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Effects of oviposition in a non-host species on foraging behaviour of the parasitoid *Cotesia glomerata*

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Abstract

1. Parasitoids lay their eggs in or on a host, usually another insect. During foraging, parasitoids can encounter insects that differ in terms of host suitability and quality. At one extreme end of this spectrum are non-hosts that are unsuitable for offspring development.

2. Non-hosts are generally ignored but parasitization does occur and occasionally also results in egg deposition. Here, the authors investigate how oviposition in a non-host influences subsequent foraging behaviour of a parasitoid and whether this is mediated by learning. The study system consists of the endoparasitoid *Cotesia glomerata* and the presumed non-host caterpillar *Mamestra brassicae*.

3. In the presence of *Pieris brassicae* hosts and/or their traces (frass), *C. glomerata* inserted its ovipositor into *M. brassicae* caterpillars. Eggs were deposited, but all eggs disappeared within 96 h, confirming the non-host status of *M. brassicae*. In contrast to the expectation, there was no memory retention after oviposition in a non-host and parasitoids did not alter their behaviour with respect to non-host contacts and ovipositions. Instead, *C. glomerata* became more motivated to forage on a non-host infested leaf.

4. The authors propose that egg deposition in non-hosts by *C. glomerata* might be due to their high egg load, which is thought to make parasitoids less selective on host quality, especially when they have few reproductive opportunities. In such cases, fitness costs to individual females are low. Egg deposition in non-hosts might ultimately lead to host range expansion if parasitoids overcome the defence response of non-hosts over evolutionary time.

KEYWORDS
cabbage, foraging behaviour, herbivore-induced plant volatiles, host specialisation, parasitoid–host interactions

INTRODUCTION

Parasitoid wasps (Hymenoptera) lay one or more eggs in or on the body of another organism, mostly insects. Successful development of parasitoid offspring only occurs in suitable hosts, ultimately leading to the death of the host (Godfray, 1994). The diversity and number of species used by a certain parasitoid describes its host range (Heimpel et al., 2021), which comprises host species along a spectrum of suitability or quality in terms of offspring development and fitness (Heimpel et al., 2003; Monticelli et al., 2019). Individuals of the same host species can also vary in suitability for a parasitoid, for example, due to host age, host size or when a host individual has already been

parasitized (Heimpel et al., 1996; Islam & Copland, 2000; van Lenteren & Bakker, 1975). Although parasitoid females are expected to prefer hosts that are most suitable for development, oviposition in less-suitable or low-quality hosts is often observed (Hopper et al., 2013; Monticelli et al., 2019). In this light, oviposition in organisms that are not suitable for offspring development (i.e. non-hosts) may be seen as the most extreme case (Arthur et al., 1972; Chabaane et al., 2015; de Bruijn, Vet, & Smid, 2018; Takasu & Lewis, 2003). There are clear costs of oviposition in non-hosts because eggs and time are spent without reproductive gain (Abram et al., 2019). To date, it remains unclear if and how oviposition in non-hosts may benefit parasitoids.

Parasitoids have evolved behavioural mechanisms to locate and evaluate their host(s) and these mechanisms may also allow parasitoids to discriminate host and non-host organisms (Godfray, 1994). Discrimination may occur at different stages of the host location and acceptance process. At a distance, specialist parasitoids may discriminate host- from non-host infested plants using host-specific herbivore-induced plant volatiles (HIPVs), while generalist parasitoids may also be attracted to HIPVs induced by non-host insects (van Oudenhove et al., 2017). Closer by, information from frass, silk, honeydew or saliva is involved in host recognition, and contact chemical cues may allow parasitoids to discriminate between host and non-host organisms, as has been found for Cotesia sesamiae and C. flavipes (Obonyo et al., 2010). After inserting the ovipositor, female parasitoids decide whether or not to deposit eggs, how many eggs and which sex ratio to lay, based on the presence of certain cues and the absence of deterrents (Hays & Vinson, 1971; McDonald et al., 2015; Vinson, 1975, 1998). Indeed, females of Cotesia kariyai lay fewer eggs in the non-host Spodoptera littoralis, suggesting that they perceive their lower quality using internal cues (Aikawa et al., 2020).

Despite these mechanisms, recognition of non-hosts or discrimination between hosts and non-hosts does not always occur in parasitoids. Parasitoids may not have evolved the ability to recognise non-hosts, as appears to be the case in native parasitoids laying eggs in exotic pest insects that are unsuitable for offspring development (Abram et al., 2014; Kruitwagen et al., 2021). However, oviposition in non-hosts also occurs in the native habitat of parasitoids, in the presence of hosts (Bukovinszky et al., 2012; Chabaane et al., 2015; de Bruijn, Vet, & Smid, 2018; Meisner et al., 2007). Indeed, host- and non-host species frequently feed on the same plant individual or even on the same leaf at the same time (Dicke et al., 2009; Vos et al., 2001). Information related to hosts and non-hosts is thus often mixed, such as frass or silk, or the composition of HIPVs may be altered (de Rijk et al., 2013). This complicates parasitoid foraging and particularly discrimination between hosts and non-hosts. Under these conditions, oviposition in non-hosts may simply be considered mistakes but they may also present an opportunity to parasitoids to adjust their subsequent foraging behaviour through learning.

Parasitoids are well known for their ability to adjust their behaviour based on prior experience and different types of learning may occur (Haverkamp & Smid, 2020). Of these, associative learning is probably the most studied type. Females of many species of parasitoids can indeed learn to associate environmental cues, such as HIPVs

emitted by the food plant of the host with a reward, an oviposition in the host (Fukushima et al., 2001; Little et al., 2019; Smid & Vet, 2016). After this learning experience, the association between reward and plant odours is stored as memory that alters foraging behaviour and can improve foraging efficiency. Interestingly, in studies of parasitoid associative learning, researchers commonly use preferred or high-quality hosts, while much less is known about the effects of oviposition experience with hosts of lower quality or non-hosts on memory formation and subsequent foraging behaviour. Host quality may determine the value of the reward in oviposition learning, as it appears to affect memory consolidation in Cotesia glomerata and the egg parasitoid Trichogramma evanescens (Kruidhof et al., 2012), but not in the species Nasonia vitripennis and Nasonia giraulti (Hoedjes et al., 2014). Similarly, oviposition in non-hosts may either result in decreased interest in the non-host or associated volatiles, as is the case in C. kariyai (Aikawa et al., 2020; Steven et al., 2019), or to increased searching intensity, as found in the egg parasitoid Telonomus podisi (Bertoldi et al., 2021).

Here, we address the question how oviposition in non-hosts influences subsequent foraging behaviour of a parasitoid and whether this is mediated by learning. We use the parasitoid C. glomerata L. (Hymenoptera: Braconidae) that uses Pieris brassicae L. and Pieris rapae L. (Lepidoptera: Pieridae) as its main hosts (Le Masurier & Waage, 1993; Vos et al., 2001). C. glomerata's foraging and learning behaviour and memory dynamics have been studied extensively. Females of this parasitoid species can associate an oviposition in a host with nearby HIPVs. Long-term memory is formed after a single learning experience with the preferred, high-quality, host P. brassicae, while a less persistent memory type is formed after oviposition in the lower quality host Pieris rapae (Kruidhof et al., 2012). Reward value is thus known to affect memory formation but it is not known how non-hosts influence learning and memory. C. glomerata has been observed to attack and deposit eggs in the presumed non-host Mamestra brassicae L. (Lepidoptera: Noctuidae), especially (but not only) in the presence of Pieris or their traces (e.g. frass) (Bukovinszky et al., 2012; de Bruijn, Vet, et al., 2021; de Bruijn, Vet, & Smid, 2018; de Bruijn, Vosteen, et al., 2021). These caterpillar species feed on the same brassicacious plant species and individuals but it is not known at which frequencies they co-occur (Vos et al., 2001). Interestingly, C. glomerata is also strongly attracted to HIPVs of plants infested with M. brassicae (de Rijk et al., 2013; Geervliet et al., 1996; Vosteen et al., 2019). In the present study, we test the following predictions: (1) C. glomerata deposits eggs in M. brassicae but these eggs do not survive; (2) C. glomerata forms associative memory after an oviposition in M. brassicae in the presence of host traces; and (3) this memory leads to reduced contact with and oviposition in M. brassicae caterpillars.

MATERIALS AND METHODS

Insects

For all experiments, first instar P. brassicae host caterpillars, first instar M. brassicae non-host caterpillars and C. glomerata females of 3–5 days old were used. Brussels sprouts plants (Brassica oleracea

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L. var. gemmifera cultivar Cyrus) were used to rear both caterpillar species and for all experiments. All insects were reared at the Laboratory of Entomology, Wageningen University, The Netherlands, in a greenhouse with a L16:D8 photoperiod (both natural and artificial light), at 21 ± 1°C and 50%–70% relative humidity. Each year, insect colonies were re-established with individuals collected from cabbage fields near Wageningen.

C. glomerata females were obtained by transferring cocoons from the laboratory colony to a mesh cage (30 × 30 × 30 cm, Bugdorm-1 Insect rearing cage, type DP1000, Megaview Science, Taiwan) with water and honey, which was placed in a climate cabinet (21 ± 1°C, photoperiod L16:D8, 50–70% relative humidity). Two days after emergence, females were selected from these cages and transferred to a new cage with water and honey, until use in experiments.

Egg deposition and survival

To test the first prediction, we determined whether *C. glomerata* deposits eggs in the presumed non-host *M. brassicae* (further referred to as Mamestra) and whether these eggs could survive, by dissecting parasitized Mamestra caterpillars. To stimulate parasitoids to insert their ovipositor into Mamestra (further referred to as 'sting'), we mimicked a situation of a non-host encounter near a host by using leaves with fresh host traces, that is, frass, silk and feeding damage of *P. brassicae* (Pieris), which are known to provoke stinging in *C. glomerata*.

A 5-week-old *B. oleracea* plant was infested with approximately 200 first instar Pieris caterpillars, which were distributed over four leaves in groups of approximately 50 caterpillars. After 24 h, these infested leaves were detached from the plant and Pieris caterpillars were removed from the leaves. A first instar Mamestra caterpillar was placed on a leaf with fresh Pieris traces using a fine paint brush. A single female parasitoid was released on the leaf. When we observed a sting that lasted for at least 2 s (to allow for potential egg deposition), the caterpillar was replaced with a new one until a single female had stung four caterpillars. Stings that lasted less than 2 s only happened very rarely and were not considered to be successful. These caterpillars were discarded. A total of 280 stung caterpillars were collected in 1 day using this process. Thirty caterpillars were dissected immediately after stinging (0 h, see below), the remaining 250 were randomly distributed over 10 plastic boxes (13 × 17 × 6.5 cm), each housing 25 parasitized caterpillars. Each group was provided with four fully expanded leaves of 8-week-old *B. oleracea* plants. Holes were punctured in the plastic lid for ventilation and a layer of absorbent paper was placed under the lid to absorb moisture that condensed on the lid. The plastic boxes were placed in a climate cabinet (21 ± 1°C, photoperiod L16:D8, 50%–70% relative humidity). After 0, 24, 48, 72 and 96 h, 30 caterpillars were randomly selected from the boxes and dissected in a drop of water to count parasitoid eggs. Dissections were done with a pair of fine tweezers and an insect pin in a glass Petri dish, using a stereomicroscope with backlight illumination. We quantified healthy eggs, that is, eggs that did not show signs of encapsulation. Eggs that were (partially) encapsulated were sometimes observed but assumed to not survive and they were not counted.

Memory retention after oviposition in a host or non-host

In the second experiment, we assessed whether an oviposition in the Pieris host caterpillar or Mamestra non-host caterpillar led to the formation and retention of associative memory 24 h after the experience, by testing if the parasitoid prefers or avoids the volatiles offered during oviposition. We use the term oviposition for stings (of at least 2 s) that presumably led to egg deposition, but without confirming this (except for a subset of caterpillars, see below).

Parasitoid females were given an associative learning experience with a classical conditioning procedure, where they could learn to associate a single oviposition with either a 4% vanilla or 4% coffee odour extract (Nielsen-Massay Vanillas Intl., Leeuwarden, the Netherlands) using a protocol developed by de Bruijn, Vet, Jongsma, et al. (2018). These extracts were presented as a globular sphere of odorized agarose (A9539-500 g, Sigma) on a micro pestle (SIAL501ZZ0, Sigma-Aldrich), on which Pieris frass and silk were applied. A single parasitoid was collected in a glass vial and brought into contact with Pieris traces to trigger oviposition behaviour, upon which either a Pieris or Mamestra caterpillar was offered on the tip of a fine paint brush to let the parasitoid oviposit. After an oviposition, parasitoids were placed in a small cage (17.5 × 17.5 × 17.5 cm, Bugdorm-41515 Insect Cage, type BD41515, Megaview Science, Taiwan) with water and honey. These cages were placed in a climate cabinet (21 ± 1°C, photoperiod L16:D8, 50%–70% relative humidity) until testing. Four treatments were used; parasitoids oviposited in either Mamestra or Pieris, on either vanilla or coffee scented agarose. Per treatment, 90 parasitoids were conditioned. This procedure, with a single oviposition in Pieris, is known to result in the formation of associative memory, resulting in a clear preference for the conditioned odour that is maintained for at least 24 h (de Bruijn, Vet, Jongsma, et al., 2018). We expected that an oviposition in the non-host caterpillar Mamestra would be an adverse experience, leading to avoidance of the conditioned odour.

Testing for memory retention was done in the high-throughput individual T-maze, with video recording and tracking software (de Bruijn, Vet, Jongsma, et al., 2018). The system consists of 36 identical two-choice arenas, each with 1% vanilla-scented agarose on one side and 1% coffee-scented agarose on the other side. Per run, 9 parasitoids that had oviposited in Mamestra and 9 parasitoids that oviposited in Pieris were individually released in 1 of the 36 two-choice arenas (18 arenas were not used), 24 h after conditioning, and their behaviour was recorded for 10 min. From this recording, we extracted first choice, that is, whether a parasitoid first walked into the vanilla- or the coffee-scented agarose zone. In two subsequent runs on the same day, a pair of reciprocally conditioned parasitoids (one vanilla conditioned parasitoid and one coffee conditioned parasitoid) were tested at the same position in the high-throughput system (i.e. in the same of the 36 two-choice arenas). Since the ability to retain this information could in theory depend on the number of deposited eggs, we dissected a subset of *P. brassicae* and *M. brassicae*. Twenty caterpillars of each species, 10 per conditioning odour, were
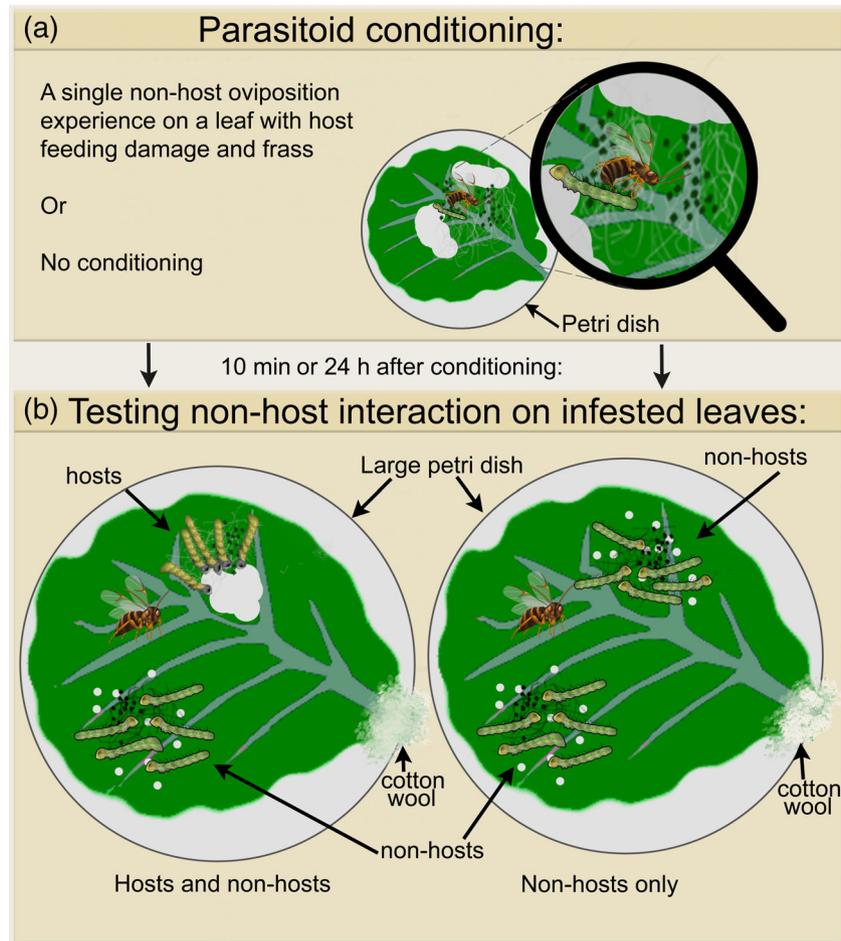
FIGURE 1 Overview of the experimental procedure to test how *Cotesia glomerata* parasitoids interacted with *Pieris brassicae* host and *Mamestra brassicae* non-host caterpillars after oviposition experience in a first instar *M. brassicae* caterpillar. Parasitoids were conditioned in a Petri dish where they were either allowed to oviposit once in a *M. brassicae* non-host on a leaf with feeding damage and frass of the host *P. brassicae*, that is, given a Mamestra experience, or they were kept unconditioned (a). Parasitoid contact with and oviposition in caterpillars was then tested on a detached *Brassica oleracea* leaf as depicted in (b). These leaves were infested 24 h prior with either 5 host caterpillars and 5 non-hosts caterpillars or with only 10 non-hosts. See the text for a more detailed description

dissected within 24 h after the oviposition, to determine the number of parasitoid eggs.

On patch foraging behaviour following oviposition in a non-host

Third, we tested how *C. glomerata* foraged on a leaf with host and non-host caterpillars after oviposition in a non-host caterpillar (Figure 1). Parasitoids were kept unconditioned or given one Mamestra oviposition experience, hereafter called a Mamestra experience. A Mamestra experience consisted of a single oviposition in Mamestra as described above for the first experiment. We expected that a Mamestra experience would influence the number of contacts with and ovipositions in Mamestra non-hosts in the subsequent behavioural assay. Parasitoids were assayed either 10 min after the Mamestra experience, or on the following day. Parasitoids tested 24 h after conditioning were stored overnight in a small cage with water

and honey, as described above. Time after conditioning does not apply to unconditioned wasps. Parasitoids of this group were tested at the same time as those with a Mamestra experience.

Parasitoid behaviour was observed in a glass Petri dish (20 cm diameter, 5 cm high) with a *B. oleracea* leaf that was infested 24 h before, with either 5 Mamestra and 5 Pieris caterpillars or with 10 Mamestra caterpillars to assess the effect of host presence and absence. We infested leaves by placing groups of 5 caterpillars in a clip cage, of which two were attached to a fully expanded leaf of a 5-week-old *B. oleracea* plant. Just prior to the assays, the infested leaf was detached from the plant, its petiole was wrapped in moist cotton wool, and it was placed upside-down in the glass Petri dish. We made sure that 10 caterpillars were present on the leaf, and we replaced missing caterpillars if needed.

A single parasitoid was then released inside the Petri dish, at the centre of the lid. A hand-held computer (Workabout Pro 3, Zebra Technologies Corp., Lincolnshire, IL, USA) with behaviour recording software (The Observer XT 10, Noldus Information Technology B. V.,

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Wageningen, The Netherlands) was used to record parasitoid foraging behaviour during an observation period of 10 min. We assessed whether a parasitoid responded, that is, whether it started foraging by contacting the infested leaf, and whether it contacted and subsequently oviposited in the offered host and non-host caterpillars. After 10 min, the parasitoid was removed, and caterpillars were replaced if any ovipositions had occurred. A single leaf was used to test the three corresponding parasitoid treatments (in randomised order) in either the presence or absence of Pieris hosts, and then discarded. The experiment was replicated 21–24 times with parasitoids given Mamestra experience (10 min or 24 h before) and 19–23 times with unconditioned parasitoids.

Statistical analyses

All analyses were done in R version 3.5.0 (R Core Team, 2018). To test our prediction that *M. brassicae* is a non-host for *C. glomerata*, parasitoid egg survival over time, that is, the number of eggs at different time points, was tested with a zero-inflated generalised linear model (GLM) with a negative binomial distribution (log link-function) and time as a continuous fixed factor, using R package pscl (Jackman, 2020). Likelihood ratio tests were used for model comparisons in this GLM and those described below.

We next tested the prediction that *C. glomerata* retains associative memory after an oviposition in *M. brassicae*. Performance Index (PI) scores of binomial first choice data were used as indices of memory retention as described in de Bruijn, Vet, Jongsma, et al. (2018). PI scores were assigned by combining the first choice of a vanilla and a coffee conditioned parasitoid that were tested in the same two-choice arena, in two subsequent runs. If both parasitoids chose the odour on which they were conditioned, a PI of 100 was assigned, one correct choice resulted in a PI of 0, and if neither parasitoid made a choice for their conditioned odour, a PI of −100 was assigned. If one or both parasitoids did not make a choice within 10 min, no PI was formed for this particular pair and the data of the two-choice arena was discarded. One-sample Wilcoxon signed rank tests were used to test if the PI scores of parasitoids that oviposited in Mamestra, and those that oviposited in Pieris, significantly deviated from 0 ($\mu = 0$), to evaluate the null hypothesis of no memory retention. We used a 2-sided binomial test to evaluate whether choice (preference) of *C. glomerata* between the conditioned and control odour deviated from a 50:50 distribution. The number of eggs deposited in Mamestra and Pieris in combination with the two odours (vanilla and coffee) was compared with a GLM (assuming a Poisson distribution and using a log link-function) using host and odour as fixed factors.

In the third experiment, we tested the prediction that an oviposition experience in *M. brassicae* (Mamestra experience) influences the likelihood of *C. glomerata* contacting caterpillars and ovipositing, either in the presence or absence of the host caterpillar *P. brassicae*. We first assessed the effect of time after Mamestra experience, using only data of parasitoids with Mamestra experience (excluding unconditioned parasitoids). Time after oviposition (10 min vs. 24 h) and host

presence were used as fixed factors in full factorial generalised linear models (binomial distribution, logit link-function), for the three following response variables separately: (1) parasitoid response (number of parasitoids that started foraging out of total number of parasitoids), (2) caterpillar contact (number of parasitoids that contacted a caterpillar out of those that started foraging) and (3) oviposition (number of parasitoids that oviposited in a caterpillar out of those that contacted a caterpillar). In these models, contact with and oviposition in caterpillars irrespective of species was considered. Because there was no effect of time after Mamestra experience on any of the response variables (see Results section and Table S1), we then pooled data of all experienced parasitoids to compare them with unconditioned parasitoids. In this set of full factorial GLMs, host presence and experience (Mamestra experience vs. unconditioned) were used as fixed factors to evaluate their effects on the same three foraging parameters, that is, caterpillar species was not considered in the contact and oviposition parameters. In a fourth full factorial GLM, we evaluated the effect of host presence and experience on oviposition in Mamestra caterpillars (number of parasitoids that oviposited in Mamestra out of those that contacted a Mamestra caterpillar). In GLMs with a significant interaction effect, a least-square means post hoc comparison with a Bonferroni error correction was used to test for pairwise differences between groups (Lenth, 2016). Finally, we used the subset of parasitoids that foraged in the presence of Pieris to study the effect of Mamestra experience on caterpillar-specific interactions. In this situation, parasitoids could contact only one of the two caterpillar species or both species. We used experience as a fixed factor and used the following binary response variables: (1) contact with Mamestra (the number of parasitoids that contacted only Mamestra or Mamestra and Pieris out of those parasitoids that contacted at least one caterpillar), (2) oviposition in Mamestra (the number of parasitoids that oviposited only in Mamestra or in Mamestra and Pieris out of those ovipositing at least once).

RESULTS

Egg deposition and survival in *M. brassicae*

After *C. glomerata* stung *M. brassicae*, 90% of the caterpillars contained parasitoid eggs (0 h after oviposition). The number of eggs found in Mamestra caterpillars decreased rapidly during the first 72 h and after 96 h there were no unencapsulated egg left (Figure 2). Emerged parasitoid larvae were never observed. This shows that *M. brassicae* is indeed an unsuitable species for offspring development of *C. glomerata*, thus a non-host.

Memory retention after oviposition in a host or non-host

An oviposition in a Pieris host resulted in significant memory retention (one-sample Wilcoxon signed rank test, $V = 360$, $p < 0.001$), as

demonstrated by a clear preference of *C. glomerata* for the conditioned odour (binomial test, $p < 0.001$, Table 1). In contrast to our expectation, oviposition in the non-host *Mamestra* did not result in memory retention after 24 h (one-sample Wilcoxon signed rank test, $V = 388.5$, $p = 0.322$) and parasitoids did not discriminate between the two odours (binomial test, $p = 0.20$). In the subset of dissected caterpillars, almost all contained parasitoid eggs (Pieris: 95%; Mamestra 90%; $n = 20$ for each species). In caterpillars with eggs, the number of eggs was not influenced by species or the odour presented during oviposition (GLM: interaction $X^2 = 0.048$, $p = 0.83$; odour $X^2 = 3.085$, $p = 0.08$; species $X^2 = 1.865$, $p = 0.17$; mean $\pm$ SE eggs per caterpillar: Pieris 22.5 $\pm$ 1.8; Mamestra 20.4 $\pm$ 1.3).

On patch foraging behaviour following oviposition in a non-host

In this experiment, we tested whether parasitoids changed their foraging behaviour after having oviposited once in a non-host *Mamestra* caterpillar. Time after experience (10 min or 24 h) did not significantly
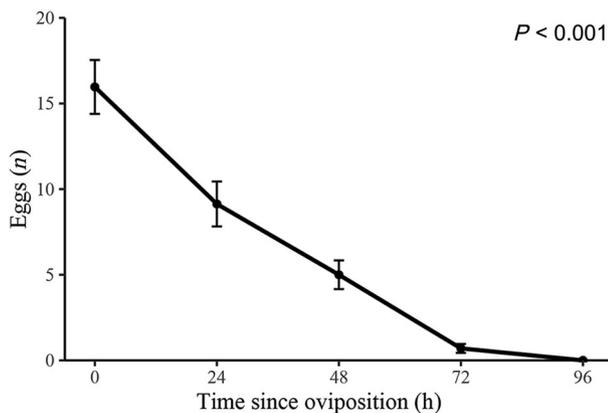

FIGURE 2 Number of healthy (i.e. unencapsulated) eggs of the parasitoid *Cotesia glomerata* in first instar *Mamestra brassicae* caterpillars at different time points after oviposition. The *p*-value is based on a negative binomial zero-inflated generalised linear model and indicates a significant difference in egg numbers over time. Per time point, 30 caterpillars were dissected and error bars depict the standard error of the mean. Encapsulated eggs were sometimes observed but not quantified

influence either of the three foraging parameters: foraging response (i.e. the proportion of females that started foraging on the infested leaf), caterpillar contact or oviposition. There were no significant interactions between host presence and time after Mamestra experience either (Table S1).

Compared to unconditioned parasitoids, the effect of prior oviposition in a non-host on parasitoid response depended on the presence of Pieris hosts (GLM, Interaction $X^2 = 4.514$, $p = 0.034$, Figure 3a and Table S2). Parasitoids with a Mamestra experience were more motivated to search on the infested leaf than unconditioned parasitoids in the absence of Pieris hosts (GLM post hoc, $z = 2.362$, $p = 0.018$) but this was not the case in the presence of hosts (GLM post hoc, $z = -0.644$, $p = 0.520$). The percentage of parasitoids contacting a caterpillar (of either species) was significantly higher when both Pieris and Mamestra were present (GLM, $X^2 = 23.768$, $p < 0.001$), but was neither influenced by experience, nor by the interaction (Figure 3b and Table S2 for details of statistics). The same results were found for oviposition: a higher percentage of parasitoids oviposited in a caterpillar (of either species) when both Pieris and Mamestra were present (GLM, $X^2 = 27.180$, $p < 0.001$), while experience and the interaction term had no significant effect (Figure 3c and Table S2 for details of statistics). With respect to oviposition in Mamestra following contact with the non-host, parasitoids were more likely to oviposit in Mamestra in the presence than in the absence of Pieris hosts (GLM, $X^2 = 3.859$, $p = 0.049$), but there was no effect of prior experience or the interaction (Figure 3d and Table S2).

Finally, we evaluated how Mamestra experience influenced *C. glomerata*'s interaction with Mamestra in the presence of Pieris hosts, that is, using the subset of parasitoids that foraged in arenas with host and non-host caterpillars. Overall, 17 of 38 Mamestra-experienced parasitoids (45%) contacted the non-host (either by contacting only Mamestra or by contacting Pieris and Mamestra), while 6 of the 17 unconditioned parasitoids (35%) contacted the non-host caterpillars (GLM, Experience $X^2 = 0.435$, $p = 0.510$, Table S3). Similarly, the percentages of parasitoids that oviposited in Mamestra were comparable in Mamestra-experienced and unconditioned parasitoids (respectively, 33% [12 of 36] and 21% [3 of 14], GLM, Experience $X^2 = 1.480$, $p = 0.224$). Considering parasitoids that only oviposited in a single caterpillar species, *C. glomerata* had a strong preference for contacting Pieris as compared to Mamestra caterpillars (76% [31 of

TABLE 1 Preference for the conditioned odour and memory retention (Performance Index, PI) of *Cotesia glomerata* 24 h after associative learning with an oviposition in either *Pieris brassicae* (Pieris) or *Mamestra brassicae* (Mamestra)

Oviposition experience	Preference for the conditioned odour			Performance index		
	95% CI	*p*-value[a]	*n*[b]	Avg $\pm$ SE	*p*-value[a]	*N*[b]
Pieris	0.56–0.74	<0.001	122	31.1 $\pm$ 7.9	<0.001	61
Mamestra	0.47–0.64	0.20	136	8.8 $\pm$ 8.8	0.32	68

[a]Binomial tests (two-sided) were used to test whether parasitoid choice deviated significantly from a 50:50 distribution over the conditioned and the control odour; one-sample Wilcoxon signed rank tests were used to test if the PI differed significantly from 0.
[b]Sample sizes (*n* = number of individual parasitoids; *N* = pairs of parasitoids conditioned on either vanilla or coffee scent).

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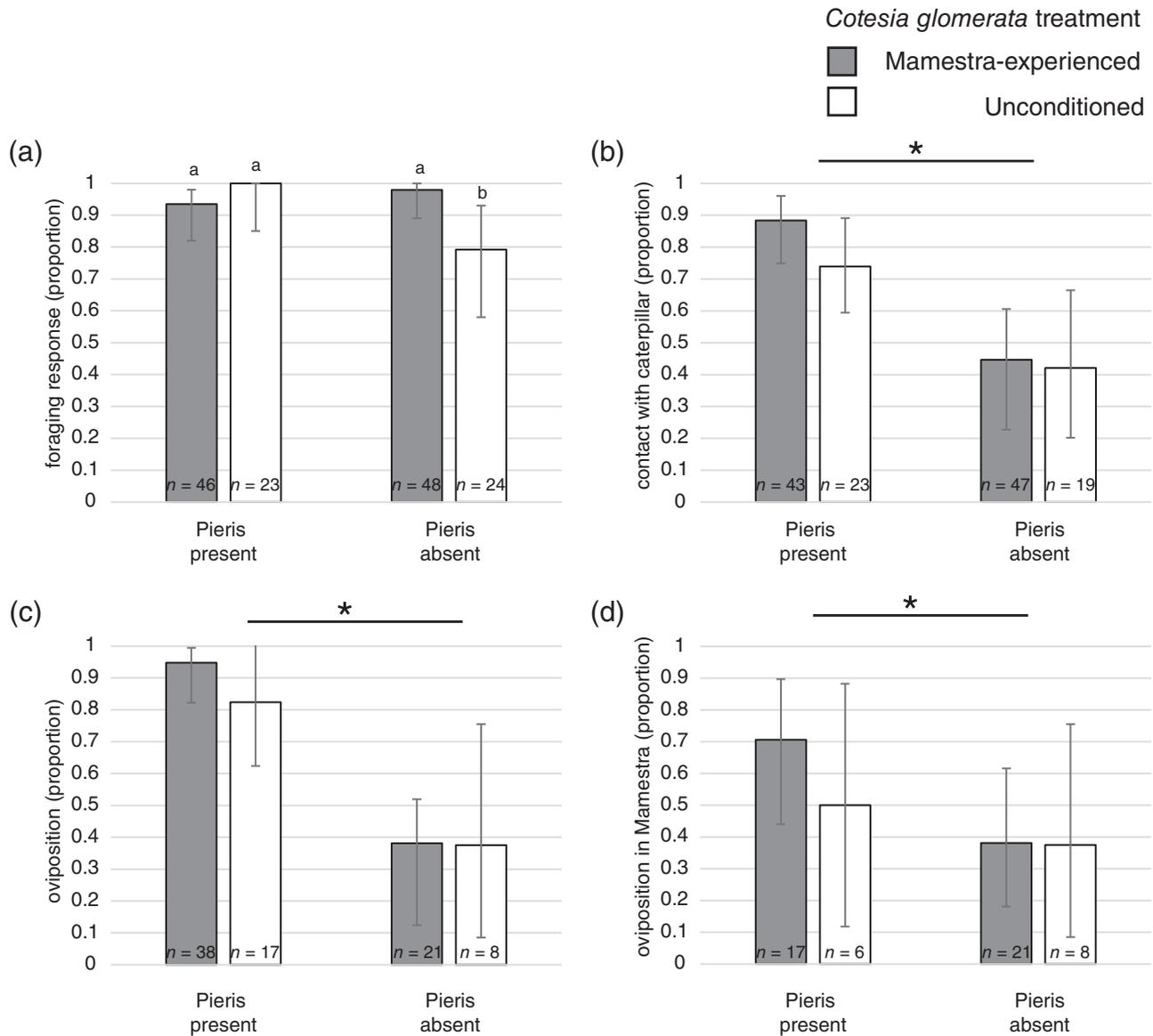Entomological
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FIGURE 3 Effect of Mamestra experience on foraging behaviour of *Cotesia glomerata* on an infested leaf. The percentage of parasitoids with oviposition experience in the non-host *Mamestra brassicae* (Mamestra experience, blue) or without experience (unconditioned, orange) that: (a) started foraging on the infested leaf with Mamestra caterpillars in the presence or absence of hosts (Pieris caterpillars), (b) made contact with a caterpillar of either species, (c) subsequently oviposited in either caterpillar species, and (d) oviposited in Mamestra after contacting a caterpillar of this species. Error bars reflect binomial 95% confidence intervals. Significant main effects are indicated with asterisk symbol (GLM, $p < 0.05$). Different letters above bars in (a) indicate significant pairwise differences between groups (Tukey's test, $p < 0.05$, for details of statistics see Table S2). *n* values inside bars represent the sample size

41], binomial test, $p = 0.001$), and the same was found for oviposition (85% [35 of 41], $p < 0.001$).

DISCUSSION

In this study, we investigated the interaction between the endoparasitoid *C. glomerata* and the presumed non-host *M. brassicae*. Our results demonstrated that *M. brassicae* is indeed a non-host for *C. glomerata*. Although *C. glomerata* deposits eggs in *M. brassicae* caterpillars, none of the eggs developed in *M. brassicae*. Our findings did

not support our other two predictions since *C. glomerata* did not adjust its foraging behaviour after oviposition in the non-host *M. brassicae*. We did not observe memory retention in *C. glomerata* of volatiles encountered during oviposition in the non-host, and parasitoids did not alter their behaviour with respect to contact with and oviposition in *M. brassicae*. Instead, after oviposition in the non-host, parasitoids became more motivated to forage on an infested leaf with only non-hosts. Apparently, a single oviposition in an unsuitable host species is not stored in memory as an adverse event.

Despite egg deposition by 90% of *C. glomerata* parasitoids in *M. brassicae*, all deposited eggs disappeared within 4 days.

This observation conflicts with the general assumption that parasitoids do not deposit eggs in non-host species or, in the case of gregarious parasitoids, that they adjust their egg numbers (McDonald et al., 2015; Vinson, 1975, 1976). For example, Bukovinszky et al. (2012) suggested that *C. glomerata* deposits a smaller number of eggs in the non-host *M. brassicae* than in the host *P. rapae* but this may have been caused by dissecting the caterpillars approximately 36 h after exposure to parasitoids, and thus the majority of eggs may have disappeared already. In our study, clutch sizes of *C. glomerata* in the non-host *M. brassicae* were comparable to those in the host *P. brassicae* (immediately after egg deposition), as was also found by Chabaane et al. (2015) for clutch sizes of *C. glomerata* in the non-host *S. littoralis*.

We then addressed the effect of oviposition in a non-host on memory formation and foraging behaviour of *C. glomerata*. In contrast to our expectation, we found no memory retention for the conditioned odour after oviposition in *M. brassicae*, using an approach that is well known to result in associative memory after an oviposition in the host *P. brassicae* (de Bruijn, Vet, Jongsma, et al., 2018; Kruidhof et al., 2012), which was also confirmed in our experiment. The absence of associative learning after conditioning with oviposition in a non-host, in combination with vanilla odour and host frass, has also been found in the parasitoid *Microplitis croceipes* (Takasu & Lewis, 2003). Our findings and those of Takasu and Lewis (2003) suggest that parasitoids can detect the unsuitability of non-hosts to some extent: in both cases eggs are deposited but no memory is formed for the conditioned odour. This suggests that triggers for egg release and memory formation are different. Triggers for egg deposition are generally amino acids and sugars found in the haemolymph (Arthur et al., 1969, 1972). Triggers for memory formation are also expected to be present in the haemolymph, but the identity of these triggering compounds is still unknown and requires more research. In *C. glomerata*, host frass plays an important role in recognition of the host and formation of associative memory. Although oviposition in a host in the absence of frass does not increase the foraging response of *C. glomerata*, exposure to host frass alone leads to memory retention after 24 h in this parasitoid (Bleeker et al., 2006; Geervliet et al., 1998). Interestingly, our experiments show that associative learning with host frass can be suppressed by oviposition in a non-host (see also de Bruijn, Vet, et al., 2021; Takasu & Lewis, 2003). Alternatively, the lack of memory formation may result from conflicting experiences: the presence of host frass is perceived as a reward, while oviposition in the non-host is not.

A single oviposition in a non-host on a leaf with feeding damage and frass of the host did also not influence the frequency of contact with and oviposition in *M. brassicae* of *C. glomerata* in a subsequent behavioural assay. In the absence of hosts, almost half of all parasitoids (irrespective of prior experience) contacted a Mamestra caterpillar and this also led to oviposition. In the presence of hosts, *C. glomerata* interacted more frequently with Mamestra, although there was a clear preference for Pieris caterpillars. It is possible that multiple oviposition experiences would lead to behavioural changes in

M. croceipes and *C. glomerata*. Repeated ovipositions in hosts, particularly when spaced in time, are known to lead to more persistent memory in parasitoids (Gols et al., 2012; Hoedjes et al., 2011), but further research should confirm if this is also the case with oviposition in non-hosts. In addition, it would be important to investigate if a rewarding oviposition in a host influences the interaction of *C. glomerata* with *M. brassicae*. Interestingly, early work on discrimination of *Pseudeucoila bochei* showed that parasitoid females learn to discriminate against already parasitized *Drosophila* larvae by experiencing oviposition in unparasitized larvae (van Lenteren & Bakker, 1975) and similar findings have since been reported for other parasitoid species, for example, *Microplitis demolitor* (Takano & Schellhorn, 2021).

Only one foraging parameter was affected by oviposition in a non-host in the behavioural assay, but not in the direction that we expected. *C. glomerata* parasitoids with Mamestra experience were more motivated to forage on an infested leaf with non-hosts than unconditioned parasitoids. Exposure to hosts and/or their traces is known to increase the motivational state of parasitoids (Bleeker et al., 2006; McAuslane et al., 1991; Papaj & Prokopy, 1989; Takasu & Lewis, 2003), and it is possible that our finding resulted from exposure to host traces during oviposition in *M. brassicae*. Alternatively, this increased motivation may be a non-specific effect, resulting from changes in the parasitoid that are triggered by egg release but unrelated to the caterpillar species in which they oviposit. Disentangling the effects of exposure to host frass and oviposition in the non-host on the behaviour of *C. glomerata* asks for controls of oviposition in a non-host (or another way of egg release) in the absence of host frass. However, in our laboratory experiments, oviposition in the absence of host frass happened only a few times and it was not considered a feasible control. Based on what is known about the distribution of hosts and non-hosts (Vos et al., 2001) and *C. glomerata* behaviour (attraction to HIPVs and how much time is spent on plants with non-hosts) (de Bruijn, Vosteen, et al., 2021), *C. glomerata* encounters *M. brassicae* in the presence or absence of hosts in nature. Further studies should investigate the relative frequencies of these encounters and the role of (host) frass in acceptance behaviour of *C. glomerata* because it appears to be particularly important in this parasitoid.

Based on our observations, we cannot yet conclude whether *C. glomerata* is unable to discriminate against *M. brassicae* or whether discrimination depends on factors that we have not yet investigated. Moreover, we cannot assess whether other parasitoid species would respond similarly. Importantly, theoretical and experimental studies on discrimination of parasitoids between parasitized and healthy hosts have shown that parasitoid age and egg load are important drivers of host discrimination and acceptance. A high egg load may be associated with few current reproductive opportunities and can lead to the acceptance of hosts of lower quality, such as already parasitized hosts, and even non-hosts (Godfray, 1994; Heimpel & Casas, 2008; Hopper et al., 2013). Islam and Copland (2000) for example, found that parasitoids with a high egg load accepted parasitized hosts just as frequently as unparasitized hosts, while parasitoids with a low egg load oviposited in parasitized hosts less

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frequently. In such cases, the waste of resources might outweigh the costs of retaining and maintaining high numbers of eggs as well as the costs of discriminating (e.g. in terms of time spent) between high- and low-quality hosts in the case of superparasitism or between hosts and non-hosts as in our study on *C. glomerata*. *C. glomerata* indeed has a very high fecundity, it emerges with 500–800 eggs, continues to produce eggs throughout its lifetime and when females are not allowed to oviposit, they can hold up to 2000 eggs within 2–11 days after emergence (Le Masurier, 1991). In our experiment (and see also Takasu & Lewis, 2003; Meisner et al., 2007; Chabaane et al., 2015), well-fed mated parasitoid females of several days old were used, which likely had a high egg load. It should be noted though that even parasitoids with high egg loads may become egg-limited, depending on clutch size. Future research should elucidate the effects of female age and egg load on the acceptance of non-hosts and whether this phenomenon occurs in parasitoid species that have a lower fecundity.

Parasitoid foraging behaviour is known to be highly context dependent (Heimpel & Casas, 2008) and is shaped by what parasitoids encounter in their habitat (Vet et al., 1995). Although many parasitoid species are known to be specifically attracted to plants on which suitable host species occur (Geervliet et al., 1996; Vet et al., 1995), these plants are often also used by non-hosts (Dicke et al., 2009; Vos et al., 2001), making non-host encounters (in combination with host frass) inevitable events in nature. Our study showed that *C. glomerata* frequently deposits eggs in the non-host *M. brassicae*, especially in the presence of host frass, which may be an adaptation to allow quick 'decisions' on oviposition. Remarkably, *C. glomerata* does not form associative memory following oviposition in *M. brassicae*, suggesting that females detect the difference between host and non-host but only after inserting their ovipositor and releasing eggs. Egg deposition in non-hosts may be due to a combination of *C. glomerata*'s host-acceptance behaviour and its high egg load, which makes parasitoids less selective on host quality. An alternative, or additional, explanation might be that the observed interactions between *C. glomerata* and *M. brassicae* represent only a snapshot in coevolutionary time, and hence the evolutionary arms-race, between the two species. Although we can only speculate, it is possible that *M. brassicae* or its ancestor was perhaps once a suitable host, but has evolved the ability to defend itself against *C. glomerata* or its ancestor. Vice versa, it is also possible that oviposition in non-hosts in *C. glomerata* may lead to an expansion of its host range over evolutionary time when the parasitoid evolves the ability to overcome caterpillar defence and evolves behaviour that leads to more directed acceptance of the new species as has been suggested for the seed weevil *Callosobruchus maculatus* (Cheng et al., 2008).

AUTHOR CONTRIBUTIONS
Jessica A.C. de Bruijn developed the idea for the study, the research question and the experimental design. The experiments were conducted by Jessica A.C. de Bruijn, with the help of BSc students (Elliot Martin, Lorenzo Castelen, Benedikt Rakotonirina-Hess, Wiene van de Bunte, Thomas Remmits and Hao Ye). Jessica A.C. de Bruijn and Jetske G. de Boer analysed the data and interpreted the results alongside Hans M. Smid, Louise E.M. Vet. Hans M. Smid made the illustration of the experimental design. Jessica A.C. de Bruijn and Jetske G. de Boer wrote and revised the manuscript, based on comments and suggestions from Hans M. Smid and Louise E.M. Vet.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study will be made openly available in Dryad repository at https://doi.org/10.5061/dryad.12jm63z0z.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

TABLE S1. Statistical overview of GLMs evaluating the effects of host presence (yes or no) and time after the Mamestra experience (time: 10 min or 24 h) on the binary response variables foraging response, caterpillar contact and oviposition

TABLE S2. Statistical overview of the GLMs, evaluating the effects of host presence (yes or no) and experience (non-host oviposition experience or unconditioned) on the binary response variables foraging response, caterpillar contact and oviposition.

TABLE S3. Number of Mamestra-experienced and unconditioned *Cotesia glomerata* parasitoids that contacted and oviposited in caterpillars in the situation where host and non-host caterpillars were present.