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Circadian control of permethrin-resistance in the mosquito *Aedes aegypti*

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ABSTRACT

Daily fluctuation of permethrin-resistance was found in adult mosquito *Aedes aegypti*, the major vector of dengue viruses in Taiwan. We hypothesized there is a relationship between resistance and the circadian clock. To test our hypothesis we correlated changes in the knock-down time (KT_{50}) response to permethrin with the expression of the pyrethroid-resistant gene *CYP9M9* and the clock gene *period* (*per*) during a 12:12 h photoperiodic cycle. Rhythmic expression of *per* peaked at early scotophase of the light–dark cycle and at early subjective night in constant darkness. The values of KT_{50} and the expression of *CYP9M9* also exhibited circadian rhythms in both susceptible and permethrin-resistant mosquito strains, from which we inferred a link to the circadian clock. The KT_{50} was significantly longer in the light than in the dark phase, and the level of *CYP9M9* mRNA was maximal in early scotophase, dropped to a minimum in the midnight and then slowly increased through the photophase. Existence of a clock control over mosquito sensitivity to permethrin was further indicated by reduced expression of *CYP9M9* and reduced mosquito resistance to permethrin after temporal silencing of the *per* gene. These data provide the first evidence on the circadian control of insect resistance to permethrin.

1. Introduction

Insects and many other organisms exhibit circadian rhythms that increase their fitness (Enright, 1970). This synchronization of crucial life processes with environmental changes such as alternation of day and night is driven by endogenous circadian clocks (Pittendrigh, 1981). The circadian clocks control behavior and physiology, for example locomotion activity, feeding behavior, hormone release, and metabolism (Hardin, 2005). Studies of the molecular mechanism of the clock gear in the mammals and insects revealed presence of homologous clock genes including *period* (*per*), *timeless* (*tim*), *clock* (*clk*), *cycle* (*cyc*), and others (Hall, 2003). Basic parts of the clockwork in the fruit fly *Drosophila melanogaster* involve CLK and CYC proteins that activate transcription of the *per* and *tim* genes. Their protein products PER and TIM accumulate in the cell nuclei, where PER represses the CLK/CYC activator, leading to the suppression of *per* and *tim* transcription. The following decline of PER and TIM allows reactivation of CLK/CYC and the feedback cycle begins again (Hardin, 2005).

Several studies reported that some insects and mites display circadian rhythm of susceptibility to toxic agents (Beck, 1963; Cole and Adkisson, 1964; Polcik et al., 1964). Results of microarray studies also suggested that circadian expression of genes is likely to be involved in the detoxification processes induced by oxidative stress in *D. melanogaster* (McDonald and Rosbash, 2001; Claridge-Chang et al., 2001; Fernanda-Ceriani et al., 2002). Recently, Krishnan et al. (2008) and Simonetta et al. (2008) demonstrated circadian regulation of the response to oxidative stress in *D. melanogaster* and the nematode *Caenorhabditis elegans*, respectively. These findings suggested that the ability to detoxify pesticides was under circadian control.

Mosquitoes are the most important vectors of infectious diseases transmitted by arthropods (Hubálek, 2008; Vasilakis and Weaver, 2008), and the mosquito *Aedes aegypti* (L.) is the main carrier of dengue fever in Taiwan (Teng et al., 2007; Kan et al., 2008). Pyrethroids are often used to control this vector and thereby suppress epidemics. The heavy use of permethrin causes outbreaks of the resistant strain of the mosquito (Lin et al., 2003). Since pyrethroids can induce oxidative stress (Vontas et al., 2001) that may be subject to circadian regulation (Krishnan et al., 2008), we hypothesized a link between the circadian clock and permethrin-resistance through the detoxification gene *CYP9M9* which belongs to cytochrome P450 family and it shows cyclic expression in *Drosophila* (McDonald and Rosbash, 2001; Poupardin et al., 2008).

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Table 1
Sequences of primers used in this experiment.

Primer name	Target gene	Sequence (from 5′ to 3′)	PCR product (bp)	Accession number
For RT-PCR				
Ae-actin-1-F	*Actin*	ACTCGCCAGCCATGTACGTC	700	AAEL001673
Ae-actin-1-R		ACAGGGAGGCCAGGATGGAG		
Aeper1-F	*Period*	GTAAGGCGCTTGCTTCATTC	435	AAEL008141
Aeper1-R		GTTGATAGCTGCCCGAAGAG		
AeCYP2-F	*CYP9M9*	ATGATCCGGACAACGAGTTC	503	AAEL001807
AeCYP2-R		TTGCTGCATCGATTCGTAAG		
For RNAi				
T7 Aeper1-F		TAATACGACTCACTATAGGGGTAAGGCGCTTGCTTCATTC	475	
T7 Aeper1-R		TAATACGACTCACTATAGGGGTTGATAGCTGCCCGAAGAG		
T7 EGFP-F		TAATACGACTCACTATAGGGTTGCATGCCTGCAGGTCGACT	600	
T7 EGFP-R		TAATACGACTCACTATAGGGTGGCGGATCTTGAAGTTCACC		

In this paper we report on the outcomes of experiments designed to test our hypothesis.

2. Materials and methods

2.1. Susceptibility to permethrin

"Susceptible" strain of mosquito *Ae. aegypti* was obtained from the Taiwan Center for Disease Control, Department of Health, Executive Yuan. The strain was originally collected in Tainan in 1987 and since then maintained at 25 ± 1 °C and 12 h of light–dark cycle. Adult mosquitoes were provided with a 10% sugar solution, and females were blood-fed on mice 1 week after eclosion. The larvae were maintained in water in a plastic container (23 cm × 17 cm × 9 cm) and fed with shattered fish food (Hai Feng Feeds Co., Ltd., Taiwan).

"Permethrin-resistant" strain was artificially selected in the laboratory according to the procedure of W.H.O. susceptibility test protocol (W.H.O. 1998). The median lethal concentration (LC_{50}) of the susceptible strain was used as the starting reference in the selection of the permethrin-resistant strain. To test mosquito susceptibility, filter paper (12 cm × 15 cm) was soaked with 1 ml of a permethrin solution in water and placed into a test tube (4.5 cm diameter and 12 cm height) to which 20–25 female adults (3–5 days old) were added. After 1 h, the adults were transferred to a holding tube and the mortality was recorded for 24 h. The numbers of knock-down individuals were recorded every 5 min. The knock-down time (KT) was measured according to the standard W.H.O. procedure and the data were analyzed by Probit-analysis (Finney, 1971).

Mosquito susceptibility to permethrin was recorded within 1 day with the same test kit and mosquito population. Red dim light was used to handle mosquitoes during the night phase. The concentration of permethrin (0.75%) was chosen as the standard W.H.O. test for the resistant strain, and 0.02% (=LC_{50} of susceptible strain) was used to test the susceptible strain. The survival rates were calculated for 24 h exposure and the results were analyzed by ANOVA followed by HSD comparison.

2.2. Daily fluctuation of gene expression

Total RNA was extracted from single mosquitoes by the TRIzol® reagent (Invitrogen) and the cDNA was synthesized by the SuperScript™ III Reverse Transcriptase (Invitrogen). Daily fluctuations of the expression of *per* and *CYP9M9* were measured by RT-PCR with gene specific primers (Table 1) designed by E-RNAi web application (Arziman et al., 2005). After total RNA extraction and reverse transcription, the cDNA sample was diluted 50 times. Each PCR reaction contained 1 μg of diluted cDNA, 1 μl of 10 mM forward and reverse primers and 4 μl of 5× Fast-Run™ Taq Master mix (PROTECH), and was adjusted to a final volume of 20 μl by sterile water. The PCR conditions were set as follows: 95 °C for 10 min followed by 32 cycles at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and finally 72 °C for 10 min. The PCR products were analyzed on 1.6% agarose gel by electrophoresis and mRNA expression was quantified by the GeneTools (Syngene Ltd., Cambridge, UK). The results were analyzed by ANOVA and followed by HSD comparison.

2.3. Silencing of period gene

The sequence of *per* from *Ae. aegypti* (VectorBase gene ID: AAE008141) was compared with the E-RNAi web application to design a highly efficient RNA interference construct (Arziman et al., 2005). The double-stranded RNAs of *per* and Enhanced Green Fluorescent Protein (EGFP) gene were synthesized by the MEGAscript® RNAi kit following the manufacturer's instructions (Ambion). Female mosquitoes were injected into thorax with 0.5 μl containing 0.5 μg double-stranded *per* or *EGFP* RNAs. The efficiency of RNA interference was assessed by RT-PCR 28 h after the injection. Effects of *period* silencing on the expression level of *CYP9M9* and on the KT_{50} against permethrin were evaluated. The results were analyzed by Student's *t*-test.

3. Results

3.1. Expression of the period clock gene

The clock gene *per* was expressed in a circadian rhythm (Fig. 1). The RT-PCR displayed a typical entrainment in the expression level of *per* under LD = 12:12 h (Fig. 1a). Calculations of the ratio between *per* mRNA and *actin* mRNA quantities revealed low level of *per* expression during photophase and high expression in the scotophase ($n = 3$ for each time point). This fluctuation displayed a peak at ZT 13 (ZT = Zeitgeber time) and a trough at ZT 5 with significant difference in the expression level ($p < 0.01$). Similar fluctuation pattern was detected under constant darkness (Fig. 1b). The results confirm circadian expression of *per* in the mosquito *Ae. aegypti*.

3.2. The existence of a rhythmic pattern of permethrin-resistance

The permethrin-resistant strain was established by selecting adult mosquitoes of the susceptible strain (LC_{50} = 196.28 ppm) that survived 24 h exposure to LC_{50} (0.02% permethrin applied to filter paper). After an 11 generation selection regime, resistance increased about 30-fold to 5897.92 ppm (LC_{50}). Since the selection pressure was kept at the same level (LC_{50} of the susceptible strain)

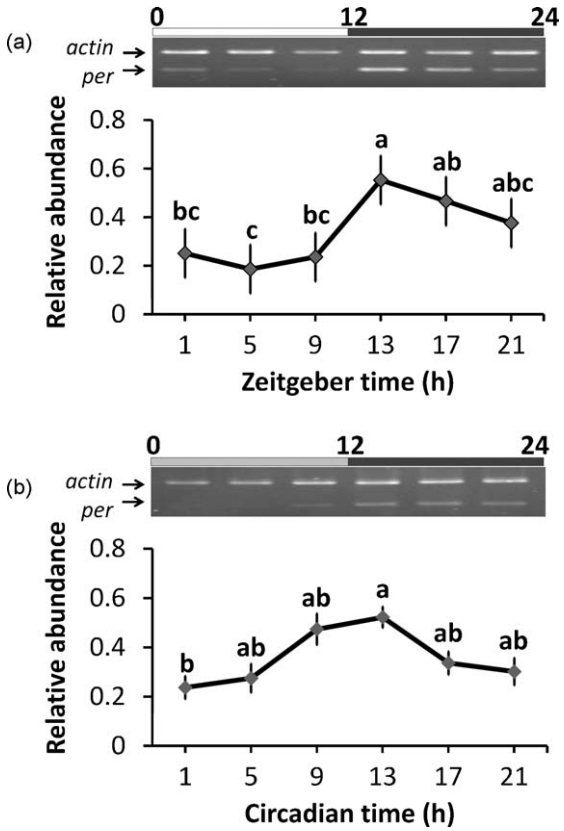

Fig. 1. Circadian expression of *period* (*per*) mRNA level in adults of the mosquito *Aedes aegypti*. (a) Daily expression of *period* gene under LD = 12:12 h and (b) constant darkness. RT-PCR products analysis by DNA electrophoresis on 1.6% agarose gels. Relative abundance of *per* mRNA expression was referred to the ratio of *per* mRNA versus *actin* mRNA expression at each time point ($n = 3$ for each time point and the vertical bars indicate standard errors). The different letters on the figure represent significant differences of the mRNA quantity (ANOVA and HSD, $p < 0.01$ for (a) and $p < 0.05$ for (b), respectively).

for each generation, the 30-fold increase of resistance could only be quantified as a median resistance (Keiding, 1986).

Differences in the median knock-down time in susceptible strain mosquitoes exposed to 0.02% permethrin at different times of day showed that the susceptibility of *Ae. aegypti* to this insecticide was subjected to circadian rhythm (Fig. 2). The mosquito showed higher susceptibility during scotophase than in the photophase; the median knock-down time was longest at ZT 9 and a consistently low during the scotophase (Fig. 2a). In constant darkness, the knock-down time was also shorter during subjective night (especially at ZT 17 and ZT 21) and longer in subjective day, with a maximum at ZT 5 and ZT 9 (Fig. 2b). This finding implied that *Ae. aegypti* increased resistance during their active state in the light phase of the photoperiod. This phenomenon was even more profound in the resistant strain (Fig. 2c).

3.3. Daily expression of CYP9M9 gene

One of the detoxification genes, *CYP9M9*, displayed daily fluctuation in the expression level with a peak at ZT 13 and a trough at ZT 17 in both susceptible and resistant strains (Fig. 3). The expression of *CYP9M9* gene was significantly higher in the permethrin-resistant strain than in the susceptible one. However, the expression levels between the peak and trough were significantly different in both resistant ($F_{5,12} = 8.0423$, $p < 0.01$) and susceptible ($F_{5,12} = 4.7192$, $p < 0.01$) strain. Daily fluctuations in the amount of detected *CYP9M9* mRNA (Fig. 3) followed similar pattern as shown for the *per* mRNA (Fig. 1a).

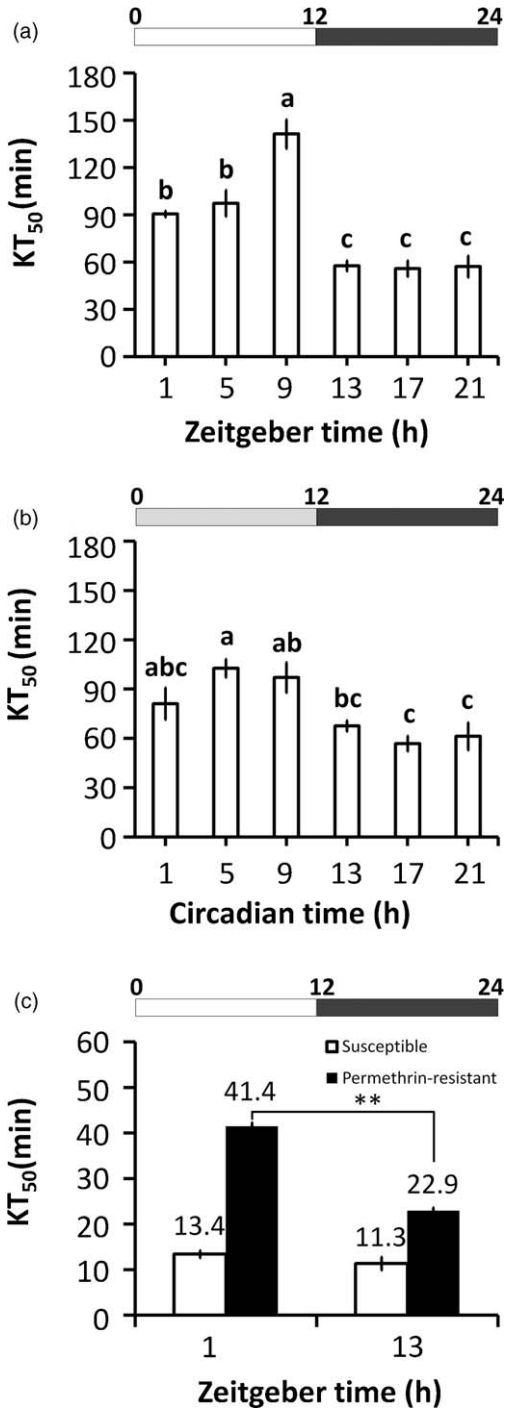

Fig. 2. Circadian change of permethrin-resistance in mosquito *Aedes aegypti*. Susceptible strain exhibited circadian rhythm in the expression of susceptibility to the LC_{50} = 196.28 ppm of permethrin under LD = 12:12 h condition (a) and constant darkness (b). (c) The susceptibility changes of susceptible and resistant strain at ZT 1 and ZT 13 against 0.75% of permethrin ($n = 20$–25 for five replicates and the vertical bars indicate standard errors). KT_{50}: median knock-down time. **Significant difference at $p < 0.001$ (Student's *t*-test).

3.4. Silencing effects of period

The injection of double-stranded *per* RNAs suppressed expression of the *per* to undetectable level within 1 day after the treatment but this silencing effect lasted 1 day only (Fig. 4). The expression level of *per* gradually recovered beginning day 2 after the injection and reached the same control level on day 4. This

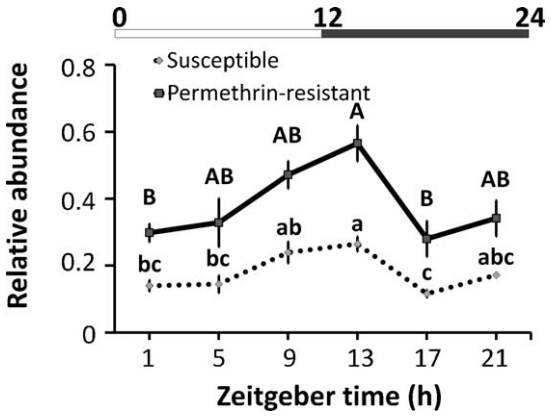

Fig. 3. Daily expression of *CYP9M9* gene in *Ae. aegypti* under LD = 12:12 h. The legend is the same as described in Fig. 1.

recovery documents an effective comparison of *per* before and after silencing effects on the same individual animal.

The subsequent effects of silencing *per* are shown in Fig. 5. Once *per* was silenced, expression of *CYP9M9* was also reduced by 2.85-fold (Fig. 5a). The overall consequence of silencing *per* expression was reflected in the significant reduction of permethrin-resistance (Fig. 5b). This finding further demonstrated the circadian regulation of permethrin-resistance. The reduction in *CYP9M9* expression and permethrin-resistant implied that *CYP9M9* might serve as underlying mechanism to permethrin-resistance in *Ae. aegypti*.

4. Discussion

The data reported in this paper strongly support our hypothesis of a link between the circadian clock and permethrin-resistance acting by way of a detoxification gene. The following points directly support our hypothesis. First, we recorded a daily rhythm in *per* expression as well as in resistance to permethrin. Second, both rhythms persisted in constant darkness. Third, we registered a daily rhythm in expression of the detoxification gene *CYP9M9*. Finally, *per* silencing led to lower expression of *CYP9M9* and reduced the resistance to permethrin. Taken together, these points make up a forceful argument in favor of our hypothesis.

Ae. aegypti is a day-active mosquito which displays diurnal locomotor behavior in a bimodal pattern as shown in *D. melanogaster* (Clopton, 1984; Gentile et al., 2006). *Ae. aegypti*'s circadian expression of *per* is similar to that of fruit fly *D. melanogaster* (Hardin et al., 1990), from which we infer an endogenous circadian clock underlying this rhythmic behavior. Since *per* controls the circadian rhythm of locomotion and feeding in *D. melanogaster* (Meunier et al., 2007), it may serve the same functions in *Ae. aegypti*.

Once we established a link between circadian clock and permethrin-resistance, our next goal was to discover a non-clock

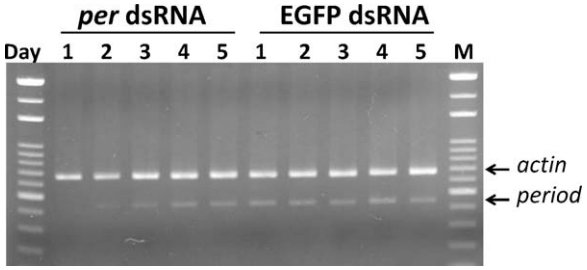

Fig. 4. The expression level of *per* mRNA at ZT 13 in the adult *Ae. aegypti* after injection of double-stranded *per* or EGFP RNAs. Arrowheads depict RT-PCR products of *per* and *actin*. The *actin* gene was used as internal control. EGFP dsRNA was injected as the control. M: DNA marker.

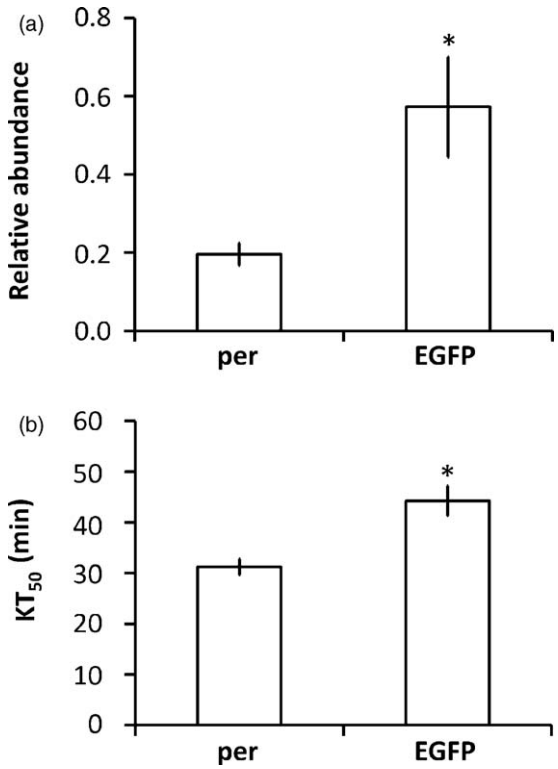

Fig. 5. The silencing effects of a clock gene *period* on (a) the expression of detoxification gene *CYP9M9* and (b) the susceptibility against permethrin at ZT 13 in resistant strain mosquito (n = 20–25 for three replicates and the vertical bars indicate standard errors). The legend was the same as described in Figs. 1 and 2. *Significant difference at $p < 0.05$ (Student's t-test).

gene down-regulated by *per*. Because CYP450 acts in mosquito pyrethroid resistance (Fogleman et al., 1998; David et al., 2005; Poupardin et al., 2008), we posed the hypothesis that *per* influences the circadian rhythm of permethrin-resistance through one or more P450s. Microarray analysis of *Ae. aegypti* detoxification genes revealed three cytochrome P450 genes including, *CYP9M9* (Poupardin et al., 2008). P450s displayed cyclic expression in fruit fly (McDonald and Rosbash, 2001). Expression of *Ae. aegypti CYP9M9* is synchronized with *per* and it is responsible for detoxifying permethrin. In addition, elevated levels of CYP450 gene expression associated with pyrethroid resistance occur in many pest insect species (Carino et al., 1994; Tomita and Scott, 1995; Liu and Scott, 1998; Kasai and Scott, 2000; Pridgeon et al., 2003; Sasabe et al., 2004; Bautista et al., 2009). Two gene silencing studies implicate cytochrome P450 enzymes in permethrin-resistance (Lycett et al., 2006; Bautista et al., 2009). In mammals, many enzymes involved in detoxification, including cytochrome P450s, are controlled by circadian clock (Gachon et al., 2006; Murakami et al., 2008). Taken with our data, these reports strongly support our hypothesis that *CYP9M9* is a *per*-regulated gene that acts in pyrethroid resistance in *Ae. aegypti* (Poupardin et al., 2008).

The injection of double-stranded RNAs is effectively demonstrating the circadian control of permethrin-resistance through *CYP9M9* in *Ae. aegypti*. Although the silencing effect of RNAi may last for different time periods depending on species (Moriyama et al., 2008; Kotwica et al., 2009) or particular genes (Fig. 4; Lee et al., 2009), a short time recovery provides a fast finding of the function of the target gene. One of the reasons to explain this time difference in silencing effect of RNAi is possibly that anti-sense of *per* has other important functions in insect such as immune response (Crosthwaite, 2004). Several findings have indicated immune responses are up-regulated with circadian clock (McDonald and Rosbash, 2001; Hardeland et al., 2003; Lee and Edery, 2008).

An effective strategy to manage *Ae. aeypti* populations is to select a time that synchronizes mosquito locomotion and feeding activity with insecticide treatments. Although there have been several attempts to determine whether susceptibility to insecticide may be influenced by the time of exposure (Beck, 1963; Cole and Adkisson, 1964; Polcik et al., 1964; Shipp and Otton, 1976; Pszczolkowski and Dobrowolski, 1999), we have seen no concrete finding until recently (Hooven et al., 2009). Two major points in this paper, the circadian rhythm of *per* expression and of permethrin-resistance, demonstrate a close connection between a clock gene and pesticide resistance. These points, taken with our finding that silencing *per* reduces resistance, support our hypothesis of a link between the central circadian clock and permethrin-resistance in *Ae. aegypti*. This study highlights the potential of "chronotoxicology" to improve the effectiveness of chemical pest management programs.

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