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Bacteria as a source of oviposition attractant for *Aedes aegypti* mosquitoes

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Abstract. Since a safe and effective mass vaccination program against dengue fever is not presently available, a good way to prevent and control dengue outbreaks depends mainly on controlling the mosquito vectors. *Aedes aegypti* mosquito populations can be monitored and reduced by using ovitraps baited with organic infusions. A series of laboratory experiments were conducted which demonstrated that the bacteria in bamboo leaf infusion produce volatile attractants and contact chemical stimulants attractive to the female mosquitoes. The results showed that the female mosquitoes laid most of their eggs (59.9 ± 8.1 vs 2.9 ± 2.8 eggs, $P<0.001$) in bamboo leaf infusions when compared to distilled water. When the fresh infusion was filtered with a 0.45µm filter membrane, the female mosquitoes laid significantly more eggs (64.1 ± 6.6 vs 4.9 ± 2.6 eggs, $P<0.001$) in unfiltered infusion. However when a 0.8µm filter membrane was used, the female laid significantly more eggs (62.0 ± 4.3 vs 10.1 ± 7.8 eggs, $P<0.001$) in filtrate compared to a solution containing the residue. We also found that a mixture of bacteria isolated from bamboo leaf infusion serve as potent oviposition stimulants for gravid *Aedes* mosquitoes. *Aedes aegypti* laid significantly more eggs (63.3 ± 6.5 vs 3.1 ± 2.4 eggs, $P<0.001$) in bacteria suspension compared to sterile R2A medium. Our results suggest microbial activity has a role in the production of odorants that mediate the oviposition response of gravid mosquitoes.

INTRODUCTION

Dengue fever (DF) is one of the most important mosquito-borne diseases that cause a serious public health problem in tropical and subtropical countries worldwide, especially in urban and semi-urban areas. A severe complication form of dengue known as dengue hemorrhagic fever causes hemorrhaging blood vessels which in rare cases, if untreated may lead to blood vessel collapse, causing a fatal condition known as dengue shock syndrome. In the absence of a safe and effective extensive vaccination program, the possible efficient way to ultimately control dengue outbreaks is reducing population abundance of the major mosquito vector, *Aedes aegypti* (WHO, 2012).

In Southeast Asia, *Ae. aegypti* is the primary vector of dengue fever. These mosquitoes are highly adaptive and breed in human-made and natural containers (Chua *et al.*, 2005). Space spraying of chemical insecticides which has been used for over 40 years as a recommended method to control *Ae. aegypti* has been a complete failure (Gubler, 2011). The failure is mainly due to the development of insecticide resistance which explains why vector control has moved from broad range, persistent chemicals to more specific biological control agents (Clements, 1992). With the dramatic rise in the number of dengue fever and dengue hemorrhagic fever cases worldwide following the spread of the vector, the need to intensify vector studies and improve vector-control methods has become urgent.

Mosquito ovitraps are used extensively as a tool to monitor, detect and control *Aedes* populations in endemic areas (Chan, 1973).

They provide an estimation of the adult population in selected areas and act as an early warning signal to prevent any possible disease outbreaks (Service, 1993). Advantages of using ovitraps to help control *Aedes* populations include their simplicity, specificity and effectiveness against container breeders such as *Ae. aegypti*, in addition to the possible use in conjunction with chemical or biological control agents such *as Bacillus thuringiensis* and *Bacillus sphaericus* (Baumann *et al.*, 1991; Park *et al.*, 2013). An understanding of the chemical ecology of the mosquitoes would help to increase the efficiency of the ovitraps used in dengue surveillance and control.

The female *Ae. aegypti* chooses to lay its eggs in multiple water- containers, a behavior called skip-oviposition (Mogi & Mokry, 1980). However, addition of organic material to the water can counteract skip-oviposition behavior and significantly increase the number of eggs laid in target containers. The leaf infusions made by fermenting a variety of organic material in water contain chemicals that attract gravid female mosquitoes and stimulate their oviposition (Trexler *et al.*, 2003; Punnusamy *et al.*, 2010; Santos *et al.*, 2010; Gopalakrishnan *et al.*, 2012). Such infusions can increase the effectiveness of the ovitraps (Regis *et al.*, 2008) and traps for capturing the adult mosquitoes (Gama *et al.*, 2007; Santos *et al.*, 2012). The chemicals (also known as semiochemicals) present in the infusions are often metabolites of microbial decomposition of organic matter (Millar *et al.*, 1992; Clements, 1999; Punnusamy *et al.*, 2008). The isolation and identification of bacteria from various attractive or stimulatory substrates, and the subsequent evaluation of the bacteria in oviposition bioassays could lead to the discovery of new sustainable source of attractants; thus, increasing the effectiveness of the ovitraps.

The majority of research on the role of microbes has focused on the oviposition attractants/repellents from different sources and/or stimulant/deterrent effects of bacteria on *Aedes* mosquitoes oviposition behavior (Ikeshoji *et al.*, 1975; Benzon & Apperson, 1988; Navarro *et al.*, 2003; Trexler *et al.*, 2003; Punnusamy *et al.*, 2008; 2010, Obenauer *et al.*, 2010). In this research our objectives are: (1) to evaluate the effect of bamboo leaf infusion on the oviposition responses of *Ae. aegypti* mosquitoes; (2) to isolate the bacterial species from bamboo leaf infusions; and (3) to ascertain whether the presence of the bacteria would increase the ovipositional rate.

MATERIALS AND METHODS

Mosquito colonies

Aedes aegypti mosquitoes used in our study were colonized from eggs collected from the field and the mosquito colonies were maintained at the insectary room in School of Medicine, University Malaysia Sabah. Adult mosquitoes were kept in 30×30×30 cm Perspex cages with a netting on two sides and an opening window (10 cm diameters) with a netting sleeve in front. The adult mosquitoes were given *ad-libitum* access to a 10% sucrose solution. Mosquitoes were kept at ca. 27°C ± 3 and at a relative humidity of ca. 75% under L:D 9:15. Larvae were once-daily fed with fish pellets. This diet was found to be the most preferable food for the larval development and for obtaining fecund females (Kasap & Demirhan, 1992).

Preparation of bamboo leaf infusions

Bamboo leaf infusions (BLIs) were prepared using senescent bamboo (*Bambusa* spp.) leaves collected from a residential area in Kota Kinabalu Sabah. The leaves were ground into particles by using a laboratory blender (Waring® Commercial Blender) set on "LOW" for 30 seconds. The ratio of plant biomass to distilled water (33.6 g per 4 L = 1X infusion) as described by Reiter *et al.* (1991) was used to make the infusion. The BLI were kept in closed bags with a headspace above (Punnusamy *et al.*, 2010) at a room temperature for one week before the experiment day.

Laboratory ovipositional bioassays

Polystyrene sterile cups used as a bioassay cups were put in the perspex cages. To eliminate visual cues presented by dark

colored BLI, black plastic sheets was used to cover the floor of each cage and the cups were covered with black sleeves. The 7-14 days-old female mosquitoes were blood fed on mice 4-5 days before the experiment day after being starved for more then 24 hours. At the end of each bioassay the eggs in each cup were collected and counted. The mosquitoes were offered a 10% sucrose solution.

(i) Oviposition bioassays using 50% bamboo leaf infusion

The 7 days old BLI were diluted 1:1 with distilled water and mixed vigorously. Six cups, three filled with 30 ml of 50% BLI and three with an equivalent volume of distilled water. The cups were randomly placed inside the experiment cages and one gravid female mosquito was released into the cage. After 24-48 hours, the bioassays were terminated and the number of eggs in each cup was counted.

A total of 13 replicates were successfully conducted for each treatment, each using a fresh female. Four other replicates in which the females did not lay any eggs were excluded.

(ii) Oviposition bioassays to filtered bamboo leaf infusion

Two sets of assays were conducted. The first set involved filtering fresh BLI through a 0.45 µm pore size syringe adapter filter to remove the microorganisms. The oviposition assays were done using the filtered vesus non filtered BLI. A total of 14 replicates were conducted for each treatment, each using a fresh female.

The second set involved filtering the fresh BLI through a 0.8 µm pore size filter membrane and the filter-retained residue were resuspended in equivalent volume of sterile distilled water. The oviposition assays were done using the filtered versus the filter-retained residue. A total of 15 replicates were conducted for each treatment, each using a fresh female.

(iii) Oviposition bioassays using bacteria isolates

To isolate the bacteria from BLI, enrichment culture was prepared by inoculating 3 ml of fresh BLI into 200 ml of Reasoner's 2A agar (R2A) broth (Reasoner & Geldreich, 1985) and incubated for two days at 28ºC. Then, enriched culture was serially diluted up to 10^{-7} with sterile peptone water 0.1% (wt/vol) and 100 µl of each of the last three dilutions was separately spread onto two replicates R2A agar plates and incubated for two days at 28ºC. Colonies with visually distinct morphologies were picked off and restreaked on R2A agar plates.

For the enumeration of heterotrophic bacteria in BLI, a standard spread plate count method was applied in triplicates on R2A agar plates. Colony Forming Unit (CFUs/mL) were enumerated after two days incubation at 28ºC. Plates with (30-300) colonies per standard-sized plate were counted.

For the oviposition bioassays, the purified bacterial isolates were grown separately in 20 ml R2A broth for two days at 28ºC, then 200 µl of each bacteria isolates were mixed in 200 ml of sterile R2A broth and incubated for two days at 28ºC. To achieve a final cell density of 10^7 cells/ml in the 30ml volumes contained in test cups the bacteria mixture was serially diluted ten-fold with sterile water. As a control, sterile R2A media was added to cups after similer dilution with sterile water. The test cups were filled either with 30 ml of each bacterial suspension or control media and randomly placed inside the experiment cages. A total of 15 replicates were conducted for each treatment, each using a fresh female.

Data analysis

The data were first tested for normality by using the Shapiro-Wilk test. For non normal data, Mann-Whitney U test was used for significance test (IBM® SPSS version 19.0). The oviposition activity index (OAI) was estimated, according to Kramer & Mulla (1979). The OAI was calculated as:

$$OAI = (Nt - Nc) / (Nt + Nc)$$

Where: Nt is the number of eggs laid in test cups and Nc is the number of eggs in control cups. Index values lay within the range of +1 to -1 with 0 indicating no response and positive values $\geq$ +0.3 indicate that the

material is an attractant, while negative values ≤ -0.3 indicate repellency.

RESULTS

In the experiment using 50% BLI versus distilled water, significantly more eggs were laid in cups containing bamboo infusion than control cups containing distilled water (95.46%, U= 7.00, P<0.001). The mean number of eggs laid by a single female *Ae. aegypti* mosquito in 50% BLI and plain water was respectively 59.9 ± 8.1 and 2.9 ± 2.8 (N=13) (Figure 1). The OAI value of BLI was + 0.84.

When given the choice between the unfiltered BLI and the BLI filtered through a 0.45 µm pore size filter membrane, the female mosquito laid significantly more eggs in cups containing the unfiltered infusion (92.85%, U=2.00, P< 0.001). The mean number of eggs laid in the unfiltered and filtered infusion by a single female mosquito was 64.1 ± 6.6 and 4.9 ± 2.6 respectively (N=14) (Figure 2). The OAI of unfiltered BLI was + 0.8.

When the BLI was filtered through a 0.8 µm pore size filter membrane and the filtrate tested against the residue that had been resuspended in sterile distilled water, the number of eggs laid in cups containing the filtrate were significantly higher than in cups that contained the resuspended residue (93.93%, U=0.00, P<0.001). The mean number of eggs laid by a single female mosquito in cups containing the filtrate and residue medium was 62.0 ± 4.3 and 10.1 ± 7.8 (N=15) (Table 1).The OAI value was +0.87.

The mean bacterial count (CFUs) in undiluted 7-day-old infusions found to be around (1.12 ± 0.45) × 10^7 CFUs/ml. Significantly more eggs (95.5%, U=2.00, P<0.001) were laid by *Ae. aegytpi* females in cups containing a mix of cultured bacterial species of a concentration around 10^7 CFUs/ml than in control cups with sterile media. The numbers of eggs laid by one female mosquitoes were 63.3 ± 6.5 in cups holding mixture of bacteria and 3.1 ± 2.4 in control cups (N=15) (Table 2). The OAI was +0.92.

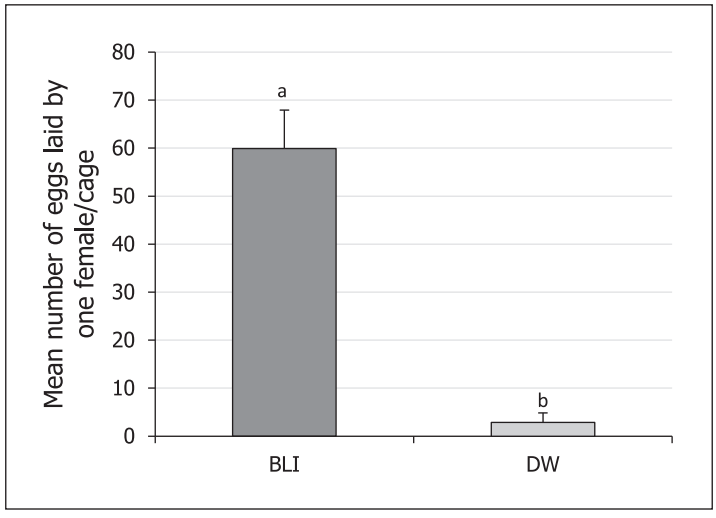

Figure 1. Mean number of eggs laid by a single *Ae. aegypti* female mosquito in 50% bamboo leaf infusion concentrations compared to distilled water. Different letters indicate significant difference between treatments (Mann Whitney U Test, p<0.001). BLI=bamboo leaf infusion (treatment), DW=distilled water (control)

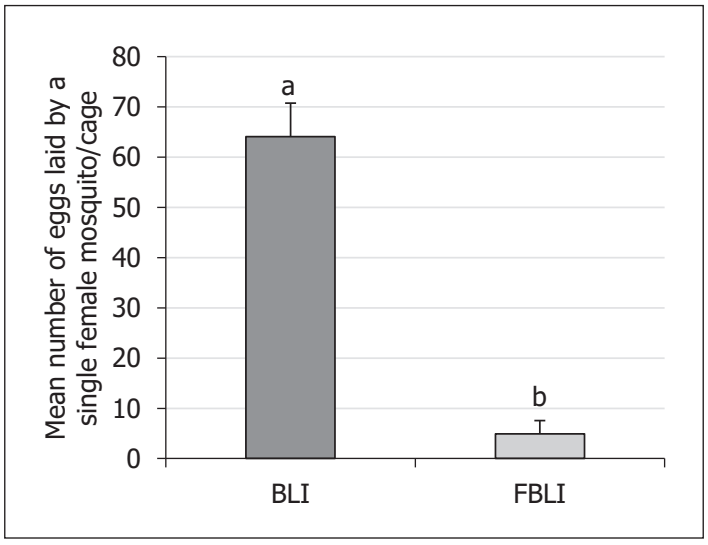

Figure 2. Mean number of eggs laid by a single *Ae. aegypti* mosquito in non-filtered versus filtered BLI. Different letters indicate significant difference between treatments shown by Mann Whitney U Test (p<0.001). FBLI= bamboo leaf infusion filtered through a 0.45 µm pore size filter membrane

Table 1. Mean number of eggs laid by a single *Ae. aegypti* female mosquito when offered a chioce between filtrate obtained from filtering BLI through a 0.8 µm pore size filter membrane and the residue resuspended in sterile distilled water. Fifteen single-female assays were conducted. BLI = filtered bamboo leaf infusion. Mann Whitney U statistic was significant (P<0.001). OAI = Oviposition activity index

Medium	N	Total no. of eggs	Mean (± SE)	% of eggs	OAI
BLI filtrate	15	930	62 (± 4.3)	93.9%	+0.87
Residue from filtering BLI	15	60	4 (± 1.8)	6.1%	

Table 2. The mean number of eggs laid by a single *Ae. aegypti* female mosquitoe when offered a chioce between a R2A medium containing a mixture of bacteria isolated from bamboo leaf infusion and a sterile R2A medium. Fifteen single-female assays were conducted. Mann Whitney U statistic was significant (P<0.001). OAI=Oviposition activity index

R2A medium	N	Total no. of eggs	Mean (± SE)	% of eggs	OAI
with bacteria	15	949	63.27 (± 6.58)	95%	+0.92
without bacteria	15	46	3.07 (± 2.44)	5%	

DISCUSSION

The oviposition activity of a female mosquito can be divided into two stages. First, it must be attracted to an oviposition medium, and second there must be chemical biological stimulants in the medium which will stimulat the actual oviposition process (Dethier *et al.*, 1960).

Semiochemical cues are used, in part, by gravid *Ae. aegypti* females to select an oviposition site to lay their eggs. These include volatile attractants/repellents togather with contact stimulants/deterrents which most often originate from fermenting or decomposit organic material (Millar *et al.*, 1992; Trexler *et al.*, 2003).

Our study showed that gravid female *Ae. aegypti* mosquitoes were highly attracted and stimulated to oviposit in the BLI with OAI of + 0.84 indicating that BLI has an attractant activity. Similer results was observed previously by Ponnusamy *et al.* (2008). Other workers using infusions made by fermenting a variety of plant species have been reported to be also active towards gravid *Ae. aegypti* in laboratory and field bioassays (Reiter *et al.*, 1991; Chadee *et al.*, 1993; Rawlins *et al.*, 1998; Trexler *et al.*, 1998; Polson *et al.*, 2002; Sant'Ana *et al.*, 2006).

When the microorganisums (i.e, bacteria, protozoa, fungi) were physically removed by 0.45 µm pore size membrane, the gravid female mosquitoes chose to lay their eggs in the unfiltered BLI. This indicates that the chemical stimuli for mosquitoes oviposition including keromones are associated with the microorganisums present in BL infusions and that these stimuli were filtered out together with the microorganisms rather than being solubilized in the infusion.

However, when a 0.8 µm pore size membrane was used to filter the BLI and the residue was resuspended in sterile distilled water the mosquitoes prefered to oviposit in the BLI filtrate. It would appear that microorganisms larger than the 0.8 µm pore size (i.e. fungi and yeast) were trapped or retained on the upper surface of the filter paper while the smaller microorganizms (i.e. most of the bacteria) passed through the filter paper. In addition, the OAI of +0.87 suggests that bacteria rather then other micro-organisms pesent in the BLI are the oviposition stimulants and/or attractants.

Ponnusamy *et al.* (2008) demostrated that sterile filtered BLI, prepared by using a 0.22 µm pore size filter membrane, was still attractive to mosquitoes when compared to distilled water. Nevertheless, it is important to note that the volatile chemicals emanating from a plant infusion which attract the females mosquitoes may not necessarily function as oviposition stimulants (Ponnusamy *et al.*, 2010).

Significant variation in the abundance and species composition of microbial populations is known to occur on the surface of leaves from the same trees (Brunel *et al.*, 1994; Yang *et al.*, 2001; Lambais *et al.*, 2006). Ponnusamy *et al.* (2010) mentioned that such variations found in microbial composition in different experimental plant infusions result in differences in the amount of volatile odorants produced and consequently affect the responses of *Ae. aegypti* toward the plant infusions.

From the experiment using different bacteria isolate concentrations it appears that the concentration of the bacteria influences the decisions made by the female mosquitoes, as Ponnusamy *et al.* (2010) have also found. The OAI value of + 0.92 indicates a high attractant activity of the bacteria mixture. However, the stimulatory mix of bacteria at higher concentrations can become a deterrent as Ponnusamy *et al.* (2008) have shown that a higher concentrations of bacteria i.e. 10^9 cell/ml deterred oviposition.

Our findings and observations agree with the recent invistigations conducted by Ponnusamy and his coworkers (2008, 2010) on *Ae. aegypti* mosquitoes. Several previous studies have determined bacteria and/or its metabolits to be an effective oviposition attractant and/or stimulant in mosquitoes using different methods. (Ikeshoji *et al.*, 1975; Hasselschwert & Rockett, 1988; Benzon & Apperson, 1988; Pavlovich & Rockett, 2000; Navarro *et al.*, 2003; Trexler *et al.*, 2003; Ponnusamy *et al.*, 2008).

It is clear that semiochemicales play a crucial role in the selection of oviposition sites by *Ae. aegypti* female mosquitoes.

Using these semiochemicals to increase the number of eggs laid in target containers would likely enhance the sensitivity of ovitraps that are used to detect and monitor the activity of *Ae. aegypti* in endemic areas. When these chemical cues are effectivelly applied, it may provide promising results in the control and monitoring of *Aedes* populations.

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