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### A 3-year survey on parasitism of *Halyomorpha halys* by egg parasitoids in northern Italy

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1 **A 3-year survey on parasitism of *Halyomorpha halys* by egg parasitoids in northern Italy**

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11

12 **Abstract**

13 A 3-year survey was carried out to assess the impact of egg parasitoids on *Halyomorpha halys* in  
14 northern Italy. In total, 1826 *H. halys* egg masses were collected in the 3 years, and parasitoid adults  
15 emerged from 12% of eggs in 2016 and 2017, and from 21% in 2018. *Anastatus bifasciatus* was the  
16 main species emerging from *H. halys* eggs at all sites and years, confirming its ability to develop on  
17 fresh *H. halys* eggs. Only a few adults of native scelionid species emerged: *Trissolcus kozlovi* (first  
18 record in Italy), *T. semistriatus*, *T. basalis* and *Telenomus turesis*. In addition, a few adults of the  
19 Nearctic hyperparasitoid *Acroclisoides solus* (first record in Europe) were obtained from *H. halys* eggs  
20 collected at different sites. In 2018, for the first time an adventive population of *Trissolcus japonicus*  
21 was also recorded at one site, where the parasitism rate by the parasitoid species complex was overall  
22 higher than in 2016 and 2017. An additional supplemental survey at other sites of northern Italy in  
23 2018 revealed the presence of *T. japonicus* and *Trissolcus mitsukurii*. The distribution and abundance  
24 of *T. japonicus*, *T. mitsukurii* and *A. solus* should be further investigated. Their host range and their  
25 interaction with native egg parasitoids, especially with *A. bifasciatus*, should be also assessed to better  
26 understand their potential role in biological control of *H. halys*.

27

28 **Keywords:** Brown marmorated stink bug, *Anastatus bifasciatus*, *Trissolcus kozlovi*, *Trissolcus*  
29 *japonicus*, *Trissolcus mitsukurii*, *Acroclisoides solus*

30

31 **Key message:**

- 32 • Egg parasitoids emerged from 12 and 21% of *Halyomorpha halys* eggs collected in 2016-2017  
33 and 2018, respectively.
- 34 • *Anastatus bifasciatus* was the predominant parasitoid species, while native scelionid species were  
35 only occasionally found emerging from *H. halys* eggs.
- 36 • *Trissolcus kozlovi* and *Acroclisoides solus* are recorded for the first time in Italy and Europe,  
37 respectively.

- 38 • In 2018, *Trissolcus japonicus* and *Trissolcus mitsukurii* were also found emerging from field-  
39 collected *H. halys* eggs.

40

#### 41 **Author Contribution Statement**

42 LT, STM, MGP conceived and designed the research. GC, STM, MGP, SS and SV conducted the  
43 research. FT identified the parasitoids. MP performed molecular analysis. STM, LT and FT wrote the  
44 manuscript. All authors read and approved the final manuscript.

45

#### 46 **Introduction**

47 The brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), is a highly  
48 invasive harmful pest on several crops, as well as a nuisance pest in urban landscapes, when the adults  
49 invade human-made structures, often in very large numbers, to overwinter inside protected  
50 environments (Haye et al. 2015b). The species is native to East Asia, and it is now present in North  
51 America (the USA and Canada) and Europe, and most recently also in South America, in Chile  
52 (Leskey and Nielsen 2018). Moreover, other regions in both hemispheres with suitable climatic  
53 characteristics for the spread of *H. halys* are at risk of invasion (Zhe et al. 2017; Kriticos et al. 2017).  
54 In the invaded areas, *H. halys* has caused severe economic damage on many crops, among which  
55 pome and stone fruit, corn, hazelnut have been the most seriously affected (Rice et al. 2014; Maistrello  
56 et al. 2017; Bosco et al. 2018; Leskey and Nielsen 2018). Crop protection from *H. halys* damage  
57 mainly relies on intensive use of broad-spectrum insecticides, which does not always give a  
58 satisfactory control due to the pest's large host range, high mobility, and knockdown and recovery  
59 from many insecticides (Kuhar and Kamminga 2017; Leskey and Nielsen 2018). Their use also  
60 interferes with the largely adopted integrated pest management strategies, and leads to negative  
61 consequences on environment and human health. Therefore, research has been focused on generally  
62 more environmental-friendly control methods such as biological control.

63 In the native area, *H. halys* populations are attacked by a complex of egg parasitoids mainly belonging  
64 to Scelionidae and Eupelmidae (Lee et al. 2013). Within the egg parasitoid guild, *Trissolcus japonicus*  
65 (Ashmead) (syn. *T. halyomorphae* Yang; Talamas et al. 2013) is the predominant species, showing  
66 parasitism rates ranging from 50 to 80% in Beijink and Hebei provinces (China) (Yang et al. 2009,  
67 2015; Zhang et al. 2017), and is therefore considered a good candidate for biological control of this  
68 pest. Since 2014, adventive populations of *T. japonicus* have been found in North America in  
69 Maryland (Talamas et al. 2015), and since then, its presence has been assessed in 10 states in the USA,  
70 and research is now going on to establish its impact on *H. halys* and on other native stink bugs in field  
71 (Milnes et al. 2016; Hedtrom et al. 2017; Morrison et al. 2018).

72 In Europe, classical biological control with the introduction of exotic biological control agents, such as  
73 *T. japonicus*, is currently not allowed, because of risks of potential non-target effects and restrictions  
74 due to the Nagoya protocol (Cock et al. 2010). In the meantime, several studies have been conducted

75 in the invaded areas on native natural enemies, especially egg parasitoids. In North America and  
76 Europe, three principal groups of hymenopteran parasitoids attack *H. halys* eggs: Scelionidae  
77 (*Telenomus* spp., *Trissolcus* spp. and *Gryon* spp.), Eupelmidae (*Anastatus* spp.), and Encyrtidae  
78 (*Ooencyrtus* spp.) (Abram et al. 2017). The relative prevalence of different parasitoid species  
79 associated with *H. halys* eggs seems to be habitat dependent, since *Telenomus podisi* Ashmead is the  
80 most abundant species in field/vegetable crops, while *Anastatus* spp. and *Trissolcus* spp. are  
81 predominant in ornamental, forest, and seminatural/urban habitats (Abram et al. 2017). However, data  
82 have been collected using different sampling methods (e.g., collecting field laid egg masses or  
83 exposing fresh or frozen sentinel egg masses), and this can affect which parasitoid species are detected  
84 and their emergence rates (Abram et al. 2017). Specifically, sentinel egg masses seem to show lower  
85 parasitism levels, probably due to the absence of host-associated infochemical cues (Jones et al. 2014;  
86 Abram et al. 2017; Rondoni et al. 2017).

87 In a preliminary survey in NW Italy in 2015, a high concentration of *H. halys* egg masses was  
88 found on maple trees in a suburban landscape, and *Anastatus bifasciatus* (Geoffroy) emerged from  
89 16% of the eggs (Abram et al. 2017). Therefore, this study aimed to assess if and which native egg  
90 parasitoids were able to adapt to the exotic stink bug and evaluate the parasitism rate of *H. halys* eggs  
91 in field conditions. To estimate the impact of egg parasitoids, *H. halys* egg masses were collected and  
92 examined in a 3-year survey in northern Italy, as sites located in suburban and agricultural landscapes  
93 not subject to insecticide pressure.

94

## 95 **Materials and methods**

### 96 **Field collection on *H. halys* egg masses**

97 Field surveys to assess the parasitism of *H. halys* eggs were conducted during a 3-year period from  
98 2016 to 2018. In 2016 and 2017, *H. halys* egg masses were collected at 10 suburban sites in Piedmont  
99 (NW Italy) (Table 1). The sites were all characterized by the presence of maple (*Acer* spp.) not subject  
100 to any insecticide applications. Field surveys were carried out three times (every 2 weeks, from early  
101 August to early September) in 2016 and five times (two additional times compared to 2016, in mid-  
102 June and mid-July) in 2017. During surveys, canopies of maple between 1.5 and 2.5 m from the  
103 ground were inspected for the presence of egg masses of *H. halys* using a 4-step foldable aluminum  
104 ladder at each site for 30 min. Leaves with egg masses of *H. halys* as well as of other stink bugs were  
105 removed and transferred to the laboratory.

106 In 2018, field surveys were carried out at four sites in Piedmont (NW Italy), two of them already  
107 inspected in 2016 and 2017 (Table 1). The four sites were all characterized by the presence of high  
108 populations of *H. halys* and of various host plants besides maple, such as ash (*Fraxinus excelsior* L.),  
109 walnut (*Juglans regia* L.), pear (*Pyrus communis* L.), dogwood (*Cornus sanguinea* L.), linden (*Tilia*  
110 spp.), hazelnut (*Corylus avellana* L.), bee-bee tree [*Tetradium daniellii* (Bennett) Hartley], tree of  
111 heaven [*Ailanthus altissima* (Miller) Swingle], Eurasian smoketree (*Cotinus coggyria* Scopoli), fig

112 (*Ficus carica* L.), corn (*Zea mays* L.) and black locust (*Robinia pseudoacacia* L.). Field surveys were  
113 carried out three or four times (in mid-June, mid-July, late August and mid-September) (Table 1).  
114 During surveys, canopies of all host plants between 1.0 and 2.5 m from the ground were inspected for  
115 the presence of egg masses of *H. halys* using a 4-step foldable aluminum ladder at each site for 4 h.  
116 Leaves with egg masses were removed and transferred to the laboratory. At the end of the season, in  
117 mid-September, an additional survey was carried out at six of the 10 sites inspected in 2016 and in  
118 2017 and at further 23 sites in Piedmont and Lombardy (northern Italy), located at the foot of the Alps  
119 (sites in which at least one egg mass was found are reported in Table 1). All field-collected *H. halys*  
120 egg masses were transferred to the laboratory.

121

### 122 **Laboratory rearing of *H. halys* egg masses**

123 In laboratory, the field-collected egg masses were first separated into two groups: I) egg masses with  
124 at least one egg still closed and apparently viable, and II) completely vacated egg masses. The first  
125 ones were placed individually in plastic Petri dishes (60 mm diameter), and reared in controlled  
126 climate chambers at  $24\pm 1^\circ\text{C}$ ,  $65\pm 5\%$  RH and 16:8 h L:D, until all nymphs and/or parasitoids emerged.  
127 The egg masses were checked every 2 days to record any emergence of bug nymphs and separated  
128 according to their taxa and sexed. For the most abundant taxa, sex ratio was also calculated as the  
129 mean percentage of female offspring for each egg mass from which the parasitoid species had emerged  
130 in laboratory. All parasitoids were then stored in 99% ethanol prior to identification.

131 In all 3 years, at the end of the season, all collected egg masses were inspected under a Leica stereo  
132 microscope S6D with a magnification up to  $40\times$  to assess the fate of all eggs. Following Morrison et  
133 al. (2016) and Jones et al. (2017), egg fate categories were assigned to individual eggs within each egg  
134 mass with some adjustments: (1) hatched, where *H. halys* emerged from the vacated egg, recognizable  
135 for the presence of at least one of these characteristics: attached open lid, egg buster, incision line of  
136 the lid (Javahery 1994; Yang et al. 2009); (2) parasitized, where parasitoid emergence had occurred,  
137 recognizable for different coloration and opening of the vacated egg: non incision line of the lid but a  
138 hole with irregular margins (Yang et al. 2009); (3) sucked, where the egg was empty and one or more  
139 stylet sheaths protruded the egg; (4) broken, where egg was empty and the chorion was broken in at  
140 least one place; (5) unhatched, where a direct cause of mortality could not be properly diagnosed.

141 Additionally, parasitized eggs from which parasitoids had emerged were ascribed to a parasitoid group  
142 based on some characteristics observed after parasitoid emergence in laboratory: (1) Eupelmidae and  
143 Pteromalidae leave larval waste and pupal exuviae fragments inside the egg that make the egg appear  
144 dark inside, and a hole with different irregular margins (Jones et al. 2017, personal observations)  
145 (group 1, ESM1); (2) Scelionidae leave the egg nearly empty with a creamy compact waste  
146 accumulated against the corion and some typical half-moon shaped residuals of chewed chorion  
147 around the exit hole (Yang et al. 2009) (group 2, ESM2).

148 Furthermore, to evaluate the overall efficacy of the emerged egg parasitoids, the three indices  
149 proposed by Bin and Vinson (1991) were applied at each survey in the 3 years. The 'discovery  
150 efficiency' (i.e., percentage of egg masses containing parasitized eggs) was calculated as the number of  
151 egg masses discovered by the parasitoid (at least one parasitized egg) over the total number of egg  
152 masses. The 'exploitation efficiency' was calculated as the number of parasitized eggs over the total  
153 number of eggs within the discovered egg masses. Finally, the 'parasitoid impact' or parasitism rate  
154 (i.e., percentage of parasitized eggs) was calculated as the number of parasitized eggs over the total  
155 number of field-collected eggs. In each year, the three indices were then compared among sampling  
156 dates via a binomial distribution model with a logit link function, using the general linear model  
157 (GLM) procedure of the software IBM SPSS® Statistics 25 (IBM Corp., NY, USA). Means were then  
158 separated at  $P < 0.05$  using the Bonferroni test under the GLM procedure.

159

### 160 **Parasitoid identification and characterization**

161 Ethanol-stored specimens were dried and glued on card points for morphological analyses. A Leitz  
162 large-field stereo microscope TS with magnification up to 160× and a spotlight Leica CLS 150× were  
163 used for morphological diagnosis. For Eupelmidae, individuals were identified using the keys  
164 proposed by Askew and Nieves-Aldrey (2004); for Pteromalidae, individuals were identified following  
165 the description of Grissell and Smith (2006) and compared with the holotype kindly provided by the  
166 National Museum of Natural History, Smithsonian Institution (Washington, DC, USA). For  
167 Scelionidae, Mylar paper was used as filter to diffuse the spotlight and allow inspection of the  
168 specimens' microsculpture, which contains important features necessary for the species-level  
169 identification. *Telenomus* species were determined using the keys of Kozlov and Kononova (1983) and  
170 Johnson (1984), and *Trissolcus* species were identified using the keys by Talamas et al. (2017),  
171 Kononova (2014, 2015), and Kozlov and Lê (1977). Moreover, *Trissolcus* specimens were compared  
172 with pictures of holotype and paratypes made available via Specimage (specimage.osu.edu) by  
173 Norman Johnson (The Ohio State University, USA) and in Talamas et al. (2017). All the specimens  
174 used for morphological analysis were deposited in the Dipartimento di Scienze Agrarie, Forestali e  
175 Alimentari, Italy (DISAFA).

176 Molecular analyses were performed to confirm some morphological identifications, as a routine  
177 procedure, and also to characterize some parasitoids belonging to Scelionidae and Pteromalidae.  
178 Genomic DNA was extracted according to Garipey et al. (2014), and the barcode region of the  
179 cytochrome oxidase I (COI) gene was amplified for Scelionidae using universal PCR primers for  
180 insects LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198 (5'-TAA ACT  
181 TCA GGG TGA CCA AAA AAT CA-3') (Folmer et al 1994), and for Pteromalidae using the PCR  
182 primers LCO1490 and HCOOUT (5'-CCA GGT AAA ATT AAA ATA TAA ACT TC-3') (Carpenter  
183 1999). As described in Stahl et al. (2019c), the PCR was performed in a 50 µl reaction volume: 2 µl of  
184 DNA, 37.9 µl molecular grade water, 5 µl 10X Quiagen PCR buffer, 3 µl dNTPs (25 mM each), 1.5 µl

185 MgCl<sub>2</sub>, 0.2 µl of each primer (0.3 µM each), 0.2 µl *Taq* DNA Polymerase (Qiagen, Hilden, Germany).  
186 Thermocycling conditions were optimized to shorten reaction times and included initial denaturation  
187 at 94°C for 300 s, followed by 35 cycles of 94°C for 30 s, annealing at 52°C for 45 s and extension at  
188 72°C for 60 s; then further 600 s at 72°C for final extension. PCR products were purified using a  
189 commercially available kit (QIAquick PCR Purification Kit, Qiagen GmbH, Hilden, Germany)  
190 following the manufacturer's instructions and sequenced by a commercial service (Genechron S.r.l.,  
191 Rome, Italy). The sequences were compared with the GenBank database using the Basic Local  
192 Alignment Search Tool (<http://www.ncbi.nlm.nih.gov/BLASTn>). All sequences that showed a  
193 similarity lower than 99% were deposited in the GenBank database. All residual DNA is archived at  
194 DISAFA.

195

## 196 **Results**

### 197 **Field-collected egg masses**

198 At the 10 sites surveyed in 2016 and 2017, a total of 671 and 436 *H. halys* egg masses (17,545 and  
199 11,370 eggs) were collected, respectively (Table 2). Despite the higher number of surveys in 2017 (5)  
200 than in 2016 (3), an approximately similar number of egg masses was found at most of the 10 sites in  
201 the 2 years. In 2018, excluding the final survey of mid-September (sites from 13 to 24, Table 1),  
202 overall 469 and 155 *H. halys* egg masses (11,890 and 4035 eggs) were collected at the four sites  
203 surveyed three-four times and at the five sites surveyed at the end of the season, respectively (Tables 1,  
204 2). Most *H. halys* egg masses were generally collected from mid-August to mid-September in the 3  
205 years (Fig. 1).

206

### 207 **Egg fate and parasitism rate**

208 Overall more than 60% of *H. halys* eggs hatched or had already hatched in the first 2 years (64.5% and  
209 62.9% in 2016 and 2017, respectively), while only 46.8% of eggs hatched or had already hatched in  
210 2018, with the lowest value of 27.9% at site 6 (Table 2). At the same, the overall parasitoid impact was  
211 also similar in the first 2 years (12.7% and 11.8% in 2016 and in 2017, respectively), even if highly  
212 variable among the sites, while it was higher in 2018 (19.0%), with the highest value of 34.3% at site 6  
213 (Table 2). In 2016 and in 2017, the total parasitism, including eggs from which parasitoids had  
214 emerged, was almost exclusively attributable to parasitoids of group 1 (i.e., Eupelmidae and  
215 Pteromalidae), with few emergences of parasitoids of group 2 (i.e., Scelionidae) at sites 4 and 5 (Table  
216 2). In 2018, the parasitism due to group 1 was overall similar (13.6%), whereas that one due to group 2  
217 increased to 5.4%, with the highest value of 15.6% at site 6 (Table 2). Percentages of sucked, broken  
218 and unhatched eggs were quite variables among sites and years (Table 2). Overall, the incidence of  
219 sucked eggs was low in the 3 years (0.4% in 2016, 3.0% in 2017, and 2.6% in 2018) (Table 2). The  
220 incidence of broken eggs was low in 2016 (1.7%), while it increased in 2017 (7.5%), and in 2018

221 (8.7%) (Table 2). The incidence of unhatched eggs was generally higher (20.8% in 2016, 14.8% in  
222 2017 and 23.0% in 2018) (Table 2).

223 Although most egg masses were collected from mid-August to mid-September, the incidence of  
224 parasitized eggs was variable during the season (Fig. 1). The discovery efficiency was variable in the 3  
225 years (30.1% in 2016, 23.0% in 2017, and 41.8% in 2018). However, while in 2016 and in 2017 no  
226 differences were found in relation to the period, in 2018 discovery efficiency was significantly higher  
227 in late August, mainly due to the site 6 (Table 3). The exploitation efficiency increased over the 3 years  
228 (39.8% in 2016, 44.3% in 2017, and 50.8% in 2018) (Table 3). The parasitoid impact showed the same  
229 trend as exploitation efficiency in 2016 and in 2017, whereas in 2018 increased throughout the season  
230 following the same trend of the discovery efficiency (Table 3). In particular, at site 6 the parasitoid  
231 impact increased from 1.3% in mid-June to 40.8% in mid-September.

232

### 233 **Parasitoid species composition and abundance**

234 In the laboratory, from egg masses collected at sites 1-12 (Table 1), 1548, 896 and 1215 hymenopteran  
235 adults emerged in 2016, 2017 and 2018, respectively, belonging to six species (Table 2). Among these  
236 parasitoid species, *A. bifasciatus* was the predominant species emerging from *H. halys* eggs in all  
237 years (97.5% in 2016, 99.4% in 2017 and 78.6% in 2018), and on all sites and dates (Tables 2 and 3,  
238 Fig. 1). In laboratory, *A. bifasciatus* emerged for 4-8 week period after nymph emergence and showed  
239 a mean percentage of female offspring for each egg mass of  $63.9 \pm 1.45\%$ . By contrast, few scelionids  
240 emerged in the 2 years: *Telenomus turesis* Walker at site 5 in 2016 and at site 11 in 2018 (0.1% of  
241 emerged parasitoids in both years); *Trissolcus kozlovi* Ryakhovskii only at sites 4 but always in all  
242 years (0.7% of emerged parasitoids in 2016, 0.4% in 2017, and 0.1% in 2018); *Trissolcus basalis*  
243 (Wollaston) and *Trissolcus semistriatus* (Nees von Esenbeck) [syn. *Trissolcus grandis* (Thomson):  
244 Talamas et al. 2017)] only at site 11 in 2018 (0.2% of emerged parasitoids for both species); *T.*  
245 *japonicus* in higher number on maple as well as on other plants only at site 6 in 2018 (20.8% of  
246 emerged parasitoids) (Table 2). The ratio between parasitoid *Acroclisoides solus* Grissell & Smith  
247 (Hymenoptera: Pteromalidae) emerged occasionally at site 10 in 2016 (1.7%) and at site 6 in 2017  
248 (0.1%).

249 In the survey conducted at 23 sites in Piedmont and Lombardy in mid-September 2018, a total of 92  
250 egg masses (2403 eggs) were collected at 12 sites (Table 1). In laboratory, 420 hymenopteran adults  
251 emerged, of which *A. bifasciatus* was the most abundant (50.0%), followed by *T. japonicus* (36.9%),  
252 *A. solus* (8.3%), and *Trissolcus mitsukurii* (Ashmead) (4.8%), which was found only in Lombardy  
253 (Fig. 3).

254

### 255 **Parasitoid species characterization**

256 Molecular analyses confirmed the identity of *T. japonicus* and *T. mitsukurii* emerged from *H. halys*  
257 (100% similarity with GenBank sequence, accession no. AB971832, and 99% similarity with



258 GenBank sequence, accession no. AB971831, respectively). Since sequences of *T. kozlovi* and *A. solus*  
259 were not present in the GenBank database, all sequences obtained from specimens identified as *T.*  
260 *kozlovi* and *A. solus* by morphological analyses were deposited into the GenBank database  
261 [MH521283 for *T. kozlovi* emerged from *H. halys*, MH521284 for *T. kozlovi* emerged from *Palomena*  
262 *prasina* L., MH521285 for *A. solus* emerged from *Arma custos* (F.)] (Table 4).

263

## 264 **Discussion and conclusions**

265 In the 3-year surveys (sites from 1 to 12, Table 1), parasitism rate of *H. halys* eggs, with successful  
266 native parasitoid emergence, was stable and overall lower than 20%, consistent with data from  
267 previous surveys reviewed by Abram et al. (2017). The predominant parasitoid species was the  
268 generalist *A. bifasciatus*, already known to be able to develop on *H. halys* viable eggs (Haye et al.  
269 2015A; Roversi et al. 2016; Abram et al. 2017; Costi et al. 2019; Stahl et al. 2018, 2019a). Similarly,  
270 other species of the genus *Anastatus* have been found to develop on fresh *H. halys* eggs in the area of  
271 origin (Lee et al. 2013; Zhang et al. 2017) as well as in North America (Ogburn et al. 2016; Dieckhoff  
272 et al. 2017; Jones et al. 2017; Morrison et al. 2018). In our study, *A. bifasciatus* emerged from *H. halys*  
273 eggs collected at all sites and in all years, showing a wide distribution and suitability to be considered  
274 as a candidate for augmentative releases in Europe (Haye et al. 2015A; Stahl et al. 2018, 2019a).  
275 Moreover, female offspring of *A. bifasciatus* emerging from field-collected eggs was higher (over  
276 60%) than what was obtained in the laboratory (Stahl et al. 2018), further confirming the suitability of  
277 the egg masses laid in the field. However, in the 3-year surveys, the overall impact of *A. bifasciatus* on  
278 *H. halys* eggs did not increase. Continuous augmentative releases of *A. bifasciatus* may accelerate  
279 local population growth, and increase parasitism of *H. halys* eggs, even if the current release strategy  
280 could not effectively suppress the pest (Stahl et al. 2018, 2019b).

281 Concerning the native scelionid species, very few individuals were found overall, consistent with  
282 previous studies demonstrating the inability of native parasitoids to develop on this exotic host (Haye  
283 et al. 2015a, Abram et al 2014). However, the four scelionid species obtained in our study were found  
284 for the first time to emerge from *H. halys* eggs laid in the field. Furthermore, the presence of *T. kozlovi*  
285 in Italy is reported for the first time. In particular, *T. kozlovi* is extremely similar to *T. japonicus* both  
286 morphologically (Talamas et al. 2017) and genetically (91% similarity), and laboratory trials are  
287 currently underway to confirm the ability of *T. kozlovi* to develop on fresh *H. halys* eggs. However,  
288 similarity to *A. bifasciatus*, the impact of *T. kozlovi* on *H. halys* eggs as well as its distribution did not  
289 increase in the 3 years, although it was found emerging from eggs of other bug species at different  
290 sites (data not shown here). This fact suggests a lower attractiveness and/or suitability of *H. halys* eggs  
291 for its development, even if higher than for other native scelionid species.

292 The discovery of adventive populations of the exotic *T. japonicus* in 2018 confirmed its presence in  
293 Italy, as observed in the same year by Sabbatini Peverieri et al. (2018). From its first record in Europe,  
294 in Switzerland in 2017 (Stahl et al. 2019c), the species could spread, according to its potential

295 distribution (Avila and Charles 2018) and following *H. halys* presence. At the Piedmont site where *T.*  
296 *japonicus* was found for the first time on maple as well as on other plants in 2018 (site 6), comparing  
297 data of the 3 years show that the impact of the exotic parasitoid on *H. halys* eggs was additive to that  
298 of *A. bifasciatus*, which indeed increased in turn. This observation confirms that the two species can  
299 coexist and act synergistically in controlling *H. halys*, as predicted in the previous laboratory trials  
300 (Konopka et al. 2017b). Moreover, the presence of *T. japonicus* could be favorable for native scelionid  
301 species by providing them a lifeline as hyperparasitoids (Konopka et al. 2017a), but the interactions  
302 between the exotic and the native species should be evaluated in the following years. If the lifeline  
303 hypothesis was confirmed, a positive correlation between *T. japonicus* presence and successful  
304 development of native scelionid species on *H. halys* eggs would be expected. Consequently, the spread  
305 and establishment of the exotic parasitoid could lead to an increased emergence rate of indigenous  
306 parasitoids from *H. halys* eggs. However, a molecular approach on single eggs, as developed by  
307 Garipey et al. (2014, 2019) and Konopka et al. (2019), would be needed to distinguish if the native  
308 scelionid species behaved as hyperparasitoid of *T. japonicus* or as a direct parasitoid of *H. halys*.  
309 Furthermore, the impact of *T. japonicus* on non-target host species should be investigated especially in  
310 the field, as it was found emerging from other native pentatomid species in laboratory in the USA and  
311 Europe (Hedstrom et al. 2017; Botch and Delfosse 2018; Haye et al. 2019), as well as in field in China  
312 (Zhang et al. 2017). Further studies on *T. japonicus* distribution, spread and impact on *H. halys* and  
313 non-target pentatomid species, including zoophagous species, in our area are therefore needed.  
314 Similarly, distribution, spread and impact should be assessed also for *T. mitsukurii*, found emerging  
315 from *H. halys* egg masses collected in Lombardy, as well as in other regions by Sabbatini Peverieri et  
316 al. (2018). In Japan, this species is reported as the main egg parasitoid of *H. halys*  
317 (Arakawa and Namura 2002) and *Nezara viridula* (L.) (Arakawa et al. 2004), which is also wide  
318 spread in Italy. Therefore, the potential for competition between *T. mitsukurii* and *T. basalis* on *N.*  
319 *viridula* eggs (Jones 1988; Nishimoto et al. 2015) needs to be taken in account. Moreover, *T.*  
320 *mitsukurii* was reported in Iran on eggs of bug species such as *Eurygaster integriceps* Puton,  
321 *Acrosternum arabicum* Wagner, *Acrosternum breviceps* (Jakovlev) and *Brachynema germari* Kolenati  
322 (Mohammadpour et al. 2016); therefore, its distribution and host range could be wider than supposed.  
323 Finally, the interactions among native and exotic parasitoids and stink bugs are further complicated by  
324 the presence of *A. solus*, which is known to be a hyperparasitoid of *Trissolcus* spp. (Grissell and Smith  
325 2016; Garipey et al. 2014). This species described in North America is here recorded in Europe for the  
326 first time. Its presence could represent a threat for both *T. japonicus* and *T. mitsukurii*, but potentially  
327 also for all the native species. In our study, it emerged not only from *H. halys* eggs but also from other  
328 pentatomid species, such as *A. custos* and *P. prasina*, collected at three sites in Piedmont and one in  
329 Lombardy, showing to be widespread in North Italy. Moreover, *Acroclisoides* sp. was recorded from  
330 *H. halys* eggs in China (Lee et al. 2013).

331 During the 3-year surveys, besides the parasitized eggs, also sucked and broken eggs of *H. halys* were  
332 recorded. These sucked and broken eggs are generally ascribed to the feeding activity of sucking and  
333 chewing predators, respectively, and from our collection data we could not attribute them to a specific  
334 predatory arthropod. Actually, few studies have been carried out on the impact of predators under field  
335 conditions; however, the incidence of sucked and broken eggs recorded in NW Italy was consistent  
336 with what was observed in another Italian area (Costi et al. 2019) as well as in North America, where  
337 the predation varied widely among the states and crops (Morrison et al. 2016; Ogburn et al. 2016).  
338 In conclusion, during the 3 years, the impact of native parasitoid species on *H. halys* eggs did not  
339 increase. As expected, the most abundant species, *A. bifasciatus*, is a generalist parasitoid, with a wide  
340 host range and a limited population-level impact on the exotic host. In fact, indigenous parasitoids  
341 often fail to immediately adapt to the invasive species, but if subject to a strong selective pressure,  
342 they can evolve the capacity to develop successfully on the new host. This could be the case of native  
343 scelionid parasitoids, which can attack *H. halys* eggs, but fail to develop (Abram et al. 2014).  
344 However, this process may take a long time, and currently, after the discovery of adventive  
345 populations of *T. japonicus* and *T. mitsukurii*, the situation has been changing, as highlighted by the  
346 increased parasitism rate at the site where, together *A. bifasciatus*, also *T. japonicus* was detected in  
347 2018. Long-term studies will be therefore necessary to assess the effects of the interaction between  
348 native and exotic parasitoids and hyperparasitoids on *H. halys* control.

349

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355

### 356 **Compliance with ethical standards**

357 Conflict of interest The authors declare that they have no conflict of interest.

358

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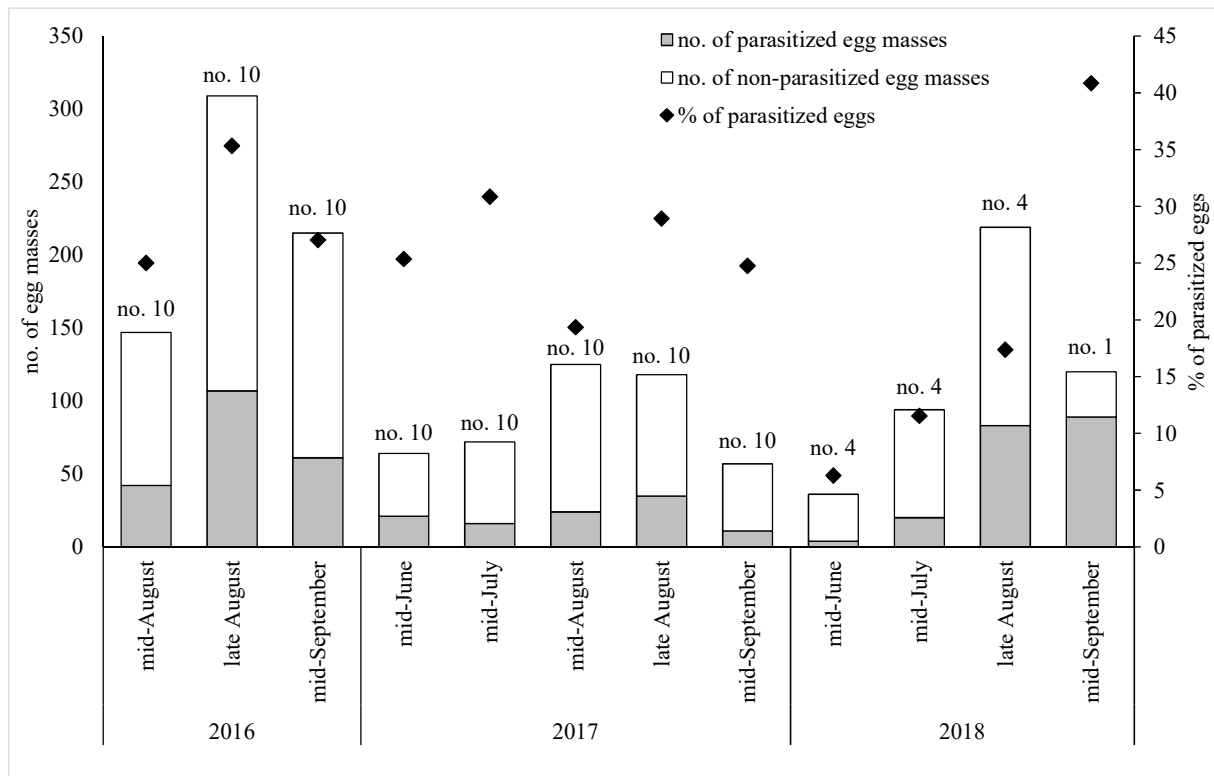
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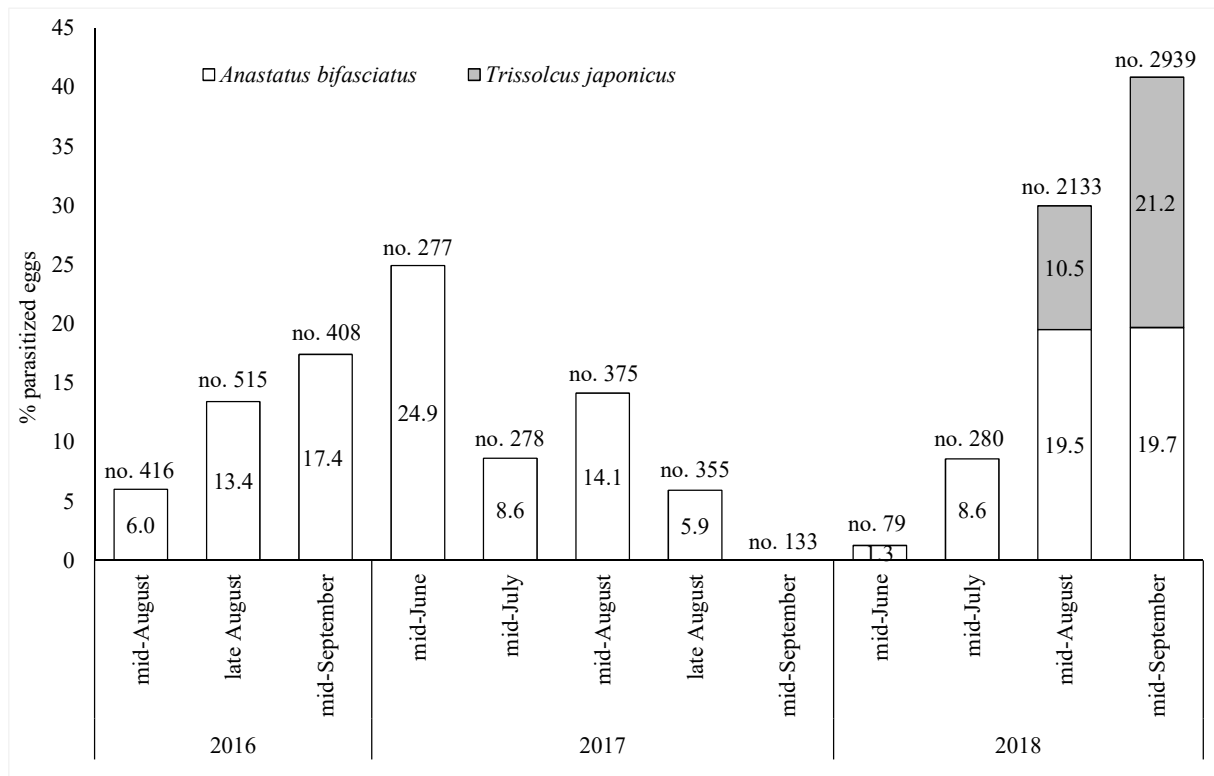




549

550 **Figure 1.** Total number of field-collected *Halyomorpha halys* egg masses, parasitized and  
 551 non-parasitized ones, and respective parasitism rate of eggs per sampling date at the sites  
 552 surveyed more than once in 2016, 2017, and 2018. Numbers of surveyed sites in each date are  
 553 displayed above bars.

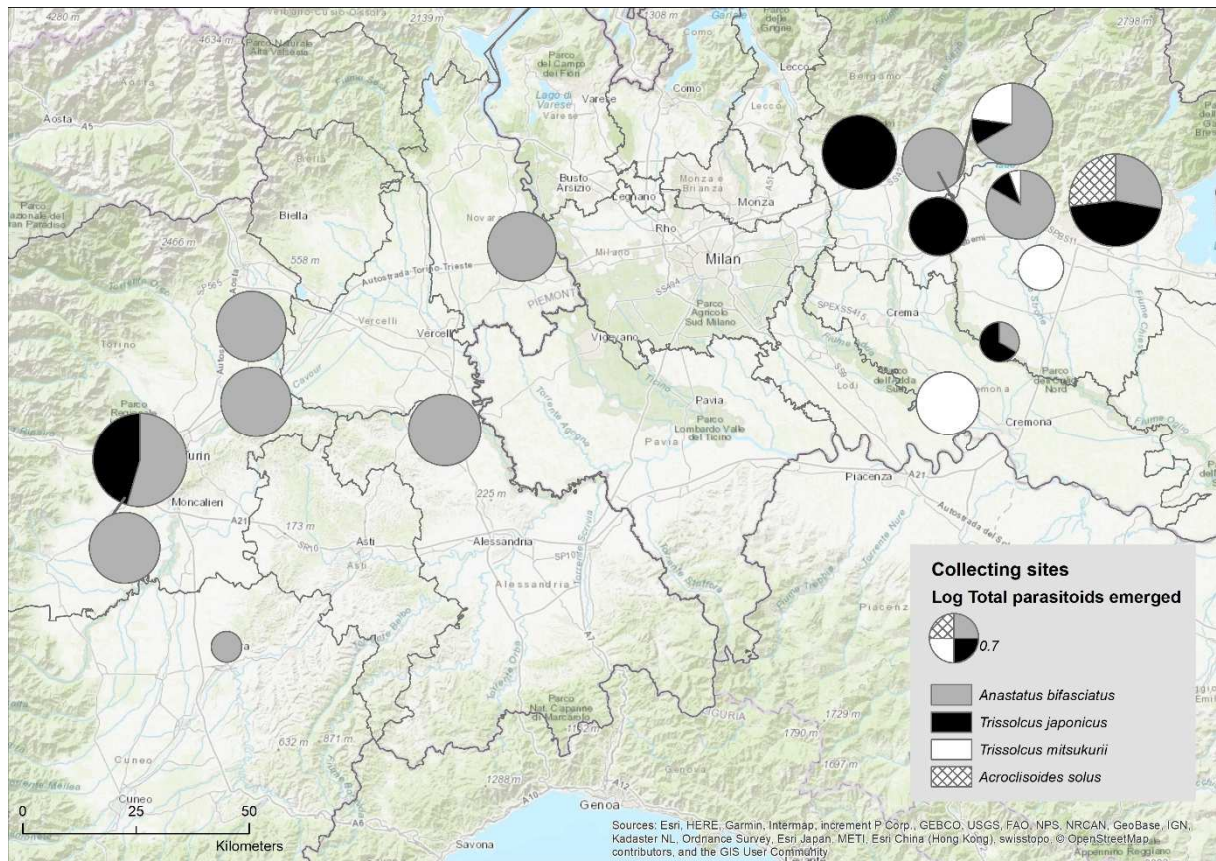
554



556

557 **Figure 2.** Parasitism rate of *Halyomorpha halys* eggs due to *Anastatus bifasciatus* and  
 558 *Trissolcus japonicus* observed at site 6 in the three-year survey. Numbers of observed eggs in  
 559 each date are displayed above bars.

560



562

563 **Figure 3.** Proportions of emerged parasitoid species from field collected *Halymorpha halys*  
564 eggs in the sites surveyed in mid-September 2018. Pie chart variation size is normalized by log.  
565

566 **Table 1.** Sites in North Italy, where *Halyomorpha halys* egg masses were collected in 2016, in  
 567 2017 and in 2018.

Id	Site (province)	Coordinates	Altitude (m asl)	2016	2017	2018
1	Bra (CN)	44°42'23.6"N 7°50'31.9"E	286	3	5	1
2	Carrù (CN)	44°28'42.6"N 7°52'37.2"E	363	3	5	
3	Casale Monferrato (AL)	45°08'35.6"N 8°26'47.4"E	117	3	5	1
4	Cavour (TO)	44°46'52.4"N 7°22'59.2"E	295	3	5	3
5	Chivasso (TO)	45°11'42.8"N 7°54'54.6"E	182	3	5	1
6	Grugliasco (TO)	45°03'51.5"N 7°35'30.3"E	287	3	5	3
7	Manta (CN)	44°36'38.7"N 7°29'18.1"E	400	3	5	1
8	Orbassano (TO)	44°59'57.3"N 7°33'01.4"E	266	3	5	1
9	Trofarello (TO)	44°58'48.6"N 7°45'08.7"E	243	3	5	
10	Pinerolo (TO)	44°53'16.8"N 7°20'06.1"E	370	3	5	
11	Chieri (TO)	45°02'28.2"N 7°50'03.9"E	335			3
12	Narzole (CN)	44°36'58.7"N 7°51'56.0"E	300			3
13	Collegno (TO)	45°04'26.5"N 7°35'27.6"E	297			1
14	Cameri (NO)	45°30'31.7"N 8°39'41.7"E	166			1
15	Candia (NO)	45°19'05.5"N 7°53'58.9"E	232			1
16	Concesio (BS)	45°35'40.1"N 10°13'49.2"E	194			1
17	Fenili Belasi (BS)	45°28'27.6"N 10°07'44.9"E	98			1
18	Quinzano d'Oglio (BS)	45°18'54.1"N 10°00'32.1"E	54			1
19	Tagliuno (BG)	45°38'22.4"N 9°53'48.4"E	223			1
20	Grumello Cremonese (BG)	45°11'40.7"N 9°51'39.9"E	52			1
21	Bergamo (BG)	45°41'39.0"N 9°41'44.6"E	257			1
22	Chiuduno (BG)	45°39'13.3"N 9°50'28.9"E	223			1
23	Gorlago (BG)	45°40'42.7"N 9°49'47.0"E	242			1
24	Paderno Franciacorta (BS)	45°35'13.2"N 10°04'26.6"E	182			1

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570 **Table 2.** Numbers of *Halyomorpha halys* egg masses and eggs collected at the 12 sites  
 571 surveyed from 2016 to 2018, with respective percentages of hatched, parasitized (total, and  
 572 attributable to the group 1, i.e. Eupelmidae and Pteromalidae families, or to the group 2, i.e.  
 573 Scelionidae family), sucked, broken and unhatched eggs, and numbers and species of  
 574 parasitoids emerged in laboratory.

Site	year (no. of surveys)	no. of egg masses	no. of eggs	% hatched	% parasitized (group 1; group 2)	% sucked	% broken	% unhatched	no. and species of parasitoids emerged in laboratory*
1	2016 (3)	74	1953	78.39	7.48 ( 7.48; 0.00)	0.00	1.08	13.06	72 <i>Ab</i>
	2017 (5)	63	1629	72.31	2.27 ( 2.27; 0.00)	2.52	8.90	14.00	16 <i>Ab</i>
	2018 (1)	10	282	78.01	0.35 ( 0.35; 0.00)	1.77	10.28	9.57	1 <i>Ab</i>
2	2016 (3)	17	472	65.04	14.83 (14.83; 0.00)	0.00	0.00	20.13	39 <i>Ab</i>
	2017 (5)	9	220	63.64	17.73 (17.73; 0.00)	0.00	8.18	10.45	28 <i>Ab</i>
3	2016 (3)	83	2195	59.09	18.50 (18.50; 0.00)	0.18	1.91	20.32	300 <i>Ab</i>
	2017 (5)	36	998	52.00	18.74 (18.74; 0.00)	10.52	2.51	16.23	110 <i>Ab</i>
	2018 (1)	24	649	46.53	10.63 (10.63; 0.00)	9.71	7.86	24.96	23 <i>Ab</i>
4	2016 (3)	81	2033	55.44	13.13 (12.59; 0.54)	1.23	4.57	25.63	149 <i>Ab</i> , 11 <i>Tk</i> , 19 <i>As</i>
	2017 (5)	64	1704	60.97	10.09 ( 7.34; 2.76)	0.00	17.90	11.03	92 <i>Ab</i> , 4 <i>Tk</i>
	2018 (3)	143	3588	55.52	10.67 (10.62; 0.06)	0.72	12.35	20.60	232 <i>Ab</i> , 1 <i>Tk</i>
5	2016 (3)	181	4847	66.45	9.53 ( 9.51; 0.02)	0.87	0.74	22.41	376 <i>Ab</i> , 1 <i>Tt</i>
	2017 (5)	19	490	65.92	11.02 (11.02; 0.00)	12.86	4.49	5.71	30 <i>Ab</i>
	2018 (1)	40	1059	52.69	12.18 (12.18; 0.00)	8.50	3.21	23.51	20 <i>Ab</i>
6	2016 (3)	54	1339	72.67	12.32 (12.32; 0.00)	0.00	1.19	13.82	69 <i>Ab</i>
	2017 (5)	53	1418	64.03	11.78 (11.78; 0.00)	2.47	5.22	16.50	139 <i>Ab</i> , 1 <i>As</i>
	2018 (4)	213	5431	27.88	34.32 (18.76; 15.56)	3.09	7.60	27.10	498 <i>Ab</i> , 253 <i>Tj</i>
7	2016 (3)	44	1070	63.36	16.64 (16.64; 0.00)	0.00	0.47	19.53	143 <i>Ab</i>
	2017 (5)	29	706	78.05	4.96 ( 4.96; 0.00)	3.54	1.70	11.76	21 <i>Ab</i>
	2018 (1)	5	119	59.66	14.29 (14.29; 0.00)	0.00	13.45	12.61	--
8	2016 (3)	74	1978	56.98	17.69 (17.69; 0.00)	0.05	2.53	22.75	252 <i>Ab</i>
	2017 (5)	86	2171	58.68	19.58 (19.58; 0.00)	1.52	5.39	14.83	306 <i>Ab</i>
	2018 (1)	76	1926	45.74	15.78 (15.42; 0.36)	1.82	8.10	28.71	21 <i>Ab</i>
9	2016 (3)	24	652	76.07	6.13 ( 6.13; 0.00)	0.15	2.91	14.72	24 <i>Ab</i>
	2017 (5)	43	1143	45.93	15.75 (15.75; 0.00)	3.67	4.90	29.75	19 <i>Ab</i>
10	2016 (3)	39	1006	54.67	14.02 (14.02; 0.00)	0.00	0.89	30.42	85 <i>Ab</i> , 8 <i>As</i>
	2017 (5)	34	891	77.55	5.50 ( 5.50; 0.00)	0.11	8.42	8.42	130 <i>Ab</i>
11	2018 (3)	88	2248	65.88	10.90 (10.63; 0.27)	1.29	6.43	16.19	144 <i>Ab</i> , 3 <i>Tb</i> , 2 <i>Ts</i> , 1 <i>Tt</i>
12	2018 (3)	25	623	69.34	3.53 ( 3.37; 0.16)	0.00	14.45	12.36	16 <i>Ab</i>

575 \**Ab*: *Anastatus bifasciatus*, *Tk*: *Trissolus kozlovi*, *As*: *Acroclisoides solus*, *Tt*: *Telenomus turesis*, *Tj*:  
 576 *Trissolcus japonicus*, *Tb*: *Trissolcus basalis*, *Ts*: *Trissolcus semistriatus*

577 **Table 3.** Mean ( $\pm$ SE) discovery efficiency, exploitation efficiency, and parasitoid impact on *Halyomorpha halys* egg masses collected in each sampling  
578 date (no. = sites) in the three-year survey. In each column, values followed by the same letter are not significantly different (Bonferroni test,  $P < 0.05$ ,  
579 under GLM procedure with binomial distribution and logit link).

Date	2016 (no. = 9-10 <sup>a</sup> )			2017 (no. = 5-10 <sup>a</sup> )			2018 (no. = 3-4 <sup>a</sup> )		
	Discovery efficiency (no.=10)	Exploitation efficiency (no.=9-10)	Parasitoid impact (no.=10)	Discovery efficiency (no.=7-10)	Exploitation efficiency (no.=5-9)	Parasitoid impact (no.=7-10)	Discovery efficiency (n=4)	Exploitation efficiency	Parasitoid impact (n=4)
Mid-June				(7) 25.46 $\pm$ 8.29	(5) 71.72 $\pm$ 6.43 a	(7) 18.99 $\pm$ 6.67 a	14.58 $\pm$ 7.12 b	(3) 47.02 $\pm$ 22.02 ab	5.03 $\pm$ 2.93 c
Mid-July				(9) 34.24 $\pm$ 9.62	(9) 52.29 $\pm$ 12.35 a	(9) 15.95 $\pm$ 4.80 b	17.05 $\pm$ 5.86 b	(3) 48.37 $\pm$ 3.39 a	8.50 $\pm$ 3.00 b
Mid-August	26.55 $\pm$ 4.64	(9) 45.11 $\pm$ 7.14 a	12.37 $\pm$ 3.28 b	(10) 32.29 $\pm$ 4.77	(8) 49.39 $\pm$ 5.42 b	(10) 9.22 $\pm$ 1.84 c			
Late August	36.78 $\pm$ 4.57	(10) 44.45 $\pm$ 4.63 a	17.13 $\pm$ 3.08 a	(10) 29.36 $\pm$ 6.41	(9) 39.52 $\pm$ 5.84 c	(10) 11.25 $\pm$ 2.15 bc	31.59 $\pm$ 11.28 a	(4) 43.02 $\pm$ 4.76 b	13.94 $\pm$ 5.51 a
Mid-September	28.11 $\pm$ 4.34	(10) 26.10 $\pm$ 4.04 b	8.32 $\pm$ 2.18 c	(9) 27.50 $\pm$ 11.83	(5) 18.32 $\pm$ 5.41 d	(9) 4.74 $\pm$ 2.33 d			
Wald $\chi^2$	2.950	102.392	139.568	6.318	151.281	242.350	14.574	8.250	96.560
df	2	2	2	4	4	4	2	2	2
P	0.229	<0.001	<0.001	0.177	<0.001	<0.001	0.001	0.016	<0.001

580 <sup>a</sup>sites varied from 2 to 11 depending on the presence of egg masses (discovery efficiency and parasitoid impact) and on the presence of parasitized ones (exploitation  
581 efficiency).

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584 **Table 4.** Sampling information and GenBank Accession Number for the deposited sequences generated from this study.

Species	sex	country	region	year of collection	host	GenBank Accession Number	ID code
<i>Trissolcus kozlovi</i>	m	Italy	Piedmont	2016	<i>Halyomorpha halys</i>	MH521283	DISAFA-draw1466-HYM-0482
<i>Trissolcus kozlovi</i>	f	Italy	Piedmont	2017	<i>Palomena prasina</i>	MH521284	DISAFA-draw1466-HYM-0481
<i>Acroclisoides solus</i>	f	Italy	Piedmont	2017	<i>Arma custos</i>	MH521285	DISAFA-draw1466-HYM-0480

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