Parasitism of frozen *Halyomorpha halys* eggs by *Trissolcus japonicus*: applications for rearing and experimentation

Hanna McIntosh[a], David M. Lowenstein[b], Nik G. Wiman[b], Jessica S. Wong[a,b], and Jana C. Lee[a]

[a]Horticultural Crops Research Unit, USDA-ARS, Corvallis, USA; [b]Department of Horticulture, Oregon State University, Corvallis, USA

Contact: Hanna McIntosh, hrmcintosh@wisc.edu, Department of Entomology, University of Wisconsin-Madison, WI, USA

Abstract

The brown marmorated stink bug, *Halyomorpha halys* (Stål), has become a well-known pest to growers and homeowners since its 1996 introduction to the United States. A classical biocontrol program is under development using the egg parasitoid *Trissolcus japonicus*. Widespread implementation of biocontrol requires efficient mass rearing, which is constrained by availability of fresh *H. halys* eggs. In this study, parasitism rate, development time, sex ratio, and size were compared between wasps reared on fresh versus frozen, newly-laid (<1 d old) versus variably-aged (0-3 d old), and frozen egg masses stored ≤4 y. Frozen eggs yielded 56-65% fewer wasps, with parasitism rate decreasing 1-3% per month stored. Parasitism rate, sex ratio, and development time were comparable between newly-laid and variably-aged eggs. Freezing eggs for any duration did not affect sex ratio or weight of emerged wasps, but delayed emergence 5-6 d. To simulate deployment of sentinel eggs in the field, we incubated frozen eggs at 20 and 30 °C for 1-9 d before exposing them to *T. japonicus,* then evaluated parasitism trends. *T. japonicus* parasitism rate decreased 5-8% per day incubated, unhatched wasps increased 9% per day incubated, and sex ratio was not impacted. Variably-aged, frozen, and longer-stored eggs can be used for *T. japonicus* rearing and experimentation without affecting emerged wasp sex ratio or size within one generation, but have lower parasitism and slower development. Frozen sentinel eggs are effective <3-5 d, especially in hot conditions.

Keywords: biocontrol, samurai wasp, parasitoid, stink bug, BMSB, host acceptance

Introduction

The brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), has become a well-known pest to growers and homeowners since its introduction to the United States in 1996 (Hoebeke & Carter, 2003). Subsequently, the insect was introduced to Canada (Fogain & Graff, 2011), Europe (Wermelinger, Wyniger, & Forster, 2008), and South America (Fáundez & Rider, 2017) in just over 20 years. It has >170 known host plants including economically-important crops and ornamentals (Bergmann et al., 2013). *Halyomorpha halys* nymphs and adults can disperse quickly by walking (Lee, Nielsen, & Leskey, 2014). Adults can fly 2-5 km per day (Lee & Leskey, 2015; Wiman, Walton, Shearer, Rondon, & Lee, 2014), and can move easily between cultivated and wild host plants (Leskey & Nielsen, 2017). Feeding by adults and nymphs causes reduced crop quality or yield loss (Nielsen & Hamilton, 2009). A severe infestation in the Mid-Atlantic region in 2010 caused $37 million in losses in apple (American / Western Fruit Grower, 2010), with some growers losing over 80% of their crop (Leskey & Hamilton, 2010). In response, broad-spectrum insecticide application increased fourfold, increasing risk of secondary pest outbreaks because of detrimental effects on natural enemy populations (Leskey & Nielsen, 2017). Chemical-based management is costly for growers and may only provide limited efficacy. A concerted effort is required to develop alternative approaches for managing *H. halys*. Control within an integrated pest management (IPM) framework may reduce pest populations while limiting non-target effects. One such option is classical biological control, which is currently under development for *H. halys,* with potential for landscape-scale control.

Trissolcus japonicus (Ashmead) (Hymenoptera: Scelionidae), or the samurai wasp, is an egg parasitoid of *H. halys* that can kill up to 70% of *H. halys* eggs in their native range (Yang, Yao, Qiu, & Li, 2009). The wasp has a fast development time, many more generations than its host, and a high female to male ratio. These factors make *T. japonicus* an excellent candidate biological control (biocontrol) agent. The wasp has been studied under quarantine in the United States since 2007 (Rice et al., 2014)Due to its ability to parasitise other Pentatomidae eggs in its native range (Zhang et al., 2017). However, in 2014 and 2015, adventive populations genetically distinct from those in quarantine were found in Maryland (Talamas et al., 2015) and Washington state (Milnes et al., 2016), and have since been detected in Virginia, West Virginia, Delaware, New Jersey, New York, the District of Columbia (Leskey & Nielsen, 2017), Oregon (Hedstrom et al., 2017, Ohio, and Pennsylvania (Northeastern IPM Center, 2018), expanding the potential for implementing biocontrol research in the field. Host-specificity tests indicate that *T. japonicus* could complete development in a subset of native Pentatomidae eggs in its introduced North American range (Hedstrom et al., 2017; Botch & Delfosse, 2018). As *T. japonicus* populations become established in new areas, ongoing research seeks to develop specific methods for biocontrol, including release strategies, augmentation, conservation, monitoring, and rearing.

T. japonicus is ideally reared on fresh, newly-laid *H. halys* egg masses, but rearing and experimentation is constrained by the availability of such eggs. Typically, newly-laid (<1 d old) egg masses are collected from a lab colony and are prioritised for use in experiments and rearing. Excess egg masses can be frozen for future use. Recently-frozen egg masses are used in experiments that require freeze-killed egg masses or replication exceeding colony production, such as sentinel egg studies (Herlihy, Talamas, & Weber, 2016). No studies have

compared *H. halys* egg mass quality and *T. japonicus* development between fresh versus frozen eggs. Freezing and storing egg masses could reduce potential attractiveness to parasitoids through physical and physiological changes. Frozen *H. halys* egg masses have reduced lipid levels and turn dark grey after thawing (Skillman & Lee, 2017). Additional changes seen in other parasitoid-host systems may include loss of critical host volatiles or cues, degradation of non-volatile surface chemicals, change in surface or interior texture, desiccation, or degradation of nutrients (Godfray, 1994). Changes in these egg mass characteristics can impact host location and selection, parasitoid development and survival, sex ratio, fecundity, and longevity, but it is unknown how developing on frozen eggs may affect *T. japonicus*. In sentinel egg studies, prolonged exposure to heat in the field may impact parasitoid host location and parasitism. This affects the interpretation of study results, and influences the active time of sentinel egg masses, or the amount of time that eggs remain a suitable host for oviposition and development in the field. Furthermore, host age is another important component of host quality, with older eggs developing host defenses that may deter successful parasitism (Godfray, 1994). Newly-laid fresh and frozen egg masses are used for several applications, but the inability to collect egg masses on a daily basis leads to accumulation of variably-aged 'weekend eggs' ranging from 0-3 d in age. It is unclear if these variably-aged egg masses are of sufficient quality for experiments or rearing.

Our study addresses gaps in knowledge critical to rearing *T. japonicus* for biocontrol. This paper includes five experiments encompassed in three objectives that examined the suitability of frozen, longer-stored, and variably-aged (0-3 d old) *H. halys* egg masses for *T. japonicus* rearing, experimentation, and for sentinel egg studies. We predicted that the use of these lower quality egg masses would result in lower parasitism rates, slower development time, male-biased sex ratios, and smaller wasps than higher quality eggs. Objective 1a compared fresh versus frozen, and newly-laid versus variably-aged egg masses. Objective 1b examined the potential for frozen egg masses to prolong development time of wasps in a no-choice environment. Objective 2 evaluated a potential decrease in quality and suitability of egg masses stored frozen up to four years with experiments under high (Objective 2a) and low (Objective 2b) parasitism pressure. Finally, Objective 3 investigated how heat exposure affects suitability of *H. halys* egg masses for *T. japonicus* parasitism using a no-choice incubation study to mimic degradation of sentinel egg masses in the field.

Materials and methods

For a detailed description of the experimental conditions for Objectives 1-3, see Table 1.

Table 1. Experimental conditions for Objectives 1-3.

Study	Treatments	Total sample size	Insect colonies	Wasps	Arena	Exposure time	Summary
Obj. 1a	<1 d fresh <1 d frozen 0-3 d fresh 0-3 d frozen	66 cohorts	USDA	25-50, mixed sex & age	colony cup	3-4 d	Assessment of frozen & variably-aged eggs
Obj. 1b	0-3 d fresh 0-3 d frozen	20 egg masses	USDA	4♀ + 1♂, mixed age	vial	24 h	No-choice comparison of fresh & frozen eggs
Obj. 2a	frozen 77-1352 d	36 egg masses	USDA	10♀ + 4♂, mixed age	cup	4 d	Assessment of longer-stored frozen eggs with high parasitism pressure
Obj. 2b	frozen 3-490 d	75 egg masses	OSU	1♀, mated, 2 d old	petri dish	3-5 d	Assessment of longer-stored frozen eggs with low parasitism pressure
Obj. 3	frozen eggs incubated 1-9 d at 20 °C and 30 °C	106 egg masses	OSU	2♀, mated <36 h old	vial	24 h	Heat exposure of frozen sentinel eggs

H. halys *colonies*

We reared *H. halys* in two colonies—one in a growth chamber at the USDA ARS Horticultural Crops Research Unit laboratory (Corvallis, OR), and a second in a growth chamber at Oregon State University (OSU; Corvallis, OR). Both were started from wild-collected *H. halys*, and were replenished with new adults throughout the year, so that all individuals were wild-caught, not lab-reared. From May to November, adults and nymphs were collected via beat sheet at multiple sites across the Willamette Valley. The USDA bugs were collected primarily from holly (*Ilex aquifolium),* and OSU bugs from holly, catalpa (*Catalpa spp.*), linden (*Tilia spp.*), and maple (*Acer spp.*). During winter months, diapausing adults were collected from homeowners in the same region, usually found aggregated on building exteriors, in garages, or wood piles. Both colonies were contained in 29.5 x 29.5 x 30.5 cm plastic mesh cages (Bug Dorm, BioQuip, Rancho Dominguez, CA) inside growth chambers (21-22 °C, 16:8 L:D, 60-70% RH) at either facility. Each cage included 20-80 adults. The USDA colony was provided carrots, jelly beans, raw unsalted peanuts, sunflower seeds, and a water wick. The OSU colony was provided sunflower seeds, holly fruits and leaves, tomato, grapes, a water wick, and occasionally fresh jalapeño or bean plants. For egg collection in both colonies, egg masses were wetted, removed with a spatula, affixed to filter paper, and stored in plastic petri dishes. Fresh egg masses were either immediately used, or stored at -80 °C. Egg masses were collected every week day.

T. japonicus *colonies*

The USDA *T. japonicus* laboratory colony was established in 2017 from field populations collected from sentinel and wild egg masses in Portland, Oregon in 2016. The OSU colony was established in the same way from field populations in 2016 and 2017. Both colonies were contained in 16 oz. plastic-lined paper soup cups with plastic lids (Huhtamaki Inc., Espoo, Finland). Colony cups were stored inside 29.5 x 29.5 x 30.5 cm Bug Dorm cages. The

USDA colony was stored in the lab (~21 °C, natural day length conditions, ~60% RH). The OSU colony was kept in a growth chamber (24 °C, 16:8 L:D, 70% RH). In both colonies, each cup contained ~25-50 wasps. A small piece of filter paper soaked in 50% honey-water solution was given to the USDA colony on weekdays, and the same solution was brushed inside OSU colony cup lids. The USDA colony cups were given 4-8 egg masses per week, and the OSU cups were given 2-3, using fresh when available, and supplementing with frozen egg masses when necessary. After one week, egg masses were removed from both colonies and stored in 50 mm lockable plastic petri dishes for emergence. Emerged wasps were returned to the colony cups as necessary, or stored in separate soup cups and fed as described previously until needed for experimental use.

Objective 3 experiments predated the field populations of *T. japonicus* in Oregon and utilised a quarantined population of the wasp maintained in the USDA APHIS PPQ certified quarantine facility on the OSU campus in Corvallis. The quarantine population was initiated with 6 parasitised egg masses that were attacked by 'Beijing' strain *T. japonicus* at the USDA ARS Beneficial Insects Introduction Research Laboratory in Newark, DE in 2011. Wasp colonies were maintained according to Hedstrom et al. (2017; 20 °C, 16:8 L:D, 60-70% RH).

Objective 1a: assessment of frozen and variably-aged eggs

Trissolcus japonicus in colony cups from the USDA colony were used to assess differences between fresh and frozen, and newly-emerged and variably-aged egg masses. Colony cups were exposed to a total of four treatments varying egg type and age: <1 d old fresh, <1 d frozen, 0-3 d fresh, and 0-3 d frozen. Frozen egg masses had been stored for ≤1 year. Sets of replicates were generated on a given day by placing 3-8 egg masses of mixed treatments into each colony cup. Due to limited availability, all treatments were not always present in each cup. This was repeated on different days for a total of 7 groups of egg masses per cup, totaling 257 egg masses parasitised. All wasps and egg masses were from USDA colonies. After 3-4 d, egg masses were removed from cups, placed into 50 mm lockable plastic petri dishes, placed in a growth chamber (21 °C, 16:8 L:D, 60% RH), and monitored for emergence. Upon emergence, wasps were identified to sex and counted. For analysis, data was grouped into 66 cohorts, or all egg masses of a given treatment parasitised in the same cup on the same date.

Objective 1b: no-choice comparison of fresh and frozen eggs

To determine development time of *T. japonicus* reared on fresh and frozen eggs, four females and one male were exposed to one egg mass inside 25 x 95 mm plastic vials, totaling 10 repetitions per egg type treatment, or 20 egg masses. We used mixed-age wasps to mimic age heterogeneity in a population or colony. Females were mated, but had not been exposed to host eggs in their lifetime. One egg mass was attached to the underside of each vial's lid. All egg masses were 0-3 d old when collected, and frozen egg masses were stored for <1 yr. All wasps and egg masses were from USDA colonies. Wasps were removed from the vial after 24 h, vials were moved to a growth chamber (21 °C, 16:8 L:D, 60% RH), and monitored daily for emergence. A total of 321 emerged wasps were removed and frozen at -80 °C. At a later date, wasps were identified to sex and counted. To evaluate size, wasps were dried in the same growth chamber for 2 d inside plastic microplates covered by a paper towel. Dry weight was determined to stabilise after 2 d of drying (HM pers. obs.). Size was determined by dry

weight at 1.0 µg (Orion Cahn c-35 microbalance, Thermo Fisher Scientific, Waltham, MA) and by measuring the right or left hind tibia with a microscope.

Objective 2a: assessment of longer-stored frozen eggs (high parasitism pressure)

To assess suitability of prolonged frozen storage, we exposed *T. japonicus* to egg masses stored 77-1352 d. We released 10 female and 4 male wasps into a soup cup with three frozen egg masses laid in the same year, totaling 36 egg masses. All egg masses were 0-3 d old at the time of collection, and had been stored at -80 °C. We used mixed-age wasps to mimic age heterogeneity in a population or colony. Females were mated, but had not been exposed to host eggs in their lifetime. Wasps that died were replaced to ensure even parasitism pressure. All wasps and egg masses were from USDA colonies. After 4 d, egg masses were transferred into separate 50 mm lockable plastic petri dishes, and stored in a growth chamber (21 °C, 16:8 L:D, 60% RH) for emergence. A total of 267 emerged wasps were frozen at -80 °C. Wasps were later identified to sex, counted, then dried, weighed, and measured as described previously.

Objective 2b: assessment of longer-stored frozen eggs (low parasitism pressure)

In a similar study with lower parasitism pressure and wasps from the OSU colony, frozen egg masses were presented singly to a mated, >2 d old female *T. japonicus* within a 60 x 90 mm petri dish, totaling 75 egg masses. All egg masses were <1 d old at the time of collection, and stored at -80 °C until use in this experiment. The storage duration of the egg masses ranged from 3-490 d. All wasps and egg masses were from OSU colonies. Wasps were removed from the petri dishes after 3-5 d, and eggs were monitored for emergence in a growth chamber (24 °C, 16:8 L:D, 70% RH). Upon emergence, wasps were identified to sex and counted.

Objective 3: heat exposure of frozen sentinel eggs

To assess the impact of heat exposure on sentinel eggs, frozen egg masses were incubated in growth chambers at 20 °C or 30 °C for 1-9 d, after which they were placed in separate 10-dram plastic vials and presented to two <36 h old, mated female *T. japonicus*. This was a repeated for a total of 122 egg masses. All egg masses were stored frozen for 1-2 y. All wasps and egg masses were from OSU colonies. Wasps were allowed to parasitise the incubated egg masses for 24 h, after which the wasps were removed and the vials held in growth chambers (20 °C, 16:8 L:D, 60% RH) until emergence. Emerged wasps were then identified to sex and counted. After 30 d, each egg mass was dissected and number of unhatched *T. japonicus* was recorded.

Statistical analysis

In all studies, parasitism rate and sex ratio were calculated as proportions: [number of parasitised eggs / total number of eggs] and [number of emerged females / total number of emerged wasps]. All data were analysed in R version 3.4.4 (R Core Team, 2018). Untransformed data met the assumptions of models used, unless specified. Random effects were selected according to experimental parameters, and overfitted models were then pared down by screening for singularity (parameters with variances near zero) and removing effects

with standard deviations <10e-6. Generalised linear models were conducted using *glm*, linear mixed models with *lmer* from the 'lme4' package (Bates, Maechler, Bolker, & Walker, 2015), and generalised linear mixed models with *glmer* from 'lme4'. Type III Chi-square test fixed effect tables were produced from mixed models with *Anova* from the 'car' package (Fox & Weisbert, 2011).

Objective 1a: assessment of frozen and variably-aged eggs

Parasitism rate and sex ratio were compared between egg type and egg age treatments with separate generalised linear mixed models with binomial distributions. Development time data was log-transformed and compared between egg type and egg age treatments with a linear mixed model. Since development time was recorded on a per wasp basis, data were grouped by cohort, with cohort and colony cup as random effects.

Objective 1b: no-choice comparison of fresh and frozen eggs

Parasitism rate and sex ratio data were compared between egg type treatments with separate generalised linear models with binomial distributions. Development time data was log-transformed and compared by egg type treatments with a linear mixed model, and data were collected on a per wasp basis and grouped by replicate (a single egg mass) as a random effect. Emerged wasp weight and tibia length were compared between egg type treatments and between emerged wasp sex, and data were grouped by replicate (a single egg mass) as a random effect.

Objective 2a: assessment of longer-stored frozen eggs (high parasitism pressure)

Parasitism rate and sex ratio data were compared by days stored with separate generalised linear models with binomial distributions. Emerged wasp weight and tibia length were compared by days stored, and data were grouped by replicate (a single egg mass) as a random effect. Trends from models with significant fixed effects were evaluated by linear regression using *lm*. Adjusted R-square values are reported for all regressions.

Objective 2b: assessment of longer-stored frozen eggs (low parasitism pressure)

Parasitism rate and sex ratio data were compared by days stored with generalised linear models with binomial distributions. Trends from models with significant fixed effects were evaluated by linear regression using *lm*.

Objective 3: heat exposure of frozen sentinel eggs

Unhatched wasps was defined as the proportion of attacked eggs that did not hatch, calculated by [eggs with unhatched parasitoids / (eggs with hatched parasitoids + eggs with unhatched parasitoids)]. Parasitism rate, unhatched wasps, and sex ratio were compared by incubation temperature and days incubated with separate generalised linear models with binomial distributions. Trends from models with significant fixed effects were evaluated by linear regression using *lm*. Two linear regressions were run for models where incubation temperature was a significant effect, one for each temperature. If incubation temperature was not a significant effect, data were grouped into one linear regression.

Results

Objective 1a: assessment of frozen and variably-aged eggs

When given a choice between fresh and frozen eggs, parasitism rate was lower in frozen egg masses (Table 2), with an average of 80% of fresh and only 20% of frozen eggs parasitised (Figure 1a). There was no difference in parasitism rate between <1 d and 0-3 d old egg masses of either egg type. Wasps reared on frozen eggs took 3-4 d longer to develop than those reared on fresh eggs (Figure 2a). Egg age had no effect on development time. Finally, there was no difference in sex ratio among all four treatments, where 70-86% of emerged wasps were female (Figure 3a).

Table 2. Results for Objective 1a.

Type III Wald χ2 tests		Parasitism rate		Sex ratio		Development time	
Effect	df	χ2	Pr>χ2	χ2	Pr>χ2	χ2	Pr>χ2
Egg type	1	15	**<0.001**	0.0041	0.95	8.4	**0.004**
Egg age	1	1.8	0.18	0.25	0.62	0.85	0.36

Linear models of parasitism rate, sex ratio (% female) of emerged wasps, and development time for fresh and frozen egg masses of two ages exposed to *T. japonicus* colony for 4 d. Bold values are statistically significant.
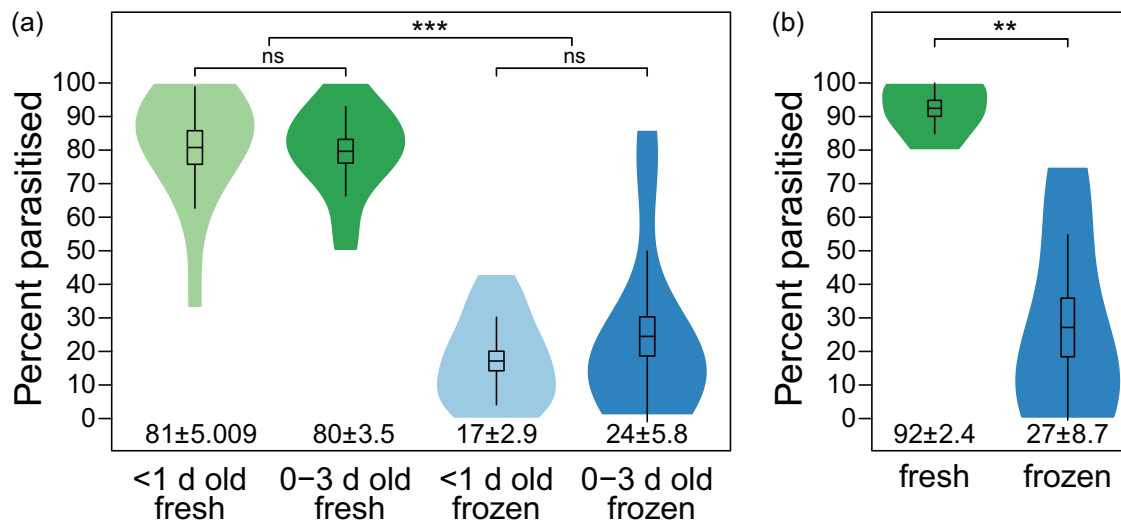

Figure 1. Kernel density plots of parasitism rate for egg masses exposed to *T. japonicus* for 4 d (a, Obj. 1a) or 24 h (b, Obj. 1b). Internal boxplots show mean and standard error; whiskers extend to standard deviations. Mean ± standard errors are printed for each treatment. Per results of linear models: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns not significant.

Objective 1b: no-choice comparison of fresh and frozen eggs

In our no-choice study, parasitism rate of fresh eggs was significantly higher than frozen (Table 3), with approximately 92% of fresh and 27% of frozen eggs parasitised, and overall higher parasitism rates than the previous study (Figure 1b). Wasps emerged from fresh eggs 4-7 d faster than from frozen eggs. Also observed in our colony (HM, pers. obs.), males emerged 1-2 d earlier than females from fresh and frozen eggs (Figure 2b). There was no
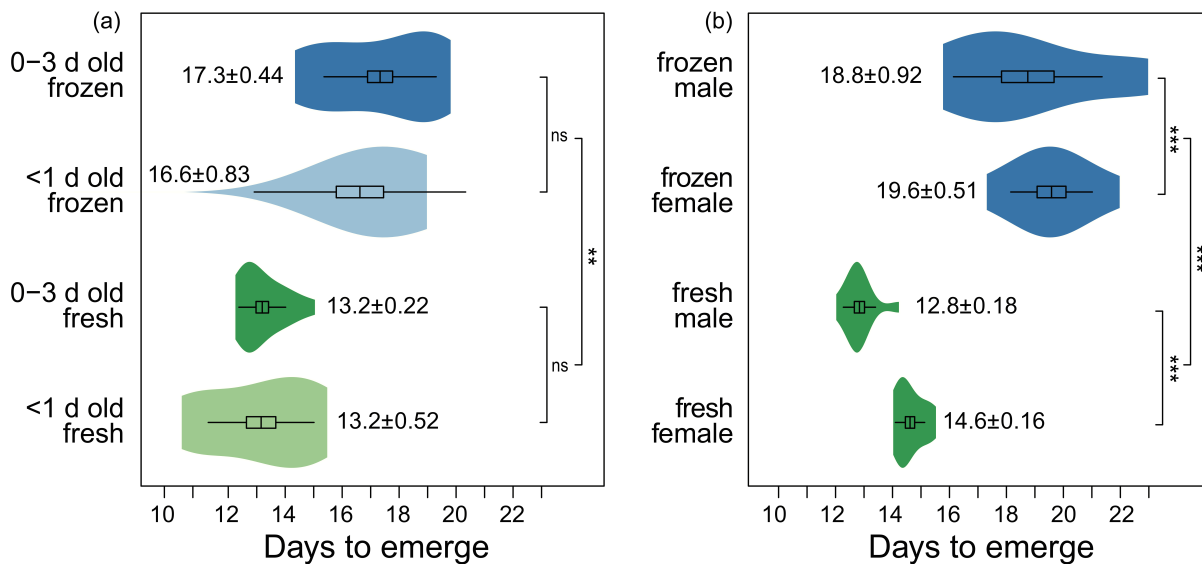
Figure 2. Kernel density plots of development time for *T. japonicus* emerged from egg masses exposed to *T. japonicus* for 4 d (a, Obj. 1a) or 24 h (b, Obj. 1b). Internal boxplots show mean and standard error; whiskers extend to standard deviations. Mean ± standard errors are printed for each treatment. Per results of linear models: *** p < 0.001, ** p < 0.01, * p < 0.05, ns not significant.
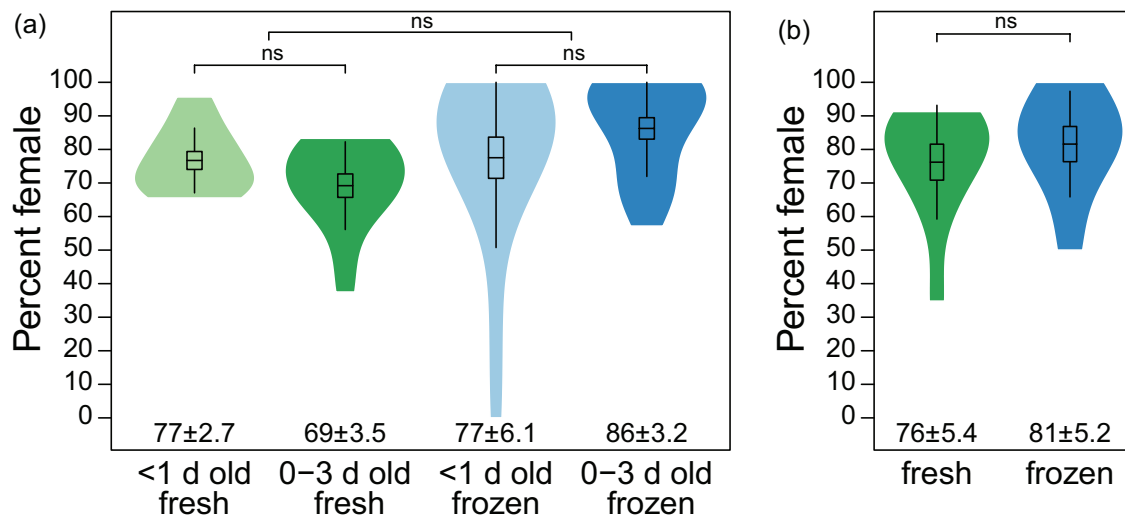

Figure 3. Kernel density plots of sex ratio for *T. japonicus* emerged from egg masses exposed to *T. japonicus* for 4 d (a, Obj. 1a) or 24 h (b, Obj. 1b). Internal boxplots show mean and standard error; whiskers extend to standard deviations. Mean ± standard errors are printed for each treatment. Per results of linear models: *** p < 0.001, ** p < 0.01, * p < 0.05, ns not significant.

significant difference in sex ratio, where 76-82% of emerged wasps were female in both treatments (Figure 3b). Egg type did not affect emerged wasp weight, with an average weight of 0.15 µg. Across treatments, *T. japonicus* females were heavier than males (Figure 4a). In contrast, tibia length was different between egg types but not between sexes. Wasps developed on fresh eggs had longer tibias than those developed on frozen (Figure 4b).

Table 3. Results for Objective 1b.

Type III Wald χ2 tests		Parasitism rate		Sex ratio		Development time		Weight		Tibia length	
Effect	df	χ2	Pr>χ2	χ2	Pr>χ2	χ2	Pr>χ2	χ2	Pr>χ2	χ2	Pr>χ2
Egg type	1	9.9	**0.002**	0.0204	0.89	179	**<0.001**	0.013	0.91	24	**<0.001**
Sex	1	nd	nd	nd	nd	102	**<0.001**	80	**<0.001**	0.47	0.5

Linear models of parasitism, sex ratio of emerged wasps, developmental time, emerged wasp weight and hind tibia length for egg masses exposed to *T. japonicus* for 24 h. Bold values are statistically significant. No data collected are indicated with 'nd'.
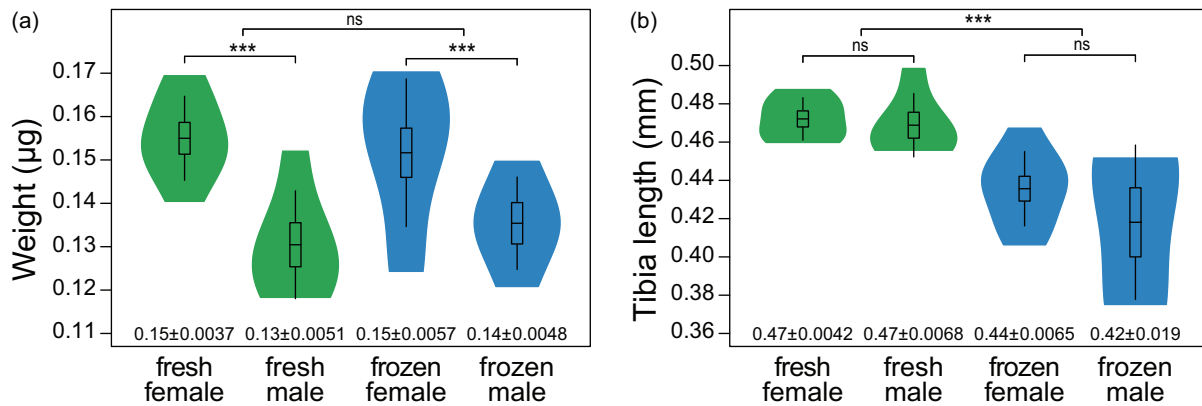

Figure 4. Kernel density plots of weight (a) and hind tibia length (b) for *T. japonicus* emerged from egg masses exposed to *T. japonicus* for 24 h (Obj. 1b). Internal boxplots show mean and standard error; whiskers extend to standard deviations. Mean ± standard errors are printed for each treatment. Per results of linear models: ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; ns, not significant.

Objective 2a: assessment of longer-stored frozen eggs (high parasitism pressure)

Storing frozen egg masses reduced parasitism rate by 1.2 ± 0.35 % (mean ± se) per month stored ($R^2 = 0.23$, Table 4). Overall average parasitism rate was 27 ± 0.05 % (mean ± se). There was no significant difference in sex ratio, but it is notable that there were more females emerging from longer-stored eggs. Emerged wasp weight was not impacted by frozen storage, but was greater for females than males. Tibia length was marginally different, with hind tibia length decreasing 0.47 ± 0.13 μm per month stored ($R^2 = 0.05$).

Objective 2b: assessment of longer-stored frozen eggs (low parasitism pressure)

Consistent with results from Objective 2a under high parasitism pressure, parasitism rate of longer-stored frozen eggs was also reduced at low parasitism pressure (Table 4).

Objective 3: heat exposure of frozen sentinel eggs

The parasitism rate of incubated egg masses was higher at 20 °C than at 30 °C (Table 5). Number of days incubated was also a significant effect, with parasitism rate reduced 8 % per day incubated at 20 °C and 5 % per day incubated at 30 °C (Figure 5a). The number of unhatched wasps increased by 8 % per day incubated at both temperatures (Figure 5b). Finally, there was no significant different in sex ratio by incubation temperature or days incubated (Figure 5c).

Table 4. Results for Objective 2.

	Type III Wald χ2 tests		Parasitism rate		Sex ratio		Weight		Tibia length	
Study	Effect	df	χ2	Pr>χ2	χ2	Pr>χ2	χ2	Pr>χ2	χ2	Pr>χ2
Obj. 2a	Days stored	1	4.3	**0.038**	0.26	0.61	1.5	0.22	4.8	**0.028**
	Sex	1	nd	nd	nd	nd	103	**<0.001**	0.30	0.58
Obj. 2b	Days stored	1	4.04	**0.044**	0.39	0.53	nd	nd	nd	nd

Linear models of parasitism rate, sex ratio (% female) of emerged wasps, emerged wasp weight and hind tibia length for egg masses stored frozen for up to 4 years, and exposed to *T. japonicus* for 4 d. Bold values are statistically significant. No data collected are indicated with 'nd'.Percent of eggs parasitised decreased 2.6 ± 0.79 % per month stored ($R^2 = 0.12$). Overall average parasitism rate was 53 ± 0.04 % (mean ± se). There was no significant difference in sex ratio.

Table 5. Results for Objective 3.

Type III Wald χ2 tests		Parasitism rate		Unhatched parasitoids		Sex ratio	
Effect	df	χ2	Pr>χ2	χ2	Pr>χ2	χ2	Pr>χ2
Temperature	1	3.9	**0.049**	0.62	0.43	0.23	0.63
Days incubated	1	15	**<0.001**	17	**<0.001**	0.74	0.39

Generalised linear models of parasitism rate, unhatched parasitoids and sex ratio for egg masses incubated at 20 and 30°C for 1–9 d. Bold values are statistically significant.
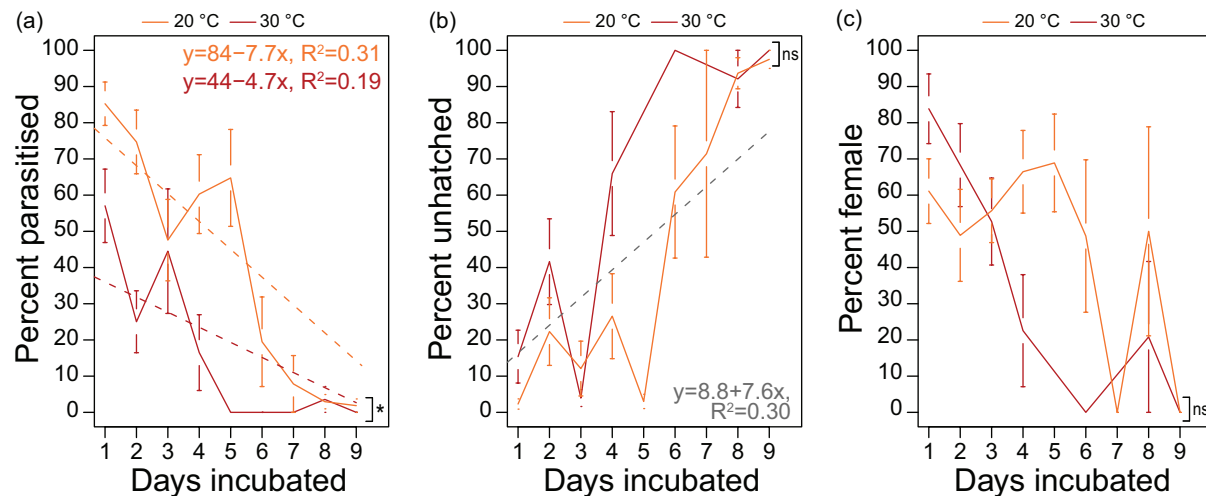

Figure 5. Parasitism rate (a), unhatched wasps (b) and sex ratio (c) of frozen egg masses incubated at 20 and 30°C for 1–9 d. Dots and whiskers indicate the mean and standard error. Dotted lines show linear regressions for generalised linear models where days incubated was a significant effect. Models where temperature was a significant effect include two linear regressions. Asterisks denote significant differences between temperature treatments per results of linear models: *p < 0.05; ns, not significant.

Discussion

Frozen and longer-stored *H. halys* egg masses are suitable for use in *T. japonicus* rearing and experimentation, but there are clear limitations. *Trissolcus japonicus* parasitised frozen egg masses, but emergence was substantially lower. Storing frozen eggs longer resulted in overall lower emergence, around 27-53 %, and decreased emergence by 1-3 % per month. This result

was consistent at high and low parasitism pressure, with wasps from two colonies, under slightly different experimental conditions at two locations. Freezing eggs also slowed *T. japonicus* emergence by 5-6 d. These effects vary between Scelionid parasitoid-host systems. *Trissolcus nigripedius* parasitised 55 % fewer frozen *Dolycoris baccarum* eggs than fresh, and parasitism rate decreased further for eggs frozen longer (Mahmoud & Lim, 2007). Contrarily, *Trissolcus basalis* showed no reduction in parasitism rate of *Nezara viridula* eggs frozen <30 d, but eggs stored frozen >30 d had slightly reduced emergence (<2 %; Wright & Diez, 2011). *Trissolcus semistriatus* showed no difference in parasitism of four heteropterans' eggs stored frozen one month, but parasitism rate declined when eggs were stored for 2-5 months (Kivan & Kilic, 2005). *Trissolcus semistriatus* also developed more slowly on frozen eggs (Kivan & Kilic, 2005). Overall, lower emergence should be accounted for when using frozen egg masses for rearing *T. japonicus*, especially eggs stored for longer durations.

Our study cannot elucidate whether the reported reduction in emergence is due to fewer eggs laid in frozen and longer-stored egg masses, or if such egg masses cause poor parasitoid survival. Mahmoud & Lim (2007) distinguished between overall parasitism rate (including unhatched wasps) and emergence rate (percent of parasitised eggs that emerged), finding no significant difference in emergence rate between fresh and frozen eggs. This may suggest that wasps have equal survival in frozen and fresh eggs, but lay fewer eggs in frozen egg masses. On the other hand, reduced parasitism rate and slower development may be explained by reduced nutritional quality of eggs due to freezing. Frozen *H. halys* egg masses have lower lipid levels than fresh (Skillman & Lee, 2017), and *Ephestia kuehniella* eggs with reduced triglycerides impacted *Trichogramma brassicae* fecundity and longevity (Kishani Farahani, Ashouri, Zibaee, Abroon, & Alford, 2016). Further experimentation in our parasitoid-host system could illuminate the cause of lower and slower emergence. For instance, future experiments would count hatched and unhatched parasitoids to determine if lower emergence in frozen and longer-stored eggs is due to reduced survival.

Potential sex ratio bias from rearing on frozen eggs has critical impacts for colony success, but freezing *H. halys* eggs for any duration did not impact sex ratio of emerged *T. japonicus*. In all experiments, emerged wasps were 76-86 % female, consistent with the 83 % female sex ratio found by Hedstrom et al. (2017). Similarly, the sex ratio of *T. nigripedius* was the same for wasps reared on eggs frozen 0, 8, 20, and 60 d (Mahmoud & Lim, 2007). Contrarily, the sex ratio of *T. basalis* was male-biased from rearing on frozen eggs (Wright & Diez, 2011).

Sex allocation theory predicts the preferential placement of female eggs into higher quality host eggs and males into lower quality host eggs to increase female survival (Charnov, 1981). This might explain why fewer *T. basalis* females emerged from frozen eggs. However, *T. japonicus* does not employ this strategy. While not significant, slightly more *T. japonicus* females emerged from frozen and longer-stored egg masses. It is possible that *T. japonicus* actually allocates more females into lower quality eggs, with greater numbers increasing the likelihood of female survival. Regardless, using frozen and longer-stored egg masses in rearing *T. japonicus* will not immediately affect the colony sex ratio.

Despite potential impacts of freezing on host egg quality, weight of the first generation of *T. japonicus* was not impacted by rearing on frozen or longer-stored egg masses. However, wasps that developed on frozen eggs had shorter hind tibias, with a small decrease in tibia length as eggs were stored longer. A change in hind tibia length without a

change in weight is puzzling, possibly pointing to a change in body proportions due to development on frozen eggs, or a result that is not biologically significant. Mahmoud & Lim (2007) also found a slight decrease in hind tibia length for *T. nigripedius* reared on *D. baccarum* eggs refrigerated for >20 d, but did not measure wasp weight. While limited by reduced parasitism rate and slower emergence, using frozen and longer-stored *H. halys* egg masses for *T. japonicus* rearing will not substantially impact colony sex ratio or size of the first generation of wasps.

Next, we showed that variably-aged egg masses are suitable for *T. japonicus* rearing and experimentation. While host age is an important component of host quality, 0-3 d old eggs were equivalent to newly-laid eggs, with no change in parasitism rate, sex ratio, and development time when fresh or frozen. Similarly, Qiu, Yang, & Tao (2007) reported no difference in parasitism rate of *H. halys* eggs up to 2.5 d old, a slight reduction in parasitism rate of 3 d old eggs, and a substantial drop for eggs older than 3 d. Yang et al. (2018) recently examined *T. japonicus* parasitism rate and development time on fresh, fertilised *H. halys* eggs that were 1 d, 3 d, and 5 d old. Emergence rate and development time were the same for 1 d and 3 d old eggs, but emergence rate was lower and development time was longer for 5 d old eggs. Further, Yang et al. (2018) found a decrease in the number of female offspring in 5 d old eggs, but the sex ratio was the same for 1 d and 3 d old eggs. The results of these three different studies suggest that *T. japonicus* can compete with *H. halys* host defense for at least 3 d. Results are likely temperature-dependent, since unparasitised *H. halys* egg development time varies from 3 d at 30 °C to 22 d at 15 °C (Nielsen, Hamilton, & Matadha, 2008). Interestingly, other egg parasitoids of pentatomids have the ability to attack and develop from egg masses until just before emergence of the neonate host (Cusumano, Peri, Amodeo, McNeil, & Colazza, 2013). Ultimately, host quality of 0-3 d old *H. halys* egg masses can be considered equivalent to newly-laid egg masses.

Finally, prolonged heat exposure has clear impacts on sentinel egg masses. Rate of parasitism was reduced at high temperatures, and was further reduced the longer egg masses were exposed. This may be due to lower parasitoid survival, since the percent of unhatched parasitised eggs increased by 8 % per day incubated. Skillman & Lee (2017) showed that glycogen levels increased and sugar levels decreased for frozen egg masses incubated for 4 and 7 d at 22.5 °C and 34 °C. Poor parasitoid survival may be due to poor host nutritional quality. This could explain why Qiu et al. (2007) found reduced longevity of *T. japonicus* developed at higher temperatures. We conclude that frozen sentinel egg masses will best reflect natural rates of parasitism when left in the field for short periods: <5 d at 20 °C and <3 d at 30 °C. It is also necessary to dissect all sentinel eggs to improve accuracy of results, since unhatched parasitoids are common. While the active time of frozen sentinel egg masses is short, they may actually remain attractive to wasps for longer than fresh sentinel egg masses in some circumstances. For example, *H. halys* eggs hatch in 3 d at 30 °C (Nielsen et al., 2008), so the active time of fresh sentinel egg masses at that temperature is 2 d or less, which is slightly shorter than for frozen eggs. However, at 20 °C, fresh sentinel egg masses have a much longer active time than frozen, as it takes eggs around 11.5 d to hatch. Besides the time for eggs to hatch, it is unclear whether heat affects fresh egg masses differently than frozen.

Using frozen *H. halys* egg masses for *T. japonicus* rearing and experimentation will yield a lower rate of parasitism and slower development time, but will not result in deleterious short-term effects on emerged wasp sex ratio or size. We offer several

recommendations for considering the use of frozen egg masses. We suggest using frozen and longer-stored eggs as a supplement in rearing when availability of fresh, newly-laid eggs is low, but we advise against complete replacement of fresh eggs. Storage time of frozen eggs should be minimised. To yield approximately 50% parasitism, eggs can be stored frozen 4-7 months. However, if egg availability is low, a storage duration of 17-25 months would yield approximately 25% parasitism. In the absence of fresh eggs, frozen eggs are suitable for experimental applications where a lower emergence rate is acceptable. Also, experiments should require only one generation of emerged *T. japonicus*, as we did not study the impact of frozen and longer-stored eggs on the fitness of subsequent generations of wasp offspring. Experiments must account for decreased parasitism, and should continue to separate analyses of parasitism by fresh and frozen egg masses. Egg masses can be up to 3 d old when used fresh or frozen, and are suitable for colony and experimental use. Finally, for best results in sentinel egg studies, frozen sentinel egg masses should be deployed for short durations, ideally <3 d. Overall, our findings provide a better understanding of the utility of frozen, longer-stored, and variably-aged egg masses, exhibit the temporal limitations of frozen sentinel egg masses, and contribute to important foundations for *H. halys* biocontrol.

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Declaration of interest statement

No potential conflict of interest was reported by the authors.

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