VORACITY AND PREY PREFERENCE OF PHILIPPINE POPULATION OF *TOXORHYNCHITES SPLENDENS* WIEDEMANN (DIPTERA: CULICIDAE) AMONG *AEDES* SPP (DIPTERA: CULICIDAE) AND *CULEX QUINQUEFASCIATUS* SAY (DIPTERA: CULICIDAE)

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Abstract. Mosquito-borne diseases are a major burden in the Philippines for centuries due to the lack of effective and sustainable vector control measures. Sightings of the elephant mosquito, *Toxorhynchites splendens* in the country were recorded since the 1940's. It has not been mentioned, however, in any control programs to date. The prey consumption by this larval predator for *Aedes aegypti* (Linnaeus, 1762) (Diptera: Culicidae), *Ae. albopictus* (Skuse, 1894) (Diptera: Culicidae), and *Culex quinquefasciatus* Say (1823) (Diptera: Culicidae) was, therefore, studied under a "with choice" and "without choice" situation to initially assess its capability under local conditions. The average cumulative voracity of larval *Tx. splendens* was higher for *Ae. aegypti* than for *Ae. albopictus* at all densities, with 70% consumed by the fourth instar. An increase in predation relative to increase in density was observed for all *Toxorhynchites* instars. Consumption of males and females did not vary in both prey species. Percent preys consumed per number of preys offered declined as density increased suggesting a satiation point. When offered a mix of different instars of *Ae. aegypti* larvae, *Tx. splendens* first and second instar larvae consumed the younger instars, but fourth instar *Tx. splendens* preferred older larvae and pupa, and when offered a mix of larvae belonging to three different species, the majority of *Tx. splendens* instars preferred *Ae. aegypti* and *Ae. albopictus* over *Cx. quinquefasciatus*. Our results suggest that *Tx. splendens* has very good potential in controlling dengue-carrying mosquitoes under Philippine conditions.

Keywords: *Aedes* spp, *Toxorhynchites splendens*, mosquito control, prey preference, voracity

INTRODUCTION

The Philippines is placed fourth highest from 2008-2012 on the list of countries with incidence of dengue and dengue hemorrhagic fever in Southeast Asia, with an average of 117,065 cases yearly (Edillo *et al*, 2015). More recently, Philippines' CDC reported 12 novel cases of the emerging Zika from various provinces (Santos, 2016). Since the establishment of a control program by the Department of Health, various entomological surveys

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of communities around the country have been initiated, especially during vector surges in the wet months of August to November (Shultz, 1993). Attempts in controlling dengue remain challenging as existing vaccines still need improvement and therapy is only symptomatic (WHO, 2017). Population growth, urbanization, requirement for surveillance improvement, and limited success of vector control measures are correlated with increasing disease incidences (Bravo *et al*, 2014).

Control strategies in the country focus on the most widespread mosquito vectors *Aedes aegypti* (Linnaeus) (Diptera: Culicidae) and *Ae. albopictus* (Skuse) (Diptera: Culicidae) (WHO, 2007). Approaches include fumigation, use of permethrin-treated bed nets and introduction of *Mesocyclops* sp and *Gambusia* sp. However, attempts to fully control vector populations have remained a major burden.

Alternative control measures using biological agents have recently gained interest especially those aimed at eliminating pre-adult stages. The predatory elephant mosquito, *Toxorhynchites* spp (Diptera: Culicidae), has shown great potential as larval carnivores of immature mosquitoes for the following reasons: (i) all larval stages are carnivorous and prey upon larvae and pupae of other mosquito species (Padgett and Focks, 1981); (ii) carnivorous larval stage may last for three weeks or more (Rubio and Ayesta, 1984); (iii) larvae are able to withstand desiccation and starvation for long periods and are able to survive on non-prey diet (Dodge, 1964; Trpis, 1979; Steffan and Evenhuis, 1981); (iv) larvae nearing pupation exhibit a "killing without eating" behavior in certain species (Corbet and Griffiths, 1963; Taylor, 1989; Collins and Blackwell, 2000); (v) adults are highly mobile and non-hematophagous; (vi) adult females may live up to seven weeks and have a lifetime egg production (Focks *et al*, 1979); and (vii) females oviposit on small natural and artificial water containers where target prey breeds and can be used in tandem with chemical control (Collins and Blackwell, 2000).

Mass releases of this predatory mosquito through inoculation or augmentation can be used to introduce or boost naturally occurring populations in the environment without causing any detrimental effect (Collins and Blackwell, 2000). Biocontrol attempts using *Toxorhynchites* spp have long been carried out with some success in many neighboring countries such as Malaysia (Nyamah *et al*, 2011) and Singapore (Chan, 1968), and in Japan (Toma and Miyagi, 1992). However, no attempt has been initiated in the Philippines. Hence, the voracity of *Tx. splendens* was determined with and without choice of size and species of target preys.

MATERIALS AND METHODS

Mosquitoes

Starting population of *Tx. splendens* was established from immature stages collected in College of Forestry and Natural Resources, University of the Philippines Los Banos (UPLB), Laguna from August 2013 to February 2016. Oviposition traps composed of 24-cm wide 8-cm deep black pans fixed on top of a 3-foot wood block were set around the study area for gravid females. Sampling was conducted by scooping eggs into a water-containing vessel, and larvae and pupae were transferred using a modified asepto-syringe fitted with a rubber extension especially for water-containing habitats. All samples were brought to the laboratory at the Institute of Weed Science, Entomology and Plant Pathology, College of Agriculture and Food Science, UPLB for rearing. Species confirmation was performed by extracting DNA from 2-3 legs of some adult specimens and processed using Jena Bioscience® Animal and Fungal kit (Jena, Germany). PCR was performed as previously described (Kumar *et al*, 2007) and sent to 1st Base (Selangor, Malaysia) for sequencing. Rearing procedures were adapted from Furumizo and Rudnick (1978) for *Tx. brevipalpis* using *Ae. aegypti* as prey.

Ae. aegypti and *Ae. albopictus* egg strips were initially requested from the Insect Pathology and Storage Pests Laboratories, UPLB. Eggs were soaked in dechlorinated tap water and hatched larvae were fed with brewer's yeast. Adult females were given blood meals from a restrained guinea pig every two days. Egg rafts of *Cx. quinquefasciatus* were collected in ovitraps infused with rice hay. Rearing was conducted in cages covered with a black cloth and blood meals were provided by a restrained chick or dove.

Voracity for *Aedes* spp larvae assay

Experiments were divided into voracity (without choice) and host preference (with choice). For without choice situation, a newly-hatched *Toxorhynchites* wriggler was given daily five *Ae. aegypti* or *Ae. albopictus* larvae of the same instar. After 24 hours, the number of eaten larvae was counted and replaced with a new set of five preys. This was conducted throughout the larval period. This protocol was also performed at prey densities of 10, 20, 40, and 60. *Tx. splendens* larvae alone served as negative control, with mortality counts used to correct the number of eaten larvae in both trials using Sun-Shepard's formula (Puntener, 1981). Average number of dead individuals in the negative control was subtracted from the total average of wrigglers killed or consumed in the corresponding treatment of the same replicate. Two trials were conducted for each prey density and species with at least 15 replicates for each experiment.

For the with choice situation, experiments were divided into two parts. In one part, *Tx. splendens* larvae were exposed to a set of different *Ae. aegypti* larval instars, and in the other part, predator larvae was exposed to a set of preys composed of a mix of three different species. *Tx. splendens* in the second, third, and fourth instar stages were given a diet of 10 *Ae. aegypti* daily until it reaches the desired age. Prior to the experiment proper, *Tx. splendens* wrigglers were starved for 12 hours after hatching or molting. The same protocol was also used for the negative control.

Preference for prey instar and species assay

A single first instar predator was introduced into a container with 20 *Ae. aegypti* larvae (five individuals from each of the four larval instars and five pupae). Uneaten preys after 24 hours were counted and this procedure was repeated until the predator molted and reached pupation.

In a single container, each *Toxorhychites* larva was given five individuals per species of larval prey (*Ae. aegypti, Ae. albopictus, Cx. quinquefasciatus*) belonging to the same instar. Uneaten preys after 24 hours were removed, killed, processed, and mounted on glass slides for species determination. This was carried out until the larva molted into the next instar and later pupated.

Statistical analysis

Shapiro-Wilk test in SPSS version 23.0 (IBM, Armonk, NY) was employed to test for non-normal data distribution of both voracity and preference. Additional tests were conducted using non-parametric Mann-Whitney *U* test involving data on sex of adult mosquitoes and Kruskall-Wallis for variables related to density and species.

RESULTS

Voracity

Predatory consumption of *Ae. aegypti* by all *Tx. splendens* instars was positively influenced by prey density in both *Ae. aegypti* (Kruskal-Wallis, Sig. <0.0001) and *Ae. albopictus* (Kruskal-Wallis, Sig. <0.001) (Table 1). The average total number of wrigglers eaten by each *Tx. splendens* during its entire larval stage was higher for *Ae. aegypti* compared to *Ae. albopictus* larvae on a daily amount of 5, 10, 20, 40, and 60 preys. However, with the early instars, consumption was higher for *Ae. albopictus* and voracity for only *Ae. aegypti* plummeted during the third and fourth instars.

The fourth instar was the most voracious of the larval stages at all densities of both preys. A single fourth instar *Tx. splendens* consumed preys ranging from a minimum of 47 to a maximum of 222 *Ae. aegypti* and 39 to 222 *Ae. albopictus* over the entire duration of its stadium. The number of individuals eaten by the fourth instar constituted 59-66% (*Ae. aegypti*) and 59-70% (*Ae. albopictus*) of the total number of prey consumed during the entire larval period. However, as observed that on a daily basis, voracity was lower when given 5 preys per day and consumption was comparable with the daily rates of the other instars. This lower daily consumption, consequently, was compensated by the long duration of the fourth instar in this type of experiment.

Although the mean consumption of the *Tx. splendens* larva on a daily basis and in total were positive in relation to density, the percent prey consumed over the total number of prey given was descending for all instars. In the fourth instar, the decrease started at prey density of 10. This could be the point where the larva has reached its satiation. Male and female *Tx. splendens*, as determined after emergence, did not vary in the mean number of consumed *Ae. aegypti* and *Ae. albopictus* for all larval instars (Fig 1).

Preference for prey instar

First instar *Tx. splendens* was able to consume 3.36 first instar *Ae. aegypti* but not any fourth instars or pupae. Second and third instar *Tx. splendens* were able to attack fourth instar and pupae of *Ae. aegypti*, but to a lesser extent. Fourth instar *Tx. splendens*, on the other hand, readily preyed upon fourth instar and pupae of *Ae. aegypti* and attacked first to second instars to a lesser degree. Non-parametric test using Kruskal-Wallis indicated that the voracity of different *Tx. splendens* instars was not the same for the different instars of *Ae. aegypti* (Sig. <0.0001). As the age of larval *Tx. splendens* increased, it favored late instars of *Ae. aegypti*, and conversely, younger *Tx. splendens* larvae preferred to attack younger instars of *Ae. aegypti* (Fig 2).

Preference for prey species

First, second, and fourth instar *Tx. splendens* larvae consumed, in general, more of the two *Aedes* spp larvae compared to *Cx. quinquefasciatus*. However, the third instar *Tx. splendens* preferred otherwise (Fig 3). These differences could have accounted for the indifference found on the preference of *Tx. splendens* over a specific species of prey (Kruskal-Wallis, Sig. = 0.98).

When grouped by instar, the consumption for each species is significantly different among *Ae. aegypti* (Kruskal-Wallis, Sig. <0.0001), *Ae. albopictus* (Sig.

Table 1
Voracity of the four different *Toxorhynchites splendens* instars for *Aedes aegypti* and *Ae. albopictus* at different prey densities.

Prey density (number of prey/ *Toxorhynchites* wriggler)	First		Second		Third		Fourth		Total	
	Mean ± SD	Total	Mean ± SD	Total	Mean ± SD	Total	Mean ± SD	Total	Mean ± SD	Total
5[c]	3 ± 1[a]	7	3 ± 1	8	2 ± <1	18	2 ± <1	48	2 ± <1	80
	3 ± 1[b]	8	2 ± 1	7	2 ± <1	12	2 ± <1	39	2 ± <1	67
10[d]	5 ± 2	9	4 ± 1	9	4 ± 1	26	6 ± 1	90	5 ± 1	134
	7 ± 2	9	6 ± 2	9	4 ± 1	11	6 ± 1	65	6 ± 1	94
20[e]	5 ± 1	9	4 ± 1	11	6 ± 1	35	9 ± 1	119	7 ± 1	175
	7 ± 2	16	4 ± 1	13	4 ± 1	20	7 ± 1	94	6 ± 1	144
40[f]	9 ± 2	18	10 ± 2	20	10 ± 1	46	15 ± 2	175	13 ± 1	259
	11 ± 1	21	10 ± 1	25	12 ± 2	52	15 ± 1	178	12 ± 1	277
60[g]	10 ± 3	13	13 ± 2	45	13 ± 3	55	21 ± 2	222	17 ± 2	334
	11 ± 3	17	14 ± 2	23	13 ± 3	56	21 ± 1	222	15 ± 1	317

[a]*Aedes aegypti*. [b]*Ae. albopictus*. [c]Total number of set-ups using *Ae. aegypti*, $n = 29$; *Ae. albopictus*, $n = 35$. [d]*Ae. aegypti*, $n = 52$; *Ae. albopictus*, $n = 34$. [e]*Ae. aegypti*, $n = 31$; *Ae. albopictus*, $n = 33$. [f]*Ae. aegypti*, $n = 30$; *Ae. albopictus*, $n = 30$. [g]*Ae. aegypti*, $n = 32$; *Ae. albopictus*, $n = 30$. *Aedes* preys given were of proportionate size or the same larval instar as the *Toxorhynchites* wriggler.

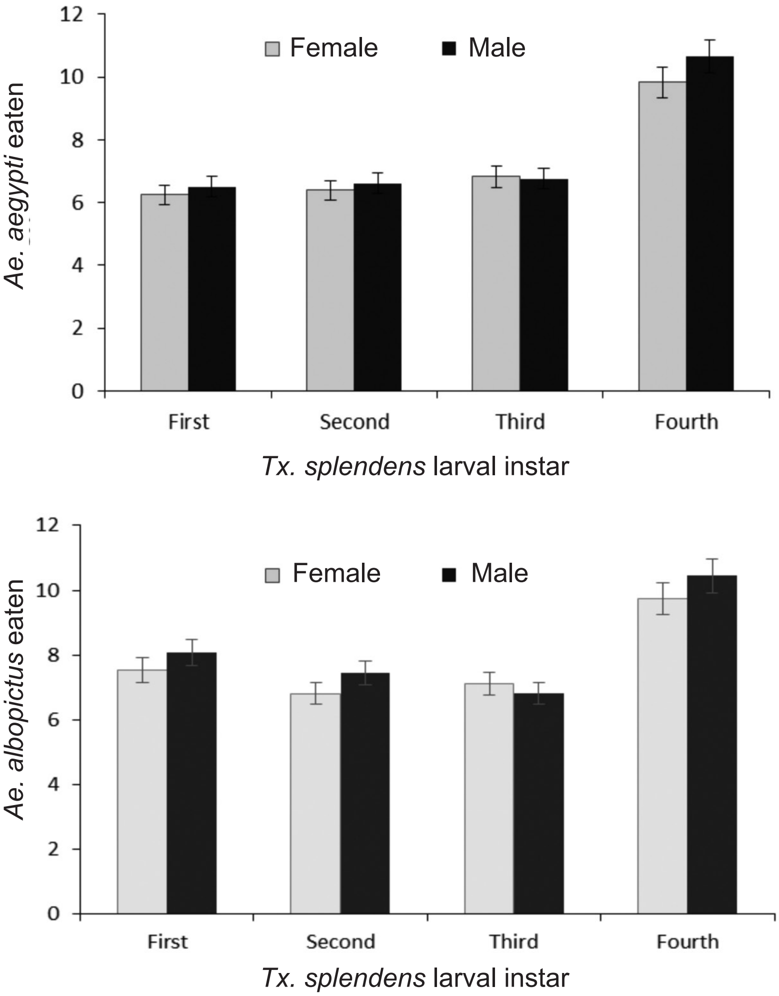

Fig 1–Mean daily consumption of *Aedes aegypti* and *Ae. albopictus* larvae by male and female *Toxorhynchites splendens* during the four larval stages at all prey densities. *Aedes* preys given were of proportionate size or the same larval instar as the *Toxorhynchites* wrigglers (*Ae. aegypti*, *n*=174; *Ae. albopictus*, *n*=163).

= 0.025), and *Cx. quinquefasciatus* (Sigs. = 0.002). Second and fourth *Tx. splendens* instars consumed more prey, regardless of the species, compared to the first and third instars.

DISCUSSION

Our findings on the voracity of *Tx. splendens* for *Ae. aegypti* and *Ae. albopictus* are comparable to that obtained by Begum *et al* (1988) where the average number of prey eaten during the larval life span is 257 ± 21. Trpis (1973) in Kenya showed *Tx. brevipalpis* consume 154 - 358 *Ae. aegypti* during the course of its larval stage. On the other hand, a lower number is obtained for *Ae. albopictus* in comparison to the 389 prey larvae eaten by *Tx. splendens* (Toma and Miyagi, 1992). Difference in the consumption was only more evident in the last two stadia and were more uniform in the first and second stadia in both males and females (Rubio and Ayesta 1984). Differences in the range of minimum numbers of preys consumed could be attributed to the number of larvae offered and effects of ambient temperature. Although Corbet (1963) stated that fourth instar *Tx. brevipalpis condrati* is able to reduce feeding at low food availability, the experimental conditions in the current study involved replenishment of eaten prey, thereby, even at a low prey density, *Toxorhynchites* larvae still had a constant supply of prey *per se*.

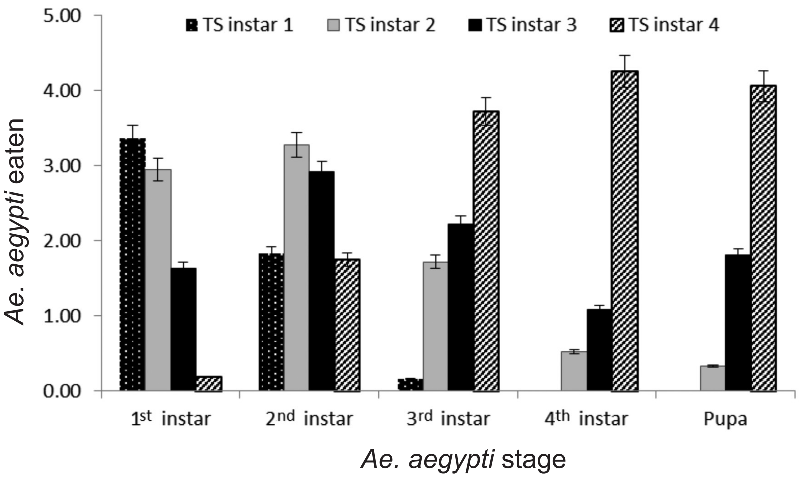

Fig 2–Prey preference of first to fourth *Toxorhynchites splendens* instar when given a mix of first to fourth instars and pupae of *Aedes aegypti* as prey within 24 hours. *Aedes* preys given were of proportionate size or the same larval instar as the *Toxorhynchites* wrigglers (n = 36).

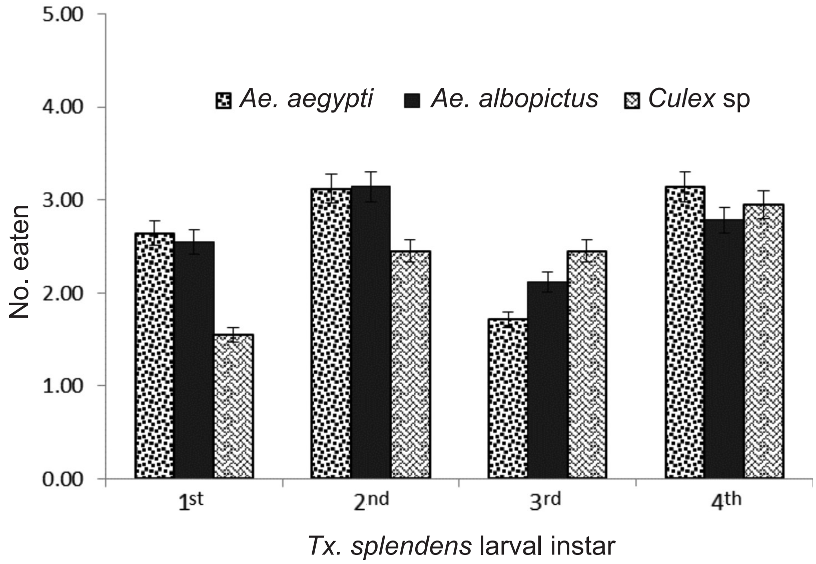

Fig 3–Prey species preference of the four different larval instars of *Toxorhynchites splendens* when given a mix of *Aedes aegypti, Ae. albopictus,* and *Culex quinquefasciatus*. Preys offered were of proportionate size or the same larval instar as the *Toxorhynchites* wrigglers (n = 42).

The large number of preys consumed by the fourth instar compared to the younger instars could be attributed to its higher nutritional requirement, especially the energy needed during the non-feeding pupal period (Crans and Slaff, 1977). Trimble and Smith (1978) reported the minimum amount of prey consumed by the fourth instar of *Tx. brevipalpis* larva is 60-70% of its total consumption during the whole larval stage. This last instar is considered to have the most intense phagoperiod according to the rate of biosynthesis across many species of mosquitoes (Timmerman and Briegel, 1998).

Foraging theory suggests that predators learn to adjust consumption based on the energy content and size of the prey in light of its specific nutritional requirements. Schmidt *et al* (2012) explained that the previous nutritional experience of generalist predators, such as the wolf spider, pre-determines its choice of prey. It has long been understood that the potential energy provided by the target prey is the major factor influencing predator foraging behavior (Whelan and Schmidt, 2007). However, more in depth studies revealed that it could be further broken down into the prey's impact on the predator's growth, development, distribution, and survival (Schmidt *et al*, 2012). As a result, most predators regulate food intake to maximize nutrition and prevent excessive consumption of prey with less nutritional value. Even after capture, predators still regulate ingestion to match their metabolic intake (Mayntz *et al*, 2005).

In the field, *Toxorhynchites* larva food source is limited by the rate at which potential prey hatch and develops. These are highly influenced by the prey's oviposition rate as well as water level (Russo, 1986). The low rate of consumption exhibited by *Tx. splendens* in the study by Russo (1986) compared to other species, such as *Tx. theobaldi* (Dyar and Knab, 1906), could be attributed to the habitat where this species thrives. Unlike *Tx. theobaldi* has adapted to a drier environments, and therefore, consume large amounts of food during the short time available, while *Tx. splendens* has a constant supply of prey food in the wetter tropical countries.

The preference for a prey of the same instar or body proportion was also observed in *Tx. brevipalpis* Theobald (1901), where in the presence of younger instars, fourth instar *Toxorhynchites* preferred to consume third and fourth instar prey larvae and pupae (Lounibos, 1979). Also, the consumption of fourth instar *Ae. aegypti* is higher than of pupae and dramatically higher than of first instars when offered altogether (Padgett and Focks, 1981). However, when offered separately, *Tx. rutilus* is able to eat 93 first instars compared to 10 fourth instars and 7 pupae (Coquillet, 1896).

Toxorhynchites larvae do not actively seek out prey and prey capture mostly relies on the collisions between itself and its prey (Zuharah *et al*, 2015). Among the four larval instars and pupae offered, the fourth instar *Aedes* larva has the highest activity and often "grazes" on the body of *Toxorhynchites* larva, thereby increasing interaction compared to the more lethargic pupa (Zuharah *et al*, 2015; Nyamah *et al*, 2011). The preference over the younger prey instars was theorized to be based on the optimal foraging strategy, which explains that attacking a larger prey requires a lower attack rate. The decrease in time needed to assault a prey outweighs the added energy needed for a longer handling time. In this way, *Toxorhynchites* larva may have evolved this mode of predation as it is more economical as regards time and energy than stalking smaller preys even though they are easier to consume (Lounibos, 1979).

The voracity of *Tx. splendens* for *Ae. aegypti* and *Ae. albopictus* increased as it reached the second instar, then dropped during the third instar and slightly increased again as it reached the last instar. Predatory insects such as *Toxorhynchites* spp are expected to increase in voracity as they prepare for the high energy-consuming pupal stage as observed in most cases (Chan, 1968; Furumizo and Rudnick, 1978), making the low consumption during the third instar, a peculiarity. During this stage, larvae were observed to passively rest near the container's edge. This thigmotactic behavior is theorized to be a manifestation of anxiety (Schnörr *et al*, 2012). Also, unlike other instars that readily attack approaching preys, this instar prefers to observe passing *Aedes* larvae by moving its head towards the prey's direction without attacking. Although unclear, these behaviors could be attributed to the development of the compound eyes, which is usually initiated during this stage as observed with other dipterans (Wolff and Ready 1993; Ashburner *et al*, 2005). While *Toxorhynchites* larvae depend on movement-based signals (Zuharah *et al*, 2015) for detecting prey, this development of eyes may be a novel process in the growing larva, which could also explain its thigmotaxis.

Voracity of *Tx. splendens* for *Clue* was similar to that observed for the *Aedes* varied in such *Toxorhynchites* sp is able to consume 570 *Cx. quinquefasciatus* during its lifetime (Urmila *et al*, 2000), a number greater than most studies using *Aedes* (Steffan and Evenhuis, 1981; Toma and Miyagi, 1992). Comparing the three prey species in our study, *Cx. quinquefasciatus* was observed to be consumed the most, probably owing to its higher chance of contact stemming from its higher movement activity especially by the third instar larva.

In conclusion, predatory preference of *Tx. splendens* is an important factor which contribute to high reduction in Aedine populations. The use of this predatory mosquito will be instrumental to the success of any dengue and dengue hemorrhagic fever control effort in the Philippines.

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