

Effects of Visual Silhouette, Leaf Size and Host Species on Feeding Preference by Adult Emerald Ash Borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae)

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Abstract

The emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) is an invasive species recently established in North America. In large arena bioassays, when given a choice among live green ash, *Fraxinus pennsylvanica* Marsh and artificial ash saplings that were hidden or exposed from view, beetles preferred live trees (either visible or hidden) compared to artificial trees that had similar visual silhouettes, confirming that olfactory cues are used to locate hosts. Examination of the effect of leaf size revealed that large leaves attracted more beetles than medium-sized leaves that in turn attracted more beetles than small leaves of the same age. Beetles also consumed more of the large leaves in terms of total leaf area than either medium or small leaves, but the proportion of foliage that beetles consumed relative to total available leaf area, did not differ. When newly emerged adults were fed on green and Manchurian ash, *Fraxinus mandshurica* Rupr., foliage in a no-choice assay, beetles that were given green ash consumed significantly more foliage compared to those that fed on Manchurian ash, but neither longevity nor beetle body weight differed. Our results suggest that while beetles might use olfactory cues to identify suitable hosts, visual cues also play a role in landing and feeding behavior. Manchurian ash might have greater nutritive value or resistance than green ash, necessitating lower consumption and therefore less damage in nature.

The emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), an invasive pest of ash *Fraxinus* spp. was discovered in the United States of America in 2002 (Haack et al. 2002, Poland and McCullough 2006). It has killed over 40 million ash trees in Michigan and tens of millions in at least 11 surrounding states (Poland and McCullough 2006, EAB Info 2009). *A. planipennis* is also established in Ontario and Quebec, Canada (CFIA 2009). Feeding and breeding are restricted to *Fraxinus* spp. (Cappaert et al. 2005, Anulewicz 2006). Outbreaks of *A. planipennis* have not been recorded on ash (*Fraxinus* spp.) in its native range in the far east (China, Japan and Korea) (Wei et al. 2004), where it sporadically occurs on Chinese ash, *F. chinensis* Roxb., and Manchurian ash, *F. mandshurica* Rupr. Adults undergo maturation feeding on leaf margins for about two weeks, after which they mate and lay eggs in bark crevices (Bauer et al. 2004). Tree mortality usually occurs when larval feeding within phloem, cambium and outer xylem, of branches and tree trunks disrupts translocation of water and nutrients to the crown (Poland and McCullough 2006).

Herbivorous insects generally use a combination of visual and olfactory cues to locate suitable host plants (Prokopy and Owens 1983, Borden et al. 1986). Long range visual cues such as vertical trunk silhouettes or emitted visual spectra

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from bark may provide general stimuli for landing and intercepting insects in flight (Campbell and Borden 2006). Olfactory cues may be more specific and can help insects discriminate among closely related hosts and nonhosts, particularly when choosing an unsuitable host can have potentially lethal consequences (Pureswaran et al. 2004, Pureswaran and Borden 2005). Suitable hosts may be identified by their distinct attractive volatile profiles or emitted stress volatiles that indicate their susceptibility to colonization. Volatile cues from unsuitable hosts or nonhosts may be repellent by being associated with plant defenses, toxins or antifeedants that herbivores may not have evolved to overcome (Paré and Tumlinson 1999). Integration of visual and olfactory stimuli during the host selection process permits efficient foraging by insects in heterogeneous environments containing a medley of odors (Borden et al. 1986).

The relative role of vision *versus* olfaction in the host and mate finding behavior of *A. planipennis* has received considerable attention over the past few years (Francese et al. 2005, Rodriguez-Saona et al. 2006, 2007, Lelito et al. 2007, Crook et al. 2008a, b, de Groot et al. 2008, Pureswaran et al. 2009a, b), with the goal of employing behavioural strategies of the beetle to manage its populations (Francese et al. 2008). There is evidence that adult *A. planipennis* locate hosts using a combination of visual and olfactory cues. For example, beetles preferred to land on purple panels when box traps with different colored panels were suspended among ash trees, compared to black, green, blue, red, silver, white or yellow, indicating that vision is involved in host location (Francese et al. 2005, 2008). Visual cues are also used in mate finding. Males tend to hover near tree canopies and land directly on other beetles (Rodriguez-Saona et al. 2007), as well as odor-free models, but preferring to remain on females and mating (Lelito et al. 2007). The olfactory potential of *A. planipennis* has been investigated by scanning electron microscopy of antennal sensilla (Crook et al. 2008b) as well as gas chromatographic-electroantennographic detection analyses (GC-EAD) of volatiles from ash trees (Crook et al. 2008a, Rodriguez-Saona et al. 2006, de Groot et al. 2008). Electrophysiological responses were obtained for 16 green leaf volatiles emitted from host trees (Rodriguez-Saona et al. 2006). Manuka oil, which contains four antennally-active sesquiterpenes found in volatiles from ash bark, was found to be attractive to the beetle (Crook et al. 2008a). Beetles also demonstrated feeding preferences in behavioral assays. When given a choice among green ash (*F. pennsylvanica* Marsh), black ash (*F. nigra* Marsh), white ash (*F. americana* L.), blue ash (*F. quadrangulata* Michx.), European ash (*F. excelsior* L.) and Manchurian ash (*F. mandshurica* Rupr.) foliage, they preferred to distribute themselves and feed on green, black and white ash compared to blue, European and Manchurian ash (Pureswaran and Poland 2009b). Manchurian ash from the beetle's native range was clearly the least preferred (Pureswaran and Poland 2009b), implying that it might contain repellent compounds or antifeedants that beetles might be inclined to avoid.

In this study, we investigated the importance of vision and olfaction in the host finding behavior of *A. planipennis* in a large arena bioassay performed outdoors using real and artificial trees that were either hidden or exposed from view, and we tested the effect of adding bark volatiles to hidden artificial trees. We also tested whether a short-range visual cue such as leaf size had a significant effect on beetle distribution and feeding, by giving beetles a choice among small, medium and large leaves of the same species, evergreen ash, *F. uhdei* (Wenzig) Linghesh. We then examined adult performance on a novel host (green ash from North America) *versus* a host from the beetle's original geographic range (Manchurian ash from Asia) by quantifying feeding, body weight and longevity of freshly emerged beetles that fed on these hosts for two weeks.

Materials and Methods

Role of visual silhouette in host location

To examine the effect of visual silhouette in host location in August 2006, we released beetles in an outdoor arena where they could choose among live ash

saplings that were exposed or hidden from view, or artificial trees that were constructed to resemble the visual silhouettes of real trees. Real trees were live green ash that were approximately one meter tall. They were planted in 18 cm long $\times$ 18 cm wide $\times$ 30 cm tall pots with hi-porosity soil mix (Baccto; Michigan Peat Co., Houston, TX), watered daily with a hose until the soil was moist, and fertilized weekly with a 20-20-20 N-P-K Scotts Peters Professional water-soluble fertilizer (Marysville OH). Seedlings were grown in a greenhouse at $\sim 25^\circ\text{C}$, under natural light supplemented with 400W high-pressure sodium lamps. Artificial nursery trees were created using 2.5-cm diameter PVC pipe painted taupe for the stem into which branch and leaf material from an artificial indoor fig tree that possessed dark green, plastic foliage was inserted. The amount and size of the branch and leaf material was similar to live trees. Treatments were 1) real sapling exposed (visual and olfactory cues), 2) artificial sapling exposed (visual cue), 3) real sapling hidden from view behind a vented screen (olfactory), 4) artificial sapling with an ash bark blend (visual and artificial olfactory cues). The bark blend consisted of manuka oil (Coast Biologicals, Ltd., Manukau City, Auckland) released from five 0.4 ml microcentrifuge tubes (Thermo Fisher Scientific, Inc., Waltham, MA) at a rate of 2 mg/day per tube or a total combined release rate of 10 mg/day. We wanted to supply a partial olfactory cue to determine if it increased attraction to an artificial visual cue.

A white paper arena three meters in diameter was constructed using 1.5-m-wide rolled paper supported by T-posts. Treatments were arranged in randomized complete blocks in a ring either inside or outside the circular paper arena, depending on whether they were intended to be visible or not. Real saplings were hidden behind a paper screen that was ventilated with three holes 10 cm in diameter that obscured the visual silhouette but allowed volatiles to permeate through them. The arena was enclosed in a clear plastic canopy two meters high that was supported by metal poles about 45 cm outside the arena to minimize escape of beetles from the arena. The experiment was set up in a grassy field in an agricultural area of Eaton County, MI, with no ash trees within an approximate radius of 1000 m.

Treatments were placed 0.5m apart. One hundred and twenty adult beetles (60 of each sex) that were less than 5 days from emergence and were starved for 24h were released from a Petri dish, from the ground, in the middle of the arena. The number of beetles that had settled on each real or artificial tree, or on the paper screen in front of hidden trees was recorded every 20 minutes for 4h. Four replicates were set up in randomized complete blocks in the arena at any given time, and the experiment was re-randomized and run five times (three morning, 8-12h and two afternoon, 12-16h trials) to obtain a total of twenty replicates. The number of beetles that had settled on each treatment in the large arena bioassay were transformed by $\log_{10}(x+1)$ followed by repeated measures analysis of variance (PROC ANOVA) and the REGW multiple comparisons procedure (Day and Quinn 1989).

Effect of leaf size on feeding

In July 2006, to determine the effect of leaf size on beetle feeding, we compared complete leaves of the same age from three size classes, small ($34 \pm 4 \text{ cm}^2$), medium ($75 \pm 5 \text{ cm}^2$) and large ($161 \pm 13 \text{ cm}^2$). All leaves were collected approximately 4 weeks after flushing and were fully expanded, but contained different number of leaflets that determined the differences in overall leaf surface area. Leaves were cut from greenhouse saplings that were 2-4 years old, and placed individually in glass vials containing water. Each leaf array was arranged in a row in random order, and enclosed within a metal screen cage ($90 \times 90 \times 90 \text{ cm}$). For each of 10 replicates (taken from 10 different trees), 30 male beetles (~ 5 days old) were released into each cage and residence time and leaf consumption were monitored for 48h.

Residence time was measured by recording the number of beetles on each treatment at 2hr intervals for a total of 9 daytime observations over the 48h trial. All observations were made during the day, when beetles are most active (Rodriguez-Saona et al. 2007). Leaf area consumed was determined by comparison of scans right before, and again at the end of the experiment (Winfolia software, Regent Instruments Inc. 2003). The average number of beetles landing on small, medium and large leaves over a 48h period, the total leaf area consumed, and the arcsine square root transformed proportion of leaf area consumed were analyzed by analysis of variance (PROC ANOVA) followed by the REGW multiple comparisons procedure to determine the effects of leaf size on host selection behavior by beetles (Day and Quinn 1989).

Effect of host species (green versus Manchurian ash) on feeding

In July 2007, to assess performance of beetles on a host exotic to them *versus* a native host, we fed freshly emerged adults from the day of emergence on green ash (exotic to the beetle) or Manchurian ash (native to the beetle) foliage, and monitored them until they died. We chose green and Manchurian ash because they were the most and least preferred, respectively, in feeding bioassays (Pureswaran and Poland 2009b).

Forty beetles of each sex were placed individually in 295 ml plastic beverage containers with either a green or Manchurian ash leaf in a vial of water for feeding. Each leaf was changed weekly, and was retrieved after the end of the first and second week. Leaves were scanned before and after feeding at weeks 1 and 2 (as described above) to quantify the area of each leaf consumed. All beetles were weighed individually on emergence, and surviving beetles were weighed again at two weeks to determine the weight change associated with feeding on either species. Beetles were monitored over their lifetime, and mortality was recorded daily to ascertain if feeding on green *versus* Manchurian ash affected beetle longevity. We also tried to mate beetles that were fed on both species to assess fecundity. However, only a few beetles successfully mated, so data were insufficient to draw valid conclusions.

The proportion of green ash versus Manchurian ash foliage consumed was arcsine square root transformed and the total amount and proportion consumed over two weeks were subjected to analysis of variance (PROC GLM) with species and sex as the main effects and a species $\times$ sex interaction. Differences in weight at emergence and at week two were calculated and the number of days that beetles survived was transformed by $\log_{10}(x+1)$ and similarly analyzed. Where there was no effect of sex, data from males and females were pooled for REGW multiple comparisons (Day and Quinn 1989). All analyses were performed using SAS Institute Inc. version 9.1 (2002-2003) statistical software, and $\alpha = 0.05$.

Results

Role of visual silhouette in host location

About 50% of beetles, when released, flew up towards the canopy of the arena and tended to follow the position of the sun during the course of the day. The rest flew around within the arena and either landed on the ground or on the experimental trees. There was a significant treatment effect for every observation across time (Repeated Measures ANOVA: $F = 9.4$; $df = 3, 76$; $P < 0.0001$) (Fig. 1, Table 1). Exposed real trees had the highest number of beetles that settled on them in all 12 observations over four hours (Fig. 1, Table 1). When hidden from view, the number of beetles on real trees was not significantly different from exposed trees in 10 out of 12 observations. Artificial trees had the fewest beetles on them irrespective of whether they were baited with the bark blend (Fig. 1, Table 1). There was no effect of time (Wilk's $\lambda = 0.87$; $F = 0.77$; $df = 11, 66$; $P = 0.67$) nor a treatment $\times$ time interaction (Wilk's $\lambda = 0.68$; $F = 0.82$, $df = 33, 195$; $P = 0.75$) across observations.

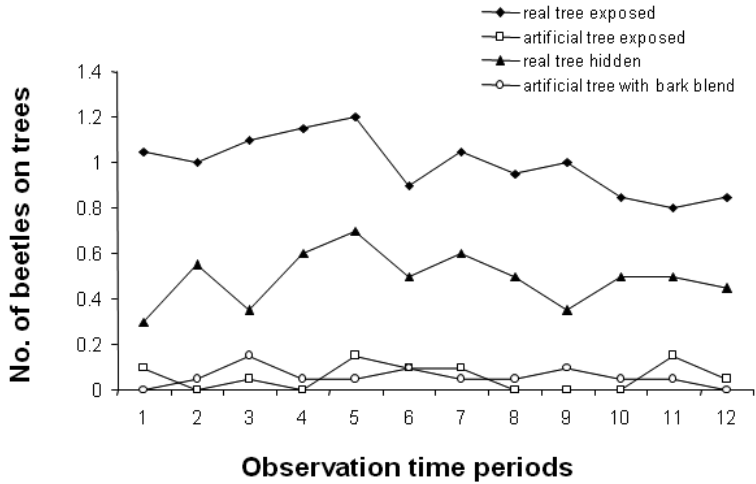


Figure 1
Number of *A. planipennis* observed on treatments with visual and/or olfactory cues. Twelve observations were made at 20 minute intervals over four hours. Four replicates were set up at any given time and repeated five times to obtain a total of twenty replicates. See Table 1 for corresponding results of repeated measures ANOVA and multiple comparisons tests.

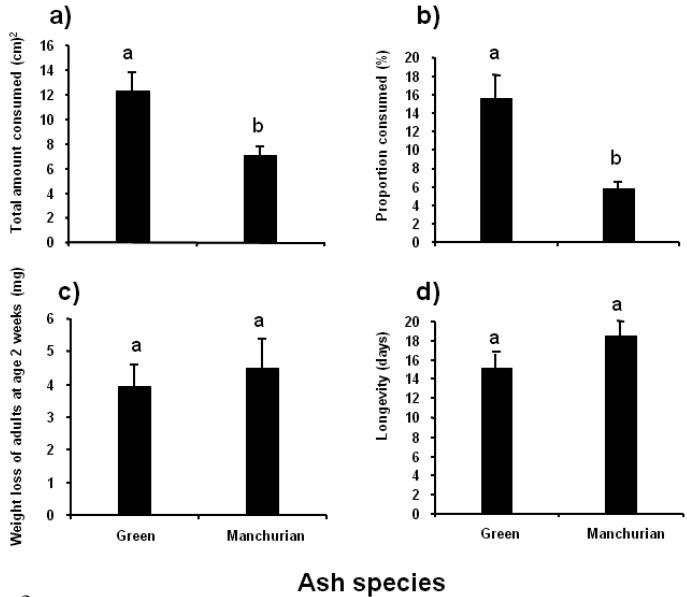


Figure 2
Total amount of foliage consumed (a), proportion of foliage consumed (b), weight loss in adults at age two weeks (c) and longevity (d) of *A. planipennis* when fed from emergence on green *versus* Manchurian ash (n=40). Amount of foliage consumed and weight lost were measured for the first two weeks after emergence. Beetles were monitored until they died for longevity. Bars with the same letter are not significantly different, REGW-multiple comparisons test, $P < 0.05$.

Table 1. Mean (SE) number of *A. planipennis* over time on real or artificial ash trees in the host location experiment. Results of repeated measures ANOVA followed by REGW multiple comparisons test (n=20). Within a column, means followed by the same letter among treatments (rows) are not significantly different.

Treatment	Mean (SE) number of beetles on trees over time											
	Observation period											
	1	2	3	4	5	6	7	8	9	10	11	12
Real tree	1.05	1.0	1.1	1.15	1.2	0.90	1.05	0.95	1.0	0.85	0.80	0.85
exposed	(0.38) a	(0.36) a	(0.36) a	(0.38) a	(0.41) a	(0.3) a	(0.43) a	(0.35) a	(0.34) a	(0.34) a	(0.27) a	(0.25) a
Artificial tree	0.10	0.0	0.05	0.0	0.15	0.10	0.10	0.0	0.0	0.0	0.15	0.05
exposed	(0.06) b	(0.0) c	(0.05) b	(0.0) b	(0.11) b	(0.07) b	(0.07) b	(0.0) b	(0.0) c	(0.0) b	(0.15) b	(0.05) b
Real tree	0.30	0.55	0.35	0.60	0.70	0.50	0.60	0.50	0.35	0.50	0.50	0.45
hidden	(0.11) b	(0.18) ab	(0.13) b	(0.17) a	(0.19) a	(0.11) ab	(0.15) a	(0.15) ab	(0.11) ab	(0.14) a	(0.11) a	(0.14) a
Artificial tree	0.0	0.05	0.15	0.05	0.05	0.10	0.05	0.05	0.10	0.05	0.05	0.0
with bark blend	(0.0) b	(0.05) bc	(0.08) b	(0.05) b	(0.05) b	(0.07) b	(0.05) b	(0.05) b	(0.05) bc	(0.05) b	(0.05) b	(0.0) b

Effect of leaf size on feeding

There was a significant effect of leaf size on the number of beetles that settled on them, with large leaves attracting more beetles (2.2 ± 0.16) than medium sized leaves (1.2 ± 0.13), that in turn attracted more beetles (0.61 ± 0.08) than small leaves (Repeated Measures ANOVA $F = 11.2$; $df = 2, 27$; $P = 0.0003$ and REGW multiple comparisons procedure $P < 0.05$). Beetles also consumed more of the large leaves ($15.1 \pm 2.3 \text{ cm}^2$) in terms of total leaf area than either medium ($7.8 \pm 2.1 \text{ cm}^2$) or small leaves ($3.8 \pm 0.5 \text{ cm}^2$) ($F = 10.10$; $df = 2, 27$; $P = 0.0005$). However, the proportion of foliage (percentage) that beetles consumed relative to total available leaf area, did not differ based on leaf size (large: $10.3 \pm 2.0 \%$, medium: $9.8 \pm 2.2 \%$, small: $12.1 \pm 1.7 \%$) ($F = 0.47$; $df = 2, 27$; $P = 0.6$).

Effect of host species (green versus Manchurian ash) on feeding

The total amount of foliage consumed over the two week period ($F = 9.89$; $df = 1, 76$; $P = 0.002$) as well as the proportion consumed ($F = 10.4$; $df = 1, 76$; $P = 0.002$) were higher for beetles that consumed green ash compared to those that consumed Manchurian ash (Fig. 2a, b). After emergence, beetles lost weight as they aged. Two weeks after emergence, 33 and 53% of beetles fed on green and Manchurian ash respectively, had survived. However, there was no difference in weight loss between beetles that were fed on either species ($F = 0.08$; $df = 1, 30$; $P = 0.78$) (Fig. 2c). There was also no difference in longevity between beetles that were fed on green or Manchurian ash ($F = 2.78$; $df = 1, 76$; $P = 0.1$) (Fig. 2d).

Discussion

Agrilus planipennis tended to distribute themselves and stay on or near real trees whether or not they were exposed from view compared to artificial trees with similar visual silhouettes (Fig. 1, Table 1), suggesting that olfactory cues from host trees might serve to attract beetles to them. Artificial trees with an olfactory signal (i.e., with Manuka oil added to them) were not as attractive as real trees. Our bioassay used 5-day-old adult beetles, which are sexually immature and spend most of their time feeding on foliage. It appears that the strongest attractive cue for *A. planipennis* under 5 days of age is light (Rodriguez-Saona et al. 2007), in response to which they take off and fly above the canopy, presumably to disperse and find new hosts. On average, 0.33% of released beetles distributed themselves on the treatments of our experiment, and the rest landed on the roof of the arena. Nevertheless, a few beetles did settle on artificial trees that provided only a visual signal. It is possible however, that artificial trees emitted different visual spectra compared to real trees that made them less attractive. Rodriguez-Saona et al. (2007) found that beetles tended to land on leaves exposed to the sun and preferred leaves damaged by conspecific feeding compared to intact leaves. In other studies, Francese et al. (2005, 2008) found that *A. planipennis* adults were attracted to purple panels on box traps in greater numbers than to panels of other colors indicating that both visual and olfactory cues are probably involved in host finding.

The ability to combine visual, olfactory and gustatory cues is advantageous for herbivorous insects searching for suitable host plants (Prokopy and Owens 1983, Borden et al. 1986). It is likely that the absence of gustatory stimulants from artificial trees in our experiment prevented beetles from staying on them even if they did land in response to visual stimuli. While long-range visual cues might serve to orient beetles to potential hosts, our results (Fig. 1) indicate that short-range visual, olfactory and gustatory cues would further determine whether or not beetles stay on the host and proceed to feed.

Short-range visual cues such as leaf size and shape can also influence the feeding preference of insects (Brown and Lawton 1991, Rivero-Lynch et al. 1996). In a previous study on host preference among six different ash species (green,

black, white, blue, European and Manchurian ash), we examined leaf area as a covariate in our analyses and found a significant effect of amount (but not proportion) of leaf area consumed by female beetles (Pureswaran and Poland 2009b). A similar result was obtained in this study in which more beetles settled on large leaves compared to small ones but the proportion of foliage consumed relative to total available leaf area, did not differ. Therefore, it appears that while a short-range visual cue such as leaf size might influence initial settling behavior perhaps by providing a larger visual stimulus, it does not influence feeding preference by beetles.

Although there are limitations to data acquired from artificial laboratory experiments and tests in enclosed arenas, results do provide some insight into behavior and choices an insect might make in nature. When we assessed gustatory preference and beetle performance in a no-choice situation, *A. planipennis* fed on the novel host, green ash, to a greater extent than on Manchurian ash with which they co-evolved (Fig. 2a, b). Surprisingly, however, their performance (weight and longevity) did not differ in either case (Fig. 2c, d). This is consistent with previous feeding trials in which three North American ashes (green, white and black ash) were consistently preferred over Manchurian ash (Pureswaran and Poland 2009b) when *A. planipennis* was given a choice among six different ash species. The lack of evidence for superior adult survivorship on green ash is intriguing and suggests that further studies should investigate possible differences in nutritional quality between the two ash species and interactions between nutrition and allelochemicals.

Many herbivorous insects are known to compensate for low nutrition by greater consumption (Scriber and Slansky 1981). Manchurian ash contains some unique phenolics in the phloem that are not present in green ash (Eyles et al. 2007), that may function as antifeedants or digestibility reducers in *A. planipennis* larvae. However, allelochemicals can interact with nutrition levels, and when nutritional quality of food is optimal, the negative effects of allelochemicals on insect performance can diminish (Simpson and Raubenheimer 2001). We hypothesize that in combination with low resistance of green ash to beetle feeding, its lower nutritional quality compared to Manchurian ash might play a role in greater feeding by *A. planipennis* on this host species.

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