LAPORAN AKHIR PROJEK PENYELIDIKAN JANGKA PENDEK

FINAL REPORT OF SHORT TERM RESEARCH PROJECT

UNIVERSITI SAINS MALAYSIA

PEJABAT PENGURUSAN & KREATIVITI PENYELIDIKAN

Sila kemukakan **dua (2) salinan** laporan akhir ini melalui Jawatankuasa Penyelidikan di Pusat Pengajian dan Dekan/ Pengarah/ Ketua Jabatan kepada Pejabat Pengurusan dan Kreativiti Penyelidikan (RCMO)

1. **Nama Ketua Penyelidik: Wan Fatma Zuharah Wan Musthapa**
Name of Research Leader

☐ Profesor Madya/ *Assoc. Prof.* ☒ Dr./ *Dr.* ☐ Encik/Puan/Cik *Mr/Mrs/Ms*

2. **Pusat Tanggungjawab (PTJ): School of Biological Sciences**
School/Department

3. **Nama Penyelidik Bersama:**
Name of Co-Researcher

4. **Tajuk Projek: The influence of mosquito predators *Toxorhynchites* species on four species of mosquitoes**
Title of Project

5. **Ringkasan Penilaian**/*Summary of Assessment*:	Tidak Mencukupi *Inadequate* 1	2	Boleh Diterima *Acceptable* 3	Sangat Baik *Very Good* 4	5
i) **Pencapaian objektif projek:** *Achievement of project objectives*	☐	☐	☐	☐	✓
ii) **Kualiti output:** *Quality of outputs*	☐	☐	☐	☐	✓
iii) **Kualiti impak:** *Quality of impacts*	☐	☐	☐	☐	✓
iv) **Pemindahan teknologi/potensi pengkomersialan:** *Technology transfer/commercialization potential*	☐	☐	✓	☐	☐
v) **Kualiti dan usahasama :** *Quality and intensity of collaboration*	☐	☐	☐	✓	☐
vi) **Penilaian kepentingan secara keseluruhan:** *Overall assessment of benefits*	☐	☐	☐	☐	✓

6. Abstrak Penyelidikan
(Perlu disediakan di antara 100 - 200 perkataan di dalam **Bahasa Malaysia dan juga Bahasa Inggeris**. Abstrak ini akan dimuatkan dalam Laporan Tahunan Bahagian Penyelidikan & Inovasi sebagai satu cara untuk menyampaikan dapatan projek tuan/puan kepada pihak Universiti & masyarakat luar).

Abstract of Research
(An abstract of between 100 and 200 words must be prepared in Bahasa Malaysia and in English). This abstract will be included in the Annual Report of the Research and Innovation Section at a later date as a means of presenting the project findings of the researcher/s to the University and the community at large)

As attached (Appendix A)

7. Sila sediakan laporan teknikal lengkap yang menerangkan keseluruhan projek ini. [Sila gunakan kertas berasingan]
Applicant are required to prepare a Comprehensive Technical Report explaining the project. (This report must be appended separately)

See Appendix B

Senaraikan kata kunci yang mencerminkan penyelidikan anda:
List the key words that reflects your research:

Bahasa Malaysia	*Bahasa Inggeris*
Toxorhychites	*Toxorhynchites*
Kawalan Biologi	Biocontrol
Nyamuk	Mosquito

8. Output dan Faedah Projek
Output and Benefits of Project

(a) * **Penerbitan Jurnal**
Publication of Journals
(Sila nyatakan jenis, tajuk, pengarang/editor, tahun terbitan dan di mana telah diterbit/diserahkan)
(State type, title, author/editor, publication year and where it has been published/submitted)

Two manuscripts have been submitted:

(1) Wan Fatma Zuharah[1,2] · Nur Aishah Yusof[1] · Nik Fadzly[1] · Hamady Dieng[1] · Abu Hassan Ahmad[1] · Sazaly Abu Bakar[3] Risky behaviors: Effects of *Toxorhynchites splendens* (Diptera: Culicidae) predator on the behavior of three mosquito species. Journal of Insects Behaviour (Accepted with changes)

(2) Nurhafiza Mohamad[1] and Wan Fatma Zuharah[1,a] Influence of container design on predation rate of classical bicontrol agent, *Toxorhynchites splendens* (Diptera:Culicidae) against dengue vector. Tropical Biomedicine (under review)

(b) **Faedah-faedah lain seperti perkembangan produk, pengkomersialan produk/pendaftaran paten atau impak kepada dasar dan masyarakat.**
State other benefits such as product development, product commercialisation/patent registration or impact on source and society.

From our study, *Tx. splendens* has been proved as one of the potential biocontrol agent in controlling the population of *Aedes* especially in artificial containers. The use of this biocontrol agent has potential in replacing some of vector control program mainly use of the chemical insecticide, which may harm human health and cause resistance to the mosquitoes. This potential agent has been accepted by communities and has been released in Subang Jaya as one of the promising alternative for mosquito control program.

* Sila berikan salinan/*Kindly provide copies*

(c) **Latihan Sumber Manusia**
Training in Human Resources

i) Pelajar Sarjana:
Graduates Students
(Perincikan nama, ijazah dan status)
(Provide names, degrees and status)

NurHafiza Mohamad, PhD, on-going

ii) Lain-lain: NurAishah Yusof, BSc. (2012), Graduated
Others

9. Peralatan yang Telah Dibeli:
Equipment that has been purchased -Non-

Dr. Wan Fatma Zuharah Binti Wan Musthapa
Lecturer
School of Biological Sciences
Universiti Sains Malaysia

Tandatangan Penyelidik
Signature of Researcher

23/7/13

Tarikh
Date

Komen Jawatankuasa Penyelidikan Pusat Pengajian/Pusat
Comments by the Research Committees of Schools/Centres

Output kajian yang memuaskan

24/7/13.

TANDATANGAN PENGERUSI
JAWATANKUASA PENYELIDIKAN
PUSAT PENGAJIAN/PUSAT
Signature of Chairman
[Research Committee of School/Centre]

PROFESSOR SITI AZIZAH MOHD NOR
Deputy Dean
(Research)
School of Biological Sciences
Universiti Sains Malaysia
11800 USM, Penang

Tarikh
Date

APPENDIX A: ABSTRACT

THE INFLUENCE OF MOSQUITO PREDATORS *TOXORHYNCHITES* SPECIES ON FOUR SPECIES OF MOSQUITOES

With the difficulties in finding new biocontrol candidates for mosquitoes, improving the efficacy of existing biological agents is one of the major current issues in conservation biological control. In this prospect, a better understanding of the predatory and predation-risk behavioral responses is relevant and important in understanding the factors contributed to the successful of biological control program. Here, we investigated the ability of, *Toxorhynchites splendens* predator and its potential as biocontrol agent in controlling the mosquito populations especially the dengue vector mosquitoes in Malaysia. Our study had revealed that this agent preferred to consume on *Aedes aegypti* even in the presence of other mosquito species. With the presence of more abundance prey (mosquito larvae), more prey will be consume due to the close contact and more opportunity for *Tx. splendens* to ambush the prey. Our data suggested that the maximum consumption number by a *Tx. splendens* predator was 9 individual within 24 hours. "Thrashing" and "browsing" activities were greater in *Ae. aegypti* larvae, which was found to be the most vulnerable activities to the predator. Such water movements are likely correlated with vulnerability. The successful of *Tx. splendens* predation rate also correlated with the releasing habitat especially in man-made containers. We found that, *Tx. splendens* predate efficiently more on prey larvae when they co-existed together in vertical type container compared to the horizontal container, regardless of water volume. In which, allows less hiding space for the prey. As conclusion, this leads to a great finding that *Tx. splendens* have fine chances in controlling dengue hemorrhagic vector, *Aedes aegypti,* and with the information on suitable releasing technique to enhance the ability and capability of this biocontrol agent in the nature.

APPENDIX B: COMPREHENSIVE TECHNICAL REPORTS

This study was proposed to evaluate the effectiveness of biocontrol agent, *Toxorhynchites splendens* on mosquito populations that normally found in Malaysia especially *Aedes* species, vector of dengue fever. Even, this biocontrol has been known and researched since 1930s, but less literatures and studies have been recorded. This is due to the new emergence of other biocontrol agents that more attractive to the researcher communities. Due to this matter, the real ability of *Tx. splendens* has been left behind without knowing their true abilities to control the mosquito populations. Therefore, I was called to understand the ability of this agent in term of the behaviour of both predator (*Tx. splendens*) and also the prey (mosquitoes), in order to create the working successful relationship and make this biocontrol agent to become one of the effective control strategies in the future. This biocontrol agent was chose as this agent has been found to be one of the safest control methods, no effect on non-target organisms, not causing any resistance problem, ability to control huge population of prey and their co-existed together with the target prey (mosquitoes).

Here, we evaluated four different angles and objectives in understanding the ability of *Tx. splendens*.

Two objectives have been translated in manuscript submitted to Journal of Insect Behaviour (accepted with changes). Here, we found that in the presence of different mosquito species, *Toxorhynchites splendens* had showed primary preference towards *Ae. aegypti*, even when *Ae. albopictus* and *An. sinensis* were offered together in the present study (Figure 1).

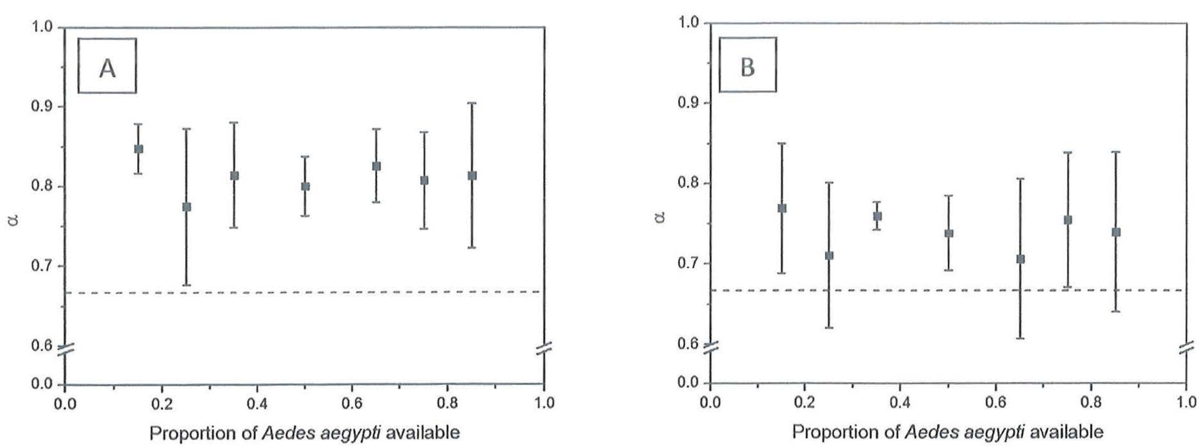

Figure 1: The preference of *Tx. splendens* for *Ae. aegypti* larvae compared to (A) *Ae. albopictus* larvae, and (B) *Anopheles sinensis*, indicated by (α) (±SE). The broken line indicates no preferences for either mosquito larvae, at $\alpha = 0.667$

To explain the preference of *Tx. splendens* towards *Ae. aegypti* and other prey species, we then looked on the behaviour of preys in response to the *Tx. splendens*. The behaviour of organism is an important factor in answering why some of the prey species become vulnerable to predation. In this study, we score the behavior categories from 1 to 4 for activities and 5 to 8 for positions as follows: (1) resting; (2) browsing; (3) filtering; (4) thrashing; (5) surface; (6) bottom; (7) wall; and (8) middle, which were then modeled as being dependent on prey species (*Ae. aegypti, Ae. albopictus* and *An. sinensis*), and treatments (control, free-roaming predator, and residual kairomones remnant). Based on our findings, we concluded that the most vulnerable prey, *Ae. aegypti* exhibited significantly high frequency of "thrashing" activities at the "wall" position when facing the predator (Figure 2). This behaviour of thrashing cause the movement of prey bodies with may attract the predator and get more attacked rate by the *Tx. splendens* predator.

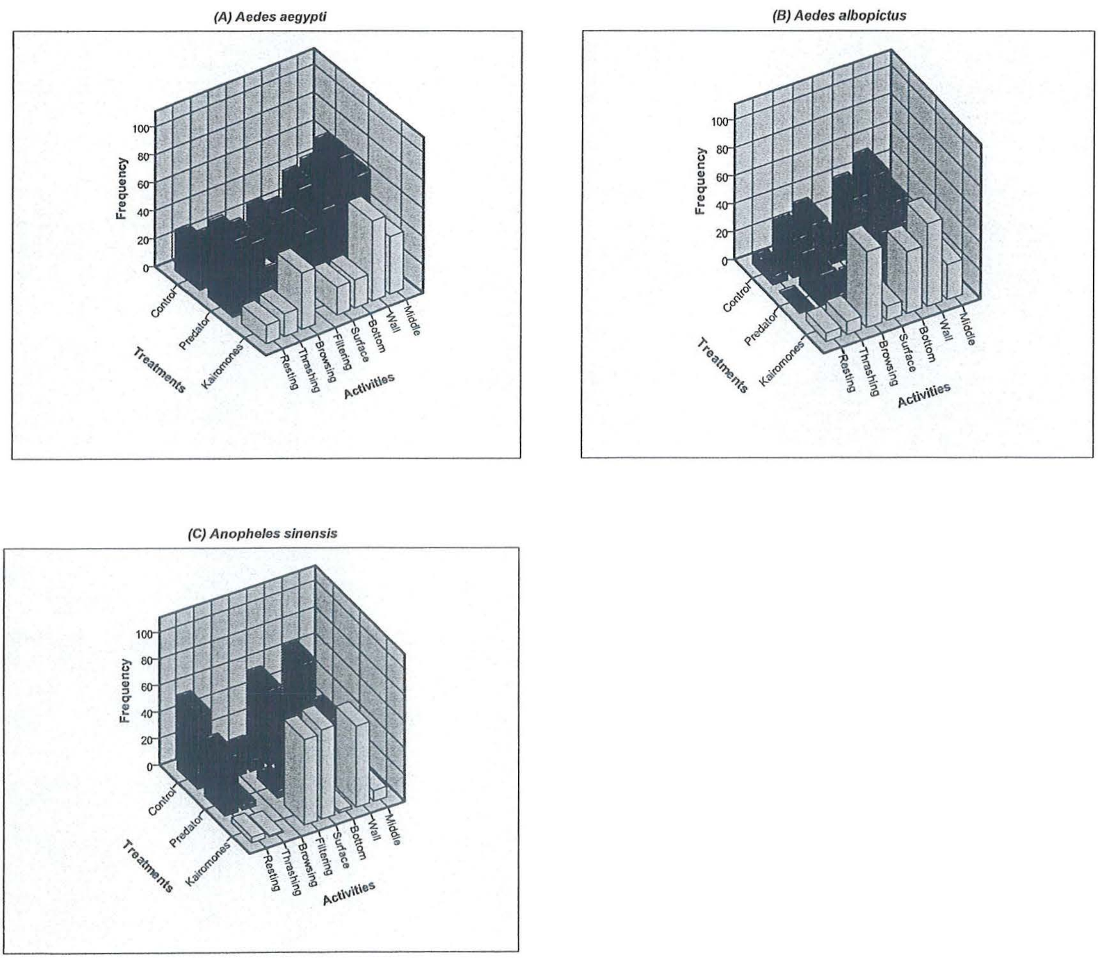

Figure 2. Behavior of three species of mosquito larvae (A) *Ae. aegypti,* (B) *Ae. albopictus* and (C) *An. sinensis* in response to various treatments of control (absence of predator), with predator and predator's kairomones only.

Other two objectives of study have been reported recently in another manuscript submitted to Tropical Biomedicine (under review). In these studies, we investigated the effectiveness of *Tx. splendens* larvae by researching on the maximum number of prey (*Ae. albopictus*) that can be consumed within 24 hours. We also manipulated containers variation with the differences of water volumes that may influence the successful predation rate of *Tx. splendens* larvae. Here, we discovered that one *Tx. splendens* predator may consumed maximum of nine individuals of *Ae. albopictus* larvae in 24 hours (Figure 3). We also found that in higher abundance of *Ae. albopictus* prey, more prey will be consumed. This is due to the close contact between predator and prey and makes it as an easy catches for this ambush predator.

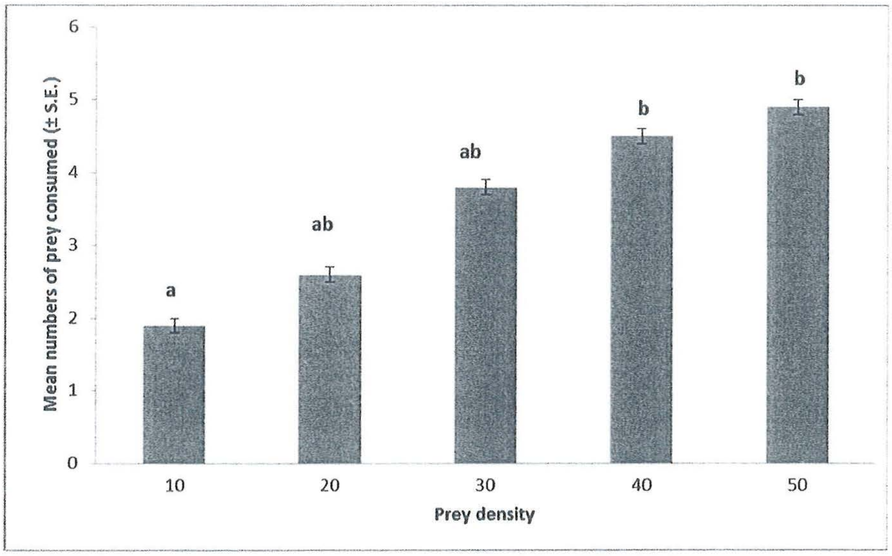

Figure 3. Mean numbers (± S.E.) of *Ae. albopictus* prey consumed by third instar larvae of *Tx. splendens* predator after 24 h exposure. Different letters indicated significant difference among the results (Tukey's HSD multiple comparison test, $p<0.05$).

In our observation, this biocontrol predator is found in almost all types of containers available with restriction that the container should be close to the resting habitat areas; around the vegetation. Therefore, we conducted a study on the different types of container; rubber tire, tin can, plastic drinking glass, and bamboo stumps in order to understand the ability of *Tx. splendens* in different habitats. Due to different rainfall receives in our country, we manipulated the water volumes with (1) full (300ml), (2) half full (150ml), (3) 1/4 full (75ml), and (4) 1/8 full (37.5ml).

Our results indicated that the numbers of prey consumed by *Tx. splendens* in rubber tire were significantly higher (Tukey's HSD, $p<0.05$) (Figure 4). The results indicated that regardless of water volumes used in the container, the predation of *Tx. splendens* was higher in horizontal type of container (rubber tire) as compared to vertical type of containers (bamboo stump, tin cans and plastic drinking glass).

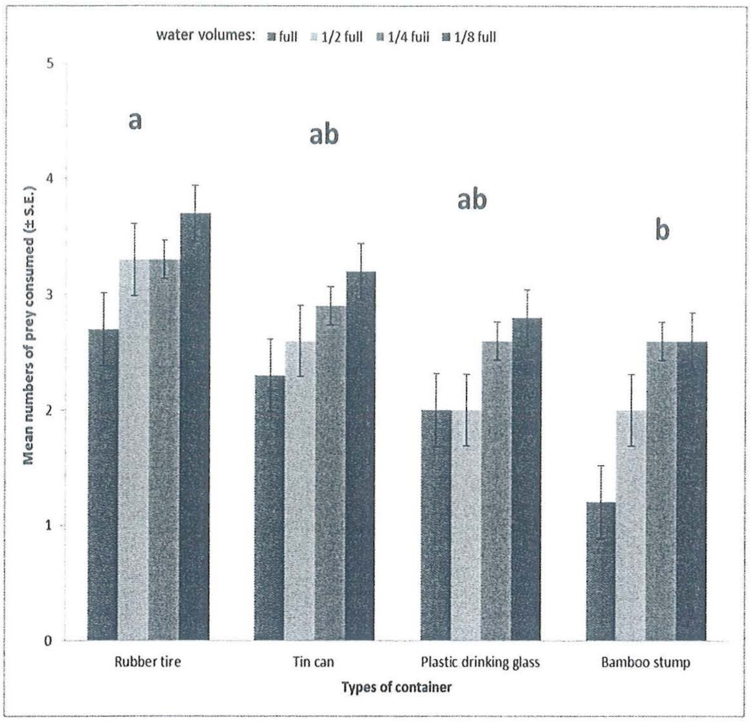

Figure 4. Mean numbers (± S.E.) of *Ae. albopictus* prey consumed after 24 h by third instar larvae of *Tx. splendens* predator in various types of container contains different water volumes. Different letters indicated significant difference among the groups (Tukey's HSD multiple comparison test, $p<0.05$).

In conclusion, behavioral response and positioning of prey are two important factors that contribute to the successful of *Tx. splendens* as biocontrol agent. Whereas, oviposition habitat or introduce containers is also important in enhancing the ability of Tx. splendens to reach the maximum performance as biocontrol agents. This agent has been proved in our study, to have significant on reducing dengue hemorrhagic fever vector, *Ae. aegypti* and *Ae. albopictus.*

Informations on this particular predatory *Tx. splendens* larva are important in understanding the factors contributed to the successful of biological control program. This information also can be use in future to maximize the ability of this biocontrol agent in vector control program and can be use as the safest way to control dengue vector populations in Malaysia.

This study was also presented in four international conferences as below:

- Mohamed N and **Zuharah WF**. The predation of giant mosquito (*Toxorhynchites splendens*) in different densities of prey, types of container and water volumes. 48th Annual Scientific Conference of the Malaysian Society of Parasitology and Tropical Medicine. 27-28 March 2012. Grand Season Hotel, Kuala Lumpur.

- Mohamed N and **Zuharah WF**. The prevelance of the mosquito predator, *Toxorhynchites splendens* larvae along with its prey, *Aedes albopictus* larvae in Malaysia. Entomology 2012. 11-14 November 2012. Knoxville Convention Centre, Tennesse, USA

- **Zuharah WF,** Dieng H, Fadzly N, Ahmad AH, Rajen S, Gurbel SS, Maniam T, Rattanam AR, AbuBakar S. The effectiveness of *Toxorhynchites splendens* as biocontrol agents in various integrated managements. LRGS Dengue and Colloquium and Workshop. 11-14 June 2013. Universiti Malaya, Malaysia.

- Mohamad N, **Zuharah WF**. Field observation of the predatory mosquito, *Toxorhynchites splendens* in various types of container. Seminar on Sustainable Agriculture and Natural Resources. 9 April 2013. Universiti Sains Malaysia, Malaysia.

Gmail by Google

Wan Fatma Zuharah < wanfatma@gmail.com>

Major Revisions requested JOIR-D-13-00048

1 message

Ethel Dionela (JOIR) < ethel.dionela@springer.com> Thu, Jun 27, 2013 at 12:48 AM
To: Wan Fatma Zuharah bt Wan Musthapa <wfatma@usm.my>

Dear Wan Fatma,

We have received the reports from our advisors on your manuscript, "Risky behaviors: Effects of Toxorhynchites splendens (Diptera: Culicidae) predator on the behavior of three mosquito species", which you submitted to Journal of Insect Behavior.

Based on the advice received, I feel that your manuscript could be reconsidered for publication should you be prepared to incorporate major revisions. When preparing your revised manuscript, you are asked to carefully consider the reviewer comments which can be found below, and submit a list of responses to the comments. You are kindly requested to also check the website for possible reviewer attachment(s).

In order to submit your revised manuscript, please access the following web site:

http://joir.edmgr.com/

Your username is: wfatma
Your password is: fadzly22

We look forward to receiving your revised manuscript before 23 Dec 2013.

Sincerely,

Dr. Thomas L. Payne, Editor
Journal of Insect Behavior
College of Agriculture, Food
and Natural Resources
University of Missouri -- Columbia
Columbia, MO 65211-7120

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COMMENTS FOR THE AUTHOR:

Reviewer #1: The underlying idea of the manuscript Risky behaviors: Effects of Toxorhynchites splendens (Diptera: Culicidae) predator on the behavior of three mosquito species is interesting and worth of study. Quantitative analysis methods were also appropriate for the collected data. Nevertheless, this study needs to improve many aspects to meet the expectations of a sound experiment for hypothesis testing and to better frame the study and its implications in the context of insect behavior and public health.

1 - The language requires strong editing. The whole manuscript language must be revised by a native speaker the way it is written makes it very difficult to understand.

Manuscript for consideration for publication in Tropical Biomedicine

Wan Wan Musthapa

Sent: Monday, July 01, 2013 6:54 PM
To: editor@msptm.org
Attachments: Mohamad and Zuharah-Contai~1.doc (161 KB) ; Mohamad and Zuharah et al.~1.pdf (83 KB)

Dear Editor of Tropical Biomedicine,

Here I attached the manuscript entitle "Influences of container design and water volume on predation rate of *Toxorhynchites splendens* (Diptera:Culicidae) as biocontrol agent against dengue vector" to be considered for publication in your journal.

Please contact me if you need anything else and thank you for considering this manuscript.

Regards,
Dr. Wan Fatma Zuharah Wan Musthapa (PhD),
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Universiti Sains Malaysia,
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Penang,MALAYSIA.
Phone: +60-(0)4-6538888 ext.6130
Fax: +60-(0)4-6565125

Influence of container design on predation rate of classical bicontrol agent, *Toxorhynchites splendens* (Diptera:Culicidae) against dengue vector

Nurhafiza Mohamad[1] and Wan Fatma Zuharah[1,a]

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Abstract

Toxorhynchites splendens larvae are natural predator to dengue vector mosquito larvae, *Ae. albopictus*. This study was carried out to evaluate the predation rate of *Tx. splendens* third instar larvae on *Ae. albopictus* larvae in 24 h. Each predator was offered with prey density between 10 to 50 individuals. Predation rate of *Tx. splendens* were also tested with two manipulated factors; various types of container and different water volumes. The predation rate of *Tx. splendens* was evaluated in man-made containers (tin cans, plastic drinking glasses and rubber tires) and natural container (bamboo stumps) which filled with different water volumes (full, half full, 1/4 full, and 1/8 full). The results showed that the predator significantly consumed more *Ae. albopictus* when more numbers of prey available in the container (F=3.935, df=4, p=0.008). The prey consumption of *Tx. splendens* was significantly higher in rubber tire as compared to other types of container (F=3.100, df=3, p=0.029) due to the horizontal design of the container. However, there was no significant effect of water volumes on predation rate of *Tx. splendens* larvae (F=1.736, df=3, p=0.162). We concluded that the finding in this study can contributed to understand the relevant factors in improving the efficiency of *Tx. splendens* predatory larvae as one of the promising biological control agent. In which, we found that the best target container is in horizontal position with wide opening and can be introduced either in wet and dry seasons.

volumes, depend on the amount of the rainfall received. The numbers of *Ae. albopictus* prey larvae available on these containers are also various.

The objective of our study is to investigate the maximum number of prey (*Ae. albopictus*) that can be consumed by third instar larvae of *Tx. splendens* within 24 hand the effects of containers variations together with water volumes in influencing the successful predation rate of *Tx. splendens* larvae. Informations on this particular predatory *Tx. splendens* larvae is important in understanding the factors contributed to the successful of biological control program. This information also can be use in future to maximize the ability of this biocontrol agent in vector control program in Malaysia.

MATERIALS AND METHODS

Predators and prey colonies

The predatory larvae of *Tx. splendens* and *Ae. albopictus* prey were collected from Durian Valley (05° 47'N, 100° 25'E) and Persiaran Sungai Emas (05° 36'N, 100° 30'E) in Penang Island, Malaysia by placing 20 oviposition traps in each areas prior to experiment. Durian Valley is a small forest situated in the middle of Universiti Sains Malaysia while Persiaran Sungai Emas is a small shrubbery area nearby Batu Ferringhi beach. Both areas are surrounded with heavy vegetations, providing the most suitable sylvan environment for both species to inhabit in the oviposition traps. Both species were brought back to laboratory for identification and cultured in the laboratory at 23 ± 1°C and 40 ± 1% RH, respectively. In order to have enough predator and prey samples at the same instar stage, we cultured both mosquito species until F1 generation.

For *Ae. albopictus* prey, about 100-150 larvae of *Ae. albopictus* were placed in enamel pan and fed with 0.5 gram of fine ground larval food made of 2 : 1 : 1 : 1 of dog biscuits, dried cow's liver, yeast and milk powder, daily until reached pupal stage. The pupae were collected and placed in the mosquito cage to allow them to become an adult. Adults were fed with 10% of sucrose solution with addition of vitamin B complex and allowed to mate. Females were then blood fed with a laboratory mouse for 12 h and allows to oviposit in moist oviposition substract made of filter paper with a cone shape placed on a petri dish.

For the *Tx. splendens* predator, the larvae were reared individually to avoid cannibalism. Each larvae were fed with ten larvae of *Ae. albopictus* daily. Same instar stage of *Ae. albopictus* prey larvae were fed to same instar stage of *Tx. splendens* predator larvae (i.e 1st instar prey larvae feed to 1st instar predator larvae). When the predators reached the pupal stage, they were collected and placed in a cage. They were permitted to become an adult, allowed to mate and supplied with 10% of sucrose solution plus vitamin B complex as food source. After few days, the females laid eggs (F1 generation) on the water surface in the provided black colour cup.

A healthy third instar larvae of predator and prey were selected for the experiment. Since the level of satiation can influence the predatory consumption rate of *Tx. splendens*, the selected predator larvae were starved for 24 h prior to prey consumption experiment.

Study designs

Effects of prey densities on predation of Tx. splendens

INTRODUCTION

Dengue disease is rank as the most important mosquito borne viral disease in the world by World Health Organization (WHO, 2012). As the dengue vaccines are still under development stages, the available method to prevent this disease is by controlling the vector mosquito. *Aedes aegypti* and *Aedes albopictus* mosquitoes are two major vectors involve in the transmission of dengue disease in many urban areas of South-east Asia (Smith, 1956; Hammon, 1966; Rudnick, 1967). Both of these vectors are known as container breeders and can be found in all types of natural and artificial containers in and around human habitations (Chan *et al.*, 1971; Focks, 2007). These larval habitat containers are commonly treated with chemical larvicide to control the population of these vectors.

Chemical control are widely used in vector control programme, however, the method always fraughting with some problems such as an increasing in chemical and labor costs and reluctant by the publics to have any chemical in their domestic water containers (Focks, 2007). Futhermore, the vectors mosquitoes were reported have been developed resistant to several pesticides. Bioassay data demonstrate that resistance to organophosphates (temephos) and pyrethroids is widespread in *Ae. aegypti*, and resistance has also been reported in *Ae. albopictus* (WHO, 2012). Due to these reasons, the search of alternative methods for vector control has become more imperative (Collins & Blackwell, 2000).

One of the potential alternative approaches is to use the biological control agents such as *Toxorhynchites* spp. mosquitoes (Collins & Blackwell, 2000). *Toxorhynchites* spp. is a container breeder and found in wide variety of both artificial and natural containers

(Steffan & Evenhuis, 1981). They were reported to co-exist together with *Ae. aegypti* and *Ae. albopictus* in bamboo stumps, rubber tires, earthen-ware jars and tin cans (Yasuno & Tonn, 1970; Trpis, 1973; Nyamah *et al.*, 2011). *Toxorhynchites* spp. mosquitoes were emphasized as ideal biological control agent because the adults do not feed on blood. Therefore they cannot act as vectors of diseases (Trimble, 1983). The adult only feed on the plant nectar and the larvae are predatory on other mosquito larvae that share the same habitat.

The female of *Toxorhynchites* spp. has an ability to search out distributed containers which are not accessible to chemical control method without supervision (Collins & Blackwell, 2000; Focks, 2007). However, the introduction of female adult as biological agent in several previous studies showed little success in controlling the target prey species because females oviposited their eggs into other available habitats rather than the target habitats (Focks *et al.*, 1979; Focks *et al.*, 1983; Bailey *et al.*, 1983; Toohey *et al.*, 1985). Therefore, Collins and Blackwell (2000) recommended the introduction of *Toxorhynchites* spp. larvae to infested containers is more suitable than releasing the adult.

Toxorhynchites larvae are predacious during all larval instars (Collins & Blackwell, 2000; Focks, 2007). The predation rates on several species of *Toxorhynchites* larvae were previously reported depend on some factors including container size, prey size, prey type, water temperature and light level (Collins & Blackwell, 2000; Focks, 2007). *Toxorhynchites splendens* is one of the species of the genus that can be found in Malaysia. This predatory larvae are potentially biological control agent that can be released in a lot of outdoor containers to control the population of *Ae. albopictus* (Nyamah *et al.*, 2011). From our preliminary survey, these outdoor containers are either natural or man-made containers and varieties in types, sizes and designs. They are generally occupied with different water

Square plastic containers sized 18.5 cm length x 12.0 cm width x 6.50 cm height filled with 300ml of seasoned water were used for this study. Seasoned water is tap water that was left standing for more than 48 h to reduce the chlorine content (Zuharah and Lester, 2010). Five treatments were established based on densities of *Ae. albopictus* prey; (1) 10, (2) 20, (3) 30, (4) 40, and (5) 50. Each treatment container was then exposed with one individual of *Tx. splendens* predator. The numbers of prey consumed by predator were recorded after 24 h post-treatment. No prey was found died in control treatment (without any predator). Experiment was replicated for ten times.

Data were tested for normality using one sample Kolmogorov-Smirnov test. All data were log transformed (x+1) prior to analysis to satisfy the assumptions of ANOVA. In order to test the effects of prey density on predation, we ran a one-way Analysis of Variance (ANOVA) and mean differences were separated with Tukey's HSD multiple comparison test by using SPSS version 20.0 (SPSS 2012). The numbers of prey consumed by a predator in 24 h served as dependent variable and prey density served as factor.

Effects of container variations and water volumes on predation of Tx. splendens

In this study, different types of containers which contained different water volumes were tested as variables that may influence predation strategies. We had chose variety of containers that represent man-made containers (tin cans, rubber tire, and plastic drinking glass) and natural container (bamboo stumps) as tested containers. Four types of containers used for this study were; (1) tin cans (height: 10.5 cm, diameter: 7.4 cm), (2) plastic drinking glasses (height: 12.5 cm, diameter: 7.8 cm), (3) short length motocycle rubber tires (ca. 31.0 cm long and with an opening of 5.5 cm wide), and (4) bamboo stumps (height: 17.0 cm, diameter: 5.0 cm). Each type of container was then filled with different volumes of seasoned water at; (1) full (300ml), (2) half full (150ml), (3) 1/4 full (75ml), and (4) 1/8 full (37.5ml).

Ten larvae of *Ae. albopictus* prey were placed in each container types with different water volume prior to experiment with acclimation time of 1 h before the introduction of *Tx. splendens* predator. One individual of *Tx. spendens* predatory larvae was added into each container. The numbers of prey consumed by the predator were observed and recorded after 24 h. Experiment was replicated for ten times. No mortality was recorded for control treatment (without predator in it).

Data was tested for normality using a one sample Kolmogorov-Smirnov test. All data were log transformed (x+1) prior to analysis to satisfy the assumptions of ANOVA. In order to test the effects of preferable containers and water volume choices on predation of *Tx. splendens*, we ran a two-way Analysis of Variance (ANOVA) by using SPSS version 20.0 (SPSS 2012). Mean differences were separated with Tukey's HSD multiple comparison test. The numbers of prey consumed by predator were served as dependent variable while types of containers and water volumes served as fixed factors.

RESULTS

Effects of prey densities on predation of Tx. splendens

The maximum number of prey larvae consumed by a predator in 24 h was nine individuals. The result showed that the number of prey consumed by *Tx. splendens* larvae in 24 h was significantly higher in higher prey densities (40 and 50 preys) compared to lower densities ($F=3.935$, $df=4$, $p=0.008$) (Figure 1). This indicated that more prey will be consumed by *Tx. splendens* predator when more prey were available.

Effects of container types and water volumes on predation of Tx. splendens

The types of container had a major effect on the predation of *Tx. splendens*. The predatory larvae consumed more *Ae. albopictus* prey in rubber tire, followed by tin can, plastic drinking glass and bamboo stump (Figure 2). The two-way ANOVA showed that the number of prey consumed by *Tx. splendens* larvae were significantly difference between different types of container ($F=3.100$, $df=3$, $p=0.029$). However, there was no significant effect of water volumes on predation rate of *Tx. splendens* larvae ($F=1.736$, $df=3$, $p=0.162$) and the interaction between types of container and water volumes was also non-significant ($F=0.431$, $df=9$, $p=0.917$) (Table 1). Post-hoc comparisons showed that the numbers of prey consumed in rubber tire were significantly higher than the results in bamboo stump (Tukey's HSD, $p<0.05$) (Figure 2). The results indicated that regardless of water volumes used in the container, the predation of *Tx. splendens* was higher in horizontal type of container (rubber tire) as compared to vertical type of containers (bamboo stump, tin cans and plastic drinking glass).

DISCUSSION

The third instar larvae of *Tx. splendens* was found significantly consumed more *Ae. albopictus* larvae at higher density when various densities of prey were offered in the container. In our present study, the maximum number of prey consumption in 24 h was at nine prey larvae per predator. The prey density was found to be the significant factor which affected the consumption rate of *Toxorhynchites* spp. larvae (Padgett & Focks, 1980; Dominic & Das, 1998). All instars larvae of *Tx. splendens* and *Tx. towadensis* were reported inhibited functional response type II, where the numbers of prey consumed increased with increasing prey density and reached a plateau at a certain prey density

(Russo, 1983; Yasuda, 1995; Dominic & Das, 1998). The prey consumptions of fourth instar larvae of *Tx. brevipalpis* and *Tx. rutilus rutilus* were found linearly correlated with prey density (Lounibos, 1979; Padgett & Focks, 1980; Hubbard *et al.*, 1988). Russo (1986) reported that fourth instar larvae of *Tx. amboinensis, Tx. rutilus rutilus, Tx. brevipalpis and Tx. splendens* consumed large numbers of prey larvae presented in the container to achieve the weight required for their pupation. However, we found that third instar larvae of *Tx. splendens* only consumed a maximum of nine *Ae. albopictus* prey larvae in order for them to get full or reach satiation.

The strategy of *Tx. splendens* feeding behavior plays a big role in the successful of limiting prey populations. The feeding behavior of *Tx. splendens* has been described as 'opportunistic' because this predatory larvae were not searching for prey, but the larvae ambushed prey as it came within their mandibles (Furumizo & Rudnick, 1978; Russo, 1986). In present of large numbers of prey per container, we suggested that the foraging space between both predator and prey become closer. In this condition, we observed that active *Ae. albopictus* larvae are busy searching for foods and ignored the presence of *Tx. splendens* predator. While the predator took this opportunity to ambushed the prey. The predator ambush hunting strategy become easier, resulting in higher prey captures and prey consumption in 24 h in response to higher density of prey available in the water. The predator undulated toward a cluster of prey and attacked within 2 minutes (Russo, 1986).

There are many factors that affect the rate of prey consumption during larval development of the predator including container sizes and container types. In previous study by Focks and Padgett (1980), the prey consumption of fourth instar larvae of *Tx. rutilus rutilus* was found inversely with the container size. Contradictory, Dominic and Das (1998) reported that the attack rate of all instar larvae of *Tx. splendens* were not affected by the size of containers. Our study found that the prey consumption of *Tx. splendens* was influenced by the characteristic of the container. Our study suggested that the ambush hunting strategy by *Tx. splendens* predator was more efficient in horizontal type of container (rubber tire) as compared to vertical type of container (bamboo stump). The reason is because both prey and predator are foraging for food in the same close flat area, resulting in higher contact rate and leads to more attack by predator. On contrary, in vertical types of containers, *Ae. albopictus* foraging foods at the bottom of container while the predatory larvae usually ambushed prey at the water surface (Horsfall, 1955) caused less contact between both organisms. Futhermore, the feeding behavior of *Tx. splendens* which described as 'sit and wait' for the prey (Furumizo & Rudnick, 1978; Russo, 1986) may lead to low prey consumption in vertical types of container.

The prey consumption was found has no association with water volumes, in which the increasing of water volume was not affected the comsumption rate of prey by *Tx. splendens* predator. The similar results were also reported on *Tx. splendens* tested in the container filled with different water volumes at 2.0L and 0.5L (Dominic & Das, 1998). This finding suggested that *Tx. splendens* was achievably to released in tropical areas, such as in Malaysia which receives rainfall throughout the year. Since the prey consumption did not affected by the water volumes in the containers, therefore the prey control by the predator will be consistent eventhough in the dry and rainy seasons.

In conclusion, *Tx. splendens* has been proved as one of the potential biocontrol agent in controlling the population of *Ae. albopictus* especially in artificial containers. The finding in the present study can contributed to understand the factors that may influence and enhancing the effectiveness of *Tx. splendens* predatory larvae as biological control agent. In which, we found that the best target container is in horizontal position with wide opening and can be introduced either in wet and dry seasons; regardless of water volumes. However, more studies need to be done to evaluate the effect of other environment factors on prey consumption in the field conditions. Implementation of the third instar larvae of *Tx. splendens* to control *Ae. albopictus* in the breeding containers hopefully can be one of the successful solution to fight dengue vector.

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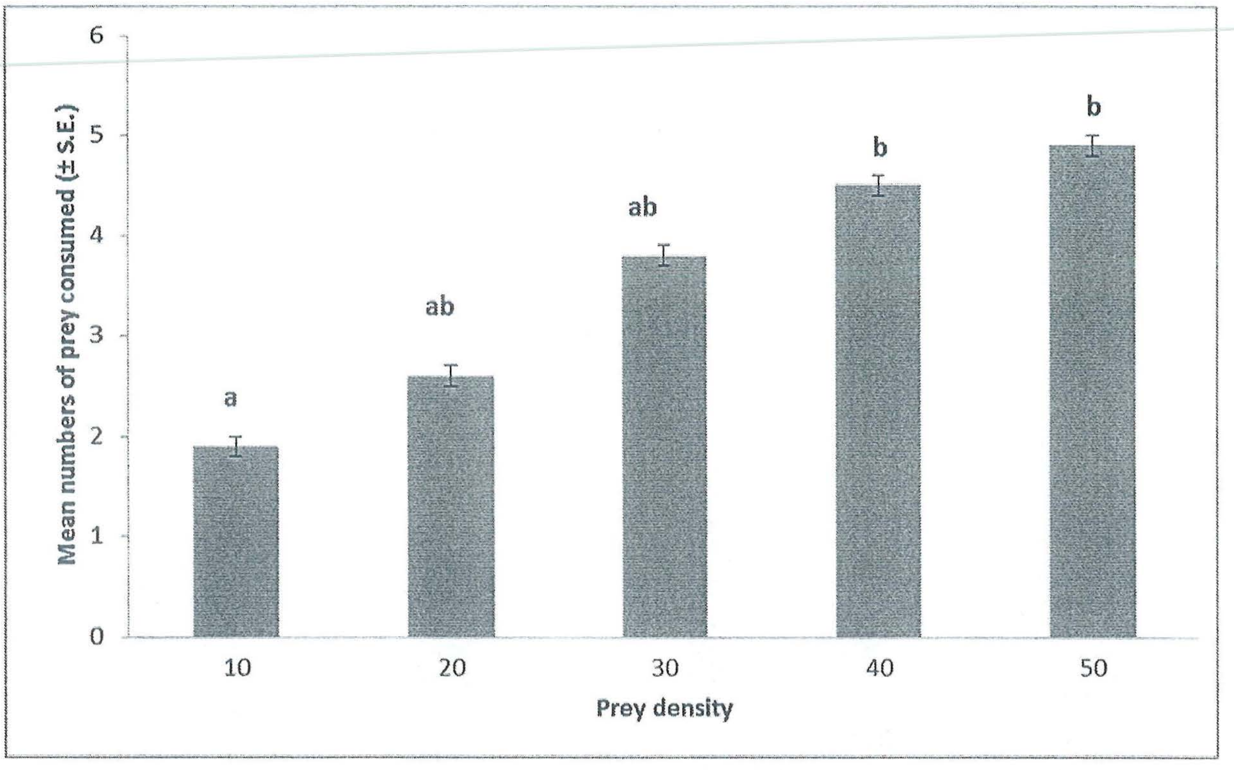

Figure 1. Mean numbers (± S.E.) of *Ae. albopictus* prey consumed by third instar larvae of *Tx. splendens* predator after 24 h exposure. Different letters indicated significant difference among the results (Tukey's HSD multiple comparison test, $p<0.05$)

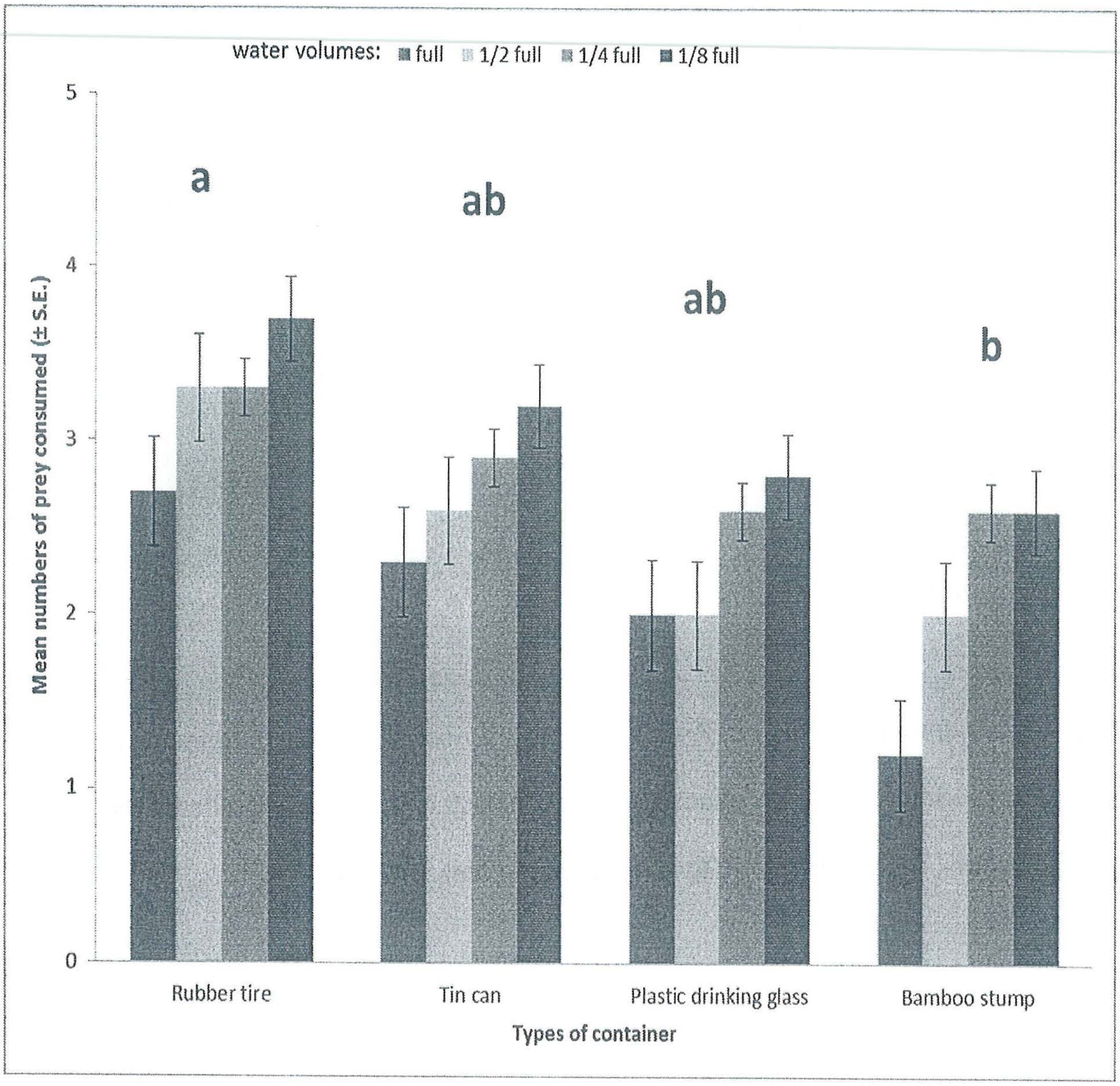

Figure 2. Mean numbers (± S.E.) of *Ae. albopictus* prey consumed after 24 h by third instar larvae of *Tx. splendens* predator in various types of container contains different water volumes. Different letters indicated significant difference among the groups (Tukey's HSD multiple comparison test, $p<0.05$).

Table 1. Results of two-way ANOVA analysis between the numbers of *Ae. albopictus* prey consumed after 24 h by third instar larvae of *Tx. splendens* predator in different types of container filled with different level of water volumes. S^2=type III sum of square, df=degree of freedom, MS=mean squared values. Significant values are in bold.

Source	S^2	df	MS	F	Significance
Types of container	0.613	3	0.204	3.100	**0.029**
Water volumes	0.343	3	0.114	1.736	0.162
Types of container * water volumes	0.255	9	0.28	0.431	0.97

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Risky behaviors: Effects of Toxorhynchites splendens (Diptera: Culicidae) predator on the behavior of three mosquito species

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Abstract:	With the difficulties in finding new biocontrol candidates for mosquitoes, improving the efficacy of existing biological agents is one of the major current issues in conservation biological control. In this prospect, a better understanding of the predation-risk behavioral responses of preys is relevant. Here, we investigated prey choices by Toxorhynchites splendens by determined behavioral responses of Aedes aegypti, Aedes albopictus and Anopheles sinensis larvae to the predator. We found that the predator prefers to consume Ae. aegypti larvae over the two other mosquito preys. The observed vulnerability patterns could be explained on the basis of a difference in behavioral responses of the three prey types to the presence of the predator. "Thrashing" and "browsing" activities were greater in Ae. aegypti larvae. Such water movements are likely correlated with vulnerability. With the increasing of water disturbances, Ae. aegypti larvae are exposed to enhanced risk of predator attack. In contrast, Ae. albopictus and An. sinensis larvae exhibited low-risk behaviors, spending most of the time on the "wall" position near the edges of the container habitats. Collectively, these data suggest that Ae. aegypti has less ability to perceive cues from predation and to modify behavior to reduce risk of predation risk compared to Ae. albopictus and An. sinensis. This leads to a great finding that Tx. splendens have fine chances in controlling dengue hemorrhagic vector, Aedes aegypti even when this species co-exist together with other species in water bodies.

Risky behaviors: Effects of *Toxorhynchites splendens* (Diptera: Culicidae) predator on the behavior of three mosquito species

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Abstract

With the difficulties in finding new biocontrol candidates for mosquitoes, improving the efficacy of existing biological agents is one of the major current issues in conservation biological control. In this prospect, a better understanding of the predation-risk behavioral responses of preys is relevant. Here, we investigated prey choices by *Toxorhynchites splendens* by determined behavioral responses of *Aedes aegypti, Aedes albopictus* and *Anopheles sinensis* larvae to the predator. We found that the predator prefers to consume *Ae. aegypti* larvae over the two other mosquito preys. The observed vulnerability patterns could be explained on the basis of a difference in behavioral responses of the three prey types to the presence of the predator. "Thrashing" and "browsing" activities were greater in *Ae. aegypti* larvae. Such water movements are likely correlated with vulnerability. With the increasing of water disturbances, *Ae. aegypti* larvae are exposed to enhanced risk of predator attack. In contrast, *Ae. albopictus* and *An. sinensis* larvae exhibited low-risk behaviors, spending most of the time on the "wall" position near the edges of the container habitats. Collectively, these data suggest that *Ae. aegypti* has less ability to perceive cues from predation and to modify behavior to reduce risk of predation risk compared to *Ae. albopictus* and *An. sinensis.* This leads to a great finding that *Tx. splendens* have fine chances in controlling dengue hemorrhagic vector, *Aedes aegypti* even when this species co-exist together with other species in water bodies.

Key words: *Aedes*- Behavior – Biocontrol – Dengue – Mosquito - *Toxorhynchites*

Introduction

Regardless of the control efforts done by various parties, mosquitoes still plays a major role in transmitting vector borne diseases in many parts of the world, with over one million cases of death in children annually due to malaria infection (World Health Organization 1999; Breman 2001). While, with an estimated 50-100 millions of dengue and dengue haemorrhagic fever cases a year worldwide are linked to the spread of vector *Aedes aegypti* and *Aedes albopictus* (Monath 1994; Gubler and Meltzer 1999).

Several factors are contributing to the increasing number of mosquito borne disease. Reckless and rampant use of chemical insecticide in pest control sector has given rise to the problem of resistance in insect (Collins and Blackwell 2000; Impoinvil et al. 2007; Rafikov et al. 2009; Wijesinghea et al. 2009; Nyamah et al. 2011). Studies showed that in Malaysia, *Ae. aegypti* has developed temephos resistance (Lee 1998), while in South Vietnam tolerant towards permethrin, lambda-cyhalothrin and deltamethrin has been detected (Vu and Nguyen 1999). Chemical insecticide causes many concerns over the increasing resistant traits in mosquitoes and also to the negative impact towards the environment. Currently, biological control has received much attention and interest as an alternative control method for mosquito borne diseases' vector (Collins and Blackwell 2000; Focks 2007; Wijesinghea et al. 2009; Nyamah et al. 2011).

Toxorhynchites predatory larva is one of the biological control agents and is primary predator to mosquito larvae. *Toxorhychites* and mosquito larvae frequently co-existed together and share the same habitat in common aquatic ecosystem (Steffan and Evenhuis 1981). When preparing to feed or hunt, *Toxorhynchites* larva will position its body angle into a horizontal point. When a prey draws near within the larva's striking distance, it will laterally hit and seize the prey with its mandibles. The prey is then typically consumed within minutes and prey capture can occur either on the surface or at the bottom of the container (Steffan and Evenhuis 1981).

Predator behavior affects the morphology, behavior and life history of the prey, acting as a persistent selective force (Lima and Dill 1990; Kats and Dill 1998; Wisenden 2000). Predation occurrence would most definitely change the facultative behavior of a particular mosquito larva which later on would affect its susceptibility to a predator (Juliano and Gravel 2002). The ability to identify and avoid potential predators can be considered as a survival advantage (Mirza and Chivers 2003). Then, there is also evidence of evolution in behavioral response in the prey when they are exposed to consistent predation risk, suggesting that the predator-prey behavior is adaptive (Blaustein et al. 2000; Juliano and Gravel 2002). According to Juliano (2009), mosquito prey larvae have already acquired evolvement of response mechanisms to avoid predation by their natural enemies. In small container system; modified behavior is the key device of anti-predator reaction. In general, predation events and interspecific competition is influenced by behavior and behavioral change of an organism (Kesavaraju and Juliano 2004).

In this study, we examined the preferences of *Toxorhynchites splendens* towards three different species of vector mosquito larvae (*Ae. aegypti, Ae. albopictus, and Anopheles sinensis*) and behavioral changes in response to predatory *Tx. splendens* larva and also its residual kairomones remnant.

Materials and methods

Predator and prey colonies

Predatory mosquito (*Toxorhynchites splendens*) was obtained from Vector Control Research Unit (VCRU), Universiti Sains Malaysia. T*oxorhynchites splendens* are unusually large mosquitoes;

the wingspan may exceed 12 mm while the body length may exceed 7 mm. Larvae are generally dark brown or reddish in appearance, with very conspicuous hairs on the abdomen. The head capsule is quite thick and contains powerful mandibles. Fourth instar larvae (sizes from 6 to 9 mm) was used for the experiment.

Whereas, late 3rd and early 4th instar larvae of *Ae. aegypti, Ae. albopictus* and *An. sinensis* (VCRU strain) were utilized as prey.

Prey preferences

To study the prey preferences of *Toxorhynchites splendens* towards the three different species of mosquito, the experiment was conducted using a total of 20 preys in 500 ml of seasoned or aged tap water in containers measuring 6.5 x 17.5 x 11 cm (height x length x width). The ratios of mosquito larvae offered to a predator were: 0:20; 3:7; 5:15; 7:13; 10:10; 13:7; 15:5; 17:3; 20:0 (*Ae. aegypti: Ae. albopictus*). A similar ratio was also applied for *Ae. aegypti: An. sinensis*. In order to understand the hunting process of *Tx. splendens, Anopheles* species was choosing because of different feeding behavior from *Aedes* species. *Anopheles* species mostly feeding on suspended particles on water surface (Ye-ebiyo et al. 2003), whereas *Aedes* species feeding technique is between submerged feeding and also feeding near or at the water surface (Merrit et al. 1992).

After 24 hours exposure, the *Tx. splendens* predator was removed from the container and the remaining number of prey left was counted and identified under a light microscope. The experiment was conducted in laboratory conditions with temperature of 26 ± 1°C and 65-85% humidity. Each experiment was replicated six times.

Prey preferences were determined by using Manly's α (Manly, 1974) equation with Chesson's (1982) alteration to account for prey depletion:

$$\alpha = \frac{\ln(N_{Ae} - C_{Ae}) / N_{Ae}}{\ln((N_{Ae} - C_{Ae}) + \ln(N_{An} - C_{An}) / N_{An})}$$

(1)

where *N* is the initial number and *C* is the number of larvae consumed of *Ae. aegypti (Ae)* and *An. sinensis (An)*. We also can predict the preferences (α) for each predator with this multiplicative formula:

$$\alpha_a = \frac{\alpha_{Ae}}{\alpha_{Ae} + \alpha_{An} - (\alpha_{Ae}\alpha_{An})} \quad (2)$$

Where α_a s the predicted preference for *Ae. aegypti*, α_{Ae} nd α_{An} re attack constants for *Ae. aegypti* and *An. sinensis*, respectively.

Predator avoidance behavior

Three different treatments were applied: (1) control; without any predator; (2) when prey were placed with a free-roaming predator; and (3) when prey were placed in water which contains residual predator's kairomones but without the actual predators in order to study the avoidance behavior of *Ae. aegypti, Ae. albopictus* and *An. sinensis*. For residual kairomones preparation, a predator was released in 500 ml seasoned water and fed with 10 mosquito larvae for 24 hours prior to the start of the treatment. Feeding is crucial to simulate the kairomones release by injured prey (Dodson et al. 1994; Kats and Dill 1998; Kusch et al. 2004) and production of remnants exists from predation event (Kesavaraju and Juliano 2004).

Mosquito larva prey was placed in a plastic container filled with 500 ml seasoned water. After approximately 10 minute's period of acclimation time, a *Tx. splendens* predator was added into the same container. The behaviors and positions of prey were recorded for 30 minutes or until it was captured. We categorized the behavior into four types of activity: (1) resting – larva neither feeding or moving; (2) browsing – larva propelled along the surface of the container by the movements of their mouthparts; (3) filtering – larva floating in the water column propelled by the movements of their mouthparts; and (4) thrashing – vigorous lateral movements of the larval body, propelling themselves through the water. Four positions were also categorized as; (1) surface – spiracular siphon of the larva in contact of the water-air interface; (2) bottom – larva within 1 mm of the bottom of the container; (3) wall – larva within 1 mm from any surface of the container walls; and (4) middle – larva more than 1 mm from any surface of the container and not in contact with the water surface. All experiments were conducted in laboratory conditions with temperature of 26 ± 1^{o}C and 65-85% humidity. All treatments were replicated six times.

The behavioral data were analyzed using multinomial logistic regression in IBM SPSS 20.0 (2012). We score the behavior categories from 1 to 4 for activities and 5 to 8 for positions as follows: (1) resting; (2) browsing; (3) filtering; (4) thrashing; (5) surface; (6) bottom; (7) wall; and (8) middle, which were then modeled as being dependent on prey species (*Ae. aegypti, Ae. albopictus* and *An. sinensis*), and treatments (control, free-roaming predator, and residual kairomones remnant).

Results

Prey preferences study

Result showed that *Tx. splendens* consumed more of *Ae. aegypti* larvae in a treatment when varied ratios of *Ae. aegypti* and *Ae. albopictus* were offered (Fig. 1). When *Ae. aegypti* larvae were mixed with *An. sinensis*, similar result was also obtained. In which, *Tx. splendens* preferred *Ae. aegypti* compared to *An. sinensis* (Fig. 2). This can be inferred by observing both of Fig. 1 and Fig. 2 where all the values of preference (α) lies above the broken line, α = 0.667 which signified that *Tx. splendens* preferred *Ae. aegypti* larvae over other two species.

Predator Avoidance Behavior

The multinomial logistic likelihood ratio test shows in Table 1 displayed significant effects between species (x^2 = 49.36, df = 2, *P* = 0.000), type of treatments (x^2 = 49.36, df = 2, *P* = 0.000), and activities exhibit by larvae (x^2 = 219.54, df = 7, *P* = 0.000).

The most vulnerable larvae to *Tx. splendens* predation, *Aedes aegypti* showed considerably high frequency of "thrashing" activities at the "wall" position when facing the predator. In absence of predator (control treatment), more "resting" activity at "surface", "wall" and "middle" positions were exhibited. However, in residual kairomones treatment, *Ae. aegypti* exhibited more "browsing" activity (Fig. 3a). In contrast, *Ae. albopictus* displayed less activity and positioning in occurrence of predator (Fig. 3b). While *An. sinensis* chose safer activities of "resting" in presence of predator similar to in control condition (Fig. 3c).

The Cox and Snell's pseudo statistic showed that less than half the variation in prey behavior was explained by the model (R_2=0.35). Table 2 shows the parameter estimates from the model that shows each factor tested was compared with reference factor. Based on the multinomial logistic regression, *Ae. albopictus* prey was prone to display more "browsing" behavior (odds ratio = 10.67, *df* = 1, *P* = 0.001) at the "bottom" (odds ratio = 17.50, *df* = 1, *P* =

0.000) and "wall" positions (odds ratio = 6.68, *df* = 1, $P = 0.010$) compared to reference category; *Ae. aegypti*. However, no significant differences were observed between all treatments for both *Aedes* species ($P > 0.05$).

In predator treatment, there was a significant difference in behavior between *Ae. aegypti* and *An. sinensis* (odds ratio = 27. 95, *df* = 1, $P = 0.000$), with *An. sinensis* larvae showed high frequency of "resting" (odds ratio = 63.51, *df* = 1, $P = 0.000$) at the "surface" (odds ratio = 99.72, *df* = 1, $P = 0.000$) and "wall" of the container (odds ratio = 67.42, *df* = 1, $P = 0.000$) in response towards predation risk posed by *Tx. splendens.* This result display of defensive behavior by *An. sinensis* is thought to serve as lowering the chances of being hunted by prospective predator.

Discussions

Toxorhynchites splendens had showed primary preference towards *Ae. aegypti*, even when *Ae. albopictus* and *An. sinensis* were offered together in present study. *Toxorhynchites splendens* prefer to attack *Ae. aegypti* even at small number when combined with the more abundant of other alternative prey species such as *Ae. albopictus* and *An. sinensis.* In our study prey switching towards higher density of certain species did not occur, which mean that *Tx. splendens* still preferred to consume *Ae. aegypti.* We suggested that *Tx. splendens* is very effective predator and has a strong ability to control *Ae. aegypti*, the main vector of the dengue hemorrhagic fever in Malaysia.

However, if the predator demonstrates a strong preference on one particular prey species, the prey is said to be able to endure the highest level of predation (Bonsall and Hassell 1999). In our case, *Ae. aegypti* populations can be estimated by the existence of *Tx. splendens* predator as it's become a favorable prey even at low density. Thus, when predation is more aggressive on the superior prey competitor, the inferior prey competitor may be able to coexist through a keystone predator effect (Paine 1966).

Predator preference is predicted to shift according to prey density availability (Mauck and Coble 1971; Savino and Stein 1989) and thought to happen through mechanisms of density-dependent predation and switching (Holling 1965; Murdoch and Oaten 1975; Hassell and Comins 1978). Holt and Lawton (1994) pointed out that an apparent mutualism can occur between competing prey species when the presence of either one would lower the predation rates on the other. For example, selective predation of *C. appendiculata* and *T. rutilus* on *Ae. albopictus* may also reduce predation on *Ae. triseriatus*, enabling this species to propagate in numbers (Griswold and Lounibos 2005). However, in this study, *Tx. splendens* showed primary interests to consume *Ae. aegypti* even more proportion of *Ae. albopictus* and *An. sinensis* were offered. Based on density-dependent theory, the low density population will be leave alone and high population will get decimated to a minimum number. In which allows the low density population to growth. But, no evidence in our study supported this theory. We had confirmed that the predation desire by *Tx. splendens* caused by the behavior and positioning of *Ae. aegypti* prey when facing the predator which attract predators towards them and become more vulnerable.

More than 70% of *Ae. aegypti* larvae captured by *Toxorhynchites* larvae were occurred when the predaceous larvae were not in contact with the water surface (Russo 1986) and were relatively motionless, waiting to ambush the prey (Steffan and Evenhuis 1981). Sometimes, they would swim towards a group of prey larvae and the most attacks were on swimming larvae (Russo 1986; Linley and Darling 1993; Griswold and Lounibos 2005). Generally, T*oxorhynchites* larvae spend most of their time immobile, with the degree varying according to species (Clements 1999). There are three different mechanisms of prey capture displayed by *Toxorhynchites* larvae depending on its nature: (1) staying inactive and waiting for sub-surface prey to approach within striking distance; (2) swimming towards a particular prey that was trapped on the water surface; and (3) illustrating continual prey-finding activity after grabbing a floating egg (Clements 1999).

From our observation, *Tx. splendens* favored the first mechanism to approach prey. Instead of swimming towards potential prey option, predatory *Tx. splendens* larvae would lie motionless on the bottom of a particular container and waited for prey to swim across and captured them. Due to the active 'thrashing' behavior flaunted by *Ae. aegypti* in the water, they became un aware of predator existence and easily got captured by the predator. This hunting mechanism is suitable for *Toxorhynchites*, which is a phytotelmata breeder, meaning that rigorous movement is not an option in a small and restricted space. According to Clements (1999), the characteristic of a striking behavior of *Toxorhynchites* larvae also differs according to the position of approaching prey. When prey is situated directly in front of *Toxorhynchites* larva's head, the strike movement involved a rapid displacement of the head towards the prey though extension of the neck by over 1 mm. Alternatively, if the prey approaches *Toxorhynchites* larva from the side or behind, the strike took form of a rapid, lateral bending that moved the predator's head towards the prey.

Both *Ae. aegypti* and *Ae. albopictus* larvae displayed almost similar frequency of behavioral activities. However, *Ae. albopictus* larvae displayed high occurrence at "wall" position in contrast to *Ae. aegypti*. This evidence suggesting that prey situated near the edge of the container was less susceptible than any alternative prey species which are constantly moving. Sih (1979) also stated that among all the larvae that were captured by the predators, 98% were positioned more than 38 mm from the edge of the container, meaning that they can be found within the central of 70% of the surface area of the container. Thus evidence also applied in our study to describe the incidence of vulnerability of *Ae. aegypti* larvae to predator due to the prey position which were likely to be found at the middle, bottom and surface of the container during treatments.

Due to the attracting behavior exhibited by *Ae. aegypti* which comprised of "thrashing" and constantly "browsing" for food sources at the "surface" and "middle" of the container, it was not a surprise that *Tx. splendens* preferred to consume on *Ae. aegypti* larvae instead of *An. sinensis* that continually adopted a low risk behavior of "resting" at the "surface" and "wall" positions. Zuharah and Lester (2011) found that *Aedes notoscriptus* appeared to be more visible and more attractive to predators by exhibiting thrashing behavior because of vigorous movement attracted predators. According a study conducted by Nyamah et al. (2011), *Ae. albopictus* larvae was reported to be moving actively, contrary to *Culex fuscocephala* (Theobald) and this behavioral characteristics causes *Tx. splendens* to prey on them. In our study, when *Ae. aegypti* larva was placed with a free roaming *Tx. splendens* larva in predator treatment, *Ae. aegypti* was seen to be displaying it "thrashing" behavior, thus making it more vulnerable towards predation events.

Such behavior could be attributed to the "threat sensitivity hypothesis" which stated that a particular prey species would change their avoidance reaction according to the degree of the threat (Helfman, 1989). *Anopheles sinensis* definitely displays such reaction, where there was a significant behavior displayed between *An. sinensis* and *Ae. aegypti* larvae when they were placed with predatory *Tx. splendens*. *Anopheles sinenis* larva was seen to be "resting" at the "surface" of the container "wall". According to Juliano and Reminger (1992) this resting behavior was the least risky behavior in the presence of a potential predator. However, it is also possible that the "wall' position displayed by *An. sinensis* was also due to its natural larval behavior where *Anopheles* larvae were said to demonstrate negative thigmotaxis, a tendency to maintain bodily contact with solid object and its locomotion reduced (Clements 1999). For instance, larvae of *An. minimus* and *An. maculatus*, when placed in an experimental water flow channel, anchored themselves to the edge (Muirhead-Thomson 1940) and this ability is due to its dorsal brush setae modified to form hooks which can be used to cling to any solid objects (Lamborn 1921).

Aquatic organisms usually received warning about prospective predation events by means of visual (Chivers et al. 2001) and chemical information known as kairomones, which can be released by injured prey (Dodson et al. 1994; Kats and Dill 1998; Kusch et al. 2004), predation events, predators (Kesavaraju et al. 2007) solid residues from predation events on either conspecifics or competing prey (Kesavaraju and Juliano 2004) and feces from predator that fed on conspecifics (Brown et al. 1955a, 1955b, 1966). There is also evidence suggesting that mosquito larval contact with solid residues while foraging were able to provide signal to the presence of predation threat (Kesavaraju and Juliano 2010). However, predation risk cues in aquatic systems can degrade if they are not replenished by additional predation events, and prey thus may alter their reactions depending on the degradation level (Ferrari et al. 2005). In our study, *Ae. aegypti, Ae. albopictus* and *An. sinensis* seems to display risky behavior of "thrashing" and "browsing" activities in kairomones treatments. It's possible that the 24 hours residual kairomones from *Tx. splendens* is not strong enough to elicit their avoidance behavior towards predators to alarm the existence of the predator in the water body. Therefore, these larvae were freely exhibiting their normal activities without any concern of predator presence.

In conclusion, behavioral response and positioning of prey are two important factors that contribute to the successful of *Tx. splendens* as biocontrol agent. This agent has been proved in our study, to have significant on reducing dengue hemorrhagic fever vector, *Ae. aegypti*.

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Table 1 Results from multinomial logistic regression likelihood ratio test from the model on three different effects. Significant values are in bold.

Effects	X^2	**df**	**Significance**
Species	49.36	4	**0.000**
Treatment	49.36	4	**0.000**
Activities	219.54	14	**0.000**

Table 2 Results from multinomial logistic regression showing nominal parameter estimates from the model. The references category was *Ae. aegypti*. Significant values are in bold.

	B	SE	Wald	*df*	Sig.
Aedes albopictus					
Treatments					
Control	0.096	0.144	0.443	1	0.506
Predator	0.273	0.184	2.218	1	0.136
Kairomones	0	-	-	0	-
Activities					
Resting	0.912	0.338	7.299	1	**0.007**
Thrashing	0.485	0.237	4.178	1	**0.041**
Browsing	0.726	0.222	10.667	1	**0.001**
Filtering	0.340	0.000	-	1	-
Position					
Surface	0.458	0.287	2.542	1	0.111
Bottom	0.914	0.219	17.499	1	**0.000**
Wall	0.508	0.917	6.679	1	**0.010**
Middle	0	-	-	0	-
Anopheles sinensis					
Treatments					
Control	0.282	0.165	2.940	1	0.086
Predator	1.016	0.192	27.946	1	**0.000**
Kairomones	0	-	-	0	-
Activities					
Resting	2.268	0.285	63.514	1	**0.000**
Thrashing	0.860	0.485	3.145	1	0.076
Browsing	1.277	0.634	4.057	1	**0.044**
Filtering	24.986	8061.007	0.000	1	0.998
Position					
Surface	2.783	0.279	99.717	1	**0.000**
Bottom	0.833	0.316	6.940	1	**0.008**
Wall	2.110	0.257	67.415	1	**0.000**
Middle	0	-	-	0	-

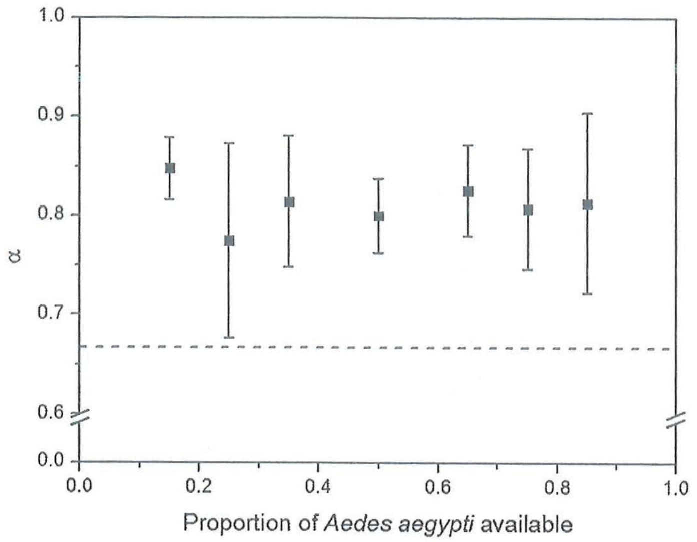
Figure-caption list

Fig. 1: The preference of *Tx. splendens* for *Ae. aegypti* larvae compared to *Ae. albopictus* larvae, indicated by (α) (±SE). The broken line indicates no preferences for either mosquito larvae, at α = 0.667

Fig. 2: The preference of *Tx. splendens* for *Ae. aegypti* larvae compared to *An. sinensis* larvae, indicated by (α) (±SE). The broken line indicates no preferences for either mosquito larvae, at α = 0.667

Fig. 3. Behavior of three species of mosquito larvae (A) *Ae. aegypti,* (B) *Ae. albopictus* and (C) *An. sinensis* in response to various treatments of control (absence of predator), with predator and predator's kairomones only.

Fig. 1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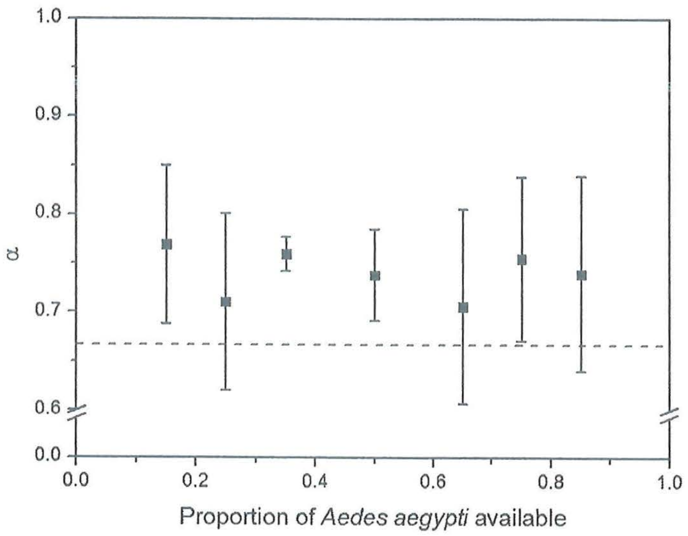
Proportion of *Aedes aegypti* available

Fig. 2



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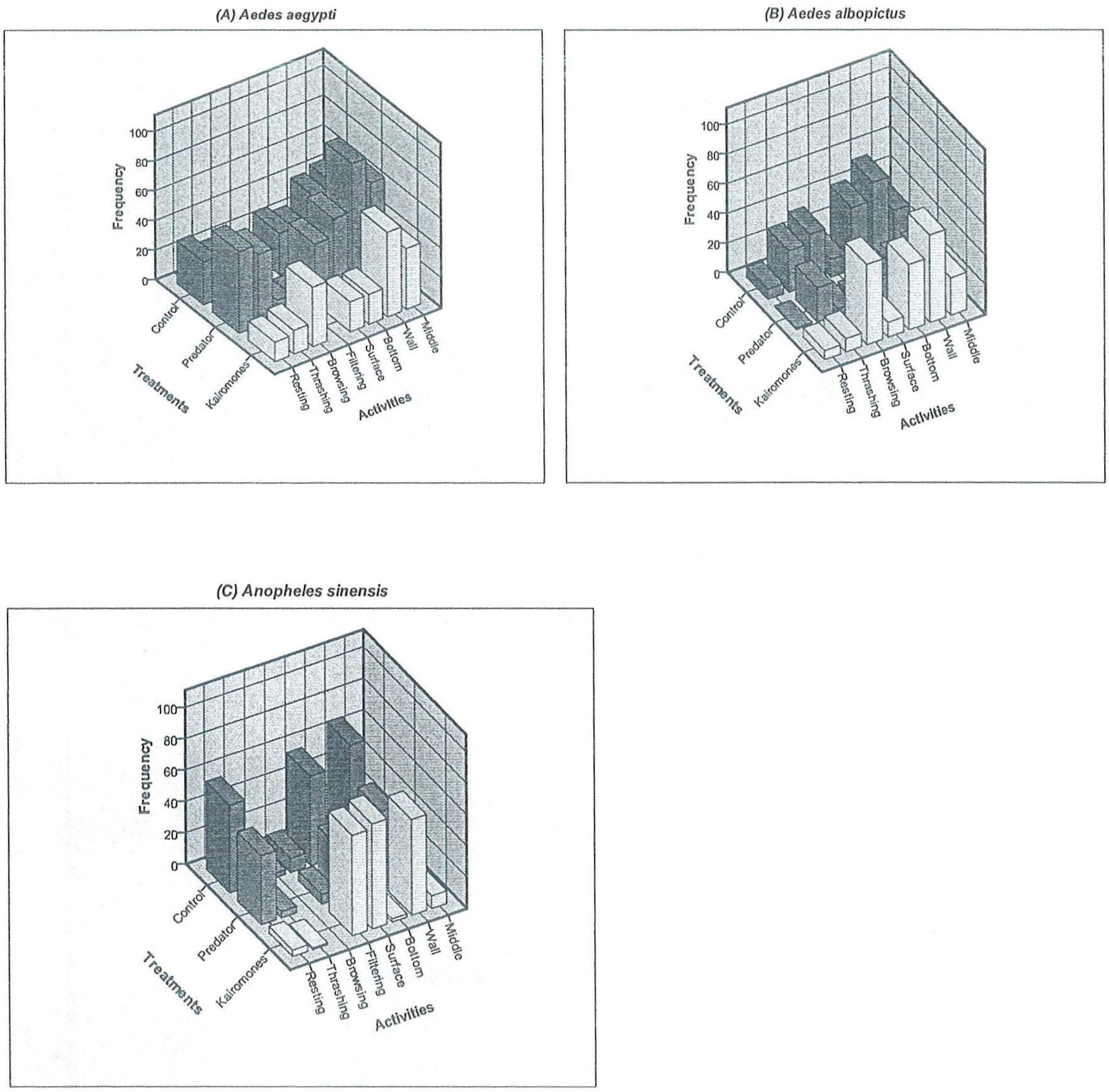
Proportion of *Aedes aegypti* available

Fig. 3


(A) Aedes aegypti
(B) Aedes albopictus
(C) Anopheles sinensis
Frequency
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80
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Treatments
Control
Predator
Kairomones
Activities
Resting
Thrashing
Browsing
Filtering
Surface
Bottom
Wall
Middle

Figure 1
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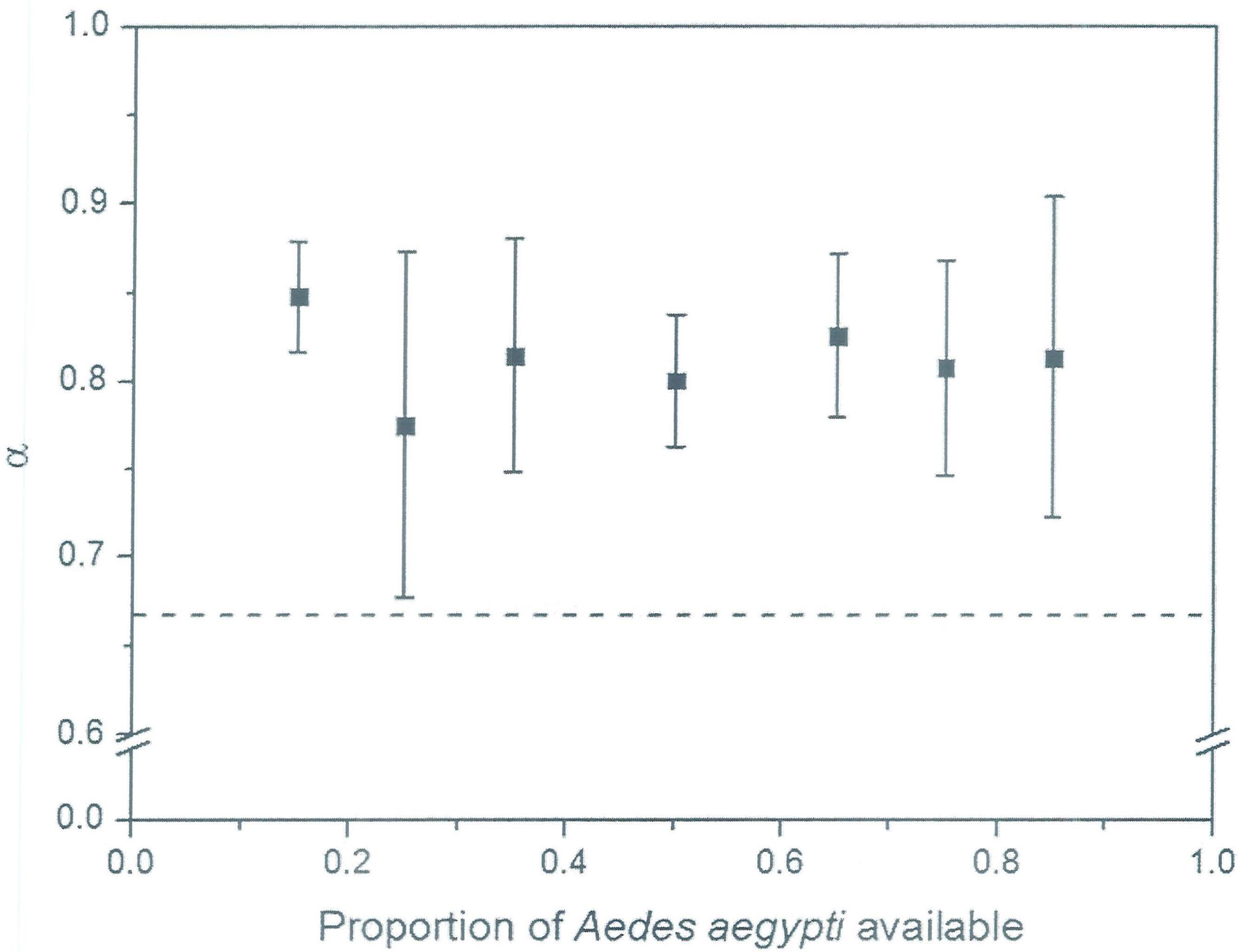

Figure 2
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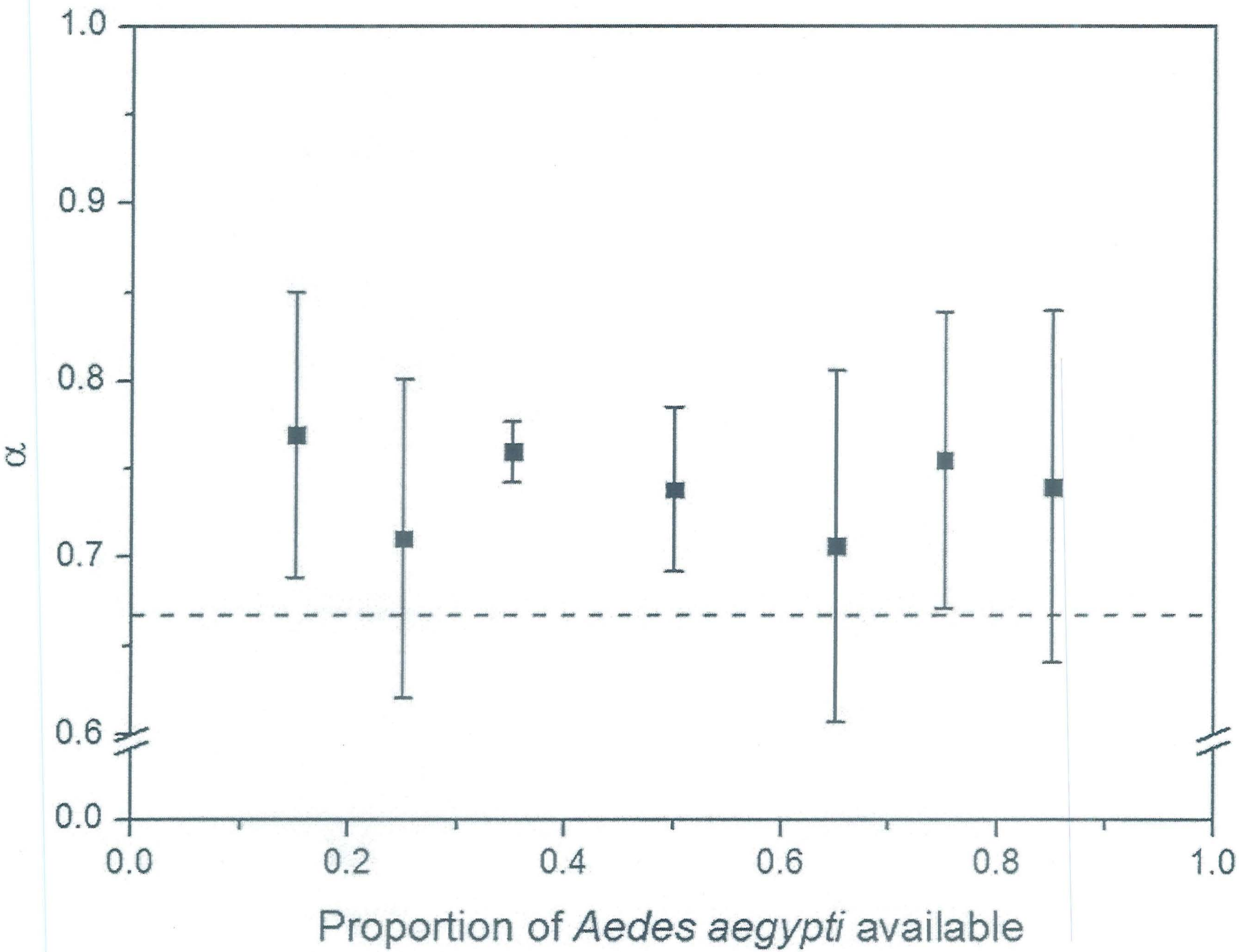

Figure 3
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