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Comparing Methods for Monitoring Establishment of the Emerald Ash Borer (*Agrilus planipennis*, Coleoptera: Buprestidae) Egg Parasitoid *Oobius agrili* (Hymenoptera: Encyrtidae) in Maryland, USA

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Abstract: The emerald ash borer, *Agrilus planipennis* Fairmaire (EAB), is an invasive beetle that has caused widespread mortality of ash trees in North America. To date, four parasitoids have been introduced in North America for EAB biological control, including the egg parasitoid *Oobius agrili* Zhang & Huang (Hymenoptera: Encyrtidae). Monitoring EAB egg parasitism is challenging because female beetles oviposit in bark crevices and EAB eggs and *O. agrili* are small (<1 mm in diameter). Consequently, multiple methods have been developed to recover this parasitoid. Here we compared two methods, visual surveys and bark sifting, used to monitor establishment of *O. agrili* in Maryland, USA. From 2009 to 2015, a total of 56,176 *O. agrili* were released at 32 sites across the state. In 2016, we surveyed nine of the study sites for *O. agrili* establishment using both methods. We compared the amount of time spent searching for eggs separately in each method, and also analyzed the effects of years-post release, total number of parasitoids released, and median month of release, on percent parasitism of EAB eggs, and the percentage of trees per site with parasitized EAB eggs. We found that visually surveying ash trees for EAB eggs was more efficient than bark sifting; the percent parasitism observed using the two methods was similar, but visually surveying trees was more time-efficient. Both methods indicate that *O. agrili* can successfully establish populations in Maryland, and June may be the best month to release *O. agrili* in the state. Future research should investigate EAB phenology in the state to help optimize parasitoid release strategies.

Keywords: ash trees; biological control; Buprestidae; Encyrtidae; invasive species

1. Introduction

Invasive arthropods represent a serious threat to forest ecosystems worldwide [1–3]. In the USA, invasive woodboring insects in particular are increasing in frequency [4]. These insects can cause extensive economic and environmental damage [2,5], yet their management is challenging because they often have cryptic life stages which are difficult to observe and target.

Of the invasive woodboring insects in the USA, emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) is especially damaging [6–10]. EAB is native to northeastern Asia and is thought to have been accidentally introduced to North America sometime in the 1990s [11,12]. Management strategies for EAB include trunk injections of pesticides, removal of infested trees, and biological control [11,13,14]. To date, biological control of EAB in North America has

involved the release of three parasitoids of EAB larvae (*Tetrastichus planipennisi* Yang (Hymenoptera: Eulophidae), *Spathius agrili* Yang (Hymenoptera: Braconidae), and *S. galinae* Belokobylskij & Strazanac (Hymenoptera: Braconidae)), and one parasitoid of EAB eggs (*Oobius agrili* Zhang & Huang (Hymenoptera: Encyrtidae)) [13,15].

The establishment and effectiveness of EAB larval parasitoids has been the subject of several studies [16–20], but comparatively little is known regarding the establishment of the egg parasitoid *O. agrili*. Previous research on *O. agrili* has taken place in Michigan [21,22], New York [23], and Kentucky [18], where generally it appears as though populations of this parasitoid are successfully establishing. Nonetheless, monitoring the recovery of *O. agrili* remains especially challenging because of the size of both *O. agrili* and EAB eggs (<1 mm in diameter), as well as the location of the eggs in bark crevices. Consequently, a range of methods has been developed and tested for assessing EAB egg mortality [21,23–28]. These methods include EAB egg sentinel logs [22] and envelopes [27], yellow pan traps [23], visual surveys, and bark sifting [21].

Improving the efficiency of *O. agrili* monitoring is paramount to the EAB biological control program. Although some studies have simultaneously compared multiple methods for monitoring *O. agrili* [21,23], the extent to which results depend on environmental factors such as climate, habitat, and *O. agrili* release protocols remains unclear. Consequently, more data are needed to further refine the *O. agrili* monitoring process. For instance, Abell et al. [21] compared visual surveys and bark sifting in Michigan, finding that the bark sifting method revealed considerably higher *O. agrili* parasitism. Additionally, Parisio et al. [23] compared various methods in New York, including egg sentinel logs and yellow pan traps, and found that yellow pan traps recovered more *O. agrili* than egg sentinel logs. These methods all have different benefits associated with them, including variation in financial costs and labor. Comparing methods within the same study should help to guide practitioners in the field, especially those operating with limited resources.

In the present study, our objective was to monitor *O. agrili* establishment and EAB egg parasitism rates across Maryland and to compare two different methods: visual surveys and bark sifting. These two methods were selected because they are among the most cost-effective to implement, and because of their previous use by Abell et al. [21]. Conducting the research in Maryland enabled us to compare our results to those of Abell et al. [21] in Michigan and determine how *O. agrili* populations respond to different climates and environments in the USA. The results from the present study should help to improve the efficiency of *O. agrili* monitoring.

2. Materials and Methods

2.1. Parasitoid Releases

Oobius agrili released in the present study were obtained from the population maintained at the USDA APHIS EAB Biocontrol Facility in Brighton, MI, USA [29]. Releases of *O. agrili* in Maryland began in the summer of 2009, and by 2015 this parasitoid had been released at 32 sites (Figure 1a). Release methods for *O. agrili* included use of logs and inverted cups attached to trees allowing parasitoids to emerge naturally from EAB eggs, as well as direct releases of adult parasitoids [30]. By 2015, 56,176 *O. agrili* had been released in Maryland overall (Table 1; Supplementary Table S1).

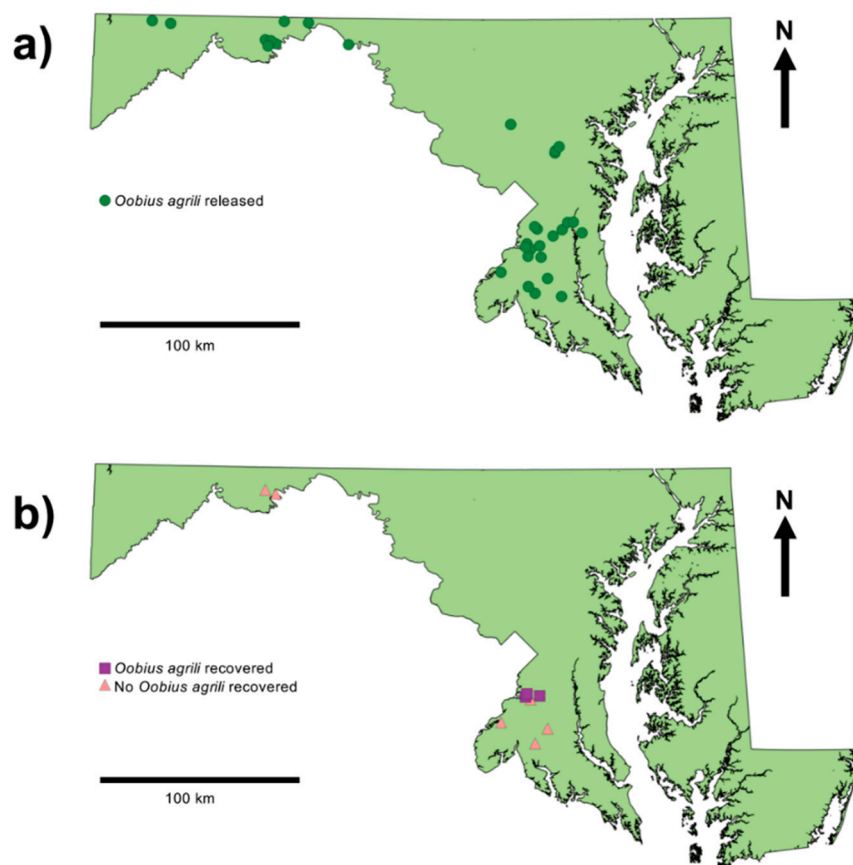


Figure 1. Map of *Oobius agrili* (a) release and (b) recovery sites in Maryland, USA. Nine of the 32 release sites were sampled for *O. agrili* recovery in the present study. Parasitized eggs were found at three of the sites sampled, and *O. agrili* parasitism was confirmed (through an emerging adult) from one site.

Table 1. Summary of *Oobius agrili* releases in Maryland by year from 2009 to 2015. Shown are number of releases per year, mean \pm SE *O. agrili* per release, total number released, and the earliest and latest date of release.

Year	No. of Releases	Mean (\pm SE)	Total	Earliest	Latest
2009	2	182.0 \pm 39.0	364	3 June	1 July
2010	5	145.2 \pm 28.5	726	26 May	22 June
2011	16	167.0 \pm 6.8	2672	25 May	30 June
2012	10	87.9 \pm 36.9	2286	8 June	14 September
2013	10	181.1 \pm 75.1	6518	30 May	3 July
2014	24	675.4 \pm 96.2	16,210	20 June	11 September
2015	39	702.6 \pm 62.0	27,400	18 June	10 September

2.2. Sampling Methods

We surveyed *O. agrili* recovery in late March and early April 2016 at nine of the 32 release sites throughout Maryland (Figure 1b). Recovery sites were selected based on logistics and geography, including urban as well as rural areas. Two different sampling methods were used to monitor *O. agrili* recovery in the present study: visual surveys for EAB eggs on ash trees in the field, and sifting ash bark in the laboratory. Both methods were utilized at all sites.

For the visual survey method, we selected green ash trees (*Fraxinus pennsylvanica* Marshall) that had apparent external signs of EAB infestation (i.e., EAB exit holes, woodpecker damage, epicormic growth, and reduced crown condition). Visual surveys were conducted for 30 min per tree, and on 10 trees per site, for a total of 90 green ash trees (mean diameter at breast height \pm standard error

= 16.28 ± 1.05 cm). Using a utility knife, we picked away at the surface layer of bark between approximately 0.5 and 1.5 m high on the trees to expose cracks and crevices where EAB females typically oviposit. We recorded the number of EAB eggs observed and whether or not the eggs were parasitized (indicated by egg discoloration, with eggs turning a dark brown/black, as opposed to their normal light brown color). All EAB eggs were collected and taken to the laboratory where they were stored in environmentally controlled incubation chambers (25 °C, 65% relative humidity, 16:8 light:dark photoperiod). Any parasitoids that emerged from eggs were then identified.

For the bark sifting method, we scraped off a 10×20 cm section of bark on each green ash tree using a drawknife. Bark sifting was conducted immediately after visual surveys on a random subsample of five of the visually surveyed trees per site, for a total of 45 trees (mean diameter at breast height \pm standard error = 17.88 ± 1.28 cm). We scraped off the same area of bark on each tree to attempt to standardize the amount collected. A small plastic sheet was placed on the ground beneath the tree to catch falling bark, which was then collected in plastic bags and returned to the laboratory for exhaustive inspection using a microscope. The time spent sifting through bark was recorded to enable us to compare the two sampling methods in terms of efficiency. Bark samples were also kept in environmentally controlled incubation chambers to collect and identify any parasitoids that emerged.

2.3. Data Analyses

First, we examined if the recovery sites were independent from each other by using Mantel tests. We then used generalized linear models with binomial error distributions to test the effects of years-post release, total number of parasitoids released, and median month of release, on percent parasitism of EAB eggs, and the percentage of trees per site with parasitized EAB eggs. For this analysis, parasitism from both sampling methods was pooled together. These tests were followed by Tukey HSD tests when there were significant main effects. Lastly, we used a generalized linear model with Gaussian error distribution to compare the amount of time spent searching for eggs separately in each method. For this analysis, we only included trees where both sampling methods had been used. To ensure that all models fit the data ($p > 0.05$), model fits were assessed using Pearson tests. All analyses were conducted using R3.3.2 [31].

3. Results

3.1. Summary

All of the adult parasitoids that emerged in the present study were identified as *O. agrili*. Mantel tests indicated that there were no significant relationships between the distance between sites and egg parasitism (Mantel $r = -0.118$, $p = 0.867$) or trees with egg parasitism (Mantel $r = -0.197$, $p = 0.848$). Therefore, we considered the recovery sites to be independent for subsequent analyses.

We found parasitized EAB eggs at three of the nine sites. At those three sites, mean percent parasitism per tree was 13.03%; mean percent parasitism per tree across all sites was 5.16%. There was a significant effect of the number of years post-release on percent parasitism (LR = 28.48, df = 1, $p < 0.001$; Figure 2a) and the percentage of trees with parasitized eggs (LR = 4.81, df = 1, $p = 0.028$; Figure 2b), with both increasing over time. Mean percent parasitism per tree reached 29.11%, and the percentage of trees with parasitized eggs reached 40%, at the site where *O. agrili* releases had been conducted seven years prior. However, neither the total number of parasitoids released (LR = 0.01, df = 1, $p = 0.916$; Figure 2c), nor median month of release (LR = 0.65, df = 1, $p = 0.421$; Figure 2e), significantly affected percent parasitism. Similarly, there was no significant effect of the total number of parasitoids released (LR = 0.03, df = 1, $p = 0.857$; Figure 2d) or median month of release (LR = 0.89, df = 1, $p = 0.345$; Figure 2f) on the percentage of trees with parasitized eggs.

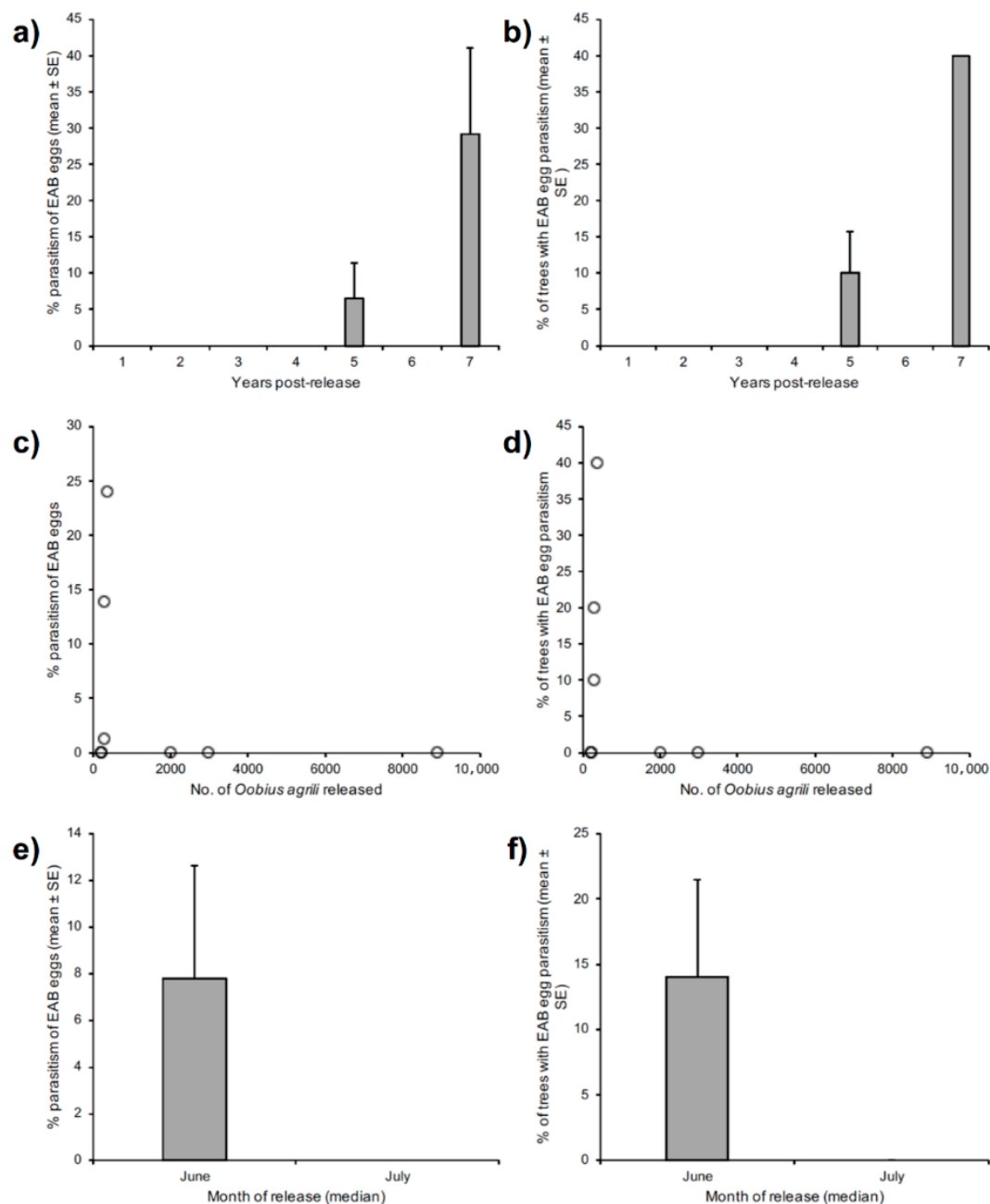


Figure 2. Mean percent parasitism per tree, and mean percentage of trees with parasitized emerald ash borer eggs, by years-post release (a,b), total number of parasitoids released (c,d), and median month of release (e,f), across the nine recovery sites. Data pooled from both survey methods. Black lines represent standard error.

3.2. Comparison of Sampling Method Efficiency

The visual survey method detected 235 EAB eggs, of which 13 were parasitized (5.53%). EAB eggs were found on 62 of the 90 trees (68.89%), and five of those trees had parasitized EAB eggs. The bark sifting method detected 125 EAB eggs, of which five were parasitized (4%). EAB eggs were found on 33 of the 45 trees (73.33%), and three of those trees had parasitized EAB eggs. The mean weight of bark sampled was 4.12 ± 0.34 g.

There were no significant differences in percent parasitism (LR = 0.42, df = 1, $p = 0.518$; Figure 3a) or the percentage of trees with egg parasitism (LR = 0.07, df = 1, $p = 0.798$; Figure 3b) between the

two methods. However, there was a difference in time per sample ($LR = 19.65$, $df = 1$, $p < 0.001$) when comparing the methods, with almost double the amount of time spent processing (in the laboratory) each bark sample (59.64 ± 6.69 min) compared with the visual survey in the field (30 ± 0 min).

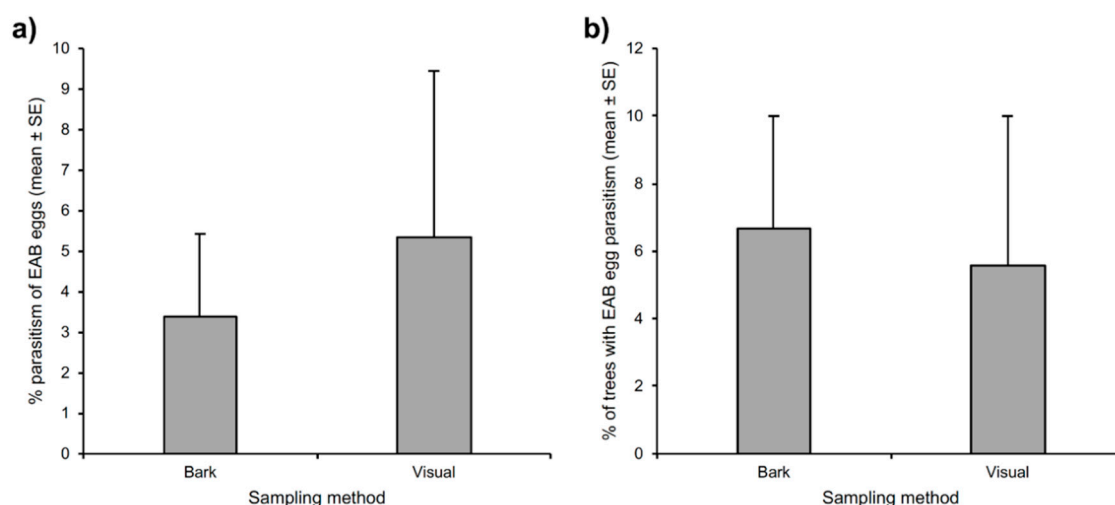


Figure 3. Comparison of results using visual survey and bark sifting methods. Shown are percent parasitism (a), and percentage of trees with parasitized emerald ash borer eggs (b), across the nine recovery sites. Black lines represent standard error.

4. Discussion

Oobius agrili adults were recovered with both visual survey and bark sifting methods from one site, and parasitized EAB eggs were found at a further two sites. The highest EAB egg parasitism was found at the site where the longest time had passed since the initial release. Generally, rates of *O. agrili* recovery, and parasitism of EAB eggs, were slightly low in comparison with other studies [21,22]. However, percent parasitism at the site sampled seven years post-release was comparable to parasitism found in previous studies elsewhere [21,22]. Thus, these results indicate that it is at least possible for *O. agrili* to successfully establish populations in Maryland.

Parasitism was only detected at sites within Prince George's County, MD. Interestingly, the only other *O. agrili* recovered in Maryland in a separate study was also in Prince George's County [9]. All of the adult parasitoids that emerged in the present study were identified as *O. agrili*. Although adult parasitoids were not collected from all of the parasitized eggs, given the lack of native parasitoids thus far observed attacking EAB eggs [9,21] it is likely that this parasitism can still be attributed to *O. agrili*.

Many factors need to be considered when designing a sampling protocol, such as personnel availability, field conditions, and financial resources. However, if choosing between the two methods used in the present study, visually surveying trees for EAB eggs appears to be a more efficient method than collecting bark samples from the field and processing them in the laboratory. This finding contrasts with the results of Abell et al. [21], who found that the bark sifting method was more effective at detecting EAB egg parasitism. The findings of Abell et al. [21] could be explained by their bark sifting method collecting a larger area of bark than in the present study (10×100 cm and 10×50 cm compared with 10×20 cm used here).

Somewhat surprisingly, the number of parasitoids released, and the median month of release, did not significantly affect *O. agrili* recovery. Indeed, the lack of effect from median month of release was surprising because *O. agrili* was only recovered from sites where June was the median month of release. Erring on the cautious side, we would still suggest that *O. agrili* releases in Maryland and nearby areas take place in June to increase the likelihood of establishment. The total number of parasitoids released may be less important than timing, because *O. agrili* need to be released when EAB oviposition is at its peak, which is June–July in the Midwest [11,32] and likely similar in Maryland.

Another possible explanation for the trends observed in parasitoid recovery could relate to *O. agrili* diapause patterns. If *O. agrili* are released later in the summer there is less time for them to produce multiple generations before entering diapause to overwinter [33,34]. Additionally, *O. agrili* are also sensitive to variation in climate [35], which means that seasonal changes in weather patterns could strongly affect the establishment of this parasitoid.

We appreciate that the present study was relatively limited in the number of sites sampled and methods compared. However, our results demonstrate that *O. agrili* can establish populations in the Mid-Atlantic region of the USA, and highlight the importance of timing for parasitoid releases. Further, the present study shows that although different *O. agrili* monitoring methods may yield similar results, there are clear contrasts in the efficiencies of each method.

5. Conclusions

Egg parasitoids have been implicated in, or deployed for, biological control of other agricultural and forest pests such as brown marmorated stink bug, *Halyomorpha halys* Stål (Hemiptera: Pentatomidae) [36], and eucalyptus longhorned borer, *Phoracantha semipunctata* Fab. (Coleoptera: Cerambycidae) [37]. Biological control of EAB appears to be able to slow ash mortality [38,39]. Even with the comparative lack of research on *O. agrili*, targeting EAB eggs for biological control could provide an additive effect to parasitism by larval parasitoids, and lower the number of EAB larvae boring into trees. Future research should further investigate the phenology of EAB in Maryland [32], with a view to further optimizing biological control release strategies.

Supplementary Materials: The following are available online at <http://www.mdpi.com/1999-4907/9/10/659/s1>, Table S1: Summary of *Oobius agrili* release data in Maryland by site from 2009–2015. Shown are year of release, number of releases, mean \pm SE *O. agrili* per release, total number released, and the earliest and latest date of release. Bold type indicates sites surveyed for recovery, and asterisks indicate sites where *O. agrili* were recovered.

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