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Research article

Laboratory Evaluation of the Toxicity of Systemic Insecticides to Emerald Ash Borer Larvae

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Abstract

Emerald ash borer (*Agrilus planipennis* Fairmaire) (Coleoptera: Buprestidae), an invasive phloem-feeding insect native to Asia, threatens at least 16 North American ash (*Fraxinus*) species and has killed hundreds of millions of ash trees in landscapes and forests. We conducted laboratory bioassays to assess the relative efficacy of systemic insecticides to control emerald ash borer larvae in winter 2009 and 2010. Second- and third-instar larvae were reared on artificial diet treated with varying doses of emamectin benzoate (TREE-äge, Arborjet, Inc., Woburn, MA), imidacloprid (Imicide, J. J Mauget Co., Arcadia, CA), dinotefuran (Safari, Valent Professional Products, Walnut Creek, CA), and azadirachtin (TreeAzin, BioForest Technologies, Inc., Sault Ste. Marie, Ontario, and Azasol, Arborjet, Inc., Woburn, MA). All of the insecticides were toxic to emerald ash borer larvae, but lethal concentrations needed to kill 50% of the larvae (LC_{50}), standardized by larval weight, varied with insecticide and time. On the earliest date with a significant fit of the probit model, LC_{50} values were 0.024 ppm/g at day 29 for TREE-äge, 0.015 ppm/g at day 63 for Imicide, 0.030 ppm/g at day 46 for Safari, 0.025 ppm/g at day 24 for TreeAzin, and 0.027 ppm/g at day 27 for Azasol. The median lethal time to kill 50% (LT_{50}) of the tested larvae also varied with insecticide product and dose, and was longer for Imicide and Safari than for TREE-äge or the azadirachtin products. Insecticide efficacy in the field will depend on adult and larval mortality as well as leaf and phloem insecticide residues.

Key words: emerald ash borer, larval mortality, systemic insecticide, toxicity, lethal concentration

The emerald ash borer, *Agrilus planipennis* Fairmaire, first identified in southeast Michigan, USA, and Windsor, Ontario, Canada, in 2002 (Haack et al. 2002, Cappaert et al. 2005, Poland and McCullough 2006), has become the most destructive and costly forest insect to invade North America (Aukema et al. 2011, Herms and McCullough 2014). To date, populations of emerald ash borer have been found in at least 25 U.S. states and two Canadian provinces (EAB Info 2015). Although emerald ash borer acts as a secondary pest in its native range in Asia, colonizing severely stressed or dying ash trees (Yu 1992), it has killed hundreds of millions of North American ash in urban and forested settings (Herms and McCullough 2014, EAB Info 2015). Interspecific differences in emerald ash borer host preference have been documented (Anulewicz et al. 2007, Pureswaran and Poland 2009, Tanis and McCullough 2012), but virtually all North American and European ash species appear to be threatened (Anulewicz et al. 2008, European and Mediterranean Plant Protection Organization [EPPO] 2013, Herms and McCullough 2014).

Systemic insecticides are used increasingly to protect landscape trees from a wide variety of insect pests, including emerald ash borer. Depending on the product, systemic insecticides may be applied via trunk injection, as a soil drench around the base of the tree, or as a basal bark spray (Herms et al. 2009). Systemic insecticides are translocated within xylem from the roots or base of the tree up the trunk and into canopy branches and leaves (Mota-Sanchez et al. 2009, Tanis et al. 2012, Aćimović et al. 2014). In contrast to cover sprays of broad-spectrum insecticides, systemic products reduce applicator exposure, eliminate concerns about spray drift, and minimize impacts on nontarget organisms.

When emerald ash borer was initially identified, only a few systemic insecticides were registered for ornamental trees. Field trials with those products yielded inconsistent results, and many trees treated annually with these products succumbed 2–3 yr after nearby nontreated trees (McCullough et al. 2005, 2007; Herms and McCullough 2014). Since then, however, several new systemic insecticide products have been developed and application technology has improved.

An emamectin benzoate-based insecticide, first registered in the United States in 2010, is currently the most effective product available (Herms and McCullough 2014), providing two to three years of nearly complete emerald ash borer control (Herms et al. 2010, Smitley et al. 2010a, McCullough et al. 2011, McCullough and Mercader 2012). Many municipalities, as well as private landowners, are now using this product, both to protect individual trees, and in integrated emerald ash borer management programs (McCullough et al. 2015, Mercader et al. 2015). Economic analyses have shown that treating landscape ash trees in alternate years with the emamectin benzoate insecticide is highly cost effective when compared to costs of preemptive tree removal or removing trees as they die (Kovacs et al. 2010, 2014; McCullough and Mercader 2012; Vannatta et al. 2012). Treating a portion of trees in a given area with emamectin benzoate can slow the rate of emerald ash borer population growth and the progression of ash mortality (Mercader et al. 2011, 2015; McCullough et al. 2015).

Other options for protecting landscape ash trees include neonicotinoid products containing imidacloprid or dinotefuran. Imidacloprid products must be applied annually, and efficacy varies considerably depending on the product, application method, and timing (Herms et al. 2009). Dinotefuran, a highly soluble, new-generation neonicotinoid, typically is applied to ash trees as a basal trunk spray and can effectively protect most ash trees if applied annually (McCullough et al. 2011, Herms et al. 2014).

Recently developed azadirachtin insecticides, applied via trunk injection, are also used for emerald ash borer control in the United States and Canada (Herms and McCullough 2014). These products are not toxic to adult beetles, but may impair reproduction and appear to control young larvae. They provide one to two years of tree protection, depending on local emerald ash borer density (McKenzie et al. 2010).

Studies to evaluate the ability of systemic insecticides to protect ash from emerald ash borer have included bioassays with adult emerald ash borer beetles, foliage residue analyses, visual evaluation of tree canopy condition, and quantification of larval density on selected branches or entire trees (McKenzie et al. 2010, Smitley et al. 2010a, McCullough et al. 2011). Adult emerald ash borer beetles feed on foliage throughout their 3–6-wk life span, and females must feed for at least 2–3 wk before oviposition begins. When adult beetles feed on treated trees, foliar insecticide residues can be acutely toxic and kill the beetles rapidly (e.g., emamectin benzoate, dinotefuran), inhibit feeding, and trigger intoxicated or knockdown behavior (e.g., imidacloprid), or reduce viable egg production (e.g., azadirachtin) (McCullough et al. 2011, 2015).

The relative toxicity of systemic insecticide products for emerald ash borer larvae, however, is largely unknown. Larvae feed in serpentine galleries on phloem and cambium (Cappaert et al. 2005), presumably minimizing their exposure to the insecticides in xylem tissue. When movement of ^{14}C-labeled imidacloprid in green and white ash trees was monitored for up to two years postinjection, there was no evidence that imidacloprid moved into phloem (Mota-Sanchez et al. 2009, Tanis et al. 2012). Nevertheless, in studies that involved debarking branches or trees, larval densities were lower on treated trees than on nearby nontreated trees and on trees treated with emamectin benzoate, larvae were frequently absent (Smitley et al. 2010a, McCullough et al. 2011, Herms and McCullough 2014). Reduced larval densities on treated trees are at least partially attributable to mortality of adult females prior to oviposition (McCullough et al. 2011). It seems likely, however, that adult females could feed on leaves of nearby or adjacent nontreated trees, then lay viable eggs on a treated tree. In this situation, a systemic insecticide must affect larvae, as well as adults, to protect the tree from injury. Larval galleries often score the outer sapwood, perhaps exposing larvae to the toxic compounds in the xylem. Our objective was to assess the relative toxicity of emamectin benzoate, imidacloprid, dinotefuran, and azadirachtin to emerald ash borer larvae using artificial diet treated with different doses of each insecticide.

Materials and Methods

We conducted laboratory bioassays with second- and third-instar larvae reared on artificial diet incorporating a range of doses of emamectin benzoate, imidacloprid, dinotefuran, or azadirachtin in winter 2009 and 2010. Artificial diet, which included autoclaved and ground white ash (*Fraxinus americana* L.) phloem, was modified from Blossey et al. (2000) (Table 1). The insecticide formulations tested included TREE-äge (4% emamectin benzoate, Arborjet, Inc., Woburn, MA), Imicide (10% imidacloprid, J. J Mauget Co., Arcadia, CA), Safari (20% dinotefuran, Valent Professional Products, Walnut Creek, CA), TreeAzin (5% azadirachtin, BioForest Technologies, Inc., Sault Ste. Marie, Ontario), and Azasol (6% azadirachtin, Arborjet, Inc., Woburn, MA). Insecticide formulations and blank formulations of inert ingredients for TREE-äge, Imicide, and Safari without the active insecticidal ingredient were supplied by the respective companies. Bioassays were conducted with second- and third-instar larvae carefully extracted from logs

Table 1. Ingredients of the artificial diet for *A. planipennis* larvae

Ingredient	Amount (g)	Sources (vendor)
Portion 1 (preautoclave)		
Deionized water[a]	27.56	
Agar (from *gracillarius*)	8.67	Moorhead and Co.
Yeast extract	7.22	Sigma Aldrich
Sucrose	10.83	BioServ
Wesson's salt	2.53	Bioserv
Casein	11.63	Fonterra
Portion 2 (postautoclave)		
Ash phloem	100	Self made
Vanderzant vitamin mix	3.2	Bioserv
Sorbic acid	0.817	Bioserv
Methyl paraben	0.417	Bioserv
Total	372.88	
Moisture level	61.03%	

[a] Deionized water was reduced by 10 g in Portion 1 (preautoclave), and 10 g of insecticide diluted in deionized water was added to Portion 2 (postautoclave) in the amended diet.

Steps for preparation of artificial diet

1. Disinfect the tools and work area with 70% ethanol.
2. Measure the preautoclave dry ingredients into a clean 1,500-ml beaker, and stir to mix well.
3. Slowly add the deionized water while stirring. Cover the beaker with aluminum foil and autoclave for 15 min, using the slow exhaust cycle to prevent the liquid from boiling over.
4. Measure the postautoclave ingredients keeping the phloem powder separate from the other ingredients.
5. After removing the beaker from the autoclave, keep it covered and let it cool at room temperature for about 5 min. Then remove the foil and stir while adding the postautoclave dry ingredients.
6. Slowly add the phloem powder while stirring until it is all incorporated. Cover the beaker and let the diet cool completely.
7. To complete the mixing process, while wearing disposable lab gloves, knead the diet by hand inside the beaker. At this point it should crumble easily and can be packed into dishes.

cut in October 2009 and December 2010 from infested green ash (*Fraxinus pennsylvanica* Marsh.) trees harvested in large forested parks in Ingham and Clinton Counties, MI. For each bioassay, an equal number of second- and third-instar larvae were assigned to each treatment.

We tested five insecticide doses and a nontreated control, plus a blank formulation control for TREE-äge, Imicide, and Safari. No formulation blank was available for TreeAzin or Azasol; instead, we included a sixth insecticide dose an order of magnitude higher. Insecticide doses were based on preliminary foliar residue levels observed and injection rates used in previous field studies (Phil Lewis, personal communication). Subsequently, McCullough et al. (2011) reported residue levels in ash foliage, determined by ELISA analyses, ranged from 2.2 to 11.1 ppm for TREE-äge, 0.5 to 8.5 ppm for Imicide, and 0.7 to 6.5 ppm for Safari. McKenzie et al. (2010) reported mean foliar residues of TreeAzin in small (<5 cm dbh) ash trees, determined by liquid chromatography-mass spectrometery (LCMS), ranged from 11.2 ppm at 7 d to 0.81 ppm at 55 d postinjection. Doses in our tests ranged from one or two orders of magnitude higher than reported foliar residue levels in field studies, down to a few orders of magnitude lower. This range of doses was designed to produce response curves ranging from little mortality on the lowest dose to complete or nearly complete mortality on the highest dose. Doses ranged from 10 to 0.01 ppm for Imicide and TREE-äge, 50 to 0.05 ppm for Safari, and 100 to 0.01 ppm for the two azadirachtin products (Table 2). Serial dilutions were prepared in distilled water for each insecticide to obtain the range of desired doses.

Diluted insecticides replaced a portion of the water in the artificial diet recipe. Prepared diet was loosely packed into individual small Petri dishes (100 mm × 15 mm) with one larva per dish and 30 to 36 replicates of each insecticide dose. Larvae, which were visible tunneling in the diet, were checked two times per week to determine if they were dead, moribund, or alive. After 4 wk, larvae were removed from the diet and, if alive, placed on fresh diet of the appropriate dose. During the transfer to fresh diet, larvae were weighed, and shed exuviae or evidence of molting was noted. Larvae were allowed to feed for up to 72 d or until all larvae feeding on diet with the highest insecticide doses had died.

Percent mortality for each treatment was corrected for control mortality using Abbott's formula (Abbott 1925). The formulation blank served as the control if available; water served as the control for TreeAzin and Azasol. For each insecticide, lethal concentration (LC_{50}) and lethal time (LT_{50}) values were calculated using probit analysis (PROC PROBIT, SAS V 9.4, SAS Institute 2012). Posttreatment larval weight at the time of transfer to fresh diet after ~4 wk of feeding (transfer weight) was compared among insecticide doses by an analysis of covariance using PROC GLIMMIX (SAS Institute, 2012) with insecticide dose (treatment) as a fixed effect, and initial weight and treatment × initial weight as covariates. The distribution was set as gamma, the link function as log, and the Kenward–Roger's approximation was used to compute the denominator degrees of freedom to correct for bias in general linear mixed models and reduce the Type 1 error rate (Kenward and Rogers 1997, Stroup 2012). Differences among insecticide dose treatments were compared using the Tukey Kramer comparison procedure (LSMeans, SAS Institute 2012). If the treatment × initial weight covariate was significant, then an unequal slopes model was used and means comparisons were evaluated at three different levels of the covariate corresponding to the median, 10th, and 90th percentile values of initial weight. An α level of 0.05 was used for all statistical analyses.

Table 2. Median lethal times (LT_{50}) in days for *A. planipennis* second- and third-instar larvae fed artificial diet treated with various concentrations of emamectin benzoate (TREE-äge), imidacloprid (Imicide), dinotefuran (Safari), and azadirachtin (TreeAzin and Azasol)

Insecticide	Dose (ppm)	*N*	Slope ± SE	LT_{50} (d)	95% CI	χ^2
TREE-äge	1.0	30	2.53 ± 0.64	23.1	17.4–38.2	12.4[a]
	10.0	30	3.67 ± 0.60	14.9	11.8–18.2	14.6[a]
Imicide	0.1	30	1.69 ± 0.25	71.0	57.1–99.5	10.3[a]
	1.0	30	4.55 ± 0.52	48.5	45.3–52.6	3.0[a]
	10.0	30	3.80 ± 0.36	43.8	40.6–47.8	15.4[a]
Safari	0.5	30	1.24 ± 0.21	34.8	27.5–48.1	7.7[a]
	5.0	30	1.96 ± 0.76	55.1	33.3–2360	87.1
	50.0	30	2.45 ± 0.27	27.7	24.5–31.5	11.9[a]
TreeAzin	0.01	30	2.64 ± 1.03	52.2	33.9–849.8	0.13[a]
	0.1	30	3.63 ± 1.10	13.7	4.3–35.0	29.1
	1.0	30	9.24 ± 1.93	12.1	10.7–13.2	0.15[a]
	10.0	30	10.02 ± 2.69	10.9	9.2–11.9	0.01
Azasol	0.01	36	2.33 ± 0.49	64.4	47.7–123.5	8.67[a]
	0.1	36	1.72 ± 0.53	57.3	35.2–962	12.16[a]
	1.0	36	4.86 ± 0.48	15.9	14.4–17.5	8.98[a]
	10.0	36	5.64 ± 0.56	16.3	14.9–17.8	6.7[a]
	100.0	36	6.77 ± 0.84	11.2	10.1–12.3	8.5[a]

[a] χ^2 value not significant at the $\alpha = 0.05$ level, indicating a good fit of the probit model. The observed mortality was not significantly different from the expected mortality generated by the concentration–mortality response curve.

Results

Insecticide Toxicity

All of the insecticides were toxic to emerald ash borer larvae (Tables 2 and 3). The median lethal time to kill 50% (LT_{50}) of the larvae varied with insecticide product and dose. For TREE-äge, the LT_{50} was ~15 d for the highest dose (10 ppm) and 23 d for the 1.0 ppm dose. At the low doses of 0.1 ppm and 0.01 ppm, larval mortality was minimal throughout the entire 60-d bioassay and confidence limits could not be determined.

For the 10.0 and 1.0 ppm doses of Imicide, LT_{50} values were similar, ranging from roughly 44 to 49 d, compared to 71 d for the 0.1 ppm dose (Table 2). Mortality remained low at the lowest dose (0.01 ppm) during the entire trial and confidence limits could not be determined.

For Safari, LT_{50} values were roughly 28 and 35 d for larvae on diet with 50.0 and 0.5 ppm doses, but was 55 d at the 5.0 ppm dose (Table 2), which is comparable to foliar residue levels reported from field studies. Little larval mortality was observed at the lowest dose (0.05 ppm), and the LT_{50} was estimated to be 363 d.

Bioassays with the two azadirachtin products (TreeAzin and Azasol) yielded similar results. The LT_{50} values ranged from 10 to 16 d for doses of 100, 10, and 1.0 ppm (Table 2) and even at the lowest dose of 0.01 ppm, LT_{50} values were 52 d for TreeAzin and 64 d for Azasol (Table 2). At the 0.1 ppm dose, the two formulations were less consistent; LT_{50} values were 13 d for TreeAzin and 57 d for Azasol.

The median lethal concentration required to kill 50% (LC_{50}) of the tested larvae also varied among insecticides and over time. For all insecticides, at early observation dates (<20 d), mortality was low across all doses and confidence limits could not be determined (Table 3). For TREE-äge, the earliest observation period for which the probit model fit the concentration–mortality curve was at day

Table 3. Median lethal concentration (LC_{50}) in ppm of emamectin benzoate (TREE-äge), imidacloprid (Imicide), dinotefuran (Safari), and azadirachtin (TreeAzin and Azasol) for *A. planipennis* second- and third-instar larvae fed insecticide-treated artificial diet

Insecticide	Days of feeding	*N*	Slope ± SE	LC_{50} (ppm)	95% CI	χ^2	LC_{50}/mg (ppm/mg)
TREE-äge	14	30	0.60 ± 0.20	5.5	2.00–46.5	0.13	
	22	30	0.57 ± 0.30	2.04	–	4.27	
	29	30	1.89 ± 0.30	1.3	0.80–2.10	1.14	
	32	30	1.89 ± 0.33	0.34	0.18–0.60	1.63[a]	0.0238[b]
Imicide	50	30	0.59 ± 0.25	1.49	–	7.85	
	63	30	0.63 ± 0.12	0.27	0.09–0.69	1.60[a]	0.0151[b]
Safari	40	30	0.30 ± 0.37	43.69	–	22.58	
	46	30	0.39 ± 0.11	0.55	0.06–2.20	2.52[a]	0.0304[b]
	49	30	0.54 ± 0.10	0.52	0.12–1.46	3.82[a]	0.0287[b]
	56	30	0.60 ± 0.20	0.67	–	7.85	
TreeAzin	11	30	0.75 ± 0.11	3.65	1.70–8.50	2.67	
	16	30	0.77 ± 0.38	0.40	0.008–4.1	2.97	
	24	30	1.53 ± 0.27	0.40	0.02–0.69	1.11[a]	0.0250[b]
	28	30	7.90 ± 374	0.13	–	0.00	
Azasol	12	36	0.51 ± 0.14	28.8	2.15–8661	7.97	
	16	36	0.53 ± 0.13	6.17	0.43–8344	7.96	
	27	36	1.30 ± 0.35	0.32	0.009–7.7	14.02[a]	0.0267[b]
	32	36	1.02 ± 0.28	0.17	0.007–3.26	13.1	

[a] χ^2 value not significant at the $\alpha = 0.05$ level, indicating a good fit of the probit model. The observed mortality was not significantly different from the expected mortality generated by the concentration–mortality response curve.

[b] LC_{50}/mg was calculated by dividing the LC_{50} by the average initial fresh weight of larvae used for the bioassay.

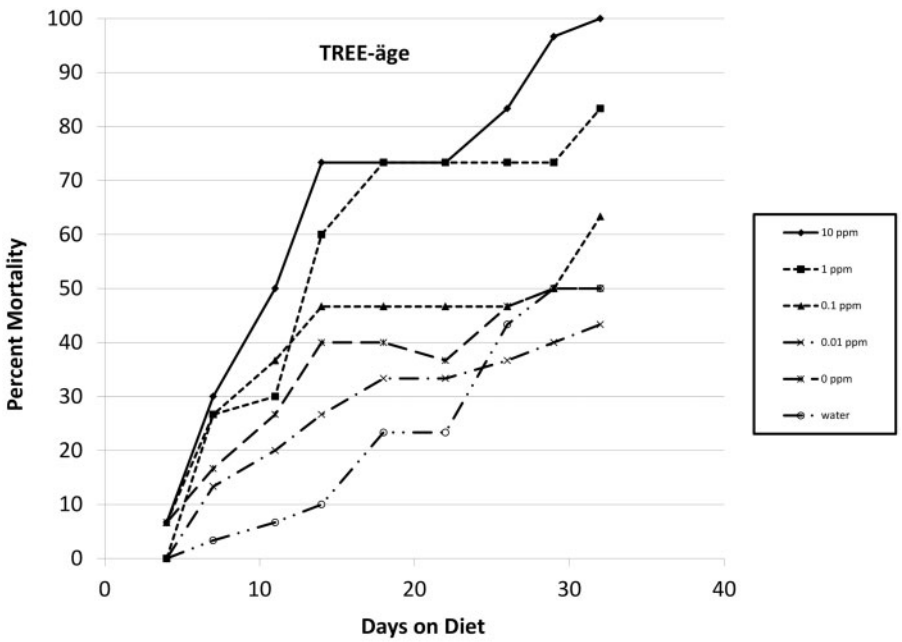

Fig. 1. Cumulative percentage mortality for *A. planipennis* larvae fed on artificial diet treated with various doses of TREE-äge in fall 2009 ($N = 30$).

32 and the LC_{50} standardized by average initial larval weight was estimated to be 0.0238 ppm/g. The LC_{50} values at the earliest observation periods with a good fit of the probit model were 0.0151 ppm/g at day 63 for Imicide, 0.0304 ppm/g at day 46 for Safari, 0.0250 ppm/g at day 24 for TreeAzin, and 0.0267 ppm/g at day 27 for Azasol.

Cumulative Mortality

Cumulative mortality of emerald ash borer increased more rapidly on successively higher doses of all the insecticides. Mortality reached 100 and 83% for larvae fed on diet treated with the two highest doses of TREE-äge (10 and 1 ppm, respectively) by 32 d of feeding (Fig. 1), while larval mortality at the two low doses (0.1 and 0.01 ppm) was similar to that of larvae feeding on the formulation blank (0 ppm) and water control diets (Fig. 1). In diets with Imicide (Fig. 2), 80% of larvae feeding on diet with the two highest doses (10 ppm and 1 ppm) and 70% of larvae on the 0.1 ppm dose diet had died by day 54. At the lowest dose (0.01 ppm), larval mortality on day 54 was only 43%, similar to mortality of larvae on the formulation blank (0 ppm) and water control diet (Fig. 2). Mortality of larvae on diets with Safari exceeded 80% at 45 d of feeding for the

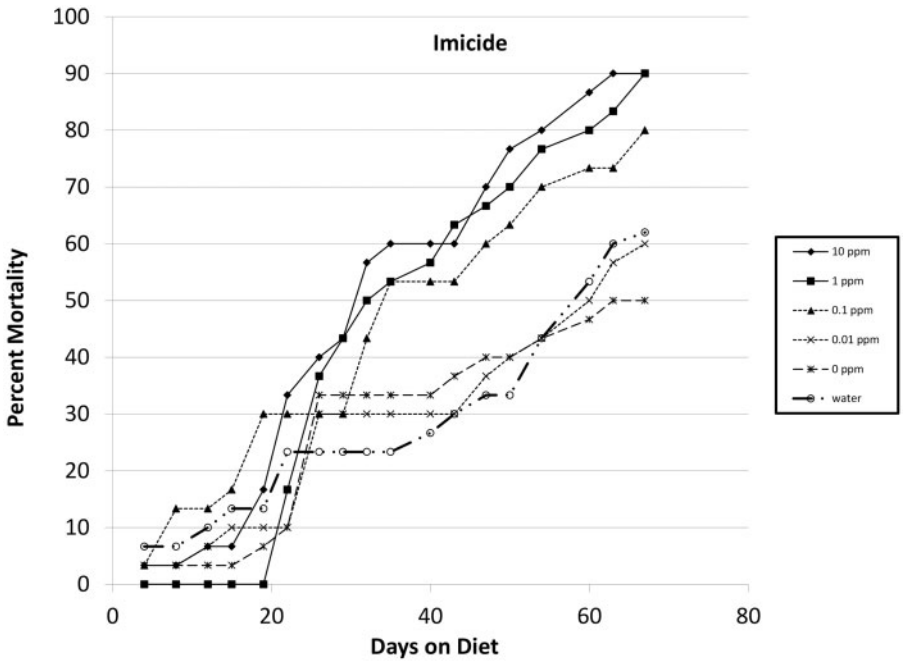

Fig. 2. Cumulative percentage mortality for *A. planipennis* larvae fed on artificial diet treated with various doses of Imicide in fall 2009 ($N = 30$).

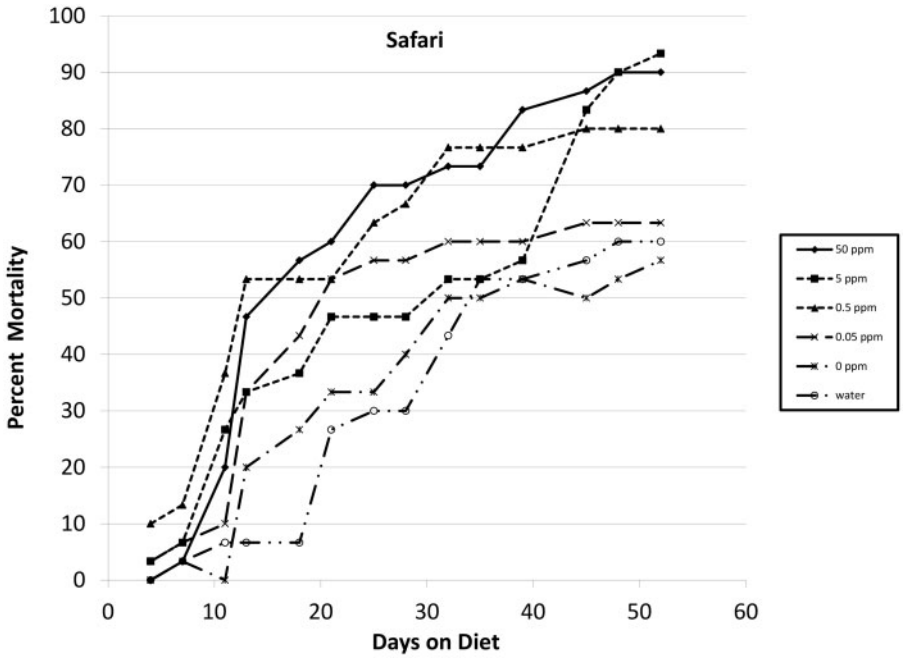

Fig. 3. Cumulative percentage mortality for *A. planipennis* larvae fed on artificial diet treated with various doses of Safari in fall 2009 ($N = 30$).

three highest doses (50, 5, and 0.5 ppm) and was 64% on the lowest dose (0.05 ppm), which was slightly higher than mortality on the formulation blank (56%) and water (50%) control diets (Fig. 3). All larvae on artificial diet treated with the three highest doses (100, 10, and 1 ppm) of TreeAzin had died by day 18. At lower doses, 83% died on the 0.1 ppm dose and 60% died on the lowest dose (0.01 ppm) by day 18, while larval mortality on the control diet was 50% (Fig. 4). Similarly, by day 27, larval mortality on diet with Azasol reached 100% for the highest dose (100 ppm) and 95% for the next two highest doses (10 and 1 ppm) within 27 d (Fig. 5), while mortality on day 27 reached 42 and 36 % on the lowest doses (0.1 and 0.01 ppm, respectively) and 33% on the water control (Fig. 5).

Larval Weight

Larvae that fed on formulation blank (0 ppm) or water control diets for 32 d gained weight, but weight of larvae that fed on diet treated with 1, 0.1, or 0.01 ppm doses of TREE-äge did not increase (Fig. 6). All larvae fed on the highest dose (10 ppm) had died by day 32 when larvae were transferred to fresh diet. Larval weight after 32 d of feeding differed among TREE-äge doses ($F = 8.25$; df = 4,18;

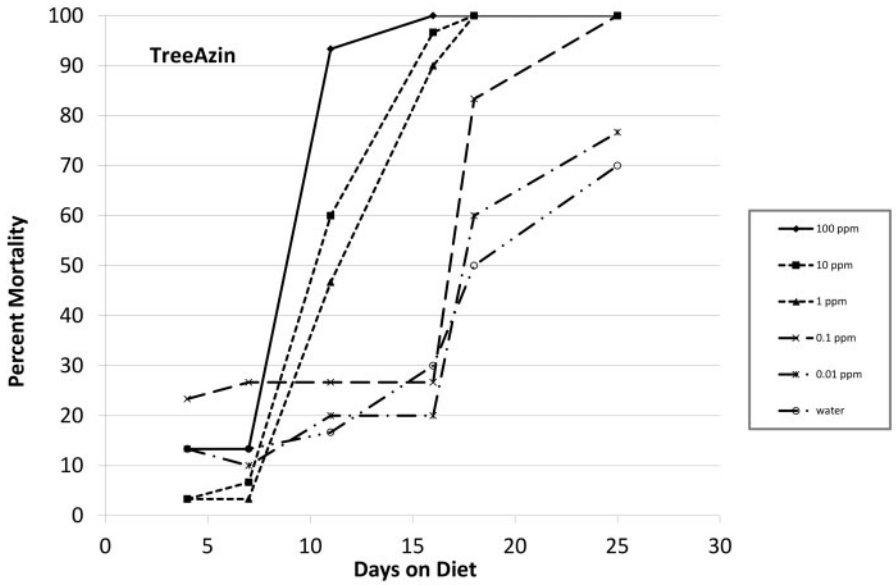

Fig. 4. Cumulative percentage mortality for *A. planipennis* larvae fed on artificial diet treated with various doses of TreeAzin in fall 2009 ($N = 30$).

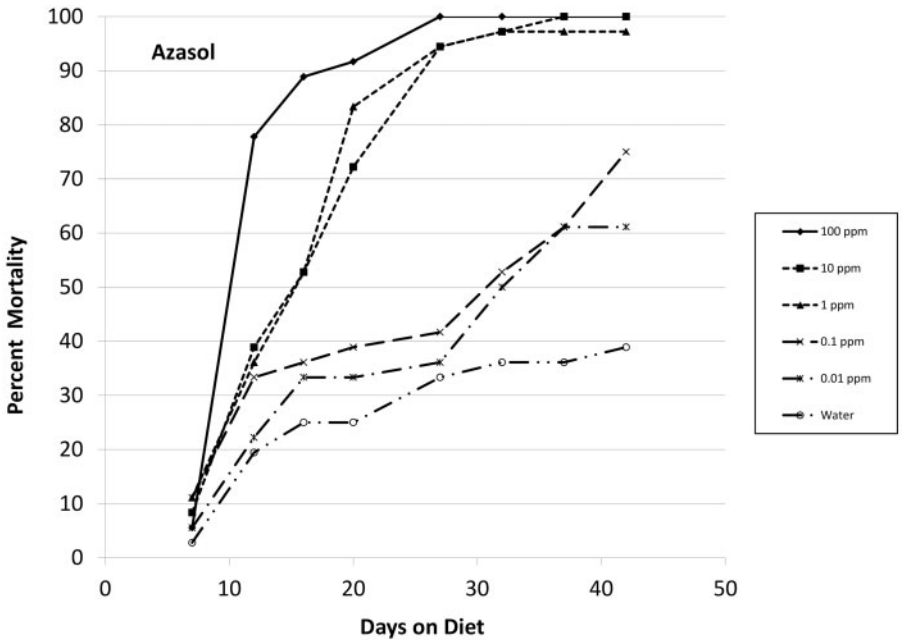

Fig. 5. Cumulative percentage mortality for *A. planipennis* larvae fed on artificial diet treated with various doses of Azasol in fall 2010 ($N = 36$).

$P = 0.0006$). The initial weight covariate was also significant ($F = 145.87$; df = 1,33; $P < 0.0001$), but the treatment × initial weight covariate was not ($F = 1.32$; df = 4,16; $P = 0.3$). Weight on day 32 was greater for larvae on the formulation blank (0 ppm) and water control diets than for larvae fed on diet treated with any dose of TREE-äge (Fig. 6).

Larvae on diet with the formulation blank, water control, or the lowest dose (0.01 ppm) of Imicide had gained weight by day 34 when transferred to new diet, while larvae neither gained nor lost weight on diets treated with higher doses (0.1, 1, or 10 ppm) of Imicide (Fig. 7). Larval weight on day 34 differed among Imicide doses ($F = 45.23$; df = 5, 39; $P < 0.001$). Because both covariate terms were significant (initial weight $F = 97.29$; df = 1,83; $P < 0.0001$; initial weight × treatment $F = 10.62$; df = 5,39; $P < 0.0001$), an unequal slopes model was used and treatment differences were evaluated at three levels of the covariate corresponding to the 10th percentile, median, and 90th percentile for initial weight. At the 10th percentile (i.e., relatively small larvae) and at the median for initial weight, larval transfer weight was significantly greater for larvae fed on the water control diet and diet treated with the lowest dose (0.01 ppm) of Imicide compared to the other treatments, and larval weight on day 34 was greater for larvae fed on the formulation blank (0 ppm) diet than on diets with 0.1, 1, or 10 ppm doses of Imicide. At the 10th percentile for initial weight, larval weight on day 34 was also greater for larvae fed on diet treated with 0.1 ppm Imicide than on diets treated with the 1 or 10 ppm dose.

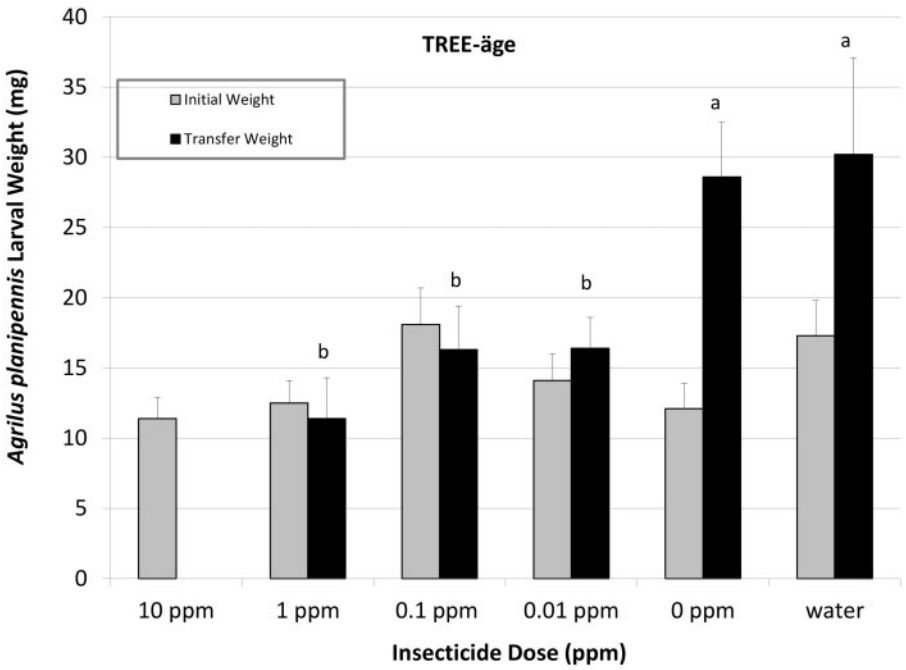

Fig. 6. Mean (+SE) initial larval weight and weight at the time of transfer to fresh diet (transfer weight) of *A. planipennis* fed for 32 d on artificial diet treated with various doses of TREE-äge insecticide. Bars for transfer weight with the same letter are not significantly different among insecticide dose treatments with initial weight as a covariate (Tukey Kramer LSmeans comparison procedure, $P > 0.05$; $N = 30$).

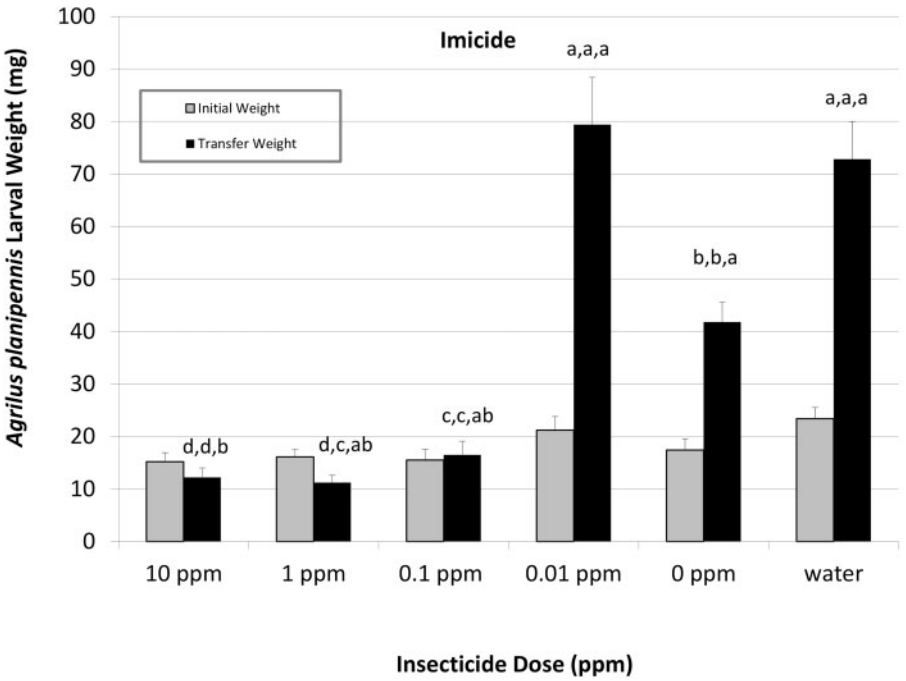

Fig. 7. Mean (+SE) initial larval weight and weight at the time of transfer to fresh diet (transfer weight) of *A. planipennis* fed for 34 d on artificial diet treated with various doses of Imicide insecticide. Bars for transfer weight are topped by three letters to indicate significant differences among insecticide dose treatments at three levels of the covariate corresponding to 10th percentile, median, and 90th percentile for initial weight. Bars with the same letter at a given covariate percentile are not significantly different at that percentile (Tukey Kramer LSmeans comparison procedure, $P > 0.05$, $N = 30$).

At the 90th percentile for initial weight (i.e., relatively large larvae), larval weight on day 34 was greater for larvae fed on the formulation blank, water control and lowest dose (0.01 ppm) of Imicide compared to larvae fed on diets with 0.1, 1, or 10 ppm doses of Imicide (Fig. 7). Differences in larval weight on day 34 among Imicide doses were more pronounced at the 10th percentile for initial weight (smaller larvae at the start of the bioassay) than at the 90th percentile (larger larvae at the start of the bioassay).

Larvae gained weight when fed for 31 d on the formulation blank or water control diet but lost weight when diets incorporated

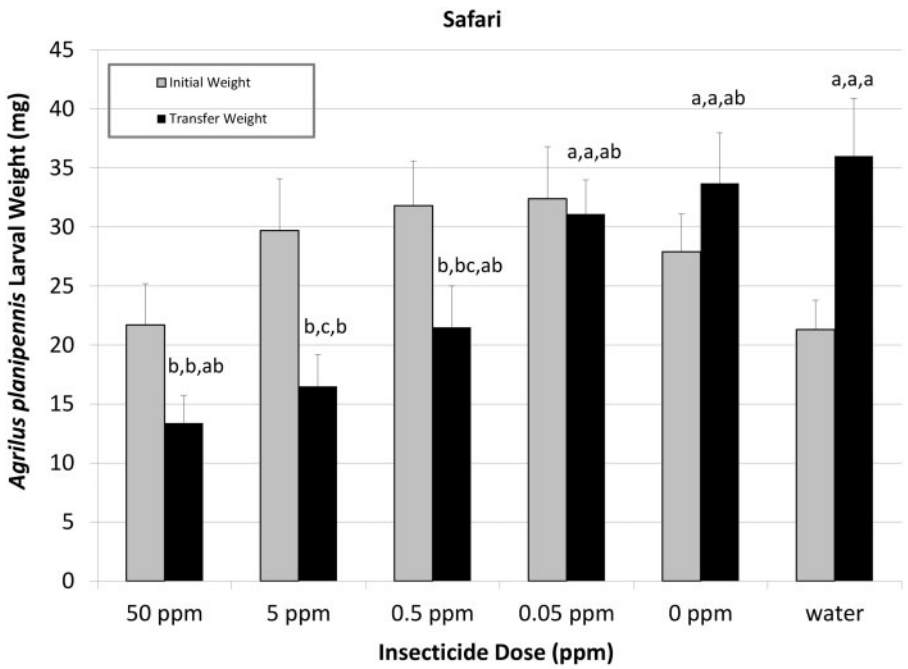

Fig. 8. Mean (+SE) initial larval weight and weight at the time of transfer to fresh diet (transfer weight) of *A. planipennis* fed for 31 d on artificial diet treated with various doses of Safari insecticide. Bars for transfer weight are topped by three letters to indicate significant differences among insecticide dose treatments at three levels of the covariate corresponding to 10th percentile, median, and 90th percentile for initial weight. Bars with the same letter at a given covariate percentile are not significantly different at that percentile (Tukey Kramer LSmeans comparison procedure, $P > 0.05$, $N = 30$).

various doses of Safari (Fig. 8). Larval weight at the time of transfer to fresh diet differed among Safari doses ($F = 14.76$, df = 5,27, $P < 0.0001$). Both covariate terms were significant (initial weight $F = 161.1$, df = 1,57, $P < 0.0001$; initial weight × treatment $F = 4.98$, df = 5,26, $P = 0.003$), so an unequal slopes model was used and means comparisons were analyzed at three levels of the covariate corresponding to 10th percentile, median, and 90th percentile values of initial weight. At the 10th percentile and median for initial weight, larval weight on day 31 was significantly greater for larvae fed on diets with the lowest dose (0.05 ppm Safari), and the formulation blank (0 ppm) or water control diet, compared to larvae fed on diet with 0.5, 5, or 50 ppm doses of Safari. At the median percentile for initial weight, larval transfer weight was lower for larvae fed on the diet treated with the 5 ppm dose than for those fed on diet treated with the 50 ppm dose. At the 90th percentile for initial weight, larval transfer weight was significantly greater for larvae fed on the water control diet than on the diet treated with 5 ppm dose of Safari and was intermediate for larvae fed on all other doses (Fig. 8). Differences in larval transfer weight among Safari insecticide dose treatments were more pronounced at the 10th percentile for initial weight (smaller larvae at the start of the bioassay) than at the 90th percentile (larger larvae at the start of the bioassay).

All larvae fed on diet treated with the four highest doses of TreeAzin (100, 10, 1, and 0.1 ppm) and 77% of those fed on the lowest dose (0.01 ppm) had died by day 25, before larvae were transferred to fresh diet; therefore, transfer weights could not be compared. For the Azasol bioassay, larvae were transferred to fresh diet after 24 d of feeding. Even then, all larvae fed on diet with the two highest Azasol doses (100 and 10 ppm) had died and only one remained alive on the 1 ppm dose. Larvae on the water control diet and diets treated with 0.1 or 0.01 ppm doses of Azasol gained weight and larval weight at the time of transfer to fresh diet did not differ among Azasol doses ($F = 0.25$; df = 2,30; $P = 0.78$; Fig. 9).

Discussion

All of the insecticide products were toxic to emerald ash borer larvae when incorporated into artificial diet at doses similar to foliar residue levels reported in field studies, but the lethal concentrations and time to mortality varied among the insecticide products. Previous research indicated TREE-äge was acutely toxic to adult emerald ash borer (Herms et al. 2009, Smitley et al. 2010a, McCullough et al. 2011). McCullough et al. (2011) reported that up to 97% of adults died within 24 h and 100% died within 4 d of feeding on leaves excised from trees injected with TREE-äge. Density of larval galleries was also significantly lower in TREE-äge-injected trees, which had an average of only one live larva per tree, than in nontreated control trees, suggesting TREE- äge is also toxic to larvae. However, dead larval cadavers were rarely found when entire trees were debarked. Similarly, Smitley et al. (2010a) found no live emerald ash borer larvae in dissected branches of trees treated with TREE-äge and rarely found dead larvae even when treated trees were surrounded by heavily infested, nontreated trees. Although many emerald ash borer adults would be killed after feeding on treated trees, it is doubtful that no eggs are deposited on the trees because adults may also feed on nearby nontreated trees. Therefore, it seems likely that TREE-äge must kill neonates before they have excavated galleries, resulting in cadavers that are too small to find during dissection. Results from our bioassays support this idea. Larvae did not gain weight when fed diet with any dose of TREE-äge, suggesting that they consumed very little diet. Despite little feeding, larvae died fairly quickly, indicating an acute lethal effect of TREE-äge. Similarly, in leaf-feeding bioassays, emerald ash borer adults caged with leaves from trees treated with TREE-äge produced virtually no frass and took only a few bites from leaves before they died (McCullough et al. 2011).

Larvae survived longer on diet treated with the neonicotinoid insecticides, Imicide and Safari, compared to diets with TREE-äge or the azadirachtin products. Cumulative mortality approached but did

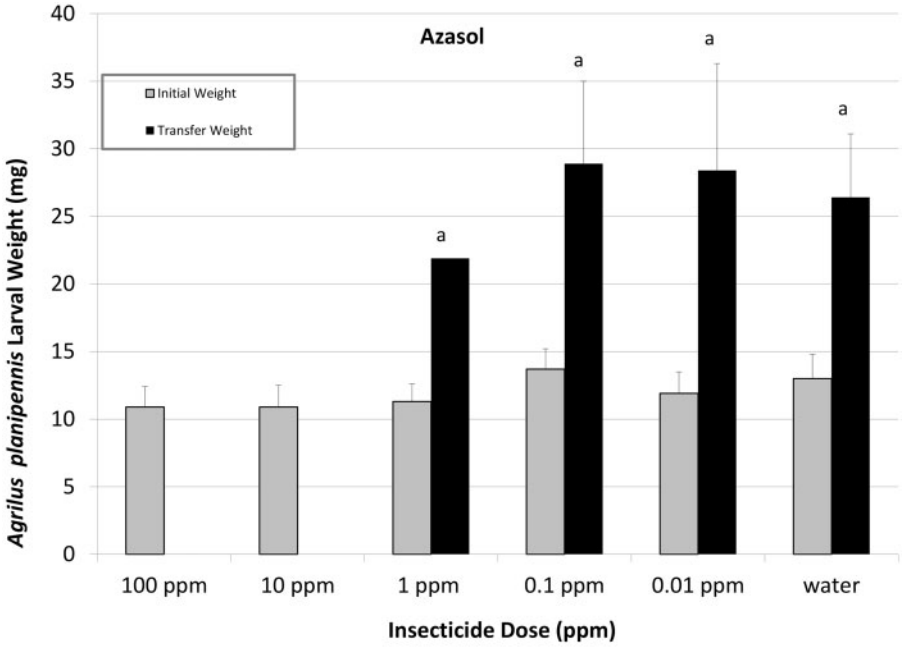

Fig. 9. Mean (+ SE) initial larval weight and weight at the time of transfer to fresh diet (transfer weight) of *A. planipennis* fed for 24 d on artificial diet treated with various doses of Azasol insecticide. Bars for transfer weight with the same letter are not significantly different among insecticide dose treatments with initial weight as a covariate (Tukey Kramer LSmeans comparison procedure, $P > 0.05$; $N = 36$).

not reach 100%, even at the highest doses of Imicide and Safari by the end of the bioassays, indicating the neonicotinoids were less toxic to emerald ash borer larvae than TREE-äge. Similarly, McCullough et al. (2011) reported larval density was 57–68% lower on trees treated for two years with Imicide or Safari than on nontreated control trees, and adult emerald ash borer mortality ranged from 44–65% after 4 d of feeding on leaves from trees treated with the neonicotinoids, as compared to nearly complete mortality in trees treated with TREE-äge. Smitley et al. (2010b) found that canopies of trees treated with a soil drench and trunk injection of imidacloprid appeared healthier than nontreated control trees, but had significantly more larvae in dissected branches than trees treated with TREE-äge.

Antifeedant effects of imidacloprid have been well documented for an array of insect species including Cerambycidae such as Asian longhorned beetle (*Anoplophora glabripennis* (Motchulsky)), and cottonwood borer (*Plectodera scalator* (F.)) (Poland et al. 2006a), the Asian citrus psyllid (*Diaphorina citri* Kuwayama) (Hemiptera: Psyllidae) (Boina et al. 2009), sweetpotato whitefly (*Bemisia tabaci* (Gennadius)) (Hemiptera: Aleyrodidae) (He et al. 2011), and Japanese beetle, *Popillia japonica* Newman (Coleoptera: Chrysomelidae) (Van Timmeren et al. 2011). Our data similarly indicate Imicide acts as an antifeedant for emerald ash borer larvae. Although larvae gained weight on diet treated with the lowest doses of Imicide, they did not gain weight on diets treated with higher doses. Differences in larval weight at the time of transfer to fresh diet among insecticide doses were more pronounced for larvae that were smallest at the start of the bioassay (the 10th percentile for initial weight) compared to larvae that were relatively large (90th percentile for initial weight). This indicates small larvae on the control diet and diet treated with the lowest dose of Imicide continued to grow throughout the bioassay, while larger larvae had approached their final size. Although larvae consuming diet with high concentrations of Imicide did not feed or gain weight, mortality was prolonged and several larvae were not yet dead by the end of our experiment. This is typical of many wood-boring larvae that are able to remain alive with little or no feeding for prolonged periods of time (Linsley 1943, Smith 1962, Haack and Slansky 1986). Similarly, McCullough et al. (2011) found most adult emerald ash borer exhibited signs of intoxication when fed leaves from trees treated with Imicide and took several days to die.

The active ingredient of Safari, dinotefuran, has also been shown to have antifeedant effects in some insects such as the invasive stink bug *Bagrada hilaris* (Hemiptera: Pentatomidae) (Palumbo et al. 2015) and the wheat aphid *Sitobion avenae* (F.) (Hemiptera: Aphididae) (Miao et al. 2014). Our results suggest Safari can act as an antifeedant on emerald ash borer larvae. Larvae gained weight on the control diets but lost weight on diet treated with any dose of Safari. Many adult emerald ash borer fed leaves from trees treated with Safari died after only a few bites, typically after regurgitating, a pattern which was not observed for other insecticide treatments (McCullough et al. 2011). It is possible that regurgitation may explain the weight loss of larvae that fed on diet treated with Safari.

The two azadirachtin products killed larvae relatively quickly at doses similar to typical foliar residue levels recorded in field studies. McKenzie et al. (2010) reported azadirachtin inhibited larval development, reducing adult emergence, but did not cause mortality of adult beetles. Azadirachtin acts as a growth regulator and interferes with molting hormones and larval development in several orders of insects (Rembold et al. 1982). Development of emerald ash borer from egg to fourth instar on similar artificial diets was ~8 wk (Keena et al. 2015), representing an average of about 14 d per developmental instar. The LT_{50} of 11 and 16 d for TreeAzin and Azasol, respectively, and the high cumulative percentage mortality by 18 to 27 d is similar to the time required for development to the next larval instar. Therefore, mortality likely coincided with timing of the next larval molt.

We found emerald ash borer larvae gained weight when fed on diet treated with any dose of Azasol and larval weights at the time of transfer to fresh diet were similar for all Azasol doses and the

nontreated control diet. All larvae had died on the two highest doses and only one larva was alive on the next highest dose (1 ppm) at the time of transfer to fresh diet. Even the single living larva at the 1 ppm dose had gained approximately the same weight as larvae on the nontreated control diet. This suggests that there was no antifeedant or regurgitating effect of the diet, and larvae fed and grew until they died, presumably at the time of molting if a lethal dose had been consumed. Although azadirachtin has antifeedant effects in many insects (Isman et al. 1990), Rembold et al. (1982) found larval weight gains of *Epzlachnu vurivestis* Muls. (Coleoptera: Coccinellidae) fed leaves treated with azadirachtin and *Apis mellifera* L. (Hymenoptera: Apidae) treated with topical azadirachtin applications were comparable to controls.

When small ash trees were injected in a previous study, foliar residue levels of TreeAzin decreased quite rapidly from an average of 11.2 ppm seven days after injection to 0.81 ppm 55 d postinjection (McKenzie et al. 2010). In our bioassays, larvae died relatively quickly on diet treated with either of the two azadirachtin products and LT_{50} values ranged from 10 to 16 d at the 1.0 or 10 ppm doses and from 13 to 52 d at the 0.01 or 0.1 ppm doses. If phloem residue levels are similar to those in foliage and decline at a similar rate, residues could remain at toxic levels long enough for significant larval mortality. McKenzie et al. (2010) found fewer complete galleries in trees injected with moderate doses of TreeAzin (≥1.7 mg (AI)/cm dbh) than in control trees injected with solvent only. They found no complete galleries in trees injected with higher TreeAzin doses (≥13.6 mg (AI)/cm dbh) and no larvae survived beyond the second instar.

Insecticide susceptibility may vary with larval development. For instance, Yu (1983) reported sixth-instar larvae of fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae), were more tolerant of several insecticides than third instars and tolerance was related to developmental changes in mid-gut oxidases. The scarcity of emerald ash borer larval galleries and cadavers in debarked ash trees treated with TREE-äge (Smitley et al. 2010a, McCullough et al. 2011) suggests that neonates must be killed before they have excavated discernable galleries and while they are too small to be recovered. We used second- and third-instar larvae for our bioassays due to difficulty in handling and poor survival of neonates and first-instar larvae on artificial diet. Further research with improved artificial diets is needed to more thoroughly assess insecticide toxicity for neonates and small larvae. Larvae used in our bioassays were dissected from trees cut in late fall, a time when larvae would otherwise begin to cease feeding for the winter. Nevertheless, the larvae placed on diet were active, fed readily, gained weight on the control diet, and survived at rates similar to those in other diet-rearing studies (Keena et al. 2015).

Due to the prolonged mortality of larvae on diets treated with the neonicotinoids, Imicide and Safari, the bioassays were extended for a longer duration than bioassays with TREE-äge or the azadirachtin products, TreeAzin or Azasol. The average number of molts per larva increased with decreasing insecticide dose and larvae survived longer than those on diet with the other insecticides (data not shown). Some larvae survived on the lowest doses of Imicide or Safari for over 60 d and continued to feed and molt. Individual larvae fed the highest dose of Imicide molted only once, whereas some larvae on diet treated with the lowest dose of Imicide molted up to four times. Similarly, no larvae fed the highest dose of Safari molted but at the lowest dose of Safari, larvae molted up to four times. On the control diet, some larvae molted up to five times, indicating that larvae can continue to feed and molt on artificial diet in the absence of cues that induce pupation. Supernumerary molting has been observed for other insects reared on artificial diet. Keena et al. (2010) found that the number of Asian longhorned beetle larvae with extended instars varied with rearing temperature and at 25 and 30°C, several larvae went through 14 to 20 molts. Improved rearing methods for emerald ash borer on artificial diet include a chill period, ~70 d after egg hatch, during which larvae are transferred to 10°C for 84 d then returned to 25°C to induce pupation (Keena et al. 2015).

Probit models for LT_{50}s could not be fit for low doses of any of the insecticides due to high mortality on the control diets (i.e., control-corrected mortality was zero or negative). Although larval mortality on control diet by the end of the bioassay was higher than what we would have preferred, it was comparable to that achieved on similar artificial diets (Keena et al. 2015). Recent improvements in artificial diet and rearing methods (Keena et al. 2015) have improved survivorship to some degree. Nevertheless, mortality in our bioassays was greater on successively higher doses of all of the insecticides, allowing estimation of significant LC_{50} and LT_{50} values.

It is important to note that we cannot assume foliar residue levels reflect phloem residue levels. Systemic insecticides are translocated in the xylem (Mota-Sanchez et al. 2009, Tanis et al. 2012) and reach the phloem either by diffusion across the cambium or through transport in transverse rays. Further research is required to determine if and how systemic insecticides may move to the phloem and whether phloem residue levels are consistently correlated with xylem or foliar residue levels. Insecticide doses used in our bioassays were based on foliar residue levels determined using ELISA for Imicide, Safari, and TREE-äge (McCullough et al. 2011) and LCMS for TreeAzin (McKenzie et al. 2010), typically 3–10 wk after products were applied according to their label rates. Although ELISA is useful for detection and semiquantitative analysis, matrix effects caused by tree tissues and metabolites that interact with the antibody-binding site may cause false positives or overestimate concentrations (Skerritt and Rani 1996, Eisenback et al. 2009). Such effects are minimized by sample dilution. Dilution factors to circumvent matrix effects of ELISA tests vary greatly among different plant tissues; for instance, the minimum dilution factors ranged from 20 for grape vine xylem fluid (Byrne et al. 2005) to 100 for hemlock tissue (Eisenback et al. 2009) to 800 for green peppers (Watanabe et al. 2004). Both LCMS and High Performance Liquid Chromatography (HPLC) provide residue concentrations without matrix effects and allow separate identification and quantification of insecticide metabolites, but require more time and expensive analytical equipment. Poland et al. (2006b) reported mean residue levels determined by LCMS of 0.34 ppm in leaves and 0.33 ppm in twigs of willow trees injected with Imicide at 0.135 g A.I./cm dbh using Mauget capsules. Ugine et al. (2012) injected maple trees with Imicide at 0.87 g A.I./cm dbh and determined residues using ELISA with a dilution factor of 40× and further dilution of 100× to 1,000× for samples with high concentrations. They reported a wide variation in leaf (0–25 ppm) and twig bark (0–12 ppm) residue levels. The ranges of estimated residue levels in ash foliage determined by ELISA with a 20× dilution factor for TREE-äge (2.2–11.1 ppm), Imicide (0.5–8.5 ppm), and Safari (0.7–6.5) (McCullough et al. 2011) were quite similar and were also similar to the range of residue levels determined by LCMS for TreeAzin (0.81–11.2 ppm; McKenzie et al. 2010). The range of treatment doses we tested included intermediate concentrations that fell within the reported ranges for foliar residues and doses that were higher or lower by one or two orders of magnitude.

While all of the insecticide products exhibited toxicity to emerald ash borer larvae, TREE-äge and the azadirachtin products, TreeAzin and Azasol, killed emerald ash borer larvae more quickly

than the neonicotinoids, Imicide and Safari. Although the azadirachtin products killed larvae quickly, they do not appear to kill emerald ash borer adults (McKenzie et al. 2010). On the other hand, TREE-äge is highly toxic to emerald ash borer adults, and Imicide and Safari are also quite toxic to adults (McCullough et al. 2011, Herms and McCullough 2014). The combined effect of adult and larval mortality may enhance the efficacy of these products in the field.

Acknowledgments

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