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Impact of *Trichogramma* parasitoid age on the outcome of multiparasitism in the factitious host eggs of Chinese oak silkworm, *Antheraea pernyi*

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Abstract

The age of *Trichogramma* parasitoids could influence biological traits which are of great importance for effectiveness of mass rearing systems. Recent studies demonstrated that *Trichogramma ostriniae* (TO) could parasitize *Antheraea pernyi* eggs through multiparasitism along with other *Trichogramma* species, although few adults could emerge successfully. It is important to characterize the factors modulating the performance of TO on *A. pernyi* eggs, notably in order to try enhancing its quantitative emergence through multiparasitism for further use in biological control. In this context, irrespective of host age, we tested different ages of TO females (from 0.5-day- to 6-day-old postemergence) in multiparasitism with *Trichogramma chilonis* (TC) on *A. pernyi* eggs. The results of the present study confirmed that TO parasitism on *A. pernyi* eggs was largely age dependent. Higher percentage of multiparasitism and higher number of emerged TO adults were recorded in the treatment where 3-day-old TO females were used for parasitization with TC on *A. pernyi* host. In addition, we also tested different female ratios of both *Trichogramma* species to find out an optimal ratio for use in mass production. The highest percentage of multiparasitism, emergence and optimum emerged number of TO (per *A. pernyi* egg) was observed with the ratio 3:2 (TO:TC) per 1 host egg. This study demonstrated that the age of TO females can significantly affect their performance in multiparasitism of large *A. pernyi* eggs. These results prompt for more research in order to develop an effective multiparasitism-based production of TO at the industrial scale.

Keywords *Trichogramma chilonis* · *Trichogramma ostriniae* · Biological control · Parasitoid development · Mass rearing

Key messages

- Few studies documented the factors affecting rearing of *Trichogramma ostriniae* on the large eggs of Chinese oak silkworm, *A. pernyi*.
- We evaluated the impact of *Trichogramma ostriniae* age on multiparasitism with *Trichogramma chilonis* at different parasitoid ratios in *A. pernyi* eggs.
- The multiparasitism capability of *Trichogramma ostriniae* on *A. pernyi* host was dependent of parasitoid age, with 3-day-old parasitoid females showing highest performance on *A. pernyi*.
- Parasitoid female ratio of 3:2 (*Trichogramma ostriniae*: *Trichogramma chilonis*) per *A. pernyi* egg was found optimal for potential mass rearing. These results provide useful information about mass production of two *Trichogramma* species using multiparasitism method.

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Introduction

The Trichogrammatidae family consists of about 650 parasitoid species from which some have been successfully used against numerous insect pests for over 100 years (Smith

1996; Tabone et al. 2010; Yuan et al. 2012; Chailleux et al. 2013; Du et al. 2017; Guo et al. 2019; Zang et al. 2021). These parasitoids are key biological control agents against various agricultural pests in China (Zang et al. 2021). Among these, *Trichogramma ostriniae* Pang & Chen is abundant and used against Asian corn borer *Ostrinia furnacalis* Guenee (Lepidoptera: Crambidae) since the early 1950s (Gardner et al. 2012; Li et al. 2018; Zang et al. 2021).

Ostrinia furnacalis is a key economic pest of corn in China, causing an average of 6–9 million tons yield losses per year (Wang et al. 2014). Currently, about 5.5 million hectares of corn fields are treated annually with *T. ostriniae*, *T. chilonis* Ishii, *T. dendrolimi* Matsumura and other *Trichogramma* species across northeastern China (Zang et al. 2021). Inundative releases of *T. ostriniae* against *O. furnacalis* in corn are on average 75,000–120,000 per hectare, and result in over 90% parasitism and successful control of the pest (Wang et al. 2000).

Trichogramma ostriniae is a key biological control agent for *O. furnacalis* in China and is usually mass-produced on rice moth *Corcyra cephalonica* Stainton (Lepidoptera: Pyralidae) eggs. However this host is not cost effective owing to the small size of eggs that could support the development of only one parasitoid per egg (Liu et al. 1998). In addition, the quality of these eggs for parasitoid rearing declines when the eggs are stored for more than 2–3 weeks (Wang et al. 1998; Wu et al. 2018). Another biological control agent, *Trichogramma chilonis*, can be mass-produced on eggs of Chinese oak silkworm, *Antheraea pernyi* Guérin-Méneville (Lepidoptera: Saturniidae) (Wang et al. 2005). Currently, *A. pernyi* is considered as the best factitious host for the mass production of *Trichogramma* parasitoids because of high parasitism rate, low cost of production, and easy to store and transport (Huang et al. 2013; Zang 2015; Zhang et al. 2017, 2018; Zang et al. 2021). Due to their large size, *A. pernyi* eggs can enable 60–260 *Trichogramma* adults to be produced per host egg (Zang et al. 2021). Although *A. pernyi* host eggs are widely accepted, they also come across some limitations where thick and hard chorion is one of these limitations. Several *Trichogramma* species like *T. embryophagum* Hartig, *T. evanescens* Westwood, *T. brassicae* Bezdenko and *T. piceum* Dyurich could parasitize *A. pernyi* eggs but they often fail to chew an emergence hole (Hassan et al. 2004). This failure is due to the thick and hard chorion of *A. pernyi* eggs; parasitoid adults are trapped inside the host egg and they die. *Trichogramma ostriniae* is one of the key species that faces these limitations (Li et al. 2018).

The oviposition behavior of parasitoids is an important component in their biology which determines their role in ecosystems and their economic importance (Reznik et al. 2001; Wang et al. 2019). Parasitoid species having all of their egg complement mature upon emergence are referred

as pro-ovigenic, while those that do not are syn-ovigenic; egg maturation strategies are related to parasitism mode (Jervis et al. 2001). *Trichogramma ostriniae* are among syn-ovigenic parasitoids, carrying only a fraction of mature eggs in ovaries after emergence, and egg maturation tends to increase as the age increases till 3rd day of adult life (Chen et al. 2005). Li et al. (2018) reported multiparasitism of *T. ostriniae* (newly emerged < 8 h) with *T. dendrolimi* on *A. pernyi* host. Their results confirmed that *T. ostriniae* can multiparasitize *A. pernyi* eggs but only few adults were able to emerge per host egg. Therefore, in the context of possible development of mass production of *T. ostriniae* using a multiparasitism-based method, further experiments are needed to evaluate how factors such as parasitoid age could modulate emergence rate from *A. pernyi* host (during multiparasitism). Several studies demonstrated that *Trichogramma* parasitoid age could impact parasitism and progeny emergence from different host eggs in monoparasitism (Garcia et al. 2001, Hegazi and Khafagi 2001; Hohmann and Luck 2004; Makee 2005; Ayvaz et al. 2008; Pizzol et al. 2012). However, parasitoid developmental rates and age-related oviposition patterns (syn-ovigenic or pro-ovigenic) could also have influence on the outcomes of multiparasitism between parasitoids (Cusumano et al. 2012; Harvey et al. 2013). Multiparasitism occurs when an already parasitized host is attacked again by a parasitoid of the same or different species (Yamamoto et al. 2007; Desneux et al. 2009; Harvey et al. 2009; Keinan et al. 2012; Harvey et al. 2013; Velzen et al. 2016). In a previous study we demonstrated that rearing host species significantly affected the outcomes of multiparasitism between egg parasitoids (Iqbal et al. 2019). Parasitoids reared on large host eggs produced more progeny in multiparasitism as compared to small host-reared parasitoids. However, it remains unclear whether parasitoid age could have an impact on multiparasitism. Published literature lacks the information related to the effect of parasitoid age on multiparasitism; therefore, to our knowledge, this study is the first to evaluate these aspects.

Trichogramma chilonis is among the few parasitoids which can efficiently be mass reproduced on *A. pernyi* eggs through monoparasitism. The most abundant parasitoid species attacking eggs of *O. furnacalis* are *T. ostriniae*, *T. dendrolimi* and *T. chilonis* which are distributed throughout China (Wang et al. 2005). A previous study indicated that *T. dendrolimi* exhibited higher adaptation to host eggs of *A. pernyi* than *T. ostriniae* mostly during multiparasitism, where interspecific competition inside the large egg could significantly suppress *T. ostriniae* (Li et al. 2019). Therefore, to test the hypothesis that parasitoid age affects the outcome of multiparasitism, we selected *T. chilonis* to test in multiparasitism with *T. ostriniae*. Here, we evaluated different age limits (in days) of *T. ostriniae* in multiparasitism with *T. chilonis* to shed light on the impact of *T. ostriniae* age on

parasitism of *A. pernyi* host. We also tested different female ratios of both species to identify optimal ratio for application in mass production system. The present study provides useful information on possible methods for *T. ostriniae* industrial production, and potential further use against *O. furnacalis* (and other lepidopteran pests).

Materials and methods

Parasitoids

Egg parasitoids *T. chilonis* (TC) and *T. ostriniae* (TO) were collected in 2011 from rice fields using sentinel egg masses of rice striped stem borer, *Chilo suppressalis* Walker (Lepidoptera: Crambidae) in Changchun, Jilin province, China. *Trichogramma* species were identified from male genital capsule using electronic microscope micrograph and were further confirmed by sequence analysis of rDNA-ITS2 (GeneBank Accession Nos. of FR828481 and FR750277 for TO and TC, respectively) (Pinto 1992; Stouthamer et al. 1999). Under laboratory conditions 25 ± 1 °C, $65 \pm 5\%$ RH and a 14:10 (L:D) photoperiod, the rearing of both parasitoid species was carried out on *C. cephalonica* eggs for several generations. Parasitoids and host voucher specimens were preserved in the Institute of Biological Control, Jilin Agricultural University, Changchun, Jilin province, China.

Chinese oak silkworm host

Cocoons of Chinese oak silkworm *A. pernyi* were collected from Yongji city (Jilin Province, China) and were shifted to Institute of Biological Control, Jilin Agricultural University in December 2018. Cocoons were stored till February 2019 for near 3 months at temperature of -4 °C. Then, the cocoons were hanged in emergence rooms for incubation at a temperature of 25 ± 1 °C. Mature and unmated female moths of *A. pernyi* were collected after emergence from cocoons and were kept for 2–3 days at a temperature of 4 °C in refrigerator. Host eggs for experiments were collected by dissecting the abdomen of mature female moths, washed with distilled water and were kept under room temperature to dry. Immature greenish eggs were removed, and healthy large eggs were collected for experiments after they became dry. Five large eggs of *A. pernyi* collected within 8 h were then pasted using non-toxic glue on a strip-shaped egg card (5 cm \times 0.5 cm) and were used in the experiments.

TO age impact in multiparasitism with TC on *A. pernyi* host

Parasitoid colonies were raised on *C. cephalonia* eggs under laboratory conditions of 25 ± 1 °C, $65 \pm 5\%$ RH and a 14:10

(L:D) photoperiod. *Trichogramma ostriniae* (both male and female) emerged from *C. cephalonica* eggs were kept in a glass tube of 3.5 cm in diameter and 10 cm long to reach the various ages, i.e., 0.5-, 1-, 2-, 3-, 4-, 5- and 6-day-old postemergence without host eggs. All adult parasitoids were fed with drops of 20% honey solution till they were used in the experiments. *Trichogramma chilonis* wasps emerged within 12-h from *C. cephalonica* eggs were used in all experiments. The same age TC wasps were used throughout experiments, because regardless age factor TC can parasitize *A. pernyi* eggs and can emerge successfully under monoparasitism.

Specific wasp ratio having twenty-five TO females and five TC females [25:5 (TO:TC)] per replicate were used to evaluate the parasitism capability of different ages of TO females in multiparasitism with TC on *A. pernyi* large host eggs. Different ages TO females and 0.5-day-old TC females were introduced simultaneously into glass tube of 3.5 cm in diameter and 10 cm long, containing five host eggs of *A. pernyi* (host eggs were pasted 1 cm apart on a strip-shaped card with the help of non-toxic glue). Five host eggs were kept constant throughout all experiments. Both *Trichogramma* species were allowed to parasitize *A. pernyi* eggs for 48 h under laboratory conditions. After wasp removal, parasitized eggs of *A. pernyi* were cut out individually and each egg was enclosed in a glass tube held in incubation chamber per conditions already defined for laboratory rearing till parasitoids developed. After 6–7 days of wasp removal, the *A. pernyi* eggs held in incubation chamber changed its chorion color from pure white to dark gray, where these eggs were considered as multiparasitized by TO and TC. Further confirmation of multiparasitized eggs was made at the time of wasp emergence per egg. Test for each age limit of TO was repeated 10 times in multiparasitism with TC. Total of 250 females of TO, 50 females of TC and 50 *A. pernyi* host eggs were tested in 10 replications for each age limit experiment.

Screening of different wasp to wasp ratios of both *Trichogramma* species in multiparasitism on *A. pernyi*

Different ratios of females of both *Trichogramma* species were tested in order to optimize emergence of TO from *A. pernyi* eggs in multiparasitism. In these experiments, all the TO female wasps used were 3-day-old postemergence (higher parasitism rate of *A. pernyi*, reported here in results section) and TC female wasps were 12-h-old postemergence. Different tested ratios were 15:10 (TO:TC), 20:10 (TO:TC) and 25:5 (TO:TC). For each ratio test, female wasp of TO and TC was introduced simultaneously into glass tube of 3.5 cm in diameter and 10 cm long, containing five host eggs of *A. pernyi* (host eggs were pasted 1 cm apart on a strip-shaped card with the help of non-toxic glue). Test for

each ratio was repeated 15 times. The rest of the procedure was the same as mentioned above for the TO age evaluation experiments.

Studied parameters

The percentage of multiparasitism was documented as the number of eggs multiparasitized/total number of eggs offered $\times 100$. The percentage of multi-species emergence for each ratio was documented as the number of eggs with holes/total number of parasitized eggs $\times 100$. Total number of adults emerged per *A. pernyi* egg were recorded for each species. Total number of adults emerged for TO and TC per *A. pernyi* were also recorded. Developmental times were recorded as the number of days from parasitism to adult wasp emergence. At end, parasitized eggs from which no wasp emerged were dissected and pupae per egg, dead wasps inside egg were recorded under microscope. The emergence holes on the surface of egg were also recorded.

Data analysis

In all those treatments where different age limits of TO were used in multiparasitism with TC, comparison of percentages of multiparasitized eggs, the developmental time of two species, the number of emerged adults for specific species per egg and their female progeny, and the number of parasitoids of both species emerging from the same egg of the host was performed by using a one-way ANOVA (SAS Institute 2010); where recorded data for all the parameters showed significant differences among all treatments in ANOVA, the mean numbers of all the parameters were compared among

the tested ratios using Tukey's honestly significant difference (HSD) test ($\alpha=0.05$). Before an ANOVA was performed, the percentages of multiparasitized eggs, adult emergence, and female progeny were arcsine \sqrt{x} transformed to homogenize variances and were subjected to Shapiro–Wilk test. The same analysis was done for experiment where different female ratios were tested.

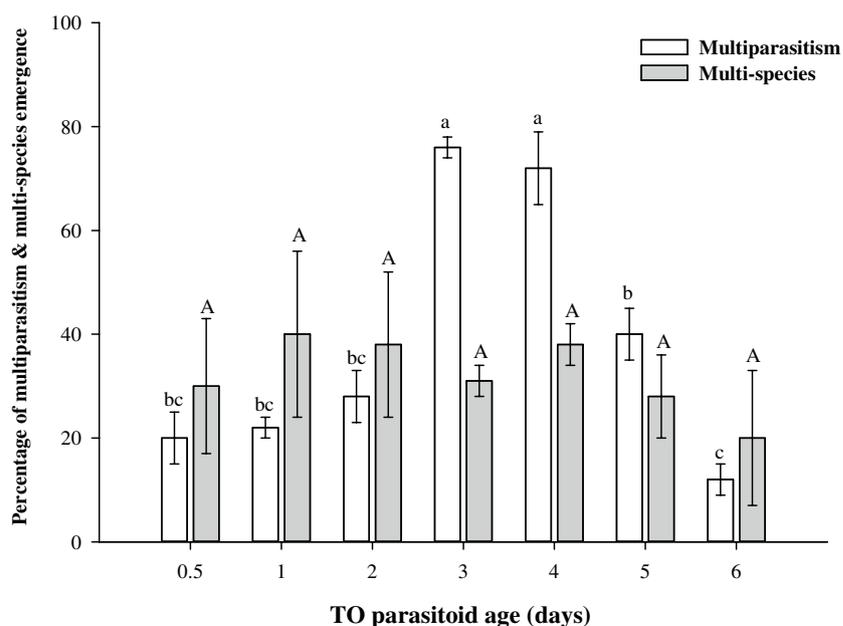
Results

TO age impact in multiparasitism with TC on *A. pernyi* host

Parasitoid age significantly affected parasitism of TO in multiparasitism conditions with TC on *A. pernyi* ($F_{6,63}=29.101$; $P<0.0001$; Fig. 1). The treatments where 0.5-day-, 1-day- and 2-day-old TO females were used for parasitizing *A. pernyi* eggs with TC showed significantly lower percentages of multiparasitism (20%, 22% and 28%, respectively) than when 3-day- and 4-day-old females were used (76% and 72%, respectively). The percentages of multiparasitism were decreased to 40% and 12% in the treatments where 5-day- and 6-day-old TO females were used for parasitization, respectively.

The percentages of multi-species emergence in all the treatments were similar ($F_{6,63}=0.387$; $P=0.8844$; Fig. 1). The percentage of emergence in all treatments was dependent on TC species, where the same number of TC females was offered in all treatments in multiparasitism.

Fig. 1 Mean percentage (\pm SE) of multiparasitism and multi-species emergence from *A. pernyi* eggs for different *Trichogramma ostrinia* (TO) age treatments (0.5-day- to 6-day-old). *Trichogramma chilonis* (TC) age was 0.5-day-old. Lowercase and capital letters indicate significant differences among the treatments (Tukey's HSD test, $P<0.05$)



Similar to multiparasitism, number of emerged TO offspring per *A. pernyi* egg showed significant differences in all treatments where various age limits of TO females were used for parasitizing *A. pernyi* host during multiparasitism with TC ($F_{6, 40} = 4.558$; $P < 0.0001$; Fig. 2). Significantly high number (29.50) TO offsprings emerged per *A. pernyi* egg in the treatment where TO females used for parasitism had an age limit of 3-day-old. That's why we selected TO females 3-day-old age limit in the second set of experiments. No TO offspring emerged per *A. pernyi* egg in the treatment where the TO females used for parasitizing had an age limit of 0.5-day-old. The emerged offspring number of TC per *A. pernyi* egg was similar among all the treatments ($F_{6, 40} = 1.351$; $P = 0.2579$; Fig. 2). Furthermore, significant differences were found in the number of parasitoids of both species emerging from the same host egg among the treatments ($F_{6, 40} = 4.014$; $P = 0.0001$; Fig. 2).

Screening of different wasp to wasp ratios of both *Trichogramma* species on *A. pernyi* host under multiparasitism

Parasitism and emergence rate

No significant differences were found when comparing percentage of multiparasitism among the tested ratios ($F_{2, 42} = 2.549$; $P = 0.0902$; Fig. 3). Percentage of multi-species emergence showed significant differences among the tested ratios ($F_{2, 42} = 7.764$; $P < 0.0001$; Fig. 3). Significantly highest percentage of multi-species emergence of 71% was recorded in the treatment where wasps to wasps

ratio was 15:10 (TO:TC). Significantly lower percentages of multi-species emergence were recorded in the treatments where wasps to wasps ratios were 20:10 (TO:TC) and 25:5 (TO:TC) (49% and 40%, respectively).

Emerged wasps per *A. pernyi* egg

The number of TC adults emerged per *A. pernyi* egg showed significant differences in multiparasitism with TO among the tested ratios (Table 1). The total offspring emerged for TC in the tested wasp to wasp ratios 15:10 (TO:TC), 20:10 (TO:TC) and 25:5 (TO:TC) were 36.62, 29.81 and 27.18, respectively ($F_{2, 76} = 4.869$; $P < 0.0001$). For TO, emerged offspring numbers did not show significant differences when comparing the mean values among tested wasp to wasp ratios (Table 1). The total emerged TO offsprings per *A. pernyi* egg were 22.08, 25.13 and 26.36 for the ratios 15:10 (TO:TC), 20:10 (TO:TC) and 25:5 (TO:TC), respectively ($F_{2, 76} = 0.804$; $P = 0.4514$).

The total number of adult wasps of both species emerging from the same *A. pernyi* egg also did not show significant differences in all tested wasp to wasp ratios ($F_{2, 76} = 1.286$; $P = 0.2824$; Table 1). The total number of adult wasps of both species emerging from the same *A. pernyi* egg in the wasp to wasp ratios of 15:10 (TO:TC), 20:10 (TO:TC) and 25:5 (TO:TC) were 58.70, 54.94 and 53.54, respectively. When emergence of parasitoids from *A. pernyi* eggs accomplished, dissection of eggs was carried out under microscope to record inside egg data. The total number of immature pupa (unidentified) inside per egg showed significant differences among the tested ratios ($F_{2, 42} = 8.093$; $P < 0.0001$;

Fig. 2 Mean number (\pm SEs) of parasitoids emerged per egg (per species and in total) for different *Trichogramma ostriniae* (TO) age treatments (0.5-day- to 6-day-old). *Trichogramma chilonis* (TC) age was 0.5-day-old. Lowercase, capital and italic letters indicate significant differences among the treatments (Tukey's HSD test, $P < 0.05$)

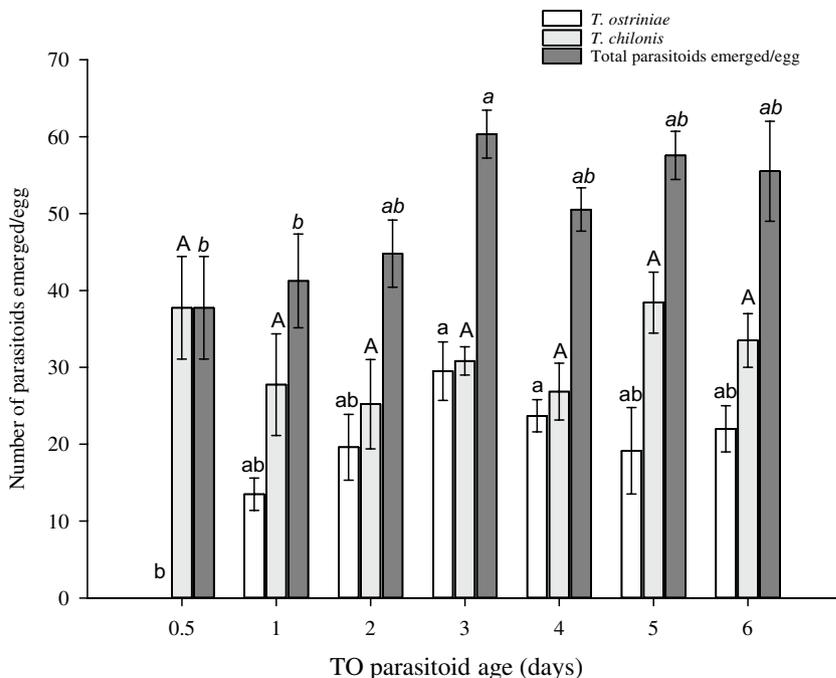


Fig. 3 Mean percentages (\pm SE) of multiparasitism and multi-species emergence from *A. pernyi* eggs for different wasp to wasp ratios (*Trichogramma ostrinae* (TO):*Trichogramma chilonis* (TC)). Lowercase and capital letters indicate significant differences among the tested ratios (Tukey's HSD test, $P < 0.05$)

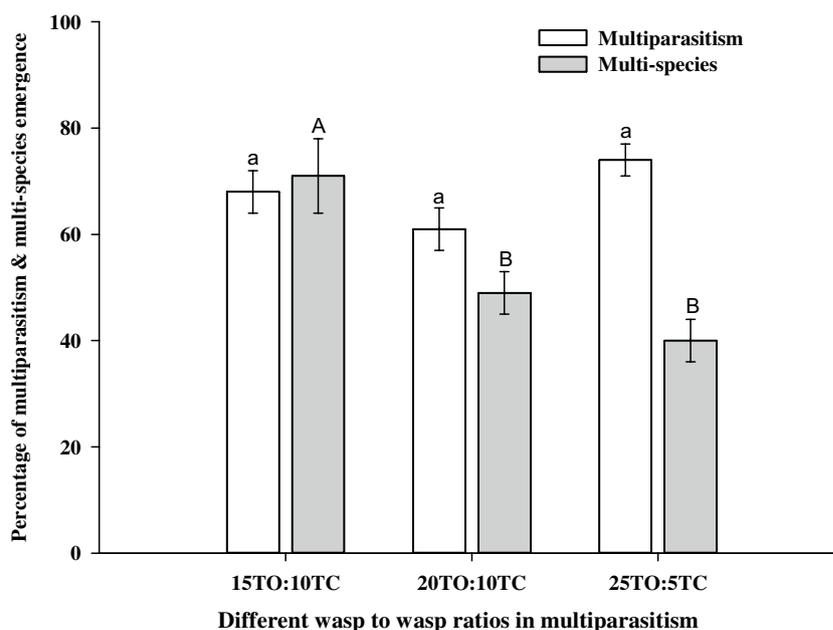


Table 1 Different biological parameters of *Trichogramma ostrinae* (TO) and *T. chilonis* (TC) multiparasitizing *Antheraea pernyi* eggs

Parameters		Different wasp ratios of both <i>Trichogramma</i> species in multiparasitism		
		15TO:10TC	20TO:10TC	25TO:5TC
No. of emerged TO and TC/egg	TO	22.08 \pm 2.50 a	25.13 \pm 2.33 a	26.36 \pm 2.61 a
	TC	36.62 \pm 2.28 a	29.81 \pm 2.11 ab	27.18 \pm 2.25 b
Development rate	TO	12.82 \pm 0.40 a	13.15 \pm 0.14 a	12.57 \pm 0.12 a
	TC	12.60 \pm 0.18 a	12.71 \pm 0.15 a	12.32 \pm 0.09 a
Average dead wasps/egg	TO	8.45 \pm 2.73 b	14.50 \pm 2.29 ab	17.93 \pm 2.49 a
	TC	2.84 \pm 1.41 a	3.39 \pm 1.07 a	3.16 \pm 0.85 a
Female rate	TO	0.82 \pm 0.00 a	0.80 \pm 0.00 a	0.81 \pm 0.01 a
	TC	0.85 \pm 0.00 a	0.83 \pm 0.00 a	0.85 \pm 0.01 a
Total no. of wasp emerged		58.70 \pm 2.36 a	54.94 \pm 2.32 a	53.54 \pm 2.60 a

3-day-old TO with 0.5-day-old TC. For each parameter, means \pm SEs are reported. Each value followed by different lower-case letters was significantly different among the tested ratio treatments (Tukey's HSD test, $P < 0.05$)

Fig. 4A). Significantly less number 1.17 of immature pupa per *A. pernyi* egg was recorded for the wasp to wasp ratio 15:10 (TO:TC). Significantly high numbers 3.22 and 4.07 of immature pupae per *A. pernyi* egg were recorded for the ratios 20:10 (TO:TC) and 25:5 (TO:TC), respectively. Similarly, significant different numbers of dead adults were found per egg among the tested wasp to wasp ratios, specifically for the TO species ($F_{2,42} = 3.645$; $P < 0.0001$; Table 1). The numbers of dead wasps per egg for TO species were 8.45, 14.50 and 17.93 in the tested wasp to wasp ratios 15:10 (TO:TC), 20:10 (TO:TC) and 25:5 (TO:TC), respectively. The number of dead adults per egg recorded for TC was statistically similar among all of the tested wasp to wasp ratios ($F_{2,42} = 0.058$; $P = 0.9436$) (Table 1).

Pre-emergence time of both species did not show significant differences in all the tested wasp to wasp ratios (Table 1). Pre-emergence time for TO species was 12.82, 13.15 and 12.57 days in the ratios 15:10 (TO:TC), 20:10 (TO:TC) and 25:5 (TO:TC), respectively ($F_{2,74} = 0.674$; $P = 0.5127$). Similarly, the pre-emergence time for TC was 12.60, 12.71 and 12.32 days in the ratios 15:10 (TO:TC), 20:10 (TO:TC) and 25:5 (TO:TC), respectively ($F_{2,75} = 1.26$; $P = 0.2896$).

Furthermore, the female rates of emerged offsprings for both parasitoid species in multiparasitism showed no significant differences (Table 1). The offspring population of TO emerging from *A. pernyi* eggs always showed at minimum 80% of females in all tested wasp to wasp ratios

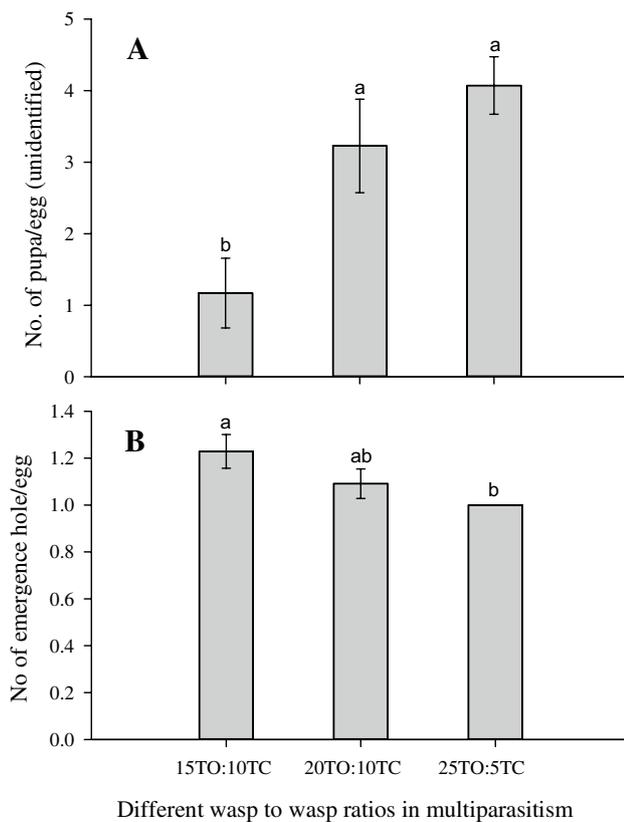


Fig. 4 **A** mean number (\pm SEs) of pupa, and **B** mean number (\pm SEs) of emergence holes per host egg among different wasp to wasp ratios (*Trichogramma ostriniae* (TO):*Trichogramma chilonis* (TC)). Lowercase letters indicate significant differences among the tested ratios (Tukey's HSD test, $P < 0.05$)

($F_{2,74} = 0.674$; $P = 0.5127$). Similar results were observed for TC ($F_{2,75} = 1.26$; $P = 0.2896$) (Table 1). Emergence holes made by *Trichogramma* parasitoids on the surface of *A. pernyi* egg showed significant differences in numbers among the tested ratios ($F_{2,76} = 3.541$; $P < 0.0001$; Fig. 4B). Significantly highest number (1.22) of emergence holes per egg was recorded for the 15:10 (TO:TC). Significantly lower numbers 1.09 and 1.00 of emergence holes per egg were recorded for 20:10 (TO:TC) and 25:5 (TO:TC) ratios, respectively.

Discussion

Parasitoids are often involved in extrinsic and/or intrinsic competitive interactions (Cusumano et al. 2012), including in mass rearing conditions (e.g. in *Trichogramma* parasitoids, Zang et al. 2021). We demonstrated that two parasitoid species, TO and TC, can parasitize successfully the large egg host, *A. pernyi*, through multiparasitism, and particular conditions were identified to maximize parasitoid production in

such condition (e.g. wasp to wasp ratios and optimal parasitoid age). These results match with our previous study (Iqbal et al. 2019) reporting multiparasitism of *T. leucaniae* Pang & Chen and TC, and in which we suggested that both parasitoid species can be mass reared on *A. pernyi* through multiparasitism. Similarly, Li et al. (2018) reported multiparasitism of TO and *T. dendrolimi* on *A. pernyi*, with *T. dendrolimi* presence increasing the emergence rate of TO from this host. In overall, parasitoid species sharing the same host sources can be involved in multiparasitism, and this appears being common in egg parasitoids (Yamamoto et al. 2007; Mandour et al. 2008; Cabello et al. 2011; Cusumano et al. 2016).

Trichogramma ostriniae age impacts on multiparasitism

The age of *T. ostriniae* females had an impact on the outcome of multiparasitism with TC on the host *A. pernyi*. We observed lower percentages of multiparasitism in treatments where newly emerged and young TO females were used for parasitizing *A. pernyi* eggs with TC, and higher percentages of multiparasitism were observed when TO females were 3- and 4-day-old (postemergence). It means that young TO females did not parasitize as many eggs as older females. These results match with those of Pizzol et al. (2012), who tested different ages *T. cacoeciae* Marchal adult females parasitizing eggs of *Lobesia botrana* Denis and Schiffermüller (Lepidoptera: Tortricidae). Their results showed that parasitoid ages had impact on parasitism, as 1-day-old females produced fewer parasitized eggs than 2, 3, and 4-day-old females. In our experiments, we observed a strong choosiness behavior in young TO females on *A. pernyi* host. Young TO females did not show strong attractiveness toward *A. pernyi* eggs, notably the newly emerged females. This behavior may be due to the low ovarian load in the young TO females. *Trichogramma ostriniae* are among syn-ovigenic parasitoids exhibiting few mature eggs in ovaries after eclosion (Chen et al. 2005). Papaj (2000) reported that low egg load in early age of parasitoids is often responsible for high choosiness behavior during encountering hosts. We noticed that TO females deprived for 3 and 4 days from parasitization of *A. pernyi* host, severely attacked *A. pernyi* host after provision for parasitism and did not show strong choosiness behavior. This behavior suggest that these older TO females had high ovarian load. Barnay et al. (1999) reported that *T. cacoeciae* foraging activity was influenced by ovarian load, and that it could lead to a higher discovery rate in case of older *T. cacoeciae* females (when compared to younger females). The old parasitoid females had higher ovarian pressure because of inhibiting high number of mature eggs in ovaries, which consequently resulted into weaker choosiness behavior toward host (Klomp et al. 1980). Other factors such as endogenous circadian rhythm (Wang et al. 2019),

temperature (Pizzol et al. 2010), or plants of targeted hosts (Thiery and Desneux 2018) may also affect multiparasitism through modulating the oviposition behavior in *Trichogramma* parasitoids.

In parasitoids, fecundity usually shows a gradual increase until it reaches a maximum rate and then a gradual decrease occurs until reproduction ceases shortly before death (Jervis and Kidd 1996; Pizzol et al. 2012; Biondi et al. 2013). Hegazi and Khafagi (2001) reported that efficiency of parasitization by host-deprived females of *T. cacoeciae* varied with ages, where parasitization rate was higher for 1–5-day-old females than 6-day-old females on *Sitotroga cerealella* Olivier (Lepidoptera: Gelechiidae) eggs, and the parasitization rate then declined as the females aged. Similarly, in our study, a drastic decline in parasitism rates (after a peak) was observed in the treatments where 5 and 6 days deprived TO females were used for parasitization of *A. pernyi* host with TC. We assumed that TO females deprived from *A. pernyi* host for such a long period of time (i.e., more than 4 days) resulted in intensive oosorption and consequently the parasitization ability of TO females decreased. The variation in multiparasitism rates in our results did confirm that parasitism ability of TO on *A. pernyi* host was largely age dependent (Fleury and Bouletreau 1993; Godfray 1994; Garcia et al. 2001; Makee 2005; Huang et al. 2017).

Moreover, comparison of percent emergence from *A. pernyi* host among treatments did not show any significant differences, likely because the same number of TC was offered in multiparasitism with TO in all the treatments. More specifically, the emergence rate was dependent on the competent parasitoid species (i.e. TC), as previously reported by studies on multiparasitism in *Trichogramma* parasitoids (Li et al. 2018; Iqbal et al. 2019). *Trichogramma ostriniae* species benefited using the emergence holes made by *T. dendrolimi* in Li et al. (2018) and *T. leucaniae* by TC (Iqbal et al. 2019). Furthermore, significant differences were found in emerged number of TO adults per egg. No TO individual wasp emerged per egg in the first treatment where TO females used for parasitization were 0.5-day-old. By contrast, significantly more TO adults emerged per egg in the treatment where TO females used for parasitizing were 3-day-old. These results are consistent with that of Pizzol et al. (2012) where *T. cacoeciae* emergence increased according to ovipositing females age, with the highest emergence rate observed for 3-day-old females and the lowest emergence rate for 1-day-old females. It has been reported that when oviposition was prevented by host deprivation, *T. cacoeciae* and *T. brassicae* Bezdenko stored approximately twice as many eggs as found in newly emerged female (Volkoff and Daumal 1994). Our results showed that when host deprivation duration for TO was prolonged for 3 days, the parasitism ability and progeny production were markedly increased on *A. pernyi* host.

Screening of different wasp to wasp ratios of both *Trichogramma* species in multiparasitism on *A. pernyi* host

The percent multiparasitism in all the tested ratios was similar. High parasitism rates were observed because high densities of parasitoid wasps were offered in multiparasitism of *A. pernyi* eggs. Our results are in line with that of Huang et al. (2017) where increase in *T. dendrolimi* wasps to host eggs of *O. furnacalis* resulted in high parasitism rate. Watt et al. (2015) evaluated different ratios of parasitoid *Spathius galinae* Belokobylskij & Strazanac (Hymenoptera: Braconidae) and its host *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), and found that parasitism rate was positively associated with increasing parasitoid to host ratio. Because of the large size of host *A. pernyi* eggs, we offered high densities of parasitoid wasps. Indeed, eggs of the factitious host *A. pernyi* are large enough for multiple *Trichogramma* parasitoids to develop (having approximate 3180 µm length and 2684 µm width, Iqbal et al. 2019).

Biological parameters such as emergence, number of dead TO adults per egg, number of immature pupae (unidentified) and number of emergence holes per host egg varied among the tested TO:TC ratios. We found that the optimal wasp to wasp ratio was 15:10 (TO:TC) when they were offered for parasitization of 5 *A. pernyi* eggs under laboratory conditions. Therefore, the optimal ratio of multiparasitism of TO:TC to host eggs of *A. pernyi* for being used for mass production is likely close to 3:2:1. We assumed that optimal emergence of TO adults, low number of dead TO adults per egg, and high number of emergence holes per egg in the ratio of 3:2 (TO:TC) was likely due to a low interspecific intrinsic competition in *A. pernyi* egg at such ratio. *Trichogramma chilonis* did not perform more aggressively in our present study of multiparasitism on *A. pernyi* eggs as *T. dendrolimi* did in previously reported study of Li et al. (2018). Such low aggressiveness of TC in multiparasitism conditions may facilitate the emergence of TO adults from *A. pernyi* egg. Indeed, TC does not show strong specificity to *A. pernyi* as host, when compared to *T. dendrolimi*, and therefore, only few strains of TC can be reared on using this host (Hassan et al. 2004; Huang et al. 2013; Hou et al. 2018). Similar results were found when studying multiparasitism of TC and *T. leucaniae* on *A. pernyi* egg (Iqbal et al. 2019). Shi and Liu (2003) stressed that multiparasitism could help different parasitoids to use same host resources more effectively. Within-host competition among gregarious parasitoids that can also result in the phenomenon of ‘resource sharing’ in which adults from both species can emerge from the same multiparasitized hosts (Magdaraog et al. 2012). We also suspect that severe intrinsic competition occurred inside *A. pernyi* host in some of the tested ratios, i.e., 2:1 (TO:TC) and 5:1 (TO:TC), which resulted notably in high number of

dead TO adults inside host egg, and low number of emergence holes on host egg surface. So, it is notable that these limitations could result from the high population densities of maternal parasitoid during parasitism of *A. pernyi* eggs. A similar conclusion was reached by Hassan et al. (2004) for *T. dendrolimi*, TC, *T. cacoeciae*, and *T. embryophagum* developing on *A. pernyi* host: occurrence of high levels of superparasitism could result in low emergence rate, and high number of dead larvae, pupae and adults inside *A. pernyi* egg. Han et al. (1993) reported that interspecific competition could decrease the survivorship of one or more parasitoid species in multiparasitism. An optimal parasitoid to host ratio is one of the most important prerequisites in mass production of parasitoids (Wei et al. 2016). Moreover, the total number of parasitoids of both species emerging from the same egg of *A. pernyi* did not show any significant differences. *Antheraea pernyi* eggs could enable producing 50 to 260 *T. dendrolimi*, TC or *T. leucaniae* per individual host egg, but mostly about 60–70 *Trichogramma* adults often develop in each *A. pernyi* egg (Wang et al. 1998, 2014; Liu et al. 2000; Huang et al. 2013; Zang 2015; Iqbal et al. 2019).

The pre-emergence time for the offspring of both species did not show significant differences. The offspring of both species emerged at the same time (day) from *A. pernyi* eggs. We considered that TO completed its development inside *A. pernyi* egg faster because of multiparasitism with TC. Previous study by Li et al. (2018) reported that the pre-emergence time for TO was 17 days in monoparasitism whereas it decreased to 12 days in multiparasitism conditions (with *T. dendrolimi*). These changes in the pre-emergence time could be considered as an advantage of multiparasitism technique on *A. pernyi* host which could ensure the availability of two different parasitoid species at a same time in field.

Inundative releasing of parasitoids against insect pests depends on their cost-effective mass rearing (Wei et al. 2016; Khanzada et al. 2019; Morais et al. 2019; Picciau et al. 2019; Suarez et al. 2019; Zang et al. 2021), aside their compatibility with other management methods potentially also used in Integrated Pest Management systems and/or in organic farming (e.g. Kogan 1998; Desneux et al. 2007; Ragsdale et al. 2011; Biondi et al. 2018; Mohammed et al. 2018). From all above discussed results, we could conclude that the parasitism ability of TO on *A. pernyi* host is largely age dependent, i.e., TO females deprived from parasitism activity for 3 days (postemergence) not only could parasitize *A. pernyi* host in large amount but also can emerge successfully in optimum number in multiparasitism with TC. Our present study provided useful information on the biology of TO on *A. pernyi* host under multiparasitism. However, further evaluation of environmental conditions, wasp longevity, fecundity and fitness parameters of *Trichogramma* parasitoids produced through multiparasitism

technique will need to be studied in detail for effective practical developments.

Author contributions

AI, ND and LSZ conceived and designed research. AI, YYH and YMC conducted experiments. AI analyzed data. AI, AA, LSM and LSZ wrote the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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