Suitability and Accessibility of Immature *Agrilus planipennis* (Coleoptera: Buprestidae) Stages to *Tetrastichus planipennisi* (Hymenoptera: Eulophidae)

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ABSTRACT *Tetrastichus planipennisi* Yang (Hymenoptera: Eulophidae), a gregarious larval endoparasitoid, is one of three biocontrol agents from Asia currently being released in the United States to combat the invasive emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae). The current protocol for rearing *T. planipennisi* involves presenting the wasps with artificially infested ash sticks made by placing field-collected larvae into shallow grooves beneath flaps of bark. Although third and fourth instars are readily accepted by *T. planipennisi* in these exposures, the suitability of younger or older developmental stages, which are often more readily available in the field, has not been tested. In this study, we used both artificially infested ash sticks and naturally infested ash logs to test which emerald ash borer developmental stages (second to fourth instars, J larvae [preprepupae], prepupae, and pupae) are most suitable for rearing *T. planipennisi*. *T. planipennisi* parasitized all stages except for pupae, but parasitized fewer J larvae and prepupae in naturally infested logs than in artificially infested ash sticks. This is probably because, in naturally infested ash logs, these stages were confined to pupal chambers excavated in the sapwood and may have been largely beyond the reach of ovipositing *T. planipennisi*. The number of *T. planipennisi* progeny produced was positively correlated (logarithmic) with host weight, but this relationship was stronger when J larvae and prepupae were excluded from the data set. Fourth instars yielded the most parasitoid progeny, followed by, in approximately equal numbers, J larvae, prepupae, and third instars. Second instars yielded too few parasitoid progeny to benefit rearing efforts.

KEY WORDS classical biological control, concealed host, exotic, forest pest, non-native

Since being first detected near Detroit, MI, and Windsor, Ontario, Canada, in 2002 (Haack et al. 2002), the emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), a phloem feeder native to Asia, has killed millions of ash (*Fraxinus* spp.) trees in northeastern North America and is expected to kill many millions more over the next decade (Kovacs et al. 2010). Bringing emerald ash borer under control is currently one of the most important challenges facing forest entomologists in North America given the ecological, cultural, esthetic, and economic importance of the sixteen ash species endemic to the continent (Cappaert et al. 2005, Poland and McCullough 2006, Gandhi and Herms 2010, Kovacs et al. 2010). Achieving this objective, however, has proven difficult because efforts to eradicate emerald ash borer are largely ineffective; chemical control options are expensive, temporary, and not suited for large-scale application; and endemic predators and parasitoids have only negligible effects on emerald ash borer populations (Cappaert et al. 2005, Poland and McCullough 2006, Duan et al. 2009). Clearly, additional intervention is needed.

A biological control program for emerald ash borer is under way involving the following three hymenopteran species associated with emerald ash borer in Asia: *Oobius agrili* Zhang & Huang (Encyrtidae), an egg parasitoid; *Spathius agrili* Yang (Braconidae), a gregarious larval ectoparasitoid; and *Tetrastichus planipennisi* Yang (Eulophidae), a gregarious larval endoparasitoid (Bauer et al. 2009). *T. planipennisi* seems particularly promising given its high reproductive potential, averaging >50 offspring per late-instar host in the laboratory (Ulyshen et al. 2010), and at least four generations per year in China (Liu et al. 2007). Levels of parasitism by *T. planipennisi* reported from China also are encouraging, averaging 22.4% and reaching as high as 65% (Liu et al. 2003, 2007; Yang et al. 2006).

The emerald ash borer life cycle starts in midsummer when mated females, which have maturation-fed on ash foliage for ≈2 wk, begin to lay eggs under bark flakes or in crevices on trunks and branches of ash trees. Upon hatching, the first instars chew through

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the bark until they reach the phloem layer where they feed continuously for several months, often spanning two seasons in relatively healthy ash trees (Cappaert et al. 2005). After larvae complete three molts, the mature fourth instars excavate pupation chambers in the sapwood or outer bark. Within the pupation chambers, the fourth instars fold in half, forming "J larvae" (also called preprepupae). The following year, these J larvae will prepupate and then pupate. Adults typically emerge in late spring or early summer after the leaves are fully flushed (Cappaert et al. 2005).

The current protocol for rearing *T. planipennisi* involves presenting the wasps with artificially infested ash sticks made by placing field-collected larvae into shallow grooves beneath flaps of bark (Liu and Bauer 2007, Ulyshen et al. 2010). Although *T. planipennisi* readily parasitizes fourth instars in these exposures (Ulyshen et al. 2010), the suitability of other developmental stages, which are often more readily available in the field, has not been explored. The purpose of this study was to assess the suitability of different emerald ash borer developmental stages to *T. planipennisi* in the laboratory using both artificially infested and naturally infested ash. In addition to aiding rearing efforts, the information gained from this study will both help optimize the timing of field releases and predict the impact of *T. planipennisi* on emerald ash borer populations after establishment.

Materials and Methods

Parasitoids. A laboratory colony of *T. planipennisi*, originally collected in 2008 from Liaoning province of China, was used in this study. Only naïve wasps that had not been presented with hosts were used in experiments. They were generally >1 wk old at the time of use and were presumed to have mated as mating activities were observed almost immediately after female emergence and both sexes were held together before use in the experiments.

Artificially Infested Ash Sticks. Using no-choice assays, we carried out two laboratory experiments to determine which emerald ash borer stages were suitable to *T. planipennisi.* The first assay tested the different larval stages and the second assay tested pupae. Emerald ash borers used in both experiments were collected from infested ash trees in the field and were weighed before use.

Experiment 1. Different larval stages (second to fourth instars, J larvae, or prepupae) were inserted into sticks (for details, see Ulyshen et al. 2010) and presented to female *T. planipennisi* in laboratory arenas. Each arena consisted of a ventilated plastic box containing a vial of water with a cotton wick and a 12-well culture plate half-filled with water and covered with a single stretched sheet of Parafilm. Small x-shaped cuts were made in the Parafilm above every other well in the culture plate to allow six emerald ash borer-infested ash sticks (≈1 cm in diameter by 10 cm in length) to be held in an upright position. Each stick contained a single emerald ash borer larva belonging to one of the five larval stages. Six mated female *T. planipennisi* (i.e., 1:1 host:parasitoid ratio) were added to each arena. To reduce fungal growth, the sticks were gently scrubbed under running tap water, sealed at both ends with paraffin, held in a 0.05% bleach bath for ≈5 min, and rinsed with running tap water for 15 min. There were six replicate arenas for each emerald ash borer stage with the six sticks in each arena containing the same stage. The arenas were held in an environmental growth chamber (daytime and nighttime temperatures cycling between 25 ± 2 and 20 ± 2°C, respectively; 65 ± 10% RH; and a photoperiod of 14:10 [L:D] h). The wasps were provisioned with drops of honey on the screen-covered ventilation holes as needed. The sticks were dissected after a 2-wk exposure period, and the emerald ash borer larvae were placed individually in petri dishes (50 by 9 mm, with tight-fit lid; Falcon 351006, Becton Dicksinson Labware, Franklin Lakes, NJ) lined with moistened filter paper. Data collected were percentage of parasitism and the average number of *T. planipennisi* progeny per host for each replicate. We used SAS (SAS Institute 1990) to perform analyses of variance on square root-transformed data to determine differences in percent parasitism (all replicates included) or in the average number of parasitoid progeny per host (only replicates yielding progeny data included) among the different developmental stages. Tukey's studentized range test was used to separate means. We also performed regression analyses on square root-transformed progeny production data to explore the relationship between progeny number and host weight.

Experiment 2. Because emerald ash borer pupae are very fragile and difficult to collect undamaged from the field, prepupae were collected and held in an incubator (25°C, ≈75% humidity, and a photoperiod of 16:8 [L:D] h) until pupation. The resulting pupae were inserted into sticks as described above in experiment 1. Five sticks, each containing one pupa, were placed in alternating diagonal directions (i.e., to minimize contact among sticks) inside a 473-ml clear plastic drinking cup with a snap-top lid. A 5.8-cm-diameter hole was cut in the lid, which held a piece of fine screening over the hole when closed. Five mated female *T. planipennisi* (i.e., 1:1 host:parasitoid ratio) were then added to each cup. For the purpose of comparison, the same assays were carried out simultaneously using fourth instars. There were five replicate arenas for both stages. The arenas were held in an incubator (25°C, ≈75% humidity, and a photoperiod of 16:8 [L:D] h) with drops of honey added to the screens when needed. After 12 d, the sticks were dissected and the pupae or fourth instars were placed individually in petri dishes (50 by 9 mm) lined with moistened filter paper. Data collected were percentage of parasitism and average number of progeny per host for each replicate.

Naturally Infested Ash Logs. Thirty-six green ash, *Fraxinus pennsylvanica* Marshall, logs (each 7–9 cm in diameter by ≈20 cm in length) were cut in the field and returned to the laboratory. To maintain moisture within the logs, several layers of damp paper towels

were held against one end of each log using Parafilm, whereas the other end was sealed with paraffin. Each log was wrapped six to eight times in a spiral using a length of curling ribbon (≈0.5 cm in width), under which emerald ash borer females readily oviposit, and exposed to two to three pairs of emerald ash borer in ventilated 3.8 liter (1-gal) jars for ≈1 wk in an environmental growth chamber (daytime and nighttime temperatures cycling between 25 ± 2 and 20 ± 2°C, respectively; 65 ± 10% RH; and a photoperiod of 14:10 [L:D] h). The number (mean ± SE) of eggs laid on each log was 11.7 ± 1.6 (range, 2–49). The logs were then incubated at the same settings, with their ends submerged in plastic trays filled to a depth of ≈2 cm with water. The larvae were held 7.5 ± 0.2 wk (range, 5.4–10.4) to develop in the logs before being exposed to 12 or 24 gravid *T. planipennisi* females and two to 12 males (i.e., variable parasitoid:host ratio). The infested logs were placed in ventilated 3. 8 liter (1-gal) jars with the wasps for ≈2 wk. Subsequently, the logs were dissected and the emerald ash borers were placed individually in petri dishes (50 by 9 mm) with moistened filter paper. On average, 7.8 ± 0.9 (range, 1–25) larvae, at one to three stages of development, were recovered per log. Because these assays used naturally infested logs, varying widely in host densities, developmental stage distributions, and parasitoid:host ratios, the data from the 36 exposures were pooled into a single data set. Any record for which there was incomplete information was removed from the data set. Likelihood ratio chi-square tests were performed using JMP 8.0.1 (SAS Institute 2008) to compare the rates at which different host stages were parasitized. We also performed analysis of variance (ANOVA) on square root-transformed data to determine whether there were differences in the number of *T. planipennisi* progeny produced per host among the different developmental stages. Tukey's studentized range test was used to separate means.

Results

Artificially Infested Ash Sticks. *Experiment 1.* The parasitism rate by *T. planipennisi* was similar among the emerald ash borer developmental stages tested ($F = 0.8$; df = 4, 25; $P = 0.60$) (Fig. 1A). The number of parasitoid progeny produced per host, however, did differ among host developmental stages ($F = 4.1$; df = 4, 20; $P = 0.01$), with fourth instars yielding significantly more progeny than second instars (Fig. 1B). There was a positive relationship (logarithmic) between the number of parasitoid progeny and host weight (Fig. 2A), particularly when J larvae and prepupae were excluded from the data set (Fig. 2B).

Experiment 2. Although *T. planipennisi* parasitized 44 ± 9.8% of fourth instars, producing 42.0 ± 12.7 progeny per host, none of the pupae were parasitized.

Naturally Infested Ash Logs. *T. planipennisi* parasitized all emerald ash borer developmental stages tested except for prepupae (Table 1). Parasitism rate

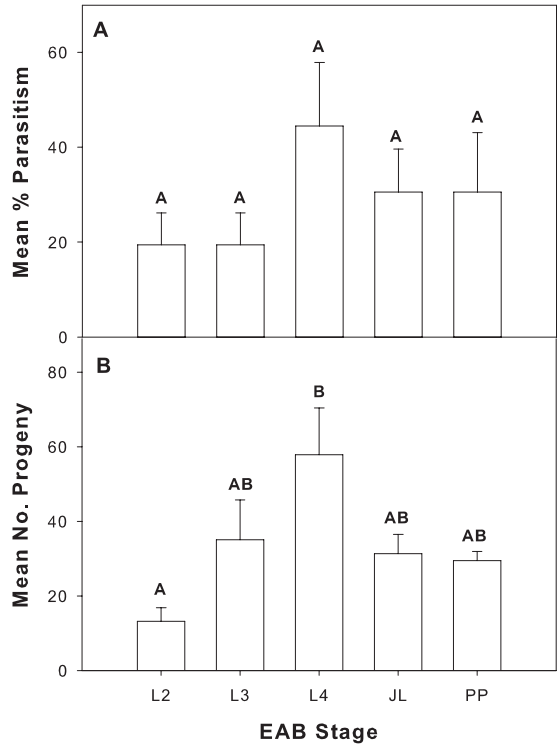

Fig. 1. Mean ± SE ($n = 6$) percentage of parasitism by *T. planipennisi* (A) and number of progeny per host (B) in no-choice assays using ash sticks artificially infested with different emerald ash borer (EAB) developmental stages (L2–L4, second to fourth instars; JL, J larvae; PP, prepupae). Bars with different letters are significantly different based on Tukey's studentized range test ($\alpha = 0.05$) using square root-transformed data (untransformed data are presented).

differed among host stages (likelihood ratio $\chi^2 = 55.3$, df = 4, $P < 0.0001$), being significantly lower for J larvae and prepupae than for third or fourth instars (Table 1). The number of parasitoid progeny produced per host also differed among host stages ($F = 8.5$; df = 3, 87; $P < 0.0001$), with fourth instars and J larvae yielding significantly more progeny than third instars (Table 1).

Discussion

In artificially infested sticks, *T. planipennisi* parasitized all immature stages of emerald ash borer tested, except pupae. In addition, parasitism rates were similar among the different host stages. In naturally infested logs, by contrast, *T. planipennisi* failed to parasitize prepupae and parasitized only three J larvae (≈5%). Several factors may have contributed to this discrepancy. First, J larvae and prepupae were likely less accessible to *T. planipennisi* (ovipositors rarely exceed 2.5 mm) in naturally infested logs because they were confined to pupal chambers excavated in the sapwood. In the artificially infested sticks, these hosts were placed in shallow grooves directly under the bark. Second, the J larvae and prepupae inserted ar-

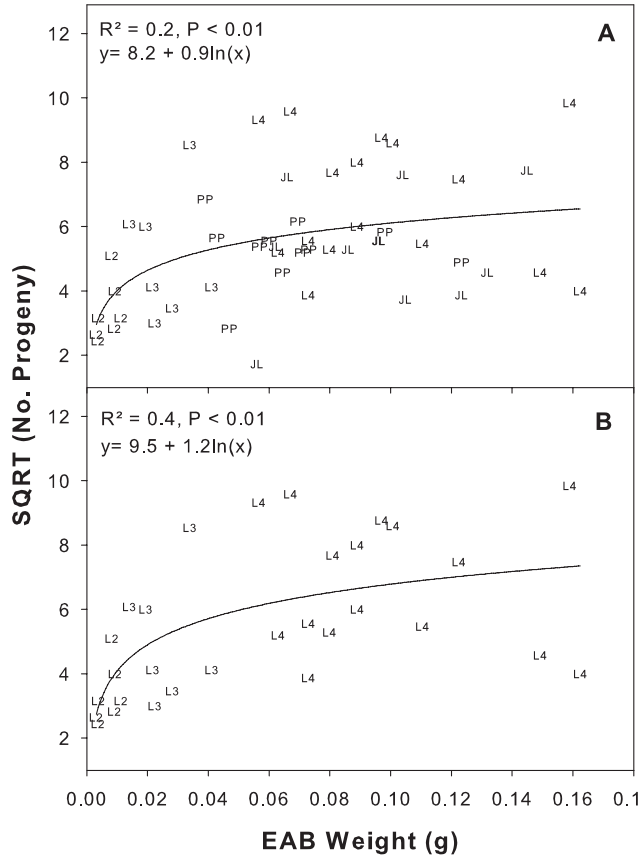

Fig. 2. Relationship between the square-root-transformed number of *T. planipennisi* progeny produced per emerald ash borer (EAB) host and host weight (data from artificially infested sticks). All developmental stages were included in the analysis (A), whereas J larvae and prepupae are excluded from the analysis (B). The symbols represent the different developmental stages (L2–L4, second to fourth instars; JL, J larvae; PP, prepupae).

tificially into sticks may have generated more noise (e.g., chewing, moving) than those in naturally infested logs. Vibrational cues may be important for host location in *T. planipennisi*, as for many other parasitoid species attacking concealed hosts (Godfray 1994, Duan and Messing 2000). Finally, because more than one developmental stage was present in each naturally infested log, *T. planipennisi* may have avoided J larvae and prepupae when other stages were available. Given these results, we recommend that field releases of *T. planipennisi* be made, when possible, before emerald ash borer populations reach J larval and prepupal stages.

Table 1. Response of *T. planipennisi* to logs naturally infested with various immature stages of emerald ash borer

Host stage[a]	Host no.	Hosts parasitized		Progeny per host[b,c] (mean $n \pm$ SE)
		n	%[d]	
L2	4	1	25.0ba	6
L3	65	24	36.9a	20.7 ± 1.7a
L4	134	66	49.3a	44.7 ± 3.7b
JL	59	3	5.1bc	67.5 ± 23.5b
PP	15	0	0bc	0

[a] Emerald ash borer developmental stages: L2–L4, second to fourth instars; JL, J larvae; PP, prepupae.

[b] Means with different letters next to them are significantly different based on Tukey's studentized range test ($\alpha = 0.05$).

[c] Because only one L2 and no PP were parasitized, these stages were not included in the analysis. Also, due to missing data, $n = 64$ and $n = 2$ for L4 and JL, respectively.

[d] Percentages with different letters next to them are significantly different based on likelihood ratio chi-square tests ($\alpha = 0.05$).

More *T. planipennisi* offspring were produced from large hosts than small hosts as shown by Liu et al. (2007). We attribute this pattern to *T. planipennisi* laying fewer eggs in small hosts (i.e., as opposed to cannibalism among larvae competing for limited resources) given that progeny consistently become smaller, not fewer, when resources are limited (M.D.U. and J.J.D., unpublished data). The relationship between progeny number and host weight, however, was stronger when J larvae and prepupae were excluded from the analysis. The number of progeny produced from these stages was more variable and often lower than expected based on weight alone. Therefore, *T. planipennisi* probably uses cues other than host weight when allocating larvae. For example, vibrational cues are used by many parasi-

Table 2. Estimated total number (females)[a] of *T. planipennisi* produced per emerald ash borer provided (mean percentage of parasitism × mean progeny produced per host) in the current study and in a previous study (Ulyshen et al. 2010)

Host stage	Current study		Ulyshen et al. (2010)	
	Small sticks[b]	Logs[c]	Small sticks[b]	Large sticks[d]
L2	3 (2)	2 (1)	NA	NA
L3	7 (5)	8 (6)	NA	NA
L4	26 (19)	22 (16)	26 (19)	36 (26)
JL	10 (7)	3 (2)	NA	NA
PP	9 (6)	0 (0)	NA	NA
P	0 (0)	NA	NA	NA

NA, not applicable.

[a] Estimates for the number of females are based on the observation that, on average, 72% of *T. planipennisi* offspring are female (M.D.U., personal observation). These estimates should be interpreted with caution, however, considering that sex ratio may vary among host stages (see Discussion).

[b] Artificially infested ash sticks, ≈1 cm in diameter by 10 cm in length.

[c] Naturally infested ash logs, ≈8 cm in diameter by 20 cm in length.

[d] Artificially infested ash sticks, ≈4.5 cm in diameter by 10 cm in length.

toids of concealed hosts (Godfray 1994). The intensities and patterns of emerald ash borer-feeding sounds may differ with host size and among different developmental stages. Because they are preparing to pupate, for example, J larvae and prepupae may produce fewer and/or less intense sounds than actively feeding larvae of the same weight. Furthermore, pupae may never produce sounds audible to *T. planipennisi*, possibly explaining why none were parasitized in this study. Research is under way to explore this possibility.

The results from this study have important implications for rearing *T. planipennisi* by using artificially infested ash. In Table 2, we estimate the number of *T. planipennisi* progeny produced per exposed host for each developmental stage (i.e., mean percentage of parasitism × mean progeny produced per host). Clearly, fourth instars yield the most progeny, followed by, in about equal numbers, J larvae, prepupae, and third instars. Although *T. planipennisi* will accept second instars, these yield too few parasitoid progeny to benefit mass-rearing operations. The number of progeny produced per fourth instar in this study was identical to that produced per fourth instar by Ulyshen et al. (2010) when the same-sized sticks (i.e., ≈1 cm in diameter) were used (Table 2). However, Ulyshen et al. (2010) found more progeny are produced per host in larger-diameter sticks (i.e., ≈4.5 cm) (Table 2). Consequently, sticks used in rearing operations should be larger in diameter than those used in the current study.

We also estimated the number of female progeny produced per host provided based on previous rearing data collected without respect to host stage (Table 2). These estimates should be interpreted with caution, however, considering that sex ratio is another factor likely to vary among host stages. Many parasitoid species produce a greater proportion of males from small or low-quality hosts than from large or high-quality hosts (Werren 1984). If *T. planipennisi* exhibits the same tendency, there would be even more reason to avoid small hosts in rearing efforts.

The results from this study indicate that although *T. planipennisi* will parasitize most emerald ash borer developmental stages provided in artificially infested sticks in the laboratory, the species will not necessarily parasitize the same stages in naturally infested material. Our results show, for example, that J larvae and prepupae in naturally infested logs are not parasitized. These stages are probably too deep within the wood to be reached by *T. planipennisi*. Bark thickness also will limit access to hosts. Given the findings from this study and ovipositor-length considerations, it can be predicted that the impact of *T. planipennisi* on emerald ash borer in the field will be largely limited to phloem-feeding larval stages in relatively small-diameter (thin-barked) trees.

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