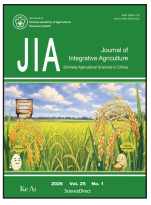
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RESEARCH ARTICLE

Performance and functional responses of the thelytokous and arrhenotokous strains of *Neochrysocharis formosa* to *Tuta absoluta*, a globally severe tomato pest

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Highlights

- The thelytokous (TH) and arrhenotokous (AR) strains preferentially parasitize and attack 1st instar host larvae over later instars.
- Variations in host-killing by *N. formosa* produce distinct functional responses to *T. absoluta*.
- Parasitism by TH and AR and host-stinging by TH display type III functional responses.
- Host-feeding by TH and AR and host-stinging by AR display type II functional responses.

Abstract

The native thelytokous (TH) and arrhenotokous (AR) strains of *Neochrysocharis formosa* (Westwood) (Hymenoptera: Eulophidae) are promising biocontrol agents against the invasive tomato pest *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). This study assessed the performance and preferences of these strains in choice experiments involving five host instar ratios and evaluated their functional responses to seven densities of 1st instar larvae (5 to 40 hosts). In host-attacking behavior assays, an increasing proportion of 1st instar larvae led to a significant rise in host mortality rates for both strains. Both strains exhibited strong preferences for parasitizing and attacking 1st instar larvae over later instars, with the TH strain demonstrating significantly greater host-killing efficacy than the AR strain. Functional response experiments revealed that the attack rates of both strains were positively correlated with host density. Parasitism by both strains and host-stinging behavior by the TH strain showed type III functional responses, while host-feeding by both strains and host-stinging by the AR strain followed type II functional responses. Early establishment of the TH strain in tomato agroecosystems could enhance the management of *T. absoluta*. These findings provide critical insights into the functional dynamics of the TH and AR strains of *N. formosa* that can inform the development of effective biocontrol programs for this globally significant pest.

Keywords: tomato leafminer, Eulophidae, Gelechiidae, idiobiont endo-parasitoid, choice experiment, biological control

1. Introduction

Insect parasitoids have been a cornerstone of pest management in agriculture and forestry for decades (Godfray 1994). Reproduction in parasitoid wasps typically follows two distinct modes: sexual reproduction (arrhenotoky) and asexual reproduction (thelytoky) (Cook 1993). Thelytokous parasitoids produce exclusively female offspring, which is particularly advantageous as only females actively parasitize and kill host pests. This reproductive mode offers higher population growth rates compared to arrhenotokous parasitoids (Stouthamer 1993) and reduces production costs (Heimpel and Lundgren 2000; Sivinski 2013). These economic benefits make thelytokous parasitoids ideal candidates for large-scale mass-rearing (Heimpel and Lundgren 2000; Sivinski 2013; Xu *et al.* 2022) and enhance their utility as biological control agents (Stouthamer 1993;

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Sivinski 2013).

One of the most challenging pests of tomato cultivation worldwide is the South American tomato leafminer, *Tuta absoluta* (Meyrick), also known as *Phthorimaea absoluta* (Chang and Metz 2021). Native to Peru, *T. absoluta* (Lepidoptera: Gelechiidae) has rapidly spread to over 110 countries and regions (Soares and Campos 2020, last updated on 6 February 2025). Its management is notoriously difficult due to the concealed feeding behavior of its larval stages, high reproductive capacity with overlapping generations, and resistance to many insecticides (Urbaneja *et al.* 2013; Biondi *et al.* 2018; Cherif *et al.* 2019; Guedes *et al.* 2019). Without effective control measures, *T. absoluta* infestations can lead to 80–100% losses in tomato production (Desneux *et al.* 2010; Biondi *et al.* 2018; Zhang *et al.* 2021). Therefore, this pest significantly threatens global tomato production (Lietti *et al.* 2005; Desneux *et al.* 2022). In this context, biological control using natural enemies such as predators and parasitoids offers a sustainable and feasible alternative to chemical control (Guleria *et al.* 2020).

Globally, approximately 100 hymenopteran parasitoids have been associated with *T. absoluta*. Among them, three species, including one larval parasitoid (*Neochrysocharis formosa* (Westwood)) and two egg parasitoids (*Trichogramma dendrolimi* (Matsumura) and *T. exiguum* (Girault)), have been reported in both native and invaded regions (Ferracini *et al.* 2019; Desneux *et al.* 2022). Moreover, four *Trichogramma* species, *Trichogramma chilonis* Ishii, *T. ostriniae* Pang et Chen, *T. japonicum* Ashmead (Jiang *et al.* 2024; Li *et al.* 2024), and *T. pintoi* (Girault) (Fu *et al.* 2023), have been recently reported as promising candidates for controlling *T. absoluta* in newly invaded regions such as China.

Neochrysocharis formosa (Hymenoptera: Eulophidae) is a polyphagous, host-feeding idiobiont endoparasitoid native to Asia (Wang *et al.* 2012; Guleria *et al.* 2020). It parasitizes a wide range of leaf-mining and gall-forming insects across several orders, including Lepidoptera (e.g., Gracillariidae, Nepticulidae, Gelechiidae, and Lyonetiidae), Diptera (e.g., Agromyzidae), and Coleoptera (Arakaki and Kinjo 1998; Luna *et al.* 2011; Xu *et al.* 2022). Its presence has been documented in various crops, including tomato, eggplant, melon, celery, and others in the Cucurbitaceae, Fabaceae, and Brassicaceae families (Arakaki and Kinjo 1998; Wen *et al.* 1999; Luna *et al.* 2011; Wang *et al.* 2012). Notably, *N. formosa* achieves natural control rates exceeding 50% against leaf miner larvae, such as *Liriomyza* species (Tryon and Poe 1981; Cabello *et al.* 1994). Field observations of *N. formosa* parasitizing *T. absoluta* larvae have been reported in South America, including Argentina (Luna *et al.* 2011); Europe (Zappalà *et al.* 2013), including Italy (Ferracini *et al.* 2012), France (Biondi *et al.* 2013), and Spain (Gabarra *et al.* 2014); Africa, including Algeria (Dehliz and Guénaoui 2015); and Asia, including Iran (Sohrabi *et al.* 2014), India (Guleria *et al.* 2020), and China (Wang *et al.* 2024). These findings highlight its potential as a biocontrol agent for pests such as *Liriomyza sativa* (Ye *et al.* 2023) and *T. absoluta* (Guleria *et al.* 2020; Wang *et al.* 2024).

The thelytokous (TH) and arrhenotokous (AR) reproductive modes of *N. formosa* occur sympatrically in regions such as Japan and China (Arakaki and Kinjo 1998; Weintraub *et al.* 2017; Yang *et al.* 2017; Xu *et al.* 2022). These strains exhibit three host-killing behaviors — parasitism (oviposition), host-feeding, and host-stinging — and show preferences for different larval instars depending on the host species (Liu *et al.* 2015; Xuan *et al.* 2022; Ye *et al.* 2023; Wang *et al.* 2024). For instance, both strains prefer the late 2nd and early 3rd instar larvae of *L. sativa* (Ye *et al.* 2023) but target all larval instars of *T. absoluta* under no-choice conditions, with a preference for the 1st instars (Wang *et al.* 2024).

Since its first detection in China in 2017 (Zhang *et al.* 2020), *T. absoluta* has rapidly spread to over 20 provinces (Han *et al.* 2019; Zhang *et al.* 2021; Li *et al.* 2025) and successive disastrous outbreaks have occurred in some regions, such as northwestern, southwestern, northern, and eastern China (Zhang *et al.* 2022; Li *et al.* 2025). In some regions (including southwestern China), over 80% of tomato cultivation areas have been infested by this notorious leafminer (Li *et al.* 2025), which poses a grave threat to agricultural productivity. With its multiple overlapping generations (10–12 per year) and simultaneous occurrence of various life stages in the same field (Urbaneja *et al.* 2013; Biondi *et al.* 2018; Cherif *et al.* 2019), *T. absoluta* is very challenging to manage. In tomato ecosystems, *N. formosa* is the most prevalent parasitoid associated with *T. absoluta* (Luna *et al.* 2011; Guleria *et al.* 2020; Desneux *et al.* 2022; Wang *et al.* 2024), so it is a promising candidate for conservation biological control strategies within integrated pest management (IPM) frameworks (Bodino *et al.* 2019; Desneux *et al.* 2022; Han *et al.* 2024; Wang *et al.* 2024).

Effective biocontrol requires a comprehensive evaluation of local natural enemies, particularly in systems where multiple larval instars coexist in field scenarios (Cornell and Hawkins 1993; Gabarra *et al.* 2014). Functional response analysis, which evaluates the relationship between host density and predation or parasitism rate, is a fundamental tool for assessing a natural enemy's pest control potential (Fellowes *et al.* 2005; Laumann *et al.* 2008; Dannon *et al.* 2010; Garay *et al.* 2015; Chen *et al.* 2016). However, information on the functional responses of *N. formosa* strains to the most promising host larval instar remains limited.

This study aims to address that knowledge gap by: (1) assessing the performance and preferences of the TH and AR strains of *N. formosa* toward the four larval instars of *T. absoluta* under choice conditions; and (2) determining the functional response types exhibited by both strains when exposed to varying densities of 1st instar *T. absoluta* larvae.

2. Materials and methods

2.1. Insect cultures

Tuta absoluta The *T. absoluta* colonies used in this study were originally collected from tomato fields located at an organic vegetable production base in Hongta, Yuxi, Yunnan, Southwest China (24°21′N, 102°32′E; 2,108.4 m a.s.l.) at the end of 2018. These colonies were reared for over 50 generations under controlled laboratory conditions at the National Agricultural Biosafety Science Center, Institute of Plant Protection, Chinese Academy of Agricultural Sciences

(IPP, CAAS), Beijing, China. The rearing conditions were maintained at (26±1)°C, a relative humidity of (40–60)%, and a photoperiod of 14 h light/10 h dark.

The *T. absoluta* population was sustained on potted tomato plants (cultivar: Shouyan PT326; sourced from Shouguang, Shandong, China) with 6–8 well-developed true leaves. The plants were housed in mesh cages (40 cm×40 cm×60 cm; mesh size: 150 μm×150 μm). To generate synchronized larval populations of the four different instars, two tomato plants per cage were exposed to 40–50 *T. absoluta* adults (female:male sex ratio 1:1) for a 24-h oviposition period. This exposure protocol was repeated once every 24 h. After egg-laying, the adults were removed, and larvae at the desired developmental stages were collected for subsequent experiments.

Neochrysocharis formosa Colonies of the TH and AR strains of *N. formosa* were separately established in 2015 and 2016 from Beijing and Hainan, China, respectively (Yang *et al.* 2020; Wang *et al.* 2024). Both strains were reared on late second and early third instar larvae of *Liriomyza sativae* Blanchard, which were cultured on kidney bean (*Phaseolus vulgaris* L.) plants. To minimize environmental and genetic variability, the colonies of each strain were maintained separately for over 30 generations under standardized conditions of (26±1)°C, (40–60)% relative humidity, and a 14 h light/10 h dark photoperiod. The purity of these strains was routinely verified every two months using polymerase chain reaction (PCR) with DNA barcoding targeting the *cytochrome c oxidase subunit I* (*COI*) gene (Yang *et al.* 2017).

2.2. Performance and preferences of the TH and AR strains of *N. formosa* in choice experiments

Previous no-choice experiments indicated that both the TH and AR strains of *N. formosa* prefer to parasitize younger (1st and 2nd instar) *T. absoluta* larvae (Wang *et al.* 2024). Based on these findings, younger instars were included in higher proportions for the choice experiments in this study. Five host-instar ratios were evaluated: Ratio 1, L1:L2:L3:L4=20:0:0:0; Ratio 2, L1:L2:L3:L4=15:5:0:0; Ratio 3, L1:L2:L3:L4=10:5:3:2; Ratio 4, L1:L2:L3:L4=5:10:3:2; and Ratio 5, L1:L2:L3:L4=5:5:5:5 (Appendix A). The four larval instars were differentiated and separated according to body length and head capsule width (Zhang *et al.* 2023).

Females of the TH and AR strains, aged 72–96 h and actively ovipositing (Ye *et al.* 2023), were individually introduced into Petri dishes for 48 h under the same controlled conditions used for insect rearing. Each Petri dish contained a tomato leaflet infested with 20 *T. absoluta* larvae spanning the specified instars and a 10 mL agar gel layer (1% w/v) to maintain leaflet freshness.

Petri dishes were covered with cling film perforated with small holes (~0.3 mm diameter) (Yang *et al.* 2020; Wang *et al.* 2024). After 48 h, the female wasps were removed, and the parasitized, fed-upon, stung, and surviving larvae were counted under a stereomicroscope 48 h later. Three host-killing behaviors (parasitism, feeding, and stinging) were classified following established protocols (Ye *et al.* 2023; Wang *et al.* 2024). A total of 20–22 females (replicates) from each strain were assessed across the five host-instar ratios. The percentage (%) of killed hosts was calculated as: (Number killed/Total number provided)×100%.

2.3. Functional responses of the TH and AR strains of *N. formosa* to 1st *T. absoluta* larvae

Functional response experiments focused exclusively on first-instar *T. absoluta* larvae, as this stage is highly preferred by both strains (Wang *et al.* 2024). Females of both strains at peak oviposition age were individually exposed to tomato leaflets infested with varying densities of first instar larvae (5, 10, 15, 20, 25, 30, or 40 larvae per leaflet). Preliminary trials determined 40 larvae as the maximum density for ensuring that the attack asymptote could be reached and the functional response parameters accurately estimated. The experimental conditions were identical to those used in the choice experiments.

Killing behavior of the TH and AR strains of *N. formosa* against *T. absoluta* at different densities Petri dishes containing 10 mL of agar gel (1% w/v) to maintain leaflet freshness were covered with cling film perforated with small holes (~0.3 mm in diameter) and placed in climate-controlled chambers under the same conditions used for insect rearing. Females of both *N. formosa* strains were allowed to oviposit, feed on, or sting the *T. absoluta* larvae for 48 h. After this period, the female parasitoids were removed. The parasitized, fed on, or stung host larvae were maintained under the same conditions for an additional 48 h to evaluate the attack responses at varying host densities. Assays where the female parasitoid was found dead at the end of the experiment were excluded. A total of 20–24 individual females (TH: 20–23 replicates; AR: 20–24 replicates) were successfully evaluated and recorded for each strain and host density.

Fitting of functional response model of the TH and AR strains of *N. formosa* to *T. absoluta* As the most effective way to distinguish type II and III functional responses (Neter *et al.* 1974; Glantz *et al.* 1990), data were fit to a logistic regression model between host density offered (N_0) and host density killed (*Ne*) (i.e., the number of hosts parasitized, fed on, or stung) to characterize the type of functional response according to formula (1) (Juliano 2001; Okuyama 2013):

$$\frac{Ne}{N_0}=\frac{\exp(P_0+P_1N_0+P_2N_0^2+P_3N_0^3)}{1+\exp(P_0+P_1N_0+P_2N_0^2+P_3N_0^3)} \quad (1)$$

where *Ne* is the number of host larvae killed (i.e., hosts parasitized, fed on, or stung), and N_0 is the initial host larval density offered. P_0, P_1, P_2, and P_3 are the constant, linear, quadratic, and cubic parameters to be estimated, respectively. A positive linear parameter (P_1>0) with a negative quadratic parameter (P_2<0) is a type III functional response, while a negative linear parameter P_1 (P_1<0) implies a type II functional response (Juliano 2001). In this case, only type II or type III functional responses were obtained, so Holling's disc equations for type II (formula 2) and type III (formula 3) functional responses were fit to estimate the attack rate and handling time, and the best searching density, respectively (Holling 1959; Wang *et al.* 2016; Guleria *et al.* 2021):

$$Ne=aN_0T/(1+aT_hN_0) \quad \text{(type II)} \quad (2)$$

where a is attack rate (or instantaneous search rate), T is total experimental duration (i.e., 2 days), and T_h is handling time; and

$$Ne=a'\exp(-b/N_0) \quad \text{(type III)} \quad (3)$$

where a' is the maximal number of host larvae that could be attacked (i.e., hosts parasitized, fed on, or stung) during the time interval (i.e., T=2 days), and b is the best searching density.

The attack rate per handling time (a/T_h) and the theoretical maximum parasitizing rate ($Ne_{max}=T/T_h$), as well as the best search density (b) were calculated.

2.4. Statistical analysis

For the choice experiments, differences in host-attacking behaviors (percentages parasitized, fed on, stung, and total killed) across different larval instar ratios were analyzed using nonparametric one-way ANOVA (Kruskal-Wallis H test). Independent two-sample Mann-Whitney U tests were used to compare the total percentages of hosts killed by the TH and AR strains, as the data did not meet the necessary normality and homoscedasticity assumptions. Paired-sample t-tests were applied to evaluate differences in total mortality between the strains across the five choice tests. Two-way ANOVA with Tukey's post hoc tests was performed to assess differences in host-killing behaviors (parasitizing, feeding, and stinging) between the two strains across larval instar ratios.

In the functional response experiments, differences in the numbers of parasitized, fed-on, and stung larvae between the two strains at different host densities were evaluated using Mann-Whitney U tests.

Percentage data were arcsine-transformed before analysis. Statistical significance was set at P<0.05. All analyses were conducted using DPS software (DPS DP_v. 9.01) (Tang and Zhang 2013).

3. Results

3.1. Performance and preference of the TH and AR strains of *N. formosa* for four larval instars of *T. absoluta* in choice experiments

Performance As the proportion of 1st instar larvae decreased, the total percentages of hosts parasitized (TH strain: $F_{4, 98}$=32.531, P<0.0001; AR strain: $F_{4, 97}$=45.845, P<0.0001), fed on (TH strain: $F_{4, 98}$=47.982, P<0.0001; AR strain: $F_{4, 97}$=22.006, P<0.0001), and stung (TH strain: $F_{4, 98}$=12.197, P<0.0001; AR strain: $F_{4, 97}$=18.623, P<0.0001) by the TH and AR strains significantly decreased (Fig. 1-A and B).

In cases where the hosts provided were exclusively 1st instar larvae (Ratio 1: 100% 1st instar larvae; Appendix A), no significant differences were detected among the three host-killing behaviors (TH strain: $F_{2, 57}$=0.834, P=0.4395; AR strain: $F_{2, 57}$=0.975, P=0.3835). However, as the proportion of 1st instar larvae decreased in Ratios 2–5 (Fig. 1-A and B), host-stinging became more prominent than either host-parasitizing or host-feeding for both strains. Higher percentages of host-stinging compared to host-parasitizing and/or host-feeding were observed in TH for each ratio (Ratio 2: $F_{2, 57}$=7.249, P=0.0016; host-stinging *vs*. host-parasitizing, P=0.0232; host-stinging *vs*. host-feeding, P=0.0015. Ratio 3: $F_{2, 60}$=11.244, P<0.0001; host-stinging *vs*. host-parasitizing, P=0.0007; host-stinging *vs*. host-feeding, P=0.0002. Ratio 4: $F_{2, 63}$=31.632, P<0.0001; host-stinging *vs*. host-parasitizing, P<0.0001; host-stinging *vs*. host-feeding, P<0.0001. Ratio 5: $F_{2, 57}$=7.636, P=0.0012; host-stinging *vs*. host-parasitizing, P=0.0015; host-stinging *vs*. host-feeding, P=0.0110) (Fig. 1-A). Similar results were observed in the AR strain (Ratio 2: $F_{2, 57}$=5.606,

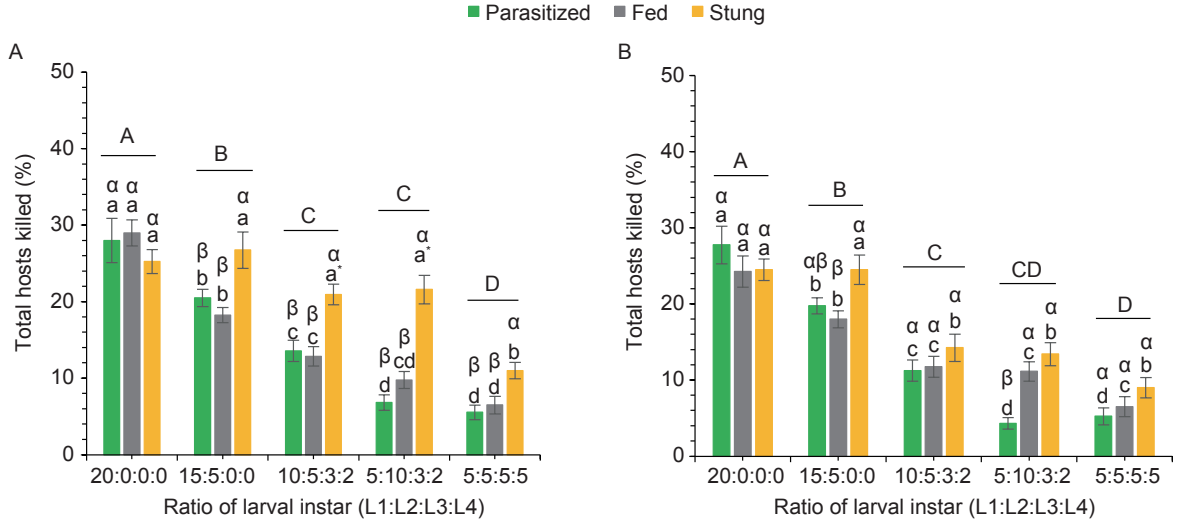


Fig. 1 Mortality of host larvae caused by each host-killing behavior of the thelytokous (TH; A) and arrhenotokous (AR; B) strains of *Neochrysocharis formosa* at different ratios of 1st, 2nd, 3rd, and/or 4th larvae (L1–L4) of *Tuta absoluta* over 48 h. Different lowercase letters in the same host-killing behavior indicate significant differences between different host larval instar ratios (P<0.05, K independent samples Kruskal-Wallis H test). Different lowercase Greek letters in the same host larval instar ratio indicate significant differences between different host-attacking behaviors (P<0.05, K independent samples Kruskal-Wallis H test). Different uppercase letters above the horizontal lines indicate significant differences between host larval instar ratios (P<0.05, two-way non-parametric analysis of variance). *, significant differences between the TH and AR strains in the same host larval instar ratio and the same host-killing behavior (P<0.05, Mann-Whitney U test). Bar is SE (n=20–22).

P=0.006; host-stinging *vs*. host-parasitizing, *P*=0.0536; host-stinging *vs*. host-feeding, *P*=0.0057. Ratio 4: $F_{2, 63}$=14.803, *P*<0.0001; host-stinging *vs*. host-parasitizing, *P*<0.0001) (Fig. 1-B).

Significant differences in host choice behavior were observed across all ratios for the TH ($F_{2, 4}$=5.421, *P*<0.0001) and AR ($F_{2, 4}$=3.014, *P*=0.0029) strains of *N. formosa* (Fig. 1-A and B). For the TH strain, the host-attacking ability was the highest in Ratio 1, followed by Ratio 2, with Ratios 3 and 4 showing intermediate performance and the lowest ability observed in Ratio 5. For the AR strain, the host mortality was also the highest in Ratio 1, followed by Ratios 2 and 3, with the lowest values observed in Ratio 5; but no significant differences were detected between Ratios 3 and 4 or Ratios 4 and 5 (Fig. 1-A and B).

Significant or marginally significant differences between the TH and AR strains in host choice behaviors were detected in two ratios, Ratios 3 and 4. In Ratio 3, the total percentage of host-stinging by the TH strain was significantly (*t*=2.9971, *df*=39, *P*=0.0047) greater than that by the AR strain. In Ratio 4, the total percentages of parasitoid-induced and sting-induced mortality by the TH strain were marginally significantly (*t*=1.9729, *df*=42, *P*=0.0551) and significantly (*t*=3.3920, *df*=42, *P*=0.0015) higher than those by the AR strain, respectively. No significant differences (*P*≥0.0840) were observed in the other ratios (Fig. 1). Furthermore, the total percentages of parasitoid-induced (*t*=2.4555, *df*=4, *P*=0.0700) and sting-induced (*t*=2.7395, *df*=4, *P*=0.0519) mortality by the TH strain were marginally significantly higher than those by the AR strain, but no significant difference was detected in feed-induced mortality (*t*=0.9265, *df*=4, *P*=0.4066) (Fig. 1).

The percentages of total hosts killed by the TH strain were significantly higher than those by the AR strain in Ratio 3 (*t*=3.1144, *df*=39, *P*=0.0034) and Ratio 4 (*t*=2.7556, *df*=42, *P*=0.0086) (Fig. 2). Moreover, the total host mortality caused by the TH strain was significantly higher than that by the AR strain (*t*=4.1223, *df*=4, *P*=0.0146; paired samples *t*-test) (Fig. 2).

Preference Females of both the TH and AR strains preferred to parasitize, feed on, and sting 1st instar *T. absoluta* larvae regardless of the host larval instar ratios. Similar results were observed when the percentage of total host mortality was evaluated (Table 1). At Ratio 1, where only 1st instar larvae were present, no significant differences were found between the three host-killing behaviors for either the TH ($F_{2, 57}$=0.834, *P*=0.4395) or AR ($F_{2, 57}$=0.975, *P*=0.3835) strains of *N. formosa*.

At Ratio 2, where 1st and 2nd instar larvae coexisted in a ratio of 15:5, there were no significant differences in the three host-killing behaviors for either the TH ($F_{2, 57}$=2.167, *P*=0.1239) or AR ($F_{2, 57}$=2.049, *P*=0.1383) strains with respect to the 1st instar larvae. However, significant differences were detected for the 2nd instar larvae. For the TH strain, the percentage of host-stinging ($F_{2, 57}$=12.698, *P*<0.0001) was significantly higher than either host-parasitizing (*P*=0.0002) or host-feeding (*P*<0.0001). Similarly, for the AR strain, the percentage of host-stinging ($F_{2, 57}$=8.608, *P*=0.0005) was significantly higher than either host-parasitizing (*P*=0.0031) or host-feeding (*P*=0.0013) (Table 1).

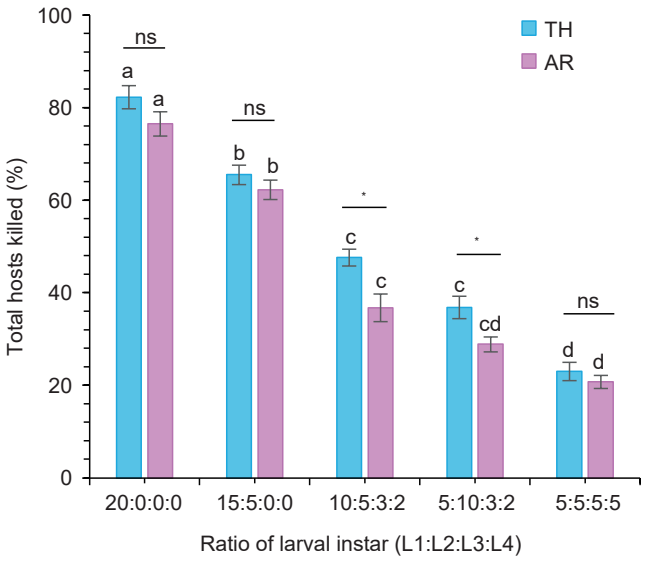


Fig. 2 Mortality of host larvae caused by combined host-killing behaviors of the thelytokous (TH) and arrhenotokous (AR) strains of *Neochrysocharis formosa* at different ratios of 1st, 2nd, 3rd, and/or 4th larvae (L1–L4) of *Tuta absoluta* over 48 h. Different lowercase letters in the same parasitoid strain indicate significant differences between different host larval ratios (*P*<0.05, K independent samples Kruskal-Wallis *H* test). *, above the horizontal lines indicate significant differences between the TH and AR strains (*P*<0.05, Mann-Whitney *U* test); ns, not significant.

At Ratio 3, where larvae of four instars were present in a ratio of 10:5:3:2, no significant differences were observed in the three host-attacking behaviors for the 1st (TH: $F_{2, 60}$=0.722, *P*=0.4900; AR: $F_{2, 57}$=0.258, *P*=0.7737), 3rd (TH: $F_{2, 60}$=1.522, *P*=0.2266; AR: $F_{2, 57}$=0.500, *P*=0.6092), or 4th instar larvae (AR: $F_{2, 57}$=0.500, *P*=0.6092). However, significant differences were observed for the 2nd instar larvae, where the TH strain ($F_{2, 60}$=24.444, *P*<0.0001) showed a higher percentage of host-stinging than either host-parasitizing (*P*<0.0001) or host-feeding (*P*<0.0001). Similarly, the AR strain ($F_{2, 57}$=8.717, *P*=0.0005) exhibited higher host-stinging percentages compared to host-parasitizing (*P*=0.0036) or host-feeding (*P*=0.0010) (Table 1).

At Ratio 5, where all instar larvae were present in equal proportions (5:5:5:5), no significant differences were detected between the three host-killing behaviors for the 1st (TH: $F_{2, 57}$=0.081, *P*=0.9227; AR: $F_{2, 57}$=0.186, *P*=0.8309), 3rd (TH: $F_{2, 57}$=1.900, *P*=0.1589; AR: $F_{2, 57}$=2.353, *P*=0.0620), or 4th instar larvae (TH and AR: $F_{2, 57}$=1.000, *P*=0.3742). However, significant differences were observed for the 2nd instar larvae. The TH strain ($F_{2, 57}$=20.934, *P*<0.0001) exhibited a higher percentage of host-stinging than either host-parasitizing (*P*<0.0001) or host-feeding (*P*<0.0001). The AR strain ($F_{2, 57}$=4.120, *P*=0.0213) also demonstrated a higher percentage of host-stinging compared to host-parasitizing (*P*=0.0211) (Table 1).

At Ratio 4, where the larvae were present in the ratio of 5:10:3:2, the host-attacking behavior patterns were notably different. For the TH strain, the percentage of host-stinging

Table 1 Percentages of hosts parasitized, fed on, and stung by the thelytokous (TH) and arrhenotokous (AR) strains of *Neochrysocharis formosa* at different ratios of the 1st, 2nd, 3rd, and/or 4th instar *Tuta absoluta* larvae (20 larvae in each ratio) in choice experiments for 48 h

Strain-host instar ratio (L1:L2:L3:L4)[1]	Host instar	TH strain				AR strain			
		Percentage of parasitized larvae (%)	Percentage of fed larvae (%)	Percentage of stung larvae (%)	Percentage of total killed larvae (%)[4]	Percentage of parasitized larvae (%)	Percentage of fed larvae (%)	Percentage of stung larvae (%)	Percentage of total killed larvae (%)[4]
TH/AR–20:0:0:0	1st	28.00±2.89 αA	29.00±1.69 αA	25.25±1.56 αA	82.25±2.50A	27.75±2.47 αA	24.25±2.06 αA	24.50±1.40 αA	76.50±2.62 A
	2nd	–	–	–	–	–	–	–	–
	3rd	–	–	–	–	–	–	–	–
	4th	–	–	–	–	–	–	–	–
TH/AR–15:5:0:0[2]	1st	26.33±1.41 aαA	23.67±1.23 aαA	28.33±2.04 aαA	78.33±2.04 aA	25.67±1.39 aαA	23.67±1.57 aαA	28.00±1.57 aαA	77.33±2.18 aA
	2nd	3.00±2.19 bβA	2.00±1.38 bβA	22.00±4.57 aαA	27.00±4.42 bA	2.00±1.38 bβA	1.00±1.00 bβA	14.00±3.87 bαA	17.00±3.91 bA
	3rd	–	–	–	–	–	–	–	–
	4th	–	–	–	–	–	–	–	–
TH/AR–10:5:3:2[3]	1st	25.71±2.81 aαA	22.90±2.30 aαA	26.70±1.60 aαA	75.24±2.55 aA	21.00±2.50 aαA	23.50±2.70 aαA	23.00±2.70 aαA	67.50±5.42 aA
	2nd	2.86±1.56 bβA	3.80±1.80 bβA	27.60±4.20 aαA	35.24±4.12 bA	1.00±1.00 bβA	0.00±0.00 bβB	9.00±2.70 baB	10.00±3.08 bB
	3rd	0.00±0.00 baA	3.20±2.20 baA	4.80±2.60 baA	7.94±3.18 cA	1.67±1.67 baA	0.00±0.00 baA	1.70±1.70 baA	1.67±1.67 bA
	4th	0.00±0.00 bA	0.00±0.00 bA	0.00±0.00 bA	0.00±0.00 cA	2.50±2.50 baA	0.00±0.00 baA	2.50±2.50 baA	2.50±2.50 bA
TH/AR–5:10:3:2[3]	1st	24.55±3.46 aβA	27.30±3.60 aαβB	38.20±3.70 aαA	84.55±4.15 aA	15.45±2.61 aβB	41.80±4.50 aαA	32.70±3.80 aαA	90.00±3.16 aA
	2nd	1.36±1.00 bβA	5.00±1.40 bβA	20.00±2.90 baA	26.36±3.52 bA	0.91±0.63 bβA	0.90±0.60 bβB	10.50±2.10 baB	12.27±2.46 bB
	3rd	0.00±0.00 bβA	3.00±2.10 bβA	13.60±3.60 baA	16.67±3.64 bcA	0.00±0.00 baA	1.50±1.50 baA	0.00±0.00 caB	1.52±1.52 bB
	4th	0.00±0.00 bA	0.00±0.00 bA	0.00±0.00 cA	0.00±0.00 cA	0.00±0.00 bA	0.00±0.00 bA	0.00±0.00 cA	0.00±0.00 bA
TH/AR–5:5:5:5[3]	1st	22.00±3.81 aαA	22.00±4.10 aαA	24.00±3.10 aαA	68.00±4.68 aA	19.00±3.69 aαA	22.00±4.80 aαA	22.00±4.30 aαA	63.00±3.91 aA
	2nd	0.00±0.00 bβA	3.00±1.60 bβA	16.00±2.80 aαA	19.00±3.07 bA	2.00±1.38 bβA	4.00±1.80 baβA	10.00±2.70 baA	16.00±3.11 bA
	3rd	0.00±0.00 baA	1.00±1.00 baA	3.00±1.60 baA	4.00±2.34 bcA	0.00±0.00 baA	0.00±0.00 baA	3.00±1.60 baA	3.00±1.64 bcA
	4th	0.00±0.00 baA	0.00±0.00 baA	1.00±1.00 baA	1.00±1.00 cA	0.00±0.00 baA	0.00±0.00 baA	1.00±1.00 baA	1.00±1.00 cA

[1] L1, L2, L3, and L4, represent the 1st, 2nd, 3rd, and 4th instar larvae of *Tuta absoluta*, respectively.
[2] Mann-Whitney *U* test.
[3] K independent samples Kruskal-Wallis *H* test.
[4] Percentage of total killed larvae (%)=((Number of parasitized larvae+Number of fed larvae+Number of stung larvae)/Total number of larvae provided)×100%
Data are mean±SE (n=20–22 female parasitoid individuals). Different lowercase letters within the same column and the same strain-host larval ratio indicate significant differences between the 1st and 2nd instar larvae (P<0.05, Mann-Whitney *U* test) or between the four larval instars (P<0.05, K independent samples Kruskal-Wallis *H* test). Different lowercase Greek letters in the same row and the same parasitoid strain indicate significant differences between the three host-killing behaviors (P<0.05, K independent samples Kruskal-Wallis *H* test). Different uppercase letters within the same row and the same host-killing behavior denote significant differences between the TH and AR strains (P<0.05, Mann-Whitney *U* test). –, not available.

for 1st instar larvae was higher than host-parasitizing (P=0.0235). For the AR strain, the percentages of host-stinging (P=0.0090) and host-feeding (P<0.0001) for 1st instar larvae were higher than host-parasitizing. When the 2nd instar larvae were encountered, the percentages of host-stinging for both the TH (P<0.0001) and AR (P<0.0001) strains were higher than either host-parasitizing or host-feeding. For the 3rd instar larvae, the TH strain showed higher host-stinging percentages than either host-parasitizing (P=0.0004) or host-feeding (P=0.0073), whereas no significant differences were detected for the AR strain (Table 1).

Significant differences between the TH and AR strains were also detected when host-attacking behaviors were compared. At Ratio 3, the TH strain exhibited significantly higher percentages of feeding (t=2.1693, df=39, P=0.0423), stinging (t=3.6819, df=39, P=0.0008), and killng (t=4.8123, df=39, P<0.0001) on 2nd instar larvae compared to the AR strain. At Ratio 4, the TH strain parasitized a higher percentage of 1st instar larvae than the AR strain (t=2.1146, df=42, P=0.0404), while the AR strain fed on a higher percentage of 1st instar larvae than the TH strain (t=2.5200, df=42, P=0.0156). The TH strain also exhibited higher percentages of feding (t=2.6125, df=42, P=0.0141), stnging (t=2.6868, df=42, P=0.0103), and killng (t=3.2940, df=42, P=0.0020) on 2nd instar larvae. For the 3rd instar larvae, the TH strain had higher percentages of stinging (t=3.8129, df=42, P=0.0010) and killng (t=3.8456, df=42, P=0.0006) on them than the AR strain. However, no significant differences were observed between the TH and AR strains at Ratios 1, 2, or 5 (P>0.05) (Table 1). These findings demonstrate that the ability of the TH strain to control *T. absoluta*

is greater than that of the AR strain, especially when the 2nd and 3rd instars are present.

3.2. Functional responses of the TH and AR strains of *N. formosa* to *T. absoluta*

The numbers of *T. absoluta* larvae parasitized, fed on, or stung by the TH and AR strains of *N. formosa* increased as host density rose, and this trend was consistent across all behaviors (Fig. 3). However, the percentage of larvae attacked decreased with increasing host density but showed notable differences between the strains and among the three host-attacking behaviors (Fig. 4). A significant interaction between parasitoid strain and host density was observed for the parasitism rates ($F_{1, 6}$=2.384, P=0.017), with the TH strain parasitizing more larvae than the AR strain, particularly at higher densities (15–40 larvae). Specifically, the TH strain exhibited significantly higher parasitism rates than the AR strain at host densities of 15 (t=3.5291, df=39, P=0.0011), 25 (t=2.5578, df=45, P=0.0156), 30 (t=3.0496, df=38, P=0.0048), and 40 (t=2.0842, df=38, P=0.0439) larvae (Fig. 3). To ensure that the functional response curve approached its upper asymptote, host density was increased up to 40 larvae for both strains. In contrast, the numbers of host-feeding and host-stinging events also rose with host density (P≤0.0010), but these behaviors did not differ significantly between the TH and AR strains (P>0.05) (Fig. 3).

Logistic regression analyses revealed that the functional responses of host-feeding by the TH strain (P_1=–0.1129), as well as host-feeding (P_1=–0.0948) and host-stinging (P_1=–0.0779) by the AR strain, followed the type II response model. Conversely, the responses for host-parasitizing (P_1=0.0218, P_2=–0.0001) and host-stinging (P_1=0.0458, P_2=–0.0001) by the TH strain, as well as host-parasitizing (P_1=0.0380, P_2=–0.0021) by the AR strain, adhered to the type III response model (Juliano 2001) (Appendix B). Thus, the functional responses of the TH and AR strains showed distinct behavioral dynamics. Host-feeding in both strains and stinging in the AR strain were better modeled by a type II curve, while parasitism in both strains and stinging in the TH strain aligned more closely with a type III curve (Table 2; Fig. 3).

In the type II functional response, the host-feeding rate (a) of the TH strain (0.2549) was higher than that of the AR strain (0.2257) of *N. formosa*, while the handling time (T_h) of the TH strain (0.1786) was shorter than that of the AR strain (0.1904) (Table 2). Similar results were observed in the type III functional response, in which the host-parasitizing rate (a) and best host-searching density (b) of the TH strain (0.1059 and 9.2163, respectively) were greater than those of the AR strain (0.1031 and 9.1599, respectively) of *N. formosa*, and the handling time (T_h) of the TH strain (0.1063) was shorter than that of the AR strain (0.1291) (Table 2).

The theoretical maximum host-killing rates, calculated as $1/T_h$, revealed that the TH strain achieved higher daily rates of host-feeding (11.1982 larvae/day) and host-parasitizing (9.4037 larvae/day) than the AR strain (10.5042 and 7.7470 larvae/day, respectively). Conversely, the AR strain demonstrated a significantly higher maximum rate of host-stinging (28.9855 larvae/day) compared to the TH strain (9.5898 larvae/day) (Table 2).

4. Discussion

This study provides novel insights into the killing behavior of the TH and AR strains of *N. formosa* against the invasive pest

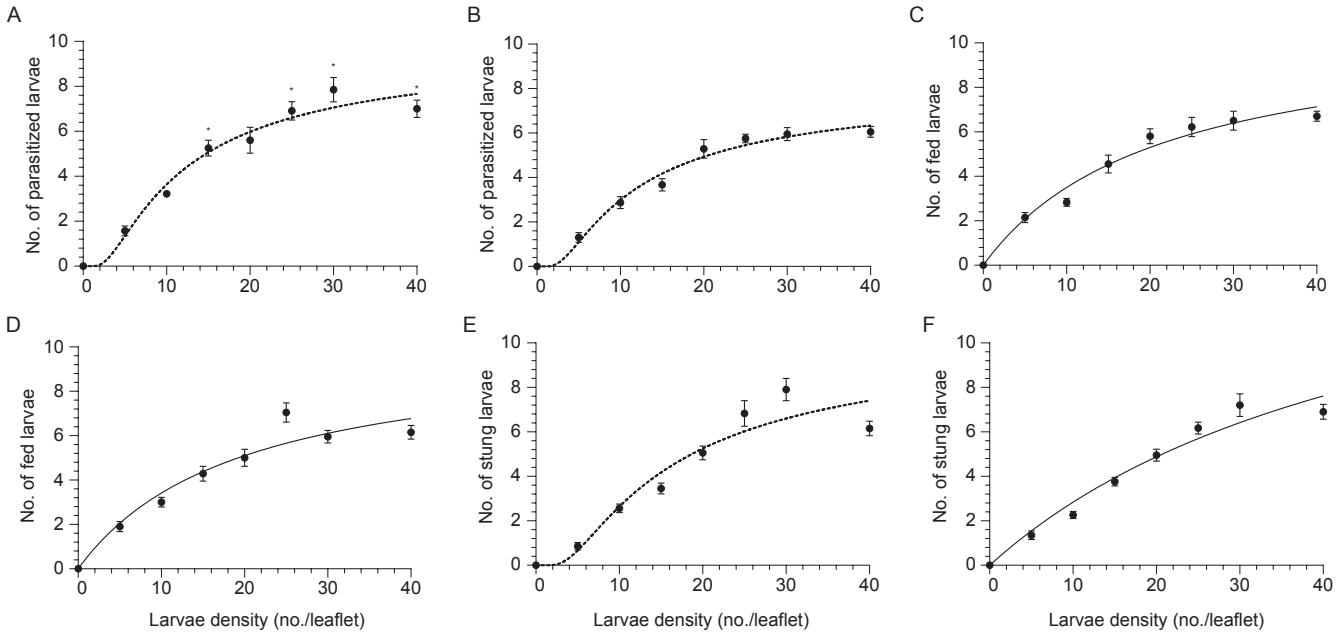


Fig. 3 Functional responses of the thelytokous (TH; A, C and E) and arrhenotokous (AR; B, D and F) strains of *Neochrysocharis formosa* based on number of parasitized (A and B), fed (C and D), and stung (E and F) larvae. The round dots represent the mean number of *Tuta absoluta* larvae attacked by the TH and AR strains (n=20–23 and 20–24 female parasitoids, respectively) of *N. formosa* across various host larval densities. Solid lines represent type II functional responses, while dotted lines represent type III functional responses. *, P<0.05 (Mann-Whitney U test).

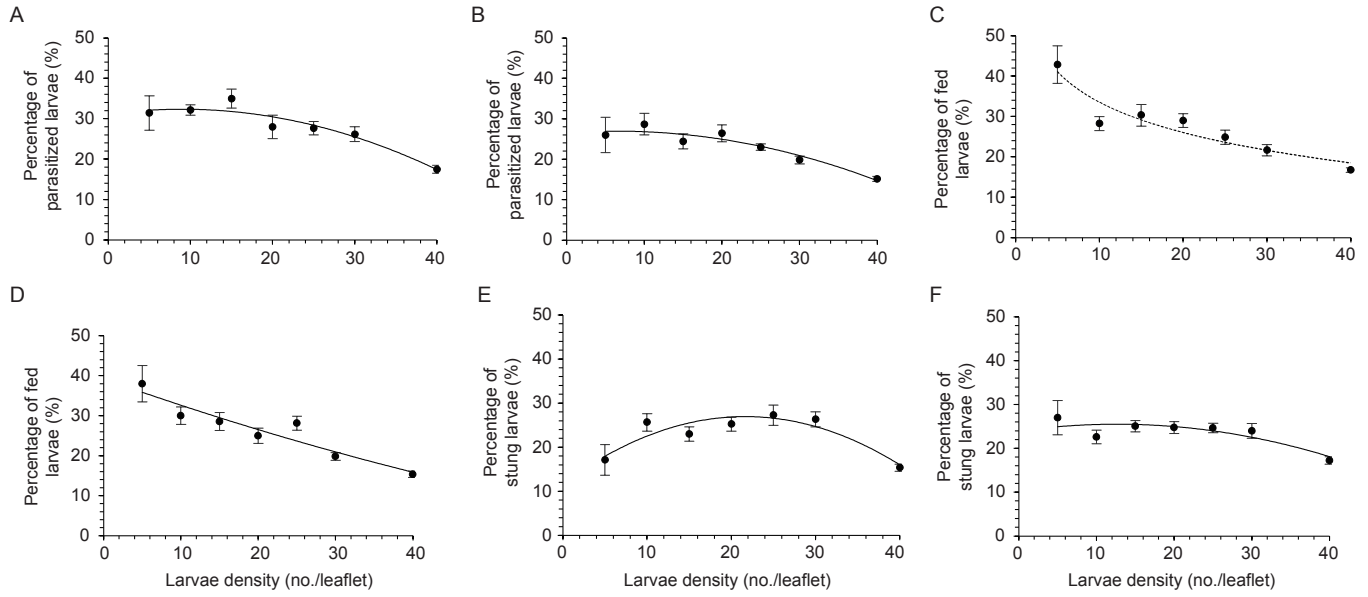


Fig. 4 Functional responses of the thelytokous (TH; A, C and E) and arrhenotokous (AR; B, D and F) strains of *Neochrysocharis formosa* based on percentage of parasitized (A and B), fed (C and D), and stung (E and F) larvae. Round dots represent the mean percentage of *Tuta absoluta* larvae attacked by the TH and AR strains at different host larval densities. Solid lines represent quadratic polynomial functions, and dotted lines are logarithmic functions.

T. absoluta. It is the first to compare the performance and preferences of these strains under laboratory conditions that simulate field-like scenarios, such as overlapping generations and the coexistence of 1st to 4th instar host larvae (Biondi *et al.* 2018). Both strains demonstrated strong potential for killing and parasitizing 1st instar larvae and, less frequently, 2nd instars (both TH and AR strains) and 3rd and 4th instars (AR strain only), with parasitism rates≤3% for the older instars. These findings suggest that the 1st instar larvae of *T. absoluta* are more suitable targets for the development and pest management potential of *N. formosa*.

In choice experiments with both strains, the proportion of 1st instar larvae significantly influenced the host-attacking behavior. As the proportion of 1st instar larvae increased, the percentage of hosts killed by both strains rose significantly, highlighting the potential effectiveness of releasing these parasitoids early in the *T. absoluta* infestation cycle when 1st instar larvae dominate. Both strains consistently preferred to parasitize, feed on, and sting 1st instars over older larvae.

Despite their biological differences, both strains achieved high host-killing rates, with the TH strain killing 68.00–84.55% and the AR strain killing 63.00–90.00% of available hosts within 48 h. These corresponded to total host-attacking rates of 13.60–16.91 larvae for the TH strain and 12.60–18.00 larvae for the AR strain, underlining their value as biological control agents (Bodino *et al.* 2019; Wang *et al.* 2024). Furthermore, the TH strain exhibited a significantly greater pest attacking capacity than the AR strain, especially at Ratios 3 and 4, where the proportions of 1st instar larvae (i.e., the most preferred larval stage) were lower and the TH strain showed significantly higher rates of parasitism (to 1st instars) and/or host-feeding, stinging, and killing to 2nd and/or 3rd instars compared to the AR strain. Host-feeding and stinging are key components of the attack strategies of both strains, particularly when the host stages available are unsuitable for oviposition (Jervis *et al.* 1996, 2008).

These results align with prior findings from Wang *et al.* (2024), which showed that the host-attacking capacity in both strains was strongly influenced by host larval instar, with a clear preference for 1st instars in no-choice experiments. However, this contrasts with observations by Guleria *et al.* (2020) in India, where *N. formosa* populations preferred 2nd instar larvae for parasitism. Genetic differences between the *N. formosa* populations in China and India, as indicated by mitochondrial *COI* gene analysis (Xu *et al.* 2022), may explain these discrepancies. Note that the genetic distances between the TH and AR strains from China and the Indian populations (0.034–0.043 and 0.024–0.038, respectively) exceed the species delimitation threshold (≥0.02) defined by DNA barcoding (Hebert *et al.* 2003).

Host larval instar significantly impacts parasitoid attack rates, survival, and sex ratios (Li *et al.* 2006; Jervis *et al.* 2008; Kant *et al.* 2012; Wang *et al.* 2024). While both the TH and AR strains of *N. formosa* can parasitize, feed on, and sting all instars, their attacking potential was the highest for 1st instars. Both our choice experiments and the no-choice experiments of Wang *et al.* (2024) confirm that 1st instars are preferred for oviposition, probably because early instars are less immunologically developed, reducing the risk of host resistance (Brodeur and Vet 1995; Strand and Pech 1995). Further studies are needed to determine whether *N. formosa* uses chemical cues or other mechanisms to distinguish between larval instars.

In functional response assays, our laboratory experiments revealed distinct functional responses for different host-killing behaviors of the same strain of *N. formosa* when targeting *T. absoluta* larvae. For example, the host-stinging behavior of the TH strain and parasitism by both the TH and AR strains

Table 2 Functional response models and parameters, including attack rate (*a*), handling time (T_h), best searching density (*b*), and theoretical maximum (Na_{max}) of the thelytokous (TH) and arrhenotokous (AR) strains of *Neochrysocharis formosa* for parasitizing, feeding on, and stinging 1st instar larvae of *Tuta absoluta*

Strain	Functional response type	Host-killing behavior	Functional response equation	*a* (larvae d^{-1})	T_h (d)	*b*	Na_{max}	Correlation coefficient
TH	Type III	Host-parasitizing	$Ne=9.4037\exp(-9.2163/N_0)$	0.1059	0.1063	9.2163	9.4037	0.9861
TH	Type II	Host-feeding	$Ne=0.5098N_0/(1+0.0485N_0)$	0.2549	0.1786	–	11.1982	0.9720
TH	Type III	Host-stinging	$Ne=9.5898\exp(-12.3790/N_0)$	0.1287	0.1043	12.3790	9.5898	0.9803
AR	Type III	Host-parasitizing	$Ne=7.7470\exp(-9.1599/N_0)$	0.1031	0.1291	9.1599	7.7470	0.9896
AR	Type II	Host-feeding	$Ne=0.4514N_0/(1+0.0403N_0)$	0.2257	0.1904	–	10.5042	0.9902
AR	Type II	Host-stinging	$Ne=0.2776N_0/(1+0.0096N_0)$	0.1388	0.0690	–	28.9855	0.9938

–, not available.

exhibited density-dependent type III functional responses, whereas host-feeding by both strains and host-stinging by the AR strain demonstrated inversely density-dependent type II responses. The type III functional response is widely recognized for its stabilizing effect on host-parasitoid interactions, enhancing the likelihood of effective pest population regulation (Hassell 2000; Fernández-Arhex and Corley 2003; Yazdani and Keller 2016; Bodino *et al.* 2019). However, the low instantaneous attack rates observed for both strains in parasitism suggests a limited capacity to respond efficiently to sharp increases in pest density (Bodino *et al.* 2019). Nevertheless, parasitoids with type III functional responses can excel at controlling pests under low-density conditions (Hassell *et al.* 1977). This indicates that the TH strain could be particularly effective in managing *T. absoluta* populations early in the growing season, especially at the peak vulnerability periods of tomato when infestation levels are low, such as flowering or fruit set, primarily through host-parasitism and host-stinging. In contrast, the AR strain exhibited slightly weaker performance, especially in high-pest-pressure periods, as only its host-parasitism behavior displayed a type III functional response, and a lower attack rate and longer handling time were observed.

Our findings regarding the type III functional responses of the TH strain's parasitism and host-stinging behaviors align with previous research on *Necremnus tutae* Ribes and Bernardo, another larval parasitoid of *T. absoluta* in the Mediterranean region (Bodino *et al.* 2019). This species is not currently available for commercial use, but it remains the only one mass-reared in bio-factories (Desneux *et al.* 2022).

Conversely, the host-stinging behavior of the AR strain and the host-feeding behaviors of both the TH and AR strains exhibited type II functional responses, characterized by steady increases in the number of larvae attacked with rising host density (Juliano 2001). Type II functional responses are often associated with a limited ability to adjust to varying host densities and are generally considered less stable for managing host-parasitoid interactions (Hassell 2000; Bodino *et al.* 2019). However, previous studies have demonstrated that some parasitoids exhibiting this type of response, such as *Encarsia formosa* Gahan on *Trialeurodes vaporariorum* (Westwood) (van Roermund *et al.* 1997) and *Anagyrus kamali* Moursi on *Maconellicoccus hirsutus* Green (Sagarra *et al.* 2000), have successfully served as biological control agents (Fernández-Arhex and Corley 2003). Furthermore, the potential instability of host-parasitoid populations arising from type II responses remains controversial (Fernández-Arhex and Corley 2003; Kidd and Amarasekare 2012; Bodino *et al.* 2019).

The higher attack rate and shorter handling time estimated for the TH strain in host-feeding, compared to those of the AR strain, underscore its ability to swiftly attack large numbers of larvae even at high host densities, which is a critical trait for effective pest control under heavy infestations (Bodino *et al.* 2019). The AR strain's capacity to sting a higher theoretical maximum number of host larvae further highlights its control potential against *T. absoluta*. Type II functional responses are common among eulophids and have been previously reported for *Necremnus cosmopterix* Ribes and Bernardo against *T. absoluta* (Bodino *et al.* 2019) and the India population of *N. formosa* targeting the 1st, 2nd and 3rd larval instars of *T. absoluta* (Guleria *et al.* 2021), where all three host-attacking behaviors — parasitism, host-feeding, and host-stinging — exhibited type II responses. These variations are likely attributable to differences in species (Bodino *et al.* 2019) or strain (Guleria *et al.* 2021; Xu *et al.* 2022). Both the TH and AR strains of *N. formosa* are present in the field in China (Yang *et al.* 2017), while the AR strain is likely the sole representative in India. Despite their genetic similarity, individuals of the AR strain from China and India differ with genetic distances of 0.024–0.038 (Xu *et al.* 2022).

The functional response parameters for parasitism and host-feeding were superior for the TH strain compared to the AR strain, with higher attack rates and lower handling times, indicating greater efficiency in these behaviors. Conversely, the AR strain outperformed the TH strain in host-stinging, which was characterized by a higher attack rate and shorter handling time, making it more efficient in this behavior. These findings suggest that the TH strain is better suited to stabilize pest-natural enemy dynamics, particularly in controlling *T. absoluta* populations through parasitism and host-feeding (Hassell 2000; Yazdani and Keller 2016). While functional response types alone may not fully predict the efficacy of a natural enemy (Fernández-Arhex and Corley 2003; Tazerouni *et al.* 2019), parameters such as attack rate and handling time offer valuable insights. Thus, a natural enemy with a higher attack rate and shorter handling time is expected to be more effective for pest control (Tazerouni *et al.* 2019; Guleria *et al.* 2021).

As one of the most commonly occurring species/strains

in tomato agroecosystems (Wang *et al.* 2012; Guleria *et al.* 2020), the TH strain of *N. formosa* (exhibiting type III functional responses of host-parasitizing and host-stinging and higher host-killing capacity than the AR strain) might be more suitable as a potential effective natural enemy in conservation biocontrol programs against *T. absoluta* in the open field; however, augmentative release strategies in greenhouses could be considered (Hassell *et al.* 1977). The early establishment of the species/strain in tomato fields, with a subsequent increase in their residential populations, could help keep *T. absoluta* populations under the economic threshold of 1% damaged fruits (Cocco *et al.* 2015), thereby preventing the pest populations from getting out of control (Bodino *et al.* 2019).

Therefore, to develop a successful biological control program, other factors, such as behavioral interactions among natural enemies, release timing and rate, parasitoid quality, and environmental conditions such as temperature and photoperiod, must also be considered to fully evaluate the effectiveness of a natural enemy (Fernández-Arhex and Corley 2003; Tazerouni *et al.* 2019).

5. Conclusion

The current study demonstrates that as the proportion of first-instar *T. absoluta* larvae increased, the percentages of hosts killed by both the TH and AR strains of *N. formosa* rose significantly, with a preference for first-instar larvae over the later stages. The TH strain exhibited a superior host-killing capacity under choice conditions and a density-dependent (type III) functional response, suggesting its early establishment in tomato agroecosystems could enhance pest management. These findings provide crucial insights into the biocontrol potential of the TH and AR strains and emphasize the need for further research on key ecological factors and multitrophic interactions to optimize their use.

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Declarations of competing interest

The authors declare that they have no conflicts of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

Ethical statements

All applicable international, national and institutional guidelines for the care and use of animals were followed.

Appendices associated with this paper are available at https://doi.org/10.1016/j.jia.2025.04.023

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