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Locomotor activity in *Aedes aegypti* with different insecticide resistance profiles

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ABSTRACT

OBJECTIVE: To evaluate locomotor activity in four field populations of *Ae. aegypti* with different insecticide resistance profiles from the state of São Paulo for two years.

METHODS: This study comprised the susceptible Rockefeller strain and four populations from São Paulo, Brazil: two considered populations with "reduced susceptibility" to pyrethroids (Campinas and Marília), and two "resistant populations" (Santos and Ribeirão Preto). First, 2016 and 2017 eggs from these five populations were hatched in laboratory. Virgin females underwent experiments under laboratory conditions at 25°C, with 12:12h light/dark (LD) photoperiod; 24-hour individual activity was recorded using a locomotor activity monitor (LAM).

RESULTS: In females from 2016 field populations, both resistant populations showed significant more locomotor activity than the two reduced susceptibility populations and the Rockefeller strain ($p < 0.05$). As for females from 2017 field populations, reduced susceptibility populations showed a significant increased locomotor activity than the Rockefeller strain, but no significant difference when compared to Santos resistant population ($p > 0.05$).

CONCLUSIONS: Our results indicate that insecticide-resistant *Ae. aegypti* populations show increased locomotor activity, which may affect the transmission dynamics of their arboviruses.

DESCRIPTORS: *Aedes*. Insecticide Resistance. Locomotion. Disease Vectors.

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INTRODUCTION

Aedes (*Stegomyia*) *aegypti* (Linnaeus, 1762) is a mosquito highly adapted to the urban environment, often found inside human dwellings and their surroundings[1–3]. This diurnal and anthropophilic species is the primary vector of several arboviruses, such as dengue, chikungunya, and Zika – all mainly transmitted to humans through the bite of an infected female *Ae. aegypti*[3].

Among the measures adopted to control *Ae. Aegypti*, using chemical insecticides has been proposed as chemical control, eliminating or relocating breeding sites as mechanical control and applying *Bacillus thuringiensis insraelensis (Bti)* larvicide as biological control[4]. With dichloro-diphenyl trichloroethane (DDT) development in 1939 new prospects loomed regarding the species, including its eradication in Brazil and neighboring countries by 1958[5-7]. In general, all chemical insecticide classes act on mosquito's central nervous system (CNS), inducing the "knockdown effect"[8].

Besides the organochlorine class (OC), which contains DDT, chemical organophosphate (OP), carbamate (CA), and pyrethroid (PY) insecticides are also used against *Ae. aegypti* and other mosquito vectors[8,9]. Whereas OC and PY act by destabilizing the balance and passage of sodium and potassium ions though the voltage-gated sodium channel (NaV)[10], OP and CA act by phosphorylating acetylcholinesterase (AChE), preventing acetylcholine (Acetyl-COA) degradation and thus disrupting the following neuron action potential[8].

The massive use of chemical insecticides may lead different *Ae. aegypti* populations to develop resistance. The first case of DDT-resistance in *Ae. aegypti* populations was reported in Cúcuta, Colombia, in 1957[7]. Besides resistance, DDT also incur a long residual action in the environment, affecting agriculture and aquatic ecosystems, besides having a hazardous effect on human health, so that its use in the Americas declined in the early 1970s[9,11].

In Brazilian *Ae. Aegypti* populations, insecticide resistance was first observed in 1994, with reduced susceptibility to organophosphates[12]. Due to *Ae. aegypti* decreased sensitivity to PY and OP, several populations of this mosquito have been monitored in São Paulo[12], indicating high levels of PY resistance, especially in the cities of Santos and Ribeirão Preto[13,14], highly urbanized areas. In turn, *Ae. aegypti* populations from Marília and Campinas showed low levels of PY-resistance and susceptibility to OP insecticides[13,14].

The World Health Organization (WHO) classifies mosquitoes susceptibility profile according to mortality, as follows: > 98% mortality is deemed susceptible; < 98% as suspected resistance; 90–97% as suspected presence of resistant genes; and < 90% as confirmed presence of resistant genes[15]. Mosquito vectors acquire resistance to PY through the structural alteration of specific genes due to random and non-synonymous substitutions, encoding altered target proteins and reducing insecticides molecules binding[16]. Such mutations, known as "knockdown resistance" (*Kdr)*, were first detected in *Musca domestica* as a leucine-to-phenylalanine substitution (*L*1014*F*) and are passed down to new generations[16,17]. Two mutations are widespread among Brazilian *Ae. aegypti* populations: phenylalanine-to-cysteine (F1534C) and valine-to-isoleucine (V1016I) substitution – both related to the phenotypic profile of pyrethroid resistance[14,18,19].

The daily activities of mosquito vectors, such as mating, blood-feeding, flight, and oviposition, are controlled by a circadian clock[20]. *Ae. aegypti* often present a diurnal and bimodal locomotor activity, with morning and afternoon peaks[2]. However, insemination, blood-feeding[21], and dengue infection[22] may influence this species' locomotor activity and consequently its arbovirus transmission dynamics[23]. Brito et al. (2013) found *Ae. aegypti* with *Kdr* mutations to show a significant increase in locomotor activity when compared to the Rockefeller strain[24]. Considering that, studies evaluating the locomotor activity of resistant populations are necessary for providing a better understanding of the effects of insecticide resistance on *Ae. aegypti* locomotor activity.

We assess the hypothesis that insecticide-resistant *Ae. aegypti* populations present an increased locomotor activity, potentially favoring arboviruses transmission dynamics. This study sought to assess locomotor activity of four *Ae. aegypti* populations with different insecticide resistance profiles from the state of São Paulo over two years.

METHODS

Population Profiles

Aedes aegypti populations from São Paulo, Brazil, were selected by the phenotype profile of insecticide resistance. Based on bioassays tests[14] and following WHO criteria[15], populations from the municipalities of Campinas (22°54'26"S; 47°3'48"W) and Marília (22°12'50"S; 49°56'45"W) were considered as presenting "reduced susceptibility" to pyrethroid (PY), whereas populations from Santos (23°56'41"S; 46°19'49"W) and Ribeirão Preto (21°10'39"S; 47°48'37"W) were considered "resistant" (Figure 1). We also tested the Rockefeller strain (from the Centers for Disease Control and Prevention-CDC, San Juan, Puerto Rico), a laboratory reference strain susceptible to insecticides and frequently used for monitoring *Ae. aegypti* field populations biological responses[13]. For presenting a > 98% mortality to pyrethroids (susceptible), this strain enables the comparison with reduced-susceptibility and resistant populations. *Ae. aegypti* populations from 2016 and 2017 underwent experiments, except for Ribeirão Preto, which was only tested in 2016. Eggs were collected using ovitraps during Spring – the pre-epidemic season. The susceptibility/resistance status of non-blood-fed female *Ae. aegypti* (2–5 days old) was evaluated based on mortality rates obtained from bioassays using insecticide-impregnated papers, according to WHO methodology[14,15]. Insecticide dose was established as the double of 99% lethal dose obtained to a susceptible strain.

Mosquito Rearing

For hatching, eggs from each population were placed in 10 x 10 x 5 cm labeled trays containing 400 mL of tap water and 0.2 mg of fish food (Tetramin®). Larvae were kept in 30 x 20 x 5 cm labeled trays with 1 L of tap water and 20 mg of fish food. Hatching trays were kept in an incubator at 25° Celsius and 70% relative humidity with 12 hours of light and 12 hours of dark (LD 12:12). All pupae were placed in individual small containers

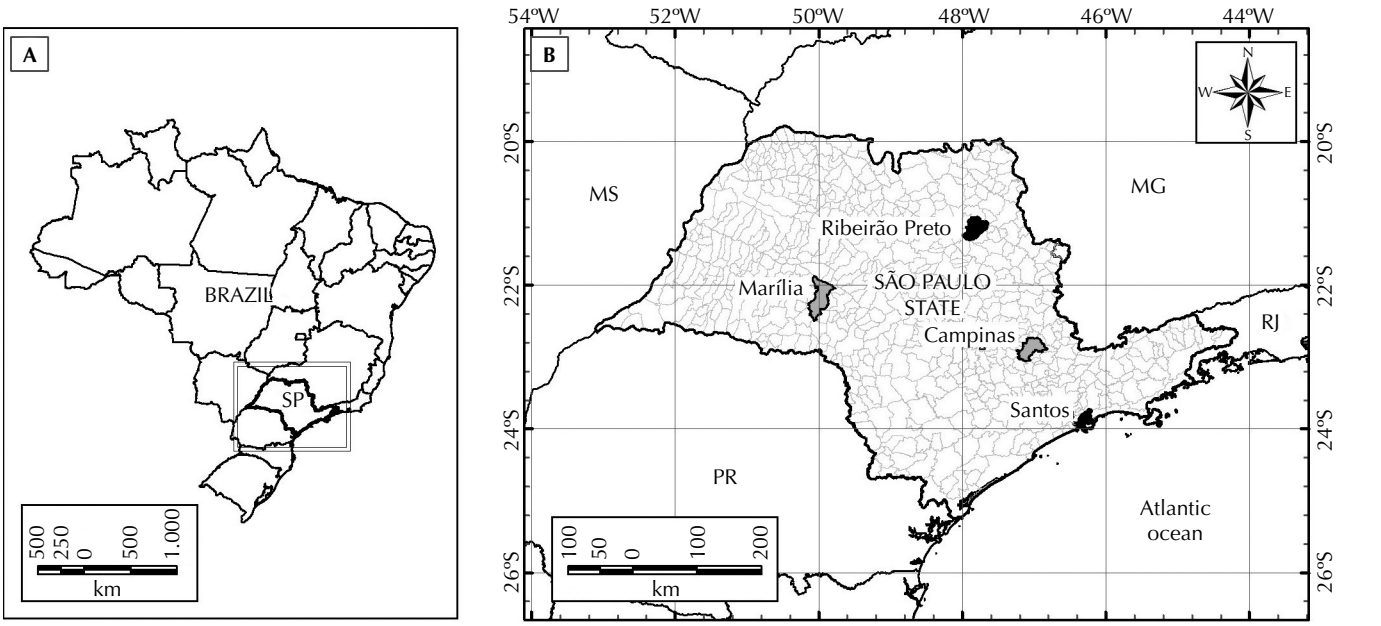

Figure 1. *Ae. aegypti* population profile according to insecticide resistance. (A) Brazil Map highlighting the state of São Paulo. (B) São Paulo municipalities with the *Ae. aegypti* populations tested. Reduced susceptibility to pyrethroids (grey – Campinas and Marília) and resistant (black – Santos and Ribeirão Preto).

with 5 mL of tap water until emergence, ensuring virgin females. Then, females from each population were transferred to labeled cages and kept in the same incubator under the same photoperiod, temperature, and humidity as the rearing phase.

Analysis of Locomotor Activity

Three-to-four-days-old females from all tested populations (Campinas, Marília, Santos, Ribeirão Preto and Rockefeller) were anesthetized in ice and then placed in individual glass tubes (1 cm x 10 cm). Adults were fed by a 10% sucrose-soaked cotton plug. Glass tubes were sealed with Parafilm® and placed in the locomotor activity monitor (TriKinects Inc, Waltham, MA, USA), which was equipped with infrared beam and detectors, counting every time mosquitoes broke the beam and measuring their activity-rest pattern[25,26]. All monitors were placed within a Precision Scientific Incubator Model 818, under LD 12:12 and constant temperature of 25ºC. In our experiments, light functioned as the *Zeitgeber* (ZT) – a Germanic term that means "time giver" or synchronizer[21]. In a LD 12:12 cycle, for example, ZT02 corresponds to 2 hours after lights are on and ZT14 to 2 hours after lights are off.

Each female's total activity was individually recorded for seven consecutive days, at 30-minute intervals, using the DAMSystem data acquisition software. At least two replicate experiments were conducted for all populations in 2016 and 2017 (except for Ribeirão Preto, which only comprised populations from 2016). Only females that survived until the sixth day of the experiment were analyzed (Table 1), and data from the second to the fifth day of the experiment were used for analysis.

William's mean (Wm) was calculated as an estimate of the central tendency activity during each 30 minute-interval[21,16]. William's mean is a modification of geometric mean related to data log-transformation to accommodate zero values by adding 1 to all data values and then subtracting 1 from the mean[27,28].

Statistical Analysis

The means of total activity (24 hours), diurnal activity without lights-on (excluding the first 30 minutes of the diurnal phase lights-on due to the "startle response effect" resulting from the dark-light transition)[21], and half-day activity (HDA; ZT06 to ZT12 interval) were calculated for each mosquito. Each mosquito mean HDA, calculated using all log (N+1) values related to every ZT06-ZT12 30-min interval, was employed in statistical analyses. We chose half-day activity for statistical analyses because *Ae. aegypti* shows

Table 1. Means of female *Ae. aegypti* locomotor activity according to insecticide resistance profile.

Year	Profile	Population	n	Mean (SD)		
				Total activity (24h)	Diurnal activity without lights-on (11.5h)	HDA (ZT06 to ZT12)
2016	Reduced	Campinas	95[b]	0.43 (0.16)	0.68 (0.26)	0.83 (0.28)
	susceptibility	Marília	104[b]	0.43 (0.19)	0.62 (0.25)	0.79 (0.26)
	Resistant	Santos	68[a]	0.48 (0.17)	0.73 (0.25)	0.91 (0.28)
		Ribeirão Preto	73[a]	0.57 (0.21)	0.83 (0.30)	0.97 (0.29)
	Susceptible	Rockefeller	75[a]	0.45 (0.16)	0.62 (0.21)	0.78 (0.23)
2017	Reduced	Campinas	96[b]	0.58 (0.20)	0.83 (0.31)	0.96 (0.33)
	susceptibility	Marília	43[a]	0.46 (0.18)	0.74 (0.29)	0.91 (0.31)
	Resistant	Santos	63[a]	0.56 (0.20)	0.86 (0.32)	1.00 (0.35)
	Susceptible	Rockefeller	72[a]	0.45 (0.16)	0.58 (0.22)	0.73 (0.24)

n: total number of individuals tested; SD: standard deviation; HDA: half-day activity.
[a] Two replicates.
[b] Three replicates.

greater activity during this time of the day[2,29]. Previous studies have also used this method to calculate the total, diurnal, and nocturnal *Ae. aegypti* locomotor activity means for statistical analysis[21,27].

After calculating mean HDA, the Kolmogorov-Smirnov test was use to assess data normality. Once normality assumptions were satisfied, the parametric independent samples T-test was used to verify differences between replicas of each population in each year (when three replicas were present, one-way ANOVA was used). Independent samples T-test was also used to compare possible differences within the same population between years (temporal variation). One-way ANOVA and Fisher's LSD *post-hoc* test compared locomotor activity between populations for the two years evaluated and among all five tested populations. Populations with reduced susceptibility or with resistant profile were not grouped for statistical analyses. All statistical analyses were performed in the software Statistical Package for the Social Science (SPSS), version 17, with $\alpha < 0.05$.

RESULTS

We assessed 542 virgin females from four *Ae. aegypti* field populations (resistant and susceptible) and 147 the Rockefeller strain (Table 1). Table 1 shows the number of females within each tested population for 2016 and 2017 and the means of total activity, diurnal activity without lights-on, and half-day activity (HDA – ZT06 to ZT12 interval). All populations presented higher means in the ZT06-ZT12 interval, when *Ae. aegypti* showed more locomotor activity.

Figure 2 shows locomotor activity of populations with reduced susceptibility, from Campinas (A) and Marília (B), resistant populations, from Santos (C) and Ribeirão Preto (only 2016) (D), and for the Rockefeller strain (E), all for 2016 and 2017, during the four days of experiment (days 2-5). All populations showed a diurnal and bimodal locomotor activity pattern, peaking at lights-on and near lights-off (Figure 2 A-E).

The 2017 populations from Campinas and Marília showed significantly increased locomotor activity during light phase when compared to 2016 populations (T-test, ZT06 to ZT12 interval; $t = -2.991$, $p = 0.003$ and $t = -2.479$, $p = 0.014$ respectively), suggesting a temporal variation (Figures 2A and B). However, we found no significant differences in the locomotor activity of Santos and Rockefeller populations between 2016 and 2017 (T-test, ZT06 to ZT12 interval; $t = -1.555$, $p = 0.122$ and $t = 1.305$, $p = 0.194$ respectively) (Figures 2C and E). As for Riberião Preto, no temporal comparison was possible as we only had data regarding 2016 (Figure 2D). The replicates of each population in 2016 and 2017 presented no significant difference (ANOVA, $p > 0.05$ and t-test, $p > 0.05$).

Table 2 shows Fisher's LSD *post-hoc* results for *Ae. aegypti* populations with reduced susceptibility, resistant populations, and Rockefeller strain. In 2016, the Rockefeller strain showed a higher second peak compared to Marilia and Campinas (Figure 3A). However, both reduced susceptibility populations showed no significant difference in ZT06-ZT12 interval means when compared to the Rockefeller strain ($p = 0.234$ and $p = 0.815$, respectively) (Table 2). We also verified no significant differences between the populations of Marilia and Campinas ($p = 0.296$) (Table 2).

Populations from Santos and Ribeirão Preto presented significantly increased locomotor activity when compared to the Rockefeller strain, especially during the anticipation period (from ZT06 to ZT12) (Figure 3B). ZT06-ZT12 interval means were significantly higher in resistant populations than in the reduced susceptibility populations from Campinas ($p = 0.044$ and $p < 0.001$, respectively) and Marília ($p = 0.003$ and $p < 0.0001$, respectively), and in the Rockefeller strain ($p = 0.003$ and $p < 0.0001$, respectively) (Table 2). However, we found no significant difference between the 2016 populations from Santos and Ribeirão Preto ($p = 0.175$) (Table 2).

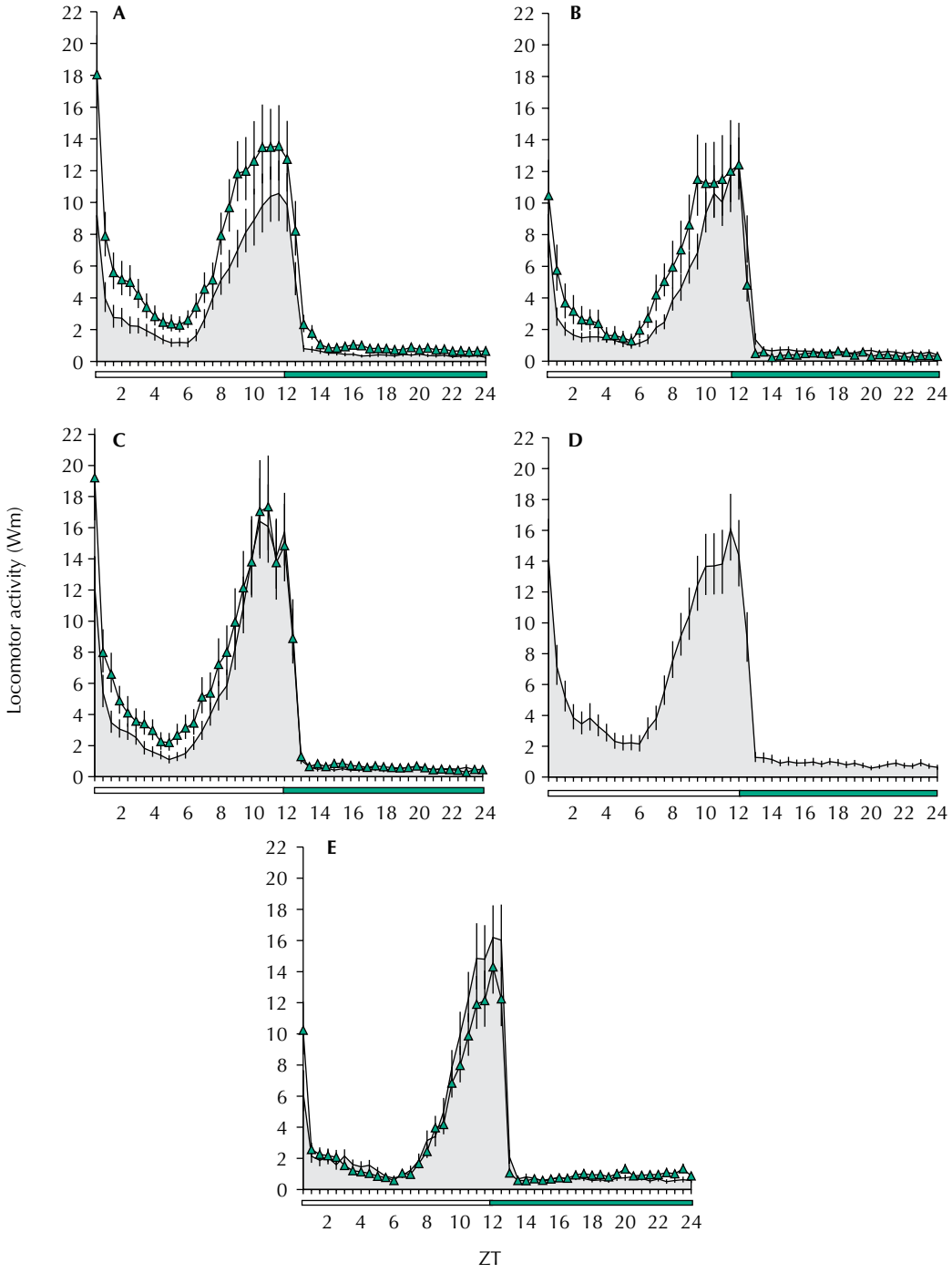

Figure 2. Locomotor activity of *Ae. aegypti* populations in 2016 and 2017. Locomotor activity of *Ae. aegypti* populations with reduced susceptibility (A – Campinas and B – Marília), resistant (C – Santos and D – Ribeirão Preto), and Rockefeller strain (E) in 2016 (grey area) and 2017 (line with triangles), under LD 12:12, at 25°C. Error bars are represented by solid (2016) and dashed (2017) lines. White bar represents the photophase and black bar the scothophase. ZT: *Zeitgeber* time; ZT0: time the light turns on; ZT12: time the light turns off.

Although 2017 populations from Campinas and Marília showed no significant differences in locomotor activity between them ($p = 0.379$) (Table 2), when compared to the Rockefeller strain locomotor activity was significantly increased ($p < 0.0001$ and $p = 0.003$, respectively), especially during the photophase (Figure 3C).

Contrary to 2016, Campinas and Marília populations did not differ significantly from Santos resistant population ($p = 0.446$ and $p = 0.150$, respectively) (Figure 3D; Table 2). Santos population differed significantly from the Rockefeller strain ($p < 0.0001$).

Table 2. Comparison between each population half-day activity (ZT06-ZT12) mean in relation to different insecticide resistance profiles.

Profile	Populations (I *versus* J)	2016		Populations (I *versus* J)	2017	
		Mean Difference (I-J)	p		Mean Difference (I-J)	p[a]
Reduced susceptibility/ Susceptible	Camp *versus* Mar	0.040	0.296	Camp *versus* Mar	0.050	0.379
	Camp *versus* Rock	0.049	0.234	Camp *versus* Rock	0.232	**< 0.001**
	Mar *versus* Rock	0.009	0.815	Mar *versus* Rock	0.182	**0.003**
Reduced susceptibility/ Resistant	Camp *versus* San	-0.085	**0.044**	Camp *versus* San	-0.038	0.446
	Camp *versus* Rib	-0.147	**< 0.001**	Mar *versus* San	-0.088	0.150
	Mar *versus* San	-0.125	**0.003**			
	Mar *versus* Rib	-0.186	**< 0.001**			
Susceptible/ Resistant	San *versus* Rock	0.135	**0.003**	San *versus* Rock	0.270	**< 0.001**
	Rib *versus* Rock	0.196	**< 0.001**			
Resistant	San *versus* Rib	-0.061	0.175			

Camp: Campinas; Mar: Marilia; San: Santos; Rib: Ribeirao Preto; Rock: Rockefeller.
[a] Results with ANOVA One-way and Fisher's LSD post hoc test.

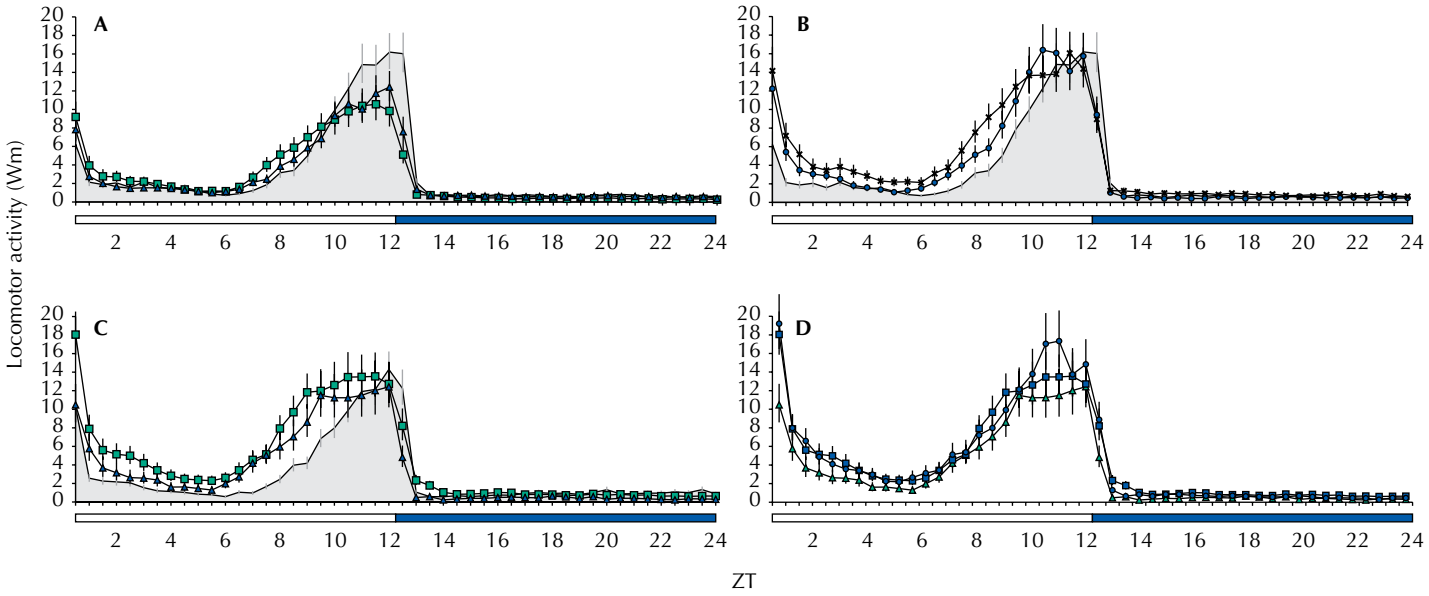

Figure 3. Locomotor activity of *Ae. aegypti* populations. 2016: (A) Populations with reduced susceptibility from Campinas (line with squares; error bars, solid line) and Marília (triangles; error bars, dark dashed line); (B) Resistant populations from Santos (circles; error bars, solid line) and Ribeirão Preto (crosses; error bars, dark dashed line). 2017: (C) Populations with reduced susceptibility from Campinas and Marília. (D) Resistant population from Santos and susceptible populations from Campinas and Marília. Locomotor activity of susceptible Rockefeller strain in 2016 and 2017 is represented in A, B, and C by a shaded grey area with dashed error bar. White bar represents the photophase and black bar the scothophase. ZT: *Zeitgeber* time; ZT0: time the light turns on; ZT12: time the light turns off.

DISCUSSION

Ae. aegypti resistant populations presented increased locomotor activity when compared to two populations with reduced susceptibility and to the susceptible Rockefeller laboratory strain. We also verified a temporal variation in the locomotor activity of both reduced susceptibility populations (Campinas and Marília): these populations showed lower locomotor activity than Santos and Ribeirão Preto resistant populations in 2016, but not in 2017. Chemical insecticides are still widely used to control *Ae. aegypti* mosquitoes, acting on their central nervous system, especially in axonal regions and at the voltage-gated sodium channel (NaV). Yet, the indiscriminate use of different insecticides classes has spurred random mutations, responsible for the development of insecticide resistance phenotype in different *Ae. aegypti* populations around the world[18,30].

Brito et al. (2013) evaluated several life-cycle aspects in a resistant *Ae. aegypti* strain with *Kdr* mutations, using the Rockefeller strain as control group. According to the authors, when compared to the susceptible allele, the mutation probably has a deleterious maintenance cost or a higher fitness cost. In their study, the *Kdr* strain showed a significantly increased locomotor activity during the light phase when compared to the control group. Our results corroborate those reported by Brito et al. (2013), as *Ae. aegypti* populations from Ribeirão Preto and Santos, classified as resistant, showed more locomotor activity than populations with reduced susceptibility and the Rockefeller strain, especially in 2016[24].

The 2016 populations from Campinas, Marília, and Rockefeller showed similar locomotor activity – significantly lower than that presented by the resistant populations from Santos and Ribeirão Preto. However, locomotor activity increased in Campinas and Marília in 2017, presenting significantly different values than the Rockefeller strain but not than Santos populations. This suggests a temporal variation that may be explained by changes in the pyrethroid mortality phenotype of these populations. In 2016, the mortality rate was 67% for the Campinas population and 96% for the Marília population; as for 2017, these values were 53% and 52%, respectively [Macoris MLG unpublished data]. Santos resistant population showed a lower mortality rate (28% in 2016 and 40% in 2017) than Campinas and Marília populations [Macoris MLG unpublished data]. This data could justify this population greater locomotor activity in relation to the Rockefeller strain in the two evaluated years, and in relation to populations with reduced susceptibility in 2016.

Previous studies have described temporal variation in resistance alleles frequency for all tested populations. In 2001, Santos showed a 0.24 frequency for the resistant allele 1016Ile[19]. Ten years later, resistant allele frequencies were 0.77 for Campinas, 0.53 for Marília, and 0.86, for Santos [19]. In 2013, Ribeirão Preto presented a 0.68 frequency for the resistant allele 1016Ile, and 0.80 for 1534Cys [Nakazato BM and Bracco JE unpublished data]. In 2014, the last follow-up data, Campinas and Marília populations presented a 0.63 frequency for both 1016Ile and 1534Cys mutations. In the same year, both Santos and Ribeirão Preto populations also presented two fixed mutations (frequency 1.00)[15]. This finding may explain the similar locomotor activity results presented by Santos and Ribeirão Preto populations in 2016.

Few studies investigated the impact of insecticide resistance on *Ae. aegypti* locomotor activity, either in populations from São Paulo or elsewhere. Thus, our findings may help shedding light on several aspects directly involved in this mosquito's activity, such as blood-feeding, flight, and oviposition. We observed an increased locomotor activity of female *Ae. aegypti,* possibly contributing biologically to their broader dispersion, greater number of blood meals, or any other aspect affecting arboviruses transmission dynamics. A mosquito vectorial capacity is calculated considering several variables. Host-feeding, for examples, exert greater influence on a pathogen's basic reproductive rate (*R*0) than the abundance of mosquitoes[31]. Studies have suggested that the vector-biting rate is the most influential parameter for Zika virus transmission dynamics, causing the virus to invade a susceptible population[32]. DENV-2-infected female *Ae. aegypti* have been reported to present more locomotor activity than uninfected controls[22]. Such increased activity could raise infected mosquitoes biting rate, which, according to a mathematical model, could unfold into dengue outbreaks with greater numbers of primary and secondary infections and more severe biennial epidemics[23]. Thus, control measures should target reducing the vector-biting rate[32]. Mosquitoes circadian rhythms are mainly regulated by circadian clock neurons in the brain[33], and mutations at sites in the mosquito central nervous system targeted by molecular pyrethroid are the major causes of resistance to this insecticide class[24]. Considering that, further studies should investigate whether these mutations are also associated with changes in locomotor activity.

Insecticide resistance in *Anopheles* mosquitoes, the main vectors of *Plasmodium* species, is a genuine concern throughout Africa, especially regarding *Anopheles gambiae*[34]. Different species of *Anopheles* mosquitoes resistant to insecticide also pose a concern for malaria control in other continents, including the Americas[35]. Few laboratory studies have compared

the daily activities of resistant and susceptible *Anopheles* females[36–38]. Resistant *Anopheles gambiae* mosquitoes, for example, the main malaria vector in Africa, showed a shift in peak biting periods from 21:00-22:00hrs to 03:00-04:00hrs[36]. A study evaluated the flight activity of resistant and susceptible *Anopheles stephensi* females under laboratory conditions[37], finding resistant females to show lower flight activity in the first two days than susceptible females[37], differently than that found in our study. *Anopheles aquasalis* females fed with blood containing Ivermectin also showed a decrease in locomotor activity[38]. Further studies should be conducted on this important subject.

As insecticide-resistant populations pose a growing obstacle to vector control programs, our results may contribute to other studies aimed at elucidating aspects involving insecticide resistance, arbovirus infection, and locomotor activity in this mosquito vector.

CONCLUSIONS

Ae. aegypti populations with insecticide resistance profile showed increased locomotor activity. In 2017, populations with reduced susceptibility presented an increase in the locomotor activity pattern, indicating a temporal change in relation to the previous year. The locomotor activity pattern in the populations from Campinas and Marília did not differ significantly from that found for Santos resistant population.

Our results indicate that insecticide-resistant *Ae. aegypti* populations show increased locomotor activity, which may affect their arboviruses transmission dynamics by increasing dispersion, number of blood meals, and other ecological parameters. Such results can shed light on these mechanisms action and effect in the context of an arbovirus epidemic, while helping to improve vector control strategies, innovations, and the epidemiology of this and other viral infections transmitted by *Ae. aegypti*.

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