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RESEARCH ARTICLE

Assessment of the susceptibility status of *Aedes albopictus* (Diptera: Culicidae) from Interior, Sandakan and Tawau divisions of Sabah, Malaysia based on WHO diagnostic doses of larvicides

Elia-Amira, N.M.R.[1], Chen, C.D.[1,2*], Low, V.L.[3], Lau, K.W.[1], Haziqah-Rashid, A.[1], Amelia-Yap, Z.H.[3], Lee, H.L.[4], Azidah, A.A.[1], Sofian-Azirun, M.[1]

[1]Institute of Biological Sciences, Faculty of Science, Universiti Malaya, 50603 Kuala Lumpur, Malaysia
[2]Rimba Ilmu Botanical Garden, Universiti Malaya, 50603 Kuala Lumpur, Malaysia
[3]Higher Institution Centre of Excellence (HICoE), Tropical Infectious Diseases Research and Education Centre (TIDREC), Universiti Malaya, 50603 Kuala Lumpur, Malaysia
[4]Medical Entomology Unit, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia
*Corresponding author: chen_ctbr@um.edu.my

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ABSTRACT

Susceptibility status of *Aedes albopictus* (Skuse) sampled from residential areas in Interior, Sandakan and Tawau divisions of Sabah, Malaysia, was evaluated based on the WHO-recommended doses of organochlorine and organophosphate larvicides. To determine susceptibility status, larval bioassays were carried out and post 24-hour mortalities based on WHO resistance classifications were adopted. The results demonstrated that *Ae. albopictus* larvae were resistant toward 5 out of the 8 larvicides tested. Larvae from all populations were resistant against bromophos, fenitrothion, malathion, temephos and dichloro-diphenyltrichloroethane (DDT), with mortalities ranging from 0.00 to 89.33%. Dieldrin, on the other hand, could induce 100.00% mortalities in all populations, followed by fenthion and chlorpyrifos, with mortalities ranging from 97.33 to 100.00% and 81.33 to 100.00% respectively. Despite most populations exhibiting similitude in their resistance status, larvae from Sandakan exhibited the highest resistance level whereas the lowest level was observed in Keningau. In view of the inadequacy of some larvicides in controlling *Ae. albopictus* in this study, integrated management such as insecticide rotation or combination of interventions is warranted.

Keywords: *Aedes albopictus*; organochlorine; organophosphate; Sabah; Malaysia.

INTRODUCTION

To control *Aedes* mosquitoes, insecticide application targeting the immatures is often a popular option because larvae are the weakest link in *Aedes* life cycle and can be easily eradicated as compared to adults (Vector Disease Control International [VDCI], 2017). In terms of practicality, larvicide application could be easily mimicked by the community, thus it could reduce the reliance on health authorities to eradicate *Aedes* (Centers for Disease Control and Prevention [CDC], 2020). Nevertheless, the biggest hindrance in using insecticides is the continuous resistance build up (Ong, 2016). Described as the ability of insects to tolerate the deadly concentration for normal individual, resistance is a serious adversity that hampers many *Aedes* control programs and causes tremendous economy loss (WHO, 1957; Lum *et al*., 2008).

In Malaysia, insecticide resistance in *Aedes* mosquitoes was recorded as early as in the 1970th and ever since, numerous literatures depicted the severity of resistance in Malaysia, especially in the congested and developed cities in West Malaysia (Thomas, 1970; Selvi *et al*., 2006; Chen *et al*., 2013; Rahim *et al*., 2017). Nevertheless, a very miniscule number of studies have been conducted in the East part of Malaysia, specifically in Sabah.

The present authors investigated the larvicide and adulticide resistance status of *Ae. albopictus* in Sabah, Malaysia, and discovered substantial resistance against various types of insecticides (Elia-Amira *et al*., 2018, 2019). However, in the prior larvicidal study, only two divisions of Sabah (West Coast and Kudat divisions) were researched, leaving another three divisions unexplored. Therefore, this study aimed to determine the resistance status of *Ae. albopictus* larvae collected from six districts, representing

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the Interior, Tawau and Sandakan divisions against the eight WHO-recommended doses of larvicides from the organophosphate and organochlorine classes.

MATERIALS AND METHODS

Study Sites

Aedes albopictus was collected from 19 residential areas, across six districts located in Interior, Tawau and Sandakan divisions, Sabah, Malaysia (Figure 1). Collection sites were pooled based on district to give rise to the final six field samples. Collections were mostly conducted in suburban areas, with few rural areas involved. Suburban areas were the city outskirts (approximately of 10 km distance) with some basic infrastructures and moderately populated. Rural areas, on the other hand, were further away from the city (approximately 30 km distance) with lesser populations and infrastructures (Elia-Amira *et al*., 2018, 2019). Most study sites were the residential areas situated nearby palm oil plantations, except for a few localities in Sandakan and Tawau (Table 1).

Collection Method

Aedes collections were performed using ovitraps as described in our previous studies (Elia-Amira *et al*., 2018, 2019). Briefly, a 300 ml slightly tapered cylindrical plastic container (6.5 cm D base × 7.8 cm D opening × 9.0 cm H) was painted black and a paddle (10 cm H × 2.5 cm L × 0.3 cm W) serving as an oviposition site was put in each container. Dechlorinated tap water was filled in each ovitrap unit at 5.5 cm level and placed randomly outside the house compound, 25 cm apart from each other and on a ground level. In total, the numbers of ovitraps placed in a locality should exceed 10% of the total number of houses in that particular area. Ovitrap collection was conducted 5 days after the placement (Lee, 1992; Chen *et al*., 2005).

Colonization of Mosquitoes

To colonize *Ae. albopictus* larvae, method previously described was adopted (Elia-Amira *et al*., 2018). Collected ovitraps were processed in an insectarium, where their contents and paddles were transferred individually in each plastic container (6.5 cm D base × 7.8 cm D opening × 9.0 cm H). Clean

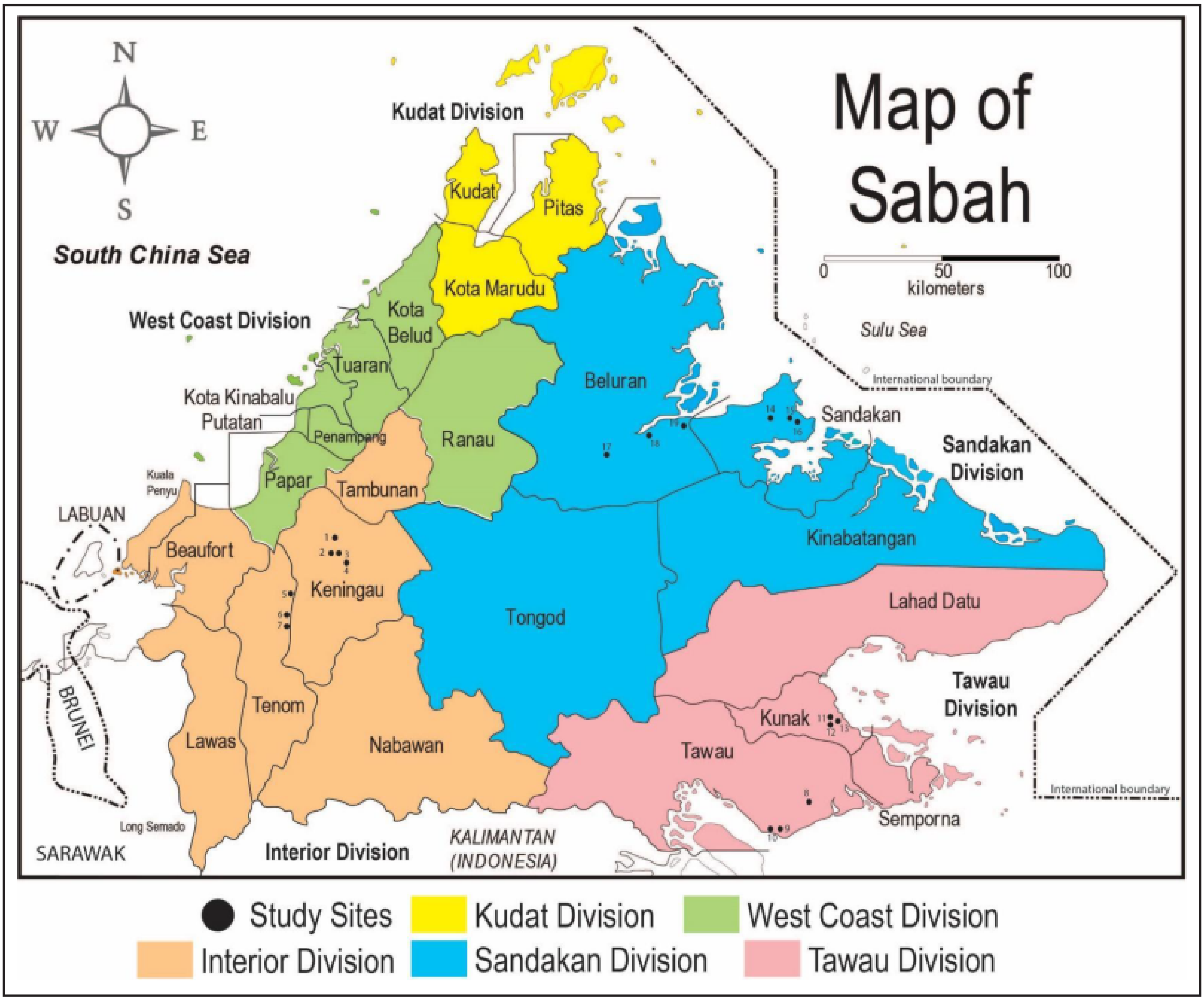

Figure 1. Location of study sites in Sabah, Malaysia. (1) Pekan Keningau; (2) Kampung Keningau; (3) Kampung Tuarid Taud; (4) Kampung Pampang; (5) Taman Ko-Pelajaran; (6) Jalan Ulu Kallang; (7) Pekan Tenom; (8) Taman Sawit; (9) Kampung Tinagat; (10) Taman Joying; (11) Simpang Empat Kunak; (12) Pekan Kunak; (13) Kampung Cenderawasih; (14) Kampung Gum-Gum; (15) Labuk Square; (16) Taman Utama; (17) Kampung Piuludan; (18) Kampung Ranpek Muanad; (19) Pekan Beluran.

Table 1. Geographical and ecological description of study sites

Division	District	Study Site	Coordinate	Elevation (m a.s.l.)	Residential Environment	Landscape
Interior	Keningau	Pekan Keningau	N 5°20"10.702", E 116°09’17.302"	283	Suburban	Nearby palm oil plantations
		Kampung Keningau	N 5°22’12.238", E 116°09’05.522"	362	Suburban	Nearby palm oil plantations
		Kampung Tuarid Taud	N 5°20’37.201", E 116°10’04.001"	284	Suburban	Nearby palm oil plantations
		Kampung Pampang	N 5°19’16.335", E 116°09’04.193"	261	Suburban	Nearby palm oil plantations
	Tenom	Taman Ko-Pelajaran	N 5°09’45.043", E 115°57’38.779"	223	Suburban	Nearby palm oil plantations
		Jalan Ulu Kallang	N 5°08’56.014", E 115°57’22.132"	201	Suburban	Nearby palm oil plantations
		Pekan Tenom	N 5°07’27.576", E 115°56’33.796"	193	Suburban	Nearby palm oil plantations
Tawau	Tawau	Taman Sawit	N 4°21’33.553", E 118°06’00.143"	61.0	Suburban	Nearby palm oil plantations
		Kampung Tinagat	N 4°14’23.426", E 117°58’02.539"	142.5	Suburban	Nearby palm oil plantations
		Taman Joying	N 4°14’44.311", E 117°53’45.084"	11.5	Suburban	Nearby town
	Kunak	Simpang Empat Kunak	N 4°40’25.679", E 118°12’53.941"	63	Suburban	Nearby palm oil plantations
		Pekan Kunak	N 4°40’58.568", E 118°14’48.729"	12	Suburban	Nearby palm oil plantations
		Kampung Cenderawasih	N 4°39’01.420", E 118°12’16.372"	156	Rural	Nearby palm oil plantations
Sandakan	Sandakan	Kampung Gum-Gum	N 5°53’32.750", E 117°55’16.334"	8	Suburban	Nearby palm oil plantations
		Labuk Square	N 5°53’09.434", E 118°01’17.739"	10	Suburban	Nearby town
		Taman Utama	N 5°51’46.108", E 118°03’33.816"	16	Suburban	Nearby town
	Beluran	Kampung Piuludan	N 5°48’45.309", E 117°37’41.806"	16	Rural	Nearby palm oil plantations
		Kampung Ranpek Muanad	N 5°49’41.507", E 117°33’24.764"	8	Rural	Nearby palm oil plantations
		Pekan Beluran	N 5°53’49.716", E 117°33’27.564"	10	Suburban	Nearby palm oil plantations

dechlorinated tap water and beef liver powder were added to each container to support the optimal growth of the larvae. Larvae were then reared to adulthood and sorted in cages (30 cm H × 30 cm L × 30 cm W) by species and localities. Only *Ae. albopictus* that was abundantly sampled as compared to *Ae. aegypti*, thus only a single species was tested in this study. Mosquitoes were supplemented with sucrose and B-complex solution and females of aged three days old were blood-fed using a BALB/C mouse. After blood feeding, oviposition sites made from plastic cups (4 cm D base × 7.5 cm D opening × 8 cm H) containing 200 ml dechlorinated tap water and lined with a 15 cm diameter filter papers were prepared for them to lay eggs. Laid eggs were removed for air drying at room temperature. Dried filter papers were later immersed into dechlorinated tap water in 1.5 L rectangular plastic containers (7.5 cm H × 16 cm L × 15 cm W) in order to hatch the eggs. Hatched larvae were designated as F1 and fed with beef liver powder. At least 50% of water in the containers was removed and replenished with clean water every two days to ensure the water was free from accumulated food debris. Late third or early fourth instar larvae of F1 were sorted out and subsequently used for larval bioassays. A laboratory strain of *Ae. albopictus* from the Institute for Medical Research (IMR), Kuala Lumpur, which has been colonized under insecticide free condition for 71 generations was used as reference strain.

Larvicide

Larvae were tested against the diagnostic doses of eight larvicides, namely bromophos (0.05 mg/L), malathion (0.125 mg/L), fenthion (0.025 mg/L), fenitrothion (0.02 mg/L), temephos (0.012 mg/L), chlorpyrifos (0.012 mg/L), dichloro-diphenyltrichloroethane (DDT, 0.012 mg/L), and dieldrin (0.050 mg/L) (WHO, 1981). In the case where the procured larvicide was too concentrated and the needed diagnostic dose was quite low, a serial dilution was carried out to produce lower dose of stock solution. One-time serial dilution of temephos was carried out in order to lower its procured dose of 312.5 mg/L to 31.25 mg/L (stock solution) and then the needed volume for its diagnostic dose (0.012 mg/L) was calculated using the dilution formula. The procured doses for other larvicides were as follows; bromophos (31.25 mg/L), malathion (8%), fenthion (31.25 mg/L), fenitrothion (31.25 mg/L), chlorpyrifos (6.25 mg/L), DDT (4%), and dieldrin (1%). Larvicides were procured from the WHOPES Collaborating Centre in Universiti Sains Malaysia, Penang, Malaysia.

Larval Bioassay

Larval bioassays were conducted in accordance with the WHO standard method (WHO, 1981). Bioassay was performed in a 300 ml cylindrical cup and a total of 25 late third or early fourth instar larvae were used. Larvae were exposed to the diagnostic dose of each larvicide, in a 250 ml dechlorinated tap water. For control, 1 ml ethanol was added to 249 ml dechlorinated tap water. Each bioassay test was replicated at least three times and was carried out at room temperature of 27±2°C and 75±10% relative humidity. Mortality was tabulated at 24 hours post exposure. Larvae were considered dead if they cannot move after being probed and sank to the bottom of the cup.

Data Analysis

Percentage of mortality was calculated by dividing the number of dead larvae by the total number of larvae tested. Mortality at 24-hour post treatment was used to determine the susceptibility status, where; 98-100% mortality indicated susceptibility, <98% mortality suggested the possibility of resistance that needs to be further confirmed, and <90% mortality suggested resistance (WHO, 2016). Should the mortality percentage of the control group had exceeded 5%, it was corrected using the Abbot's formula (WHO, 2016).

RESULTS

Mortality percentages of *Ae. albopictus* larvae are tabulated in Table 2. Among all organophosphate larvicides tested, fenthion was the most effective larvicide, with mortalities ranging from 97.33 to 100.00% in five populations. Chlorpyrifos, whereas was moderately effective, with half of the populations proven to be susceptible against it (81.33 to 100.00%). On the other hand, *Ae. albopictus* larvae from all populations were indiscriminately resistant toward bromophos, fenitrothion, malathion and temephos, with mortalities ranging from 0.00 to 89.33%. As for organochlorine larvicides (i.e., DDT and dieldrin), differing resistance status was observed. *Aedes albopictus* was resistant towards DDT with zero mortalities recorded for all populations, whereas 100% mortality was observed in dieldrin test.

Most *Ae. albopictus* populations exhibited same resistance trend, albeit various mortality ranges were observed. All populations exhibited total mortalities against malathion and DDT; and zero mortalities against dieldrin. *Aedes albopictus* from Sandakan showed the highest resistance level whereas Keningau population had the lowest resistance level.

DISCUSSION

All *Ae*. *albopictus* populations in the present study were resistant toward DDT, yet, susceptible against dieldrin. DDT has a stable half-life of more than 30 years, whereas dieldrin stands at approximately five years of persistence which may decipher resistance towards DDT and susceptibility towards dieldrin (WHO, 1989; Sava *et al*., 2007). Reintroduction of organochlorines via farming or illicit use of unregistered foreign household insecticide products may have reignited resistance (Ramachandran & Mourin, 2006). For instance, there were reports affirming illegal uses of organochlorine pesticides in farming in Kundasang, Sabah and Cameron Highlands, Pahang (Ramachandran & Mourin, 2006; Hossain *et al*., 2010). Furthermore, prior studies also detected substantial amount of organochlorine in water bodies and soil surrounding those agricultural areas (Zakaria *et al*., 2003; Saadati *et al*., 2012). In addition to their already persistent nature, incidence like this may further contribute to a rampant resistance towards organochlorines.

Majority of *Ae. albopictus* populations were susceptible toward fenthion, singling out only one population with possible resistant status (Tawau). Fenthion is a broad-spectrum insecticide used in many sectors to control sucking and biting pests (Vagi *et al*., 2017). Many previous publications depicted fenthion as a potent organophosphate insecticide, causing significant susceptibility among *Aedes* (Sharma *et al*., 2004; Mukhopadhyay *et al*., 2006; Tikar *et al*., 2008). Regardless of its potency, countries such as India and Australia have banned its use, whilst the USA has classified it under 'Restricted Use Pesticide' (United States Prevention Environmental Protection Agency [US EPA], 2001; Lloyd *et al*., 2010; Bonvoisin *et al*., 2020). The ban and restriction were partly because of its harsh toxicity towards the environment, especially on non-target organisms such as wild birds (US EPA, 2001). In Malaysia, fenthion, however, is more commonly used as an active ingredient in adulticiding as opposed to

Table 2. Percentage mortality of *Ae. albopictus* from Interior, Tawau and Sandakan Divisions, Sabah, Malaysia at 24 h post treatment against various larvicides

Study Sites	Larvicides							
	Organophosphates						Organochlorine	
	Bromophos 0.05 mg/L	Chlorpyrifos 0.012 mg/L	Fenitrothion 0.02 mg/L	Fenthion 0.025 mg/L	Malathion 0.125 mg/L	Temephos 0.012 mg/L	DDT 0.012 mg/L	Dieldrin 0.05 mg/L
	Percentage of Mortality (% ± SE)							
Reference Strain	82.67 ± 0.67 M	100.00 ± 0.00 S	100.00 ± 0.00 S	100.00 ± 0.00 S	0.00 ± 0.00 R	26.67 ± 0.88 R	0.00 ± 0.00 R	100.00 ± 0.00 S
	Interior Division							
Keningau	58.67 ± 2.60 R	100.00 ± 0.00 S	89.33 ± 1.20 R	100.00 ± 0.00 S	0.00 ± 0.00 R	5.33 ± 1.33 R	0.00 ± 0.00 R	100.00 ± 0.00 S
Tenom	56.00 ± 2.89 R	100.00 ± 0.00 S	77.33 ± 1.76 R	100.00 ± 0.00 S	0.00 ± 0.00 R	6.67 ± 1.67 R	0.00 ± 0.00 R	100.00 ± 0.00 S
	Tawau							
Tawau	33.33 ± 0.88 R	86.67 ± 0.88 R	77.33 ± 0.88 R	97.33 ± 0.33 P	0.00 ± 0.00 R	0.00 ± 0.00 R	0.00 ± 0.00 R	100.00 ± 0.00 S
Kunak	49.33 ± 0.88 R	94.67 ± 0.88 P	76.00 ± 0.58 R	100.00 ± 0.00 S	0.00 ± 0.00 R	0.00 ± 0.00 R	0.00 ± 0.00 R	100.00 ± 0.00 S
	Sandakan							
Sandakan	21.33 ± 0.33 R	81.33 ± 0.33 R	68.00 ± 0.58 R	100.00 ± 0.00 S	0.00 ± 0.00 R	0.00 ± 0.00 R	0.00 ± 0.00 R	100.00 ± 0.00 S
Beluran	42.67 ± 2.33 R	100.00 ± 0.00 S	81.33 ± 0.33 R	100.00 ± 0.00 S	0.00 ± 0.00 R	0.00 ± 0.00 R	0.00 ± 0.00 R	100.00 ± 0.00 S

P – possible resistance (mortality < 98%), R– resistance (mortality < 90%), S – susceptible (mortality ≥ 98%) as determined by WHO (2016).

larviciding (Ong, 2016). Similar to other states in Malaysia, temephos and *Bacillus thuringiensis* have also been used in larviciding programs in Sabah (Vythilingam & Wan-Yusoff, 2017).

On the other hand, Keningau, Tenom and Beluran populations were susceptible toward chlorpyrifos. Classified as moderately hazardous by WHO, chlorpyrifos is not a routine insecticide in *Aedes* control in Malaysia, as it is more common as an agricultural insecticide (Fang *et al*., 2006; WHO, 2010). In Malaysia, chlorpyrifos has been used by farmers in Cameron Highlands to control fungi in their cabbage farms and as a residual treatment against cockroaches in restaurants by pest control professionals (Lee, 1998; Ismail *et al*., 2017). Resistance against chlorpyrifos, may have transgressed due to the cross exposure from agricultural or residential areas.

All populations of *Ae. albopictus* larvae were resistant against bromophos, fenitrothion, malathion and temephos, with varying mortality percentages. Bromophos is also not included in the current regime of *Aedes* control in Malaysia and it is more synonymous as an agricultural pesticide (Wightman & Whitford, 1982). As of previously, bromophos was applied as a grain-protectant (wheat, barley) against grain-infesting insects and as housefly control in barns (Hansens *et al*., 1968; Green *et al*., 1970). However, to date, in Malaysia, there is nil record on bromophos use in agricultural sector (Wan-Norafikah *et al*., 2020). Resistance against bromophos could have stemmed from cross-resistance to other commonly used organophosphate insecticides, as they have overlapping mechanism of action (Li & Liu, 2010). It is also possible that bromophos-resistant *Aedes* mosquitoes may have been transported from other countries via ships or planes (WHO, 2017). Nevertheless, widespread bromophos resistance in *Aedes* larvae was also documented (Elia-Amira *et al*., 2018; Haziqah-Rashid *et al*., 2018; Wan-Norafikah *et al*., 2020).

Like fenthion, fenitrothion is also a broad-spectrum insecticide, used concurrently in many agricultural and public health sectors (Price & Weighton, 1971; Ong, 2016). In agricultural sectors, it is in fact a registered protective insecticide used in the treatment of stored grains against pests like rice weevil (Vásquez-Castro *et al*., 2012). It was also used in public health sector to control *Anopheles* in Indonesia and *Aedes* in Thailand (Bang *et al*., 1969; Gandahusada *et al*., 1984). Similarly, in Malaysia, fenitrothion is commonly used in adulticiding program, and inter-changeably with fenthion, to control *Aedes* mosquitoes (Ong, 2016). Its routine and extensive uses in many sectors, may have triggered development of resistance against it among *Aedes* mosquitoes (Hidayati *et al*., 2011; Elia-Amira *et al*., 2018; Haziqah-Rashid *et al*., 2018; Wan-Norafikah *et al*., 2020).

On the other hand, malathion and temephos are the staples in *Aedes* eradication programs in Malaysia. Temephos has been relentlessly used in Malaysia since 1970's and considered a precedent in larviciding program (Chen *et al*., 2005). Malathion, in contrast, has been commonly used in *Aedes* adulticiding program in Malaysia (Ong, 2016). However, in 1996, malathion was put to a halt, as communities unwelcomed its unpleasant smell and oily residues, sticking on the wall and floor and was later replaced by water-based pyrethroid fogging formulations (Health Technology Assessment Section, Medical Development Division, Ministry of Health Malaysia, 2018). Nonetheless, use of malathion in rotation with pyrethroid formulation was adopted in recent *Aedes* control program in Malaysia (Wan-Norafikah *et al*., 2020). Despite being mainly used as an adulticide, malathion could be trapped in larval breeding areas during fogging and somehow offering some exposure to the larvae (Ong, 2016). This warrants the investigation of larval resistance towards malathion. Nevertheless, evidence of temephos and malathion resistance among *Ae. albopictus* larvae are plethora and most cited their decades of use as the main reason for resistance development (Chen *et al*., 2005; Shafie *et al*., 2012; Elia-Amira *et al*., 2018).

Aedes albopictus larvae in all study sites generally displayed the same resistance pattern, despite some variations in mortality percentages were observed. Sandakan population was found to be the most resistant, exhibiting the least larval mortalities among all studied populations. In contrast, Keningau population showed the lowest resistance level. Sandakan is the third most populous city in Sabah (409,056 human population) and it is considered as one of the major towns in Sabah. In comparison, Keningau is in the Interior division of Sabah, which is governed by variegated landscapes with scattered and less populous population as compared to other districts (177,735 human population) (Department of Statistics Malaysia, 2010; Hawkes *et al*., 2019). Densely populated city is usually a dengue hub with high *Aedes* population and higher level of insecticide use (Li *et al*., 2018; Majid *et al*., 2020). Sandakan is no exception, as it recorded higher severe dengue incidence as compared to other districts (in this study), possibly explaining the higher resistance level among *Ae. albopictus* population (Murphy *et al*., 2020).

In this study, heterogeneity in toxicity levels can be observed with various degrees of mortalities. In comparison with our previous study, there were also some slight discrepancies in resistance status of *Ae. albopictus* larvae against the same larvicides. For instance, resistant towards fenthion in the current study was less pronounced, with mortalities ranging between 97.33 to 100.00%, as opposed to 74.67 to 100.00% mortalities reported previously (Elia-Amira *et al*., 2018). *Aedes albopictus* in search for blood meal or oviposition sites may have led to their wide dispersal and subsequently given rise to progenies with various toxicity tolerance (Jirakanjanakit *et al*., 2007; Tikar *et al*., 2008).

Furthermore, insecticide resistance in this study may not just solely contributed by their heavy reliance in *Aedes* control, but also through their parallel use in other sectors, notably, agricultural (Reid & McKenzie, 2016). Being the biggest palm oil-producing state in Malaysia, Sabah's landscape has been heavily defined by palm oil plantations (Dayang Norwana *et al*., 2011). The same goes with most study sites in this study that were mostly residential areas situated nearby palm oil plantations (except few localities in Sandakan and Tawau) (Table 1). As such, to control pest infestation in the plantations, organophosphates such as malathion and chlorpyrifos have been usually applied (Myzabella *et al*., 2019). Unlike fogging and larviciding in *Aedes* control that are usually performed by authorized health personnel in a regulated manner, pesticide application in plantation areas, has been usually supervised by the plantation company or the owner themselves (Ong, 2016). The decision regarding pesticide concentration, frequency of application or type of pesticides were all decided internally (Wan-Norafikah *et al*., 2020). Aggravatingly, in small scale plantations, farmers were oblivious on correct handling of pesticide application (Sulaiman *et al*., 2019). They tend to re-use the same pesticide and increase its concentration when it is no longer effective. They also usually ignored the spray direction of pesticides and disposed the containers irresponsibly, which may increase the risk of water and soil contaminations (Ntow *et al*., 2006; Afari-Sefa *et al*., 2015). And lead to cross exposure to public health pests such as *Aedes*, making them resistant to these insecticides.

In short, this study uncovered the inadequacy of various larvicides in the control *Ae. albopictus* larvae in Sabah, Malaysia. Insecticide management such as rotation and combination of interventions, needs to be properly planned, taking into account the cross exposure from agricultural sector.

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Conflict of Interest

The author declares that they have no conflict of interests.

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