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RESEARCH ARTICLE

Assessment of *Ceratitis capitata* (Diptera, Tephritidae) pupae killed by heat or cold as hosts for rearing *Spalangia cameroni* (Hymenoptera: Pteromalidae)

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Abstract

In this work, we study the suitability of using dead medfly *Ceratitis capitata* pupae, killed by heat- or cold-shock, for the mass rearing of *Spalangia cameroni*, a pupal parasitoid of key pests. 100% mortality of medfly pupae could be accomplished with cold-shock at −20°C for 60 min or with heat-shock at 55°C for 30 min. Neither parasitism percentage nor sex ratio of the offspring differed significantly among heat-shocked, cold-shocked and untreated pupae. In addition, there was no significant difference in the percentage of parasitoids that aborted (♂♂ or ♀♀) among pupal treatments. Some of the pupae were covered with peat because the third larval instar of the medfly buries itself before pupation. However, the buried pupae were not parasitised at a greater or lesser rate than those not covered with peat. The percentage of parasitism was also unaffected by whether the pupae had been killed recently or had been stored at between 4°C and 6°C over 15 or 30 days. The use of dead hosts and later storage permitted the following: (a) the use of hosts over long periods of time; (b) a rapid increase in parasitoid numbers and (c) the availability of pupae killed at the most suitable postpupation times for the production of parasitoids. Furthermore, in biological control projects, the use of dead parasitised pupae in the field avoids the risk of enhancing the pest and allows an increase in parasitism in the field through the use of pupae treated with cold- or heat-shock.

Introduction

Spalangia cameroni Perkins (Hymenoptera, Pteromalidae) is a solitary, primary ectoparasitoid of the pupae of various dipteran pests. Currently, it is one of the parasitoids most widely used for the biological control of house flies, *Musca domestica* Linnaeus, and stable flies, *Stomoxys calcitrans* (Linnaeus), species that are harmful in intensive (confined) raising of livestock and birds (Novartis Animal Health Inc., Perkins Ltd; Protecnet). Species of *Spalangia*, together with the genus *Muscidifurax*, are now being used in countries such as Denmark, USA, Australia, Costa Rica, Colombia and Argentina in inundative releases. Such releases result in sufficiently high parasitism to control populations of these dipterans at sustainable levels (Morgan & Patterson, 1990; Geden *et al*., 1992; Crespo *et al*., 1998; Kaufmann *et al*., 2001; Steenberg *et al*., 2001; Skovgard & Nachman, 2004; Geden & Hogsette, 2006). In Spain, in 2003, a population of the parasitoid *S. cameroni* was found parasitising pupae of the fruit medfly *Ceratitis capitata* (a dipteran species, in which larvae in the final instar bury themselves in the ground to pupate) in the field. Adults of *S. cameroni* were obtained in the laboratory emerging from pupae of the medfly found in the field (Falcó *et al*., 2004, 2006). This is the first record of *S. cameroni* as a parasitoid of *C. capitata*. In the Valencian Institute of Agrarian Research (Instituto Valenciano de Investigaciones Agrarias, IVIA, Valencia, Spain), using the 'fruit medfly' *C. capitata* (Wiedmann) as host, we have

been rearing this parasitoid on a semi-massive basis since 2003 with a view to developing it for use in biological control against the fruit medfly.

Biological control of the fruit medfly by inundative releases of *S. cameroni* initially requires massive rearing of the parasitoid. Although the production of large numbers of parasitoids and hosts (pupae) is relatively simple (Morgan, 1981), the peculiar biology of *C. capitata* means that it is not economically viable to maintain large populations of parasitoids during seasons in which releases are not performed (winter, autumn and beginning of spring). As a result, it is necessary to increase production when it becomes expedient to release parasitoids. To achieve a substantial increase, one must necessarily wait for several generations to be born (Geden & Kaufmann, 2007). This, together with the problems (e.g. increases in humidity, the presence of fungi and difficulties in extracting parasitoid) that occur in the rearing terraria caused by the emergence of flies from non-parasitised pupae, prompted us to carry out the present study. Under the environmental conditions in which the study was carried out, the pupal phase lasts 8 days. However, despite using 2-day-old pupae that were withdrawn 48–72 h after their exposure to the parasitoids, possible lags in rearing and imbalances in the environmental variables in the rearing chamber mean that Diptera sometimes emerge in the rearing boxes of flies from non-parasitised pupae. In addition, we took into account the advantages of avoiding releases of non-parasitised host pupae into the field and of being able to establish populations of exotic parasitoids at locations where live hosts are not available (Pickens & Miller, 1978; Geden *et al*., 2006; Geden & Kaufmann, 2007; Ognabola & Fadamiro, 2008).

Here, we report how to improve rearing by: (a) the collection of dead *C. capitata* pupae by cold- and heat-shock treatment, and storage of these pupae at 4–6°C for 30 days; and (b) ensuring the acceptance of the treated pupae by *S. cameroni*.

The use of dead host pupae killed by cold treatment has, to date, been considered unsuitable for rearing *Spalangia* spp. (Morgan *et al*., 1986; Roth *et al*., 1991; Klunker & Fabritius, 1992; Floate, 2002). Nevertheless, recently cold-killed pupae of *M. domestica* have been successfully used in *S. cameroni* rearing (Geden & Kaufmann, 2007). In the present work, we analysed the effect of host pupae (*C. capitata*) killed by cold treatment on the development of *S. cameroni* and, hence, the suitability of these pupae for massive rearing of the parasitoid.

To date, the use of host pupae killed by heat for massive rearing has only been tested by Geden & Kaufmann (2007), where they used *M. domestica* as a host for *S. cameroni*. Here, we also analyse the effect of *C. capitata* pupae killed by heat on the development of *S. cameroni*.

The appropriateness of using dead or live *C. capitata* pupae (killed either by heat or cold) in massive rearings of *S. cameroni* is of particular relevance, since the first description and rearing of this parasitoid on medfly is very recent (Falcó *et al*., 2006).

Materials and methods

To carry out the experiments, pupae of *C. capitata* and adults of *S. cameroni* were obtained between 2007 and 2008 from the IVIA, where they had been reared following the methodology of Pérez-Hinarejos & Beitia (2008).

The experiments were carried out in plastic boxes (20 cm × 15 cm × 10 cm) with a cloth-covered window for ventilation in the lid. As a nutritional supplement, the parasitoids were offered water, honey and sugar by placing small pieces of soaked cotton in Petri dishes. *C. capitata* pupae of approximately 2 days of age were exposed to parasitism by placing the pupae in 5 cm × 5 cm × 1 cm Petri dishes, which were oriented centrally in the boxes. The boxes were placed in a climatic chamber (Sanyo MLR 350), with conditions of 21–24°C, 55–80 RH and 16L:8D photoperiod to await the emergence of the parasitoids. Emergences were first examined at 21 days, since in the environmental conditions under which the experiments were performed, the development of *S. cameroni* up to the imago stage lasted 28–29 days in the case of ♂♂ and 34–35 days in the case of ♀♀.

In all cases, 10 pupae were exposed to each parasitoid pair per day. Overall, there were 18 days of exposure in the five experiments (4 days, Monday–Thursday, in the first 2 weeks and 5 days, Monday–Friday, in the two following weeks, with exposure to untreated pupae during the intervening days).

In all cases, control cold/heat (room temperature, control) exposure was performed simultaneously. Accordingly, 300 pupae per day were used. The details of the experimental design (types of pupae used in each experiment, daily repeats, length of exposure, covered or not covered with peat, etc.) are shown in Table 1.

Determination of heat and cold temperatures lethal to hosts

To determine the minimum temperatures of heat and cold that would cause 100% mortality in the pupae, 2-day-old pupae were divided into five groups of 20 pupae each (total of 100 pupae per temperature/time exposure treatment), in glass Petri dishes (5 cm × 5 cm × 2 cm). For the heat treatment, the pupae were placed in

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Table 1 Details of experimental design (types of pupae (A) = A1: pupae recently killed; A2: pupae stored for 15 or 30 days; Groups of pupae (B) = B1: a group of 10 pupae, either cold-killed, heat-killed, or control, is exposed to a pair (♂ and ♀) of parasitoids; B2: a group of 100 pupae, either cold-killed, heat-killed or control, is exposed to 10 pairs (10 ♂♂ and 10 ♀♀) of parasitoids; C = daily repeats; D = days of exposure; E = total number of pupae used per day; F = pupae number exposed per parasitoid pair; G = pupae not covered with peat (G1)/pupae covered with peat (G2). In the five experiments, the pupae were opened and examined for aborted dipteran and parasitoid pupae

	A		B	C	D	E	F	*G*
Experiment 1	A1		B1	10	18	300	10	*G1*
Experiment 2	A1		B2	1	18	300	10	*G1*
Experiment 3	A1		B2	1	18	300	10	*G2*
Experiment 4		A2(15)	B2	1	18	300	10	*G1*
Experiment 5		A2(30)	B2	1	18	300	10	G1

Table 2 Mortality of pupae of approximately 2 days of age because of exposure to heat/cold [29 January 2008 (exposure)/8 February 2008 (examination of emergence. Up to that date they remained on Petri dishes in a climatic chamber, at 21–24°C, 55–80% RH and a 16L:8D photoperiod]; $n = 5$ groups of 20 pupae for each treatment

T(°C)	Exposure (min)	Mean (SE) of mortality (%)
Cold		
−20	10	2.0 (1.2)
−20	30	29.0 (7.1)
−20	60	100 (0.0)
−20	70	100 (0.0)
Control		9.0 (1.9)
Heat		
50	15	6.0 (3.6)
50	30	58.0 (9.0)
55	15	44.0 (5.3)
55	30	100 (0.0)
60	15	100 (0.0)
60	30	100 (0.0)
Control		2.0 (1.2)

a Heraeus oven (model UT 6120) at one of three temperatures (50°C, 55°C or 60°C) for either 15 or 30 min. In the cold treatment, the pupae were exposed to −20°C for 10, 30 or 60 min (Table 2). The choices of age of the pupae, temperature, duration of exposure and number of repetitions were based on the previous experiments with other hosts of *S. cameroni* (Geden & Kaufmann, 2007). The treated pupae were then transferred to a climatic chamber under the above-described environmental conditions. Untreated pupae, placed in the climatic chamber, were used as controls.

Effect of pupal treatment on parasitoid production

To analyse the effect of pupal treatment on the production of parasitoids, pupae were subjected to conditions closest to critical, i.e. both hot and cold that caused 100% mortality with the shortest exposure time (Table 2) (a) cold treatment (−20°C for 60 min) and (b) heat treatment (55°C for 30 min). Five experiments were conducted, each of them differing in the value of a single experimental treatment:

Experiment 1: parasitism of recently killed pupae. A group of 10 pupae, either cold-killed, heat-killed or controls, were exposed to a pair of parasitoids (one ♂ and one ♀). Each group of pupae was placed on a Petri dish base inside the above-mentioned parasitoid boxes. In this experiment, 30 groups (10 cold-killed, 10 heat-killed and 10 controls) of 10 pupae each were used over 18 days. This experiment was performed in combination with Experiment 2 to assess possible competition effects, e.g. expression of agonistic behaviour and excessive exploitation. All pupae were opened and examined for aborted dipteran and parasitoid pupae in Experiments 1–5, even though doing so was extremely laborious and Experiments 1 and 2 gave almost identical results.

Experiment 2: parasitism of recently killed pupae. A group of 100 pupae, either cold-killed, heat-killed or controls, were exposed to 10 pairs of parasitoids over 18 days. This experiment was to observe the effects of possible competition because of interference and exploitation, and to avoid the low rate of parasitism that occurs when pupae are subjected to only one pair of parasitoids (unpublished data).

Experiment 3: parasitism of recently killed pupae. A group of 100 pupae, either cold-killed, heat-killed, or controls, were exposed to 10 pairs of parasitoids over 18 days. Pupae were first covered with peat because third-stage instar of *C. capitata* bury themselves in the ground in order to pupate and *S. cameroni* will burrow to depths of a few centimetres in search of hosts. Accordingly, the location of pupae may affect parasitism.

Experiment 4: parasitism of pupae killed and subsequently stored for 15 days under refrigeration (4–6°C). A group of 100 pupae, either cold-killed or heat-killed, was exposed to 10 pairs of parasitoids over 18 days.

Experiment 5: parasitism of pupae killed and subsequently stored for 30 days under refrigeration (4–6°C). A group of 100 pupae, either cold-killed or heat-killed, was exposed to 10 pairs of parasitoids over 18 days.

In all cases, the adult parasitoids that were used in experiments were collected from emergence dishes when they were less than 1-day-old and were 6–8 days old at the beginning of the experiment. Original and replacement adults were kept at 21–24°C, 55–80 RH and a 16L:8D photoperiod, in a terrarium with water, sugar and honey prior to use. At the end of the experiments, the adults were 17–18 days old.

In all experiments, analysis of variance (one-way and two-way ANOVA) was used to establish the relationships between the different measured responses (parasitism among treatments, parasitism with 1 or 10 pairs of parasitoids, aborted parasitoids among the three types of treatments, parasitism on pupae covered with peat and parasitism on stored pupae). Since the criterion of data normality was already met, data were not transformed prior to analysis. In addition, it was observed that the distribution of residuals was close to normality. Also, on one occasion, a Kruskal–Wallis test was implemented. All analyses were performed using the SPSS statistical package (12.0).

Results and discussion

Determination of heat and cold temperatures lethal to hosts

The exposure times and least extreme temperatures that led to 100% mortality of the pupae of *C. capitata* were 30 min at 55°C for heat and 60 min at −20°C for cold (Table 2). The heat threshold is similar to that reported for *M. domestica* by Geden & Kaufmann (2007). Those authors established the minimum temperature thresholds leading to 100% mortality in the housefly as 15 min at 55°C for heat and 10 min at −80°C for cold.

Effect of pupal treatment on parasitoid production

Experiment 1

In this experiment, where 10 pupae were presented to one pair of parasitoids, parasitism (without taking into account the aborted parasitoids) did not differ significantly among the three treatment types of pupae (one-way ANOVA: $F_{2,45} = 0.178$, $P = 0.837$; untreated pupae: $\overline{x} = 42.5\%$, cold-treated pupae: 46.8%, heat-treated pupae: 41.3%). Sex ratio also did not differ among treatments ($F_{2,45} = 0.177$; $P = 0.838$; 58.6% female for cold-treated pupa; 63.6% female for heat-treated pupae; 50% female for control pupae). During this assay, the aborted parasitoid pupae accounted for 4.3% of cold-treated pupae; 2.0% of heat-treated pupae and 10.5% of control pupae. In the case of untreated pupae, the aborted host pupae accounted for 7%, which was not significantly different from other treatments (Kruskall–Wallis: $X^2(2) = 4.939$, $P = 0.085$; for females only: $X^2(2) = 3.262$, $P = 0.196$). In this experiment, we did not observe significant differences with regard to abortion in the different assays of the experiment (type of treatment/general parasitism/female parasitism).

Experiment 2

In this experiment, in which 100 pupae were exposed to 10 pairs of parasitoids, parasitism (without taking into account the aborted parasitoids) was 34.6% (cold-treated pupae), 38.9% (heat-treated pupae) and 42.8% (untreated pupae), which were not significantly different (one-way ANOVA: $F_{2,51} = 2.345$, $P = 0.106$). This is similar to the results obtained by Geden & Kaufmann (2007) for *Muscidifurax raptor* Girault and Sanders (Hymenoptera, Pteromalidae) on *M. domestica* which, like *S. cameroni,* is a solitary pupal parasitoid of certain fly species. Similar to our results, parasitism of *M. raptor* on *M. domestica* is greater in heat-treated pupae (49%) than in those cold-treated (40.5%), with the exception that in the latter case the differences between the parasitism obtained with each treatment were significant. Parasitism of *S. cameroni* on *M. domestica* does not demonstrate significant differences between heat-treated and untreated pupae, whereas between cold-treated and heat-treated and between control- and heat-treated pupae significant differences have been reported (Geden & Kaufmann, 2007). These results are similar to those reported by the same authors using *M. raptor*, and differ from those obtained by us, where parasitism, although greater on heat-treated pupae, did not differ significantly from that seen with cold-treated pupae or that of control pupae. The sex ratio did not differ significantly among pupal treatments (60% females for cold-treated pupae, 60.5% for heat-treated pupae, 46.3% for control pupae; $F_{2,51} = 1.177$, $P = 0.316$). These observations are consistent with those from treated pupae of *M. domestica* with the parasitoids *M. raptor* and *S. cameroni* (Geden & Kaufmann, 2007). Those authors reported 62–64% of females in the case of *M. raptor* and 65–70% in the case of *S. cameroni*.

The percentage of parasitoid offspring that were aborted did not differ significantly among pupal treatments (5.2% for cold-treated pupae; 5.05% for heat-treated pupae and 5.77% for control pupae; one-way ANOVA, aborted parasitoids: $F_{2,51} = 0.291$, $P = 0.749$; aborted females: $F_{2,51} = 0.297$, $P = 0.744$).

Although the relative results from the different types of pupae obtained during this experiment were of the same order as those obtained in Experiment 1, the total absolute parasitism obtained during Experiment

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1 was significantly lower (two-way ANOVA: $F_{1,96} = 420.73$, $P \leq 0.0001$). Furthermore, the two-way ANOVA did not reveal any significant effect of pupal treatment on parasitism or any significant interaction between pupal treatment and number of pairs of parasitoids ($F_{2,96} = 1.76$, $P = 0.177$; $F_{2,96} = 2.19$, $P = 0.117$).

Experiment 3

In this experiment, where 100 pupae were exposed to 10 pairs of parasitoids and the pupae were covered with peat, parasitism (without taking into account the aborted parasitoids) was 35.7% (cold-treated pupae); 38.8% (heat-treated pupae) and 43.9% (untreated). With regard to the proportion of parasitism giving rise to females, there was no significant interaction between pupal treatment and whether they were covered with peat (pupae obtained in this experiment) or not (pupae obtained in Experiment 2) (two-way ANOVA: $F_{2,102} = 1.104$, $P = 0.335$). There also was no significant effect of covering with peat ($F_{1,102} = 0.766$; $P = 0.383$) or of pupal treatment ($F_{2,102} = 2.248$, $P = 0.111$).

Experiments 4 and 5

In these experiments, parasitism (without taking into account the aborted parasitoids) was 36.6–35.5% (cold-treated pupae stored for 15–30 days) and 36.8–35.6% (heat-treated pupae stored for 15–30 days). With regard to the proportion of parasitism, there was no significant interaction between the effect of exposure of the pupae to heat or cold and whether they were freshly killed or had been stored for 15 or 30 days (two-way ANOVA: $F_{2,102} = 0.417$, $P = 0.660$). Furthermore, there were no significant differences in parasitism between fresh pupae and pupae killed and stored for 15 or 30 days ($F_{2,102} = 0.134$, $P = 0.875$), or between the different pupal treatments ($F_{1,102} = 0.518$, $P = 0.473$).

Although previous results have indicated that parasitism obtained from *S. cameroni* using hosts cold-killed and stored at 4°C is significantly different from that obtained using live pupae (Floate, 2002), the results of our study are in agreement with recent findings from Geden & Kaufmann (2007). Using cold-killed pupae stored at 4°C for a period of up to 2 months, those authors obtained parasitism approximately 80% of that obtained with *S. cameroni* on live pupae.

The results of our study also indicate that heat is a good method for killing *C. capitata* pupae, which can be stored later at 4–6°C. Killing the pupae by heat-shock or cold-shock does not result in significantly less parasitism by *S. cameroni* than that obtained with live pupae. Similar results to those reported here have been recently obtained by Geden & Kaufmann (2007), using the same protocol on the same parasitoid acting on *M. domestica*.

The possibility of employing dead hosts has advantages both for rearing and for the subsequent use of parasitoids in projects involving biological or integrated control (Geden & Kaufmann, 2007). In our case, the following should be noted in relation to parasitoid rearing: (a) dead, non-parasitised hosts do not give rise to adults. Accordingly, in the parasitoid-rearing boxes adult flies never emerge, thus excessive humidity is avoided and likewise the appearance of fungi; (b) dead hosts can be stored at any time and can be used over long periods of time. Thus, although the medfly under ambient rearing conditions has a pupal stage of approximately 8 days duration, the pupae killed by heat or cold can be used by *S. cameroni* over at least 48 days. This long period allows the pupae to be used in rearing, in order to rapidly increase parasitoid production. It should be noted that under normal conditions an increase in parasitoid production requires 8–10 generations. With treated pupae, this can be achieved in only two generations; (c) dead pupae can be used in rearing carried out at sites where the host does not exist or is not available (Geden *et al*., 2006); (d) treated pupae also have an advantage over live ones in that they can be killed at the precise moment of 'postpupation', when they are most suitable for the production of parasitoids. This age may vary, depending on the species of parasitoid (Petersen & Matthews, 1984; Morgan *et al*., 1989, 1991). In our case, for the pupae subjected to heat- and cold-shock, it was approximately 48 h, because the pupae used in traditional rearing with live pupae are of that age (this is the most suitable age for preventing the emergence of dipteran imagos while they are still exposed to the parasitoids, owing to some lag in the rearing of hosts). Nevertheless, for use in massive rearing of *S. cameroni*, future studies should analyse the greater or lesser suitability of pupae as a function of age.

In relation to recommendations for use as biological controls, the following should be noted: (a) hosts killed by heat or cold and exposed to parasitoids can be used directly in the field against the pest to be eradicated. In the case of *C. capitata*, treated pupae of this host subjected to parasitism by *S. cameroni* can be placed in the field since the pupae that have not been parasitised will not increase the level of the pest, as no dipteran adults emerge from them. Another advantage of using dead fly pupae is that in the summer period they are not easily parasitised by other species with shorter development times than *S. cameroni*. However, a species such as *Nasonia vitripennis* (Walker) may out-compete *S. cameroni* in a few generations and render the whole parasitoid production process unviable; (b) the use of dead pupae allows the level of parasitism in the field to be easily raised. In this regard, it should

be noted that owing to physiological constraints, in many species the pupae are only suitable for parasitism over short periods of time. The period may even vary with variations in temperature (Geden & Kaufmann, 2007). In our case, it is very advantageous to be able to have and deposit treated pupae in the field because, unlike live pupae that are only suitable for parasitism over a defined space of time, they can be parasitised over longer periods, hence increasing the proportion of parasitism in periods of low parasite activity.

In conclusion: (a) parasitism obtained with *S. cameroni* using heat- or cold-killed hosts stored at 4°C for at least 48 days did not differ significantly from that obtained using live pupae; (b) the methods described here allow parasitoids to be produced for use against a key pest of fruit trees in the Mediterranean basin at low cost and with simple equipment.

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