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Does the fundamental host range of *Trissolcus japonicus* match its realized host range in Europe?

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Abstract

Unintentional introduction of natural enemies has increased in recent years due to a massive rise in global trade and tourism. One such natural enemy is the Asian egg parasitoid *Trissolcus japonicus*, a promising agent for classical biological control of *Halyomorpha halys*. In Europe, adventive *T. japonicus* populations have been detected in Switzerland, Italy and Germany. Host specificity testing demonstrated that its fundamental host range is fairly broad; however, it is unclear whether spatial or temporal refuges reduce parasitism of non-target species in the field. To address this, the realized host range of *T. japonicus* was assessed over three years by exposing sentinel egg masses of *H. halys* and 18 non-target species and collecting naturally laid egg masses in Switzerland and Italy. In total, 15 of 18 non-target species were successfully parasitized by *T. japonicus* in the field, confirming its broad fundamental host range. However, most non-target species were less parasitized by *T. japonicus* than *H. halys*, profiting from either partial temporal or spatial refuges from parasitism. Species with an unusual life cycle and the same ecological niche as *H. halys*, such as *Pentatoma rufipes*, which was the most parasitized non-target species in both countries, potentially face an increased risk of parasitism. In contrast, beneficial non-target effects may occur for the invasive pest, *Nezara viridula*, which suffered high non-reproductive mortality induced by *T. japonicus*. In both cases, life table studies will be needed to determine the impact of non-target parasitism and the potential consequences at the population level.

Keywords Biological control · Egg parasitoids · Non-target effects · Risk assessment · *Halyomorpha halys* · Ecological host range

Key message

- The Asian parasitoid *Trissolcus japonicus* associated with *Halyomorpha halys* is spreading in Europe
- Its realized host range was assessed in Switzerland and Italy using sentinel and wild egg masses
- Species in the same ecological niche as *H. halys* are more likely to be attacked by *T. japonicus*
- DNA analysis of *Nezara viridula* eggs showed non-reproductive mortality caused by *T. japonicus*
- *Pentatoma rufipes* is regularly attacked, but the impact at population level remains unknown.

Introduction

The brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) is native to Asia and arrived in North America and Europe in the late 1990s and early 2000s, respectively (Leskey and Nielsen 2018). The first records from Europe (Switzerland, Liechtenstein) date back to 2004 (Hoebeke and Carter 2003; Arnold 2009; Haye et al. 2014), but at least two additional, independent introductions were later detected in Italy and

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Greece (Gariepy et al. 2015; Cesari et al. 2018). Since its arrival, it has spread through most of Europe's mainland (Claerebout et al. 2018), and due to climate change, it has the potential to further expand its range (Kriticos et al. 2017; Stoeckli et al. 2020). Because of its polyphagous behaviour, *H. halys* quickly became an invasive pest of a wide variety of tree fruit, nut, vegetable, and field crops in Europe, particularly in Italy and Georgia, where it has caused severe economic losses in tree fruit (e.g. apples, peaches, pears) and nuts (hazelnuts) (Maistrello et al. 2017; Bosco et al. 2018).

As the impact of native natural enemies on invasive *H. halys* populations in Europe and North America is generally low (Abram et al. 2017), *H. halys* was identified as promising target for classical biological control in the invaded range. Surveys for natural enemies that have co-evolved with *H. halys* in its native range revealed that it is mostly attacked by egg parasitoids, among which the samurai wasp, *Trissolcus japonicus* (Ashmead) (Hymenoptera: Scelionidae), was identified as the most promising candidate for classical biological control, with parasitism levels ranging from 30 to 90% (Yang et al. 2009; Lee et al. 2013; Zhang et al. 2017).

However, prior to the release of exotic biological control agents into a new environment, it is important to consider the potential for unintended non-target effects such as host range expansion to native or beneficial species (van Lenteren et al. 2003). Accordingly, host specificity testing of *T. japonicus* has been carried out under containment conditions in North America and Europe (Hedstrom et al. 2017; Botch and Delfosse 2018; Haye et al. 2020; Sabbatini Peverieri et al. 2021). Overall, these studies came to similar conclusions that *T. japonicus*' fundamental (physiological) host range is restricted to the Pentatomoidea, but the parasitoid is capable of successfully parasitizing the eggs of several pentatomids other than *H. halys*, as well as a few species of scutellerids. These studies also showed that most non-target species, compared to *H. halys*, were usually accepted at lower rates and were less suitable for development of *T. japonicus* offspring. In addition, incorporating more complexity and the parasitoid's host finding behaviour in laboratory experiments (host chemical footprints, cage trials with plants) seemed to further increase the likelihood that *H. halys*, more often than non-target species, was detected and parasitized. In Europe, developmental suitability of non-target host species for *T. japonicus* was demonstrated in no-choice tests by successful offspring emergence from 11 of 13 non-target species tested (Haye et al. 2020). Whereas most non-target species were less accepted than *H. halys*, four native heteropteran species [*Palomena prasina* (L.), *Rhaphigaster nebulosa* (Poda), *Pentatoma rufipes* (L.), and *Arma custos* (F.)] were accepted at rates comparable to the target host. In a later study, nine out of 16 non-target species were accepted and suitable for *T. japonicus* development, confirming the potentially higher risk of non-target parasitism for *P. prasina* and *R. nebulosa* (Sabbatini Peverieri et al. 2021).

As laboratory risk assessment studies for *T. japonicus* in Europe were underway, adventive (self-introduced) populations of *T. japonicus* were discovered in Switzerland, Italy, and Germany (Sabbatini Peverieri et al. 2018; Stahl et al. 2019b; Moraglio et al. 2020; Dieckhoff 2021). A large-scale survey conducted in northern Italy and parts of Switzerland in 2019 demonstrated that within just three years, *T. japonicus* has rapidly spread into all types of habitats where *H. halys* is present (Zapponi et al. 2021). There is no doubt that *T. japonicus* will continue to quickly expand its range in Europe as predicted by bioclimatic envelope models (Avila and Charles 2018).

Accurately reproducing the complex factors that influence host searching and acceptance behaviour of parasitoids in their natural environment can be difficult. As such, the *fundamental* host range of a biological control agent is often greater than its *realized* (ecological) host range, which is the current and evolving set of host species used for successful reproduction in nature (Cameron and Walker 1997; Morehead and Feener 2000; Froud and Stevens 2003; Haye et al. 2005; Barratt et al. 1997, 2010; Cameron et al. 2013). Assuring host specificity from laboratory testing remains challenging, and it has been advocated that laboratory observations should be combined with field observations to provide a basis for correctly interpreting fundamental host range estimations (Onstad and McManus 1996; Hopper 2001; Kuhlmann and Mason 2003). After completing fundamental host range studies for *T. japonicus* in Europe, it remained unclear if there are behavioural or phenological barriers to parasitism, such as habitat preferences, competition with native egg parasitoids, or oviposition periods of non-target species, which may exist and reduce parasitism of those species that were identified as highly suitable hosts in laboratory tests. However, the unintentional introduction of *T. japonicus* into Europe now provides a unique opportunity to validate retrospectively estimates of fundamental host range with realized 'post arrival' host range as it manifests over time, and determine whether enemy-free space exists in nature, and provides protection to species considered suitable under laboratory conditions.

The objective of the present study was to determine the realized host range of *T. japonicus* by exposing sentinel egg masses of *H. halys* and native non-target species in areas of Switzerland and Italy where the parasitoid was first established and compare these results with its predicted fundamental host range (Haye et al. 2020; Sabbatini Peverieri et al. 2021). As sentinel egg masses are sometimes less exploited in the field (Jones et al. 2014), naturally laid egg masses were also collected in order to assess parasitism and parasitoid species composition under more natural conditions. In addition, because egg masses of the invasive *Nezara* *viridula* (L.) (Hemiptera: Pentatomidae) suffered from high (non-reproductive) host mortality induced by *T. japonicus* in laboratory tests (Haye et al. 2020), we aimed to quantify this often neglected aspect of non-target impact under field conditions.

Material and methods

Selection, source and rearing of stink bug species

To assess the realized host range of *T. japonicus* in Europe, *H. halys* and 12 non-target species tested by Haye et al. (2020) [Pentatomidae: *Acrosternum heegeri* Fieber, *A. custos*, *Carpocoris fuscispinus* (Boheman), *Dolycoris baccarum* (L.), *Graphosoma lineatum* (L.), *N. viridula*, *P. prasina*, *P. rufipes*, *Peribalus strictus* (F.), *Piezodorus lituratus* (F.), *R. nebulosa*; Scutelleridae: *Eurygaster maura* (L.)] were selected for exposure of sentinel egg masses. In addition, three pentatomids [*Carpocoris mediterraneus* Tamanini, *Carpocoris purpureipennis* (De Geer), *Eurydema ventralis* Kolenati] tested by Sabbatini Peverieri et al. (2021) and three species that had not been part of previous laboratory testing [Pentatomidae: *Eysarcoris venustissimus* (Schrank), *Eurydema ornata* (L.); Scutelleridae: *Eurygaster austriaca* (Schrank)] were added to the test list, for a total of 18 non-target species.

Stink bug colonies (Hemiptera: Pentatomidae, Scutelleridae) were first established in the laboratories of DISAFA (Italy) and CABI (Switzerland) in 2018–2019 from field-collected adults as previously described (Moraglio et al. 2020, 2021a, b; Haye et al. 2020). In subsequent years, new adults were collected in the same way and at the same sites to maintain the laboratory colonies or to establish new ones. Species were identified using the keys by Derjanschi and Péricart (2005), Belousova (2007), Péricart (2010), Wyniger and Kment (2010), Ribes and Pagola-Carte (2013), Lupoli et al. (2013), and Neimorovets (2020). Adults were then reared, each species separately, in gauze cages (BugDorm-4090 Insect Rearing Cage, 47.5 × 47.5 × 47.5 cm; MegaView Science, Taichung, Taiwan) with the bottoms covered with white absorbent paper. At DISAFA, herbivorous pentatomids were fed with broad bean and fennel seedlings, hazelnuts without shells, and apples. At CABI, pentatomids were fed with corncobs, green beans, sunflower seedlings, a mix of wild flowers and fruit-bearing branches of common ivy. Diet for predatory pentatomids was complemented with adults and larvae of *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae). Scutellerids collected on wheat were supplied with wheat ears (Feekes growth stages: 10–11; Miller 1992) and wet cotton. All food sources were periodically replaced. Mass rearing was carried out in controlled climatic chambers at 24±1 °C, 65±5% RH, and 16L:8D.

Exposure sites for sentinel egg masses

In Italy, egg masses of *H. halys* and non-target species were exposed in Piedmont (NW Italy), from April to October in 2019 and from May to September in 2020 in one of the first sites where *T. japonicus* was found in 2018 (Moraglio et al. 2020). The site, located in a peri-urban area near Turin (Grugliasco, 45.064358, 7.591715), is a park with several stink bug host plants, such as maple, poplar, mulberry, elder, and others, surrounded by crops (hazelnut, pome and stone fruits, wheat).

In Switzerland, stink bug egg masses were exposed at three sites in the Cantons Basel-Stadt, Zurich, Bern, and Ticino between May and September from 2019 to 2021. In the canton Ticino, eggs were exposed in an organic apple orchard and bordering natural areas in the centre of Manno (46.031119, 8.922250), where *T. japonicus* was first discovered in Europe in 2017 (Stahl et al. 2019b). In the city of Basel, eggs were exposed at the St. Alban Anlage (47.552206, 7.599038), an urban park with a high tree diversity (including maple and catalpa) that has been highly infested with *H. halys* over the past nine years. In Zurich, eggs were exposed in an urban park ('Zürichhorn', 47.354245, 8.552178) on the north shore of the lake of Zurich, where *H. halys* was first found in Europe in 2007 (Wermelinger et al. 2008). At both urban sites, the presence of *T. japonicus* was confirmed in the first year of the study, and accordingly, the exposure of non-target species was only started at end of summer 2019. Additional small exposures were conducted at an urban site in the city of Bern (46.963518, 7.447849), where *T. japonicus* was first discovered in the last year of the study (2021).

Preparation and field exposure of sentinel egg masses

Rearing cages were checked daily for freshly laid egg masses. Egg masses laid on the absorbent paper or on fennel leaves were collected with the substrate on which they were laid and glued on a green plastic tag (16 × 1.3 cm PVC plant labels, Hortima AG, Hausen, Switzerland) with a thin layer of neoprene adhesive (Bostik® Superchiaro, UHU Bostik S.p.A., Milan, Italy). Egg masses laid on the cage gauze or on broad bean and ivy leaves were gently removed from the substrate on which they were laid, and then directly glued on the tag, because the egg masses would easily detach from the drying leaves once they were cut, and consequently get lost. Number of glued egg masses of each species and the number of intact eggs per mass were counted. Empty, sucked, broken or collapsed eggs were not included and removed when possible.

Italy: Freshly laid egg masses were collected, glued, and exposed daily from Monday to Friday between April and October in 2019 and 2020, according to their availability (ESM1). The prepared egg mass tags were immediately hung on the host plants on which the bugs were, or could be, present at that time. Eggs of *E. maura* and *E. austriaca* were exposed on wheat. Eggs of *R. nebulosa* were exposed on *Populus alba* L. (April) and on *Prunus avium* (L.) (May and June), and eggs of *N. viridula* were exposed on *Morus alba* L. until the end of June and on *Sambucus nigra* L. from July onwards. Eggs of all other bug species were exposed on *P. avium* and *M. alba* from April until the end of June and on *Acer* spp. from July to October. Tags were attached with a plastic ring to branches of selected trees at ca 1.5–2 m height and on wheat to the stems under the ears. Exposed egg masses were no older than 72 hours and recollected after 2–4 days. In 2019, additional potted broad bean and fennel plants with egg masses laid directly on the underside of their leaves were exposed on a support placed under the trees on which egg tags were exposed. Overall, in NW Italy, 135 *H. halys* egg masses and 574 egg masses of non-target species (including 24 and 21 exposed directly on the potted bean and fennel plants, respectively) were exposed in 2019, whereas 37 *H. halys* and 726 egg masses of non-target species were exposed in 2020 (Table 1).

Switzerland: Newly laid egg masses were collected on a daily basis and refrigerated at 6 °C until exposure (no longer than 14 days), which means they were no longer viable for nymph development, but remained viable for parasitoid development (Wong et al. 2021). This was necessary, as field sites in Basel, Zurich and Ticino, where *T. japonicus* occurred naturally, were too far away for continuous egg exposure like in Grugliasco. In Basel and Zurich, tags with glued egg masses of bug species living on trees, i.e. *N. viridula, P. prasina*, *P. rufipes*, and *R. nebulosa*, were hung on cigar trees (*Catalpa bignonioides* Walter), Asian maple (*Acer palmatum* Thunberg), tulip trees (*Liriodendron tulipifera* L.), beech (*Fagus sylvatica* L.) and holly (*Ilex aquifolium* L.). Tags were attached to branches with a plastic ring at ca. 3m height. Egg masses of these four species were exposed in parallel on the same trees and if possible in the same numbers (no more than 5 egg masses per species/per tree). Egg masses of all other species, which feed primarily on herbs, were exposed in weedy patches or wild flower meadows within or in the vicinity of the parks. Plastic tags were attached to the weeds with paper clips. As the presence of *T. japonicus* was only discovered in Basel and Zurich by chance in the first year of the study, only egg masses of *H. halys* and *P. rufipes* were exposed in late August 2019. In the Ticino, egg masses of *H. halys* and non-target species were exposed in parallel on plastic tags and potted plants (*Vicia faba* L., *A. palmatum*, *Fragaria x ananassa* Duchesne, *Hedera helix* L.) in the first year of the study (2019). Potted plants were either placed underneath the trees or hung in the apple trees at 1.5 m height. Since egg parasitism on tags and potted plants did not differ but predation on eggs was much higher on potted plants, from 2020 onwards, eggs were only exposed on plastic tags. Eggs of bug species living on trees were either exposed on apple trees within the orchard or on trees (*Robinia pseudoacacia* L., *Acer pseudoplatanus* L. and *Corylus avellana* L.) in natural habitats nearby. Egg masses of all other species were exposed on weeds growing underneath the apple trees or on weeds in a wild flower meadow 150m southeast of the orchard, where non-target species naturally occurred. Towards the end of the study in 2021, *T. japonicus* had also dispersed to the city of Bern, and accordingly, eggs of *H. halys* and *P. rufipes* were exposed in Bern on maple trees in August/September of the same year. All sentinel egg masses were recollected after 4 days and brought back to the laboratory. The number of exposures at each site depended on the year, bug species and availability of egg masses (ESM1). Since all native species used in the study are univoltine and, with the exception of *P. rufipes*, overwinter as adults, egg exposures were primarily conducted in June during their peak oviposition periods. Only exposures of *P. rufipes* egg masses were conducted at the end of the summer due to its unusual phenology with oviposition starting in August. Over the period of three years (2019–2021) 498, 397 and 259 *H. halys* egg masses and 390, 762 and 468 non-target egg masses were exposed in Switzerland, respectively (Table 2).

Collection of wild stink bug egg masses

In Italy, throughout the exposure period of sentinel egg masses, weekly surveys were conducted at the same site to collect naturally laid egg masses of *H. halys* and non-target pentatomids and scutellerids. Egg masses were collected by visual inspection on the host plants present in the site (maple, hazelnut, cherry, elder, and ash) as described in Moraglio et al. (2021a).

In Switzerland, wild egg masses of *H. halys*, *N. viridula*, and *P. prasina* were collected primarily from catalpa trees in the cities of Basel and Zurich based on visual inspection of the foliage following the exposure of sentinel egg masses. In addition, *H. halys* egg masses were also collected from catalpa trees in the city of Bern (2020/21) and Biel (2021) after increasing infestations had been reported from both cities. Egg masses of *P. rufipes* were collected from maple trees in the urban areas of Bern in early autumn 2021 after the first *T. japonicus* had been detected in May of the same year.

All field-collected egg masses were transferred to the laboratory and reared for parasitoid adults and bug nymphs. In Italy, egg masses from which parasitoids had already

Table 1 Parasitism and predation of sentinel pentatomid and scutellerid egg masses exposed at Grugliasco, Italy, between 2019 and 2020 (T = on plastic tags; P = on potted plants)

		2019						2020					
Stink bug species	Exposure method	n (egg masses)	Total no. of eggs	% parasitism *Trissolcus japonicus*	% parasitism *Trissolcus mitsukurii*	% parasitism other parasitoids	% predation	Total no. of eggs	n (egg masses/eggs)	% parasitism *Trissolcus japonicus*	% parasitism *Trissolcus mitsukurii*	% parasitism other parasitoids	% predation
Pentatomidae													
Acrosternum heegeri	T	19	305	2.62	0	6.23	54.10	–	–	–	–	–	–
	P	3	59	0	0	0	76.27						
Arma custos	T		–	–	–	–	–	21	333	30.03	0	32.43	4.20
Carpocoris mediterraneus	T	7	86	23.26	32.56	2.33	13.95	30	427	28.10	0	11.94	36.30
Carpocoris purpureipennis	T	32	416	7.69	0	3.61	24.04	12	178	1.69	0	29.21	32.02
	P	4	58	0	0	0	25.86						
Dolycoris baccarum	T	44	967	0	0	4.34	25.75	59	1363	13.94	0	15.11	46.37
	P	1	38	0	0	0	55.26						
Eurydema ornata	T	–	–	–	–	–	–	9	188	0	0	0	0
Eurydema ventralis	T	–	–	–	–	–	–	49	1126	0	0	0.18	1.15
Eysarcoris venustissimus	T	–	–	–	–	–	–	12	189	1.06	0	0	95.77
Graphosoma lineatum	T	15	205	0	0	6.83	48.29	164	2145	12.82	0	18.14	28.07
	P	1	13	0	0	0	0.00						
Halyomorpha halys	T	111	2761	10.11	0	9.92	18.98	37	831	45.73	0	2.17	9.63
	P	24	613	0	0	1.96	27.41						
Nezara viridula	T	63	3742	0	0	2.97	14.54	151	11165	0.05	0	2.36	19.38
	P	4	238	0	0	0	6.30						
Palomena prasina	T	84	1781	13.87	0	24.26	8.48	45	770	14.68	0	27.40	13.64
	P	7	130	0	0	13.85	14.62						
Pentatoma rufipes	T	7	85	3.53	15.29	0	34.12	26	328	76.83	0	0.61	4.88
Peribalus strictus	T	8	101	0	0	6.93	19.80	40	495	15.76	0	30.30	13.74
	P	1	14	0	0	0.00	0.00						
Piezodorus lituratus	T	76	1471	4.55	0	7.48	7.75	82	1321	2.95	0	22.71	1.67

Table 1 (continued)

Stink bug species	Exposure method	2019 n (egg masses)	Total no. of eggs	% parasitism *Trissolcus japonicus*	% parasitism *Trissolcus mitsukurii*	% parasitism other parasitoids	% predation	2020 Total no. of eggs	n (egg masses/eggs)	% parasitism *Trissolcus japonicus*	% parasitism *Trissolcus mitsukurii*	% parasitism other parasitoids	% predation
Rhaphigaster nebulosa	T	7	94	0	0	5.32	1.06	8	92	10.87	0	0	17.39
Scutelleridae													
Eurygaster austriaca	T	20	240	0	0	1.67	2.92	–	–	–	–	–	–
Eurygaster maura	T	171	2256	0	0	3.24	16.45	18	230	1.74	0	40.00	28.70

partially emerged were included in the analysis, whereas in Switzerland, only complete egg masses (no emergence prior to collections) were included.

Laboratory rearing of sentinel and wild egg masses

Both sentinel and field-collected egg masses were placed individually in plastic Petri dishes (∅ 60 mm) and reared at 24±1 °C, 65±5% RH and 16L:8D until all bug nymphs or parasitoid adults had emerged. Egg masses were checked every 2 days, and emergence of nymphs and parasitoids was recorded. Parasitoids were sexed, sorted by family (Scelionidae, Eupelmidae, Encyrtidae and Pteromalidae) and stored in 99% ethanol. Parasitoids were identified to species level using the keys by Peng et al. (2020) for genus *Anastatus* Motschulsky, Samra et al. (2018) and Triapitsyn et al. (2020) for genus *Ooencyrtus* Ashmead, Johnson (1984) and Kozlov and Kononova (1983) for genus *Telenomus* Haliday, Talamas et al. (2017) and Tortorici et al. (2019) for genus *Trissolcus* Ashmead and Sabbatini Peverieri et al. (2019) for genus *Acroclisoides* Girault and Dodd (hyperparasitoids). All identified specimens were deposited in the collection of the Dipartimento di Scienze Agrarie, Forestali e Alimentari (DISAFA), University of Torino, Italy. At the end of each season, all egg masses were inspected under a stereo microscope at up to 40x magnification to assess their fate. Following Moraglio et al. (2021a), the following egg fate categories were assigned to individual eggs within each egg mass: (1) hatched—a bug nymph had emerged; (2) parasitized—a parasitoid had emerged; (3) preyed—the egg was empty or partly destroyed due to the attack of a sucking or chewing predator; and (4) unemerged—undamaged, but nothing had emerged (ESM 2, 3).

Non-reproductive mortality of N. viridula *induced by* T. japonicus

As laboratory tests have shown that *T. japonicus* frequently attacks and kills eggs of *N. viridula* but cannot complete development (Haye et al. 2020), unemerged eggs (undamaged, but no nymphs or parasitoids emerged) from *N. viridula* egg masses were stored in ethanol for molecular analysis. Molecular forensic analysis was applied to unemerged eggs from the following collections: (1) a subsample of 2019 *N. viridula* sentinel egg masses (n = 40 out of 67 exposed) from Italy; (2) all 2019 *N. viridula* wild egg masses collected in Italy (n = 12); and (3) all 2019 *N. viridula* wild egg masses collected in Switzerland (n = 4). Since all other non-target species tested in Haye et al. (2020) were suitable for parasitoid development, non-reproductive mortality was only analysed in *N. viridula*.

Genomic DNA was extracted and amplified from individual unemerged eggs using Scelionidae-specific primers,

Table 2 Parasitism and predation of sentinel stink bug egg masses exposed in Switzerland at Manno (MaO = apple orchard; MaN = natural site), Basel (Ba), Zurich (Zu) and Bern (Be) between 2019 and 2021 (T = on plastic tags; P = on potted plants)

Host species	Location	Exposure method	n (egg masses)	Total no. of eggs	2019						2020					2021	
					% parasitism *T. japonicus*	% parasitism other parasitoids	% predation	n (egg masses)	Total no. of eggs	% parasitism *T. japonicus*	% parasitism other parasitoids	% predation	n (egg masses)	Total no. of eggs	% parasitism *T. japonicus*	% parasitism other parasitoids	% predation
Pentatomidae																	
C. fuscispinus	MaO	T	20	267	0	7.49	25.47	16	328	0	6.71	11.28	7	88	0	0	7.25
	MaO	P	10	184	13.59	31.52	13.04	–	–	–	–	–	–	–	–	–	–
	MaN	T	–	–	–	–	–	16	204	0	27.45	14.71	11	168	0	0	17.26
	Ba	T	–	–	–	–	–	8	101	0	16.83	1.98	22	346	0	1.73	3.47
	Zu	T	–	–	–	–	–	9	143	0	15.38	32.87	17	275	0	0	12.73
D. baccarum	MaO	T	23	528	0	1.33	47.16	30	763	0	15.20	16.91	15	276	0	7.61	6.52
	MaO	P	49	1140	0	5.79	37.02	–	–	–	–	–	–	–	–	–	–
	MaN	T	–	–	–	–	–	30	630	0	27.30	35.71	10	234	0	21.37	15.81
	Ba	T	–	–	–	–	–	72	1676	0	4.95	29.89	60	1334	0	2.32	7.50
	Zu	T	–	–	–	–	–	15	425	0	5.41	57.41	28	651	0	0	52.53
G. lineatum	MaO	T	4	57	0	3.51	3.51	15	255	0	9.41	3.53	8	129	0	9.30	0
	MaN	T	–	–	–	–	–	14	223	0	29.15	26.01	8	8/128	0	39.84	2.34
	Ba	T	–	–	–	–	–	51	677	0	12.11	25.41	6	6/85	0	2.35	38.82
	Zu	T	–	–	–	–	–	13	210	0	8.10	4.29	–	–	–	–	–
H. halys	MaO	T	124	2789	2.94	1.47	15.56	39	929	0	1.61	19.48	15	376	0	3.99	12.77
	MaO	P	206	4557	1.05	1.87	20.96	–	–	–	–	–	–	–	–	–	–
	MaN	T	–	–	–	–	–	63	63/1453	2.48	2.34	15.35	21	512	6.05	0.39	12.70
	MaN	P	64	1417	0	0.56	24.28	–	–	–	–	–	–	–	–	–	–
	Ba	T	95	1921	5.78	3.80	18.84	138	3405	6.46	2.20	13.25	117	2876	0	1.74	2.71
	Zu	T	95	1748	10.98	0.80	48.23	69	1682	3.21	1.55	14.74	51	1152	3.99	0.26	10.68
	Be	T	–	–	–	–	–	88	2112	0	5.87	9.47	55	1416	0.78	3.74	6.00
N. viridula	MaO	T	4	271	0	0	0.37	–	–	–	–	–	–	–	–	–	–
	MaO	T	12	853	0	1.17	41.27	–	–	–	–	–	–	–	–	–	–
	MaN	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Ba	T	–	–	–	–	–	67	3974	0	0.81	11.52	–	–	–	–	–
	Zu	T	–	–	–	–	–	5	378	0	0	0.79	–	–	–	–	–
P. prasina	MaO	T	35	715	2.66	13.71	20.00	30	661	0	9.83	15.58	15	207	0	29.95	8.70
	MaO	T	116	2466	0.57	10.83	37.19	–	–	–	–	–	–	–	–	–	–
	MaN	T	–	–	–	–	–	20	362	1.66	25.14	32.60	15	292	0	5.48	55.14

Table 2 (continued)

Host species	Location	Exposure method	n (egg masses)	Total no. of eggs	2019						2020					2021	
					% parasitism *T. japonicus*	% parasitism other parasitoids	% predation	n (egg masses)	Total no. of eggs	% parasitism *T. japonicus*	% parasitism other parasitoids	% predation	n (egg masses)	Total no. of eggs	% parasitism *T. japonicus*	% parasitism other parasitoids	% predation
	MaN	P	37	786	0	11.32	49.75	–	–	–	–	–	–	–	–	–	–
	Ba	T	–	–	–	–	–	61	1694	4.49	19.83	5.90	72	1518	2.96	14.82	1.98
	Zu	T	–	–	–	–	–	10	153	10.46	17.65	22.22	29	643	0	19.75	5.75
P. rufipes	MaO	T	–	–	–	–	–	10	142	0	11.97	69.72	–	–	–	–	-
	MaN	T	–	–	–	–	–	18	258	16.67	5.43	37.60	25	396	2.02	18.18	10.86
	Ba	T	26	388	17.53	16.49	43.56	20	302	3.97	8.28	17.55	29	415	6.27	0	6.02
	Zu	T	19	262	21.76	7.63	34.35	25	353	3.68	0	30.31	29	430	3.26	0	6.51
	Be	T	–	–	–	–	–	–	–	–	–	–	29	418	0	0	6.46
P. strictus	MaO	T	12	169	0	5.92	29.59	30	439	0	7.06	17.31	7	104	0	0	0
	MaO	P	17	289	0	11.42	27.68	–	–	–	–	–	–	–	–	–	–
	MaN	T	–	–	–	–	–	30	411	0	11.44	36.74	–	–	–	–	–
	Ba	T	–	–	–	–	–	59	766	0	6.66	24.81	13	177	0	7.34	4.52
	Zu	T	–	–	–	–	–	12	146	0	0.00	62.33	3	42	0	0	30.95
P. lituratus	MaO	T	–	–	–	–	–	14	201	0	4.41	13.72	2	39	0	2.56	0
	MaN	T	–	–	–	–	–	13	226	0	8.85	23.01	–	–	–	–	–
	Ba	T	–	–	–	–	–	35	580	0	8.45	3.28	8	136	0	0	2.21
	Zu	T	–	–	–	–	–	12	218	0	2.29	30.73	–	–	–	–	–
R. nebulosa	MaO	T	1	14	0	0	0	–	–	–	–	–	–	–	–	–	–
	MaO	P	5	69	0	20.29	7.25	–	–	–	–	–	–	–	–	–	–
	Ba	T	–	–	–	–	–	2	26	0	15.38	3.83	–	–	–	–	–

following the protocols described by Gariepy et al. (2019). All conditions for amplification of scelionid DNA were the same, except a single round of PCR was performed using 5 µL of template DNA, instead of the original protocol described and implemented by Gariepy et al. (2014, 2019) in which two rounds of PCR (each using 1 µL of template DNA or PCR product, respectively) were performed. Additionally, as *Anastatus bifasciatus* (Geoffroy) (Hymenoptera: Eupelmidae) was reared from sentinel and field-collected *N. viridula* egg masses, a separate PCR was performed on these same samples using *Anastatus*-specific PCR primers, following the protocol described by Stahl et al. (2019a).

All PCR products were visualized with a QIAxcel Advanced automated capillary electrophoresis system (Qiagen, Hilden, Germany) using the QX DNA Fast Analysis Kit. QIAxcel screengel software (version 1.2.0) was used to score the results, and only samples with the expected fragment size (~562-bp for Scelionidae and ~270-bp for *A. bifasciatus*) and >0.1 relative fluorescent units signal strength were considered positive. All unemerged sentinel and field-collected eggs that were positive for Scelionidae DNA were purified with ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) following the manufacturer's protocol. PCR products were bidirectionally Sanger-sequenced using an ABI 3730 DNA analyser (Robarts Research Institute, London Regional Genomics Centre, Ontario, Canada) with the same primers used in the PCR reactions. Forward and reverse sequences were assembled and sequences were edited using CodonCode Aligner, version 9.0.1 (CodonCode Corporation, Centreville, MA, USA). The *Anastatus* primers were only used diagnostically to detect the presence of parasitoids within this genus. However, as a precaution, all field-collected specimens which yielded *Anastatus*-positive PCR fragments were purified and sequenced (as described above) to confirm their identity.

All specimen and sequence data were uploaded to the Barcode of Life Data System (BOLD) v4.0 (http://www.boldsystems.org/) under the public project "Egg Parasitoids Associated with *Nezara viridula* in Europe" (Project code: EPNEZ), and sequences were also deposited in GenBank (accession no. OQ320837-OQ321705). Barcode Index Numbers (BINs) (Ratnasingham and Hebert 2013) were assigned to the scelionid sequences, and the BIN registry was used for taxonomic identification. If species-level identification was not available in the BIN registry, the sequence was screened though the BOLD identification engine to obtain genus or family-level identification. As *Anastatus*-specific PCR products are only ~270-bp in length, the resulting DNA sequences are considered non-barcode compliant (<500-bp in length) and are not assigned a BIN in BOLD; identification of *Anastatus*-specific sequences was therefore accomplished using the BOLD Identification Engine.

For each collection [(1) 2019 sentinels collected in Italy; (2) 2019 wild egg masses collected in Italy; and (3) 2019 wild egg masses collected in Switzerland], the total and mean proportion of non-parasitized unemerged and parasitized unemerged eggs was calculated. Overall parasitoid species composition in unemerged eggs for each collection was calculated based on the number of individual eggs assigned to a given species or taxonomic unit (based on molecular analysis), divided by the total number of eggs that yielded positive PCR results for parasitoid DNA.

Statistics

Because the phenology and oviposition periods and thus also the exposure periods of univoltine non-target species only partially overlapped with the oviposition period of the bivoltine *H. halys* (May to September), the proportions of eggs parasitized by *T. japonicus* were only compared for those months in which the non-targets and targets were exposed simultaneously. For example, when sentinel eggs of *P. rufipes* were exposed in August and in September, these were compared to *H. halys* eggs exposed in the same two months instead of all *H. halys* eggs exposed (May to September). Proportions of sentinel *H. halys* and non-target eggs parasitized by *T. japonicus* were compared with a two-sample test for equality of proportions with continuity correction using the proportion test function of the stats package in R version 3.6.1 (R Core Team 2019) (ESM4). Proportions of sentinel eggs parasitized by *T. japonicus* when exposed on plants or tags in Manno in 2019 was compared with Chi-square tests in R.

Results

Parasitism of sentinel eggs by T. japonicus

In Tables 1 and 2, the parasitism data for each bug species have been pooled for each year and locality. Accordingly, parasitism data refer to the total number of eggs exposed at each site in each year (total parasitism). Figures 1, 2, 3, 4 show the comparison of proportions of sentinel *H. halys* and non-target eggs parasitized by *T. japonicus* when exposed simultaneously; in other words, only data from those months in which sentinel eggs of both target and non-target species were exposed were compared (ESM 4).

Italy: In total 963 and 4,289 parasitoids were reared from sentinel eggs of *H. halys* and non-target species, respectively (EMS 2). *Trissolcus japonicus* parasitized sentinel egg masses exposed on *P. avium*, *M. alba* and *Acer* spp. between the second week of May and the second week of September in both years. In 2019, in addition to *H. halys*, six out of 14 non-target species were parasitized by *T. japonicus* (Table 1; Figs 1, 2). Overall parasitism (all exposures throughout the season combined) of *H. halys* and *P. prasina* eggs by *T. japonicus* was 8.3% (*n* = 3,374 eggs) and 12.9% (*n* = 1,911), respectively. When both species were exposed simultaneously, parasitism of *P. prasina* (13.3%) was significantly higher (Fig. 1, EMS4). Parasitism of *P. rufipes* and *H. halys* was low (<10%), but not significantly different (Fig. 1, EMS4). The highest level of *T. japonicus* parasitism was found in *C. mediterraneus* (23.3%). Parasitism of other non-target species by *T. japonicus* was minimal, ranging from 0 to 7.7% (Fig. 2, EMS4).

In 2020, the number of non-target species parasitized by *T. japonicus* increased to 13 out of 15 (Table 1). Compared to 2019, parasitism of *H. halys* eggs by *T. japonicus* increased from 10.1% to 45.7%. At the same time, total parasitism of *P. prasina* (14.7%) and *C. mediterraneus* (28.1%) eggs remained stable or increased just slightly. Parasitism of *D. baccarum*, *G. lineatum* and *R. nebulosa* increased from 0% in 2019 to 13.9%, 12.8% and 10.9% in 2020, respectively (Table 1). When sentinel eggs of *A. custos, P. prasina*, *R. nebulosa*—three of the species that were attacked at the same level as *H. halys* in no-choice tests (Haye et al. 2020)—were exposed simultaneously with *H. halys*, parasitism by *T. japonicus* was significantly higher for *H. halys* eggs (Fig. 1). However, as in 2019, parasitism of *H. halys* and *P. rufipes* was not significantly different.

Switzerland: In total 1,449 and 3,324 parasitoids were reared from sentinel eggs of *H. halys* and non-target species,

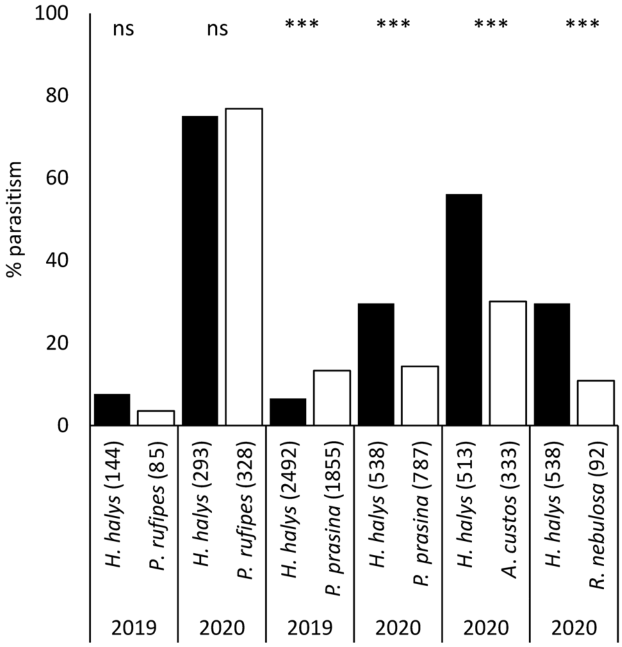


Fig. 1 Parasitism of sentinel eggs of *H. halys* and non-target species in Grugliasco (I) that were attacked at same level as *H. halys* in laboratory tests (Haye et al. 2020; Sabbatini Peverieri et al. 2021) (two-sample test for equality of proportions; ns = not significant, * = $P <$ 0.05; ** = $P <$0.001; *** = $P <$0.0001; for details see ESM4)

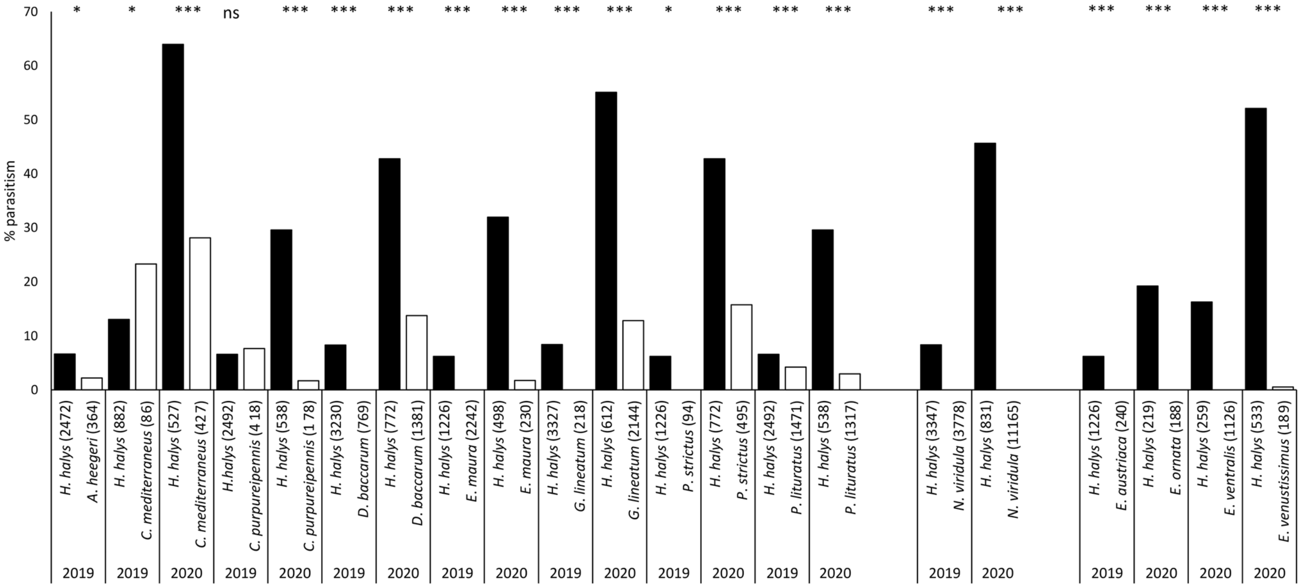
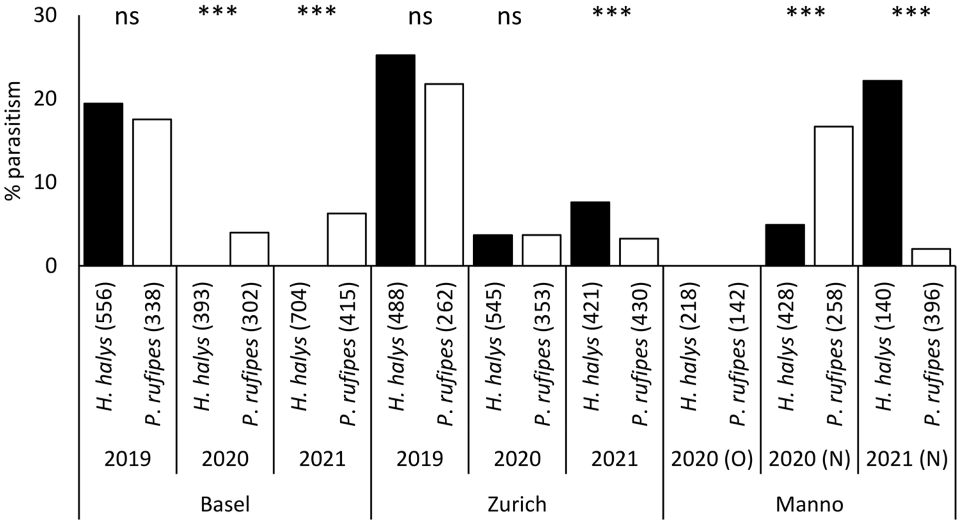


Fig. 2 Parasitism of sentinel eggs of *H. halys* and non-target species in Grugliasco (I) that were less attacked than *H. halys* in laboratory tests (grouped on the left), attacked but not suitable for development (*N. viridula*) (centre) and four additional species that were not part of previous laboratory testing (grouped on the right) (Haye et al. 2020; Sabbatini Peverieri et al. 2021); (two-sample test for equality of proportions; ns = not significant, * = $P <$ 0.05; ** = $P <$0.001; *** = P <0.0001; for details see ESM4)

Fig. 3 Parasitism of sentinel eggs of *H. halys* and *P. rufipes* by *T. japonicus* at three locations in Switzerland (O = apple orchard; N = natural site) (two-sample test for equality of proportions; ns = not significant, * = $P < 0.05$; ** = $P < 0.001$; *** = $P < 0.0001$; for details see ESM4)



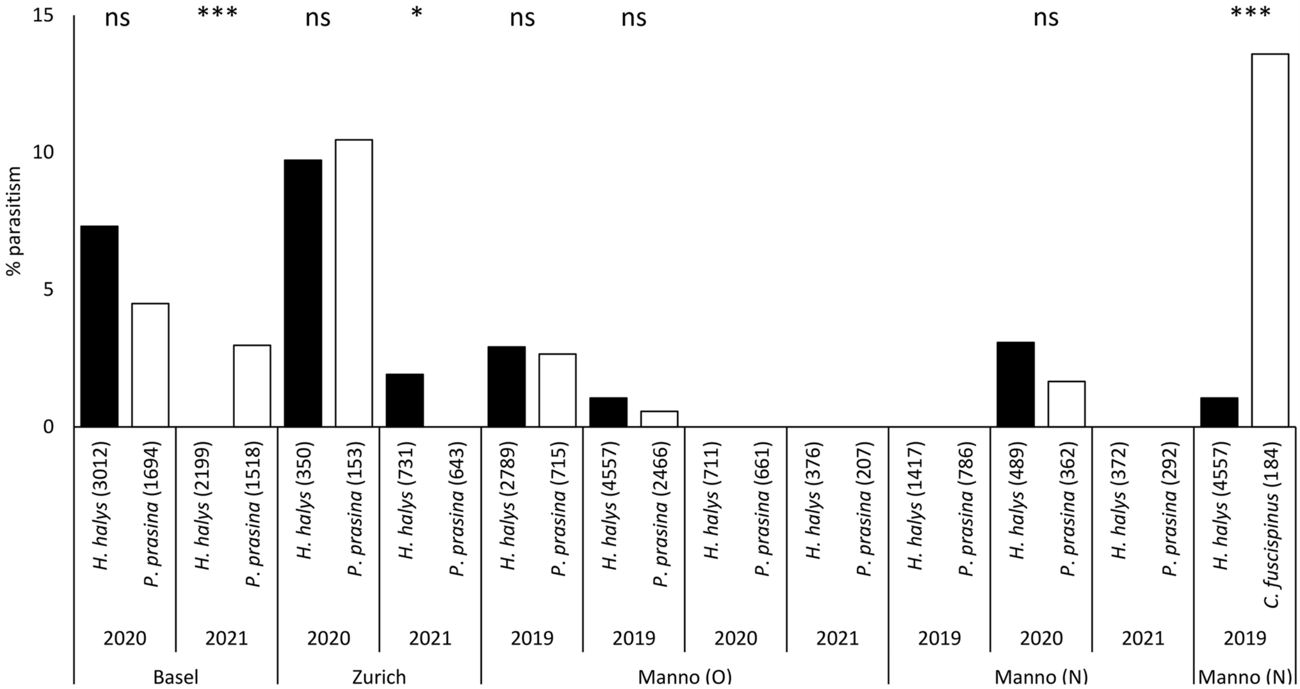


Fig. 4 Parasitism of sentinel eggs of *H. halys*, *P. prasina* and *C. fuscispinus* by *T. japonicus* at three locations in Switzerland (O = apple orchard; N = natural site) (two-sample test for equality of proportions; ns = not significant, * = $P < 0.05$; ** = $P < 0.001$; *** = $P < 0.0001$; for details see ESM4)

respectively. Over the course of three years, *T. japonicus* was recorded from *H. halys* and three non-target species, including *P. prasina*, *P. rufipes* and *C. fuscispinus* (Table 2; Figs. 3, 4). In Zurich, overall parasitism of sentinel *H. halys* eggs by *T. japonicus* was 11.0% in 2019, but decreased to 3.2% and 4.0% in the two following years (Table 2). Parasitism of *H. halys* in Basel was 5.8% and 6.5% in 2019 and 2020, respectively, but no *T. japonicus* were detected in 2021. Similarly, in the apple orchard in Manno, where *T. japonicus* was originally discovered, parasitism of sentinel eggs on tags was 2.9% in 2019, but no parasitism was detected in the two following years. However, in nearby natural habitats in Manno, *T. japonicus* was still present in 2020 and 2021, causing 2.5% and 6.1% parasitism, respectively. When eggs were exposed simultaneously on tags and plants in Manno (Table 2), significantly higher parasitism by *T. japonicus* on tags was observed for *H. halys* (χ^2 = 34.35, df=1, $P <$ 0.0001) and *P. prasina* (χ^2 = 21.58, df=1, $P < 0.0001$). A significantly higher level of parasitism by *T. japonicus* on plants was only observed for *C. fuscispinus* (χ^2 = 35.86, df=1, $P < 0.0001$). Eggs of *D. baccarum* were not parasitized by *T. japonicus*, independent of the exposure method.

Pentatoma rufipes was the non-target species most frequently parasitized by *T. japonicus*. Particularly, in 2019, overall parasitism by *T. japonicus* in Zurich and Basel was 17.5% and 21.8%, respectively (Table 2). When eggs of both species were exposed in parallel at both sites in August 2019, parasitism of *H. halys* and *P. rufipes* by *T. japonicus* was not significantly different (Fig. 3). In the two following years, parasitism by *T. japonicus* dropped significantly at both sites and only reached 3.3% and 6.3% in 2021 (Fig. 3). Remarkably, in Basel *T. japonicus* was only reared at low levels from *P. rufipes* sentinel eggs exposed in August 2020 and 2021, but not from *H. halys*. When exposed simultaneously in 2020 at Manno, parasitism of *P. rufipes* eggs by *T. japonicus* (16.7%) was significantly higher than parasitism of *H. halys* eggs (4.9%) (Fig. 3), whereas in 2021, it was nearly the opposite situation (*H. halys*: 22.1%, *P. rufipes*: 2.0%). Parasitism of *P. prasina* was generally low at all sites (<5%) (Table 2), and only in 2020, parasitism levels in Zurich reached more than 10%. In this year, *T. japonicus* parasitism of *H. halys* and *P. prasina* sentinel eggs exposed simultaneously in Zurich was not significantly different (Fig. 4). *Carpocoris fuscispinus* was only parasitized (13.6%) when exposed on plants at Manno in 2019 (Table 2, Fig. 4), whereas *H. halys* eggs exposed simultaneously were significantly less attacked (1.1%).

Parasitism of wild **H. halys** *and non-target egg masses by* **T. japonicus**

In total, 845 *H. halys* egg masses and 142 egg masses from eight non-target species were collected in Italy and Switzerland. *Trissolcus japonicus* was the most common parasitoid emerging from *H. halys* egg masses (65.1% to 100% of the emerged parasitoids) (Table 3). In Basel, parasitism in 2019 was 43.2% but declined to 22.3% and 13.3% in 2020 and 2021, respectively. In Zurich, a similar decline in parasitism was observed from 2020 (21.3%) to 2021 (13.9%). In Italy, parasitism of *H. halys* eggs in 2019 was 14.4% and increased to 17.3% in 2020. Since many parasitoids had already emerged in the field before the collections, the actual parasitism level is likely underestimated. *Trissolcus japonicus* was also reared from wild egg masses of *P. prasina* in Italy and Switzerland. In Italy, parasitism of *P. prasina* by *T. japonicus* was very low (<2%) and only three individuals emerged from a total of 13 egg masses in the laboratory. However, 85 parasitoids had already emerged in the field, and thus, parasitism was likely higher. In Switzerland, two out of 13 *P. prasina* egg masses were parasitized by *T. japonicus* (16.4% egg parasitism). In addition, *T. japonicus* was reared from two out of 12 wild egg masses of *P. rufipes* collected in Bern (Switzerland) in 2021 (16.9% egg parasitism), whereas six were attacked by native parasitoids (43.4%). Other non-target species living on weeds, e.g. *D. baccarum*, *Carpocoris* sp. or *P. lituratus,* were exclusively parasitized by native European egg parasitoids (Table 3).

Non-reproductive mortality of **N. viridula** *induced by* **T. japonicus**

The majority of the *N. viridula* sentinel eggs (*n* = 2,056) exposed in Italy in 2019 (69%) were unemerged (no nymph or parasitoid emerged), with a mean proportion of 72.3% (±5.2SE) unemerged eggs per egg mass. A total of 22 of the 40 egg masses (55%) contained at least one unemerged egg that was parasitized, with a mean proportion of 24.2% (±5.6SE) unemerged eggs containing parasitoid DNA (including Scelionidae and *Anastatus*). Parasitoid species composition in unemerged eggs is shown in Table 4. Of the scelionids, *T. japonicus* dominated the species composition, and accounted for 60.7% of parasitized, unemerged eggs (*n* = 364), followed by *Trissolcus cultratus* (Mayr) (16.8%), and *Trissolcus mitsukurii* (Ashmead) (11.5%), another exotic egg parasitoid of *H. halys* present in Italy (Zapponi et al. 2021). Approximately, 2.2% of parasitized eggs were specimens which were positive for scelionid DNA, but yielded low-quality DNA sequences which precluded their identification. The remaining 8.8% of parasitized eggs yielded PCR products consistent with the genus *Anastatus* and are presumably *A. bifasciatus* based on the fact that this was the only *Anastatus* species reared from *N. viridula* (Table 5).

Unemerged eggs from all naturally laid, field-collected egg masses in Italy and Switzerland in 2019 were processed and sequenced for scelionid and *Anastatus* DNA. From the Italian samples, only five of the 12 egg masses were parasitized. Approximately, 22% of the 1002 individual eggs were unemerged, with a mean proportion of 23.9 (±4.9SE) unemerged eggs per egg mass. The mean proportion of unemerged eggs that were positive for parasitoid DNA was 28.8% (±6.3SE). The dominant parasitoid species was *T. japonicus* (63.8%), followed by *Anastatus* (32.0%) (Table 4). All samples that were positive for *Anastatus* DNA were sequenced (*n* = 30), and 68% yielded high-quality sequences consistent with *A. bifasciatus*. The remaining 32% failed to sequence; however, based on the fact that other eggs within the same egg mass produced high-quality *A. bifasciatus* sequences, it is highly likely that all *Anastatus* detected here were indeed *A. bifasciatus*. *Trissolcus basalis* (Wollaston) occupied 2.1% of the parasitoid species composition, and another 2.1% of the samples positive for scelionid

Table 3 Parasitoid species composition of wild stink bug egg masses collected in Italy (Gr = Grugliasco) and Switzerland (Ba = Basel; Be = Bern; Bi = Biel; Zu = Zurich) between 2019 and 2021 at sites where adventive populations of *T. japonicus* are present; [1] in Switzerland egg masses which showed partial emergence of parasitoids in the field were not included; [2] *Tel. sp1* = *Telenomus* sp1

Bug species	Year	Country	Location	n. egg masses	n. eggs	Preyed	Unhatched eggs	nymphs	Empty parasitiz. eggs, emerged in the field[1]	Total parasitoids emerged in lab	% parasitism by *T. japonicus*	Parasitoid species composition (%)									
												Scelionidae							Eupelmidae	Encyrtidae	Pteromalidae
												Tr. japonicus	*Tr. mitsukurii*	*Tr. kozlovi*	*Tr. cultratus*	*Tr. basalis*	*Tel. turesis*	*Tel. sp.1*[2]	*An. bifasciatus*	*O. telenomicida*	*Acr.*sinicus
Pentatomidae																					
Carpocoris sp.	2019	I	Gr	2	28	0	6	13	0	9	0	0	0	0	0	0	100	0	0	0	0
	2020	I	Gr	2	28	16	5	0	0	7	0	0	0	0	0	0	100	0	0	0	0
D. baccarum	2019	I	Gr	1	28	28	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2020	I	Gr	3	84	3	1	56	0	24	0	0	0	0	0	0	100	0	0	0	0
H. halys	2019	I	Gr	253	6580	716	919	2956	532	1457	14.4	65.2	2.2	0	0.3	0	0	0.1	32.2	0	0.1
	2020	I	Gr	416	10677	1547	1500	3135	1662	2833	17.3	65.1	0	0	0	0	0	0	34.7	0.1	0.1
	2019	CH	Ba	26	625	23	120	164	-	297	43.2	90.9	0	0	0	0	0	0	8.1	0	1.0
	2020	CH	Ba	35	935	30	74	622	-	209	22.4	100	0	0	0	0	0	0	0	0	0
	2021	CH	Ba	8	203	3	51	122	-	27	13.3	100	0	0	0	0	0	0	0	0	0
	2019	CH	Zu	21	574	26	87	316	-	145	21.3	84.1	0	0	0	0	0	0	0	0	15.9
	2020	CH	Zu	44	1148	168	121	693	-	166	13.9	96.4	0	0	1.2	0	0	0	0	2.4	0
	2020	CH	Be	32	823	29	105	690	-	0	0	0	0	0	0	0	0	0	0	0	0
	2021	CH	Be	7	183	24	24	133	-	2	0	0	0	0	100	0	0	0	0	0	0
	2021	CH	Bi	3	76	20	2	48	-	6	7.9	100	0	0	0	0	0	0	0	0	0
N. viridula	2019	I	Gr	12	1158	177	195	689	15	82	0	0	0	0	0	73.2	0	0	26.8	0	0
	2020	I	Gr	17	1779	205	698	737	3	136	0	0	0	0	0	6.6	0	0	92.6	0	0.7
	2019	CH	Ba	14	984	0	508	162	-	314	0	0	0	0	0	99.7	0	0	0.3	0	0
	2020	CH	Ba	15	1192	110	501	144	-	437	0	0	0	0	0	100	0	0	0	0	0
	2021	CH	Ba	4	353	11	5	237	-	0	0	0	0	0	0	0	0	0	0	0	0
P. prasina	2019	I	Gr	11	298	70	28	126	59	15	0.7	13.3	0	0	0	0	0	0	86.7	0	0
	2020	I	Gr	2	57	0	20	10	26	1	1.8	100	0	0	0	0	0	0	0	0	0
	2021	CH	Ba	2	56	0	0	28	-	28	50.0	100	0	0	0	0	0	0	0	0	0
	2019	CH	Zu	8	197	2	72	69	-	56	13.7	48.2	0	0	0	0	1.8	14.3	0	0	35.7
	2020	CH	Zu	3	82	48	0	2	-	31	0	0	0	0	100	0	0	0	0	0	0
P. rufipes	2021	CH	Be	12	166	1	14	52	-	100	16.9	28.0	0	13.0	59.0	0	0	0	0	0	0
P. lituratus	2020	I	Gr	14	176	0	56	21	38	61	0	0	0	0	0	0	0	77.0	23.0	0	0
R. nebulosa	2019	I	Gr	5	70	7	14	41	3	5	0	0	0	0	0	0	0	0	100	0	0

Table 3 (continued)

Bug species	Year	Country	Location	n. egg masses	n. eggs	Preyed	Unhatched eggs	nymphs	Empty parasitiz. eggs, emerged in the field[1]	Total parasitoids emerged in lab	% parasitism by *T. japonicus*	Parasitoid species composition (%)									
												Scelionidae							Eupelmidae	Encyrtidae	Pteromalidae
												Tr. japonicus	*Tr. mitsukurii*	*Tr. kozlovi*	*Tr. cultratus*	*Tr. basalis*	*Tel. turesis*	*Tel. sp.1*[2]	*An. bifasciatus*	*O. telenomicida*	*Acr.*sinicus
	2020	I	Gr	11	146	35	35	52	12	12	0	0	0	0	0	0	0	0	100	0	0
Scutelleridae																					
E. maura	2019	I	Gr	4	56	0	1	55	-	0	0	0	0	0	0	0	0	0	0	0	0

Table 4 Parasitism and parasitoid species composition in unemerged (no parasitoid or nymph hatched) sentinel (S) and field-collected (F) *N. viridula* eggs based on molecular forensic analysis

	Parasitism of unemerged eggs						Parasitoid species composition (%) in unemerged eggs							
	Year	n (egg masses/eggs)	# masses parasitized (%)	# eggs unemerged (%)	total # parasitized (%)	Mean proportion parasitized (%±SE)	*Tr. japonicus*	*Tr. mitsukurii*	*Tr. cultratus*	*Tr. basalis*	*Te.turesis*	*Telenomus sp.*	*A. bifasciatus*	Scelionidae DNA, no sequence
Italy (S)	2019	40/2056	22 (55)	1414 (69)	364 (25.7)	24.2 ± 5.6	60.7	11.5	16.8	0	0	0	8.8	2.2
Italy (F)	2019	12/1002	5(42)	221 (22)	94 (42.5)	28.8 ± 6.3	63.8	0	0	2.1	0	0	32.0	2.1
Switzerland (F)	2019	14/984	13 (93)	508 (52)	376 (74.0)	68.0 ± 4.8	75.3	0	0.5	22.1	0.3	0.3	0	1.5

Table 5 Species composition (%) of egg parasitoids other than *T. japonicus* (see Tables 1 and 2) emerging from sentinel eggs exposed in Italy and Switzerland. (N = no. of parasitoids reared; [1]non-native species; [2]potentially non-native) (all years combined)

Hosts	Country	N	Parasitoid species composition (%)												
			Scelionidae									Eupelmidae			Pteromalidae
			Tr. mitsukurii[1]	*Tr. cultratus*	*Tr. basalis*	*Tr. semistriatus*	*Tr. scutellaris*	*Tr. belenus*	*Tr. kozlovi*	*Tel. turesis*	*Tel.* sp.1	*Anast. gansuensis*[1]	*Anast. bifasciatus*	*Anast. japonicus*[2]	*Acr. sinicus*[2]
Pentatomidae															
Acrosternum heegeri	I	19	0	0	0	0	0	0	0	0	78.9	0	21.1	0	0
Arma custos	I	108	0	0	0	0	0	0	0	0	31.5	0	68.5	0	0
Carpocoris fuscispinus	CH	159	0	0	0	49.1	0	19.5	0	17.6	0	4.4	9.4	0	0
Carpocoris mediterraneus	I	81	34.6	0	0	0	0	0	0	0	8.6	0	56.8	0	0
Carpocoris purpureipennis	I	67	0	19.4	0	0	0	0	0	0	62.7	0	17.9	0	0
Dolycoris baccarum	I	248	0	14.1	0	0	0	0	0	0	57.3	0	28.6	0	0
	CH	569	0	0	4.0	79.3	0	0	0	16.5	0	0	0.2	0	0
Eurydema ventralis	I	2	0	0	0	0	0	0	0	0	100	0	0	0	0
Graphosoma lineatum	I	403	0	0	0	0	0	0	0	0	63.3	0	36.5	0	0.2
	CH	255	0	0	11.0	76.1	0	4.3	0	7.8	0	0.8	0	0	0
Halyomorpha halys	I	304	0	0	0	0	0	0	0	0	0	0.7	99.3	0	0
	CH	441	0	33.8	9.5	0	0	0	0	0	0.7	0.2	54.2	1.6	0
Nezara viridula	I	375	0	0	0	0	0	0	0	0	1.9	0	98.1	0	0
	CH	42	0	0	31.0	0	0	0	0	0	0	0	69.0	0	0
Palomena prasina	I	661	0	53.1	0	0	0	0	0	1.5	25.4	1.7	18.3	0	0
	CH	1358	0	62.2	4.4	3.2	0	0	0	11.5	10.3	0	6.8	1.6	0

Table 5 (continued)

Hosts	Country	N	Parasitoid species composition (%)												
			Scelionidae									Eupelmidae			Pteromalidae
			Tr. mitsukurii[1]	*Tr. cultratus*	*Tr. basalis*	*Tr. semistriatus*	*Tr. scutellaris*	*Tr. belenus*	*Tr. kozlovi*	*Tel. turesis*	*Tel.* sp.1	*Anast. gansuensis*[1]	*Anast. bifasciatus*	*Anast. japonicus*[2]	*Acr. sinicus*[2]
Pentatoma rufipes	I	15	86.7	0	0	0	0	0	0	0	0	0	13.3	0	0
	CH	212	4.2	50.5	16.0	0	0	5.7	6.6	0	0	0	17.0	0	0
Peribalus strictus	I	157	0	4.5	0	0	0	0	0	0	68.2	0	27.4	0	0
	CH	185	0	0	24.9	37.8	0	14.1	0	17.8	3.8	0	0	1.6	0
Piezodorus lituratus	I	410	0	11.5	0	0	0	0	0	0.5	77.6	0	10.5	0	0
	CH	84	0	0	0	75.0	0	23.8	0	1.2	0	0	0	0	0
Rhaphigaster nebulosa	I	5	0	0	0	0	0	0	0	0	100	0	0	0	0
	CH	18	0	100	0	0	0	0	0	0	0	0	0	0	0
Scutelleridae															
Eurygaster austriaca	I	4	0	0	0	100	0	0	0	0	0	0	0	0	0
Eurygaster maura	I	165	0	0	0	59.4	1.2	10.3	0	26.7	0	0	2.4	0	0
No of hosts per parasitoid species			3	9	7	8	1	6	1	9	15	4	20	3	1

DNA yielded low-quality sequence data, precluding their identification.

Of the 14 *N. viridula* egg masses collected in 2019 in Switzerland, 13 masses were parasitized. A total of 984 individual eggs were obtained, 508 were unemerged, with a mean proportion of 53.6 (±5.2SE) unemerged eggs per egg mass. Approximately, 74% of unemerged eggs were positive for parasitoid DNA (mean 68.0%±4.8SE), and the species composition is shown in Table 4. The dominant parasitoid species detected was *T. japonicus* (75.3%). *Trissolcus basalis*, *T. cultratus*, and *T. turesis* occupied 22.1%, 0.5%, and 0.3% of the parasitoid species composition (respectively), and an unidentified species of *Telenomus* (0.3%) was sporadically detected in the species composition. The remaining 1.5% of the species composition consisted of specimens which were positive for scelionid DNA but failed to produce identifiable DNA sequences. The unidentified *Telenomus* species (BIN ADW5015) produced a high-quality, barcode compliant DNA sequence, but no species-level match was available in BOLD and GenBank. This is likely because voucher specimens of this species have not yet been sequenced, and as such, no barcode matches are available in public DNA databases.

Parasitism by other parasitoids

In Italy, overall parasitism of sentinel eggs by native egg parasitoids in 2019 was below 10% in most species, and only in *P. prasina,* it reached 24.3% (Table 1). In parallel to the increase in parasitism of *H. halys* by *T. japonicus* in 2020, however, parasitism by native species also increased for nine non-target species. Remarkably, parasitism of *H. halys* did not follow this trend and actually decreased, whereas parasitism of *N. viridula* remained stable at low level.

In Switzerland, parasitism of *H. halys* by native parasitoids (mainly *A. bifasciatus*) did not exceed 4.0% in all years (Table 2). In contrast, parasitism of native stink bugs such as *P. prasina* and *P. rufipes* was much higher, reaching maximum 30.0% and 18.2%, respectively. Parasitism of *C. fuscispinus* (one of the few other native stink bugs that was attacked by *T. japonicus* in Switzerland) by native parasitoids, reached up to 31.5% (when exposed on plants).

Associations of parasitoids other than *T. japonicus* and their host obtained by sentinel egg exposure is shown in Table 5. Apart from *T. japonicus*, eight native Scelionidae, three Eupelmidae and one Pteromalidae (hyperparasitoid) were reared from sentinel egg masses. *Trissolcus mitsukurii* was reared from the two native hosts *P. rufipes* (15.3%) and *C. mediterraneus* (32.6%) in 2019, whereas in 2020, it was not detected (Tables 1, 3). In addition, *T. mitsukurii* was also reared from a single egg mass of *P. rufipes* in the Canton Ticino (Table 5), representing the first record of this species in Switzerland.

In general, each *Trissolcus* species was associated with 1 to 9 host species, whereas the two *Telenomus* species were reared from 9 to15 hosts, respectively. The highest number of hosts for a given parasitoid species (20) was recorded for *A. bifasciatus*. Two additional *Anastatus* species that have not previously been reported in Europe were sporadically reared from sentinel non-targets; however, further collections would be necessary to clarify trends in their host ranges. *Anastatus gansuensis* Chen & Zang was reared in low numbers from *H. halys* in Italy and Switzerland, and also *P. prasina* in Italy, and *C. fuscispinus* and *G. lineatum* in Switzerland, whereas *Anastatus japonicus* Ashmead emerged from *H. halys*, *P. prasina* and *P. strictus* in Switzerland.

Predation of sentinel eggs

In both Italy and Switzerland, predation of sentinel eggs exposed on tags was highly variable among species, sites and years (Tables 1, 2). In 2019, in Italy (all species combined), predation of eggs exposed on tags was ranging from 1.1% to 54.1% (mean 20.5±4.2% SE) (Table 1), whereas on plants, it was 0% to 76.3% (mean 25.7±9.7% SE). For nearly all species, overall predation was higher than overall parasitism. In five out of eight stink bug species that were exposed on tags and plants, predation was higher on plants. In 2020, predation levels ranged from 0% to 95.8% (mean 22.1±6.0% SE). In 2019, in nine out of 14 species, predation levels were higher than overall parasitism, whereas in 2020, it was only six out of 16 (Table 1).

In Switzerland, predation levels of eggs exposed on plant tags were similar to those observed in Italy (Table 2), averaging 23.9±5.0% (SE) and 22.0±2.8% (SE) in 2019 and 2020, respectively. In 2021, however, predation was much lower than in previous years, averaging 12.0±2.7%. Predation was the highest (28.8±4.1% SE), when eggs were exposed on plants in 2019.

Discussion

Unintentional introduction of natural enemies has increased in recent years due to massive increase in global international trade and tourism (Gippet et al. 2019). The recent establishment of such natural enemies provides a unique opportunity to validate estimates of fundamental (laboratory) host range with realized (ecological) post-arrival host range as it manifests over time, particularly for those species that, based on laboratory experiments, would have been considered non-specific and not approved for release [e.g. *Trichomalus perfectus* (Walker), *Leptopilina japonica* Novković & Kimura)] (Haye et al. 2015; Beers et al. 2022; Puppato et al. 2022; Weber et al. 2021). Among these species is also the samurai wasp, *T. japonicus*, which was first recorded in Europe in 2017 (Stahl et al. 2019b).

Studies of the fundamental host range of *T. japonicus* in Europe had shown that 13 out of 21 non-target species tested were suitable for successful development of *T. japonicus* offspring in no-choice tests (Haye et al. 2020; Sabbatini Peverieri et al. 2021). In the present study, 15 out of 18 non-target species exposed in Italy and Switzerland were successfully parasitized by *T. japonicus* in the field, confirming its high potential to develop on a wide variety of hosts as predicted by its fundamental host range. Among the 15 suitable hosts was also an additional species, *E. venustissimus*, that had not been part of previous host range studies. In addition, *T. japonicus* was reared from a single egg mass of *N. viridula*, although this host was not suitable for development in previous laboratory no-choice tests.

Remarkably, in Grugliasco, a much larger number of non-target hosts was parasitized, including many species that were not attacked in Switzerland, which likely resulted from different methodologies, climate conditions or *H. halys* densities. Whereas in Grugliasco, all egg masses, except those of *E. maura* and *E. austriaca*, were exposed on trees, where most likely natural *H. halys* populations were present, in Switzerland, egg masses of stink bugs usually feeding on weeds or bushes (e.g. *D. baccarum*, *P. lituratus*, *P. strictus*) were not exposed on trees but in their natural habitats, weedy patches or meadows, where *H. halys* usually does not occur. The absence of *T. japonicus* in the weedy habitats in Switzerland agrees with earlier studies that *T. japonicus* was more frequently found in woodland or lightly wooded urban habitats than for example ground crops like soybean (Herlihy et al. 2016; Quinn et al. 2019a, b, 2021). This arboreal habitat preference may explain the differences in the number of non-targets attacked in the two regions, and consequently, some non-target species parasitized in Grugliasco could be 'false positives' based solely on exposure locations that are not realistic in nature. Similarly, it has been suggested that ecological refuges from parasitism (e.g. habitats at higher altitude) have mitigated the population-level impacts of a closely related egg parasitoid, *T. basalis* on native Hawaiian stink bug species (Johnson et al. 2005). In addition, at Grugliasco, a remarkable increase in *H. halys* parasitism by *T. japonicus* from 10% in 2019 to 45% in 2020 was observed, and at the same time, non-target parasitism by *T. japonicus* increased significantly. The reason for this drastic increase remains unknown, but the number of wild *H. halys* eggs collected at Grugliasco in 2019 and 2020 was 6,580 and 10,677, respectively, indicating that more host eggs were available in 2020, which may have supported an increase in *T. japonicus* populations and subsequently parasitism of *H. halys* and non-target species.

Although the realized host range comprised 15 non-target species, the number of hosts attacked alone does not necessarily suggest an increased risk of ecological damage. As suggested by van Driesche and Hoddle (2017), parasitism of native species at levels that do not significantly lower the long-term density of a non-target species (<10%) should be considered as 'host use', while rates of more than 50%, if widespread and sustained over time, may have negative population-level impacts for non-target species. Milnes and Beers (2019) showed that in a field paired-host assay in North America, the rate of parasitism on three native species was significantly less than that on *H. halys* eggs and concluded that the potential impact of *T. japonicus* on these pentatomids is probably minimal. Similarly, the large majority of non-target species exposed in the present study was clearly less parasitized by *T. japonicus* than *H. halys*. In both countries, the forest bug, *P. rufipes*, was the most parasitized non-target species with higher levels of parasitism particularly observed in Basel (18%) and Zurich (22%) in 2019, and in Manno (17%) and Grugliasco (77%) in 2020. The reasons for this are likely based on its phenology and the overlap of its feeding niche (trees) with *H. halys*. Whereas most European pentatomids overwinter as adults and lay their eggs in May/June, *P. rufipes* overwinters as second instar nymph and lays its eggs in August/September (Powell 2020). Field studies in China (Zhang et al. 2017) and the USA (Quinn et al. 2021) have shown that parasitism of sentinel *H. halys* eggs by *T. japonicus* is continuously increasing from May to August due to its rapid development and its ability to produce several generations per year. The same seasonal increase of parasitism of *H. halys* from 19% in May to 83% in September 2020 (ESM 5) was observed at Grugliasco in 2020, and accordingly, by the time *P. rufipes* started laying eggs (August), *T. japonicus* populations had likely built up and likely reached their maximum. Consequently, sentinel *P. rufipes* eggs were likely more frequently encountered by *T. japonicus* than any of the univoltine non-target species that lay their eggs in spring when *T. japonicus* populations are still fairly low. It should also be considered that the Grugliasco site did not represent the natural habitat for *P. rufipes*; in fact, no wild egg masses were ever found in the two years. In Bern, where *T. japonicus* was first detected in spring 2021, parasitism by this exotic parasitoid of wild *P. rufipes* egg masses collected in autumn at the same site was 16.9%, whereas parasitism by the native parasitoid *T. cultratus*, which can act as hyperparasitoid of *T. japonicus* (Konopka et al. 2017), was 35.5%. To what extent the native *T. cultratus*, which is the dominant parasitoid of *P. rufipes*, may prevent intense *T. japonicus* parasitism remains unknown, but ongoing life table studies in Europe may help to understand if the arrival of *T. japonicus* could have potential negative impacts at population level.

Compared to *P. rufipes*, the green stink bug *P. prasina*, which was among the four species that were parasitized at the same level as *H. halys* in laboratory tests (Haye et al.

2020; Sabbatini Peverieri et al. 2021), was attacked at relatively low levels (sentinel eggs: <11% in Switzerland and <15% in Italy). Parasitism of the few wild *P. prasina* egg masses found at Basel and Zurich combined was higher though (3 out of 10), but the sample size was very low. In contrast to *P. rufipes*, the oviposition period of *P. prasina* started in late April, when *T. japonicus* populations had not built up yet. Accordingly, a negative impact of *T. japonicus* at population level is not expected. Maximum parasitism of *R. nebulosa,* which starts laying eggs even before *P. prasina* in early April, was 11% in Grugliasco in 2020, but it should be noted that the amount of sentinel *R. nebulosa* egg masses available for this study was very limited. The predatory species *A. custos* was only exposed in July and August 2020 at Grugliasco and the higher level of parasitism by *T. japonicus* (30%) was likely due to the late exposure, given the timing with higher populations of this parasitoid in the field.

Previous laboratory host range tests had indicated that the exotic common green stink bug, *N. viridula*, is not suitable for parasitoid development, although regularly attacked by *T. japonicus* (Haye et al. 2020; Sabbatini Peverieri et al. 2021). Instead, oviposition in the unsuitable host induced a significant level of non-reproductive mortality. In the present study, the analysis of unemerged eggs from field-collected *N. viridula* egg masses with molecular tools showed that a high proportion of eggs contained *T. japonicus* DNA, confirming that non-reproductive mortality induced by egg parasitoids can indeed be an important mortality factor of invasive stink bugs (Abram et al. 2019; Kaser et al. 2018). As *N. viridula* is an invasive pest in Europe, any impact resulting in mortality of this pest would be considered beneficial.

Botch and Delfosse (2018) demonstrated that *T. japonicus* females reared on non-target hosts showed reduced host specificity and suggested that future efforts should be made to investigate the ability of *T. japonicus* to reproduce long term on non-target species when *H. halys* eggs are limited. Such a situation is currently observed in northern Switzerland, where *H. halys* populations have consistently declined since 2019 (Ackermann et al. 2022). However, concerns that such a decrease in *H. halys* could result in a host shift and substantial parasitism of alternative non-target hosts such as *P. rufipes* have not been validated during the course of the present study. In fact, in Switzerland the opposite trend was observed and with declining *H. halys* populations in 2020 and 2021, *T. japonicus* parasitism of sentinel *P. rufipes* eggs also declined.

Since it is impossible to collect large (meaningful) numbers of wild (field-laid) egg masses of native European non-target species, sentinel egg masses were used in the present study instead (Milnes et al. 2019; Quinn et al. 2019b). This can underestimate parasitism, parasitoid abundance and species richness as shown for *H. halys* by Jones et al. (2014). For example, in 2021 in Basel, *T. japonicus* was only reared in very low numbers from sentinel *P. rufipes* and *P. prasina* eggs but not from *H. halys*. However, parasitism of wild *H. halys* egg masses collected in the same area and year was 13%. One reason for the observed difference could be that 'oviposition sites' chosen for the exposure of sentinel eggs may not have matched with those that would have been chosen naturally by the stink bugs. Since sentinel egg masses were exposed on plastic tags, lower parasitism may have resulted from a lack of important chemical cues used for host location by *T. japonicus* (Arif et al. 2021; Malek et al. 2021). On the contrary, if these cues were absolutely essential, one would not have expected high levels of parasitism of sentinel eggs as observed in Grugliasco in 2020 or China (Zhang et al. 2017). However, at some sites (Grugliasco, Basel, Zurich), wild *H. halys* populations were already present on the trees selected for the exposure of sentinel eggs, likely compensating for the lack of chemical cues.

Peterson et al. (2022) demonstrated that feeding and oviposition by *H. halys* induced direct and systemic changes in volatile compound emissions from peach trees and tree of heaven and speculated that such changes in plant volatile compounds could be used by natural enemies such as *T. japonicus*. In the first year of the study (2019), we had exposed egg masses laid on host plants in the laboratory, but we did not find increased parasitism on plants compared to sentinel eggs exposed on plastic tags. However, egg predation on potted plants was extremely high, and we cannot exclude that at least some parasitized egg masses had been eaten by predators, thereby masking the potential effect of plant volatiles on parasitism.

The potential impact of *T. japonicus* on *H. halys* or specific non-target species cannot be summarized by interactions between species pairs because these species are entangled in diverse communities and are exposed to a multitude of ecological factors (Sentis et al. 2022). The large majority of European stink bug species lay their eggs in spring when *T. japonicus* populations are low, and can therefore gain partial temporal refuge from parasitism. In addition, species living on herbaceous hosts in the ground cover can escape intensive parasitism due to the preference of *T. japonicus* for woody habitats, thereby providing a spatial refuge for those species occupying such habitats. On the other hand, species sharing the same ecological niche as *H. halys* (trees) are more likely to be attacked by *T. japonicus*, but a potential increased risk should only be considered for those species with an unusual life cycle, such as *P. rufipes* or *Picromerus bidens* (L.) (Hemiptera: Pentatomidae), that lay their eggs in late summer when the majority of parasitism by *T. japonicus* takes place. Incorporating ecological factors such as habitat specificity, host and parasitoid phenology, host density and competition with native parasitoids or predators, can help to yield a more realistic scenario of potential risks for non-target species, but such field studies will only be possible where the biological control agents have become established—either through intentional releases or accidental establishments.

Author contribution

TH and LT conceived and designed research. LT, TH, CM, FT and STM conducted field experiments and analysed data. LT, STM and TH collected and reared non-target species for establishing laboratory colonies. TG did the molecular analysis of stink bug eggs. FT identified all egg parasitoids. TH, STM, TG and LT wrote the manuscript. All authors read and approved the manuscript

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Data availability All data generated or analysed during this study are included in this published article and its supplementary information files.

Declarations

Conflict of interest All authors declare that there is no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals (vertebrates) performed by any of the authors.

Consent to participate Informed consent was obtained from all individual participants included in the study.

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