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EFFECT OF PHOTOPERIOD ON PERMETHRIN RESISTANCE IN *AEDES AEGYPTI*

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ABSTRACT. Living organisms have been exposed to light–dark cycles that allowed them to adapt to different ecological niches. Circadian cycles affect hormone release, metabolism, and response to xenobiotic compounds. Current studies have shown that insect susceptibility to toxic agents depends on circadian cycles, mainly because the biochemical processes involved in detoxification and responses to oxidative stress are modulated by this process. The goal of this study was to determine the effect of photoperiod on resistance to permethrin in *Aedes aegypti*. Collections of *Ae. aegypti* from 4 locations in Yucatan, southern Mexico, were subjected to 2 different photoperiod schemes: dark (0 h light:24 h dark) and natural photoperiod (12 h light:12 h dark). The comparison of both photoperiods was evaluated with respect to permethrin resistance using bottle bioassays and by monitoring the possible mechanism related such as enzymatic activity and by the frequency of 2 knockdown resistance mutations in the voltage-dependent sodium channel gene (V1016I and F1534C). The susceptible strain was used as a reference. The mosquitoes in dark photoperiod showed a reduction in resistance to the pyrethroid. The α-esterases and glutathione S-transferase enzymatic activities showed lower levels in the dark photoperiod, and the frequencies of V1016I knockdown resistance mutation showed significant difference between photoperiod schemes.

KEY WORDS *Aedes aegypti*, detoxifying enzymes, *kdr*, I1016, C1534, permethrin, photoperiod

INTRODUCTION

Most living organisms, from bacteria to animals, are under the influence of the circadian rhythms. These are responsible for several mechanisms such as control of blood pressure, body temperature, hormone levels, and number of immune cells in the blood in animals and other systems in the organisms (Ishida et al. 1999). The rhythm is entrained by the environment, for example, through light stimuli (Schibler 2007). In several organisms, circadian rhythms are maintained through autoregulatory feedback loops in a central oscillator that are well conserved between insects and mammals (Giebultowicz 2001, Stanewsky 2003, Tobback et al. 2011).

The molecular machinery of the circadian clock has been extensively studied in the fruit fly, *Drosophila melanogaster* Meigen. There are several clock genes involved in the generation of the rhythmicity (Hardin 2006, Sandrelli et al. 2008). One major loop is that formed by the genes period (*per*), timeless (*tim*), Clock (*Clk*), and cycle (*cyc*) (Stanewsky 2002, Hardin 2006). Circadian genes have been cloned in species closely related to *D. melanogaster*, such as *Anopheles gambiae* Giles (Holt et al. 2002) and *Aedes aegypti* (L.) (Nene et al. 2007).

Aedes aegypti is considered an urban mosquito and shows endophilic and anthropophilic behavior (Meireles-Filho and Kyriacou 2013), and it is the principal urban vector of dengue, chikungunya, Zika, and yellow fever viruses. Circadian rhythms in mosquitoes have been extensively studied, both in the wild and under laboratory conditions. Mosquitoes show a remarkable variation in locomotor activity behavior that can be affected by several factors such as nutrition and mating status, temperature, and light intensity (Clements 1999). The study of the circadian rhythms of arthropod vectors is of epidemiological relevance and important for disease control (Meireles-Filho and Kyriacou 2013).

Several studies have reported that some insects and mites display a circadian rhythm for susceptibility to toxic agents (Beck 1963, Cole and Adkisson 1964, Polcik et al. 1964). There is evidence that the effects of organophosphate, organochlorine, and pyrethroid pesticides on various pest insect species vary with the time of day during which they are applied (Sullivan et al. 1970, Eesa et al. 1995, Pszczolkowski and Dobrowolski 1999).

Although expression levels of detoxifying enzymes seem to remain constant until induced, microarray studies in several model species suggest that several xenobiotic metabolizing genes are expressed in daily rhythms such as glutathione S-transferase (GST) genes and cytochrome P450 superfamily of genes (Ptitsyn et al. 2011). Extensive studies of circadian gene expression reveal rhythms in the expression of multiple genes involved in the

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toxicological response of flies (Wijnen and Young 2006) and mammals (Yan et al. 2008).

Yang et al. (2010) showed that the knockdown time (KT_{50}) in a selected strain population of *Ae. aegypti* was significantly longer in the light than in the dark phase, and that the mRNA level of *CYP9M9* (detoxification gene) was maximal in early scotophase, dropped to a minimum at midnight, and then slowly increased during the photophase. The 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.

Pyrethroids are the most common insecticides used for vector control. Permethrin was used in Mexico for >10 consecutive years (1999–2010) (Flores et al. 2013). Evidence of resistance to permethrin in *Ae. aegypti* populations in Mexico due to enzymatic mechanisms and knockdown-resistant (*kdr*) mutations in the voltage-gated sodium channel *para* gene have been reported (Flores et al. 2005, 2006, 2009, 2013; Saavedra-Rodríguez et al. 2007; Ponce et al. 2009; Siller et al. 2011; Aponte et al. 2013). Considering that this insecticide can induce oxidative stress (Vontas et al. 2001) and may be subject to circadian regulation (Krishnan et al. 2008), the objective of this study was to determine the effect of photoperiod on permethrin resistance and the mechanisms involved in *Ae. aegypti*.

MATERIALS AND METHODS

Mosquitoes

Aedes aegypti field populations were collected in 2012 from Yucatan State in southern Mexico; the localities sampled were: Vergel, Motul, Vergel III (20°57′08″N, 89°34′93″W), and Uman (20°53′77″N, 89°44′07″W). The New Orleans (NO) strain was used as a susceptible reference strain.

Laboratory colonies were established from larvae collected from natural breeding sites and maintained at 25 ± 4°C and 12:12 h light–dark (L:D) photoperiod. Pupae were placed in 250-ml flasks in cages (30 × 30 cm) until the adults emerged (parental generation). Sexing was performed by observing antennas having long hairs abundant in males unlike females. The male mosquitoes were fed with 10% sugar solution and the females on rats (*Rattus norvegicus* (Berkenhout)) as a source of blood. The females and males were placed in the same cage for mating and the production of eggs. These eggs corresponded to the F_1 generation, which were used in the light treatments and all bioassays. F_1 eggs were placed in plastic containers with dechlorinated water along with powdered liver protein as a food source provided daily for the subsequent larval stage. One group of adults was maintained in 12:12 h L:D photoperiod and another group to a complete dark period (0:24 h L:D), where cages were covered with a black voile fabric for the scotophase. All strains were established and maintained at 25 ± 2°C and 70 ± 2% RH.

Bioassays

Nonbloodfed F_1 females from field populations and susceptible NO strain (1–3 days old) were used in the bottle bioassays (Brogdon and McAllister 1998), in which a 250-ml Wheaton® bottle contained 1 ml of an acetone solution of technical-grade insecticide (ChemService, West Chester, PA). The bottle was capped and shaken to ensure uniform coverage and dried for an hour at room temperature. The insecticide tested was permethrin (40.1% *cis*–58.7% *trans*). Doses of AI (μg/bottle) were predetermined to obtain a range of knockdown and 24-h mortality rates from 0% to 99%. The number of different doses tested varied from 5 to 8 μg/bottle, with 3 repetitions per dose and 20 females per repetition.

The numbers of knocked-down mosquitoes were recorded every 10 min up to 1 h. After 1 h of exposure, all mosquitoes were gently transferred to recovery containers without insecticide and were offered a cotton ball soaked with a sugar solution. Mortality was recorded at 24 h. A dim red light (Omegon 33137; Nimax, Landsberg, Germany) was used to handle mosquitoes during the dark phase. Both bottles and recovery containers were kept at 24 ± 2°C and 70% RH.

We determined the concentration causing 50% knockdown (KC_{50}) following 1 h of exposure. The concentration causing 50% mortality (LC_{50}) was estimated 24 h after exposure to permethrin. The knockdown and lethal times (KT_{50} and LT_{50}) were also determined with the data obtained at KC_{50} and LC_{50} for each population/condition. The values of all parameters (KC_{50}, LC_{50}, KT_{50}, and LT_{50}) with 95% confidence limits were calculated using a quick calculator software program (http://sourceforge.net/projects/irmaproj/files/) with logistic regression.

Enzyme assays

Sixty females from each population in each photoperiod treatment and NO reference strain were individually homogenized in 100 μl of 0.01 M potassium phosphate buffer, pH 7.2, and the homogenate resuspended in 2 ml of the same buffer. Next, 100 μl were transferred to microtiter plates; each sample was analyzed in triplicate on each plate. We then quantified the activities of α- and β-esterases, mixed-function oxidases (MFOs), and glutathione S-transferases (GSTs) according to the methods proposed by Brogdon (1989), Brogdon and Barber (1990), and Brogdon et al. (1997). Absorbance was measured using a UVM-340 microplate reader (ASYS Hitech GmbH, Eugendorf, Austria), and triplicate values were averaged. Protein concentration was determined using the method proposed by Brogdon (1984), and in some cases, it was necessary

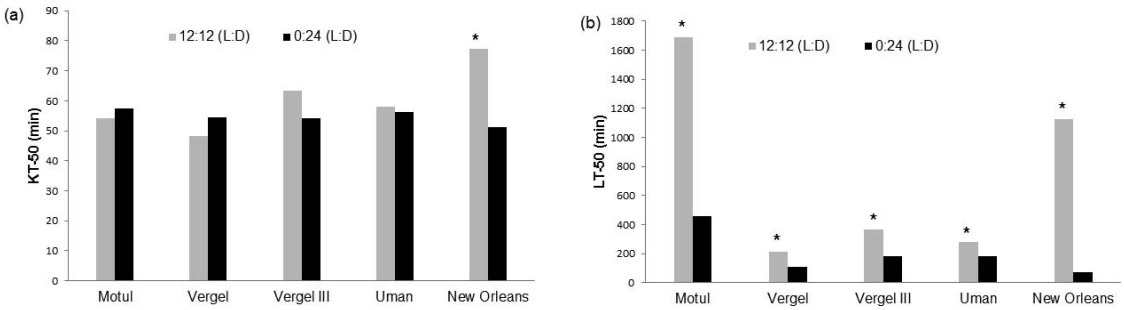

Fig. 1. Photoperiod change and permethrin resistance in *Aedes aegypti*. (a) The median knockdown time was significantly longer in the 12:12 h photoperiod only in the susceptible New Orleans strain. (b) Differences in the median lethal time in all mosquito strains showed that the susceptibility of *Ae. aegypti* was subject to change of photoperiod.

to dilute the homogenates because of variation in the size of the mosquitoes.

The data from each enzyme assay were analyzed by the nonparametric Kruskal–Wallis test ($P < 0.05$), comparing the mean activity between the populations in each treatment.

DNA isolation and genotype determination

DNA was isolated from each mosquito by the salting-out technique (Coen et al. 1982) and suspended in 50 µl of buffer (10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid [EDTA, pH 8.0]) and stored at –70°C. Genotypes at the I1016 locus were determined in a single-tube polymerase chain reaction (PCR) using the 2 different "allele-specific" primers and the reverse primer (Saavedra-Rodríguez et al. 2007) (Invitrogen, Carlsbad, CA). Products were subjected to agarose gel electrophoresis in a 1.5% (w/v) UltraPure™ agarose (Invitrogen) gel poured with Tris borate–EDTA (89 mM Tris-borate and 2 mM EDTA, pH 8.3). The DNA fragments were fractionated by electrophoresis for 30 min at 90 V alongside a 25-bp DNA ladder (Invitrogen).

For the F1534C mutation, allele-specific PCR was carried out in an Eco™ real-time thermal cycler (Illumina, San Diego, CA), to determine the genotypes by analysis of the denaturation curves following the protocols described by Yanola et al. (2011).

We included 3 controls for each PCR plate: a homozygous susceptible control (V1016/V1016 susceptible NO strain) and a homozygous resistant strain (I1016/I1016 resistant strain from Isla Mujeres, Mexico). For the F1534C mutation, we used a homozygous susceptible control (F1534/F1534 susceptible NO strain) and homozygous resistant strain (C1534/C1534 resistant strain).

The genotypic frequencies for the 1016 and 1534 loci were calculated by dividing the number of individuals with a given genotype by the total number of mosquitoes analyzed. Frequencies of field populations, as well as those subjected to 12:12 h or 0:24 h photoperiod, were compared.

RESULTS

KC_{50}, LC_{50}

Overall, slightly higher values of KC_{50} were obtained with the 12:12 h photoperiod compared with 0:24 h for the populations tested including the NO strain. The KC_{50} values for the populations of Motul and Vergel, under a 12:12 h photoperiod, were 0.44 and 0.53 µg/bottle, respectively. The values obtained when females were exposed to a 0:24 h photoperiod were 0.51 and 0.67 µg/bottle, respectively, but there was an overlap in the confidence intervals, indicating no significant difference between the KC_{50} values between the 2 photoperiods. For Vergel III and Uman, KC_{50} values for the individuals with a 12:12 h photoperiod were 11.99 and 13.75 µg/bottle, respectively, which were significantly higher than the KC_{50} values obtained with a 0:24 h photoperiod (9.35 and 10.50 µg/bottle, respectively). For the susceptible NO strain, the KC_{50} under a 12:12 h photoperiod was significantly higher (0.47 µg/bottle), compared with the KC_{50} of 0.22 µg/bottle obtained with 0:24 h (Table 1).

The LC_{50} values under a 12:12 h photoperiod for all populations tended to be higher than those obtained with 0:24 h. However, the differences were significant for Vergel, Uman, and the susceptible NO strain. The LC_{50} values in a 12:12 h photoperiod (0.59, 12.77, and 0.29 µg/bottle, respectively) were significantly higher than the values obtained with a 0:24 h photoperiod (0.16, 7.9, and 0.18 µg/bottle, respectively) (Table 1).

The median knockdown time was significantly longer in the 12:12 h photoperiod only in the susceptible NO strain (Fig. 1a). Differences in median lethal time in all mosquito strains showed that the susceptibility of *Ae. aegypti* was subject to circadian rhythm (Fig. 1b). This finding implied that *Ae. aegypti* was more resistant (based on LT_{50}) during their active state in the 12:12 h photoperiod.

Mean values for α- and β-esterases, MFOs, and GST of Motul, Vergel, Vergel III, Uman, and NO are shown in Table 2. The results indicated that α-esterase and GST activities were significantly higher

Table 1. Knockdown concentrations (KC_{50}) and lethal concentrations (LC_{50}) of permethrin against *Aedes aegypti* strains at different photoperiods.[1]

	12:12 h					0:24 h				
Strain	*N*	KC_{50} (95% CI)	b ± EE	LC_{50} (95% CI)	b ± EE	*N*	KC_{50} (95% CI)	b ± EE	LC_{50} (95% CI)	b ± EE
Motul	475	0.4413 (0.4086–0.4765)	2.9234 (0.2716)	0.4868 (0.3952–0.5995)	2.0928 (0.2195)	352	0.5056 (0.4673–0.5471)	3.2046 (0.3904)	0.4352 (0.3943–0.4802)	1.092 (0.2670)
Vergel	539	0.5315 (0.4492–0.6287)	1.2551 (0.1176)	0.5907 (0.4969–0.7022)	0.7999 (0.1027)	538	0.6730 (0.5798–0.7810)	1.4096 (0.1276)	0.1627 (0.1097–0.2445)	1.186 (0.1134)
Vergel III	597	11.9950 (10.5729–13.6086)	1.7052 (0.1595)	7.3497 (6.4790–8.3370)	1.2601 (0.1142)	584	9.3523 (8.3788–10.4385)	2.0385 (0.1679)	6.6366 (5.5010–8.0080)	1.689 (0.1446)
Uman	471	13.7500 (13.3778–14.1328)	7.9282 (0.8034)	12.769 (12.3914–13.158)	7.4571 (0.8834)	657	10.496 (9.824–11.213)	2.7577 (0.2621)	7.8671 (7.1320–8.6780)	2.220 (0.2212)
New Orleans	477	0.4697 (0.4278–0.5159)	2.4543 (0.2792)	0.2875 (0.2475–0.3340)	1.5442 (0.1504)	477	0.2236 (0.2079–0.240)	3.099 (0.2912)	0.1767 (0.1679–0.1859)	5.409 (0.5536)

[1] CI, confidence interval; EE, 2 slope ± SE.

Table 2. Mean absorbance corrected by protein (± SD) values from the biochemical assays carried out on the 4 strains of *Aedes aegypti* and New Orleans strain with modified photoperiod.[1]

	α-Esterases		β-Esterases		MFO		GST	
Strain	12:12 h	0:24 h	12:12 h	0:24 h	12:12 h	0:24 h	12:12 h	0:24 h
Motul	0.731* (0.095)	0.579 (0.078)	0.998* (0.140)	0.993 (0.141)	0.212 (0.035)	0.228 (0.042)	0.083* (0.038)	0.056 (0.032)
Vergel	0.647* (0.033)	0.626 (0.044)	0.915 (0.057)	0.945 (0.090)	0.188 (0.022)	0.277 (0.038)	0.058* (0.014)	0.039 (0.011)
Vergel III	0.891* (0.078)	0.834 (0.088)	1.145 (0.131)	1.253 (0.107)	0.197 (0.026)	0.206 (0.018)	0.092* (0.022)	0.075 (0.029)
Uman	0.925* (0.099)	0.913 (0.060)	1.218* (0.129)	1.169 (0.126)	0.284* (0.045)	0.248 (0.033)	0.113* (0.039)	0.074 (0.025)
New Orleans	0.725* (0.056)	0.624 (0.042)	0.973 (0.098)	1.078 (0.157)	0.198* (0.032)	0.192 (0.036)	0.042* (0.011)	0.035 (0.007)

[1] GST, glutathione S-transferase; MFO, mixed-function oxidase.
* Mean values are significantly higher ($P < 0.05$) compared with 0:24 h photoperiod.

Table 3. Genotype and allelic frequency of V1016I and F1534C of *Aedes aegypti* subjected to change in photoperiod.[1]

Strain–photoperiod	*N*	RR (I/I)	RS (I/V)	SS (V/V)	% RR alleles	% alleles I1016 (field population)	*N*	RR (C/C)	RS (C/F)	SS (F/F)	% RR	% alleles C1534 (field population)	*N*
Motul 12:12	15	7	0	8	46.7	48	25	3	5	7	36.7	60	14
Motul 0:24	20	6	5	9	42.5			6	6	6	50		
Vergel 12:12	15	1	0	14	6.7	79	50	3	6	6	40	100	30
Vergel 0:24	15	1	2	12	13.3			3	9	3	50		
Vergel III 12:12	20	20	0	0	100	75	14	20	0	0	100	54	25
Vergel III 0:24	18	7	0	11	38.9			18	0	0	100		
Uman 12:12	32	9	1	22	29.7	80	25	32	0	0	100	97	18
Uman 0:24	54	45	8	1	90.7			47	1	0	99.0		

[1] *N*, number of mosquitoes analyzed; SS, susceptible mosquitoes; RS, heterozygous; RR, homozygous mutant.

in the 12:12 h photoperiod compared with 0:24 h photoperiod in all populations tested. Table 3 shows the number of mosquitoes with each genotype and the I1016 and C1534 allele frequencies, along with 95% confidence interval for each photoperiod scheme. Overall, the proportion of RR (resistant homozygous) alleles for C1534 were higher in all populations analyzed, regardless of the photoperiod treatment (0:24 h and 12:12 h) compared with I1016. A Pearson χ^2 test to compare the frequencies of genotypes for I1016 and C1534 indicated significant differences between photoperiods in genotypes for I1016 ($P < 0.001$) but not for C1534. The proportion of the C1534 genotype found in the strains in the 2 photoperiods was similar, suggesting that this particular *kdr* mutation does not appear to be mediated by the circadian cycle. These results support our hypothesis that susceptibility is linked to the circadian clock in *Ae. aegypti*. This study shows that esterase and the mutation V1016I have changes in their expression and frequency, respectively, but these mechanisms require further studies.

DISCUSSION

A number of factors can influence the susceptibility of insects to poisonous chemicals, for example, temperature, humidity, age, sex, and weight. Superimposed upon the effects of these factors are daily changes in physiology and behavior which may also influence insecticide susceptibility levels (Bainbridge et al. 1982). Circadian rhythms of mammalian sensitivity to drugs were first recorded by Halberg in 1960 and 1970 (Halberg 1960, 1970). At different times of the 24-h cycle, mice were found to react differently to equivalent doses of ethanol. Highest susceptibility was recorded (in 12:12 h L:D) toward the end of the light period and lowest susceptibility close to midnight. A circadian susceptibility–resistance cycle to ethanol persisted in mice kept in continuous darkness for several days. The data reported in this paper in *Ae. aegypti* support our objective of a link between the photoperiod and insecticide resistance. The following points support this hypothesis: first, the results of knockdown resistance ($RRKC_{50}$) and postrecovery at 24 h ($RRLC_{50}$) obtained in our study are consistent with those reported by Yang et al. (2010), who proposed that mosquitoes are more susceptible during the dark phase of the photoperiod and show less sensitivity during the light phase, because this is considered the time of their active state. Second, enzyme data support previous studies in which the expression was found to be regulated by circadian clocks in *Ae. aegypti* (Ptitsyn et al. 2011) and for GST in *An. gambiae* (Rund et al. 2013).

The results support our hypothesis that susceptibility is linked to photoperiod effect in *Ae. aegypti*. This study shows that esterase and the mutation V1016I have changes in their expression and frequency, respectively. This study highlights the potential of chronotoxicity (Pszczolkowski and Dobrowolski 1999) to improve the effectiveness of chemical pest management programs.

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