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Egg load mediates target and non-target host acceptance and self-superparasitism in *Trissolcus japonicus*: identifying an optimal window for augmentative release

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Abstract Effective augmentative biological control requires releasing parasitoids physiologically prepared to exploit target hosts while minimizing non-target acceptance and redundant host use. We first tested how female age and mating status shape egg load the samurai wasp, Trissolcus japonicus (Ashmead) and how these physiological traits in turn mediate three behaviors: acceptance of target (Halyomorpha halys Stål) and non-target (Antheraea pernyi Guérin-Méneville) host eggs, and self-superparasitism of the target host. Egg load rose from approximately 10 eggs at emergence to a plateau of 35-41 eggs by day 6 remaining stable over the following 21 days. Acceptance (attack rate) of target host eggs increased sharply with age, from 45.7% at emergence to near saturation (~100%) within two days. Newly emerged females already accepted non-target host eggs (7.0%); this rate peaked at 29.2% at days 4-6. Self-superparasitism followed a similar trajectory: absent at emergence (0%), rare at day 2 (2.1%), clearly expressed at 4-6 days (18.5%), and rising only slightly thereafter (32.2% at 22-27 days). Experimental egg depletion reduced non-target host acceptance and self-superparasitism, confirming egg load as a proximate driver of both. Mating status had no significant effect on egg load, target or non-target host acceptance, or self-superparasitism, indicating that it is not a major determinant of these traits. Collectively, our findings suggest an adult age of approximately 2–4 days as a promising release window that balances high target host acceptance and increasing egg availability with low non-target risk and redundant host use.

Keywords:·*Trissolcus japonicus*; Egg load; Female age; Host acceptance;·Self-superparasitism; Ecological risk

Introduction

The brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), is an invasive agricultural pest that damages fruit, vegetable and field crops across its invaded range (Rice et al. 2014; Leskey and Nielsen 2018; Pajač Beus et al 2024). Its movement between crops and surrounding habitats complicates management, while reliance on broad-spectrum insecticides can disrupt integrated pest management (Leskey et al. 2012; Leskey et al. 2014). The samurai wasp, *Trissolcus japonicus* (Ashmead) (Hymenoptera: Scelionidae), is a leading candidate for the biological control of *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), as it is a primary egg parasitoid of this pest in its native range (Yang et al. 2009; Zhang et al. 2017; Haye et al. 2020). Its establishment outside its native range and advances in mass-rearing have increased opportunities for its use in augmentative biological control (Talamas et al. 2015; Sabbatini Peverieri et al. 2018, 2020; Bittau et al. 2021; Li et al. 2023). An important consideration in planning such releases is how female state influences target acceptance, non-target host acceptance (Sabbatini Peverieri et al. 2021) and self-superparasitism, as understanding these behaviors can help identify females best suited for release.

High target host acceptance is desirable because females that delay oviposition may be killed by natural enemies before exploiting available host eggs (Meyhöfer and Klug 2002). However, target host acceptance alone may be insufficient to identify suitable females for release. A state associated with greater willingness to oviposit could also increase acceptance of non-target host eggs and superparasitism (i.e., repeated oviposition into previously parasitized hosts) (Fletcher et al. 1994; Sirot et al. 1997). Both behaviors waste eggs and handling time; moreover, non-target attack poses ecological risks to non-target species (Babendreier et al. 2003), whereas superparasitism reduces the reproductive efficiency of solitary parasitoids (Visser 1993).

Female age and egg load are key determinants of parasitoid oviposition behavior, as host-use decisions depend on current egg availability and future reproductive opportunities (Minkenberg et al. 1992; Heimpel and Rosenheim 1998; Rosenheim 1999; Jervis et al. 2008), ultimately shaping host acceptance and superparasitism (Roitberg et al. 1992; Fletcher et al. 1994; Sirot et al. 1997; Tunca et al. 2017; de Bruijn et al. 2022). In addition, mating has been reported to influence female physiological condition and to modify host utilization patterns in parasitoids (Nishimura 1997; Liu and Hao 2019; Li et al. 2021). Therefore, investigating the effects of female age, egg load and mating status may help us understand host acceptance, non-target host acceptance and self-superparasitism in *T. japonicus*, yet the

roles of these factors in shaping these behaviors remain unclear.

To date, assessments of non-target host acceptance in *T. japonicus* have focused almost exclusively on other hemipterans, particularly pentatomids (Yang et al. 2009; Zhang et al. 2017; Haye et al. 2020; Hepler et al. 2020), leaving it largely unknown whether females accept eggs of insects from non-hemipteran orders.The Chinese oak silkmoth, *Antheraea pernyi* Guérin-Méneville (Lepidoptera: Saturniidae), provides a well-suited probe of this question. Its larvae feed on oak foliage (Liu et al. 2010), and *H. halys* likewise uses oaks as woody host plants and overwintering sites (Rice et al. 2014; Bosco et al. 2020), so the two species may share host plants and occur in proximity. However, *A. pernyi* eggs differ from *H. halys* eggs in size, shape and coloration and belong to a different insect order, making them a deliberately distant, morphologically dissimilar non-target with which to probe the limits of *T. japonicus* host acceptance. Furthermore, *T. japonicus* has been observed performing repeated oviposition sequences on a single egg of *Hypsithocus hudsonae* (Saunders et al. 2021). This atypical behavior is of concern, as the underlying causes of such self-superparasitism in this species remain largely unknown.

Here, we addressed three specific questions: (1) how female age and mating status affect egg load in *T. japonicus*; (2) how these factors and egg load influence the acceptance of target and non-target host eggs as well as self-superparasitism; and (3) whether these responses define a candidate release window that best reconciles high target host acceptance with low non-target risk and self-superparasitism.To address these questions, we quantified egg load across female ages and mating states and compared the corresponding behavioral responses of *T. japonicus* on both target host, *H. halys* eggs and non-target host, *A. pernyi* eggs. To our knowledge, this is the first study to report that *T. japonicus* attempts to attack moth eggs, and to evaluate the effect of egg load on self-superparasitism in its target host.

Materials and methods

Insects

Host eggs: Adults of *H. halys* were collected in Baiwang Mountain Park (116°15′31″E, 40°01′43″N) and Fragrant Hills Park (116°10′21″ E, 39°59′28″ N), Haidian District, Beijing. Insects were transferred into tent shaped rearing cages (60 cm × 60 cm × 60 cm) maintained in a climatic room at 25±2 °C with a 16L:8D photoperiod, and 55-60% RH to establish laboratory colonies. Green beans (*Phaseolus vulgaris* L.), sweet corn (*Zea mays* L.), and soy bean seedlings (*Glycine max* (L.) Merr.) were provided as food.

Non-target host eggs: *Antheraea pernyi* eggs were purchased from Fucai *Trichogramma* Production Professional Cooperative (Liaoning, China) and maintained at −20 °C until needed. To select high-quality

individuals for bioassays, the eggs were subjected to a water flotation test in a 200-mL beaker. To prevent contamination, the beaker was pre-cleaned with water and ethanol, followed by air drying at room temperature. Floating eggs were discarded, and the sinking, high-quality eggs were retained.

Parasitoids: *Trissolcus japonicus* were obtained from *H. halys* egg masses collected in the wild. The wasps were then reared in a clear Perspex box (25 × 25 × 25 cm) and fed with 20% honey-water solution under the same rearing conditions mentioned above. To maintain the laboratory population, egg masses produced by the above-mentioned stinkbug population were provided.

Egg load dynamics across female physiological states

To determine the egg load of *T. japonicus* females, dissections were performed across different mating statuses (mated and unmated) and age groups (0, 2, 4, 5, 6, 8, 12, and 27 post-emergence). Female wasps were first killed by freezing at −80 °C. Each specimen was then individually transferred to a glass slide using distilled water as the dissection medium. Under a stereomicroscope (Olympus SZ51, Japan) equipped with a digital camera system (MC-2000, Beijing Jieying Technology Co., Ltd., China), the abdomen of the wasp was carefully ruptured using fine-tipped forceps. The eggs were thereby released into the water, where they were subsequently counted. In addition, to document ovarian morphology, a single ovary was dissected intact from mated, oviposition-naive females at 0, 1, 2, 4, 6, and 27 days post-emergence and photographed using the same imaging system. Subsequently, the maximum width of each unilateral ovary was measured using ToupView software for females aged 0, 6, and 27 days, with at least four replicates per age group.

Host and non-target acceptance assays

To evaluate the impact of physiological states on the egg acceptance behavior of *T. japonicus*, mated and unmated female wasps without prior oviposition experience at various ages (0, 2, 4-6, 8-12, and 22-27 days old) were prepared. Egg cards were constructed using a non-toxic adhesive. For the host egg cards, intact, naturally laid fresh *H. halys* egg masses (2-3 days old, containing 22-28 eggs per mass) were used. For the non-target host egg cards, six *A. pernyi* eggs, previously selected via a flotation test, were arranged horizontally in a standardized configuration.

All behavioral bioassays were conducted between 14:00 and 19:00. To minimize temporal and observer bias, treatments were tested in a randomized order by a single observer. The assays for host and non-target host eggs were conducted as two independent experiments using separate batches of wasps. The bioassays were performed in Petri dishes (6 cm diameter) previously cleaned with 75% ethanol. A

single female wasp was gently introduced into the dish containing its respective egg card, and her behavior was directly observed for 30 min.

If a parasitoid spontaneously contacted the eggs within the initial 30-min window, it was recorded as a "spontaneous contact." For individuals that failed to make contact within this period, manual assistance was provided to guide them to the eggs to ensure that host acceptance could be assessed independently of search failure. Subsequent inspection and attack behaviors were examined for all individuals, regardless of whether contact occurred spontaneously or with manual assistance. Inspection behavior was characterized by the female wasp slowly crawling over a host egg, continuously drumming its chorion with her antennae, and circling around it. Attack behavior was defined as a wasp drills host egg with her ovipositor. Searching duration and inspection duration were also examined. For parasitoid that spontaneously contacted an egg, searching duration was defined as the time elapsed between her introduction and the first contact an egg with her antenna. The inspection duration was calculated as the total time the wasp remained in contact with the eggs, excluding the time spent actively attacking with ovipositor.

In the host assays, the recorded parameters included contact rate, inspection rate, attack rate, and searching duration. At least 15 replicates were performed per treatment, with a total of 260 wasps tested. In the non-target host egg assays, contact rate, inspection rate, attack rate, searching duration, and inspection duration were recorded. At least 25 replicates were performed per physiological state treatment, with a total of 355 wasps tested. In both assays, each replicate consisted of one egg card and one wasp, neither of which was used in any other replicate.

$$\text{Contact rate (\%)} = \frac{\text{Number of parasitoids that contacted the eggs}}{\text{Total parasitoids tested}} \times 100\%$$

$$\text{Inspection rate (\%)} = \frac{\text{Spontaneously inspecting parasitoids} + \text{Manually assisted inspecting parasitoids}}{\text{Total parasitoids tested}} \times 100\%$$

$$\text{Attack rate (\%)} = \frac{\text{Spontaneously drilling parasitoids} + \text{Manually assisted drilling parasitoids}}{\text{Total parasitoids tested}} \times 100\%$$

Self-superparasitism assays under host limitation

To assess the effect of varying physiological states on superparasitism behavior when host resources are limited, a new cohort of oviposition-naïve female wasps was prepared, comprising both mated and unmated females in each of five age groups (0, 2, 4–6, 8–12, and 22–27 days old). Host egg cards were

constructed by affixing four *H. halys* eggs in a square configuration and placed into Petri dishes (6 cm diameter).

A single female wasp was introduced into the arena and continuously guided manually to contact the host eggs until she initiated her first attack. Once the initial attack commenced, all manual interventions were ceased, and the wasp's behavior was continuously observed for the subsequent 1 hour.

Superparasitism events were identified and recorded based on the characteristic host-marking behavior exhibited by the wasp immediately following oviposition. A host egg was considered superparasitized if the same female wasp marked it more than once during the assay. During the 1-hour observation period, the incidence of superparasitism (superparasitism marking rate) was recorded. Furthermore, the number of supernumerary oviposition (i.e., the number of superparasitized mark) was quantified by subtracting the total number of available hosts (4) from the total number of observed host-marking events. A minimum of 17 replicates per physiological state treatment were conducted, yielding a total of 278 wasps tested.

Egg-depletion manipulation

To obtain egg-depleted individuals, each female wasp was provided with a cluster of 15-20 unparasitized fresh *H. halys* eggs (1-2-day-old) and continuously observed in a petri dish (6 cm diameter). The wasp was immediately removed from the arena upon successfully parasitizing 10 host eggs. and designated as egg deprived individual, whereas untreated wasps serving as controls.

To assess the impact of egg load on non-target host acceptance behavior, 8-day-old mated females without prior oviposition experience were tested in either egg deprived or egg replete (control) states following the previously described protocol for non-target host egg acceptance. Only the attack rate and inspection rate were recorded. A minimum of 35 replicates per group were conducted for each treatment.

To evaluate the effect of egg load on superparasitism under limited host availability, 6-day-old and 10-day-old mated females without prior oviposition experience were tested in either egg-deprived or egg replete states following the previously described superparasitism assay protocol. Sample sizes were 18 replicates per treatment for the 6-day-old cohort and 24 replicates per treatment for the 10-day-old cohort.

Statistical analysis

All analyzes were performed in Python v3.12.3 (SciPy v1.18.1, statsmodels v0.15.0), with female age class and mating status as fixed factors, each female as one independent observation, and $\alpha = 0.05$. Binary outcomes (contact, inspection, attack and superparasitism) were analyzed with Firth's penalized

logistic regression. The full model included the age × mating interaction, which was tested with a profile penalized likelihood-ratio test in which the coefficients of the tested term were constrained to zero within the full design matrix (Firth 1993; Heinze and Schemper 2002); Wald tests and profile likelihood confidence intervals are reported alongside. Non-significant interactions were removed and main effects tested in the additive model.

Latency to first contact, inspection duration and the number of eggs per superparasitising event were log10 (x + 1)-transformed and analyzed by two-way ANOVA (type II) including the age × mating interaction; eggs per event was restricted to the three age classes in which superparasitism occurred. Egg load was analyzed on untransformed counts over eight age classes, for which variance was homogeneous (Levene test); a log10(x + 1) analysis gave the same conclusions. Ovary size was compared by one-way ANOVA followed by Tukey's HSD, with a Kruskal–Wallis test as a robustness check.

Age classes were compared pairwise with two-sided Fisher's exact tests (incidence data) or Tukey's HSD tests (continuous data), with one Benjamini–Hochberg family pre-specified for each outcome: ten comparisons when ages were pooled over mating status, and twenty-five when a mating-status effect was retained. Egg-load comparisons used Tukey's HSD across the eight age classes (28 pairs). Effects of egg depletion on inspection of non-host eggs, attack and superparasitism were tested with two-sided Fisher's exact tests. Arithmetic means ± SE are reported throughout.

Results

Egg load dynamics

Egg load in female *T. japonicus* varied significantly with age ($F_{7,212} = 78.33$, $p < 0.001$), whereas neither mating status ($F_{1,212} = 0.34$, $P = 0.561$) nor the age × mating status interaction ($F_{7,212} = 1.91$, $P = 0.070$) had a significant effect (Fig. 1). Mean egg load increased significantly from ~10 in newly emerged females to a peak of 37–40 by day 6, and remained stable (~35–41 eggs) across older age groups up to day 27 ($p > 0.05$).

The age groups used in the subsequent behavioral assays (0, 2, 4-6, 8-12, and 22-27 days) spanned the observed age-related increase in egg load and the subsequent apparent plateau. Within this sequence, the 4-6-day group represented females approaching the plateau, although egg load still increased over this interval.

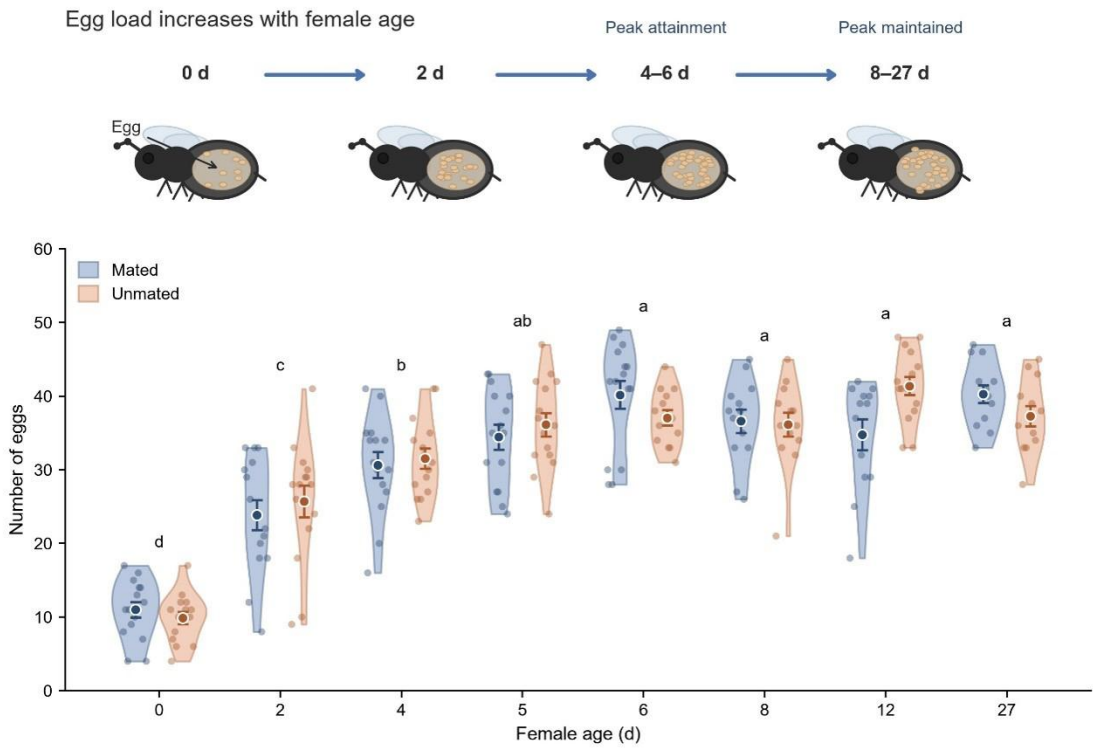


Fig. 1. Effects of female age and mating status on ovarian egg load in *Trissolcus japonicus* females at 0–27 days post-emergence. Error bars represent means ± SE. Data and error bars are shown separately for mated and unmated females. Letters indicate comparisons among age groups with mating statuses pooled; age groups sharing the same letter do not differ significantly ($p > 0.05$).

Age-related changes in ovarian morphology

Descriptive observations of ovarian morphology in female *T. japonicus* were consistent with the age-related changes in egg load (Fig. 2). As eggs accumulated, the ovaries expanded markedly in older females (6 and 27 days old) compared with newly emerged females ($F_{2,9}$=39.530, $p < 0.001$), whereas no significant difference in ovary size was observed between 6- and 27-day-old females.

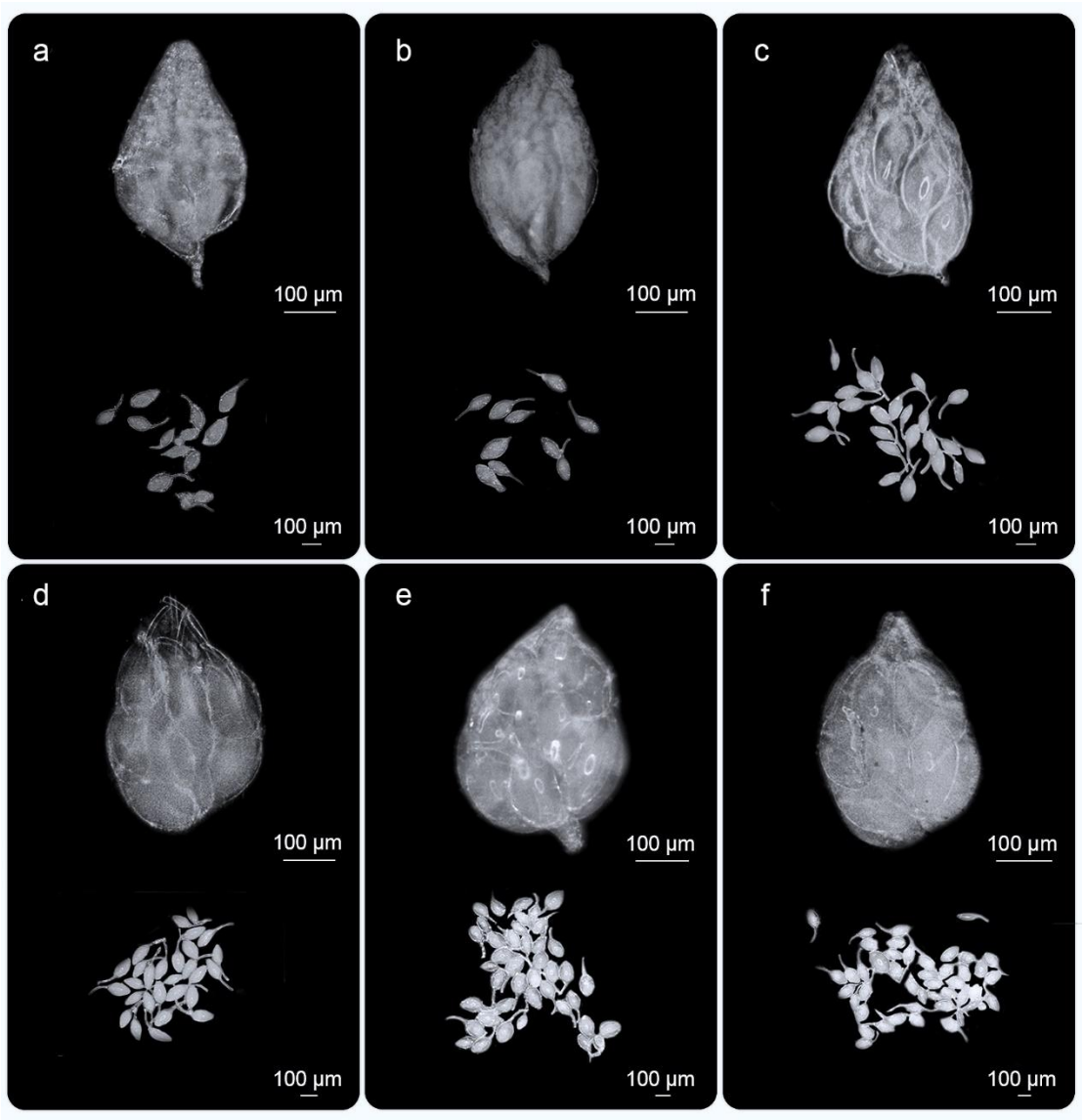


Fig. 2 Ovarian development of mated, oviposition-inexperienced *Trissolcus japonicus* females across different ages post-emergence: (a) 0 days, (b) 1 day, (c) 2 days, (d) 4 days, (e) 6 days, and (f) 27 days. For each age, the upper image shows one freshly dissected unilateral ovary, and the lower image shows the mature eggs dissected from the paired ovaries of the same female (scale bars = 100 μm).

Responses to target host eggs

The host-acceptance behavioral cascade of *T. japonicus* females toward target host eggs was strongly driven by early post-emergence age progression, whereas mating status and the age × mating status interaction had no significant effects on any behavioral parameter (Fig. 3). Specifically, the probabilities of host contact, inspection, and attack all increased sharply following emergence and stabilized at peak levels beyond 2 days of age (Fig. 3a–c).

When pooled across mating statuses, contact rates more than doubled from 37.1% in newly emerged

females to 78.6% in 2-day-old females (Fig. 3a). Similarly, both inspection and attack rates approached saturation by day 2, rising from 74.3% to 95.2% and from 45.7% to 95.2%, respectively, with no significant variations among older age cohorts (Fig. 3b, c).

In contrast, total searching duration remained constant across all treatments, averaging 494.6 ± 31.5 s regardless of female age or mating status (Fig. 3d).

Table 1. Effects of female age and mating status on responses to target host eggs

Response variable	**n***	**Factor**	**Test method*****	**Statistic**	**df****	***p***
Contact	260	Age	Firth, profile LR	24.47	4	<0.001
		Age × mating status	Firth, profile LR	5.53	4	0.237
		Mating status	Firth, profile LR	0.64	1	0.423
Inspection	260	Age	Firth, profile LR	20.18	4	<0.001
		Age × mating status	Firth, profile LR	1.29	4	0.864
		Mating status	Firth, profile LR	0.50	1	0.479
Attack	260	Age	Firth, profile LR	55.55	4	<0.001
		Age × mating status	Firth, profile LR	0.52	4	0.972
		Mating status	Firth, profile LR	2.19	1	0.139
Searching duration	191	Age	ANOVA (type II)	0.02	4, 181	0.999
		Age × mating status	ANOVA (type II)	0.95	4, 181	0.434
		Mating status	ANOVA (type II)	0.25	1, 181	0.616

*n, number of females analyzed;

**df, degrees of freedom (effect, residual for F tests).

*** Firth, Firth penalized logistic regression with profile penalized likelihood-ratio tests; ANOVA, two-way analysis of variance (type II) on log10(x + 1)-transformed data; Fisher exact, two-sided Fisher's exact test. Searching duration was recorded only for females that contacted the egg.

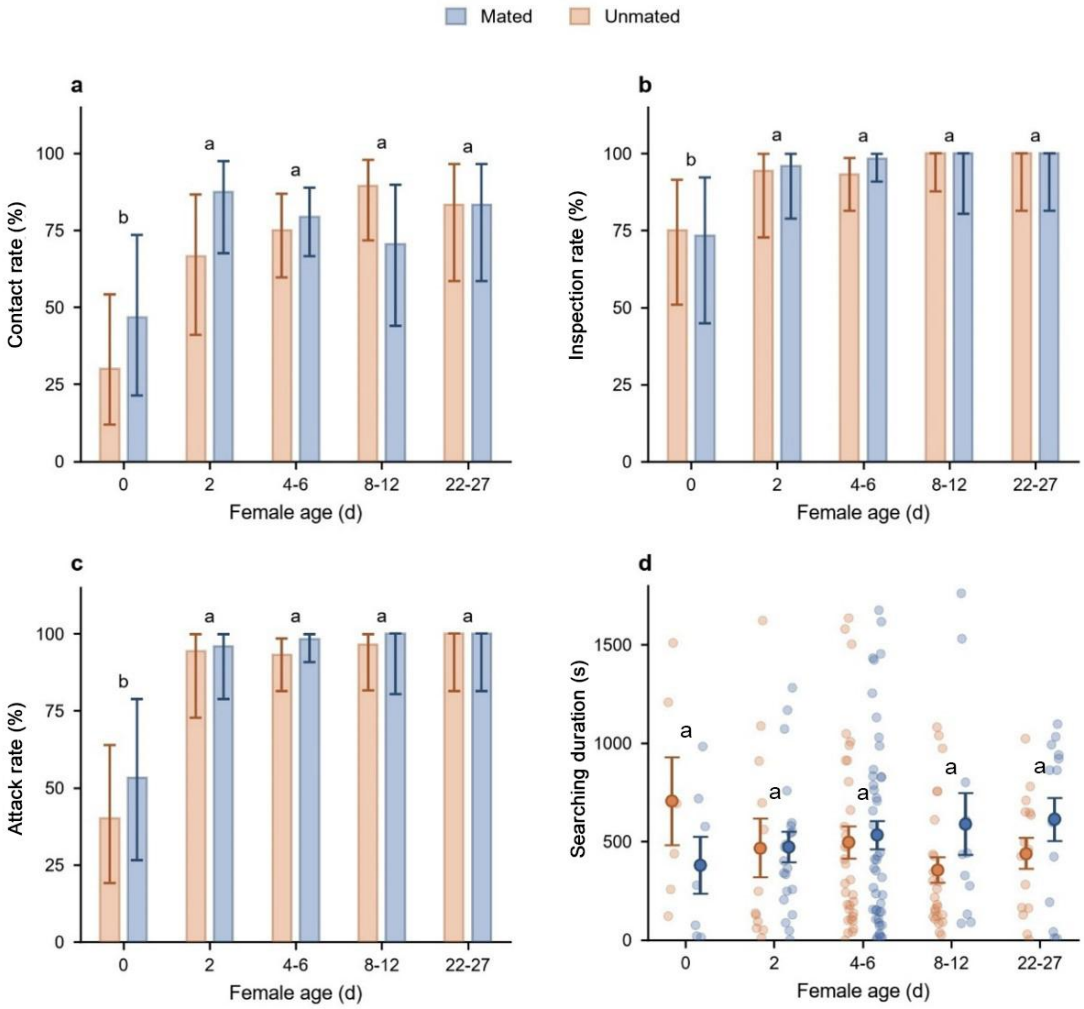


Fig. 3. Effects of female age and mating status on behavioral responses of *Trissolcus japonicus* to target host eggs (a) Incidence of contact; (b) incidence of inspection; (c) incidence of attack; (d) searching duration, respectively. Error bars in (a–c) represent Wilson 95% confidence intervals and (d) represent means ± SE. In panels (a–d), data and error bars are shown separately for mated and unmated females. Letters indicate comparisons among age groups with mating statuses pooled; age groups sharing the same letter do not differ significantly ($p > 0.05$).

Responses to non-target host eggs

Similar to patterns observed with host eggs, female behavioral responses to non-target host eggs were significantly modulated by age and egg load, but remained completely independent of mating status and the age × mating status interaction (Fig. 4; Table 2).

While the probability of initially contacting non-host eggs was invariant across all treatments (averaging 81.4%), subsequent inspection and attack behaviors were strongly age-dependent (Fig. 4a-c).

Pooled across mating statuses, inspection rates rose significantly from 70.2% at emergence to 88.3% at 4–6 days of age, stabilizing thereafter (Fig. 4b). Attack rates exhibited a parallel trajectory, significantly surging from 7.0% at emergence to 29.2% at 4–6 days of age before reaching a plateau (Fig. 4c; Fig. S1). Conversely, temporal investments, including searching duration (465.6 ± 25.7 s; Fig. 4d) and inspection duration (80.7 ± 4.4 s; Fig. 4e), were unaffected by age, mating status, or their interaction.

Furthermore, physiological egg load played a critical role in mediating non-target acceptance. Although egg-depleted females still occasionally attacked non-host eggs (Supplementary Fig. S1), their inspection (31.4%) and attack (5.7%) rates were significantly reduced compared to control females carrying intact egg loads (64.9% and 24.3%, respectively; Fig. 4f, g).

Table 2. Effects of female age and mating status on responses to non-target host eggs

Response variable	n*	Factor	Test method***	Statistic	df**	*p*
Contact	355	Age	Firth, profile LR	0.38	4	0.984
		Age × mating status	Firth, profile LR	1.75	4	0.781
		Mating status	Firth, profile LR	0.84	1	0.358
Inspection	355	Age	Firth, profile LR	24.04	4	<0.001
		Age × mating status	Firth, profile LR	8.04	4	0.090
		Mating status	Firth, profile LR	2.75	1	0.097
Attack	355	Age	Firth, profile LR	19.70	4	<0.001
		Age × mating status	Firth, profile LR	2.00	4	0.735
		Mating status	Firth, profile LR	0.42	1	0.517
Searching duration	289	Age	ANOVA (type II)	0.76	4, 279	0.553
		Age × mating status	ANOVA (type II)	1.99	4, 279	0.096
		Mating status	ANOVA (type II)	0.30	1, 279	0.583
Inspection duration	289	Age	ANOVA (type II)	0.79	4, 279	0.536
		Age × mating status	ANOVA (type II)	1.97	4, 279	0.099
		Mating status	ANOVA (type II)	1.74	1, 279	0.188
Inspection after egg depletion	72	Control vs depleted	Fisher exact	—	—	0.005
Attack after egg depletion	72	Control vs depleted	Fisher exact	—	—	0.047

*n, number of females analyzed;

**df, degrees of freedom (effect, residual for F tests).

***Firth, Firth penalized logistic regression with profile penalized likelihood-ratio tests; ANOVA, two-way analysis of variance (type II) on log10(x + 1)-transformed data; Fisher exact, two-sided Fisher's exact test. Searching and inspection durations were recorded only for females that contacted the egg.

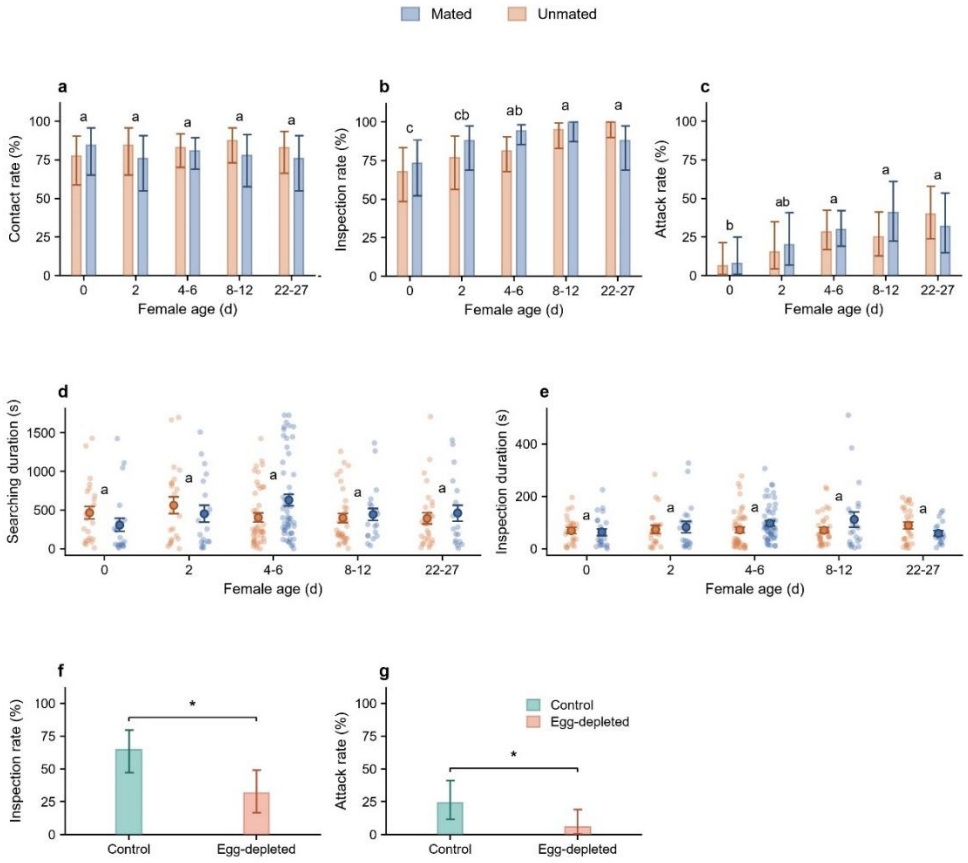


Fig. 4. Effects of female age, mating status, and prior egg depletion on behavioral responses of *Trissolcus japonicus* to non-target host eggs. (a–e) Incidence of contact, incidence of inspection, incidence of attack, searching duration, and inspection duration, respectively, by female age and mating status; (f-g) Incidence of inspection and attack in egg-depleted versus non-depleted females at 6 and 10 days of age. Error bars in panels (a–c) and (f–g) represent Wilson 95% confidence intervals, whereas those in panels (d) and (e) represent means ± SE. In panels (a–e), data and error bars are shown separately for mated and unmated females. In panels (a–e), letters indicate comparisons among age groups with mating statuses pooled; groups sharing the same letter do not differ significantly ($p > 0.05$).

Self-superparasitism under limited host availability

Female age significantly modulated superparasitism behavior in *T. japonicus* under host-limited conditions, whereas mating status and the age × mating status interaction had no significant effects (Fig.

5a; Table 3). Females prioritized unparasitized hosts, typically initiating superparasitism marking only after all four available host eggs were parasitized. Pooled across mating statuses, superparasitism marking rates were significantly lower in newly emerged (0-day-old: 0.0%) and 2-day-old (2.1%) females than in older cohorts (4–6 days: 18.5%), with no significant changes at older ages (8–12 days: 16.4%; 22–27 days: 32.2%). Furthermore, among superparasitizing females, superparasitism marking number (2.43 ± 0.18) was unaffected by age, mating status, or their interaction (Fig. 5b).

Subsequent egg-depletion assays confirmed that physiological egg load drives this behavior (Fig. 5c). Egg depletion abolished superparasitism marking, whereas non-depleted controls exhibited marking incidences of 16.7% and 19.0% at 6 and 10 days of age, respectively; this divergence was statistically significant at 10 days but not at 6 days.

Table 3. Effects of female age and mating status on superparasitism under limited host availability

Response variable	**n***	**Factor**	**Test method*****	**Statistic**	**df****	***p***
Superparasitism marking	278	Age	Firth, profile LR	26.45	4	<0.001
		Age × mating status	Firth, profile LR	0.67	4	0.955
		Mating status	Firth, profile LR	1.66	1	0.197
Eggs per marking event	37	Age	ANOVA (type II)	0.16	2, 31	0.849
		Age × mating status	ANOVA (type II)	0.41	2, 31	0.669
		Mating status	ANOVA (type II)	0.15	1, 31	0.698
Marking after egg depletion, 6 d	36	Depleted vs non-depleted	Fisher exact	—	—	0.229
Marking after egg depletion, 10 d	66	Depleted vs non-depleted	Fisher exact	—	—	0.043

*n, number of females analyzed;

**df, degrees of freedom (effect, residual for F tests).

***Firth, Firth penalized logistic regression with profile penalized likelihood-ratio tests; ANOVA, two-way analysis of variance (type II) on log10(x + 1)-transformed data; Fisher exact, two-sided Fisher's exact test. Marking events were less in 0- and 2-day-old females, so eggs per marking event were analyzed over the three older classes.

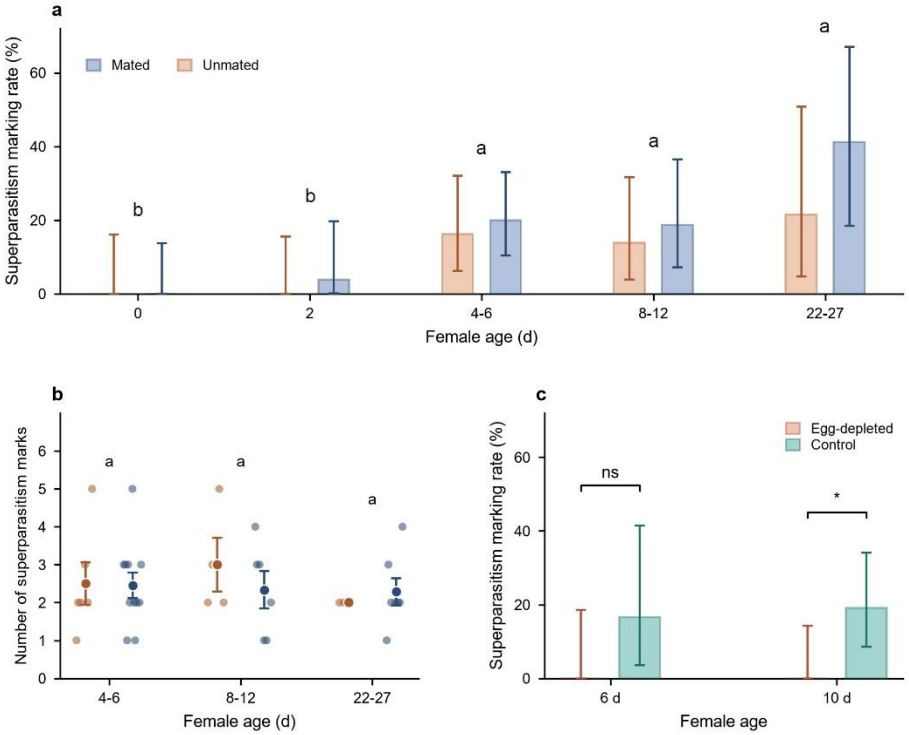


Fig. 5. Effects of female age, mating status, and prior egg depletion on superparasitism behavior of *Trissolcus japonicus* under host-limited conditions. (a) Incidence of superparasitism marking by female age and mating status; (b) Number of superparasitism marks per superparasitized host egg; data shown only for age groups (≥4–6 days) in which superparasitism occurred; (c) Incidence of superparasitism marking in egg-depleted versus non-depleted females at 6 and 10 days of age. Error bars in panels (a) and (c) represent Wilson 95% confidence intervals. In panel (a), error bars are shown separately for mated and unmated females. In panels (a) and (b), letters indicate comparisons among age groups with mating statuses pooled; groups sharing the same letter do not differ significantly ($p > 0.05$).

Discussion

To minimize non-target risks and maximize release efficacy, it is essential to understand how the physiological state of *T. japonicus* influences its target host non-target host acceptance and superparasitism. By combining behavioral assays with egg-load quantification and depletion manipulations, this study demonstrates that these behaviors are driven by egg load. Prior to the egg-load plateau, acceptance of both target and non-target hosts, alongside superparasitism, increased with female age. However, when egg load remained constant, advancing age did not promote non-target acceptance or superparasitism; conversely, egg-load depletion significantly reduced both behaviors. By contrast,

316 mating status had no effect on any behavioral traits. These results provide vital mechanistic insights for
317 optimizing the field release of *T. japonicus*.

318 Newly emerged *T. japonicus* females carried approximately 10 eggs, mostly immature eggs
319 characterized by incomplete posterior structures and fragile chorions. Total egg load increased rapidly
320 with age before stabilizing at a plateau, confirming a synovigenic strategy in which females emerge with
321 a partial egg complement and continue maturing eggs after emergence (Jervis et al. 2001). This pattern
322 aligns with previous findings in *T. japonicus* and its congener *T. mitsukurii*, where egg loads peak within
323 the first week post-emergence and remain stable thereafter (Sabbatini Peverieri et al. 2020; Paul et al.
324 2022). Notably, aging *T. japonicus* females did not show the late-life decline in egg load typically
325 associated with egg resorption. In contrast, females of the scelionid *Gryon pennsylvanicum* displayed
326 egg changes suggestive of resorption after 5–9 days of host deprivation (Vogt and Nechols 1993). Such
327 inconsistencies may reflect interspecific variation in the resorption response to host deprivation. Notably,
328 immature eggs were present in both young and old females, suggesting that the capacity for egg
329 maturation is retained over a substantial part of the adult stage. Whether the plateau in egg load reflects
330 a dynamic balance between oocyte maturation and resorption remains an open question. We did not use
331 microscopy at sufficient resolution to identify structural changes associated with egg resorption.
332 Therefore, this question cannot be answered by the present study.

333 Although newly emerged females carried very few mature eggs, they accepted both target and non-
334 target host eggs, demonstrating that host recognition is innate at emergence rather than requiring post-
335 emergence experience (Vos and Vet 2004). Moreover, the spontaneous attack on lepidopteran non-target
336 host eggs is unlikely to represent a novel behavior induced by physiological stress. A more plausible
337 explanation is that the shape, microstructure, and chemical cues on the surface of *A. pernyi* eggs share

features with those of the natural host (Vinson 1976). Identifying which of these similarities elicit oviposition willingness toward moth eggs in a specialist of pentatomic hosts is therefore an important question for future work. We acknowledge that the laboratory-prepared non-target host eggs may exhibit differences compared to wild, naturally laid eggs. Nevertheless, *A. pernyi* was utilized in this study primarily to provide a lepidopteran host model that contrasts sharply with *H. halys* eggs, thereby illustrating the potential for *T. japonicus* to inflict deleterious effects on the eggs of non-hemipteran species.

Nevertheless, behavioral acceptance of both target and non-target host eggs by *T. japonicus* was tightly coupled with age-related dynamics in early-life egg load. Acceptance of fresh *H. halys* eggs increased rapidly and plateaued by day 2 post-emergence, whereas acceptance of non-target eggs continued to rise until egg load plateaued at days 4–6. In addition, our egg-depletion assays show that experimental reduction of egg load sharply decreased non-target host acceptance. These findings support the egg-limitation model: as egg load increases, the acceptance threshold for lower-quality or non-target hosts decreases (Iwasa et al. 1984; Minkenberg et al. 1992; Fletcher et al. 1994; Heimpel et al. 1996; Heimpel and Rosenheim 1998). Interestingly, non-target acceptance did not increase further in older females (days 22–27). Given the exceptional adult longevity of *T. japonicus* (2–3 months; Sabbatini Peverieri et al. 2020), 22–27-day-old females may not yet experience “time limitation” to trigger further threshold relaxation, although this remains speculative. In addition, we noticed variation in non-target acceptance among broods from different mothers reared on *H. halys* eggs, hinting that genetic or other factors may also influence host-acceptance strategies (Pavlík 1993).

In *T. japonicus*, females normally rely on post-oviposition marking to recognize eggs they have already parasitized (Rosi et al. 2001). In our behavioral assays, we used repeated marking on the same

host as an indicator of self-superparasitism. Subsequent dissections of these multiply marked host eggs confirmed that the vast majority indeed contained two or more parasitoid eggs, verifying that repeated marking reflected egg deposition (Fig. S2). Furthermore, we observed that, in the vast majority of cases, females began to self-superparasitize only after all four host eggs had been parasitized and marked once. This pattern indicates that self-superparasitism did not appear to arise from a failure of host discrimination.

Superparasitism on a limited supply of host eggs was directly related to egg load. Newly emerged females with low egg loads never superparasitized, only one such event was recorded at 2 days after emergence, and superparasitism became frequent once the egg-load plateau had been attained (4–6 days). Once part of the egg load had been depleted, superparasitism ceased altogether. These observations identify egg load as the principal determinant of self-superparasitism in *T. japonicus*, in line with models in which superparasitism depends on egg load (Sirot et al. 1997; Tunca et al. 2017). Because only one offspring can survive per host in this solitary parasitoid (Yang et al. 2009), depositing multiple eggs in a single fresh, suitable host yields minimal benefit to the female. Why high egg load promotes superparasitism behavior remains unclear. One plausible hypothesis is that mature egg accumulation and ovarian distension provide an internal physiological cue that increases oviposition motivation (Gou et al. 2014; Medrano and Collins 2023). In addition, our egg-depletion treatment combined reduced egg load with prior oviposition experience, whereas control females were naive. However, because oviposition-naive young females (0–2 d) with naturally low egg loads also avoided superparasitism, internal egg load—rather than experience alone—remains the most parsimonious explanation.

Across the egg-load assessments and all behavioral assays, mating status had no significant effect on any measured response, suggesting that it plays a limited role in the increase in egg load, host

acceptance, and superparasitism in the synovigenic parasitoid *T. japonicus*. Previous studies reported that mating accelerates egg maturation, shortens female lifespan, and modifies host-use patterns (Nishimura 1997; Liu and Hao 2019; Li et al. 2021), but such effects may be species-specific or dependent on experimental conditions.

In conclusion, host and non-target host acceptance and superparasitism in *T. japonicus* are primarily driven by early-life changes in female egg load. For *H. halys* biological control, our findings identify an optimal release window of 2–4 days post-emergence, when target host acceptance has peaked and females carry enough eggs to parasitize an entire host egg mass, while non-target host attacks and superparasitism remain rare. These findings apply primarily to the initial post-release period, before females expend their eggs and associated risks decline. While relevant to both release strategies, optimizing release age is particularly critical for inundative releases, which deploy large numbers for immediate pest suppression, compared with inoculative releases that rely on smaller founding populations for multi-generational control. If logistical constraints prevent release within 2–4 days, releasing females before 22 days is a practical alternative, as risk-associated behaviors did not escalate further up to 22–27 days. Although these laboratory no-choice findings require field validation and consideration of other age-dependent traits (e.g., dispersal, searching capacity, and reproductive potential), our results remain support prioritizing the release of young females.

Author contributions

Z.-J.S, J.-P.Z and Y.-X.L planned and designed the research. Z.-J.S, F.C and Y.W performed experiments and analyzed data. Z.-J.S drafted the manuscript, Y.-X.L and J.-P.Z revised the manuscript.

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Supplementary materials

Supplementary material associated with this article can be Found.

Conflict of interest

The authors declare that they have no conflict of interest.

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