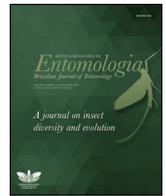




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Effects of temephos resistance on life history traits of *Aedes albopictus* (Skuse) (Diptera: Culicidae), a vector of arboviruses



Junaid Rahim, Abu Hassan Ahmad\*, Alhaji Hamisu Maimusa

Universiti Sains Malaysia, School of Biological Sciences, Minden, Malaysia

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ABSTRACT

The effects of temephos resistance on the fitness cost of the wild populations of *Aedes albopictus* was evaluated. The larvae of two wild populations were exposed to the diagnostic dose of 0.02 mg and 0.012 mg/L. The larvae which survived after the 24 h exposure to diagnostic dose were considered as resistant and F<sub>1</sub> generations were chosen for the comparisons of fitness parameters with the control strain, which includes larval developmental time, adult longevity, fecundity, wing length and hatchability. We found that temephos resistance had negative effects on larval developmental time which was longer for Gelugor strain among the populations with the median range of 10 days and a shorter longevity was observed with the median range of 13 days for males and 16 days for females. Whereas, an effective reduction of 29.8 and 38.6% was observed in fertility and fecundity of Gelugor strain as compared to control strain. In contrast, no clear differences were found in biological parameters of Balik Palau and USM strain, except fecundity and fertility with a reduction of 13.4 and 15.5%, respectively. Whereas, no significant differences were seen in the wing size between the populations with the mean length (mm) of 2.40 for Gelugor, 2.44 for Balik Palau and 2.46 for USM control ( $p > 0.05$ ). Present results indicated that the temephos resistance is associated with the developmental and reproduction potential of resistant population of *A. albopictus* and the fitness has been compromised.

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Introduction

In spite of tremendous vector control efforts, mosquito vector-borne diseases are flourishing around the globe, causing a significant impact on the humans in terms of mortality and morbidity (Lounibos, 2002). Among the viral infections, dengue virus is a major growing and is the most common public health threat with an estimated 390 million infections yearly (Bhatt et al., 2013). In the case of Malaysia, a recent seroprevalence study showed that 55% of the total population have previously been infected by dengue by 2013 (Chew et al., 2016).

The mosquito *Aedes (Stegomyia) albopictus* (Skuse, 1894) known as the Asian tiger mosquito is an invasive species. Laboratory studies have proven its capability of diffusing 32 disease causing pathogens (Vanlandingham et al., 2016), including the three most chief viruses, namely zika (Wong et al., 2013), dengue (Gratz, 2004), and chikungunya (Chomposri et al., 2016). This vector species has the most widespread distribution and is established in every part of the world except Antarctica (Kraemer et al., 2015). Apart from its

ability to transfer diseases causing agents, this mosquito also has a great biting nuisance impact on quality of human's life (Halasa et al., 2014). For these reasons, this mosquito species stands out among the vectors and has gained the primary interest of medical entomologist (Goubert et al., 2016).

*Aedes albopictus* is recognized as an outdoor breeding species, secondary vector for dengue and major for Chikungunya infections (Delatte et al., 2008). However, in Penang Island, this species has successfully adopted to indoor breeding environment (Dieng et al., 2010). In Malaysia, this mosquito has also been implicated as the main vector of dengue (Lee and Rohani, 2005), chikungunya (Noridah et al., 2007), and is one the most dominant species with the evidence of DENV transovarial transmission in larvae (Rohani et al., 2014). To date, the current major dengue control efforts heavily depends on the application of insecticides against the vector species (Schechtman and Souza, 2015), due to the inadequacy of an effective antiviral treatment (Flipse and Smit, 2015).

The continued use and uneven distribution of insecticide against a particular species, in results, have the chances that a proportion of the population may tolerate the toxic effect of the insecticide due to several factors (Sanil and Shetty, 2012). These factors could be the environmental, targeted population peculiarities, low effectiveness, and improper application of the insecticide (Silva et al., 2009).

\* Corresponding author.

E-mail: [stegoculex@gmail.com](mailto:stegoculex@gmail.com) (A.H. Ahmad).

Insecticide resistance in arthropods is an important issue and is considered as a serious threat in vector control programmes (Nauen, 2007). Resistance in *A. albopictus* is growing against the insecticides that are frequently being used in its control and is reported in different countries (Vontas et al., 2012).

Temephos, an organophosphate, is the only approved chemical larvicide which is frequently being used in Malaysia for controlling the container-breeding *Aedes* larvae as a restraint tool for the past 43 years (MOH, 2008; Chen et al., 2013). However, tolerance towards temephos has also been documented from Malaysia (Ishak et al., 2015; Rahim et al., 2016). To handle the toxic effect of insecticides, insects use different tactics such as behavioural, physiological and genetic expressions (Kliot and Ghanim, 2012). These actions when compared with their susceptible counterparts are costly to resistant insects in terms of reduction in fecundity, survival time, body size, an increase in the developmental time (Fang et al., 2011) and susceptibility to predation (Berticat et al., 2004; Chan and Zairi, 2013). In the development of effective and usable vector control policies, familiarity with the reproductive strategies of a targeted species are crucial that is responsible for the diseases transmission in humans (Shaw et al., 2015).

It is believed that, in the stressed conditions if the fitness cost is high the resistant individuals will take more time to spread in a population than their susceptible counterparts. Several studies have reported about the fitness cost in several insect species associated with the insecticide resistance (Lee, 2000; Kliot and Ghanim, 2012), including mosquitoes (Sanil and Shetty, 2012; Brown et al., 2013; Jaramillo-O et al., 2014). In contrast, some testified that no apparent biotic disadvantages in the resistant insects population (Okoye et al., 2007; Bielza et al., 2008; Lyons et al., 2016).

Therefore, in any integrated pest management programme, understanding the biological status of vector population associated with insecticide resistance can be advantageous for the virus transmission dynamics or to restrict the spread of resistant individuals. The present study is designed as an attempt to answer the first question raised by Rivero et al. (2010) by considering on the effects of temephos resistance on the *A. albopictus* life history traits mainly focusing on, larval developmental time, adult longevity, fecundity, body size and egg hatchability. In the series of experiments under temephos selection pressure, using three wild collected populations that previously showed dissimilar patterns of resistance in the field (Rahim et al., 2016).

## Material and methods

### Mosquito strains

The wild *A. albopictus* strains used in this study were collected from three localities situated in Penang Island, Malaysia. One from Gelugor (5°25'00"N, 100°19'00"E), that was previously found resistant to temephos, and the second that showed incipient resistance was collected from the Balik Pulau (5°21'N, 100°14'E) (Rahim et al., 2016). Another wild *A. albopictus* (USM) population was collected within the main campus area of Universiti Sains Malaysia, USM Penang, which has previously been used in different biological studies (Nur Aida et al., 2008; Nur Aida et al., 2011; Maimusa et al., 2016). Purposely, this strain after screening of insecticides susceptibility status was taken as a control in these experiments. All wild populations were collected using the ovitraps and larval collection.

### Laboratory rearing

The card board paddles containing eggs and the larvae were taken to the laboratory and treated under the controlled environmental conditions. In general, prior to acclimatize in water,

eggs were allowed to complete embryonic cycle for 48 h in the insectary maintained at the environmental conditions of relative humidity  $80 \pm 10$ , temperature  $28 \pm 2$  and a photoperiod of 12 h day and night. After that, the eggs were hatched in plastic containers containing dechlorinated water. Larvae were fed on the diet of ground beef liver, milk powder, yeast and a pet food at the ratio of 1:1:1:2. Upon pupation, the wild samples were shifted in the separate cages covered with thin muslin cloth and identified as *A. albopictus* after the adult emergence based on their morphological attributes (Rattananarithikul et al., 2010). Adults were kept *ad libitum* on a diet of 10% sugar solution supplied via soaked cotton wick, and replaced every 3 days. To initiate a uniform colony of all the population, once a day, females were allowed to take a blood meal for a period of 1 h on restrained mice (Approval no. USM/IACUS/2017/(107) (843)), and an oviposition substrate was provided into each cage.

### Larval bioassays

Prior to exposing the populations for temephos selection pressure ( $F_1$ ) generation, the resistance level and lethal concentration of both the strains were evaluated through dose–response bioassays following The World Health Organization (WHO) standard procedure (WHO, 2005). In the bioassays, 8 working concentrations of temephos solutions were prepared, and the bioassays on wild populations were performed as described previously for the susceptible strain (Rahim et al., 2016), known as VCRU (Vector Control Research Unit) strain. Briefly, each test was carried out in 6 replicates, containing 25 larvae per replica, among them 4 were treated with temephos solution and the other two with ethanol as control. Bioassays were repeated thrice on different days.

Lethal concentrations were calculated using the Probit analysis (Finney, 1971). The resistance ratio (RR) values for  $F_1$  generations were calculated by dividing the  $LC_{50}$  values of the wild collected populations with the corresponding  $LC_{50}$  values of susceptible strain (VCRU).

### Temephos selection pressure

Thousands of late 3rd instar larvae of both the populations were exposed to the diagnostic concentration of 0.02 mg/L for 24-h, with the rearing density of 1 thousand larvae per litre in an enamel pan. This diagnostic dose was established previously in our laboratory (Rahim et al., 2016). Those individuals which survived during the exposure period were considered as resistant and rinsed with the water. Subsequently, these individuals were raised to adults for next generations as described above. In the case of Balik Pulau, the strain was exposed to 0.012 mg/L because of high mortality rates against diagnostic dose. We preferred these doses because 100% mortalities were observed during the preliminary experiments with the Ministry of Health (MOH) operational dose of 1 mg/L and half of this dose (MOH, 2008).

Here it is worth mentioning that during all the experiments described below, all the populations were used simultaneously where USM population served as a control. To avoid environmental variations, experiments on all the populations were accomplished under the identical laboratory conditions.

### Larval developmental time

After the exposure to the selected temephos concentrations on populations, the 8 h old larvae of  $F_2$  progenies of each insecticide selected strain were segregated in 8 plastic cups (30 larvae each) containing 250 ml of dechlorinated water, accompanied with the same numbers of reference (USM) strain. Larvae were fed daily on larval food 0.2 g. Water was changed on alternate days or whenever

required. Developmental time was scored daily until the pupal emergence. Using pipettes, emerging pupae were collected into a separate plastic cups and shifted to adult rearing cages.

#### Longevity test

Males and females at the ratio of 30:30 aged between 5 and 8 h were selected randomly after the emergence and housed in the four separate cages with the dimensions of (30 cm × 30 cm × 30 cm) provided with adult diet. Blood meals were offered to females 3 times in a week. The bases of cages were fitted with the clean white papers to observe the mortalities clearly. Dead individuals were removed daily and mortality rates were recorded until the death of all individuals.

#### Fecundity test

Three days old females were offered a blood meal for 2 h. After that the blood engorged 10 females were randomly selected with the same number of males and shifted into the 10 cages (18 cm × 18 cm × 24 cm). After that females were allowed to take blood meal twice daily for a period of 2 h (9:00–10:00 a.m. and 16:00–17:00 p.m.). To count number of eggs, the oviposition cups were replaced daily from each cage and eggs laid by each female were counted under a dissecting microscope (Meiji EMZ; Techno co. Ltd, Tokyo, Japan) and stored at ambient temperature (Bellini et al., 2013). Adult food of 10% sugar solution was provided ad libitum. However, due to the early death of males in these experiments and considering the female *A. albopictus* being not a monogamist (Oliva et al., 2013), males of the same progenies were provided until the death of each female. The mean values of the total eggs laid by each female were taken as measures of fecundity. These experiments were repeated three times using same number of mosquitoes.

#### Wing length

Upon the death of the females in the fecundity test, one wing of each female was used for body size measurement irrespective of the side. The wings were removed from the thorax and were mounted on the slide, shielded with a cover slip. Adopting (Zuharah et al., 2016), the length of the each wing was measured from axillary incision to the extreme apical margin, keeping the fringe scales out from measurement. The measurements were made under the stereomicroscope (Olympus BX41; Olympus, Tokyo, Japan).

#### Fertility test

The eggs harvested during the above-mentioned fecundity test, filter paper containing eggs per female and population were immersed in the larval tray containing half litre of dechlorinated water. Eggs from the filter papers were gently removed and seeded into the water using a small soft and humid paint brush (Brito et al., 2013), keeping in mind that brushing do not effect *A. albopictus* eggs (Zheng et al., 2015). The number of emerged larvae per population were counted and separated 5 times after each 3rd day.

#### Data analysis

All statistical data obtained were analyzed using to SPSS version 22 for analysis. Kolmogorov and Shiperowilks normality tests were used prior to analysis. For larval developmental time and longevity test (males and females) comparison between the populations were carried out using the Kruskal–Wallis test followed by pair-wise comparison among individuals' groups. Comparison between the wings lengths were performed using the one-way ANOVA, followed

**Table 1**

LC<sub>50</sub> and RR values of all populations against temephos with 95% C.I.

Population	LC <sub>50</sub> mg/L (95% C.I.)	RR	n
VCRU	0.005 (0.004–0.005)	–	400
Gelugor	0.018 (0.013–0.039)	3.6	300
Balik Palau	0.010 (0.009–0.011)	2	299
USM	0.007 (0.006–0.009)	1.4	300

LC, lethal concentration; C.I., confidence interval; RR, resistance ratio; n, sample size.

**Table 2**

Larval developmental time taken by all wild strains.

Population	n	Larval developmental time in days Median (IQR) <sup>a</sup>	p-Value	χ <sup>2</sup> (df)
Balik Palau	230	8 (4–11)	<0.001	344.2(2)
Gelugor	210	10 (7–14)		
USM	232	7 (3–11)		

<sup>a</sup> IQR, interquartile range; n, sample size.

by Tukey's multiple comparison test. For the fertility and hatchability test descriptive statistics were used to calculate the mean values and minimum and maximum range. Whereas, the effective reduction (ER%) was calculated as described elsewhere (Zuharah et al., 2016).

## Results

Table 1 shows the LC<sub>50</sub> values for all the wild populations with their resistance ratio compared with the VCRU strains calculated by Probit analysis. Offspring of wild *A. albopictus* showed resistance ratio values with the increase of 3.6, 2 and 1.4 folds for Gelugor, Balik Pulau and USM populations respectively. Comparison between the strains revealed that the Gelugor population was more resistant to temephos followed by Balik Palau and USM.

#### Larval developmental time

Comparison between the larval developmental times of each population was performed until the pupal emergence time. A significant difference between the larval developmental times was observed in the overall populations (Table 2). A pairwise comparisons between each population revealed that USM strain was significantly associated with both Gelugor (χ<sup>2</sup> = 281.6, df = 1, p < 0.001) and Balik Palau (χ<sup>2</sup> = 7.1, df = 1, p = 0.006) strains. However, larval developmental time for Gelugor strain was longest (median = 10 days) compared to USM (median = 7 days) while for Balik Palau strain it was around median of 8 days.

#### Adult longevity

Significant differences were seen between the overall longevity of adults (p < 0.001). In all strains females lived longer than males. The days survived by the males were recorded higher in USM population followed by Balik Palau and Gelugor, with the median ranges of 18, 17 and 13 days respectively. Whereas, females of Gelugor strain lived significantly shorter with the longevity of 16 days as compared to Balik Palau and USM with the median range of 20 and 21 days respectively (Table 3). A reduction in the adult longevity of both the sexes has been observed in Gelugor strain (χ<sup>2</sup> = 74.1, df = 1, p ≤ 0.001). Comparison between males (χ<sup>2</sup> = 33.3, df = 1, p ≤ 0.001) and females (χ<sup>2</sup> = 44.9, df = 1, p ≤ 0.001) showed a significant decrease in the Gelugor population as compared to USM. In contrast, no significant differences were observed between the gender and overall adult longevity of Balik Palau and USM population (p > 0.05).

**Table 3**

Difference between each gender among populations and difference between overall adults longevity as compared to (USM).

Gender	Total longevity in days						$\chi^2$ (df)	p-Value
	USM		Gelugor		Balik Pulau			
	n	Median (IQR) <sup>a</sup>	n	Median (IQR)	n	Median (IQR)		
Male	120	18 (1–30)	120	13 (1–23)	120	17 (1–32)	33.3 (2)	<0.001
Female	120	21 (1–37)	120	16 (1–27)	120	20 (1–35)	51.4 (2)	<0.001
Total longevity Median (IQR)	240	21 (1–37)	240	14 (1–27)	240	19 (1–35)	81.1 (2)	<0.001

<sup>a</sup> IQR, interquartile range; n, sample size.

### Wing length

The mean wing length of USM, Balik Palau and Gelugor strains were  $2.46 \pm 0.03$ ,  $2.44 \pm 0.02$  and  $2.40 \pm 0.01$ , respectively. A decrease in the wing length of both the populations was observed as compared to USM, but statistically it was insignificant between the populations ( $p > 0.05$ ).

### Fecundity test

Females of USM strain laid the high number of eggs with a mean number of  $92.90 \pm 9.4$  eggs per female. Whereas, the females of Balik Pulau and Gelugor were fecund with the mean values of  $84.93 \pm 4.6$  and  $65.20 \pm 4.2$  eggs respectively. The effective reduction with the percentages of 29.8 and 13.4% was noted for Gelugor and Balik Pulau as compared to USM (Table 4).

### Hatchability

The mean hatching rate of USM strain was  $77.16 \pm 8.1$  eggs per female, whereas, Gelugor and Balik Pulau strains scored a mean range of  $47.36 \pm 3.5$  and  $67.93 \pm 3.9$  respectively. A decrease in hatchability of 38.6 and 15.5% was observed in Gelugor and Balik Pulau strains respectively, as compared to the USM strain (Table 4).

### Discussion

In the current study, we evaluated the effects of temephos resistance on several life history traits of three wild *A. albopictus* populations, which showed different resistance patterns in the field. Among the populations two were assayed with the exposure of temephos lethal concentrations. Fitness cost associated with insecticide resistance is widely studied in insects using coisogenic strains where the strains were exposed to specific time period depending on their life stage. The effect of insecticide resistance on the fitness cost of the other mosquito vector species has been reported. However, studies on fitness cost of *A. albopictus* are not documented in literature (Smith et al., 2016). Our results show that the survival of the *A. albopictus* after the exposure to the lethal concentration of temephos, affects the life history traits of resistant population. Similarly, several other studies conducted on the *A. aegypti* have reported the different life span in the resistant populations (Martins et al., 2012; Jaramillo-O et al., 2014).

In the current study, an increase in the larval developmental time of Gelugor resistant strain was observed as compared to USM strain, in support of these results an increase in the larval developmental time was reported in temephos resistant strain of *A. aegypti* from Brazil (Diniz et al., 2015). Beside this the sublethal effect of several other insecticides can also affect the larval developmental time (Shaalan et al., 2005). In contrast to these findings, a study conducted in Thailand on a knockdown resistant strain of *A. aegypti* found no differences in the larval developmental time although the involved mechanism was known (Plernsub et al., 2013). The dissemination of mosquito population in the field mainly depends on

the developmental time (Martins et al., 2012). However, in natural environment the dynamics of a population development, such as prolong larval stage may effects the adaptive advantages of an individual due to several extrinsic factors, such as elimination of breeding habitats (Berticat et al., 2004), and the presence of predators or parasites can reduce the larval survival rate (Agnew and Koella, 1999) which results in the reduction of generations.

Adult longevity of female mosquitoes is one of the key features of the vectorial capacity which plays a major role in the virus transmission. For example, in case of *A. aegypti* the extrinsic incubation period for DENV replication in the salivary glands is 7–12 days depending on the environmental conditions and female must survive this period for transmission of virus. In our findings longevity of Gelugor resistant strain was significantly shorter than that of Balik Pulau and USM strains. Similar results were seen in the temephos (Belinato et al., 2012; Diniz et al., 2015) and pyrethroids resistant strains (Martins et al., 2012) of *A. aegypti* and *Culex pipiens pallens* (Li et al., 2002). In contrast to these findings no differences were observed in the two dieltrin resistant malaria vectors *Anopheles gambiae* and *Anopheles stephensi* (Rowland, 1991). Similarly, Okoye et al. (2007) reported that resistant females of *Anopheles funestus* had a longer life span than the susceptible strains. Reduction in the female longevity have the chances to reduce the gonotrophic cycles and number of progenies, whereas reduced longevity of male may lead to reduction in the mating opportunities. These factors could reduce the frequency of blood feeding and pathogen diffusion in short-lived mosquitoes (Silva et al., 2009).

Wing length is considered as the best indicator of the body size of the mosquitoes. Longer the wing size larger will be the body size of the mosquitoes and it often correlates with the egg laying potential of the females and their flight range. It has been documented that larger females tend to lay more eggs as compared to their smaller counterparts (Blackmore and Lord, 2000). Similarly, Armbruster and Hutchison (2002) found a significant association between the wing length and fecundity in *A. albopictus*. However, in the current study no significant difference in the wing length among the populations was observed, although there was a slight difference in the size of the wings. Even though Gelugor strain took longer larval developmental time and it is evident that the prolonged larval developmental time has negative effect on the body size of the adult mosquitoes in high larval density conditions (Wiwatanaratnabutr and Kittayapong, 2009). Our results support the findings of Jaramillo-O et al. (2014) where they found no difference in the wing lengths in lambda-cyhalothrin resistant strains of *A. aegypti*.

In Malaysia, permethrin, deltamethrin, malathion and temephos are frequently being used for the control of dengue vectors. A recent study from Malaysia has reported the metabolic resistance mechanism responsible for pyrethroids resistance in *A. albopictus* due to over production of cytochrome P450 (Ishak et al., 2016), and it is evident that the increase in the activity of metabolic resistance mechanism has developed the cross resistance between pyrethroids and organophosphate based on the detoxifying enzymes (Martins et al., 2012). Moreover, a high knock

**Table 4**

Mean number of eggs and mean number of hatched eggs per female with the minimum and maximum ranges and Effective reduction percentages.

Population	n	Mean ± S.E. Number of eggs per female	Range	ER % <sup>a</sup>	Mean ± S.E. Number of hatched larvae per female	Range	ER % <sup>a</sup>
USM	30	92.90 ± 9.4	11–207		77.16 ± 8.1	8–165	
Gelugor	30	65.20 ± 4.2	21–121	29.8	47.36 ± 3.5	11–99	38.6
Balik Palau	30	84.93 ± 4.6	35–140	13.4	67.93 ± 3.9	16–108	15.5

<sup>a</sup>ER, effective reduction; n, sample size.

down resistance ratio was also observed in the Gelugor adult strain against permethrin and deltamethrin (unpublished data). Taken together, these results suggest that the resistance mechanism involved in Gelugor strain exhibit a reduction in fitness. However, the impact of insecticide resistance on the vectorial capacity of this vector species is unclear, as it was found associated in a pyrethroids resistant strain of *An. gambiae* having a negative impact on its competency to transmit *Plasmodium falciparum* (Alout et al., 2013).

It is also an important issue that the studies on fitness cost were carried out in the laboratory control conditions that are not the representatives of the ecological conditions in the field. Several environmental factors have been found to influence on the survival and development of mosquito immatures. The quantity and quality of food, larval density, temperature and humidity are the main stress factors that could be deleterious for resistant populations in the field and more likely these factors in optimum conditions may under-estimated the physiological costs (Belinato and Martins, 2016).

Besides the negative effects of resistance on developmental time, longevity and reproduction, changes in behavioural aspects has also been reported in the mosquitoes. The number of eggs laid by females directly relate to the amount of ingested blood. For example, 15% reduction in the amount of ingested blood was observed in the temephos resistant females of *A. aegypti* as compared to their susceptible control and as a result deposited 21% fewer eggs (Belinato et al., 2012). In our results, we noticed a reduction in both fecundity and hatchability of both strains, where the proportion of laid and viable eggs by Gelugor females was lesser than the other two strains. Similar results were observed in *Cx. pipiens pallens* (Li et al., 2002) and a pyrethroids selected F<sub>9</sub> generation of *A. aegypti* (Martins et al., 2012) with a reduction of 30% in viable eggs. In contrast to these, a study conducted on pyrethroid resistant malaria vector, *An. funestus* showed some reproductive advantages in resistant individuals as compared to susceptible control (Okoye et al., 2007).

In the current study, the exposure time was 24-h and the survived larvae where considered as resistant. Several studies have reported the sublethal effects of insecticides on the biological parameters using a fixed exposure time to the sublethal concentrations. However, behaviourally the larvae of mosquitoes are unable to avoid the toxic effects in an insecticide treated container as adults do in the nature (Chareonviriyaphap et al., 2013). The reproductive success of an individual is dependent on genetic background, environmental conditions, and interactions between these. One factor which is increasingly recognized to have a profound impact on individual success is the environmental conditions experienced by their parents (Grech et al., 2007).

It can be concluded that in the natural conditions with the long exposure to sublethal doses may have massive negative impact on the surviving individuals. The developmental time, shorter longevity and reduction in reproductive performance are the factors against the maintenance and dispersion of the resistant individuals, and this would be the reason that the resistant individuals are always considered latent in the field. Further studies are needed to investigate the impact of insecticide resistance on

the physiology, behaviour and especially the vectorial capacity of *A. albopictus*. Results from the present study can be useful to predict the population dynamics of this vector species in the areas, where insecticide resistance is reported and resistance management is needed.

### Conflicts of interest

The authors declare no conflicts of interest.

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