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## Asia Pacific Journal of Public Health

### Resistance of *Aedes aegypti* (Diptera: Culicidae) populations to deltamethrin, permethrin and temephos in Cambodia

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Manuscript Type:	Original Manuscript
Keywords:	<i>Aedes aegypti</i> , Cambodia, insecticide, mosquito, resistance, vector control
Abstract:	<p>Dengue fever is a major public health concern, including 185,000 annual cases in Cambodia. <i>Aedes aegypti</i> is the primary vector for dengue transmission and is targeted with insecticide treatments. This study characterized the insecticide resistance status of <i>Ae. aegypti</i> from rural and urban locations. The susceptibility to temephos, permethrin and deltamethrin of <i>Ae. aegypti</i> was evaluated in accordance with WHO instructions. All the field populations showed lower mortality rate to temephos compared to the sensitive strain with Resistance Ratio 50 (RR50) varying from 3.3 to 33.78 and RR90 from 4.2 to 47 compared to the sensitive strain, demonstrating a generalized resistance of larvae to the temephos in Cambodia. <i>Ae. aegypti</i> adult populations were highly resistant to permethrin regardless of province or rural/urban classification with an average mortality of 0.02%. Seven of the eight field populations showed resistance to deltamethrin. These results are alarming for dengue vector control, as widespread resistance may compromise the entomological impact of larval control operations. Innovative vector control tools are needed to replace ineffective pesticides in Cambodia.</p>

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5 2 **and temephos in Cambodia.**  
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39 16 **Keywords.** *Aedes aegypti*; Cambodia; insecticide; mosquito; resistance; vector control.  
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## 17 Introduction

18 Dengue fever is a major public health concern, with estimates of 400 million cases every year in urban,  
19 suburban and rural tropical areas.<sup>1</sup> In Cambodia, around 185,000 cases are estimated annually.<sup>2</sup> The  
20 primary vector for dengue transmission is *Aedes aegypti* which favors environments where water  
21 storage is abundant and solid waste disposal is deficient.<sup>3</sup> As *Ae. aegypti* is implicated in the  
22 transmission of arboviruses such as Zika, Chikungunya and Yellow fever<sup>4</sup>, vector control strategies that  
23 target *Ae. aegypti* populations may have an major public health impact. Many insecticides have been  
24 used in order to control *Ae. aegypti* populations, but little information exists on the susceptibility of  
25 Cambodian populations to the most commonly used insecticides.

26 As early as 1955, DDT residual spray was used in the first malaria eradication pilot in Snuol district<sup>5</sup>. DDT  
27 was again used in public health programs targeting malaria and dengue in urban and rural areas and at  
28 UNHCR refugee camps along the Cambodia-Thailand border from 1981 to 1987, after which it was no  
29 longer imported<sup>6</sup>. Pyrethroids, particularly permethrin and deltamethrin, were introduced to Cambodia  
30 in the late 1980s and 2000 for the control of malaria (impregnation of bednets) and dengue (thermal  
31 fogging and ULV sprays), respectively<sup>6</sup>. Since 1992, Temephos has been imported with roughly 200 tons  
32 per year used mainly for larval control of dengue vectors<sup>6</sup>. In 1966, Mouchet and Chastel showed total  
33 susceptibility of *Ae. aegypti* to DDT, fenthion, malathion and diazinon insecticides, but observed  
34 resistance to dieldrin and gamma HCH<sup>7</sup>. More recently, *Ae. aegypti* resistance to temephos was also  
35 investigated during two field studies in Cambodia<sup>8</sup>. The resistance pattern and future of temephos is  
36 increasingly important as this larvicide has been the main dengue control strategy used by National  
37 Dengue Control Program (NDCP) for more than 20 years and for biannual larvicide campaigns since  
38 2001<sup>3,6</sup>.

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3 39 Using the WHO diagnostic dose (0.02mg/L), the Phnom Penh population tested in 2001 was found to be  
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5 40 resistant to temephos, while Kampong Cham population was still susceptible. More recently, among  
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7 41 seven *Ae. aegypti* populations, six were found to be resistant to temephos with mortality ranging from  
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9 42 11.02% up to 88.62% at the WHO diagnostic concentration (To Seta, Pers. Comm.). While it seems clear  
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11 43 that that temephos resistance among *Ae. aegypti* populations has increased over time in Cambodia, the  
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13 44 patterns between rural and urban areas are as delineated.

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17 45 While pyrethroid and organophosphate insecticides are used in the national malaria and dengue control  
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19 46 programs, significant use of insecticides (including larvicides, repellents, space sprays, treated materials  
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21 47 and coils) at home and in the private sector results in unquantifiable use of insecticides. Coupled with  
22  
23 48 the lack of information on adult resistance status in Cambodia and long-term usage of space spraying by  
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25 49 pest control companies and public health authorities, the need for characterizing the susceptibility of  
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27 50 *Ae. aegypti* to pyrethroids is urgent. This study aims to characterize the insecticide resistance status for  
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29 51 immature and adult stages of *Ae. aegypti* collected from rural and urban Cambodian environment. Eight  
30  
31 52 field populations were tested using WHO test procedures against the most commonly used insecticides  
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33 53 in Cambodia which include temephos (for immature stages) and deltamethrin/permethrin (for adult  
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35 54 stages).

## 35 55 **Material and Methods**

### 36 56 Mosquito collection

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43 57 Four different geographical areas in Cambodia were selected for field sample collections (Phnom Penh,  
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45 58 Kampong Cham, Battambang and Siem Reap). Two urban villages and two rural villages were selected as  
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47 59 collection points within each village. Villages were selected by NDCP according to geographical  
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49 60 representation, dengue incidence and recent use of temephos (within the previous two years)  
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51 61 (Supplementary File 1). Twenty five households were randomly selected within each village and all  
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3 62 containers were inspected for larvae and pupae using direct pipetting for small containers and sweep  
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5 63 net method for large containers<sup>9</sup>. Collected larvae/pupae were pooled by location (rural/urban) in each  
6  
7 64 province and transported to an insectary.

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9  
10 65 Larvae and pupae were reared in standard conditions (temperature:  $28 \pm 1^\circ\text{C}$ ; relative humidity:  $75 \pm$   
11  
12 66  $25\%$ ; photoperiod: 12 hours day/night) in 24.8 x 19.7 x 3.8 cm standard white plastic larval tray  
13  
14  
15 67 containing 2 liters of purified water and fed with half a teaspoon of grounded fish food daily until adult  
16  
17 68 emergence. Adult *Aedes* were separated from other species by direct aspiration and each population  
18  
19 69 was separated by location (total of 8 populations from 4 Provinces).

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21  
22 70 For both larvae and adults assays, a USDA reference susceptible strain<sup>10</sup> was used as positive and  
23  
24 71 negative control with water and ethanol in plastic beakers.

#### 25 26 27 72 Rearing of F1 larvae for testing

28  
29  
30 73 Adult *Aedes* mosquitos from parental generations were reared at standard conditions and fed with 10%  
31  
32 74 sucrose solution. All populations were also provided with lab reared mice for blood meal once every  
33  
34 75 three days for 3-4 hours. Eggs from the F1 generation were collected on white filter paper and placed  
35  
36 76 inside black plastic cups. Eggs were dried and stored in envelopes and later sent to the laboratory. F1  
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38 77 eggs were immersed in water according to assay needs for testing procedures and larvae were reared as  
39  
40 78 previously described.

#### 41 42 43 79 *Ae. aegypti* larval bioassays

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46  
47 80 In accordance with WHO instructions<sup>11</sup>, late third instar larvae of F1 generation were used for  
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49 81 determining the resistance of mosquito larvae to temephos.

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52 82 Temephos (Sigma, Pestanal analytical grade, 250 mg) was diluted in ethanol to produce a stock solution  
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54 83 of 1000 mg/L. The main stock solution was diluted into several working concentrations better suited for

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3 84 testing. All solutions were stored in glass bottles and labeled accordingly. To obtain each of these  
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5 85 concentrations the adequate volume of temephos was pipetted from stock solutions, adding the  
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7 86 remaining amount of solution with ethanol into each beaker containing 99 ml of water. Four replicates  
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10 87 were used for every concentration, and each replicate consists of 25 larvae.

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13 88 Six temephos concentrations (0.2, 0.05, 0.03, 0.02, 0.01, 0.004 mg/L) were used to determine Lethal  
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15 89 Concentration (LC) 50/95 (e.g. the necessary concentrations needed to kill 50%/95% of mosquito  
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17 90 larvae). Resistance ratios (RR50 and RR95) were calculated dividing  $LC_{50}$  and  $LC_{95}$  rates from *Ae. aegypti*  
18  
19 91 field populations by the  $LC_{50}$  and  $LC_{90}$  rates of the USDA susceptible strain.

#### 22 92 *Ae. aegypti* adult bioassays

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24  
25 93 Insecticide resistance screening for adult mosquitos was conducted using the WHO tube assay<sup>11</sup>. Two  
26  
27 94 synthetic pyrethroids; permethrin and deltamethrin, at diagnostic concentrations appropriate for *Aedes*  
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29 95 mosquitoes were used. WHO tube kit and impregnated permethrin (0.25%), deltamethrin (0.03%) and  
30  
31 96 piperonyl butoxide for synergist assay (PBO 4%) papers were obtained from Vector Control Research  
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33 97 Unit at the University of Science, Penang, Malaysia. Diagnostic and synergist concentrations were  
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35 98 chosen following WHO recommendations<sup>11</sup>.

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39 99 For this bioassay, each tested population used four tubes containing Permethrin (0.25%), four tubes  
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41 100 containing deltamethrin (0.03%), and four control tubes containing silicone oil paper. Twenty-five adults  
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43 101 at least 3 days old and non-blood fed female mosquitoes were introduced into each tube lined with  
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45 102 untreated paper (holding tube) for 60 minutes. Mosquitoes were then transferred into the exposure  
46  
47 103 tube and exposed to impregnated paper for 60 minutes. Mosquito Knock Down (KD) was measured at  
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49 104 the end of the exposure, after which mosquitoes were transferred back to the tube without insecticide.  
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51 105 Mortality was counted at the end of a 24 hours period and the resistance status was interpreted  
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53 106 according to the WHO protocol.  
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3 107 Insecticide-synergist assay using piperonyl butoxide (PBO) was conducted to measure the effect of pre-  
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5 108 exposure to a synergist on the expression of insecticide resistance. Adult *Aedes* were pre-exposed to this  
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7 109 synergist for one hour before exposure to insecticide. KD and mortality were recorded the same way as  
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10 110 standard tests.

### 11 112 Data management and statistical analysis

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15 112 Knock down and mortality were registered at 1 hour and 24 hours post-exposure respectively. RRs for  
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17 113 larvae and adult mosquitos were calculated by dividing the average mortality found in each field  
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19 114 population by the mortality obtained with the USDA susceptible reference strain.

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23 115 For larvae results, LC50 and LC90 were obtained by plotting the mortality using log probit analysis.

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26 116 Statistical analysis (ANOVA and mean comparison) were completed to compare the mortality of adults  
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28 117 to permethrin and deltamethrin with or without the use of PBO. Graphs and data analysis were done  
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30 118 with R software<sup>12</sup>.

## 31 119 **Results**

### 32 120 Larval bioassays

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36 121 The overall bioassay results for larvae are presented in Table 1. The highest LC50 and LC90 values were  
37  
38 122 obtained with Battambang urban populations (LC<sub>50</sub>=0.125±0.004 mg/L and LC<sub>90</sub>= 0.221±0.008 mg/L) and  
39  
40 123 Kampong Cham (Table 1). Phnom Penh and Siem Reap, the LC<sub>50</sub> and LC<sub>90</sub> were lowest with LC<sub>50</sub> values  
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42 124 comprised between 0.012 mg/L (Siem Reap rural) and 0.020 mg/L (Phnom Penh rural).

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46 125 The RR for urban and rural populations of Siem Reap and Phnom Penh provinces were mostly above the  
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48 126 threshold which is defined as a resistant population with RR ≥ 5. RR values of Kampong Cham and  
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50 127 Battambang urban and rural populations were two and nine-fold higher than the threshold, respectively.  
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52 128 While these results may be linked to the continued distribution of temephos and consequent exposure  
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3 129 of populations to this chemical, it is of great concern that 2 out of 4 populations in these two provinces  
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5 130 registered RRs twice as high as the defined resistance threshold (Kampong Cham Rural, RR=13.0;  
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7 131 Battambang rural, RR=11.2) and one registered a RR 6 times higher than the defined threshold  
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9 132 (Battambang urban, RR=33.6).

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13 133 Higher lethal doses ( $LC_{50}$  or  $LC_{90}$ ) are needed to kill *Ae. aegypti* larvae from Battambang and Kampong  
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15 134 Cham populations as depicted on the four mortality curves on the right side of the graph compared to  
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17 135 Siem Reap and Phnom Penh populations (Figure 1). Lastly all the field populations showed higher  
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19 136 mortality curve patterns compared to the sensitive strain over a range of concentrations (Figure 1).

#### 20 21 22 137 Adult bioassays

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25 138 Results showed a very high level of resistance to permethrin regardless of province or rural/urban  
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27 139 classification (Figure 2; Supplementary File 2). The average mortality to permethrin at the WHO  
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29 140 diagnostic dose is  $2.22\% \pm 0.02$  for all the populations. While all populations showed resistance to  
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31 141 permethrin, six of the eight populations showed no mortality to permethrin at all. The additional two  
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33 142 Kampong Cham populations had 1.1% and 3.9% of mortality. Adult bioassays showed a significant  
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35 143 difference in mortality to permethrin depending on the population and the presence of PBO ( $F=3.35$ ;  
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37 144  $df=8$ ;  $p=0.003$ ), particularly a significant increase in mortality from 1.1% to 18.6% in rural population  
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39 145 from Kampong Cham province (Supplementary File 2).

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44 146 Seven of the eight field populations had a percentage below 90% of mortality due to deltamethrin,  
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46 147 meaning that these populations are resistant. The average mortality of *Ae. aegypti* populations from  
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48 148 Phnom Penh and Siem Reap provinces ranged between 4.0% and 8.3% only. A significant difference in  
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50 149 mortality to deltamethrin among the five highest mortality populations (>52%) tested were observed in  
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52 150 the presence of PBO ( $F=7.20$ ;  $df=8$ ;  $p<0.0001$ ).

#### 53 54 55 56 151 **Discussion**

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3 152 Resistance to temephos: implications for public health  
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6 153 Observed *Ae. aegypti* resistance to temephos is consistent with a recent study where 6 of 7 populations  
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8 154 showed similar resistance in Cambodia (To Seta, pers. comm.). The RR50 range of the 8 populations to  
9  
10 155 temephos between 3.8 and 33.6 reflects the intensity of insecticide control. In Thailand, despite  
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12 156 mosquito resistance to deltamethrin and permethrin, temephos is still an effective insecticide to control  
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14 157 *Ae. aegypti* larvae<sup>13</sup>. On the basis of data showing temephos resistance in Phnom Penh over 17 years<sup>8</sup>, a  
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16 158 review of prevention and control strategies should be conducted and highlight the effects of reliance on  
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18 159 a single method of control (e.g. high levels of temephos use in Cambodia<sup>14</sup> may compromise the  
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20 160 entomological impact of larval control operations).  
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24 161 *Bacillus thuringiensis* var. *israelensis* (Bti) was tested with success in 2005 around Phnom Penh<sup>15</sup>. A new  
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26 162 Bti strain AM65-52 was tested in 2016 against *Ae. aegypti* field population from Kandal province that  
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28 163 was resistant to temephos. Results showed a reduction in the number of pupae over 13 weeks, with an  
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30 164 average 70% reduction during the 8 first weeks<sup>16</sup>. The use of the *Poecilia reticulata* (guppy) fish to  
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32 165 control *Aedes* populations in water storage was tested in 2008 and after one year a 79% reduction in  
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34 166 *Aedes* larvae in community was observed with a presence of guppies in only 57% of the containers<sup>17</sup>. In  
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36 167 2008, a new formulation of pyriproxifen was tested in water containers against *Ae. aegypti* in Phum  
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38 168 Thmei near Phnom Penh<sup>18</sup>. The study identified an inhibition of adult emergence in treated jars reaching  
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40 169 90% for 20 weeks, and remaining above 80% until the end of the study (34 weeks). In Kampong Cham  
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42 170 Province in 2008 water jars were covered with LLIN Permanet 2.0 (insecticide = deltamethrin) without  
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44 171 significant reductions in mosquitoes<sup>17</sup>, possibly explained by the strong resistance to deltamethrin that  
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46 172 we observed in *Ae. aegypti* adults. A large-scale randomized trial comparing guppy and COMBI  
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48 173 (Communication for Behavioural Impact) in Kampong Cham showed 92.5 % reduction in larval-positive  
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50 174 containers and 76%-88% coverage with guppies after one year. A recently completed cluster  
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52 175 randomized control trial showed that an integrated vector management approach using guppy fish  
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3 176 (*Poecilia reticulata*), a new slow release pyriproxyfen matrix (Sumilarv® 2MR), and community  
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5 177 engagement through a clear Community for Behavioral Impact (COMBI) strategy reduced indoor adult  
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7 178 density roughly 50% as compared to the control arm<sup>19</sup>. All of these methods focused on key containers,  
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9  
10 179 especially water cement jars that produced approximately 95% of *Ae. aegypti* larvae and pupae<sup>9</sup> and  
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12 180 should be considered in Cambodia as a cost-effective replacement of temephos.

#### 15 181 Resistance to permethrin but susceptible to deltamethrin

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18 182 *Ae. aegypti* deltamethrin-resistant populations have been described in different countries in Asia<sup>20</sup>, Latin  
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20 183 America<sup>21</sup>, Africa<sup>22</sup>, Oceania<sup>23</sup>, and the Caribbean<sup>24</sup>. In our study, *Aedes aegypti* populations were either  
21  
22 184 totally resistant to deltamethrin (with two populations exhibiting zero mortality) or had tolerance  
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24 185 patterns. Recently, the same pattern was observed in Thailand where *Ae. aegypti* F1 females were  
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26 186 susceptible to deltamethrin, but resistant to permethrin<sup>13</sup>. A substantial geographic variation exist to  
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28 187 pyrethroid resistance, with lower adult resistance levels in Asia, Africa and the USA. However there is  
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30 188 250-fold resistance to deltamethrin in Thailand<sup>25</sup>.

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34 189 In this study, an extremely strong resistance to permethrin was observed both with/without PBO which  
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36 190 seems to indicate that the resistance is already fixed. Comparatively, the result with deltamethrin and  
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38 191 deltamethrin + PBO suggest the involvement of detoxifying enzymes. However, generally multiple  
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40 192 resistance between pyrethroids are possible and it can be expected that there is a *kdr* mutation for  
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42 193 resistance in both insecticides. As the mechanisms of resistance between permethrin and DDT are  
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44 194 expected to be the same, via a *kdr* mutation<sup>26</sup>, the already existing DDT-resistance<sup>7</sup> may explain the  
45  
46 195 current fixed resistance observed with permethrin. There are several *kdr* mutations common in *Aedes*  
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48 196 species that synergize with each other when they are associated<sup>27</sup>. Heterozygous V1016G, and F1534F  
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50 197 and F1534C mutants were found in Thailand<sup>28</sup>, and the same mutation was also described southern  
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52 198 China with V1016G mutants<sup>29</sup>. There is substantial variation in *kdr* in the Southeast Asian region that has  
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199 effects on resistance (arising from different combinations of three mutations - S989P, V1016G and  
200 F1534C - in *Ae. aegypti*). Although there are other mutations detected in *Ae. aegypti* they do not appear  
201 to have effect on resistance based on current evidence. For example, combinations of F1534, C1534C,  
202 V1016G, S989P<sup>29</sup> are present in Cambodia and may act together with metabolic resistance. The  
203 resistance patterns to deltamethrin and permethrin in the Cambodian villages fit with the variation in  
204 frequencies of the three mutations and especially in low 989/1016 but high 1534 in permethrin (but not  
205 deltamethrin) resistant locations, but higher 989/1016 in Phnom Penh and Siem Reap (perhaps in  
206 combination with 1534).

207 Our results question the resistance mechanisms. Indeed, the absence of correlation between  
208 permethrin and deltamethrin may involve different effects induced by type I Pyrethroid (permethrin)  
209 and a pseudo pyrethroid (nonester pyrethroid; deltamethrin), and so different resistance mechanisms<sup>30</sup>

#### 210 Limitations and conclusion

211 We acknowledge the lack of baseline data on temephos distribution in the villages sampled. While  
212 temephos distribution has been acknowledged as the main outbreak response tool in Cambodia<sup>3</sup>, the  
213 timing and concentrations used in the villages sampled in this study were not discriminated. Hence, we  
214 cannot fully characterize the existing pre-conditions of each village in terms of previous larviciding  
215 activities, but temephos distribution is organized annually at a national and province scales. Likewise,  
216 pyrethroid based interventions like thermal fogging, long lasting insecticide nets (LLIN) usage and  
217 pyrethroid based aerosol spray use was not characterized during field collection, limiting the possibility  
218 to ascertain potential drivers for the resistance patterns registered.

219 Nevertheless, our results and those of neighboring countries are alarming. From a regional point of  
220 view, it seems essential to rapidly change control methods and replace temephos with another larvicide  
221 that remains to be determined. Finally, and perhaps most worrying, it seems that in the event of an

222 epidemic the adulticides used in the Southeast Asia region are no longer effective. We must quickly find  
223 an alternative.

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For Peer Review

319 Table 1. Mean Lethal Concentration (LC) 50 and LC90 ( $\pm$  SE) of 8 *Aedes aegypti* larval populations with  
 320 temephos in Cambodia. RR50 and RR90 represent the resistance ratio of the field populations compared  
 321 to the USDA susceptible reference strain. <sup>a</sup> USDA strain: LC50 = 0.0037  $\pm$  0.00008 mg/L ; LC90= 0.0047  $\pm$   
 322 0.0001mg/L

Environment	Populations <sup>a</sup>	LC50 (SE)	RR50	LC90 (SE)	RR90
Urban	Phnom Penh	0.020 (0.0006)	5.4	0.028 (0.0008)	6.0
	Siem Reap	0.014 (0.0008)	3.8	0.020 (0.0008)	4.2
	Kampong Cham	0.031 (0.0012)	8.4	0.052 (0.0025)	11.1
	Battambang	0.125 (0.0044)	33.8	0.221 (0.0082)	47.0
Rural	Phnom Penh	0.014 (0.0007)	3.8	0.031 (0.0011)	6.6
	Siem Reap	0.012 (0.0006)	3.3	0.021 (0.0010)	4.4
	Kampong Cham	0.048 (0.0015)	13.0	0.066 (0.0029)	14.0
	Battambang	0.041 (0.0015)	11.1	0.064 (0.0031)	13.6

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324 Supplementary File 2. Percentage of mortality ( $\pm$  SE) of 8 *Aedes aegypti* adult populations to  
 325 Deltamethrin and Permethrin. In bold are represented the significant differences of mortality between  
 326 bioassays with and without PBO.

<i>Ae. aegypti</i> populations	Without PBO		With PBO	
	Deltamethrin	Permethrin	Deltamethrin; p-value	Permethrin; p-value
Battambang rural	88.0 (5.1)	0.0 (0.0)	<b>97.8 (2.6); 0.014</b>	3.3 (6.5); 0.355
Battambang urban	59.6 (3.7)	0.0 (0.0)	<b>80.6 (9.2); 0.006</b>	1.2 (2.4); 0.355
Kampong Cham rural	70.0 (8.9)	1.1 (2.2)	71.8 (15.6); 0.844	<b>18.6 (4.4); 0.0003</b>
Kampong Cham urban	90.8 (2.3)	3.9 (5.4)	<b>98.8 (2.4); 0.003</b>	7.5 (3.4); 0.300
Phnom Penh rural	7.1 (2.1)	0.0 (0.0)	9.9 (7.6); 0.509	0.0 (0.0); -
Phnom Penh urban	8.3 (9.0)	0.0 (0.0)	7.3 (7.1); 0.867	0.0 (0.0); -
Siem Reap rural	6.3 (5.6)	0.0 (0.0)	<b>52.3 (12.6); 0.0006</b>	0.0 (0.0); -
Siem Reap urban	4.0 (3.1)	0.0 (0.0)	<b>24.6 (16.8); 0.047</b>	0.0 (0.0); -
USDA Sensitive Strain	100 (0.0)	100 (0.0)	100 (0.0); -	89.4 (21.2); 0.355

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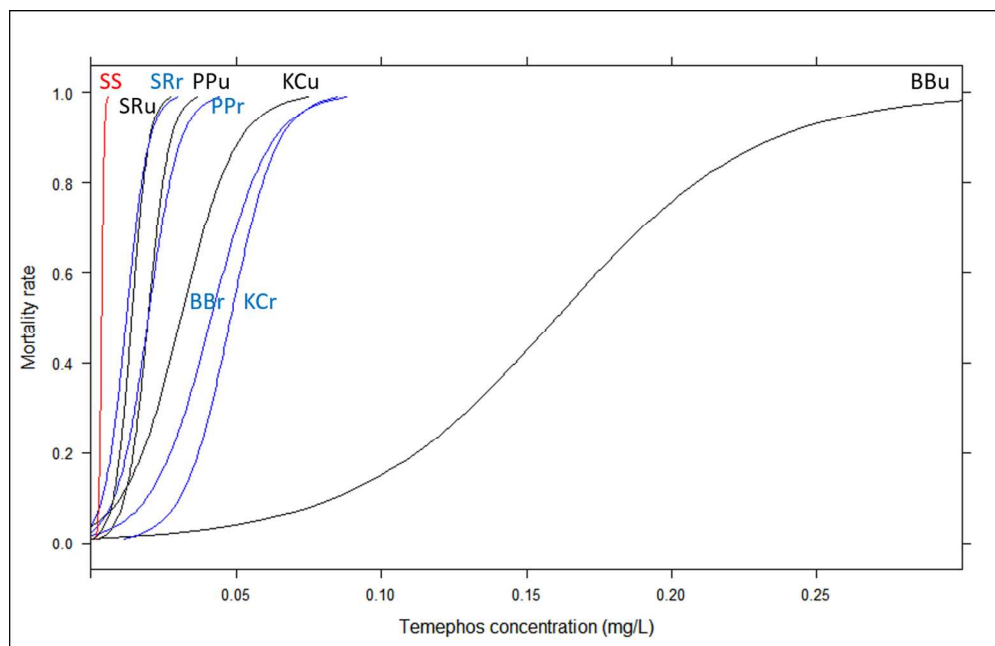


Figure 1. Mortality rate of *Aedes aegypti* larvae to temephos in the 4 provinces. The 4 urban populations are represented in black, the rural populations in blue. The red line is the Sensitive strain (SS). BB Battambang, KC Kampong Chan, SR Siem Reap, PP Phnom Penh. The small letters - "r" and "u" represent rural and urban areas, respectively.

228x147mm (150 x 150 DPI)

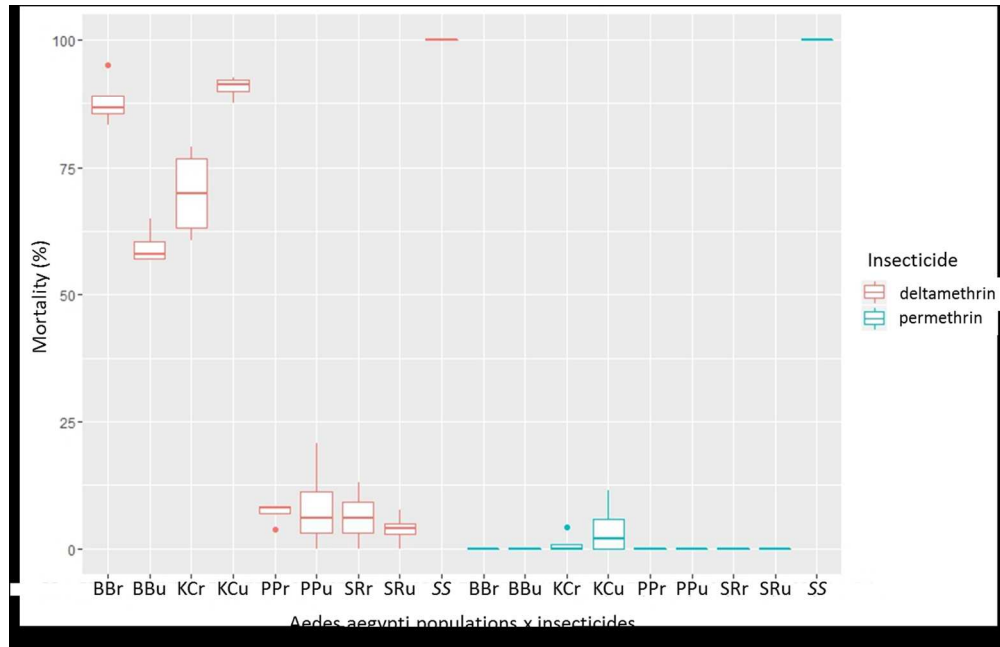
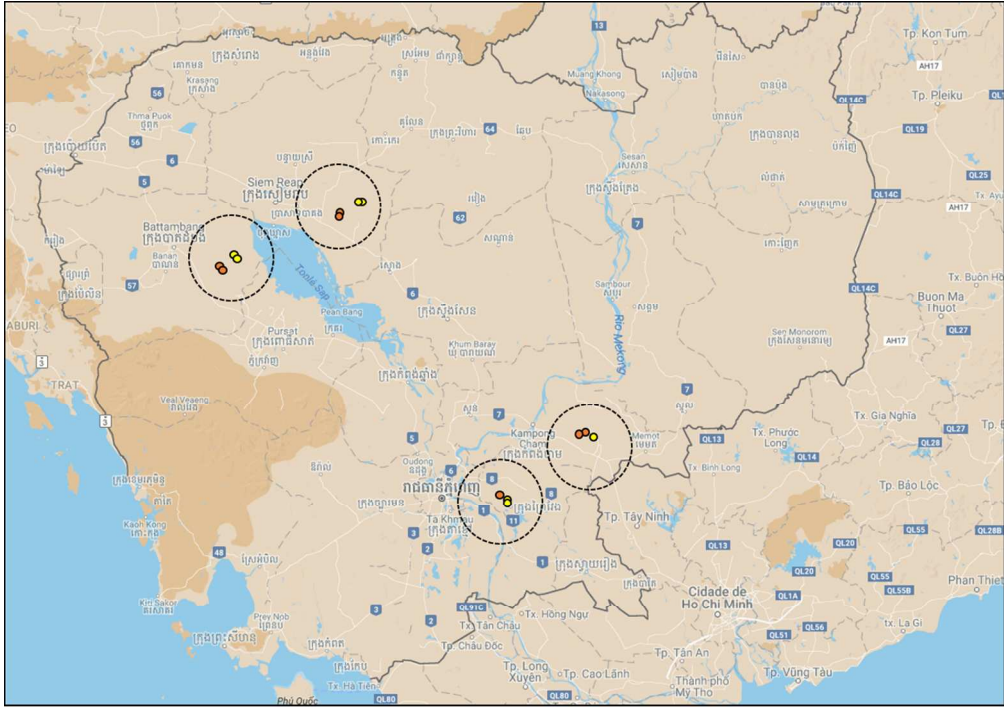


Figure 2. Mortality of *Aedes aegypti* populations to Deltamethrin and Permethrin. BB represents Battambang; KC Kampong Cham; PP Phnom Penh; SR Siem Reap; SS USDA Sensitive Strain. The small letters - "r" and "u" represent rural and urban areas, respectively.

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3 Figure 1. Mortality rate of *Aedes aegypti* larvae to tempehos in the 4 provinces. The 4 urban populations  
4 are represented in black, the rural populations in blue. The red line is the Sensitive strain (SS). BB  
5 Battambang, KC Kampong Chan, SR Siem Reap, PP Phnom Penh. The small letters - "r" and "u" represent  
6 rural and urban areas, respectively.  
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12 Figure 2. Mortality of *Aedes aegypti* populations to Deltamethrin (0.03%) and Permethrin (0.25%)  
13 following recommended WHO diagnostic doses. BB represents Battambang; KC Kampong Cham; PP  
14 Phnom Penh; SR Siem Reap; SS USDA Sensitive Strain. The small letters - "r" and "u" represent rural and  
15 urban areas, respectively.  
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22 Supplementary File 1. Location of collection sites in the 4 different provinces in Cambodia: Battambang,  
23 Siem Reap, Kampong Cham and Phnom Penh. The orange and yellow circles represent field collections in  
24 rural and urban areas, respectively.  
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8 **1 Resistance of *Aedes aegypti* (Diptera: Culicidae) populations to deltamethrin, permethrin**  
9 **2 and temephos in Cambodia.**

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12 **3 Abstract.**

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15 4 Dengue fever is a major public health concern, including 185,000 annual cases in Cambodia. *Aedes*  
16 5 *aegypti* is the primary vector for dengue transmission and is targeted with insecticide treatments. This  
17 6 study characterized the insecticide resistance status of *Ae. aegypti* from rural and urban locations. The  
18 7 susceptibility to temephos, permethrin and deltamethrin of *Ae. aegypti* was evaluated in accordance  
19 8 with WHO instructions. All the field populations showed lower mortality rate to temephos compared to  
20 9 the sensitive ~~strains~~strain with Resistance Ratio 50 (RR50) varying from 3.3 to 33.78 and RR90 from 4.2  
21 10 to 47 compared to the sensitive strain, demonstrating a generalized resistance of larvae to the  
22 11 temephos in Cambodia. *Ae. aegypti* adult populations were highly resistant to permethrin regardless of  
23 12 province or rural/urban classification with an average mortality of 0.02%. Seven of the eight field  
24 13 populations showed resistance to deltamethrin. These results are alarming for dengue vector control, as  
25 14 widespread resistance may compromise the entomological impact of larval control operations.  
26 15 Innovative vector control tools are needed to replace ineffective pesticides in Cambodia.  
27 16 **Keywords.** *Aedes aegypti*; Cambodia; insecticide; mosquito; resistance; vector control.



## 17 Introduction

18 Dengue fever is a major public health concern, with estimates of 400 million cases every year in urban,  
19 suburban and rural tropical areas.<sup>1</sup> In Cambodia, around 185,000 cases are estimated annually.<sup>2</sup> The  
20 primary vector for dengue transmission is *Aedes aegypti* ~~that~~which favors environments where water  
21 storage is abundant and solid waste disposal is deficient.<sup>3</sup> As *Ae. aegypti* is implicated in the  
22 transmission of arboviruses such as Zika, Chikungunya and Yellow fever<sup>4</sup>, vector control strategies that  
23 target *Ae. aegypti* populations may have an major public health impact. Many insecticides have been  
24 used in order to control *Ae. aegypti* populations, but little information exists on the susceptibility of  
25 Cambodian populations to the most commonly used insecticides.

26 As early as 1955, DDT residual spray was used in the first malaria eradication pilot in Snuol district<sup>5</sup>  
27 ~~followed by. DDT was again used in~~ public health programs targeting malaria and dengue in urban and  
28 rural areas and at UNHCR refugee camps along the Cambodia-Thailand border ~~during~~from 1981 to 1987,  
29 after which ~~DDT~~it was no longer imported<sup>6</sup>. Pyrethroids, particularly permethrin and deltamethrin, were  
30 introduced to Cambodia in the late 1980s and 2000<sup>7</sup>, for the control of malaria (impregnation of bednets)  
31 and dengue (thermal fogging and ULV sprays), respectively<sup>6</sup>. Since 1992, Temephos ~~was~~has been  
32 imported ~~from 1992 to present~~ with roughly 200 tons per year used mainly for larval control of dengue  
33 vectors<sup>6</sup>. In 1966, Mouchet and Chastel showed total susceptibility of *Ae. aegypti* to DDT, fenthion,  
34 malathion and diazinon insecticides, but observed resistance to dieldrin and gamma HCH<sup>7</sup>. More  
35 recently, *Ae. aegypti* resistance to temephos was also investigated during two field studies in  
36 Cambodia<sup>8</sup>. The resistance pattern and future of temephos is increasingly important as this larvicide has  
37 been the main dengue control strategy used by National Dengue Control Program (NDCP) for more than  
38 20 years and for biannual larvicide campaigns since 2001<sup>3,6</sup>.

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9 39 Using the WHO diagnostic dose (0.02mg/L), the Phnom Penh population tested in 2001 was found to be  
10 40 resistant to temephos (~~LC50=0.02mg/l and LC95=0.03mg/L~~), while Kampong Cham population was still  
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12 41 susceptible (~~LC50=0.009mg/l and LC95=0.015mg/L~~). More recently, among seven *Ae. aegypti*  
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14 42 populations, six were found to be resistant to temephos with mortality ranging from 11.02% up to  
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16 43 88.62% at the WHO diagnostic concentration (To Setha, Pers. Comm.). While it seems clear that that  
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18 44 temephos resistance among *Ae. aegypti* populations has increased over last year's time in Cambodia, the  
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20 45 patterns between rural and urban areas are not clear as delineated.  
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22 46 While pyrethroid and organophosphate insecticides are used in the national malaria and dengue control  
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24 47 programs, significant use of insecticides (including larvicides, repellents, space sprays, treated materials  
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26 48 and coils) at home and in the private sector results in unquantifiable use of insecticides. Coupled with  
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28 49 the lack of information of on adult resistance status in Cambodia and long-term usage of space spraying  
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30 50 by pest control companies and public health outbreak response, it is timely to characterize authorities,  
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32 51 the need for characterizing the susceptibility of *Ae. aegypti* to ~~pyrethroids~~ pyrethroids is urgent. This  
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34 52 study aims to characterize the insecticide resistance status for immature and adult stages of *Ae. aegypti*  
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36 53 collected from rural and urban Cambodian environment. ~~Using WHO test procedures, 8~~ Eight field  
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38 54 populations were tested ~~with~~ using WHO test procedures against the most commonly used insecticides  
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40 55 in Cambodia: which include temephos (for immature stages), and deltamethrin ~~and~~ /permethrin (for  
41  
42 56 adult stages).

## 57 **Material and Methods**

### 58 Mosquito collection:

59 Four different geographical areas in Cambodia were selected for field sample collections: ~~(Phnom Penh,~~  
60 Kampong Cham, Battambang and Siem Reap. ~~Within each Province, two~~. Two urban villages ~~in an~~  
61 urban setting and two rural villages were selected as collection ~~point~~. points within each village. Villages

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9 62 were selected by ~~Cambodian National Center for Entomology, Parasitology, and Malaria Control~~  
10 63 ~~(CNM)NDCP~~ according to geographical representation, dengue incidence and ~~history of~~ recent use of  
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12 64 temephos (~~within the~~ previous two years) (Supplementary File 1).  
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14 65 ~~In each village, 25 Twenty five~~ households were randomly selected ~~within each village~~ and all containers  
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16 66 were inspected for larvae and pupae, using direct pipetting for small containers and sweep net method  
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18 67 for large containers<sup>9</sup>. Collected larvae/pupae were pooled by location (rural/urban) in each province and  
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20 68 transported to an insectary.  
21  
22 69 Larvae and pupae were reared in standard conditions (temperature: 28±1°C; relative humidity: 75 ±  
23  
24 70 25%; photoperiod: 12 hours day/night) in 24.8 x 19.7 x 3.8 cm standard white plastic larval tray  
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26 71 containing 2 liters of purified water and fed with half a teaspoon of grounded fish food daily until adult  
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28 72 emergence. Adult *Aedes* were separated from other species by direct aspiration and each population  
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30 73 was separated by location (total of 8 populations from 4 Provinces).  
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32 74 For both larvae and adults assays, a USDA reference susceptible strain<sup>10</sup> was used as positive and  
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34 75 negative control with water and ethanol in plastic beakers. ~~LC50 and LC90 results obtained were used to~~  
35 76 ~~calculate Resistance Ratios (RR).~~  
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37 77 Rearing of F1 larvae for testing:  
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40 78 Adult *Aedes* ~~mosquitomosquitos~~ from parental ~~generationgenerations~~ were reared at standard  
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42 79 conditions and fed with 10% sucrose solution. All populations were also provided with lab reared mice  
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44 80 for blood meal once ~~in~~ every three days for 3-4 hours. Eggs ~~offrom the~~ F1 generation were collected on  
45 81 white filter paper ~~and~~ placed inside black plastic cups. Eggs were dried and stored in envelopes and later  
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47 82 sent to ~~the~~ laboratory. F1 eggs were ~~later~~ immersed in water according to assay needs for testing  
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49 83 procedures and larvae were reared as previously described.  
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84 *Ae. aegypti* larval bioassays:

85 In accordance with WHO instructions<sup>11</sup>, late third instar larvae of F1 generation were used for

86 determining the resistance of mosquito larvae to temephos. ~~Larvae showing any abnormalities were~~  
87 ~~discarded before the experiment.~~

88 Temephos (Sigma, Pestanal analytical grade, 250 mg) was diluted in ethanol to produce a stock solution  
89 of 1000 mg/L. The main stock solution was diluted into ~~other solutions several working concentrations~~  
90 better suited for testing, ~~denominated as stock solutions.~~ All solutions were stored in glass bottles and  
91 labeled accordingly. ~~To determine Lethal Concentrations 50 and 90 (LC 50, LC 90), six temephos~~  
92 ~~concentrations were used: 0.2, 0.05, 0.03, 0.02, 0.01, 0.004 mg/L.~~ To obtain each of ~~this concentration,~~  
93 ~~we pipetted these concentrations~~ the adequate volume of temephos ~~was pipetted~~ from stock solutions,  
94 ~~completing adding~~ the remaining ~~value amount of solution~~ with ethanol into each beaker containing 99  
95 ml of water. Four replicates were used for every concentration, and each replicate ~~consist~~consists of 25  
96 larvae.

97 Six temephos concentrations (0.2, 0.05, 0.03, 0.02, 0.01, 0.004 mg/L) were used to determine Lethal  
98 Concentration (LC) 50/95 (e.g. the necessary concentrations needed to kill 50%/95% of mosquito  
99 larvae). Resistance ratios (RR50 and RR95) were calculated dividing LC<sub>50</sub> and LC<sub>95</sub> rates from *Ae. aegypti*  
100 field populations by the LC<sub>50</sub> and LC<sub>90</sub> rates of the USDA susceptible strain.

101 *Ae. Ae. aegypti* adult bioassays:

102 *aegypti* adult bioassays

103 Insecticide resistance screening for adult ~~mosquito~~mosquitos was conducted using the WHO tube  
104 assay<sup>11</sup>. Two synthetic pyrethroids; permethrin and deltamethrin, at diagnostic concentrations  
105 appropriate for *Aedes* mosquitoes were used. WHO tube kit and impregnated permethrin (0.25%),

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9 106 deltamethrin (0.03%) and piperonyl butoxide for synergist assay (PBO 4%) papers were obtained from

10 107 Vector Control Research Unit (~~VCRU~~) inat the University of Science (~~USM~~), Penang, Malaysia. Diagnostic  
11  
12 108 and synergist concentrations were chosen following WHO recommendations<sup>11</sup>.

13  
14 109 For this bioassay, each tested population ~~useused~~ four tubes ~~contained~~containing Permethrin (0.25%),  
15  
16 110 four tubes ~~contained~~containing deltamethrin (0.03%), and four control tubes ~~contained~~containing  
17  
18 111 silicone oil paper. Twenty-five ~~adult with~~adults at least 3 days old and non-blood fed female mosquitoes  
19  
20 112 were introduced into each tube lined with untreated paper (holding tube) for 60 ~~min~~minutes.

21  
22 113 Mosquitoes ~~were~~ then transferred into ~~the~~ exposure tube and exposed to impregnated paper for 60  
23  
24 114 ~~min~~minutes. Mosquito Knock Down (KD) ~~mosquito were counted~~was measured at the end of the  
25  
26 115 exposure, after which mosquitoes were transferred back to the tube without insecticide. Mortality was  
27  
28 116 counted at the end of ~~a~~ 24 hours period and the resistance status was interpreted according to the WHO  
29  
30 117 protocol.

31 118 Insecticide-synergist assay using piperonyl butoxide (PBO) was conducted to measure the effect of pre-  
32  
33 119 exposure to a synergist on the expression of insecticide resistance. Adult *Aedes* were pre-exposed to  
34  
35 120 this synergist for one hour before exposure to insecticide. KD and mortality were recorded the same  
36  
37 121 way as standard tests.

38  
39 122 Data ~~Management~~management and statistical analysis:

40  
41 123 Knock down and mortality were registered at ~~4h~~1 hour and ~~24h~~24 hours post-exposure respectively.  
42  
43 124 RRs for larvae and ~~adults mosquito~~adult mosquitos were calculated by dividing the average mortality  
44  
45 125 found in each field ~~populations~~population by the mortality obtained with the USDA susceptible  
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47 126 reference strain.

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9 127 For larvae results, LC50 and LC90 were obtained by plotting the mortality using log probit analysis. LC50  
10 128 ~~and LC90 results obtained from field populations were then divided by results obtained in USDA strain~~  
11 129 ~~to obtain RRs for each field population.~~

12  
13  
14 130 Statistical analysis (ANOVA ~~then~~and mean comparison) were ~~realized~~completed to compare the  
15  
16 131 mortality of adults to permethrin and deltamethrin with or without the use of PBO. Graphs and data  
17  
18 132 analysis were done with R software<sup>12</sup>.

## 19 20 133 21 22 134 **Results**

### 23 24 135 Larval bioassays:

25  
26 136 The overall bioassay results for larvae are presented in Table 1. The highest LC50 and LC90 values were  
27  
28 137 obtained with Battambang urban populations ~~with~~(LC<sub>50</sub>=0.125±0.004 mg/L and LC<sub>90</sub>= 0.221±0.008 mg/L;  
29  
30 138 ~~followed by ) and~~ Kampong Cham (Table 1). ~~These two outlying provinces are distant from big urban~~  
31  
32 139 ~~centers and have experienced large outbreaks and significant outbreak responses. Hence, in~~ Phnom  
33  
34 140 Penh and Siem Reap, the LC<sub>50</sub> and LC<sub>90</sub> were lowest with LC<sub>50</sub> values comprised between 0.012 mg/L  
35  
36 141 (Siem Reap rural) and 0.020 mg/L (Phnom Penh rural).

37  
38 142 The RR for urban and rural populations of Siem Reap and Phnom Penh provinces were ~~mainly~~mostly  
39  
40 143 above the threshold which is defined as a resistant population with RR ≥ 5. RR values of Kampong Cham  
41  
42 144 and Battambang urban and rural populations were two and nine-fold higher than the threshold,  
43  
44 145 respectively. While these results may be linked to the continued distribution of temephos and  
45  
46 146 consequent exposure of populations to this chemical, it is of great concern that 2 out of 4 populations in  
47  
48 147 these two provinces registered RRs twice as high as the defined resistance threshold (Kampong Cham  
49  
50 148 Rural, RR=13.0; Battambang rural, RR=11.2) and one registered a RR 6 times higher than the defined  
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52 149 threshold (Battambang urban, RR=33.6).

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9 150 Higher lethal doses (LC<sub>50</sub> or LC<sub>90</sub>) are needed to kill *Ae. aegypti* larvae from Battambang and Kampong  
10 151 Cham populations as depicted on the four mortality curves on the right side of the graph compared to  
11 152 Siem Reap and Phnom Penh populations (Figure 1). Lastly all the field populations showed higher  
12 153 mortality curve patterns compared to the sensitive strain over a range of concentrations (Figure 1).

#### 16 154 Adult bioassays:

17  
18 155 Results showed a very high level of resistance to permethrin regardless of province or rural/urban  
19 156 classification (Figure 2; Supplementary File 2). The average mortality to permethrin at the WHO  
20 157 diagnostic dose is  $2.22\% \pm 0.02\% \pm 0.0002$  for all the populations. ~~All the~~ While all populations showed  
21 158 resistance to permethrin, six of the eight populations showed no mortality to permethrin while the 2 at  
22 159 all. The additional two Kampong Cham populations had 1.1% and 3.9% of mortality. Adult bioassays  
23 160 showed a significant difference in mortality to permethrin depending on the population and the  
24 161 presence of PBO (F=3.35; df=8; p=0.003), particularly a significant increase in mortality from 1.1% to  
25 162 18.6% in rural population from Kampong Cham province (Supplementary File 2).

26 163 ~~With deltamethrin, seven~~ Seven of the eight field populations ~~have had a mortality~~ percentage below  
27 164 90% of mortality due to deltamethrin, meaning that these populations are resistant ~~to deltamethrin~~. The  
28 165 average mortality of *Ae. aegypti* populations from Phnom Penh and Siem Reap provinces ranged  
29 166 between 4.0% and 8.3% only. A significant difference in mortality to deltamethrin among the five  
30 167 highest mortality populations (>52%) tested were observed in the presence of PBO (F=7.20; df=8;  
31 168 p<0.0001).

#### 32 169 **Discussion**

33 170 Resistance to temephos: ~~implication~~implications for ~~Public Health~~public health

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9 171 | Observed *Ae. aegypti* resistance to temephos is ~~fully~~ consistent with a recent ~~analysis among study~~  
10 172 | ~~where~~ 6 of 7 populations ~~showingshown~~ similar resistance in Cambodia (To Seta, pers. comm.); ~~the~~  
11 173 | ~~RR (at LC50).~~ The RR50 range of the 8 populations to temephos between 3.8 and 33.6 reflects the  
12 174 | intensity of insecticide control. In Thailand, despite mosquito resistance to deltamethrin and  
13 175 | permethrin, temephos is still an effective insecticide to control *Ae. aegypti* larvae<sup>13</sup>. On the basis of data  
14 176 | showing ~~temephos~~ resistance in Phnom Penh ~~since over~~ 17 years<sup>8</sup>, ~~we suggest~~ a review of prevention  
15 177 | and control strategies, should be conducted and highlight the effects of reliance ~~of on~~ a single method of  
16 178 | control; ~~i. e. g. high levels of~~ temephos ~~which is the most widely used larvicide to control use in~~  
17 179 | ~~Cambodia<sup>14</sup> *Ae. aegypti*<sup>14</sup>; may compromise the entomological impact of larval control operations).~~  
18 180 | *Bacillus thuringiensis* var. *israelensis* (Bti) was tested with success in 2005 around Phnom Penh<sup>15</sup>. ~~In~~  
19 181 | ~~2016, a~~ new Bti strain AM65-52 was tested in 2016 against ~~Kandal~~ *Ae. aegypti* field population from  
20 182 | Kandal province that was resistant to temephos, ~~with~~. Results showed a reduction in the number of  
21 183 | pupae over 13 weeks, with an average 70% reduction during ~~13~~ the 8 first weeks<sup>16</sup>. The use of the  
22 184 | *Poecilia reticulata* (guppy) fish to control *Aedes* populations in water storage was tested in 2008 and  
23 185 | after one year, a 79% reduction ~~of in~~ *Aedes* larvae in community was observed with a presence of  
24 186 | guppies in only 57% of the containers<sup>17</sup>. In 2008, a new formulation of pyriproxifen was tested in water  
25 187 | containers against *Ae. aegypti* in Phum Thmei near Phnom Penh<sup>18</sup>. ~~Their main result was~~ The study  
26 188 | identified an inhibition of adult emergence in treated jars reaching 90% for 20 weeks, and remaining  
27 189 | above 80% until the end of the study (34 weeks). In ~~2008, in~~ Kampong Cham ~~province~~ Province in  
28 190 | Cambodia, 2008 water jars were covered with LLIN Permanet 2.0 (insecticide = deltamethrin) without  
29 191 | significant ~~reduction~~<sup>17</sup> reductions in mosquitoes<sup>17</sup>, possibly explained by the strong resistance to  
30 192 | delamethrin that we observed in *Ae. aegypti* adults. A large-scale randomized trial comparing guppy and  
31 193 | COMBI (Communication for Behavioural Impact) in Kampong Cham showed 92.5 % reduction in larval-  
32 194 | positive containers and 76%-88% coverage with guppies after one year. A recently completed cluster

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9 195 randomized control trial showed that an integrated vector management approach using guppy fish  
10 196 (*Poecilia reticulata*), a new slow release pyriproxyfen matrix (Sumilarv® 2MR), and community  
11 197 engagement through a clear Community for Behavioral Impact (COMBI) strategy reduced indoor adult  
12 198 density ~~by 83% (pre-versus post-intervention) or 44% (intervention-versus control arms)<sup>19</sup>, roughly 50%~~  
13  
14 198 density by 83% (pre-versus post-intervention) or 44% (intervention-versus control arms)<sup>19</sup>, roughly 50%  
15 199 as compared to the control arm<sup>19</sup>. All of these methods focused on key containers, especially water  
16 199  
17 200 cement jars that produced approximately 95% of *Ae. aegypti* larvae and pupae<sup>9</sup> and should be  
18 200  
19 201 considered in Cambodia as a cost-effective replacement of temephos.  
20 201

21 202 Resistance to permethrin but susceptible to deltamethrin

22 202  
23  
24 203 ~~Resistance of *Ae. aegypti* to deltamethrin has public health implications. *Aedes aegypti* compare~~  
25 203  
26 204 ~~favorably~~ deltamethrin-resistant populations have been described in different countries in Asia<sup>20</sup>, Latin  
27 204  
28 205 America<sup>21</sup>, Africa<sup>22</sup>, Oceania<sup>23</sup>, ~~Caraibes<sup>24</sup>. Surprisingly, we did not observe the same pattern of~~  
29 205  
30 206 ~~resistance with permethrin and the Caribbean<sup>24</sup>. In our study, *Aedes aegypti* populations were either~~  
31 206  
32 207 totally resistant to deltamethrin (with two populations exhibiting zero mortality,) or ~~with had~~ tolerance  
33 207  
34 208 patterns. Recently, the same pattern was observed in Thailand: where *Ae. aegypti* F1 females were  
35 208  
36 209 susceptible to deltamethrin, but resistant to permethrin<sup>13</sup>. ~~In a recent review (Smith et al. 2016), there~~  
37 209  
38 210 ~~is a~~ substantial geographic variation exist to pyrethroid resistance, with lower adult resistance levels in  
39 210  
40 211 Asia, Africa and the USA ~~(based on both RRs and % mortality values), although. However~~ there is 250-  
41 211  
42 212 fold resistance to deltamethrin in Thailand<sup>25</sup>.  
43 212

44 213 In this study, an extremely strong resistance to permethrin was observed both with/without PBO  
45 213  
46 214 ~~experiment that which~~ seems to indicate that the resistance is already fixed. Comparatively, the  
47 214  
48 215 ~~results result~~ with deltamethrin and deltamethrin + PBO suggest the involvement of detoxifying  
49 215  
50 216 enzymes. ~~But However,~~ generally, multiple resistance between pyrethroids are possible, ~~and we could~~  
51 216  
52 217 ~~expect the intervention of the and it can be expected that there is a~~ *kdr* mutation for resistance in both  
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9 218 insecticides. As the mechanisms of resistance between permethrin and DDT are expected to be the  
10 219 same, via a *kdr* mutation<sup>26</sup>, the already existing DDT-resistance<sup>7</sup>, may explain the current fixed resistance  
11  
12 220 observed with permethrin. There are several *kdr* mutations common in *Aedes* species that synergize  
13  
14 221 with each other when they are associated<sup>27</sup>. ~~According to the point mutations the resistant phenotype~~  
15 222 ~~could not be the same according to the pyrethroids.~~ Heterozygous V1016G, and F1534F and F1534C  
16  
17 223 mutants were ~~findfound~~ in Thailand<sup>28</sup>, and the same mutation ~~were~~was also described ~~in South~~  
18 224 ~~of southern~~ China with ~~also~~ V1016G mutants<sup>29</sup>. ~~Whilst there~~There is substantial variation in *kdr* in the  
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20  
21 225 Southeast Asian region, ~~that has effects on resistance~~ (arising from different combinations of three  
22  
23 226 mutations: ~~-~~ S989P, V1016G and F1534C ~~-~~ in *Ae. aegypti*); ~~Although~~ there are other ~~few~~ mutations  
24  
25 227 detected in *Ae. aegypti* ~~but~~ they do not appear to have effect on resistance based on current evidence.  
26  
27 228 For example, ~~combinations of~~ F1534 ~~and/or,~~ C1534C ~~and /or,~~ V1016G ~~and /or,~~ S989P<sup>29</sup> are present in  
28  
29 229 Cambodia, and ~~acting may act~~ together with metabolic resistance. The resistance patterns to  
30  
31 230 deltamethrin and permethrin in the Cambodian villages ~~would~~ fit with ~~the~~ variation in frequencies of the  
32  
33 231 three mutations and especially in low 989/1016 but high 1534 in permethrin ~~(but not deltamethrin)~~  
34  
35 232 resistant locations, ~~but~~ higher 989/1016 in Phnom ~~PengPenh~~ and Siem Reap, ~~(perhaps in combination~~  
36  
37 233 with 1534, ~~though they tend to occur on alternate chromosomes so co-occurrence is uncommon,~~  
38 234 ~~although this has certainly been observed. These will be key mutations to screen, perhaps with a little~~  
39 235 ~~sequencing to check whether others are found — the *Ae. aegypti* sodium channel is full of non-~~  
40  
41 236 ~~synonymous mutations — which is very strongly resistance-associated, and resistance links are yet to be~~  
42  
43 237 ~~established-).~~  
44  
45 238 Our results question the resistance mechanisms, ~~and even if classically expressed, the multiple~~  
46  
47 239 ~~resistance is considered as evident when talking about pyrethroids, it shouldn't or requires a different~~  
48  
49 240 ~~explanation.~~ Indeed, ~~for instance~~, the absence of correlation between permethrin and deltamethrin

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9 241 may involve different effects induced by type I Pyrethroid (permethrin) and a pseudo pyrethroid  
10 242 (nonester pyrethroid; deltamethrin), and so different resistance mechanisms<sup>30</sup>.

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13 243 Limitations and conclusion

14  
15 244 We acknowledge the lack of baseline data on temephos distribution in the villages sampled. While  
16  
17 245 temephos distribution has been acknowledged as the main outbreak response tool in Cambodia<sup>3</sup>, the  
18  
19 246 timing and concentrations used in the villages sampled in this study were not discriminated. Hence, we  
20  
21 247 cannot fully characterize the existing pre-conditions of each village in terms of previous larviciding  
22  
23 248 activities, but temephos distribution is organized annually at a national and province scales. Likewise,  
24  
25 249 pyrethroid based interventions like thermal fogging, long lasting insecticide nets (LLIN) usage and  
26  
27 250 pyrethroid based aerosol spray use was not characterized during field collection, limiting the possibility  
28  
29 251 to ascertain potential drivers for the resistance patterns registered.  
30  
31 252 Nevertheless our results as well as the results of the neighboring countries are alarming. From a regional  
32  
33 253 point of view, it seems essential to rapidly change control methods based on the use of a larvicide and  
34  
35 254 to replace the temephos with another larvicide that remains to be determined. Finally, and perhaps  
36  
37 255 more worrying, it seems that in the event of an epidemic, the adulticides used in the South East Asia  
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39 256 region are no longer effective. We must quickly find an alternative.  
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352 | Table 1. Mean Lethal Concentration (LC) 50 (~~LC50~~) and LC90 ( $\pm$  SE) of 8 *Aedes aegypti* larval populations  
 353 | with temephos in Cambodia. RR50 and RR90 represent the resistance ratio of the field populations  
 354 | compared to the USDA susceptible reference strain. <sup>a</sup> USDA strain: LC50 = 0.0037  $\pm$  0.00008 mg/L ;  
 355 | LC90= 0.0047  $\pm$  0.0001mg/L

Environment	Populations <sup>a</sup>	LC50 (SE)	RR50	LC90 (SE)	RR90
Urban	Phnom Penh	0.020 (0.0006)	5.4	0.028 (0.0008)	6.0
	Siem Reap	0.014 (0.0008)	3.8	0.020 (0.0008)	4.2
	Kampong Cham	0.031 (0.0012)	8.4	0.052 (0.0025)	11.1
	Battambang	0.125 (0.0044)	33.8	0.221 (0.0082)	47.0
Rural	Phnom Penh	0.014 (0.0007)	3.8	0.031 (0.0011)	6.6
	Siem Reap	0.012 (0.0006)	3.3	0.021 (0.0010)	4.4
	Kampong Cham	0.048 (0.0015)	13.0	0.066 (0.0029)	14.0
	Battambang	0.041 (0.0015)	11.1	0.064 (0.0031)	13.6

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Supplementary File 2. Percentage of mortality ( $\pm$  SE) of 8 *Aedes aegypti* adult populations to Deltamethrin and Permethrin. In bold are represented the significant differences of mortality between bioassays ~~realized~~ with and without PBO.

<i>Ae. aegypti</i> populations	Without PBO		With PBO	
	Deltamethrin	Permethrin	Deltamethrin; <u>p-value</u>	Permethrin; <u>p-value</u>
Battambang rural	88.0 (5.1)	0.0 (0.0)	<b>97.8 (2.6); 0.014</b>	3.3 (6.5); 0.355
Battambang urban	59.6 (3.7)	0.0 (0.0)	<b>80.6 (9.2); 0.006</b>	1.2 (2.4); 0.355
Kampong Cham rural	70.0 (8.9)	1.1 (2.2)	71.8 (15.6); 0.844	<b>18.6 (4.4); 0.0003</b>
Kampong Cham urban	90.8 (2.3)	3.9 (5.4)	<b>98.8 (2.4); 0.003</b>	7.5 (3.4); 0.300
Phnom Penh rural	7.1 (2.1)	0.0 (0.0)	9.9 (7.6); 0.509	0.0 (0.0); -
Phnom Penh urban	8.3 (9.0)	0.0 (0.0)	7.3 (7.1); 0.867	0.0 (0.0); -
Siem Reap rural	6.3 (5.6)	0.0 (0.0)	<b>52.3 (12.6); 0.0006</b>	0.0 (0.0); -
Siem Reap urban	4.0 (3.1)	0.0 (0.0)	<b>24.6 (16.8); 0.047</b>	0.0 (0.0); -
USDA Sensitive Strain	100 (0.0)	100 (0.0)	100 (0.0); -	89.4 (21.2); 0.355

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