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*In vitro* rearing of the larval-pupal parasitoid *Archytas marmoratus* (Townsend) (Diptera: Tachinidae) on oligidic diets: preliminary results. <sup>(1)</sup>

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INTRODUCTION

*Archytas marmoratus* is an oligophagous larval-pupal parasitoid of noctuid species, including important agricultural pests, such as *Agrotis ipsilon* (Hufnagel), *Helicoverpa zea* (Boddie), *Heliothis virescens* (F.) and *Spodoptera frugiperda* (J. E. Smith). It is distributed in the southern United States, Central and South America (Ravlin & Stehr, 1984).

The biology of *A. marmoratus* was described by Hughes (1975). Females oviposit in the host environment and eggs hatch immediately. The first instar larvae are planidium-type maggots. They attach themselves to the host larva, penetrate its integument and remain in an intra-tegumental position up to host pupation (Bratti *et al.*, 1993). When the host larva is parasitized in previous-to-last instars, during the moults the planidia leave the old and move to the new host cuticle. Just before the host pupates, the maggots go into the exuvial space. After ecdysis, they enter the pupa under the posterior wing pad margins, form secondary integumental respiratory funnels, moult to the second instar and proceed in their development. The larval biology of *A. marmoratus* is therefore widely dependent on host hormonal balance, as the parasitoid only moults to the second instar at host pupation, independently of host age at parasitization (Mellini, 1983, 1990). The larvae abandon the funnel after moulting to the third instar. Puparia form within the remains of the host. Even in superparasitized hosts, only one parasitoid reaches the third larval instar and pupates. Supernumerary larvae are eliminated through physiological suppression during the second stage (Reitz, 1995).

*A. marmoratus* is considered a primary candidate for use in large scale IPM programmes against the corn pests *H. zea* and *S. frugiperda* in the United States (Gross, 1994). Promising results in inundative field releases were obtained by

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Gross *et al.* (1985), Pyrah (1985) and Gross (1988, 1990). *A. marmoratus* is a beneficial of considerable practical interest also because it is highly fecund (Hughes, 1975) and can be relatively easily mass-reared both on *H. zea* (Gross & Johnson, 1985) and on the factitious host *Galleria mellonella* L. (Bratti & Costantini, 1991; Coulibaly *et al.*, 1992; Gross, 1994).

Due to its complex larval biology and behaviour, *A. marmoratus* is a difficult parasitoid to culture *in vitro* (Mellini, 1975; Campadelli & Dindo, 1987; Bratti, 1990). Bratti (1991, pub. 1994) reports that a few second instar larvae, removed from *H. zea* pupae, reached the pupal stage on the meridic diet developed by Nettles (1986) for another tachinid, *Eucelatoria bryani* (Sabr.). Subsequently (Bratti, 1993), on the same diet supplemented with cholesterol, egg yolk and *H. zea* pupal haemolymph, planidia slightly grew but did not moult. A few second instar larvae developed to pupae on the Nettles' diet integrated with 20% *H. zea* larval or pupal homogenate. No adults were obtained.

The present study reports the first results obtained by rearing *A. marmoratus* on oligidic diets based on a commercial veal homogenate for babies. This component had successfully been tested in artificial diets for *Brachymeria intermedia* (Hym. Chalcididae) (Nees) (Dindo *et al.*, 1994; Dindo & Farneti, 1996) and for *Exorista larvarum* L. (Dipt. Tachinidae) (Dindo & Farneti, unpublished data).

## MATERIALS AND METHODS

A laboratory colony of *A. marmoratus* was maintained on *G. mellonella* as described by Bratti & Costantini (1991). The colony was initiated in 1993 from the *A. marmoratus* puparia supplied by the Cotton Insect Research Laboratory of USDA at College Station, Texas.

In the present study, all the artificial diets tested for the rearing of *A. marmoratus* contained commercial veal homogenate for babies (Gerber®) as the main component. According to the indications reported on the label, besides veal this homogenate also contains water, milk proteins, starches, salt and maize oil. No information is available on the ingredient proportions. The homogenate nutrient content is the following (g/100 g): dry residue = 22.1; proteins = 12.5; lipids = 5.2; carbohydrates = 3.5; minerals = 0.9. The calorie content is 112 Kcal/100 g.

First, two oligidic diets were tested. The first (I) was composed of homogenate alone and the second (II) of 90% homogenate and 10% extract of *G. mellonella* pupae, obtained as in Bratti (1989). The pH values were 6.15 for diet (I) and 6.6 for diet (II), the osmotic pressures of the media being mOsm/Kg 312 (I) and 345 (II). Plastic 24-well plates (Nunclon, Denmark) were utilized as rearing containers. Each well contained about 0.4 cc of diet.

The experiment was performed starting from early-second or early-third instar larvae of *A. marmoratus*. In both cases, four replicates were carried out, each comprising 24 larvae per diet.

The host larvae were parasitized in last instar (full-grown larvae) by placing 4 planidia on their body surface. The second and third instar parasitoid larvae were removed from *G. mellonella* pupae which had formed respectively 24-26 or

50-55 hours earlier. All the larvae were disinfected in 0.03% sodium hypochlorite, rinsed with sterile physiological saline solution and transferred onto the diet (1 larva per well) by using a special spatula.

Subsequently, two other diets were tested. The first (A) was composed of 85.4% homogenate, 10.4% chicken egg yolk and 4.2% yeast extract. The second (B) contained 78% homogenate, 8% yolk, 4% yeast extract and 10% extract of pupae of *Calliphora vomitoria* L., a non-permissive host of *A. marmoratus*. The pH was 6.48 in diet (A) and 6.17 in diet (B), the osmotic pressure of the media being mOsm/Kg 376 (A) and 431 (B), respectively. This experiment was performed starting from early-second instar larvae only and was replicated three times. Each replicate comprised 24 larvae per medium.

All diets were supplemented with gentamycin sulphate (0.006%) to avoid bacterial contamination (Bratti & Monti, 1988) and set in 1.2% agar. After placing the larvae, the plates were sealed with Parafilm® and kept in darkness at 26°C except when they were removed for daily examinations.

Instruments and glassware were sterilized by autoclaving for 20 minutes at 120°C. All operations, including visual examinations, were performed in a laminar flow hood.

In the tests carried out starting from second instar larvae, the results were evaluated in terms of percentages of third instar larvae and of puparia. Calculations of percentages of third instar larvae (LIII) were based on the original number of larvae placed on the media. The percentages of puparia were calculated both on the number of third instar larvae (P1) and on the original number of larvae placed on the media (P2 = puparium yields).

In the tests carried out starting from third instar larvae, the results were evaluated in terms of percentages of puparia and of adults. The percentages of puparia (P) were based on the original number of larvae placed on the diets. The percentages of adults were based both on the number of puparia (A1 = % adult emergence) and on the original number of larvae placed on the diet (A2 = adult yields).

The development times up to pupation and from pupation to adult emergence were also recorded together with the puparium weights.

The results were subjected to one-way analysis of variance when a sufficient amount of data were collected. Percentages were arc sine transformed for the analysis using the values tabulated by Mosteller & Youtz (1961) according to Snedecor & Cochran (1980).

## RESULTS

### DIETS (I) AND (II)

#### 1) Tests performed starting from second instar larvae.

The mean values ( $\pm$  s.e.) of LIII were  $29.2 \pm 6$  in diet (I) and  $31.2 \pm 10$  in diet (II). The difference was not significant ( $F = 0.013$ ; d.f. 1, 6;  $p = 0.915$ ). The time between laying of the larvae on the diet and moulting to the third instar was of 2-3 days on both diets. Very few parasitoids pupated on both media. There was

no significant difference in mean values of P1 ( $F= 0.706$ ; d.f. 1,6;  $p=0.442$ ). However, a higher percentage of larvae pupated in diet (II) ( $= 11.3\pm 2$ ) than in diet (I) ( $= 4\pm 4$ ). The mean puparium yields (P2) were  $3.1\%$  ( $\pm 2$ ) in diet (I) and  $1.1\%$  ( $\pm 1$ ) in diet (II), that is, extremely low. The difference was not significant ( $F= 0.706$ ; d.f. 1,6;  $p= 0.442$ ).

The data on puparium weights and times up to pupation were too few to be subjected to statistical analysis. The puparium weights were however similar to those of the puparia usually obtained from *G. mellonella* pupae. The time between laying of the larvae on the media and pupation was of 5-6 days, comparable to the time between second larval instar and pupation usually observed *in vivo* at 26-27°C (Hughes, 1975).

The few puparia obtained in both diets were yellowish and rather misshapen. Nevertheless, all contained an adult, which regularly failed to emerge.

## 2) Tests performed starting from third instar larvae.

Only young third instar larvae were selected for the experiment. They can be distinguished because they are iso-oriented to the host pupa, whereas within 24 hours after moulting they rotate by 180° so that the anterior end of their body is facing towards the posterior end of the host pupa. Just before pupating the larvae turn round to the original position. A similar behaviour was observed by Allen (1926) for *A. analis* F. [= *A. apicifer* (Walker)].

The percentage of puparia ( $= 39.6\pm 5$ ) was significantly higher in diet (II) than in diet (I) ( $= 14.6\pm 6$ ) ( $F= 21.2$ ; d.f. 1,6;  $p= 0.004$ ). There was no significant difference in mean times between laying of the larvae on the media and pupation. These times were of  $4.8\pm 0.9$  days in diet (I) and  $4\pm 0.6$  days in diet (II) ( $F= 0.449$ ; d.f. 1,5;  $p= 0.54$ ). It should be noted that no larva pupated in diet (I) in the second replicate.

The mean weight of the puparia obtained was significantly higher in diet (II) ( $= 77.1\pm 5.6$ ) than in diet (I) ( $= 51.4\pm 8.4$ ) ( $F= 6.918$ ; d.f. 1,5;  $p= 0.048$ ). The puparia were well-shaped and pigmented.

No adult was obtained in diet (I). Some adults emerged in diet (II). The % emergence (A1) and adult yield (A2) were however very low ( $= 13.1\pm 7.6$  and  $6.3\pm 3.6$ , respectively). It should be noted that even in diet (II) no adult emerged in the second and fourth replicate.

The mean time from pupa to adult was comparable to that usually observed *in vivo* at the same temperature ( $= 12-15$  days) (Hughes, 1975).

The adults obtained were too few to perform fecundity tests.

## DIETS (A) AND (B)

On both diets, the larvae fed and survived a few days. Throughout the experiment, only 2 larvae moulted to third instar in diet (A), but eventually died. No larva moulted to third instar on diet (B).

## DISCUSSION

The diets tested were inadequate for *A. marmoratus*, which was thus confirmed to be a difficult species to rear *in vitro*. It should be noted that in the present study the artificial culture of this tachinid was carried out starting from second or third instar larvae, i.e. when parasitoid development should no longer be affected by physiological interactions with the host, which dies soon after the parasitoid moulting to the second instar (Hughes, 1975). Moreover, the oxygen requirements of the larvae, which are pneustic from the second instar onwards, were met by gelling the diets in order to prevent the parasitoids from being submerged. The second instar larvae sank into the pabulum, but always maintained the posterior pair of spiracles closely associated with the entrance hole. The third instar larvae, which in the host abandon the respiratory funnel, in the media dug wide holes so that their spiracles were in contact with air. Therefore, there were presumably no problems of oxygen deficiency. It is well known that oxygen supply is a major factor in the successful *in vitro* culture of parasitoids (Thompson, 1986; Mellini *et al.*, 1996).

Notwithstanding, only a few puparia and no adults were obtained when second instar larvae were placed on diets (I) and (II) and a very few adults were obtained even when early third instar larvae were placed on the same media. We assume that this was not so much dependent on the physiological and ethological features of the parasitic larvae, which display simple behaviour and biology in the second and third stage, but rather on the unsuitability to *A. marmoratus* of the nutrient composition of the diets employed. Dindo & Farneti (unpublished data) obtained a mean yield of 33% adults by rearing the larval parasitoid *E. larvarum* from the egg stage on the same diet (II) tested in the present study. *E. larvarum* was successfully reared on different simplified oligidic media devoid of host components, with adult yields of adults reaching as high as 50% (Mellini & Campadelli, 1995a, b; 1996; Mellini *et al.*, 1996). Mellini *et al.* (1996) have suggested that ease of *in vitro* rearing *E. larvarum* is probably related to the simple relationship between this parasitoid 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. *E. larvarum*, however, also displayed a high degree of tolerance to variations in diet composition and definitely proved to be less demanding than *A. marmoratus* in terms of nutrient requirements.

Very little is known about the nutritional needs of the parasitoids in general (Thompson, 1986), both in terms of optimal concentration of a nutrient and optimal balance between nutrients, the latter being also extremely important (Grenier *et al.*, 1986; 1994). The basic qualitative nutrient requirements of entomophages are however similar to those of free-living insects and other animals (House, 1977). We suggest that there is a relation between the degree of complexity of parasitoid larval biology and its nutritional needs throughout the post-embryonic development, i.e. the more parasitoid behaviour and relationship with the host are complicated, the more sophisticated are its nutrient requirements. As to *in vitro* rearing, the best results have so far been obtained with idiobiotic parasitoids (Grenier *et al.*, 1994). This may be related to the fact that these entomophages, besides displaying

a simple behaviour and little dependence upon host physiology, are also less demanding than koinobionts in terms of nutrient requirements.

In the tests performed in the present study starting from second instar larvae there was no difference between diet (I) and (II) as to the percentages of larvae that moulted to the third instar. In other words, these percentages were not affected by the integration with host material. This is probably due to the fact that, in *A. marmoratus*, while parasitoid moulting from the first to the second larval instar has to be triggered by the host ecdysteroids released at host pupation, the subsequent moults are not dependent upon the host hormonal balance. This situation is similar to that in *Pseudogonia rufifrons* (Wied.), a tachinid with a biological life cycle very much like that of *A. marmoratus* (Baronio & Campadelli, 1978; Mellini & Coulibaly, 1991), and in other koinobiotic larval-pupal parasitoids (Mellini, 1983). The percentages of larvae that moulted to the third instar were however quite low. This was certainly due to diet nutrient deficiency, but also to the handling of the larvae, which had to be removed from the respiratory funnels when they were taken out from hosts, so that this may have negatively affected parasitoid development and survival.

In the tests performed starting from both second and third instar larvae, as expected diet (II) proved to be a little more efficient. We believe that this was essentially due to the fact that the diet was partially enriched in nutrients (albeit not sufficiently) by the host material. The presence of host factors indispensable for successful parasitoid development (Nettles, 1990), cannot however be excluded at this stage of study.

Diets (A) and (B) were employed in order to test two media richer in nutrients. The ingredients utilized to integrate the veal homogenate were selected among those more commonly used in artificial substrates (Bratti, 1990). Diet (A) was successfully utilized for *B. intermedia* by Dindo & Farneti (1996). In diet (B) the *C. vomitoria* pupal extract was utilized as *C. vomitoria* larvae are easily and inexpensively available on the market. Moreover, this extract is easier to prepare than the one of *G. mellonella* pupae as there are no cocoons to remove. In several cases success was achieved by rearing parasitoids on artificial media containing insect material derived from non-permissive hosts (Bratti & Nettles, 1988; Bratti & Campadelli, 1993; Bratti & Coulibaly, 1995; Ferkovich *et al.*, 1991; Greany, 1986; Greany *et al.*, 1989). However, both diet (A) and (B) proved to be totally inadequate for *A. marmoratus*, being probably nutritionally unbalanced. In particular, the *C. vomitoria* extract is an unsuitable ingredient in artificial diets for this tachinid.

#### SUMMARY

*A. marmoratus* is a solitary larval-pupal parasitoid of noctuid species, including important agricultural pests. Its larval biology is widely dependent on host hormonal balance as the moult to the second instar has to be triggered by the host ecdysteroids released at host pupation. Due to this and to the complex behaviour displayed by the larvae within the host, *A. marmoratus* is a difficult parasitoid to rear *in vitro*.

In the present study, efforts were made at the artificial culture of *A. marmoratus* from either the early-second or early-third larval stage on media composed of commercial veal homogenate for babies alone (I) or 90% homogenate and 10% pupal extract of the factitious host *Galleria mellonella* (II).

As to the tests performed starting from second instar larvae, about 30% parasitoids moulted to the third instar on both media, only a very few puparia however being formed. All contained an adult, which regularly failed to emerge. As to the tests performed starting from the third instar larvae, about 40% parasitoids pupated on diet (II), only a very few adults however emerging. On diet (I), the percent of puparia was of about 14, but no adults emerged. Both diets were thus inadequate for *A. marmoratus*. It has to be pointed out that, from the second instar onwards, the larval behaviour of this tachinid becomes considerably simpler. Moreover, as the diets were set in agar, the larvae were prevented from being submerged so that their oxygen requirements were met. The failure is therefore probably ascribable to the unsuitability of the nutrient composition of the diets employed for *A. marmoratus*.

Subsequently, other two diets were tested. The first (A) was composed of veal homogenate, chicken egg yolk and yeast extract. The second (B) was composed of the same ingredients, but also contained 10% pupal extract of *Calliphora vomitoria*, a non-permissive host of *A. marmoratus*. The tests were performed starting from second instar larvae. On both media, the larvae survived a few days and only two larvae, on diet (A), moulted to the third instar. Even though they were richer in nutrients than diets (I) and (II), diet (A) and (B) were thus totally inadequate for *A. marmoratus*, being probably nutritionally unbalanced. In particular, the *C. vomitoria* extract is an unsuitable ingredient in artificial diets for this tachinid.

### Allevamento *in vitro* del parassitoide larva-pupale *Archytas marmoratus* (Townsend) su diete oligidiche: risultati preliminari

#### RIASSUNTO

*Archytas marmoratus* è un parassitoide solitario larva-pupale di lepidotteri nottuidi, alcuni dei quali di interesse agrario. È una specie a sviluppo dipendente dal bilancio ormonale dell'ospite, dal momento che la muta dalla prima alla seconda età larvale viene stimolata dagli ecdisteroidi rilasciati dall'ospite al momento dell'impupamento. Per questo motivo, e per il fatto che le larvette esibiscono, nella vittima, un comportamento alquanto complesso, tale tachinide si colloca già in partenza tra le specie particolarmente difficili da allevare *in vitro*.

In questo studio, l'allevamento di *A. marmoratus* è stato tentato a partire dallo stadio di larva di II o di III età iniziale, su substrati costituiti da solo omogeneizzato commerciale per bambini a base di carne di vitello, ovvero dal 90% di omogeneizzato e dal 10% di estratto di crisalide dell'ospite di sostituzione *Galleria mellonella*. Per quanto riguarda le prove eseguite a partire da larve di II età, su entrambi i substrati circa il 30% dei parassitoidi è mutato in III età, ma si sono formati solo pochissimi pupari. Al loro interno, si è regolarmente formato l'adulto che in nessun caso è riuscito a sfarfallare. Riguardo alle prove eseguite a partire da larve di III età, sul substrato integrato con estratto di crisalide circa il 40% dei parassitoidi si è regolarmente impupato, ma sono sfarfallati pochissimi adulti. Sulla dieta costituita da solo omogeneizzato di vitello si è impupato circa il 14% dei parassitoidi, ma nessun adulto è sfarfallato. Entrambe le diete saggiate erano dunque inadeguate per *A. marmoratus*. È da notare che, dalla II età in avanti, il comportamento delle larve di questo tachinide si semplifica notevolmente: l'insuccesso è dunque da imputare a carenze di tipo nutrizionale, più che a caratteristiche fisiche della dieta, che, comunque, grazie all'aggiunta di agar, garantiva alle larve il contatto con l'ossigeno atmosferico.

In un secondo tempo sono state saggiate altre due diete, la prima delle quali (A) costituita da omogeneizzato di vitello, tuorlo d'uovo e estratto di lievito. La seconda (B) conteneva i medesimi ingredienti, ma era integrata anche con il 10% di estratto di pupe del dittero *Calliphora vomitoria* L. Le prove sono state effettuate a partire da larve di II età. Ambedue le diete si sono dimostrate totalmente inadeguate per *A. marmoratus*. Infatti, quasi tutte le larve sono sopravvissute solo pochi giorni e solo due, sulla dieta (A), sono mutate in terza età. Entrambe le diete, quantunque più ricche delle diete (I) e (II) saggiate in precedenza, erano dunque nutrizionalmente inadeguate (probabilmente sbilanciate) in rapporto alle esigenze di *A. marmoratus*. In particolare, è da escludersi, per l'allevamento *in vitro* di questo parassitoide, l'uso di diete integrate con materiale derivato da una specie non ospite quale *C. vomitoria*.

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