




Seasonal parasitism of native egg parasitoids of brown marmorated stink bug (*Halyomorpha halys*) in Japan

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Abstract

The invasive, *Halyomorpha halys* (Hemiptera: Pentatomidae), is a severe economic insect pest native to East Asia. A strong effort has been made to identify natural egg parasitoids of *H. halys* in invaded regions, but parasitism rates reported from these studies have been inconsequentially low. To determine the species composition, phenology, and efficiency of egg parasitoids in the native region of *H. halys*, we deployed fresh and frozen sentinel *H. halys* egg masses from March through December in Kyoto, Japan. Our findings provide valuable insights on the abundance and parasitism rates of native *H. halys* parasitoids in Japan. A total of seven parasitoid species emerged from the sentinel egg masses, but *Trissolcus japonicus* had the highest parasitism rate of all parasitoids recovered (84% on fresh egg masses) and maintained the largest portion of the total parasitoid species composition (60% on fresh egg masses). The early season parasitoid community in Kyoto, Japan, is dominated by *T. japonicus*, with the first parasitism activity occurring in March. Throughout the course of the field study, *T. japonicus* also sustained a significantly higher parasitism rate on fresh *H. halys* eggs than frozen. The results from this research help expand the understanding of parasitoids in the native region of *H. halys* and hold importance for the future development of biological control programs against this invasive pest.

Keywords Biological control · *Trissolcus japonicus* · Invasive pest · Sentinel egg mass · Pseudoparasitism

Key message

- Information regarding the species richness and phenology of *Halyomorpha halys* parasitoids in the invasive pest's native range is lacking
- We conducted an intensive survey of egg parasitoids in Kyoto, Japan using sentinel *H. halys* egg masses
- Seven egg parasitoid species belonging to three genera were recovered

- Overall, *Trissolcus japonicus* was the most abundant parasitoid species, and maintained the highest parasitism rates
- These results support the implementation of *T. japonicus* in potential biological control programs against *H. halys*.

Introduction

The enemy release hypothesis states that the rapid increase in abundance and distribution that invasive species may experience upon arrival to a new region is associated with their escape from natural controlling agents in their native range (Keane and Crawley 2002). In most cases, few existing natural enemies will attack an adventive pest with much success, and the endemic species that do so are typically opportunistic generalists with a wide range of hosts (Hoddle 2004). Once established, invasive pests can cause major environmental damage and severe economic loss in agricultural industries (Pimentel et al. 2000; Messing and Wright 2006). An essential component of any successful management strategy against an invasive pest species is gaining a

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fundamental understanding of the pest's biology, behavior, and natural control in its native range (Evans and Speight 2004).

The brown marmorated stink bug, *Halyomorpha halys* (Hemiptera: Pentatomidae), is an invasive economic insect pest native to subtropical and temperate regions of East Asia including Japan, China, and Korea (Zhu et al. 2012a; Lee et al. 2013). Hitchhiking as a by-product of international commerce is the main mode of unintended introduction of the pest to foreign areas, as *H. halys* can stowaway undetected in internationally bound cargo such as machinery, used vehicles, and personal baggage (Hoebeke and Carter 2003; Garipey et al. 2014; Kriticos et al. 2017). This invasive pest has already established populations in North America, South America, and Europe (Hoebeke and Carter 2003; Wermelinger et al. 2008; Fogain and Graff 2011; Faúndez and Rider 2017), and climatic models have forecasted multiple regions as amenable for potential future invasions (Zhu et al. 2012a; Kriticos et al. 2017). Upon arrival to an uninvaded area, *H. halys* is known to spread rapidly through human activity and can establish populations throughout all climatically suitable locations in which they are exposed (Haye et al. 2015a). Monitoring and trapping *H. halys* is difficult due to the pest's exceptional mobility (Nielsen et al. 2013). This pest also has hundreds of different host crops that it is able to feed on to complete development (Lee et al. 2013; Rice et al. 2014). Furthermore, native biological control from natural enemies has been ineffective from field assessments conducted in invaded regions (Haye et al. 2015b; Ogburn et al. 2016; Dieckhoff et al. 2017). As a result, the economic damage inflicted by *H. halys* to agricultural industries in invaded areas has been severe due to damaged and thus unmarketable fruit and low crop yields (Nielsen and Hamilton 2009; Kuhar et al. 2012).

Chemical control remains the most prevalent form of management against *H. halys* in its non-native range (Leskey et al. 2012a; Kuhar and Kamminga 2017). However, pesticides are seen as a short-term solution as they can be expensive both monetarily and environmentally (Hull and VanSterner 1983; Lee et al. 2014). Frequency of insecticide applications targeting *H. halys* has increased by nearly four times dating back to when the pest first began inflicting economic injury in invaded regions (Leskey et al. 2012a). The increased use of insecticides throughout the field season has also led to secondary pest outbreaks in agricultural settings (Rice et al. 2014). In addition, *H. halys* has been shown to develop tolerance after exposure to several insecticides in laboratory trials (Leskey et al. 2012b). To sustainably mitigate the agricultural threat this adventive pest imposes in regions of invasion, the development of a robust, multi-faceted integrated pest management approach is necessary.

Biological control has emerged at the forefront of explored sustainable measures to manage *H. halys*.

Laboratory experiments have determined generalist predators have varying levels of success in providing control against different life stages of *H. halys* (Morrison et al. 2016; Pote and Nielsen 2017; Kamiyama et al. 2021a). The implementation of parasitoid wasps as a measure of control has shown more promise, as research efforts have focused on understanding the parasitism efficacy and environmental effects of prospective egg parasitoids (Lee et al. 2013; Rice et al. 2014). Field surveys exploring the presence of *H. halys* parasitoids using sentinel and naturally laid *H. halys* egg masses have been conducted in several invaded regions (Abram et al. 2017). Parasitoids from the genera *Trissolcus*, *Telenomus*, *Anastatus*, *Ooencyrtus*, and *Gyron* have been documented to successfully parasitize sentinel *H. halys* egg masses in the USA (Herlihy et al. 2016; Dieckhoff et al. 2017; Tillman et al. 2020). Similarly, a wide range of parasitoids from the genera *Trissolcus*, *Telenomus*, *Anastatus*, *Ooencyrtus*, and *Acroclisoides* have been recorded parasitizing sentinel egg masses in Switzerland and Italy (Haye et al. 2015b; Roversi et al. 2016; Moraglio et al. 2020). However, the parasitism rates inflicted by these indigenous egg parasitoids from North America and Europe are generally low, below 5% across these field studies (Abram et al. 2017; Dieckhoff et al. 2017).

Research conducted in the native range of *H. halys*—specifically China and Japan—has determined parasitoids primarily from the *Trissolcus* genus as the main *H. halys* egg parasitoids (Arakawa and Namura 2002; Lee et al. 2013), although species in the *Telenomus*, *Ooencyrtus*, and *Anastatus* genera have also been identified in successfully parasitizing *H. halys* egg masses (Zhang et al. 2017; Avila et al. 2021). Field assessments conducted in kiwifruit in central China revealed a native *H. halys* parasitoid species composition of 41% *Trissolcus japonicus* and 48% *T. cultratus* (Avila et al. 2021), whereas surveys conducted in mixed fruit orchards in northern China displayed a species composition consisting of over 90% *T. japonicus* (Zhang et al. 2017). The field parasitism rate of *T. japonicus* on *H. halys* ranges from 50 to 80% as determined by sentinel egg mass deployment in China (Yang et al. 2009; Zhang et al. 2017) and is over 90% in laboratory experiments (Haye et al. 2015b). Programs testing *T. japonicus* as an appropriate biological control agent against *H. halys* are under evaluation worldwide (Hedstrom et al. 2017; Charles et al. 2019; Milnes and Beers, 2019; Lara et al. 2019; Haye et al. 2020; Saunders et al. 2021).

The native range of currently known *H. halys* parasitoids and the overall species composition is poorly understood and projected to be underestimated due to a lack of field sampling in Asia (Avila and Charles 2018; Yonow et al. 2021). Other gaps in knowledge pertaining to *H. halys* parasitoids in Asia also exist. For example, there is limited published information available describing behaviors such as the

overwintering habits and seasonal phenology of parasitoids in the native range of *H. halys*. The presented research therefore aims to clarify the seasonal phenology and components of biological control against *H. halys* in its native range by surveying weekly for egg parasitoids in Japan. The results from this study not only establish the first phenological assessment of *H. halys* egg parasitoids in Japan, but are also expected to improve the biological knowledge of the parasitoid richness and community structure in the native range of *H. halys*. A better understanding of the parasitoid biology in the native range of *H. halys* will have implications for the development and refinement of pending classical biological control methods.

Materials and methods

Insect colonies

Halyomorpha halys used to produce egg masses for the field experiments were reared at Kyoto University in Uji, Japan. The insects were originally field collected from Tokyo, Japan. The colonies of *H. halys* were kept in plastic containers (40 cm × 15 cm × 20 cm) (Daiso, Taizo Sangyo Co., Ltd., Hiroshima, Japan) fitted with mesh lids and were provided a mixture of green beans (*Phaseolus vulgaris*), carrots (*Daucus carota* ssp. *sativus*), and raw almonds (*Prunus dulcis*). All colony containers were thoroughly checked for egg masses daily to ensure the age of each egg mass was known. The *H. halys* colonies were maintained at a temperature of 24 ± 1 °C and a relative humidity of roughly 70% in a temperature-controlled room.

Sentinel egg mass study

Three fresh and three frozen sentinel egg cards were clipped to foliage (1 m above the ground) near Kyoto University, Uji Campus in Kyoto, Japan (34°54'39" N, 135°48'09" E) every week from March 22, 2020, to December 18, 2020. Egg masses of varying age between 0 and 24 h were fixed to 3 cm × 2 cm pieces of cardstock via double-sided sticky tape (Lara et al. 2019). Exposed parts of the tape were covered with small pieces of paper towel to ensure parasitoids in the field would not get stuck, hindering potential parasitism attempts. Cold-treated egg cards were placed in a -20 °C freezer and frozen for at least one week before field deployment (Tillman et al. 2020). Sentinel cards were left in the field for 72 h and then returned to the laboratory. Upon their return, egg masses were transferred to a clear plastic snap cap vial (4.0 cm × 2.3 cm) (SKS Science, Watervliet, NY, USA). Daily observations were made of the returned egg masses for one month to observe *H. halys* nymph or parasitoids emergence (Dieckhoff et al. 2017). Sentinel egg masses

were then allowed another two months of incubation time, in which egg masses were checked three times per week to ensure potential developing parasitoids were provided sufficient time to develop and emerge. Any unemerged eggs from the sentinel egg masses were then dissected to determine the contents of the eggs: developing nymph, developing parasitoid, or undetermined (aborted egg with undifferentiated contents) (Kaser et al. 2018; Costi et al. 2020). All egg masses were kept at a temperature of 24 ± 1 °C and a relative humidity of roughly 70% in a temperature-controlled room.

Twenty control fresh egg masses were constructed in the same manner as the fresh sentinel field egg masses and then reared in a laboratory setting in order to compare *H. halys* nymphal emergence rates between the field-deployed and laboratory-reared egg mass groups. Twenty control fresh egg masses were also set up to compare the parasitism rates between fresh field-deployed sentinel egg masses and the laboratory-conducted controls. In these control parasitism experiments, only *T. japonicus* were tested since they were the only parasitoid recovered from the field in high enough numbers (see Results). *Trissolcus japonicus* colony lines were established by providing a female parasitoid emerged from a fresh sentinel egg mass with a fresh *H. halys* egg mass 24 h of age in a clear plastic snap cap vial. Female *T. japonicus* were removed following 48 h of exposure to the egg mass, and the parasitism rates were determined (see statistical analysis section). The control egg masses were reared at a temperature of 24 ± 1 °C and a relative humidity of roughly 50% in a temperature-controlled room.

Parasitoid identification

Initial identification of emerged parasitoid wasp genera was determined morphologically through the presence/absence of key features (Hirashima and Yamagishi 1981; Zhang et al. 2005; Talamas et al. 2017; Chen et al. 2019). The species of emerged parasitoid wasps was then confirmed through molecular identification for each sentinel egg mass that was successfully parasitized in the field (i.e., at least one fully emergent adult parasitoid). Genomic DNA was isolated from a whole parasitoid using QIAamp DNA extraction kit (QIAGEN, Germantown, MD, USA) per the manufacturer's instructions. Following parasitoid DNA extraction, the barcode region of the mitochondrial gene cytochrome oxidase I (*COI*) was amplified with universal insect PCR primers LCO-1490 and HCO-2198 (Folmer et al. 1994). The PCR products, ranging from 615 to 650 base pairs, were purified using a FastGene PCR extraction kit (Nippon Genetics Co., Ltd., Tokyo, Japan) and then subjected to Sanger sequencing (Eurofin Genomics Co., Ltd., Tokyo, Japan). Both forward and reverse sequences were manually trimmed and aligned using MEGA X (Kumar et al. 2018). All parasitoid *COI* sequences were then compared against existing sequences

in the GenBank database using the similarity search from the Basic Local Alignment Search Tool (BLAST) (<http://www.ncbi.nlm.nih.gov/BLAST>) to confirm the species identity of each specimen. A representative sequence from each determined parasitoid species was submitted to the GenBank database, and accession numbers were obtained.

Temperature data

Temperature data throughout the duration of the field study were obtained for the location 34°53'24" N, 135°48'00" E in Uji, Japan (Meteoblue 2021), 2 km from the location of the deployed sentinel egg masses. The data provided daily, as well as monthly minimums, maximums, and average temperatures. The temperature data were used to help relate field parasitism activity with seasonality in Kyoto.

Statistical analysis

Sentinel egg mass data were separated by season (spring, summer, and fall), unless otherwise noted (e.g., all data pooled together). The first sampling event, March 22, through May 30, was designated as “spring,” June 7 through August 28, was designated as “summer,” and September 6 through December 20 was designated as “fall.” When determining the parasitism levels of the native parasitoids, only sentinel egg masses with emerged parasitoids were included for analysis (Zhang et al. 2017). Parasitism rate was calculated by dividing the number of successfully emerged parasitoids by the total number of eggs from an egg mass. When determining nymphal emergence levels from fresh sentinel egg masses, all fresh egg masses were included for analysis (parasitized and non-parasitized). Nymph emergence rate was calculated by dividing the total number of emerged *H.*

halys nymphs by the total number of eggs from an egg mass. Developmental days for parasitoids were calculated by summing the total number of days from the first day a sentinel egg mass was deployed in the field until parasitoid emergence from the egg mass. The summed days were then added for each emerged parasitoid and finally divided by the total number of parasitoids that emerged from each parasitized egg mass to give the average developmental days.

Generalized linear models (GLMs) with a logit link function were fit to the data using the *glm* function in the *lme4* package from the RStudio program (v.3.4.1) (R Core Team 2018). The GLMs estimated the effect seasonality (spring, winter, fall), egg mass type (fresh, frozen), and the seasonality \times type interaction had on the parasitism rate and parasitoid species composition measured from the sentinel *H. halys* egg masses. Analyses were run on pooled data (all seasons and both egg mass types combined), and also data separated by season and egg mass type.

A two-way ANOVA was run to determine the effect the different egg mass types had on the developmental days between the different species of recovered parasitoids. A Tukey’s HSD post hoc test was then used to determine differences in the means between the variables at $p < 0.05$. A Welch two-sample *t* test was run to determine: (1) the difference in parasitism rates between fresh and frozen sentinel egg masses for *T. japonicus* and *T. cultratus*, (2) the difference in the *H. halys* nymphal emergence rates between the laboratory-reared and field-deployed fresh sentinel egg masses, and (3) the difference in *T. japonicus* parasitism rates between fresh sentinel and laboratory-reared control egg masses at $p < 0.05$.

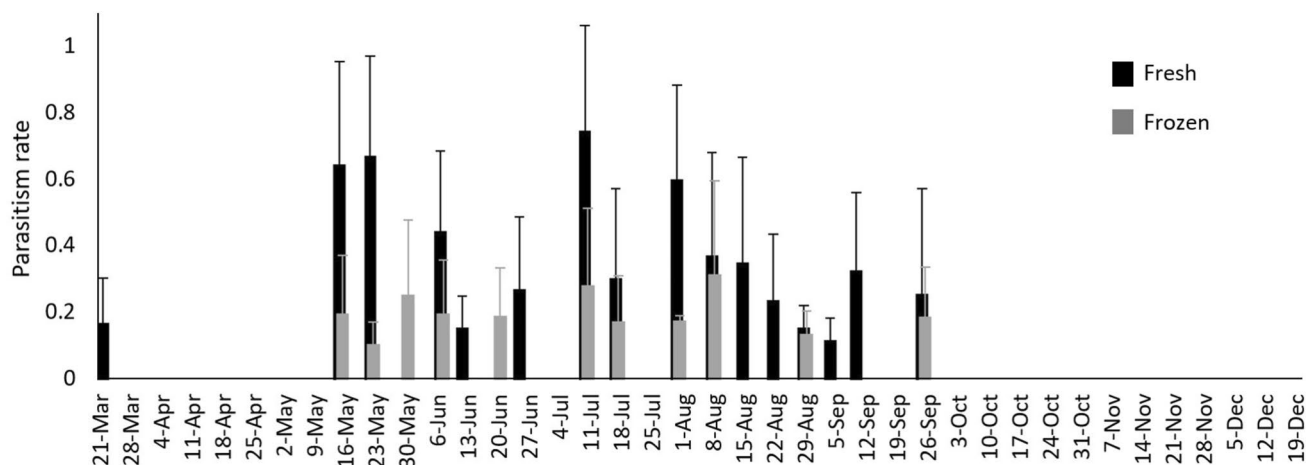


Fig. 1 Mean (\pm SE) weekly parasitism rates of native parasitoids determined from parasitized fresh and frozen field exposed sentinel *H. halys* egg masses in Kyoto, Japan, in 2020

Results

In total, 228 fresh and frozen sentinel *H. halys* egg masses (5343 eggs) were deployed in Kyoto, Japan, over 38 weeks from March through December 2020. The majority of eggs (71.3%) remained unemerged after retrieval, meaning neither *H. halys* nymphs nor parasitoids emerged from the eggs. Successful parasitism occurred on 16.2% of all the sentinel egg masses deployed. Of the successfully parasitized egg masses, 543 parasitoids emerged from 930 eggs (58.4%) (Table 2).

The first record of successful parasitism from the sentinel field *H. halys* egg cards occurred in early spring from a fresh egg mass (Fig. 1). The second record of successful parasitism occurred eight weeks later in late spring from both fresh and frozen egg masses. Parasitism was regularly recorded on sentinel egg masses from late spring until final field parasitism in early fall. The average daily temperature during the 3-day period (March 19–22) the first parasitized sentinel egg mass was in the field ranged between 10 and 13 °C, with a maximum temperature of 21 °C (Supp. Fig. 1). Throughout the year, average daily temperatures rose from early spring until late summer and then began to lower until late fall (Supp. Fig. 2).

Nymphal *H. halys* emerged from the fresh sentinel egg masses every week from early spring to late fall (Fig. 2). Nymphal emergence rate varied weekly, but was typically lowest in early spring and late fall. The highest nymphal emergence rate for a deployed set of fresh sentinel egg masses was in mid-summer (90.4%). No *H. halys* nymphs successfully emerged from the fresh egg masses deployed in the field after late November. The average nymphal emergence rate from the fresh field egg masses was 27.7%, which was significantly lower than the average nymphal emergence

rate from laboratory-reared control *H. halys* egg masses (90.5%) ($t=14.02$, $p<0.005$). Dissections of the unemerged sentinel eggs revealed 20 unemerged parasitoids from the fresh egg masses and 41 unemerged parasitoids from the frozen egg masses. Of the unemerged fresh eggs, 1.4% were developing parasitoids, 82.9% were developing *H. halys* nymphs, and 15.7% were undetermined. Of the unemerged frozen eggs, 1.6% were developing parasitoids and 98.4% were undetermined.

Morphological analysis identified parasitoids from three genera, *Trissolcus*, *Anastatus*, and *Ooencyrtus*. Molecular analysis confirmed the species of five of the emerged parasitoids, four from the genera *Trissolcus* and one from *Anastatus*. A BLAST search displayed the best similarity score (%) of the *COI* barcode sequence (base pairs in length) for the parasitoids, and a representative sequence was submitted to GenBank for each species. *Trissolcus japonicus* yielded a 650-bp sequence (accession number: MZ466407) with 99% sequence identity scores against most previously published *T. japonicus* sequences (e.g., MN615626.1). *Trissolcus cultratus* yielded a 615-bp sequence (accession number: MZ456341) with 99% sequence identity scores against most previously published *T. cultratus* sequences (e.g., AB971829). *Trissolcus mitsukurii* yielded a 628-bp sequence (accession number: MZ466409) with 99% sequence identity scores against most previously published *T. mitsukurii* sequences (e.g., MT671789.1). *Trissolcus comperei* yielded a 640-bp sequence (accession number: MZ466408) with 99% sequence identity scores against most previously published *T. comperei* sequences (e.g., MN615656.1). *Anastatus japonicus* yielded a 641-bp sequence (accession number: MZ433261) with 99% sequence identity scores against most previously published *A. japonicus* sequences (e.g., MZ433261.1). Molecular analysis could not reliably confirm the species for one other parasitoid: *Trissolcus* sp.

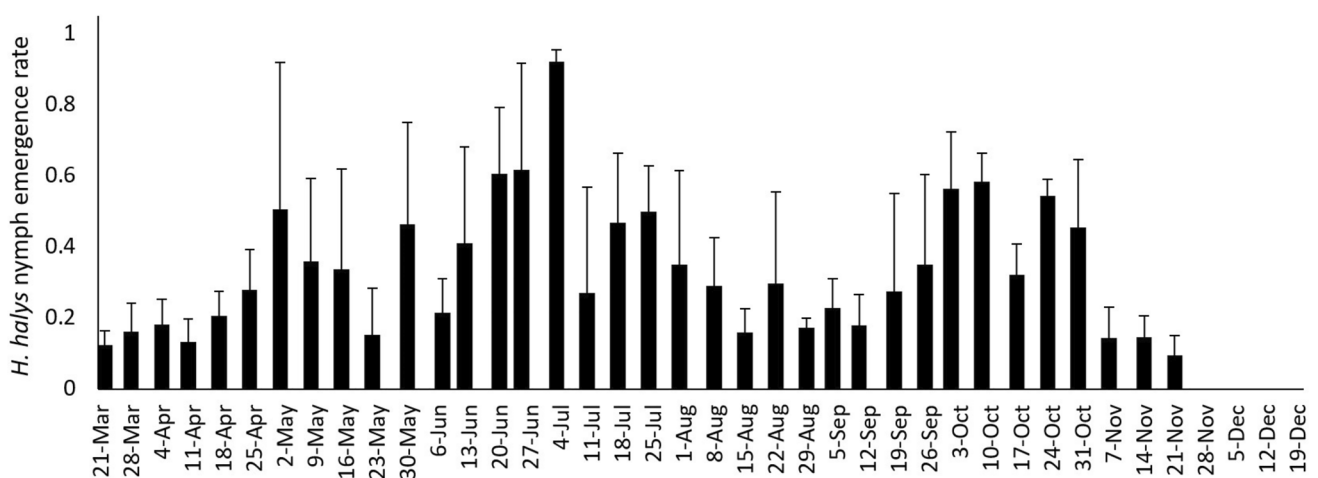


Fig. 2 Mean (\pm SE) weekly emergence rates of *H. halys* nymphs from fresh field exposed sentinel *H. halys* egg masses in Kyoto, Japan, in 2020

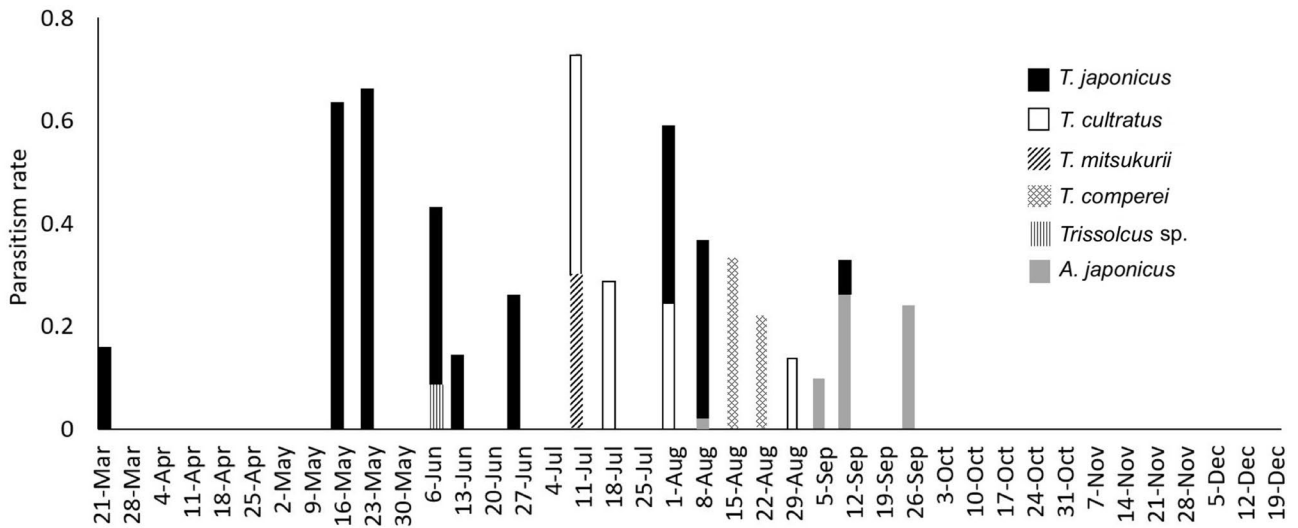


Fig. 3 Weekly parasitoid species composition and parasitism rates of native parasitoids determined from parasitized fresh field exposed sentinel *H. halys* egg masses in Kyoto, Japan, in 2020

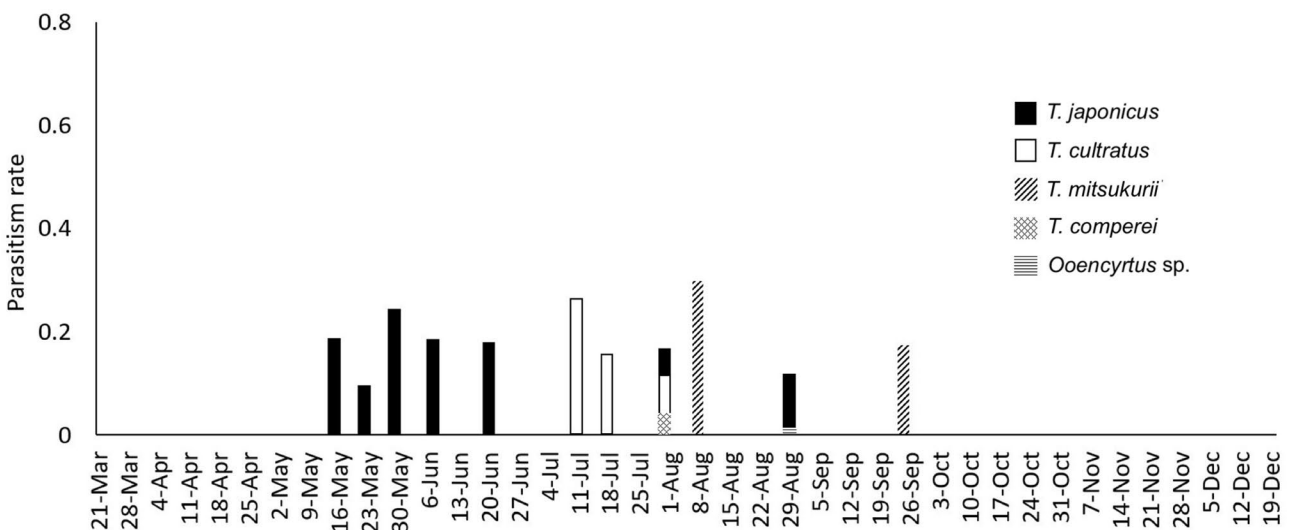


Fig. 4 Weekly parasitoid species composition and parasitism rates of native parasitoids determined from parasitized frozen field exposed sentinel *H. halys* egg masses in Kyoto, Japan, in 2020

631-bp sequence (accession number: MZ503514) with only 90% sequence identity scores against most previously published *T. plautiae* sequences (e.g., MN615614.1). Molecular analysis was not performed on *Ooencyrtus* sp. due to a low amount of successfully emerged specimens.

The first parasitoid species to successfully emerge from a fresh sentinel egg mass was *T. japonicus* (early spring), followed by *Trissolcus* sp. (early summer), then *T. cultratus* and *T. mitsukurii* (mid-summer), *A. japonicus* (late summer), and finally *T. comperei* (late summer) (Fig. 3). Similarly, *T. japonicus* was also the first parasitoid species to emerge

from a frozen sentinel egg mass (late spring), followed by *T. cultratus* (mid-summer), *T. comperei* (mid-summer), *T. mitsukurii* (late summer), and lastly *Ooencyrtus* sp. (late summer) (Fig. 4). The only parasitoid that parasitized a sentinel egg mass (fresh or frozen) during each season was *T. japonicus*.

When pooling the data, seasonality did not have a significant effect on the parasitism rate found on the sentinel *H. halys* egg masses ($Z=0.84$; $p=0.40$), but egg mass type did have a significant effect on parasitism rate ($Z=2.31$; $p=0.02$) (Table 1). The pooled data also

Table 1 Generalized linear model results estimating the effect of seasonality (spring, summer, fall), egg mass type (fresh, frozen), and the seasonality × type interaction on the parasitism rate of native parasitoids determined from parasitized fresh and frozen field exposed sentinel *H. halys* egg masses in Kyoto, Japan, in 2020

Species	Seasonality effect	Type effect	Seasonality x type effect
<i>T. japonicus</i>	Z=0.343; p=0.732	Z=2.012; p=0.044	Z=0.171; p=0.864
<i>T. cultratus</i>	N/A	Z=0.632; p=0.527	N/A
<i>T. mitsukurii</i>	Z=0.558; p=0.577	Z=0.000; p=1.000	N/A
<i>T. comperei</i>	N/A	Z=0.975; p=0.329	N/A
<i>A. japonicus</i>	Z=0.722; p=0.471	N/A	N/A
<i>Trissolcus</i> sp.	N/A	N/A	N/A
<i>Ooencyrtus</i> sp.	N/A	N/A	N/A
Species combined	Z=0.839; p=0.402	Z=2.305; p=0.021	Z=1.010; p=0.312

Table 2 Parasitism rate (± SE) of native parasitoids determined from parasitized fresh and frozen field exposed sentinel *H. halys* egg masses in Kyoto, Japan, in 2020

Species	Egg mass type	Season			
		Spring	Summer	Fall	Entire season
<i>T. japonicus</i>	Fresh	0.89 ± 0.07	0.77 ± 0.12	0.93 ± 0.00	0.84 ± 0.06
	Frozen	0.43 ± 0.10	0.32 ± 0.10	N/A	0.36 ± 0.07
	Pooled	0.72 ± 0.10	0.55 ± 0.11	0.93 ± 0.00	0.64 ± 0.07
<i>T. cultratus</i>	Fresh	N/A	0.71 ± 0.16	N/A	0.71 ± 0.16
	Frozen	N/A	0.48 ± 0.15	N/A	0.48 ± 0.15
	Pooled	N/A	0.61 ± 0.12	N/A	0.61 ± 0.12
<i>T. mitsukurii</i>	Fresh	N/A	1.00 ± 0.00	N/A	1.00 ± 0.00
	Frozen	N/A	0.89 ± 0.00	0.50 ± 0.00	0.70 ± 0.20
	Pooled	N/A	0.95 ± 0.05	0.50 ± 0.00	0.80 ± 0.15
<i>T. comperei</i>	Fresh	N/A	0.82 ± 0.18	N/A	0.82 ± 0.18
	Frozen	N/A	0.14 ± 0.00	N/A	0.14 ± 0.00
	Pooled	N/A	0.60 ± 0.25	N/A	0.60 ± 0.25
<i>A. japonicus</i>	Fresh	N/A	0.05 ± 0.00	0.62 ± 0.22	0.48 ± 0.21
	Frozen	N/A	N/A	N/A	N/A
	Pooled	N/A	0.05 ± 0.00	0.62 ± 0.22	0.48 ± 0.21
<i>Trissolcus</i> sp.	Fresh	N/A	0.21 ± 0.00	N/A	0.21 ± 0.00
	Frozen	N/A	N/A	N/A	N/A
	Pooled	N/A	0.21 ± 0.00	N/A	0.21 ± 0.00
<i>Ooencyrtus</i> sp.	Fresh	N/A	N/A	N/A	N/A
	Frozen	N/A	0.07 ± 0.00	N/A	0.07 ± 0.00
	Pooled	N/A	0.07 ± 0.00	N/A	0.07 ± 0.00
Species combined	Fresh	0.89 ± 0.07	0.69 ± 0.09	0.70 ± 0.17	0.73 ± 0.06
	Frozen	0.43 ± 0.10	0.36 ± 0.09	0.50 ± 0.00	0.40 ± 0.06
	Pooled	0.72 ± 0.10	0.55 ± 0.07	0.66 ± 0.14	0.58 ± 0.01

Spring: March–May, Summer: June–August, Fall: September–December

showed the parasitism rate was significantly higher on fresh sentinel egg masses (73.3%) than frozen (39.6%) ($t = 3.69$, $p < 0.005$). The parasitism rates were significantly higher on fresh sentinel egg masses compared to frozen for *T. japonicus* ($t = 5.023$, $p < 0.001$), and the parasitism rates were similar between fresh and frozen egg masses for *T. cultratus* ($t = 1.059$, $p = 0.339$) (Table 2). Also, the average parasitism rate of *T. japonicus* on the fresh sentinel egg masses across the entire field season (84.1%) was similar with the parasitism rate of *T. japonicus* on the control egg masses in laboratory conditions

(95.0%) ($t = 1.62$, $p = 0.131$). However, *T. mitsukurii* produced numerically the highest parasitism rate on fresh sentinel egg masses (100%). All four identified *Trissolcus* species produced average parasitism rates over 70% on fresh egg masses across the entire field season, and *Trissolcus* sp. had a parasitism rate of 21% on fresh egg masses across the entire field season. *Trissolcus mitsukurii* had numerically the highest parasitism rate on frozen sentinel egg masses across the entire field season (69.6%).

Similarly, pooling the data showed that seasonality did not have a significant on the parasitoid species

Table 3 Generalized linear model results estimating the effect of seasonality (spring, summer, fall), egg mass type (fresh, frozen), and the seasonality × type interaction on the species composition ofnative parasitoids determined from parasitized fresh and frozen field exposed sentinel *H. halys* egg masses in Kyoto, Japan, in 2020

Species	Seasonality effect	Type effect	Seasonality × type effect
<i>T. japonicus</i>	Z = 1.391; p = 0.164	Z = 1.997; p = 0.046	Z = 0.186; p = 0.853
<i>T. cultratus</i>	N/A	Z = 2.025; p = 0.043	N/A
<i>T. mitsukurii</i>	Z = 2.805; p = 0.005	Z = 0.902; p = 0.367	N/A
<i>T. comperei</i>	N/A	Z = 2.772; p = 0.006	N/A
<i>A. japonicus</i>	Z = 1.851; p = 0.064	N/A	N/A
<i>Trissolcus</i> sp.	N/A	N/A	N/A
<i>Ooencyrtus</i> sp.	N/A	N/A	N/A
Species combined	Z = 1.180; p = 0.238	Z = 2.929; p = 0.003	Z = 1.578; p = 0.115

Table 4 Parasitoid species composition of native parasitoids determined from parasitized fresh and frozen field exposed sentinel *H. halys* egg masses in Kyoto, Japan, in 2020

Species	Egg mass type	Season			Entire season
		Spring	Summer	Fall	
<i>T. japonicus</i>	Fresh	100%	42.2%	41.2%	59.7%
	Frozen	100%	35.8%	N/A	49.0%
	Pooled	100%	40.2%	33.3%	56.7%
<i>T. cultratus</i>	Fresh	N/A	33.3%	N/A	20.3%
	Frozen	N/A	36.7%	N/A	26.1%
	Pooled	N/A	34.4%	N/A	21.9%
<i>T. mitsukurii</i>	Fresh	N/A	8.0%	N/A	4.9%
	Frozen	N/A	22.9%	100%	21.6%
	Pooled	N/A	12.7%	19.0%	9.6%
<i>T. comperei</i>	Fresh	N/A	13.5%	N/A	8.2%
	Frozen	N/A	2.8%	N/A	2.0%
	Pooled	N/A	10.1%	N/A	6.4%
<i>A. japonicus</i>	Fresh	N/A	0.4%	58.8%	5.4%
	Frozen	N/A	N/A	N/A	N/A
	Pooled	N/A	0.3%	47.6%	3.9%
<i>Trissolcus</i> sp.	Fresh	N/A	2.5%	N/A	1.5%
	Frozen	N/A	N/A	N/A	N/A
	Pooled	N/A	1.7%	N/A	1.1%
<i>Ooencyrtus</i> sp.	Fresh	N/A	N/A	N/A	N/A
	Frozen	N/A	1.8%	N/A	1.3%
	Pooled	N/A	0.6%	N/A	0.4%

Spring: March–May, Summer: June–August, Fall: September–December.

composition found on the sentinel *H. halys* egg masses ($Z = 1.18$; $p = 0.24$), but egg mass type did have a significant effect on species composition ($Z = 2.93$; $p = 0.003$) (Table 3). *Trissolcus japonicus*, *T. cultratus*, *T. mitsukurii*, and *T. comperei* all successfully emerged from both fresh and frozen sentinel egg masses (Table 4). *Anastatus japonicus* and *Trissolcus* sp. only emerged from fresh egg

masses, and *Ooencyrtus* sp. only emerged from a frozen egg mass. *Trissolcus japonicus* was the only parasitoid recovered in the spring. The most abundant parasitoid from the fresh egg masses was *T. japonicus* in the summer (42.2%) and *A. japonicus* in the fall (58.8%). Across the entire field season, *T. japonicus* was the most abundant parasitoid on fresh egg masses (59.7%), followed by *T. cultratus* (20.3%). All other parasitoids made up under 10% of the species composition from fresh egg masses. *Trissolcus japonicus* was also the most abundant parasitoid on frozen egg masses cross the entire field season (49.0%), followed by *T. cultratus* (26.1%), then *T. mitsukurii* (21.6%). All other parasitoids made up under 5% of the species composition from frozen egg masses.

Developmental days prior to adult parasitoid emergence were the highest for *Anastatus japonicus* on fresh egg masses compared to all other parasitoid species ($F = 5.599$, $p = 0.001$) (Supp. Table 1).

Discussion

The results of this research elucidate the seasonal phenology and species composition of native parasitoids which target *H. halys* egg masses in Kyoto, Japan. Such information is essential for the future development and refinement of classical biological control programs against *H. halys* in invaded regions (Lara et al. 2019). The seasonal phenology of *H. halys* egg parasitoids, as measured by our study, peaks in mid-summer and ceases in early fall. The population activity trend demonstrated by the parasitoids is comparable to the seasonal population dynamics of *H. halys* in other parts of Japan, which also peaks in mid-summer and drops off in the early fall (Tsutsumi 2003; Kamiyama et al. 2021b). Similar to previous studies conducted in the native range of *H. halys*, *T. japonicus* demonstrated a high capacity for parasitizing *H. halys* (Yang et al. 2009; Zhang et al. 2017). The field parasitism rate of

T. japonicus on fresh *H. halys* egg masses was determined to be 84% (Table 2), higher than the reported field parasitism rates found in China by Yang et al. (2009) (50%) and Zhang et al. (2017) (50–80%). Parasitoids *T. cultratus* and *T. mitsukurii* also displayed relatively high parasitism rates on the fresh sentinel *H. halys* egg masses throughout the field season (71% and 100%, respectively). *Trissolcus cultratus* has been documented parasitizing sentinel *H. halys* egg masses in Switzerland, although they were only able to emerge from dead and frozen eggs (Haye et al. 2015b).

Remarkably, the first parasitism activity from our assessment occurred on March 22 from a *T. japonicus* parasitized fresh sentinel *H. halys* egg mass, months before populations of *H. halys* in Japan are expected to become active (Tsutsumi 2003; Lee et al. 2013; Kamiyama et al. 2021b). This is the first record of successful parasitism by any parasitoid species on *H. halys* eggs as early as March. Field studies conducted in central Italy, northern China, and the southeastern USA invariably reported the first parasitism of *H. halys* in May (Roversi et al. 2016; Zhang et al. 2017; Tillman et al. 2020). These former surveys, however, did not begin sampling for parasitoids until May, meaning earlier parasitism is feasible in other regions. The minimum threshold temperature for *T. japonicus* development is estimated at 12.2 °C when reared on *H. halys* eggs (Yang et al. 2009), and 12.4 °C when reared on *Glaucias subpunctatus* eggs, another phytophagous Pentatomidae native to Japan (Kuki et al. 2019). March temperatures in Kyoto did meet this developmental temperature threshold; however, the second record of successful parasitism occurred on May 15, nearly two months after the first record. There was no sign of parasitoid activity between the first two parasitism records as no developing parasitoids were found from the dissections of the unemerged sentinel egg masses during this time, supporting the notion that the first female *T. japonicus* breaking diapause in the early spring was more of an anomaly.

The first parasitism in March resulted in both male and female progeny, evidencing the original female *T. japonicus* was mated prior parasitism (Kuki et al. 2019). It is unknown whether the female was mated prior to overwintering or after breaking diapause. Other insects such as leaf beetles and grasshoppers are able to mate before entering diapause and lay fertile eggs after overwintering without mating again in the spring (Stevens and McCauley 1989; Zhu et al. 2012b). *Trissolcus biproruli* (Hymenoptera: Scelionidae), a parasitoid of spined citrus bug, demonstrated successful parasitism up to three weeks following initial mating (James 1988). Furthermore, laboratory and semi-field experiments conducted by James (1988) revealed that adult *T. biproruli* do not appear to enter a reproductive diapause while overwintering, and are capable of winter parasitism if exposed to suitable environmental conditions. Yet, further research investigating the overwintering habits of *T. japonicus* is

necessary to confirm if the initial female *T. japonicus* parasitizes the sentinel *H. halys* egg mass without prior spring mating.

The parasitism rate on the fresh *H. halys* sentinel egg masses was significantly higher than the frozen egg masses in Kyoto, Japan, which differed from previous studies that reported similar parasitism rates between fresh and frozen sentinel egg masses in the southeastern USA, and higher rates on frozen than fresh in the eastern USA (Herlihy et al. 2016; Tillman et al. 2020). Additionally, field studies performed in Europe showed that native European parasitoids can successfully emerge from frozen *H. halys* egg masses, but fail to develop on fresh egg masses (Haye et al. 2015b; Roversi et al. 2016). Egg parasitoids must overcome defensive chemical compounds on the host's eggs, as well as the immune response of the developing host embryo in order to achieve successful parasitism (Tognon et al. 2017). Parasitoids generally accomplish this by probing their ovipositor and injecting venom and/or symbiotic viruses into the host's eggs (Strand et al. 1985; Zhu et al. 2018; Abram et al. 2019). Endemic parasitoids often lack the ability to overcome an invasive host's defensive response to complete successful parasitism (Haye et al. 2015b; Roversi et al. 2016). However, cold-sterilization has been reported to disable a host's natural defensive mechanisms, resulting in higher parasitism rates (Haye et al. 2015b). In the present study, *T. cultratus* had similar parasitism rates between fresh and frozen sentinel egg masses, whereas *T. japonicus* had a higher parasitism rate on fresh than frozen. Egg parasitoids have limited resources and a short time period when host eggs are viable for parasitism, so female parasitoids depend heavily on chemical volatiles from adult hosts and egg masses (Conti and Colazza 2012; Bertoldi et al. 2019; Malek et al. 2021). The suitability for parasitoid development decreases with egg mass freezing (Wong et al. 2020); however, the chemical effect of cold-sterilizing egg masses is currently unknown.

Trissolcus japonicus was the dominant early season parasitoid emerging from sentinel *H. halys* egg masses (100% of the total spring parasitoid species composition), indicating the parasitoid faces little competition or simply outcompetes other egg parasitoids in the spring. Over the course of the entire field season, the parasitoid species richness in Kyoto expands, as *T. japonicus* made up 59.7% of the overall parasitoid species complex from fresh egg masses, followed by *T. cultratus* (20.3%). Our results are similar to research done in central China, which determined the two most abundant parasitoids attaching fresh *H. halys* egg masses were *T. japonicus* (41% relative abundance) and *T. cultratus* (48% relative abundance) (Avila et al. 2021). Zhang et al. (2017) reported a *H. halys* parasitoid species composition in northern China dominated by *T. japonicus* (90% of total species composition), with no other parasitoid species reaching 5%. In the spring in Kyoto, the *H. halys* parasitoid species

composition detected from our assessment is similar to the *T. japonicus* heavy distribution reported from northern China, whereas over the course of an entire field season, the species composition is more comparable to that of central China. Parasitoids from the genus *Trissolcus* dominate the overall parasitoid species composition in the native range of *H. halys*, but sentinel egg mass studies in invaded regions including Europe and the USA typically show more even distributions among *Anastatus* sp., *Ooencyrtus* sp., *Telenomus* sp., and *Trissolcus* sp. (Roversi et al. 2016; Abram et al. 2017; Dieckhoff et al. 2017; Zhang et al. 2017; Moraglio et al. 2020; Tillman et al. 2020; Avila et al. 2021). Regardless, the parasitism rate of *H. halys* egg masses by native parasitoid species in invaded regions is inconsequentially low, ranging between 2 and 7% (Talamas et al. 2015; Dieckhoff et al. 2017). Also, the invasive *H. halys* can act as an evolutionary trap for indigenous parasitoids in regions of recent invasion, as generalist parasitoids may unsuccessfully attempt to parasitize the eggs of the *H. halys*, leading to a decrease in the native egg parasitoid numbers (Abram et al. 2014; Costi et al. 2020). It is worth noting that in our study only one specimen per parasitized egg mass was sent in for molecular analysis, meaning *H. halys* egg masses that were potentially hyperparasitized by multiple species may have been overlooked (Konopka et al. 2017).

In the current study, there is evidence that factors from the field have a negative effect on the nymphal emergence rates of the fresh sentinel *H. halys* egg masses. Nymphal emergence rates of the fresh egg masses in the field were reduced by approximately 75% in comparison with the laboratory-reared controls. Studies which highlight the life cycle of *H. halys* denote the developmental temperature threshold for eggs to be within 15 °C and 33 °C (Nielsen et al. 2008). Overnight temperatures in March and April, as well as November and December, routinely fell below the lower-temperature developmental limit for eggs (Supp. Fig. 2), indicating that ambient temperature in Kyoto may have been the culprit for the low nymph emergence rates early and late in the season. However, when temperatures were maintained within the developmental threshold, nymphal emergence rates remained lower than the controls, suggesting different reasons for the reduced field hatch rates. We speculate biotic factors, aside from successful parasitism, as the main facilitator of the reduced *H. halys* field emergence. In the sentinel egg masses, unemerged eggs (neither *H. halys* nymphs or parasitoids emerged) were the most abundant on returned sentinel cards. One possible explanation for the high amount of unemerged eggs is that these eggs may have been visited by parasitoids and parasitism was attempted, but not successful in terms of producing progeny. Non-reproductive parasitism has commonly been observed in several groups of insects including Pentatomidae in field experiments (Abram et al. 2019; Kamiyama et al. 2019; Costi

et al. 2020). Pseudoparasitism is one such phenomenon of parasitoid-induced host egg abortion, in which physical disruption and/or infection during the parasitism effort leads to host mortality, but ultimately no parasitoids emerge from the host egg (Abram et al. 2016, 2019). Although only about 1.5% of the unemerged sentinel eggs from our study revealed developing parasitoids, non-reproductive parasitism is often difficult to identify and quantify even through egg dissections (Jones et al. 2014; Cornelius et al. 2016).

This research reveals the native seasonal phenology and species composition of *H. halys* parasitoids in Kyoto, Japan, and offers support for a classical biological control approach as a form of management against the pest. Although this study highlights the high parasitism rate and abundance of *T. japonicus*, further research is still necessary to gain a better understanding of the phenology and behavior of parasitoids in the native range of *H. halys*, and how this may translate to invaded regions. We acknowledge the amount of caution and level of assuredness that must be attained before implementation of a classical biological control scheme. Therefore, we present these results to fill gaps in knowledge and supplement the groundwork for potential future programs in invaded regions.

Author contributions

MTK conceived and designed the research. MTK conducted the research and analyzed the data. MTK and CCSY wrote the manuscript. KM, TH, and CCYS secured funding for the research. All authors read and approved the manuscript.

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Availability of data and material All the sequences have been deposited in GenBank with accession numbers provided in the main text. All parasitoid samples can be made accessible upon request to the first and corresponding authors.

Code availability All codes will be made available upon request.

Declarations

Conflict of interest All authors report no potential conflict of interest.

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