Effects of *Wolbachia* Infection, Parasitic Order, And Time Interval On The Outcome of Inter-Strain Intrinsic Competition Between Bisexual Strain And *Wolbachia*-Induced Thelytokous Strain of Egg Parasitoid, *Trichogramma Dendrolimi*

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Research Article

Keywords: egg parasitoids, intraspecific competition, superparasitism, Early Acting Competitive Superiority Hypothesis, symbiont

Posted Date: January 11th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-981919/v1>

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Abstract

Parasitoids may deposit a second clutch of eggs on a host that has been parasitized by the same parasitoid species. This is termed “superparasitism”. *Wolbachia* infection increases the superparasitism frequency of *Trichogramma* females. We investigated the outcome and fitness cost of inter-strain intrinsic competition between *Wolbachia*-infected thelytokous strain (TDW) and uninfected bisexual strain (TDB) of *Trichogramma dendrolimi*. To determine the developmental rate of both strains, the size of immature stages of *T. dendrolimi* offspring at different times after parasitization was measured in single parasitism and superparasitism conditions. The results showed increased superparasitism by *Wolbachia*-infected females compared to uninfected females, regardless of the time interval between oviposition; *Trichogramma* females were unable to distinguish between host eggs previously parasitized by TDB or TDW females. When the first oviposition was performed by TDB females, the TDB offspring outcompeted TDW offspring deposited later. However, although the TDW offspring was deposited 8 h earlier than the TDB offspring, it gained no advantage over TDB offspring. Regardless of parasitism conditions, the differences in development rate and time between TDW and TDB offspring were not significant. This study revealed that albeit TDW females presented a higher tendency of superparasitism than TDB females, TDB offspring outcompeted TDW offspring even when the latter was deposited 8 h earlier. The negative effects of *Wolbachia* infection on the competitive ability of TDW offspring inside the host eggs were due to offspring's low fitness rather than delayed development.

Introduction

Trichogramma wasps are a group of egg parasitoid species worldwide used in biological control programs through inundative release of mass-reared wasps for the fast control of lepidopteran pests in agriculture and forestry (Zhou et al. 2019a; Ferguson et al. 2020; Zang et al. 2020). The infection by the intracellular bacterial symbiont *Wolbachia* induces parthenogenetic thelytoky of *Trichogramma*, which allows females to produce almost 100% female offspring from unfertilized eggs (Huigens et al. 2000; Wu et al. 2020). Thelytokous *Wolbachia*-infected *Trichogramma* species may be more suitable in the pest control than their bisexual uninfected counterparts. The infected wasps may have a higher population increase rate as the offspring are almost all female, and the colonization of infected wasps is easier to handle because females can produce generations of fertile offspring without mating under field conditions. Some heritable traits can be fixed in infected strains of *Trichogramma* because infected offspring females are homozygous diploid, which enables a selection and establishment of optimal lines of *Trichogramma* in biological control programs (Stouthamer 1999; Ebrahimi et al. 2019; Zhou et al. 2019).

Generally, the resource of suitable hosts fluctuates across seasons and is often limited for certain parasitoid species in field conditions (Lindsey and Stouthamer 2017). For example, female wasps, which are short-lived, prefer to remain longer on a host patch and deposit more eggs on hosts because they may fail to find new hosts (Sheng et al. 2014; Robert et al. 2016a, b). Intraspecific competition may occur when the female wasp deposits a second clutch of eggs on a host that has been parasitized by the same parasitoid species. This is termed “superparasitism” (Van Alphen and Visser 1990; Zhou et al. 2019b). The superparasitism in *Trichogramma* wasps is expected to be more frequent in the augmentative biological control programs when the inundative number of *Trichogramma* wasps is released in fields, and in the mass-rearing procedure of *Trichogramma* production when the host eggs are supplied in excess compared to the number of wasps (Wang et al. 2015; Huang et al. 2017; Zhou et al. 2020).

Interestingly, previous studies indicated that *Wolbachia* infection in *Trichogramma* females induced memory loss (Farahani et al. 2017; 2021) and declined host discrimination (Pan et al. 2007; Abrun et al. 2021; Zhang et al. 2021) of infected females compared to their uninfected counterparts. In *T. brassicae* (Farahani et al. 2015) and *T. dendrolimi* (Liu et al. 2018), *Wolbachia*-infected females presented a higher probability of superparasitism than uninfected females. It is worth noting that some pathogens are able to manipulate host wasps to perform superparasitism, which facilitates their horizontal transmission among parasitoid offspring that share the same host. For example, the proclivity of *Nasonia vitripennis* to superparasitize increased with the presence of a male-killing heritable symbiont, *Arsenophonus nasoniae* (Parratt et al. 2016). The *Leptopilina boulardi* Filamentous virus (LbFV) is reported to manipulate the behavior of the parasitoid host *L. boulardi* females, which tend to lay additional eggs in a parasitized host (Patot et al. 2010). In multiparasitism (i.e. the host is parasitized by different parasitoid species) or superparasitism conditions, previous studies have reported inter- and intra-specific horizontal transmission in the parasitoid offspring that shared the same host egg (Schilthuisen and Stouthamer 1997; Huigens et al. 2004; Farokhi et al. 2010; Pan et al. 2011). Thus, it may be an adaptive strategy of *Wolbachia* that manipulate wasp behaviors to increase wasps' tendency to superparasitize hosts and increase the opportunity for horizontal transmission among *Trichogramma* offspring (Farahani et al. 2015; Zhou et al. 2019b). However, the superparasitism benefits for intrinsic intra-specific competition remain unknown.

Once the hosts are superparasitized, parasitoid offspring compete for host resources either by scramble competition for the acquisition of shared host nutrition or by contest competition for the monopolization of host resources through excluding competitors. The Early Acting Competitive Superiority Hypothesis predicts that the early acting parasitoid offspring may outcompete the later parasitoid offspring, because either the first active parasitoid larva would have consumed most of the host body fat before pupation (superiority in scramble competition) or the first active larva may kill the eggs or larvae and monopolize the host (superiority in contest competition) (Harvey et al. 2013). Thus, the outcome of the intrinsic competition of parasitoid offspring largely depends on two factors, the order and time lag between the first and second oviposition by the wasps and the time spent on the embryological development.

Considering the short life span and limited host resources in field conditions, the wasps may not hesitate to deposit their eggs in already parasitized hosts. For the *Trichogramma* host, the tendency of superparasitism in *Wolbachia*-infected wasps leads to high intraspecific competition with the native bisexual population of *Trichogramma* in field conditions, especially when *Wolbachia*-infected wasps are inundatively released in augmentative biological control programs. Additionally, the intraspecific competition has been a serious problem in the mass-rearing procedure of thelytokous *Wolbachia*-infected strains of *Trichogramma*, and subsequently affects the quality of offspring wasps. The high tendency for superparasitism of *Wolbachia*-infected *Trichogramma* has been reported (Farahani et al. 2015; Liu et al. 2018), and current investigations should focus on *Wolbachia*-infected and uninfected *Trichogramma* offspring sharing the same superparasitized host.

Trichogramma wasps are often found as a facultative gregarious parasitoid. For example, *T. dendrolimi* females deposit tens of offspring eggs on a larger lepidopteran egg, such as those of *Antheraea pernyi* (Guérin-Méneville) and *Samia cynthia* (Drury) (Iqbal et al. 2019, 2020; Wang et al. 2020), but often deposit one offspring egg on small host eggs, such as eggs of rice moth, *Corcyra cephalonica* (Stainton) (Zhang et al. 2010; Wang et al. 2015). It seems impossible to control the condition in which superparasitism occurs in relatively larger host eggs parasitized by *Trichogramma* wasps because *Trichogramma* wasps allocate a random number of offspring eggs during oviposition. Furthermore, the ancestry of offspring wasps produced by the first or the second mother is hard to determine.

In this study, the eggs of *C. cephalonica* were chosen because *Trichogramma* wasps often deposit only one offspring egg per oviposition, which facilitates and increases the accuracy of the superparasitism determination of host eggs. Moreover, we investigated the outcome and fitness cost of inter-strain intrinsic competition between *Wolbachia*-infected thelytokous strain (TDW) and uninfected bisexual strain (TDB) of *T. dendrolimi* offspring females. To measure the developmental rate of both *T. dendrolimi* strains in single parasitism and superparasitism conditions, the immature stages of *T. dendrolimi* offspring were photographed at several time intervals after the parasitization. The results may elucidate the carry-over effects of superparasitism behavior of host *Trichogramma* induced by *Wolbachia* and provide the reference for the application of *Wolbachia*-infected thelytokous *Trichogramma* in biological control programs.

Material And Methods

Insects

The *Wolbachia*-infected thelytokous strain (TDW) and the *Wolbachia*-uninfected bisexual strain (TDB) of *T. dendrolimi* were originally isolated from the eggs of *Dendrolimus punctatus* (Walker) (Lepidoptera: Lasiocampidae) collected from a forest of Chinese red pine, *Pinus massoniana* Lamb (Coniferales: Pinaceae), located at the southern end of the Changbai Mountains in Huanren County (125°25' E, 41°10' N), Liaoning Province, China, on April 16, 2017 (Liu et al. 2019). The *Wolbachia* infection of TDW strain was detected using specific primers (81F: 5'-TGGTCCAATAAGTGATGAAGAAAC-3' and 691R: 5'-AAAAATTAAACGCTACTCCA-3') for the *wsp* gene of *Wolbachia* (Zhou et al. 2020). Thereafter, *T. dendrolimi* females were reared on *C. cephalonica* eggs for the next generations. The host insects (*C. cephalonica*) were reared on a semi-artificial diet (Yang et al. 1990). The fresh host eggs (1 day old without UV irradiation) were glued on an A4 card with Arabic gum. A small square (1 cm × 1 cm) with a group of 50 eggs was gently cut from the A4 card and used in this experiment. White cards with a group of ca. 300 host eggs with UV-irradiation were used to test the fecundity of offspring females in the experiment.

All organisms were reared in a climate chamber (MLR-352H-PC; Panasonic, Japan) at 25 ± 1 °C, photoperiod of 16:8 h (light: dark), and 75 ± 5% relative humidity.

Observation of the immature offspring

To determine the developmental stage of TDB or TDW offspring in single-parasitized or superparasitized hosts at different times after parasitization, a group of 300 TDB or TDW females were transferred into a glass tube (75 mm length×12 mm diameter) containing the egg card with ca. 300 host eggs for 10 min. The eggs were cut out individually from the cards and then dissected at 0.5, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 60, 72, 96, 144, and 196 h after the parasitization under a stereomicroscope (SV6; Zeiss, Germany). The single-parasitized and superparasitized host was recognized by the presence of one and two *Trichogramma* offspring, respectively, in a host egg. To collect the images of the immature stages of *Trichogramma* offspring with unchanged body size, the samples of *Trichogramma* offspring were treated with Carnoy's reagent for 2 h. The samples were dehydrated in a gradient of ethanol and glycerol solution according to the procedures described in our previous study (Ning et al. 2019). Then, three samples at each stage were photographed using a camera with a macro lens (SMZ25; Nikon, Japan). The length and width of each sample were measured. All images were edited using the Adobe Illustrator CS6 software.

Experimental protocol

Two factors were considered in the experiment: the order of parasitism (TDW-TDB: TDW females parasite the host egg first, and TDB females parasite after; TDB-TDW: TDB females parasite the host egg first, and TDW females parasite after) and the time interval between two parasitizations (0.5, 8, 16, and 32 h).

A TDB or TDW female (1 day old) was introduced into a Durham tube (30 mm length×6 mm diameter) containing an egg card with 50 host eggs. The oviposition behavior was observed and recorded under the stereomicroscope (SV6; Zeiss). The oviposition behavior of the female parasitoid was confirmed when single oviposition was detected by the fluctuating abdominal movement (Guo 1993). The location of the parasitized host egg in the egg card was marked with a surgical skin marking pen (0.5 mm tips, T3023; Tondaus, Dongguan, China) immediately after the oviposition behavior occurred. The wasp's behavior was observed continuously for 30 min, after which the wasp was removed. The superparasitized eggs and excess fresh eggs or parasitized eggs were removed gently by a tungsten needle (0.04 mm tips). Only 15 fresh eggs and 15 parasitized eggs were kept on the egg card. After 0.5, 8, 16, or 32 h of the first parasitism, the same egg card was supplied to the female of the counterpart strain to parasitize on the host eggs for 30 min. The oviposition behavior and location of each oviposition event were observed and recorded under the stereomicroscope. The host eggs were then maintained in the Durham glass tube until the eggs blackened, which indicated the prepupal stages of *Trichogramma* offspring (Flanders 1937). The blackened eggs were cut out from the egg card and individually transferred into a new Durham tube for emergence. After wasp emergence, the blackened host eggs were dissected to determine the existence of a dead body of *Trichogramma* offspring. The superparasitism of the host egg was finally confirmed by the observation of oviposition frequency and the number of offspring wasps allocated in an egg. However, the parasitism type of 46 host eggs failed to be identified because the number of offspring wasps allocated in an egg was not consistent with the oviposition frequency. The development time of each wasp was recorded every 12 h. The emerged female was supplied with an egg card and replaced daily (ca. 300 eggs) for parasitization. The wasps were supplied with a 10% honey solution via the cotton thread. The

fecundity of a female individual was recorded by the number of offspring deposited during the life cycle of the female. The longevity of the emerged female wasps was recorded every 12 h until death. Before DNA extraction, the body size of offspring females was measured as the length of the right hind tibia.

The aborted *Trichogramma* offspring and the dead offspring wasps were frozen at -20 °C. The DNA content of a single individual of aborted offspring or emerged wasp was extracted according to the Chelex-100 method (Sumer et al. 2009). Every wasp or aborted offspring was ground in a 1.5 mL tube containing 50 µL 5% Chelex-100 mixed with 2.5 µL proteinase K (20 mg mL⁻¹). The tube was maintained at 55 °C for 1 h, and then at 99 °C for 10 min. Thereafter, the samples were centrifuged at 15,000 rpm for 10 min. The extracted DNA was stored at -20 °C. The *COI* sequences of each sample were amplified using PCR according to the procedure described by Liu et al. (2019). The PCR products were sequenced by Invitrogen Trading Co, Ltd. (Shanghai, China). The *Trichogramma* strain of the offspring was identified using the different *COI* sequences of the TDW strain and TDB strain of *T. dendrolimi*. The frequencies of inter- and intra-strain-superparasitism were recorded according to the *COI* sequence of the wasps that emerged from a host egg. The survival proportion of TDW or TDB offspring was calculated as the number of emerged wasps from the offspring.

In the experiment, all single-parasitized host eggs by TDB or TDW females were collected as the control groups; the survival proportion, adult longevity, body size, development time, and fecundity of these offspring females were recorded.

Statistical analysis

The total proportion of superparasitism and the proportion of inter-strain superparasitism of the second-visiting wasps influenced by the time interval and *T. dendrolimi* strain were analyzed using the generalized linear models (GLM) with quasibinomial distribution. The GLM with quasi-Poisson distribution was applied to analyze adult longevity, fecundity, and development time of the offspring as influenced by the order of parasitism, time interval, and *T. dendrolimi* strain. The GLM with Gaussian distribution was used to analyze the body size of offspring in reference to the order of parasitism, time interval, and *T. dendrolimi* strain. A logistic growth model was used to analyze the developing rate of offspring body length and body width influenced by *T. dendrolimi* strain and parasitism type (single parasitism and superparasitism) as a function of development time (0.5, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 60, 72, 96, 144, and 196 h). The model is given by the following equations:

$$y = \frac{k}{1 + ae^{-bt}} \quad (1)$$

$$T_1 = \frac{1}{b} \ln \frac{2 + \sqrt{3}}{a} \quad (2)$$

$$T_1 = \frac{1}{b} \ln \frac{2 - \sqrt{3}}{a} \quad (3)$$

$$V_m = \frac{bk}{4} \quad (4)$$

$$T_m = -\frac{\ln a}{b} \quad (5)$$

where a and b are parameters, k is the upper bound of offspring body length or width, T_1 is the time to germinate, T_2 is the time of fully grown, V_m is the maximum developing rate, and T_m is the time of offspring with maximum developing rate (Swishchuk and Wu 2003).

All study analyses were conducted using R software version 4.0.2 (R Development Core Team 2020).

Results

Superparasitism

The proportion of superparasitism was significantly affected by *T. dendrolimi* strain ($\chi^2 = 82.86$, $df = 1$, $P < 0.001$) and time interval ($\chi^2 = 27.03$, $df = 3$, $P < 0.001$), but was not affected by the interaction between *T. dendrolimi* strain and time interval ($\chi^2 = 0.82$, $df = 3$, $P = 0.85$). The proportion of superparasitism by TDW female (0.5 h interval: $64.12 \pm 2.88\%$; 8 h interval: $66.97 \pm 2.68\%$; 16 h interval: $73.59 \pm 2.56\%$; 32 h interval: $56.62 \pm 3.51\%$) was significantly higher than that by TDB female (0.5 h interval: $39.50 \pm 4.39\%$, $z = 4.53$, $P < 0.001$; 8 h interval: $38.40 \pm 4.46\%$, $z = 5.28$, $P < 0.001$; 16 h interval: $53.01 \pm 4.29\%$, $z = 4.18$, $P < 0.001$; 32 h interval: $32.30 \pm 4.65\%$, $z = 3.93$, $P < 0.001$), regardless of time interval. The proportion of superparasitism by TDW or TDB female at 32 h interval was significantly lower than that at 16 h interval (TDW: $z = 3.93$, $P < 0.001$; TDB: $z = 3.15$, $P = 0.0088$), but it was not lower than that at 0.5 h interval (TDW: $z = 1.67$, $P = 0.34$; TDB: $z = 1.12$, $P = 0.68$) and 8 h interval (TDW: $z = 2.37$, $P = 0.082$; TDB: $z = 0.94$, $P = 0.78$) (Fig. 1-S1).

The proportion of inter-strain superparasitism was not affected by time interval ($\chi^2 = 8.01$, $df = 3$, $P = 0.053$), *T. dendrolimi* strain ($\chi^2 = 0.39$, $df = 1$, $P = 0.53$), or their interaction ($\chi^2 = 1.18$, $df = 3$, $P = 0.76$) (Fig. 1-S2).

Survival proportion of offspring

The survival proportion of offspring from inter-strain superparasitized hosts was significantly affected by the interaction of the order of parasitism, *T. dendrolimi* strain, and time interval ($\chi^2 = 32.90$, $df = 3$, $P < 0.001$). When the order of parasitism was TDB-TDW, the survival proportion of TDW offspring (0.5 h interval: $83.97 \pm 1.06\%$; 8 h interval: $83.07 \pm 1.10\%$; 16 h interval: $83.02 \pm 1.12\%$; 32 h interval: $81.90 \pm 1.16\%$) was significantly lower than that of TDB offspring (0.5 h interval: $89.03 \pm 0.71\%$, $z = 4.08$, $P < 0.001$; 8 h interval: $89.72 \pm 0.70\%$, $z = 4.08$, $P < 0.001$; 16 h interval: $90.40 \pm 0.57\%$, $z = 5.12$, $P < 0.001$; 32 h interval: $91.54 \pm 0.52\%$, $z = 8.43$, $P < 0.001$), irrespective of the time interval. The survival proportion of TDW offspring was not affected by time interval. The survival proportion of TDB offspring with 32 h interval was significantly higher ($z = 2.88$, $P = 0.021$) than that with 0.5 h interval, but not significantly higher than that of 8 h ($z = 2.17$, $P = 0.13$) and 16 h intervals ($z = 1.50$, $P = 0.44$) (Fig. 2-S1).

When the order of parasitism was TDW-TDB, the survival proportion of TDB offspring ($88.63 \pm 0.96\%$) at 0.5 h interval was significantly higher than that of TDW offspring ($83.18 \pm 1.51\%$, $z = 3.13$, $P = 0.0017$), but the differences were not significant in the offspring at 8 h (TDB: $86.72 \pm 0.92\%$; TDW: $84.77 \pm 1.24\%$; $z = 1.23$, $P = 0.22$) and 16 h intervals (TDB: $84.46 \pm 1.12\%$; TDW: $86.26 \pm 1.08\%$; $z = 1.15$, $P = 0.25$). The survival proportion of TDB offspring ($81.44 \pm 1.39\%$) was significantly lower than that of TDW offspring at 32 h interval ($87.20 \pm 0.99\%$, $z = 3.48$, $P < 0.001$). The survival proportion of TDW offspring was not affected by the time interval. The survival proportion of TDB offspring at 0.5 h interval was significantly higher than that at 16 h ($z = 2.76$, $P = 0.030$) and 32 h intervals ($z = 4.36$, $P < 0.001$) (Fig. 2-S2). In the control groups, the survival proportion of TDB offspring ($91.73 \pm 0.58\%$) was significantly higher than that of TDW offspring ($83.97 \pm 0.98\%$, $z = 4.58$, $P < 0.001$) (Fig. 2-S3).

Offspring body size

The body size of offspring female was significantly affected by the interaction of the order of parasitism, *T. dendrolimi* strain, and time interval ($\chi^2 = 147.83$, $df = 3$, $P < 0.001$). When the order of parasitism was TDB-TDW, the body size of TDW female (0.5 h interval: $116.09 \pm 1.61 \mu\text{m}$; 8 h interval: $108.56 \pm 1.31 \mu\text{m}$; 16 h interval: $101.65 \pm 0.81 \mu\text{m}$; 32 h interval: $100.97 \pm 1.45 \mu\text{m}$) was significantly smaller than that of TDB female (0.5 h interval: $125.30 \pm 0.62 \mu\text{m}$, $z = 4.98$, $P < 0.001$; 8 h interval: $127.92 \pm 2.25 \mu\text{m}$, $z = 19.36$, $P < 0.001$; 16 h interval: $129.03 \pm 0.54 \mu\text{m}$, $z = 16.37$, $P < 0.001$; 32 h interval: $130.58 \pm 1.10 \mu\text{m}$, $z = 29.61$, $P < 0.001$), irrespective of the time interval. The body size of TDW female at 0.5 h interval was significantly bigger than that at 8 h ($z = 4.13$, $P < 0.001$), 16 h ($z = 8.25$, $P < 0.001$), and 32 h intervals ($z = 8.54$, $P < 0.001$). The body size of TDW females at 8 h interval was significantly bigger than that at 16 h ($z = 3.95$, $P < 0.001$) and 32 h intervals ($z = 4.29$, $P < 0.001$). The body size of TDB female at 0.5 h interval was significantly smaller than that at 32 h interval ($z = 2.93$, $P = 0.018$), but not significantly smaller than that at 8 h ($z = 1.37$, $P = 0.52$) and 16 h intervals ($z = 2.10$, $P = 0.15$) (Fig. 3-S1).

When the order of parasitism was TDW-TDB, the body size of TDW females ($116.50 \pm 0.86 \mu\text{m}$) was significantly smaller than that of TDB females ($123.99 \pm 1.72 \mu\text{m}$, $z = 3.22$, $P = 0.0013$) at 0.5 h interval; however, the body size of TDW females at 16 h interval ($120.61 \pm 0.96 \mu\text{m}$) and 32 h interval ($125.11 \pm 2.50 \mu\text{m}$) was significantly bigger than that of TDB females at 16 h ($114.67 \pm 0.95 \mu\text{m}$, $z = 2.77$, $P = 0.0055$) and 32 h intervals ($106.70 \pm 2.25 \mu\text{m}$, $z = 8.46$, $P < 0.001$). The difference in the body size between TDW ($117.39 \pm 0.98 \mu\text{m}$) and TDB ($119.46 \pm 1.22 \mu\text{m}$) females was not significant ($z = 0.98$, $P = 0.33$). The body size of TDW female at 32 h interval was significantly bigger than that at 0.5 h interval ($z = 3.89$, $P < 0.001$) and 8 h interval ($z = 3.65$, $P = 0.0015$), but not significantly bigger than that at 16 h interval ($z = 2.10$, $P = 0.15$). The body size of TDB females at 0.5 h interval was significantly bigger than that at 16 h interval ($z = 4.13$, $P < 0.001$) and 32 h interval ($z = 7.56$, $P < 0.001$), but not significantly bigger than that at 8 h interval ($z = 2.01$, $P = 0.18$) (Fig. 3-S2). In the control groups, the body size of TDW female ($146.93 \pm 2.34 \mu\text{m}$) was significantly smaller than that of TDB female ($154.28 \pm 2.34 \mu\text{m}$, $z = 2.23$, $P = 0.026$) (Fig. 3-S3).

Offspring fecundity

The fecundity of offspring female was significantly affected by the interaction of the order of parasitism, *T. dendrolimi* strain, and time interval ($\chi^2 = 151.94$, $df = 3$, $P < 0.001$). When the order of parasitism was TDB-TDW, the fecundity of TDW females (0.5 h interval: 43.44 ± 1.87 ; 8 h interval: 39.38 ± 1.91 ; 16 h interval: 31.89 ± 1.09 ; 32 h interval: 32.94 ± 3.39) was significantly lower than that of TDB females (0.5 h interval: 91.60 ± 2.45 , $z = 9.92$, $P < 0.001$; 8 h interval: 96.50 ± 2.17 , $z = 11.50$, $P < 0.001$; 16 h interval: 97.95 ± 4.85 , $z = 14.84$, $P < 0.001$; 32 h interval: 100.78 ± 4.66 , $z = 14.63$, $P < 0.001$), regardless of the time interval. The fecundity of TDW females at 0.5 h interval was significantly higher than that at 16 h ($z = 3.44$, $P = 0.0033$) and 32 h intervals ($z = 3.06$, $P = 0.012$), but not at 8 h interval ($z = 1.10$, $P = 0.69$). The fecundity of TDB females was not significantly influenced by the time interval (Fig. 3-S4).

When the order of parasitism was TDW-TDB, the fecundity of TDW females at 0.5 h (39.93 ± 2.45) or 8 h intervals (46.33 ± 2.26) was significantly lower than that of TDB females within the same intervals (86.14 ± 4.95 , $z = 10.10$, $P < 0.001$; 87.47 ± 4.07 , $z = 9.65$, $P < 0.001$, respectively); however, the fecundity of TDW females at 32 h interval (70.18 ± 2.39) was significantly higher than that of TDB females in the same interval (45.69 ± 1.73 , $z = 6.00$, $P < 0.001$). The difference was not significant in the fecundity of females at 16 h interval (TDW: 60.12 ± 2.69 ; TDB: 57.65 ± 3.12 ; $z = 0.62$, $P = 0.54$). The fecundity of TDW females at 0.5 h or 8 h interval was significantly lower than that of TDW females at 16 h or 32 h interval; however, the fecundity of TDB females at 0.5 h or 8 h interval was significantly higher than that of TDB females at 16 h or 32 h interval (Fig. 3-S5). In the control groups, the fecundity of TDW females (102.40 ± 5.63) was significantly lower than that of TDB females (158.75 ± 10.52 , $z = 4.80$, $P < 0.001$) (Fig. 3-S6).

Offspring longevity

The longevity of females was significantly affected by the interaction of the order of parasitism, *T. dendrolimi* strain, and time interval ($\chi^2 = 43.20$, $df = 3$, $P < 0.001$). When the order of parasitism was TDB-TDW, the longevity of TDW females (0.5 h interval: 5.25 ± 0.43 d; 8 h interval: 4.19 ± 0.38 d; 16 h interval: 3.89 ± 0.34 d; 32 h interval: 3.00 ± 3.31 d) was significantly lower than that of TDB females (0.5 h interval: 9.07 ± 0.58 d, $z = 5.24$, $P < 0.001$; 8 h interval: 9.57 ± 0.62 d, $z = 7.35$, $P < 0.001$; 16 h interval: 9.42 ± 0.53 d, $z = 8.51$, $P < 0.001$; 32 h interval: 10.01 ± 0.56 d, $z = 10.33$, $P < 0.001$), regardless of the time interval. The longevity of TDW females at 0.5 h interval was significantly higher than that at 32 h ($z = 4.27$, $P < 0.001$), but not significantly higher than that at 8 h ($z = 1.84$, $P = 0.26$) and 16 h intervals ($z = 2.49$, $P = 0.061$). The longevity of TDB females was not influenced by the time interval (Fig. 4-S1).

When the order of parasitism was TDW-TDB, the longevity of TDW females at 0.5 h (5.13 ± 0.68 d) or 8 h intervals (5.50 ± 0.64 d) was significantly lower than that of TDB females at 0.5 h (8.00 ± 0.88 d, $z = 2.57$, $P = 0.010$) or 8 h intervals (7.47 ± 0.77 d, $z = 1.96$, $P = 0.049$); however, the longevity of TDW females at 32 h interval (7.82 ± 0.79 d) was significantly higher than that of TDB females at the same interval (4.00 ± 0.58 d, $z = 3.79$, $P < 0.001$). The difference was not significant in the longevity of females at 16 h interval (TDW: 6.29 ± 0.71 d; TDB: 5.12 ± 0.64 ; $z = 1.23$, $P = 0.22$). The longevity of TDW females was not influenced by the time interval. The longevity of TDB females at 0.5 h ($z = 3.80$, $P < 0.001$) or 8 h intervals ($z = 3.50$, $P = 0.0026$) was significantly higher than that of TDB females at 32 h interval, but was not higher than that of TDB females at 16 h interval ($z = 2.68$, $P = 0.037$; $z = 2.33$, $P = 0.091$) (Fig. 4-S2). In the control groups, there was no significant difference in the longevity of TDW and TDB females (TDW: 8.35 ± 0.78 d; TDB: 10.45 ± 0.88 d; $z = 1.79$, $P = 0.074$) (Fig. 4-S3).

Development time of offspring

The development time of the offspring was not affected by *T. dendrolimi* strain ($\chi^2 = 0.26$, df = 1, $P = 0.61$), order of parasitism ($\chi^2 = 0.030$, df = 1, $P = 0.86$), and time interval ($\chi^2 = 4.86$, df = 3, $P = 0.18$) (Figs. 4-S4, 4-S5, 4-S6).

Developmental Rate Of Offspring

Logistic growth models showed that the rate of development and upper bound of offspring body length was not different between TDB offspring (single parasitism: $V_m = 15.82$ $\mu\text{m/h}$, $T_m = 27.68$ h, $k = 555.05 \pm 17.09$ μm ; superparasitism: $V_m = 10.81$ $\mu\text{m/h}$, $T_m = 24.67$ h, $k = 445.99 \pm 16.33$ μm) and TDW offspring (single-parasitism: $V_m = 15.08$ $\mu\text{m/h}$, $T_m = 27.93$ h, $k = 466.48 \pm 16.71$ μm ; superparasitism: $V_m = 11.20$ $\mu\text{m/h}$, $T_m = 25.11$ h, $k = 459.86 \pm 15.01$ μm), regardless of parasitism type. The rate of development and upper bound of the offspring body length in single parasitism was significantly higher than that in superparasitism, regardless of *T. dendrolimi* strain (Table 1; Figs. 5–7).

Table 1

Logistic growth parameters of length and width of TDB or TDW offspring in single parasitism or superparasitism

Variables	Parameters	Single parasitism						Superparasitism					
		TDB			TDW			TDB			TDW		
		Estimate±SE	t	P	Estimate±SE	t	P	Estimate±SE	t	P	Estimate±SE	t	P
Length	α	23.47±8.72 a	2.69	0.0098	19.56±6.31 a	3.10	0.0032	10.93±3.54 b	3.09	0.0033	11.55±3.39 b	3.38	0.0011
	β	0.11±0.014 a	7.99	<.001	0.11±0.012 a	8.62	<.001	0.097±0.014 a	7.01	<.001	0.097±0.012 a	7.99	<.001
	k	555.05±17.09 a	32.47	<.001	566.48±16.71 a	33.89	<.001	445.99±16.33 b	27.31	<.001	459.86±15.01 b	30.59	<.001
	T1 (h)	16.13	—	—	15.56	—	—	11.09	—	—	11.59	—	—
	T2 (h)	39.24	—	—	40.31	—	—	38.25	—	—	38.63	—	—
	Vm (μm/h)	15.82	—	—	15.08	—	—	10.81	—	—	11.20	—	—
	Tm (h)	27.68	—	—	27.93	—	—	24.67	—	—	25.11	—	—
Width	α	118.01±67.31 a	1.75	0.086	85.64±36.76 a	2.33	0.024	58.69±28.87 a	2.03	0.048	50.29±21.9 a	2.29	0.025
	β	0.15±0.019 a	7.93	<.001	0.14±0.015 a	9.79	<.001	0.13±0.017 a	7.78	<.001	0.13±0.015 a	8.62	<.001
	k	312.39±8.35 a	37.40	<.001	317.16±7.11 a	44.63	<.001	263.93±7.86 b	33.59	<.001	263.97±7.27 b	36.43	<.001
	T1 (h)	22.55	—	—	21.67	—	—	20.89	—	—	20.10	—	—
	T2 (h)	39.74	—	—	39.88	—	—	40.86	—	—	40.46	—	—
	Vm (μm/h)	11.96	—	—	11.47	—	—	8.70	—	—	8.54	—	—
	Tm (h)	31.14	—	—	30.78	—	—	30.87	—	—	30.28	—	—

Notes: Different uppercase letters indicate the significant difference ($P<0.05$) among the same parameter in different treatment

Similar to the offspring body length, the development rate and upper bound of the offspring body width was not different between TDB offspring (single-parasitism: $V_m = 11.96$ $\mu\text{m/h}$, $T_m = 31.14$ h, $k = 312.39 \pm 8.35$ μm ; superparasitism: $V_m = 8.70$ $\mu\text{m/h}$, $T_m = 30.87$ h, $k = 263.93 \pm 7.86$ μm) and TDW offspring (single-parasitism: $V_m = 11.47$ $\mu\text{m/h}$, $T_m = 30.78$ h, $k = 317.16 \pm 7.11$ μm ; superparasitism: $V_m = 8.54$ $\mu\text{m/h}$, $T_m = 30.28$ h, $k = 263.97 \pm 7.27$ μm), regardless of parasitism type. The developing rate and upper bound of the offspring body length in single parasitism was significantly higher than that in superparasitism, regardless of *T. dendrolimi* strain (Table 1; Figs. 5–7).

Discussion

Similar to previous studies (Farahani et al. 2015; Liu et al. 2018; Zhou et al. 2019b), our results confirmed that *Wolbachia*-infected females had a tendency for superparasitism when compared to uninfected females, regardless of the time interval of oviposition. Under field conditions, when encountering a depleted patch in which most host eggs are already parasitized, parasitoid females have to decide whether to reject the parasitized hosts or to superparasitize the current hosts. To avoid intra-specific competition, parasitoid wasps label the host with host marking pheromones (HMP) along with oviposition behaviors (Godfray 1994; Hubbard et al. 1999). Newly emerged wasps experience both healthy and parasitized hosts and learn to recognize the HMP, which allows them to distinguish the two. Two hypotheses may explain the tendency of superparasitism of *Trichogramma* wasps induced by *Wolbachia* infection: 1) *Wolbachia*-infected females do not produce HMP (Amat and Bernstein 2009). If *Wolbachia*-infected females lack HMP, both *Wolbachia*-infected and uninfected females are expected to more frequently superparasitize on the host eggs that have been parasitized by *Wolbachia*-infected females. However, our results do not support this hypothesis—there was no difference in the proportion of inter-strain superparasitism between TDB and TDW. In fact, *Trichogramma* females were unable to distinguish the host eggs previously parasitized by TDB or TDW females. 2) *Wolbachia* infection in *Trichogramma* females induce the loss of memory. Some studies showed that the memory retention of *Trichogramma* wasps is induced by *Wolbachia* infection. For example, the memory duration is significantly longer in *Wolbachia*-infected *T. brassicae* than in their uninfected counterparts (Farahani et al. 2017, 2021; Abrun et al. 2021). Owing to the memory loss of *Wolbachia*-infected *Trichogramma* wasps, the wasps are expected to forget cues related to HMP and lose the ability to distinguish healthily and parasitized hosts (Ueno and Tanaka, 1996; Farahani et al. 2017).

Our results showed a higher proportion of superparasitism for both TDW and TDB females at 16 h interval than at 32 h interval. Generally, the decision of parasitoid females relies on different cues produced by hosts and conspecific individuals. The rise in superparasitism of wasps over time is dependent upon the state of the hosts (Kishinevsky and Keasar 2015). Some parasitoids prefer recently parasitized hosts than the hosts that have been parasitized for a longer time because the quality of the parasitized hosts is expected to decline along with the development and consumption of the parasitoid offspring (Kishinevsky and Keasar 2015). However, in many other wasps, the tendency for superparasitism increases with the increase in time since the previous oviposition. Parasitoid wasps tend to reject the recently parasitized hosts that carry a clear cue of HMP but may accept the hosts with faded cues that were parasitized much earlier (Nufio and Papaj, 2001). Our results suggested that the decision-making of the wasps was influenced by the breakdown of repellent HMP and the decline in host quality with time. In mass-rearing procedure and field release of *Trichogramma* wasps, healthy hosts are scarce and rarely encountered when certain host eggs are supplied to a large number of wasps; the cue of HMP and memory of *Trichogramma* fade overtime when the hosts are exposed to the wasps for a long period; parasitoid females are expected to accept parasitized hosts and then reduce the quality of offspring wasps, which may negatively affect the control efficacy against pests.

The present results partly support the Early Acting Competitive Superiority Hypothesis. Duval et al. (2018) reported that the second clutch of *T. euproctidis* offspring was overcompeted by the first clutch for tens of hours intervals but gained advantages over the first clutch within 1 h interval. In this study, when the first oviposition was performed by TDB females, the TDB offspring outcompeted TDW offspring deposited by the later females, which was reflected by the higher survival proportion, extended longevity, bigger body size, and higher fecundity. However, even though the TDW offspring was deposited 8 h earlier than the later TDB offspring, TDW offspring gained no advantage over the TDB offspring. A potential explanation is that the hatching time of *Wolbachia*-infected *Trichogramma* eggs is longer than that of uninfected *Trichogramma* eggs. The first active larva of uninfected *Trichogramma* may subsequently consume more resources than infected larva or even kill the later competitor and then monopolize the host resource. For example, Heslin and Merritt (2005) reported that the larvae of *T. carverae* Oatman & Pinto were able to kill and siphon their siblings. As we known, *T. dendrolimi* larvae have not been reported to initiate aggressive attacks similar to *T. carverae* larvae. A Previous study reported that approximately 35% of embryos of infected *Trichogramma* remained at the mitotic stage even at 48 h after oviposition, while the embryos of their uninfected counterparts could develop to the cellular blastoderm stage within 6 h (Tagami et al. 2001). Another study showed that the infected offspring of *T. kaykai* took half a day longer than uninfected offspring to hatch (Hohmann et al. 2001). In contrast, the differences in the development rate and time between infected and uninfected offspring in the present study were not significant regardless of parasitism conditions. Thus, we suggest that the superiority of TDB to TDW offspring is due to the effects *Wolbachia* exerted on the fitness of *T. dendrolimi*. The results of the control groups also showed that, in single parasitism, infected offspring presented lower survival proportion, smaller body size, and lower fecundity compared with those of uninfected offspring. Similar results were also found in other *Trichogramma* species with large bodies (Stouthamer and Luck 1993; Hohmann et al. 2001; Miura and Tagami 2004; Russell and Stouthamer 2011; Zhou et al. 2020). The negative effects of *Wolbachia* on host fitness may subsequently reduce the competitive ability of infected *Trichogramma* offspring against uninfected *Trichogramma* offspring inside the host eggs. Even though *Wolbachia*-infected *T. dendrolimi* wasps could not benefit from *Wolbachia*, *Wolbachia* may gain more opportunities to spread due to the reduced competitive ability of the infected *Trichogramma* offspring. In superparasitized or multi-parasitized hosts, uninfected *Trichogramma* offspring or other parasitoid species may be able to siphon the dead body and excrement of infected *Trichogramma* offspring. Some solitary parasitoids, such as *Telenomus* spp, even kill the infected *Trichogramma* offspring and consume the dead bodies. Then, *Wolbachia* may be horizontally transmitted to other *Trichogramma* individuals or individuals of other parasitoid species (Huigens et al. 2004).

In this study, we occasionally observed the repeated stinging of *Trichogramma* females on a host egg, which is similar to the infanticide behavior described by Duval et al. (2018) in *T. euproctidis*. However, the females performed this behavior both in healthy host eggs and in parasitized host eggs and deposited a random number of offspring eggs. Thus, it was impossible to determine whether this was the oviposition or infanticide behavior. Moreover, the parasitism type of the host was hard to determine. Studies should investigate in more detail this mechanism to describe the complex parasitic behaviors of *Trichogramma* in future.

In conclusion, the results partly support the Early-Acting Competitive Superiority Hypothesis. The negative effects of *Wolbachia* on the competitive ability of infected *Trichogramma* offspring inside the host eggs were due to the low fitness of the offspring rather than the delay in offspring development. Future studies should focus on the effects of *Wolbachia* infection on the outcome of inter-strain intrinsic competition of *Trichogramma* offspring in field conditions to provide insights into the application of *Wolbachia*-induced thelytokous *Trichogramma* in biological control programs.

Declarations

Acknowledgements

This research was funded by the Projects of National Natural Science Foundation of China (32102275, 32172476, 31972339), the Projects of Guizhou Tobacco Corporation (201936, 201937, and 201941), the Major Projects of China National Tobacco Corporation (110202001032 (LS-01)), the Agricultural Science and Technology Innovation Program (CAAS-ZDRW202108), Natural Science Foundation of Liaoning Province (2020-BS-137), and the Fundamental Research Funds for the Universities of Liaoning Province (LR2019061, LJKZ0646). We should thank ELSEVIER Language Editing Services for improving the language.

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Figures

Figure 1

Proportion of superparasitism (S1) and inter-strain superparasitism (S2) as influenced by time interval and *Trichogramma* strain. Difference upper case letters indicate significant difference among different time interval of TDB females. Difference lower case letters indicate significant difference among different time interval of TDW females. "ns", "*", "**", and "***" indicate insignificant difference ($P > 0.05$), significant difference at 0.05, 0.01, and 0.001 level, respectively, between TDB and TDW.

Figure 2

Survival proportion of *Trichogramma* offspring as influenced by order of parasitism, time interval, and *Trichogramma* strain (TDB-TDW: S1; TDW-TDB: S2); and the control group (S3). Difference upper case letters indicate significant difference among different time interval of TDB females. Difference lower case letters indicate significant difference among different time interval of TDW females. "ns", "*", "**", and "***" indicate insignificant difference ($P>0.05$), significant difference at 0.05, 0.01, and 0.001 level, respectively, between TDB and TDW.

Figure 3

Body size (TDB-TDW: S1; TDW-TDB: S2; CK: S3) and fecundity (TDB-TDW: S4; TDW-TDB: S5; CK: S6) of *Trichogramma* offspring female as influenced by order of parasitism, time interval, and *Trichogramma* strain; and the control group. Difference upper case letters indicate significant difference among different time interval of TDB offspring females. Difference lower case letters indicate significant difference among different time interval of TDW offspring females. "ns", "*", "**", and "***" indicate insignificant difference ($P>0.05$), significant difference at 0.05, 0.01, and 0.001 level, respectively, between TDB and TDW.

Figure 4

Adult longevity (TDB-TDW: S1; TDW-TDB: S2; CK: S3) and development time (TDB-TDW: S4; TDW-TDB: S5; CK: S6) of *Trichogramma* offspring as influenced by order of parasitism, time interval, and *Trichogramma* strain; and the control group. Difference upper case letters indicate significant difference among different time interval of TDB offspring. Difference lower case letters indicate significant difference among different time interval of TDW offspring. "ns", "*", "**", and "***" indicate insignificant difference ($P > 0.05$), significant difference at 0.05, 0.01, and 0.001 level, respectively, between TDB and TDW.

Figure 5

Images of immature stage of TDB (A) and TDW (B) offspring in single-parasitism at different developmental time.

Figure 6

Images of immature stage of TDB (A) and TDW (B) offspring in superparasitism at different developmental time.

Figure 7

Length of immature offspring of TDB and TDW offspring as influenced by developmental time in single-parasitism (TDB: A; TDW: C) and superparasitism (TDB: B; TDW: D)

Figure 8

Width of immature offspring of TDB and TDW offspring as influenced by developmental time in single-parasitism(TDB: A; TDW: C) and superparasitism condition(TDB: B; TDW: D). Lines were fitted by logistic growth models.