Sri Lanka J. Aquat. Sci. 28(2) (2023): 65 - 74 https://doi.org/10.4038/sljas.v28i2.7608

Oviposition-Site Selection and Egg-Hatching of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) in Leaf Infusions of Three Invasive Plants Found in Sri Lanka

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Received: 10.05.2023 Revised: 07.09.2023 Accepted: 13.09.2023 Online: 15.09.2023

Abstract *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) are the main human arboviral vectors in Sri Lanka. The oviposition behaviour of *Aedes* mosquitoes determines their survival and population dispersion. Using varying concentrations of plant leaf infusions is a vital technique in determining mosquito oviposition behaviour. The objective of this study was to evaluate the oviposition behavior and egg-hatching rate of *Ae. aegypti* and *Ae. albopictus* to three leaf infusions of three invasive plants found in Sri Lanka. The oviposition media were prepared using guinea grass (*Megathyrsus maximus*), alligator weed (*Alternanthera philoxeroides*) and Austroeupatorium (*Austroeupatorium inulifolium*). Each oviposition medium was prepared in four different concentrations: 25%, 50%, 75%, and 100%. The ovitraps were placed in a rubber plantation in Rathnapura district, Palmadulla, Sri Lanka in order to determine the oviposition site selection towards those leaf infusions. Different plant infusions have a significant effect on the oviposition and egg hatch rate of *Ae. aegypti* and *Ae. albopictus.* Accordingly, the highest number of eggs has been laid in ovitraps with a 25% concentration of *M. maximus* infusion. The ovitrap positivity index values were highest in *A. philoxeroides* at 25%, 50% and 75% concentrations; *M. maximus* at 25%, 50% and 100% concentrations; and *A. inulifolium* at 25% and 75% concentrations. Despite the type of infusion and concentration, the egg-hatching rate was highest on the second day of rearing. *Ae. aegypti* and *Ae. albopictus* responded differently to different plant infusions based on the plant species and biomass employed. The study shows that organic matter infusions composed of guinea grass improve trap effectiveness and attract females more effectively than ovitraps using aerated tap water. The use of leaf infusions of guinea grass at 25% concentration as an attractant in combination with lethal substances to control container-breeding mosquito species is suggested as a strategy in integrated pest management.

Keywords: oviposition behavior, container breeding mosquitoes, integrated pest management

INTRODUCTION

Major human illnesses, including dengue, yellow fever, and chikungunya, are often transmitted by the mosquitoes *Aedes aegypti* and *Aedes albopictus*. Out of all these diseases, dengue is currently the most common mosquito-borne viral disease in Sri Lanka (Gunathilaka *et al.* 2023). Clinical dengue fever has been documented in Sri Lanka since the turn of the 20th century, and it was serologically verified in 1962 (Senaratne & Noordeen 2014). Since 1989, dengue hemorrhagic fever (DHF) has become widespread in Sri Lanka (Vitarana 1997).

Since *Aedes* mosquitoes are container breeders, the gravid females attach their eggs to the wall of the water-filled containers just above the water surface. They examine water quality and pick the best breeding site using visual (colour, texture, brightness), olfactory (semiochemicals), and gustatory cues (Millar *et al*. 1992; Navarro *et al*. 2003; Ponnusamy *et al*. 2008; Day 2016). Infusions emit volatile compounds that function as chemical signals for gravid mosquitos, assisting in oviposition-site selection. Optimum oviposition theory indicates that gravid insects lay their eggs in settings that maximize their offspring's performance (Scheirs & De Bruyn 2002). Previous investigations revealed that gravid female mosquitoes preferred containers with high-quality leaf material over containers with no leaf material or low-quality leaf material (Reiskind *et al.* 2009). Furthermore, the oviposition-site selection and development of mosquitoes are often affected by different physicochemical parameters of water such as temperature, turbidity, and pH in the oviposition site (Christiansen-Jucht *et al.* 2014). With respect to temperature, it has been reported that the larval growth rates of many mosquito species are slowed down by temperatures below 14–16°C and over 30°C (Christiansen-Jucht *et al.* 2014). Moreover, most mosquito larvae live in a pH range of 3.3–10.5 in nature (Christiansen-Jucht *et al*. 2014).

Organic infusions made from a range of plant species, grasses, and plant-associated materials have been generally used to examine oviposition behaviour and larval development of *Aedes* mosquitoes (Reiter *et al*. 1991; Chadee 2009; Rawlins *et al*. 1998; Polson *et al*. 2002). In addition to the infusions made from actual plant matter, other organic substances, such as sod and pelletized plant-based animal feeds, have been fermented to create infusions that were attractive to gravid *Aedes* mosquitoes. (Lampman & Novak 1996; Ritchie 2001; Allan & Kline 1995) have shown that female *Ae. aegypti* lay noticeably more eggs on conspecific or *Ae. Albopictus* on egg-containing oviposition strata than on egg-free strata. *Ae. albopictus* and *Ae. aegypti* are hard to monitor since adults are slow to respond to light traps (Service 1977) and sampling larvae take a long time. The use of oviposition attractants/stimulants to increase the sensitivity of ovitraps, as well as gravid female traps (Reiter *et al.* 1991) has significant potential. Ovitraps are frequently used for population monitoring and surveillance (Chadee 2009; McHugh & Hanny 1990). Infusions or substances that both evoke long-range orienting of gravid females to possible oviposition locations and promote oviposition once at the oviposition site are particularly important for this use.

Rubber plantations of Palmadulla in Rathnapura, Sri Lanka are occupied with some invasive flora. The invasive flora is capable of out-compete the other flora and dominating an ecosystem that would create different threats. In a nation like Sri Lanka, the introduction of alien invasive species, whether purposefully or accidentally, may have a huge detrimental impact on biodiversity (Bambaradeniya 2002).

Hence, it was hypothesized that leaf infusions prepared from invasive flora positively affect the oviposition and larval development of *Aedes* species. Thus, the objective of the present study was to determine the effects of leaf infusions of *Megathyrsus maximus (Family: Poaceae)*, *Alternanthera philoxeroides (Family: Amaranthaceae)* and *Austroeupatorium inulifolium* (Family: Asteraceae) on oviposition-site selection and egg-hatching rate of *Ae. aegypti* and *Ae. albopictus*.

MATERIALS AND METHODS

Test insects, study period and study area

Aedes aegypti and *Ae. albopictus* were the two mosquito species selected to assess oviposition behaviour and larval performance in leaf infusions of three common alien invasive flora: *M. maximus* (Maana), *A. philoxeroides* (Kimbulwenna) and *A. inulifolium* (Pathan Palu). The study was conducted from November 2021 to January 2022 and the ovitraps were set up in a rubber estate at Palmadulla, Rathnapura, Sri Lanka (6°36'50.5"N; 80°33'03.3"E).

Preparation of leaf infusions

Thirty grams of leaves were washed, and dehydrated (24h at 100ºC) and the cut leaves of three plant species were placed in bottles containing 2.0 l of distilled water for seven days in anaerobic conditions. To maintain anaerobic conditions, the lids of the bottles were closed without letting the solution contact with air. The resulting infusion was diluted to 25%, 50%, 75% and 100% using distilled water to prepare four concentrations. (Santos *et al*. 2010).

Effect of different plant leaf infusions on oviposition, egg hatch rate and species attraction of Aedes sp.

(i) Ovitrap Positivity Index and Egg Density Index

Black colour cylindrical plastic cups (200 ml) with a diameter of 7.0 cm and a depth of 6.0 cm were used to prepare the ovitraps. The cups were soaked in a water bath for 1 week to avoid the bad plastic odour. A filter paper strip of 22.0 cm in length and 5.0 cm width was placed along the interior circumference of the plastic cup, which acted as the substrate for egg-laying (Gunathilaka *et al.* 2023). Forty-five ovitraps (4 concentrations and 1 control with 3 replicates for each leaf species) were placed in the study site (field) in a completely randomized manner. One-third of each ovitrap was filled with leaf infusion while distilled water was used as the control. After four days, the egg sheets were collected, air-dried and brought to the laboratory. The whole experimental procedure was repeated three times.

The egg sheets of each cup were carefully observed for egg laying and the number of eggs laid was counted. The Ovitrap Positivity Index (OPI) and Egg Density Index (EDI) were determined using the following equations (Gomes 1998).

$$\text{Ovitrap Positivity Index (OPI)} = \frac{\text{Number of positive ovitraps}}{\text{Number of ovitraps set}} \times 100$$

$$\text{Egg Density Index (EDI)} = \frac{\text{Total number of eggs}}{\text{Total number of positive ovitraps}}$$

(ii) Egg hatch rate

The egg sheets collected from the rubber plantation were placed in a 2-litre plastic tray (23.0 cm × 18.0 cm × 5.0 cm) separately for each sheet. Two-thirds of the tray was filled with aerated tap water (until the egg sheets were fully immersed). The number of first instar larvae that hatched out each day was recorded for four days to determine the hatch rate.
After pupation, pupae were transferred to another container that contained aged tap water and were kept inside the mosquito-rearing cage until they emerged.

(iii) Species attraction

The pupae were finally transferred to a 500 ml beaker having aerated tap water and placed inside a mosquito-rearing cage (30 cm × 30 cm× 30 cm) until the adult emergence. A piece of cotton wool was soaked in ethyl acetate and placed inside the mosquito-rearing cage which was covered with a polythene bag to kill the emerging mosquitoes. The dead mosquitoes were then collected and identified at the species level using a low-power stereo microscope at 7X magnification. The pictorial keys were used for the identification of mosquitoes associated with dengue virus transmission (Rueda 2004).

DATA ANALYSIS

Data were analyzed using MINITAB 19. First, the data were subjected to the Anderson-Darling normality test to check if they followed a normal distribution. The interaction between different plant leaf infusions and concentrations was determined using a two-way ANOVA for OPI and EDI separately. A two-way PERMANOVA was used to analyze the egg hatch rate data to determine how plant species and concentration interacted. The attraction of Aedes species for various concentrations of plant leaf infusions were assessed using a two-way ANOVA.

RESULTS

The maximum ovitrap positivity index values were obtained by *A. philoxeroides* at 25%, 50% and 75% concentrations and M. maximus at 25%, 50% and 100% concentrations, and A. *inulifolium* at 25% and 75% concentrations. The minimum ovitrap positivity index value was obtained by *A. philoxeroides* at 100% concentration. The maximum egg density index value was obtained by *M. maximus* at 25% concentration and the minimum value was obtained by *A. inulifolium* at 100% concentration (Table 1).

Table 1 Mean number of eggs, Ovitrap positivity index, and Egg density index values (AP-*Alternanthera philoxeroides*, MM-*Megathyrsus maximus*, AI-*Austroeupatorium inulifolium*)

Infusion type	Concentration (%)	Number of eggs (Mean ± SE)	OPI	EDI
AP	25	4.0±0.0	100.0	4.0
	50	7.3±0.7	100.0	7.3
	75	7.7±0.7	100.0	7.7
	100	2.7±2.7	33.3	4.0
MM	25	22.0±5.0	100.0	22.0
	50	4.3±0.9	100.0	4.3
	75	3.7±1.9	66.7	5.5
	100	5.3±1.9	100.0	5.3
AI	25	11.7±4.3	100.0	11.7
	50	2.3±1.2	66.7	3.5
	75	6.0±0.6	100.0	6.0
	100	1.7±1.2	66.7	2.5
Control	N/A	10.8±2.1	77.8	13.9

There was no discernible difference in egg density among the plant species and hence the plant infusions utilized had no effect on the mosquito egg density ($F = 1.24$, $df = 2$, $P > 0.05$). However, there was a significant combined impact of infusion type and their amounts on the mosquito oviposition (Two-Way ANOVA, $F = 3.03$, $df = 6$, $P<0.05$) and the egg densities were significant at varied infusion concentrations ($F = 6.13$, $df = 3$, $P<0.05$). The gravid females respond significantly ($P=0.03$) to different plant infusions and concentrations by ovipositing more when given different concentrations and different plant infusions (Fig 1).

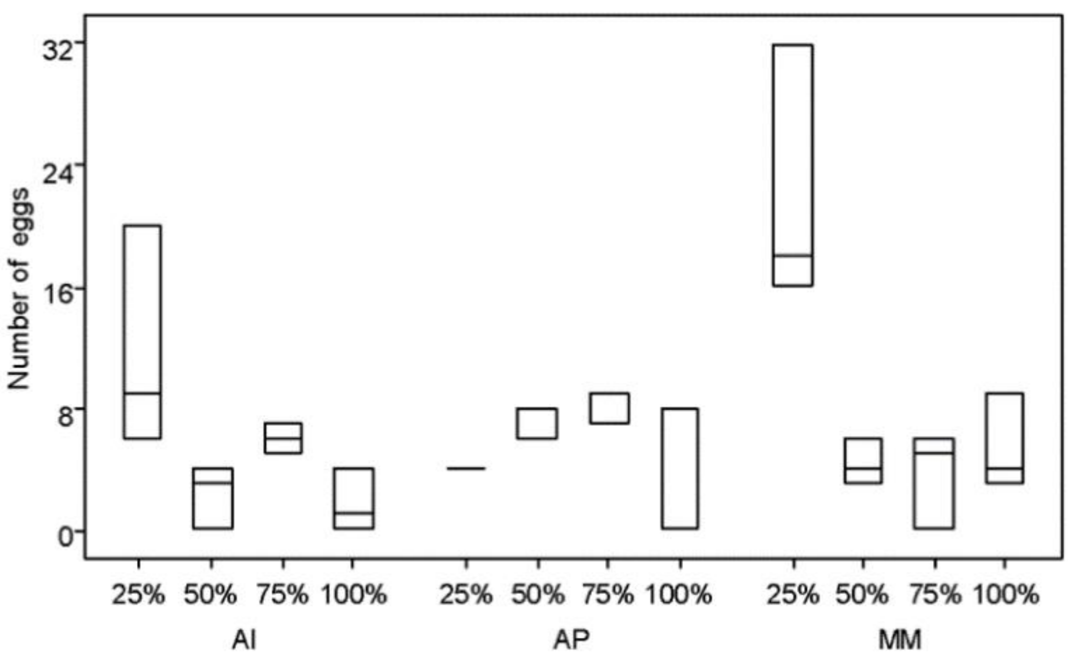

Fig 1 The egg density index analysis for three species of common alien invasive flora; *Austroeupatorium inulifolium*, *Alternanthera philoxeroides* and *Megathyrsus maximus*.

There were significant differences in the egg hatch rate with the infusion type (F=4.70; DF=2; *P*<0.05) and concentration (F=4.12; DF=3; *P*<0.05). The interaction of plant species and concentration shows a significant difference in the daily hatch rate (Two-way PERMANOVA, F=4.28, df=6, P<0.05). In all four concentrations 25%, 50%, 75% and 100% the emergence of larvae was highest during the second day of rearing. On the second day of rearing, the highest hatch rates were observed for *A. philoxeroides* at a 25% concentration, *M. maximus* at a 50% concentration, and *A. inulifolium* at a 75% concentration. However, contrastingly *M. maximus showed the highest hatch rate at 100% concentration during the first day of rearing* (Fig 2*).*

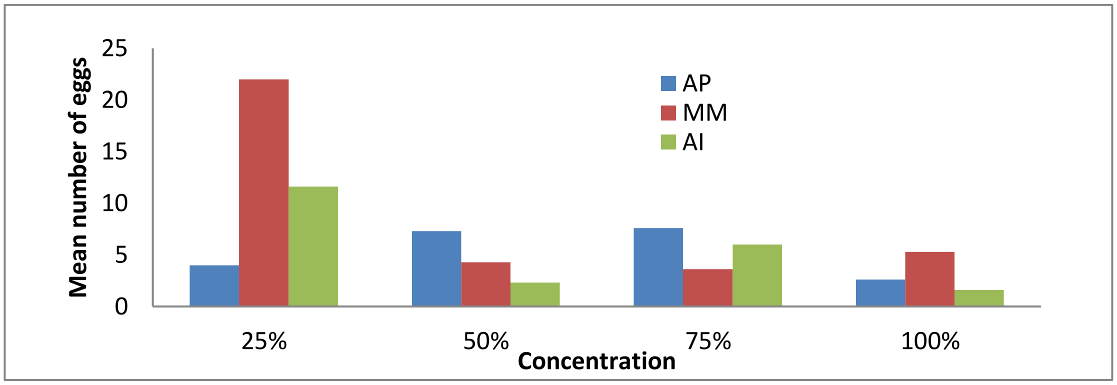

Fig 2 Mean number of eggs vs. concentration

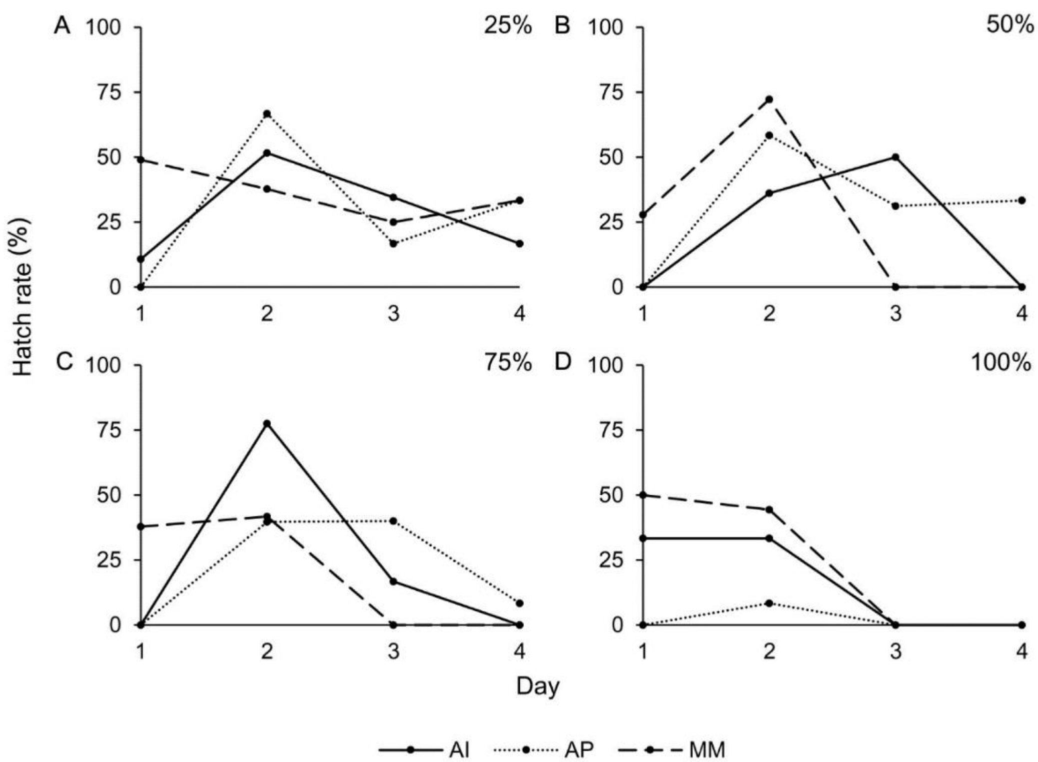

Fig 3 Daily hatch rate of *Aedes* eggs under four different concentrations of leaf infusions

The leaf infusions were attractive to gravid females of both *Ae. aegypti* and *Ae. albopictus*. The mosquitoes exhibit different responses to various concentrations of each plant species. When considering the concentration and species in *A. inulifolium,* there were significant differences in the species attracted to the different infusion concentrations (F=3.92, df=3, P<0.05) and there was a significant difference in plant species with the species attraction (F=8.64, df=1, P<0.05). However, there was no significant combined effect between concentration and species (F=0.64, df=3, P>0.05; Fig 3).

Similarly, in *A. philoxeroides* there were significant differences in the infusion concentration and the species attraction (F=8.68, df=3, P<0.05) and there was a significant difference in plant species with the species attraction (F=37.43, df=1, P<0.05). Also, there was a significant combined effect between concentration and species (F=6.95, df=3, P<0.05; Fig 3).

Moreover, there were significant differences in the infusion concentration and the species attraction in *M. maximus* (F=77.32, df=3, P<0.05) and also a significant difference in plant species with the species attraction (F=280.3, df=1, P<0.05). However, a significant combined effect between concentration and species was not observed (F=1.47, df =3, P>0.05; Fig 3).

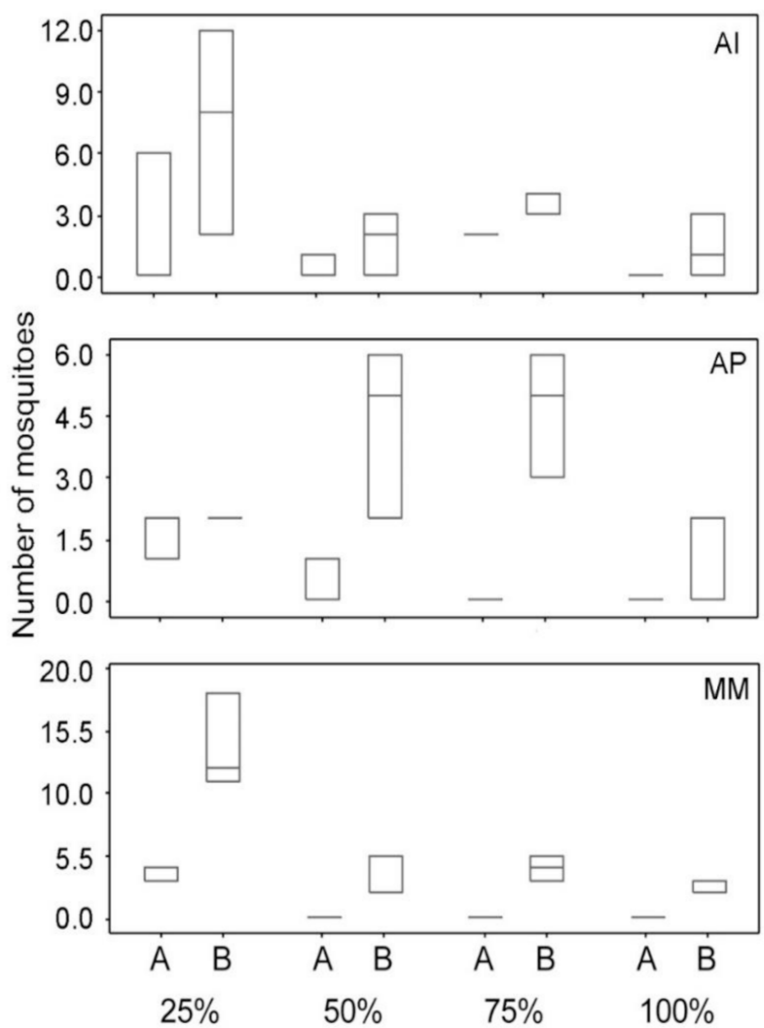

Fig 4 Species attraction analysis

DISCUSSION

Currently, the suitability of plant infusions is widely used for evaluating ovitrap enhancement. Rice straw, grass, mango leaf, and banana leaf infusions are widely used for such studies, (Gopalakrishnan *et al*. 2012). The results of this study strongly support those of previous researchers who found that *Ae. aegypti* mosquitoes lay eggs more frequently in ovitraps with hay infusion than in those with plain water. Similar results were observed in guinea grass at a 25% concentration. According to this study, it can be concluded that grass species such as rice straw, grass hay, and guinea grass may contain some chemicals that can induce a positive oviposition response in *Ae. albopictus (Dormont et al. 2021b).* In contrast, these compounds induced repellency at high concentrations (Allan & Kline 1995), which shows the same results as the present study (Reiter *et al*., 1991) This may be explained by the fact that leaf infusions include a complex blend of substances that impact both mosquito oviposition performance and gravid females' choice of oviposition sites. Hence, the present study revealed that Guinea grass infusion with a 25% concentration was observed as the most suitable type of infusion to be used in ovitrap surveillance.

Mosquitoes select their oviposition sites depending on physical attributes such as colour, substrate texture, odorants, and other chemical compounds (Day 2016). The response of gravid mosquitoes to various infusions varied greatly from batch to batch, even under uniform fermentation conditions. These variances may lead to variations in the microbial populations in the experimental plant infusions, as well as corresponding variations in the amounts and kinds of odorants generated. Notably, *Ae. aegypti* or *Ae. albopictus* were not particularly attracted to infusions made from *A. philoxeroides* leaves. These findings confirm the critical significance of the plant species that are utilized to make infusions. The results of this study suggest that plant infusions may not always be a reliable source of attractive odorants and that each batch of infusion should be tested to ensure that it is behaviorally active before being applied in the field. Because volatile compounds that attract females may not always serve as oviposition stimulants, it should be emphasized that the attraction of gravid females to odorants that emerge from a plant infusion does not always result in enhanced oviposition. (Gubler 1971)

The present study found that the ovipositional rate of gravid *Ae. aegypti* females was high in low concentrations and vice-versa confirming the findings of Reiter *et al*. (1991). More *Aedes* eggs were laid in ovitraps with a lower concentration of guinea grass infusion than in a greater concentration. This may be explained by the fact that leaf infusions include a complex blend of substances that impact both mosquito oviposition performance and gravid females' choice of oviposition sites. However, the mean number of eggs obtained from the control is lower than the mean number of eggs found in guinea grass at a 25% concentration. The level of infusion attractiveness may vary depending on a variety of factors. An infusion can change from becoming an attractant to being a repellant depending on the amount of protein and microorganisms present (Gubler 1971). According to the study, *A. philoxeroides* showed the lowest oviposition rate and that may be because it contains some proteins or microorganisms that have repellent action against gravid mosquitoes.

The *M. maximus* had a higher oviposition rate at 25% concentration. With a 25% concentration, *A. inulifolium* had the second-highest number of eggs. The information presented here indicated that although most organic infusions utilized as attractants in mosquito traps have an unpleasant odour, the fetid *M. maximus* may attract *Aedes* females to sites where they lay their eggs. As shown both in the lab (Navarro *et al*. 2003) and in the field, the process of bacterial growth with subsequent secondary metabolite synthesis affects the attractiveness of organic infusions. Certain active compounds in the infusions, which are regarded as additional sources of attractant, may potentially have an impact on the significant ovipositional variability within and between the numerous infusions tested.

The emergence of larvae from the eggs was highest during the second day of rearing. However, a decreasing hatch rate was observed with time. Mosquito larvae may be directly affected by toxicity from decomposing plant matter (David *et al.* 2000), and when the concentration of plant infusion is high, it may have a more toxic effect on the larvae. In aquatic and terrestrial systems, a variety of leaf species may act as a more diverse resource base for microorganisms (Cardinale *et al.* 2007). As a result, they may provide either an increased abundance or diversity of microbes, resulting in a superior diet for mosquito larvae. Complementary or self-selecting feeding, which uses a variety of resources to give a consumer better nutrition, may enable quicker larval growth (Greenstone 1979; Waldbauer & Friedman 1991).

The larval density shows a significant difference with the different plant infusions. The highest larval density was observed in *M. maximus* at a lower concentration. Therefore, *M. maximus* also known as guinea grass, may produce optimum conditions for larval development at lower concentrations. It may be possible to achieve a high egg density through guinea grass infusion. This circumstance would be affected by the resultant low larval density in *A. philoxeroides.* Therefore, the oviposition choice of gravid *Aedes* mosquitoes is low on *A. philoxeroides* leaf infusion. This observation indicates that *A. philoxeroides* may contain fewer oviposition attractants than *M. maximus.*

The number of eggs collected using ovitraps and their corresponding OPI and EDI indices demonstrate how effective this technique is at tracking these vectors. The removal of eggs from the environment is comparable to the use of a control agent to manage immature stages. This increases the efficacy and safety of this strategy for mosquito control efforts. This technique might monitor vectors and help with vector management since a significant number of eggs were discovered in the various control agents without repellents (Silva *et al.* 2018). Hence, with reference to the present study, it demonstrates that the usage of organic matter infusions, primarily those composed of guinea grass, can enhance the effectiveness of the trap and function as a female attractant, and it is more effective than ovitraps with aerated tap water, which was used as a control.

Studies have shown that female *Ae. aegypti* lay noticeably more eggs on conspecific or *Ae. albopictus* egg-containing oviposition strata than on egg-free strata (Allan & Kline 1995). *Ae. albopictus* and *Ae. aegypti* are hard to monitor since adults are slow to respond to light traps (Service 1977) and sampling larvae take a long time. The use of oviposition attractants/stimulants to increase the sensitivity of ovitraps as well as gravid female traps (Reiter *et al.* 1991) has significant potential. Ovitraps are frequently used for population monitoring and surveillance (Chadee 2009; McHugh & Hanny 1990). Infusions or substances that both evoke long-range orienting of gravid females to possible oviposition locations and promote oviposition once at the oviposition site are particularly important for this use. Our results indicated a greater attraction of *Ae. albopictus* than *Ae. aegypti* to guinea grass infusion and *Austroeupatorium* infusion.

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