



Intrinsic competition between two European egg parasitoids of the brown marmorated stink bug

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Abstract

Following the accidental introduction and spread of the invasive polyphagous agricultural pest *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), the two European egg parasitoids *Anastatus bifasciatus* (Geoffroy) (Hymenoptera: Eupelmidae) and *Ooencyrtus telenomicida* (Vassiliev) (Hymenoptera: Encyrtidae) have been investigated for inundative biological control. Since the competitive outcome between the two generalist parasitoids is difficult to predict, intrinsic competition was investigated with a time-course development study. Both species readily oviposited in *H. halys* eggs containing eggs and early instar larvae of the competitor, but oviposition decreased when eggs contained late instar larvae and pupae. *Ooencyrtus telenomicida* offspring emergence from multiparasitized eggs was significantly lower than that from rearing controls, independent of the order of parasitization. *Anastatus bifasciatus* offspring emergence was not influenced by the presence of *O. telenomicida* when it parasitized as the first species, but emergence was decreased after oviposition in eggs containing *O. telenomicida* larvae and pupae. There was no indication that *O. telenomicida* can act as a facultative hyperparasitoid of *A. bifasciatus*. These results suggest that *A. bifasciatus* is the superior intrinsic competitor and no or minor negative implications for *A. bifasciatus* are expected if released in combination with *O. telenomicida*.

KEYWORDS

Anastatus bifasciatus, biological control, *Halyomorpha halys*, invasive species, multiparasitism, *Ooencyrtus telenomicida*

1 | INTRODUCTION

Interspecific competition between parasitoid species is a common phenomenon that can influence species coexistence and community structure (Cusumano, Peri, & Colazza, 2016; Godfray, 1994). In terms of biological control, an increased diversity of natural enemies can lead to null, additive, antagonistic or synergistic effects (Casula, Wilby, &

Thomas, 2006) and thus, advantages and disadvantages of using single or multiple species against a single target have been long discussed in biological control and more generally in multitrophic interaction studies (Bográn, Heinz, & Ciomperlik, 2002; Heinz & Nelson, 1996; Pemberton & Willard, 1918; Denoth, Frid, & Myers, 2002).

Many egg parasitoids in the genera *Anastatus* Motschulsky (Hymenoptera: Eupelmidae) and *Ooencyrtus* Ashmead

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(Hymenoptera: Encyrtidae) are highly polyphagous, attacking both lepidopteran and hemipteran hosts, and sometimes even share the same hosts. In fact, species from both genera have been used as biological control agents against the same pests, e.g., *Anastatus japonicus* Ashmead, *Ooencyrtus corbetti* Ferrière and *O. phongi* Trjapitzin, Myartseva & Kostjukov against the litchi stink bug, *Tessaratoma papillosa* Drury (Hemiptera: Pentatomidae) (Halperin, 1990; Hirose, 1964; Nanta, 1988; Liu, Zhang, Gu and Zhang, 2000) or *A. japonicus* and *O. kuvanae* Howard against the gypsy moth, *Lymantria dispar* L. (Lepidoptera: Erebidae) (Parker, 1933).

In Europe, species from both genera, *A. bifasciatus* (Geoffroy) and *O. telenomicida* (Vassiliev), have been reared from eggs of the invasive Asian brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) (Costi, Haye, & Maistrello, 2019; Haye, Fischer, Zhang, & Garipey, 2015; Roversi et al., 2016), which was first introduced into Switzerland in the early 2000s (Wermelinger, Wyniger, & Forster, 2008) and has since spread into many European countries (reviewed in Leskey & Nielsen, 2018). In the past couple of years, the highly polyphagous *H. halys* has become a key pest of fruit crops and hazelnut in Italy and Georgia (Bosco, Moraglio, & Tavella, 2017; Maistrello et al., 2017). There is a range of parasitoid species native to Europe that attack *H. halys* eggs, but so far only *A. bifasciatus* and *O. telenomicida* have been shown to terminate *H. halys* nymph development and successfully develop on viable *H. halys* eggs (Haye et al., 2015). Consequently, studies on inundative biological control of *H. halys* using *A. bifasciatus* and *O. telenomicida* have been initiated in Europe (e.g., Stahl, Babendreier, & Haye, 2019a). The outcome of inundative releases will highly depend on the host searching behaviour (Rondoni et al., 2017) and parasitism capacity of both species, but also on their ability to succeed in interspecific competition. Detrimental effects of parasitoid competition on biological control may arise if both parasitoids are mass released simultaneously against *H. halys* in European fruit orchards, where one species is released while the other species naturally occurs or where both species overlap in their natural distribution.

In earlier studies investigating the competition between either *A. bifasciatus* or *O. telenomicida* and Asian and European *Trissolcus* Ashmead species (Hymenoptera: Platygasteridae), it was demonstrated that both species were superior intrinsic (=larval) competitors (Cusumano, Peri, Vinson, & Colazza, 2011; Konopka, Haye, Garipey, & McNeil, 2017). In addition, since both are strongly synovigenic and have a similar life history, it remains difficult to predict the outcome of competition between *A. bifasciatus* and *O. telenomicida* (Cusumano, Peri, Vinson, & Colazza, 2012; Stahl, Babendreier, & Haye, 2019). Exactly this was investigated in the present paper, addressing the following questions: (a) Do *A. bifasciatus* and *O. telenomicida* females accept eggs previously parasitized by the other species?; (b) Are previously parasitized eggs suitable for development of either species?; (c) Is the outcome of the larval competition dependent on the order of parasitization?; (d) Which species is the superior larval competitor and what are the implications for inundative biological control?

2 | MATERIAL AND METHODS

2.1 | Insect rearing

2.1.1 | Hosts

A colony of *H. halys* was established at CABI Switzerland in 2012 from individuals collected in Zurich and Basel, Switzerland. Groups of 50 adults were kept in gauze cages ("BugDorm-4090 Insect Rearing Cage 47.5 × 47.5 × 47.5 cm," MegaView Science Co. Ltd., Taichung, Taiwan) at 26°C, 70% RH, and a 16L:8D photoperiod. Adults and nymphs were fed twice a week with a diet of corn, beans, apples, peanuts and carrots. Fresh branches of cherry trees (*Prunus avium* L.), common ash (*Sorbus aucuparia* L.), or common ivy (*Hedera helix* L.) were offered when available as oviposition substrate and additional food source. Folded pieces of black mesh (separation fleece for pots, Windhager AG, Hünenberg, Switzerland) placed at the bottom of the cages served as additional oviposition substrate. Egg masses were collected on a daily basis and used for the competition assays. To provide the two parasitoid species with optimal rearing conditions and avoid confounding results due to acclimatization to *H. halys* as a host before the assays, they were reared on species that were evaluated to be closer to their natural hosts before *H. halys* spread in Europe.

A colony of *Nezara viridula* L. (Hemiptera: Pentatomidae) was established from individuals collected at various places in the Canton of Ticino, Switzerland in 2017. Both nymphs and adults were reared on corn, beans and potted broad bean plants (*Vicia faba* L., Fabaceae) in the same type of cages and under the same conditions as *H. halys*. *Nezara viridula* eggs were collected daily to maintain the *O. telenomicida* colony.

A colony of *Dendrolimus pini* L. (Lepidoptera: Lasiocampidae) was established in 2016 from commercially obtained eggs collected at Slatiňany, Czech Republic. The caterpillars were reared in the same cages as described above and provided with fresh pine branches twice a week. Newly emerged moths were provided with pine branches as oviposition substrate and eggs were collected on a daily basis to maintain the *A. bifasciatus* colony.

2.1.2 | Parasitoids

A colony of *A. bifasciatus* was established in 2013 from sentinel *H. halys* egg masses exposed in the Canton of Valais, Switzerland (Haye et al., 2015), and specimens from the original colony were identified by Lucian Fusu (Alexandru Ioan Cuza University). Freshly emerged *A. bifasciatus* adults were collected daily and transferred to 100 × 115 mm mesh-top cylindrical plastic containers placed above a 90 × 20 mm Petri dish filled with honey-water solution. The container and the Petri dish were connected through two cotton wicks and stored in an incubator set at a light/temperature cycle of L 16 hr/20°C and D 8 hr/15°C. Every second day, the honey-water solution was exchanged, and new drops of honey

added to the mesh of the container. Seven days after emergence, when preliminary studies showed the highest host acceptance, fresh (<24 hr old) or cold-stored (<24 hr old when stored at 10°C for a maximum of 3 days) *D. pini* eggs glued onto cardboard pieces were placed on the bottom of the container and replaced every second day. Parasitized *D. pini* eggs were kept at 26°C, 70% RH, and a 16L:8D photoperiod.

A colony of *O. telenomicida* was originally established in 2008 from *N. viridula* sentinel egg masses placed in tomato fields around Palermo, Italy. Freshly emerged *O. telenomicida* adults were collected daily and transferred to plastic tubes (7 cm height and 2.5 cm diameter) plugged with a sponge and kept in an incubator set at a light/temperature cycle of L 16 hr/20°C and D 8 hr/15°C. Adults were fed with honey droplets and provided with fresh (<24 hr old) or cold-stored (<24 hr old when stored at 10°C for a maximum of 3 days) *N. viridula* egg masses every second day seven days after adult emergence. Parasitized *N. viridula* eggs were kept at 26°C, 70% RH, and a 16L:8D photoperiod.

2.1.3 | Experimental design

Single fresh (<24 hr old) *H. halys* eggs, which were glued individually on 1 cm × 1 cm black cardboard pieces with “Cementit” (merz + benteli AG, Niederwangen, Switzerland) and placed in plastic Petri dishes (5 × 1 cm), were used in the competition studies. Individual *H. halys* eggs were offered instead of egg masses to increase the probability of *A. bifasciatus* oviposition and to reduce non-concurrent host feeding by *A. bifasciatus* females (Konopka et al., 2017; Stahl, Babendreier, & Haye, 2019). Randomly selected *A. bifasciatus* females (naïve, mated, 7 days old) were observed for a maximum of three hours until probing and removed once they had left the egg for fifteen minutes. An egg was counted as “probed” when females had inserted their ovipositor into the egg. Since *A. bifasciatus* females do not mark parasitized host eggs, successful oviposition was measured indirectly by keeping a proportion of the probed eggs as rearing control ($n = 65$) or destructively by dissections of probed eggs ($n = 180$). Previous dissections had revealed *O. telenomicida* eggs up to 24 hr (interval 0 day) and *A. bifasciatus* eggs up to 48 hr (intervals 0–2 days) after oviposition into fresh (<24 hr old) *H. halys* eggs, when stored at 26°C. After that, parasitoid larvae hatched and grew, and *H. halys* eggs contained late instar larvae of either species at day 9 and pupae of either species at day 14 after oviposition.

To assess the outcome of competition, *O. telenomicida* females (naïve, mated, 7 days old) were provided with single eggs that were probed by *A. bifasciatus* 0, 1, 2, 3, 4, 5, 9 or 14 days earlier. In preliminary tests, *O. telenomicida* showed very little oviposition within the first seven hours of observation. Consequently, instead of observing *O. telenomicida* females during egg exposure, *H. halys* eggs were checked for parasitism after 24 hr. In contrast to *A. bifasciatus*, eggs parasitized by *O. telenomicida* can easily be recognized without dissections by the respiratory egg stalk (one per egg) protruding from the host egg chorion (Takasu and Hirose, 1988); the number of

egg stalks was recorded. Single, unparasitized *H. halys* eggs (<24 hr) were offered to individual *O. telenomicida* females ($n = 210$) as controls, i.e., in the absence of competitors. In addition, fresh *H. halys* eggs ($n = 125$) were kept as quality control of the host eggs. The entire experiment was also conducted vice versa, i.e., by offering egg parasitized by *O. telenomicida* to females of *A. bifasciatus*, using the same time intervals as above. Due to the 24-hr exposure time for *O. telenomicida*, the intervals were encompassing a range of 24 hr with the 0 day interval representing 0–24 hr, 1 d interval 24–48 hr, etc. For each time interval, 20 replicates were targeted, but due to reduced acceptance of older eggs by *O. telenomicida* fewer replicates were achieved for time intervals >4 days. All assays were carried out at 26°C, 70% RH, and a 16L:8D photoperiod. Multiparasitized *H. halys* eggs as well as rearing controls were kept at the same conditions and checked daily for the number, sex and species of emerged parasitoids.

2.1.4 | Statistical analyses

Host probing (only *A. bifasciatus*) and oviposition (*A. bifasciatus* and *O. telenomicida*) were compared within species using a generalized linear mixed model (GLMM) with a binomial error distribution (logit link function) with the different treatments (time intervals) as independent variable and the number of *O. telenomicida* egg stalks as random variable where *O. telenomicida* was exposed first. A Tukey's all-pair comparison was run as a post hoc test. The same GLMM was repeated for both parasitoid species measuring the effect of multiparasitism on adult emergence. The number of progenies was compared between parasitoid species with a Mann–Whitney *U* test. All statistics were carried out with R, version 3.2.3 (R Core Team, 2017) and the development environment RStudio (RStudio Team, 2016). For the GLMMs, the package “lme4” (Bates et al., 2015) and for the Tukey's post hoc tests the package “multcomp” (Hothorn et al., 2017) were employed.

3 | RESULTS

3.1 | Probing and oviposition

Ooencyrtus telenomicida oviposition occurred in *H. halys* eggs containing different developmental stages of *A. bifasciatus* (Figure 1). When females were provided with *H. halys* eggs that had been parasitized by *A. bifasciatus* 0, 1, 2, 3, or 4 days previously, oviposition did not significantly differ from controls, but oviposition significantly decreased (<20%) when time after parasitism by *A. bifasciatus* was greater than four days (binomial GLM, $df = 8, 294, \chi^2 = 164.1, p < .001$) (Figure 1).

No significant differences were found for *A. bifasciatus* females probing *H. halys* eggs that had been parasitized 0 to 14 days previously by *O. telenomicida*, or unparasitized controls (binomial GLMM, $df = 8, 1,292, \chi^2 = 5.3, p = .729$) (Figure 2). However, in terms of

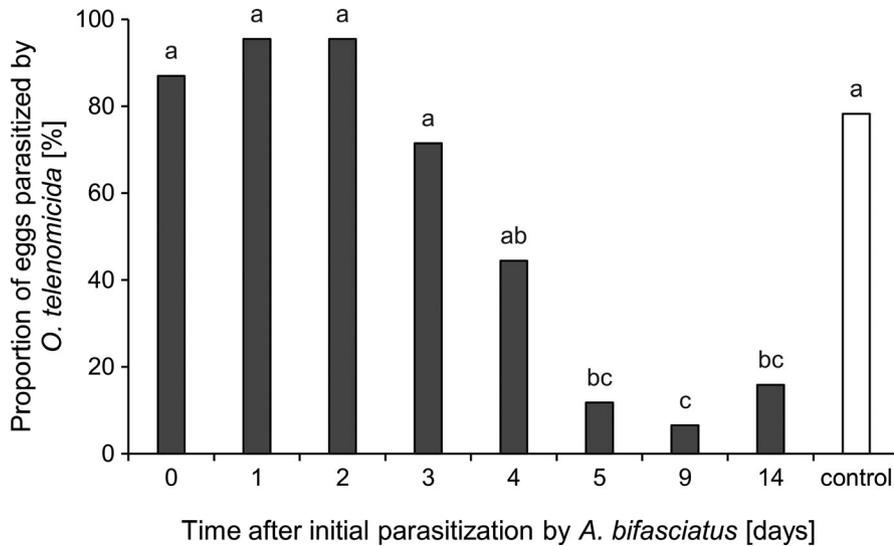


FIGURE 1 Proportion of *H. halys* eggs parasitized by *O. telenomicida* when eggs were exposed to *A. bifasciatus* 0–14 days before. Unparasitized, fresh (<24 hr) *H. halys* eggs were used as controls. Bars sharing the same letter are not significantly different by Tukey-adjusted mean separations (binomial GLM, $df = 8$, 294, $\chi^2 = 164.1$, $p < .01$)

oviposition, *A. bifasciatus* females discriminated among *H. halys* eggs containing different developmental stages of *O. telenomicida* (binomial GLMM, $df = 8$, 171, $\chi^2 = 36.2$, $p < .001$) (Figure 3). No significant differences were found among treatments where eggs were parasitized by *O. telenomicida* 0 to 9 days before and controls (unparasitized eggs) (Tukey's all-pair comparisons, all $p > .05$) (Figure 3). However, oviposition in eggs that were parasitized 14 days before was significantly lower (20%) than in controls (90%) (Tukey's all pair comparisons, $p = .0054$) (Figure 3).

3.1.1 | Emergence

In controls, i.e., from eggs parasitized by *O. telenomicida* in the absence of competitors ($n = 210$), 70% of all parasitized eggs gave rise to *O. telenomicida* adults ("Ot rearing control," Figures 4 and 5). In the dissection controls for *A. bifasciatus*, 90% of all observed probing events ("probed eggs"; $n = 180$) resulted in oviposition ("Ab dissection control" Figure 4), while only 65% of "probed" eggs ($n = 65$) gave rise to *A. bifasciatus* in rearing controls ("Ab rearing control" Figures 4 and 5), suggesting that some mortality occurs during development.

When multiparasitism of the same egg occurred, both *O. telenomicida* and *A. bifasciatus* could sometimes complete

development on *H. halys* eggs previously parasitized by the other species, but overall far more *A. bifasciatus* than *O. telenomicida* adults emerged (Mann-Whitney *U* test, $W = 216.5$, $p < .001$, $n = 16$) (Figures 4 and 5). The developmental outcome depended on the order of parasitization and the time interval between the parasitizations by the two competing species. In all cases, a proportion of multiparasitized eggs produced no parasitoids at all (Figures 4 and 5).

When *A. bifasciatus* was the first species to parasitize *H. halys* eggs, offspring production at the time intervals 0 to 4 days was not significantly different from rearing controls regardless of subsequent attack by *O. telenomicida* (binomial GLMM, $df = 4$, 108, $\chi^2 = 0.857$, $p = .9306$) (Figure 4). In contrast, in the same treatments, the offspring emergence of *O. telenomicida* was significantly lower (0%–5%) than in rearing controls (70%) (binomial GLMM, $df = 5$, 188, $\chi^2 = 103.1$, $p < .001$) (Figure 4). Little emergence of either species was recorded from the few multiparasitized eggs (due to low acceptance by both species) with five to fourteen days between initial parasitization events (Figure 4). These treatments (5, 9, 14 days) were excluded from the statistical analysis. Both female and male *A. bifasciatus* and *O. telenomicida* offspring was recorded. In all treatments with over ten *A. bifasciatus* offspring (0–4 days), the sex ratio was at least 0.1 (proportion females).

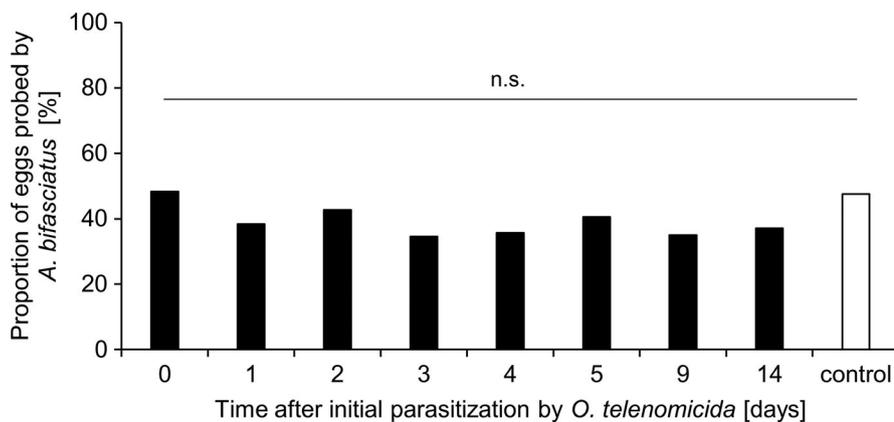


FIGURE 2 Proportion of *H. halys* eggs "probed" by *A. bifasciatus* when eggs were parasitized by *O. telenomicida* 0–14 days before. Unparasitized, fresh (<24 hr) *H. halys* eggs were used as controls. There were no significant differences between treatments (binomial GLMM, $df = 8$, 1,292, $\chi^2 = 5.3$, $p = .729$)

FIGURE 3 Proportion of *H. halys* eggs previously parasitized by *O. telenomicida* and subsequently probed by *A. bifasciatus*, assessed from dissections. Unparasitized, fresh (<24 hr) *H. halys* eggs probed by *A. bifasciatus* were used as controls. Sample sizes equal 20 in each treatment and the control; bars sharing the same letter are not significantly different by Tukey-adjusted mean separations (binomial GLMM, $df = 8, 171, \chi^2 = 36.2, p < .001$)

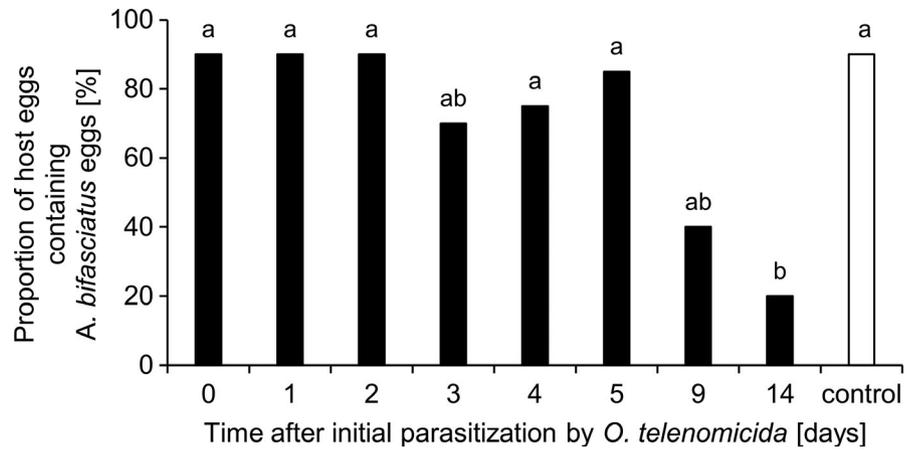


FIGURE 4 Proportion of *H. halys* eggs giving rise to *A. bifasciatus* (Ab), *O. telenomicida* (Ot), *H. halys* (Hh) nymphs or nothing (dead eggs), following probing by *A. bifasciatus* (1st) and subsequent parasitism by *O. telenomicida* (2nd) at different time intervals between attacks. Sample sizes are given in brackets. Bars sharing the same letter are not significantly different from each other; capital letters show result for *A. bifasciatus*, and non-capitalized letters for *O. telenomicida*

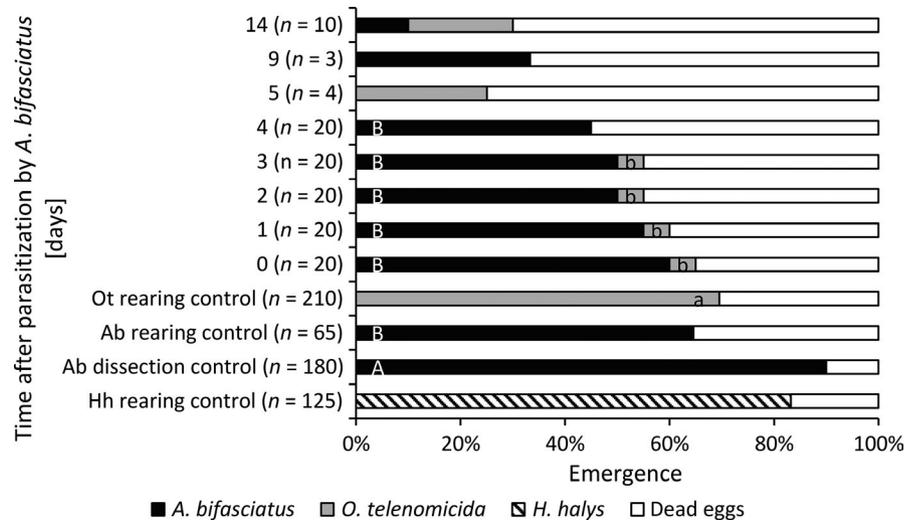
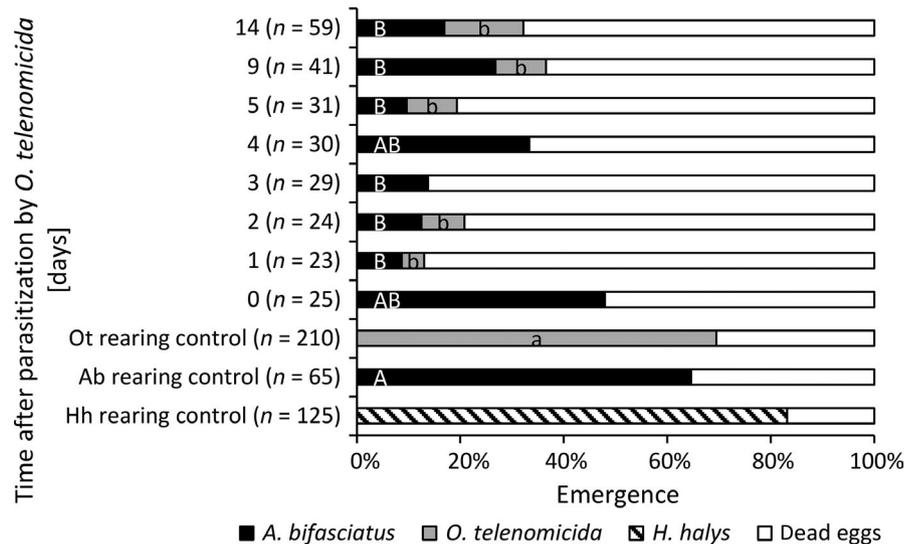


FIGURE 5 Proportion of *H. halys* eggs giving rise to *A. bifasciatus* (Ab), *O. telenomicida* (Ot), *H. halys* (Hh) nymphs or nothing (dead eggs), following parasitism by *O. telenomicida* (1st) and subsequent probing by *A. bifasciatus* (2nd) at different time intervals between attacks. Sample sizes are given in brackets. Bars sharing the same letter are not significantly different; capital letters show result for *A. bifasciatus*, and non-capitalized letters for *O. telenomicida*



When *O. telenomicida* was the first species to parasitize *H. halys* eggs, multiparasitized eggs yielded no or very few *O. telenomicida* offspring (max. 4%) compared to controls (70%) (binomial GLMM, $df = 8, 464, \chi^2 = 235.3, p < .001$) (Figure 5). In contrast to the reverse order of parasitization (*A. bifasciatus* attacking first), multiparasitism also had a negative impact on *A. bifasciatus* offspring production

(binomial GLMM, $df = 7, 322, \chi^2 = 19.2, p = .00763$). When probing by *A. bifasciatus* occurred shortly after oviposition by *O. telenomicida* (0 day), *A. bifasciatus* offspring production (48%) was not significantly different from controls (65%), but significantly reduced in all other treatments apart from day 4, varying from 8% to 33%. Due to the negative impact on both parasitoid species, the proportion

of dead eggs (no emergence) was up to 87% (Figure 5). Both female and male *A. bifasciatus* and *O. telenomicida* offspring were recorded. Female *A. bifasciatus* progeny only emerged from eggs parasitized by *A. bifasciatus* immediately after their exposure to *O. telenomicida* (0 days).

4 | DISCUSSION

Interspecies competition of parasitoids can cause changes in natural communities and influence the outcome of biological control programs (Meiners & Peri, 2013). This study is the first assessing the outcome of intrinsic competition between two European egg parasitoids, *A. bifasciatus* and *O. telenomicida*, considered for inundative releases against invasive brown marmorated stink bug in Europe. Apart from competition for *H. halys* eggs after inundative releases, competition may also naturally occur since the ecological host ranges of the two parasitoid species overlap, including hosts such as *Iphioides polairius feisthamelii* (Duponchel) (Lepidoptera: Papilionidae) and *N. viridula* (Cusumano, Peri, Boivin, & Colazza, 2015; Stahl, Babendreier, & Haye, 2018; Stefanescu, Pintureau, Tschorsnig, & Pujade-Villar, 2003).

Both *A. bifasciatus* and *O. telenomicida* females parasitized eggs that were previously attacked by the other species, but multiparasitism was dependent on the time interval between the two parasitization events. As very different developmental stages of the competitors meet each other inside the host eggs at these different times, it is likely that this factor plays an important role for the outcome of the competition. Fresh host eggs containing eggs or early larval stages of their competitors were considered highly suitable for oviposition by both species, but oviposition noticeably declined when parasitoids were offered older host eggs containing late instar larvae or pupae. Since *O. telenomicida* females frequently rejected to oviposit in eggs containing mid and late instar larvae (5, 9 days) or pupae (14 days) of *A. bifasciatus*, females might be responding to internal markers such as an injected secretion, physical changes, or changes in the hemolymph (reviewed in Vinson, 1976), by the means of sensory receptors (chemical or mechanical) on the ovipositor (Hermann & Douglas 1976). Parker (1933) conducted similar competition experiments with *O. kuvanae* and *A. japonicus*, egg parasitoids of the gypsy moth, and explained the decrease in oviposition by *O. kuvanae* with the movement of the larger *A. japonicus* larvae, touching its ovipositor and hindering its oviposition, which caused the *Ooencyrtus* females to leave the host eggs eventually. In *A. bifasciatus*, reduced oviposition was only observed for eggs containing late instar larvae (9 days) or pupae (14 days) of *O. telenomicida*, which might relate to the morphology of their larvae. The respiratory stalk of the parasitoid egg remains attached to the *O. telenomicida* larva as it grows and only becomes disconnected shortly before pupation, which might restrict the movement of the larvae and thus hinder active defense against oviposition of *A. bifasciatus*. On the other hand, the frequent oviposition into host eggs containing eggs or young larvae of competitors suggests that females of both species were

not deterred from oviposition, despite possibly limited success of offspring emergence.

Since parasitoids often share the same hosts, many species have developed mechanisms to avoid competition or to win against competitors. In an earlier study, it was demonstrated that *O. telenomicida* females mediated physiological suppression by injecting factor(s) prior to the egg deposition which may alter the ooplasm inside the eggs of its host, *N. viridula*, making it unsuitable for larval development of its competitor *Trissolcus basalis* (Wollaston) (Cusumano et al., 2012). Remarkably, in the same study the emergence of *T. basalis* from multiparasitized eggs was always strongly reduced, regardless of whether host eggs were first parasitized by *T. basalis* and then punctured by *O. telenomicida*, or vice versa. Similarly, *O. pityocampae* (Mercet) was the superior intrinsic competitor over *Trissolcus agriopae* Kozlov & Lê inside eggs of the green stink bug, *Brachynema signatum* Jakovlev (Hemiptera: Pentatomidae) (Cusumano et al., 2011). However, the results of the present study suggest that the factors (potentially including a venom) injected by *O. telenomicida* did not have the same advantageous effects when competing with *A. bifasciatus*. The offspring emergence of *O. telenomicida* was drastically reduced (to <10%) in multiparasitized eggs, regardless of the time interval or the oviposition order. The mechanism preventing the development of *O. telenomicida* remains unknown. It is not known if *Anastatus* species have venom glands, but it seems likely since they were found in other Eupelmidae like *Eupelmus orientalis* (Crawford) and *E. urozonus* Dalman (Doury, Bigot, & Periquet, 1997; Moretti & Calvitti, 2014). It is also conceivable that mechanical damage inflicted by the probing itself, or substances other than venom injected together with the egg or substances produced by the larvae could arrest development of competing larvae (Thompson, 1980; Dahlman 1991; Cusumano et al., 2018). On the other hand, first instar *Anastatus* larvae are equipped with simple sharp mandibles and a bifurcate tail (Parker & Thompson, 1925), which makes them highly mobile, and thus, they would be able to quickly reach competitors inside their hosts to destroy them (Harvey, Poelman, & Tanaka, 2013).

Interestingly, the outcome of intrinsic competition was less uniform for *A. bifasciatus*. If *A. bifasciatus* was the first species to oviposit and the same eggs were subsequently parasitized by *O. telenomicida* no later than 4 days after, no negative effects on *A. bifasciatus* emergence was observed. In other words, the piercing of the egg and possibly the injection of venom by *O. telenomicida* females did not affect *A. bifasciatus* development. Thus, it was confirmed that *A. bifasciatus* is a superior larval competitor, as was also shown when the intrinsic competition between *A. bifasciatus* and *Trissolcus japonicus* (Ashmead) was investigated (Konopka et al., 2017). However, when the order of species was reversed and *O. telenomicida* parasitized eggs prior to *A. bifasciatus*, the outcome of competition drastically changed. If *A. bifasciatus* parasitized the eggs immediately after *O. telenomicida* (0 and 24 hr), *A. bifasciatus* remained the superior competitor and only *A. bifasciatus* offspring developed. In contrast, if parasitized eggs were attacked by *A. bifasciatus* at least 24 to 48 hr later (1 day), only few *A. bifasciatus* emerged and nearly all multiparasitized eggs were dead (no parasitoid emergence). Although it is

not entirely clear what caused the sudden change, one plausible scenario might be that the hatching of the *O. telenomicida* larvae within the first 24 to 48 hr had changed the physiology of the host in a way that *A. bifasciatus* larvae were not able to develop, before being killed by the ovipositing *A. bifasciatus* female. This change could be due to substances released by secretions during egg hatching (e.g., Mackauer, 1959), by the larvae (e.g., Moretti & Calvitti, 2014) or by consumption of host resources to an extent that might be detrimental to the development of *A. bifasciatus*. Evidence for the latter point is provided by Stahl et al. (2018), showing that *H. halys* eggs are near the lower size limit allowing complete female development. This is further supported by the lack of female *A. bifasciatus* progeny when oviposition occurred after the *O. telenomicida* larva had hatched and presumably started to consume the host resources. However, none of these hypotheses explains the comparatively high *A. bifasciatus* emergence from 4-days-old eggs.

It has been demonstrated in multiple systems that *Ooencyrtus* species can act as facultative hyperparasitoids, mainly of *Trissolcus* species (Cusumano et al., 2011; Mohammadpour, Jalali, Michaud, Ziaaddini, & Hashemirad, 2014), and the same was assumed for *O. kuvanae* hyperparasitizing hibernating *A. japonicus* larvae in gypsy moth eggs (Howard & Fiske, 1911). However, Parker (1933) was not able to confirm this and concluded that the few emerging *O. kuvanae* developed on *Anastatus* larvae that were dead or dying at the time. Since parasitism in *A. bifasciatus* rearing controls was lower than in dissection controls, suggesting some baseline mortality occurs in *A. bifasciatus* larvae, the few *O. telenomicida* emerging in our experiments may have not only resulted from eggs without *A. bifasciatus* oviposition, but also from eggs containing dead *A. bifasciatus* stages. Accordingly, our study supports the conclusion provided by Parker (1933), i.e., that we did not find any evidence that *Ooencyrtus* species can act as facultative hyperparasitoids of *Anastatus* species.

In this study, individual *H. halys* eggs were used instead of egg masses. Limiting the number of host eggs to one increased the level of self-superparasitism of *O. telenomicida*. This additional factor of intraspecific competition may have confounded the results for the interspecific competition. However, despite self-superparasitism, 70% of the parasitized *H. halys* eggs yielded *O. telenomicida* in the rearing control. Since superparasitized eggs were randomly distributed over all treatments, the conclusion that *O. telenomicida* offspring emergence was negatively influenced by *A. bifasciatus* parasitization before and after remains the same. In reverse, we cannot exclude that the presence of several *O. telenomicida* reduced the offspring emergence of *A. bifasciatus* simply due to food limitation. Additional experiments, using host eggs containing only single *O. telenomicida* larvae, will be necessary for clarification.

Using individual eggs is also not representative of a field setting where *H. halys* eggs are present in clusters of around twenty-eight eggs and parasitoid females can choose between different eggs. Considering the egg laying behaviour of *A. bifasciatus* (Stahl, Babendreier, Marazzi, et al., 2019) and *O. telenomicida* (Roversi et al., 2016) to only parasitize a portion of a *H. halys* egg mass, they might be able to avoid larval competition even if they find the same

egg mass. This would depend on the females' ability to recognize a parasitized egg and judge it unsuitable, which our (no-choice) experiments only suggest for eggs containing later instar larvae and pupae of the respective competitor. Several *H. halys* egg masses in Northern Italy have recently been shown to give rise to both *A. bifasciatus* and the adventive *T. mitsukurii* (Ashmead) (Scaccini et al., 2020). This has so far not been reported for the combination of *A. bifasciatus* and *O. telenomicida*. A possible reason for this is that in contrast to *A. bifasciatus*, *O. telenomicida* has not been retrieved from *H. halys* egg masses in Europe without prior releases (Costi et al., 2019; Haye et al., 2015; Moraglio et al., 2020; Scaccini et al., 2020; Stahl, Babendreier, Marazzi, et al., 2019), which could indicate that *H. halys* is not a preferred host for *O. telenomicida*. However, the parasitoid species have a significant geographical overlap in parts of Europe that have been invaded by *H. halys* (Noyes, 2014) in addition to sharing a multitude of host species apart from *H. halys* (Noyes, 2014; Stahl et al., 2018). Consequently, intrinsic competition could impact biological control efforts and naturally occurring parasitoid populations. For example, inundative releases of *A. bifasciatus* could lead to overflow of the released parasitoids into natural habitats and the potentially ensuing intrinsic competition could reduce *O. telenomicida* populations in native pentatomid hosts (van Lenteren & Loomans, 2006). Another aspect of the interactions between the two parasitoid species, extrinsic competition was not explored. In contrast to many *Trissolcus* species, neither *A. bifasciatus* nor *O. telenomicida* adults seem to exhibit guarding behaviour after oviposition and females of the two species have even been observed parasitizing the same *H. halys* egg masses simultaneously in a laboratory setting (JMS, personal observation). Consequently, even if females of *A. bifasciatus* and *O. telenomicida* met in a field setting, influence of adult competition on parasitoid emergence is expected to be negligible.

In conclusion, the intrinsic competition experiments between *A. bifasciatus* and *O. telenomicida* suggest that negative effects on the latter species may occur when co-existing in the same habitat or released simultaneously, whereas inundative biological control with *A. bifasciatus* would likely not be affected. The effects of extrinsic competition remain to be elucidated. A wider geographical distribution across the areas invaded by *H. halys* and being a superior intrinsic competitor favour *A. bifasciatus*, but in terms of biological control the success of inundative releases will depend on the actual parasitism level that each of the two species can achieve under field conditions.

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CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

AUTHOR CONTRIBUTION

TH, DB and JS conceived research. JS conducted experiments. SC and MF contributed parasitoids. JS analysed data and conducted statistical analyses. JS wrote the manuscript; all authors edited it. TH and DB secured funding. All authors read and approved the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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