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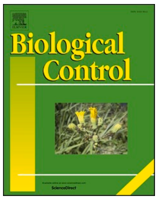

Susceptibility of European *Agrilus* beetle species to the biocontrol agents of Emerald Ash Borer in the laboratory

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HIGHLIGHTS

- *Tetrastichus planipennisi* and *Spathius galinae* parasitised *Agrilus biguttatus* larvae in no-choice laboratory assays.
- *Oobius agrili* parasitised *Agrilus biguttatus* and *A. convexicollis* eggs in no-choice laboratory assays.
- This study is the first to show parasitism of a non-target *Agrilus* species by *Tetrastichus planipennisi.*

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ABSTRACT

The emerald ash borer (EAB, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) is devastating ash trees in its invaded range of North America and spreading rapidly towards Western Europe from European Russia and Ukraine. To allow a rapid response when the beetle is detected, slow its spread and protect trees as part of a wider integrated pest management programme, pre-emptive biological control strategies that are suitable for Great Britain and the rest of Europe must urgently be developed. Three classical hymenopteran biological control agents have been mass-reared and released within North America to control EAB: the egg parasitoid *Oobius agrili* Zhang and Huang (Hymenoptera: Encyrtidae), and the larval parasitoids *Tetrastichus planipennisi* and *Spathius galinae* Belokobylskij & Strazanac (Hymenoptera: Braconidae). This study presents data on the risk these parasitoid wasps might pose to British *Agrilus* beetles. "No-choice" laboratory assays tested the susceptibility of *A. biguttatus* Fabricus eggs and larvae and *A. sulcicollis* Lacordaire and *A. convexicollis* Redtenbacher eggs to the parasitoids. *Oobius agrili, T. planipennisi* and *S. galinae* all attacked *A. biguttatus,* and offspring were produced. *Oobius agrili* also attacked the eggs of *A. convexicollis,* but not *A. sulcicollis*. This study is the first to show parasitism of a non-target species by *T. planipennisi.* Further work is needed to fully assess the non-target risk of these parasitoids for release using more ecologically relevant tests, such as 'choice', semi-field and chemical ecology assays on the attacked *Agrilus* species.

1. Introduction

The emerald ash borer beetle (EAB, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae)) has killed hundreds of millions of ash trees (*Fraxinus* spp.) after its accidental introduction to North America. First detected in the Great Lakes Region in 2002 (Detroit, Michigan, in May-June, and in Ontario, Canada, in July, respectively), it was probably introduced in wood packaging material (Haack et al., 2002; Siegert et al., 2014). In its native range, in temperate regions of north-eastern Asia, the species is an occasional, secondary pest of weakened native ash trees, as well as a pest of non-native ash plantations and ornamental trees (Wang et al., 2010b; Dang et al., 2022). To date, EAB has spread to 36 US states and 6 Canadian provinces (Haack et al., 2002; Canadian Forest Service, 2003; USDA APHIS, 2023). Of the American ash species EAB has so far encountered, only blue ash seems to show resistance (Kelly et al., 2020; Cipollini and Morton, 2023; Sun et al., 2024). While

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genotype and environmental factors such as ash density and tree condition may influence the timeline of tree death, the mortality rate in mature North American ash stands during the first wave of the EAB invasion was measured as 98-99% in some observational studies (Knight et al., 2013; Klooster et al., 2014; Steiner et al., 2019). The few, apparently resistant "lingering" ash trees are the subject of resistance breeding programmes in the United States (Koch et al., 2012; Koch et al., 2015).

Although EAB is currently absent from most of Europe, human-mediated transport could allow it to reach Great Britain and other parts of Northern Europe despite natural barriers (BenDor et al., 2006). EAB was also found in Moscow in 2005 (Izhevskii and Mozolevskaya, 2010) and has since spread north to St. Petersburg and west into Ukraine as far as Kiev (Drogvalenko et al., 2019; Musolin et al., 2021; EPPO, 2023). Native ash hosts (European ash, *Fraxinus excelsior* L., narrow-leaved ash, *F. angustifolia* Vahl, and manna ash, *F. ornus* L.) are present across much of Europe (Orlova-Bienkowskaja and Bieńkowski, 2022). The impact of EAB in northern parts of Europe, including Great Britain, may be mediated by a relatively cool summer maritime climate. Given its recent establishment as far north as St. Petersburg, EAB will probably be able to develop in milder areas of Great Britain (Volkovitsh and Suslov, 2020). Southern England appears to be increasing in suitability for *Agrilus* species, potentially due to warmer summers and increasing host stress; six species of *Agrilus* have recently established from continental Europe, and species already present have become more widespread and abundant (Bantock and Ashby, in press; Alexander, 2003; Duff, 2020; Williams et al., 2023). Similar trends of northwards spread of *Agrilus* spp. have also been observed in Scandinavia (Pedersen and Jørum, 2009). The impact of EAB may be reduced if it has a two-year rather than one-year lifecycle, as has been observed in northerly areas of North America and European Russia (Cappaert et al., 2005; Orlova-Bienkowskaja and Bieńkowski, 2016).

The impact of EAB may also depend on the suitability of European ash (*Fraxinus excelsior* L.) as a breeding resource. The available evidence on the susceptibility of European ash is mixed. Observational studies in Russia and Ukraine, where green ash *F. pennsylvanica* Marsh. is extensively planted in reforestation / landscaping projects, suggest lower susceptibility in *F. excelsior* than *F. pennsylvanica,* with EAB attacking only stressed *F. excelsior* or trees adjacent to *F. pennsylvanica* plantings (Straw et al., 2013; Orlova-Bienkowskaja et al., 2020). The spread of EAB into Europe poses an enormous risk to European ash trees, especially given the potential synergistic interaction with ADB, and it is important to take advantage of the window before the arrival of the pest to prepare appropriate management strategies. Biological control represents one of very few economically feasible strategies to control the beetle within European forests, and pre-emptive work to identify suitable biological control agents (BCAs) and obtain approval for their release is urgently needed (Avila et al., 2023; Hoddle, 2023). The introduction of an exotic BCA requires extensive research, risk assessments, and regulatory approval, a process that can take years. Consequently, biological control programmes are typically implemented only after a pest is well-established in an invaded area. Pre-emptive work on biological control, targeting pest threats on the horizon, aims to expediate responses to invasive pests by obtaining pre-release approval, allowing rapid deployment upon the arrival of a pest. This strategy can reduce pest population densities and spread early on in the invasion, minimising environmental and economic impacts, and potentially aiding in both pest containment and eradication within localised areas (Avila et al., 2023).

Due to the rapid dispersal ability of EAB and the likelihood of a lag between its arrival and detection, its eradication is unlikely to be possible in the UK, much like in North America, where the high abundance of susceptible ash species facilitated rapid EAB population growth (BenDor et al., 2006; Duan et al., 2023a). Early signs of EAB infestation are cryptic and very similar to those of ADB. Initial large-scale efforts in North America to eradicate EAB through a combination of intensive surveys, removal and destruction of infested trees, including "trap trees", ash tree and wood movement restrictions and the establishment of "firebreaks" were futile in preventing EAB spread and population buildup (Herms and McCullough 2014; McCullough 2015; Inward and Straw, 2021). While effective chemical control options have been developed for urban and high-value trees, these are impractical for large-scale use in forests (Kenis et al., 2017; Duan et al., 2023a). The recent discovery in England of *Agrilus convexicollis* Redtenbacher (Coleoptera: Buprestidae) also shows how easy it is for *Agrilus* spp. to remain undetected (Bantock and Ashby, in press). A species often found alongside and associated with the EAB, *A. convexicollis* is native to Europe and was first identified in Russia in 2007 (Orlova-Bienkowskaja and Volkovitsh, 2015). Since then, its distribution has expanded, and it was recently collected at multiple locations in southern England, first at two sites in the West Thames area of Surrey, and subsequently in additional areas within Surrey and Essex (Bantock and Ashby, in press, F. Cole and K. Reed, pers. obs.).

An integrated management strategy including the rapid deployment of effective biological control agents may allow the survival of some ash trees in Europe if the majority were to be killed by ADB and EAB. Introduced parasitoid wasps have successfully controlled invasive insect pests in different temperate forest ecosystems (Yang et al., 2014; MacQuarrie et al., 2016). Effective biological control agents of invasive pests should be able to establish in the invaded area and considerably reduce the impact of the pest whilst having minimal non-target impacts on native fauna (Barratt et al., 2010). Non-target impacts of introduced parasitoids may be both direct (parasitism) and indirect (hybridisation, competition for resources / hosts) (Kenis et al., 2017). Guidance and frameworks now exist, detailing these potential impacts and guiding the pre-emptive screening process (Barratt et al., 2016; Avila et al., 2023; Hoddle, 2023). Estimating the potential for species interactions is important to predict the risk of non-target parasitism: are the parasitoid and prey likely to come in contact, e.g. on a shared plant host? The taxonomic relatedness of potential prey to the natural host may also be also important; for example, an aphid parasitoid was shown to preferentially parasitise species that are more closely related to its natural host, including within a genus (Desneux et al., 2012). Other considerations for which potential prey species to test include availability / feasibility of testing (can a species be collected? Can it be reared?) and the cultural value of the species (Barratt et al., 2016).

A recent feasibility study determined that three parasitoid wasps are potentially suitable for pre-emptive biological control of EAB in Europe (Horrocks et al., 2024). These are *Oobius agrili* Zhang and Huang (Hymenoptera: Encyrtidae) and *Tetrastichus planipennisi* Yang (Hymenoptera: Eulophidae), which control EAB in China, and *Spathius galinae* Belokobylskij & Strazanac (Hymenoptera: Braconidae), which attacks EAB in the Russian Far East (Zhang et al., 2005; Liu and Bauer 2007; Yang et al., 2006; Wang et al., 2016). *Oobius agrili* is a solitary, parthenogenic egg parasitoid (no males are present in its introduced range), whereas *T. planipennisi,* an endoparasitoid, and *S. galinae,* an ectoparasitoid, develop gregariously on late instar larvae; all three species are typically multivoltine (Liu and Bauer, 2006; Bauer and Liu, 2007; Belokobylskij et al., 2012). None of these species are currently present in Europe.

Post-release monitoring suggests that these parasitoids have established and spread widely from their release points in the USA and are exerting a significant reduction on EAB population growth and numbers on trees, particularly on regenerating ash (Duan et al., 2022). After the introduction of *S. galinae* and *T. planipennisi,* EAB larval density in ash trees fell by 76% in ash-dominated stands across three states from 2015-2022 (Duan et al., 2022). *Spathius galinae* has so far appeared to be the most effective of the three parasitoids at reducing EAB populations, although *O. agrili* is difficult to evaluate because both it and EAB eggs are small and cryptic (Duan et al., 2018, 2022, 2023a,b; Gould et al., 2022). There is great interest in determining the suitability of these insects for release against EAB in Europe (Avila et al., 2023).

Draft risk assessments have been written to accompany a licensing application for the release of the three parasitoids in Great Britain; these identified particular knowledge gaps around the risk of parasitism of non-target hosts (N. Audsley, unpublished.). The results of host specificity testing in North America are summarised in Gould et al. (2015) and Duan et al. (2023a). Bauer and Liu (2007) report that *O. agrili* parasitised eggs of three of six non-target North American *Agrilus* species tested, and the non-target eggs parasitised were similar in size to EAB (*A. anxius* Gory*, A. bilineatus* Weber*,* and *A. ruficollis* F.). In follow-up choice tests, *O. agrili* preferred EAB eggs to non-target eggs. Duan et al. (2015) report that *S. galinae* only attacked one of five non-target *Agrilus* species tested (*A. auroguttatus* Schaeffer), and that the parasitism rate on *A. auroguttatus* was lower than on EAB. Liu and Bauer (2007) report that *T. planipennisi* did not attack any of five tested non-target *Agrilus* species. Non-*Agrilus* test spp. were not attacked by any of the parasitoids, suggesting all three parasitoids are specific to the *Agrilus* genus (Bauer and Liu, 2007; Liu and Bauer, 2007; Duan et al., 2015). Regulatory approval was granted to release *O. agrili* and *T. planipennisi* in 2007, and *S. galinae* in 2015 (Gould et al., 2015; Duan et al., 2023b). As far as the authors are aware, post-release monitoring in North America has concentrated on determining whether the insects have established rather than quantifying non-target impacts on non-ash tree species (Duan et al., 2019, 2023a). Nor has testing been conducted on the host specificity of *Agrilus* species that occur in Europe, although *T. planipennisi* appeared to be host-specific in all tests conducted.

This study presents data from experiments on three parasitoids, *O. agrili, S. galinae,* and *T. planipennisi,* in relation to three *Agrilus* species, *A biguttatus* Fabricus (Coleoptera: Buprestidae)*, A. sulcicollis* Lacordaire (Coleoptera: Buprestidae) and *A. convexicollis*. These data provide initial evidence towards understanding the potential risks these parasitoids may pose to non-target organisms in Europe and will inform the licensing for their release in Great Britain.

2. Materials and methods

For this study we tested the native *A. biguttatus* because the beetle occupies a broadly similar niche to EAB (Evans et al., 2007), developing on the stems of mature *Quercus* spp., and because collection and rearing methods have been developed to research its role in acute oak decline, in which it is implicated (Brown et al., 2015; Reed et al., 2018). We tested the abundant, non-native *A. convexicollis*, which colonises *Fraxinus* twigs $<$ 3cm diameter, because it develops on ash, and would be likely to encounter the BCAs (Orlova-Bienkowskaja and Volkovitsh, 2015). We also tested *A. sulcicollis,* another abundant, non-native *Agrilus* species which colonises the stems and branches of *Quercus* spp. (Jendek and Grebennikov, 2009).

No-choice assays were carried out to determine whether the three parasitoids of EAB would attack eggs of the three *Agrilus* test species. EAB was employed as a positive control. Egg lengths of 20 *A. biguttatus, A. sulcicollis* and *A. convexicollis* eggs, randomly selected from batches laid by at least 5 different females, were measured using a dissecting microscope. Egg lengths for EAB, *A. biguttatus*, *A. sulcicollis,* and *A. convexicollis* ranged from 1.0 to 1.2 mm, 1.0 to 1.2 mm, 0.7 to 1.0 mm, and 0.8 mm to 1.0 mm, respectively (Chamorro et al., 2012).

All experiments were conducted in a PHcbi MLR-352H Climate Chamber at 25̊C, 70% RH, and a 16:8 day:night light cycle (PHC Europe B.V.), to approximate the conditions used by the Canadian Forest Service in mass-producing EAB parasitoids (Natural Resources Canada, 2019).

Agrilus biguttatus, A. sulcicollis and *A. convexicollis* eggs were produced at Forest Research, Alice Holt, Farnham. To culture *A. biguttatus* and *A. sulcicollis,* briefly, oak trees were felled when dormant to collect pre-pupal larvae that had finished feeding. Slabs of outer bark and sapwood were brought to Alice Holt and kept within large mesh emergence cages. Adults were collected as they emerged (June-July). They were placed in plastic jars, where they fed on freshly cut oak leaves with the stems kept in water containers and mated. Water and 20% sugar-water solutions were provided on cotton wool. Eggs were laid onto sheets of blue paper towel placed under the cages, usually under the water or sugar-water containers. The cages were refreshed twice-weekly, at which time eggs were collected and either used immediately for experiments with *O. agrili* or incubated at 17-22.5∘C for production of larvae. See (Reed et al., 2018) for further details of collection and culturing methods. Adult *A. convexicollis* beetles, collected from ash tree leaves, were provided with freshly cut ash leaves and plain water. They were kept in mixed pairs of up to 10 within 32 oz round deli cups (Northampton Reptile Centre, UK) and provided with fresh ash leaves. Following the methods described in (Natural Resources Canada, 2019), screen-door mesh (openings: 1.7 mm) covered by filter paper provided a suitable egg-laying substrate. Cages were refreshed twice weekly, and the eggs collected and either used immediately or stored at 10∘C until use. *Oobius agrili* and EAB eggs were supplied by the USDA APHIS EAB Biocontrol Facility or the Canadian Forest Service. *Oobius agrili* were shipped as pupae within parasitised EAB eggs laid on filter paper, while EAB eggs were shipped on the day of collection from the USDA, or hand-carried on the day after collection from the Canadian Forest Service. The shipments arrived within 4 days, and eggs were used in experiments with *O. agrili* on the day of arrival.

Immediately after their emergence, *O. agrili* females were placed with eggs of EAB, *A. biguttatus* or *A. sulcicollis,* in one of two methods. First, either 1 or 2 parasitoids were placed with 1 to 3 eggs (*A. biguttatus* rarely lays single eggs; usually eggs are laid in batches) within 30ml plastic vials, at an approximately 1:1 ratio (n = 18 *A. biguttatus* and 13 EAB replicates) (1:1 ratio egg experiments) (Table 1). Second, according to advice given by the Canadian Forest Service in rearing *O. agrili*, eggs and parasitoids were combined at a 1 parasitoid: 10 egg ratio within 290 ml reusable plastic drinks cups (Natural Resources Canada, 2019) (n = 10 *A. biguttatus,* 2 *A. sulcicollis* and 2 EAB replicates) (1:10 ratio egg experiments) (Table 1). *Agrilus convexicollis* eggs (<4 days old) were tested separately to answer the yes/no question of whether *O. agrili* would parasitise them. Tests were run in 32 oz round deli cups (Northampton Reptile Centre, UK) with a 1:10 parasitoid-to-host ratio (20 *A. convexicollis* eggs to 2 parasitoids). In all setups, a 1 cm^2 piece of honey-soaked paper towel provided food and moisture to the parasitoids.

After 1 week, in all setups, the parasitoids were removed, and the eggs were checked for the presence of a small breathing tube / stalk protruding from the egg surface; this stalk shows parasitism by *O. agrili* has occurred and is usually visible several days after oviposition by the parasitoid. The exposure period was chosen to allow for a period of pre-oviposition maturation feeding, and because the age at which the novel species' eggs might become attractive to the parasitoids was unknown; for instance, EAB eggs older than 4 days are less suitable (Natural Resources Canada, 2019; Duan et al., 2014). Parasitised eggs were left to develop for a further 6 weeks and were checked several times a week for adult emergence. Un-parasitised eggs were left to develop for 10 days to determine their viability (presence of hatching *Agrilus* larvae).

The susceptibility of the larvae of *A. biguttatus* to parasitism by *S. galinae* and *T. planipennisi* was also tested through no-choice assays.

Table 1
Number of viable *Agrilus* eggs exposed to *Oobius agrili*, number of eggs parasitised, and number of new adults that emerged, by *Agrilus* species and experimental method (1:1 ratio or 1:10 ratio egg experiments).

Agrilus species	Method	No. viable *Agrilus* eggs	No. eggs parasitised	No. new parasitoid adult females
A. biguttatus	1:1	29	12	9
	1:10	321	101	76
A. sulcicollis	1:1	-	-	-
	1:10	46	0	0
A. planipennis (EAB)	1:1	15	10	5
	1:10	65	50	48

Maximum larval lengths for EAB and *A. biguttatus* have been recorded as 34 mm and 36 mm, respectively (Chamorro et al., 2012; Reed et al., 2018). To culture *A. biguttatus* larvae*, Q. robur* (10-15cm DBH) were cut, sectioned into 30cm lengths, and kept in water outdoors at ambient temperatures for approximately 14 days to allow constitutive defences to reduce sufficiently for larval colonisation. Egg batches, calculated as 2-3 days from hatching according to their day degree accumulation, were inserted onto holes cut into the phloem of the logs with a cork borer, and the cork plug gently replaced. The logs were incubated in trays of water at 25◦C. See (Reed et al., 2018) for further details of rearing methods. Further larvae were collected directly from an infested oak root-stump in Suffolk and identified morphologically by K. Reed. EAB larvae were cultured at Alice Holt from eggs supplied by the USDA-APHIS or the Canadian Forest Service, using the same methods as described for *A. biguttatus* larvae except that larvae were cultured on *F. excelsior* branch or stem sections 3-12 cm diameter. Third or fourth instar larvae of *A. biguttatus* and EAB were removed from their host material and one larva was individually re-inserted into a small (2-5 cm diameter, 15 cm length) freshly cut oak or ash stick, respectively (approx. 10 cm high, 2-3 cm diameter) by cutting a channel slightly wider and longer than the larva ∼ 2mm into the sapwood with a chisel and mallet, removing the channel of sapwood, inserting the larva head-down, replacing the outer bark and wrapping the stick with Parafilm® (Reed et al., 2018).

Spathius galinae and *T. planipennisi* were shipped as adults by the USDA APHIS EAB Biocontrol Facility in plastic drinking cups. After shipment, male and female larval parasitoids were kept within 290 ml plastic cups with ultrafine mesh hot-glued onto the lids and allowed to mate for at least 7 days. Honey was streaked onto the mesh to provide food and moisture. One larva-colonised bolt and 1-2 female parasitoids and 1 male (where possible) were placed in cups. The parasitoids were removed after one week and the cups were monitored for emergence of new parasitoids. After 6 weeks, the bolts were destructively sampled to determine the viability of the *Agrilus* larvae and check for the presence of parasitoids that failed to emerge.

2.1. *Statistical analyses*

To determine whether there was a higher rate of parasitism or emergence on either beetle species (*A. planipennis* or *A. biguttatus*), either generalized linear models or generalized linear mixed-effects models (glmer(), in the “lme4” package (Bates et al., 2015) were fitted. The binomial family was specified in all models. In the 1:1 ratio egg experiments, the responses were the proportion of viable eggs parasitised and the proportion of adults emerging from parasitised eggs. The explanatory variables were beetle species (EAB or *A. biguttatus*, hereafter referred to as “species”), number of female wasps per vial, the interaction between species and number of wasps, and the medium on which the eggs were laid (bark or paper, hereafter referred to as “medium”). In the 1:10 ratio egg experiments, the responses were parasitism (yes/no) of individual eggs and emergence of adults (y/n) from the parasitised eggs. The explanatory variables were beetle species and number of female wasps per cup, and a random effect for batch (cup) was fitted; the interaction between the main effects was not included due to insufficient data. In the *T. planipennisi* and *S. galinae* larval experiments, due to lower replication, the only response tested was parasitism of the larva (yes/no), and the explanatory variables were beetle species, number of female wasps exposed, and their interaction. For all models, the significance of the explanatory variables was tested using the Anova() function in the car package (Fox and Weisberg, 2018). Model goodness-of-fit was tested in the simulationOutput() function in the DHARMA package and the distribution of residuals inspected visually (Hartig 2021). Post-hoc comparisons were made using emmeans (Lenth 2021). No statistical analyses were conducted on the *A. convexicollis* study.

All analyses were performed in R 4.3.2 (R Core Team 2023).

3. Results

All three parasitoid species were able to parasitise *A. biguttatus* and produce offspring that developed and emerged successfully from this host. Parasitism of *A. biguttatus* eggs by *O. agrili* occurred in both the 1:1 and 1:10 ratio egg experiments, and subsequent parasitoid offspring were able to develop and emerge as adults, while no eggs of *A. sulcicollis* were parasitised (Table 1). While multiple breathing stalks were present on many individual eggs of *A. biguttatus* and EAB, only one adult (female) ever emerged from each parasitised egg. Both *S. galinae* and *T. planipennisi* parasitised at least 2 larvae of *A. biguttatus*; again, adult offspring of both species emerged (Table 2). In the experiment with *A. convexicollis*, 17 out of 20 eggs produced breathing stalks indicating parasitism by *O. agrili*, and adult offspring emerged from 7 of the eggs.

The analyses of the egg data suggested a higher parasitism rate of EAB eggs in the 1:1 ratio egg experiments may have been observed with more data, and a higher rate was observed in the 1:10 ratio egg experiments ($X^2 = 3.5$, df $= 1$, $p = 0.06$; $X^2 = 10$, df $= 1$, $p = 0.002$, 1:1 and 1:10 ratio egg experiments, respectively; proportion $= 0.8 \pm 0.11$, 0.3 ± 0.07 in EAB vs *A. biguttatus*). In the 1:1 ratio egg experiments, there was a suggestion that more eggs were parasitised on paper than oak bark ($X^2 = 3.2$, df $= 1$, $p = 0.07$), and that more eggs were parasitised when more wasps were present ($X^2 = 3.5$, df $= 1$, $p = 0.06$). Number of wasps did not affect parasitism outcome in the plastic cup setup ($X^2 = 2.6$, df $= 1$, $p = 0.1$). The interaction between species and number of wasps did not affect parasitism rate in the 1:1 ratio egg experiments ($X^2 = 0.38$, df $= 1$, $p = 0.54$). When emergence from parasitised eggs was compared, in the 1:1 ratio egg experiments, more new wasps emerged when more female wasps were present ($X^2 = 5.4$, df $= 1$, $p = 0.02$). There was no effect of beetle species ($X^2 = 0.01$, df $= 1$, p $= 0.9$), medium ($X^2 = 2.1$, df $= 1$, $p = 0.15$) or the interaction between beetle species and number of wasps ($X^2 = 0.54$, df $= 1$, $p = 0.46$). In the 1:10 ratio egg experiments, there was a suggestion of less successful emergence from *A. biguttatus* eggs ($X^2 = 3.3$, df $= 1$, $p = 0.07$). Number of wasps did not influence emergence rate ($X^2 = 3.3$, df $= 1$, $p = 0.75$).

Beetle species did not influence parasitism rate by either *S. galinae* or *T. planipennisi* ($X^2 = 0.04$, df $= 1$, p $= 0.83$; $X^2 = 1$, df $= 1$, $p = 0.32$, respectively). *Tetrastichus planipennisi* was less likely to parasitise a larva if more than one female was present ($X^2 = 5.8$, df $= 1$, $p = 0.02$). Number of wasps did not influence parasitism rate in *S. galinae* ($X^2 = 0.04$, df $= 1$, $p = 0.85$).

4. Discussion

The findings of the present study suggest that the release of *O. agrili, T. planipennisi* and *S. galinae* in Great Britain or elsewhere in Europe involves a risk of parasitism of *A. biguttatus* eggs and larvae, and *A. convexicollis* eggs. Further work is needed to determine the risk these parasitoids would pose to *A. biguttatus* and other *Agrilus* species in a

Table 2

Number of viable *Agrilus* larvae exposed to *Tetrastichus planipennisi* and *Spathius galinae,* parasitism rates, and number of new adults that emerged from parasitised larvae.

***Agrilus* species**	**Parasitoid**	**Results**		
		No. viable *Agrilus* larvae	**No. larvae parasitised**	**Sex ratio of new parasitoid adults (F:M)**
A. planipennis (EAB)	*Tetrastichus planipennisi*	5	2	47: 15
A. biguttatus		28	2	90: 19
	Parasitoid	**No. viable Agrilus larvae**	**No. larvae parasitised**	**Sex ratio of new parasitoid adults (F:M)**
A. planipennis (EAB)	*Spathius galinae*	5	1	10: 1
A. biguttatus		26	6	33: 7

woodland setting.

In this study, *O. agrili* appeared to prefer the eggs of EAB to those of *A. biguttatus,* and a potential negative effect of oak bark was observed. The parasitism rate on EAB may also have been higher if eggs were freshly laid, and not shipped from North America. Together, these findings suggests that *O. agrili* might not be likely to parasitise *A. biguttatus* on oak trees.

Oobius agrili did not parasitise *A. sulcicollis* in these assays. Its eggs are considerably smaller than those of EAB (in North American trials eggs similar in size to those of EAB were parasitised), and it appears to be less closely related to EAB than *A. biguttatus* according to a phylogenetic study (Bauer and Liu, 2007; Kelnarova et al., 2018). However, results from the study with *A. convexicollis*, whose eggs are similar in size to *A. sulcicollis*, suggest that parasitism by *O. agrili* may be irrespective of egg size and phylogenetic proximity. Insect eggs are generally inconspicuous and produce minimal or no volatiles (Fatouros et al., 2008). As a result, parasitoids that target eggs often rely on an 'information detour,' using oviposition-induced plant cues or other herbivore signals, such as host sex pheromones, to locate their hosts (Vet and Dicke, 1992; Fatouros et al., 2008). The cues, including potential semiochemicals, that *O. agrili* relies on to locate *A. planipennis* eggs are still unknown (Quinn et al., 2022). Although initial phylogenetic studies indicated that *A. convexicollis* is more distantly related to EAB (Kelnarova et al., 2018), parasitism by *O. agrili* still occurred. This indicates that *O. agrili*'s host selection may be influenced more by the hosts' ecological niche, as both EAB and *A. convexicollis* feed on ash trees, rather than solely by phylogenetic relationship or egg size. However, a field study in North America found no difference in parasitism between sentinel EAB eggs deployed on *F. pennsylvanica* and fringetree, *Chionanthus virginicus* L., indicating that *O. agrili* may not rely solely on host tree species for egg parasitism, or may accept a broader host tree range (ash and fringetree are both Oleaceae) (Quinn et al., 2022). Further laboratory and field studies are needed to better understand *O. agrili's* host-seeking behaviour.

Parasitism of non-target North American *Agrilus* species in laboratory assays has been observed for *S. galinae* and *O. agrili,* but not for *T. planipennisi* (see Section 1), and so this study is the first to confirm the risk to non-target species from this larval parasitoid. In this study, the parasitism rates of *S. galinae* and *T. planipennisi* on EAB and *A. biguttatus* larvae did not differ, although replication was limited.

Direct non-target impacts of insect biological control agents on native insects are difficult to detect or quantify in the field but may have significant impacts on populations. For example, the tachinid fly *Compsilura concinnata* Meigen (Diptera: Tachinidae), a generalist parasitoid which was introduced to North America to control various forest pests, appears to be implicated in the decline of native saturniid moths (Boettner et al., 2000). Threats to non-target *Agrilus* species, a charismatic group with an important ecological niche as early colonisers of dying and dead woody material, would be taken into consideration by the regulatory body that grants licenses to release non-native biological control agents.

The parasitism of *A. biguttatus* and *A. convexicollis* which was observed in the laboratory may not be replicated in a woodland setting. Parasitoids are attracted first to long-range olfactory or visual cues of suitable host trees, and only subsequently to short-range olfactory cues produced by the ovipositing females, or vibrations produced by larval feeding (Tumlinson et al., 1993; Liu and Bauer, 2006; Bauer and Liu, 2007; Wang et al., 2010a). The volatiles produced by the tree hosts of native British *Agrilus* species such as oak (*A. biguttatus*), pear and hawthorn (*A. sinuatus*) may not be attractive to the parasitoids or may be less attractive than those produced by ash trees. In this study, "no-choice" experiments were conducted, and the behaviour of the parasitoids may differ when a choice between hosts is permitted. A criticism of host-range tests is that confinement in small spaces for extended periods prevents parasitoids from exhibiting natural host-searching behaviours, such as foraging, which allows the rapid abandonment of patches containing sub-optimal hosts, potentially leading to an overestimation of non-target parasitism (Gómez Marco et al., 2023). For example, in no-choice conditions, parasitoids may lay eggs on or in non-target hosts as substitutes when optimal hosts are unavailable (Yang et al., 2008). Negative results from laboratory tests, showing no parasitism, provide strong evidence that a species is not a host in natural environments, while positive results remain less definitive and harder to interpret (Charles et al., 2019). Separately, the bark thickness of oak trees may preclude parasitism of *A. biguttatus,* at least by *T. planipennisi,* in a woodland setting. *Agrilus biguttatus* larvae develop mainly within the main stem of oak trees, and *T. planipennisi* is unable to penetrate thick bark due to its relatively short ovipositor (Abell et al., 2012).

Interestingly, *T. planipennisi* females appeared to be less likely to parasitise larvae if multiple females were present within a cup. If replicated in further studies, this finding would have implications for mass rearing of this species.

To verify the likelihood of the EAB parasitoids attacking *A. biguttatus* and *A. convexicollis*, future work on these species should include "choice" assays, in which parasitoids are able to choose between EAB and the non-target species and because these are conducted in small arenas without ecological context further work should follow, including behavioural olfactometry. Olfactometry may help determine the attractiveness of non-ash-feeding *Agrilus* species to the parasitoids. More complex, semi-field testing which is more representative of the woodland heterogeneity would also be required. Further research is also needed into the risk to other potential hosts, particularly *Agrilus* that colonise trees. Other knowledge gaps that require investigation include additional potential non-target impacts the three parasitoids may have within Great Britain and Europe, such as their hybridisation potential with congeneric native parasitoids, and the potential impacts of competition with native insects for resources. The suitability of both the British climate and attractiveness of *F. excelsior* as host trees for the parasitoids remain uncertain, as is the influence of ash dieback disease on host suitability and attractiveness (Barratt et al., 2010; N. Audsley, unpublished). Factors such as the economic, environmental, and social costs of releasing the parasitoids must be weighed against the expected benefits of their protection of ash trees.

Moreover, the potential for natural enemies of *Agrilus* species present in the European range of *F. excelsior* to control EAB should be pre-emptively explored, particularly in light of the findings of this study. Parasitoids of *Agrilus* species present Great Britain and Europe may attack and help control EAB on its arrival. While native natural enemies in North America have had limited impact on EAB due to its rapid population growth during the early stages of invasion (Sadof et al., 2017; Duan et al., 2023a), they may have more potential in Europe, particularly if European ash is less susceptible (Herms and McCullough, 2014). For example, *Spathius polonicus* Niezabitowski (Hymenoptera: Braconidae), a congener of *S. galinae,* was found to have parasitised 30 of 54 large-instar larvae collected in Moscow Province (Orlova-Bienkowskaja and Belokobylskij, 2014). This species is thought to be widely, although usually scarcely distributed within Europe, and as it is already present within England (although not yet collected and cultured) it may be a more ecologically and environmentally suitable candidate for augmentative release as a control of EAB (Broad and Barclay, in press).

CRediT authorship contribution statement

Katy Reed: Methodology, Investigation, Formal analysis, Writing – original draft. **Freya Cole:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Neil Audsley:** Funding acquisition, Conceptualization. **Anastasia Uglow:** Investigation. **Rachel Down:** Resources. **Kerry Barnard:** Investigation. **Daegan Inward:** Writing – review & editing, Supervision.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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