Egyptian Journal of Biological Pest Control, 18(2), 2008, 347-351

Side Effects of Azadirachtin Fed Preys on the Diving Predator, *Eretes sticticus* Linnaeus (Coleoptera: Dytiscidae)

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(*Received: October 25 and Accepted*: *November 11, 2008*)

ABSTRACT

The effects of the indirect exposure of predators to neem active ingredient, azadirachtin were tested by feeding larvae and adults of *Eretes sticticus* Linnaeus on azadirachtin.-fed fourth-instar larvae of *Culex pipiens*. Also, searching behavior of the adult predator when contacted with residue from contaminated surfaces was considered. Firstly, susceptibility of the fourth instar mosquito larvae to azadirachtin was determined in laboratory bioassays. Lethal doses of 0.045, 0.06 and 0.25 ppm, were obtained representing LC_{25}, LC_{50} and LC_{90}, respectively. The experiments showed that, 0.045 ppm azadirachtin treatments had no significant effects on the predator *E. sticticus*. Both azadirachtin treatments 0.06 and 0.25 ppm had negative effects on the immature survival, development and consumption. Reductions in the percentage survival and consumption with prolonged in development of the immature instars were obtained. Also, the predator adults' fed-preys in contaminated surfaces significantly negatively affected their searching activity and consumption rates. The results suggest that, although the bio-substance, azadirachtin, has been though useful in biological control, assuming that the indirect effects of this compound are not enhanced by any direct exposure of the natural enemies to the chemical in the field. Application of this bio-substance still should be careful, especially in high concentrations, if persistent natural enemy populations are required. The rate of azadirachtin application needs to be adjusted because consumption of the compound by the preys resulted in negative effects on their predators.

Key Words: Azadirachtin, Biopesticide, Aquatic insect, *Eretes sticticus*, *Culex pipiens*, Biocontrol.

INTRODUCTION

In recent years, the use of environment-friendly and easily biodegradable natural insecticides of plant origin has received much attention for control of medically important arthropods. Mosquitoes transmit a wide range of human and animal pathogens; management of these pests using synthetic chemicals has failed because of insecticide resistance and environmental pollution (Scott, 1999; Hemingway *et al*., 2004 and Boyer *et al*., 2006). Consequently, intensive efforts have been made to find alternative methods of control (Pates and Curtis, 2005). Plant-derived materials are usually safer and more ecologically acceptable. They must be tested, however to judge there efficacy against the target hosts. Therefore, application of easily degradable botanicals for the control of mosquitoes is recommendable.

Compounds derived from the neem tree, *Azadirachta indica* A. Juss (Meliaceae), have been shown to be effective against a broad range of insect pests (Schmutterer, 1990 and 1995). Due to their relative selectivity, neem products recommended for many pest management (IPM) programs (Morgan, 2004). Bioactive compounds in the neem kernel extracts showed considerable promises for control of culicine mosquitoes (Rao *et al.*, 1995 and Senthil Nathan *et al*., 2005). Azadirachtin, a tetranortriter-penoid compound is considered the most important active principles contained in neem kernels (Mordue and Blackwell, 1993). Biological control agents for mosquito larvae include the predaceous aquatic insects. The predaceous water beetles (Fam.: Dytiscidae) are of the largest and most commonly encountered groups of aquatic predatory beetles (Larson, 1985). Dytiscids generally prefer slow moving or stagnant water, such as ponds, lakes, dams and pools at the edges of streams. *Eretes sticticus* Linnaeus (Coleoptera – Dytiscidae) is an active diving beetle in searching and catching mosquito larvae where both immature and adults are predators of mosquito larvae. Several studies have investigated the potential for adverse effects of neem-based insecticides on aquatic arthropods (Kreutzweiser, 1997; Dunkel and Richards, 1998; Kreutzweiser *et al.* 2000 and Scott and Kaushik, 2000). As part of a program to investigate the usefulness and environmental effects of neem-based insecticide, azadirachtin, on the control of *Culex pipiens* larvae, determination of the potential of adverse effects on the predator, *E. sticticus* was investigated.

The work reported here evaluates the % mortality of azadirachtin-treated fourth instar larvae of *C. pipiens.* Further, survivorship, developmental duration and consumption rate of immature *E. sticticus* after being fed on treated-larvae were studied. Consumption rate and searching activity of the predatory adults towards the treated *C. pipiens*

larvae were also determined.

MATERIALS AND METHODES

Insects

A laboratory strain of *C. pipiens* was reared in standard insectary conditions (24 ± 2°C, 12 h/12 h light/dark period, tap water) and used for all experiments. Larvae were reared in the laboratory in 1-liter plastic containers, where mixture of Tetramin® and yeast was added to each container as larval food. Water in the containers was replaced every 7-days. Adults of the dytiscid beetle, *E. sticticus* were kept in 1- m^3 tanks filled with tap water and provided with mosquito larvae as preys. The predator larvae were individually reared in 2-liter plastic cups filled with distilled water, where they were provided with 10 – 20 *C. pipiens* larvae every 2 or 3 days.

Bioassay

Newly molted fourth instar larvae of *C. pipiens* were used for the bioassay tests. The commercial neem-based bio-insecticide, azadirachtin, was purchased from Tween 80, Sigma, USA. Prey larvae were pre-exposed to non-lethal doses of azadirachin to evaluate its short-term impact on *Culex* larvae. Homogenous biological samples (including 100 same-sized fourth instar *Culex* larvae with standard Tetramin and yeast) were exposed for 24 h to different doses of azadirachtin to estimate the lethal mortality. Disposable vials, each contained 20 equal-sized fourth-instar larvae in 100-ml aqueous medium infused with various concentrations of azadirachtin were used. One hundred unexposed mosquito larvae were used as control. For each sample analyzed, bioassays were performed in five replicates. Mortality was recorded after 24 h and corrected with Abbott's formula (Abbott, 1925). Percentage mortality was analyzed using probit analysis for lethal dose determination according to Finney (1971).

Indirect Effect of neem formulation, azedirachtin on the larval stages of *Eretes sticticus*

Experiments were conducted to determine whether the treated *C. pipiens* larvae have an effect on the survivor, developmental duration and consumption rate of individuals of different three larval instars of *E. sticticus*. The treatments consisted of predator's larvae that fed on mosquito larvae post-treated with different concentrations of azadirachtin (LC_{25}, LC_{50} and LC_{90}). Newly hatched predator larvae (1^{st} instar) were individually provided with 24 h post-treated mosquito larvae. The predator larvae were checked daily and the biological parameters were recorded. Once predator larvae had reached the following instar, new treated mosquito larvae and so a new predator larval instar was tested. Twenty treated mosquito larvae/day were used with azadirachtin concentration LC_{25}, and LC_{50} while 100 prey larvae/day were used with the high concentration (LC_{90}). Each experiment was replicated twice over time using 10 *E. stacticus* larvae/instar for each prey-treatment.

Effect of prey-treatment on the adult predator

Adults of the predatory beetle from the stock colony were placed individually in glass bucket of 10-liter capacity, filled with distilled water. To determine if neem formulation, azadirachtin affected their searching activity test solutions of lethal and sublethal concentrations (LC_{25}, LC_{50} and LC_{90}) for mosquito preys were prepared in glass buckets of 10-liter capacity. Twenty mosquito larvae (4^{th} instar) were released in treated water, contained Tetramin® and yeast as food for the larvae. Predator adults from the stock culture were introduced individually into the glass buckets after 24 h of treatment. Both mosquito larvae and adult predators were tested only once. Predator searching time (latency to capture preys, handling time (the time from capture to end of feeding) were recorded. Searching time was measured as the time elapsed between introduction of prey and successful capture. Comparisons between treatments and control for the adult predator activity were made. Percentage of consumed preys was also recorded. The experiment was conducted five times for each treatment. Control treatments consisted of distilled water mixed with Tetramin® and yeast, mosquito larvae and predator adults were used only once. All experiments were carried out at room temperature of 24 ± 2°C, 75 ± 10 % R.H. and a photoperiod of 12 : 12 (L : D).

Data analysis

Immature predator developmental periods, survival rate, consumption rate with the adult consumption and searching activity were analyzed using one-way analysis of variance (ANOVA), and means separated by Duncan's multiple range test when F-value was significant (SAS institute, 2001).

RESULTS AND DISCUSSION

Neem-formulation (azadirachtin) dosage mortality

Toxicity of azadirachtin towards 4^{th} instar larvae of *C. pipiens* is shown in table (1). The effect on mosquito larval mortality was concentration

Table (1): Dosage mortality of neem formulation (active ingredients), azadirachtin, toward fourth-instar *Culex pipiens* larvae.

Lethal Conc.	Azadirachtin Conc. (ppm)	Slope	95 % confidence limits
LC_{25}	0.045	3.41	0.036 – 0.051
LC_{50}	0.060	2.73	0.054 – 0.069
LC_{90}	0.250	2.20	0.098 – 0.350

Table (2): Developmental duration of the larval instars of *Eretes sticticus* after feeding azadirachtin-treated fourth instar of *Culex pipiens* larvae.

Treatment	Developmental periods (mean ± SE) days			
	1st	2nd	3rd	Total
LC_{25}	6±0.3^{ab}	7.7±0.42^{ab}	10.4±0.6a^{b}	24.1±0.5^{a}
LC_{50}	6.6±0.2b	8.3±0.2^{8b}	12.3±0.5^{b}	27.2±0.5^{b}
LC_{90}	6.9± 0.3^{b}	9±0.31 b	12.6±0.6^{b}	28.5±0.37^{b}
Control	5.3±0.4a	6.8±0.4 ^{a}	9.7±0.18^{a}	21.8±0.6^{a}
F	4.492	6.355	8.135	38.53
P-value	0.012	0.0025	0.0007	<0.0001

- Means followed by the same letter, in the same column, are not significantly different (P>0.05).

Table (3): Survival rate (%) of larval instars of *Eretes sticticus* fed on azadirachtin-treated fourth instar of *culex pipiens* larvae.

Lethal Conc.	Predator larval instar		
	1st	2nd	3rd
LC_{25}	82.6 ±1.3ab	85.6 ± 0.5a	85.8 ± 0.6a
LC_{50}	72 ± 0.86b	75.7 ± 0.6b	77 ± 1.1b
LC_{90}	60 ± 1.6b	63 ± 0.8c	68 ± 1.6c
Control	86.2 ± 1.1	88 ± 0.7a	89 ± 0.8a
F	82.33	289.1	72.93
P-value	<0.0001	<0.0001	<0.0001

- Means followed by the same letter, in the same column, are not significantly different (P>0.05).

Table (4): Effect of azadirachtin-treated fourth instar larvae of *Culex pipiens* on the consumption rate of the predator, *Eretes sticticus* stages.

Treatment	Mean no. of consumed preys (±SE)			
	Larvae (instars)			Adult
	1st	2nd	3rd	
LC_{25}	4.2±0.42^{ab}	5.8±0.26^{ab}	8.8±0.51^{ab}	8.4±0.64^{ab}
LC_{50}	3.8±0.18^{b}	5.4±0.53^{b}	7.8±0.5^{b}	7.6±0.42^{bc}
LC_{90}	3.2±0.36^{b}	4.6±0.65^{b}	7.2 ± 0.4^{b}	6.8 ± 0.51^{c}
Control	5.2±0.42^{a}	6.4±0.37^{a}	10.2±0.64^{a}	10.1±0.51^{a}
F	5.789	6.887	8.381	6.976
P-value	0.004	0.0017	0.0005	0.0015

- Means followed by the same letter, in the same column, are not significantly different (P>0.05).

dependent. When the larvae were treated by neem-formulation, azadirachtin, they exhibited slower movement in the water. Probit analysis indicated that percent mortality at 0.045 ppm was 25% and it was further increased to 50 % at 0.06 ppm. On the other hand, 90 % mortality was estimated at 0.25 ppm concentration.

Indirect effects of neem extract, azadirachtin:

1- Immature predator

The effects of different lethal concentrations (LC_{25}, LC_{50} and LC_{90}) of neem extract on the developmental period of 1st, 2nd and 3rd larval instar of *E. sticticus* are shown in table (2). Obtained data showed that, sub-lethal concentration (LC_{25}) had no significant effect on the developmental periods as compared with the control ones. On the other hand, significant differences were obtained considering all lethal concentrations (F = 4.492, P = 0.012 for first instar), (F= 6.355, P= 0.0025 for second instar) and (F= 8.135, P= 0.0007 for third instar). The total developmental periods of the larval stage were 1.1, 1.25 and 1.31 times longer for preys treated with LC_{25}, LC_{50} and LC_{90}, respectively, in comparison with non-treated preys (control).

In addition, neem formulation was observed to have a significant effect on the percentage survivorship for each of the predator larval instars (Table 3). The percentage of predator survival was dependent on the neem active ingredient (azadirachtin) concentration-treated preys. Data indicated that, increasing the prey-lethal concentration from LC_{25} to LC_{50} declined the percentage survivor of the first instar of the predator from 79.6 to 86.2%. Also, decreasing in the percentage survivor of the second and third instars of the predator was obtained with increasing the prey-lethal concentration. Data in table (3) also shows that, survivor of the predator larvae in the early instars was lower than that in the older instars.

2- Consumption rate of the predator

The mean number of preys consumed by the larvae and adults of *E. sticticus* are presented in table (4). In general, surviving *E. sticticus* consumed fewer azadirachtin-treated preys than those offered untreated ones. The mean number of preys for each immature and adult individual predator varied significantly depending on the neem-fed prey concentration. Preys exposed to lower concentration (LC_{50}) consumed proportionately more than those treated with high concentration (LC_{90}). Statistically, significant differences (P<0.05) were obtained in comparison of the different treated-preys consumed and those of non-treated consumed by both

Table (5): Effect of azadirachtin- treated fourth instar *Culex pipiens* larvae and contaminated surfaces on the searching activity of the adults of the predator *Eretes sticticus.*

Predator activity	Treatment					
	LC_{25}	LC_{50}	LC_{90}	Control	F	P-value
Searching time (min.)	26.8 ± 1.2a	22±0.9[b]	19.3±0.7[b]	29±1.2[a]	19.73	<0.0001
Handling time (min.)	3.9±0.28[ac]	4.6±0.37[c]	5.2±0.5[b]	3.6±0.4[a]	3.583	0.028

- Means followed by the same letter, in the same raw, are not significantly different (P>0.05).

immature and adult predator (Table 4).

3- Adult predator searching activity

Adult *E. sticticus* showed differences in searching and handling times towards neem-treated and non-treated preys (Table 5). Obtained data showed that, adult predator required less time in searching treated preys. The searching time also differed according to the lethal neem concentration used in the prey treatments. Preys treated with high lethal concentration showed less activity and slow moving than those treated with sub-lethal concentration or those of non-treated. The minimum searching time of the adult predator was obtained with preys treated by LC_{90} neem-extract. On the other hand, the predator handling time towards treated preys was longer than that obtained towards non-treated preys. Statistically, significant differences ($P<0.05$) were obtained considering searching activity and handling times of the predator towards neem-treated and non treated preys (Table 5).

Biocontrol agents comprise an important element of many integrated pest management (IPM) programs, but many synthetic pesticides affect them negatively (DeBach and Rosen, 1991 and Mordue and Blackwell, 1993). Biopesiticides provide an alternative to synthetic pesticides; the neem-based biopesticides has attracted considerable attention from IPM researchers and practitioners (Schmutterer, 1997). Present pesticide testing procedures are designed to test two routes of exposure of an organism, contact with residue from contaminated surfaces and consumption of contaminated preys.

Exposure of fourth instar *C. pipiens* larvae to sub-lethal dose (LC_{25}) of the azadirachtin had no negative effect on the survival, development and even consumption rate of the immature predator. On the other hand, high concentrations tested (LC_{50} and LC_{90}) of azadirachtin, had negative effects on the immature and adult predator individuals. Decreases in the percentage survival and in the consumption rate with an increase in the developmental period of the immature *E. sticticus* were obtained after being fed on treated preys. For adult predator, although a decrease in the searching time was obtained, an increase in the handling time was recorded. These may be a result of the slow motion of the treated preys and or of their unfavorable taste.

Toxicity of azadirachtin and various neem extracts against insect pests has been widely demonstrated, however, several reports indicate also variable susceptibility of parasitoids and predators, although in most cases they are comfortingly less susceptible than their hosts and preys (Schmutterer, 1997). Also, nymphs and larvae of some natural enemies are more susceptible to direct contact with azadirachtin under laboratory conditions (Mordue and Blackwell, 1993) which may signify certain limitations to the use of neem in IPM. Considering the aquatic environments, Kreutzweiser *et al.* (2002) obtained a significant treatment effect of azadirachtin on the natural zooplankton communities. Other studies have shown that formulation contains active ingredients of neem-based insecticides can contribute substantially to effects on aquatic invertebrates (Dunkel and Richard, 1998). According to Helson *et al.* (1999), azadirachtin typically expresses growth regulating of antifeedant effects on target insects and this may have induced early mortality of target insects at higher concentrations. In the present study, the delayed reductions in the development of the *E. sticticus* larvae may be indicative of growth-regulating effects of azadirachtin, while the more acute response (mortality) may reflect toxicity of formulation ingredients or other neem constituents.

Further work is required to establish specific application rates at which behavioral responses begin to occur and the relative effects of increased survival and decreased foraging efficancy on mosquito population depression under field conditions where the presence of alternative preys may further affect the predatory ability.

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