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Research

Larval Age and Nutrition Affect the Susceptibility of *Culex quinquefasciatus* (Diptera: Culicidae) to Temephos

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Abstract

Larval age and nutrition significantly affected the insect's physiology. These influences are important when rearing a population of vectors that is used to monitor the resistance level, in which standardized conditions are crucial for a more harmonized result. Little information has been reported on the effects of larval age and nutrition on the susceptibility of insects to insecticides, and therefore, we studied the effects on the susceptibility of *Culex quinquefasciatus* Say's (Diptera: Culicidae) larvae to temephos by comparing the median lethal concentration (LC_{50}) after 24 hr between the second and fourth instar larvae and between the larvae that fed on protein-based and carbohydrate-based larval diets. The susceptibility of the larvae was significantly affected by the larval diets, as the larvae that fed on protein-based beef food and milk food demonstrated significantly higher LC_{50} value compared with the larvae that fed on carbohydrate-based food: lab food and yeast food. The larval diet interacted significantly with the larval age: while the second instar larvae were susceptible to temephos when supplied with carbohydrate-based food, the second and fourth instar larvae had no significant effect when supplied with protein-based diets, implying that a protein-rich environment may cause the mosquito to be less susceptible to temephos. This study suggested the importance of standardizing nutrition when rearing a vector population in order to obtain more harmonized dosage–response results in an insecticide resistance monitoring program. Future research could focus on the biochemical mechanism between the nutrition and the enzymatic activities of the vector.

Key words: larval age, larval nutrition, susceptibility, temephos, *Culex quinquefasciatus* Say

Culex quinquefasciatus Say (Diptera: Culicidae) is the principal vector of lymphatic filariasis, and vector control is regarded as one of the cost-effective approaches available in controlling the disease (Maizels and Denham 1992). However, the control of the vector faces significant challenges due to the development of insecticide resistance (Maria et al. 2000). The insecticide resistance levels in field populations of the *Cx. quinquefasciatus* were measured in order to select appropriate insecticides for vector control (WHO 2016). To determine the resistance level, standardized guidelines and procedures were proposed by the World Health Organization (WHO 2005, IRAC 2011, WHO 2016) and Centers for Disease Control and Prevention (CDC) to harmonize the testing procedures conducted and the data from different laboratories and institutions. The resistance level was typically studied by comparing the median lethal concentration (LC_{50}) between the susceptible and field strains to obtain the resistance ratio (RR). By obtaining the larvae from both susceptible and field strains, some of the rearing conditions, for example, humidity and temperature, the number of larvae, and size of the container, were stated in the WHO (2016) larvicide testing. Although the WHO testing procedure suggested third to fourth instar in the bioassay testing of larvae (WHO 2016), the effect of using different larval ages in a bioassay is not fully studied. Moreover, the nutrition of the larval diet is not mentioned in the procedures (WHO 2005, WHO 2016) even though the information is crucial. Kivuyo et al. (2014) demonstrated that the pupation rates and sex ratios of emerging *Anopheles gambiae* senso stricto (Diptera: Culicidae) adults were significantly affected by five types of diets, and the survival rate of the mosquito was significantly higher when the larvae were fed with Tetramin fish food. Linenberg et al. (2016) determined that the larval diet significantly affected the *Anopheles coluzzii*'s (Diptera: Culicidae) physiology, in which the larval diet affected the mosquito's development and permissiveness to *Plasmodium* infection. Different diets during rearing may affect the dosage–response of the mosquito, even though the species and testing circumstances were standardized at a certain range. This has been demonstrated in separate studies that tested the LC_{50} of *Ae. aegypti* L. (Diptera: Culicidae) larvae on temephos; when Ponlawat et al. (2005) and McAllister et al. (2012) used ground fish food (approximately 40%

protein) and Bellinato et al. (2016) used ground cat food (~20% protein) in maintaining the susceptible and field strains of *Ae. aegypti*, their LC_{50}s were markedly different from each other. Therefore, this study aimed to determine the significance of larval age and diet in rearing *Cx. quinquefasciatus* larvae on the median lethal concentration (LC_{50}) of temephos.

Materials and Methods

Insects

The experiment was carried out on susceptible strain-WHO/VCRU *Cx. quinquefasciatus* Say larvae. The mosquitoes had not been exposed to any insecticides since 1995, and the colony was maintained at 27 ± 2°C and 75 ± 5% relative humidity in insectariums in the Vector Control Research Unit (VCRU), Universiti Sains Malaysia. The egg rafts were collected in a 2-liter aluminum enameled container (diameter: 30 cm, height: 5 cm) that contained 1 liter of dechlorinated water. For the larval age component, second and fourth instar larvae were used, and previous studies suggested the size of the mosquito may influence susceptibility of insecticide (WHO 2013). Therefore, the age was determined based on both the body length (2nd instar 0.4–0.5 mm, and 4th instar 0.8–0.9 mm, determined by a compound microscope with calibrated eyepiece graticule) and the day of emergence from the eggs. For the nutrition component, 5 g of diet was added to 1 liter water of rearing container. Four larval diets: VCRU/lab food (LF), beef food (BF), yeast food (YF), and milk food (MF) were fed to the larvae daily, beginning from the day the larvae hatched from the eggs. The LF was the control for the experiment because it is the standard larval diet for rearing the WHO/VCRU strain of *Cx. quinquefasciatus* Say in the VCRU, Universiti Sains Malaysia.

Larval Diet Preparation and Proximal Analysis

The brewing yeast powder (Sigma, Malaysia), milk powder (Dutch Lady Milk Industries Berhad, Malaysia), and dog biscuit (Alpo, Malaysia) were purchased from the distributor, while beef powder was obtained by grinding dried, raw beef liver. All the feeds were previously filtered through a brass frame test sieve with pore size 45 µm before being mixed into their respective ratios according to their dry weight. The foods were different in their ratios of yeast, beef powder, milk powder, and dog biscuit. The nutrition of the larval diet was previously categorized into protein- and carbohydrate-based by purposely increasing the proportion of a certain content. The control LF contained yeast, beef powder, milk powder, and dog biscuit in a ratio of 1:1:1:3; BF contained beef powder and dog biscuit in a ratio of 3:1; YF contained yeast and dog biscuit in a ratio of 3:1; and MF contained milk powder and dog biscuit in a ratio of 3:1.

To categorize the larval diet into protein- and carbohydrate-based diets, a slightly modified AOAC (2000) proximate analysis was carried out on the larval diets. For moisture, approximately 4.00 g of the food samples was measured on an electrical balance before and after drying in an oven. The moisture level was indicated by the difference in mass, and it was converted into a percentage. Later, the dried samples were subjected to protein analysis using the Kjeldahl method. Initially, approximately 0.10 g of sample was first digested with concentrated sulfuric acid and catalyst in a Kjeldahl flask. The products were later cooled down at room temperature, and sodium hydroxide was added into the flask. The flask was placed into a distillation connection unit, and the distillate was mixed with boric acid and few drops of methyl red. The distillate mixture was titrated with 0.40% hydrochloric acid, and the protein was calculated as a percentage. Determination of lipid content was performed following the Soxtec method described by AOAC (2000). Food samples were weighed at approximately 0.20 g, and 4 ml of solvent petroleum ether was used for the extraction. First, the mixture was homogenized by using an ultrasonic homogenizer (Fisherbrand Model 505 Sonic Dismembrator, ThermoFisherSci, Malaysia) and filtered with a Buchner funnel. The filtrate was transferred to a separating funnel and shaken with 20 ml of distilled water. The mixture was allowed to settle overnight, and the ether part was removed and dried in the oven at 60°C for 8 hr. The residue weight was the lipid and was expressed as a percentage. For the ash content, a dry ashing method was used to determine the content. The samples were placed in a pre-weighted ceramic cup and incinerated at 550°C for 8 hr in a furnace. The inorganic material was cooled and weighed, and the ash content was expressed as a percentage. The total carbohydrate content (including fibers) in the samples was calculated by subtracting the other constituents (protein, fat, water, and ash) from the total weight of the food.

Bioassay and Temephos Preparation

The medium lethal concentration (LC_{50}) is preferable in this study over the discriminating dose (the two-times higher LC_{95}) due to the lower accuracy of the LC_{95} on its near endpoint plot on the dose–response curve (Jørgen 2004, IRAC 2011). Therefore, the comparison of LC_{50} for susceptible and field strains in the form of an RR is more reliable for providing information on the resistance level (IRAC 2011). The bioassays followed the insecticide resistance monitoring guidelines proposed by the WHO (2005). Distilled water was used as the solvent to prepare nine dilution rates that ranged from 1 to 9 µg/liter from 1.00 mg/liter stock solution of temephos (PESTANAL, analytical standard, Sigma-Aldrich, Malaysia). Batches of 25 *Cx. quinquefasciatus* larvae were transferred by a screen loop to a 354.88 ml paper cup that contained 100 ml of dechlorinated water. Five replicates were prepared for each concentration, and the controls were set up simultaneously with the dechlorinated water only. The test containers were held at a photoperiod of 12:12 (L:D) h. After 24 h of exposure, larval mortality was recorded, and dead larvae were considered those that could not be induced to move.

Statistical Analysis

To classify the larval diets into protein-based (relatively higher protein content) and carbohydrate-based (relatively higher carbohydrate content), the nutrition content of the diets was one-way analyzed by their variances, and their significance was compared with the least significant difference as post hoc using SPSS 17.0.

For the bioassay, the mortality of *Cx. quinquefasciatus* larvae was corrected with Abbott's formula (Abbott 1925), and the concentration–mortality data were subjected to probit analysis (Finney 1971) using SPSS 17.0 to obtain the LC_{50} of the mosquito. The differences and interaction between the factors of larval age and diet were first determined by two-way factorial analysis of variance (two-way ANOVA, SPSS 17.0), and where there were significant differences in those dependent factors, the differences were compared using the 95% confidence interval (CI) of the LC_{50}, in which no overlap indicated rejection of the null hypothesis (Gordon et al. 2015). Moreover, to determine the correlation between the protein and LC_{50}, linear regression was performed on the protein content of the diet and the LC_{50} of the mosquitoes on temephos.

Results

As predicted before the experiment, the protein-based larval diet of BF and MF contained significantly higher protein content than

the carbohydrate-based diet of LF and YF after standard testing proximate analysis by AOAC (Table 1). For the fat content, YF that contained yeast as the major component had the lowest fat content among the four diets. Ash and moisture were not considered nutrients, as the ash refers to the inorganic material left over after the food was burned at a high temperature (600–800°C) in a furnace (FAO 2003), and, therefore, not used for comparison.

Larval diet significantly affected the LC_{50} of *Cx. quinquefasciatus* larvae on temephos ($F_{3,32}$ = 841.47) at $P < 0.001$ level, in that the protein-based diet generated significantly higher LC_{50} value than the carbohydrate-based diet (Table 2).

On the other hand, the two-way analysis of variance also showed that both larval age and nutrition interacted significantly on the LC_{50} of *Cx. quinquefasciatus* larvae on temephos ($F_{3,32}$ = 30.31, $P < 0.001$). This finding was supported when we compared the CI of LC_{50} between the 2nd and 4th instar larvae that fed on different larval diets. However, for the comparison of the CI of the LC_{50} value, larval age was influenced positively by nutrition, although 4th instar larvae had a significantly higher LC_{50} than 2nd instar larvae (for the carbohydrate-based diet, Table 2); nevertheless, when a high protein larval diet was supplied to the mosquito 2nd instar larvae, the LC_{50} was not significantly different than with the 4th instar larvae. This strongly suggested a direct interaction between the protein content and the LC_{50} value. From the result of the linear regression (Table 3), the general form of the regression equation for the LC_{50} versus protein content was

$$y = a + bx$$

where a is the y intercept, b is the change in LC_{50} per change of protein content, and x is the protein content. The slopes (b) were positive, which indicates that as the protein content increases, the LC_{50} value increases. Moreover, the slopes for the second instar larvae were greater than for the fourth instar larvae ($P < 0.05$), suggesting that the effect of protein content exerted greater effect on the LC_{50} of the second instar larvae than on that of the fourth instar larvae.

Discussion

Nutrition significantly affects insect physiology. Protein and carbohydrates in the larval diet are two keys macronutrients for the physiology of insects (Lichtenstein and Russell 2005), and we demonstrated that the protein- and carbohydrate-based larval diet significantly affected the susceptibility of *Cx. quinquefasciatus* larvae, in that a protein-based diet caused the larvae to be less susceptible to temephos. This was concurred by the studies of Briegel (1990) and Telang and Wells (2004), who showed that the fitness-related traits of *Ae. aegypti* were strongly influenced by their nutritional environment, and Owusu et al. (2017), who also reported that nutrition was the major factor that affected the mortality and body weight of *An. gambiae* and *Anopheles stephensi* larvae on permethrin.

Standardization of larval nutrition to obtain a harmonized LC_{50} value is important in an insecticide resistance monitoring program. With manipulation of nutrition of the larval diet in this study, the susceptibility of *Cx. quinquefasciatus* larvae to temephos was greatly affected. Povey et al. (2009) showed how the immune system of Spodoptera exempta W. (Lepidoptera: Noctuidae) against bacterium *Bacillus subtilis* infection when the diet was enhanced with protein. Rivero et al. (2011) demonstrated the insecticide resistant larvae *Culex pipiens* Linnaeus (Diptera: Culicidae) with overproduction of esterases, the ratio of protein: carbohydrate is high. Nevertheless, contrary to this study, Kulma et al. (2013) investigated the correlation of three food regimes (different in quantity from the normal diet) and age on the susceptibility of *An. gambiae* to DDT and suggested that food regimes have a smaller effect than the larval age of *An. gambiae*. However, this study manipulated the nutrient content (protein and basic carbohydrates) rather than quantity, and we have demonstrated that larval diets' nutrition, especially a protein-based diet, exerted significantly greater effect than larval age (when we showed that the slope of the second instar was significantly greater than that of the fourth instar, Table 3, and that the CI of LC_{50} of the second instar was not significant with the fourth instar when supplied with the protein-based diet, Table 2). This implicated that the high protein content in larval diets could assist the younger age of the larvae in balancing out the effect of temephos, as the protein is the most important nutrient for synthesizing the insecticide's detoxifying enzyme (Sarkar et al. 2009). Although biochemical bioassay was not conducted in this study, as the protein-based diet may have supplied a higher level of amino acid, the larvae that fed on the protein-based diet could have generated a higher level of detoxifying enzymes such as glutathione *S*-transferase (GST) family enzymes (Daniel and Vasilis 2004); as the enzyme increased, the ability to resist insecticides was elevated.

We highlighted how the application of a protein-rich diet may decrease the susceptibility of young *Cx. quinquefasciatus* larvae to temephos due to the potential of young larvae becoming more resistant to temephos if the organic environment (such as a sewage system) is able to provide a high level of protein. As reported by Richards et al. (2017), *Culex spp.* mosquitoes were 15 times more likely to show resistance than *Aedes spp.* mosquitoes and *Cx. quinquefasciatus* that prefers to breed in containers rich in organic matter, and the nutrients in the breeding water may affect the susceptibility of the mosquito to the larvicide. The results of the present study suggested that the likelihood of *Culex spp.* to resist insecticides was better due to the protein-rich environment.

Larval age affected *Cx. quinquefasciatus* larvae susceptibility to temephos when carbohydrate-based diets were provided. Although studies have reported that the susceptibility of adult mosquitoes to insecticide increased as they became older (Chouaibou et al. 2012), this study showed that the younger group of larvae (second instar)

Table 1. Proximate analysis of the artificial larval diet, mean percentage % ± SE

	Moisture	Protein	Fat	Carbohydrate	Ash
LF	6.91 ± 0.01	26.21 ± 0.39a	9.49 ± 0.20a	48.35 ± 0.21a	5.94 ± 0.12
BF	8.53 ± 0.75	40.69 ± 0.42b	12.20 ± 0.15a	31.05 ± 0.37b	5.32 ± 0.73
YF	6.45 ± 0.06	28.48 ± 0.43a	4.45 ± 0.23b	50.89 ± 0.46a	6.11 ± 0.04
MF	4.98 ± 0.00	37.41 ± 0.24b	10.31 ± 0.20a	38.34 ± 0.51b	6.12 ± 0.34

Different alphabets in columns indicate there are significantly different for the artificial diet at $P < 0.05$.
SE (standard error); LF (lab food); Bf (beef food); YF (yeast food); MF (milk food).

Table 2. The medium lethal concentration (LC_{50}) values of temophos for second and fourth instar of *Cx. quinquefasciatus* larvae on different artificial larval diet

	LC_{50}, µg/liter (95% CI)‡			
	Second instar	Slope ± SE	Fourth instar	Slope ± SE
LF	3.26 (3.12–3.40)†	1.48 ± 0.27	5.08 (4.99–5.17)*,†	4.08 ± 0.47
BF	5.85 (5.66–6.04)‡	2.10 ± 0.30	6.23 (6.03–6.43)‡	5.21 ± 0.76
YF	3.37 (3.22–3.52)†	4.07 ± 0.58	4.74 (4.61–4.87)*,†	1.91 ± 0.27
MF	6.03 (5.95–6.11)‡	3.56 ± 0.39	6.26 (6.06–6.46)‡	2.16 ± 0.31

Different alphabets in columns indicate there are significantly different for the artificial diet at $P < 0.05$.

CI (confidence interval); SE (standard error); LF (lab food); Bf (beef food); YF (yeast food); MF (milk food).

*Significantly different between second and fourth instar larva at $P < 0.05$.

†Larval diet was significantly different, $F_{3, 32} = 841.47$, $P < 0.001$; second and fourth instar larvae were significantly different, $F_{1, 32} = 726.08$, $P < 0.001$; interaction between age and larval diets was significant, $F_{3,32} = 30.31$, $P < 0.001$.

‡Significance test based on the confidence interval at a 95% level, in which no overlap of the two intervals indicates rejection of the null hypothesis.

Table 3. Result of regression of LC_{50} and larval diets' protein content

	Parameter estimate		r^2
Age (instar)	*a* (*y* intercept)	*b* (slope)	
Second	–2.24 (0.53)	0.21*a* (0.02)	0.56 (0.43)
Fourth	1.18 (0.52)	0.14*b* (0.02)	0.82 (0.42)

General form of regression is $y = a + bx$, where y is the LC_{50} of *Cx. quinquefasciatus* on temephos, a is the y intercept, b is the change in LC_{50} per change of protein percentage, x is the protein content. Numbers in parentheses are standard error of the estimates. Means numbers in same column followed by different letters are significantly different at $P < 0.05$.

was more susceptible to temephos compared with the older larvae (fourth instar). This may be because the physiological responses of the immature and adult stages of the mosquito toward stimuli were different, and this was supported by Céline et al. (2015), who noted that *An. gambiae*'s adult reproductive traits reacted significantly differently in either larval or adult growing temperatures. Nevertheless, the present study only involved two stages of immaturity, and more larval stages need to be involved for a more comprehensive investigation.

For monitoring insecticide resistance, dosage–mortality bioassays on *Cx. quinquefasciatus* that estimate the mosquito's susceptibility in the field have to be accurate. Therefore, the larval diets that are used for *Cx. quinquefasciatus* must adequately simulate the nutritional conditions in the field, which is rich in organic matter. However, most larval diets supplied to mosquito larvae are carbohydrate-based such as wheat-grain or yeast powder with pet food (Ponlawat et al. 2005, McAllister et al. 2012, Bellinato et al. 2016), and based on our data, carbohydrate-based diets such as these are likely overestimating the susceptibility of *Cx. quinquefasciatus* by generating nutritional stress on the mosquito. Additionally, the present data also suggest that the standard testing procedures should standardize the diet for rearing the mosquito population.

Conclusions

In conclusion, the nutrition of larval diets affected the LC_{50} of *Cx. quinquefasciatus* larvae on temephos. The susceptibility of younger larvae (2nd instar) could be decreased when high-protein-content larval diets are applied in the rearing environment. Future research could focus on molecular analysis (amino acid profiles) and biochemical assay to determine the correlation between the detoxifying enzyme and the susceptibility of the mosquito.

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