Soil-applied imidacloprid is translocated to landscape flowers and reduced survival of adult *Coleomegilla maculata*, *Harmonia axyridis*, and *Hippodamia convergens* lady beetles, but not two species of butterflies

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ABSTRACT Integrated pest management programs (IPM) promote the use of multiple cultural, biological, and chemical tactics to reduce the abundance of pest insects, while conserving pollinators and beneficial insects. Much research has focused on the impact of systemic neonicotinyl insecticides on the colony health and foraging behavior of bees, but few papers considered these impacts on pollen and nectar-feeding beneficial insects, such as parasitoids, lacewings, lady beetles, and butterflies. Contact insecticides only contaminate pollen and nectar of flowers that are open at the time of spraying. In contrast, soil-applied neonicotinyl insecticides are translocated to pollen and nectar of flowers for a longer duration, often months. Neonicotinyl seed-treated crops (imidacloprid, 0.11 mg/canola seed and 0.625 mg/corn seed) translocate less than 10 ppb to nectar and pollen. However, higher rates of soil-applied imidacloprid are used in urban landscapes and nurseries (270-300 mg/3gallon pot, 67g imidacloprid applied to soil surface for 24 diam. tree), which should result in residues higher than the 10 ppb in nectar and pollen compared to seed-treated crops. This study shows that the translocation of imidacloprid from soil (300 mg) to flowers of *Asclepias curassavica* resulted in imidacloprid residue in nectar of 6,000 ppb for 1X and 10,000 ppb for 2X treatments. A second imidacloprid soil application 7 months after the first resulted in residues of 21,000 ppb in 1X and 45,000 ppb in 2X treatments. Consequently, landscape use of imidacloprid applied to flowering plants can result in 697 to 1,162 times more imidacloprid in milkweed nectar compared to a seed treatment, where most research has focused. These higher residue levels caused significant mortality in both 1Xand 2X treatments at day 12 in three lady beetle species, *Coleomegilla maculata*, *Harmonia axyridis*, and *Hippodamia convergens*, but not a fourth species, *Coccinella septempunctata*. Survival and fecundity of two nymphalid butterfly species, monarch, *Danaus plexippus* and painted lady, *Vanessa cardui*, were not reduced when free-ranging butterflies foraged on 1X and 2X treated milkweed plants or when butterflies were force-fed lower amounts of imidacloprid (0 ppb, 15 ppb, or 30 ppb imidacloprid). However, larval survival was significantly reduced on plants treated with soil-applied imidacloprid at1X and 2X treatments. Consequently, the use of systemic, neonicotinyl insecticides, such as imidacloprid, increased the insecticide's duration in

pollen and nectar, and increased the insecticide's exposure to beneficial insects, thereby increasing the risk of mortality. Consequently, the use of systemic insecticides is incompatible with the principles of IPM.

KEY WORDS IPM, neonicotinyl, neonicotinoid, imidacloprid, residue, flower nectar, landscape, beneficial insects, lady beetles, butterflies

INTRODUCTION

Integrated Pest Management (IPM) is a decision making process to manage pests that uses cultural, chemical, biological control and biorational insecticides that conserve beneficial insects (Smith and Krischik 1999, 2000). When systemic neonicotinyl insecticides were first registered their use was embraced in IPM due to their low mammalian toxicity (Tomizawa and Casida 2005; Aliouane et al. 2009) and the use of soil applications, thereby reducing the nontarget effects on beneficial insects from foliar spraying. However, the amount and duration of neonicotinyl insecticides in pollen and nectar and their effects on beneficial insects were not studied. After spraying a contact insecticide, new flowers that open will not contain residues. In contrast, systemic insecticides applied to the soil remain in the plant and are expressed in pollen and nectar for a much longer duration (Doering et al. 2004 a,b,c; Maus et al. 2004a; Doering et al. 2005 a,b, CA DPR 2009, Aliouane et al. 2009; Blacquiere et al. 2012, Goulson 2013).

The neonicotinyl class of systemic insecticides can be applied to seeds, soil, bark, and foliage. The neonicotinyl insecticide imidacloprid was first registered in the 1990's and presently is the second most widely used agrochemical in the world (Pollak 2011). Systemic, neonicotinyl insecticides have continued to be registered, with active ingredients thiamethoaxam, clothianidin, dinotefuran, and sulfoxaflor (Cutler et al. 2013), all of which are translocated to pollen and nectar and are highly toxic to bees (EFSA 2012). In the U.S., over 907,185 kg of imidacloprid, clothianidin, and thiamethoxam are used on 58 million ha of 178 million ha of cropland (Pilatic 2012).

Recently, the translocation of systemic neonicotinyl insecticides into nectar and pollen has been suggested as one of the factors contributing to Colony Collapse Disorder (CCD) and the decline in honey bees (Frazier et al. 2011, Blacquiere et al. 2012) and bumblebees (Goulson 2013). In 2013 the European Union's (EU) European Food Safety Authority (EFSA) banned the use of neonicotinyl insecticides on flowering plants for two years to identify the risk that systemic neonicotinyl insecticides caused to bees. The EFSA review paper on the risk of neonicotinyl treatments identified the lack of studies on residue in crops and landscape plants as a deficit (EFSA 2012).

Neonicotinyl insecticide residues in pollen and nectar differ widely depending on the way it is applied to crops and landscapes. Seed treatments result in relatively low levels, less than 10 ppb, in pollen and nectar from use of 0.625 mg imidacloprid on corn and 0.11 mg on canola seed (Gaucho, BayerCropScience, Research Triangle Park, NC) (Goulson 2013, Bonmatin et al. 2005,

Girolami et al. 2009, Krupke et al. 2012, EFSA 2012). Imidacloprid residue in pollen from seed-treatments was 4.4 to 7.6 ppb in canola, 3 ppb in sunflower and 3.3 ppb in maize (Schmuck et al. 2001, Scott-Dupree and Spivak 2001, Bonmatin et al. 2005, EFSA 2012). Imidacloprid residue in nectar from seed-treatments was 0.8 ppb in canola and 1.9 ppb in sunflower (Schmuck et al. 2001, Scott-Dupree and Spivak 2001). Thiamethoxam and clothianidin seed treatments also resulted in low levels in pollen and nectar. Residue in corn pollen contained 1 to 7 ppb thiamethoxam and 1 to 4 ppb metabolite CGA322704 (clothianidin). Thiamethoxam residue in canola was 1 to 3.5 ppb in pollen and 0.65 to 2.4 ppb in nectar, and the metabolite CGA322704 (clothianidin) was not detected (Pilling et al. 2013).

In numerous studies, seed treatments of imidacloprid and clothianidin did not result in residues in pollen or nectar at sufficient levels to reduce honey bee colony health (Cutler and Scott-Dupree 2007, Chauzat et al. 2006, Cresswell et al. 2010, EFA 2012, Goulson 2013, Pilling et al. 2013). A 4 year study on thiamethoxam seed-treatments on corn and canola reported no adverse effects on honey bee colony health (mortality, foraging behavior, colony strength, colony weight, brood development, and food storage levels) and overwintering (Pilling et al. 2013).

The imidacloprid field crop rate is 4 mg/ sq ft (AdmirePro, Gaucho, BayerCropScience, Research Triangle Park, NC) which is a higher rate than what is applied to seed treatments and should result in higher residue in flowering crops. In squash, residues in pollen were 14.7 ppb for imidacloprid and 12.9 ppb for thiamethoxam and in nectar were 10.3 ppb for imidacloprid and 11.6 ppb for thiamethoxam (Stoner and Eitzner 2012). In pumpkin, residues in pollen were 31.8 ppb for imidacloprid, 34.7 ppb for dinotefuran, and 25.2 ppb for thiamthoxam and in nectar were 9.1 ppb for imidacloprid, 7.0 ppb for dinotefuran, and 4.3 ppb for thiamethoxam, with maximums of 122 ppb in pollen and 18 ppb in nectar (Dively and Kamel 2012). There are no field studies that investigate these levels of neonicotinyl insecticides on bee foraging and survival.

In ornamental flowering plants grown for residential landscapes, a soil application of imidacloprid is 300 mg for a 3 gallon pot (Marathon 1%G or Bayer Advanced Tree and Shrub, Bayer CropScience). This is a 400 times higher rate when compared to a seed treatment rate on corn (0.675 mg /seed), and a 75 times higher rate when compared to a field crop rate (4 mg/sg ft rate). In trees, a soil surface drench under the canopy permits 67 g imidacloprid for a 61cm (24 in) diameter at breast height (dbh) tree, and requires 45 g imidacloprid for a 41 cm (16 in) dbh tree. If we calculate the area under a 61cm (24 in) tree to be 1.5 sq m, then the amount of imidacloprid applied is 4,188 mg/sq ft compared to 4 mg/sq ft in agriculture, a 1047 times greater amount. If these higher amounts of insecticide used in urban landscapes are translocated to pollen and nectar, then bees and other beneficial insects should be negatively impacted.

Indeed, Bayer's research on imidacloprid translocation from soil to flowers of landscape plants found very high imidcloprid levels. Doering et al. (2005b) found 1,038–2,816 ppb in dogwood, *Cornus mas*, flowers at 17 months after application. Other studies by Bayer found residues of 27-

850 ppb in rhododendron flowers at 6 months after application, and residues of 19 ppb at 3 to 6 years after application (Doering et al. 2004b,c); residues of 66-4,560 ppb in serviceberry, *Amelanchier* spp., flowers at 18 months after application; residues of 1038-2816 ppb in dogwood, *Cornus mas*, flowers at 17 months after application; and residues of 5 ppb in horsechestnut, *Aesculus hippocastanum,* flowers at 18 months after application (Doering et al. 2004a, 2005a,b; Maues et al. 2004a). The California Department of Pesticide Regulation reported dead bumblebee containing 146 ppb imidacloprid residue when little leaf linden, *Tilia chordate*, were treated with a soil drench of imidacloprid at a golf course (CA DPR 2009).

The initial research performed by USDA APHIS to understand the effects of imidacloprid trunk injections on flowers, found that maple, *Acer* spp., and horse chestnut, *Aesculus hippocastanum*, flowers collected from trees that were trunk injected with imidacloprid 10-12 months earlier had residues of 130 ppb in one sample and 30-99 ppb in five samples. The report went on to discuss the potential of 130 ppb to cause bee mortality (USDA APHIS 2003). Eucalyptus trees treated with an imidacloprid soil injection (Merit 75WP label rate, Bayer CropScience) at five months pre-bloom expressed 660 ppb imidacloprid in nectar (Paine et al. 2011), levels that killed two beneficial wasp parasitoids, the braconid larval parasitoid, *Syngaster lepidus* (oral LC50 288 ppb imidacloprid) and the encyrtid egg parasitoid, *Avetianella longoi* (oral LC50 212 ppb imidacloprid). This is similar to the oral LC50 for honey bees of 185 (CA EPA 2009) to 192 ppb imidacloprid (Fischer and Chalmers 2007). In 2013 in Oregon, linden trees (*Tilia* spp.) sprayed with dinotefuran at flowering caused mortality of 50,000 bumblebees (Oregon Department of Agriculture, 2013). Turf treated with clothianidin (Arena 50 WDG; Valent, Walnut Creek, CA) resulted in residues of 171 ppb in nectar, residue levels that reduced colony health and foraging of the bumblebee *Bombus impatiens*. These studies provide evidence that systemic neonicotinyl insecticides used in urban, residential landscapes can be translocated to pollen and nectar at sufficient levels to alter behavior and later cause mortality in bees and beneficial insects.

Also, other systemic insecticides are translocated to pollen and nectar and are toxic to beneficial insects. When the systemic organophosphate dimethoate (dimethoate EC, 0.1% AI, no company given) was sprayed on foliage it caused 40% mortality when honeybees fed on flower nectar from California bluebell, *Phacelia campanularia,* borage, *Borago officinalis,* and Argentine rape, *Brassica napus*. Nectar from flowers that opened post-spray was toxic to honey bees (Jaycox 1964). In another study, a foliar spray of dimethoate (Cygon 2E, 23.4% AI, American Cyanaimid Company, Wayne, NJ) on containerized alfalfa, *Medicago sativa*, resulted in 16,000 ppb in uncovered florets and 5,000 ppb in covered florets. After 2 weeks, 1,000 ppb dimethoate was found in both covered and uncovered florets. Sucrose water containing 1,000 ppb dimethoate killed 8% of honeybees when fed for 7 days and 10,000 ppb dimethoate killed 82% of honey bees in 1 day (Barker et al. 1980). Aldicarb, a systemic organophosphate insecticide (Temik 15 G, 15% AI, Bayer Crop Science, Research Triangle Park, NC) applied to the soil of container-grown cotton in the greenhouse was translocated to the extrafloral nectaries and reduced the flight response and longevity of the parasitoid *Microplitis croceipes* (Stapel et al.

2000). Metabolites of aldicarb (Temik, no treatment level provided) were found in orange nectar at 3000 ppb (Bullock and Townsend 1992).

There are currently very few published studies investigating imidacloprid residue in flowers from soil-applied landscape rates and imidacloprid's impact on survival of nectar and pollen feeding beneficial insects, such as lady beetles and butterflies. Our objectives are: (1) determine the imidacloprid residue in flowers of *Asclepias curassavica* treated with1X and 2X label rates of soil-applied imidacloprid; (2) determine the effects of soil-applied imidacloprid and its translocation to flowers on survival of four species of adult lady beetles, *Coleomegilla maculata*, *Coccinella septempunctata*, *Harmonia axyridis*, and *Hippodamia convergens*; (3) determine the effects of soil-applied imidacloprid on adult survival, fecundity, and egg viability of nectar feeding free-ranging adult monarch, *Danaus plexippus*, and painted lady, *Vanessa cardui*, butterflies; (4) determine the effects of force-fed imidacloprid (0, 15 and 30 ppb) in 30% sucrose on adult survival, fecundity, and egg viability of butterflies; and (5) determine the effects of soil-applied imidacloprid on butterfly larval survival.

Materials and Methods

Milkweed Plants for Residue Analysis and Butterfly and Beetle Bioassays

Mexican milkweed, *Asclepias curassavica*, has small, open flowers that produce large amounts of nectar that attract beneficial insects (Wyatt and Broyles 1994). Plugs of Mexican milkweed were purchased from North Creek Nurseries (Landenburg, PA), Rush Creek Growers (Spring Valley, WI), and Michell's (King of Prussia, PA). Two plugs were planted in 15-cm-diameter black plastic pots (Belden Plastics, Roseville, MN) containing Sunshine SB 500 Universal growing media (Sun Gro Horticulture, Bellevue, WA). Plants were watered daily, and fertilized weekly with a dilute concentration (1g/100ml) of Peter's general purpose water soluble fertilizer (20%N- 8%P2O5-16%-K2O) (Allentown, PA). Imidacloprid was applied to the soil approximately three weeks prior to the start of the experiment to allow time for translocation. Granular imidacloprid was applied to the soil at the label rate (1X treatment, 300 mg) and twice label rate (2X treatment, 600 mg). Since the Marathon label permits repeated application, and imidacloprid persists for a long time in leaves, we wanted to determine residue in flowers from a second soil application at 7 months after the first application for 1X and 2X treatments (Marathon 1%G, Olympic Horticultural Products, Mainland, PA).

Residue Analysis

Open flowers were collected at mid-day from approximately 30 different milkweed plants (6-10-ml scintillation vials for each plant, each containing around 50 flowers/vial) and stored on ice. Imidacloprid was applied on 18 May 2007: and flowers were collected post application at 26 days (14 June 2007), 37 days (25 June 2007), and 51 days (9 July 2007). A fourth group of plants received a second application of imidacloprid at 7 months after the first application (first applied

26 February and reapplied on 27 September 2007) and flowers were collected 234 days post first application on 19 October 2007.

Vials were stored in an ultralow freezer at -80°C until shipment in October of 2007 to Enviro-Test Laboratory (ALS Laboratory Group, Edmonton, Canada) for determination of levels of imidacloprid parent compound and hydroxy and olefin metabolites. For residue analysis, each sample was added to 1.0 ml of water in a 50 ml culture tube, placed in an ultrasonic bath for 2 min, then placed on a wrist shaker for 2 hr, filtered, partitioned with dichloromethane, filtered, and evaporate to dryness. The residue was dissolved in 20% acetonitrile/0.1% acetic acid and brought to 1 ml, frozen, and then extracted with acetonitrile and concentrated with a rotovaporator. The samples were then analyzed by Liquid Chromatography-Mass Spectrometry LC/MS (PE Sciex API 3200 or 4000 Q-trap system) with variant solvent delivery system, and Agilent Automatic Sample Injector. The operating conditions were a YMC-ODS-AM column, 5 µm particle size, 40 ºC, mobile phase A 0.1% acetic acid in water and mobile phase B 0.1% acetic acid in acetonitrile, flow rate 0.5 ml/min, and injection volume 15 µl. Gradient was 0 min 90% A, 10% B; 6.5 min 30% A, 70% B; 8.0 min 50% A, 50% B; 13 min 90% A, 10% *B.* The standards were received from Bayer CropSciences (Research Triangle Park, NC) (imidacloprid lot no. 0625200305, purity 99.2%; hydroxy lot no. 072620061 purity 96.8%; olefin lot no. 12192000301, purity 79.8%). The spiking standards were prepared in 20% acetonitrile/0.1% acetic acid. Samples were fortified with imidacloprid, hydroxy, and olefin at 0.05 and 0.10 ppm. Retention time was 7.75 min for imidacloprid (mass transition 256 to 209), 7.36 for hydroxy (mass transition 272 to 225) and 7.24 min for olefin (mass transition 254 to 207). The limit of quantification for imidacloprid, hydroxy, and olefin was 0.05 ppm based on a 1.0 g sample and final volume of 1.0 ml. The average recovery of imidacloprid, hydroxy, and olefin was 95%, 74%, and 96% respectively at 0.05, 0.10, and 15 ppm.

After the residue analysis was done in 2007, the lab was sold and could no longer process further samples. In order to make certain that the residue analysis from the ALS laboratory was accurate, in October 2011we had the same samples shipped and analyzed by the USDA AMS Gastonia, NC lab using their Quenchers standard method (Lehotay 2005). The comparison of the residue from the two labs is presented in Table 1.

Lady beetle Adult Survival Bioassays

Three species of lady beetle were collected from the field for bioassays: seven-spotted lady beetle (*Coccinella septempunctata*, Crookston, MN, July 2007), pink lady beetle (*Coleomegilla* maculate, British Columbia, CA, June 2007), and multicolored Asian lady beetle (*Harmonia axyridis*, Rochester, MN, Nov 2007 and overwintered into 2008). In May 2007, convergent lady beetle (*Hippodamia convergens*) were ordered from Rincon-Vitova Insectaries (Ventura, CA). Beetles were reared in mesh cages (30 cm x 30 cm x 30 cm, BioQuip, Rancho Dominquez, CA) and for protein were supplied organic cat food for protein (Wellness brand, beef and chicken canned soft food) and artificial bee pollen (Mann Lake, Hakensack, MN) smeared onto 35 mm

diameter petri dishes (3/cage). Also, petri dishes were filled with apple slices for moisture (3/cage). Four tubes with water (Aquatube, Syndicate Sales, Kokomo, IN) and four tubes with 50% honey-water were provided. The top of the screened cage was covered with thin lines of honey. Beetles fed freely for at least three weeks prior to the bioassays.

Three replicate experiments with adult lady beetles were performed simultaneously. Each experiment had five treatments with at least ten bioassay containers per treatment each containing ten lady beetles. The five treatments were: control flowers-control flowers (C-C), control flowers-1X treated flowers (C-1X label rate), 1X treated flowers -1X treated flowers (1X-1X label rate), control flowers-2X treated flowers (C-2X, twice label rate), and 2X treated flowers -2X treated flowers (2X-2X, twice label rate). Treatments C-1X and C-2X were performed to determine if lady beetles could avoid feeding on flowers from imidacloprid-treated plants. Bioassay containers were 10 cm x 2 cm (diameter) Aquapic (Syndicate Sales, Inc., Kokomo, Indiana) water tubes. In the plastic cap of each water tube a 0.5 ml centrifuge tube with a hinged plastic cap and pointed end was inserted. A hole was made in the pointed end to accommodate a flower stalk. Water was placed inside the centrifuge tube to keep the flower hydrated. Flowers were changed every day to insure food availability. All bioassay chambers were kept in laboratory incubators and maintained under a photoperiod of 12:12 (L: D) h, 25 °C, and 70 to 75% RH. Bioassays were conducted on convergent lady beetle (June 15, 2007), seven-spotted lady beetle (24 August 2007), pink lady beetle (1 August 2007), and Asian lady beetle (1 June 2008).

Butterfly Colonies

Monarch butterfly (*Danaus plexippus*) pupae were purchased from the Monarchs in the Classroom Laboratory (University of Minnesota, St. Paul, MN) and Gulf Coast Butterfly Co. (Naples, FL). Prior to the start of the experiment, newly emerged adult monarchs were checked for protozoan (*Ophryocystis elektroscirrha*) spores as infection can reduce lifespan (Altizer and Oberhauser 1999) and infection is common in laboratory-reared butterflies (Leong et al. 1997). Transparent tape was applied to the abdomen, then placed on a white sheet of paper and evaluated under the microscope at 30-40 X for spores. Diseased monarch butterflies were discarded. Healthy, non-infected monarch butterflies were fed 30% sucrose solution and placed in individual glassine envelopes in an incubator at 11ºC, under 60-70% RH, for a maximum of one week, until released in greenhouse cages. Painted lady butterfly (*Vanessa cardui*) pupae were purchased from Clearwater Butterfly Co. (Chuluota, FL). Pupae were attached with dress pins thru the terminal attachment fibers to coffee filters that were hung on the sides of mesh cages (60 x 60 x 120 cm (tall), BioQuip, Rancho Dominguez, CA) in the greenhouse. All butterfly studies were performed in the greenhouse with 16:8 (L:D) photoperiod and daytime/nighttime temperatures of 20°/16° C.

Butterflies Free-Ranging: Adult Survival, Fecundity, and Egg Viability

Monarch and painted lady butterflies were housed in mesh cages (60 x 60 x 120 cm) that contained six to eight pots of flowering Mexican milkweed. Sponges with 30% honey water were attached to cage frames for butterfly feeding to prevent dehydration in case nectar was limited during the heat of the day.

For monarch butterflies, four replicate experiments were performed, each consisting of three treatments (C, 1X and 2X) with three cages per treatment. Replicate 1began on 5 July 2005 (plants were treated on15 June 2005) and included six male and six female butterflies per cage. Replicate 2 began on 19 August 2005 (plants were treated on 29 July 2005) and Replicate 3 began on 12 September 2005 (plants were treated on 22 August 2005) and both included ten male and ten female butterflies per cage. Replicate 4 began on 17 October 2005(plants were treated on 26 September 2005) and included eight male and eight female butterflies per cage. All experiments ran until ≤10% of the initial population remained. Dead adults were frozen and later sexed. In monarchs, sex was determined by the presence of scent glands on the hind wings of the males (Hausman 1951) and by identifying male genitalia. Data on egg viability and fecundity of free-ranging adult monarch butterflies is lacking, since neonates died on treated plants.

For painted lady buterflies, three replicate experiments were performed, each consisting of three treatments (C, 1X and 2X). Replicate 1 began on 16 May 2007 (plants were treated on 25 April 2007) and included three cages per treatment that contained five to eight butterflies per cage. Replicate 2 and 3 began on 26 June 2007 (plants were treated on 4 June 2007) and included five cages per treatment that contained 16 to 19 butterflies per cage. All experiments ran until ≤10% of the initial population was remaining. Dead adults were frozen and later sexed by inspection of the forelegs; female forelegs have spines that are used in drumming during oviposition, while male forelegs lack this character and are brush-like (Ackery et al. 1998) and by identifying male genitalia. The number of eggs counted on the host plants were divided by number of females alive at the time of egg collection to determine fecundity (every third 3rd day for Replicates 1 to 3). Eggs were cut off the host plant, the foliage around the eggs was trimmed, and the eggs were kept in 150 mm petri dishes on control leaves to determine percentage hatch.

Butterflies Force-Fed Imidacloprid-Treated Syrup: Adult Survival, Fecundity, and Egg Viability

Experimental conditions were the same as for the section above except flowers were removed from control plants and butterflies were force-fed every other day with 30% sucrose solution containing either 0 ppb (C), 15 ppb (1X), or 30 ppb (2X) imidacloprid. The solution was made by adding 0.15 g (1X) or 0.30 g (2X) of analytical grade imidacloprid (99% purity, ChemServices, West Chester, PA) to 1 liter distilled water and then diluted to the correct concentration. Butterflies were force-fed by weighing them down using a 1 cm hex nut on a 96-well tissue culture plate (Linbro/Titertek, Flow Laboratories, Mclean, VA) that held approximately 0.25 ml of sucrose solution. The butterfly proboscis was extended with a pin, and butterflies were allowed to drink from the solution until they withdrew 3 times.

For monarchs, four replicate experiments were performed, each consisting of three treatments (C, 1X and 2X) with five cages per treatment, each containing eight males and eight females. Dates of replicate experiments were: Replicate 1 (19 July 2006); Replicate 2 (28 August 2006); Replicate 3 (22 October 2006) and Replicate 4 (2 March 2007). All experiments ran until ≤10% of the initial population was remaining. Dead adults were frozen and later sexed. The number of eggs counted on the host plants was divided by the number of females alive at the time of egg collection to determine fecundity (every third day for Replicates 1 to 3). Eggs were cut off the host plant, the foliage around the eggs was trimmed, and the eggs were kept in 150 mm petri dishes on control leaves to determine percentage egg hatch.

For painted lady butterflies, three replicate experiments were performed, each consisting of three treatments (C, 1X and 2X) with five cages per treatment. Dates of replicate experiments were: Replicate 1 (2 March 2007) contained 12-16 butterflies per cage, Replicate 2 (31 March 2007) contained 10-18 butterflies per cage, and the Replicate 3 (12 May 2007) contained 13-17 butterflies per cage. All experiments ran until ≤10% of the initial population was remaining. Dead adults were frozen and later sexed. The number of eggs counted on the host plants was divided by the number of females alive at the time of egg collection to determine fecundity (every third day for Replicates 1 to 4). Eggs were cut off the host plant, the foliage around the eggs was trimmed, and the eggs were kept in Petri dishes (150 mm) on control leaves to determine percentage egg hatch.

Host Plants for Butterfly Larval Feeding Experiments

Mexican milkweed was the larval host plant for monarch butterflies. For painted lady butterflies we needed to determine a suitable larval host plant. Painted lady butterflies are polyphagus and larval host plants include thistles (Asteraceae), nettles (Urticaceae) (Garrigan 1994, Janz 2005), soybean and lupine (Fabaceae) (Garrigan 1994, Kelly and Debinski 1998), and hollyhock (Malvaceae) (Janz 2005). In no-choice preference studies, larval plant preference was investigated by cutting nine discs from three leaves and placing them in 150 mm petri dishes for 6 -first-instar painted lady larvae to feed upon. Host plants were: globe thistle, *Echinops ritro*, stinging nettle, Urtica dioica, soybean, *Glycine max*, lupine, *Lupinus* spp., hollyhock, *Alcea rosea*; and dandelion, *Taraxacum officinale* (Leitner's Garden Center, St. Paul, MN; Linder's, Falcon Heights, MN; Gertens, Inver Grove Heights, MN). After 24 hours, the dishes containing globe thistle had the highest total area eaten so it was chosen as the larval host plant. Three plugs of *E. ritro* were planted in 30-cm-diameter black plastic pots containing Sunshine SB 500 Universal growing media in 73 pots. Plants were watered as needed, and fertilized weekly with a dilute (1g/100ml) concentration of Peter's general purpose water soluble fertilizer (20%N-8%P2O5-16%K2O). Plants were started in the greenhouse and granular imidacloprid was applied to the soil at the label rate (1X, 30 g) and twice label rate (2X, 60 g) (Marathon 1%G, 1% AI, Olympic Horticultural Products, Mainland, PA) approximately three weeks prior to the start of the experiment to allow time for translocation of systemic imidacloprid. A twice label rate,

2X, treatment was used since imidacloprid is often reapplied in greenhouses and urban landscapes.

Butterfly Larval Survival

Neonates were placed on whole intact plants that experienced no prior feeding to evaluate larval survival. Imidacloprid was applied approximately three weeks prior to placing neonates on leaves. For monarch and painted lady butterflies, three replicate experiments were performed per species, each consisting of three treatments (C, 1X and 2X) with ten cages per treatment, each containing thirty larvae. For monarchs, dates of replicate experiments and soil treatments were: Replicate 1 (15 March 2007, soil treated 9 February 2007); Replicate 2 (18 April 2007, soil treated 9 March) and Replicate 3 (23 May 2007, soil treated 20 April 2007). For painted lady butterflies, dates of replicate experiments and soil treatments were: Replicate 1 (29 May 2007, soil treated 7 May 2007); and Replicates 2 and 3 (5 July 2007, soil treated 14 June 2007). Larval survival was recorded every three days until 10% of the larvae remained.

Statistical Analysis

Data from replicate experiments were combined for analysis. Survival data presented in the figures and fecundity data were analyzed by ANOVA and Levene's test to determine homogeneity of variance. If variances were unequal, a Welch test was used (JMP, SAS 2005). Also, data were analyzed with one-way ANOVA for treatment, replicate, and replicate by treatment interactions using PROC GLM (SAS 2004). Means were compared with Tukey's HSD test. For adult and larval survival, a repeated measures ANOVA was performed. The samples (cages) were random, the treatments were fixed, and the repeated measure was time (days) (SAS 2004). Comparison of the Canadian and USDA methods of residue analysis was performed with a paired t-test and replicates were combined as they were not significantly different. Residues of imidacloprid and olefin and hydroxyl metabolites were analyzed by one-way ANOVA, Levene’s test, Welch’s test, and means compared with TukeyKramer MRT.

Results

Residue analysis

Residue analysis in our previous papers (Krischik et al. 2007) was performed by the ALS Laboratory in Edmonton, Alberta, Canada , but the lab stopped offering the service in 2011. In order to confirm that the ALS method produced similar residue levels to the currently approved USDA Quenchers Method, the same samples were shipped from ALS lab to the USDA AMS lab in Gastonia, NC. The results are statistically similar for both methods for the imidacloprid parent compound and olefin metabolite, although the hydroxyl metabolite was at higher levels with the Canadian method. However, the olefin and hydroxyl metabolites were both very small, approximately 2 % of the imidacloprid residues (Table 1).

Residue analysis on a composite sample of 1 g Mexican milkweed, *Asclepias*, flowers showed imidacloprid residues of 6.03 ± 1.01 ppm in1X label rate, and 10.4 ± 4.61ppm in 2X label rate flowers (Table 2). A second imiacloprid application 7 months later in September after the first resulted in more than double the imidacloprid residue; 21.67 ± 2.45ppm in 1X label rate, and 45.89 ± 3.74 ppm in 2X label rate flowers (Table 2). Higher imidacloprid residues from a September soil application may be due to slower vegetative growth rates in September and more imidacloprid was concentrated in the flowers rather than spread out over new leaves. Imidacloprid residue in 1X (6.03 ± 1.01 ppm) and 2X (10.4 ± 4.61ppm) treatments were similar to our residue data on buckwheat, *Fagopyrum esculentum*, for 1X (6.60 ± 1.00 ppm) and 2X (12.3 ± 2.70 ppm) 2X (Table 3).

Lady beetle Adult Survival Bioassays

Imidacloprid significantly reduced survival of four species of lady beetles when analyzed for all 14 days (Figure. 1, Table 4, repeated measures).

When analyzed at day 3, two species had significantly lower survival in 1X and 2X treatments than the control, but by day 12, three species had significantly lower survival in 1X and 2X treatments than controls (Table 4, Figure 2). In seven-spotted ladybeetles, *Coccinella*, survival at day 3 and day 12 showed no significant difference between control and imidacloprid treatments Survival in pink lady beetles, *Coleomegilla*, at day 3 showed significant differences in 1X treatments and controls, but survival in 2X treatments was similar to controls. However, by day 12, 1X and 2X treatments were significantly different from controls. Survival in Asian lady beetles, *Harmonia*, at day 3 showed no significant differences among treatments, but at day12 all treatments were significantly different than controls. Survival in convergent lady beetles, *Hippodaemia*, at day 3 and 12, showed significant differences in all imidacloprid treatments compared to controls (Figure 2, Table 4). It did not appear that beetles could detect imidacloprid and avoid feeding on it, since by day 12, survival of beetles in 1X-C and 1X-1X and 2X-C and 2X-2X treatments were not significantly different for the three beetle species (Figure 2, Table 4).

Butterflies Free-ranging and Force-Fed Imidacloprid-Treated Syrup: Adult Survival, Fecundity, and Egg Viability

Imidacloprid did not reduce the survival of free-ranging and force-fed butterflies (Figure 3 repeated measures, 4 and 5). Monarch, *Danaus*, adults, lived longer when fed imidacloprid containing 30% sucrose solution than when free-ranging (Figure 3-5).

Imidacloprid did not reduce the number of eggs produced and egg viability of free-ranging painted lady, *Vanessa*, butterflies and force-fed monarch, *Danaus*, and painted lady butterflies ((free-ranging butterflies (painted lady number of eggs, C = 260.4 ± 53.6, 1X = 278.0 ± 32.8, and 2X = 270.2 ± 44.6; F = 0.15; df = 2, 27; P = 0.8650; and painted lady % egg viability, C = 63.7 ± 3.3, 1X = 63.5 ± 5.9, and 2X = 62.3 ± 4.0, F = 0.03; df = 2, 27; P = 0.9668); force-fed monarch and painted lady butterflies (monarch number of eggs, C = 437.5 ± 51.8, 1X =433.6 ± 57.3, and

2X = 385.3± 56.5; F = 0.79; df = 2, 57; P = 0.4618; and monarch % egg viability, C = 45.9 ± 5.5, 1X = 42.3 ± 5.9 and 2X = 41.4 ± 5.8; F = 1.23; df = 2, 56; P = 0.3029; painted lady number of eggs, C = 260.4 ± 53.6, 1X = 278.0 ± 32.8, and 2X = 270.2 ± 44.6; F = 0.06; df = 2, 27; P = 0.9411; and painted lady% egg viability, C = 70.3 ± 4.0, 1X = 65.8 ± 4.4, and 2X = 76.8 ± 3.7; F = 1.75; df = 2, 27; P = 0.1960)).

Butterfly Larval Survival

Survival of monarch, *Danaus*, and painted lady, *Vanessa*, larvae fed 1X and 2X imidacloprid-treated plants was significantly reduced compared to that of larvae fed controls (Figure 6). By day 14 most monarch larvae on 1X and 2X treatments were killed. Some larvae on control plants lived until day 21, but died before pupating. By day 14 painted lady larval survival was 70% on controls, 43% on 1X, and 19% on 2X treatments. Percentage pupation of painted lady larvae was 22.3 ± 8.0% on controls, 2.5 ± 2.5% on 1X, and 0% on 2X treatments.

Discussion

The Mexican milkweed presented in this paper and the buckwheat residue analysis presented in our lab's 2007 paper (Krischik et al. 2007) were performed by the ALS Laboratory in Edmonton, Alberta, CA. The Mexican milkweed flowers also were analyzed by USDA AMS Gastonia, NC using their Quenchers standard method (Table 1). Both species of plants showed similar amounts of imidacloprid in nectar. In Mexican milkweed residues in 1X treatments were 6.03 ± 1.01 ppm and in 2X were 10.4 ± 4.61 ppm while in buckwheat, residues in 1X were 6.60 ± 1.00 ppm and in 2X were12.3 ± 2.70 ppm (Krischik et al. 2007, Table 3). In Mexican milkweed, a second imidacloprid application 7 months later in September resulted in more than double the imidacloprid residue; imidacloprid residues were 21.67 ± 2.45 ppm in 1X treatments and were 45.89±3.74 ppm in 2X treatments (Table 3). This may be due to slower growth rates in September, which resulted in more imidacloprid that was concentrated in the flowers rather than spread out over new growth.

Residues from landscape applications of soil applied imidacloprid resulted in much higher residue in flower nectar compared to imidacloprid residues in nectar of less than 10 ppb in seed treatments (EFSA 2013, Goulson 2013) or a maximum residue of 121 ppb imidacloprid in pumpkins from a crop application treatment (Dively and Kamal 2012). Although few research papers were found on imidacloprid levels in nectar over time, imidacloprid was documented to have a duration of at least 12 months in leaves in container and urban landscape plants: 12 months on linden (Frank et al. 2007, Johnson and Williamson 2007), poplar (Tenczar and Krischik 2007), and ash (McCullough et al. 2003); 24 months on hemlock (Cowles et al. 2006) and cotoneaster (Szczepaniec and Raupp 2007).

Initially at day 3, lady beetles did not show reduced survival on Mexican milkweed flowers treated with soil-applied imidacloprid, as the beetles were very well fed prior to the start of the experiment. By day 12, imidacloprid reduced survival of three out of four species of lady beetles.

Lady beetles did not avoid feeding on imidacloprid treated flowers as there was not a significant difference between 1X-1X and 1X-C and 2X-2X and 2X-C treatments. Consequently, soil-applied imidacloprid is translocated to pollen and nectar and will kill flower feeding beneficial insects, such as lady beetles.

In previous studies in our laboratory, the parasitoid, *Anagyrus pseudococci* (Krischik et al. 2007), and the green lacewing (Rogers et al. 2007) had altered behaviors, such as trembling and lack of coordination that resulted in reduced survival when feeding on buckwheat flowers and Mexican milkweed flowers from plants treated with a 1X and 2X landscape rate of soil-applied imidacloprid (Krischik et al. 2007). The pink lady beetle when confined in mesh cages on flowers showed reduced survivorship (sunflower) and reduced movement on three plant species (sunflower, *Helianthus annus* 'Big Smile'; chrysanthemum, *Chrysanthemum morifolium* 'Pelee'; and dandelion, *Taraxacum officinale*, Smith and Krischik 1999).

In this study, imidacloprid did not appear to effect survival of adult monarchs or painted lady butterflies that were either free-flying or force-fed. The use of 15 ppb (1X) and 30 (2X) ppb imidacloprid treatments for treated sugar syrup experiments was substantially less than what free-ranging butterflies were experiencing on intact plants of 6, 030 ppb(1X)and 10,400 ppb (2X). Imidacloprid may not alter behavior in butterflies, as they may not metabolize the insecticide, instead excreting it unchanged, as was shown for phytochemicals present in the host plant of *Papilio glaucus* (Frankfater et al. 2005) and *Orgyia leucostigma* (Kopper et al. 2002). Survival for adult monarch butterflies force-fed imidacloprid was higher than free-ranging butterflies, but painted lady butterflies survived similarly in both studies. It is possible that monarch butterflies could not forage adequately in cages for plant nectar experiments, perhaps due to interference with cage walls, as monarch are twice the size of painted lady butterflies.

Larval painted ladies and monarchs by 7 days had significantly higher mortality when feeding on leaves treated with 1X and 2X landscape rates compared to controls. Recent studies demonstrate that volunteer plants growing near seed-treated crops can contain imidacloprid residues (Krupke et al. 2012, Goulson 2013). There is growing concern that seed treatments have no economic benefit to plants, but a very costly environmental benefit to beneficial insects. In its review, the Center for Food Safety (2014) reports that 19 scientific peer-reviewed studies that looked at whether seed treatments increased yields, the number of bushels they can grow per acre, eight found no improvement and 11 said results were inconsistent.

The risk of pollen from transgenic corn landing on milkweed plants and killing monarch butterflies was critically evaluated by numerous researchers after Losey et al. 1999 demonstrated 56% mortality in monarch larvae fed for 4 days on Mexican milkweed leaves dusted with one type of transgenic corn pollen. That type of transgenic corn was no longer planted and others did not have the same mortality on monarch larvae (Sears et al. 2001). The potential risk of mortality from imidacloprid is higher for monarch larvae feeding on milkweed plants near seed-treated crops.

Few studies outside our laboratory document the effects of soil-applied imidacloprid on nectar and pollen-feeding beneficial insects. A few studies showed indirect effects on beneficial insects when attacking prey insects feeding on imidacloprid-treated plants. The minute pirate bug, *Orius insidiosus* died when feeding on imidacloprid seed-treated soybeans (Seagraves and Lundgren 2012). Lab studies with *Vicia faba* plants treated with soil-applied imidacloprid showed that *Hippodamia undecimnotata* had reduced survival and egg numbers when fed aphids reared on treated plants (Papachristos and Milonas 2013). Trees treated with imidacloprid decreased predatory mite numbers and altered their behavior, but phytophagous mite numbers increased (foliar sprays, Beers et al. 2005; trunk injection, Szczepaniec et al. 2011). Also, imidacloprid trunk injections decreased feeding and mobility of predatory insects, such as the spidermite destroyer, *Stethorus punctillum* and green lacewing, *Chrysoperla* rufilabris (Szczepaniec et al. 2011).

Residues from foliar sprays of systemic insecticides killed or altered behavior of non-target beneficial insects. In citrus, foliar sprays of imidacloprid was found to reduce survival of lacewings (*Chrysoperla* sp.) and red scale parasitoids (*Comperiella bifaciata* and *Aphytis* sp.) (Mo and Philpot 2003).The label rate of imidacloprid foliar spray (240 FS, 240 g AI/L, Mobay Corp., Pittsburg, PA), when applied to petri dishes and left to dry for 48-72h, resulted in 84.2% mortality of big-eyed bug G. punctipes and 78.3% *Hippodamia convergens*, in 48-72 h (Mizell and Sconyers 1992). The label rate of imidacloprid foliar spray (35 SC, 35% AI, 0.25L/ha, Nanjing Essence Fine Chemical Co., China) when applied to a petri dish and left to dry, killed 37% of 2 d old green lacewing larvae (Rezaei et al. 2006). The searching and resting behavior of the seven-spotted lady beetle was disrupted by dimethoate residues on bean plants, *Vicia faba* (Singh et al. 2001). After dimethoate and acephate were sprayed in a lemon orchard for citrus thrips, populations of the parasitoid, *Aphytis melinus*, were reduced for 3 weeks, and populations of predatory mite *Amblyseius tularensis* were reduced for 11 weeks, while phytophagous citrus red mite *Panonychus cirri* populations increased (Philips et al. 1987).

Six days after dimethoate was sprayed on cereal grains, a beneficial chrysomelid beetle, *Gastrohysa polygoni*, experienced up to 76% mortality and survivors had reduced fecundity (Kjaer and Jepson 1994).

Abamectin (Vertimec 1.8% EC, 1.8% AI, Syngenta, New Ryde, NSW, Australia) and chlorfenapyr (Rampage 10% SC, 10% AI) sprayed in test tubes and allowed to dry, killed 68% (abamectin) and 100% (chlorfenapyr) of the parasitoid *Cotesia plutellae*, a parasitoid of the diamondback moth, *Plutella xylostella* (Miyata et al. 2001). Dry spray residues of the label rate of pymetrozine spray (1 kg/ha of 25WP) in petri dishes resulted in 37% mortality of two day old green lacewing larvae, and 50% reduction in adult egg production (Rezaei et al. 2006). Aphids sprayed in the field with dimethoate and later fed to predators in lab bioassays, killed 100% of larvae and 65% of adult seven-spotted lady beetles (Singh et al. 2004).

Imidacloprid has been shown to reduce foraging and colony health of bees. The actual estimated oral imidacloprid LD50 for foraging honeybees is 185 ppb (CA EPA 2009) and 192 ppb (Bayer, Fischer and Chalmers 2007). Oral toxicity of imidacloprid to honey bees was 370 ppb at 72 h, while the olefin metabolite was more toxic (290 ppb) and the hydroxy metabolite less toxic (2060 ppb) compared to imidacloprid (Suchail et al. 2000, 2001). Bayer Chemical researchers demonstrated that there was no effect on honey bees at <20 ppb (Schmuck 1999, Schmuck et al. 2001), while at levels >20 ppb behavior was changed, as measured by a reduction in recruitment to food sources (Schmuck 1999). Imidacloprid reduced the orientation of honey bees at 25 ppb (Lambin et al. 2001). Foraging bees reduced their visits to feeders containing imidacloprid-treated syrup at 6 ppb (Colin et al. 2004) and 50 ppb (Kirchner 1999). Reduction in recruitment was postulated as a result of decrease in effectiveness of dances at the hive to recruit bees (Kirchner 1999).

In field studies honey bee foraging was reduced at 15 ppb imidacloprid (Schneider et al. 2012), 5 ppb clothianidin (Schneider et al. 2012), and 67 ppb thiamethoxam (Henry et al. 2012). Foraging was reduced at 10 ppb imidacloprid for *Bombus terrestris* (Gill et al. 2012, Mommaerts et al. 2010) and 30 ppb imidacloprid for *B. impatiens* (Morandin and Winston 2003).Whitehorn et al. 2012 showed that queenright colonies of *B. terrestris* fed 0.7 and 1.4 ppb imidacloprid in sugar syrup for 2 weeks in the lab and then monitored in the field for 6 weeks, could not recover from imidacloprid effects, colony weight was lower by 8% and 12% and queen production by 85% and 90%, respectively, compared to controls. *Bombus impatiens* displaced away from their nests in the field were impaired in their ability to orient to landmarks after being fed 5 ng/bee (50 ppb) imidacloprid (Averill 2011). Gill et al. 2012 found that *B.terrestris* fitted with RFID (radio frequency identification tags) and fed 10 ppb imidacloprid in sugar syrup for 4 weeks had significantly more workers (50%) that did not return to the colony. Worker foraging performance, particularly pollen collecting efficiency, was significantly reduced which led to increased colony demand for food as shown by increased worker recruitment to forage and less time spend on brood care. In the field, imidacloprid seed-treated sunflowers reduced return of *B. terretris* by 10% (Tasei et al. 2001). Larson et al. 2012 found that queenright colonies of *B. impatiens* did not avoid foraging on clothianidin-treated clover (114 ppb nectar) and showed reduced foraging activity and increased worker mortality in the hives within five days. Colonies showed a trend for fewer workers and males, no queen production, reduced number of wax pots, and reduced colony weight compared to controls. Reduced colony weight is related to worker foraging and behavior.

The European Union's Food Safety Authority review paper on the risk of neonicontinyl treatments identified as a knowledge deficit the lack of studies on residue in crops and landscape plants (EFSA 2012). This study shows that the translocation of imidacloprid from soil (300 mg) to flowers of Mexican milkweed produced imidacloprid residue in nectar of 6,030 ppb for 1X and 10,400 ppb for 2X treatments. Consequently, landscape use of imidacloprid applied to flowering plants can result in 697 to 1,162 times more imidacloprid in milkweed nectar

compared to a seed treatment, where most research has focused. These residue levels caused significant mortality from both 1Xand 2X treatments at day 12 in three lady beetle species, *Coleomegilla maculata*, *Harmonia axyridis*, and *Hippodamia convergens* as well as monarch and painted lady larvae. Consequently, the use of systemic, neonicotinyl insecticides, such as imidacloprid, increased the risk of mortality in beneficial insects. Systemic insecticides are not compatible with pollination, biocontrol and IPM programs and discontinuing their use systemic merits advocacy.

Figure 1. Survival of adults of four species of lady beetles that were fed flowers from Mexican milkweed, *Asclepias curassavica*, plants that were untreated (C-C) or treated with 1X (1X-C, 1X-1X) or 2X (2X-C, 2X-2X) label rate of soil-applied imidacloprid (Marathon 1%G). Flowers were presented in opposite ends of a tube cage (Table 4, repeated measures, *Coccinella* survival, F = 2.8; df = 4, 440; P = 0.0273; *Coleomegilla* survival, F = 8.9; df = 4, 540; P = 0.0001; *Harmonia* survival, F = 10.1; df = 4, 880; P = 0.0001; and *Hippodamia* survival, F = 103.2; df = 4, 820; P = 0.0001).

Figure 2. Survival of adults of four species of lady beetles at days 4 and 12 that were fed flowers from Mexican milkweed, *Asclepias curassavica*, plants that were untreated (C-C) or treated with 1X (1X-C, 1X-1X) or 2 X (2X-C, 2X-2X) label rate of soil-applied imidacloprid (Marathon 1%G). Flowers were presented in opposite ends of a tube cage (Table 4, day 3, *Coccinella*

survival, F = 0.6; df = 4,105; P = 0.6435; *Coleomegilla* survival, F = 3.7; df = 4,103; P = 0.0071; *Harmonia* survival, F = 1.4; df = 4,135;P = 0.2516; *Hippodamia* survival, F = 23.9; df = 4,135; P = 0.0001; day 12, *Coccinella* survival, F = 1.2; df = 4,105; P = 0.3271; *Coleomegilla* survival, F = 37.5; df = 4,103; P = 0.0001; *Harmonia* survival, F=18.4; df = 4,135; P = 0.0001; *Hippodamia* survival, F = 50.4;df = 4,135; P = 0.0001). Beetles did avoid feeding on imidacloprid, since by day 12, survival of beetles in 1X-C and 1X-1X and 2X-C and 2X-2X treatments were not significantly different for the three beetle species.

Figure 3. Survival of adults of two species of butterflies that were free-ranging and fed on nectar from flowering Mexican milkweed, *Asclepias curassavica* (AC), that was untreated (C), treated with label rate (1X) and twice label rate (2X) of soil-applied imidacloprid (Marathon 1%G) or when force-fed 30% sucrose solution (S) containing 0 ppb (C), 15 ppb (1X) and 30 ppb (2X) imidacloprid (repeated measures, free ranging monarch, *Danaus*, survival, F = 0.46; df = 2, 99; P = 0.6314; free ranging painted lady, *Vanessa*, survival, F = 1.86; df = 2, 150; P = 0.1606; force-fed monarch survival, F = 2.45; df = 2, 360; P = 0.0878; force-fed painted lady survival, F = 1.28; df = 2, 225; P = 0.2799).

Figure 4. Survival of adults of two species of butterfly at days 7, 15, 21, 29 for monarch and painted lady butterflies that were free-ranging and fed on nectar from Mexican milkweed, *Asclepias curassavica*, that was untreated (C), treated with label rate (1X) and twice label rate (2X) of soil-applied imidacloprid (Marathon 1%G) (free ranging monarch, *Danaus*, survival day 7, F = 2.77; df = 2, 29, P = 0.09; day 15, F = 0.70; df = 2, 29. P = 0.51; free ranging painted lady, *Vanessa*, survival day 7, F = 0.43; df = 2, 39, P = 0.43; day 15, F = 0.29; df = 2, 39, P = 0.65; Day 21, F = 0.70; df = 2, 39, P = 0.29; Day 29, F = 0.04; df = 2, 39, P = 0.75).

Figure 5. Survival of adults of two species of butterfly at days 7 and 15 for monarchs and days 7, 15, 21, and 29 for painted lady butterflies that were force-fed 30% sucrose solution containing 0 ppb (C), 15 ppb (1X) and 30 ppb (2X) of imidacloprid (force-fed monarch, *Danaus*, survival day 7, F = 0.39; df = 2, 57, P = <0.68; day 15, F = 0.34; df = 2, 57, P = 0.72; day 21, F = 0.15; df = 2, 57, P = 0.86; day 29, F = 0.10; df = 2, 57, P = 0.27; force fed-painted lady, *Vanessa*, survival day 7, F = 0.13; df = 2, 27, P = 0.88; day 15, F = 0.35; df = 2, 27, P = 0.71; day 21, F = 0.07; df = 2, 27, P = 0.93; day 29, F = 0.08; df = 2, 27, P = 0.93).

Figure 6. Survival of larvae at days 7, 14, and 21 for monarchs, when fed Mexican milkweed, *Asclepias curassavica*, and painted lady, butterflies when fed, globe thistle, *Echinops ritro*, that was untreated (C), treated with label rate (1X) and twice label rate (2X) of imidacloprid (Marathon 1%G). Monarch, *Danaus*, larval survival day 7: F = 631.1; df = 2, 147, P = 0.0001; day 14: F = 620.4; df = 2, 147, P = 0.0001; day 21: F = 200.5; df = 2, 147, P = 0.0001; Painted lady, *Vanessa*, larval survival day 7: F = 8.27; df = 2, 33, P = 0.0014; day 14: F = 4.71; df = 2, 33, P = 0.0167; day 21: F = 6.04; df = 2, 33, P = 0.0062; and repeated measures, larval survival monarch, F = 511.10; df = 2, 600; P 0.0001; repeated measures, larval survival painted lady, F = 3.76; df = 2, 144; P = 0.0262).

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Table 1. Imidacloprid, hydroxy, and olefin residue (ppm) in nectar extracted from 1g of Mexican milkweed *Asclepias curassavica* flowers after a 1X and 2X soil application of imidacloprid (Marathon 1%G). Comparison of residue analysis performed at ALS Laboratories, Edmonton, Alberta, CA. and USDA AMS Lab, Gastonia.

USDA-AMS-NSL (ppm)				**ALS, Canadia (ppm)**		
trt	**Imid**	**5-Hydroxy**	**Olefin**	**Imid**	**5 - Hydroxy**	**Olefin**
Replicate 1						
untreated	0.0121	0	0	0	0	0
untreated	0.0135	0	0	0	0	0
untreated	0.0216	0	0	0	0	0
untreated	5.46	0.208	0.453	3.63	0.338	0.186
Replicate 2						
untreated	0.0117	0	0	0	0	0
untreated	0.0060	0	0	0	0	0
untreated	0.0091	0	0	0	0	0
untreated	0.0184	0	0	0.04	0	0
Replicate 1						
1X	7.57	0.157	0.386	8.20	0.565	0.405
1X	10.40	0.177	0.495	9.40	0.565	0.390
1X	6.43	0.241	0.471	4.80	0.720	0.500
1X	5.44	0.198	0.410	5.10	0.510	0.360
1X	7.57	0.157	0.386	8.20	0.565	0.405
Replicate 2						
1X	8.80	0.527	0.946	24.00	3.400	2.900
1X	22.50	1.470	2.540	26.00	3.500	2.700
1X	32.50	1.590	2.340	34.00	4.600	2.700
Replicate 1						
2X	14.70	0.393	0.504	16.00	0.710	0.410
2X	15.60	0.436	0.609	18.00	0.940	0.710
2X	8.02	0.256	0.437	6.40	0.300	0.200
2X	6.93	0.192	0.460	4.60	0.250	0.180
2X	43.30	1.230	2.520	68.00	8.200	4.300
Replicate 2						
2X	36.10	1.990	2.520	42.00	6.100	2.700
2X	53.70	2.680	3.140	42.00	5.900	3.200
2X	34.00	1.790	2.110	32.00	4.200	1.900
2X	43.30	1.230	2.520	68.00	8.200	4.300
Paired t-test	1.1087	3.2989	1.6533	--	--	--
n	24	24	24	--	--	--
P	0.2790	0.0031	0.1119	--	--	--

Table 2. Imidacloprid, hydroxy, and olefin residue (ppm) in nectar extracted from 1g of Mexican milkweed *Asclepias curassavica* flowers after a 1X and 2X soil application of imidacloprid (Marathon 1%G). Residue analysis was performed at ALS Laboratories, Edmonton, Alberta, CA.

Treatments	**Rep 1 21-d**	**Rep 2 37 d**	**Rep 3 51 d**	**Rep 1-3 mean 21-51 d**	**Rep 1-3 Mean 2nd trt 7 mos mean 234 d**
Imidacloprid (ppm mean ± SE)**					
untreated	0.0 ± 0.0c	0.00 ± 0.00b	1.20 ± 1.20c	0.40± 0.40c	0.00 ± 0.00c
1X	8.00 ± 0.85b	5.33 ± 0.68a	4.87 ± 0.67b	6.03 ± 1.01b	21.67 ± 2.45b
2X	17.00 ± 1.41a	7.33 ± 3.30a	9.07 ± 0.38a	10.40 ± 4.61a	45.89 ± 3.74a
ANOVA, *F*, (df) *P*	259.20 (2,4) 0.0001	11.39 (2,6) 0.0091	22.72 (2,6) 0.0016	25.86 (2,22) 0.001	79.00 (2,24) 0.0001
Levene's Test *F*, (df), *P*	0.02, (2,4) 0.0000	7.4702, (2,6) 0.0091	3.79, (2,6) 0.0864	5.60, (2,22) 0.0107	11.22 (2, 24) 0.042
Welch's Test *F*, (df), *P*	182.64 (2,1.33) 0.0236	79.59 (2,3) 0.0042	24.84 (2,3.44) 0.0090	37.23, (2,12) 0.001	103.36, (2,10) 0.0001
PROC GLM, *F*, (df), *P* trt	NA	NA	NA	102.60, (2,4) 0.0001	82.94 (2,4) 0.0001
F, (df), *P* rep	NA	NA	NA	17.03, (2,4) 0.0001	1.52 (2, 4) 0.2447
F, (df), *P* trt x rep	NA	NA	NA	8.46, (2,4) 0.0007	1.04, (2,4) 0.4153
Hydroxy metabolite (ppm mean ± SE)					
untreated	0.00 ± 0.0b	0.00± 0.00a	0.11± 0.11a	0.00 ± 0.00b	0.00 ± 0.0c
1X	0.56 ± 0.3a	0.55 ± 0.48a	0.64 ± 0.23a	0.61 ± 0.08a	3.07 ± 0.35b
2X	0.83 ± 0.16a	0.62 ± 0.16a	0.62 ± 0.06a	0.65 ± 0.10a	5.97 ± 0.36a
ANOVA *F*, (df), *P*	67.47 (2,4) 0.0008	4.32 (2,6) 0.0688	4.07 (2,6) 0.0713	19.84 (2,22) 0.0001	102.21 (2,24) 0.0001

Levene's Test *F*, (df), *P*	0.00, (2,4) 0.0000	11.62 (2,6) 0.0086	4.49 (2,6) 0.0642	2.40 (0.22) 0.1142	1.07 (2, 24) 0.3590
Welch's Test *F*, (df), *P*	66.77 (2,1.33) 0.0441	42.95 (2,3) 0.0552	6.93 (2,3.44) 0.0632	28.21 (2,12) 0.0001	90.86 (2,14) 0.0001
PROC GLM, *F*, (df), *P* trtl	NA	NA	NA	18.12, (2,4) 0.0001	118.68, (2,4) 0.0001
F, (df) *P* rep	NA	NA	NA	0.26, (2,4) 0.7744	1.39, (2,4) 0.2755
F, (df) *P* trt x rep	NA	NA	NA	0.44, (2,4) 0.7788	1.77, (2,4) 0.1781
Olefin metabolite (ppm mean ± SE)					
untreated	0.0 ± 0.0b	0.08 ± 0.1b	0.06± 0.06b	0.03 ± 0.02b	0.0 ± 0.00c
1X	0.39 ± 0.02a	0.33 ± 0.2a	0.31 ± 0.06a	0.37 ± 0.03a	2.37 ± 0.31b
2X	0.58 ± 0.23a	0.42 ± 0.08ab	0.48 ± 0.00a	0.45 ± 0.07a	3.48 ± 0.30a
ANOVA *F*, (df), *P*	15.85 (2,4) 0.0125	6.96 (2,6) 0.0273	18.84 (2,6) 0.0026	27.37 (2,22) 0.0001	50.42 (2,24) 0.0001
Levene's Test *F*, (df), *P*	0.09 (2,4) 0.0001	10.80 (2,6) 0.0103	1.84, (2,6) 0.2389	3.18, (0.22) 0.0613	1.36 (2, 24) 0.2763
Welch's Test *F*, (df), *P*	508.99, (2,1.33) 0.0120	44.96, (2,3) 0.0088	15.64 (2,3.44) 0.0206	46.621 (2,12) 0.0001	38.426 (2,14) 0.0001
PROC GLM *F*, (df), *P* trt	NA	NA	NA	33.07 (2,4) 0.0001	45.07 (2, 4) 0.0001
F, (df), *P* rep	NA	NA	NA	0.68, (2,4) 0.5196	0.35, (2,4) 0.7119
F, (df), *P* trt x rep	NA	NA	NA	1.59 (2,4) 0.2253	0.69 (2,4) 0.6082

Means in the same column followed by different letters are significantly different, Tukey-Kramer MRT,α = 0.05

Table 3. Comparison of imidacloprod, hydroxy, and olefin residue (ppm) in nectar extracted from 1g flowers of Mexican milkweed, *Asclepias curassivica,* and buckwheat, *Fagopyrum esculentum*, flowers after a 1X and 2X soil application of imidacloprid (Marathon 1%G).

	untreated	**1X**	**2X**
Imidacloprid ppm (mean ± SE)			
Buckwheat 21d	0.00 ± 0.00	6.60 ± 1.00	12.30 ± 2.70
Milkweed 21 d	0.40 ± 0.40	6.03 ± 1.01	10.40 ± 4.61
Milkweed, 2nd applic 7 mo	0.00 ± 0.00	21.67±2.45	45.89 ± 3.74
Hydroxy metabolite ppm (mean ± SE)			
buckwheat 21d	0.00 ± 0.00	1.08 ± 0.20	1.94 ± 0.40
milkweed 21 d	0.00 ± 0.00	0.61 ± 0.08	0.65 ± 0.10
milkweed 2nd applic 7 mo	0.00 ± 0.00	3.07 ± 0.35	5.97 ± 0.36
Olefin metabolite ppm (mean ± SE)			
buckwheat 21d	0.00 ± 0.00	0.20 ± 0.10	0.51 ± 0.10
milkweed 21 d	0.03 ± 0.02	0.37 ± 0.03	0.45 ± 0.07
milkweed 2nd applic 7 mo	0.0 ± 0.00	2.37 ± 0.31	3.48 ± 0.30

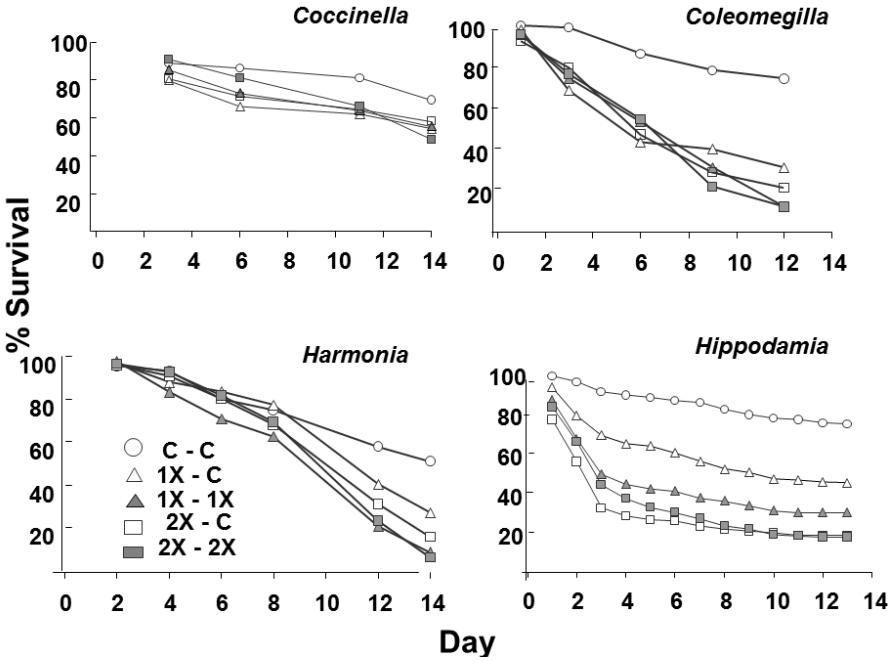
*****data from Krischik, V. A., et. al. 2007. Soil-applied imidacloprid translocated to nectar and kills nectar-feeding *Anagyrus pseudococci* (Hymenoptera: Encyrtidae). Environ. Entomol. 36: 1238-1245.

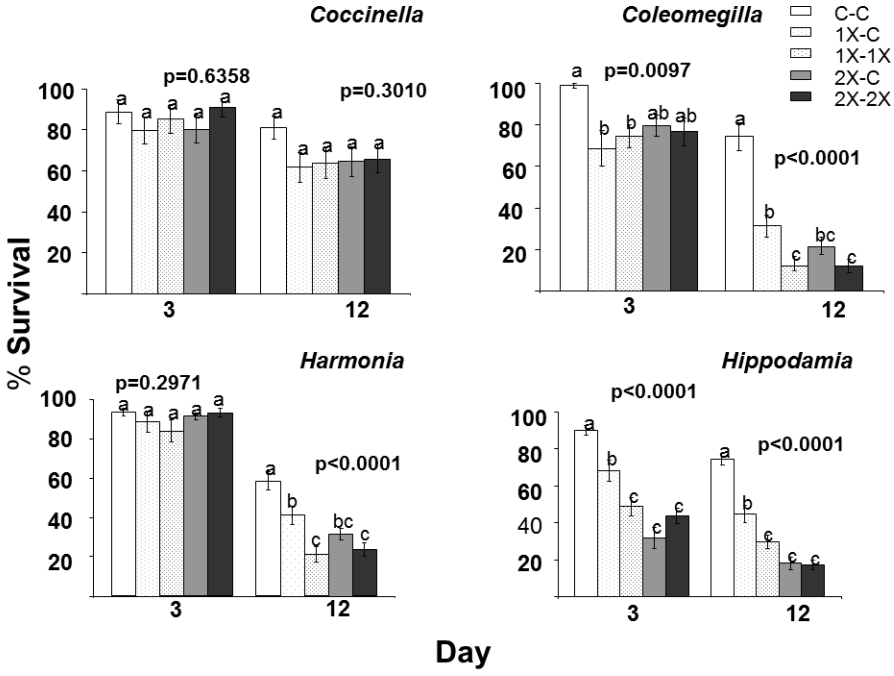
Table 4. Survival of four species of lady beetles that were fed Mexican milkweed, *Asclepias curassavica*, flowers that were untreated (C-C) or treated with 1X or 2 X label rate of soil-applied imidacloprid (Marathon 1%G). Flowers were presented as pairs in opposite ends of a tube cage (1X-C, 1X-1X, 2X-C, 2X-2X).

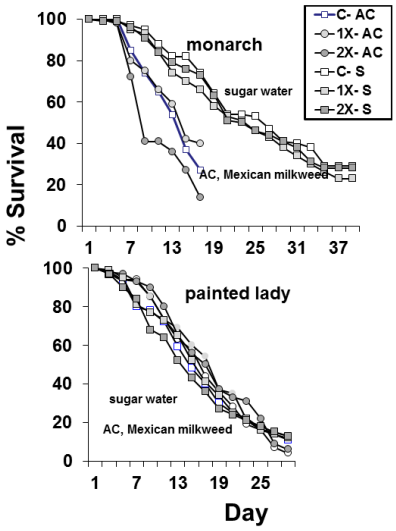
Treatment	***Coccinella***	***Coleo megilla***	***Harmonia***	***Hippodamia***
Day 3 (mean ± SE)				
C-C	88.9 ± 5.7a	98.9 ± 1.1a	93.5 ± 1.8a	90.0 ± 2.5a
1X-C	79.9 ± 6.7a	68.6 ± 8.5b	88.4 ± 5.0a	68.3 ± 5.6b
1X-1X	85.4 ± 6.8a	74.5 ± 5.5b	83.9 ± 5.6a	49.2 ± 4.9c
2X-C	80.6 ± 6.8a	79.6 ± 5.1ab	91.3 ± 1.8a	32.1 ± 5.7c
2X-2X	90.9 ± 4.5a	76.6 ± 6.6ab	99.3 ± 2.3a	43.8 ± 4.1c
ANOVA, *F* (df), *P*	0.6 (4,115) 0.6358	3.5 (4,113) 0.0097	1.2 (4,145) 0.2971	23.2 (4,145) 0.0001
Levene's test *F* (df), *P*	0.9 (4,115) 0.4756	9.4 (4,113) 0.0001	6.5 (4,145) 0.0001	8.0 (4,145) 0.0001

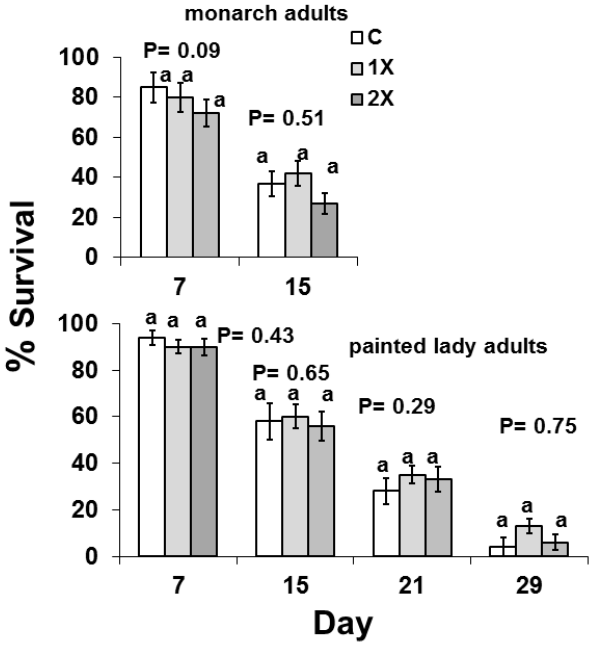
Welch' s test *F* (df), *P*	0.7 (4,57) 0.5856	12.2 (4,49) 0.0001	0.9 (4,70) 0.4487	40.3 (4,70) 0.0001
Model *F* (df), *P*	0.8 (14,105) 0.7156	2.4 (14,103) 0.0071	3.2 (14,135) 0.0002	7.8 (14,135) <0.0001
Trt *F* (df), *P*	0.6 (4,105) 0.6435	3.7 (4,103) 0.0071	1.4 (4,135) 0.2516	23.9 (4,135) 0.0001
Rep *F* (df), *P*	0.7 (2,105) 0.4847	1.9 (2,103) 0.1576	5.7 (2,135) 0.0043	1.3 (2,135) 0.2849
Trt x Rep *F* (df), *P*	0.8 (8,105) 0.5842	1.8 (8,103) 0.0965	3.6 (8,135) 0.0009	1.5 (8,135) 0.1719
Day 12 (mean ± SE)				
C-C	81.3 ± 5.7a	74.4 ± 5.8a	58.5 ± 4.6a	74.6 ± 3.1a
1X-C	61.8 ± 7.3a	31.4 ± 5.3b	41.1 ± 4.8b	45.0 ± 4.7b
1X-1X	63.9 ± 7.5a	12.1 ± 2.6c	21.4 ± 4.1c	30.0 ± 3.6c
2X-C	64.6 ± 7.6a	21.5 ± 4.2bc	31.7 ± 3.2bc	18.3 ± 3.5c
2X-2X	65.9 ± 7.1a	12.0 ± 3.1c	23.8 ± 3.6c	17.5 ± 2.7c
ANOVA, *F* (df), *P*	1.2 (4, 115) 0.0301	33.9 (4,113) 0.0001	13.9 (4,145) 0.0001	44.1 (4,145) 0.0001
Levene's test *F* (df), *P*	1.5 (4,115) 0.2032	4.2 (4,113) 0.0033	1.5 (4,145) 0.1984	2.8 (4,145) 0.028
Welches test *F* (df), *P*	1.6 (4,57) 0.1893	25.7 (4,55) 0.0001	11.8 (4,72) 0.0001	57.5 (4,72) 0.0001
Model *F* (df), *P*	0.65 (14,105) 0.8159	12.1 (14,103) 0.0001	9.5 (14,135) 0.0001	16.6 (14,135) 0.0001
Trt *F* (df), *P*	1.2 (4,105) 0.3271	37.5 (4,103) 0.0001	18.4 (4,135) 0.0001	50.4 (4,135) 0.0001
Rep *F* (df), *P*	0.5 (2,105) 0.6296	1.5 (2,103) 0.2345	22.8 (2,135) 0.0001	2.9 (2,135) 0.0589
Trt x Rep *F* (df), *P*	0.4 (8,105) 0.8963	2.2 (8,103) 0.031	1.7 (8,135) 0.1166	3.1 (8,135) 0.0027
Repeated Measures (mean ± SE)				
C-C	81.4 ± 3.5a	87.6 ± 2.3a	72.0 ± 1.5a	83.8 ± 1.0a
1X-C	65.5 ± 3.5b	56.3 ± 2.2b	67.8 ± 1.5b	58.8 ± 1.0b
1X-1X	69.4 ± 3.5b	53.4 ± 2.2b	57.8 ± 1.5c	42.7 ± 1.0c

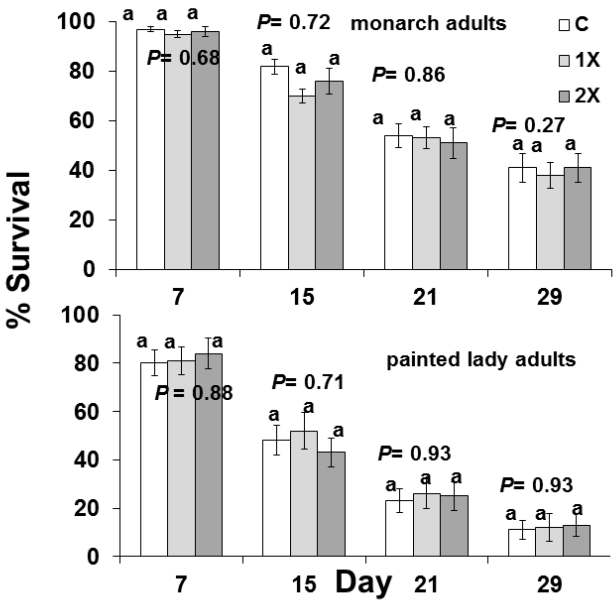
2X-C	68.7 ± 3.5b	54.0 ± 2.2b	67.2 ± 1.5b	29.4 ± 1.0d
2X-2X	71.7 ± 3.5b	52.3 ± 2.2b	59.6 ± 1.5c	33.4 ± 1.0e
Days ***F* (df), *P***	1.5 (3,440) 0.2146	29.4 (4,540) 0.0001	52.1 (6,880) 0.0001	17.1 (12,1820) 0.0001
Trt ***F* (df), *P***	2.8 (4,440) 0.0273	8.9 (4,540) 0.0001	10.1 (4,880) 0.0001	103.2 (4,1820) 0.0001
trt*days ***F* (df), *P***	0.4 (12,440) 0.9736	1.3 (16,540) 0.177	2.5 (24,880) 0.0001	0.9 (48,1820) 0.6998
Rep ***F* (df), *P***	0.9 (1,440) 0.3392	13.3 (1,540) 0.0003	65.8 (1,880) 0.0001	52.4 (1,1820) 0.0001
rep*days ***F* (df), *P***	0.1 (3,440) 0.9469	1.8 (4,540) 0.1191	15.4 (6,880) 0.0001	0.8 (12,1820) 0.6835
rep*trt ***F* (df), *P***	2.5 (4,440) 0.0446	1.8 (4,540) 0.1284	7.1 (4,880) 0.0001	32.9 (4,1820) 0.0001
Rep*trt*days *F* (df), *P*	0.2 (12,440) 0.9962	0.8 (16,540) 0.6879	1.9 (24,880) 0.0057	0.4 (48,1820) 0.9997

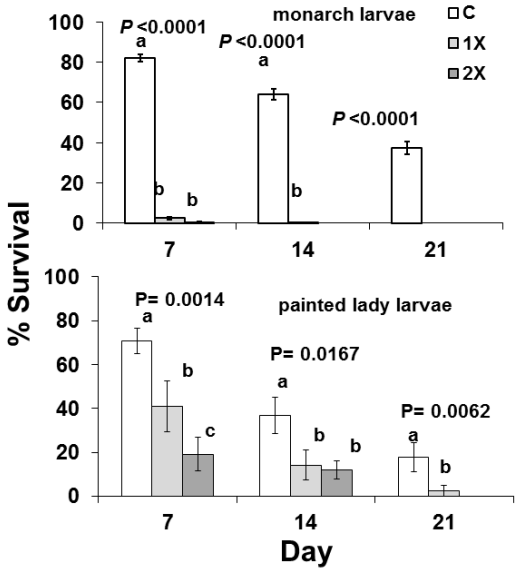

Coccinella
Coleomegilla
Harmonia
Hippodamia
% Survival
Day
C - C
1X - C
1X - 1X
2X - C
2X - 2X


Coccinella
Coleomegilla
Harmonia
Hippodamia
% Survival
Day
C-C
1X-C
1X-1X
2X-C
2X-2X
p=0.6358
p=0.3010
p=0.0097
p<0.0001
p=0.2971
p<0.0001
p<0.0001
p<0.0001


C- AC
1X- AC
2X- AC
C- S
1X- S
2X- S
monarch
sugar water
AC, Mexican milkweed
painted lady
sugar water
AC, Mexican milkweed
% Survival
Day


monarch adults
C
1X
2X
P= 0.09
P= 0.51
painted lady adults
P= 0.43
P= 0.65
P= 0.29
P= 0.75
% Survival
Day


% Survival
monarch adults
C
1X
2X
P= 0.72
P= 0.68
P= 0.86
P= 0.27
painted lady adults
P= 0.88
P= 0.71
P= 0.93
P= 0.93
Day
7
15
21
29


% Survival
monarch larvae
C
1X
2X
P <0.0001
P <0.0001
P <0.0001
painted lady larvae
P= 0.0014
P= 0.0167
P= 0.0062
Day
7
14
21