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

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Abstract. *Toxorhynchites splendens* larvae are a natural predator of dengue vector mosquito larvae, *Aedes 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 prey at a 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 experiment was evaluated in man-made containers (tin cans, plastic drinking glasses and rubber tires) and natural container (bamboo stumps) which were filled with different water volumes (full, half full, 1/4 full, and 1/8 full). The prey density and the characteristics of the container were found as significant factors which influence the predation rate of *Tx. splendens*. The predator consumed significantly more prey at higher prey densities (40 and 50 preys) compared to the lowest density (10 preys) (F=3.935, df=4, p=0.008). The results showed significantly higher consumption in horizontal shaped container of rubber tire than in vertical shape of bamboo stumps (F=3.100, df=3, p=0.029). However, the water volume had no significant effect on predation rate of *Tx. splendens* (F=1.736, df=3, p=0.162). We generally suggest that *Tx. splendens* is best to be released in discarded tires or any other containers with horizontal shape design with wide opening since *Tx. splendens* can become more effective in searching prey in this type of container design. This predator is also a suitable biocontrol candidates to be introduced either in wet and dry seasons in Malaysia.

INTRODUCTION

Dengue fever is ranked as the most important mosquito borne viral disease in the world by World Health Organization (WHO, 2012). As dengue vaccines are still under development, the best available method to prevent this disease is by controlling the vector mosquito. *Aedes aegypti* and *Aedes albopictus* mosquitoes are two major vectors involved 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 is widely used in vector control programmes. However, the method is always fraught with some problems such as increasing chemical and labour costs and reluctantance by the public to have any chemicals in their domestic water containers (Focks, 2007). Futhermore, vector mosquitoes have developed resistantance to several pesticides. Bioassay data demonstrate that resistance to organo-phosphates (temephos) and pyrethroids is widespread in *Ae. aegypti*, and resistance has also been reported in *Ae. albopictus* (WHO, 2012). For these reasons, the search of alternative methods for vector control has become more imperative (Collins & Blackwell, 2000).

One of the potential alternatives to chemical control approaches is to use biological control agents such as *Toxorhynchites* spp. mosquitoes (Collins & Blackwell, 2000). *Toxorhynchites* spp. are container breeders and found in wide variety of both artificial and natural containers (Steffan & Evenhuis, 1981). *Toxorhynchites* spp. are reported to co-exist 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 have been 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 adults only feed on 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 difficult to treat effectively using chemicals (Collins & Blackwell, 2000; Focks, 2007). However, the introduction of female adults as biological control agents in several previous studies showed little success in controlling the target prey species because females oviposited their eggs into available habitats other than the target habitats (Focks *et al.*, 1979, 1983; Bailey *et al.*, 1983; Toohey *et al.*, 1985). Therefore, Collins & Blackwell (2000) recommended that 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 of several species of *Toxorhynchites* larvae were previously reported to depend on 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. These predatory larvae are potentially biological control agents that can be released in many 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 vary in type, size and design. They generally contain different water volumes, depending on the amount of the rainfall received. The numbers of *Ae. albopictus* prey larvae available in these containers also varies.

The objective of our study was to investigate the maximum number of prey (*Ae. albopictus*) that can be consumed by third instar larvae of *Tx. splendens* within 24 hrs and the effects of container properties together with water volumes in influencing the successful predation rate of *Tx. splendens* larvae. Information on this particular predatory *Tx. splendens* species is important in understanding the factors contributing to a successful of biological control programme. This information also can be used in the future to maximize the ability of this biocontrol agent in vector control programme in Malaysia.

MATERIALS AND METHODS

Predators and prey colonies

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

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 pupation. The pupae were collected and placed in an adult cage to allow them to eclose. 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 allowed to oviposit in moist oviposition substrate made of filter paper with a cone shape placed on a petri dish.

Toxorhynchites splendens larvae were reared individually to avoid cannibalism. Each larvae was fed with ten larvae of *Ae. albopictus* daily. The same instar stage of *Ae. albopictus* were fed to the same instar stage of *Tx. splendens* larvae (i.e., 1st instar prey larvae feed to 1st instar predator larvae). When the *Tx. splendens* reached the pupal stage, they were collected and placed in a cage. They were permitted to become adults, allowed to mate and supplied with 10% of sucrose solution plus vitamin B complex as food source. After a 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 experiments.

Study designs

Effects of prey densities on predation of Tx. splendens

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 & 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 *Tx. splendens* predator. The numbers of prey consumed by predator were recorded after 24 h post-treatment. No dead prey were found in the control treatment (without any predator). The experiment was replicated 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) followed by Tukey's HSD multiple comparison test of means 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 under laboratory condition. We chose a variety of containers that represent man-made containers (tin cans, rubber tyre, and plastic drinking glass) and a natural container (bamboo stumps). 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 tyres (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* were placed into each container type with different water volumes and allowed 1 h acclimation prior to the introduction of a single *Tx. splendens* larva per container. The number of prey consumed by the predator was observed and recorded after 24 h. The experiment was replicated ten times. No mortality was recorded for the 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 container type and water volume on predation of *Tx. splendens*, we ran a two-way Analysis of Variance (ANOVA) by using SPSS version 20.0 (SPSS 2012) followed by Tukey's HSD multiple comparison test of treatment means. The number of prey consumed served as dependent variable while container type and water volume 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 results showed that the number of prey consumed by *Tx. splendens* larvae in 24 h was significantly higher in higher prey densities (40 and 50 prey) compared to the lowest density offered at ten prey per container ($F=3.935$, $df=4$, $p=0.008$) (Figure 1). This indicated that more prey will be consumed by *Tx. splendens* predator when more preys were available.

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

The type of container had a major effect on the predation of *Tx. splendens*. The predatory larvae consumed more prey in rubber tyre, 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 was significantly different between container types ($F=3.100$, $df=3$, $p=0.029$). 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 number of prey consumed in rubber tire was significantly higher than 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 rate of *Tx. splendens* was higher in horizontal type of container (rubber tyre).

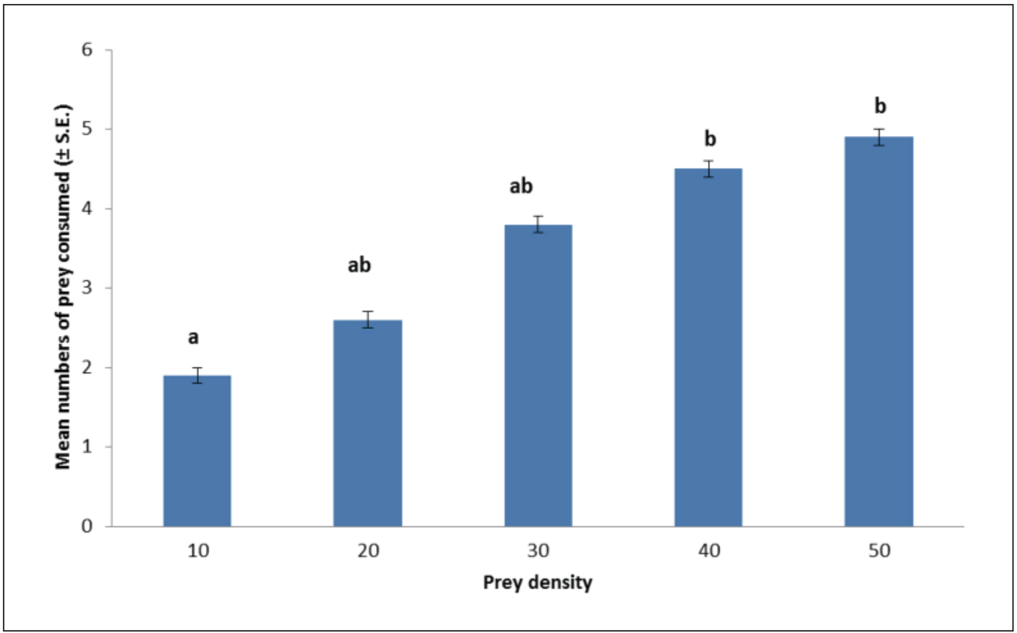

Figure 1. Mean numbers (± S.E.) of *Ae. albopictus* prey consumed by third instar larvae of *Tx. splendens* predator after 24 h exposure. Different letters indicated significant difference among the results (Tukey's HSD multiple comparison test, $p<0.05$)

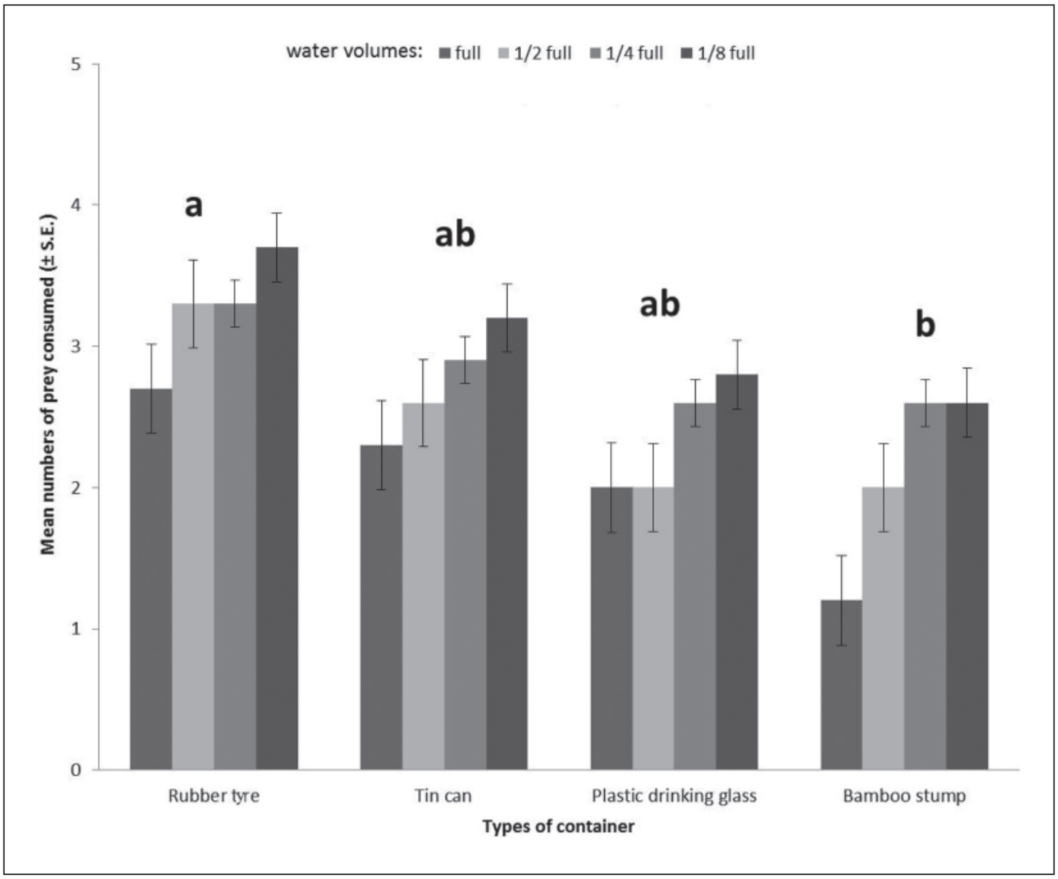

Figure 2. Mean numbers (± S.E.) of *Ae. albopictus* prey consumed after 24 h by third instar larvae of *Tx. splendens* predator in various types of container contains different water volumes. Different letters indicated significant difference among the groups (Tukey's HSD multiple comparison test, $p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

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

DISCUSSION

The third instar larvae of *Tx. splendens* were found to consume significantly more *Ae. albopictus* larvae when higher density of prey was offered in the container. At these densities of 50 prey, the maximum number of prey consumption in 24 h was at nine prey larvae per predator. The prey density was reported 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 *Toxorhynchites towadensis* were reported to exhibit a type II functional response, 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). Russo (1986) reported that fourth instar larvae of *Toxorhynchites amboinensis*, *Toxorhynchites rutilus rutilus*, *Toxorhynchites 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 the third instar larvae of *Tx. splenden* consumed a maximum of nine *Ae. albopictus* prey. From our study, the degree of *Tx. splendens* predation was found relatively depends on prey density and also the design of the container which was associated with hiding place available for prey.

The strategy of *Tx. splendens* feeding behavior plays a big role in the successful limiting of prey populations. The feeding behavior of *Tx. splendens* has been described as 'opportunistic' because these predatory larvae do not search for prey, but instead the larvae ambush prey that come into contact with their mandibles (Furumizo & Rudnick, 1978; Russo, 1986). In the present study with a large number of prey per container, we suggest that the foraging space between both predator and prey becomes closer. In this condition, we observed that active *Ae. albopictus* larvae are busy searching for food and ignored the presence of *Tx. splendens* predator. The predator took this opportunity to ambush the prey. In response to higher density of prey available in the water, the predator ambush hunting strategy becomes easier, resulting in higher prey captures and prey consumption in 24 h.

Although the feeding behavior of some 'sit and wait' predators can be changed (Gullan & Cranston, 2005), this phenomenon was not observed during other instars larvae of *Toxorhynchites* except in fourth instar larvae (Yasuda, 1995). When the predator turned into fourth instar larvae and the prey was at low density, the predator may change their feeding behaviour from waiting to active searching the prey (Russo, 1986; Yasuda, 1995). In response to an increase in hunger level and capture time, the fourth instar larvae of *Tx. splendens* will undulate toward a cluster of prey and attack within two minutes (Russo, 1986; Yasuda, 1995).

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

We suggest that *Tx. splend*ens larvae are best introduced in tyres or containers with similar wide opening as observed in this study. Tyres are a favourable breeding habitat for many vector mosquitoes and *Ae. albopictus* was the most abundant species found in this type of breeding habitat (Yee, 2008). Since, the increase in prey density can lead to high prey consumption by *Tx. splendens* predator, we predict that this predator may be able to significiantly reduce the prey population, especially in tyre habitats. This goal supports the aim of biological control to bring pest densities within acceptable limits even if elimination is not possible (Ann Hajek, 2004).

One of the abilities of *Tx. splendens* larvae is that they are able to consume and attack prey as soon as they are released into the target containers. Direct impact on reduction of prey population can immediately be seen once larvae of *Tx. splendens* are introduced in breeding habitat compared to adult. The introduction of third instar larvae is more appropriate than first or second instar because third instar is stronger, able to resist starvation and can consume more prey larvae (Furumizo & Rudnick, 1978). The life length for third and fourth instar larvae ranged between 2-5 and 4-9 days (Furumizo & Rudnick, 1978), which can provide effective control period up to 14 days.

No association was found between the prey consumption and water volume. Increasing the water volume did not affect the consumption rate of prey by *Tx. splendens* predator. Similar results were also reported for *Tx. splendens* tested in containers filled with different water volumes at 2.0L and 0.5L (Dominic & Das, 1998). This finding suggests that *Tx. splendens* can be released in tropical areas, such as in Malaysia which receives rainfall throughout the year. Since the prey consumption was not affected by the water volume in the containers, the prey control by the predator should be consistent in both the dry and rainy seasons.

In field conditions, the abundance of *Ae. albopictus* is not consistent in breeding habitats. The estimation of prey consumed by *Tx. splendens* provided in this study is an important guideline to determine suggested numbers of predator needed to be to released in target container. However, more studies under field conditions need to be performed to evaluate the effect of other biotic and abiotic factors on predatory ability of *Tx. splendens*. We believed *Tx. splendens* is a promising biocontrol agent that can be introduced into many types of discarded containers, especially in tyres. We also postulate that the *Tx. splendens* will work well as a biocontrol agent under Malaysian climate conditions. Since *Tx. splendens* larvae are indigenous species that can be easily found naturally in this country (Furumizo & Rudnick, 1978; Collins & Blackwell, 2000), mass-rearing and application of this predator in the target containers to control *Ae. albopictus* are more achievable. We hope that implementation of the *Tx. splendens* larvae in *Ae. albopictus* breeding containers can be one of the successful solution to fight dengue vectors in future.

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