Multiple Routes of Pesticide Exposure and the Risk of Pesticides to Biological Controls: A Study of Neem and the Sevenspotted Lady Beetle (Coleoptera: Coccinellidae)

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ABSTRACT Microcosms were used to illuminate community-level interactions among the pea aphid, *Acyrthosiphon pisum* Harris (Homoptera: Aphididae), and the sevenspotted lady beetle, *Coccinella septempunctata* L., after a spray exposure to a commercial neem pesticide. The instantaneous rate of increase (r_i) of pea aphid populations was used as a response variable in a 2 × 3 factorial experiment. Microcosms were treated with 100 ppm (mg/liter) or 600 ppm azadirachtin, the active ingredient in the commercial neem insecticide, or with water in the presence or absence of adult or 4th instar lady beetles. We compared our results with those of a previous study where *C. septempunctata* was exposed to only 1 route of exposure, direct application. Results from the previous study indicated that 100 ppm of the neem insecticide was sublethal ($<LC_1$) and 600 ppm was equivalent to the LC_{62} for 4th instars. Both concentrations caused no mortality to adults based on direct application. However, in the current study, *C. septempunctata* was exposed to direct sprays, residues on leaves, and pesticide-contaminated prey. Population growth rates (r_i values) of the aphid populations 4 d after treatment were compared with 2-way analysis of variance. The pesticide alone and the predator alone caused a significant decrease in aphid population growth rates. However, no significant ($P > 0.05$) interaction between the predator and the pesticide was detected indicating that the chemical and biological control agents are not working synergistically. Furthermore, exposure to the pesticide in micrososms significantly reduced or completely eliminated oviposition in adult *C. septempunctata*, and all of the larval lady beetles exposed to 100 or 600 ppm died within 10 d of treatment. Based on these results, we question the value of toxicity tests where only 1 route of pesticide exposure is considered.

KEY WORDS lady beetles, ecotoxicology, exposure, microcosms, neem

AFTER YEARS OF strict reliance on chemical pesticides, natural enemies of insect pests are now recognized as essential players in long-term pest control. This shift in attitude toward biological control agents has led to the development of a new generation of less broadly biocidal, botanical pesticides (Croft 1990, Schmutterer 1990) that are promoted as toxic to pest species yet benign to their predators and parasitoids. The introduction of these selective pesticides in turn has inspired a change in the motivation supporting toxicological research. Rather than study the harm pesticides do to insect pests, the general trend is to focus on their harmlessness to natural enemies (Brown 1977).

The International Organization for Biological Control (IOBC) has developed a standard protocol for the analysis of the impact of pesticides on nontarget organisms (Hassan 1989). The IOBC recommends a tiered testing procedure in which pesticides are first tested on beneficial organisms in the laboratory (Hassan 1985). If no harmful effect is observed, the pesticide is considered safe for use in integrated pest management (IPM) programs. If the pesticide is found to be toxic in the laboratory, tests are performed in semifield trials, where nontarget organisms are exposed to treated, caged plants under simulated or natural field conditions. Pesticides that cause no toxic effects in semifield trials are then recommended for use in IPM, and those that still harm beneficial species are tested again in field trials.

The protocol prescribed by the IOBC is based on the assumption that pesticides found to be harmless to a beneficial species in laboratory tests will be harmless to the same organism in the field (Hassan 1989). In the laboratory, individual test organisms of uniform age are either exposed to dried residue on treated surfaces or directly sprayed and moved to a clean surface, depending on the expected means of exposure in the field, and monitored until mortality or a reduction in predation or parasitism is observed. These tests, however, are designed to assess the effects of only 1 route of pesticide exposure—contact with a treated surface—whereas in the field, beneficial organisms may receive exposure from 3 sources: direct exposure to spray droplets, uptake of

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residues through contact with contaminated surfaces, and oral uptake from contaminated food sources (Longley and Stark 1996). In addition, traditional tests discount the consequences of differential susceptibility among population age structure (Stark and Wennergren 1995, Kareiva et al. 1996), density-dependent population regulation (van Leeuwen et al. 1985, 1987; McNair et al. 1995), and interspecific relationships (Marshall 1962, 1969).

Pesticides derived from the neem tree, *Azadirachta indica* A. Juss, appear to be promising pesticides for use in IPM programs for some pest species. Neem pesticides are reported to provide broad-spectrum control of >200 species of insect pests (Ascher 1993), yet remain compatible with beneficial species (Schmutterer 1990, National Research Council 1992). Neem insecticides are candidates for use in the control of the pea aphid, *Acyrthosiphon pisum* Harris, the major insect pest of commercially grown peas in western Washington (Stark and Rangus 1994). Recent studies support the use of neem insecticides to control aphids (Stark and Rangus 1994; Lowery et al. 1993; Lowery and Isman 1993, 1996), but their effects on coccinellid predators of aphids are not well documented. Kaethner (1991) found that sevenspotted lady beetle, *Coccinella septempunctata* L. larvae were susceptible to neem only after direct exposure to pesticide sprays; contact with dried residues caused no mortality or sublethal effects. Lowery and Isman (1994) found that adult eclosion of *Coccinella undecimpunctata* L. (Coleoptera: Coccinellidae) was completely inhibited after exposure of larvae to neem-treated, aphid-infested foliage for 1 wk in the laboratory. However, in field tests, application of a neem insecticide did not alter the prespray ratio of predators to pests, leading them to conclude (as the IOBC dictates) that neem-based pesticides are appropriate for IPM programs. In a previous study, Banken and Stark (1997) found that Neemix, a commercial neem insecticide, was virtually nontoxic to sevenspotted lady beetle larvae exposed to direct sprays.

These conflicting results about the susceptibility of lady beetles to neem and the apparent importance of exposure routes in determining toxicity, led us to ask, How important are exposure methods for the estimation of pesticide toxicity? We addressed this question by using terrestrial microcosms. Microcosms are reproducible, model ecosystems maintained within controlled environmental conditions (Morgan and Knacker 1994). Although they are limited by their simplicity (Morgan and Knacker 1994), microcosms are useful tools for predicting the effects of contaminants on natural populations (Cairns 1983, Kimball and Levin 1985) because they enable ecotoxicologists to evaluate the effects of a pesticide on whole systems rather than their individual components.

Materials and Methods

Insects. Fourth-instar and adult *C. septempunctata* were obtained from a colony maintained for 9 mo in a free-standing growth chamber at the University of Washington, Department of Zoology. 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 growth chamber set to the same conditions as described above. At these conditions, all aphids existed as females and reproduced parthenogenetically.

Chemical. The insecticide we evaluated in this study was Neemix 4.5 (W. R. Grace, Columbia, MD). Neemix 4.5 is a natural insecticide derived from the neem tree that contains 4.5% azadirachtin, the primary active ingredient.

Bioassay. Potted pea plants were grown in a greenhouse. When plants were ≈15 cm tall, they were thinned to a density of 8–10 plants per 20-cm-diameter pot to ensure adequate coverage of leaf surfaces during pesticide application. Plastic sleeve cages 30 cm tall and 18 cm in diameter were secured over the pots to enclose the plants. Each pot of peas was infested with 10 adult apterous pea aphids. A piece of organdy was secured around the top of the sleeve cage with a rubber band. Microcosms were transferred to a growth chamber set to the same conditions as described above. Because aphid population growth was highly variable, populations were allowed to grow for 8 d to ensure the starting population density would be high enough to sustain predation by the lady beetles. In addition, this time interval allowed the populations to reach a stable age distribution (Banken 1996), which is "the only sound basis on which to make comparison between different values for rates of increase" (Birch 1948). The average initial aphid density (N_0) on the day of treatment (day 0) was determined by destructive sampling of 4 microcosms to be 304 ± 41 (mean ± SE) aphids.

Treatments were arranged in a 2 × 3 factorial experiment so that aphids were exposed to 1 of 6 combinations of pesticide and predators: (1) water only, (2) 5 adult lady beetles and water, (3) 5 larval lady beetles and water, (4) pesticide only, (5) 5 adult lady beetles and pesticide, and (6) 5 larval lady beetles and pesticide. Preliminary studies indicated that 5 adult lady beetles were sufficient to decrease significantly the aphid population inside a microcosm without causing the population to go to extinction. Each treatment was replicated 4 times per trial.

Lady beetles were added to the appropriate cages (no distinction was made between males and females) and all microcosms were transferred outside for pesticide application. A Norgren backpack sprayer (model R81200LNKA, R&D Sprayers, Opelousas, LA) set at 30 psi was used to apply either water or a Neemix solution in water containing 100 ppm of azadirachtin. This concentration was suble-

thal ($<LC_1$) to 4th instars based on direct exposure (Banken 1996, Banken and Stark 1997) and caused no mortality to adults (J.D.S., unpublished data). The nozzle of the sprayer was passed over the microcosms at a rate of ≈0.5 m/s and at a height of ≈43 cm, just a few centimeters over the top of each sleeve cage, so that the volume of spray received by the plants in each microcosm was ≈16 ml.

All microcosms were returned to the growth chamber after pesticide application. After 4 d, pots were removed and all of the aphids were counted. The 4-d time interval was chosen because it provided enough time for population growth to occur but not enough time for aphids to kill the pea plants. Aphid population growth rate was determined as the instantaneous rate of increase (r_i) for each aphid population with the equation

$$r_i = (\ln[N_t/N_0])/t,$$

where N_0 is the initial number of individuals in the population, and N_t is the number of individuals in the population at the end of the time interval, t (measured in days) (Walthall and Stark 1997). Values of r_i that are positive indicate a growing population, when r_i is zero the population is neither growing nor declining, and when r_i is negative the population is in decline and headed towards extinction.

Coccinella septempunctata eggs on pea foliage and sleeve cage walls were counted in each microcosm containing adult beetles. Larvae and adults were collected after the aphid population census and maintained in plastic cups. *C. septempunctata* were fed live untreated pea aphids daily until the end of the study. Survivorship of the treated and untreated larvae was monitored daily until day 11 after treatment, when all larvae had either eclosed to the adult stage or died. Adult survivorship was monitored for 10 d after treatment.

The entire procedure was repeated with a 600-ppm solution of Neemix. This concentration was equivalent to the LC_{62} for 4th instars based on direct spray exposure (Banken 1996) and caused no mortality to adults (J.D.S., unpublished data). The average initial aphid density for this test was 403 ± 81 (mean ± SE), which was not significantly different from the initial aphid density in the test performed with 100 ppm of Neemix. All adults as well as larval lady beetles were collected after the aphid population census and their survivorship was monitored as previously described until day 11.

Data Analysis. The instantaneous rates of increase of aphid populations exposed to 100 or 600 ppm of azadirachtin were analyzed with 2-way analyses of variance (ANOVA) by the general linear model (GLM) procedure. Normality and equal variance of the data were tested before analysis by using the Kolmogorov-Smirnov test for normality and the Levene Median test for equal variance (Glantz 1992, Sigmastat 1994). The 100-ppm data set passed the normality ($P = 0.79$) and equal variance test ($P = 0.25$) test. The 600-ppm data set also passed the normality ($P = 0.65$) and equal variance test ($P = 0.25$) test.

Data from the 2 × 3 factorial experiment were analyzed to investigate possible interactions between Neemix (present or absent) and *C. septempunctata* (absent, larvae, or adults). Means were separated by the least significant difference (LSD) test ($P \leq 0.05$) (SAS Institute 1985). Egg production by treated and untreated adults and the initial aphid densities of the 2 experiments were compared with t-tests (Sigmastat 1994).

Results

The r_i of aphid populations exposed to larval and adult lady beetles and the 2 concentrations of pesticide are shown in Fig. 1. Overall, results of a 2-way ANOVA found that exposure to 100 ppm of Neemix significantly reduced the r_i of the aphid populations ($F = 28.23$, df = 1, $P = 0.0002$), irrespective of the presence or absence of the lady beetles. Predation by lady beetles also significantly reduced aphid population growth ($F = 10.52$, df = 2, $P = 0.003$), both with and without the pesticide. Mean separation with an LSD test indicated that adult lady beetles significantly reduced the r_i of aphids ($P < 0.05$), but larval lady beetles did not ($P > 0.05$). No significant interaction between 100 ppm of Neemix and the predators was detected at the $P \leq 0.05$ level ($F = 4.04$, df = 1, $P = 0.07$).

Aphid r_i was significantly reduced by 600 ppm of Neemix ($F = 39.40$, df = 1, $P = 0.0001$) in the presence or absence of predators (Fig. 1). Predation also significantly reduced aphid r_i ($F = 6.40$, df = 2, $P = 0.01$), with and without the pesticide. Mean separation indicated both larvae and adults significantly ($P < 0.05$) reduced aphid r_i. There was no significant interaction between 600 ppm of Neemix and the predators ($F = 2.91$, df = 1, $P = 0.12$).

The mean ± SE numbers of *C. septempunctata* eggs collected from untreated and treated microcosms were 109 ± 26 and 31 ± 15 for the 100-ppm exposure and 152 ± 47 and 0 for the 600-ppm exposure. A t-test for differences between 2 means indicated exposure to 100 ppm of Neemix significantly reduced oviposition by adult lady beetles ($t = 2.85$, df = 5, $P = 0.036$). Exposure to 600 ppm of Neemix prevented oviposition entirely, and therefore no statistical analysis of the data was necessary.

Neemix at 600 ppm did not affect adult survivorship during a 10-d observation period. However, both concentrations of Neemix had severe qualitative and quantitative effects upon larval development and survivorship. On day 4 when the larvae were collected from the microcosms, 81.3% of the untreated larvae had begun to pupate. Only 22.6% of the treated larvae showed signs of progressing to the pupal stage. No additional reaction to the pesticide was evident until day 6. Treated larvae gradually lost their appetite for aphids and became decreasingly active, until it was difficult to judge if they were live

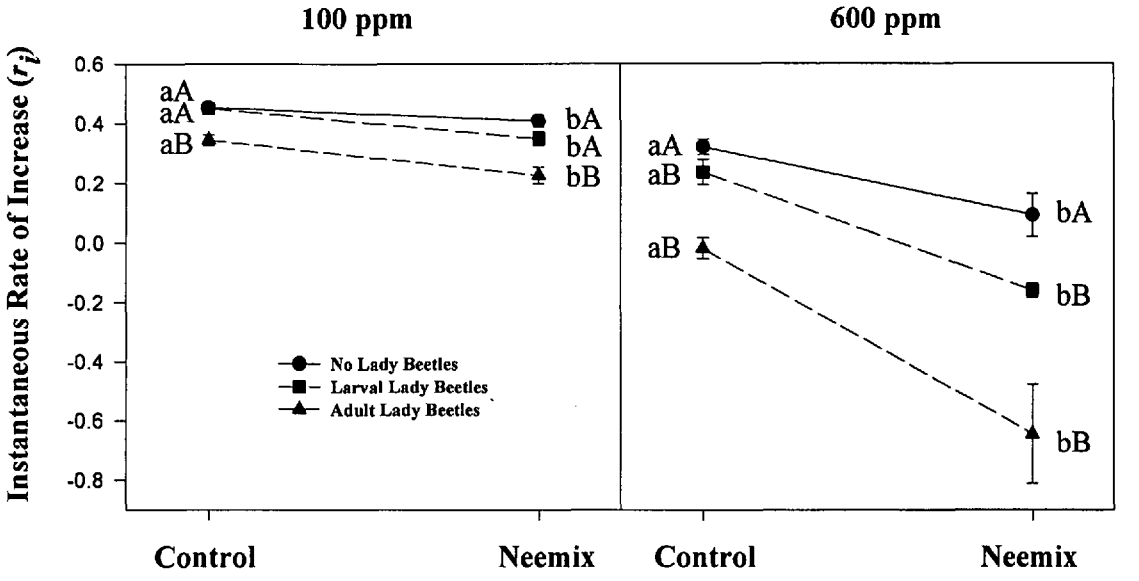

Fig. 1. Mean ± SEM instantaneous rates of increase of aphid populations exposed to larval or adult *C. septempunctata* and 100 or 600 ppm of Neemix. Means within rows (comparing control to Neemix) followed by the same lowercase letters and means within columns (comparing control larval, and adult *C. septumpunctata*) followed by the same uppercase letters are not significantly different ($P > 0.05$).

or dead. Those that developed into pupae turned black and died, or eclosed as grossly deformed, moribund adults. By day 11 posttreatment, when 91.2% of the untreated larvae had emerged from their pupal cases as healthy adults, all of the treated larvae were dead.

Discussion

No interaction between Neemix and *C. septempunctata* was detected at the $P \leq 0.05$ level. In ecological studies, however, P values up to 0.10 are often acceptable, because the complex interactions within ecological systems are highly variable (Scheiner 1993). At a P value ≤ 0.10, there was a significant interaction between the pesticide and the lady beetles treated with 100 ppm of Neemix. At 600 ppm, interaction was still not significant, but the impact of adult lady beetles on aphid r_i was visibly enhanced by treatment with 600 ppm Neemix, indicating that more replicates might statistically verify an interaction between *C. septempunctata* and Neemix.

Further investigation of the community-level impact of Neemix cast doubt on the selectivity of this pesticide to *C. septempunctata*. All larval lady beetles exposed to 100 ppm failed to pupate and died even though this concentration of Neemix was found to be sublethal ($<LC_1$) in a previous study (Banken and Stark 1997). However, in the previous study, larvae were exposed only to direct sprays and then moved to a clean environment.

In our microcosm studies, lady beetles and aphids were exposed to pesticides as they would be in the field. Lady beetles received direct contact with spray droplets during pesticide application, residual contact with contaminated foliage, and oral uptake from ingesting contaminated aphids. The cumulative effects of the 3 routes of exposure greatly increased the toxicity of this pesticide to *C. septempunctata*. Realistic exposure of beneficial species is, therefore, critical when estimating the potential risk of a pesticide.

The importance of different routes of pesticide exposure to terrestrial organisms has been discussed by Mullié and Everts (1992) with the linyphiid spider *Oedothorax apicatus* (Blackwall) (Acari: Linyphiidae). In their study, *O. apicatus* was treated topically with deltamethrin, exposed to deltamethrin-treated soil, or fed deltamethrin-treated fruit flies. Residual uptake from treated soil was found to be the most important route of exposure in terms of insecticide uptake. Uptake of deltamethrin through contaminated food contributed very little to the body burden of this product. Mullié and Everts (1992) then conducted another study where all 3 routes of exposure were combined. Uptake through feeding again was not important in terms of body burden and the authors suggested that exposure to residues led to decreased feeding activity. In contrast to the study by Mullié and Everts (1992), abamectin was found to be very toxic to the spider *Phidippus audax* (Hentz) after ingestion of treated *Heliothis zea* (Boddie) (Roach and Moore 1988).

Kaethner (1991) discovered that 2 neem insecticides prepared in the laboratory, the 1st containing 1,000 ppm of azadirachtin and the 2nd containing 250 ppm of 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, when the larvae were exposed to direct sprays, mortality and morphogenetic defects occurred. Based on these results, Kaethner (1991) stated that neem insecticides can be considered harmless to *C. septempunctata*. Although we did not do separate studies to determine the toxicity of neem when ingested only, or after exposure to residues only, susceptibility of *C. septempunctata* after exposure to 3 routes of exposure to Neemix was much greater than if this species was only exposed to direct sprays (Banken and Stark 1997).

Bakker and Jacas (1995) found that laboratory tests of pesticide toxicity were not always predictive of effects in the field. Laboratory results of both Kaethner (1991) and Banken and Stark (1997) indicated that neem was not toxic to *C. septempunctata* when indeed it is. This error would be a case of a type A error where a pesticide is determined to be harmless in the laboratory but then found to be harmful in the field (Bakker and Jacas 1995). Jepson (1993) recognized that laboratory susceptibility data alone discount the effects of natural levels of exposure and are, therefore, insufficient to predict effects of a contaminant in the field. Indeed, traditional laboratory bioassays (Banken 1996, Banken and Stark 1997) greatly underestimated the impact of Neemix on *C. septempunctata*.

Laboratory bioassays also can overestimate the effects of a contaminant on a population if they fail to account for the protection provided by natural foliage during spray application (Robertson and Worner 1990). In the field, spray deposition declines throughout the canopy and is reduced on the undersides of leaves as a consequence of sheltering by vegetation (Cilgi and Jepson 1992, Kjaer and Jepson 1995). In the current study, Neemix was applied to the pea plants within the microcosms in the same manner that it would have been applied in the field. Therefore, aphids low in the vegetation or on the undersides of pea leaves should have received less exposure to Neemix than aphids higher up within the canopy or on the plant stems. Subsequently, aphids in the lower canopy would be less affected by the pesticide than aphids in the upper canopy, which would diminish the total effect of the pesticide on their growth.

Microcosm toxicity tests can detect subtle, sublethal effects that might be overlooked in traditional laboratory bioassays. Although survivorship of adult lady beetles was unaffected, exposure to Neemix resulted in a severe reduction in fecundity or complete sterility depending on the concentration. Toxicity tests that assess survivorship only would miss effects on reproduction that might eventually lead to the extinction of the population.

Our results have demonstrated that pesticides that appear to be harmless to organisms when tested with conventional laboratory tests are not necessarily harmless to the same species when they are exposed within terrestrial microcosms. The influence of natural routes of pesticide exposure and protection provided by foliage may have subsequent effects on reproduction and survivorship that would not be detected in the laboratory tests prescribed by the IOBC. Microcosms are, therefore, efficient tools in assessing the effects of pesticides on nontarget species at the community level.

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