Assessment of the biological control complex and seasonal phenology of *Halyomorpha halys*

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Chapter 1: General introduction

1.1 Invasive species and their impacts

The world today is faced with a growing concern regarding the rapid spread of globally invasive pest species [1]. Invasive species are able to rapidly increase their abundance and distribution upon arrival to a new region due to the escape from natural controlling agents in their native range [2]. The estimated costs linked with nonindigenous pests including control measures, agricultural losses, and ecosystem damages can be immense. This intensifying economic and environmental injury caused by invasive species is primarily driven by increasing international trade and rising global temperatures [3,4].

A major component in establishing a successful control approach against an invasive pest species is gaining a deep understanding of the pest's biology and behavior in both its native and introduced areas [5]. Chemical control remains the most prevalent form of management against invasive pests, however, pesticides are seen as a short-term solution as they can be expensive both monetarily and environmentally [6]. To sustainably mitigate the agricultural threat adventive pest species impose in regions of invasion, the development of robust, multi-faceted integrated pest management approaches are necessary.

1.2 *Halyomorpha halys*

In this thesis, the seasonal phenology and natural control of brown marmorated stink bug (*Halyomorpha halys*) in its native range was researched. This insect pest is native to subtropical and temperate regions of East Asia including Japan, China, and Korea [7,8], and already invaded North America, South America, and Europe

[9,10,11,12]. Climatic models have also forecasted multiple regions as amenable for potential future invasions [8,13]. Upon arrival to an uninvaded area, *H. halys* can spread rapidly through human activity and establish populations throughout all climatically amenable locations in which they are exposed [14]. This invasive pest is highly polyphagous, capable of feeding and completing development on hundreds of different host crops including: cultivated fruit, vegetables, row crops, and ornamentals [7,15]. The wide range of suitable climates and large number of host plants make *H. halys* a particularly difficult invasive species to monitor and control.

In Japan, *H. halys* is recognized more as an arboreal pest of cypress trees and other timber sources, and will opportunistically feed on fruit crops as a secondary food source [16,17]. Although, specific accounts of financial losses due to feeding damage from the pest are not easily accessible and therefore scarce in formal published literature [7]. By most published accounts, *H. halys* persists as an occasional outbreak pest in its native range [14], with damage levels varying between fruit crops [7]. This pest also generally has higher populations in the Northern regions of Japan [16]. A better understanding of the biology and behavior of *H. halys* in its native range will have strong implications towards the development and refinement of future control programs.

1.3 Biological control of *Halyomorpha halys*

In order to lessen the intensity of chemical control used to manage *H. halys*, alternative, more sustainable means of control are being investigated. Once such management strategy is biological control, which aims to use natural enemies to suppress pest populations. The predatory ability of generalist predators of *H. halys* from invaded regions have been assessed, and some species of Orthoptera and Hemiptera have had success preying on *H. halys* at its egg and nymphal stage [18,19]. There is, however, a noticeable lack of information regarding predators of *H. halys* in its native range. The ability of parasitoids to parasitize *H. halys* egg masses has also been investigated, with parasitism rates remaining inconsequentially low in field studies conducted in invaded regions [20,21]. Conversely, field surveys conducted in China reported much higher rates of parasitism on *H. halys* egg masses [22]. An assessment measuring the parasitism rates of *H. halys* egg parasitoids in the field in Japan has yet to be published, and the ability of native Japanese parasitoids to act as potential agents of biological control for *H. halys* is largely unknown.

1.4 Trap monitoring and phenology of *Halyomorpha halys*

To gain a better understanding of the phenology of *H. halys,* several field monitoring studies have been conducted. This includes studies done in both the native range [7] and invaded range of *H. halys* [23,24], where the population dynamics of *H. halys* vary by region. The seasonal phenology also seems to change based on the monitoring attractant used in the field traps [7,25]. Further research is necessary to determine the most effective monitoring methods of *H. halys,* and to better understand the factors influencing their phenology. Once the seasonal population dynamics of *H. halys* can be effectively measured, monitoring data can be compiled and analyzed to generate models capable of predicting seasonal population trends [26]. Such models capable of forecasting changing pest populations in the field hold valuable importance for sustainable management as the timing and frequency of subsequent control measures can be implemented more efficiently.

1.5 Objectives and outline of this thesis

This thesis delves into refining the philosophies behind managing globally invasive pest insects by researching their native behavior and biology, using *Halyomorpha halys* as a model organism. This was accomplished by focusing on the main questions: In the native range of *H. halys* 1) what are their natural sources of control, 2) what is their seasonal phenology, and 3) can their population trends be modelled and predicted? Chapter 2 of this thesis investigated the biological control potential of native natural enemies by evaluating the predatory ability of Japanese *Crematogaster* ants against *H. halys*. In laboratory bio-assays, the *Crematogaster* ants were able to effectively prey upon early immature stages of *H. halys,* but were unable to prey upon on the later immature stages or eggs. Chapter 3 concentrated on discovering the species composition and parasitism rates of *H. halys* egg parasitoids by conducting field egg mass assessments. Overall, seven species of *H. halys* egg parasitoid were identified, and *Trissolcus japonicus* displayed the highest parasitism rates. Chapter 4 of this thesis established a baseline seasonal phenology of *H. halys* in Japan by analyzing eleven years of black light, incandescent light, and pheromone monitoring traps. It was determined that black light traps produced the most representative population curve of *H. halys.* Chapter 5 then used the black light monitoring data to construct predictive phenology models, which revealed *H. halys* populations follow a predictable trend in Japan with activity peaking in the mid-summer. The abundance of *H. halys* as described by trap counts was also deemed to be strongly influenced by temperature. Finally, chapter 6 summarizes the results and findings of this thesis, and discusses the large-scale implications. This thesis fills gaps in knowledge pertaining to the native behavior and natural control of *H. halys*, and carries strong implications towards the improvement of management practices against this globally invasive pest.

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Chapter 2: Predatory ability of native *Crematogaster* ants against *Halyomorpha halys*

2.1 Introduction

Formicidae (ants) constitute an important biotic part of the environment, providing valuable ecosystem services including plant defense [1,2] and consuming/scavenging [3,4]. Several species of ants have been recognized for their predatory ability and are considered valuable biological control agents in many different ecosystems [5]. Ants are unique in comparison to many of the previously researched *Halyomorpha halys* predators. For example, ants are eusocial insects capable of eliciting a rapid colony response, allowing for predation of a wide variety of generally much larger and stronger prey compared to the individual ants [6,7]. Arboreal ants from the *Crematogaster* genus are one group that notably displays this foraging behavior, as large groups of *Crematogaster* workers will quickly seize and fragment their prey while occasionally depositing venom [8]. Few *Crematogaster* species (*C. scutellaris, C. rogenhoferi*) have been explored in their potential to prey on and potentially act as controlling agents against invasive stink bugs including *H. halys* [4,9]. In lab experiments, *C. scutellaris*, a native European *Crematogaster* species, was able to lower the survivorship of *H. halys* first and second instars by 85 – 95% [4]. In another study done by Wu et al. (2018), *C. rogenhoferi* had the ability to physically damage and consume the eggs of the invasive litchi stink bug, *Tessaratoma papillosa*, in field trials. Also, several laboratory and field studies have demonstrated chewing predators have had more success than sucking predators in controlling different life stages of *H. halys* [10,11,12,13,14], another reason ants merit further investigation as predatory biological control candidates. Several native ant species from the genus *Crematogaster* are widely distributed across Japan [15], although their potential as suitable agents of biological control against *H. halys* is unknown.

Our presented study is the first to explore the biological control prospects of native Japanese *Crematogaster* ants by determining their predatory effect on *H. halys* egg emergence and nymph survivorship. In this work, two *Crematogaster* species were selected as the test subjects: *C. matsumurai* (Hymenoptera: Formicidae), and *C. osakensis* (Hymenoptera: Formicidae). *Crematogaster matsumurai* is broadly distributed around Japan [15], and being arboreal [16], is assumed to be in frequent close contact with the pest. *Crematogaster matsumurai* have been reported to as a predator of a braconid parasitoid of lepidopteran larvae [17], and the red-necked longhorn beetle (Sunamura et al., 2020). Another study found that approximately 38-56% of solid food items retrieved by *C. matsumurai* were arthropod fragments [16]. *Crematogaster osakensis* is another ant species thought to occupy the same habitat as *H. halys*, with it being common to forested areas of western Japan [18]. As a ground dwelling ant species, *C. osakensis* use a cooperative prey retrieval strategy when foraging, where the worker ants retrieve the entire prey object together without prior fragmentation [19]. The ubiquitously invasive Argentine ant, *Linepithema humile*, was also included in this research as a positive control due to its hostile nature and aggressiveness in food recruitment [20,21,22]. After its first detection in Japan in 1993, *L. humile* has spread throughout the country while disrupting many indigenous ant communities [23,24]. *Linepithema humile* has also been recorded in the field as a predator of fruit and forest insect pests [25,26], so it was inferred to have a strong predatory ability against *H. halys*. Further information detailing the distribution overlap of *C. matsumurai*, *C. osakensis*, and *L. humile* with *H. halys* in Japan can be viewed in Figure 2.1. The research objectives for this work were to: 1) measure the predatory ability and *H. halys* egg mass hatch reduction efficacy of *C. matsumurai* and *C. osakensis* on *H. halys* eggs, and 2) determine the predatory ability *C. matsumurai* and *C. osakensis* have on nymphal survivorship of *H. halys*.

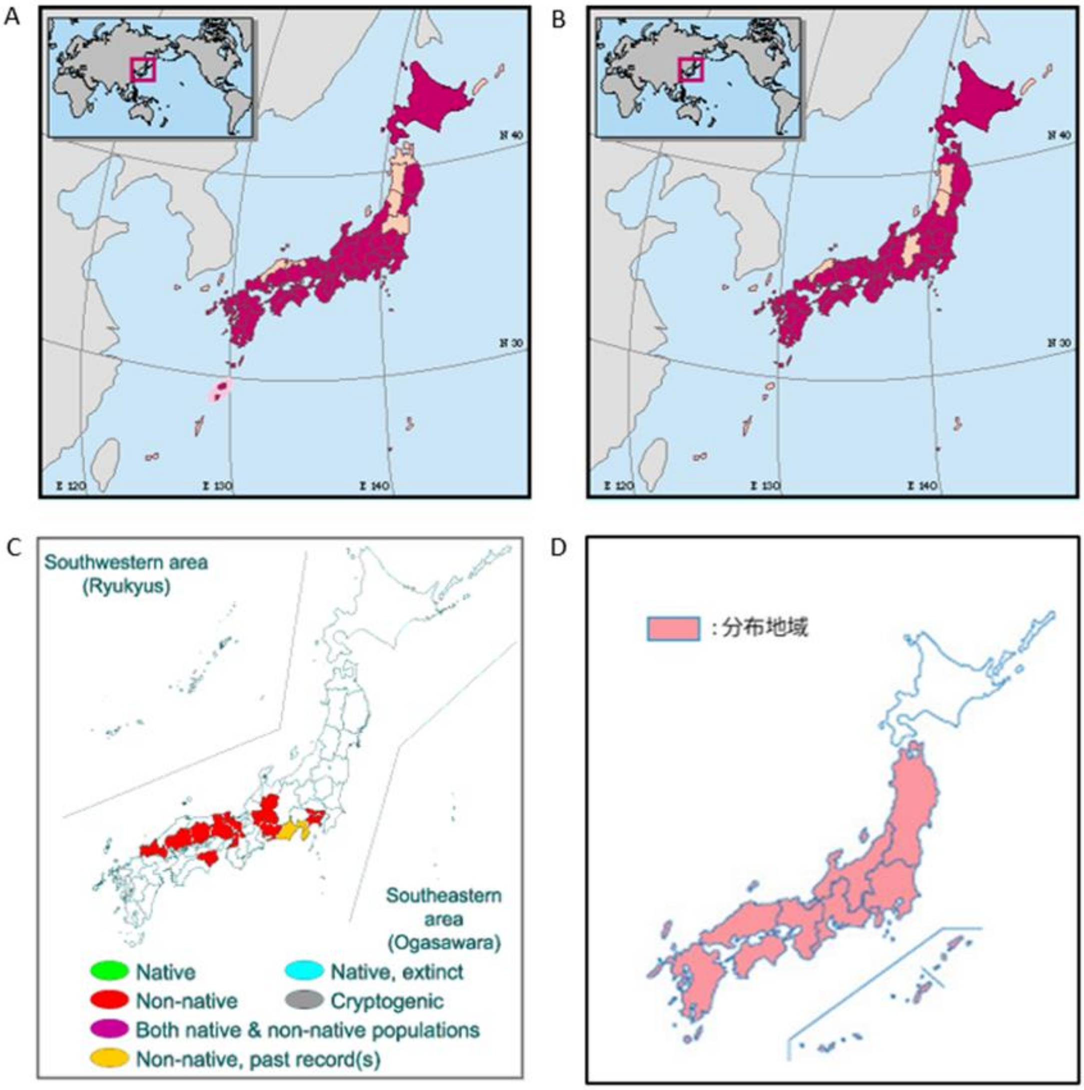

Figure 2.1 Distributions of the two *Crematogaster* species, and *Linepithema humile* in relation to *Halyomorpha halys* in Japan. A: *C. matsumurai*, B: *C. osakensis*, C: *L. humile*, and D: *H. halys*. Note: actual distribution for *L. humile* is greater than what is shown in (C), with five additional prefectures added by 2020. Images retrieved from: the Japanese Ant Image Database Group, National Institute for Environmental Studies (Japan), and Environmental Control Center Co., Ltd. (Japan).

2.2 Materials and methods

2.2.1 Insect colonies

Halyomorpha halys were collected from Tokyo, Japan and maintained at Kyoto University in Uji, Japan. The colonies of *H. halys* were lab-kept in plastic containers (Daiso, Taizo Sangyo Co., Ltd., Hiroshima Prefecture, Japan) fitted with mesh lids. The *H. halys* were provided moist cotton for water, and a mixture of green beans (*Phaseolus vulgaris*), carrots (*Daucus carota ssp. sativus*), and raw almonds (*Prunus dulcis*), which were replaced twice per week. The stink bug colonies were maintained at a temperature of 24 ± 1ºC and a relative humidity of roughly 70% in a temperature controlled room.

Ant colony fragments (roughly 100 workers each) were kept in lid-less plastic containers (Daiso, Taizo Sangyo Co., Ltd., Hiroshima Prefecture, Japan) with the sides lined with baby powder (Johnson & Johnson, Inc., Skillman, NJ, U.S.A.) to prevent individuals from escaping. The ants were provided with moist cotton for water, beetle jelly (KB Farm, Saitama Prefecture, Japan), and dead cockroaches from a separate lab-kept colony. *Crematogaster matsumurai* specimens used in the experiments were field collected in natural areas near Kyoto University in Uji, Japan. *Crematogaster osakensis* specimens were obtained from a lab colony originally kept at Konan University in Kobe, Japan. Colonies of *Linepithema humile* were field collected from Port Island in Kobe, Japan. All ant colonies were maintained at a temperature of 24 ± 1ºC and a relative humidity of roughly 50% in a temperature controlled room.

2.2.2 Predation bio-assay protocol

Prior to each experiment, the ant colonies were starved for four days before exposure to *H. halys* since ants have been documented increasing their foraging effort after four days of starvation [27]. One egg mass (roughly 28 eggs), or five immature *H.*

halys of the same nymphal stage (first through fifth instar) were placed in a small uncovered Petri dish and left in the *C. matsumurai, C. osakensis,* or *L. humile* colony containers (22cm x 16cm x 12cm) for 48h (Figure 2.2). The sides of the containers were brushed with baby powder also to prevent the *H. halys* nymphs and ants from escaping the arenas. Negative controls were run in the same manner, but placed in containers without ants. Six replicates were run for the egg masses and each instar stage across all treatments, each replicate consisting of an individual ant colony fragment from each ant species tested. Following 48h of exposure, *H. halys* egg masses or nymphs were removed from the containers and mortality rates were measured. Egg masses were reared and number of hatched nymphs were counted. Consumed and unemerged eggs were also documented. Nymphs, if recovered, were removed from the ant containers after the 48h and kept in individual containers. Nymphs were classified as surviving if they lived for seven days post ant exposure [28]. Nymphs were classified as dead if they were consumed during the exposure time, died during the seven-day post exposure period, or not recovered (individual completely consumed). Nymphs not recovered were assumed to have been completely consumed rather than having escaped because among all the negative control replicates, all nymphal *H. halys* of every instar treatment tested (n = 150) remained in the bio-assay containers following the 48hr period.

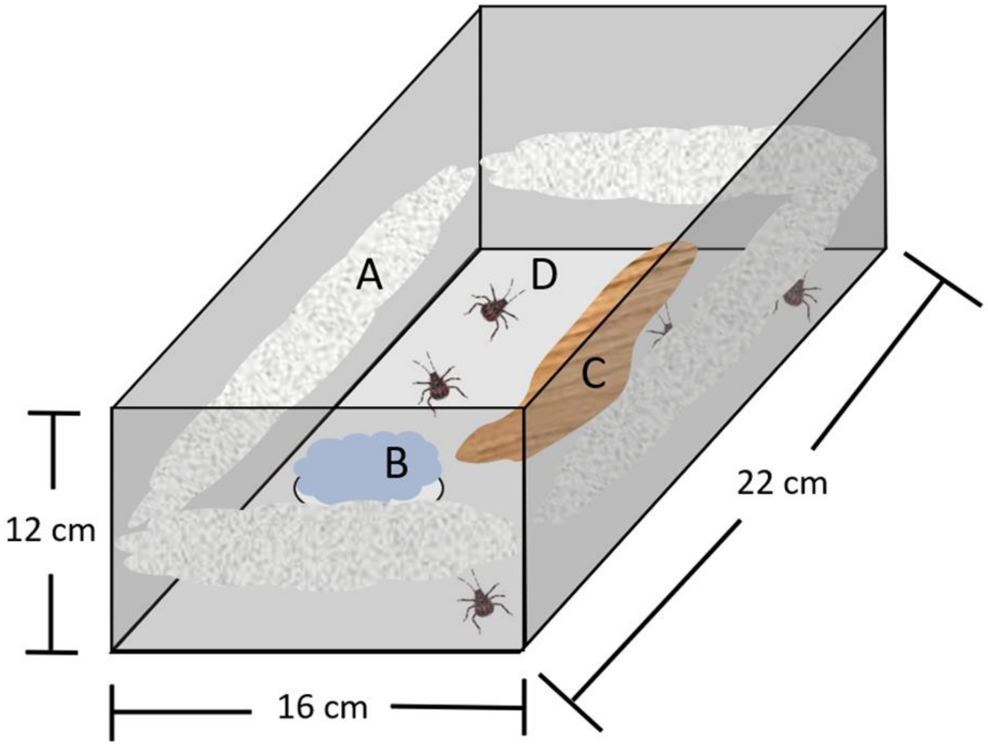

Figure 2.2 Diagram of bio-assay arena. A: Baby powder spread on side of arena to prevent specimens from escaping; B: Moist cotton; C: Substrate used as harborage for ant colonies; D: Nymphal *H. halys*.

2.2.3 Statistical analysis

Data were analyzed using the RStudio program [29]. A one-way ANOVA was run to assess the effect the different treatments (*C. matsumurai, C. osakensis*, *L. humile*, or negative control) had on the emergence rate of *H. halys* eggs following the exposure time. A two-way ANOVA was run to determine the effect the different treatments (*C. matsumurai*, *C. osakensis*, *L. humile*, or negative control) had on the survival rate of first, second, third, fourth, and fifth instar *H. halys* following the exposure time. A Tukey's HSD post-hoc test was then implemented to separate the means of the variables at $p < 0.05$.

2.3 Results

No ant species was able to successfully prey on the *H. halys* egg masses. All ant species tested (both species of *Crematogaster* and the *L. humile*) investigated the egg masses (antennal probing, walking on top, and biting), although no evidence of actual predation was observed at the completion of the bio-assays (Figure 2.3). For example, we did not find remnants of chewed eggs as recorded in similar lab experiments with *H. halys* predators exposed to eggs masses [10,12]. Numerically, *H. halys* emergence rates were the lowest for the egg masses exposed to *L. humile* at 57% (71 emerged nymphs of 124 total eggs), egg masses exposed to *C. osakensis* had the second lowest emergence rate at 69% (100 emerged nymphs of 144 total eggs), egg masses exposed to *C. matsumurai* had the second highest emergence rate at 82% (108 emerged nymphs of 132 total eggs), and the negative control egg masses had the highest emergence rate at 90% (126 emerged nymphs of 140 total eggs) (Figure 2.4). Emergence rates of the egg masses exposed to *L. humile* were significantly lower than the negative control egg masses, and the emergence rates of the egg masses exposed to *L. humile, C. matsumurai* and *C. osakensis* were all similar. Egg mass emergence rates were also similar between egg masses exposed to *C. matsumurai, C. osakensis,* and the unexposed controls.

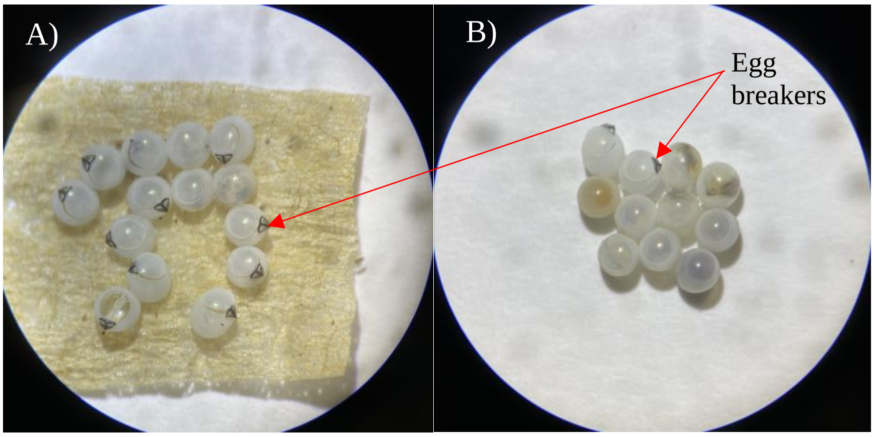

Figure 2.3 *H. halys* egg masses following 48hr exposure to: A) *C. matsumurai*; or B) *L. humile*. Egg breakers, designated by the red arrows, indicate a nymph emerged from the egg.

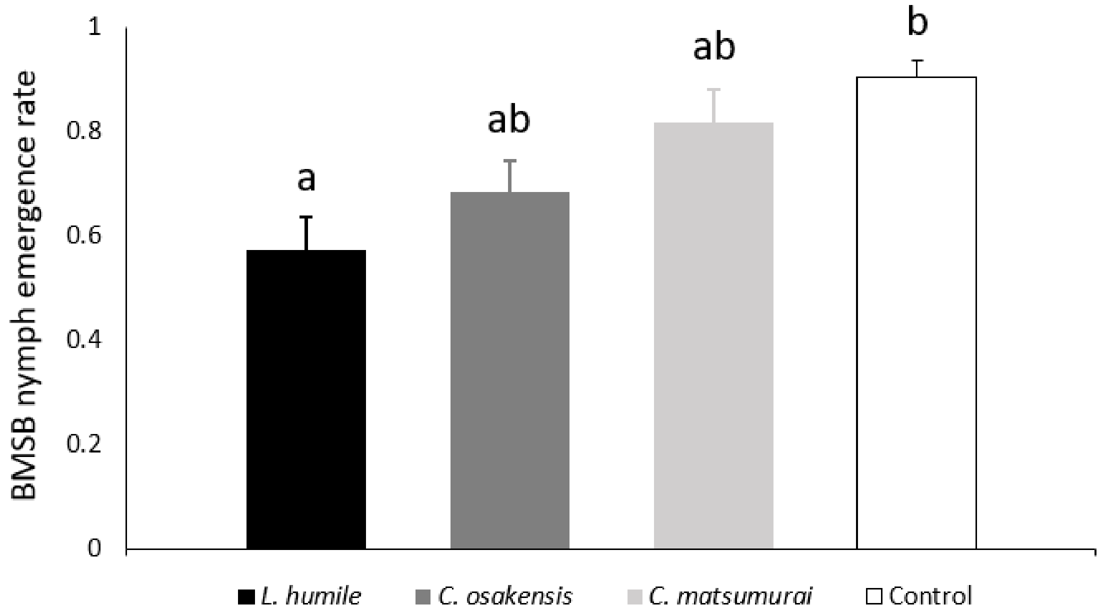

Figure 2.4 Emergence rate (±SE) with standard error bars of H. halys egg masses following 48hr exposure to *L. humile*, *C. osakensis*, *C. matsumurai*, or controls. Different lowercase letters indicate significant differences of emergence rates at $p < 0.05$ (Tukey's HSD).

All ant species were observed actively subduing, killing, then consuming *H. halys* nymphs, with varying levels of success between ant species and nymphal instar stage (Figure 2.5). During successful predation, the *C. matsumurai* workers individually fragmented the still-living *H. halys* nymphs, then brought back the smaller portions of the nymph (legs, antennae, etc.) to the main colony. The *C. osakensis* and *L. humile* workers primarily collectively swarmed and killed the nymphs first, then brought the prey back to the main colony. The survival rates of *H. halys* generally increased from first instar to fifth instar after exposure to *C. matsumurai* and *C. osakensis* with no first instars surviving and 100% of the fifth instars surviving (Table 2.1). All nymphs at each *H. halys* instar stage were preyed upon at 100% after the 48h exposure to the positive control group, *L. humile*. The survival rate was similar across all stages of *H. halys* from the unexposed negative control groups, near 100% survival throughout experimentation.

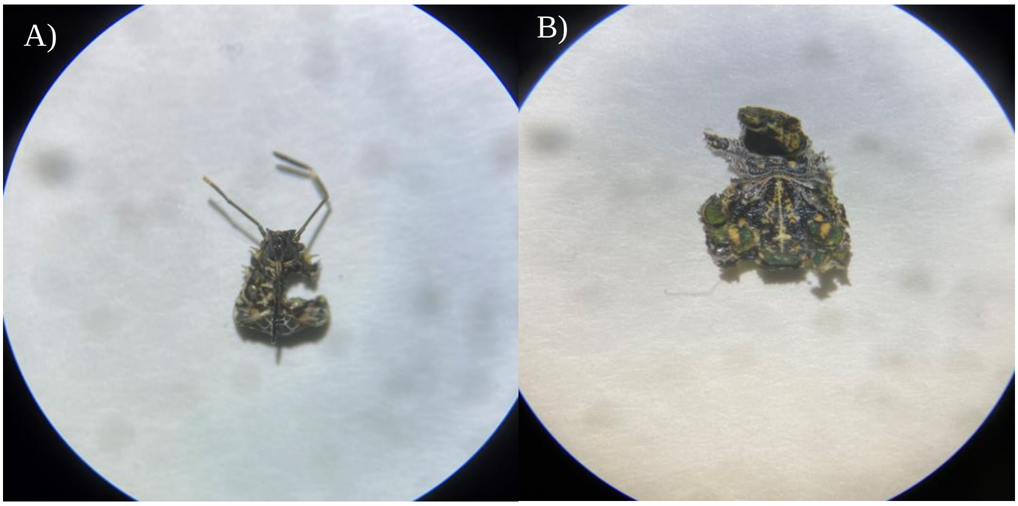

Figure 2.5 A) Third instar *H. halys* nymph following 48hr exposure to *C. matsumurai*; B) Fourth instar *H. halys* nymph following 48hr exposure to *L. humile*.

Table 2.1 Survival rate (±SE) of *H. halys* nymphs after exposure to ants and controls

		Ant species				
H. halys metric	Instar Stage	*L. humile*	*C. osakensis*	*C. matsumurai*	Control	ANOVA
Survival rate $n = 30$	First	0 ± 0 a; A	0 ± 0 a; A	0 ± 0 a; A	1 ± 0.04 a; B	$F_{4,100}$ = 151.7; $p < 0.001$
	Second	0 ± 0 a; A	0.1 ± 0.07 a; A	0.4 ± 0.05 b; B	1 ± 0.03 a; C	
	Third	0 ± 0 a; A	0.6 ± 0.07 b; B	0.7 ± 0.04 c; B	1 ± 0 a; C	
	Fourth	0 ± 0 a; A	0.7 ± 0.07 b; B	0.9 ± 0.04 d; C	1 ± 0 a; C	
	Fifth	0 ± 0 a; A	1 ± 0 c; B	1 ± 0 d; B	1 ± 0 a; B	
ANOVA			$F_{3,100}$ = 703.3; $p < 0.001$			

Different lowercase letters within a column designate significant differences in *H. halys* survival rate between instar stages at $p < 0.05$ (Tukey's HSD). Different uppercase letters within a row designate significant differences in *H. halys* survival rate between ant species and the negative control at $p < 0.05$ (Tukey's HSD).

2.4 Discussion

Overall, *C. matsumurai* and *C. osakensis* did not consume any of the *H. halys* egg masses. This result is similar to the findings from lab experiments done with the European native ant, *Crematogaster scutellaris*, which also was not recorded in consuming *H. halys* eggs [4]. In addition to the lack of predation, the Japanese *Crematogaster* ants also did not have a significant effect on reducing the *H. halys* nymphal emergence from egg masses in comparison to the unexposed control egg masses. One explanation why *C. matsumurai* and *C. osakensis* did not consume any *H. halys* eggs through the course of the study may be chemically based. It has been documented that *H. halys* eggs masses contain chemicals that manipulate the behavior of other insects, including repelling some species of parasitoid wasps (e.g., *Telenomus podisi* and *Trissolcus erugatus*) [30]. It is conceivable that the natural chemicals on the *H. halys* eggs exposed to the ants in the experiments provided a protective safety barrier, causing the ants to disregard the eggs as a food source. However, several other generalist chewing predators including Orthoptera, Dermaptera, Coleoptera, and Neuroptera have been noted in consuming *H. halys* egg masses [10,12]. Also, another species of *Crematogaster, C. rogenhoferi*, has been observed successfully rupturing and consuming the eggs of litchi stink bug (*Tessaratoma papillosa*) during field observations [9]. *Crematogaster rogenhoferi* is another arboreal ant species native to southeast Asia including parts of China and Taiwan, and shares the same subgenus (Crematogaster) as *C. matsumurai* [31]. Our study, along with similar research, suggests that ants in general have difficulty consuming *H. halys* eggs, as ants from *H. halys'* native range (*C. matsumurai, C. osakensis*) as well as ants from its invaded range (*C. scutellaris, L. humile*) do not consume *H. halys* eggs [4]. Given these conclusions, we concede that *Crematogaster* ants probably do not provide significant contributions to the natural control of the egg stage of *H. halys* in Japan. Egg parasitoid wasps specifically have been recognized as being much more effective sources of management against *H. halys* at the egg stage according to studies done in Japan and China [32,33,34]. These parasitoids are capable of producing high rates of parasitism on H. halys eggs in the field, up to 80.1% in one species of parasitoid, *Trissolcus japonicus* [34].

The native Japanese *Crematogaster* ants had much more success consuming *H. halys* nymphs as opposed to their eggs. However, the predatory ability of the *Crematogaster* ants was reduced significantly when exposed to later instar nymphs, with survival rates of fourth and fifth instar *H. halys* ranging from 93% to 100% for *C. matsumurai* and from 67% to 100% for *C. osakensis*. This result is similar to the findings of a laboratory study that determined *C. scutellaris* can reduce *H. halys* early (first and second) instar survival to 9% and later (third through fifth) instars to 50% [4]. First instar *H. halys* nymphs, in addition to being smaller and more vulnerable to predation than later-staged nymphs, commonly aggregate together and remain stationary following eclosion [35]. Also, second instar *H. halys* nymphs were determined to have significantly shorter walking distances than third and fourth instars during laboratory experiments [33]. The relatively stationary nature of the first and second instar nymphs likely caused them to serve as more accessible forms of prey compared to later instars. This provided the *Crematogaster* ants a greater opportunity to organize a collaborative attack, ultimately lowering the survival rates. Conversely, the more mobile and mature third through fifth instar nymphs were better suited to evade predation attempts, which likely resulted in the higher survival rates. Third instar *H. halys* nymphs are also capable of producing similar amounts of natural semiochemicals as adults [36], and these volatiles can act as defensive compounds which repel and prompt avoidance from generalist predators [37,38]. Such semiochemicals may have been emitted from the later instar *H. halys* nymphs during their exposure to *C. matsumurai* and *C. osakensis*, further contributing to the low predation rates. Although the Japanese *Crematogaster* ants showed difficulty in controlling the survival of late instar *H. halys* nymphs, the ants were successful in consuming the early instars suggesting they probably do provide natural control of *H.*

halys in its native region in some capacity. We also acknowledge that the *H. halys* nymphs being confined to an arena in a lab setting reduces their mobility advantage and decreases the resource input for the foraging ants, meaning the predatory ability of these ants may be reduced in a natural setting. Future field studies are necessary to confirm the presence and potential impact that these ants have against *H. halys* in agricultural areas

A somewhat unexpected, yet nonetheless noteworthy outcome from our research was the remarkable ability of *L. humile* to prey on and control *H. halys* nymphs at all instar stages. The *L. humile* colonies completely consumed every *H. halys* nymph exposed to them over every replicate, inducing a nymphal survival rate of 0%. The *L. humile* colonies were able to effectively prey on the later instar *H. halys* nymphs despite their increased mobility and defensive compound emissions. Unlike *C. matsumurai* and *C. osakensis, L. humile* were also able to reduce the nymphal emergence of the *H. halys* eggs in compassion to the unexposed control egg masses. Although no egg predation was recorded, the excessive probing and disrupting of the eggs by the ants may have accounted for the reduced hatch rates. Previous studies have determined that *L. humile* are adept and vigorous predators of a wide variety of insects including parasitoid wasps, moth larvae, and termites [26,40,41]. *Linepithema humile* have even been documented in the field as having a significant predatory effect on pest insects including the Mediterranean fruit fly and pine processionary moth [25,26]. However, *L. humile* is also considered an invasive pest itself outside of its native range of Argentina [41,42]. Due to their aggressive nature and exploitative competitiveness, *L. humile* are highly proficient at displacing native ants following an invasion, designating them unfit for any classical biological control prospects [43,44,45]. Still, this result evidences that ants in general should not be overlooked in their predatory ability and potential for implementation in biological control programs, provided the appropriate situation.

The outcome of our research holds importance in devising novel integrated pest management strategies against the invasive *H. halys* by exploring the predatory success of natural enemies in the pest's native range. Overall, *C. matsumurai* and *C. osakensis* did not consume or negatively influence the emergence rates of *H. halys* eggs. As a result of this predatory ineffectiveness at the egg stage, it is expected other natural enemies are involved in the multifaceted biological control community managing *H. halys* in its native region [32,34]. The Japanese *Crematogaster* ants did demonstrate a significant ability to control early (first, second, and third instar) *H. halys* nymphs, indicating they potentially play a role as a natural enemy against the pest at the early nymph life stage. We propose that *C. matsumurai* and *C. osakensis* are not the main agents of biological control against *H. halys* in its native region, but instead potentially work in an additive effort with other natural enemies such as egg parasitoids which are more successful in managing *H. halys* at different life stages. Future research efforts should focus on identifying effective native biological agents of *H. halys* in its egg, late-instar, and adult stages to maximize the efficiency of controlling methods in invaded regions.

2.5 References

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Chapter 3: Seasonal parasitism of native egg parasitoid of *Halyomorpha halys*

3.1 Introduction

Chemical control remains the most prevalent form of management against *Halyomorpha halys* in its non-native range [1,2]. However, pesticides are seen as a short-term solution as they can be expensive both monetarily and environmentally [3,4]. Frequency of insecticide applications targeting *H. halys* have increased by nearly four times dating back to when the pest first began inflicting economic injury in invaded regions [1]. The increased use of insecticides throughout the field season has also led to secondary pest outbreaks in agricultural settings [5]. In addition, *H. halys* has been shown to develop tolerance after exposure to several insecticides in lab trials [6]. 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*. Lab experiments have determined generalist predators have varying levels of success in providing control against different life stages of *H. halys* [7, 8,9]. 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 [5,10]. 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 [11]. Parasitoids from the genera *Trissolcus, Telenomus, Anastatus, Ooencyrtus*, and *Gyron* have been documented to successfully parasitize sentinel *H. halys* egg masses in the US [12,13,14]. Similarly, a wide range of parasitoids form the genera *Trissolcus, Telenomus, Anastatus, Ooencyrtus,* and *Acroclisoides* have been recorded parasitizing sentinel egg masses in Switzerland and Italy [15,16,17]. However, the parasitism rates inflicted by these indigenous egg parasitoids from North America and Europe are generally low, below 5% across these field studies [11,13].

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 [10,18], although species in the *Telenomus, Ooencyrtus,* and *Anastatus* genera have also been identified in successfully parasitizing *H. halys* egg masses [19,20]. Field assessments conducted in kiwifruit in central China revealed a native *H. halys* parasitoid species composition of 41% *Trissolcus* japonicus and 48% *T. cultratus* [20], whereas surveys conducted in mixed fruit orchards in northern China displayed a species composition consisting of over 90% *T. japonicus* [19]. The field parasitism rate of *T. japonicus* on *H. halys* ranges from 50 – 80% as determined by sentinel egg mass deployment in China [19,21], and is over 90% in lab experiments [15]. Programs testing *T. japonicus* as an appropriate biological control agent against *H. halys* are under evaluation worldwide [22,23,24,25,26,27].

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 [28,29]. Other gaps in knowledge pertaining to *H. halys* parasitoids in Asia also exist. For example, there is limited published information available describing behavior 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.

3.2 Materials and methods

3.2.1 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 (40cm x 15cm x 20cm) (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 ± 1ºC and a relative humidity of roughly 70% in a temperature controlled room.

3.2.2 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 0-24hr were fixed to 3cm x 2cm pieces of cardstock via doublesided sticky tape [25] (Figure 3.1). 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 -20ºC freezer and frozen for at least one week before field deployment [14]. Sentinel cards were left in the field for 72hr, and then returned to the lab. Upon their return, egg masses were transferred to a clear plastic snap cap vial (4.0cm x 2.3cm) (SKS Science, Watervliet, NY, U.S.A.). Daily observations were made of the returned egg masses for one month to observe *H. halys* nymph or parasitoids emergence [13]. Sentinel egg masses were then allowed another two months of rearing 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) [30,31]. All egg masses were reared at a temperature of 24 ± 1ºC and a relative humidity of roughly 70% in a temperature controlled room.

Figure 3.1 Sentinel *H. halys* egg cards. Top: Cold-treated (frozen) set; Bottom: Fresh set.

Twenty control fresh egg masses were constructed in the same manner as the fresh sentinel field egg masses, then reared in a lab setting in order to compare *H. halys* nymphal emergence rates between the field-deployed and lab-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 lab 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. *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 24hr of age in a clear plastic snap cap vial. Female *T. japonicus* were removed following 48hr 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 ± 1ºC and a relative humidity of roughly 50% in a temperature controlled room.

3.2.3 Parasitoid identification

Initial identification of emerged parasitoid wasp genera was determined morphologically through the presence/absence of key features [32,33,34,35]. The species of emerged parasitoid wasps was then confirmed through molecular identification for each sentinel egg mass that was successfully parasitized in the field (had at least one fully emergent adult parasitoid). Genomic DNA was isolated from a whole parasitoid using a commercial QIAamp DNA extraction kit (QIAGEN, Germantown, MD, U.S.A.) 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 [36]. 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 [37]. All parasitoid CO1 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.

3.2.4 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 [38], 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 was used to help relate field parasitism activity with seasonality in Kyoto.

3.2.5 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 [19]. 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 [39]. The GLMs estimated the effect seasonality (spring, winter, fall), egg mass type (fresh, frozen), and the seasonality x 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 lab-reared and field-deployed fresh sentinel egg masses, and 3) the difference in *T. japonicus* parasitism rates between fresh sentinel and lab-reared control egg masses at $p < 0.05$.

3.3 Results

In total, 228 fresh and frozen sentinel *H. halys* egg masses (5,343 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 (Figure 3.2). Of the successfully parasitized egg masses, 543 parasitoids emerged from 930 eggs (58.4%) (Table 3.1).

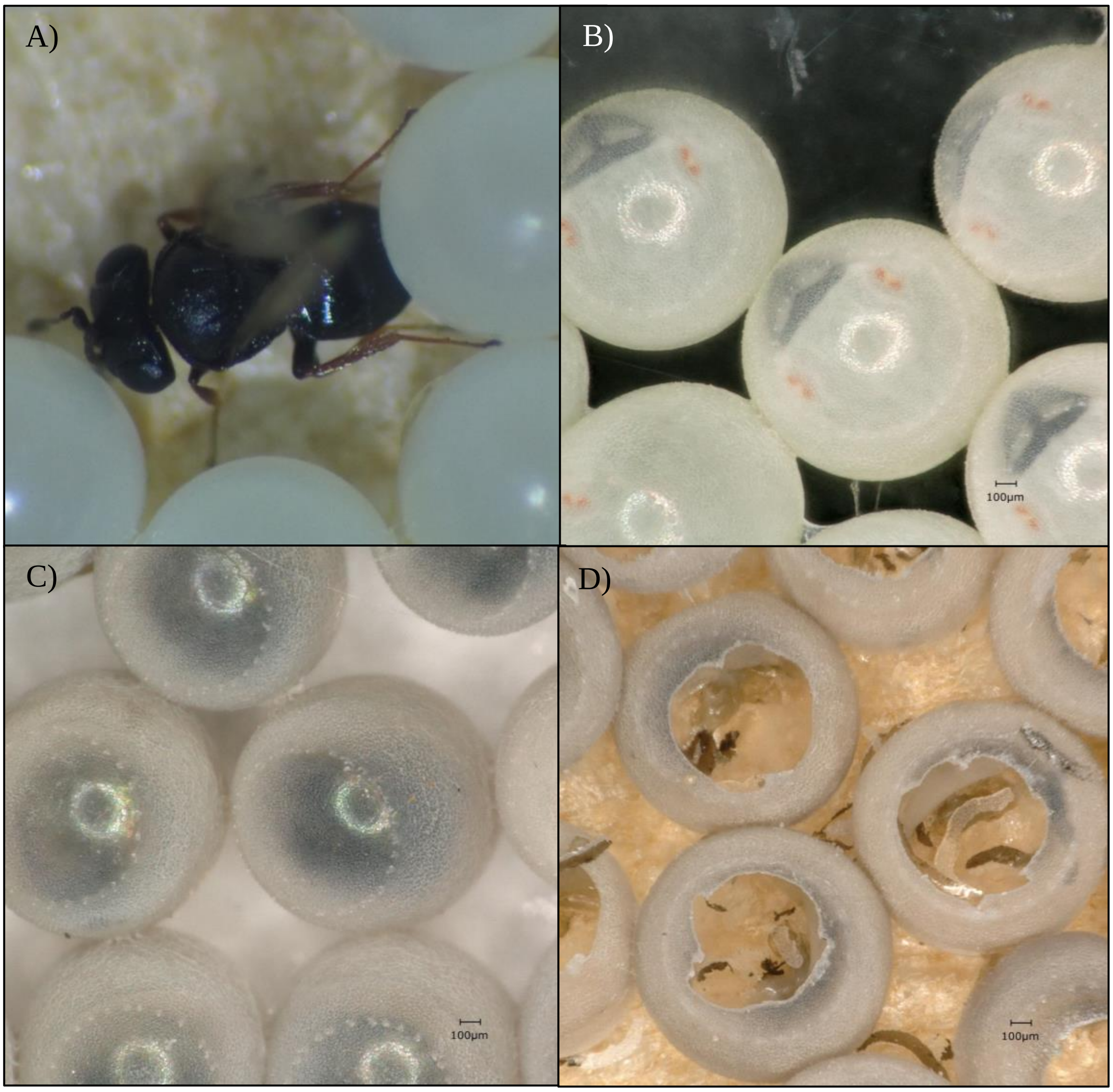

Figure 3.2 A) Egg parasitoid *T. japonicus* parasitizing *H. halys* egg mass; B) Non-parasitized *H. halys* eggs (egg breakers forming); C) Parasitized *H. halys* eggs (nymphal parasitoids developing); D) *H. halys* eggs post parasitoid emergence (top of eggs chewed through).

Table 3.1 Parasitism rate of native *H. halys* egg parasitoids

		Season			
Species	Egg mass type	Spring	Summer	Fall	Entire season
T. japonicus	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
T. cultratus	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
T. mitsukurii	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
T. comperei	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
A. japonicus	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
Trissolcus 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
Ooencyrtus 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

Parasitism rate (± SE) of native parasitoids determined from parasitized fresh and frozen field exposed sentinel *H. halys* egg masses in Kyoto, Japan in 2020. (Spring: March – May, Summer: June – August, Fall: September – December).

The first record of successful parasitism from the sentinel field *H. halys* egg cards occurred in early spring from a fresh egg mass (Figure 3.3). 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 10ºC and 13ºC, with a maximum temperature of 21ºC. Throughout the year, average daily temperatures rose from early spring until late summer, then began to lower until late fall.

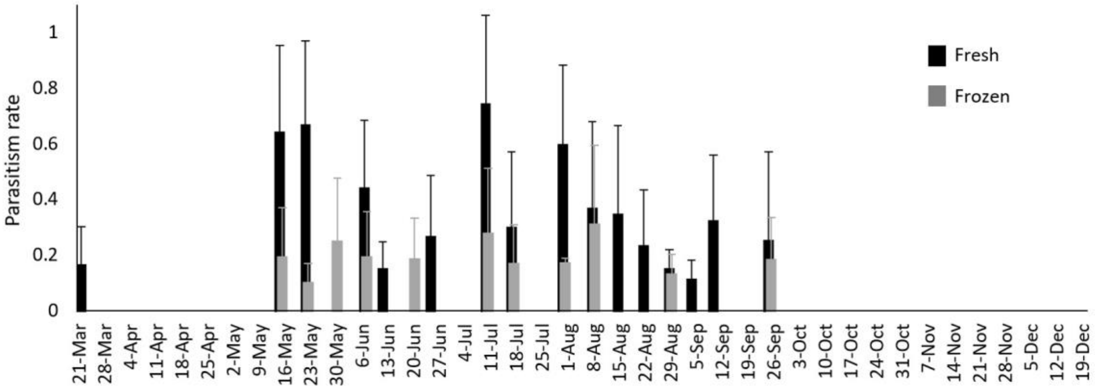

Figure 3.3 Mean weekly parasitism rates with SE bars of native parasitoids determined from parasitized fresh and frozen field exposed sentinel *H. halys* egg masses in Kyoto, Japan in 2020.

Nymphal *H. halys* emerged from the fresh sentinel egg masses every week from early spring to late fall (Figure 3.4). 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 lab-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.

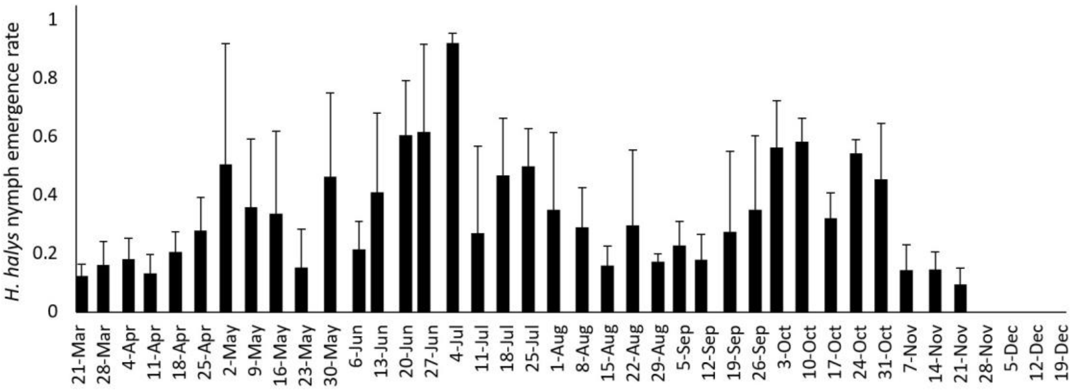

Figure 3.4 Mean weekly emergence rates of *H. halys* nymphs with SE bars from fresh field exposed sentinel *H. halys* egg masses in Kyoto, Japan in 2020.

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 CO1 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*. 631 bp sequence (Accession number: MZ503514) 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) (Figure 3.5). 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) (Figure 3.6). The only parasitoid that parasitized a sentinel egg mass (fresh or frozen) during each season was *T. japonicus*.

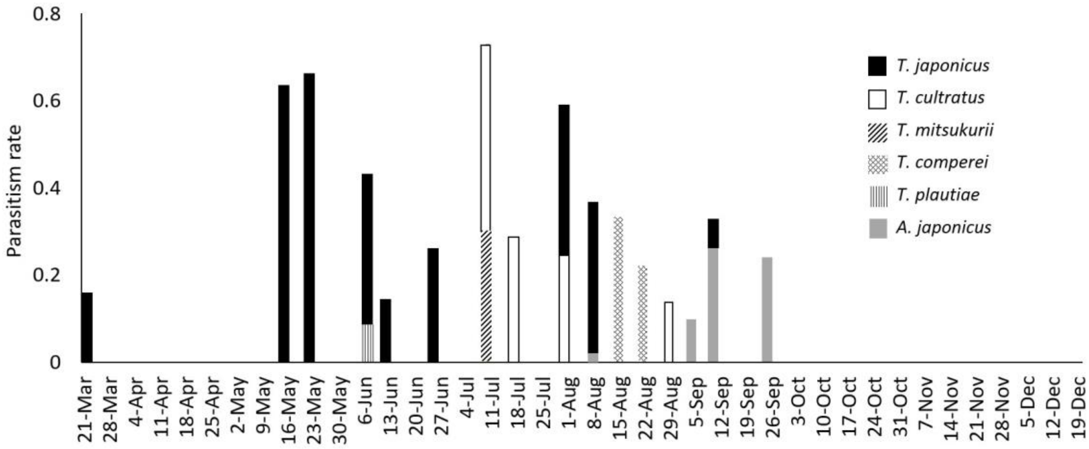

Figure 3.5 Weekly parasitoid species composition and parasitism rates of native parasitoids determined from parasitized fresh field exposed sentinel *H. halys* egg masses in Kyoto, Japan in 2020.

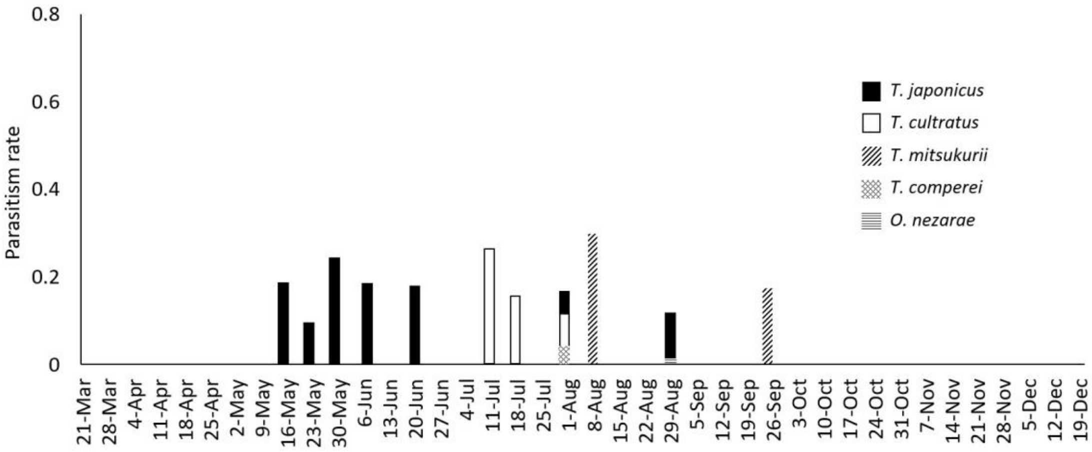

Figure 3.6 Weekly parasitoid species composition and parasitism rates of native parasitoids determined from parasitized frozen field exposed sentinel *H. halys* egg masses in Kyoto, Japan in 2020.

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 3.2). The pooled data also 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 3.1). 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 lab 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%).

Table 3.2 Effect of seasonality and egg mass type on parasitism rate as estimated by the generalize linear model

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

Parasitism rate (± SE) of native parasitoids determined from parasitized fresh and frozen field exposed sentinel H. halys egg masses in Kyoto, Japan in 2020. (Spring: March – May, Summer: June – August, Fall: September – December).

Similarly, pooling the data showed that seasonality did not have a significant on the parasitoid species 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 3.3). *Trissolcus japonicus, T. cultratus, T. mitsukurii,* and *T. comperei* all successfully emerged from both fresh and frozen sentinel egg masses (Table 3.4). *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.

Table 3.3 Effect of seasonality and egg mass type on parasitoid species composition as estimated by the generalize linear model

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

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

Table 3.4 Species composition of native *H. halys* egg parasitoids

		Season			
Species	Egg mass type	Spring	Summer	Fall	Entire season
T. japonicus	Fresh	100%	42.2%	41.2%	59.7%
	Frozen	100%	35.8%	N/A	49.0%
	Pooled	100%	40.2%	33.3%	56.7%
T. cultratus	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%
T. mitsukurii	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%
T. comperei	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%
A. japonicus	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%
Trissolcus 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%
Ooencyrtus 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%

Parasitoid species composition of native parasitoids determined from parasitized fresh and frozen field exposed sentinel *H. halys* egg masses in Kyoto, Japan in 2020. (Spring: March – May, Summer: June – August, Fall: September – December).

3.4 Discussion

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 [10,40,41]. 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 US invariably reported the first parasitism of *H. halys* in May [14,17,19]. 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 12.2ºC when reared on *H. halys* eggs [21], and 12.4ºC when reared on *Glaucias subpunctatus* eggs, another phytophagous Pentatomidae native to Japan [42]. 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 [42]. 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 [43,44]. *Trissolcus biproruli* (Hymenoptera: Scelionidae), a parasitoid of spined citrus bug, demonstrated successful parasitism up to three weeks following initial mating [45]. Furthermore, lab and semi-field experiments revealed that adult *T. biprorufi* do not appear to enter a reproductive diapause while overwintering, and are capable of winter parasitism if exposed to suitable environmental conditions [45]. Yet, further research investigating the overwintering habits of *T. japonicus* is necessary to confirm if the initial female *T. japonicus* parasitized 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 US, and higher rates on frozen than fresh in the eastern US [12,14]. 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 [15,17]. 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 [46]. Parasitoids generally accomplish this by probing their ovipositor and injecting venom and/or symbiotic viruses into the host's eggs [47,48,49]. Endemic parasitoids often lack the ability to overcome an invasive host's defensive response to complete successful parasitism [15,17]. However, cold-sterilization has been reported to disable a host's natural defensive mechanisms, resulting in higher parasitism rates [15]. 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 [50,51,52]. The suitability for parasitoid development decreases with egg mass freezing [53], 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) [20]. The *H. halys* parasitoid species composition in northern China dominated by *T. japonicus* (90% of total species composition), with no other parasitoid species reaching 5% [19]. 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 [19,20]. 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 U.S. typically show more even distributions among *Anastatus sp., Ooencyrtus sp., Telenomus sp., and Trissolcus sp.* [11,13,14,16,17]. Regardless, the parasitism rate of H. halys egg masses by native parasitoid species in invaded regions is inconsequentially low, ranging between 2% and 7% [13,54]. 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 [31,55]. 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 [56].

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 to the lab reared controls. Studies which highlight the life cycle of *H. halys* denote the developmental temperature threshold for eggs to be within 15º and 33º C [57]. Overnight temperatures in March and April, as well as November and December, routinely fell below the lower temperature developmental limit for eggs, 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. Non-reproductive parasitism has commonly been observed in several groups of insects including Pentatomidae in field experiments [31,58,59]. Pseudoparasitsm 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 [49,58]. 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 [60,61].

This research reveals the native seasonal phenology and species composition of *H. haly*s 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 [62]. Therefore, we present these results to fill gaps in knowledge and supplement the groundwork for potential future programs in invaded regions.

3.5 References

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Chapter 4: Monitoring trap attractant comparisons and seasonal phenology of *Halyomorpha halys*

4.1 Introduction

Invasive pest species continually present major economic and environmental challenges to natural and agricultural systems around the world [1]. The estimated costs associated with nonindigenous insect pests such as environmental damages, agricultural losses, and management measures exceed $14 billion per year (USD) in the US alone [2]. Following the detection of an invasive species, effective and reliable monitoring methods are an essential component of integrated pest management (IPM) programs [3]. Understanding pest populations through field monitoring allows for more efficient implementation of control measures, including timing and frequency of pesticide applications [4]. However, invasive pest species monitoring can be difficult due to gaps in knowledge of a pest's behavior and ecology from their native range [3].

Establishing an effective yet dependable monitoring method is imperative for future control schemes of any invasive pest species [5]. However, reliably monitoring and detecting *H. halys* continues to be particularly challenging, stemming from the pest's cryptic nocturnal activity and excellent mobility [3,6,7]. Past studies in the US have focused on determining the most effective *H. halys* monitoring method by testing pheromone attractants, black light traps, sticky traps, sweep netting, and visual sampling [6,8,9,10,11]. Currently, black pyramid traps and sticky traps (clear and yellow) baited with the *H. halys* aggregation pheromone [12], in combination with the aggregation pheromone synergist, methyl (E, E, Z)-2, 4, 6-decatrienoate (MDT) from the brown-winged green bug, *Plautia crossota stali* [13,14], are the recommended monitoring method from trapping studies conducted in the eastern US [5,11,15]. However, this *H. halys* monitoring recommendation is specific to agricultural and orchard systems in a certain region, and ongoing research is being conducted to determine more efficient monitoring methods optimized for different settings [3,7]. In Japan, light traps and MDT pheromone traps appear to be the most common forms of *H. halys* monitoring, and the pest's phenology changes depending on the monitoring method [13,16]. From the limited published reports concerning the phenology of *H. halys* in Japan, light traps reveal peak abundance in the summer [16], whereas MDT pheromone traps evidence highest adult activity in the fall [17]. More information about the pest's phenology in its native range is necessary for refining current management practices. Also, the effectiveness of established monitoring and detection techniques may vary with abiotic and biotic factors, emphasizing the importance of investigating different *H. halys* trapping methods in different regions.

The Kyoto Prefecture Agriculture, Forestry, and Fisheries Technology Center has produced 11 years of monitoring data from black light, incandescent light, and pheromone traps from three locations in Kyoto, Japan. The monitoring data was comprised of five-day *H. halys* trap counts from May through October each year. This information is essential for filling gaps in knowledge pertaining to the phenology and monitoring methods of this invasive pest in its native range. Using this dataset, we first determined the seasonal population dynamics of *H. halys* in Japan. Then, we compared the mean adult *H. halys* trap counts from the black light, incandescent light, and pheromone monitoring traps. After evaluating over a decade's worth of *H. halys* monitoring data from Japan, we believe this work can contribute to the future management of this invasive pest species.

4.2 Materials and methods

4.2.1 Field sites

The *H. halys* adult monitoring traps were located at three agricultural research stations in Kyotanabe, Kameoka, and Kyotango City, Japan (Figure 4.1). Each agricultural facility stationed one black light trap, one incandescent light trap, and one pheromone trap. Kyotanabe is located in the southern portion of the Kyoto Prefecture, Kameoka in the central, and Kyotango in the northern portion. Each research station sparsely cultivated various field crops on a small scale for research purposes. These crops mainly included cucurbits and cereal grains such as rice. Pesticides were rarely applied to the crops; there were typically only one or two applications per year. A hand sprayer was used to apply the pesticides, which targeted general pest insects. Monitoring data was accumulated for 11 years from 2010 to 2020.

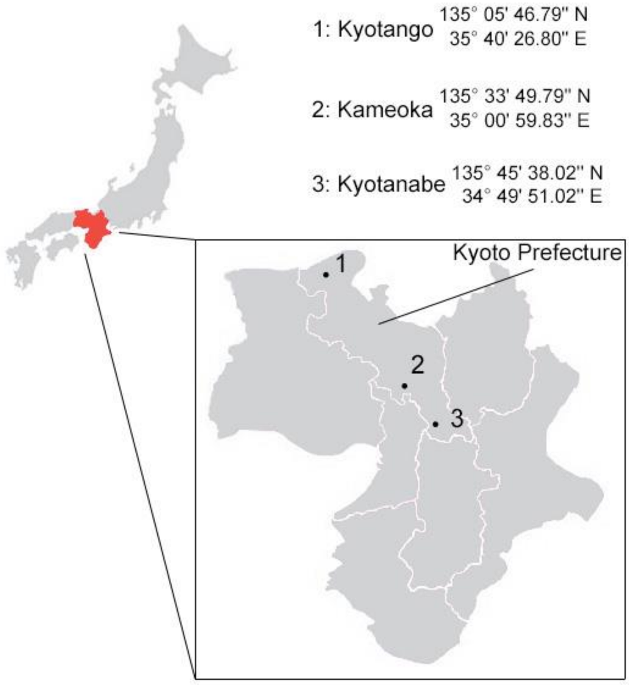

Figure 4.1 Map and GPS coordinates of the three *H. halys* monitoring sites. The three agricultural research were all located within the Kyoto Prefecture in Japan.

4.2.2 Black light and incandescent light monitoring traps

Two pest outbreak prediction light traps (model MT-7-N, Ikeda Scientific Co., Ltd., Tokyo, Japan) were used to monitor populations of adult *H. halys* at each of the three trapping locations. Light trap monitoring for *H. halys* took place from May 1 through October 31 at each site from 2010 to 2020. The steel light traps measured 92 cm x 92 cm x 200 cm and weighed 85 kg. At each site, the black light traps were installed with a 20-watt black light, and the incandescent light traps were installed with a 60-watt incandescent light. The black lights and incandescent lights were programmed to automatically turn on by a timer at 6:00 PM and turn off at 6:00 AM every day. The black light and incandescent light traps at each site were positioned in open areas near crop plantings, and spaced at least 50 m from each other. Each light trap had a catch basin that automatically rotated every five days, allowing each trap catches to be divided into separate five-day total counts. Upon a collection event, the monitoring trap contents were collected and the total number of adult *H. halys* were counted and recorded. A total of 36 individual five-day trap counts were recorded per year (six times each month, over the six-month trapping period).

4.2.3 Pheromone monitoring traps

One yellow plastic insect attractor trap (Senkei Chemical Co., Ltd., Kagoshima, Japan) was used to monitor adult *H. halys* populations at the three sites. Pheromone trap monitoring for *H. halys* took place from May 1 through October 31 at each site from 2010 to 2020. The plastic traps were baited with commercial *Plautia crossota stali* aggregation pheromone methyl (E, E, Z)-2, 4, 6-decatrienoate (MDT) lures (Senkei Chemical Co., Ltd., Kagoshima, Japan), and water was added to the trap basin to trap specimens. The *P. C. stali* pheromone lures are the most commonly used attractant lure used in Japan for monitoring adult *H. halys* [13,16]. Traps were hung from a steel pole roughly 1.5 m off the ground in open areas near crop plantings. Pheromone traps were spaced at least 50 m from light traps. The pheromone traps were checked every 7 – 10 days throughout the monitoring period each year, at which time specimens were removed from the trap basin and adult *H. halys* counted and recorded. Water was replenished in the basin upon every collection event, and pheromone lures were replaced every other month per the manufacturer's recommendation.

4.2.4 Data analysis

Five-day adult *H. halys* count totals from the black light and incandescent light traps were recorded from 2010 to 2020 for each of the three sites. In the case of the pheromone traps, the adult *H. halys* trap count was standardized to every five days to conform with the five-day collection frequency of the light traps. Data from all years and sites were pooled for analysis.

The dataset was analyzed using the RStudio program [18]. In order to compare the ability of the different traps throughout the field season, the trapping period each year was divided into three seasons: early summer (May – June), mid-summer (July – August), and fall (September – October). The data did not conform to the assumptions of a normal distribution due to a high amount of zero trap counts. Chi-square analyses were deemed appropriate for the zero-inflated data [19,20], and a chi-square test was run to determine differences in trap counts between the season and trap type factors. Estimated marginal means [21], for the trap counts were produced using the *emmeans* function from the *emmeans* package. The *pairs* function was then used to group the means, and a '*tukey*' adjustment was incorporated in the post-hoc analysis to separate the means of the factors at $p < 0.05$.

A zero-inflated negative binomial (ZINB) model was generated using the *glmmTMB* function from the *glmmTMB* package using the RStudio program to correct for the overdispersion caused by an overabundance of zero counts in the monitoring data. The model implemented a logit-link from the family '*nbinom2*'. This framework allowed us to estimate the relative effectiveness each monitoring trap type (black light, incandescent light, pheromone lure) had on attracting *H. halys* adults, as well as the influence each trap type had on producing a zero count. The ZINB incorporated the factor 'trap type' as a random effect, and the factors 'site', 'year', and the 'site * year' interaction as fixed effects. A reduced ZINB model containing only the effect of trap type was also produced. A likelihood ratio test was performed on the full and reduced ZINB models, and the full model was deemed as the best fitting model ($\chi^2 = 157.04$, $p < 0.001$) [22]. Compared to the reduced model, the full model also had lower Akaike information criterion (AIC) and Bayesian Information Criterion (BIC) scores, further evidencing it as the more parsimonious model [22]. The ZINB generated coefficient estimates for each trap type in a conditional model and a zero-inflation model. In the conditional model, a positive coefficient estimate indicates higher *H. halys* abundance in response to trapping method, whereas a negative coefficient estimate indicates lower abundance [23,24]. In the zero-inflation model, a positive coefficient estimate indicates a higher chance of encountering a zero *H. halys* count in response to trapping method, whereas a negative coefficient estimate indicates lower chance of encountering a zero count [25].

4.3 Results

In total, there were 3,049 instances of a recorded zero count from the *H. halys* monitoring traps out of 3,551 total collection events across all trap types, sites, and years. At the Kyotanabe site, no *H. halys* were recovered from the incandescent light traps in 2010 and 2011, and none were recovered from the pheromone traps in 2010, 2011, 2013, 2015, 2017, 2018, and 2019. At Kameoka, no *H. halys* were recovered from the incandescent light traps in 2010, 2011, and 2013, and none were recovered from the pheromone traps in 2011, 2012, and 2013. At Kyotango, no *H. halys* were recovered from the incandescent light traps in 2010, and none were recovered from the pheromone traps in 2015, 2018, and 2020. The single high adult *H. halys* count was 63 for the black light traps, 21 for the incandescent light traps, and 2.1 (standardized for a five-day period) for the pheromone traps. The total mean five-day adult *H. halys* count was 1.1 for the black light traps, 0.2 for the incandescent light traps, and 0.05 for the pheromone traps (Table 4.1).

Table 4.1 Mean (±SE) five-day adult *H. halys* trap counts

	Season			
Trap type	Early summer	Mid-summer	Fall	Total
Black light	0.08 ± 0.02 A; a	2.99 ± 0.35 A; b	0.20 ± 0.04 A; a	1.09 ± 0.12 A
Incandescent	0.07 ± 0.02 A; a	0.45 ± 0.08 B; b	0.03 ± 0.01 B; a	0.22 ± 0.03 B
Pheromone	0.09 ± 0.01 A; a	0.02 ± 0.01 C; b	0.03 ± 0.01 B; b	0.05 ± 0.01 C
Chi-square	$\chi^2 = 4.86$; $p = 0.09$	$\chi^2 = 45.69$; $p < 0.001$	$\chi^2 = 23.91$; $p < 0.001$	$\chi^2 = 46.08$; $p < 0.001$

Different uppercase letters within a column indicate significant differences in mean five-day adult *H.* halys trap counts between trap types (Tukey’s HSD test: $p < 0.05$). Different lowercase letters within a row indicate significant differences in mean five-day adult *H. halys* trap counts between seasons (Tukey’s HSD test: $p < 0.05$).

Across the entire monitoring period, the black light traps produced the highest five-day mean adult *H. halys* trap count, the incandescent light traps had the second highest mean count, and the pheromone traps produced the lowest mean count ($\chi^2 = 46.08$, $p < 0.001$) (Table 4.1). All three trap types had similar counts in the early summer ($\chi^2 = 4.86$, $p = 0.09$). The black light traps had the highest trap counts in the mid-summer ($\chi^2 = 45.69$, $p < 0.001$), and also in the fall ($\chi^2 = 23.91$, $p < 0.001$). Black light traps produced higher counts in the mid-summer compared to the early summer and fall ($\chi^2 = 61.02$, $p < 0.001$). Similarly, incandescent light traps had higher counts in the mid-summer than the early summer and fall ($\chi^2 = 55.15$, $p < 0.001$). Pheromone traps had higher trap counts in the early summer than the mid-summer and fall ($\chi^2 = 13.44$, $p = 0.001$).

The ZINB conditional model produced a positive coefficient estimate for the black light traps ($Z = 5.00$, $p < 0.001$), and negative coefficient estimates for the incandescent light ($Z = -2.40$, $p < 0.001$) and pheromone traps ($Z = -5.20$, $p = 0.02$) (Table 4.2). The ZINB zero-inflation model produced negative coefficient estimates for black light ($Z = -7.34$, $p < 0.001$) and incandescent light traps ($Z = -7.75$, $p < 0.001$), and a positive coefficient estimate for pheromone traps ($Z = 3.37$, $p = 0.001$).

Table 4.2 Conditional and zero-inflation model diagnostics

	Trap type	Estimate	SE	*Z*-value	*p*-value
Conditional model	Black light	1.03	0.21	5.00	< 0.001
	Incandescent light	-0.51	0.21	-2.40	< 0.001
	Pheromone lure	-0.91	0.17	-5.20	0.02
Zero-inflation model	Black light	-2.28	0.31	-7.34	< 0.001
	Incandescent light	-2.61	0.34	-7.75	< 0.001
	Pheromone lure	1.38	0.41	3.37	0.001

Coefficient estimates with standard error, and Wald z-statistics of the trap types produced by the zero-inflated negative binomial analysis on the adult *H. halys* monitoring dataset. The conditional model represents the effect trap type had on the abundance of *H. halys* (+ estimates relate to higher abundance, - estimates relate to lower abundance), and the zero-inflation model represents the effect trap type had on producing a zero count (+ estimates relate to a higher probability of influencing a zero count, - estimates relate to a lower probability of influencing a zero count).

A plot of the mean five-day trap catch data separated by trap type with site and year pooled showed that *H. halys* populations increase from the early summer until they peak in mid-summer, then quickly drop in the fall as measured by the black light traps

(Figure 4.2). Similarly, the incandescent light traps had increasing trap counts from the early summer through mid-summer, then lower trap counts in the fall. However, the peak trap count as measured by the incandescent light traps was more dampened and drawn out across mid-summer in comparison to the sharper peak produced by the black light traps. The pheromone traps had relatively low adult *H. halys* counts throughout the field season, but appeared to have highest trap counts in the early summer.

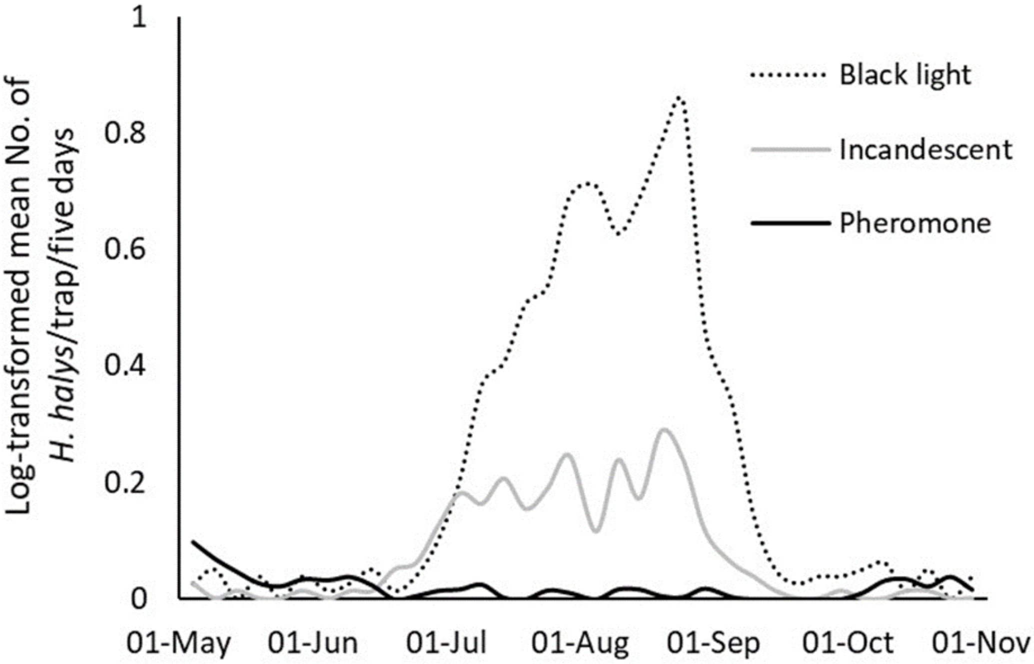

Figure 4.2 Log-transformed mean five-day *H. halys* trap catch across all sites and years for each trap type. A smoothed fit line is included to help visualize the population trends of *H. halys* for each trap type.

4.4 Discussion

The pursuit of identifying a standard, adoptable *H. halys* monitoring method which is both practical and reliable is still ongoing [7,11]. An added caveat to this issue is that it is difficult to effectively compare the phenology of *H. halys* when measured by different monitoring techniques. Implementing different trapping methods can have a significant effect on trap counts. For example, light traps with lamps emitting more UV light are more attractive to *H. halys* than standard incandescent light traps [26], and monitoring traps baited with aggregation pheromone lures alone attract less *H. halys* than traps including a pheromone synergist [27]. This study therefore establishes a seasonal phenology of adult *H. halys* in Kyoto, Japan based on adult monitoring using black light, incandescent light, and pheromone traps, and compared the mean trap counts between each respective monitoring method.

Overall, the seasonal phenology of adult *H. halys* in Kyoto is similar to the previously reported population dynamics of *H. halys* in Japan, which is described by low populations in the late spring/early summer, reaching peak activity in the late summer, then numbers dropping in the fall [16,28]. However, the population dynamics slightly differed between the trapping methods. The black light and incandescent light trap data revealed *H. halys* numbers are the highest in the mid-summer, which is comparable with previous light trap monitoring studies conducted in Japan showing *H. halys* captures typically peak in early August [16]. Black light trapping studies of *H. halys* in the Mid-Atlantic US also showed a bell-shaped population trend, with adult activity peaking in the mid-summer [6]. Unlike the light traps, the *H. halys* trap counts as measured by the *P. C. stali* aggregation pheromone synergist (MDT) traps were higher in the early summer than the mid-summer and fall. A similar monitoring study done with the MDT pheromone traps in northern Japan (Akita Prefecture) showed fluctuating *H. halys* numbers throughout the season, with a peak in activity in mid-May [17]. Monitoring research in the eastern US using the MDT pheromone in combination with *H. halys* aggregation pheromone lures also displayed early season spikes in adult *H. halys* captures in late April [29], and May [11].

The black light traps performed better than the incandescent light traps in capturing adult *H. halys*, as the black light traps had higher mean trap counts in the mid-summer and fall, as well as the highest overall mean trap count across the entire field season in Kyoto. Black light traps emit large amounts of UV radiation, which has been shown to be more attractive to nocturnal insects than light emitted with higher wavelengths (incandescent light) [30]. The increased affinity towards light lower on the wavelength spectrum also applies to *H. halys*, as evidenced by a field study which demonstrated that applying a UV-absorbing filter to UV light traps reduced their *H. halys* attractiveness by 75% compared to non-filtered traps [26]. Additionally, black light traps have been determined to be a reliable approach in tracking the spread of *H. halys* at the landscape level in the US [6]. In the current study, black light traps were the most effective trap type for producing high *H. halys* trap counts in Kyoto, as they recorded the highest mean five-day counts over the course of the field season. The black light traps also were the only trap type to generate a positive coefficient estimate in the conditional model (Table 4.2), suggesting the black light traps had a positive correlation with increased *H. halys* abundance.

The MDT pheromone traps produced the lowest mean *H. halys* trap counts over the course of the entire field season, but had numerically higher trap counts than the black light and incandescent light traps in the early summer when the *H. halys* field populations were low (Table 4.1). This result differs from previous research in the US and Japan which indicates that MDT pheromone lures have limited value early in the season when *H. halys* population densities are low, and are most attractive to *H. halys* adults late in the season (reviewed in [31]). There is existing evidence of pheromone traps being effective in detecting *H. halys* early in the field season, as MDT pheromone traps caught *H. halys* adults earlier than sweep net samples in the eastern US [8].

Additionally, monitoring work in Korea reported first adult *H. halys* capture from MDT pheromone traps as occurring in May, one month earlier than first captures from light traps which occurred in June [32]. In our work, the MDT pheromone traps had the highest probability of encountering a zero count as evidenced by the zero-inflation model (Table 4.2), and did not capture the mid-summer *H. halys* abundance peak that both types of light trap did. However, the pheromone traps were able to reliably capture *H. halys* adults in the early summer in Kyoto, indicating MDT lures may hold importance in the early season detection of *H. halys*.

The goal of this study was to compare the effectiveness of commonly used monitoring traps of adult *H. halys* in Japan, not to identify the single best trapping method. We acknowledge the fundamental differences in trapping methods, and understand environmental variables can affect trap efficacy. For example, light traps have a variable effective attraction distance for nocturnal insects, which can range from 2 m to as far as 30 m (reviewed in [33]), and several factors such as ambient light and cloud cover can also influence light trap effectiveness [34]. Pheromone traps baited with *H. halys* aggregation pheromone and MDT synergist and have a wider effective range for adult *H. halys*, up to 130 m in an open field [35], and similarly factors such as landscape type and wind speed can influence pheromone trap effectiveness [31,36]. Seasonality also appears to have a significant effect on the trap preference of adult *H. halys*, as light traps captured similar numbers of *H. halys* adults to pheromone traps early in the season in the Mid-Atlantic US, but captured more *H. halys* adults than pheromone traps during the mid-season [37]. Similarly in Kyoto, light and pheromone traps had similar adult *H. halys* counts in the early summer, but light traps had higher counts than pheromone traps in the mid-summer (Table 4.1). More recently, researchers have experimented with baiting traps with pheromone lures in combination with lights, as well as baiting traps with both *H. halys* aggregation and MDT pheromone, having variable results [7,27,37]. The affinity of *H. halys* towards different attractants throughout the field season suggests a single, best monitoring trap may not exist, and rather monitoring methods should be adapted to best suit a given locality and seasonality.

The preference of *H. halys* towards different monitoring attractants during different periods of the field season may not only be a result of environmental influence, but also the life cycle of *H. halys*. Abiotic variables such as temperature, photoperiod, and relative humidity have been determined to affect the behavior of *H. halys* [38,39,40]. After breaking diapause early in the field season, female *H. halys* require a preoviposition period of about 13 days in which a critical photoperiod is needed for ovarian development [41,42]. Adult *H. halys* then move in large numbers and aggregate on host crops to feed and replenish carbohydrates lost during diapause [43,44]. It is presumed that *H. halys* become responsive to pheromonal stimuli early in the season [29,42], and during this period, aggregation pheromone is likely produced in abundance by *H. halys* to help individuals locate to food resources [45]. This phenomenon in the life cycle of *H. halys* may explain why the MDT pheromone traps produced the highest captures in the early summer in Kyoto, compared to later in the mid-summer and fall. Furthermore, when adult *H. halys* begin to seek out overwintering sites late in the season they become unresponsive to the MDT pheromone [31], rendering the pheromone traps impractical during the pre-diapause period. Light traps on the other hand, will act as a consistent source of attraction for *H. halys* throughout the entire field season. However, the capacity of adult *H. halys* to fly decreases with decreasing temperature [36], meaning light traps may be less effective early and late in the season with lower temperatures.

To summarize, this research describes the seasonal phenology of *H. halys* in Kyoto, and to our knowledge, is the first study that compares the effectiveness of light and pheromone monitoring traps throughout the field season in Japan. Though the population dynamics of adult *H. halys* varied between monitoring methods, our work evidenced that black light traps produced the overall highest mean *H. halys* trap counts. The black light traps were also the only trap type to produce a positive coefficient estimate from the ZINB model analysis. Although, the MDT pheromone traps do show potential in acting as an early season detection method of *H. halys* in Kyoto. Future research should continue investigating the preferences of *H. halys* towards different attractants throughout the season to optimize the monitoring efficiency of this invasive pest.

4.5 References

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Chapter 5: Predictive phenology models of *Halyomorpha halys*

5.1 Introduction

Predictive phenology models play an essential role in effective integrated pest management program [1]. Several different approaches modelling the seasonal phenology of invasive pest species have been taken to determine periods of high and low populations in the field throughout the year [2,3,4]. Once the population dynamics of a pest are generally understood, steps in chemical control (timing of applications, frequency of applications, etc.), biological control (selection of natural enemies, timing of release, etc.), or other sources of management such as cultural control, can be implemented more efficiently. Robust models often include multiple factors with varying significance, and justifying their insertion or omission can be difficult. Creating models based on accumulated heat units, or degree days (DD), have become the standard tool for determining when to initiate the appropriate control methods against pest species [5]. In a biological sense, DD are a standardized way to express temperature-dependent processes in an organism's lifecycle. Phenology models scaled by DD are useful for extrapolating events including initial pest overwintering emergence and peak activity [6]. Calendar date (linear time) can restrict the predictive explanatory power of biological events in a pest's lifecycle, as field temperatures regularly fluctuate between different years and regions. For example, if a region experiences an unusually cool summer one year, that would generate a lower total DD output [7], but calendar date would progress unchanged. Although, one drawback of a

DD-based model is that not all of a pest's life-history functions are solely influenced by temperature, such are diapause triggers [8].

There have been several models produced which predict the potential range expansion of *H. halys* [9,10,11]. The process-oriented bioclimatic niche, or CLIMEX, model appears to be the most sensitive and specific in regards to fitting *H. halys* distributions to suitable climates [11]. The CLIMEX model projects areas with warm, tropical climates to be more optimal for *H. halys* populations [11]. This, however, is not the case in Japan. Populations generally dominate the cooler, mountainous areas in the northern and central regions of the country [12,13,14]. To better understand this pest's seasonal phenology in Japan, black light traps have been used as the standard to monitor their presence throughout the year [12,15]. Results of light trap monitoring show that *H. halys* experience peak activity in the field roughly between mid-July and early August.14 Efforts to measure the seasonal abundance of *H. halys* have been initiated in non-native regions as well [16,17,18]. Black light traps proved to be an effective way to monitor *H. halys* in New Jersey, and these populations similarly peaked in abundance between mid-July and early August [17]. Existing models, however, do not accurately capture the phenology of *H. halys* in Japan; there is a need for a model that describes the population dynamics of *H. halys* in its native range.

In this work, we present a zero-inflated negative binomial regression (ZINB) model, and a general additive mixed model (GAMM) based on black light trap monitoring of adult H. halys, precipitation, and temperature data from 2010-2020 in Japan. The monitoring data had an abundance of zero count data, which is a common occurrence when collecting biological count data [19,20,21]. In such instances, it is necessary to implement a zero-inflated regression method to appropriately analyze the data [22]. Zero inflation can be viewed as encountering a higher frequency of zeros than expected for fitting data to a Poisson or negative binomial distribution; ignoring zero inflation may lead to biased estimated parameters and extensive overdispersion [22]. A ZINB based model is optimal when analyzing data for a species that appears in large aggregations when present [19], such as *H. halys*. The ZINB model defines the effect selected factors exert on the seasonal phenology of *H. halys* adults in Japan, and generated a simulation which estimates the trap catch while predicting the probability of encountering a zero count during the field season. The GAMM can flexibly forecast the changing adult *H. halys* population trends and identify critical points in the pest's phenology such as first detection and peak activity throughout the field season in Japan. By integrating 11 years of *H. halys* seasonal phenology, temperature, and precipitation data from the same sites, we established two robust predictive models which advance the biological knowledge of this pest in its native region.

5.2 Materials and methods

5.2.1 Field sites

Field monitoring of adult *H. halys* took place at three agricultural research stations in Kyotanabe, Kameoka, and Kyotango City, Japan for 11 years from 2010-2020 (Figure 4.1). Kyotanabe is located in the southern part of Kyoto Prefecture, Kameoka is in the central Kyoto region, and Kyotango is in the north. The research stations cultivated various crops on a small scale for research purposes. Crops cultivated at the research stations included cucurbits and cereal grains such as rice. Pesticide sprays occurred rarely on the crops, roughly one or two applications per year. The pesticides were applied with a hand sprayer and targeted general pest insects. The GPS coordinates for each trap location are also listed in Figure 4.1.

5.2.2 Black light monitoring traps

One pest outbreak prediction light trap (model MT-7-N, Ikeda Scientific Co., Ltd., Tokyo, Japan) was used to monitor *H. halys* at each of the three sites over the 11-year period. Each steel trap measured 92 cm x 92 cm x 200 cm and weighed 85 kg. The traps were fitted with a 20 W black light which was automatically switched on via timer at 6:00 PM and off at 6:00 AM every day. Black light trap monitoring for *H. halys* occurred from May 1st to October 31st each year at each site. Every five days, the traps contents were collected and the number of adult *H. halys* were totaled and recorded. Each trap was checked a total of 36 times each year (six times per month, over the six-month trapping period).

5.2.3 Temperature and precipitation

Temperature and precipitation data from 2010-2020 were obtained from weather stations at each of the three sites. Each weather station was near (< 1 km) each corresponding *H. halys* black light trap. Daily maximum and minimum temperatures (°C) and total daily precipitation (mm) from January 1st to October 31st (end of *H. halys* monitoring) were determined for each site over the 11-year period. Degree-days (DD) were calculated by subtracting daily minimum temperatures from daily maximum temperatures, dividing by two, then subtracting 12.1 from the result. The lower threshold of 12.1°C and upper threshold of 35˚C were incorporated as Japanese *H. halys* populations have a minimum development threshold of 12.1°C [14,23], and *H. halys* development ceases at 35°C [24]. Cumulative five-day DD totals were computed for each site beginning from January 1st of each year and summed until October 31st. Total daily precipitation for each site was averaged for each five-day period from January 1st to October 31st for each site.

5.2.4 Statistical analysis

5.2.4.1 Zero-inflated negative binomial model

The zero-inflated negative binomial (ZINB) regression model was generated using the *mixed_model* function in the *GLMMadaptive* package of the RStudio program [25]. The model implemented the family "*zi.negative.binomial*" to adjust for the overdispersion and inflation of zero counts from the *H. halys* monitoring data. In the case of our research, *H. halys* monitoring methods are still being improved, meaning a zero count can result from either the influence of the covariates (true zeros), or from inefficient monitoring methods (false zeros) [22]. Fitting the data to a standard generalized model would incorrectly pool the two different types of zero counts which may have originated from separate processes [19]. Zero-inflated models can use the effect of the covariates to estimate which group a zero potentially belongs to. Principally, the probability of encountering a zero can be defined as:

$$\Pr(Y = 0) = \Pr(\text{False zero}) + [1 - \Pr(\text{False zero})] \times \Pr(\text{True zero})$$

The overall probability function for the ZINB regression is expressed as:

$$\Pr(y_i/x_i) = \begin{cases} \pi_i + (1 - \pi_i) \times (\frac{k}{\mu_i + k})^k, & \text{for } y_i = 0 \\ (1 - \pi_i) \times \int \mathrm{NB}(y_i), & \text{for } y_i > 0 \end{cases}$$

where (π_i) is given by: $\frac{e^{v + \gamma_1 \times z_{i1} + \dots}}{1 + e^{v + \gamma_1 \times z_{i1} + \dots}}$, with ($v$) as the intercept, ($\gamma$) as the regression coefficient, and (z) as the covariate (year, site, year * site, DD, and precipitation). Constant (k) is a dispersion parameter, (μ_i) is the expected mean, and the function $\int$ NB(y_i) corresponds to the probability that an observed count is not a false zero. The full model included year, site, year * site interaction, DD, and precipitation as random effects. The ZINB model as fit can only describe estimated trap counts as strictly increasing or decreasing throughout the seasonal phenology of *H. halys*, but can determine the likelihood of a random effect influencing a zero count.

5.2.4.2 Model comparisons and diagnostics

Six generalized linear models were generated and compared: a zero-inflated negative binomial full model (effect of precipitation included) (ZINB full), zero-inflated negative binomial reduced model (effect of precipitation excluded) (ZINB red.), negative binomial model (NB), zero-inflated Poisson model (ZIP), Hurdle model (H), and Poisson model (P). A Poisson model is typical for discrete count data, but does not work well with overdispersion, whereas a negative binomial distribution allows for data with high variance by incorporating an overdispersion parameter [22]. A Hurdle model discerns the positive counts from the zero counts, then models the groups separately using binary and zero-truncated approaches [20]. Lastly, a zero-inflated model also separates the positive counts from the zero counts, but additionally models the probability of measuring a false zero against all other data types (positive counts and true zeros) [22]. Maximum likelihood parameter estimations were obtained and compared to select the most parsimonious and appropriate model (Table 5.1).

Table 5.1 Generalized linear models and respective test statistics

Model	Vuong test	AIC	BIC	Log-likelihood
ZINB (full)		**2088.052**	**2244.874**	**-993.259; *df* = 31**
ZINB (red.)	$z = -1.339$; $p = 0.090$	2132.147	2279.247	-995.085; *df* = 29
NB	$z = -9.228$; $p < 0.005$	2366.819	2447.759	
ZIP	$z = -7.660$; $p < 0.005$	3297.506	3449.269	
H	$z = -7.699$; $p < 0.005$	3298.091	3449.853	
P	$z = -8.634$; $p < 0.005$	4888.671	4964.552	

Model selection criteria for the six models created. The Voung test statistics shown are results of the ZINB full model tested against all other models, with significance determined at $p < 0.05$. The log-likelihood test compares the nested ZINB full and reduced models. The bolded model (ZINB full) was selected as the best fitting model based on the selection criteria.

Vuong’s test was run to test the goodness of fit between the ZINB full model and the ZINB reduced, NB, ZIP, H, and the P models. Vuong’s test is a likelihood-based test which can be implemented for both nested and non-nested models [19,26]. The Vuong test statistic was significant from comparisons between the ZINB full and NB, ZIP, H, and the P models ($p < 0.05$) (Table 5.1). The Vuong test statistic was not significant when comparing the ZINB full and ZINB reduced models ($p = 0.09$), indicating no difference between the models. A likelihood-ratio test was performed on the nested ZINB models, and the full model produced a lower value than the reduced, indicating it being the better fitting model. Also, Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) values were determined for each model. The ZINB full model produced the lowest AIC and BIC values, recommending it as the most parsimonious model [22,27]. The inclusion of all variables to the ZINB model was justified as the full model best represented the data, and had the most explanatory power as explained by the model comparison tests.

5.2.4.3 Generalized additive mixed model

The predictive generalized additive mixed model (GAMM) was generated based on a negative binomial regression with a log-link using the *predict.gam* function in the *mgcv* package of the RStudio program [25]. The model incorporated year, site, and the year * site interaction as fixed effects, and DD and precipitation as random effects. A penalized smoothing spline function for the covariate DD was also included to determine the optimal smoothness for the model’s curve. The GAMM can be expressed as:

$$Y_{ij} \sim \text{NegBin}\ [\mu_{ij(abc)}]$$

$$g[\mu_{ij(abc)}] = \log\ [\mu_{ij(abc)}] = f\,(d_i) + \beta_j + \varepsilon_a + \varepsilon_b + \varepsilon_c + \delta_{ij}$$

$$\varepsilon_a \sim \text{N}\ (0, \sigma^2{}_a),$$

$$\varepsilon_b \sim \text{N}\ (0, \sigma^2{}_b),$$

$$\varepsilon_c \sim \text{N}\ (0, \sigma^2{}_c),$$

$$\delta_{ij} \sim \text{N}\ (0, \sigma^2)$$

In this representation, (Y_{ij}) is used to describe the seasonal trap catch of adult *H. halys* through non-linear time with DD defined by (*i*) and precipitation defined by (*j*). The influence precipitation has on trap catch is signified by coefficient (β_j), and the penalized smoothing function of DD is represented by f (di). The effects of year, site, and the year * site interaction are defined by (ε_a), (ε_b), and (ε_c) respectively. Lastly, the residual error of the count estimation is given by δ_{ij}. The GAMM has comparatively less interpretability ZINB model,40 but can more accurately predict shifting trends of the *H. halys* population over the growing season. By estimating maximums and minimums from the GAMM generated smoothing curve, the model can also designate a specific DD corresponding to critical points in the phenology of *H. halys* including first adult detection, periods of above and below average trap catch, and peak activity.

5.2.4.4 Model diagnostics

The full GAMM (all covariate effects included) was compared to a reduced model (effect of precipitation excluded) to determine the most parsimonious model. A generalized cross-validation (GCV) test was run for both models. A GSV score is used to measure the fit of a GAMM by determining an optimal smoothing parameter, where a smaller GCV score indicates a smoother, better fitting model [27]. The full model had a GCV score of 16.256 and the reduced model had a marginally lower GCV score of 16.230. An F-test was also implemented to compare the full and reduced GAMMs, and there was no significant difference between the two models (F = 0.137, p = 0.711). Overall, the full GAMM was selected since there was no obvious best fit model, to remain consistent with the effects included in the ZINB model.

5.3 Results

There was a total of 889 instances of no *H. halys* being present upon trap inspections across all sites over the 11-year monitoring period, and 290 instances of *H. halys* being present Figure 5.1). The trap catch high was 63 adult *H. halys*. The populations of *H. halys* at each site from 2010-2020 increased with DD during the field season, then peaked at the accumulation of around 1,000 DD, then began to decrease and eventually reached zero at around 1,800 DD, as illustrated by the log-transformed trap catch data (Figure 5.2).

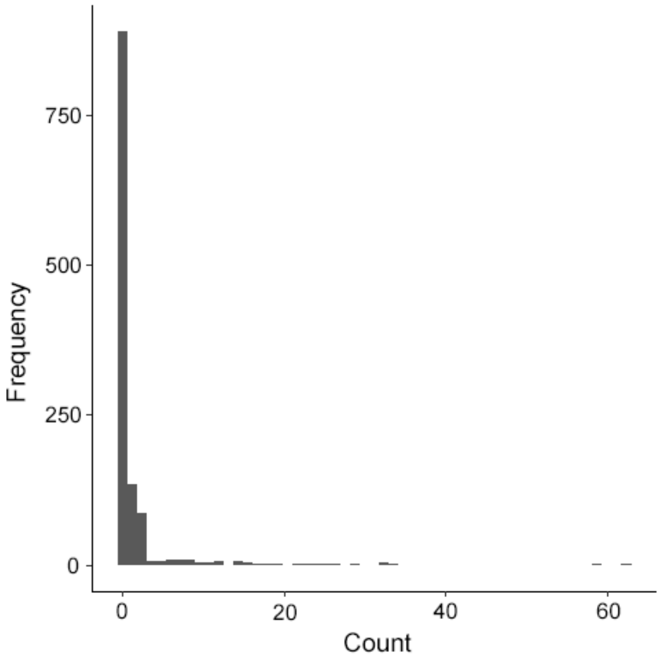

Figure 5.1 Frequency of total five-day adult *H. halys* trap counts. Low = 0 (889 instances). High: 63 (1 instance).

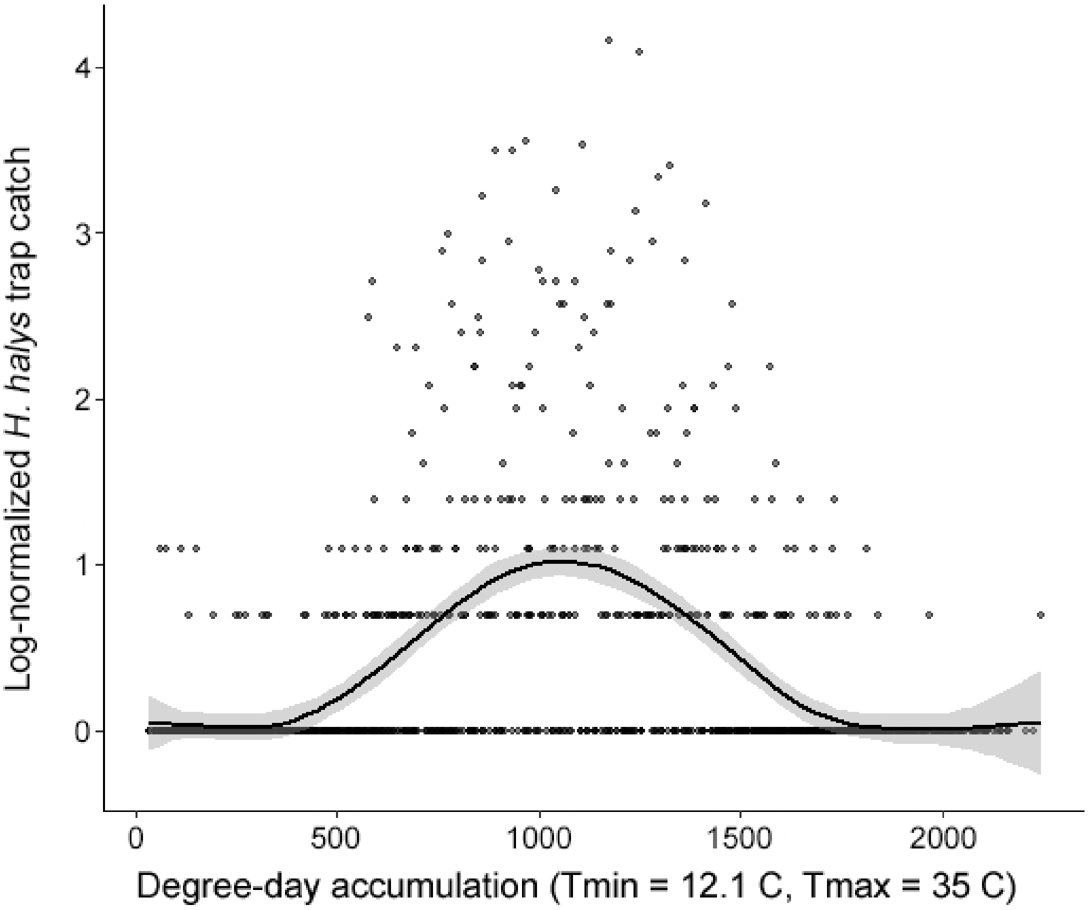

Figure 5.2 Log-normalized adult *H. halys* five-day trap catch plotted against accumulated DD averaged for the three sites over the 11-year monitoring period. A smoothed fit line for each site is displayed to help visualize the population trend of *H. halys* at each location.

5.3.1 Zero-inflated negative binomial model

The ZINB coefficient estimates for the positive count model are 0.003 for DD ($z = 14.959$, $p < 0.001$) and -0.002 for precipitation ($z = -0.910$, $p = 0.363$). At the significance level of $p < 0.05$, DD had a significant effect on *H. halys* trap catch, but precipitation did not. The positive coefficient estimate for DD indicates that *H. halys* count increases with increasing DD. The ZINB coefficient estimates for the zero-count model are 0.013 for DD ($z = 10.767$, $p < 0.001$) and 0.008 for precipitation ($z = 1.621$, $p = 0.105$). At the significance level of $p < 0.05$, DD had a significant effect on influencing a zero count of *H. halys,* but precipitation did not. The positive coefficient estimate for DD indicates that the log odds of a false zero increase as DD increases, or the probability of encountering a true zero (zero influenced by covariates) decreases as DD increases.

Simulation data sets were produced from the 11-years of data based on 1,000 simulations generated by the ZINB model. The simulations were produced by the ZINB model which predicted the probability of recording a zero-count and estimated the average number of *H. halys* per trap per five-day observational period for each site from 2010-2020. The actual probability of zero count data and average number of *H. halys* per trap per five-day observational period data for each site from 2010-2020 was plotted against the simulated data (Figure 5.3). Overall, the actual data was well captured by the ZINB simulated data for each site across every year. The ZINB model simulated data projected Kyotango to generally have the highest *H. halys* counts, followed by Kyotanabe, and then Kameoka. Kyotango also had the widest variance of predicted trap catch of the three sites. The probability of encountering a zero-count appeared to be lower from Kyotango in comparison to the other two sites. Generally, periods of higher predicted *H. halys* trap counts corresponded with a lower probability of encountering a zero count.

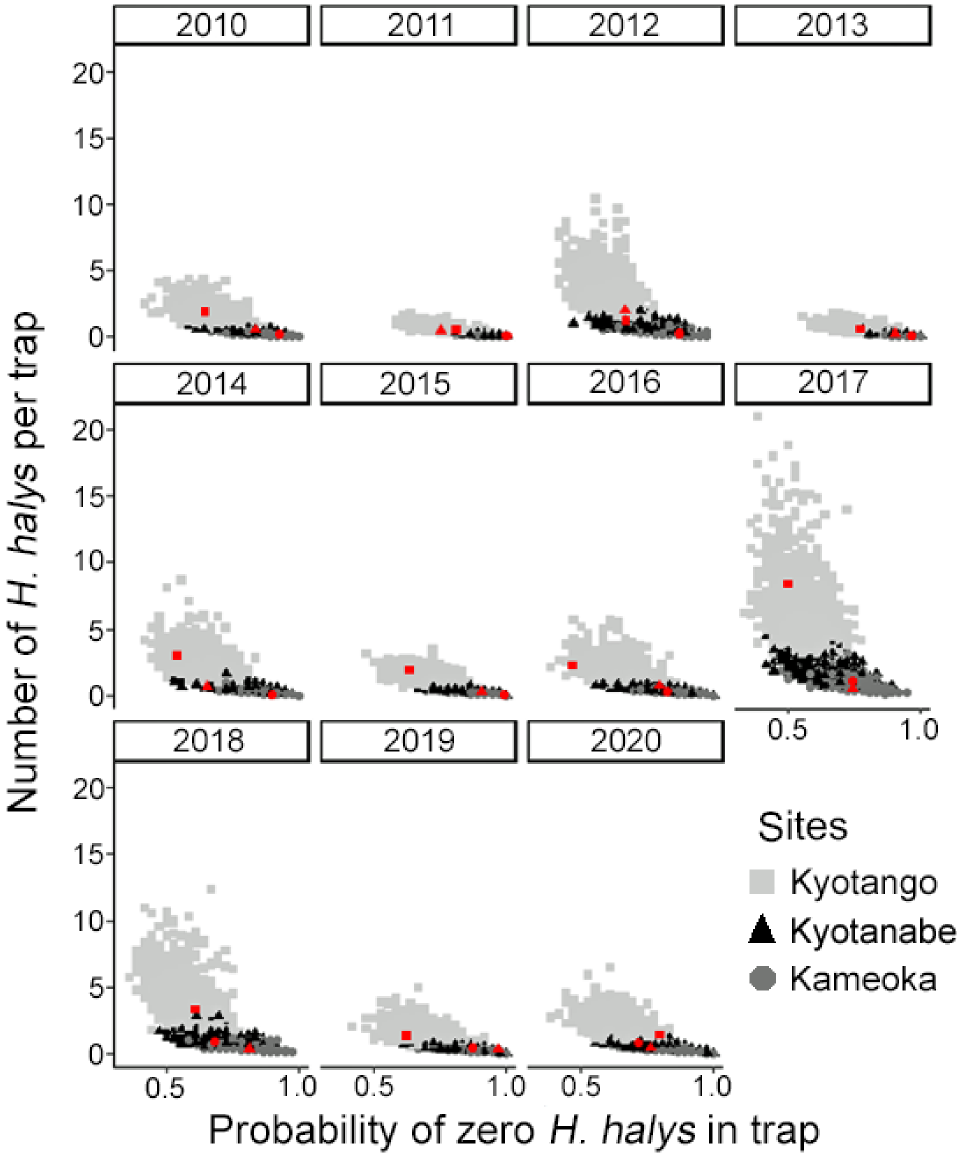

Figure 5.3 Data based on 1,000 simulations generated from the ZINB model for each site organized by year. The y-axis represents the predicted five-day average adult *H. halys* trap catch and the x-axis denotes the estimated probability of finding zero *H. halys* in a trap upon a five-day collection period. The red symbols (square, triangle, circle) on each year plot represent the actual average trap catch and zero catch probability for Kyotango, Kyotanabe, and Kameoka respectively.

5.3.2 Generalized additive mixed model

The GAMM produced a smoothed curve plot which forecasts the adult *H. halys* population trends during the field season in Japan (Figure 5.4). The GAMM smooth curve translates trap count into conditional modes (CM) which specify increasing or decreasing counts. The CM measures the population level estimations provided the factors. A positive CM indicates higher than average trap counts, and a negative CM means lower than average trap counts. The plot reveals a bell shape trend for the populations of *H. halys* scaled by DD throughout the field season. First adult detection arises at 261 DD, above average trap counts begin at 613 DD, peak activity occurs at 1,091 DD, trap counts begin to fall below average at 1,534 DD, and *H. halys* trap catch ceases at 1,826 DD.

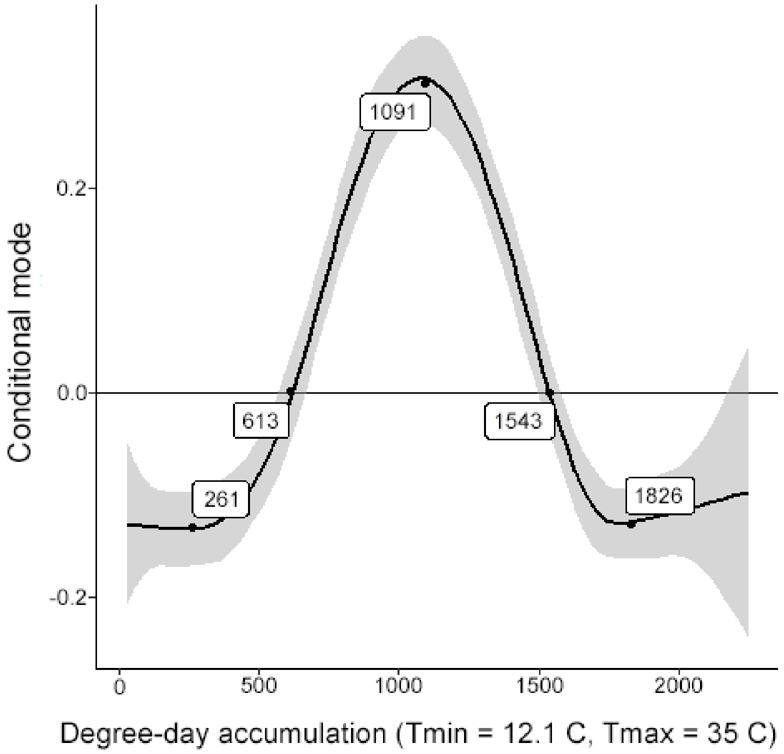

Figure 5.4 Display of the smoothed curve produced by the GAMM of adult *H. halys* trap catch in relation to accumulated DD. Critical DD values are plotted on the curve: 261 DD denotes first adult detection, 613 DD denotes beginning of above average trap catches, 1,091 DD denotes peak activity, 1,534 denotes beginning of below average trap catches, and 1,826 DD denotes the ceasing of activity. Conditional modes (CM) measure the population level estimations provided the effects. A positive CM indicates a higher-than-average estimation, and a negative CM indicates a lower-than-average estimation.

5.4 Discussion

The comprehensive results from the adult *H. halys* black light trapping are comparable to other monitoring studies done in Japan. Peak trap catch from our work occurred at around 1,000 DD, which corresponds to late July/early August at the three sites where the traps were located. These results differ from those of past studies done in Japan where peak trap catch was recorded in early September, although *H. halys* adults were monitored using pheromones traps or hand collected in these studies [28,29]. The peak activity recorded from our research more closely resembles previous *H. halys* monitoring work done in Japan with light traps, which similarly reported highest trap counts as typically occurring between mid-July and early August [15]. In the US, sweep net sampling in Pennsylvania reveals *H. halys* populations peak in early September [30], and observational data from Delaware, Maryland, New Jersey, and Virginia show populations peak in early August [31]. Differences in the population dynamics of *H. hayls* can certainly be attributed to specific regional characteristics (climate, surrounding landscape, etc.), but trapping techniques also seem to have an observable effect. The variance in the peak activity of *H. halys* as explained by multiple studies done in Japan evidences that different monitoring methods have an impact on measuring seasonal abundance, and should be considered an important factor when investigating and discussing the phenology of the pest.

The predicted probability of encountering a zero count was high (well above 50%), based on the simulations generated from the ZINB model. Typically, a zero count is not necessarily detrimental, in that it designates a period in which a target pest is not present. However, *H. halys* is a notoriously cryptic pest with exceptional mobility and that is frequently nocturnally active, rendering reliable monitoring of this pest difficult [17]. Coupled with limited available published research regarding the phenology of *H.*

halys in its native region, it is difficult to determine whether a zero count is a result of *H. halys* not being present, or a consequence of imperfect monitoring techniques due to a lack of understanding of the pest. Therefore, the ZINB model was generated to gain a better understanding of the phenology of *H. halys* in Japan, while properly navigating the excess of zero counts from the monitoring data. The model suggested that DD had a significant effect on the probability of encountering a false zero (*H. halys* present at site but not found in trap) [22], meaning a zero count later in the field season with more DD accumulated was more likely to be due to inefficient trapping as opposed to *H. halys* not being present.

The ZINB model also indicated that DD had a positive effect on *H. halys* trap catch, and that precipitation surprisingly did not have an effect. Previous work has determined that factors such as relative humidity have an influence on the behavior of *H. halys* [32,33]. However, no published studies have measured the influence precipitation has on *H. halys* phenology. Precipitation has been included as a factor in models predicting the potential range expansion of *H. halys*, which proposed uninvaded areas experiencing similar amounts of precipitation as native regions are at high risk of invasion [10,34]. Several other studies have demonstrated the importance of additional environmental factors in relation to the behavior and phenology of *H. halys*, such as heat waves and episodic cold shock events, as well as drought-like conditions [32,35]. Although temperature appears to persist as the key driver of *H. halys* development and phenology [24,36,37], future research should continue examining the potential effects that various regionally specific climatic factors have on the population dynamics of *H. halys*.

The GAMM demonstrated that *H. halys* populations in Japan increase early in the field season after first detection at 261 DD, peak at 1,091 DD, then their numbers begin to decrease and trap catch falls below average by 1,534 DD. The field abundance trend observed in Japan is similar to the results from a study conducted in the mid-Atlantic US, which *H. halys* populations monitored with black light traps also displayed a single modal peak in the middle of the field season when modelled with DD [17]. The first detection and peak activity of the mid-Atlantic US *H. halys* populations occurred respectively around 100 DD and 750 DD [17], whereas first detection and peak activity in the populations from Japan are predicted at 261 DD and 1,091 DD. The total accumulated DD from the locations sampled in the mid-Atlantic region ranged from 802 DD to 1,378 DD [17], considerably lower than the average total accumulated DD from the Kyotanabe, Kameoka, and Kyotango sites, which were 2,123 DD, 1,964 DD, and 1,796 DD, respectively. An existing phenology model suggests that a lower yearly accumulation of DD results in a lower total population density of *H. halys* [8]. Conversely, the Kyotango site from our work had the lowest average DD accumulation of the three sites, but numerically had the highest trap catch of the sites. One possibility is that since Kyotango is the farthest north of the three sites, it may have the most suitable climate for *H. halys*. In Japan, *H. halys* are more populous in the northern and central sections of the country [12,13]. Regardless, this result reveals that temperature (DD) may not be the main factor influencing population dynamics of *H. halys* in the field, and thus evidences the presence of additional important variables.

The differences in seasonal phenology between native populations of *H. halys* compared to our results from Japan may also be a result of local adaptations. The specific location *H. halys* acclimates in has an effect on the pest's behavior, including changes in cold tolerance and overwintering timing [38]. Additionally, different aspects of landscape development have been associated with *H. halys* density, as field studies have shown that *H. halys* abundance increases in more urbanized areas [39,40].

Furthermore, the differences in phenology measured from introduced *H. halys* populations may also be explained by genetics. Populations of *H. halys* originating from different native localities in Asia exhibit behavioral variations including differences in mortality temperature thresholds and number of generations per year [15,24]. Populations residing in northern China have one generation per year, whereas populations in subtropical southern China can reach up to six generations [15], and Japanese *H. halys* are typically bivoltine [12]. *Halyomorpha halys* originating from dissimilar native locations inherently possess behavioral traits most appropriate for their specific area, which may affect their seasonal phenology after invading foreign regions.

In the past, predictive DD-based models have been developed to establish guidelines and recommendations for the control of invasive insect pests [4,41,42]. There is currently no formally established economic threshold for *H. halys*, meaning insecticide applications have been strictly calendar-based [17,43]. Our DD-based models have the potential to help improve existing management programs by forecasting *H. halys* activity, allowing for more timely and efficient management practices in areas experiencing similar DD accumulations as Japan. For example, the GAMM predicted first adult *H. halys* detection at 261 DD, the beginning of above average *H. halys* populations at 613 DD, and peak activity at 1,091 DD in Japan. A linear DD model developed from lab data estimated that 147.65 DD are necessary for preoviposition period for female *H. halys,* and 537.63 DD are required for the maturation from egg to adult [24]. This information suggests that the *H. halys* found at first detection are from overwintering individuals, but the adults recovered at peak activity may be from overwintering and/or F1 generations. Therefore, to maximize the efficiency of management practices, insecticides targeting adult *H. halys* should be applied between 261 and 613 DD, whereas control methods targeting *H. halys* at all life stages should be considered from 613 DD through peak activity. Future DD models should also be designed to forecast the first egg-laying date of *H. halys* in the field. Non-chemical forms of management such as biological control are currently being investigated against *H. halys* at the egg stage [44,45], as well as the nymphal stage [46].

To summarize, the seasonal phenology of *H. halys* in Japan follows a predictable trend when scaled by DD, which we are able to forecast with the generated models. The presented ZINB and GAMM help fill in gaps of knowledge with this invasive pest by relating zero trap counts with the accumulation of DD, and establishing discrete DD points in the season corresponding to significant events in the phenology of *H. halys*. This research also evidences the need for the refinement and proceeding standardization of trapping techniques to reduce the amount of false zero counts encountered while field monitoring. It is important to note that the presented models do not incorporate all biotic and abiotic factors that impact the population dynamics of *H. halys*, such as local microclimate or anthropogenic activities [47]. We acknowledge the limitations associated with these predictive models based on 11 years of data regionally specific to Japan. However, the results of this research from the native range of *H. halys* may still be extrapolated and compared with other regions to advance the integrated pest management against this invasive pest.

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Chapter 6: Conclusions

This thesis aims to deliver information which highlights the natural biological control and the phenology of the invasive insect pest, *Halyomorpha halys,* in its native range. The objectives of this thesis are to assess 1) the predatory and parasitic biological control complex and 2) the seasonal population trends of *H. halys* in Japan. Gaining a better understanding of the pest in its native range will allow for the refinement of existing control schemes in invaded regions. The results are summarized as follows:

In Chapter 2, the predatory ability of Japanese *Crematogaster* ants against the egg and immature stages of *H. halys* was determined through bio-assays. The results indicated that the *Crematogaster* ants did not prey on the *H. halys* eggs or affect nymphal emergence, but did have a significant effect on the survival of early (first, second, and third) instar *H. halys* nymphs. *Crematogaster matsumurai* was able to limit the survival of first, second, and third instars to 0%, 40%, and 73% respectively, and *C. osakensis* was able to reduce survival rates to 0%, 13%, and 60% on first through third instars respectively. These data suggest that the generalist Japanese *Crematogaster* ants may play an important role in managing the early instar stages of *H. halys* in their native range, and likely act in an additive effort with other natural enemies which control the pest in its other life stages.

In Chapter 3, the species composition, phenology, and efficiency of egg parasitoids in the native region of *H. halys* was assessed using field deployed *H. halys* egg masses. A total of seven parasitoid species emerged from the sentinel egg masses, those from the *Trissolcus* genus being the most abundant. *Trissolcus japonicus* maintained 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 Japan is dominated by *T. japonicus,* with the first parasitism activity detected in March. These novel findings from Japan provide valuable insight on the abundance and parasitism rates of native *H. halys* parasitoids helping expand the understanding of biological control against this invasive pest.

In Chapter 4, the effectiveness of different monitoring traps and the seasonal phenology of *H. halys* was measured. To accomplish this, an 11-year collection of *H. halys* monitoring trap data from black light, incandescent light, and pheromone traps from three locations in Kyoto, Japan was analyzed. Overall, *H. halys* followed a bell-shaped population trend in Japan, with abundance peaking in the mid-summer. The attractant preference of *H. halys* varied slightly with seasonality, although of the three trap types, the black light traps produced to highest mean trap counts as evidenced by the dataset. The pheromone traps generated the lowest total mean trap counts, but displayed potential use in the reliable detection *H. halys* early in the field season. This work is expected to provide greater insight on the phenology *H. halys* in its native range, and ultimately help refine existing management programs in invaded regions.

In Chapter 5, to better understand the population dynamics of *H. halys* in its native range, we developed predictive phenological models using the black light trap monitoring data from Kyoto, Japan. An overabundance of zero counts from the data rewired the establishment of a zero-inflated negative binomial regression (ZINB) model which indicated that degree days (DD) have a significant effect on the trap catch of adult *H. halys*, and precipitation has no effect. The ZINB model also suggested that higher predicted trap counts equated to a lower probability of encountering a zero count. A general additive mixed model (GAMM) was also generated from the data which forecasts the seasonal phenology of *H. halys* as following a bell-shaped trend in Japan.

The GAMM predicted critical DD values during the field season of 261 DD for first *H. halys* adult detection and 1,091 DD for peak activity. These robust models help fill gaps in knowledge pertaining to the native phenology of *H. halys*, and ultimately help contribute to the progression of efficient management against this invasive species.

In conclusion, the findings from this thesis convey strong global impacts for improving existing management strategies for *H. halys*. This work is the first to test and prove the effectiveness of *Crematogaster* ants from the native range of *H. halys* in suppressing the survival of immature stages of the pest. This study highlights the efficiency and competitive dominance of *Trissolcus* egg parasitoids in parasitizing *H. halys* egg masses, and is also the first to report first successful field parasitism as early as March in a region where *H. halys* overwinters. This research evidenced blacklight traps as being an effective attractant for monitoring *H. halys* in its native range, and established a baseline seasonal phenology of the pest. This work is also the first to present predictive models capable of forecasting the population trends of *H. halys* in the field in Japan. Ultimately, this thesis contributes to the advancement of sustainable management practices against this globally invasive pest.

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