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M.L. DINDO, G.G. BAZZOCCHI, A. BERTELLINI, P. BARONIO

Some factors influencing oviposition in an artificial host by the pupal parasitoid *Brachymeria intermedia* (Nees)

Abstract - Attempts to identify factors capable of inducing *Brachymeria intermedia* to oviposit in an artificial host are reported. The development of an artificial substrate for *in vitro* egg laying of this hymenopterous parasitoid is also described. When presented with a choice between pupae of the factitious host *Galleria mellonella* and a blank cell in a Y tube olfactometer the females did not show a significant olfactory preference for the pupae. The response of *B. intermedia* females to *G. mellonella* pupae washed in hexane or untreated was also not significantly different. The role of kairomonal cues in the cuticle of *G. mellonella* pupae in attracting *B. intermedia* females was therefore not clear. Cylindrical host shape, on the other hand, was apparently important in inducing parasitoid oviposition. In fact, the females were observed to probe cylinder-shaped artificial substrates with their ovipositors even in the absence of chemical cue. When cylinder-shaped and purposefully flattened *G. mellonella* cuticles were exposed to parasitoid females oviposition holes were recorded only in the former and none in the latter. Cylinder-shaped artificial hosts composed of an agar-water suspension and *G. mellonella* haemolymph encapsulated in Parafilm were hence prepared and exposed to *B. intermedia* females. Some eggs were recorded in artificial pupae containing 50% or 25% haemolymph. No eggs were found in the pupae containing agar-water suspension alone, or 75% or 100% haemolymph. These results suggest that *G. mellonella* haemolymph contains ovipositional kairomones, an excess of which, however, prevents *B. intermedia* females from ovipositing.

Riassunto - *Alcuni fattori condizionanti il processo di ovideposizione in un ospite artificiale da parte del parassitoide pupale* Brachymeria intermedia *(Nees).*

Sono stati effettuati dei tentativi di individuazione di fattori in grado di stimolare il processo di ovideposizione in un ospite artificiale da parte di *Brachymeria intermedia.* Sono inoltre stati allestiti dei substrati artificiali per l'ovideposizione *in vitro* da parte di questo imenottero parassitoide. In una prova eseguita in olfattometro a due vie, le crisalidi dell'ospite di sostituzione *G. mellonella* non si sono dimostrate significativamente più attrattive rispetto al bianco nei confronti delle femmine di *B. intermedia.* Inoltre, crisalidi di *G. mellonella* previamente sciacquate in esano si sono dimostrate attrattive in modo non significativamente diverso rispetto a quelle testimoni verso le femmine del parassitoide. Pertanto, il potere attrattivo su *B. intermedia* esercitato da cairomoni della cuticola delle crisalidi di *G. mellonella* non è risultato chiaro. Viceversa, la forma cilindrica dell'ospite si è dimostrata un fattore di notevole importanza ai fini del processo di ovideposizione da parte del parassitoide. Infatti, è stato osservato che, anche in assenza di stimoli chimici, le femmine effettuavano dei tentativi di ovideposizione, inserendo la terebra, in substrati artificiali di forma cilindrica. Inoltre, quando al parassitoide sono state esposte cuticole di *G. mellonella* appiattite ad arte o che, al contrario, mantenevano la forma cilindrica che originariamente aveva la pupa, fori di ovideposizione sono stati rilevati solo in queste ultime. Pertanto, nella seconda parte del lavoro sono stati esposti alle femmine ospiti artificiali di forma cilindrica: questi erano stati allestiti utilizzando un involucro in Parafilm riempito con emolinfa di *G. mellonella* e sospensione acquosa di agar. Sono state rilevate alcune uova del parassitoide nelle crisalidi artificiali contenenti il 50% o il 25% di emolinfa. Nessun uovo è stato, al contrario, ritrovato nei substrati contenenti la sola sospensione di agar o il 75% o il 100% di emolinfa. Questi risultati suggeriscono che le femmine di *B. intermedia* vengono stimolate a deporre le uova da parte di cairomoni presenti nell'emolinfa di *G. mellonella*, un eccesso dei quali, tuttavia, sembra ostacolare il processo di ovideposizione.

Key words: pupal parasitoids, oviposition, artificial host, *Brachymeria intermedia.*

INTRODUCTION

Brachymeria intermedia (Nees) (Hymenoptera Chalcididae) is a solitary pupal parasitoid of many Lepidoptera, including *Lymantria dispar* (L.) and other important defoliators (Luciano & Prota, 1984; Cerboneschi, 1999). The complete development of this entomophage was obtained on artificial diets composed of crude components and devoid of insect material. Adult yields from egg were as high as 44% (Dindo *et al.*, 2001).

An obstacle to the continuous artificial culture of *B. intermedia* without host insect is represented by the inability of the females to directly oviposit in the diet. Despite the increased knowledge of the physical and chemical cues that influence oviposition behaviour in this species (Tucker & Leonard, 1977; Kerguelen & Cardé, 1996), the eggs to be placed on the diet still need to be collected from host insects. Dindo (1990) has however demonstrated the ability of *B. intermedia* to oviposit and develop to the adult stage in the pupae of the factitious host *Galleria mellonella* L. that have been killed by immersion in distilled water at 60°C for 12 min.

To date, a number of hymenopterous parasitoids have been induced to lay eggs in/on artificial substrates, including species belonging to the genus *Trichogramma* (Rajendram & Hagen, 1974; Grenier *et al.*, 1993; Consoli & Parra, 1999), *Oencyrtus pityocampae* (Mercet) (Masutti *et al.*, 1991), *Catolaccus grandis* (Burks) (Guerra & Martinez, 1994), *Microplitis croceipes* (Cresson) (Tilden & Ferkovich, 1988) and *Aphidius ervi* Haliday (Battaglia *et al.*, 2002). The pupal parasitoid *Itoplectis conquisitor*

(Say) was induced to oviposit in Parafilm® tubes containing host haemolymph or a variety of amino acids and magnesium chloride (Arthur *et al.*, 1972). House (1978) reported the *in vitro* oviposition and rearing of a single specimen of *I. conquisitor* in an artificial host consisting of a diet encapsulated in paraffin. No successful attempts to obtain oviposition in an artificial host by pupal hymenopterous parasitoids have since been reported.

This paper deals with (a) attempts to identify chemical and physical stimulants inducing oviposition in an artificial host by *B. intermedia,* and (b) the development of an artificial pupa for *in vitro* egg laying of this parasitoid.

MATERIALS E METHODS

INSECTS

A colony of *B. intermedia* was maintained on 2- to 4-day old *Galleria mellonella* L. pupae (Minot & Leonard, 1976a) in a rearing chamber at 26±1°C, 60% R.H., and L16:D8 photoperiod (Minot & Leonard, 1976b). The adults were fed on cotton balls soaked in a honey and water solution (20% honey) and kept in plexiglass cages (40x30x30 cm). The colony was initially established from parasitoids supplied by the laboratory of S.Nelson Thompson at the University of California, Riverside and then renewed in 2000 with adults emerged from pupae of *L. dispar* collected in the forest by Anna Cerboneschi of the Cork Experimental Station of Tempio Pausania (Italy). *G. mellonella* larvae were cultured on the diet developed by Sehnal (1966) under the conditions described by Campadelli (1973).

As in the study carried out by Grant & Noblet (1991) with the congeneric species *Brachymeria ovata* (Say), all female parasitoids were 30-40 day old and host-experienced when used.

ATTEMPTS TO IDENTIFY CHEMICAL AND PHYSICAL STIMULANTS INDUCING OVIPOSITION IN AN ARTIFICIAL HOST BY *B. INTERMEDIA*

OLFACTOMETER BIOASSAY. A Y-tube olfactometer similar to the one first described by Sabelis & van de Baan (1983) was used to examine the behavioural responses of *B. intermedia* to odours from 2 to 4 day old *G. mellonella* live pupae. Two arms of a glass Y-shaped tube (linear portion length = 20 cm; arm length = 15 cm; Ø = 1 cm) were connected to “odour chambers” (500 ml-glass flasks) in which odour sources were placed. An activated charcoal purified and re-humidified airflow (200 ml/min) was pumped into each odour chamber, and then in the arms of the olfactometer.

During the bioassay individual *B. intermedia* females were released at the base of the Y tube. A first choice was recorded upon the females reaching the centre of the Y-tube where the three arms met and choosing one direction within 5 minutes (Grant & Noblet, 1991), and then a final choice was recorded upon the females crossing the arm of the olfactometer and reaching the tip within an overall time of 8 minutes. Only final choices were taken into account for result evaluation. The odour chambers and the relevant arms were reversed after 5 runs to eliminate any possible left-right bias. The odour sources were replaced every 10 runs. To avoid possible visual cues and distractions each odour chamber was covered with a blank sheet and the bioassay room was kept in the dark. The bioassay device was illuminated from above with a lamp (100 watt) yielding about 250 lux.

Two alternative odour combinations were examined in the olfactometer: (1) five live pupae and a blank, and (2) a blank and a blank. Four replicates were performed for each odour combination, each comprising 25 females.

The results of the 4 replicates were pooled for statistical analysis and evaluated using χ^2 test to determine significant differences in choices.

RESPONSE OF *B. INTERMEDIA* TO *G. MELLONELLA* PUPAE WASHED IN HEXANE. *G. mellonella* pupae were soaked for 2 hrs in hexane in the proportion of 0.004 ml solvent/mm^2 pupal surface area (Tucker & Leonard, 1977). After washing pupae were air-dried on filter paper and presented to *B. intermedia* females. For bioassays, two pupae washed in hexane were placed in a 25-cm diameter glass Petri dish containing 1 female parasitoid. Untreated pupae were used as control. Insertion of the ovipositor by the females into the pupae within 5 min. was recorded as a response. The experiment was replicated 100 times with pupae washed in hexane and 100 times with control pupae.

A χ^2 test (2x2 table) was used to evaluate the results.

Both this bioassay and the one described above were conducted from 11.00-15.00 h (Tucker & Leonard, 1977) at approximately 26°C and 50-60% r.h.

EFFECT OF THE SUBSTRATE SHAPE ON THE OVIPOSITION BEHAVIOUR OF *B. INTERMEDIA*. Two experiments were carried out. In the first 10 cylinder-shaped artificial oviposition substrates were placed in a plexiglass cage (20x20x20 cm), each cage containing 15 *B. intermedia* females, and removed after 24 hs. The substrates consisted of 5 nicotine-free cigarette filters and 5 empty filter paper rolls (length 2 cm, diameter 0.5 cm). Upon removal from the cage the substrates were examined under a microscope to detect oviposition holes and/or eggs.

In the second experiment 20 *G. mellonella* pupae were killed by immersion in distilled water at 60°C for 12 min (Dindo, 1990) and deprived of their content with a 5 ml sterile syringe. The resulting cuticles were washed in 70% ethanol, rinsed in distilled water, air-dried on filter paper and divided into 3 groups of 10. The first group (I) comprised cuticles that still maintained a cylindrical shape resembling that of the original pupa. In the second (II) the cuticles were flattened with a glass rod before being exposed to *B. intermedia* females. The two groups of cuticles were separately placed in plexiglass cages (20x20x20 cm) (1 group per cage). Each cage contained 15 *B. intermedia* females. After 24 hrs the cuticles were removed from the cages and examined under a microscope to detect oviposition holes and/or eggs.

No statistical analysis was performed.

DEVELOPMENT OF AN ARTIFICIAL HOST FOR EGG LAYING OF *B. INTERMEDIA*

The artificial pupae measured 2 cm in length and 0.5 cm in diameter and contained 0.8-1 ml substrate. They were prepared by stretching a double sheet of Parafilm® around a metal rod (length 3.5 cm, diameter 0.6 cm) to form a capsule. The Parafilm® capsule was then slipped off the rod and sealed at one end with nylon yarn. An agar-water suspension was prepared by dissolving 0.5 g agar in 25 ml bidistilled water in a 50 ml flask which was then heated at 100°C for 10 min. The capsules were filled with *G. mellonella* haemolymph and agar-water suspension in the proportions indicated below using a 1-ml syringe. To avoid damaging the Parafilm® the suspension was left to cool down to 50°C before being injected into the capsules. Finally, the open end of each capsule was sealed with nylon yarn.

Five proportions of *G. mellonella* haemolymph (H) and agar-water suspension (A) were tested (H:A= 100%:0; 50%:50%; 25%:75%; 75%:25%; 0:100%), each corresponding to as many treatments. Six replicates were performed, each comprising 1 artificial pupa per treatment. The artificial pupae were placed in one cage (20x20x20 cm) containing 25 females. The pupae were removed from the cage after 24 hrs and dissected under a microscope to detect eggs.

No statistical analysis was performed.

RESULTS

ATTEMPTS TO IDENTIFY CHEMICAL AND PHYSICAL STIMULANTS INDUCING OVIPOSITION IN AN ARTIFICIAL HOST BY *B. INTERMEDIA*

OLFACTOMETER BIOASSAY. When presented with a choice between *G. mellonella* pupae and a blank cell 80% parasitoids made a final choice. Of the females making a final choice 58.8% chose the arm with the pupae and 41.2% the blank cell. The difference was not significant. When offered a blank cell in both arms of the Y tube olfactometer 71% females made a final choice, no significant difference in preference being observed, however, between one arm and the other (Table 1).

RESPONSE OF *B. INTERMEDIA* TO *G. MELLONELLA* PUPAE WASHED IN HEXANE. No significant difference was recorded between the attraction of *B. intermedia* females for hexane-washed or untreated *G. mellonella* pupae (Table 2). *B. intermedia* female exposure to the pupal wash was made according to Tucker & Leonard (1977). No parasitoid response was observed.

EFFECT OF THE SUBSTRATE SHAPE ON THE OVIPOSITION BEHAVIOUR OF *B. INTERMEDIA*. Oviposition holes but no eggs were observed in all the substrates of the first experiment, while no flattened cuticles in the second experiment (group II) were found to exhibit holes. Conversely, oviposition holes were recorded in all the cuticles with a cylindrical shape (group I).

Table 1 - Response of experienced Brachymeria intermedia *females to* Galleria mellonella *pupae in a Y tube olfactometer. Number of females per replicate = 25. Final choice made = number of females crossing an arm of the olfactometer up to the tip within an overall time of 8 min. The data of the 4 replicates were pooled for the analysis.*

			Replicate	**Final choice made**		χ^2	**P**	**No final choice made**
A		B						
				A	B			
G. mellonella pupae	vs	Blank						
			1	12	8			5
			2	10	10			5
			3	13	9			3
			4	12	6			7
			Total	47	33	2.45	0.118	20
Blank	vs	Blank						
			1	9	11			5
			2	6	13			6
			3	8	10			7
			4	10	4			11
			Total	33	38	0.35	0.553	29

Table 2 - Attraction of experienced B. intermedia *females to* G. mellonella *pupae washed in hexane for 2 hrs or untreated. Number of replicates= 100.*

Pupae	**Number of attracted**	**Number of non attracted**	χ^2	**P**
Washed in hexane	65	35		
Untreated	70	30		
			0.57	0.45

DEVELOPMENT OF AN ARTIFICIAL HOST FOR EGG LAYING OF *B. INTERMEDIA*

A few *B. intermedia* eggs were found in artificial pupae containing 50% or 25% haemolymph, with the highest number of eggs being recorded in "pupae" containing 25% haemolymph. No eggs were found in the pupae containing agar-water suspension alone, or 75% or 100% haemolymph.

The eggs collected from the artificial hosts and an equal number of eggs collected

Table 3 - Number of B. intermedia *eggs detected in artificial pupae made of Parafilm and filled up with* G. mellonella *haemolymph (H) and a 2% agar-water suspension (A) in different proportions (H:A). Each replicate consisted of one pupa per treatment.*

Replicate	**H:A**				
	100% : 0	50% : 50%	25% : 75%	75% : 25%	0 : 100%
1	0	1	2	0	0
2	0	2	3	0	0
3	0	2	3	0	0
4	0	0	1	0	0
5	0	0	2	0	0
6	0	2	3	0	0
Mean	0	1.17	2.3	0	0

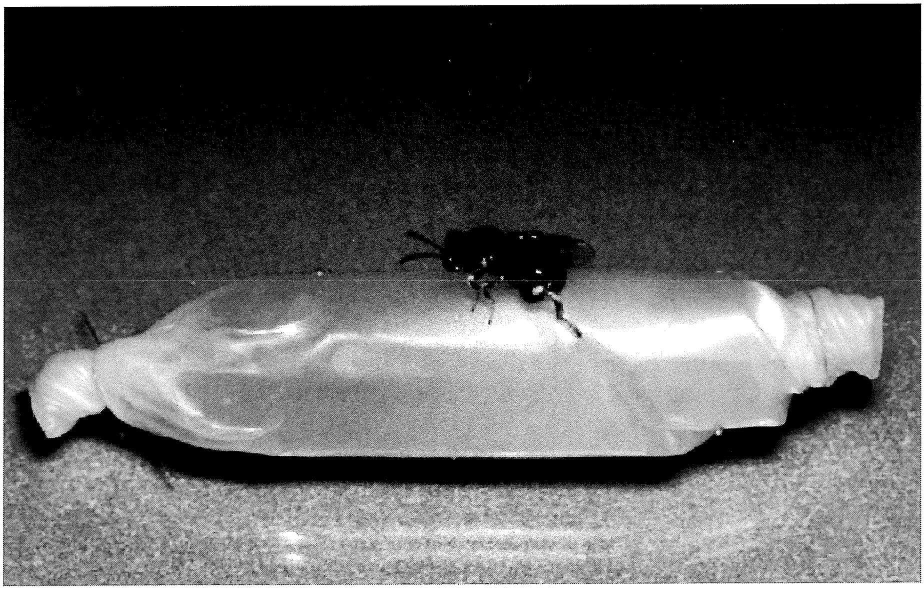

Fig. 1 - A female *Brachymeria intermedia* ovipositing in an artificial pupa. The "pupa" consists of a Parafilm capsule filled up with *Galleria mellonella* haemolymph (25%) and a 2% agar-water suspension (75%).

from superparasitized *G. mellonella* pupae were disinfected as described by Dindo *et al.* (1997) and transferred onto a diet composed of 8.9 ml commercial veal homogenate, 1.1 ml chicken egg yolk, 0.38 g yeast extract, 2 ml of a 6% agar-water suspension and 0.12 ml of a 10 mg/ml gentamicin solution (Dindo *et al.*, 2001). All eggs hatched but the parasitoids collected from the artificial pupae died within a few days because the diet was rapidly contaminated by moulds. About 45% parasitoids collected from *G. mellonella* pupae reached the adult stage. This result is consistent with the findings of Dindo *et al.* (2001).

DISCUSSION

The ultimate goal of the present research was to develop an artificial oviposition substrate for *B. intermedia*. The experiments on the chemical and physical stimulants that may influence oviposition by this parasitoid were performed with the aim of achieving this result.

In the olfactometer bioassay performed in the present study a lower number of *B. intermedia* females were found to make a final choice when offered a blank cell in both arms of the olfactometer than when presented with a choice between *G. mellonella* pupae and a blank cell. In the latter experiment a greater number of *B. intermedia* females chose the arm with the pupae than the blank cell but the difference was not significant. Moreover, the *G. mellonella* pupae that had been washed in hexane for 2 hrs remained attractive to *B. intermedia* females to the same extent as the untreated pupae, while the pupal wash did not elicit any response of the parasitoid. The attraction of *B. intermedia* females to kairomonal cues in the cuticle of the factitious host *G. mellonella* did therefore not appear to be clear from our study. However, the fact that the pupae washed in hexane maintained their capacity to attract *B. intermedia* females may be of interest in relation to the final aim of inducing oviposition by *B. intermedia* in an artificial substrate. Guerra *et al.* (1994) and Grenier *et al.* (1993, 1998), for instance, showed that hexane itself (as well as other short-chain hydrocarbons) elicits a strong oviposition activity in female parasitoids of the genus *Catolaccus* and *Trichogramma*. It will be interesting to investigate whether *B. intermedia* can also be induced to lay more eggs in hexane-coated artificial hosts, an experiment that was not performed in the present study.

The importance of physical factors including host shape has been shown to influence the oviposition behaviour of a number of parasitoids (Thompson & Hagen, 1999). In the case of *B. intermedia* the cylindrical host shape was apparently important in inducing parasitoid oviposition. In fact, even in the absence of any chemical cue, cylinder-shaped artificial substrates were found to be capable of eliciting oviposition in *B. intermedia*, although no eggs were laid following the drilling with the ovipositor into the host. This finding is further borne out by the fact that exposure of *G. mellonella* cuticles to parasitoid females led to oviposition holes being drilled only in the cuticles which maintained a cylindrial shape but not in the flattened ones. In view of this, cylinder-shaped artificial hosts containing *G. mellonella* haemolymph combined with agar-water suspension in different proportions were developed for *B. intermedia* in the second part of the study. The presence of ovipositional kairomones in the haemolymph of the insect hosts has been shown for other hymenopterous parasitoids, such as *I. conquisitor* (Hedgekar & Arthur, 1973) and *M. croceipes* (Tilden & Ferkovich, 1988). The "host" capsules were made of Parafilm®, a material already used in similar experiments (House, 1978; Guerra & Martinez, 1994). According to Guerra *et al.* (1994), Parafilm® by itself provides a chemical cue that stimulates the parasitoid female to drill into the substrate with the ovipositor, although it alone is insufficient to induce oviposition. In our study *B. intermedia* laid eggs inside the artificial host containing 50% or 25% *G. mellonella* haemolymph. No eggs were laid in the "hosts" either devoid of or containing 75% or 100% haemolymph. Therefore ovipositional kairomones seem to be present in *G. mellonella* haemolymph, but when in excess they appear to prevent *B. intermedia* females from ovipositing.

Additional research is needed in order to chemically characterise the active kairomones in *G. mellonella* haemolymph as well as to improve the artificial hosts so as to make them more effective and to obtain a higher number of *B. intermedia* eggs even in the absence of insect material. Further study is also required to control contamination in diets following the egg transfer from the artificial hosts.

It should however be pointed out that after the successful complete artificial rearing of *I. conquisitor* achieved by House in 1978 the only pupal parasitoid for which an artificial oviposition substrate has been developed is *B. intermedia.* This result may be considered as a promising step for obtaining the continuous culture of this entomophagous with exclusion of host insect.

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DR. MARIA LUISA DINDO, DR. GIOVANNI GIORGIO BAZZOCCHI, DR. ALESSANDRO BERTELLINI, PROF. PIERO BARONIO - Dipartimento di Scienze e Tecnologie Agroambientali, Via Filippo Re, 6, 40126 Bologna. E-mail: ldindo@agrsci.unibo.it

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