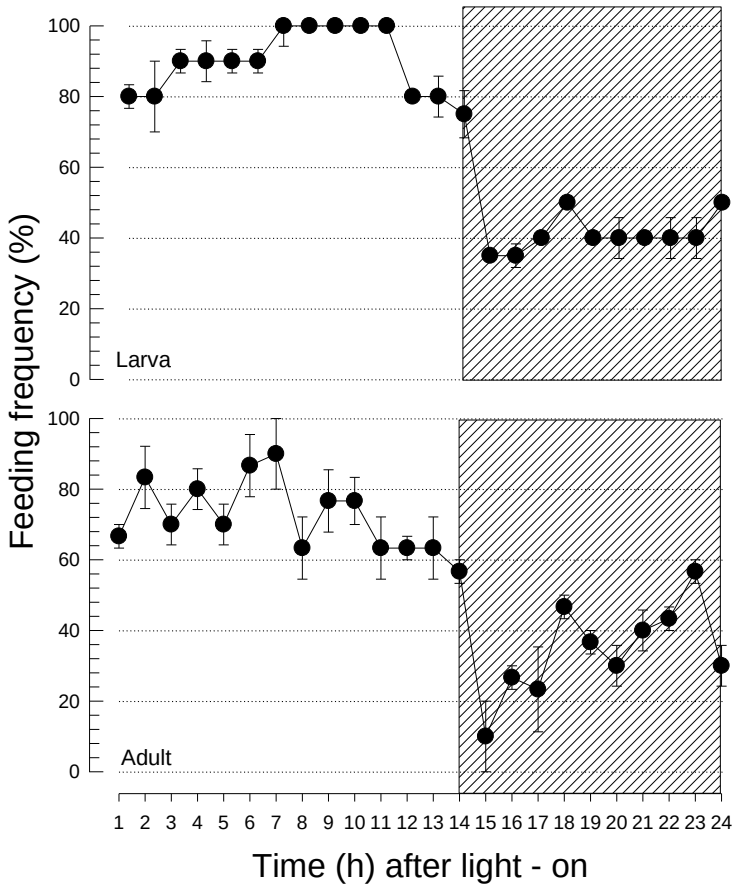

Figure 1. Diel rhythm of feeding behaviors in larvae (upper) and adults (lower) of *F. occidentalis*. Feeding behavior was determined by the stylet insertion to diet bean at an interval of every hour. Ten individuals were observed at each time point and replicated three times. Light:Dark cycle was 14:10 h. Temperature was 25 ± 2°C.

3.2. Diurnal mating behavior of F. occidentalis

Mating behavior was analyzed in a small arena (Figure 2a) at different times of day. Mating rates of the unmated adults were varied with the adult ages, in which the maximal rates were observed at

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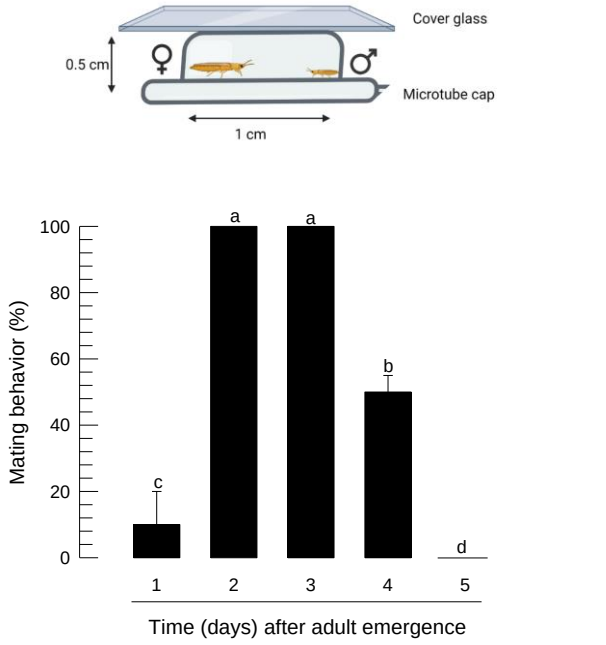
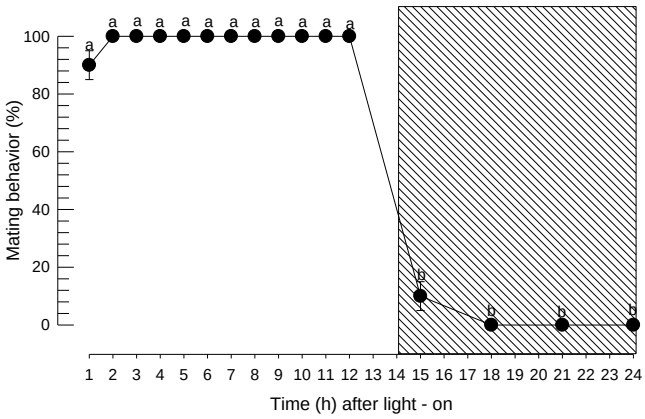
2-3 days after adult emergence. Using this active age in mating behavior, the mating rates were monitored at every hour each day (Figure 2b). Most of the mating behaviors significantly (F = 3,114.92; df = 1, 47; P < 0.0001) occurred after light-on in a statistically finding.



(a)



(b)

Figure 2. Diel rhythm of mating behavior in *F. occidentalis* adults. Mating behavior was determined by copulation between a male and a female in a dish arena (1 cm diameter). Ten pairs were observed at each time point and replicated three times. (A) Variation of mating frequency at different adult ages. Behavioral assessments were performed at photophase. (B) Diurnal mating behavior of three-day-old adults. The assessments were performed at an interval of every hour during photophase and an interval of every three hours at scotophase. Light:Dark cycle was 14:10 h. Temperature was 25 ± 2°C.

3.3. Diurnal calling behavior of F. occidentalis

The diurnal mating behavior was further supported by the calling pattern in a day (Figure 3). Both males and females at 3 days after adult emergence were attractive to both sexes. However, their calling time varied within a day. This calling behavior followed the diel rhythm. Most calls occurred at photophase and decreased at scotophase: female to female (F = 26.34; df = 1,16; P < 0.0001), female

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to male (F = 28.34; df = 1,16; P < 0.0001), male to male (F = 55.33; df = 1,16; P < 0.0001), male to female (F = 25.94; df = 1,16; P < 0.0001).

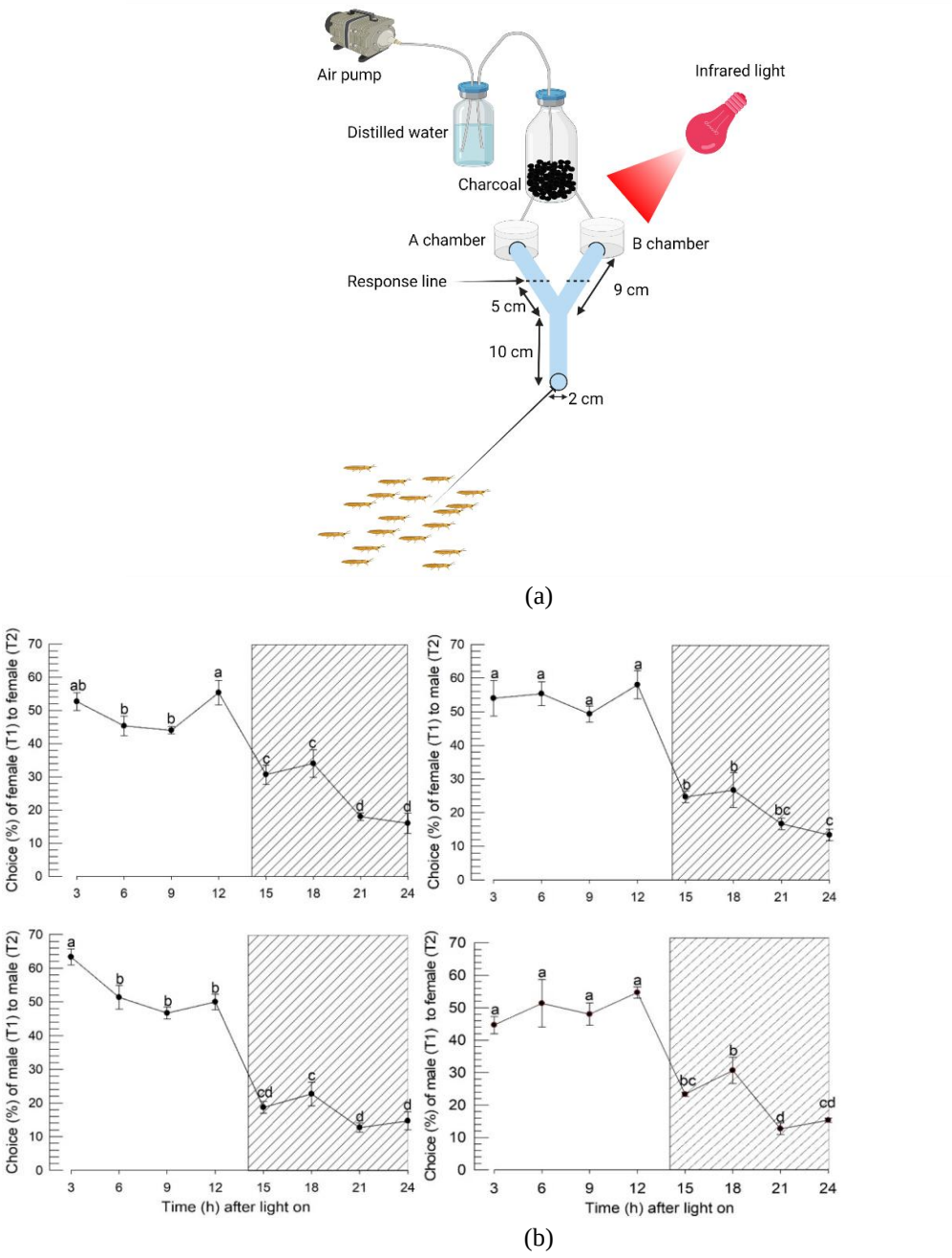

Figure 3. Diel rhythm of calling for mating. (A) Diagram of Y-tube olfactometer experiment. Test insects ('T1') for sending calling signals were inserted into the A or B chamber. Responding insects ('T2') (N = 50) were added to the base of the Y tube. The experiment was run at dark condition under infrared light. Each treatment was replicated three times. (B) Calling pattern in a day. Different letters above the standard deviation bars indicate significant differences among means at Type I error = 0.05 (LSD test).

3.4. Prediction of four genes associated with circadian clock and their expression profiles

Timeless (*TIM*), Period (*PER*), Doubletime (*DBT*), and Clock (*CLK*) are known to be some components of the circadian clock for all organisms [15]. To predict their orthologs from *F. occidentalis*, its

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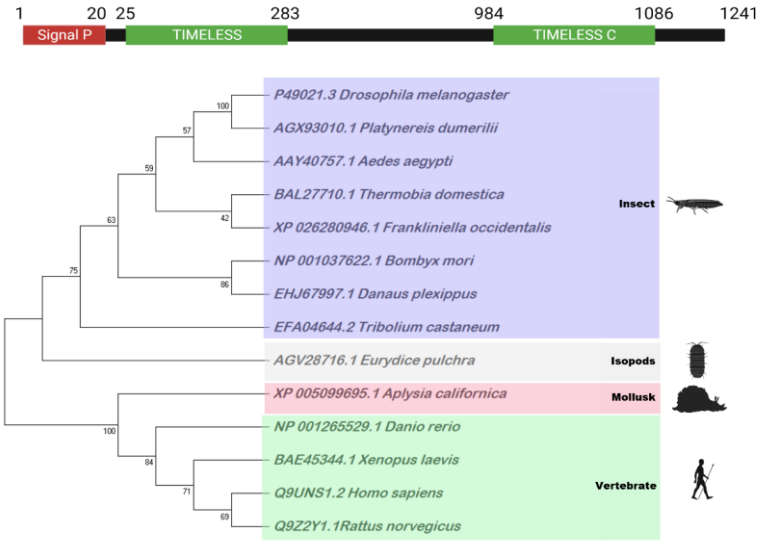
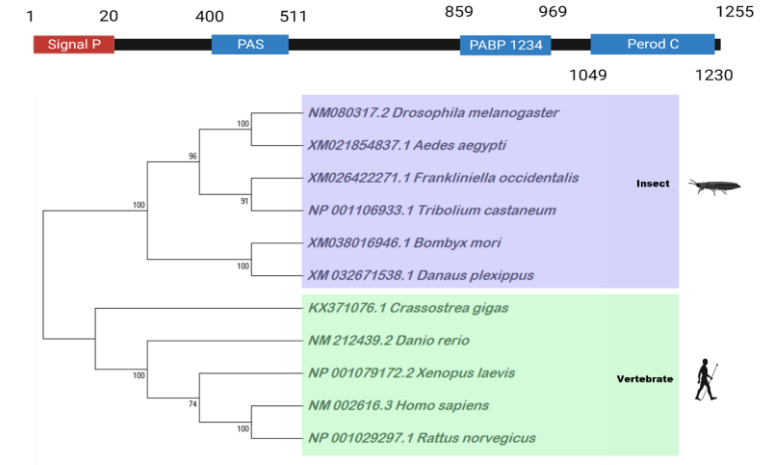
genome was interrogated. Four corresponding genes were obtained and compared with other known orthologs (Figure 4a). This phylogenetic analysis of the orthologs indicates that insect orthologs are clustered and distinct from those of other animal systems. All these circadian genes were expressed in different stages of *F. occidentalis* (Figure 4b).



(a)



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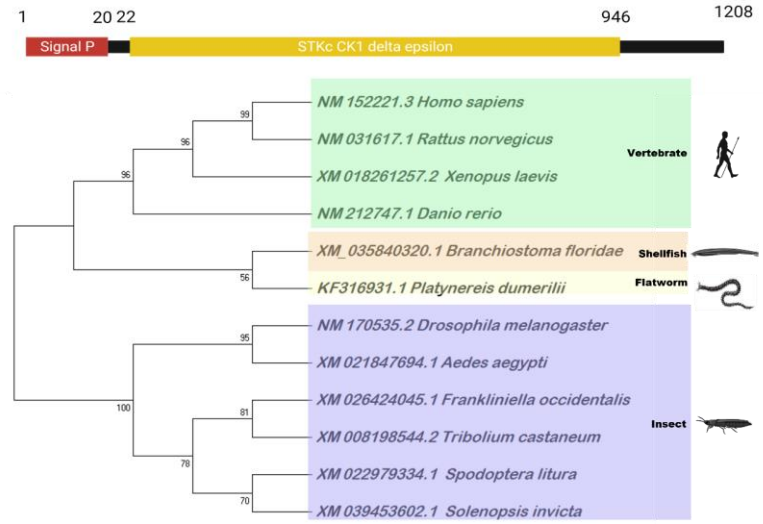

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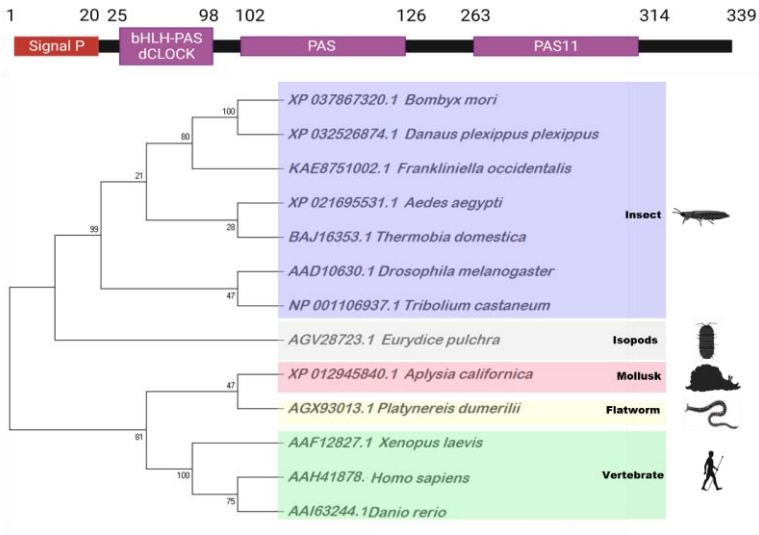

(d)

Figure 4. Phylogenetic analysis of four genes associated with circadian rhythm in *F. occidentalis*: (A) Timeless (*TIM*), (B) Period (*PER*), (C) Doubletime (*DBT*), and (D) Clock (*CLK*). The tree was generated by the Neighbor-joining method using MEGA 6.0. Bootstrapping values were obtained with 1,000 repetitions to support branch and clustering. Amino acid sequences were retrieved from GenBank, along with accession numbers. Protein domains were predicted using NCBI conserved Domain and InterPro. The N-terminal signal peptide was determined using the SignalP 5.0 server.

The expression levels of these circadian genes were assessed at intervals of every 2 h in a day in larvae and adults (Figure 5). All four genes showed diel patterns in their expression levels within a day. The diel patterns did not differ between the larval and adult stages (F = 0.12; df = 1, 239; P = 0.7200). However, the diel patterns differed between the four genes (F = 23.11; df = 3, 239; P < 0.0001). For TIM gene, the expression levels were higher at photophase compared to scotophase in larvae (F = 37.29; df = 1, 24; P < 0.0001) and adults (F = 48.87; df = 1, 24; P < 0.0001). This pattern was also detected in the expression of *PER* gene in both developmental stages: larvae (F = 22.76; df = 1, 24; P < 0.0001) and adults (F = 54.27; df = 1, 24; P < 0.0001). However, there was no significant different in the expression of *DBT* gene between photophase and scotophase in both developmental stages: larvae (F = 1.40; df = 1, 24; P = 0.2485) and adults (F = 9.40; df = 1, 24; P = 0.053). For *CLK* gene, the gene expression was also higher at photophase than at scotophase in both developmental stages: larvae (F = 31.93; df = 1, 24; P < 0.0001) and adults (F = 6.27; df = 1, 24; P = 0.0195). However, the oscillation patterns of the gene expression followed a circadian rhythmicity only in PER gene (P = 0.043 in larvae; P = 0.030 in adults) when they were assessed by a statistical algorithm to time-series data using JTK_CYCLE (Table S2).

3.5. RNAi treatments of circadian genes disrupt the circadian rhythmicity of the feeding and mating behaviors

The expression of each circadian gene was suppressed by feeding specific dsRNA (Figure 6a). The RNAi of circadian gene treatment significantly suppressed the gene expression compared to control RNAi: in *TIM* RNAi, larvae (F = 18.45; df = 1, 4; P = 0.0255) and adults (F = 13.36; df = 1, 4; P = 0.0200), in *PER* RNAi, larvae (F = 13.22; df = 1, 4; P = 0.0221) and adults (F = 13.86; df = 1, 4; P = 0.0204), in *DBT* RNAi, larvae (F = 19.89; df = 1, 4; P = 0.0112) and adults (F = 13.47; df = 1, 4; P = 0.00214), and in *CLK* RNAi, larvae (F = 18.22; df = 1, 4; P = 0.0130) and adults (F = 11.50; df = 1, 4; P = 0.0275). Under some RNAi conditions, the feeding behaviors were altered by decreasing the frequency at photophase while increasing it at scotophase (Figure 6b). For example, RNAi treatments specific to *TIM* significantly altered the feeding behavior in both developmental stages: larvae (F = 27.07; df = 1, 106;

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P < 0.0001) and adults (*F* = 26.45; df = 1, 107; *P* < 0.0001). In *PER* RNAi, the altered behavior was observed in adults (*F* = 13.66; df = 1, 106; *P* = 0.0003), but not in larvae (*F* = 0.46; df = 1, 106; *P* = 0.5007). In *DBT* RNAi, neither of the stages exhibited behavioral changes: larvae (*F* = 3.02; df = 1, 106; *P* = 0.9902) and adults (*F* = 0.31; df = 1, 106; *P* = 0.5776). In *CLK* RNAi, both stages showed significant changes: larvae (*F* = 7.16; df = 1, 106; *P* = 0.0086) and adults (*F* = 9.20; df = 1, 106; *P* = 0.0030).

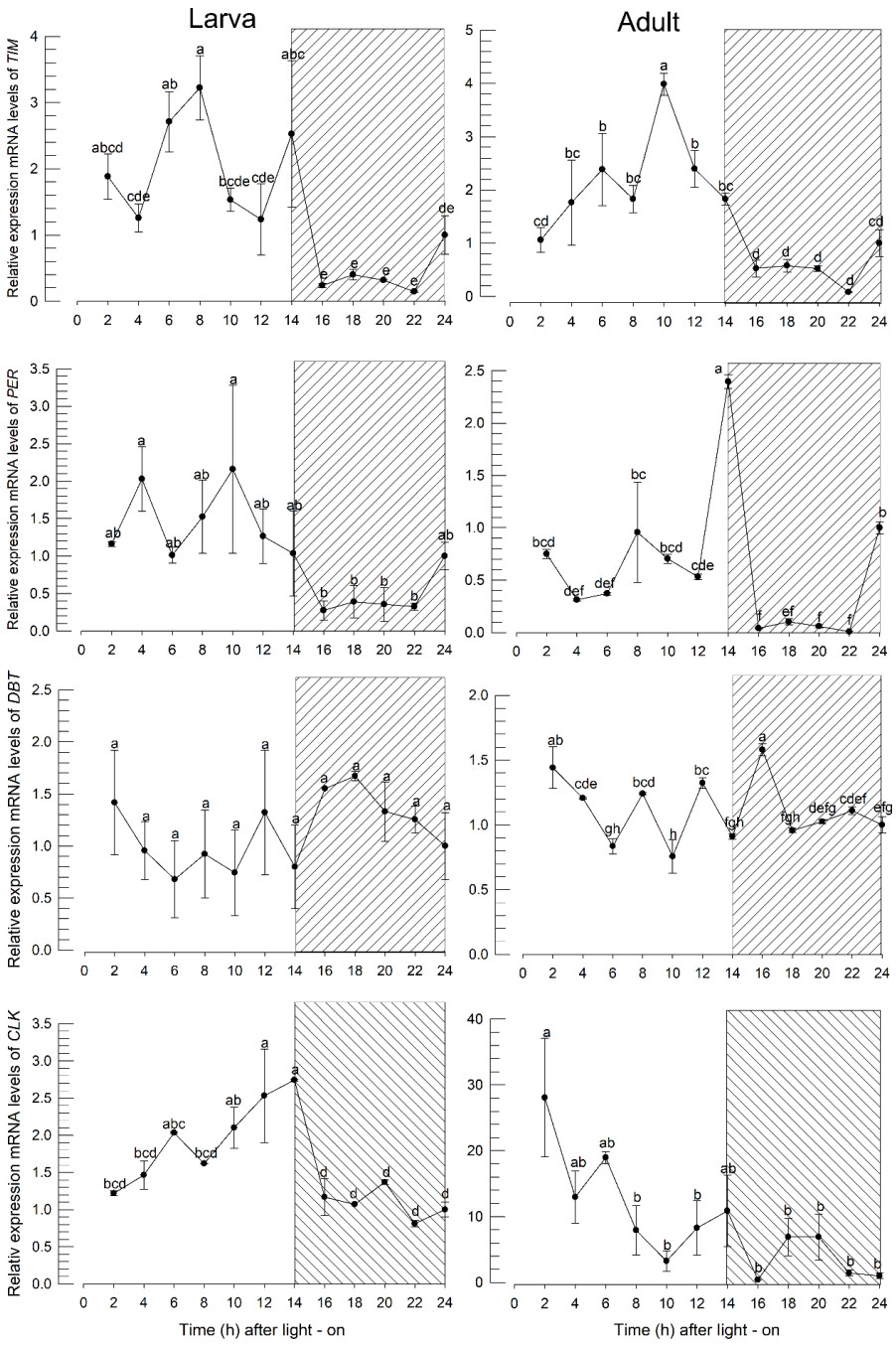

Figure 5. Expression profile of four genes associated with circadian rhythm in *F. occidentalis*: Timeless (*TIM*), Period (*PER*), Doubletime (*DBT*), and Clock (*CLK*). The experimental unit at each time point was 25 thrips to extract RNA, and each unit was independently replicated three times. The assessments were performed at every two-hour interval. The black hatch area indicates scotophase. Light:Dark cycle was 14:10 h. Temperature was 25 ± 2°C. Different letters above the standard deviation bars indicate significant differences among means at Type I error = 0.05 (LSD test).

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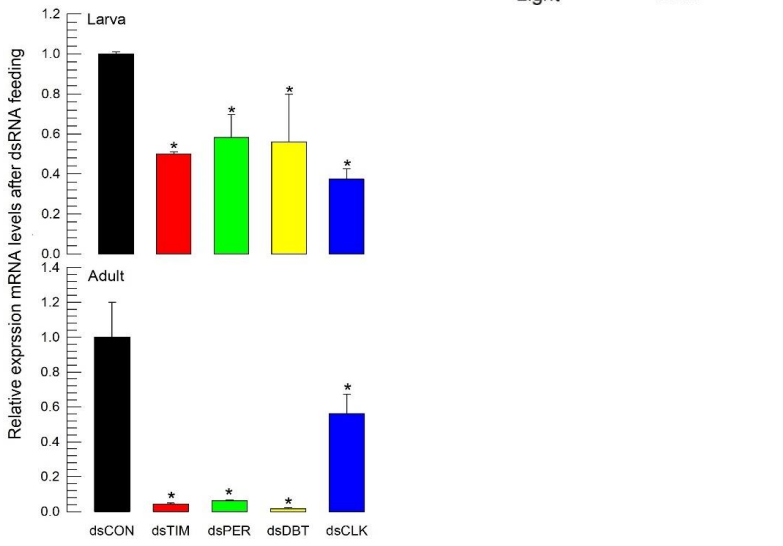
The alteration in behavior induced by RNAi treatment was also observed in adult mating behavior (Figure 6c). RNAi treatments against *TIM* and *PER* expressions significantly altered the behavior by decreasing mating behavior in photophase and increasing it in scotophase compared to control. In RNAi treatment against *DBT* expression, there was no significant difference in the behavior compared to control. In RNAi treatment against *CLK* expression, there was a significant difference in photophase, but not in scotophase.



(a) (c)

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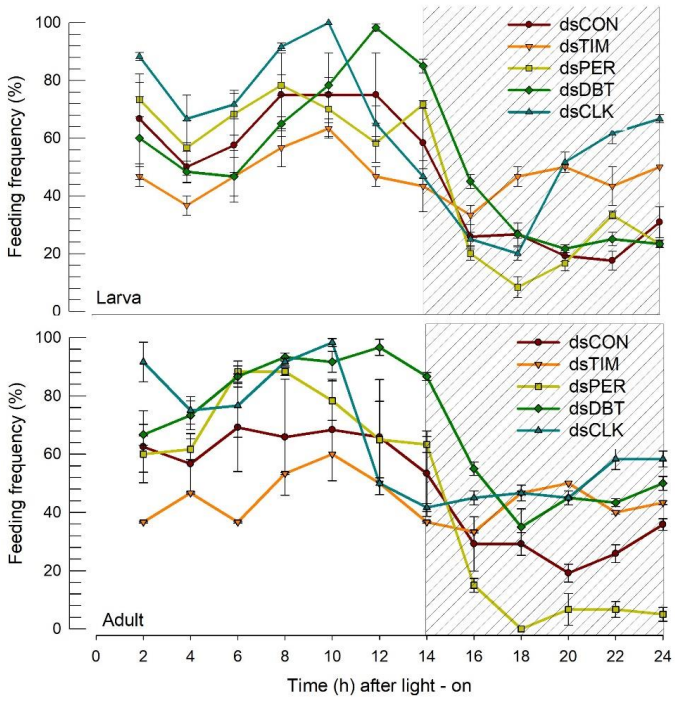

(b)

Figure 6. Alterations of feeding and mating behavior by RNA interference (RNAi) of four circadian clock genes in *F. occidentalis*: Timeless (*TIM*), Period (*PER*), Doubletime (*DBT*), and Clock (*CLK*). RNAi was performed by feeding dsRNA (dsTIM, dsPER, dsDBT, or dsCLK) specific to each circadian gene. (a) RNAi efficiency by measuring transcript levels using RT-qPCR at 24 h after dsRNA feeding. Each measurement used 25 thrips to extract RNA for RT-qPCR and was independently replicated three times. An asterisk indicates a significant difference of means compared to control ('dsCON') at Type I error = 0.05 (LSD test). (b) Alteration in feeding rhythm after feeding dsRNA. In each time point, 20 thrips as an experimental unit were used to assess feeding behavior. Each measurement was replicated three times. (c) Alteration in mating behavior after feeding dsRNA. Mating behavior was observed at 2 pm (Light) and 2 am (Dark). Ten pairs were observed at each time point and replicated three times. Different letters above the standard deviation bars indicate significant differences among means at Type I error = 0.05 (LSD test) in each light and dark condition.

4. Discussion

The feeding and mating behaviors of *F. occidentalis* followed a typical diurnal rhythmicity. Both larvae and adults exhibit similar diurnal feeding behavior. Diurnal behavior has been reported in other thrips such as *Thrips hawaiiensis*, *Scirtothrips dorsalis*, *Frankliniella schultzei*, and *Megalurothrips usitatus* infesting Mango, in which a sequential sampling at an interval of every two hours indicates the most abundant collection at midday [25]. These suggest that the feeding and mating behaviors are under the control of circadian rhythm.

Circadian behavioral rhythms are conserved in virtually all organisms, including plants [26], photosynthetic bacteria [27], *Neurospora* [28], *Drosophila* [29], and mice [30]. In particular, at a molecular level, *Drosophila* demonstrates the negative feedback regulation of transcription as a central theme of circadian rhythms [31]. The first Drosophila clock component that was identified was the *PER* gene [32]. The second essential pacemaker component that was identified was *TIM*, and both *PER* and *TIM* genes reciprocally autoregulate their transcriptions, where *TIM* and *PER* dimerization is crucial for the posttranscriptional regulation and nuclear entry of both proteins [33,34]. By contrast, both *CLK* and *CYC* belong to the basic helix-loop-helix (bHLH)–PAS (Per-Arnt-Sim) transcription

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factor family, the members of which are involved in a wide range of other life processes [35]. *Drosophila* mutants in *CLK* and *CYC* genes exhibit a retardation in the rate of transcription of the two major clock components, *PER* and *TIM* [36]. Further, *CLK* and *CYC* were found to bind the promoter containing the 21-bp E-box-containing sequence of *PER* gene [37]. CLK-CYC dimer was also found to activate transcription from promoters containing four copies of the 18-bp E-box-containing sequence from both the *PER* and *TIM* promoters in *Drosophila* [38]. Moreover, two phosphorylation sites located in a putative nuclear localization signal on *Drosophila* PER protein are phosphorylated by DBT, and these regulate the daily changes in PER stability [39]. In our current study, these four circadian components were identified from the *F. occidentalis* genome and expressed in different development stages.

The light signal triggered the feeding and mating behaviors and induced the expression of *TIM* and *PER* genes in *F. occidentalis*. For mating analysis, we used unmated male and female adults to avoid any biased behavior due to previous mating [40]. Both male and female adults were attracted to the virgin males or females. Even though males release attractants to males and females for mating [41], a recent study demonstrated that females also produce and release the aggregation pheromone in *F. occidentalis* [42]. The light cycle is the most powerful Zeitgeber entraining the circadian clock in most organisms. Insects use CRY and/or the compound eye for the light perception necessary for photic entrainment [43]. The molecular mechanism underlying CRY-dependent entrainment is currently well understood. In the cricket *Gryllus bimaculatus*, RNAi of *TIM* gene expression in the photic entrainment mechanism disrupted the entrainment to light-dark cycles, as TIM protein levels showed daily cycling characterized by an increase during the night and reduction with exposure to light [44]. In our current study, the increase of *TIM* and *PER* expression of *F. occidentalis* during photophase is explained by their autoregulation through the induction of their gene expressions by the derepressed CYC-CLK dimer in the nucleus based on the circadian clock of *Drosophila* model [45]. Whittaker and Kirk [46] also reported the increase of the thrips' behavior with increase of daylength, but they observed the significant decrease in 24-h constant light condition. This suggests the light-dark Zeitgeber entraining in the diel rhythmicity of the thrips.

Through oral administration via diet, specific dsRNAs against the four genes associated with circadian rhythm effectively suppressed their specific target genes. This led to alterations of feeding behavior when *TIM* or *PER* genes were suppressed by their specific RNAi treatments. Specific suppression of target genes through RNAi offers an efficient strategy with which to address the target gene function associated with circadian rhythm. Indeed, the dietary RNAi has been reported to be effective against *F. occidentalis* [47]. In our current study, the time shifting of the mating behavior by RNAi treatments may lead to a mismatch in locating the opposite sex and thus preventing the reproduction of diploid female progeny. Thus, the RNAi technique via diet feeding, especially of bean leaf, allows for novel tactics of *F. occidentalis* for sustainable agriculture, because it allows for greater specificity than chemical insecticides [48]. The ingestion of dsRNA can be costly because effective RNAi to kill the thrips requires continuous and repeated exposures with large amounts of dsRNA in this kind of environmental RNAi [49].

Altogether, the feeding and mating behaviors of *F. occidentalis* exhibit a typical diel rhythm, which is active at photophase. This diel rhythm is associated with PER and TIM gene expressions because their RNAi treatments altered the diel pattern. These results support the circadian control of the feeding and mating behaviors in *F. occidentalis*.

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Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: PCR primers used for this study. Table S2: Circadian thythmicity analysis of four genes of *F. occidentalis* using JTK_CYCLE algorithm.

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