Boll. Ist. Ent. "G. Grandi" Univ. Bologna, 49: 187-196, 1994.

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Qualitative improvements in the composition of oligidic diets for the parasitoid *Exorista larvarum* (L.). (*)(1)(2)

1. Introduction

The present paper, which is the third report of the series of studies conducted by us on *Exorista larvarum* (L.), further explores the possibility of optimizing oligidic diets for this tachinid with a view to its mass production for biological control purposes. This polyphagous parasite attacks the larvae of numerous plant foliage-eating species such as *Lymantria dispar* L. and *Hyphantria cunea* Drury. The latter species has been imported to Europe from North America and in the last ten years has been rapidly expanding also in Italy.

Previous studies conducted by us on the *in vitro* rearing of *Exorista* have shown that their larvae are highly adaptable to trophic substrates of simple composition and easy to prepare. Good results have in fact been obtained by Mellini *et al.* (1993 a,b) and Mellini and Campadelli (1994) with diets based on appropriately integrated bovine serum as well as by Bratti and Campadelli (1993) and Bratti and Coulibaly (1994) on tissue culture-based diets. For a number of the diets employed, no appreciable difference was noted in parasitoid production between the *in vitro* reared ones and those reared *in vivo* on the factitious host, *Galleria mellonella* L. Adults developed on an artificial pabulum were seen to be perfectly efficient, while the inseminated females were seen to be capable of attacking the *Galleria* larvae and their eggs to give rise to a new parasitoid generation.

Up till now, these have been by far the best results obtained in the rearing of Diptera Tachinidae on artificial diets. Notwithstanding these good results, our experiments are continuing in an attempt to further simplify the rearing of this parasitoid both in relation to the composition of the diet and the methods for preparing it and to the rearing techniques themselves so as to reduce the already low production costs to a minimum.

With regards the rearing techniques, we have not as yet been able to induce the female to lay its eggs directly on the artificial diet so that the entire parasitic

(*) Accepted for publication: December 14, 1994.

(1) Studies on Diptera Tachinidae. LXIV contribution.

(2) Research supported by the Project of the Italian Ministry of Agriculture and Forestry (MAF) "Lotta Biologica e integrata per la difesa delle piante agrarie e forestali".

cycle may take place on the diet itself. This objective is in actual fact not easy to achieve as *Exorista* directly contaminates the host, the females being stimulated to oviposit only on the active stages of the host when it is moving. Despite this drawback, however, the fact that macrotype eggs may be repeatedly deposited on the same larva without any limits so that superparasitization reaches incredible levels makes it extremely easy to collect these eggs, which can usually be readily removed from the integument of the host.

Generally, one of the major drawbacks in the use of artificial diets is the fact that operations must be carried out in absolutely aseptic conditions both with regards the pabulum and the containers and instruments employed, otherwise there is a risk of severely decimating or even completely destroying the biological rearing material. Notwithstanding this general drawback, however, *Exorista* has proven at least to be considerably resistant to the contamination of the pabulum by bacteria and moulds. If contamination does not occur at an early stage, many larvae, therefore, manage to reach maturity and to form puparia, even if undersized, from which, nevertheless, adults usually emerge.

Given the good results obtained with most of the diets employed and the not infrequent contaminations to which they were subject and which were responsible for the considerable decrease in the yield and weight of puparia, we have deemed it preferable to give the general mean values for the various replications rather than to elaborate the statistical data. Moreover, there were practical reasons as well for adopting this approach in this work given that the differences in productivity which can be detected by applying statistical methods were only slight and possibly misleading for the reasons given above, and that they may not be all that important if the economic aspects are also considered.

II. Materials and Methods

In preparing the diets and in rearing, aseptic methods were adopted as already described in the previous papers of this study.

The standard pabulum employed, to which the appropriate modifications were made, was that which had already given good results in the second paper of this series (Mellini *et al.*, 1993b), and comprised bovine serum (75%), *Galleria mellonella* larval homogenate (20%), and additives (5%).

The diets were invariably agarized and placed on glass Petri dishes with a diameter of 5 cms; 13 cc of pabulum and about thirty eggs were introduced. A minimum of about 0.45 g of trophic substrate (not all eggs hatch) was therefore available to each larva, an amount which was more than sufficient for larval development. This overabundance of food was necessary in order to ensure that, apart from possible contamination, the difference in the weight of the puparia eventually detected amongst the various tests did not depend on the quantitative but on the qualitative characteristics of the pabulum, even in the case of maximum parasite production.

The material was incubated up to adult emergence in a non-climatic room at temperatures between 25 and 28 C.

A. Egg disinfection. This preliminary operation is extremely important as the egg may be a vehicle for polluting microorganisms in the trophic substrate, thus altering the results.

Three substances with disinfecting properties normally employed for this purpose were compared, namely:

- Ethyl alcohol (60% proof): applied once for 7-8 seconds, followed by washing with sterile water for three times;
- 0.26% sodium hypochlorite: dipping in the solution for 1 minute and subsequent quick washing with sterile water for three times;
- 1.3% formaldehyde: applied three times for 5 minutes each, followed by washing with Schneider liquid each time.

Sterile water was used as a control, three washings being made for a total time of one minute.

In order to ensure greater uniformity, the eggs removed from the highly superparasitized larvae within an hour of deposition were gathered in a single group and then randomly distributed among the various tests.

At first, in order to ascertain whether the disinfectants were toxic, the percentage of treated eggs hatching, subdivided in groups of 20 eggs each on slightly moistened blotting paper discs, was determined. The test was repeated four times.

Next, in order to determine the efficacy of the various disinfectant solutions in preventing diet pollution during inoculation, the treated eggs were placed on standard pabulum, first in multi-cell dishes and then in Petri dishes, the tests being repeated for another four times. The diet was then checked for any pollution and the yield and weight of the puparia obtained in the various tests were determined. It should be noted that, despite all the care and attention given to these operations, the risk of secondary pollution of the diet (that is, pollution not depending on the eggs being introduced) is always possible.

B. Replacement of chrysalis with larval homogenate. These tests were performed for the purpose of further simplifying diet preparation. As *Exorista* is a parasitoid which is independent from host physiology, it does not require the ecdysteroids present in large quantities in the young chrysalises of the host. It may therefore be reasonably presumed that the necessary host material may be safely constituted by larval rather than chrysalis homogenate. The preparation of the former is easier as there are no cocoons to be opened. In fact, having emerged from the pabulum, the mature larvae are free so that they can be easily collected in large quantities.

The only significant difference between chrysalis and larval homogenate is that the former contains only small fragments of cuticle (the larger ones, clearly visible thanks to their brownish colour, being eliminated), while the latter contains, albeit in shreds, the entire cuticle as this is difficult to remove.

Two standard diets were thus compared, the only difference between them being the type of homogenate (the percentage of which being 20% in both cases), namely diet A, containing chrysalis homogenate, and diet B, containing larval homogenate.

The tests were repeated six times.

C. Enriched diets. As we were convinced that the standard diet employed by us in previous experiments tended to be poor in nutrients given that its main ingredient, bovine plasma, contains a high percentage of water (about 90%), it was decided to add protein-rich substances. The amount of serum was therefore reduced from 75 to 70%, the 5% difference being replaced with other products. Following this procedure, five diets were prepared, namely:

A. The standard diet, used as control;

B. A diet added with yeast extract;

C. A diet added with commercial Plasmon veal homogenate;

D. A diet added with lyophilized veal homogenate, which was therefore richer in nutrients than diet C;

E. A diet added with soya meal.

The tests were repeated four times.

In addition to experiments with these enriched diets, other tentative isolated tests were conducted with other diet enriching additives, namely wheat germ in place of soya meal, and de-bittered yeast extract in place of the standard extract.

D. Reduction of host homogenate quantities. The quantity of standard host homogenate, whether made from chrysalises or larvae, was reduced by half, from 20 to 10%. The 10% difference was replaced with various amounts of powdered yeast extract. It should be noted that this latter ingredient, being practically anhydrous, is much richer, for equal weights, in nutrients than the standard homogenate. In the product composition adopted by us, in fact, the protein fraction is close to 50%, the glucide one to 30%, while lipids amount to just 3-4%.

In previous experiments (Mellini *et al.*, 1993b) equally aimed at reducing the quantity of host-derived material, host homogenate was simply replaced by increasing the percentage of bovine serum, which is notoriously poor in nutrient substances.

Four tests were conducted. A and B both contained chrysalis homogenate of which B half the amount of A (the difference being made up by yeast), while C and D contained larval homogenate of which C half the amount of D (the difference also been made up by yeast).

The tests were repeated five times.

Finally, it is worth remembering that in addition to the type of diet, at least another two factors may occasionally affect puparia yield and weight despite all the care and attention given to rearing operations, namely pabulum pollution by bacteria and moulds and the formation of a thin liquid film on the pabulum surface, which hinders penetration of the newborn larvae into the trophic substrate.

In evaluating puparia yield and mean weight, those which developed with a few days' delay were not taken into consideration; their weight, in fact, was often much lower than that of the others, regardless of the diet on which they were reared. It is nevertheless worth observing that apparently normal adults emerged from a considerable percentage of underdeveloped puparia (even with a weight of just 15-20 mg as against the mean weight of 45-50 mg).

III. Results

A. Methods for egg disinfection.

1. Percentage of hatched eggs. The various replications performed by placing the eggs on moistened blotting paper indicate that embryogenesis is not adversely affected by the disinfection method employed. The percentage of hatched eggs varied from 80 to 90% in the various tests so that it may safely be said that there is no substantial difference amongst them nor with the control, which simply involved washing the eggs with sterile water. No appreciable difference was observed in the time taken for embryo development either.

2. Pollution of the pabulum. As was to be expected, pabulum pollution appeared earlier and more frequently in the tests conducted on non-disinfected eggs. Notwithstanding, moulds and bacteria were seen to be able to rapidly spread even in diets with the eggs disinfected in the various solutions. This pattern can be accounted for if one considers that pollution may derive from various sources, despite the care taken by the operators working in a laminar-flow hood and with appropriately sterilized instruments and containers.

Diet contamination may lead to a more or less sharp drop in puparia yield and weight. All considered, this situation is comparable to that occurring in nature when the parasitized host is seriously affected by infective diseases. As *in vivo*, also *in vitro* the consequences were seen to be all the more serious the earlier and the more widespread was the pollution of the trophic substrate.

3. Puparia yield. The duration of post-embryo development and the number and weight of the puparia were seen not to vary significantly in relation to the method adopted for disinfecting the eggs on condition, of course, of the pabulum not being seriously polluted at an early stage. It was however observed that a higher number of puparia with a somewhat higher weight tended to develop in the tests conducted with eggs washed with the Formalin solution.

More in general, it should also be noted that the *Exorista* larvae exhibited a good resistance to the diffusion of the microorganisms polluting the diet and an ability to penetrate into the diet where this had not been visibly altered and even to come to the surface, in the midst of thick and widespread layers of mould, without being overwhelmed, especially if at the 3rd instar.

Regarding the puparia dwarfism due to pollution of the trophic substrate, it was observed that this phenomenon does not irremediably compromise adult emergence even if the weight of the pupae drops to half their normal weight or even less.

B. Replacement of the chrysalis with the larval homogenate.

The results of the six replications in terms of general mean values are given in the following table 1.

As can be seen, there are no substantial differences between the two diets. The considerable difference in puparia yield is ascribable to the greater pollution level, a wholly casual phenomenon recorded in two replications of test A.

It may therefore safely be concluded that both larval and chrysalis homogenates are equally valid for the preparation of artificial diets for *E. larvarum*, which is an idiobiontic parasite.

Tab. 1

Diet	No. of puparia	Mean weight (mg)	Emergence(%)
A (with chrysalis homogenate)	118	49.28	67.80
B (with larval homogenate)	143	49.11	70.63

C. Enriched diets.

Considerable differences were observed amongst the various replications. These differences are mostly due to the different levels of pabulum pollution by bacteria and moulds and to the different amounts of condensate forming within the Petri dishes. It is worth stressing again, however, that these adverse factors are essentially casual and do not depend in any way on diet composition.

The following table 2 shows the results obtained.

Tab. 2

Diet	No. of puparia	Mean weight (mg)	Emergence(%)
A (standard)	58	40.56	81.00
B (+ yeast extract)	79	45.64	88.57
C (+ veal homogenate)	63	48.50	84.20
D (+ lyophilized veal)	34	49.06	88.23
E (+ soya meal)	77	59.84	81.81

A comparison of the mean values for the three parameters examined in the various tests shows that:

1. The production of puparia tends to be greater for diets B and E, which employed a pabulum enriched with yeast extract and soya meal, respectively. Moreover, growth was seen to be more rapid in trial B.

2. The weight of puparia tends to be greater in diets B, C and D, which employed a pabulum enriched with yeast, veal homogenate and lyophilized veal, respectively. The highest increase in weight can be observed in diet E, in which soya meal was employed.

3. There is no substantial difference in the percentage of emerged adults in relation to the number of puparia formed and in any case emergence is quite high, varying between 81 and 88%.

It may safely be concluded that the standard diet gives better results when enriched with yeast extract or with soya meal even when these were added in small amounts (5%).

As regards the isolated tentative tests which were also conducted, it may generally be noted that replacement of soya meal with wheat germ gave practically the same results. Similarly, no differences were observed when the standard "Sigma" yeast was replaced with the de-bittered one. Given that this latter yeast is less expensive, if this finding were to be confirmed by further experiments, it could conveniently be used instead of the standard type.

D. Reduction of the amount of homogenate.

Considering the overall mean values for the five replications, it may be seen that there were no substantial differences in puparia yield for the four diets, no appreciable differences in weight being observed either. Considerable differences, however, were recorded in the percentage of emerged adults, which was somewhat higher in the diets employing yeast, as well as in the duration of larval growth, which was shorter when the latter ingredient was used. Yeast, therefore, appears to be a valid substitute for both types of homogenates (whether chrysalis or larval), at least for the quantities employed.

As can be seen in the table 3, halving of the host material (from 20 to 10%) does not adversely affect parasite production on condition that it be compensated for by the same amount of powdered yeast.

Tab. 3

Diet	No. of puparia	Mean weight (mg)	Emergence(%)
A (20% chrysalis homogenate)	73	48.00	68.83
B (10% chrysalis homogenate + 10% yeast)	94	49.17	82.95
C (20% larval homogenate)	92	53.16	65.55
D (10% larval homogenate + 10% yeast)	88	55.35	86.41

IV. Discussion

The experiments reported in this paper have led to a further improvement and simplification in the preparation of bovine serum-based oligidic diets for *Exorista larvarum*.

Egg disinfection. First of all, particular care was given to the disinfection of the eggs removed from the integument of the larvae of the factitious host, *Galleria mellonella*. This operation is of the utmost importance as, unless properly carried out, the outcome of the experiments may be compromised from the very start. The products employed (namely, 60% proof ethyl alcohol, 0.26% sodium hypochlorite, 1.3% formaldehyde) practically gave the same results. Under the conditions in which they were employed, these products did not adversely affect embryogenesis, the percent of newborn larvae being equal to that recorded for the eggs washed with sterile water (80-90%). At the same time, they also prevented the eggs from becoming vehicles for pollution of the pabulum during the initial rearing stages when such an occurrence is fatal. Of course, these disinfectants were not capable of preventing bacteria and moulds from subsequently penetrating into the Petri dishes by other means and thus contaminating the diet. Notwithstanding, it is worth noting that the larvae of *Exorista* proved to be particularly resistant, already in the second and even more in the third instar, to the negative effects of diet pollution. Despite the fact that larval growth is slowed down and that undersized and often deformed puparia develop as mold patches and bacteria plates rapidly grow, a good percentage of apparently normal adults

nevertheless emerge. This situation is fairly similar to that occurring in natural virus or bacteria infected hosts as already reported, for example, by Mellini (1956) for the *Sturmia bella* Meig. tachinid in *Inachis io* L. lepidoptera.

Tests were then conducted using variously modified standard diets which had already given good results in two previous experiments (Mellini *et al.*, 1993a,b).

Quality of the host - derived material. Significant progress had already been made in reducing the costs of this ingredient by replacing the chrysalis extract with a simple homogenate of the same. In the present study we wished to ascertain whether this homogenate could be replaced with another larvae-based one given that, as *Exorista* is an idiobiontic parasite, it does not need the ecdysteroids of the host which are available in great quantities during the early stages of pupal growth. The use of larval material appeared in fact to be more advantageous as it is quicker to prepare given that it is not necessary to open the cocoons as is the case when preparing the chrysalis-based diet.

Findings show that the two types of homogenate give practically the same results.

Nutrient enrichment of the stan dard diet. As we thought the standard diet to be poor in nutrients, it was decided to integrate it with small amounts of substances particularly rich in proteins. The amount of bovine serum, which is made up of 90% water, was reduced from 75 to 70%, the 5% difference being made up of yeast extract (Sigma), a commercial veal homogenate (Plasmon) either of the normal or, alternatively, the lyophilized type, or soya meal. Except for the veal homogenate-enriched diet, the production of puparia tended to increase for all the others as compared to the standard diet. The mean weight of the puparia also increased, and the increase was seen to be particularly marked in the soya meal-enriched diet. Despite these differences, in the end the percentage of adults emerging from the puparia was practically the same for all enriched diets, being in all cases above 80%. The positive results obtained with the addition of small amounts of powdered yeast and soya meal have confirmed our assumption that the standard diet was too poor and encourage us to carry out further experiments employing a greater amount of the enriching ingredients.

Quantitative variations of the host - derived material. As the host-derived homogenate is comparatively more expensive than the other enriching ingredients, attempts were made to reduce its amount from 20 to 10%. The previous experiments in which host-derived homogenate reduction was associated with a corresponding increase in the amount of bovine serum were therefore continued. As the advantages to be gained by enriching the standard diet were confirmed by the results of the present experiments, yeast extract was employed as a substitute for the removed homogenate fraction. Puparia production and weight in the tests in which the homogenate (whether chrysalis or larval) had been halved did not differ from those of the other tests, while the percentage of emerged adults tended to be even greater. In experiments currently being conducted, attempts are being made to further reduce the amount of homogenate which, on the basis of the

results so far obtained, is replaced by a corresponding amount of yeast and/or egg yolk.

More in general, it should be noted that the results of the experiments reported here concerning qualitative and quantitative changes in diet composition have shown that *E. larvarum* is highly adaptable to *in vitro* rearing and that, therefore, from a practical point of view there is no need to formulate complex diets requiring an exhaustive biochemical analysis of the parasites and of their hosts.

SUMMARY

The findings of this third experimental study, the purpose of which was to simplify and to reduce the preparation costs of the already good oligidic diets previously formulated by us for *Exorista larvarum* (L.) have shown that:

A. The same results may be obtained regardless of whether egg disinfection is made using 60% proof alcohol, 0.26% sodium hypochlorite or 1.3% formaldehyde under the conditions specified herein. In fact, all these products were seen not to compromise embryogenesis, with emergence rates ranging from 80 to 90% in all cases, including that of the control simply treated with sterile water. At the same time, they prevent the eggs from acting as vehicles for bacteria and moulds, which are the main agents responsible for early pabulum alterations.

B. Host material, which makes up the standard diet by 20%, may be constituted by larval rather than chrysalis homogenate, the former being much easier to prepare and therefore less expensive. In fact, the mean weight of the puparia and the percentage of emerged adults were found to be practically identical in the two diets.

C. The standard diet comprising 75% bovine serum, albeit giving good results, seems to be rather poor in nutrients. By reducing the amount of serum to 70% and replacing the 5% difference with yeast extract or, alternatively, soya meal, the growth rate of the larva and the weight of the puparia were seen to tend to increase.

D. The amount of homogenate, whether chrysalis or larval, can be reduced from 20 to 10% without any adverse effects being detected on condition, however, that the difference be compensated for by powdered yeast extract. The mean weight of the puparia was found to be constant for both diets, while the percentage of emerged adults tended indeed to increase in the diet employing half the amount of homogenate but compensated for with yeast. The latter component, therefore, has confirmed itself to be an optimum additive for enriching oligidic diets.

Miglioramenti qualitativi nella composizione delle diete oligidiche per il parassitoide *Exorista larvarum* (L.)

RIASSUNTO

Da questo nostro terzo lavoro sperimentale, teso a semplificare e a rendere ancora più economiche le già valide diete oligidiche da noi inizialmente formulate per *Exorista larvarum* (L.), è emerso quanto segue:

A. Per la disinfezione delle uova, alcool a 60° , ipoclorito di sodio allo 0,26% e formaldeide all'1,3%, se impiegati seguendo le modalità indicate, praticamente si equivalgono. Tutti questi prodotti, infatti, non compromettono l'embriogenesi permettendo valori di schiusa varianti tra l'80 e il 90%, come nel testimonio trattato con acqua sterile. Nel contempo impediscono che le uova funzionino da veicoli per batteri e muffe responsabili di precoci alterazioni del pabulum.

B. Il materiale dell'ospite, componente delle diete standard in misura del 20%, può essere rappresentato, anzichè da omogeneizzato di crisalidi, da omogeneizzato di larve che è di più semplice preparazione e quindi più economico. Infatti, peso medio dei pupari e percentuali di sfarfallamento sono risultati praticamente uguali nelle due tesi a confronto.

C. La dieta standard formata per il 75% da siero bovino, pur dando buoni risultati, appare alquanto povera di nutrienti. Abbassando l'aliquota di siero al 70% e sostituendo il 5% mancante con estratto di lievito di birra, ovvero con farina di soia, si ottiene una tendenziale accelerazione

nell'accrescimento larvale accompagnata da un incremento ponderale dei pupari.

D. La quantità di omogeneizzato, sia di crisalidi che di larve, può essere abbassata dal 20 al 10% senza che si verifichino inconvenienti, purchè la parte mancante venga sostituita da estratto pulverulento di lievito di birra. Il peso medio dei pupari si mantiene costante nelle due tesi a confronto, mentre le percentuali di sfarfallamento tendono addirittura ad aumentare in quella con omogeneizzato dimezzato ma contenente lievito, che si è così confermato un componente integrativo ottimale.

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