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# Actual possibilities of mass production of the parasitoid *Exorista larvarum* (L.) (Diptera: Tachinidae) on oligidic diets. (*) (1)

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Our research group has been concerned for a long time with the *in vitro* rearing of insect parasitoids. Initially, the general issues involved were dealt with and five overviews giving the results progressively obtained by various authors were published [Mellini, 1975 and 1994a; Campadelli & Dindo, 1987; Bratti 1990 and 1991 (pub. 1994)]. Subsequently, experimental research was carried out, mainly on Diptera Tachinidae. In particular, a member of the research team, Mellini, has been studying the behaviour and the host-parasitoid relationship of this family for a long time and, since 1954, has published about 70 papers on this topic.

Undoubtedly, the greatest success for *in vitro* rearing has so far been obtained with Terebrantia, especially with oophagous Chalcidoidea, which have been mass-produced *in vitro* and utilized in biocontrol programmes by Chinese entomologists, first amongst whom Wu *et al.* (1982). These results, however, may depend on the fact that till now most studies concerning *in vitro* rearing as well as parasitism in general have involved Terebrants. Actually, it was our belief that tachinid flies may be cultured *in vitro* more easily than Hymenoptera Terebrantia, given their close relationship with Calliphoridae and Sarcophagidae, most of which are zoonecrophagous and zoosaprophagous. In fact, tachinid larvae are quite hardy, often displaying simple relationship with their host and, being pneustic, may grow quite rapidly. Moreover, most species are polyphagous and may readily adapt themselves to new hosts, as can be easily demonstrated in the laboratory. It may therefore not be really necessary to study the biochemical composition of the tachinid parasitoids and their victims in order to set up suitable artificial diets.

Initially, some success was achieved, mainly by Bratti and co-operators, in the rearing of *Pseudogonia rufifrons* Wied. (Bratti & Monti, 1988; Bratti, 1989; Bratti & Benini, 1991; Fanti & Bratti, 1991), *Archytas marmoratus* (Town.) (Bratti, 1993)

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and *Eucelatoria bryani* Sabr. (Bratti & D'Amelio, 1994) on meridic and sub-natural diets.

Subsequently, our research was concentrated on *Exorista larvarum* (L.), a typical idiobiont, as it was felt the time had come to test simplified, inexpensive oligidic diets, the only ones that may permit parasitoid mass production for biological control programmes. All the above mentioned tachinid species have been continuously reared for some years in our laboratory on the factitious host *Galleria mellonella* L. They display the 4 basic parasitization modes typical of Tachinids.

*E. larvarum* is a larval parasitoid of Lepidoptera, more than fifteen families having been reported amongst its hosts. It belongs to Exoristinae, which is considered the most primitive subfamily of Tachinidae (Richter, 1991). Females lay macrotype dehiscent eggs mostly upon advanced larval stages. At 25-26°C the incubation period is about 3 days. The newly-hatched larvae penetrate the host integument in front of the egg and bore into the host body, but keep the end of the last abdominal segment closely associated with the entrance hole. An integumental primary respiratory funnel is thus formed so that the posterior pair of spiracles is kept in contact with atmospheric oxygen. Parasitoid larval growth starts immediately and continues without interruptions, independently of host age at parasitization. At 27°C the larvae attain maturity in about one week and soon pupate, generally outside the host or its cocoon when this is present. As the parasitoid is gregarious, puparium weight depends both on host size and superparasitization level. In all our studies on the *in vitro* culture of *E. larvarum*, eggs were collected from heavily superparasitized *G. mellonella* larvae and then placed on the media. Removal of the eggs from host integument soon after oviposition was rather easy, after which the eggs were disinfected by dipping them three times for 5 minutes in 1.3% formaldehyde, rinsed with sterile distilled water and transferred onto the thickened diet. In fact, the original liquid diet has to be gelled by adding agar (about 1.5%) to prevent the parasitoid larvae from being submerged and dying from asphyxia. The newly-hatched larvae may therefore behave similarly in the media as they do in the host, sinking sub-vertically into the pabulum (though not immediately) and keeping the posterior part of abdomen in the entrance hole, which leads to the formation of a sort of "respiratory funnel". The funnel walls harden and become pigmented due to the action of material excreted through the anus (Gardenghi & Mellini, 1995). Third-instar larvae abandon the funnel and dig wide holes deep down into the diet, which is eventually broken into big fragments. Pupation generally occurs on the diet surface.

In six subsequent studies, the diets and rearing techniques were progressively improved, leading to more and more satisfactory results. The original diet, which had already been tested for *P. rufifrons* with poor results (Mellini *et alii*, 1991; Dindo & Campadelli, 1993), contained 75% bovine serum, 20% extract of newly-formed *G. mellonella* pupae and 5% additives (trehalose, agar, etc.). Diets were always supplemented with 0.006% gentamycin sulphate, to prevent bacterial contamination although *E. larvarum* larvae are quite resistant to diet contamination by moulds and/or bacteria, especially during the third instar. Bovine serum was

utilized as the main ingredient given that in nature some Tachinidae are known to accidentally develop at the expense of mammals, similarly to various Diptera Cyclorrhapha, capable of causing myasis (Mellini, 1992).

This diet proved indeed suitable for *E. larvarum*. Mean adult yields were quite high (36% and 59%, as determined on the basis of the original number of eggs number and on the number of hatched eggs, respectively). Moreover, parasitoid quality was good, i.e. adults mated and females readily oviposited on *G. mellonella* larvae, producing a normal second generation in the host (Mellini *et alii*, 1993a).

Next step was to replace the expensive pupal extract with the much cheaper pupal homogenate that can be easily obtained by squeezing pupae in a syringe and then removing the large pieces of cuticle (Mellini *et alii*, 1993b). This change even tended to improve parasitoid production.

Subsequently, pupal homogenate was replaced with homogenate of last-instar larvae. This permitted to simplify the technique for obtaining host material (Mellini & Campadelli, 1994). The replacement was successful because the larvae of *E. larvarum*, that is an idiobiont, are capable of developing without being triggered by the high ecdysteroid titres existing in the newly-formed pupae.

An other change was made by replacing the expensive plastic multi-well plates, required for rearing solitary parasitoids, with glass Petri dishes, in which the gregarious *E. larvarum* larvae can be cultured in groups, instead of individually. Since Petri dishes can be sterilized and, therefore, reutilized many times, they are more convenient as rearing containers for this parasitoid than plastic plates, which are disposable and can therefore only be used once. Compared to individual culture, mass-rearing in Petri dishes even resulted in qualitative improvements of parasitoid production, as mass-reared individuals were seen to reach higher puparium weights and to be more synchronized in their development (Mellini *et alii*, 1993b).

It was then demonstrated that the expensive agar, which furthermore requires careful preparation, may be advantageously replaced with previously sterilized absorbent cotton, a much cheaper physical support for the liquid diet (Mellini *et alii*, 1993b).

Further improvements were obtained by reducing the amount of bovine serum, which actually seems to be rather poor in nutrients, from 75% to 70% and making up the 5% difference with yeast extract or soya meal. This nutritional enrichment resulted in larval growth rate acceleration and in considerable increase in puparium weight (Mellini & Campadelli, 1994).

Other efforts were aimed at decreasing host material in the media. In the original diet, when pupal homogenate was reduced and replaced with equal amounts of bovine serum, puparium yields, weights and adult emergence progressively dropped. When host material was eliminated, first instar larvae were not capable of moulting, although they survived for more than one month (Mellini *et alii*, 1993b). In subsequent studies, when host homogenate (whether pupal or larval) was reduced from 20% to 10% and the 10% difference was made up with an equal amount of powdered yeast extract, adult yield tended to be higher than in the original diet containing 20% host material (Mellini & Campadelli, 1994).

Further experiments showed that in diets containing the standard amount of

75% bovine serum, *G. mellonella* larval homogenate can be completely replaced with greater or lesser success with yeast extract, fresh chicken egg yolk, a mixture of the latter ingredients (15%↔5%) or powdered pupae of *Bombyx mori* L. (Mellini & Campadelli, 1995a).

Bovine serum was also eliminated and replaced with sterile distilled water, in media containing 10% larval homogenate and a mixture of yeast and egg yolk in equal amounts (8%) (Mellini & Campadelli, 1995a).

Further studies demonstrated that: 1) trehalose (2%), which was always present in the media, can be replaced with 2% saccharose, a cheaper ingredient; 2) yeast extract is indispensable, especially in diets devoid of host material, only a few products containing high amino acid levels being, however, suitable (Mellini & Campadelli, 1995b).

Thus, the ultimate goal of eliminating the two basic ingredients of the original diet, i.e. bovine serum and *G. mellonella* homogenate, was progressively attained, with the final diets containing four cheap, widely available commercial components, namely sterile distilled water (78%), yeast extract (15→5%), fresh chicken egg yolk (5%→15%) and saccharose (2%). The highest puparium yield (53.9% as determined on the basis of the number of eggs originally placed on the diet) was obtained in the medium containing the higher yolk concentration. In all treatments, however, puparium weights were lower and similar to those usually obtained in the host *G. mellonella* (35.4-37.6 mg). Emergence rates varied between 71 and 81% in the different treatments. The adults obtained in these simple diets were capable of producing a new generation *in vivo*. Moreover, when the above mentioned diet was added with 5% homogenate of *G. mellonella* larvae, the puparia weighed more than 50 mg on average (Mellini & Campadelli, 1995b).

Other studies (Mellini & Campadelli, 1995b) were aimed at testing the suitability of powdered and skim milk and soya meal. It was thus demonstrated that skim milk can be used in place of distilled water to dissolve solid ingredients, while soya meal was found to be effective when used in small amounts (2-4%). Moreover, the use of soya meal permits to reduce agar levels from 1.5% to 1%. A more recent study (Mellini & Campadelli, 1996a) was conducted concentrating on rearing techniques rather than on diet composition. It was shown that the diet utilization index (i.e. the ratio between puparium biomass and diet weight) can be increased by reducing the diet quantity rather than multiplying the number of parasitoid eggs. In fact, when parasitoid population density increases, the number of newly-hatched larvae that escape from the medium also tends to increase. When however the diet tickness in the Petri dish is lower than 4 mm, the diet slowly tends to dry out, which results in a small decrease in puparium weight, while puparium yields are seemingly unaffected. Very recently, Mellini & Campadelli (1996b) showed that 225-175 mg is the optimal amount of medium per larva, as in such conditions the puparia obtained weighed on average 41-36 mg and the diet utilization index was about 18-20%. These values are similar to those usually observed in *G. mellonella* larvae in which a single puparium is formed (diet composition: 75% skim milk, 5% homogenate of *G. mellonella* larvae, 6% yeast powder, 12% egg yolk, 2% saccharose).

Satisfactory results at rearing *E. larvarum in vitro* were obtained also by our

co-operator A. Bratti, who worked for some time in the laboratory of Dr. W. C. Nettles in Texas, on a bilateral project between Italy and the United States funded by the Italian National Research Council. First, he utilized meridic diets (Bratti & Campadelli, 1993) and, subsequently, tissue culture media-based substrates, added with the same ingredients employed by us, albeit in different amounts (Bratti & Coulibaly, 1995). Moreover, his results showed that these diets could be effective, at least from a nutritional point of view, for two other tachinids besides *E. larvarum* (Bratti, 1994). Subsequently, he developed diets devoid of host material, based on tissue-culture media, bovine serum, yeast extract, wheat germ and 10-15% chicken egg yolk. These diets produced 51-66% adult yields and the puparia weighed more than the ones usually obtained in *G. mellonella* larvae.

*E. larvarum* was also reared on diets based on commercial meat homogenates for babies (Dindo & Farneti, unpublished data). This component had already been tested with some success in oligidic diets for the chalcidid *Brachymeria intermedia* (Nees) (Dindo *et alii*, 1994). For the artificial culture of *E. larvarum*, the homogenate was first added with 10% *G. mellonella* pupal extract and then with chicken egg yolk (10%), yeast extract (5%), saccharose (2%) and water. Adult yields (determined on the basis of the number of eggs originally placed on the media) were 40.7 and 46.7%, on the diet with and the one without host components, respectively. The mean weights of the puparia formed in both diets were however of 50-60 mg, thus higher than those usually obtained in the factitious host *Galleria*.

## Conclusions

Our research group has till now published more than 30 papers on the *in vitro* rearing of tachinids and 7 on that of chalcidids. Best results were obtained with *E. larvarum* which was easy to rear and gave high adult yields even when very simple and inexpensive diets devoid of host material were employed. To date this is the best result ever obtained with tachinids.

Powdered yeast extract with high amino acid levels proved to be a key ingredient in the promotion of parasitoid development and, in particular, larval moulting from the I to the II instar. On the other hand, chicken egg yolk appeared to be an excellent component for increasing puparium weight. In fact, the puparia obtained in diets enriched with chicken egg yolk even weighed 30-40% more than those formed in the host. In the diets, the development times from egg to adult were 5-6 days longer than those in the host, the latter being generally 12-14 days at 26°C.

The greatest drawback was that, at the beginning, a number of newly-hatched larvae appeared to jib at sinking into the pabulum. Some of them even escaped, creeping up the Petri dish walls and on the cover, where they died a few days later. Moreover, about 10% of the eggs failed to hatch, a few larvae died while growing and from 15-20% puparia adults did not emerge. On both bovine serum-based and simplified oligidic diets, adult yield rates, determined on the basis of the number of eggs placed on the media, were therefore somewhat below 50%,on

condition of the media not being widely contaminated by moulds and bacteria. These rates were similar to those usually obtained in the factitious host *G. mellonella*, when host larvae undergo parasitization at the most suitable time, i.e. within the first days after moulting to last instar. The high losses usually occurring *in vivo* are due both to the fact that straight after oviposition a number of eggs come off the host and to high superparasitization level, which often lead to the death of one or two superparasitoid larvae. It has to be pointed out that adults emerged even from dwarf, yellowish puparia, as well as from anomalous puparia with wrinkled walls and therefore with evident larval features, except for pigmentation. On the other hand, sometimes puparia which appeared normal in size, shape and pigmentation, failed to emerge as adults.

In the laboratory, the adults obtained even in the most simplified diets devoid of host material were fully viable, mated and the females oviposited on *G. mellonella* larvae. The eggs were placed on the diet and produced a normal second generation, which was in turn capable of producing a third generation *in vitro*. The efficiency of the adults obtained *in vitro*, however, has not yet been tested in the field.

We suggest that the complete elimination of host material from the diet is, from a theoretical point of view, quite important, but may not necessarily result in great practical gains. Actually, host homogenate may secure a sort of "biochemical bridge" between the two symbionts, which may enhance parasitoid production and reduce the problems possibly associated with continuous multi-generation artificial mass culture, i.e. alteration of host searching behaviour of parasitoids.It is well known, however, that the females of a number of polyphagous parasitoids prefer to parasitize hosts of the same species as the one from which they emerged. Moreover, in our studies, when diets relatively poor in nutrients were added with 5% host larval homogenate, the puparia weighed considerably more than those usually obtained *in vivo*. At moment, however, the host has necessarily to be reared in order to obtain the *E. larvarum* macrotype eggs, as oviposition by the female on artificial substrates has not yet been achieved. Therefore, the diet is not that much more expensive even if it is added with small amounts of host larval homogenate, which is easy to prepare.

It should also be noted that the preparation of the simplified diets is very easy and that the ingredients are relatively cheap so that large-scale *in vitro* production of *E. larvarum* may be close to being achieved (Mellini *et alii*, 1994). The only major drawback is that extreme care must be taken to maintain asepsis so as to avoid diet contamination. In fact, although *E. larvarum* is more resistant to contamination than other tachinids, if moulds and bacteria massively cover the diet when the larvae are still in the first or second instar, parasitoid development is retarded, puparium production is reduced and the number of dwarf puparia increased.

In general, our studies demonstrated that *E. larvarum* exhibits a rather high degree of tolerance to variations in diet composition, but less to a number of physical factors. In fact, if diet is too soft or rather too thick or, worse, if an even very thin layer of liquid is present on its surface, most parasitoids die as young first instar larvae.

As already mentioned, with three other tachinids the results were not in the least comparable to those obtained with *E. larvarum* when using the same rearing techniques and similar diets, or when more complex meridic or even sub-natural substrates were employed (Mellini, 1994). Puparium yields and weights and emergence rates were always quite lower than those usually obtained in the host, while in some cases no adults emerged from the puparia. Moreover, the time for completion of larval development was very long. We assume that this was not really so much dependent on the suitability to the parasitoid of diet nutrient compositions, but rather to the physiological and ethological features of parasitic larvae. It should first of all be borne in mind that *E. larvarum* is an idiobiont and that first-instar larvae develop regardless of host hormonal balance, whereas *Archytas marmoratus* and *Pseudogonia rufifrons* are koinobionts and moult to second larval instar only when they are triggered by the ecdysteroids released at host pupation. Moreover, *E. larvarum* larvae display a very simple behaviour, as from the beginning they anchor themselves to a primary integumental respiratory funnel. They are, therefore, always in contact with atmospheric oxygen. On the opposite, the 3 other species form secondary respiratory funnels, after staying free for some time in the host lacunoma. It is also worth noting that even the endophagous larvae of *Eucelatoria bryani*, which is an idiobiont, breathe through the host tracheae. In conclusion, the first and second instar larvae of *A. marmoratus*, *P. rufifrons* and *E. bryani* display a more complex behaviour when in the host, a behaviour which is unlikely to be reproduced in artificial diets. In fact, when in the media, young larvae end up living in an oxygen deficient conditions. As a consequence, they grow very slowly and sooner or later die. All these problems do not arise with *E. larvarum* which in the diet displays the same behaviour as in the host so that artificial rearing may actually even facilitate investigations on some aspects of larval biology. It may therefore been concluded that in view of the importance for rearing of behavioural characteristics, good results may be obtained with our diets for other tachinids which induce the formation of primary integumental respiratory funnels in the host.

SUMMARY

Attempts have been made by our research group to rear *in vitro* 4 tachinid species. Successful results, such as to permit the mass production in artificial media, however, have so far been obtained only for *Exorista larvarum*, a gregarious larval parasitoid of many Lepidoptera. Ease of rearing *E. larvarum in vitro* may be related to the simple relationship between this parasitoid, which is an idiobiont, and its host and to the fact that the larvae induce primary integumental respiratory funnels so that they may display similar behaviour in the host and in the gelled diet.

Testing was mainly carried out using oligidic diets. The complete development of *E. larvarum* was obtained on various media, the simplest of which containing distilled water (or skim milk), yeast extract, chicken egg yolk and saccharose. Rearing techniques were progressively simplified. In particular, the plastic multi-well plates, that were originally used as rearing containers, were replaced with glass Petri dishes in which the larvae can be cultured gregariously, instead of separately. Moreover, agar was substituted with cotton, a much cheaper physical support for the liquid diet.

In the most suitable media, the percentage yields of adults, based on the number of eggs placed on the diet, were similar to those usually obtained in the factitious host *Galleria mellonella* L. (≈50%). In the laboratory, adults obtained *in vitro* mated, parasitized *G. mellonella* larvae, and produced a normal second generation.

When some of the above diets were added with 5% homogenate of *G. mellonella* larvae, the puparia weighed considerably more than those usually obtained *in vivo*. For this reason, as well as to secure a sort of "biochemical bridge" between the two symbionts, we think that it may be better not to completely eliminate host material from the artificial medium. At the moment, however, the host has to be necessarily reared in order to obtain *E. larvarum* macrotype eggs to be transferred onto the diet. In fact, oviposition by the adult on artificial substrates has not yet been obtained.

## Concrete possibilità di allevamento massale su diete oligidiche del parassitoide *Exorista larvarum* (L.) (Dipt. Tachinidae)

RIASSUNTO

Il nostro gruppo di ricerca ha tentato l'allevamento *in vitro* di 4 specie di Tachinidi, ma solo con *Exorista larvarum* ha ottenuto risultati pienamente soddisfacenti e tali da rendere fattibile nell'immediato un allevamento massale di questo parassitoide a scopo di lotta biologica. Si ritiene che la facilità di allevamento di questo tachinide dipenda dal fatto che è un idiobionte e che le sue larve inducono imbuti respiratori tegumentali primari per cui possono comportarsi nel pabulum quasi come nell'ospite vivo.

Sono state saggiate soprattutto diete oligidiche. Attraverso successive prove si è giunti a formulazioni estremamente semplificate comprendenti acqua (o latte scremato), estratto polverulento di lievito di birra, tuorlo d'uovo e saccarosio. Anche le tecniche sono state semplificate: le larve vengono allevate in gruppi di svariate decine, anzichè isolate, entro capsule Petri, ed il costoso e "laborioso" agar è sostituito dall'economico cotone idrofilo che funziona come supporto scheletrico per le diete che sono, in ogni caso, liquide.

La resa in adulti è risultata, nelle diete più idonee, pari a circa il 50% delle uova messe in coltura e quindi a un livello simile a quello conseguibile nell'ospite naturale. Gli adulti così ottenuti sono in grado di attaccare, in laboratorio, le larve dell'ospite di sostituzione *Galleria mellonella* L. e di dare origine, a loro spese, ad una nuova generazione pienamente efficiente.

L'aggiunta di piccole dosi di omogeneizzato larvale di *Galleria*, a parte delle suddette diete, consente il raggiungimento di pesi notevolmente superiori a quelli conseguiti *in vivo*. Per questo motivo, e per assicurare una sorta di "ponte biochimico" tra i due simbionti, si ritiene conveniente non eliminare completamente dal substrato trofico la componente dell'ospite. D'altro canto, a tutt'oggi, questo deve essere in ogni caso allevato per poter disporre delle larve indispensabili per la ovideposizione da parte di *Exorista* e così avere a disposizione le uova macrotipiche da traslare sulle diete artificiali.

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