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Neem Seed Extract Containing Azadirachtin Affects Mortality, Growth, and Immunological Function in the Whipscorpion *Mastigoproctus giganteus* (Lucas) (Arachnida, Uropygi)

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Azadirachtin, a triterpenoid of the neem tree, *Azadirachta indica* A. Juss. (Meliaceae), has potent antifeedant and growth disrupting effects on insects (Mordue and Blackwell, 1993). Neem extracts containing azadirachtin and other related triterpene limonoids have been used with a great deal of success in the control of many phytophagous insects and mosquitos. Although there are numerous studies on the effects of azadirachtin on the physiology of molting and feeding behavior of economically injurious insects (see reviews by Schmutterer, 1990; Mordue and Blackwell, 1993), there is little information available on the effects of this compound on predatory insects and arachnids. Mansour et al. (1986) showed a relatively low toxicity of neem seed kernel extract when applied topically to the spider, *Chiracanthium mildei*. However, in their study, spiders were exposed to neem extracts through bodily contact with the treated substrate. No attempt was made to assess the oral toxicity of azadirachtin on these spiders.

In view of the important role that arachnids can play in the control of insect pests in agroecosystems (Riechert and Lockley, 1984), the present study was undertaken in order to evaluate the effects of azadirachtin on the whipscorpion *Mastigoproctus giganteus* (Lucas) (Uropygi, Thelyphonidae), a large arachnid predator commonly found in arid areas and agroecosystems in the southwestern United States and Florida (Punzo and Reeves, 2001). The average adult body length ranges from 38-47 mm. These arachnids are cursorial predators which actively pursue or ambush their prey. They are generalist predators and feed on a wide variety of arthropod prey (Punzo, 2000).

In this study I assessed the effects of neem seed extract on mortality, growth and immunological function in *M. giganteus*.

MATERIALS AND METHODS

No data exist on effects of azadirachtin on uropygids. I studied the effects of neem seed extract containing azadirachtin on their growth, development, survivorship, and immunological competency.

Immunological parameters were investigated because any impairment of immunological function will decrease the fitness of an organism even though their overall adverse effects on survival may take a considerable period of time to manifest themselves. Studies that emphasize survivorship and population dynamics may suggest that a population is thriving while the more subtle physiological effects of a toxic compound go undetected. Arthropods respond to the invasion of micro-organisms and parasites by a variety of immunological reactions, including phagocytosis by selected hemocytes, nodule formation (encapsulation), and humoral mechanisms (Ratcliffe and Rowley, 1979).

Commercially available neem seed extract containing azadirachtin (Neemix 4.5, 4.5% azadirachtin) was obtained from W.R. Grace and Co. (Columbia, Maryland).

Adult females (N = 47) containing first-instar protonymphs (N = 423) were collected in Dade Co., Florida, during March and April, 2003, and brought back to the laboratory. Each female and its progeny was housed separately in an aerated plastic container, and maintained in a Percival Model 80A environmental chamber (22°C, 65% RH, 10L : 14D photoperiod regime). Upon hatching, the young climb onto dorsum of the female's abdomen and remain their first molt, after which they climb off and begin to disperse. Upon dispersal, the young (protonymphs) were collected and housed individually in plastic containers under the same environmental conditions described above. These adults and protonymphs provided the subjects used in all subsequent experiments.

Whipscorpions were allowed to feed on prey that were injected with varying concentrations of neem seed extract (four treatment groups) immediately before they were presented to a whipscorpion. Injections were made on CO_2- anaesthetized prey using a 10-ul Hamilton syringe. Seed extract was dissolved in acetone prior to injection. Extract (2.0 µl) was injected into each animal through the intersegmental membrane immediately posterior to the cephalothorax. The concentrations of extract containing azadirachtin used in these experiments were: 0 (controls), 0.1, 1.0, and 10 mg/l as described by Barnby and Klocke (1987). These dosages represent levels commonly used in the treatment of agroecosystems. Control animals were similarly injected only with acetone. No mortality was observed in prey insects following administration of acetone or azadirachtin seed extract.

Because these arachnids feed on a variety of prey under natural conditions, several different prey species were used throughout these experiments. An additional 200 adult females (50 per treatment group) captured in the field were fed three times per week as follows: one mealworm larva (*Tenebrio molitor*) on Mon.; one juvenile cricket (*Acheta* sp.) on Wed.; followed by one grasshopper nymph (*Schistocerca americana*) on Fri. All prey items were approximately the same size (2.5 ± 0.2 mg). Protonymphs were also used, using the same time schedule,

and fed the following prey species (2.3 ± 0.4 mg) : adult fruitflies, *Drosophila melanogaster* and *D. virilis* (Carolina Biological Supply, Burlington, NC), and newly hatched crickets (*Acheta* sp.). Adults and protonymphs were maintained on this feeding schedule for a period of 30 days, after which they were fed for 20 days on prey items that had not been injected with azadirachtin. Data on mortality rates were recorded every fifth day over a 30-day period for adults and nymphs. In addition, growth parameters were assessed for protonymphs by measuring changes in mass (± 0.1 mg) and carapace width (± 0.1 mm), and the presence or absence of successful molts. These measurements were taken over a 60-day period or until the whipscorpion died. Only growth data on protonymphs that survived until they reached maturity (90 days) were used in statistical analyses. Weight measurements were taken with a Sartorius electronic balance. Carapace width measurements were taken with a Unitron dissecting microscope fitted with an ocular micrometer.

Experiments were also conducted to assess the effects of neem seed extract treatment on hematological and immunological parameters. An additional 20 adult female whipscorpions (experimental group, EG) were injected through the intersegemental membrane between the sternum and coxa of the first leg with a 1-ul suspension of *Bacillus popilliae* . Bacteria were obtained from a stock culture originally isolated from larvae of the Japanese beetle, *Popillia japonica* , and maintained in the laboratory on brain-heart infusion agar at 37°C. Pilot studies have indicated that arachnids, like insects, exhibit nodule formation and varying degrees of phagocytosis when innoculated with this and other bacterial pathogens. Control group (CG) subjects (N = 20) were injected with 1.0 ul of sterile medium. The whipscorpions were then allowed to feed on azadirachtin-treated prey (0, 0.1, 1.0, and 10 ppm) as described above for a period of three weeks. At the end of this period, hemolymph was collected from each spider with a 5-ul micropipette as described by Hoffmann (1970). For total hemocyte counts (THC), hemolymph samples were diluted in an anticoagulant solution (0.1 M EDTA, 0.1M glucose, 0.060 M NaCl, 0.03 M trisodium citrate, 0.03 M citric acid, pH 4.7, 370 mOsmol / l) in the ratio of 1 part hemolymph : 9 parts anticoagulant. The sample was then transferred directly to a hemocytometer (Fischer Scientific) for total hemocyte counts (THC) as described by Jones (1967). Differences in mean THC between the four treatment groups were analyzed for statistical significance using a one-way ANOVA and Scheffe F test (Sokal and Rohlf, 1995).

The hemocyte aggregation assay described by Gunnarsson and Lackie (1985) was used to determine whether azadirachtin blocks nodule formation *in vivo* . To summarize, hemolymph was diluted in the anticoagulant solution and strirred gently in a shaker bath to ensure an even distribution of hemocytes. The hemolymph was then examined in a hemocytometer using a Unitron phase-contrast microscope. Only hemocyte aggregates (nodules) > 30 um were scored for analysis.

RESULTS AND DISCUSSION

Ingesting prey injected with 1.0 and 10.0 mg/l of neem seed extract containing azadirachtin resulted in significant mortality over the 30-day test period (Table 1). A post-hoc Tukey's test for nonadditivity (Sokal and Rohlf, 1995) showed no significant difference in mortality ($p > 0.50$) between control animals (0 mg/l) and those ingesting prey injected with 0.1 mg/l azadirachtin, regardless of developmental stage. At 1.0

Table 1. Cumulative mortality (number) of *Mastigoproctus giganteus* protonymphs and adults over a 30-day period caused by the ingestion of prey injected through abdominal intersegmental membrane with neem seed extract (NSE).

		Conc. of NSE (ng/insect)			
	n	0	0.1	1.0	10.0
Protonymphs	100	3	6	31 *	71 **
Adult females	50	1	2	4	24 **

* significantly different from controls (* $p < 0.05$; ** $p < 0.01$)

ppm, neem seed extract caused a significant increase in mortality ($p < 0.05$) among protonymphs. Ingestion of prey injected with 10 ppm seed extract resulted in significant mortality in both protonymphs and adult females ($p < 0.01$) (Table 1). Although significantly higher mortality rates (50 -100%) have been reported for some insects reared on diets containing 1.0 - 10.0 mg/l of azadirachtin (Arnason et al., 1985; Arpaia and van Loon, 1993), the results of this study indicate that neem seed extract can cause significant mortality in whipscorpions as well. However, available information suggests that there is a wide range of tolerance among other arachnids (spiders) toward neem extracts. The wolf spider, *Lycosa pseudoannulata* , an important predator of rice pests, exhibited no mortality when exposed to 100 ug / spider of neem oil extract (Chiu, 1985). Additional studies should be conducted on various species of spiders so that we may better understand the potential impact of neem extracts on these arthropods.

Ingestion of prey treated with azadirachtin had a significant effect ($p < 0.01$, Scheffe F test, Sokal and Rohlf, 1981) on various growth parameters including mass of protonymphs and width of the cephalothorax (Table 2). Control nymphs exhibited significantly higher body weight and cephalothoracic width ($p < 0.05$) as compared to animals exposed to neem seed extract. It has been shown that overall fitness in several species of arachnids is directly related to their adult size (Spiller, 1984; Polis and McCormick, 1987). The ingestion of prey treated

with 1.0 and 10 mg/l of azadirachtin resulted in a significant decrease in the size of *M. giganteus* nymphs as shown by measurements of weight change and carapace width. Smaller whipscorpions may be more vulnerable to predation and restricted to the capture of a smaller range of prey items as has been reported for spiders (Punzo, 1989, 1991).
It should be pointed out that 53% of the animals that died were between 84 - 96 days of age . Some animals died between molts and others were found dead with partially shed exoskeletons, unable to complete the molting process. Some had partially deformed pedipalps and appendages. Although the ability of neem extracts to inhibit growth and molting in larval insects is well known (Mordue and Blackwell, 1993), its effects on molting in arachnids has not been studied. The pupae of azadirachtin-treated insects often exhibit deformities to the head and thoracic appendages. Future studies should focus on identifying the effects of azadirachtin on the physiology and biochemistry of ecdysis in arachnids.

Table 2. Effect of various concentrations (ppm) of neem seed extract on growth parameters of *Mastigoproctus giganteus* protonymphs over a 60-day period.

	Treatment groups		
	Controls	1.0 mg/l	10.0 mg/l
B W (g)	1.44 ± 0.31a	0.81 ± 0.18*	0.63 ± 0.11*
C T W (mm)	5.51 + 0.31a	3.68 + 0.21*	2.71 + 0.14*

* significantly different from controls (0 mg/l) ($p < 0.05$). BW (body weight, in g; CTW (cephalothorax width, in mm). Values represent means ± SE.

The ingestion of azadirachtin-treated prey over a three week period caused a significant decrease in the THC of adult whipscorpions. Controls exhibited a mean THC count of 41.1 x 10^3 (± 5.2 x 10^3 SE). In contrast, animals exposed to 1.0 and 10 mg/l azadirachtin exhibited a THC count of 31.9 x 10^3 (3.6 x 10^3 SE) ($p < 0.05$, Scheffe F test), and 16.7 x 10^3 (± 2.3 x 10^3 SE) ($p < 0.01$), respectively. There was no significant difference between control animals and those feeding on prey injected with 0.1 mg/l azadirachtin. When the hemolymph was examined microscopically, scattered nodules were observed in the hemolymph samples taken from controls and those exposed to 0.1 mg/l azadirachtin. The mean number of nodules observed per µl of hemolymph for these spiders was 31.5 ± 2.9 SE and 29.6 ± 2.2 SE, respectively. Animals exposed to higher concentrations of azadirachtin (1.0 and 10.0 mg/l), exhibited a significant decrease ($p < 0.01$) in the number of nodules present (11.6 ± 1.2 SE and 7.4 ± 0.5 SE, respectively). Nodules represent groups of hemocytes packed with engulfed bacteria. Nodule formation (encapsulation) is an important part of the immune response to

bacterial pathogens in arthropods. Clearly, azadirachtin has a significant adverse effect on the THC and the efficacy of the immune response in *M. giganteus* . This is the first demonstration of an effect of azadirachtin on the immune response of a uropygid.

These results indicate that neem seed extract can have deleterious effects on at least one species of uropygid. In *M. giganteus* , mortality increases and immunological competency is impaired. Furthermore, nymphs are more sensitive to azadirachtin than are adult whipscorpions. As a result, whipscorpion populations may be depressed in fields treated with azadirachtin.

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