J Insect Behav (2017) 30:343–358
DOI 10.1007/s10905-017-9614-4

Behavioural and Electrophysiological Responses of Mosquito Vectors *Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus* to an Ethyl Ester: Ethyl 2-aminobenzoate

Johirul Islam[1,2] · Sunil Dhiman[3] · Varun Tyagi[3] · Sanjukta Duarah[1] · Kamaruz Zaman[2] · Pronobesh Chattopadhyay[1]

Revised: 3 April 2017 / Accepted: 10 April 2017 /
Published online: 18 April 2017

Abstract Mosquito control using different methods remains an integral component of intervention programmes which aim to protect humans from various mosquito-borne diseases. The host seeking behaviour of mosquitoes is essentially guided by odorant receptor neurons housed in the antenna, maxillary palps and proboscis. The odorant receptor neurons are responsible for detecting chemical cues from hosts and also useful for developing sustainable mosquito-control strategies that exploit host-seeking behaviours. The present investigation evaluates host seeking behavioural responses of a novel, non-toxic and environment friendly repellent, ethyl 2-aminobenzoate against three known vector species of mosquitoes viz. *Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus* maintained in laboratory. The flight orientation of the test mosquitoes was studied using Y-tube olfactometer, whereas the antennae of adult female mosquitoes were used to investigate the effect of ethyl 2-aminobenzoate on the peripheral olfactory system using electroantennogram (EAG). The findings demonstrate that ethyl 2-aminobenzoate exhibited significant response in Y-tube olfactometer against all the three known vector species of mosquitoes. However, only *Anopheles stephensi* significantly elicited responses in EAG experiments, while the responses obtained for *Aedes aegypti* and *Culex quinquefasciatus* were not statistically

The original version of this article was revised: Those "greater than symbols" as well as "less than symbols" especially in Tables 1 & 2 which were missing in the pdf version of the article are now corrected.

✉ Johirul Islam
johirulmon@gmail.com

[1] Division of Pharmaceutical Technology, Defence Research Laboratory, Tezpur, Assam 784001, India

[2] Department of Pharmaceutical Sciences, Dibrugarh University, Dibrugarh, Assam 786004, India

[3] Division of Medical Entomology, Defence Research Laboratory, Tezpur, Assam 784001, India

significant. The results conclude that currently evaluated chemical ethyl 2-aminobenzoate has potential against some well established mosquito vector species and could be exploited to develop new and comparatively more effective anti-mosquito formulations.

Keywords Electroantennogram · ethyl 2-aminobenzoate · flight orientation · mosquito · odorant receptor neurons

Introduction

Mosquitoes use their sense of smell to avoid repellents and to find hosts, nectar, and oviposition site (Ray 2015). Vector mosquito species are adapted to feed on human blood and have a major impact on public health by transmitting diseases such as malaria, dengue, yellow fever, filariasis, chikungunya, Japanese encephalitis, etc. (Fang 2010; Lupi et al. 2013). Recently, mosquitoes have also been found to transmit Zika virus that has been declared by World Health Organisation (WHO) as a public health emergency of international concerns (Dasti 2016; Fauci and Morens 2016; Ginier et al. 2016; Jamil et al. 2016; Wiwanitkit and Wiwanitkit 2016). Even in the absence of disease pathogens, mosquitoes create displeasure and can disrupt outdoor activities (Dickens and Bohbot 2013). Many studies have proved that application of odorants can potentially reduce disease transmission by preventing mosquito-human interactions (Alpern et al. 2016; Auysawasdi et al. 2016; Das et al. 2015; Diaz 2016). Odorants particularly act via extremely sophisticated olfactory systems of mosquito with hundred of receptor proteins from three different families: (I) ionotropic receptor (Ir); (II) odorant receptor (Or) and; (III) gustatory receptor (Gr) families (Menger et al. 2015). These receptors are most likely expressed in similar numbers of classes of odorant receptor neurons (ORNs) housed in sensilla on the antenna, maxillary palps and proboscis. The axons of the ORNs project to the antennal lobe (AL) in brain's deutocerebrum, where they innervate glomeruli, probably sorting according to their expressed receptors (Ghaninia et al. 2008; Hansson and Stensmyr 2011; Lavialle-Defaix et al. 2011; Song et al. 2012).

Typically, the host-seeking mosquitoes undertake quite a number of distinct behavioural steps: activation to fly upwind, navigation of the odor plume using olfactory cues and optomotor anemotaxis, navigating along odor plumes through surging and casting, close-range navigation toward skin, and landing (Carey et al. 2010). Along the way, they constantly discriminate host odors from background, and select amongst multiple acceptable hosts. These distinctive behavioural steps are perhaps guided by distinct sets of olfactory cues that are detected by independent olfactory pathways. Certain non-olfactory attraction cues such as humidity, temperature, and visual stimuli are also incorporated by mosquitoes at close range. Although, these steps are challenging to study, they also offer numerous opportunities to reduce host-seeking behaviour (Ray 2015). Extensive investigation has already been carried out to study the role of olfactory cues in shaping mosquito behaviour in response to host odor (Allan et al. 2006; Bernier et al. 2015; Webster and Cardé 2016). In those studies, the orientation (attraction/repellency) of flying mosquitoes towards an odor source was monitored by olfactometers. In certain experiments, a mosquito landing approach was used, in which the

number of mosquitoes landing on a treated substrate (e.g. human skin) was used to guess the attraction/repellency of the compound (Dube et al. 2011; Syed and Leal 2008).

Ethyl 2-aminobenzoate (EAB, CAS: 87-25-2), a new member in the realm of entomology, has drawn significant attention in repellent research in the recent years and is being considered as an improved alternative to DEET (Afify et al. 2014; Api et al. 2015; Guda et al. 2015; Islam et al. 2017; Kain et al. 2013; Leal 2014; Raphael et al. 2013). The repellency of EAB is due to the expression of green fluorescent protein (GFP) of column glumerulus, innervated by axons of an ionotropic receptor *Ir40a* expressing neurons of sacculus, a pit like structure in the antenna of fruit fly *Drosophila melanogaster* (Kain et al. 2013). One of the most interesting facts about *Ir40a* receptor proteins is that they are also highly conserved across several agricultural pests and insects as well, such as mosquitoes, head lice, *Tribolium,* etc. (Kain et al. 2013; Raphael et al. 2013). EAB fulfils all the requirements of an ideal repellent and, in comparison with other common repellents available in the market; EAB has the advantage of being approved by Food and Drug Administration (FDA), World Health Organization (WHO) and European Food Safety Authority (EFSA). Furthermore, EAB has been listed in the 'generally recognized as safe' (GRAS) list by the Flavour and Extract Manufacturer's Association (Flavors and Fragrances 2007; Kain et al. 2013). EAB is generally used in chewing gums and beverages as grape flavouring and odor and also as fragrance in soaps, detergents, creams, lotions and perfumes (Opdyke 1979).

Present investigation involves the evaluation of EAB by testing its influence on host seeking behaviour against three well known vector species of mosquitoes namely, *Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus* under laboratory settings as per standard protocol and procedure. The investigation provides more insight on the stimulatory effect of EAB on peripheral olfactory receptors in the antenna of the female tested mosquito species using electroantennogram (EAG).

Materials and Methods

Reagents and Chemicals

Ethyl 2-aminobenzoate was purchased from Sigma Aldrich (St. Luis, USA). Acetone (HPLC grade) was purchased from Merck Ltd. (Mumbai, India). All reagents and solvents used were of analytical grade.

Mosquitoes

The laboratory reared 5–7 days old adult female *Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus* mosquitoes were obtained from the laboratory resources, Division of Medical Entomology, Defence Research Laboratory, Tezpur, Assam, India. All mosquito species were maintained at the laboratory insectary at 27 ± 2 °C, 75 ± 5% RH and 14 L:10D h of light-dark alternative cycles in standard-sized wooden cages (750 mm X 600 mm X 600 mm) with a sleeve opening on one side as described previously (Seenivasagan et al. 2010). They were provided adequate nutrition with 10% sucrose solution ad libitum. All tests were carried out with the approval from Institutional Ethics Committee (IEC) obtained prior to the initiation of the experiments.

Standard Solutions Preparation

Standard stock solution of EAB (10,000 ppm) was prepared by dissolving appropriate amount of EAB in HPLC grade acetone. The solution of standard stock was stored at 4 °C (± 0.5). Working solutions were prepared fresh everyday by properly diluting the stored stock standard solution with HPLC grade acetone.

Orientation Experiments

The flight orientation experiments were performed on non-blood fed female *Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus* mosquitoes using Y-tube olfactometer as per the method described elsewhere (Guha et al. 2014) with some modifications to different doses of test chemical. Y-tube olfactometer with stem and arms of 22 cm in length and mean internal diameter of 1 cm was used for the experiments. Each arm was separated at the fork joint at an angle of 45° (Fig. 1). Mosquitoes were exposed to the EAB concentrations to which they are readily oriented to human hand. For the orientation experiments, aliquots of EAB (50, 500, 5000 ppm) were prepared fresh everyday by properly diluting the stored stock standard solution with HPLC grade acetone. For each replicate, 20 (twenty) female mosquitoes were taken

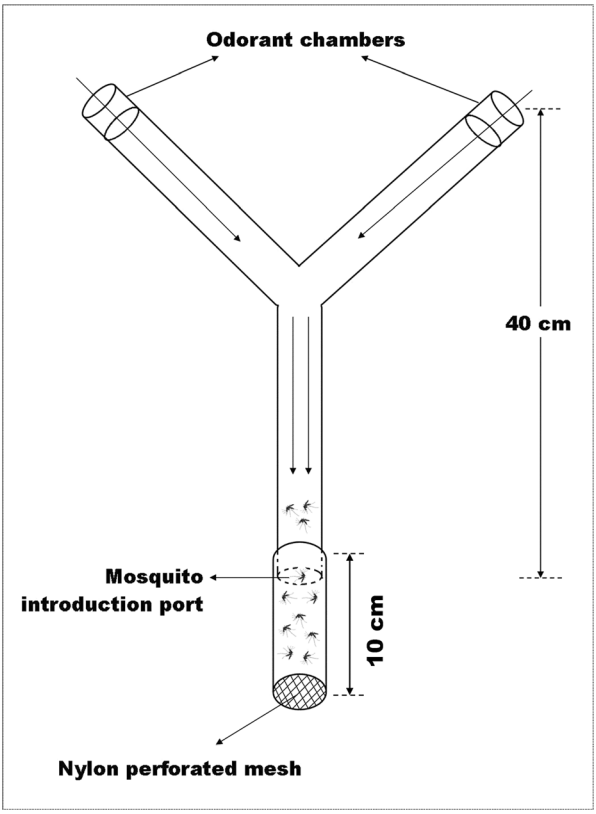

Fig. 1 Schematic drawing of customised Y-tube olfactometer with mosquito release chamber

in a customised external release chamber followed by acclimatization for 5 min. The diameter and length of the release chamber were 1.2 cm and 10 cm, respectively. The release chamber was designed in such a way that one end of the release chamber was attached to the stem of olfactometer, whereas the other end was covered with perforated nylon net to provide the mosquitoes with free access to external environment.

Fifty μL of pure EAB was applied on to a piece of adsorbent cotton and kept for about 2–3 min for complete evaporation of solvent. Then it was placed inside the treatment arm of the Y-tube olfactometer. At the same time, solvent treated (control) cotton piece was placed in the other arm of the Y-tube olfactometer. The temperature and relative humidity of the experiments was maintained at 27 ± 2 °C and 75 ± 5%, respectively. Orientation experiments for *Aedes aegypti* was conducted between 1000 h to 1600 h under high illumination. On the other hand, the orientation experiments for both *Anopheles stephensi and Culex quinquefasciatus* mosquitoes were conducted between 1900 to 2100 h at the onset of scotophase under low light condition. A card board was placed along the side of olfactometer to prevent any optical stimulation by the experimenter. The airflow was maintained at 1 L/min during the experiment by a pressure regulator, which was split into two halves by a T-splitter which carried the odor of test chemicals downwind of the olfactometer (Seenivasagan et al. 2012). At the same time, the mosquitoes were released by gently attaching one end of the release chamber to the stem of olfactometer. Five replicates (5 X 20 mosquitoes/replicate) were used for each concentration of EAB and the run time for each experiment was 5 min. Every time, a properly rinsed and dried olfactometer was used for each individual dose of the stimulus. Respective arms of the olfactometer were observed for the flight activity/orientation behaviour of the mosquitoes. The following formulas were used to calculate the preference index (PI), percentage control response (CR), percentage treatment response (TR) and percentage no response (NR) of the mosquitoes to the test chemical as described previously (Afify et al. 2014; Seenivasagan et al. 2012).

$$\text{Preference index (PI)} = \frac{T_n - C_n}{T_n + C_n}$$

$$\%\text{Control response (CR)} = \frac{C_n}{O_n} \times 100$$

$$\%\text{Treatment response (TR)} = \frac{T_n}{O_n} \times 100$$

$$\%\text{No response (NR)} = \frac{N_r}{O_n} \times 100$$

T_n Number of mosquitoes in test chamber
C_n Number of mosquitoes in control chamber
O_n Number of mosquitoes released in one replicate (i.e. 20)
N_r Number of mosquitoes not responding to either odor

Electroantennogram

The electroantennogram (EAG) experiments were performed according to the methods described in the previous study (Seenivasagan et al. 2010, 2012) with few modifications. Briefly, 5 (five) different excised antennas in five replicates of each 5–7 days old adult non-blood fed female *Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus* mosquitoes were collected and exposed to EAB to evaluate the peripheral olfactory response using an EAG instrument (Syntech, Netherland).

Antennal Preparation

Antenna of cold immobilized adult female mosquitoes were used for EAG studies. The head of each mosquito was clipped off at the neck through foremen magnum and the base of the excised head was mounted on indifferent electrode using electrode gel (Spectra 360, USA). The tip of one antenna was cut off and then connected to the recording electrode to obtain a stable base line in the oscillograph of the EAG software which indicates an ideal electrical contact of the antenna between electrodes.

Stimulus Delivery

The charcoal-filtered and humidified air (50 mL/min) was continuously delivered over the antennal preparation through a borosil glass tube (length: 15 cm, internal diameter: 0.5 cm) using a stimulus controller (Syntech, Netherland). A new hexane washed and oven dried (60 °C; 1 h) filter paper (5 cm X 1 cm) was used to adsorb the test chemical. Freshly prepared EAB concentrations (50, 500, 5000 ppm) in HPLC grade acetone were used for the experiments. EAB odor was delivered into the air stream through a side port located 10 cm from the end of the tube to ensure complete mixing of the stimulus odor with continuous air flow. A minimum quantity of air was puffed for about 0.5 s through the Pasteur pipette (Sigma) to stimulate the antenna.

Ten μL each of EAB (test chemical) concentration (50, 500, 5000 ppm) was applied on the filter paper followed by subsequent evaporation of the solvent for about 2 min. The filter paper loaded with pure EAB was then placed inside the Pasteur pipette for saturation of the air space. The stabilised isolated mosquito antenna was then presented to the puff of test concentration (stimuli) from lower to higher concentration for pulse duration of 0.5 s. For every individual stimulus a new Pasteur pipette was used and at least 1 min interval was given between each subsequent stimulation.

EAG Recording and Analysis

Each recording session was initiated by application of air, acetone (control), followed by increasing concentrations of test stimuli and terminated with reverse order of first two stimulations. To test the responsiveness of the antenna, standard acetic acid (10 μg) was puffed over the antenna at the start and finish of each recording session. The resulting amplified signals (10 X) were then directly imported into a personal computer via an IDAC interface box and an A/D converter. The EAG data were processed and analysed by using EAG software (version-2.6, Syntech, Netherland). Response to the

air puff was subtracted from the other succeeding EAG response to nullify any mechanical stimulation. The antennal responses of mosquitoes to the test chemical at different doses were compared with the solvent control based on the maximal EAG amplitude value.

Statistical Analysis

Preference index (PI), percentage control response (CR), percentage treatment response (TR) and percentage no response (NR) were calculated by the formula as described in Method section. The normalized EAG data generated in electrophysiological and flight orientation experiments were analyzed by analysis of variance (ANOVA) followed by Tukey test of multiple comparison. The difference between mean of control and treatments were examined by *t*- test to verify the significance of experimental data.

Results

Flight Orientation Responses

In Y-tube olfactometer, EAB elicited negative responses from all the three species of mosquitoes and this has been indicated by preference index (PI) (Fig. 2). The stimulus was presented in ascending order from lower to higher dose and the percentage repellency indicated a non-linear dose dependent response with increasing doses (Fig. 3). Increased numbers of mosquitoes took off from the releasing chamber and flew upwind inside the stem of Y-tube olfactometer. After reaching the fork junction, increased number of mosquitoes flew upwind into the acetone treated solvent control chamber. At least, 75% female *Aedes aegypti* mosquitoes were repelled by the plume of

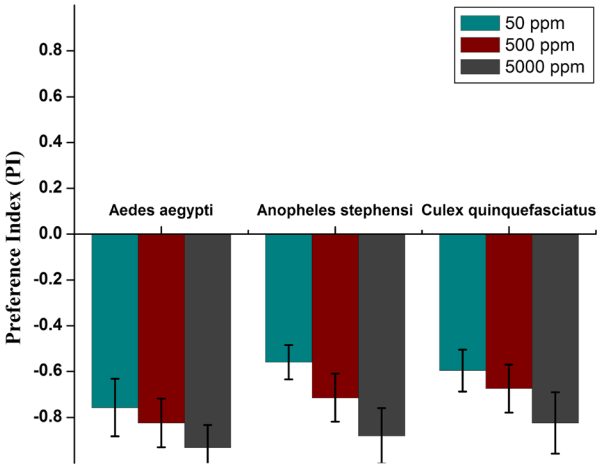

Fig. 2 Preference index of ethyl 2-aminobenzoate against *Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus*

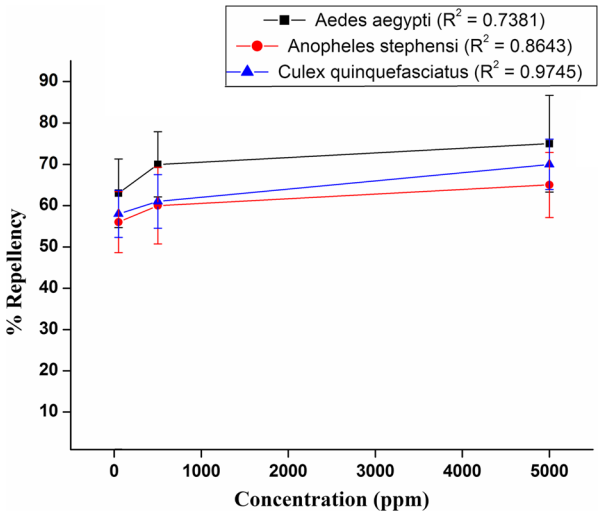

Fig. 3 Correlation between percent repellency and different concentrations of ethyl 2-aminobenzoate

5000 ppm EAB and entered the control chamber, whereas 500 and 50 ppm repelled 70 and 63% female, respectively. These results were found statistically different from control results. About 22–28% female *Aedes aegypti* remained idle inside the releasing chamber during the experiments. *Anopheles stephensi* also exhibited significant dose dependent decrease in orientation response, in which 65%, 60% and 56% female mosquitoes oriented away from the plume of 5000, 500 and 50 ppm EAB, respectively. However, 26–28% female *Anopheles stephensi* mosquitoes did not get trapped in any of the two treated upwind chambers throughout the experiments. While, 70%, 61% and 58% female *Culex quinquefasciatus* mosquitoes were significantly repelled by the plume of 5000, 500 and 50 ppm EAB, respectively. The number of *Culex quinquefasciatus* mosquitoes that did not respond throughout the experiments was between 23 and 27%. The results have been shown in Table 1.

Electroantennogram Responses

The EAG experiments were conducted to observe the sensitivity of peripheral olfactory receptors on the antennae of *Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus* mosquitoes to novel EAB (Fig. 4). The stimulus was presented in ascending order from lower to higher dose and the normalized EAG apparently indicated a non-linear dose dependent response with increasing doses (Fig. 5). A ~ 2-fold dose dependent EAG response at the highest dose of 5000 ppm was produced compared to the solvent control by the antenna of *Aedes aegypti*. The responses obtained in the EAG experiments were statistically non-significant. While, at the highest dose, antennal preparation of *Anopheles stephensi* produced almost ~3-fold higher response than the control and it was statistically significant. The EAG responses of *Culex quinquefasciatus* revealed a ~ 2-fold higher dose dependent response at the dose of 5000 ppm as compared to the response of solvent control but the response did not vary significantly (Table 2).

Table 1 Results of one-way ANOVA on flight orientation responses of *Aedes aegypti, Anophelese stephensi* and *Culex quinquefasciatus* to Ethyl 2-aminobenzoate

Flight orientation responses (Y-tube)								
Species	Concentration (ppm)	PI	CR (%)	TR (%)	NR (%)	*p*	*F*	R^2
Aedes aegypti	50	−0.75 ± 0.12	63.0 ± 8.3	9.0 ± 5.4	28.0 ± 10.95	< 0.01		
	500	−0.82 ± 0.10	70.0 ± 7.9	6.25 ± 4.7	23.0 ± 10.36	< 0.01	187.07	0.7381
	5000	−0.93 ± 0.09	75.0 ± 11.7	3.0 ± 4.47	22.0 ± 14.4	< 0.01		
Anopheles stephensi	50	−0.55 ± 0.07	56.0 ± 7.4	16.0 ± 4.01	28.0 10.36	< 0.01		
	500	−0.71 ± 0.10	60.0 ± 9.3	12.0 ± 5.7	28.0 ± 13.03	< 0.01	135.17	0.8643
	5000	−0.88 ± 0.12	65.0 ± 7.9	9.0 ± 4.1	26.0 ± 11.4	< 0.01		
Culex quinquefasciatus	50	−0.59 ± 0.09	58.0 ± 5.7	15.0 ± 5.0	27.0 ± 9.7	< 0.01		
	500	−0.67 ± 0.10	61.0 ± 6.5	12.0 ± 4.4	27.0 ± 8.3	< 0.01	117.09	0.9745
	5000	−0.82 ± 0.13	70.0 ± 6.1	7.0 ± 5.7	23.0 ± 8.3	< 0.01		

PI- Preference Index, CR- Control Response, TR- Treatment Response, NR- No Response, $\boldsymbol{p} < 0.01$ considered very Significant. All results are expressed as Mean ± SD

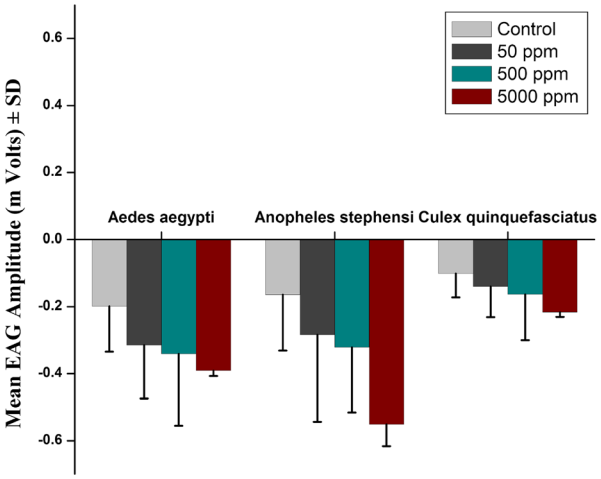

Fig. 4 Electroantennogram (EAG) responses of *Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus* to ethyl 2-aminobenzoate

Discussion

A repellent is a chemical odorant that produces a vapour barrier having an offensive taste and smell causing a responder to actively steer away from the stimulus source. Despite considerable advancement in the odorant research for reducing mosquito-borne disease transmission, there are considerable drawbacks in the attractants and repellents available currently for public use. In recent years, applicability of major essential oil based repellents such as citronella oil, lemongrass oil, eucalyptus oil, etc., as well as synthetic repellents such as diethyl phenyl acetamide (DEPA), diethyl benzamide (DEB), *N,N*-diethyl-meta-toluamide (DEET), etc. in the current market has abridged noticeably, and this can be attributed to their noxious effects, both on the environment and also on different non-target organisms (Ghosh et al. 2012). Although, many of these

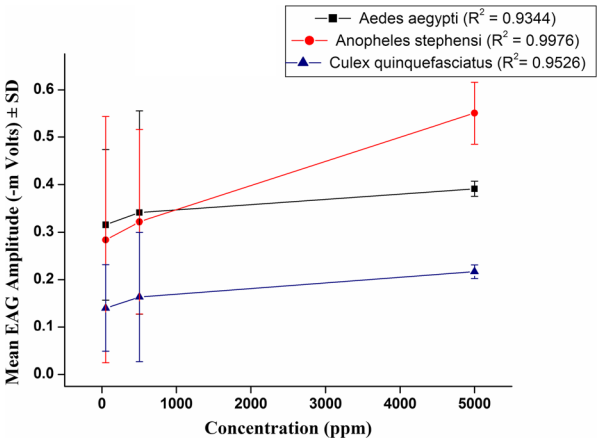

Fig. 5 Correlation between mean electroantennogram (EAG) amplitude and different concentrations of ethyl 2-aminobenzoate

Table 2 Results of one-way ANOVA on electroantennogram responses of *Aedes aegypti, Anophelese stephensi* and *Culex quinquefasciatus* to Ethyl 2-aminobenzoate

Electroantennogram responses (EAG)						
Species	Concentration (ppm)	EAG Amplitude				
		Control	EAB	p	F	R^2
Aedes aegypti	50	0.19 ± 0.13	0.31 ± 0.15	> 0.05	3.807	0.9344
	500		0.34 ± 0.21	> 0.05		
	5000		0.39 ± 0.01	> 0.05		
Anopheles stephensi	50	0.16 ± 0.16	0.28 ± 0.25	> 0.05	135.17	0.9976
	500		0.32 ± 0.19	> 0.05		
	5000		0.55 ± 0.06	< 0.05		
Culex quinquefasciatus	50	0.10 ± 0.07	0.13 ± 0.09	> 0.05	1.443	0.9526
	500		0.16 ± 0.13	> 0.05		
	5000		0.21 ± 0.01	> 0.05		

EAB- Ethyl 2-aminobenzoate, $p< 0.05$ considered significant. All results are expressed as Mean ± SD

formulations have been proved effective against mosquitoes and other biting insects but have limited use in disease-inflicted tropical countries probably for aversion to use due to their high cost, poor cosmetic quality and allied toxicity associated with their use (Chattopadhyay et al. 2015; Ray 2015). Moreover, emerging problem of development and spread of resistance against DEET, permethrin, deltamethrin, and other pyrethroids has been a serious concern (Dusfour et al. 2015; Klun et al. 2004; Meepagala et al. 2016; Stanczyk et al. 2010). Hence, there is an urgent need to identify environment friendly odorants that are capable of replacing harmful chemical pesticides in an effort to control different mosquito-borne diseases. The idea of using safer, better and economically viable anti-mosquito formulations as alternative to the existing costly and comparatively less safe natural and synthetic counterparts could be an amicable solution to scale down the negative impact on human health and environment (Dhiman et al. 2013).

EAB is a non-toxic compound that was recently revealed to elicit avoidance behaviour with host seeking *Aedes aegypti* mosquitoes and its importance has primarily been revealed as a repellent in a caged landing assay. In that assay, a non-contact version of the assay was used by inserting a human hand protected by a net in a mosquito cage, while an intermediate net treated with 10% EAB in acetone was used for the test. Thus, mosquitoes had a choice between landing and not landing on the net surface (Kain et al. 2013).

In the present investigation, we tested whether the new repellent EAB would affect (attraction/repellency) the host seeking behaviour of three major mosquito vectors. We also extended the experiment to investigate the effect of EAB on the peripheral olfactory receptors in the antenna of female *Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus* mosquitoes.

In our flight orientation experiments, the female mosquitoes displayed reduced/non-preference to the odor plume of EAB by orienting a large numbers of female mosquitoes away from the test chamber of olfactometer, which apparently revealed the repellent property of EAB against three species of mosquitoes. Altogether, 63–75% female *Aedes*

aegypti, 56–65% *Anopheles stephensi* and 58–70% *Culex quinquefasciatus* flew away from the test chamber and entered the acetone treated solvent control chamber. Although, most of the female mosquitoes flew upwind from the releasing chamber, a small proportion of the mosquitoes did not respond to either of the odor plume of EAB or the solvent. We presume that, availability of EAB at the high concentration might have saturated the olfactory receptors of mosquitoes, which in turn have affected the mosquito's decision making to specific odor plume. The behavioural response of the mosquitoes reported in the present investigation are comparable to those reported for other standard repellents previously. A recent study demonstrated that DEET repelled 39% female *Aedes aegypti* mosquitoes at the dose of 10% *w*/*v* using a two-choice Y-tube olfactometer (Afify et al. 2014). Furthermore, in another similar study conducted on the orientation behaviour, DEET (2.5% *w*/*v*) showed moderate repellency against *Aedes aegypti* (8.7%), *Aedes albopictus* (57.9%), *Anopheles minimus* (77.7%), *and Culex quinquefasciatus* (21.%), mosquito species (Sathantriphop et al. 2014). DEET has been used as a commercial repellent against mosquitoes and other hematophagous insect since long and also used as standard in evaluating the repellent activity of the new test compounds. Many studies have shown that DEET at low concentration elicits both repellent and irritant effect against many known vector mosquito (Afify et al. 2014; Sathantriphop et al. 2014; Tisgratog et al. 2011). Present results also demonstrate that the tested compound was equally effective against all the three well known mosquito vector species at considerably low concentrations range of 50–5000 ppm (0.005–0.5%), which indicates that the test compound EAB could be a promising mosquito repellent.

EAG is an extensively used technique to reveal the olfactory responses in the insect antenna to various volatile compounds (Seenivasagan et al. 2009, 2014). EAG experiments enabled the detection of several volatiles and insect pheromones important for host recognition and mating (Bezerra-Silva et al. 2016; Deletre et al. 2015; Wang et al. 2016; Ye et al. 2016).

In our EAG experiments, the antennae of *Aedes aegypti* and *Culex quinquefasciatus* elicited a dose dependent response and a ~ 2-fold fold increased EAG response was obtained with the highest dose of 5000 ppm as compared to the acetone treated solvent control. However, the results did not significantly differ from the control, which negates the correlation between the orientation and EAG experiments. It would be worth mentioning that there is no direct link between EAG and behaviour responses, neither in terms of response amplitude nor in terms of response orientation (attraction vs. repellency). A compound showing repellency does not necessarily elicit EAG responses. Inconsistencies found in the present investigation (e.g., between the behavioural and electrophysiological responses) has also been reported in the previous studies, indicating the complex behaviour of the insects involved in the repellenty evaluation (Deletre et al. 2015; Wee et al. 2008; Williams et al. 2010). For example, Deletre et al. (2015) reported the electrophysiological and behavioural responses of some bioactive compounds such as geraniol, citronellol, cinnamaldehyde, carvacrol, linalool, etc. against malaria vector *Anopheles gambiae.* In their experiments, the EAG response to carvacrol was found relatively weak, but strong behavioural responses to this compounds were observed. On the other hand, mosquitoes exhibited relatively strong EAG responses to cuminaldehyde and linalool, but were not repelled well by these compounds. Thus, these findings underscore the value of using a variety of research approaches when studying complex behaviour such as repellency at the level of the whole organism.

The data/voltage drop generated by the EAG system may be a result of the summation of the activities in different types of receptor neurons with overlapping response spectra, so that information is not obtained about the specificity of each neuron type (Wibe 2004; Wibe et al. 1997). Moreover, EAGs are very crude and low sensitivity measurement systems for odors in mosquitoes. It mainly picks up on the responses from odors that activate a large number of neurons, while the missing responses to odors that activate a small subset (Chen and Fadamiro 2007; Yang et al. 2009). EAB mainly acts via triggering the ionotropic receptor *Ir40a* expressing neurons of sacculus, in the antenna of fruit fly *Drosophila.* Even though, these receptors are highly conserved across several species of insects including mosquitoes, their occurrence and numbers are yet to be determined. We presume that, these receptors might be present in lesser number in the antenna of *Aedes aegypti* and *Culex quinquefasciatus* but may be present in large numbers at other locations of mosquitoes such as maxillary palps and proboscis due to which, probably significant dose dependent response in flight orientation experiments was obtained. On the other hand, *Anopheles stephensi* exhibited a dose dependent EAG response with the 50, 500 and 5000 ppm EAB. However, the EAG response was found significant at 5000 ppm only. The dose dependent EAG response and significant outcome may be due to the presence of most diverse olfactory receptor neurons present in *Anopheles* species (Ray 2015). Presently, although EAB has demonstrated comparable repellency to DEET, it could not elicit similar electrophysiological responses. The results indicate that in addition to the antennal reception, some other sensory neurons also play crucial role in determining the repellency response of an insect. Previous investigations have reported a dose dependent electrophysiological response of DEET in both *Aedes aegypti* and *Culex quinquefasciatus* mosquitoes (Stanczyk et al. 2010; Syed and Leal 2008) However, the DEET olfactory receptor neurons were found to display low sensitivity even at the high dose in *Anopheles*, *Aedes* and *Culex* mosquitoes (Costantini et al. 2001; Ditzen et al. 2008; Pickett et al. 2008; Stanczyk et al. 2010; Syed and Leal 2008; Xu et al. 2014).

In the conclusion, we emphasize the inclusion of more efficient and sophisticated system such as single cell recording (SCR) to reveal the specificity of each neuron type of *Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus.* We also stress on the deliberate application of modern molecular and computational tools to enable rigorous investigations of the mosquito olfactory system function considering the odorant receptors neurons of maxillary palps and proboscis of all the three type of mosquitoes. Although the results have revealed the repellent activity of EAB using laboratory reared mosquitoes, but the repellent effects are needed to be evaluated under field condition using wild mosquitoes to strengthen the present claim.

Acknowledgements One of the authors, Johirul Islam extend gratitude to Director, Defence Research and Development Establishment, Gwalior, Madhya Pradesh, India to accord permission (vide letter no. DRDE/HR-11/Lab & Lib Facility/2016 dt 05th Feb 2016), and Head, Vector Management Division for providing necessary support during experimentation. Authors are also thankful to all the sources for the direct and indirect help and the financial assistance. All anonymous reviewers are gratefully acknowledged for their specific comments that help a lot in improving this manuscript.

Compliance with Ethical Standards

Conflicts of Interest The authors declare no conflicts associated with this work.

References

Afify A, Horlacher B, Roller J, Galizia CG (2014) Different repellents for *Aedes aegypti* against blood-feeding and oviposition. PLoS One 9:e103765

Allan SA, Bernier UR, Kline DL (2006) Laboratory evaluation of avian odors for mosquito (Diptera: *Culicidae*) attraction. J Med Entomol 43:225–231

Alpern JD, Dunlop SJ, Dolan BJ, Stauffer WM, Boulware DR (2016) Personal protection measures against mosquitoes, ticks and other arthropods. Med Clin North Am 100:303–316

Api AM, Belsito D, Bhatia S, Bruze M, Calow P, Dagli ML, Dekant W et al (2015) RIFM fragrance ingredient safety assessment, ethyl anthranilate, CAS registry number 87-25-2. Food Chem Toxicol 82:S97–S104

Auysawasdi N, Chuntranuluck S, Phasomkusolsil S, Keeratinijakal V (2016) Improving the effectiveness of three essential oils against *Aedes aegypti* (Linn.) and *Anopheles dirus* (Peyton and Harrison). Parasitol Res 115:99–106

Bernier UR, Kline DL, Allan SA, Barnard DR (2015) Laboratory studies of *Aedes aegypti* attraction to ketones, sulfides, and primary chloroalkanes tested alone and in combination with L-lactic acid. J Am Mosq Control Assoc 31:63–70

Bezerra-Silva PC, Dutra KA, Santos GKN, Silva RCS, Iulek J, Milet-Pinheiro P, Navarro DMAF (2016) Evaluation of the activity of the essential oil from an ornamental flower against *Aedes aegypti*: electrophysiology, molecular dynamics and behavioral assays. PLoS One 11:e0150008

Carey AF, Wang G, Su CY, Zwiebel LJ, Carlson JR (2010) Odorant reception in the malaria mosquito *Anopheles gambiae*. Nature 464:66–71

Chattopadhyay P, Dhiman S, Borah S, Rabha B, Chaurasia A, Veer V (2015) Essential oil based polymeric patch development and evaluating its repellent activity against mosquitoes. Acta Trop 147:45–53

Chen L, Fadamiro HY (2007) Behavioral and electroantennogram responses of phorid fly *Pseudacteon tricuspis* (Diptera: Phoridae) to red imported fire ant *Solenopsis invicta* odor and trail pheromone. J Insect Behav 20:267–287

Costantini C, Birkett MA, Gibson G, Ziesmann J, Sagnon NF, Mohammed HA, Coluzzi M, Pickett JA (2001) Electroantennogram and behavioural responses of the malaria vector *Anopheles gambiae* to human-specific sweat components. Med Vet Entomol 15:259–266

Das NG, Dhiman S, Talukdar PK, Rabha B, Goswami D, Veer V (2015) Synergistic mosquito-repellent activity of *Curcuma longa*, *Pogostemon heyneanus* and *Zanthoxylum limonella* essential oils. J Infect Public Health 8:323–328

Dasti JI (2016) Zika virus infections: an overview of current scenario. Asian Pac J Trop Med 9:621–625

Deletre E, Chandre F, Williams L, Duméni C, Menut C, Martin T (2015) Electrophysiological and behavioral characterization of bioactive compounds of the *Thymus vulgaris, Cymbopogon winterianus, Cuminum cyminum* and *Cinnamomum zeylanicum* essential oils against *Anopheles gambiae* and prospects for their use as bednet treatments. Parasit Vectors 8:316–329

Dhiman S, Rabha B, Talukdar PK, Das NG, Yadav K, Baruah I, Singh L, Veer V (2013) DDT & deltamethrin resistance status of known Japanese encephalitis vectors in Assam, India. Indian J Med Res 138:988–994

Diaz JH (2016) Chemical and plant-based insect repellents: efficacy, safety, and toxicity. Wilderness Environ Med 27:153–163

Dickens JC, Bohbot JD (2013) Mode of action of mosquito repellents. Pestic Biochem Physiol 106:149–155

Ditzen M, Pellegrino M, Vosshall LB (2008) Insect odorant receptors are molecular targets of the insect repellent DEET. Science 319:1838–1842

Dube FF, Tadesse K, Birgersson G, Seyoum E, Tekie H, Ignell R, Hill SR (2011) Fresh, dried or smoked? Repellent properties of volatiles emitted from ethnomedicinal plant leaves against malaria and yellow fever vectors in Ethiopia. Malar J 10:375–388

Dusfour I, Zorrilla P, Guidez A, Issaly J, Girod R, Guillaumot L, Robello C, Strode C (2015) Deltamethrin resistance mechanisms in *Aedes aegypti* populations from three French overseas territories worldwide. PLoS Negl Trop Dis 9:e0004226

Fang J (2010) A world without mosquitoes. Nature 466:432–434

Fauci AS, Morens DM (2016) Zika virus in the Americas-yet another arbovirus threat. N Engl J Med 374: 601–604

Flavors and Fragrances (2007–2008) Catalogue (Sigma-Aldrich, 2007).

Ghaninia M, Larsson M, Hansson BS, Ignell R (2008) Natural odor ligands for olfactory receptor neurons of the female mosquito *Aedes aegypti*: use of gas chromatography-linked single sensillum recordings. J Exp Biol 211:3020–3027

Ghosh A, Chowdhury N, Chandra G (2012) Plant extracts as potential mosquito larvicides. Indian J Med Res 135:581–598

Ginier M, Neumayr A, Günther S, Schmidt-Chanasit J, Blum J (2016) Zika without symptoms in returning travellers: what are the implications? Travel Med Infect Dis 14:16–20

Guda T, Kain P, Sharma KR, Pham CK, Ray A (2015). Repellent compound with larger protective zone than DEET identified through activity screening of *Ir40a* neurons, does not require or function. bioRxiv. doi:10.1101/017145.

Guha L, Seenivasagan T, Iqbal ST, Agrawal OP, Parashar BD (2014) Behavioral and electrophysiological responses of *Aedes albopictus* to certain acids and alcohols present in human skin emanations. Parasitol Res 113:3781–3787

Hansson BS, Stensmyr MC (2011) Evolution of insect olfaction. Neuron 72:698–711

Islam J, Zaman K, Duarah S, Raju PS, Chattopadhyay P (2017) Mosquito repellents: an insight into the chronological perspectives and novel discoveries. Acta Trop 167:216–230

Jamil Z, Waheed Y, Durrani TZ (2016) Zika virus, a pathway to new challenges. Asian Pac J Trop Med 9:626–629

Kain P, Boyle SM, Tharadra SK, Guda T, Pham C, Dahanukar A, Ray A (2013) Odour receptors and neurons for DEET and new insect repellents. Nature 502:507–512

Klun JA, Strickman D, Rowton E, Williams J, Kramer M, Roberts D, Debboun M (2004) Comparative resistance of *Anopheles albimanus* and *Aedes aegypti* to N,N-diethyl-3-methylbenzamide (DEET) and 2-methylpiperidinyl-3- cyclohexen-1-carboxamide (AI3-37220) in laboratory human-volunteer repellent assays. J Med Entomol 41:418–422

Lavialle-Defaix C, Apaire-Marchais V, Legros C, Pennetier C, Mohamed A, Licznar P, Corbel V, Lapied B (2011) *Anopheles gambiae* mosquito isolated neurons: a new biological model for optimizing insecticide/repellent efficacy. J Neurosci Methods 200:68–73

Leal WS (2014) The enigmatic reception of DEET-the gold standard of insect repellents. Curr Opin Insect Sci 6:93–98

Lupi E, Hatz C, Schlagenhauf P (2013) The efficacy of repellents against *Aedes, Anopheles, Culex* and *Ixodes* spp. A literature review. Travel Med Infect Dis 11:374–411

Meepagala KM, Estep AS, Becnel JJ (2016) Larvicidal and adulticidal activity of chroman and chromene analogues against susceptible and permethrin-resistant strains. J Agric Food Chem 64:4914–4920

Menger DJ, Omusula P, Holdinga M, Homan T, Carreira AS, Vandendaele P, Derycke JL, Mweresa CK, Mukabana WR, Van Loon JJ, Takken W (2015) Field evaluation of a push-pull system to reduce malaria transmission. PLoS One 10:e0123415

Opdyke DLJ (1979) Monographs on fragrance raw materials. Pregamon Press, New York

Pickett JA, Birkett MA, Logan JG (2008) DEET repels ORNery mosquitoes. Proc Natl Acad Sci U S A 105: 13195–13196

Raphael R, Croset V, Benton R (2013) Ionotropic receptors (IRs): chemosensory ionotropic glutamate receptors in *Drosophila* and beyond. Insect Biochem Mol Biol 43:888–897

Ray A (2015) Reception of odors and repellents in mosquitoes. Curr Opin Neurobiol 34:158–164

Sathantriphop S, White SA, Achee NL, Sanguanpong U, Chareonviriyaphap T (2014) Behavioral responses of *Aedes aegypti, Aedes albopictus, Culex quinquefasciatus,* and *Anopheles minimus* against various synthetic and natural repellent compounds. J Vector Ecol 39:328–339

Seenivasagan T, Sharma KR, Sekhar K, Ganesan K, Prakash S, Vijayaraghavan R (2009) Electroantennogram, flight orientation, and oviposition responses of *Aedes aegypti* to the oviposition pheromone *n*-heneicosane. Parasitol Res 104:827–833

Seenivasagan T, Sharma KR, Ganesan K, Prakash S (2010) Electrophysiological, flight orientation and oviposition responses of three species of mosquito vectors to hexadecyl pentanoate: residual oviposition repellent activity. J Med Entomol 47:329–337

Seenivasagan T, Sharma KR, Prakash S (2012) Electroantennogram, flight orientation and oviposition responses of *Anopheles stephensi* and *Aedes aegypti* to a fatty acid ester-propyl octadecanoate. Acta Trop 124:54–61

Seenivasagan T, Guha L, Parashar BD, Agrawal OP, Sukumaran D (2014) Olfaction in Asian tiger mosquito *Aedes albopictus*: flight orientation response to certain saturated carboxylic acids in human skin emanations. Parasitol Res 113:1927–1932

Song E, Bivort B, Dan C, Kunes S (2012) Determinants of the *Drosophila* odorant receptor pattern. Dev Cell 22:363–376

Stanczyk NM, Brookfield JF, Ignell R, Logan JG, Field LM (2010) Behavioral insensitivity to DEET in *Aedes aegypti* is a genetically determined trait residing in changes in sensillum function. Proc Natl Acad Sci 107: 8575–8580

Syed Z, Leal WS (2008) Mosquitoes smell and avoid the insect repellent DEET. Proc Natl Acad Sci 105: 13598–13603

Tisgratog R, Tananchai C, Bangs MJ, Tainchum K, Juntarajumnong W, Prabaripai A, Chauhan KR, Pothikasikorn J, Chareonviriyaphap T (2011) Chemically induced behavioral responses in *Anopheles minimus* and *Anopheles harrisoni* in Thailand. J Vector Ecol 36:321–331

Wang YC, Li P, Chi D (2016) Electrophysiological and behavioral responses of *Tenebrio molitor* L. to fourteen kinds of plant volatiles. J Asia Pac Entomol 19:261–267

Webster B, Cardé RT (2016) Use of habitat odour by host-seeking insects. Biol Rev. doi:10.1111/brv.12281

Wee SL, El-Sayed AM, Gibb AR, Mitchell V, Suckling DM (2008) Behavioural and electrophysiological responses of *Pantomorus cervinus* (Boheman) (Coleoptera: Curculionidae) to host plant volatiles. Aust J Entomol 47:24–31

Wibe A (2004) How the choice of method influence on the results in electrophysiological studies of insect olfaction. J Insect Physiol 50:497–503

Wibe A, Borg-Karlson AK, Norin T, Mustaparta H (1997) Identification of plant volatiles activating single receptor neurons in the pine weevil (*Hylobius abietis*). J Comp Physiol A 180:585–595

Williams L, Blackmer JL, Rodriguez-Saona C, Zhu S (2010) Plant volatiles influence electrophysiological and behavioral responses of *Lygus hesperus*. J Chem Ecol 36:467–478

Wiwanitkit S, Wiwanitkit V (2016) Predicted pattern of Zika virus infection distribution with reference to rainfall in Thailand. Asian Pac J Trop Med 9:719–720

Xu P, Choo Y-M, De La Rosa A, Leal WS (2014) Mosquito odorant receptor for DEET and methyl jasmonate. Proc Natl Acad Sci U S A 111:16592–16597

Yang M, Dong S, Chen L (2009) Electrophysiological and behavioral responses of female beet army worm *Spodoptera exigua* (Hübner) to the conspecific female sex pheromone. J Insect Behav 22:153–164

Ye Z, Liu F, Liu N (2016) Olfactory responses of southern house mosquito, *Culex quinquefasciatus*, to human odorants. Chem Senses 41:441–447