Augmentative biological control of Halyomorpha halys using the native European parasitoid Anastatus bifasciatus: Efficacy and ecological impact

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Biological Control

Augmentative biological control of Halyomorpha halys using the native European parasitoid Anastatus bifasciatus: efficacy and ecological impact

--Manuscript Draft--

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Abstract:	We report the first large-scale augmentative biological control project carried out in Europe against Halyomorpha halys (Stål) (Hemiptera: Pentatomidae) using the native egg parasitoid Anastatus bifasciatus (Geoffroy) (Hymenoptera: Eupelmidae). During summer 2020, a total of 325,000 adults of A. bifasciatus were released at a rate of 1000 individuals/ha of orchard in 11 sites in Trentino Alto Adige (Northern Italy). Parasitism parameters were compared between release and control (no release) sites, in which at least three egg masses naturally laid by H. halys were collected (for a total of 262 egg masses). Anastatus bifasciatus and Trissolcus mitsukurii (Ashmead) (Hymenoptera: Scelionidae) were the dominant parasitoids, but parasitism by both species fluctuated widely among sites. At release sites, A. bifasciatus showed a significantly higher discovery efficiency (31.4%) and parasitism rate (16.7%) of H. halys egg masses than at control sites (1.7% and 1.2%, respectively). Parasitism by A. bifasciatus was not dependent on egg mass abundance at release sites, but at control sites a host density-dependent response was revealed by a positive relationship between parasitism and number of H. halys egg masses. On the other hand, parasitism by the adventive T. mitsukurii was not affected by either the releases of A. bifasciatus or by the abundance of H. halys egg masses per site. In conclusion, augmentative releases of A. bifasciatus contributed to increasing its parasitization of H. halys , without causing any negative effects on parasitization by naturally occurring species.
Response to Reviewers:	

Dear professor Biondi,

Thank you for your last email. According to your kind request we have modified the document of Response to Editor and Reviewers providing a point by point reply reporting also each original comment.

I’ve taken the opportunity to include in the new resubmission also the Supplementary Material (unchanged from R1) that I forgot to upload in the resubmission on May 6th.

On the behalf of all the coauthors, let me thank you once again for your time and attention.

With kindest regards,

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Response to Editors' and Reviewers' comments BCON-D-21-00637R2

A point by point reply to Editors and Reviewers in provided in the following lines in red fonts. All original comments have been reported too.

Response to Editor 's comments

L18: I think here author may need to provide a release rate rather the total number
We have changed the text as suggested.

L19: 11 sites but the abstract says 8 because I guess in three sites there were not enough egg masses, so I wonder why staing 11 locations and why mentioning the total number of parasitoids released, i.e., including those in sites without hosts
Parasitoids were indeed released in 11 sites, but only 8 of them were considered for statistical analysis because of the low number of egg masses (< 3) found in the remaining 3 sites. We would like to retain 11 sites in the highlight to stick to what was actually done.

L20 and the whole manuscript: "Parasitization parameters" is unclear
We have changed "parasitization parameters" in "parasitism parameters" throughout the text. A full description of what we intend with "parasitism parameters" is provided in lines 157-163 of the new numbering.

L30: as per author guidelines
We did our best to stick to authors' guidelines.

L37: host "discovery rate" is unclear
We have changed "discovery efficiency rate" in "discovery efficiency". This is a quite standard term when dealing with egg parasitoids exploiting hosts that lay eggs gathered in masses. See for example Saunders et al. 2022 (Retrospective host-specificity testing shows *Trissolcus basalis* (Wollaston) and the native *Trissolcus oenone* (Dodd) (Hymenoptera: Scelionidae) have overlapping physiological host ranges in New Zealand published in Biological Control 170 104926). "Discovery efficiency" has been clearly defined in lines 158-160.

L89-93: do not use weblink as citations, instead try to use formal document (much better if peer reviewed articles) use author and year and follow the guidelines
We have changed the text trying to match editor's indications.

L115: including the investigated area or not? Please specify
A specification has been added.

L127: sex ratio?.
Sex ratio has been indicated.

L135-136: this sentence as such is not informative. What is "National Biological Control Program."? How this could affect the current experiment? Why 1km min?
The minimum distance from *T. japonicus* release sites was used because it was a mandatory precaution recommended by local government. We have clearly stated that in the revised text.

L146: do authors mean 'one' trained operator per one hour per each site?
L146-150: This is a very crucial point of the research (e.g see statement in L216-7). Unfortunately, it is not well described nor very reliable., i.e., 1h of experienced operator to randomly search in an area of 78hectars seems not highly reliable. Is there at least a reference available for this methodology? Can authors justify it better and more importantly provide more details on this sampling?
More details have been provided to better explain the sampling methods.

L152: I do not think that including urban sites in the control is a good idea when comparing parasitization data between released and not released locations. The environment and the presence of parasitoid is of course totally different in the two environments (crop vs urban landscape). Of course, one of the main conditions in experimental planning is represented by the standardization of the experimental conditions among treatments, and this is not the case of the present work. Authors should take out the material and methods, results and related conclusion related to samplings in urban areas

"Urban areas" was used unproperly. We meant orchard systems quite close to scattered urban settlements as typical of the investigated areas. As it's shown in the map (fig 1) the location of no release sites is similar to that of release sites. A specification has been added in the text.

L164: how? Please explain

We have explained what we meant by" predated" and have reported how we identified the eggs attacked by generalist predators.

L194-196: I do not think that the reviewer meant to correlate the parasitism with the parasitoid release rate,

In the first round of revision, Reviewer#1 wrote "By using a different release rate on each site (lines 142-144 and Table 1S), the authors introduced a variable in the experimental design. Nevertheless, they did not take into account the release rate in the data analysis. How the parasitization level in the field correlates with the number of parasitoids released could help to understand the real impact of the augmentative release of A. bifasciatus. The authors should run this correlation analysis and discuss the results in combination with the positive correlation of the parasitization rate with the egg mass density in each site."

While the effects of egg mass density have been addressed by changing data analysis according to the suggestion of reviewer 3, we would like to retain Spearman correlation between parasitism rate of A. bifasciatus and its release rate at each site. The statement by Reviewer #1 that we followed "virtually all reviewer's comments" seemed to us an endorsement to what we did. Of course, we are ready to further modify the data analysis if needed.

L201: this section would be much better if dvided in multiple subsections

Results have been divided in 3 subsections.

L206: any voucher specimen available in a collection?

A statement about voucher specimens has been added.

L207: "empty parasitized egg masses" have not been presented in the material and method

An explanation was added in the method section. As customary, we have considered as parasitized also the eggs that were found already empty, but with clear signs of emerging holes by a parasitic wasp.

L209: as it is written, a hyperparasitoid of A. bifasciatus, T. mitsukurii and T. japonicus it seems that these are the only hosts for Acroclisoides sinicus

The sentence has been rephrased to stress that host range of A. sinicus encompass T. mitsukurii and T. japonicus

L256: 'total number'? I think authors meant per release point

"per release point" has been added.

L296: see comment in L89-93. Justifying such a crucial statement (the whole paper is based on that) with a weblink (moreover it that does not work) of an apparently "news" is not scientifically sound

The text has been changed and proper references have been cited in the effort to explain better our line of reasoning.

L373: provide statistical results in the text and not in the figure captions. While, when significant differences are present please add (1) one, two or three asteristics in the figure (depending on the significance level) and (2) the meaning of the asteriscs in the caption. When not significant add N.S. in the figure and the meaning of N.S. in the caption

The bar charts have been deleted and statistical analysis, which was modified according to the suggestion of reviewer 3, has been either reported in table 1 or in the text.

L373: spell out genus names in the figure captions

"H. halys" has been spelled out in the captions.

L373: italicize the species names in the axes labels of the figures
Done.

Authors' guidelines have not been followed properly.
-The first recommendation in the guidelines is:
"BEFORE YOU BEGIN
Ethics in publishing
Please see our information pages on Ethics in publishing and Ethical guidelines for journal publication."
And in the guidelines https://www.elsevier.com/__data/assets/pdf_file/0007/653884/Competing-Interests-factsheet-March-2019.pdf the very first potential conflict of interest mentioned is "The most obvious competing interests are financial relationships such as: ● Direct: employment". I was not able to find in the manuscript any statement about this, and this is surprising to me because some coauthors have as affiliation a private company who produce and sell the main insect studied.

We apologize for having overlooked this important point. A statement on possible conflict of interest has been added in the resubmission of the manuscript.

- Very important: funding statement is missing.
We did not receive any specific funding for this study. This has been now stated at the end of the text.

Highlights should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point).
Highlights have been shrunk to 85 characters and provided also in a separate file according to editor's suggestions.

- references for web documents are not in the proper format
Following the revision web document are no longer cited in the manuscript.

Response to Reviewer #1

The authors have improved the manuscript following virtually all reviewer's comments. I consider the manuscript acceptable in this form for publication.
We take the opportunity to thank the reviewer for the overall positive evaluations of our study and for their comments which were a spur to improve the manuscript.

Response to Reviewer #3

I have reviewed the manuscript, "Augmentative biological control of Halyomorpha halys using the native non-coevolved parasitoid Anastatus bifasciatus: efficacy and ecological impact" after it has been previously reviewed by two other evaluators.
My main takeaway is that the previous reviewers brought up excellent points about weaknesses in the data analysis that were not properly addressed during the revisions.
This is indeed quite a bit disappointing given the above statement made by reviewer #1.

The main issue, as pointed out by the first reviewer, is that the main conclusion of the paper - that parasitism increased at release sites compared to non-release sites - may be confounded by a higher density of Halyomorpha at release sites compared to non-release sites. This was not addressed; other than stating that "In fact, some release sites were characterized by a relatively low number of egg masses and despite this, the parasitization showed a trend of increase, consistent with the effect due to the parasitoid releases." In order to formally show that higher parasitism at release sites was due to the releases and not some other pre-existing characteristic of the release sites, one would have to do a formal statistical analysis - and in my opinion, this

must be done. The inclusion of Table S2 during the revision allowed me to do this myself (I put the data into a .csv and analyzed it in R software) - as the authors did, I only did this with sites where 3 or more egg masses were found. The paper needs to be heavily re-written to incorporate proper analyses, in my opinion.
We have carried out a new analysis following the suggestion of the reviewer.

1a. First of all, egg density (number per hour of searching) was significantly higher at release sites compared to non-release sites (linear model; F=6.761; p=0.0187) in the analysis. As the variance in density at release versus control sites was not equal, I repeated this with a Wilcoxon test, and the results were similar (W = 70.5, p = 0.031). This shows that at least in principle, as parasitoids are known to respond to host density in a positive way (both through their functional response and their population abundance), that higher host density at release sites could certainly be a confounding factor in the analysis. This result needs to be included in the paper so readers are aware of this potential confound. This shows that there was either bad luck or some kind of bias in site selection - I guess that maybe sites were selected based on infestation history or were the two treatments assigned randomly? This needs to be stated in the methods.
We included in the manuscript an analysis to compare abundances of BMSB egg masses in release vs control sites. However, we have carried out a parametric test (One-way ANOVA) on log transformed number of egg masses per site. The results were the same of the non-parametric test performed by the reviewer with a significantly higher abundance of eggs in release sites than in control ones.

1b. Next, I ran linear models with both site type (release or control), egg density, and their interaction as explanatory factors (I checked the residual and qq-plots to make sure a linear model was OK - it was). The results were interesting - overall, release sites had higher parasitism than control sites ($p < 0.0001$); but the effect of releasing parasitoids depended on egg density (and vice versa) (density x treatment interaction: p = 0.038). Parasitism was positively host density-dependent at control sites ($p < 0.0001$) but density-independent at release sites (p = 0.249). The estimate on the significant interaction effect shows that, as host density increases, the difference between control and release sites decreases. That is, parasitoid releases elevate parasitism above baseline (natural) levels more at low pest densities than at high pest densities. This has significant biological control implications: based on the data, releases do not do any better (proportionally) at higher host densities than lower densities - in fact, they perform best at low host densities (relative to not releasing). And, when pest pressure is higher, there is more naturally occurring parasitism, making the proportional effect of the releases smaller.
1c. I did not repeat this analysis for Anastatus discovery efficiency (or the parameters related to Trissolcus mitsukurii), but the authors should repeat it in the same way for the rest of their results and make their conclusions based on those results.
An analysis based on this line of reasoning has been performed for all the parasitism parameters and for the percentages of unhatched eggs. Because of heteroscedasticity we were forced to transform raw data in square root every now and then.

1d. I have pasted the R code I used to do the analysis at the end of this review.
We carried out all these analyses in SPSS to which we are far more familiar than to R. Anyhow we would like to express our appreciation for the time and the effort that reviewer #3 put in reanalyze our data and pasting R codes.

2. I noted that in the new correlation analysis between parasitoid release rate and parasitism level, sites were included where no egg masses found or very few (based on the number of points in the plot - 11 rather than 8). This analysis should be repeated with only the sites where sufficient egg masses were found to make a conclusion, as was done with all the other analyses.
All sites, including no release sites were used to carry out this correlation. Indeed, the control sites equals to the release of zero individuals of *A. bifasciatus*. Some point data completely overlap in the chart and so the number of circles is less than 19.

3. I disagree with the authors' response to the other reviewers that one would only expect to find an effect of release distance for exotic parasitoids and not native parasitoids. On the contrary, if released parasitoids increase parasitism levels above natural levels, and the releases actually have an effect, one would still expect to see a distance effect at release sites -- but not control sites. However it sounds like the distance-from-release-point parameter could not be measured because the releases were done in a non-standardized manner with

respect to distance from the centroid. This should be pointed out clearly in the methods, and a statement should be included as to exactly why the effect of distance from release point was not considered as an explanatory factor- readers will wonder this (as both previous reviewers, and myself did).
We understand the points made by both reviewers. However, we could not record the distance between every single egg mass and the release point. We actually did not carry out point releases. Moreover, a too low number of egg masses per site was detected to allow the inclusion of the distance from release point as a predictor. We have clearly stated this in the method section.

4. In the title: "Non-coevolved" is often used in this context but really doesn't make any sense in my opinion. We don't know much about the co-evolutionary history of specific host-parasitoid pairs in this system (demonstrating co-evolution requires experimental evidence of bi-directional selective pressures as a result of the interaction), only biogeographic co-occurrence. I suggest "Native European" or similar here and throughout.
You made a good point and we agree with you that we used the term “non-coevolved” in a rather naïve way. We have replaced “non-coevolved” with "native European" or “native to Europe” all through the text.

Augmentative biological control of *Halyomorpha halys* using the native European parasitoid *Anastatus bifasciatus*: efficacy and ecological impact

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Highlights

- Augmentative biological control of *Halyomorpha halys* was tested in Northern Italy.
- 1000 *Anastatus bifasciatus*/ha (325,000 in total) were released in 11 sites.
- Releases improved discovery efficiency and parasitism rate by *A. bifasciatus.*
- *Trissolcus mitsukurii* was not affected by the releases of *A. bifasciatus*.

Key words

Brown marmorated stink bug, Pentatomidae, invasive species, *Trissolcus mitsukurii*, egg parasitoids.

Abstract

We report the first large-scale augmentative biological control project carried out in Europe against *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) using the native egg parasitoid *Anastatus bifasciatus* (Geoffroy) (Hymenoptera: Eupelmidae). During summer 2020, a total of 325,000 adults of *A. bifasciatus* were released at a rate of 1000 individuals/ha of orchard in 11 sites in Trentino Alto Adige (Northern Italy). Parasitism parameters were compared between release and control (no release) sites, in which at least three egg masses naturally laid by *H. halys* were collected (for a total of 262 egg masses). *Anastatus bifasciatus* and *Trissolcus mitsukurii* (Ashmead) (Hymenoptera: Scelionidae) were the dominant parasitoids, but parasitism by both species fluctuated widely among sites. At release sites, *A. bifasciatus* showed a significantly higher discovery efficiency (31.4%) and parasitism rate (16.7%) of *H. halys* egg masses than at control sites (1.7% and 1.2%, respectively). Parasitism by *A. bifasciatus* was not dependent on egg mass abundance at release sites, but at control sites a host density-dependent response was revealed by a positive relationship between parasitism and number of *H. halys* egg masses. On the other hand, parasitism by the adventive *T. mitsukurii* was not affected by either the releases of *A. bifasciatus* or by the abundance of *H. halys* egg masses per site. In conclusion, augmentative releases of *A. bifasciatus* contributed to increasing its parasitization of *H. halys*, without causing any negative effects on parasitization by naturally occurring species.

1. Introduction

The brown marmorated stink bug (BMSB) *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) is a global invasive pest with a wide host range, which includes over 170 plant species. Native to eastern Asia, this bug has become a notorious pest to growers in many parts of the world (Valentin et al., 2017; Musolin et al., 2018; Rot et al., 2018; Šapina and Jelaska, 2018; Francati et al., 2021), being economically important especially in fruit and vegetable-growing regions (Leskey and Nielsen, 2018).

Programs for management of invasive pests should firstly explore biological control agents, which can contribute to pest suppression in both natural and agricultural areas (Hoddle, 2004; Cock et al., 2010). The scientific community has therefore developed a strong interest in the search and selection of natural enemies of *H. halys* (Conti et al., 2021; Rot et al., 2021). A debate has arisen on the most appropriate strategy between the use of exotic natural enemies for classical biological control or the augmentation of native, newly associated, parasitoid species (Abram et al., 2017; Zapponi et al., 2021).

In its native areas in Asia, *H. halys* populations are regulated by egg parasitoids belonging to genus *Trissolcus* (Hymenoptera: Scelionidae) and *Anastatus* (Hymenoptera: Eupelmidae) (Qiu et al., 2007; Hou et al., 2009; Avila et al., 2021). In the countries where *H. halys* has recently established, the contribution of native biological control agents has been reported as low although highly variable among habitats, seasons and sampling protocols (Cornelius et al., 2016a, 2016b; Herlihy et al., 2016; Ogburn et al., 2016; Dieckhoff et al., 2017; Jones et al., 2017; Leskey and Nielsen, 2018; Pezzini et al., 2018; Moraglio et al., 2020; Francati et al., 2021). For these reasons, the recent invasions by *H. halys* have emphasized in many countries an interest in classical biological control although this has some limitations (Conti et al., 2021) and is currently subject to regulations on the importation of exotic species (De Clercq et al., 2011). The scenario on regulation is quite complex and may differ from country to country (Bale, 2011).

Adventive populations of the Asian egg parasitoids *Trissolcus japonicus* (Ashmead) and *Trissolcus mitsukurii* (Ashmead) (Hymenoptera: Scelionidae) have been recently detected in Europe (the first one in Italy, Switzerland and Germany, the second in Italy, Western Slovenia and France) and these findings have led to reconsider classical biological control methods to manage *H. halys* (Sabbatini Peverieri et al., 2018; Stahl et al., 2019b; Moraglio et al., 2020; Scaccini et al., 2020; Zapponi et al., 2020, 2021; Bout et al., 2021; Dieckhoff et al., 2021; Rot et al., 2021). However, the use of exotic biological control agents is strictly regulated. For this reason, risk assessment evaluations for *T. japonicus* and *T. mitsukurii* are in progress, including their potential coexistence with native parasitoids (Konopka et al., 2017; Haye et al., 2020; Giovannini et al., 2021). Field releases of *T. japonicus* were authorized in Italy in 2020 in the framework of the National Biological Control Program against *H. halys*, and this represents the first officially authorized release of this parasitoid in Europe (Bittau et al., 2021; Conti et al., 2021).

Field surveys to evaluate whether indigenous parasitoids in Europe can exploit *H. halys*, leading to potential pest suppression, have been carried out in Switzerland, Italy, Georgia and recently Slovenia. Several sampling methods have been used for these surveys, including exposure of freeze-killed sentinel egg masses (Haye et al., 2015; Roversi et al., 2016; Stahl et al., 2019b; Zapponi et al., 2020), exposure of fresh sentinel egg masses, eggs laid on plants by bugs housed in field cages (Costi et al., 2019a; Zapponi et al., 2020; Rot et al., 2021) and field collections of naturally laid egg masses (Sabbatini Peverieri et al., 2018, 2019; Moraglio et al., 2020; Scaccini et al., 2020; Zapponi et al., 2020, 2021; Francati et al., 2021; Rot et al., 2021). Among native species, *Anastatus bifasciatus* (Geoffroy) (Hymenoptera: Eupelmidae) was the dominant egg parasitoid capable of completing development on *H. halys* eggs. It was also the most widespread species as it was found in nearly all the investigated sites, yet with relevant fluctuations across years. Because of its prevailing presence, *A. bifasciatus* has been proposed for augmentative biological control of *H. halys* in Europe (Haye et al., 2015). Native to Europe and currently present in the Palearctic and Nearctic regions, *A. bifasciatus* can exploit egg masses of different insect groups in the orders Hemiptera and Lepidoptera, including various pests of agronomic interest, and could play a role in limiting introduced exotic pests (Haye et al., 2015; Stahl et al., 2018). Nevertheless, little information is available on its efficacy in field conditions following inundative or inoculative releases. Some studies, although promising, were conducted releasing a low number of parasitoids and using frozen sentinel egg masses as a method to assess parasitization (Stahl et al., 2019a). Another open question is whether the releases of *A. bifasciatus* can affect parasitoid guilds and in particular the adventive populations of *T. japonicus* and *T. mitsukurii*, which have been recently recorded in some Italian regions, including Trentino Alto Adige (Zapponi et al., 2020) where this study was carried out.

The first aim of this study is to assess the efficacy of *A. bifasciatus* augmentative releases in cultivated areas of Northern Italy. The second aim is to investigate the potential impact of *A. bifasciatus* releases on the *H. halys* parasitoid guild, including adventive exotic species. As far as we are aware, this experiment represents the first large-scale augmentative biological control project carried out in Europe using *A. bifasciatus* and based exclusively on the sampling of egg masses naturally laid by *H. halys* to evaluate parasitization.

2. Material and methods

2.1 Insects

Adults of *A. bifasciatus* were provided periodically by Bioplanet srl (Cesena, Italy) in 150 ml plastic bottles containing 250 4-days old adults (sex ratio ≈ 9:1, F:M). The bottles were shipped to farmers once per week and kept at 12 °C in dark conditions until use. For the release, one bottle at a time was taken out and the cap was removed to allow parasitoid dispersion.

2.2 Release and control sites

From early June until mid / late July 2020, a total of 325,000 individuals of *A. bifasciatus* were released in 11 sites in Trentino Alto Adige region (Northern Italy). All the sites were located in agricultural areas, close to orchards that were under integrated pest management (IPM) programs (Table S1). Release sites of *A. bifasciatus* were located at a minimum distance of 1 km from areas where *T. japonicus* was released within the aforementioned National Biological Control Program against *H. halys*, following a mandatory precaution recommended by local government (Decreto 8129/2021 by Provincia Autonoma di Bolzano - Alto Adige).

Releases of *A. bifasciatus* were carried out in:

- 5 sites nearby apple orchards in South Tyrol, in the Autonomous Province of Bolzano, covering about 180 ha, between June 10th and July 29th;
- 6 sites nearby apple and kiwi orchards and vineyards in Trentino, in the Autonomous Province of Trento, covering about 145 ha, between June 10th and July 8th.

Parasitoids were released at a rate of 1000 individuals per ha of orchard (Table S1). Bottles, each containing 250 adult wasps, were opened and hung at approximately 20 m from each other on shrubs and hedgerows at the edge of the orchards, or in ecological corridors adjacent to watercourse or reforestation within the cultivated areas.

Naturally laid egg masses were searched in each site by three experienced operators of local extension services for the fixed time of 1 h per operator. Operators walked randomly up to a maximum distance of 500 m around the centroid of each release site. During samplings, leaves of wild plants (shrubs, hedgerows, herbaceous plants) and fruit trees in the orchards were inspected. Egg masses collections started two weeks after the beginning of releases and were carried out once a week until mid-September in Trentino and until mid-October in South Tyrol.

Field surveys were also performed in 21 control sites within agricultural areas, including orchard systems close to scattered urban settlements as typical of the investigated areas, where no parasitoids were released. The control sites were at least 2 km away from release sites and as similar as possible to the release sites in terms of location, altitude, and agroecosystem features. Furthermore, in control sites, egg masses were searched on the same kind of plants mentioned above for release sites.

2.3 Handling of collected egg masses

Field-collected egg masses of *H. halys* were transferred to the laboratory, stored individually in plastic Petri dishes (ø 60 mm) and kept at 25 °C, 65-70% RH and 16L:8D photoperiod. The egg masses were checked every two days for 8 weeks after collection. For each egg mass, eggs were counted and classified as: 1) hatched, when a bug nymph hatched in laboratory or a hole by a nymph already hatched in the field was detected; 2) parasitized, when an adult parasitoid emerged in laboratory or a hole by a parasitoid already emerged in the field was found; 3) unhatched, due to mortality from unknown cause; 4) predated by polyphagous predators, showing clear damage due to chewing.

To evaluate the overall performances of parasitoids, the following parasitism parameters were considered: 1) discovery efficiency (i.e. wasp ability to find egg masses), calculated as the number of egg masses discovered by parasitoids (presenting at least one parasitized egg) over the total number of egg masses collected in a site; 2) parasitism rate (= parasitoid impact), calculated as the number of emerged parasitoids over the total number of collected eggs; 3) parasitoid exploitation efficiency, calculated as the number of emerged parasitoids over the total number of eggs within the discovered egg masses (Bin and Vinson, 1990).

As suggested by Stahl et al. (2019b), measuring parasitism only by offspring emergence could lead to underestimating the real level of pest suppression. Given that the main goal of biological control is the reduction of the pest populations (van Lenteren et al., 2018), we reported the percentage of unhatched eggs (i.e. eggs that did not develop to a viable bug nymph out of the total eggs in the egg mass) as an additional indication of overall pest suppression.

2.4 Parasitoid identification

All emerged parasitoids were frozen and identified to species or genus level. Eupelmidae were identified using the keys proposed by Askew and Nieves-Aldrey (2004). Scelionidae were identified following Johnson (1984), Kozlov and Kononova (1983) and Talamas et al. (2015, 2017). The keys of Sabbatini-Peverieri et al. (2019) were used for Pteromalidae. All morphological analyses were carried out under a stereo microscope (Leica M205C; 40X).

Voucher specimens have been deposited in the Entomological Collection of Department of Agricultural and Food Sciences, University of Bologna.

2.5 Data analysis

The relative abundances of emerged parasitoids were calculated using all collected egg masses, pooling data from release and control sites. For statistical analyses of discovery efficiency, parasitism rate, exploitation efficiency and percentage of unhatched eggs (which were calculated as reported above in section 2.3) only sites in which at least three egg masses of *H. halys* had been collected were retained (Fig. 1; Table S2). One-way ANOVA on log transformed number of egg masses per site was carried out to compare egg mass abundance in release vs control sites. Two-way ANOVA considering release vs no release of *A. bifasciatus* as a fixed predictor, and number of egg masses per site as a continuous predictor were run. The interaction between predictors was tested as well. In the cases of significant interaction, a linear regression analysis of the parasitization parameter in function of the number of egg masses was run separately for releases and no-releases sites. For raw data that violated the assumption of normality and homoscedasticity, which had been verified using Shapiro-Wilk and Levene's tests, the square root transformation was used.

Spearman rank-order procedure was used to correlate discovery efficiency and parasitism rate with the total number of parasitoids released in each site pooling release and control sites.

Data analysis were carried out with IBM SPSS Statistics (version 26) (IBM corporation, Armonk, NY, USA); this software package was also used for graphical representation of data.

3. Results

3.1 Assemblages of Halyomorpha halys *natural enemies*

A total of 1641 parasitoids emerged from all collected *H. halys* egg masses from release and control sites (N=273 corresponding to 7318 eggs), including the following species: *A. bifasciatus* (N=762), *T. mitsukurii* (N=717), *Telenomus* sp. (N=4), *Trissolcus cultratus* (Mayr) (Hymenoptera: Scelionidae) (N=1), *Acroclisoides sinicus* (Huang and Liao) (Hymenoptera: Pteromalidae) (N=41). A total of 116 parasitoids, corresponding to the 7.1 % of the emerged individuals, were not identified because only empty parasitized egg masses were found. *Anastatus bifasciatus* was the dominant parasitoid, representing a relative abundance of 46,4%, followed by *T. mitsukurii* (43,7%). Concomitant parasitization by *A. bifasciatus* and *T. mitsukurii* emerging from the same *H.*

halys egg mass occurred in two egg masses collected in a release site. *Acroclisoides sinicus*, a hyperparasitoid whose host range includes *A. bifasciatus*, *T. mitsukurii* and *T. japonicus* (Sabbatini Peverieri et al., 2019), was detected with a relative abundance of 2.5%. No *T. japonicus* was recorded among the emerged parasitoids. Finally, the pressure exerted by generalist predators on *H. halys* egg masses was low, since only 1.7±0.7% of eggs were chewed.

Two egg masses of the non-target species *Nezara viridula* L. and *Palomena prasina* L. (Hemiptera: Pentatomidae) were found while monitoring *H. halys* eggs. Parasitism rates of *A. bifasciatus* on these species were 25.6% and 52.6%, respectively.

3.2. Total parasitism

A total of 262 *H. halys* naturally laid egg masses, corresponding to 7012 eggs, were taken into account for the evaluation of parasitism parameters; in particular 181 egg masses were considered from eight release sites and 81 from eleven control (no release) sites (Fig. 1). The mean number of egg masses was significantly higher (ANOVA, $F_{(1, 17)} = 10.7$; $p = 0.004$) at release sites (22.6±4.2) compared to control sites (7.36±3.57). Considering that the sampling effort was the same in all sites, these differences likely reflected the different abundance of *H. halys* in each area. The total discovery efficiency (45.4±8.2%) and the total parasitism rate (28.5±5.9%) in the release sites were not significantly higher than those in control sites (19.6±5.5% and 12.9±4,1%, respectively) (Table 1). However, the *p* value for total discovery efficiency (0.073) was close to the 0.05 significance level. The mean percentage of unhatched eggs in release sites (44.6±8.7) was almost twice as high than in control sites (23.9±5.6%), but again the difference was not supported by statistical analysis ($p = 0.12$, Table 1).

3.3. Parasitism by Anastatus bifasciatus *and* Trissolcus mitsukurii

Parasitism parameters by *A. bifasciatus* widely ranged among sites (Table S2). For example, discovery efficiencies encompassed a 0-50.0% interval while parasitism rates spanned between 0 and 29.6%. Overall, the releases of *A. bifasciatus* had a positive effect on its discovery efficiency and parasitism rate, as both parameters were significantly higher in the release sites than in the control sites (Table 1). The interactions release*number of egg masses were also significant for both parameters, demonstrating that the effects of releasing parasitoids depended on egg mass abundance. Indeed, in control sites, discovery efficiency (r^2=0.86, $F_{(1,8)}$=47.30, p< 0.001) and parasitism rate (r^2=0.89, $F_{(1,8)}$=67.10, p< 0.001) significantly increased in function of the egg mass number per sites (Fig. 2 and Fig. 3). On the other hand, the regressions were not significant in release sites (discovery efficiency r^2= 0.11, $F_{(1,6)}$=0.72, p= 0.43; parasitism rate r^2=0.23, $F_{(1,6)}$=1.78, p= 0.23), thus showing an independent response of parasitism parameters on egg mass abundance (Fig. 2 and Fig. 3). Finally, parasitism rate by *A. bifasciatus* was also positively correlated with the total number of parasitoids released in each site (r_s = 0.85 p<0.01, Fig. 4).

The discovery efficiency by *Trissolcus mitsukurii* fluctuated between 0 and 39.3%, while its parasitism rate ranged in a 0-31.6% interval. Both parasitism parameters were neither affected by the releases of *A. bifasciatus* nor by egg mass abundance per sites; the interaction release*number of egg masses did not show any significant effects as well. Therefore, parasitism by *T. mitsukurii* was not dependent on host density.

The exploitation efficiency, expressed as the number of emerged parasitoids out of the total number of eggs within the discovered egg masses, was lower for *A. bifasciatus* (median among 53 egg masses = 53.6%) than for *T. mitsukurii* (median among 29 egg masses = 92.9%) (Fig. 5).

4. Discussion

Releases of *A. bifasciatus* significantly increased the discovery efficiency and parasitism rate of *H. halys* egg masses by this native parasitoid compared to control sites. Both parasitism parameters by naturally occurring *A. bifasciatus* increased with the host density (i.e. number of egg masses) at control sites, whereas parasitism was not dependent on host density at release sites. This means that releases of *A. bifasciatus* enhanced its impact on *H. halys* also at low abundance of host egg masses. As host density increased, the difference in parasitism between control and release sites decreased.

In the control sites, foraging females of *A. bifasciatus* may have been affected by various stimuli linked to pest density, since they are known to exploit host-associated cues for egg location, like oviposition-induced plant volatiles and kairomones from male bugs and/or gravid females (Conti and Colazza, 2012; Rondoni et al., 2017). On the other hand, in release sites, the high number of released individuals likely promoted a response that was not related to host density. The parasitism rate by *A. bifasciatus* was also positively correlated with the total number of parasitoids released per release site, thus corroborating the significant effect of the augmentative releases. Parasitism by the adventive *T. mitsukurii* was similar in release and control sites. Therefore, augmentation of *A. bifasciatus* did not affect natural parasitism by *T. mitsukurii*. Although no significant differences could be detected between release and control sites for total discovery efficiency and total parasitization rate, both parameters showed a tendency to rise were *A. bifasciatus* was released, thus suggesting a possible additive effect. Exploitation efficiency of *A. bifasciatus* and *T. mitsukurii* in our study was quite in line with that previously reported (Scaccini et al., 2020; Zapponi et al., 2020, 2021).

Anastatus bifasciatus was the dominant parasitoid in most studies carried out in Northern Italy (Sabbatini Peverieri et al., 2018; Costi et al., 2019; Moraglio et al., 2020; Zapponi et al., 2020), with the exception of areas in North-eastern Italy (Scaccini et al., 2020), where an overall prevalence of

T. mitsukurii was recorded. In these areas, *A. bifasciatus* was the second most abundant parasitoid and another native species, *Trissolcus kozlovi* Rjachovskij (Hymenoptera: Scelionidae), emerged from field-collected *H. halys* egg masses (Moraglio et al., 2021b).

The potential coexistence of indigenous and exotic parasitoids of *H. halys*, which was anticipated relying on laboratory investigations (Konopka et al., 2017), was then observed in several field surveys (Sabbatini Peverieri et al., 2018; Stahl et al., 2019b; Moraglio et al., 2020; Scaccini et al., 2020; Zapponi et al., 2020, 2021; Rot et al., 2021). Our study confirmed that the native *A. bifasciatus* and the exotic *T. mitsukurii* can coexist even when *A. bifasciatus* populations are augmented for biological control. This aspect is noteworthy, considering the continuous expansion of *T. mitsukurii* and *T. japonicus* in Europe, as demonstrated by a large-scale survey recently performed in Northern Italy and Switzerland (Zapponi et al., 2021). The occurrence of adventive populations of these exotic parasitoids can lead to complex and dynamic relationships evolving over time.

Although our results indicate that releases of *A. bifasciatus* do not interfere with other parasitoids exploiting *H. halys*, parasitoid guilds are hardly predictable and different effects should be considered in the long term. For instance, augmentative releases of *A. bifasciatus* might boost the natural populations of this native species, eventually increasing its efficacy in pest suppression. On the other hand, new exotic species could be introduced accidentally or for biocontrol purpose in the areas invaded by *H. halys*. One example is the exotic *T. japonicus*, which is adventive in North-western Italy and has been recently released in several Italian regions for biological control of *H. halys*, after authorization by the Italian government (Bittau et al. 2021). Future studies will be needed to clarify the spatio-temporal dynamics of the parasitoid guild after the introduction of *T. japonicus*.

Synergistic interactions may have the potential to improve the suppression of *H. halys* populations in the long term (Leskey and Nielsen, 2018; Moraglio et al., 2020). However, in Europe the application of classical biological control is regulated by stringent risk assessments that may hamper the introduction of exotic biological control agents, boosting the exploitation of native species. The native parasitoid *A. bifasciatus* established a new association with the invasive host *H. halys*, and our data indicate a high egg mass discovery efficiency in the field, as was suggested by its positive response to volatiles associated with the new host (Rondoni et al., 2017). *Anastatus bifasciatus* had been already considered for augmentative biological control programs in Europe (Stahl et al., 2018; Stahl et al. 2019a) and for this reason it was selected for mass rearing and release in the present study.

Another aspect that should be considered when evaluating the efficacy of a parasitoid is the overall impact on the population-level suppression of the host. Usually wasp emergence rate is primarily used as a measure of parasitism rate, without considering the proportion of eggs that do not hatch following parasitoid activity, which ranges between 10-26% in *H. halys* (Abram et al., 2014, 2016; Haye et al., 2015; Cornelius et al., 2016b). Besides successful parasitism, this additional source of mortality contributes to the impact of a parasitoid on a pest (Jervis et al., 1996), and it can be ascribed to host feeding, to unsuccessful probing of the host egg that can lead to abortion, or to parasitoid incapability to complete development. Host feeding is an important biological trait of *A. bifasciatus*, and this should be considered when evaluating biological control programs using this species. Previous laboratory studies concluded that the number of eggs killed by host feeding is nearly as high as the number of eggs killed by parasitization, and can significantly contribute to the efficacy of biological control (Konopka et al., 2017; Stahl et al., 2019a). In our study, the percentage of unhatched eggs at the release sites was nearly twice as high as at control sites, although this difference was not statistically supported. Released *A.*

bifasciatus may have contributed to kill host eggs, but the overall mortality of *H. halys* eggs in field conditions is linked to a number of other factors that could introduce a bias in such evaluation. During our monitoring, very few egg masses of non-target hosts were found, including *N. viridula* and *P. prasina*. In spite of that, eggs of both species were parasitized by *A. bifasciatus*, confirming its wide host range. However, the few available data do not allow to draw any final consideration and specific studies should be carried out to acquire a more complete picture of non-target effects in the field. On the other hand, both non-target species can be also parasitized by the exotic parasitoids *T. japonicus* and *T. mitsukurii* (Haye et al., 2020; Dieckhoff et al., 2021; Giovannini et al., 2021). A polyphagous native parasitoid does not necessarily hinder other species (van Lenteren et al., 2006; van Lenteren and Loomans, 2006) and its activity does not necessarily translate into adverse effects in the field. For instance, the wide host range of *A. bifasciatus* can favour its colonization of cultivated areas. Additionally, heteropteran hosts of *A. bifasciatus* (Stahl et al., 2018, 2019b; Zapponi et al., 2020; Moraglio et al., 2021a) are mostly considered pests or very common species. Only a few potential moth species can be considered as “undesired” targets, but their occurrence is rare in agroecosystems (Masetti et al., 2017), which are the main target environments of the introductions of *A. bifasciatus*. Furthermore Stahl et al. (2018) demonstrated that quality and size of the host eggs largely affect the fitness of *A. bifasciatus*. Eggs of most lepidoptera are less suitable for *A. bifasciatus* compared to heteropteran eggs as the small size that is typical of moths (<0.7 mg) leads to emergence of mostly male offspring which would not contribute to the growth of the parasitoid population. Nevertheless, a costs and benefits balance must be figured out, considering possible non-target effects on one hand and the damage caused by *H. halys*, combined with the environmental risks due to the increased use of broad-spectrum insecticides against this pest, on the other hand.

In our augmentative biocontrol study, we assessed the efficacy of *A. bifasciatus* releases by sampling egg masses naturally laid in the field. This allowed collecting more realistic data than using sentinel eggs. Jones et al. (2014) found that parasitism rates were significantly higher on egg masses naturally laid by *H. halys* compared to sentinel egg masses. Use of sentinel eggs leads to underestimation of parasitism rate possibly because of egg mass age or handling methods that may eliminate host-finding kairomones or other stimuli (Leskey and Nielsen, 2018), including host-induced plant volatiles (Rondoni et al., 2017). Moreover, the use of freeze-killed sentinel eggs does not allow assessing the number of eggs killed by host feeding (Leskey and Nielsen, 2018). Results of other studies were consistent with these explanations. For instance, augmentative releases of *A. bifasciatus* were tested over three consecutive years in fruit orchards in Switzerland and in Italy (Stahl et al., 2019a). In this experiment, parasitization of *H. halys* sentinel eggs averaged 6% (range: 2%–16%). However, as stated by the authors, the impact of *A. bifasciatus* on *H. halys* eggs was likely underestimated because of the use of frozen egg masses. In other surveys conducted in Northern Italy using sentinel eggs obtained in the laboratory or laid in cages, *A. bifasciatus* showed very low parasitism rates (Costi et al., 2019).

In conclusion, releases of *A. bifasciatus* for augmentative biological control of *H. halys* enhanced the parasitism parameters used to evaluate the performance by this native European species, and led to an increased level of pest suppression especially in areas where pest density was low. Moreover, parasitization of the exotic *T. mitsukurii* was not affected by the release of *A. bifasciatus*. We might expect that field releases of *A*. *bifasciatus* to control the exotic pest could lead to an overall increase of biodiversity in the agroecosystems, as they promote a reduction of chemical pressure, which is one of the most detrimental factors to biodiversity (Ogburn et al., 2021). Studies are in progress to evaluate the dynamic scenario resulting from inoculative releases of the exotic *T. japonicus*, which would lead to multi-species interactions and potential additive or even synergistic effects for biological control of *H. halys*.

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Declaration of competing interest

The authors of this manuscript declare that they do not have any conflicts of interest, other than Alessia Iacovone and Marco Mosti who work for Bioplanet s.r.l. (Cesena, Italy), a biological control company which has provided the individuals of *Anastatus bifasciatus*.

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Table and Figure caption

Table 1. Discovery efficiencies, parasitism rates and percentages of unhatched eggs evaluated in 8 sites in which *Anastatus bifasciatus* was released and in 11 control (no release) sites. Only sites in which at least three naturally laid egg masses of *Halyomorpha halys* were collected are considered.

Fig. 1. Release and control (no release) sites considered for evaluating parasitism parameters. Only sites in which at least three naturally laid egg masses of *Halyomorpha halys* were collected are shown in the map.

Fig. 2. Relationship between discovery efficiencies by *Anastatus bifasciatus* (square root transformed, y-axis) and abundance of *Halyomorpha halys* egg masses (x-axis) in release and control sites (y=0.02x-0.10, *p*<0.001)

Fig. 3. Relationship between parasitism rates by *Anastatus bifasciatus (*square root transformed, y-axis) and abundance of *Halyomorpha halys* egg masses (x-axis) in release and control sites (y=0.02x-0.09, *p*<0.001).

Fig. 4. Correlation between parasitism rates by *Anastatus bifasciatus* (y-axis) and total number of parasitoids released (x-axis) in the sampled sites ($r_s = 0.85$, $P<0.05$).

Fig. 5. Exploitation efficiency of *H. halys* egg masses by *Anastatus bifasciatus* and *Trissolcus mitsukurii.* White spots and asterisks represent outliers.

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Highlights BCON-D-21-00637 R2

- Augmentative biological control of *Halyomorpha halys* was tested in Northern Italy.
- 1000 *Anastatus bifasciatus*/ha (325,000 in total) were released in 11 sites.
- Releases improved discovery efficiency and parasitism rate by *A. bifasciatus.*
- *Trissolcus mitsukurii* was not affected by the releases of *A. bifasciatus*.

Augmentative biological control of *Halyomorpha halys* using the native European parasitoid *Anastatus bifasciatus*: efficacy and ecological impact

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Key words

Brown marmorated stink bug, Pentatomidae, invasive species, *Trissolcus mitsukurii*, egg parasitoids.

Abstract

We report the first large-scale augmentative biological control project carried out in Europe against *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) using the native egg parasitoid *Anastatus bifasciatus* (Geoffroy) (Hymenoptera: Eupelmidae). During summer 2020, a total of 325,000 adults of *A. bifasciatus* were released at a rate of 1000 individuals/ha of orchard in 11 sites in Trentino Alto Adige (Northern Italy). Parasitism parameters were compared between release and control (no release) sites, in which at least three egg masses naturally laid by *H. halys* were collected (for a total of 262 egg masses). *Anastatus bifasciatus* and *Trissolcus mitsukurii* (Ashmead) (Hymenoptera: Scelionidae) were the dominant parasitoids, but parasitism by both species fluctuated widely among sites. At release sites, *A. bifasciatus* showed a significantly higher discovery efficiency (31.4%) and parasitism rate (16.7%) of *H. halys* egg masses than at control sites (1.7% and 1.2%, respectively). Parasitism by *A. bifasciatus* was not dependent on egg mass abundance at release sites, but at control sites a host density-dependent response was revealed by a positive relationship between parasitism and number of *H. halys* egg masses. On the other hand, parasitism by the adventive *T. mitsukurii* was not affected by either the releases of *A. bifasciatus* or by the abundance of *H. halys* egg masses per site. In conclusion, augmentative releases of *A. bifasciatus* contributed to increasing its parasitization of *H. halys*, without causing any negative effects on parasitization by naturally occurring species.

1. Introduction

The brown marmorated stink bug (BMSB) *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) is a global invasive pest with a wide host range, which includes over 170 plant species. Native to eastern Asia, this bug has become a notorious pest to growers in many parts of the world (Valentin et al., 2017; Musolin et al., 2018; Rot et al., 2018; Šapina and Jelaska, 2018; Francati et al., 2021), being economically important especially in fruit and vegetable-growing regions (Leskey and Nielsen, 2018).

Programs for management of invasive pests should firstly explore biological control agents, which can contribute to pest suppression in both natural and agricultural areas (Hoddle, 2004; Cock et al., 2010). The scientific community has therefore developed a strong interest in the search and selection of natural enemies of *H. halys* (Conti et al., 2021; Rot et al., 2021). A debate has arisen on the most appropriate strategy between the use of exotic natural enemies for classical biological control or the augmentation of native, newly associated, parasitoid species (Abram et al., 2017; Zapponi et al., 2021).

In its native areas in Asia, *H. halys* populations are regulated by egg parasitoids belonging to genus *Trissolcus* (Hymenoptera: Scelionidae) and *Anastatus* (Hymenoptera: Eupelmidae) (Qiu et al., 2007; Hou et al., 2009; Avila et al., 2021). In the countries where *H. halys* has recently established, the contribution of native biological control agents has been reported as low although highly variable among habitats, seasons and sampling protocols (Cornelius et al., 2016a, 2016b; Herlihy et al., 2016; Ogburn et al., 2016; Dieckhoff et al., 2017; Jones et al., 2017; Leskey and Nielsen, 2018; Pezzini et al., 2018; Moraglio et al., 2020; Francati et al., 2021). For these reasons, the recent invasions by *H. halys* have emphasized in many countries an interest in classical biological control although this has some limitations (Conti et al., 2021) and is currently subject to regulations on the importation of exotic species (De Clercq et al., 2011). The scenario on regulation is quite complex and may differ from country to country (Bale, 2011).

Adventive populations of the Asian egg parasitoids *Trissolcus japonicus* (Ashmead) and *Trissolcus mitsukurii* (Ashmead) (Hymenoptera: Scelionidae) have been recently detected in Europe (the first one in Italy, Switzerland and Germany, the second in Italy, Western Slovenia and France) and these findings have led to reconsider classical biological control methods to manage *H. halys* (Sabbatini Peverieri et al., 2018; Stahl et al., 2019b; Moraglio et al., 2020; Scaccini et al., 2020; Zapponi et al., 2020, 2021; Bout et al., 2021; Dieckhoff et al., 2021; Rot et al., 2021). However, the use of exotic biological control agents is strictly regulated. For this reason, risk assessment evaluations for *T. japonicus* and *T. mitsukurii* are in progress, including their potential coexistence with native parasitoids (Konopka et al., 2017; Haye et al., 2020; Giovannini et al., 2021). Field releases of *T. japonicus* were authorized in Italy in 2020 in the framework of the National Biological Control Program against *H. halys*, and this represents the first officially authorized release of this parasitoid in Europe (Bittau et al., 2021; Conti et al., 2021).

Field surveys to evaluate whether indigenous parasitoids in Europe can exploit *H. halys*, leading to potential pest suppression, have been carried out in Switzerland, Italy, Georgia and recently Slovenia. Several sampling methods have been used for these surveys, including exposure of freeze-killed sentinel egg masses (Haye et al., 2015; Roversi et al., 2016; Stahl et al., 2019b; Zapponi et al., 2020), exposure of fresh sentinel egg masses, eggs laid on plants by bugs housed in field cages (Costi et al., 2019a; Zapponi et al., 2020; Rot et al., 2021) and field collections of naturally laid egg masses (Sabbatini Peverieri et al., 2018, 2019; Moraglio et al., 2020; Scaccini et al., 2020; Zapponi et al., 2020, 2021; Francati et al., 2021; Rot et al., 2021). Among native species, *Anastatus bifasciatus* (Geoffroy) (Hymenoptera: Eupelmidae) was the dominant egg parasitoid capable of completing development on *H. halys* eggs. It was also the most widespread species as it was found in nearly all the investigated sites, yet with relevant fluctuations across years. Because of its prevailing presence, *A. bifasciatus* has been proposed for augmentative biological control of *H. halys* in Europe (Haye et al., 2015). Native to Europe and currently present in the Palearctic and Nearctic regions, *A. bifasciatus* can exploit egg masses of different insect groups in the orders Hemiptera and Lepidoptera, including various pests of agronomic interest, and could play a role in limiting introduced exotic pests (Haye et al., 2015; Stahl et al., 2018). Nevertheless, little information is available on its efficacy in field conditions following inundative or inoculative releases. Some studies, although promising, were conducted releasing a low number of parasitoids and using frozen sentinel egg masses as a method to assess parasitization (Stahl et al., 2019a). Another open question is whether the releases of *A. bifasciatus* can affect parasitoid guilds and in particular the adventive populations of *T. japonicus* and *T. mitsukurii*, which have been recently recorded in some Italian regions, including Trentino Alto Adige (Zapponi et al., 2020) where this study was carried out.

The first aim of this study is to assess the efficacy of *A. bifasciatus* augmentative releases in cultivated areas of Northern Italy. The second aim is to investigate the potential impact of *A. bifasciatus* releases on the *H. halys* parasitoid guild, including adventive exotic species. As far as we are aware, this experiment represents the first large-scale augmentative biological control project carried out in Europe using *A. bifasciatus* and based exclusively on the sampling of egg masses naturally laid by *H. halys* to evaluate parasitization.

2. Material and methods

2.1 Insects

Adults of *A. bifasciatus* were provided periodically by Bioplanet srl (Cesena, Italy) in 150 ml plastic bottles containing 250 4-days old adults (sex ratio ≈ 9:1, F:M). The bottles were shipped to farmers once per week and kept at 12 °C in dark conditions until use. For the release, one bottle at a time was taken out and the cap was removed to allow parasitoid dispersion.

2.2 Release and control sites

From early June until mid / late July 2020, a total of 325,000 individuals of *A. bifasciatus* were released in 11 sites in Trentino Alto Adige region (Northern Italy). All the sites were located in agricultural areas, close to orchards that were under integrated pest management (IPM) programs (Table S1). Release sites of *A. bifasciatus* were located at a minimum distance of 1 km from areas where *T. japonicus* was released within the aforementioned National Biological Control Program against *H. halys*, following a mandatory precaution recommended by local government (Decreto 8129/2021 by Provincia Autonoma di Bolzano - Alto Adige).

Releases of *A. bifasciatus* were carried out in:

- 5 sites nearby apple orchards in South Tyrol, in the Autonomous Province of Bolzano, covering about 180 ha, between June 10th and July 29th;
- 6 sites nearby apple and kiwi orchards and vineyards in Trentino, in the Autonomous Province of Trento, covering about 145 ha, between June 10th and July 8th.

Parasitoids were released at a rate of 1000 individuals per ha of orchard (Table S1). Bottles, each containing 250 adult wasps, were opened and hung at approximately 20 m from each other on shrubs and hedgerows at the edge of the orchards, or in ecological corridors adjacent to watercourse or reforestation within the cultivated areas.

Naturally laid egg masses were searched in each site by three experienced operators of local extension services for the fixed time of 1 h per operator. Operators walked randomly up to a maximum distance of 500 m around the centroid of each release site. During samplings, leaves of wild plants (shrubs, hedgerows, herbaceous plants) and fruit trees in the orchards were inspected. Egg masses collections started two weeks after the beginning of releases and were carried out once a week until mid-September in Trentino and until mid-October in South Tyrol.

Field surveys were also performed in 21 control sites within agricultural areas, including orchard systems close to scattered urban settlements as typical of the investigated areas, where no parasitoids were released. The control sites were at least 2 km away from release sites and as similar as possible to the release sites in terms of location, altitude, and agroecosystem features. Furthermore, in control sites, egg masses were searched on the same kind of plants mentioned above for release sites.

2.3 Handling of collected egg masses

Field-collected egg masses of *H. halys* were transferred to the laboratory, stored individually in plastic Petri dishes (ø 60 mm) and kept at 25 °C, 65-70% RH and 16L:8D photoperiod. The egg masses were checked every two days for 8 weeks after collection. For each egg mass, eggs were counted and classified as: 1) hatched, when a bug nymph hatched in laboratory or a hole by a nymph already hatched in the field was detected; 2) parasitized, when an adult parasitoid emerged in laboratory or a hole by a parasitoid already emerged in the field was found; 3) unhatched, due to mortality from unknown cause; 4) predated by polyphagous predators, showing clear damage due to chewing.

To evaluate the overall performances of parasitoids, the following parasitism parameters were considered: 1) discovery efficiency (i.e. wasp ability to find egg masses), calculated as the number of egg masses discovered by parasitoids (presenting at least one parasitized egg) over the total number of egg masses collected in a site; 2) parasitism rate (= parasitoid impact), calculated as the number of emerged parasitoids over the total number of collected eggs; 3) parasitoid exploitation efficiency, calculated as the number of emerged parasitoids over the total number of eggs within the discovered egg masses (Bin and Vinson, 1990).

As suggested by Stahl et al. (2019b), measuring parasitism only by offspring emergence could lead to underestimating the real level of pest suppression. Given that the main goal of biological control is the reduction of the pest populations (van Lenteren et al., 2018), we reported the percentage of unhatched eggs (i.e. eggs that did not develop to a viable bug nymph out of the total eggs in the egg mass) as an additional indication of overall pest suppression.

2.4 Parasitoid identification

All emerged parasitoids were frozen and identified to species or genus level. Eupelmidae were identified using the keys proposed by Askew and Nieves-Aldrey (2004). Scelionidae were identified following Johnson (1984), Kozlov and Kononova (1983) and Talamas et al. (2015, 2017). The keys of Sabbatini-Peverieri et al. (2019) were used for Pteromalidae. All morphological analyses were carried out under a stereo microscope (Leica M205C; 40X).

Voucher specimens have been deposited in the Entomological Collection of Department of Agricultural and Food Sciences, University of Bologna.

2.5 Data analysis

The relative abundances of emerged parasitoids were calculated using all collected egg masses, pooling data from release and control sites. For statistical analyses of discovery efficiency, parasitism rate, exploitation efficiency and percentage of unhatched eggs (which were calculated as reported above in section 2.3) only sites in which at least three egg masses of *H. halys* had been collected were retained (Fig. 1; Table S2). One-way ANOVA on log transformed number of egg masses per site was carried out to compare egg mass abundance in release vs control sites. Two-way ANOVA considering release vs no release of *A. bifasciatus* as a fixed predictor, and number of egg masses per site as a continuous predictor were run. The interaction between predictors was tested as well. In the cases of significant interaction, a linear regression analysis of the parasitization parameter in function of the number of egg masses was run separately for releases and no-releases sites. For raw data that violated the assumption of normality and homoscedasticity, which had been verified using Shapiro-Wilk and Levene's tests, the square root transformation was used.

Spearman rank-order procedure was used to correlate discovery efficiency and parasitism rate with the total number of parasitoids released in each site pooling release and control sites. Data analysis were carried out with IBM SPSS Statistics (version 26) (IBM corporation, Armonk, NY, USA); this software package was also used for graphical representation of data.

3. Results

3.1 Assemblages of Halyomorpha halys *natural enemies*

A total of 1641 parasitoids emerged from all collected *H. halys* egg masses from release and control sites (N=273 corresponding to 7318 eggs), including the following species: *A. bifasciatus* (N=762), *T. mitsukurii* (N=717), *Telenomus* sp. (N=4), *Trissolcus cultratus* (Mayr) (Hymenoptera: Scelionidae) (N=1), *Acroclisoides sinicus* (Huang and Liao) (Hymenoptera: Pteromalidae) (N=41). A total of 116 parasitoids, corresponding to the 7.1 % of the emerged individuals, were not identified because only empty parasitized egg masses were found. *Anastatus bifasciatus* was the dominant parasitoid, representing a relative abundance of 46,4%, followed by *T. mitsukurii* (43,7%). Concomitant parasitization by *A. bifasciatus* and *T. mitsukurii* emerging from the same *H.*

halys egg mass occurred in two egg masses collected in a release site. *Acroclisoides sinicus*, a hyperparasitoid whose host range includes *A. bifasciatus*, *T. mitsukurii* and *T. japonicus* (Sabbatini Peverieri et al., 2019), was detected with a relative abundance of 2.5%. No *T. japonicus* was recorded among the emerged parasitoids. Finally, the pressure exerted by generalist predators on *H. halys* egg masses was low, since only 1.7±0.7% of eggs were chewed.

Two egg masses of the non-target species *Nezara viridula* L. and *Palomena prasina* L. (Hemiptera: Pentatomidae) were found while monitoring *H. halys* eggs. Parasitism rates of *A. bifasciatus* on these species were 25.6% and 52.6%, respectively.

3.2. Total parasitism

A total of 262 *H. halys* naturally laid egg masses, corresponding to 7012 eggs, were taken into account for the evaluation of parasitism parameters; in particular 181 egg masses were considered from eight release sites and 81 from eleven control (no release) sites (Fig. 1). The mean number of egg masses was significantly higher (ANOVA, $F_{(1, 17)} = 10.7$; $p = 0.004$) at release sites (22.6±4.2) compared to control sites (7.36±3.57). Considering that the sampling effort was the same in all sites, these differences likely reflected the different abundance of *H. halys* in each area. The total discovery efficiency (45.4±8.2%) and the total parasitism rate (28.5±5.9%) in the release sites were not significantly higher than those in control sites (19.6±5.5% and 12.9±4,1%, respectively) (Table 1). However, the *p* value for total discovery efficiency (0.073) was close to the 0.05 significance level. The mean percentage of unhatched eggs in release sites (44.6±8.7) was almost twice as high than in control sites (23.9±5.6%), but again the difference was not supported by statistical analysis ($p = 0.12$, Table 1).

3.3. Parasitism by Anastatus bifasciatus *and* Trissolcus mitsukurii

Parasitism parameters by *A. bifasciatus* widely ranged among sites (Table S2). For example, discovery efficiencies encompassed a 0-50.0% interval while parasitism rates spanned between 0 and 29.6%. Overall, the releases of *A. bifasciatus* had a positive effect on its discovery efficiency and parasitism rate, as both parameters were significantly higher in the release sites than in the control sites (Table 1). The interactions release*number of egg masses were also significant for both parameters, demonstrating that the effects of releasing parasitoids depended on egg mass abundance. Indeed, in control sites, discovery efficiency (r^2=0.86, $F_{(1,8)}$=47.30, p< 0.001) and parasitism rate (r^2=0.89, $F_{(1,8)}$=67.10, p< 0.001) significantly increased in function of the egg mass number per sites (Fig. 2 and Fig. 3). On the other hand, the regressions were not significant in release sites (discovery efficiency r^2= 0.11, $F_{(1,6)}$=0.72, p= 0.43; parasitism rate r^2=0.23, $F_{(1,6)}$=1.78, p= 0.23), thus showing an independent response of parasitism parameters on egg mass abundance (Fig. 2 and Fig. 3). Finally, parasitism rate by *A. bifasciatus* was also positively correlated with the total number of parasitoids released in each site (r_s = 0.85 p<0.01, Fig. 4).

The discovery efficiency by *Trissolcus mitsukurii* fluctuated between 0 and 39.3%, while its parasitism rate ranged in a 0-31.6% interval. Both parasitism parameters were neither affected by the releases of *A. bifasciatus* nor by egg mass abundance per sites; the interaction release*number of egg masses did not show any significant effects as well. Therefore, parasitism by *T. mitsukurii* was not dependent on host density.

The exploitation efficiency, expressed as the number of emerged parasitoids out of the total number of eggs within the discovered egg masses, was lower for *A. bifasciatus* (median among 53 egg masses = 53.6%) than for *T. mitsukurii* (median among 29 egg masses = 92.9%) (Fig. 5).

4. Discussion

Releases of *A. bifasciatus* significantly increased the discovery efficiency and parasitism rate of *H. halys* egg masses by this native parasitoid compared to control sites. Both parasitism parameters by naturally occurring *A. bifasciatus* increased with the host density (i.e. number of egg masses) at control sites, whereas parasitism was not dependent on host density at release sites. This means that releases of *A. bifasciatus* enhanced its impact on *H. halys* also at low abundance of host egg masses. As host density increased, the difference in parasitism between control and release sites decreased.

In the control sites, foraging females of *A. bifasciatus* may have been affected by various stimuli linked to pest density, since they are known to exploit host-associated cues for egg location, like oviposition-induced plant volatiles and kairomones from male bugs and/or gravid females (Conti and Colazza, 2012; Rondoni et al., 2017). On the other hand, in release sites, the high number of released individuals likely promoted a response that was not related to host density. The parasitism rate by *A. bifasciatus* was also positively correlated with the total number of parasitoids released per release site, thus corroborating the significant effect of the augmentative releases. Parasitism by the adventive *T. mitsukurii* was similar in release and control sites. Therefore, augmentation of *A. bifasciatus* did not affect natural parasitism by *T. mitsukurii*. Although no significant differences could be detected between release and control sites for total discovery efficiency and total parasitization rate, both parameters showed a tendency to rise were *A. bifasciatus* was released, thus suggesting a possible additive effect. Exploitation efficiency of *A. bifasciatus* and *T. mitsukurii* in our study was quite in line with that previously reported (Scaccini et al., 2020; Zapponi et al., 2020, 2021).

Anastatus bifasciatus was the dominant parasitoid in most studies carried out in Northern Italy (Sabbatini Peverieri et al., 2018; Costi et al., 2019; Moraglio et al., 2020; Zapponi et al., 2020), with the exception of areas in North-eastern Italy (Scaccini et al., 2020), where an overall prevalence of

T. mitsukurii was recorded. In these areas, *A. bifasciatus* was the second most abundant parasitoid and another native species, *Trissolcus kozlovi* Rjachovskij (Hymenoptera: Scelionidae), emerged from field-collected *H. halys* egg masses (Moraglio et al., 2021b).

The potential coexistence of indigenous and exotic parasitoids of *H. halys*, which was anticipated relying on laboratory investigations (Konopka et al., 2017), was then observed in several field surveys (Sabbatini Peverieri et al., 2018; Stahl et al., 2019b; Moraglio et al., 2020; Scaccini et al., 2020; Zapponi et al., 2020, 2021; Rot et al., 2021). Our study confirmed that the native *A. bifasciatus* and the exotic *T. mitsukurii* can coexist even when *A. bifasciatus* populations are augmented for biological control. This aspect is noteworthy, considering the continuous expansion of *T. mitsukurii* and *T. japonicus* in Europe, as demonstrated by a large-scale survey recently performed in Northern Italy and Switzerland (Zapponi et al., 2021). The occurrence of adventive populations of these exotic parasitoids can lead to complex and dynamic relationships evolving over time.

Although our results indicate that releases of *A. bifasciatus* do not interfere with other parasitoids exploiting *H. halys*, parasitoid guilds are hardly predictable and different effects should be considered in the long term. For instance, augmentative releases of *A. bifasciatus* might boost the natural populations of this native species, eventually increasing its efficacy in pest suppression. On the other hand, new exotic species could be introduced accidentally or for biocontrol purpose in the areas invaded by *H. halys*. One example is the exotic *T. japonicus*, which is adventive in North-western Italy and has been recently released in several Italian regions for biological control of *H. halys*, after authorization by the Italian government (Bittau et al. 2021). Future studies will be needed to clarify the spatio-temporal dynamics of the parasitoid guild after the introduction of *T. japonicus*.

Synergistic interactions may have the potential to improve the suppression of *H. halys* populations in the long term (Leskey and Nielsen, 2018; Moraglio et al., 2020). However, in Europe the application of classical biological control is regulated by stringent risk assessments that may hamper the introduction of exotic biological control agents, boosting the exploitation of native species. The native parasitoid *A. bifasciatus* established a new association with the invasive host *H. halys*, and our data indicate a high egg mass discovery efficiency in the field, as was suggested by its positive response to volatiles associated with the new host (Rondoni et al., 2017). *Anastatus bifasciatus* had been already considered for augmentative biological control programs in Europe (Stahl et al., 2018; Stahl et al. 2019a) and for this reason it was selected for mass rearing and release in the present study.

Another aspect that should be considered when evaluating the efficacy of a parasitoid is the overall impact on the population-level suppression of the host. Usually wasp emergence rate is primarily used as a measure of parasitism rate, without considering the proportion of eggs that do not hatch following parasitoid activity, which ranges between 10-26% in *H. halys* (Abram et al., 2014, 2016; Haye et al., 2015; Cornelius et al., 2016b). Besides successful parasitism, this additional source of mortality contributes to the impact of a parasitoid on a pest (Jervis et al., 1996), and it can be ascribed to host feeding, to unsuccessful probing of the host egg that can lead to abortion, or to parasitoid incapability to complete development. Host feeding is an important biological trait of *A. bifasciatus*, and this should be considered when evaluating biological control programs using this species. Previous laboratory studies concluded that the number of eggs killed by host feeding is nearly as high as the number of eggs killed by parasitization, and can significantly contribute to the efficacy of biological control (Konopka et al., 2017; Stahl et al., 2019a). In our study, the percentage of unhatched eggs at the release sites was nearly twice as high as at control sites, although this difference was not statistically supported. Released *A.*

bifasciatus may have contributed to kill host eggs, but the overall mortality of *H. halys* eggs in field conditions is linked to a number of other factors that could introduce a bias in such evaluation. During our monitoring, very few egg masses of non-target hosts were found, including *N. viridula* and *P. prasina*. In spite of that, eggs of both species were parasitized by *A. bifasciatus*, confirming its wide host range. However, the few available data do not allow to draw any final consideration and specific studies should be carried out to acquire a more complete picture of non-target effects in the field. On the other hand, both non-target species can be also parasitized by the exotic parasitoids *T. japonicus* and *T. mitsukurii* (Haye et al., 2020; Dieckhoff et al., 2021; Giovannini et al., 2021). A polyphagous native parasitoid does not necessarily hinder other species (van Lenteren et al., 2006; van Lenteren and Loomans, 2006) and its activity does not necessarily translate into adverse effects in the field. For instance, the wide host range of *A. bifasciatus* can favour its colonization of cultivated areas. Additionally, heteropteran hosts of *A. bifasciatus* (Stahl et al., 2018, 2019b; Zapponi et al., 2020; Moraglio et al., 2021a) are mostly considered pests or very common species. Only a few potential moth species can be considered as “undesired” targets, but their occurrence is rare in agroecosystems (Masetti et al., 2017), which are the main target environments of the introductions of *A. bifasciatus*. Furthermore Stahl et al. (2018) demonstrated that quality and size of the host eggs largely affect the fitness of *A. bifasciatus*. Eggs of most lepidoptera are less suitable for *A. bifasciatus* compared to heteropteran eggs as the small size that is typical of moths (<0.7 mg) leads to emergence of mostly male offspring which would not contribute to the growth of the parasitoid population. Nevertheless, a costs and benefits balance must be figured out, considering possible non-target effects on one hand and the damage caused by *H. halys*, combined with the environmental risks due to the increased use of broad-spectrum insecticides against this pest, on the other hand.

In our augmentative biocontrol study, we assessed the efficacy of *A. bifasciatus* releases by sampling egg masses naturally laid in the field. This allowed collecting more realistic data than using sentinel eggs. Jones et al. (2014) found that parasitism rates were significantly higher on egg masses naturally laid by *H. halys* compared to sentinel egg masses. Use of sentinel eggs leads to underestimation of parasitism rate possibly because of egg mass age or handling methods that may eliminate host-finding kairomones or other stimuli (Leskey and Nielsen, 2018), including host-induced plant volatiles (Rondoni et al., 2017). Moreover, the use of freeze-killed sentinel eggs does not allow assessing the number of eggs killed by host feeding (Leskey and Nielsen, 2018). Results of other studies were consistent with these explanations. For instance, augmentative releases of *A. bifasciatus* were tested over three consecutive years in fruit orchards in Switzerland and in Italy (Stahl et al., 2019a). In this experiment, parasitization of *H. halys* sentinel eggs averaged 6% (range: 2%–16%). However, as stated by the authors, the impact of *A. bifasciatus* on *H. halys* eggs was likely underestimated because of the use of frozen egg masses. In other surveys conducted in Northern Italy using sentinel eggs obtained in the laboratory or laid in cages, *A. bifasciatus* showed very low parasitism rates (Costi et al., 2019).

In conclusion, releases of *A. bifasciatus* for augmentative biological control of *H. halys* enhanced the parasitism parameters used to evaluate the performance by this native European species, and led to an increased level of pest suppression especially in areas where pest density was low. Moreover, parasitization of the exotic *T. mitsukurii* was not affected by the release of *A. bifasciatus*. We might expect that field releases of *A*. *bifasciatus* to control the exotic pest could lead to an overall increase of biodiversity in the agroecosystems, as they promote a reduction of chemical pressure, which is one of the most detrimental factors to biodiversity (Ogburn et al., 2021). Studies are in progress to evaluate the dynamic scenario resulting from inoculative releases of the exotic *T. japonicus*, which would lead to multi-species interactions and potential additive or even synergistic effects for biological control of *H. halys*.

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Declaration of competing interest

The authors of this manuscript declare that they do not have any conflicts of interest, other than Alessia Iacovone and Marco Mosti who work for Bioplanet s.r.l. (Cesena, Italy), a biological control company which has provided the individuals of *Anastatus bifasciatus*.

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Table and Figure caption

Table 1. Discovery efficiencies, parasitism rates and percentages of unhatched eggs evaluated in 8 sites in which *Anastatus bifasciatus* was released and in 11 control (no release) sites. Only sites in which at least three naturally laid egg masses of *Halyomorpha halys* were collected are considered.

Fig. 1. Release and control (no release) sites considered for evaluating parasitism parameters. Only sites in which at least three naturally laid egg masses of *Halyomorpha halys* were collected are shown in the map.

Fig. 2. Relationship between discovery efficiencies by *Anastatus bifasciatus* (square root transformed, y-axis) and abundance of *Halyomorpha halys* egg masses (x-axis) in release and control sites ($y=0.02x-0.10$, $p<0.001$)

Fig. 3. Relationship between parasitism rates by *Anastatus bifasciatus (*square root transformed, y-axis) and abundance of *Halyomorpha halys* egg masses (x-axis) in release and control sites ($y=0.02x-0.09$, $p<0.001$).

Fig. 4. Correlation between parasitism rates by *Anastatus bifasciatus* (y-axis) and total number of parasitoids released (x-axis) in the sampled sites ($r_s = 0.85$, $P<0.05$).

Fig. 5. Exploitation efficiency of *H. halys* egg masses by *Anastatus bifasciatus* and *Trissolcus mitsukurii.* White spots and asterisks represent outliers.

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Fig. 1. Release and control (no release) sites considered for evaluating parasitism parameters. Only sites in which at least

Click here to access/download;Figure;Anastatus ABS - Map of sites BW (fig_1).jpg

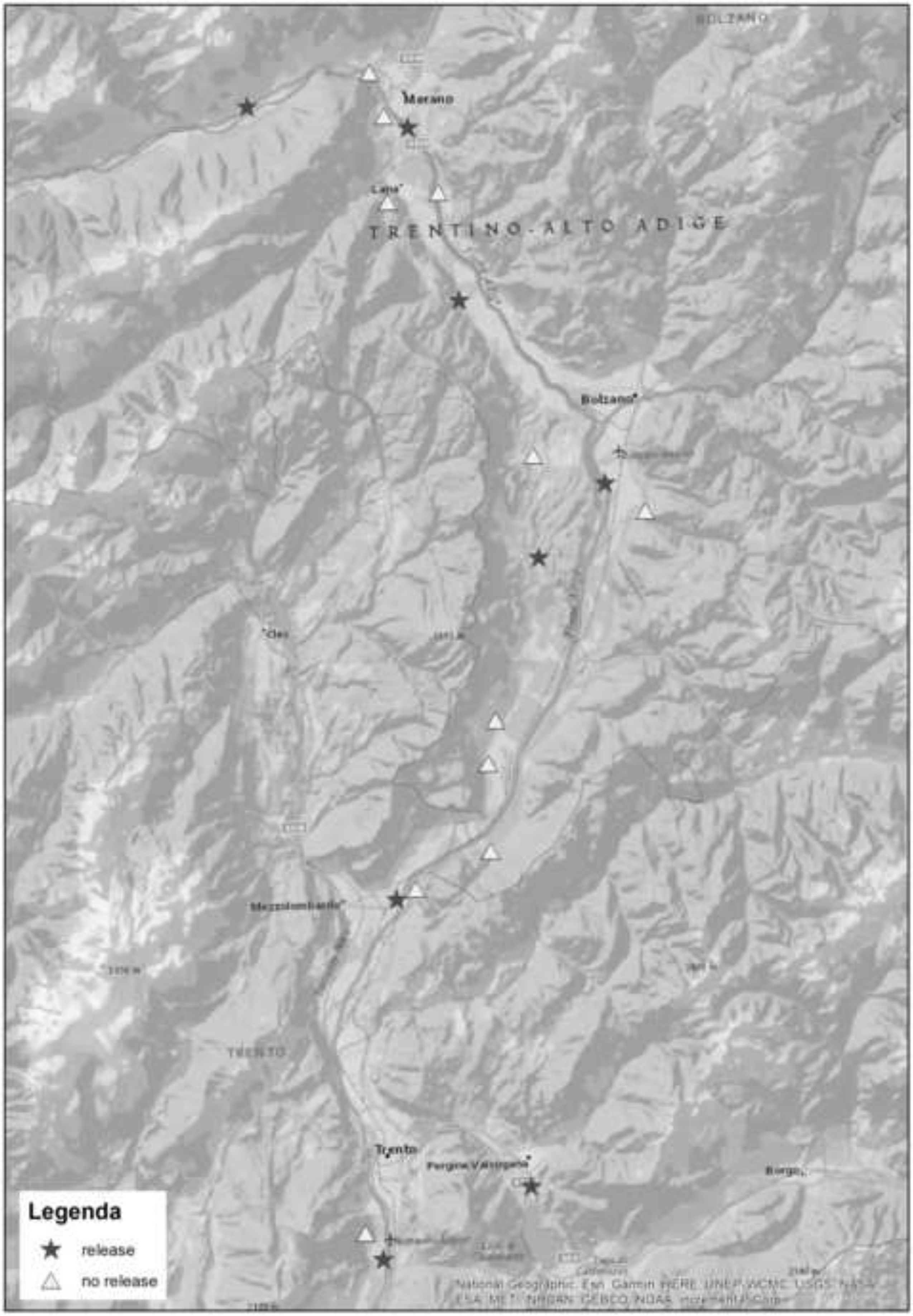
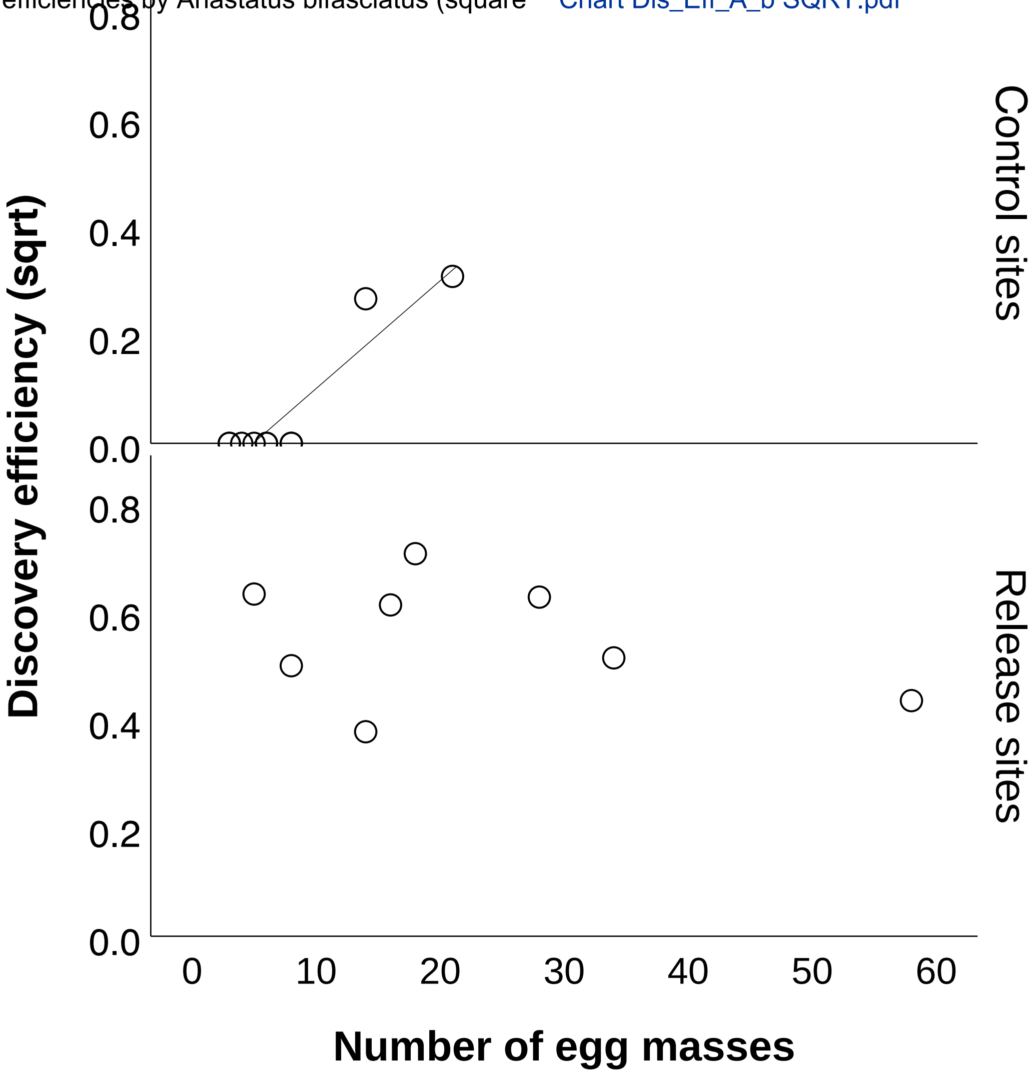


Fig. 2. Relationship between discovery
efficiencies by Anastatus bifasciatus (square



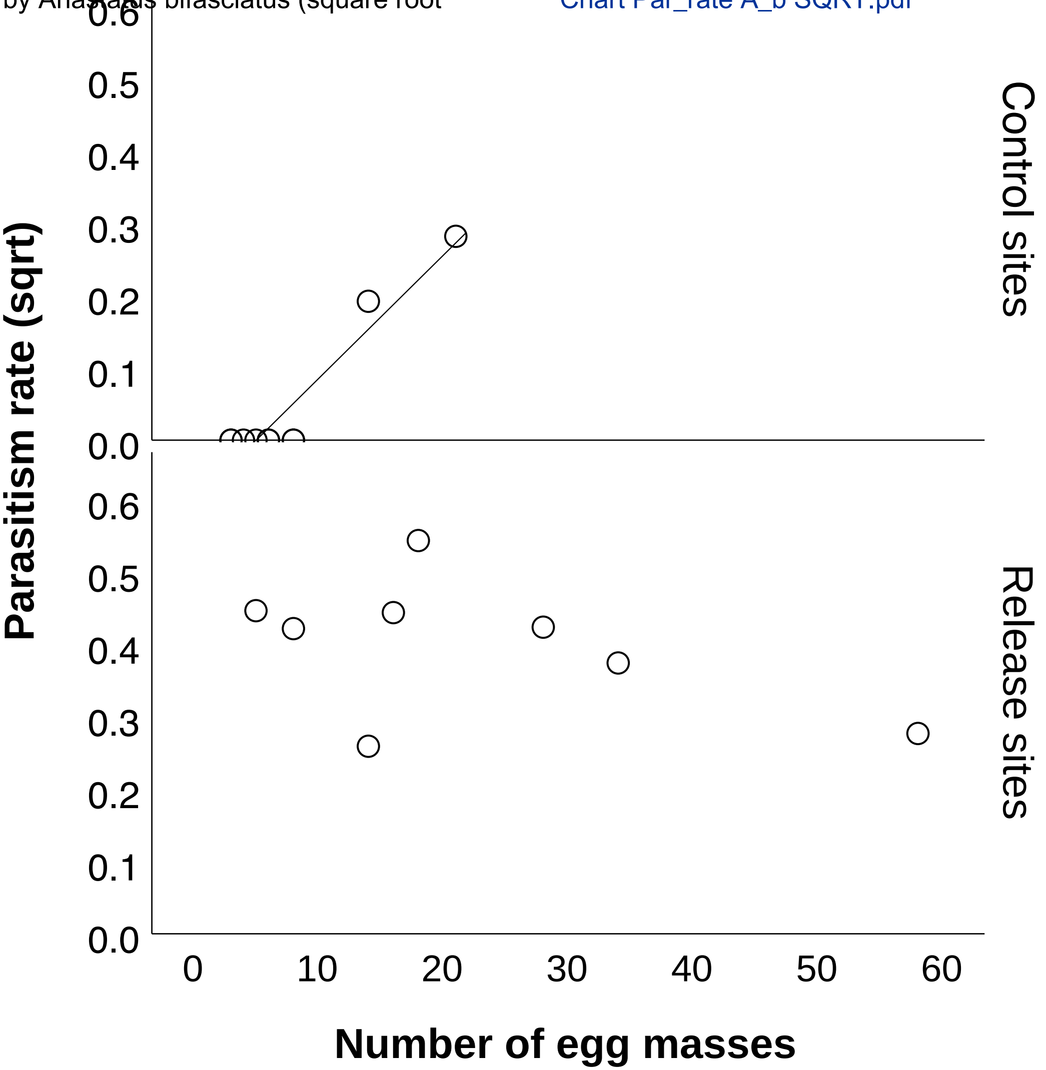

Parasitism rate (sqrt)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Control sites
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Release sites
0
10
20
30
40
50
60
Number of egg masses

Fig. 4. Correlation between parasitism rates by Anastatus bifasciatus (y-axis) and total

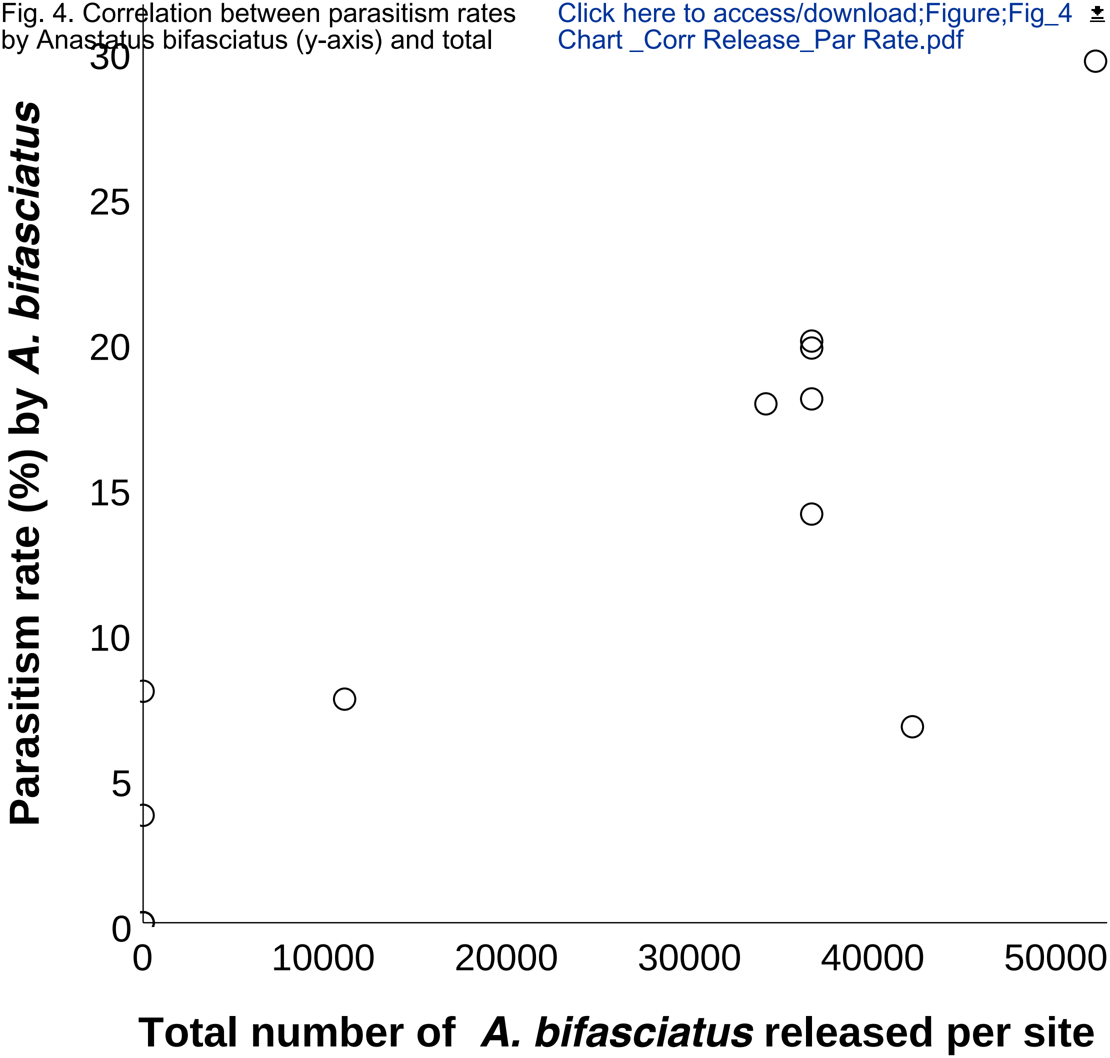


Fig. 5. Exploitation efficiency of H. halys egg masses by Anastatus bifasciatus and

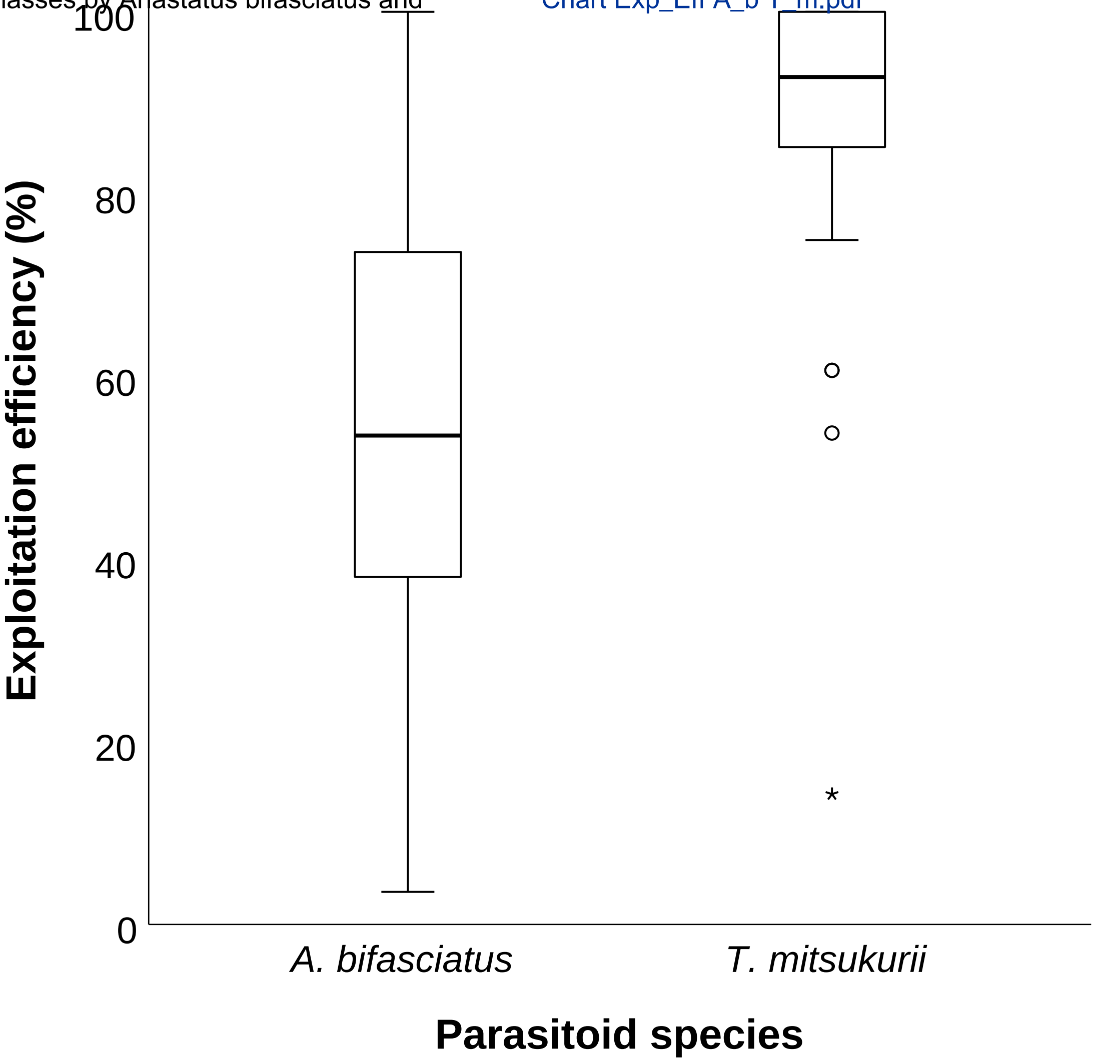


Table 1. Discovery efficiency rates, parasitism rates and percentage of unhatched eggs evaluated in 8 sites in which *Anastatus bifasciatus* was released and in 11 control (no release) sites. Only sites in which at least three naturally laid egg masses of *Halyomorpha halys* were considered.

	Marginal mean ± Standard error (%)		ANOVA Effects		
	Release sites	Control sites	*A. bifasciatus* release	Number of egg masses per sites	Release * number of egg masses
Total discovery efficiency rate	45.4±8.2	19.6±5.5	$F_{(1,15)}=3.72$ $p=0.073$	$F_{(1,15)}=0.016$ $p=0.90$	$F_{(1,15)}=0.19$ $p=0.67$
Total parasitism rate	28.5±5.9	13.0±4.1	$F_{(1,15)}=2.87$ $p=0.11$	$F_{(1,15)}=0.04$ $p=0.85$	$F_{(1,15)}=0.26$ $p=0.62$
Discovery efficiency rate by *A. bifasciatus*[1]	31.4±4.3	1.7±1.1	$F_{(1,14)}=101.9$ $p<0.001$	$F_{(1,14)}=11.5$ $p=0.004$	$F_{(1,14)}=17.6$ $p=0.001$
Parasitism rate by *A. bifasciatus*[1]	16.7±2.6	1.2±0.8	$F_{(1,14)}=105.6$ $p<0.001$	$F_{(1,14)}=12.9$ $p=0.003$	$F_{(1,14)}=23.9$ $p<0.001$
Discovery efficiency rate by *T. mitsukurii*	11.5±5.1	8.7±3.9	$F_{(1,14)}=0.0002$ $p=0.99$	$F_{(1,14)}=0.062$ $p=0.81$	$F_{(1,14)}=0.009$ $p=0.92$
Parasitism rate by *T. mitsukurii*	9.6±4.3	7.0±3.0	$F_{(1,14)}=0.0001$ $p=0.99$	$F_{(1,14)}=0.080$ $p=0.78$	$F_{(1,14)}=0.009$ $p=0.93$
Unhatched eggs	44.6±8.7	23.9±5.6	$F_{(1,15)}=2.72$ $p=0.12$	$F_{(1,15)}=0.29$ $p=0.60$	$F_{(1,15)}=0.49$ $p=0.50$

[1]: Data were square root transformed to meet ANOVA assumptions

Credit Author Statement

Alessia Iacovone: Conceptualization, Data Curation, Investigation, Writing- Original draft preparation

Antonio Masetti: Formal analysis, Visualization, Writing - Review & Editing. **Marco Mosti:** Conceptualization, Resources. **Eric Conti:** Writing - Review & Editing**. Giovanni Burgio:** Methodology, Formal analysis, Writing - Review & Editing.