Stage and Age Influence on the Susceptibility of *Coccinella septempunctata* (Coleoptera: Coccinellidae) after Direct Exposure to Neemix, a Neem Insecticide

JULIE A. O. BANKEN AND JOHN D. STARK[1]

Washington State University, Puyallup Research and Extension Center, Puyallup WA 98371

J. Econ. Entomol. 90(5): 1102–1105 (1997)

ABSTRACT The effects of Neemix 4.5 EC on predatory *Coccinella septempunctata* L. larvae were determined after direct spray exposure. First instars were treated by direct application with 0, 40, 100, 200, 400, 600, and 1,000 ppm and 4th instars were treated with 400, 600, 800, and 1,000 ppm azadirachtin, the active ingredient in Neemix. Survivorship and development of the larvae were affected in a dose-dependent manner. Symptoms of exposure included delay or prevention of pupation, blackening of the pupal case, formation of pupal–adult intermediates, and deformation of wings and elytra in adults. To account for the slow action of the pesticide, probit analysis of survivorship data was performed after all surviving larvae from each replicate had emerged as adults: day 16 for 1st instars and day 13 for 4th instars. The LC_{50} values and 95% FL for 1st and 4th instars were estimated to be 1,120 (719–3,677) ppm and 520 (405–600) ppm azadirachtin, respectively. These values were much higher than the recommended rates for control of aphids (3 weekly applications of 20 ppm), suggesting that Neemix might be used in integrated pest management programs because application of rates that control aphids should not result in appreciable mortality of predators. The slopes and intercepts of the probit regression lines were significantly different, indicating that the 2 instars responded to the pesticide differently. Fourth-instar *C. septempunctata* were innately more sensitive to the growth disrupting effects of acute exposure to Neemix than 1st instars. Disruption of morphogenetic hormone levels is more critical immediately before metamorphosis than during early instars, thus accounting for the differential susceptibility observed. It may be possible for early instars to sustain the effects of Neemix as long as the pesticide is detoxified before the onset of pupation. Our results suggest that it is extremely important to examine >1 life stage of a species to estimate the total effect of pesticides.

KEY WORDS stage/age susceptibility, neem, lady beetles, toxicity

PESTICIDES DERIVED FROM the neem tree, *Azadirachta indica* A. Juss, have recently gained popularity as components of integrated pest management. Neem derivatives have been reported to provide broad spectrum control of >200 species of phytophagous insects (Ascher 1993), yet they remain less toxic to natural enemies of insect pests than to the pests themselves (Schmutterer 1990, National Research Council 1992, Stark et al. 1992). The apparent harmlessness of neem pesticides to beneficial species has been attributed to their requirement for oral ingestion, lack of toxicity to adult insects, systemic activity, limited persistence, and antifeedant and repellent properties (Schmutterer 1990; Lowery and Isman 1993, 1995).

The principle active ingredient in neem is azadirachtin, a highly oxidized limonoid (Jacobson 1989, Mordue and Blackwell 1993). Azadirachtin acts on insect chemoreceptors to deter feeding and oviposition, and directly affects overall fitness through deleterious effects on mitosis, muscle and gut physiology, and biological rhythms. Its primary mode of action, however, is through the inhibition of release of prothoracicotropic hormones and allatotropins. The blockage of these morphogenetic peptide hormones results in disruption of ecdysteroid and juvenile hormone concentrations in the hemolymph, which in turn affects molting, metamorphosis, and reproduction (Mordue and Blackwell 1993).

Neemix, a commercial insecticide derived from the neem tree, is a candidate for use in the control of the pea aphid, *Acyrthosiphon pisum* (Harris), a key pest of commercial pea crops (Stark and Rangus 1994). If neem pesticides are to work in concert with biological control of this pest, they must be nontoxic to predatory species of the Coccinellidae, because coccinellid beetles are reported to be the principle predators of aphids (Hodek 1970, Frazer and Gilbert 1976, Kring et al. 1985, Rice and Wilde 1988).

Kaethner (1991) found that 2 neem insecticides prepared in his laboratory, the 1st containing 1000 ppm azadirachtin and the 2nd containing 250 ppm

[1] To whom all correspondence should be addressed.

0022-0493/97/1102–1105$02.00/0

azadirachtin and 3% neem oil, caused no detrimental effects to the eggs, 2nd instars, or adults of *C. septempunctata* when they were exposed to dried residues on bean leaves. However, if the larvae were exposed to direct sprays, neem was toxic to *C. septempunctata* causing mortality and morphogenetic defects. No concentration response studies were done by Kaethner (1991). Based on criteria developed by the International Organization of Biological Control (IOBC), Kaethner (1991) stated that neem insecticides can be considered harmless to *C. septempuncata* causing <30% mortality in laboratory studies (Hassan 1992).

The toxicity of different neem insecticides can vary tremendously depending on the oil content and presence of limonoids other than azadirachtin (Stark and Walter 1995). For example, neem oil greatly increased the toxicity of neem insecticides to the pea aphid (Stark and Walter 1995). Therefore, generalizations about the toxicity of neem may be impossible to make unless all formulations are equivalent. Kaethner (1991) found that the addition of neem oil to the neem insecticides he tested, did not increase mortality in *C. septempuncata*, but did cause a greater percentage of morphogenetic defects to occur.

The objective of this study was to investigate the effects of direct exposure of 2 larval stages of *C. septempunctata*, a recently established predator of the pea aphid, to a commercial neem insecticide, Neemix. We were particularly interested in whether the commercial product we evaluated would give us results similar to those obtained by Kaethner (1991) with a different neem preparation.

Materials and Methods

Insects Tested. First- (neonates ≤24 h old) and 4th-instar *C. septempunctata* were obtained from a colony maintained for 10 mo in a free-standing growth chamber in the University of Washington Department of Zoology (Seattle, WA). Environmental conditions were adjusted to 24 ± 2°C with a photoperiod of 16:8 (L:D) h.

Pea aphids were reared on potted pea plants, *Pisum sativum* L., variety Bonita, in a free-standing environmental growth chamber maintained as described above.

Chemical. The insecticide Neemix 4.5 EC ([emulsifiable concentrate] 4.5% azadirachtin) was provided by W. R. Grace, Columbia, MD.

Bioassay. Batches of 10 *C. septempunctata* larvae (1st or 4th instars) were placed in glass petri plates (10 cm diameter). The petri plates were placed in a Potter tower (Potter 1952) set at 100 kPa. One milliliter of distilled, deionized water (control) or insecticide solution was applied, which was equivalent to 2.2 μl cm^2. Larvae were immediately transferred with a camel's-hair brush to plastic petri plates (5 cm diameter) containing live pea aphids. After an initial 10-fold bracketing experiment, 1st instars were treated with 1 ml of 40, 100, 200, 400, 600, and 1,000 ppm azadirachtin and 4th instars were treated with 400, 600, 800, and 1,000 ppm azadirachtin. Three replicates were performed for each concentration using different generations of insects. Larvae were maintained in the environmental growth chamber set at the conditions described previously and fed pea aphids ad libitum, once a day until the end of the study. Survivorship was recorded daily until adult eclosion, which was 16 d after treatment for 1st instars 13 d for 4th instars.

Table 1. Toxicity of Neemix to 1st- and 4th-instar *C. septempunctata* dorsally misted with a Potter tower

Instar	No. tested	Slope ± SE	LC50 (95% FL), ppm
First	210	1.62 ± 0.46	1,120 (719–3,677)
Fourth	150	5.22 ± 1.19	520 (405–600)

Data Analysis. Concentration–mortality regressions for 1st and 4th instars were estimated by probit analysis (Finney 1971), using the SAS probit procedure (SAS Institute 1985). To account for the slow action of the pesticide, probit analysis of survivorship data was performed after all surviving larvae from each replicate had emerged as adults: day 16 for 1st instars and day 13 for 4th instars. Control mortality was corrected using Abbott's formula (Abbott 1925). Differences in toxicity were considered significant when 95% FL did not overlap. The slopes and intercepts of concentration–mortality regressions for each instar were compared with the POLO-PC maximum-likelihood procedures (LeOra Software 1987).

Results and Discussion

Neemix was more toxic to 4th instars than to 1st instars at LC_{50} (Table 1). Symptoms of exposure to Neemix in both instars were generally not evident until the onset of pupation in the controls. Death of affected organisms occurred during the larval or pupal stages, or upon adult eclosion. Morphogenetic abnormalities expressed by treated larvae were similar to those reported by Ladd et al. (1984), who described the harmful effects of contact exposure to azadirachtin on phytophagous beetles. Response to the pesticide was concentration dependent. Symptoms included an extended larval stage from 1 d at the lowest concentration tested, to no pupation at all at the highest concentration. Loss of appetite, lethargy, blackening of the pupal case, formation of pupal–adult intermediates, inability to complete pupal ecdysis, and mild to gross deformation of the elytra and wings were also observed and were concentration dependent.

Our results provide additional evidence that azadirachtin does exhibit contact toxicity to predator species, although very high concentrations of Neemix were required to have an effect on the larvae. The recommended field rate for Neemix is 20 ppm

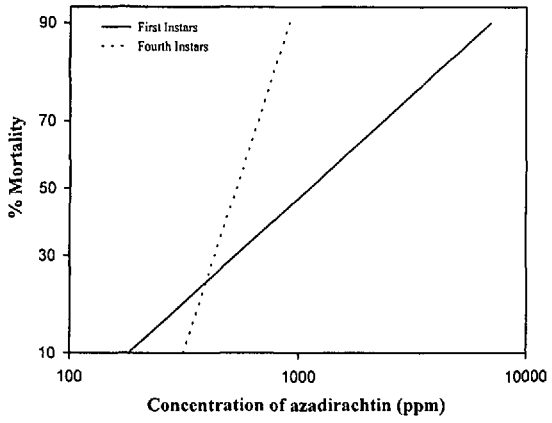

Fig. 1. Concentration-mortality curves for 1st and 4th-instar *C. septempunctata* after direct exposure to Neemix spray.

weekly for 3 wk. No toxic effect to survival of 1st or 4th instars was observed after exposure to 20 ppm.

Previous research on the toxicity of neem pesticides to lady beetles has indicated that azadirachtin must be ingested to produce a toxic effect. Lowery and Isman (1994) found no effect on survival of *Coccinella undecimpuncata* L. larvae topically treated with neem seed oil at concentrations up to 1%, but when *C. undecimpuncata* larvae were exposed to treated foliage and allowed to feed on contaminated aphids, neem seed oil reduced adult eclosion rates 100%. However, Kaethner (1991) found that direct exposure of larval *C. septempunctata* to neem sprays resulted in morphogenetic deformations and death even when their food (pea aphids) was not treated with the insecticide. Other research on phytophagous insects presents clear evidence of the contact toxicity of azadirachtin. There is evidence of a contact effect in soft-skinned larvae of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Steets 1976), and the Japanese beetle, *Popillia japonica* Newman (Ladd et al. 1984). Futhermore, Stark and Rangus (1994) found that topical application of the neem pesticide Margosan-O (W. R. Grace) to pea aphids produced lethal and sublethal effects similar to those seen in individuals which fed on treated plants, although the pesticide was slower acting when applied topically. Therefore, it appears that neem insecticides do possess contact toxicity, but toxicity may be greater when neem is ingested.

Estimations of concentration-mortality regressions for the 1st and 4th instars are shown in Fig. 1. Analysis of the slopes and intercepts of the 2 regression lines indicated that the lines were significantly different ($\chi^2 = 24.1$, df = 2, $P < 0.05$). Regression slopes estimate the change in biological activity per unit change in concentration of chemical. Parallel lines may signify that the test organisms have qualitatively identical but quantitatively different levels of detoxification enzymes (Robertson and Preisler 1992). Significantly different probit lines indicate that the 2 life stages were not responding to the pesticide in the same manner. It is generally assumed that susceptibility to xenobiotic compounds varies conversely with size (Busvine 1971). However, Neemix was more toxic to 4th instars than neonates despite their tremendous size advantage. The difference in susceptibility demonstrated by the 2 instars of *C. septempunctata* may be attributed to the timing of pesticide introduction during the hormonal cycles of the insects and the half-life of azadirachtin after absorption. During the larval stage of untreated insects, both ecdysone, a growth promoter, and juvenile hormone, a growth preventer, are produced. The 2 hormones are delicately balanced throughout the larval stage. During the final instar, however, juvenile hormone levels decrease, allowing ecdysone levels to surge, which subsequently instigates pupation in holometabolous insects (Rosomer and Stoffolano 1994). Azadirachtin is known to disrupt normal concentrations of ecdysone and juvenile hormone (Jacobson 1989, Mordue and Blackwell 1993). For example, Bidmon et al. (1987) reported that when injected into blowfly larvae, azadirachtin caused distinct alterations in larval and pupal peaks of ecdysteroids. Although ecdysteroid content in control larvae exhibited sharp, brief surges, peaks in the ecdysteroid content in treated larvae were delayed and did not decline rapidly. Disruption of morphogenetic hormone levels appear to be more critical immediately before metamorphosis than during early instars based on the results we obtained. It may be possible for early instars to sustain the effects of azadirachtin as long as the chemical is detoxified before the onset of pupation.

Our results corroborate those of Kaethner (1991). The commercial neem insecticide Neemix is much less toxic to *C. septempunctata* than to one of its hosts, the pea aphid, based on LC_{50}. However, in this study we have shown that susceptibility of *C. septempunctata* to the neem insecticide Neemix is stage and age specific. A bioassay carried out exclusively on 1st instar *C. septempunctata* would underestimate the toxic effects of Neemix to the immature forms of this predator. Toxicity studies of adults only would indicate that neem is not toxic to *C. septempunctata* (Kaethner 1991). Therefore, how do we estimate the risk of pesticides that exhibit differential toxicity to different life stages and ages? Individual-level pesticide bioassays of 1 life stage are extremely useful for obtaining information on concentrations of pesticides that affect survival, reproduction, biochemistry, and behavior (Cairns 1983). However, Stark and Wennergren (1995) warned that the total effect of a pesticide could not be estimated by testing 1 life stage when differential susceptibility among life stages was present. They suggest testing populations composed of various life stages as a means to obtain a better estimate of effect. The results presented here build on the argument presented by Stark and

Wennergren (1995). It appears that estimating toxicity of pesticides to biological controls may not be as straight forward as we think. Unless the susceptibility of various life stages is estimated, erroneous conclusions can be made. We suggest that where possible, toxicity should be estimated for 1 life stage so that better estimates of effect can be made.

Acknowledgments

We thank Garrell Long, Peter Kareiva, Bill Walthall, Malika Bounfour, Grace Jack, Barb Wood, Lily May, and Brad Klepetka for their advice and support during this study. We are indebted to Peter Kareiva (University of Washington) for providing office and laboratory facilities and for being an extraordinary mentor for J.A.O.B. We are also indebted to the Entomology Department of Washington State University and Garrell Long in particular, for providing financial support for J.A.O.B. during part of her graduate program. We also thank the Northwest Agricultural Research Foundation for providing funding for part of this study.

References Cited

Abbott, W. S. 1925. A method of computing the effectiveness of an insecticide. J. Econ. Entomol. 18: 265–267.

Ascher, K.R.S. 1993. Nonconventional insecticidal effects of pesticides available from the neem tree, *Azadirachta indica*. Arch. of Insect Biochem. Physiol. 22: 433–449.

Bidmon, J.H.J., G. Kauser, P. Mobus, and J. Koolman. 1987. Effect of azadirachtin on blowfly larvae and pupae, pp. 141–150. *In* H. Schmutterer and K.R.S. Ascher [eds.], Proceedings 3rd International Neem Conference, Nairobi. Natural Pesticides from the Neem Tree (*Azadirachta indica* A. Juss.) and Other Tropical Plants. Eschborn, Germany. GTZ.

Busvine, J. R. 1971. A critical review of the techniques for testing insecticides. Commonwealth Institute of Entomology, London.

Cairns, J., Jr. 1983. Are single species toxicity tests alone adequate for estimating environmental hazard? Hydrobiologia 100: 47–57.

Finney, D. J. 1971. Probit analysis, 2nd ed. Cambridge University Press, Cambridge.

Frazer, B. D., and N. Gilbert. 1976. Coccinellids and aphids: a quantitative study of the impact of adult ladybirds (Coleoptera: Coccinellidae) preying on field populations of pea aphids (Homoptera: Aphididae). J. Entomol. Soc. B. C. 73: 33–56.

Hassan, S. A. 1992. Guidlines for testing the effects of pesticides on beneficial organisms: Description of test methods. Bull. International Organization of Biological Control/Working Group "Pesticides and Beneficial Organisms"/West Paleartic Regional Section XV/3.

Hodek, I. 1970. Coccinellids and modern pest management. Bioscience 20: 543–552.

Jacobson, M. 1989. Focus on phytochemical pesticides, Vol. 1. The neem tree. CRC, Boca Raton, FL.

Kaethner, M. 1991. Keine Nebenwirkungen von Niempräparaten auf die aphidophagen Prädatoren *Chrysoperla carnea* (Steph.) und *Coccinella septumpuncatata* L. Anz. Schäedlingskd. Pflanzenschutz Umweltschutz 64: 97–99.

Ladd, T. L., Jr., J. D. Warthen, Jr., and M. B. Klein. 1984. Japanese beetle (Coleoptera: Scarabeidae): the effects of azadirachtin on the growth and development of immature forms. J. Econ. Entomol. 77: 903–905.

LeOra Software. 1987. POLO-PC: a user's guide to Probit Or Logit analysis. LeOra Software, Berkeley, CA.

Lowery, D. T., and M. B. Isman. 1993. Effects of extracts from neem on aphids (Homoptera: Aphididae) and their natural enemies. Proceedings of the World Neem Conference, Bangalore, India.

1994. Effects of neem and azadirachtin on aphids and their natural enemies, pp. 78–91. *In* P. A. Hedin [ed], Bioregulators for crop protection and pest control. Am. Chem. Soc. Symp. Ser. 557.

1995. Toxicity of neem to natural enemies of aphids. Phytoparasitica 23: 297–306.

Mordue (Luntz), A. J., and A. Blackwell. 1993. Azadirachtin: an update. J. Insect Physiol. 39: 903–924.

National Research Council. 1992. Neem, a tree for solving global problems. National Academy Press, Washington, DC.

Potter, C. 1952. An improved laboratory apparatus for applying direct sprays and surface films, with data on the electrostatic charge on atomized spray fluids. Ann. Appl. Biol. 39: 1–29.

Rice, M. E., and G. E. Wilde. 1988. Experimental evaluation of predators and parasitoids in suppressing greenbugs (Homoptera: Aphididae) in sorghum and wheat. Environ. Entomol. 17: 836–841.

Robertson, J. L., and J. K. Preisler. 1992. Pesticide bioassays with arthropods. CRC, Boca Raton, FL.

Rosomer, W. S., and J. G. Stoffolano, 1994. The science of entomology, 3rd ed. Wm. C. Brown, Dubuque, IA.

SAS Institute. 1985. SAS user's guide: Statistics. SAS Institute, Cary, NC.

Schmutterer, H. 1990. Properties and potential of natural pesticides from the neem tree, *Azadirachta indica*. Annu. Rev. Entomol. 35: 271–297.

Stark, J. D., and T. M. Rangus. 1994. Lethal and sublethal effects of the neem insecticide formulation, 'Margosan-O', on the pea aphid. Pestic. Sci. 41: 155–160.

Stark, J. D., and J. F. Walter. 1995. Persistence of azadirachtin A and B in soil: Effects of temperature and microbial activity. J. Environ. Sci. Health B30: 685–698.

Stark, J. D., and U. Wennergren. 1995. Can population effects of pesticides be predicted from demographic toxicological studies? J. Econ. Entomol. 88: 1089–1096.

Stark, J. D., T.T.Y. Wong, R. I. Vargas, and R. K. Thalman. 1992. Survival, longevity, and reproduction of Tephritid fruit fly parasitoids (Hymenoptera: Braconidae) reared from fruit flies exposed to azadirachtin. J. Econ. Entomol. 85: 1125–1129.

Steets, R. 1976. Die wirkung von rohextrakten aus den Meliaceen *Azadirachta indica* A. Juss auf *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) Z. Angew. Entomol. 82: 169–176.

Received for publication 4 March 1997; accepted 9 July 1997.