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Parasitoid age and host quality side effects on the parasitization behavior of *Trichogramma oleae* and *Trichogramma cacoeciae* (Hymenoptera: Trichogrammatidae)

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Abstract
Trichogramma species are known to prefer young host eggs, However, they could found themselves in environments populated in majority with old host eggs, or could become increasingly aged as they could spend days searching fruitlessly for hosts. As anticipation of their behaviours in such circumstances, One, two and three-day old *Trichogramma oleae* and *Trichogramma cacoeciae* females, were examined in order to determine the impact of alternating two different *Ephestia kuehniella* egg qualities (fresh and four-days old), on their fecundity and their offspring's survivorship. Results showed that *T. oleae* females significantly accepted more fresh eggs than older eggs, whatever was the presentation order of these eggs during both tests 1 and 2. However, *T. cacoeciae* significantly parasitized more, the eggs presented during the first half of tests 1 and 2, whatever was their quality. Also, female age effect on parasitization preferences was more visible on *T. oleae* adults, which tended to accept more deteriorated eggs when females' age increased. These results showed that both species behaved differently when put under the same circumstances.

Keywords: aging; biological control; egg parasitoids; presentation order; factitious host

1. Introduction

The tiny egg parasitoids of the genus *Trichogramma* (Hymenoptera, Trichogrammatidae), occur worldwide on a wide range of crops [1, 2] and consist of approximately 180 species [3]. Despite the fact that they are important natural enemies to over 300 species belonging to at least eight insect orders [4], they oviposit predominantly in the eggs of *Lepidoptera* [5, 6], including a large number of important agricultural and forest pests [7-9]. In fact, the use of *Trichogramma* as biological control agents has gained widespread interest throughout the world [10], partly because some species were proven successful in biological control and thus provide an alternative to the use of pesticides [11]. Indeed, they reduce both egg hatching and subsequent damage due to larval feeding [12]. However, soon after release in biological control programs, female parasitoids may not immediately encounter hosts. However, such delays may influence their subsequent patterns of parasitism [13]. In fact, these egg parasitoids are generally reported to better accept young host eggs than older ones, such as *Trichogramma thalense* which parasitize fresh *Ephestia kuehniella* (Lepidoptera: Pyralidae) eggs more readily than eggs that are a few days old [14]. Nonetheless, although host egg preference is species dependent and that some differences exist between various *Trichogramma* species [15], egg parasitoids are generally very selective because of host acceptance and successful development which are both higher in young hosts than in older ones [16-18]. In fact, insect eggs are initially nutriment-rich enlarged cells in which the nutriments are primarily in a storage form needed to initiate embryogenesis [19]. However, with an embryonic development, stored resources become scarce, and the physical structure of the host becomes increasingly complex [20]. Nonetheless, even though female is able to overcome a remarkably complex environment in such cases, through venom injection and host embryonic arrestment [21], older hosts are cuticularised, apparently resistant to penetration of venom, and do not break down easily into dissociated cells [22]. This makes the contents of young host eggs, consisting primarily of yolk, more suitable to ingestion by *Trichogramma* larvae [21]. Thus, as hosts parasitized by idiobionts do not feed or grow, their resources are effectively 'static' and progeny development is therefore dependent on the quality and amount of resources available at the at the time of oviposition [23]. As a consequence, choosing to parasitize a fresh egg over an old one seems to be a sound strategy that most egg parasitoids adopt. However, during mass-release campaigns, *Trichogramma* females could find themselves in an environment in majority populated by old host eggs, or females could become increasingly aged as they could spend days searching fruitlessly for hosts.

In this way and in order to conduct this study, two different egg qualities (fresh and four-day old eggs) were prepared. However, it is important to stress on the fact that due to the rearing complexity of *Trichogramma* species' natural hosts in the laboratory, the factitious host *Ephestia kuehniella*, which is commonly reared in different laboratories worldwide, was used for these essays. Nonetheless, this host is known for its cannibalistic behaviour, meaning that the eggs embryos must be UV killed prior to their use during the test. The presentation of these eggs of different qualities to *Trichogramma* females was alternated afterwards to one, two and three day-old *Trichogramma cacoeciae* Marchal and *Trichogramma oleae* Voegele et Pointel females, in order to understand the impact of female aging as well as of host egg qualities on *Trichogramma* fertility. Results relative to their fecundity and emergence rates are exposed below.

Materials and Methods

Species origins

Both *Trichogramma oleae* and *Trichogramma cacoeciae* species were used in this study. They were collected during the summer of 2004 in a pomegranate orchard located in the south of Tunisia [24].

Experimental protocol

Prior to the test and for at least two generations, *Trichogramma cacoeciae* (Strain Saad) and *T. oleae* were placed in culture tubes (120 mm long ×18 mm in diameter) plugged with cotton. The tubes were kept in a climatic chamber at 25 ± 1 °C; 70 ± 10% RH and L/D: 16/8. To feed the wasps, a drop of honey was placed in the tube prior to or upon their emergence. Emerged parasitoids were provided with fresh *Ephestia kuehniella* eggs (hereafter referred as "egg card"). Egg cards were prepared by sprinkling host eggs on a drop of Arabic gum on a piece of white carton paper (90 × 10 mm). The Mediterranean flour moths *Ephestia kuehniella* were produced on diets based on whole wheat flour. All host eggs were UV-killed, since the larvae are cannibalistic.

Prior to the experiment, two host qualities were prepared: freshly laid and four-day old eggs. The four-day old eggs were obtained by keeping continuously fresh UV-killed *E. kuehniella* eggs in a climatic chamber at 25 ± 1 °C; 50 ± 10% RH and L/D: 16/8 for the desired period (four days). Thus, these four-day old eggs consisted of embryos whose development stopped in the same early stage as fresh eggs. Yet, they were considered of less quality than the freshly laid ones, certainly because of the severe desiccation that egg's yolk and chorion were subject to, as well as to their dehydrated aspect. Also, newly emerged *T. cacoeciae* and *T. oleae* females were placed singly in glass test tubes (50 mm long × 12 mm in diameter) and fed with a droplet of honey to the beginning of the experiment. The glass tubes were divided into 3 groups and placed in the climatic chamber at 25 ± 1 °C; 70 ± 10% RH; L/D: 16/8. There, the three groups of inexperienced females were stored during one, two and three days, respectively, until the beginning of the test. Within each group, females were divided into two batches (n = 20 females/ batch), one for each test (see below).

In Test 1, the females were kept during two hours with an egg card on which approximately 50 freshly laid *Ephestia kuehniella* eggs were sprinkled (first part of test 1). Immediately after, the first egg card was substituted by a new one (containing approximately 50 four-day old *E. kuehniella* eggs) for another period of two hours (second part of test 1). The order of the presentation of the two types of eggs was permuted in Test 2. In both tests females were killed at the end of the experiment.

These experiments were conducted under 25 ± 1 °C; 70 ± 10% RH and L/D: 16/8. The removed egg cards were kept in a climatic chamber at 25 ± 0.5 °C, 50% RH and L/D: 18/6, until their emergence. Then, parasitized host eggs number (evidenced by blackening) as well as of emerging adults were counted. Pre-imaginal survivorship was calculated by dividing the number of adult *Trichogramma* by the number of parasitized eggs.

Data analysis

Log (for fertility) and Arcsine (for preimaginal survivorship) transformed data were analyzed using the analysis of variance, and the differences within each test were evaluated using the Tukey test and the Student's paired T-test ($P \leq 0.05$ level) with IBM SPSS® for Macintosh® Version 20.0.

Results

Relative data to *T. oleae* and *T. cacoeciae* fecundities regarding the alternation of presented egg qualities and of female ages are shown in Table 1. The latter showed that when offered fresh *E. kuehniella* eggs during the first part of test 1, one, two and three day old *T. oleae* females significantly parasitized the same amount of eggs (14,65 ± 11,07; 7,15 ± 9,49 and 11,5 ± 11,91; respectively). However, during the second half of test 1 once in contact with four-day old eggs, the three females groups behaved differently. In fact, as far as their age increases, *T. oleae* females tend to significantly parasitize more eggs of poor quality (1,35 ± 2,18; 3,45 ± 5,56 and 5,57 ± 5,92 for one, two and three-day old females, respectively). During test 2, although results show that, when in contact with four-day old eggs during its first two hours, the three *T. oleae* female groups displayed no significant differences concerning the amount of parasitized eggs, it appeared from the detailed analysis that the number of attacked eggs increases as females age increases (0,2 ± 0,7; 2,15 ± 5,55; 4,4 ± 8,47; for one, two and three-day old females, respectively). However, during the second half of test 2, three-day old *T. oleae* females significantly parasitized the lowest number of fresh eggs, in comparison to one and two-day old females (4,3 ± 7,08; 12,2 ± 11,51 and 11,3 ± 9,54; respectively). Concerning age response to egg quality alternation, *T. oleae* females groups were generally found to significantly better accept fresh eggs than four-day old eggs, whatever the order of their presentation during both tests, except for two- and three-old females during tests 1 and 2, respectively, where the amounts of parasitized fresh and aged eggs were significantly equal (table1).

Table 1: Impact of host quality alternation and female parasitoids age on *T. oleae* and *T. cacoeciae* fecundity at 25 ± 1 °C; 70 ± 10% RH; L/D: 16/8).

		Fertility *			Fertility *		
		Test 1			Test 2		
Species	Female age	1st 2h with fresh eggs	2nd 2h with 4 day old eggs		1st 2h with 4 day old eggs	2nd 2h with fresh eggs	
T. oleae	1 day old	14,65 ± 11,07 Aa	1,35 ± 2,18 Ab	P = 0,0001	0,2 ± 0,7 Ab	12,2 ± 11,51 Ba	P = 0,0001
	2 days old	7,15 ± 9,49 Aa	3,45 ± 5,56 ABa	P = 0,158	2,15 ± 5,55 Ab	11,3 ± 9,54 Ba	P = 0,0007
	3 days old	13,15 ± 12,45 Aa	5 ± 5,44 Bb	P = 0,018	4,4 ± 8,47 Aa	4,3 ± 7,08 Aa	P = 0,967
		F = 2,586; df = 2; P = 0,084	F = 2,594; df = 2; P = 0,084		F = 2,574; df = 2; P = 0,085	F = 4,101; df = 2; P = 0,022	
T. cacoeciae	1 day old	6,85 ± 7,13 Aa	4,35 ± 5,31 Aa	P = 0,341	12 ± 5,27 Aa	5,95 ± 6,29 Ab	P = 0,018
	2 days old	12,85 ± 6,75 Ba	1,6 ± 3,82 Ab	P = 0,0001	10,65 ± 6,22 Aa	7,1 ± 7,66 Aa	P = 0,246
	3 days old	10,5 ± 6,09 ABa	2,95 ± 4,58 Ab	P = 0,003	12,9 ± 6,97 Aa	3,25 ± 5,26 Ab	P = 0,001
		F = 4,112; df = 2; P = 0,021	F = 1,778; df = 2; P = 0,178		F = 0,669; df = 2; P = 0,516	F = 1,861; df = 2; P = 0,165	

Values followed by the same letter are not statistically different using mean comparisons (Tukey test within columns and paired Student's T-test within lines; $P<0.05$), Capital letters following the values represent comparisons within a column and lower case letters represent comparisons within a line. * Log transformed data were used for the means values.

Concerning *T. cacoeciae*, Table 1 shows that during the first part of test 1 when provided with fresh *E. kuehniella* eggs, two-day old females significantly parasitized the highest number (12,85 ± 6,75), followed by three-day old females (10,5 ± 6,09). However, one-day old *T. cacoeciae* females significantly parasitized the lowest number (6,85 ± 7,13) of eggs. During the second part of test 1, no significant difference was recorded between the three groups of females. In the same context, no significant differences were registered between the three group of ages when provided with either four-day old eggs or fresh eggs, during test 2. However, for the three *T. cacoeciae* female groups, we noticed the general tendency to significantly parasitize the host eggs presented during the first halves of tests 1 or 2, whatever their quality was. Nonetheless, although Table 1 shows that one- and two-day old females displayed no significant differences concerning the quality of accepted eggs during tests 1 and 2, respectively; the detailed analysis showed that the number of accepted eggs was always higher during the first part of both tests (6,85 ± 7,13 versus 4,35 ± 5,31 and 10,65 ± 6,22 versus 7,1 ± 7,66 for one-day old females during test 1 and two-day old females during test 2, respectively) (Table 1).

Obtained data on *T. oleae's* and *T. cacoeciae's* emergence rate regarding the alternation of the presented egg qualities and of female ages are summarized in Table 2. The latter showed that one, two and three-day old *T. oleae* recorded significantly similar pre-imaginal rates during all parts of tests 1 and 2. The same observation was reported for all *T. cacoeciae* age groups during both tests, except during the second half of test 2 when provided with fresh eggs. In fact, we noticed that, as far as their age increases, *T. cacoeciae* female emergence rate increased. Concerning age effect on parasitism, we noticed that one, two and three-day old *T. oleae* and *T. cacoeciae* females recorded the same emergence rate whatever eggs quality alternation was, during tests 1 and 2 (Table 2).

Table 2: Impact of host quality alternation and female parasitoids age on *T. oleae* and *T. cacoeciae* preimaginal survivorship (%) at 25 ± 1 °C; 70 ± 10% RH; L/D: 16/8).

		Pre-imaginal survivorship*			Pre-imaginal survivorship*		
		Test 1			Test 2		
Species	Female age	1st 2h with fresh eggs	2nd 2h with 4 day old eggs		1st 2h with 4 day old eggs	2nd 2h with fresh eggs	
T. oleae	1 day old	95,11 ± 4,7 A	87,08 ± 19,55 A	*	100 ± 0 A	94,28 ± 5,54 A	*
	2 days old	91,55 ± 7,9 A	81,64 ± 19,54 A	*	91,91 ± 7,02 A	94,04 ± 7,2 A	*
	3 days old	96,2 ± 5,72 A	96,77 ± 6,97 A	*	96,48 ± 3,65 A	98,14 ± 3,2 A	*
		F = 1,491; df = 2; P = 0,242	F = 2,077; df = 2; P = 0,146		F = 1,913; df = 2; P = 0,217	F = 1,252; df = 2; P = 0,299	
T. cacoeciae	1 day old	97,31 ± 5,51 A	93,85 ± 14,37 A	*	96,33 ± 4,41 A	88,26 ± 16,2 A	*
	2 days old	93,86 ± 7,46 A	100 ± 0 A	*	97,64 ± 4,41 A	93,02 ± 10,51 AB	*
	3 days old	94,82 ± 5,8 A	94,83 ± 10,61 A	*	96,57 ± 4,42 A	100 ± 0 B	*
		F = 1,057; df = 2; P = 0,356	F = 0,601; df = 2; P = 0,556		F = 0,420; df = 2; P = 0,659	F = 2,925; df = 2; P = 0,066	

Values followed by the same letter are not statistically different using mean comparisons (Tukey test; $P<0.05$) angular transformed data, Capital letters following the values represent comparisons within a column. *Arcsine transformed data were used for the means proportion.

Discussion

According to Liu *et al.* [25], even closely related species may exhibit significant differences in many activities such as oviposition. In fact, we found in this study that while the general trend of all *T. oleae* female groups was to better accept fresh eggs than low ranked ones, irrespectively of their

alternation order during tests 1 and 2, all *T. cacoeciae* female groups had the tendency to further accept all the eggs presented during the first halves of both tests, whatever their quality was, more than the eggs provided during the second halves of tests 1 and 2. Thus, following these results we assume that *Trichogramma cacoeciae* is more flexible concerning the quality of the first encountered eggs. However, *T. oleae* seemed to be more selective and laid fewer eggs until a better quality was encountered. Nonetheless, in a previous study [26], *T. oleae* was found to be more attracted by three and four-day old eggs while *T. cacoeciae* preferred more, fresh eggs. In the same context, Pizzol *et al*. [27] reported that 1-2-day-old *Lobesia botrana* (Lepidoptera: Tortricidae) eggs were more parasitized by *T. cacoeciae* than 3-4-day-old eggs.
Vinson [19] mentioned that the female's innate behaviour determines the type of cues to which it responds as well as the behaviours elicited by these cues. Thus, when a *Trichogramma* female finds preferred host eggs, it will usually stay on or near them for a long period of time until all or most of them are parasitized [8]. On the contrary, less-preferable host eggs may be totally rejected or the parasite might lay few eggs before leaving the location to search for more suitable hosts [8]. However, it appears that as a consequence of some external factors, such as captivity, a new behaviour could be acquired. In fact, hosts initially rejected will sometimes be accepted later, especially when the wasps are confined in an arena for a period of time, e.g., 24 hours [17]. In parallel, Keasar *et al*. [14] assumed that *Trichogramma* requires 45 min on average for ten ovipositions after accepting the first low ranked egg. These two statements better explain *T. oleae* females' low parasitization rates when shortly exposed (during 2 h) to four-day old eggs during tests 1 and 2 but their excessive acceptance of this egg quality up to 24 hours of exposition as reported by Ksentini *et al*. [26]. However, we noticed in this study that *T. cacoeciae* seems to adapt quickly (less than 2 h) to the provided eggs regardless of their qualities. In this context, Hegazi *et al*. [28] confirms that due to its natural urge to oviposit as early as possible, *T. cacoeciae* is able to immediately attack host eggs after its emergence. Nonetheless, although the amount of parasitized eggs during the first phases of tests 1 and 2 was generally found statistically different from those obtained during the second phases of both tests, it is important to keep in mind that *Trichogramma* females behaviour and as a consequence their parasitization rates during phase 2 might be due to phase 1 influence. In fact, according to Vinson [19], a female's previous experience remains particularly important. Indeed, thresholds for acceptance may be set by previous experience and encounters with hosts [4]. Seemingly, according to Reznik *et al*. [18], the percentage of females that oviposited during the first period of the experiment depended only on the age of the first portion of host eggs, regardless of the age of the second portion of eggs. However, the percentage of females that oviposited during the second period of the experiment depended significantly both on the age of the first and the second portions of host eggs [18]. Nonetheless, it is important to mention that experiences only increase the acceptance of a low ranked host and not a high ranked host [29]. In this way, Reznik *et al*. [18] mentioned that *Trichogramma* females much more readily accept old eggs when previously allowed to oviposit in young eggs of the same host species. This was particularly detectable with *T. oleae*, as the amount of parasitized old eggs during the second phase of test 1 was higher than the amount of four-day old eggs parasitized during the first phase of test 2. However, such behaviour remains unclear in the case of *T. cacoeciae* during the current study.
Nonetheless, this discrepancy between both *Trichogramma* species was also noted concerning the response of females as they age to the different provided eggs. In fact, as far as their age increased, *T. oleae* females tended to parasitize more eggs of poor quality during tests 1 and 2, but laid less on fresh eggs during the second half of test 2. On the contrary, age effect was generally not detectable on *T. cacoeciae* females, except during the first phase of test 1 (on fresh eggs) when one-day old *T. cacoeciae* females parasitized less eggs than two and three-day old ones. Despite these differences, the general trend of both tested species was to maintain or increase their parasitization capacities as they aged. In this context, Ayvaz *et al*. [30] confirmed that oviposition frequency of older females (56-102 h of age) is higher than that of younger females (12-35 h of age). The same tendency was observed by Vourlioti and Milonas [31] and also by Pizzol *et al*. [27] concerning 2, 3, and 4-day-old *T. cacoeciae* females that parasitized more *L. botrana* eggs than 1-day-old females. In fact, according to Ventura Garcia *et al*. [32], females that are becoming time limited through aging have different parasitization strategies by maximizing their progeny production and ovipositing a higher number of eggs as soon as suitable hosts are discovered. Indeed, wasps approaching the end of their lives probably optimized their fitness, when equalizing between the risks of being egg depleted (due to egg resorption), but with a higher longevity, or dying with a large number of oocytes (due to the high probability of short life expectancy) [30]. This suggests that, as and when they age, *Trichogramma oleae* females become less selective and tend to accept more deteriorated eggs. This might be extremely important during mass-rearing campaigns, as egg parasitoids may not immediately find suitable hosts in the field. Thus, knowing the most effective upper age limit of parasitoids is a crucial element in deciding which parasitoids age to release in the field and in storage conditions for the optimum parasitization level [29]. However, it is important to mention that *Trichogramma cordubensis* adults maintain and even increase their capacities to parasitize the same amount of eggs up to 120 h of age, and that beyond it, their fecundity decreased [30]. This might be also the case of *Trichogramma cacoeciae* and *T. oleae* during this study if they were allowed to live up to 6 days.
Yet, whatever the females' age and the provided egg qualities during this study were, we noted, generally speaking, that no significant differences concerning pre-imaginal survivorship were found for *T. oleae* and *T. cacoeciae*. These results were confirmed by Ayvaz *et al*. [29] who noticed that the adult emergence percentage is not significantly affected by parasitoid age. However, Pizzol *et al.* [27] found that parasitoid emergence increased according to the age of *T. cacoeciae* females with the highest rates observed for 3-day-old females and the lowest ones obtained with 1-day-old females.

Conclusion

In conclusion, this study showed that *Trichogramma* behaviour towards different eggs qualities offered in alternation, as well as female age influence on parasitism is species-dependent, as a significant variability in the response of both *Trichogramma* species was observed. Nonetheless, we must be careful about extrapolating the above experimental results to field situations, since these isolated females were offered alternating homogeneous patches of unnatural hosts under laboratory conditions, and were kept in containers and had no choice.

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