Monitoring the establishment and abundance of introduced parasitoids of emerald ash borer larvae in Maryland, U.S.A.

David E. Jennings^{1*}, Jian J. Duan², Dick Bean³, Juli R. Gould⁴, Kimberly A. Rice³, and Paula M. Shrewsbury¹

Abstract

Classical biological control can be an important tool for managing invasive species such as emerald ash borer (EAB), *Agrilus planipennis* Fairmaire. Emerald ash borer was first detected in Maryland in 2003, and the biological control program to manage this beetle in Maryland was initiated in 2009. Here we examine the establishment and abundance of two introduced parasitoids of EAB larvae (*Spathius agrili* Yang and *Tetrastichus planipennisi* Yang). Overall, 56,677 *S. agrili* and 191,506 *T. planipennisi* were released at 26 and 32 sites, respectively, from 2009-2014. Monitoring parasitoids involved debarking trees, and harvesting trees to place in rearing barrels, and was conducted at 47 sites (23 of which received parasitoids, and 24 of which served as controls) from 2010-2015. We recovered 77 *S. agrili* from 16 EAB larvae at six sites, and 1,856 *T. planipennisi* from 110 EAB larvae at 19 sites. Percentage parasitism by *T. planipennisi*, and the mean percentage of trees containing *T. planipennisi* broods, were positively associated with the number of years post-release of the parasitoids (reaching 11.6 % and 41.7% four years post-release, respectively). The relationship between *T. planipennisi* parasitism and tree size was best described by an exponential decrease model, with over 95% of parasitism occurring in trees with a diameter at breast height of <16 cm. In conclusion, *T. planipennisi* has established populations and dispersed in Maryland, while *S. agrili* releases have been largely unsuccessful. These findings are a step towards optimizing EAB biological control release and recovery strategies, and are particularly pertinent for other states in the Mid-Atlantic region.

Keywords: *Agrilus planipennis*; *Fraxinus* spp.; invasive species; natural enemies; *Spathius agrili*; *Tetrastichus planipennisi*

1. Introduction

Classical biological control can be an important strategy for managing invasive species (Van Driesche et al., 2010). Since the invasive emerald ash borer (EAB), *Agrilus planipennis* Fairmaire, was first detected in North America in 2002 it has killed hundreds of millions of ash trees (*Fraxinus* spp. L.) (Oleaceae) (Hermes and McCullough, 2014), and may now threaten another confamilial species, white fringe tree (*Chionanthus virginicus* L.) (Cipollini, 2015). Although effective chemical methods for protecting individual ash trees have been developed (McCullough et al., 2011; Smitley et al., 2015), at the landscape level and particularly in natural forests, biological control likely represents the most economically and environmentally feasible long-term strategy for sustainable EAB management (Bauer et al., 2015).

In 2007, USDA APHIS began releases of two EAB larval parasitoids, *Spathius agrili* Yang (Hymenoptera: Braconidae) and *Tetrastichus planipennisi* Yang (Hymenoptera: Eulophidae), in addition to the EAB egg parasitoid *Oobius agrili* Zhang and Huang (Hymenoptera: Encyrtidae), in Michigan (Gould et al., 2015). *Spathius agrili* is a gregarious idiobiont ectoparasitoid which typically has three to four generations per year, and adults typically emerge in mid- to late-summer and attack late instar EAB larvae (Yang et al., 2010). *Tetrastichus planipennisi* is a gregarious koinobiont endoparasitoid which can have up to four generations per year, and adults begin to emerge in late-spring and early-summer (Duan et al., 2011a; Liu et al., 2007). This parasitoid also attacks late instar EAB larvae (Ulyshen et al., 2010a), but is smaller than *S. agrili* and has a shorter ovipositor (Duan and Oppel, 2012; Ulyshen et al., 2010b). Consequently, *T. planipennisi* appears to be limited to parasitizing EAB larvae in trees with bark thinner than 3.2 cm (Abell et al., 2012).

61 In Michigan, *T. planipennisi* has established populations at several sites (Duan et al.,
62 2012a, 2013, 2014), and it has also been recovered from locations in Kentucky, Illinois,
63 Minnesota, New York, Ohio, and Wisconsin (Bauer et al., 2015; Davidson and Rieske, 2016;
64 Johnson et al., in press). Promisingly, these parasitoids appear to be impacting EAB population
65 growth in some areas of Michigan (Duan et al., 2015a). However, to fully evaluate the progress
66 of the EAB biological control program it is necessary to understand how these parasitoids are
67 behaving in other places with different EAB population pressures, environmental conditions, and
68 with greater spatial replication.

69 EAB was first detected in Maryland in 2003 in Prince George's County, where it arrived
70 through infested nursery stock from Michigan (Sargent et al., 2010). The initial management
71 strategy involved establishing a 0.8 km buffer zone around the detection site, from which all ash
72 trees were removed (Sargent et al., 2010). Although EAB was not successfully eradicated, these
73 efforts may have limited the spread of the beetle as it was not detected in any other counties until
74 2008 (and was restricted to counties west of the Chesapeake Bay until 2015) (Maryland
75 Department of Agriculture, 2015). During this early phase, EAB is estimated to have spread
76 approximately 1 km per year in Maryland (Sargent et al., 2010), compared with Michigan where
77 estimates were 7-8 km per year (Siegert et al., 2014). To augment the physical control methods
78 used in the state, parasitoid releases in Maryland were initiated in 2009 (Gould et al., 2015).

79 In the present study, our objectives were to examine the establishment and abundance of
80 the EAB larval parasitoids *S. agrili* and *T. planipennisi* throughout Maryland from 2009-2015.
81 Using considerable spatial and temporal replication, we wanted to determine the influence of the
82 number of years post-release (i.e., the time since parasitoid releases initially occurred), number

of parasitoids released, and tree size, on parasitism. Based upon findings from Michigan (Duan et al., 2012a, 2013, 2014, 2015a), we predicted that parasitism would increase over time, and that *T. planipennisi* would establish more successfully than *S. agrili*. Further, because *T. planipennisi* has a shorter ovipositor than *S. agrili* (Abell et al., 2012), and given the low recovery of *S. agrili* in previous studies in Maryland (Jennings et al., 2013, 2015), we predicted that parasitism generally would be higher on smaller trees.

2. Materials and methods

2.1. Parasitoid releases

Between 2009 and 2014 parasitoid releases were conducted at 32 sites throughout Maryland (Fig. 1a, Supplementary Table 1), and took place in the spring and summer and into early fall when mature (L3 and L4) EAB larvae should have been relatively abundant. These efforts resulted in 57,677 *S. agrili* and 191,506 *T. planipennisi* being released across the state (Table 1). Control sites were located at a minimum distance of 1 km from the nearest release site. Sites in the central and southern Maryland counties (e.g., Prince George's, and Charles) were typically in lowland, suburban forests where green ash (*Fraxinus pennsylvanica* Marshall) was dominant, while in western Maryland counties (e.g., Allegany, and Washington) sites were generally in more upland rural forests with a greater component of white ash (*Fraxinus americana* L.).

Parasitoid releases were conducted through: 1) direct releases of adults, and 2) deployment of green ash bolts containing parasitized EAB larvae and parasitoid pupae.

Deploying ash bolts allows parasitoid adults to emerge naturally over time, but does not enable quantification of the exact number of parasitoids released. Consequently, the number of *T. planipennisi* presented is an estimate, because the ash bolt method was used as a supplementary method to release this species at two sites (using 33 ash bolts).

2.2. Parasitoid establishment

Between 2010 and 2015, sites were monitored to assess the establishment of released parasitoids at 23 of the 32 release sites for between one to four years post-release, as well as at 24 control sites (Fig. 1b, Supplementary Table 1). Control sites were also sampled to monitor parasitoid dispersal. We classified parasitoids as established if an individual species was recovered or observed two or more years after the last release at a given site, which would indicate that the species was able to successfully overwinter and reproduce in that location. We used two methods to assess parasitoid establishment: 1) debarking of trees, either in full or the lower 3 m ($n = 238$), and 2) felling trees and placing sections into barrels for rearing ($n = 214$). Sites were sampled from late winter to early spring.

Trees were selected to be debarked or harvested only if external signs of EAB infestation were present (e.g., D-shaped exit holes, epicormic growth, bark splits, and/or woodpecker feeding damage). As trees were debarked we determined the developmental stage of all EAB based on the width of their galleries, with <2 mm wide representing L1 and L2, 2-3 mm representing L3, and >3 mm representing L4 (Wang et al., 2005). To assess the fates of EAB, the developmental stages of larvae/pupae were separated into three categories: small, early instars

(L1 and L2), mature, late instars (L3 and L4), and overwintering stages (J-shaped larvae, prepupae, and pupae). EAB larvae or galleries were also examined for evidence of parasitism, i.e., the presence of parasitoid adults, pupae, larvae, cocoons, or meconia. Fates aside from parasitization were also assigned to EAB as applicable, such as alive, diseased, exited as an adult, killed by tree resistance, and depredated by woodpeckers (where developmental stage was assigned by examining gallery width and evidence of a pupal chamber).

Trees harvested for placement in rearing barrels were cut into 50 cm sections, including any branches with a diameter >2 cm, and placed into 40.6 x 63.5 cm heavy-duty cardboard Sonotubes® (Sonoco, Hartsville, SC) in environmentally controlled rooms (25-30°C, L:D 16:8 hr) at the Maryland Department of Agriculture, Annapolis, MD. Boles were kept in barrels for 6-8 months as space allowed, without having their ends waxed or being debarked. Rearing barrels had translucent cups attached to one end to attract and collect any emerging parasitoids, and were checked daily for emergence. Voucher specimens of these parasitoids were deposited at the Maryland Department of Agriculture, Annapolis, MD, or the USDA ARS Beneficial Insects Introduction Research Unit, Newark, DE.

2.3. Data analysis

For all statistical analyses, we used data only from tree debarking. Data collected from rearing barrels were used simply to assess general patterns of establishment and dispersal across Maryland. Additionally, because of low recovery of *S. agrili*, statistical analyses were conducted only using data for *T. planipennisi*. To estimate parasitism we followed the methods of Duan et

al. (2013), such that we excluded any EAB killed by woodpecker predation, and by putative tree resistance. Further, we did not include immature larvae (L1 and L2) in our estimations as these were likely too small to have been parasitized (Ulyshen et al., 2010a). Following these methods enabled us to more easily compare our findings with those of previous studies (e.g., Duan et al., 2013).

We first examined if sites were statistically independent samples by conducting Mantel tests (with 999 iterations) to determine if there were significant relationships between the distances among sites and the proportion of parasitism on EAB by *T. planipennisi*, and the probability of a tree containing one or more *T. planipennisi* broods, using the ‘ade4’ package in R 3.2.3 (R Core Team, 2015). At release sites, we examined how the proportion of parasitism on EAB by *T. planipennisi* per tree, and the probability of a tree containing one or more broods were affected by the number of parasitoids released and the number of years post-release of the parasitoids. Because some trees were debarked in full while others had only the lower sections debarked we restricted the analysis of broods to those recovered in the lower 3 m of all trees. Data were fitted to generalized linear models with binomial error distributions (Warton and Hui, 2011), and significance was assessed using likelihood-ratio χ^2 tests in JMP 11.1 (SAS Institute Inc., 2014) followed by Tukey HSD tests if the results were significant. The statistical models used for these analyses were validated using Pearson χ^2 goodness-of-fit tests.

Additionally, using data from control and release sites, we explored how the proportion of parasitism was affected by tree diameter at breast height (DBH). To predict parasitism as a function of DBH, we compared the fits of three models (linear, second-order polynomial, and exponential decrease) to the data. Model fits were then assessed using the corrected Akaike’s

Information Criterion (AICc) (Hurvich and Tsai, 1989), again using JMP 11.1 (SAS Institute Inc., 2014).

3. Results

We found 15,057 EAB (all larvae, pupae, and adults that had exited) in 238 trees that were debarked from 2010-2015. The majority of parasitoids (96.9%) were collected from tree debarking – only 60 parasitoids (all *T. planipennisi*) out of 1,933 were recovered from rearing barrels, and from five sites.

3.1. Parasitism by *Spathius agrili*

We recovered 77 *S. agrili* (larvae, pupae, and adults) at six sites from tree debarking and harvesting (Fig. 1b). *Spathius agrili* was only recovered at release sites, and almost all of the recoveries occurred in the year immediately following releases, with *S. agrili* detected two years post-release at only two sites. Debarking of trees revealed *S. agrili* parasitism on 16 EAB larvae over the six-year period.

3.2. Parasitism by *Tetrastichus planipennisi*

Overall, 1,856 *T. planipennisi* larvae, pupae, and adults were recovered at 19 sites (Fig. 1b). This included seven control sites that were up to 5.1 km away from the closest release site, indicating that the parasitoids had dispersed from release sites. We also found *T. planipennisi* at six sites

two or more years after the last release, strongly suggesting establishment. In addition, debarking revealed 110 EAB larvae that had been parasitized by *T. planipennisi*.

From 2010-2015, the mean parasitism of susceptible EAB larvae by *T. planipennisi* per tree was $2.4\% \pm 0.5$ (range = 0-50%). Sites appeared to be independent samples, as there were no statistically significant relationships between the distances among sites and parasitism (Mantel $r = 0.059$, $p = 0.303$) or the number of broods per tree (Mantel $r = 0.008$, $p = 0.433$). *Tetrastichus planipennisi* parasitism of EAB larvae was positively associated with the number of years post-release ($\chi^2 = 9.89$, $df = 1$, $p = 0.002$; Fig. 2a), but was not significantly affected by the number of parasitoids released ($\chi^2 = 0.04$, $df = 1$, $p = 0.851$). Similarly, the number of broods per tree was also positively associated with the number of years post-release ($\chi^2 = 4.27$, $df = 1$, $p = 0.039$; Fig. 2b), but not by the number of parasitoids released ($\chi^2 = 1.08$, $df = 1$, $p = 0.300$).

We also found that parasitism was negatively associated with DBH, with it generally being much higher in smaller trees (Fig. 3). Over 95% of EAB larvae parasitized by *T. planipennisi* were found in trees with a DBH <16 cm. Indeed, the exponential decrease model (AICc = 1579.32) fitted the data better than both the second-order polynomial (AICc = 1622.64) and linear (AICc = 1632.04) models.

3.3. Summary of fates of EAB larvae

The overall fates of all EAB larvae we found in debarked trees for all years summed were: alive (39.4%), depredated by woodpeckers (32.9%), exited as adults (17.2%), killed by tree resistance (6.9%), diseased (1.9%), parasitized by any species of parasitoid (1.7%), and undetermined

predation (0.1%). Once separated by life stage however, it became apparent that parasitism was only an important source of mortality for EAB in the mature, late instar larvae stage (Table 2). Tree resistance contributed the most mortality for small, early instars, while woodpecker predation was important for mature, late instar larvae and overwintering EAB (Table 2).

4. Discussion

Both of the introduced Asian parasitoids that we examined (*S. agrili* and *T. planipennisi*) were recovered from EAB hosts infesting green and white ash trees in Maryland. However, as predicted there was a considerable difference between the two parasitoid species in terms of successful establishment and dispersal. *Spathius agrili* was recovered in relatively low numbers the year immediately following releases, and only from release sites. In contrast, *T. planipennisi* was found in increasing numbers with each year post-release, and at both release and control sites, indicating that it is successfully establishing and dispersing.

Our findings regarding the recovery and establishment of *T. planipennisi* supported our hypotheses and are generally similar to those from research conducted in Michigan. For example, parasitism of EAB larvae and the number of trees with broods of *T. planipennisi* were also positively associated with the number of years post-release (Duan et al., 2013). In Michigan, however, after four years post-release, these levels were approximately double those found in the present study in Maryland (percentage parasitism: 21.2% MI, 11.6% MD, and percentage of trees sampled containing one or more broods: 92% MI, 41.7% MD) (Duan et al., 2013). These differences could be due to variation among sites or subtle differences in release methodology

and strategy such as the numbers of parasitoids released, and the timing of releases. In particular, the staggered release approach used by Duan et al. (2013) in Michigan may have helped to enable greater numbers of *T. planipennisi* to establish there compared with Maryland.

It was not surprising to observe *T. planipennisi* successfully dispersing, as laboratory tests have indicated that they are capable of travelling up to 7 km (Fahrner et al., 2014). Our results regarding parasitism and tree DBH are similar to those from studies in Michigan and Wisconsin which found that *T. planipennisi* has difficulty parasitizing EAB larvae in trees larger than 11-12 cm DBH (Abell et al., 2012; Johnson et al., in press). While the mean rate of parasitism remains lower than the 22.4% observed in their native range of China (Liu et al., 2007), the positive relationship between parasitism and the number of years post-release suggests that there is the potential for *T. planipennisi* to ultimately suppress EAB populations in Maryland in a similar way to trends observed in Michigan (Duan et al., 2015a).

As has been observed elsewhere in northern states (Duan et al., 2012a, 2014, 2015a; Johnson et al., in press), we found little evidence that *S. agrili* was successfully establishing or dispersing even though this species can parasitize up to 50% of EAB larvae in China (Liu et al., 2003), and some experimental work had suggested that the climate in Maryland might facilitate greater parasitism by this species compared with Michigan (Ulyshen et al., 2011). Given that just three EAB larvae parasitized by *S. agrili* were found at sites two years post-release, it is perhaps more likely that this parasitism was the result of dispersing from a recent release at a nearby site rather than establishment. The causes of the lack of successful establishment by *S. agrili* remain unknown, but there is speculation that it could be related to temperatures and/or phenological mismatches with EAB in parts of the introduced range (Bauer et al., 2015; Hanson et al., 2013).

252 Additionally, the lower numbers of *S. agrili* collected could be at least partly related to the lower
253 numbers of this parasitoid released compared with *T. planipennisi*. For instance, from 2012-2014
254 only around 1,000 *S. agrili* were released per year, while almost 6,000 *T. planipennisi* were
255 released in 2015 alone. It is worth noting however, that even between 2009 and 2011, when *S.*
256 *agrili* release numbers were around 2,000-3,000 per year, recovery rates for this species were
257 still much lower than for *T. planipennisi*.

258 In the summer of 2015, USDA APHIS was approved for field releases of another
259 parasitoid of EAB larvae, *Spathius galinae* Belokobylskij & Strazanac (Hymenoptera:
260 Braconidae) (Belokobylskij et al., 2012). Like its congener *S. agrili*, *S. galinae* also parasitizes
261 late instar EAB larvae (Watt and Duan, 2014). However, *S. galinae* is native to the Russian Far
262 East which could result in this species having closer host-parasitoid phenologies in the northern
263 United States and Canada than *S. agrili* (Duan et al., 2012b). Further, extensive laboratory
264 testing suggests that this species has a narrower host range than *S. agrili* (Duan et al., 2015b;
265 Yang et al., 2008). Additionally, laboratory studies have indicated that interspecific competition
266 between *S. galinae* and *T. planipennisi* is likely to be minimal, and therefore it does not appear as
267 though releases will negatively impact *T. planipennisi* (Wang et al., 2015). Future work will
268 involve closely monitoring the progress of *S. galinae* in Maryland and elsewhere.

269 In addition to monitoring the parasitoids of EAB larvae, future research also involves
270 increasing surveys for the egg parasitoid *Oobius agrili* Zhang and Huang (Hymenoptera:
271 Encyrtidae) in Maryland. Unlike the larval parasitoids, detecting *O. agrili* can be difficult given
272 its small size, and the apparent susceptibility of sentinel eggs to predators (Jennings et al., 2014).
273 This parasitoid has established populations in Michigan (Abell et al., 2014; Duan et al., 2011b,

2012c) but recovery data from other states are rare (e.g., Parisio et al., 2015) and it remains to be seen if it has been as successful in Maryland.

Results from the present study are a step towards optimizing EAB biological control release and recovery strategies in general, and are particularly relevant for EAB management throughout the Mid-Atlantic region of the United States. In addition to Maryland, as of July 2016 EAB was known to be present in the nearby states of Virginia, West Virginia, Pennsylvania, North Carolina, and New Jersey (USDA APHIS, 2016). Our findings suggest that releases of *T. planipennisi* will likely be successful in these other states, but that recovery of sizeable numbers of this parasitoid may require continued sampling for three or more years after releases. Furthermore, to maximize the likelihood of establishment, *T. planipennisi* releases (and recovery efforts) should be conducted at sites with high numbers of small ash trees (DBH <16 cm). Ultimately, if *T. planipennisi* continues to increase in numbers over the years post-release and creates self-sustaining populations, the biological control program might be able to mitigate some of the substantial costs of the chemical treatment, or removal and replacement, of ash trees over the coming years (Kovacs et al., 2010).

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Emerald ash borer (EAB),
Agrilus planipennis

Tetrastichus planipennisi,
parasitoid of EAB larvae

Figure 1

a)

b)

Figure 2

Figure 3

456 Table 1: Mean $\pm$ 1 SE per site, total number of parasitoids released per year, and the earliest and latest dates of release in Maryland
 457 from 2009-2014.

Year	<i>Spathius agrili</i>				<i>Tetrastichus planipennisi</i>			
	Mean $\pm$ 1 SE	Total	Earliest	Latest	Mean $\pm$ 1 SE	Total	Earliest	Latest
2009	1,850 $\pm$ 80	3,700	Aug 8	Oct 28	3,246 $\pm$ 1,176	6,491	Jul 29	Oct 28
2010	2,972 $\pm$ 701	14,862	May 19	Oct 28	4,132 $\pm$ 1,534	28,926	May 25	Oct 28
2011	2,246 $\pm$ 299	24,702	May 25	Oct 28	4,002 $\pm$ 690	44,026	May 25	Oct 28
2012	682 $\pm$ 285	10,225	Jun 14	Oct 25	1,670 $\pm$ 883	23,378	Jun 14	Oct 25
2013	640 $\pm$ 185	3,201	May 10	Jun 5	4,948 $\pm$ 759	44,534	May 10	Nov 6
2014	987 $\pm$ 0	987	May 21	May 28	5,519 $\pm$ 1,370	44,151	May 21	Oct 9

458

459 Table 2: Summary of fates of emerald ash borer (EAB) larvae and pupae (adults excluded) from debarked trees ($n = 238$). Shown are
 460 percentages with n in parentheses. EAB larvae and pupae separated into three categories: SL = small, early instars (L1 and L2), ML =
 461 mature, late instars (L3 and L4), and OW = overwintering stages (J-shaped larvae, prepupae, and pupae).

Category	Alive	Diseased	Fate			
			Killed by tree	Parasitized	Predation (undetermined)	Predation (woodpeckers)
SL	35.2% (484)	2.6% (36)	61.6% (847)	0.0% (0)	0.6% (8)	0.0% (0)
ML	47.9% (1,238)	7.6% (196)	5.8% (149)	7.4% (190)	0.1% (2)	31.3% (809)
OW	49.0% (4,091)	0.5% (38)	0.4% (29)	0.7% (58)	0.1% (6)	49.5% (4,134)

462

463

Figure legends:

Fig. 1: Sites in Maryland at which introduced parasitoids of emerald ash borer larvae were: a) released, and b) recovered. *Tetrastichus planipennisi* was released at 32 sites and recovered from 19, and *Spathius agrili* was released at 26 sites and recovered from six.

Fig. 2: Abundance of *Tetrastichus planipennisi* by number of years post-release. Shown are: a) percentage parasitism (mean $\pm$ 1 SE) of emerald ash borer larvae per tree, and b) percentage of trees (mean $\pm$ 1 SE) with one or more broods. Different lowercase letters represent years post-release that were significantly different from each other ($p < 0.05$).

Fig. 3: Percentage parasitism of emerald ash borer larvae by *Tetrastichus planipennisi* by tree diameter at breast height (DBH), described by an exponential decrease model.