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In vitro rearing of the tachinid parasitoid *Exorista larvarum* with exclusion of the host insect for more than one generation

Elisa MARCHETTI, Piero BARONIO, Maria Luisa DINDO
Dipartimento di Scienze e Tecnologie Agroambientali - Entomologia, Università di Bologna, Italy

Abstract

We tested the possibility to eliminate the host insect from the line of production of *Exorista larvarum* (L.), a larval parasitoid of Lepidoptera, for more than one generation. *Galleria mellonella* (L.) larvae or a plastic sheet were provided as oviposition substrates either to parental or 1st generation females. The eggs/female laid in 45 min were dramatically fewer on the plastic sheet compared to host larvae and decreased from the parental to the 1st generation. Both the 1st and 2nd generation eggs (respectively laid by parental and 1st generation females) were removed from the oviposition substrates (host larvae or plastic sheet) and placed on a skimmed milk-based artificial medium. The percentages of hatched eggs were not significantly affected by the oviposition substrate, but the generation effect was significant and fewer eggs hatched in the 2nd than in the 1st generation. The percentages of puparia and adults were not significantly influenced either by the oviposition substrate or generation. All the 2nd generation adults obtained from eggs laid on the plastic sheet died 1-2 days after emergence, before the females oviposited, whereas the 2nd generation adults obtained from eggs removed from the host integument survived. After mating, the females laid eggs on *G. mellonella* larvae. The results confirmed that, for one generation, the out-of-host eggs are competitive with those removed from *G. mellonella* larvae to rear *E. larvarum* on an artificial medium. The quality of the *in vitro*-cultured tachinids however decreases over generations, especially if the host is completely excluded from their line of production, i.e. not even used for collecting eggs. Moreover, several factors need to be investigated for *E. larvarum* to be stimulated to lay more eggs on artificial substrates.

Key words: *Exorista larvarum*, continuous in vitro rearing, parasitoids, Tachinidae, *Galleria mellonella*.

Introduction

The females of the tachinid *Exorista larvarum* (L.), a larval parasitoid of Lepidoptera, lay macrotype eggs on the host integument. The newly hatched larvae go into the host body and continuously develop until pupation, which generally occurs outside the host larval remains (Mellini *et al*., 1993).

Although host larvae are available, in captivity many eggs are laid around the cage and are usually lost. Based on this observation, Dindo *et al*. (2007) used eggs laid out of host (on a plastic sheet) to rear *E. larvarum* on an insect material-free artificial medium, which was previously proven to be suitable for the *in vitro* culture of this tachinid (Mellini and Campadelli, 1996; Dindo *et al*., 2003; 2006). The parasitoid development of *E. larvarum* from eggs removed from the plastic sheet or from larvae of the host *Galleria mellonella* (L.) did not differ significantly in terms of hatched eggs, puparia and adult yields. It was thus shown that, at least for one generation, the *in vitro* production of *E. larvarum* may be disengaged from the steady availability of a living host, a goal that has not yet been achieved for any other tachinid that oviposits on, or in, the host body.

A principal goal of studies on *in vitro* rearing of entomophagous insects is continuous culture with elimination of the host insects, which ideally requires the direct deposition of parasitoid eggs or larvae onto the medium (Grenier *et al*., 1994). This goal has until now been achieved only for sarcophagid dipterans, which show simple relationships with their hosts, different *Trichogramma* species and a few other hymenopterans (Thompson and Hagen, 1999). The experiments described in this article were aimed at testing the possibility to eliminate the host insect from the line of production of *E. larvarum* for more than one generation, so as to add one step toward the major objective of obtaining the continuous *in vitro* culture of this tachinid.

Materials and methods

Insects

A stock-colony of *E. larvarum* was established in 1992 and augmented in 2004 with adults which had emerged from *Lymantria dispar* (L.) and *Hyphantria cunea* (Drury) larvae collected in the field in the provinces of Bologna and Modena (Emilia Romagna, Northern Italy). The colony was maintained in the laboratory using *G. mellonella* as a factitious host, according to the methods described by Dindo *et al*. (1999).

Experiment conduction

For the *E. larvarum* eggs laid out of host, a plastic (polyethylene) sheet for alimentary use weighing 35 grams per square meter (Cuki®, Torino, Italy) was used as an oviposition substrate, as in Dindo *et al*. (2007). The *in vitro* rearing was performed on the skimmed milk-based artificial medium developed by Mellini and Campadelli (1996).

Newly emerged *E. larvarum* adults (parental generation) were divided into two groups (*i* and *ii*) which corresponded to two treatments. Each group comprised an equal number of females and males (as specified below) and was placed in a plexiglass cage (40 x 30 x 30 cm) kept in a rearing chamber at 26 ± 1 °C, 70 ± 5% RH, and a 16:8 L:D photoperiod. The flies were fed on lump sugar and cotton balls soaked in a honey and water solu-

tion (20% honey), as in the standard rearing conditions. The parental females of the two treatments were intended to lay 1st generation eggs on *G. mellonella* larvae (*i*) or out of host, on the plastic sheet (*ii*). The trials were performed when the females were 5-7 day-old (Dindo *et al*., 1999). In treatment (*i*), *G. mellonella* larvae were exposed to flies for 45 min, similarly to the standard rearing (3 larvae/female as in Bratti and Coulibaly, 1995). When the larvae were removed, the eggs laid on their body were counted. In treatment (*ii*) the cage bottom was entirely covered with the plastic sheet, which was thus exposed to flies. After 45 min the sheet was removed and the eggs laid on its surface were counted. The sheet was then re-introduced in the cage for up to 24 h in order to obtain a sufficient egg number for the continuation of the experiment. The 1st generation eggs were removed either from the host larvae or the plastic sheet under a microscope, using a spatula. The larvae had previously been briefly dipped in a 60% ethanol solution, washed in distilled water, dried in blotting paper and fixed by pins in a paraffin-filled Petri dish. In both treatments, the collected eggs were dipped in distilled water on a concave slide, surface-disinfected in a 60% ethanol solution and washed twice in sterile distilled water (Bratti and Coulibaly, 1995; Dindo *et al*., 2007). The eggs were finally placed singly into the wells of 24-well multi-well plates (Nunclon, Roskilde, Denmark), which had previously been filled with artificial medium-soaked cotton balls, prepared as described by Dindo *et al*. (2003). The plates were sealed with Parafilm, wrapped in tinfoil and maintained in darkness at 26 ± 1 °C and 70% RH until puparium formation, except when they were removed for daily inspections. The instruments, glassware and cotton were autoclaved for 20 min at 120 °C. All operations (medium preparation, egg collection and transfer, daily inspections) were performed in a laminar flow hood.

When puparia formed, they were removed from the plates and placed singly into glass tubes. At emergence the 1st generation adults were counted, sexed, placed in a plexiglass cage (one per treament) and fed as previously described. Five-seven days after parasitoid emergence, either *G. mellonella* larvae (treatment *i*) or a plastic sheet (treatment *ii*) were placed into cages as oviposition substrates, for 45 min, in order to obtain 2nd generation eggs. The experiment was continued following the same procedure described for the 1st generation. At emergence, the 2rd generation adults were counted, sexed, placed in Plexiglas cages and fed as already described.

Statistical analysis

The replicates were 4 for the parental and 1st generations and 3 for the 2nd generation. The results were evaluated in terms of the following parameters: a) eggs/female laid in 45 min (parental and 1st generations); for the parental generation, in both treatments the females per replicate (the number of which is given in parentheses) were 12 (1), 11 (2), 12 (3), 10 (4); for the 1st generation the females were 8 (1), 5 (2) 7 (3) (treatment *i*) and 8 (1), 5 (2), 9 (3) (treatment *ii*); b) percentages of hatched eggs on the medium (= hatched eggs/original egg number x 100); c) percentages of puparia (= puparia/original egg number x 100); d) percentages of adults (= adults/puparia x 100); e) adult sex-ratio (males:females). Parameters b), c), d) and e) were considered for the 1st and 2nd generations. For the 2nd generation parasitoids obtained from eggs laid on the plastic sheet, the data on the adults refer to 2 replicates only, as in one replicate no puparia formed.

For the 1st generation, in treatment (*i*) the number of eggs per replicate was 48. In the other treatments the eggs/replicate varied according to availability (replicate number is given in parentheses): for the 1st generation in treatment (*ii*) they were 96 (1), 64 (2), 23 (3), 48 (4) while for the 2nd generation they were 48 (1), 27 (2), 24 (3) (treatment *i*) and 18 (1), 13 (2), 18 (3) (treatment (*ii*).

A factorial analysis of variance was made of the data (2 x 2 factors tested for the oviposition substrate and generation effect). The percentage values were transformed for the analysis using an ASN transformation (Zar, 1984). To test the independence of oviposition substrate and adult sex ratio, for each generation separate 2 x 2 contingency tables were used. Statistical tests were done with STATISTICA 6.0 (StatSoft, 2001).

Results

The eggs/female laid in 45 min were dramatically fewer on the plastic sheet compared to host larvae and decreased from the parental to the 1st generation (figure 1). The factorial analysis of variance showed that both the oviposition substrate effect ($F = 58.7$; $df = 1,10$; $P < 0.01$) and the generation effect ($F = 5.3$; $df = 1,10$; $P < 0.05$) were significant, whereas the interaction ($F = 0.4$; $df = 1,10$; $P > 0.05$) was not significant. The percentages of hatched eggs on artificial medium were not significantly affected by the oviposition substrate, but the generation effect was significant and fewer eggs hatched in the 2nd than in the 1st generation. The interaction was not significant (table 1). Neither the oviposition substrate nor the generation effects were significant for the percentages of puparia and adults. The interactions were also not significant. The percentages of puparia (especially those obtained from the eggs laid out of host) were however lower in the 2nd compared to the 1st generation (table 1). For the adults obtained from eggs

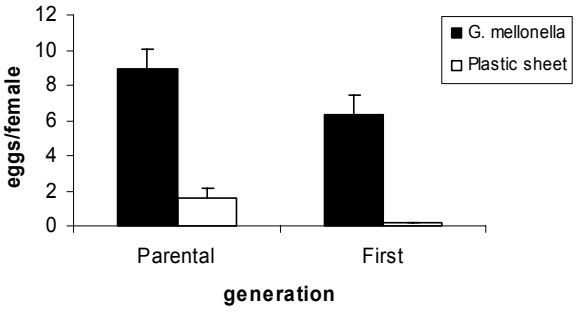

Figure 1. Eggs/female laid on *G. mellonella* larvae or on a plastic sheet by parental and 1st generation *E. larvarum* females. Vertical bars represent standard errors. The eggs were laid in 45 min.

Table 1. Percentages of hatched eggs, of puparia and adults of 1st and 2nd generation *E. larvarum* reared on artificial medium, as related to the combination of the factors "oviposition substrate" and "generation". Means ± standard errors.

Parameter	Oviposition substrate	Generation 1st	Generation 2nd	Oviposition substrate effect	Generation effect	Interaction
% hatched eggs	Host larvae	52.1 ± 2.6	38.4 ± 3.2	F = 0.26 df = 1,10 P > 0.05	F = 8.4 df = 1,10 P < 0.05*	F = 0.4 df = 1,10 P > 0.05
	Plastic sheet	52.4 ± 7.6	33.2 ± 6.4			
% puparia	Host larvae	32.3 ± 2.8	24.3 ± 4.7	F = 0.2 df = 1,10 P > 0.05	F = 4.1 df = 1,10 P > 0.05	F = 0.5 df = 1,10 P > 0.05
	Plastic sheet	34.8 ± 6.6	18.5 ± 9.8			
% adults	Host larvae	82.6 ± 3.6	60.3 ± 3.1	F = 1.1 df = 1,9 P > 0.05	F = 0.01 df = 1,9 P > 0.05	F = 0.98 df = 1,9 P > 0.05
	Plastic sheet	83.3 ± 3.4	91.7 ± 8.3			

removed from *G. mellonella* larvae (*i*) or laid on the plastic sheet (*ii*), the sex ratios (males:females) were 1:1 (*i*) and 1.2:1 (*ii*) in the 1st generation and 1.1:1 (*i*) and 1.3:1 (*ii*) in the 2nd generation. This parameter was not significantly affected by the oviposition substrate either in the 1st ($\chi^2 = 0.07$, $P > 0.05$) or 2nd generation ($\chi^2 = 0.11$, $P > 0.05$). It was not possible to continue the experiment for further generations, because all the 2nd generation adults obtained from out-of-host eggs died 1-2 days after emergence, before the females oviposited. The 2nd generation adults obtained from eggs removed from the host integument survived and, after mating, the females laid eggs on *G. mellonella* larvae. One week after emergence, these adults were transferred into the stock-colony. The parasitoid body size was not measured in this study. In all treatments, however, the adult body size as well as morphology appeared normal.

Discussion

The results confirmed that, for one generation, the eggs laid out of host are competitive with those removed from *G. mellonella* larvae to rear *E. larvarum* on an artificial medium. Two main obstacles have however to be removed to reach the main goal of excluding the host insect from the line of production of *E. larvarum* for more than one generation. The first is that the eggs laid on the plastic sheet were dramatically fewer compared to those oviposited on host larvae (and their number even decreased with generations, as below discussed). It is well known that oviposition in parasitoids is a complex process involving chemical and physical cues (Vinson, 1976; Godfray, 1994). As regards the oviposition substrate effect, in order to increase the number of *E. larvarum* eggs laid out of host, and ultimately obtain the direct parasitoid oviposition on an artificial medium, it will therefore be necessary to identify the critical cues that elicit host recognition and acceptance by this tachinid. Some knowledge is available in the literature describing how chemical and physical factors influence oviposition by tachinids that lay eggs/larvae on or in the host body. For example, a chemical that stimulated larviposition by *Eucelatoria* sp. was extracted from larval cuticles of the noctuid host *Heliothis virescens* (F.); female flies repeatedly and carefully examined artificial hosts coated with cuticle extract, but did not deposit maggots (Burks and Nettles, 1978). Weseloh (1980) showed that females of *Compsilura concinnata* (Meigen) were less attracted to freeze-dried gypsy moth larvae extracted with hexane compared to non-extracted ones and suggested that a host integumental chemical elicits host recognition. About physical stimuli, for different tachinids host movement proved to be important, though not essential, for oviposition (Monteith, 1956; Burks and Nettles, 1978; Stireman, 2002). Tanaka *et al.* (1999) showed that host shape apparently influenced oviposition by *Exorista japonica* Townsend, which laid eggs on artificial hosts consisting of semi-cylindrical black rubber tubes. Also in their study, however, flies oviposited a very few eggs on artificial compared to natural hosts. All these elements suggest that several physical and chemical oviposition cues need to be investigated for *E. larvarum* to be stimulated to lay more eggs on artificial substrates. Chemical and physical cues proved to be essential to induce oviposition by some hymenopterous parasitoids for which continuous *in vitro* culture methods have been developed (Grenier *et al.*, 1993; 1998; Guerra *et al.*, 1994; Consoli and Parra, 1999).

The second main obtstacle to the continuous *in vitro* culture of *E. larvarum* is the generation effect. In fact, for both oviposition substrates (host larvae or plastic sheet) the number of eggs laid by the *in vitro*-reared 1st generation females was significantly lower compared to the parental females. The negative generation effect on the number of eggs laid was even more evident for the females obtained from out-of-host laid eggs. Egg hatching also significantly decreased over generations, though it was not significantly influenced by the oviposition substrate. The generation effect did not significantly affect the other development parameters, though fewer 2nd generation puparia were obtained, especially from out-of host eggs. These results suggest that the quality of the *in vitro*-cultured *E. larvarum* decreases over generations, especially if the host is completely excluded from the parasitoid line of production (i.e. not even used for collecting eggs). This is also proven by the fact that the 2nd generation females and males obtained from eggs laid on the plastic sheet died soon after emergence, before any egg was laid. The maintenance

of insect quality over generations represents one of the main concern for mass culture of parasitoids and predatory insects, especially when the rearing is performed on artificial media or diets (Grenier and De Clercq, 2003; Riddick, 2008). In the case of *E. larvarum*, previous work showed the non-optimal composition of the skimmed milk-based artificial medium used in the present study, as the medium-reared females oviposited fewer eggs on *G. mellonella* larvae compared to the *in vivo*-reared ones. A correlation between the amino acid deficiency and imbalance, which was found for the *in vitro*-reared larvae, and the lower number of eggs laid was suggested (Dindo *et al*, 2006). Further biochemical parameter assessment studies may help to gain a better understanding of *E. larvarum* nutritional needs and hence change the medium composition, so as to improve *in vitro*-grown parasitoid performance, even over generations.

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Authors' addresses: Elisa MARCHETTI (elisa.marchetti3@unibo.it), Piero BARONIO (piero.baronio@unibo.it), Maria Luisa DINDO (corresponding author: marialuisa.dindo@unibo.it), DiSTA - Entomologia, *Alma Mater Studiorum* Università di Bologna, viale G. Fanin 42, 40127 Bologna, Italy.

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