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Laboratory Studies on Developmental Responses of the Filarial Vector Mosquito, *Culex pipiens pipiens* (Diptera: Culicidae), to Urea Fertilizer

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Filariasis threatens the health and economic well-being of more than a billion people worldwide. Yet, the environmental factors that condition the abundance of its principal vector, i.e., *Culex pipiens pipiens*, is poorly understood due to the greater emphasis placed on anopheline vectors of malaria. *Cx. p. pipiens* mosquitoes breed profusely in rice-farming wetlands, where urea is commonly used as fertilizer; which has been controversially reported as having varied effects on the biology of the mosquito. In order to elucidate the potential contributions of application urea, to the production of *Cx. p. pipiens* mosquitoes from rice-growing wetlands, Laboratory studies were carried out to determine the influence of urea fertilizer on survival and development of immature life stages of the mosquito. Day-old 1st instar larvae of the mosquito species were raised in urea-treated media of concentrations ranging from 2.50-15.00 g L^{-1} and monitored daily for immature life stage survival rate and duration of development. The results showed dose-dependent mortality and developmental responses of the mosquitoes to urea treatments. Total Immature Survival (TIS) reduced significantly ($p<0.05$) from 90.38±6.52% in the control mosquitoes to 65.55±28.92% among those reared in 5 g L^{-1} urea-water. Generally, mean larval survival rates were significantly lower than those of the pupal stage. Survival rates of the larval instar stages significantly reduced from L1 to L4, with few exceptions. Similarly, the duration of Total Immature Development (TID) was significantly ($p<0.05$) extended by urea from 11.57±0.20 days in the control mosquitoes to 17.44±0.94 days among mosquitoes raised in 10.00 g L^{-1} urea-water. The duration of larval instar stages (i.e., L1 to L4) also varied significantly among the treatments, with larval development terminating at L3 in the 12.50 and 15.00 g L^{-1} urea-water treatments. However, for treatments 5.00 to 12.50 g L^{-1} urea-water, the duration of the L1 larval instars did not follow the usual dose-dependent effects of urea characteristic of the results of this study. The epidemiological implications of these results were highlighted and it is hoped that the findings will facilitate the development and integration of fertilizer-application best practices in to rice-farming activities.

Key words: Breeding sites, *Culex* mosquitoes, larva, pupa, rice fields, survival rate

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INTRODUCTION

Culex pipiens pipiens is the most common cosmopolitan mosquito species and a principal vector of filariasis; a disease which threatens the health of about 1.3 billion people in 83 countries and incapacitates over 40 million people worldwide (WHO, 2004, 2011). *Cx. p. pipiens* breeds intensively in wetland rice-fields; as a result of the proliferation of lasting conducive larval habitats in such environments (Lacey and Lacey, 1990; De Plaen *et al*., 2003). Recently, studies have revealed that the propensity of *Cx. p. pipiens* mosquitoes to breed in rice-fields is not only due to the availability of breeding sites but the superiority of such larval habitats in terms of resource abundance, such as high algal growth; a major source of rich diet for rapid larval development (Sunish and Reuben, 2001). Improved algal growth in wetland rice-fields have been attributed to the use of certain agricultural chemical inputs especially Nitrogen-based fertilizers, in rice farming (Mutero *et al*., 2004). This development has made wetland rice-fields major sources of mosquito production thus posing serious threat to human health and calls to question sustainable rice production as one of the cardinal strategies for achieving the Millennium Development Goal (MDG) of food security.

However, it is known that Nitrogen-based fertilizers may be toxic to aquatic organisms (Tchounwou *et al*., 1991) and should therefore actually suppress the breeding of mosquitoes in urea-fertilized rice-fields for example. These views regarding the probable contributions of urea-fertilized wetland rice-fields to mosquito production have continued to generate controversies between agricultural and public health stakeholders in rice-growing regions of the world. Therefore, in order to shed more light on the role of urea-fertilizer application in rice-field mosquito production, this study was carried out to determine the direct effects of urea on the survival and developmental rates of *Cx, p. pipiens* under laboratory conditions.

MATERIALS AND METHODS

Source of mosquitoes and urea fertilizer: The *Cx. p. pipiens* mosquitoes used in this study were obtained from a colony maintained in the Laboratory of the Department of Biological Sciences, Federal University of Technology, Minna, Nigeria. The urea fertilizer was sourced from the Operational Office of Niger state Agricultural Development Project (NADP), Minna, Nigeria.

Mosquito handling and exposure to urea fertilizer: One hundred day-old larvae of *Cx. p. pipiens* were transferred to larval rearing-bowls (30×25 cm) containing 2.50, 5.00, 7.50, 10.00, 12.50 and 15.00 g of the urea fertilizer in 1000 mL deionized water. These doses, though much higher than the 50 kg acre^{-1} recommended (Muturi *et al*., 2007), represented the range of urea fertilizer concentration commonly applied by rice farmers in the study area (Ishiaku, pers. com.). A control experiment was set-up also containing 100 larvae in 1000 mL deionized water but without the addition of urea fertilizer. Each experimental treatment had two replicates and the whole experiment was repeated within one week of the termination of the first experiment; thus, resulting in the monitoring of 400 larvae per treatment including the control.

The larvae were fed with pulverized fish feed (Coppens®) till the 4th larval stage and the larval rearing-water was replaced every other day, with fresh preparations of urea-treated deionized water and feed, to ensure that the larvae remained exposed continuously to the intended urea concentration and to prevent scum formation in larval rearing water (Olayemi and Ande, 2008a). The experiments were monitored at 12 h intervals (i.e., 06:00, 18:00 h), when the number of dead and ecdysed larvae were counted and recorded. While, larval death was confirmed by lack of response to prodding with a glass rod, number moulted was equated with number of cast skin (i.e., exuviae) on water surface.

Data analysis: Mortality rates and duration of the life stages were determined according to the methods of Olayemi and Ande (2009). Survival rates were calculated using the formula:

$$S_i = \frac{n_i}{X_{ni-1}} \times 100$$

Where:

S_i = Survival rate of life stage
n_i = No. of individuals entering a life stage
X_{ni-1} = No. of individuals that entered the preceding life stage

For estimation of duration of life stages, the following formula was used:

$$D_i = T_i-(t_{i-1})$$

Where:

D_i = Duration of life stage
T_i = Present mean age
t_{i-1} = Previous mean age at moulting

Daily survival rates of the mosquitoes were determined by calculating the proportion of individuals alive, post-emergence, on a daily basis.

Mean survivorship and developmental data obtained were compared for statistical significance using the Chi-square test, at $p = 0.05$. The relationships between the variables investigated were determined using linear correlation coefficient.

RESULTS

Immature life stage survival rates: The effects of increasing concentrations of urea fertilizer on survivorship of the immature *Cx. p. pipiens* mosquitoes are highlighted in Table 1. Generally, dose-dependent mortality of the larvae was observed and at the highest concentrations of urea tested (i.e., 12.50 and 15.0 g L^{-1}), adult emergence of the mosquitoes was completely inhibited. Survival rates of the larvae among the urea concentrations varied significantly ($p<0.05$) in all the life stages. Total Immature Survival (TIS) reduced significantly from 90.38±6.52% in the control mosquitoes to 65.55±28.92% among those reared in 5 g L^{-1} urea-water. A similar trend of significant reduction in survival rate was observed during the pupal life stage (i.e., from 98.91±2.12% in control to 70.46±47.74% in the group of mosquitoes exposed to 7.5 g L^{-1} urea water). However, a significant ($p<0.05$) increase in pupal survival (96.43±7.14%), was recorded in the group of mosquitoes reared in the next higher concentration of urea (i.e., 10 g L^{-1}).

Mean larval survival ranged from 70.09±1.02% in the control larvae to 43.45±1.01% in the mosquitoes exposed to 15 g L^{-1} urea-water. However, there was no significant difference ($p>0.05$) between the survival rate of larvae not exposed to urea at all (i.e., control) and those reared in 2.5 g L^{-1} urea-water, as well as, between larvae treated with 12.50 and 15.00 g L^{-1} urea-water. For all concentrations, mean larval survival were significantly lower than those of pupal. For larval instars, there was a general significant reduction in survival from L1-L4, even among the control group of larvae, with the exception of the L4 larvae in treatments 2.50 and 5.00 g L^{-1} and L3 in 10.00 g L^{-1} urea-water. With the exception of the 12.50 g L^{-1} treatment, there was no significant difference ($p>0.05$) between L1 larval survival rate of the treatments and the control. Survival of the L2 larvae ranged from 80.33±8.94% in the control larvae to 23.31±4.36% in those raised in 10.00 g L^{-1} urea-water and there was no significant difference in survival between the control and 2.50 g L^{-1} urea-treatment groups of mosquitoes during this larval stage. The survivorship of the L3 larvae showed alternating significant decrease and increase with increasing urea concentration. On the other hand, survival of the L4 instar stage was significantly ($p<0.05$) higher in 2.50 and 5.00 g L^{-1} urea-water treatments (52.56±6.21 and 73.04±22.72%, respectively) than control (40.95±4.52%).

Duration of immature life stage development: Table 2 presents the influence of increasing urea concentration on the duration of immature life stages of *Cx. p. pipiens*

Table 1: Mean survival rate (%) of life stages of immature *Culex pipiens pipiens* mosquitoes, in response to increasing concentration of urea fertilizer

	Larval instar						
Urea treatment (g L^{-1})	L1	L2	L3	L4	Larval stage	Pupal stage	Total immature stage
0.00 (Control)	99.25±0.95^{b}*	80.33±8.94^{d}	59.85±9.76^{d}	40.95±4.52^{c}	70.09±1.02^{d}	98.91±2.17^{c}	90.38±6.52^{c}
2.50	98.00±2.16^{b}	81.89±2.22^{d}	32.45±4.74^{b}	52.56±6.21^{d}	66.22±0.49^{d}	91.16±10.38^{b}	84.21±1.80^{b}
5.00	91.00±0.00^{b}	28.36±10.48^{a}	43.45±13.14^{c}	73.04±22.72^{e}	58.96±2.33^{c}	73.96±44.14^{a}	65.55±28.92^{a}
7.50	91.17±7.04^{b}	48.17±7.19^{b}	43.25±6.94^{c}	25.05±19.21^{b}	51.91±1.51^{b}	70.46±47.74^{a}	66.15±31.53^{a}
10.00	97.50±0.70^{b}	23.31±4.36^{a}	44.83±20.10^{c}	29.68±16.00^{b}	48.83±1.30^{b}	96.43±7.14^{bc}	68.90±4.10^{a}
12.50	72.67±22.34^{a}	40.83±8.38^{b}	36.34±22.47^{b}	29.17±34.36^{b}	44.75±2.65^{a}	-	-
15.00	99.25±0.95^{b}	62.17±9.14^{c}	8.24±2.67^{a}	4.17±8.33^{a}	43.45±1.01^{a}	-	-

Values followed by same superscript alphabets in a column are not significantly different at $p = 0.05$

Table 2: Mean duration (days) of life stages of immature *Culex pipiens pipiens* mosquitoes, in response to increasing concentration of urea fertilizer

	Larval instar						
Urea treatment (g L^{-1})	L1	L2	L3	L4	Larval stage	Pupal stage	Total immature stage
0.00 (Control)	1.78±0.52^{ab}	1.20±0.40^{a}	2.51±0.39^{a}	4.57±0.73^{b}	10.07±1.59^{a}	1.57±0.54^{a}	11.57±0.00^{a}
2.50	1.25±0.28^{a}	1.54±0.14^{a}	3.12±0.12^{a}	4.94±0.44^{b}	10.86±0.21^{a}	3.34±0.47^{c}	14.20±0.65^{b}
5.00	2.00±0.01^{b}	3.50±0.23^{c}	4.16±0.50^{b}	2.90±0.58^{a}	12.58±1.37^{b}	2.45±0.94^{b}	15.00±2.24^{b}
7.50	2.24±0.26^{b}	1.84±0.60^{a}	4.38±0.35^{b}	4.83±3.24^{b}	13.30±3.64^{b}	2.05±1.62^{ab}	15.35±5.14^{b}
10.00	2.00±0.00^{b}	4.15±0.41^{d}	5.28±0.80^{c}	4.43±0.65^{b}	14.87±1.06^{c}	1.56±0.51^{a}	17.44±0.94^{c}
12.50	2.20±0.23^{b}	2.48±0.60^{b}	5.29±0.30^{c}	-	-	-	-
15.00	1.25±0.28^{a}	1.71±0.11^{a}	5.91±0.14^{c}	-	-	-	-

Values followed by same superscript alphabets in a column are not significantly different at $p = 0.05$

mosquitoes. The duration of Total Immature Development (TID) was significantly ($p<0.05$) extended by urea. While, the mosquitoes not exposed to urea (i.e., control) completed immature development in 11.57±0.00 days, the duration of similar development was extended by about 6 days among the mosquitoes reared in 10.00 g L^{-1} urea-water (i.e., 17.44±0.94 days), the maximum concentration at which immature development was fully completed. However, TID of the mosquitoes exposed to concentrations of urea less than 10.00 g L^{-1} were not significantly ($p<0.05$) different (range = 14.20±0.65 to 15.35±5.14 days). On the other hand, pupal stage duration was significantly extended from 1.57±0.54 days in the control group of mosquitoes to 3.34±0.47 and 2.45±0.94 days in 2.50 and 5.00 g L^{-1} treatments, respectively but not in higher concentrations. The duration of individual larval instar development, as well as, total larval development were not significantly different between the control larvae and those of 2.50 g L^{-1} urea-water treatment. Total Larval Stage duration ranged significantly from 10.07±1.59 days in the control to 14.87±1.06 days in 10.00 g L^{-1} urea-water treatment.

Generally, the duration of larval instar varied significantly among the treatments, with larval development terminating at L3 in the 12.50 and 15.00 g L^{-1} urea-water treatments. For treatments 5.00-12.50 g L^{-1} urea-water, the duration of the L1 larval instars (range = 2.00±0.00 to 2.24±0.26 days), did not follow the usual dose-dependent effects of urea that is characteristic of the results of this study. The duration of the L2 instars ranged from 1.20±0.40 days in control to 4.15±0.41 days in treatment 10.00 g L^{-1} urea-water. It is noteworthy that the duration of L1 and L2 instars in the highest concentration of urea tested were not significantly different from those of the control larvae. For all treatments, duration of the L3 larval instar was relatively long (range = 2.51±0.39 days in the control to 5.91±0.14 days in the highest treatment concentration). However, duration of L3 in the last three highest concentrations were not significantly different ($p>0.05$). The duration of the L4 instars was significantly reduced in 5.00 g L^{-1} urea-water treatment (2.90±0.58 days) than the control group (4.57±0.73 days) which in turn was not significantly different from those of other treatments that suppressed L4 larval development.

Correlation between urea concentration and larval development: Table 3 highlights correlations between increasing urea concentration and immature life stage developmental variables. For total larval stage and total immature stage, increasing concentrations of urea correlated significantly with both survival rate (r = -0.9818 and r = -0.8412, respectively) and duration of development

Table 3: Correlation between increasing urea fertilizer concentration and developmental variables of immature *Culex pipiens pipiens* mosquitoes

Immature life stage	Survival rate	Duration of development
L1	-0.3580	0.0575
L2	-0.4648	0.2854
L3	-0.7109*	0.9804*
L4	-0.7048*	-0.0746
Larval	-0.9818*	0.9923*
Pupal	-0.3093	-0.2799
Total Immature	-0.8412*	0.9590*

*Significant correlation

(r = 0.9923 and r = 0.9590, respectively). However, for both variables, the pupal stage was not significantly impacted by urea concentration (r = -0.3093 and r = -0.2799, respectively). Significant correlations in the larval instars were limited to the survival of L3 (r = 0.7109) and L4 (r = 0.7048), as well as, duration of development of L3 (r = 0.9804) instars.

DISCUSSION

The results of this study indicated dose-dependent effects of urea on mortality rate of *Culex pipiens pipiens* mosquitoes. This finding suggests that, directly, urea may be toxic to the mosquito. Earlier, Tchounwou *et al*. (1991), reported similar high toxicity of inorganic fertilizers to *Culex* mosquitoes under laboratory conditions. Yet, in the field, applications of inorganic fertilizers tend to promote mosquito production (Victor and Reuben, 2000; Mutero *et al*., 2004). It, therefore, seems that at low concentrations, perhaps at recommended doses for rice-farming, urea may not be directly toxic to mosquito larvae but, rather, enhances the growth of phyto-plankton on which the larvae feed, as well as, adjust the physico-chemical properties of rice-field mosquito larval habitats to the optimal range for mosquito immature survival and developmental rates; thus, promoting rapid mosquito population development. Therefore, since the concentrations of urea tested in this study (range = 2.50-15.00 g L^{-1}), proved toxic to the survival of *Cx. p. pipiens* larvae but were within the range applied by rice farmers in Minna, it may mean that rice-fields in the area pose no serious threat to human health, with respect to mosquito production. This assertion is further buttressed by the fact that recent investigation of productivity of mosquito larval habitats in Minna, ranked swamps (i.e., rice-fields) after drains and ponds in terms of mosquito larval density (Olayemi *et al*., 2010, 2012).

For all urea concentrations tested, mean larval survival rates were significantly lower than those of pupal. This result indicates differential susceptibility of the mosquito immature stages to the toxicity of urea. According to Yoshiaki *et al*. (1997) and Paulraj *et al*.

(2011), the younger life stages (i.e., larval) of mosquito species are more vulnerable to chemical insecticides than the older ones (i.e., pupal). Therefore, timing urea-fertilizer application in rice-fields, to coincide with when the immature mosquito populations are mostly in their larval stage, may go a long way in reducing mosquito production from such habitats.

Unlike the older larval instars (i.e., L2-L4), there was more-or-less no significant difference in the survival rates of the L1 instar among the treatments. This observation may be partially explained by the feeding behavior of mosquito larvae. Newly hatched mosquito larvae (i.e., L1) feed much less than the subsequent instar (i.e., L2-L4), as they depend more on nutrients inherited from the egg yolk and accumulation of teneral reserve takes place mainly during the L3 and L4 instar stages, when more than 90% of growth and biosynthesis take place (Timmermann and Briegel, 1996; Briegel, 2003). Being atmospheric air-breathers (Meyer, 2006) and coupled with the high impermeability characteristic of the integument of mosquito larvae (Bush *et al*., 2007), toxic substances may gain access in to their systems usually via ingestion. Therefore, it is possible that the L1 larvae were not sufficiently exposed to the urea in their environment (via. oral ingestion with food) to induce significant mortality. Also, the significant higher mortalities recorded in the L2-L4 larval instars may be due to bio-accumulation of the urea in the tissues of the larvae. According to Camargo and Alonso (2006), aquatic chemical pollutants including, inorganic fertilizers tend to accumulate in the tissues of aquatic fauna, with intensity of feeding.

The influence of urea on the duration of immature stages of *Cx. p. pipiens* mosquitoes was also counter productive and thus, confirms the toxicity of urea concentrations tested to the mosquito larvae. At high concentrations, Nitrogen-based inorganic fertilizers may interrupt nervous transmission which may be reflected as high mortality rate and prolonged larval development duration (Klowden, 2002), as observed in this study. For example, the total immature duration was significantly extended by as much as 6 days in the 10.00 g L^{-1} urea-water treatment. This result suggests that the vectorial capacity of the *Cx. p. pipiens* mosquitoes that successfully complete development and emerged from the urea-water treatments may be significantly reduced. Prolonged immature development reduces adult longevity of mosquitoes and hence, reduces the chance to take first blood meal (i.e., become infected with parasites), support parasite embryogeny and number of subsequent infectious bites (Olayemi and Ande, 2008b).

For all treatments including the control, duration of the L3 larval instar was relatively long. Usually, in mosquitoes, serious accumulation of teneral reserves for egg development in the adult stage, commences in the L3 larval instar (Briegel, 1990a, b, 2003) and may justify its relatively long duration. The dose-dependent effects of urea concentration on the survival and developmental rates of the mosquitoes were further confirmed by strong correlations between increasing urea concentrations and the developmental variables investigated. However, contrary to the expected concentration dose-dependent effects of urea on the survival and development rates of the mosquitoes, isolated cases of higher mortality and longer development rates in lower urea concentration treatments than higher ones were recorded in this study. For example, there was no significant difference between survival rates of the larvae in the 12.50 and 15.00 g L^{-1} urea-water treatments. Specifically, the L4 larval instars survived much better in urea-water treatments than the control experiment. Similarly, the duration of the development of the L1 and L2 larval instars in the highest urea concentration was not significantly different from those of the control. These results are similar to those obtained for *Cx. quinquefasciatus* exposed to some inorganic fertilizers also under laboratory conditions and such disruption in dose-dependent trend of the effects of fertilizers on survival and developmental rates of the mosquito larvae was explained as due to, among other factors, the probable acquisition of ability to compensate for damages caused by higher concentrations of the fertilizer (Muturi *et al*., 2007). Interestingly, the survival rates of the pupae were significantly higher than those of the larvae. This finding may be due to the non-feeding habit of the pupal stage and therefore, are not likely to continue the ingestion of urea-treated water that may further induce mortality. This submission, therefore, confirms the earlier suspicion that the toxicity of urea to the larvae noticed from the second larval instar stage might have been acquired orally.

The results of correlation analyses between the immature developmental indices investigated (namely, survival rate and duration of development) and increasing concentration of urea, though significant, indicated negative association for survivorship but positive for developmental duration. This finding establishes the fact that urea-fertilizer application in rice-farming influences *Cx. p. pipiens* mosquito productivity, by regulating the dynamics of survivorship and developmental rates of the larval stage, while having insignificant effects on the pupae.

CONCLUSION

Under laboratory conditions, the concentrations of urea investigated had a limiting effect on the survival of *Cx. p. pipiens* mosquito larvae. This finding contradicts the positive influence of urea on population development of rice-field breeding mosquitoes in the wild. It may, therefore, be concluded that the rice-fields in Minna may not be very productive in terms of mosquito population development thus, posing less threat to human health in the area. It is, therefore, necessary to investigate the effects of the concentrations of urea tested in this study on phytoplankton proliferation, as well as, water quality to ascertain suspected tilt in favour of mosquito larval development. Also the duration of immature stages of the mosquitoes was significantly extended by urea; with the epidemiological implication of considerably reducing adult life-span of the mosquitoes and hence, their vectorial capacity. It is hoped that the findings of this study will facilitate the development and integration of fertilizer application best-practices in to rice-farming activities, in a manner that will ensure sustainable increased rice production without significant enhancement of mosquito population development in rice fields.

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