Validation of a methodology for rearing *Spalangia cameroni* (Hymenoptera: Pteromalidae) on *Ceratitis capitata* (Diptera: Tephritidae)

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Abstract—*Spalangia cameroni* Perkins (Hymenoptera: Pteromalidae) is a pupal parasitoid of the Medfly, *Ceratitis capitata* (Wiedmann) (Diptera: Tephritidae), one of the principal pests of Spanish agriculture. *Spalangia cameroni* is a potential biocontrol agent for this pest if methods can be developed to mass-rear it effectively on *C. capitata*. Here, we report on the use of freeze-killed pupae of *C. capitata* to maintain a laboratory colony of *S. cameroni*, with a view to setting up a mass-rearing protocol. Realised fecundity, adult progeny, sex ratio, and superparasitism level were the principal parameters analysed. No significant differences were found in respect of these parameters between living or freeze-killed Medfly pupae used as hosts, although sex ratios showed a bias towards females in the case of freeze-killed pupae. Freeze-killed pupae were concluded to present the best option for the laboratory-rearing of *S. cameroni*, on account of ease of rearing, and avoidance of the emergence of Medfly adults.

Résumé—*Spalangia cameroni* Perkins (Hymnenoptera: Pteromalidae) est un parasitoïde des pupes de la mouche méditerranéenne des fruits, *Ceratitis capitata* (Wiedmann) (Diptera Thephritidae), un des ravageurs principaux de l'agriculture en Espagne. *Spalangia cameroni* pourrait devenir un agent de lutte biologique contre ce ravageur si on réussissait à trouver des méthodes efficaces d'élevage à grande échelle sur *C. capitata*. Nous décrivons l'utilisation de pupes tuées par le froid de *C. capitata* pour le maintien d'une colonie de laboratoire de *S. cameroni* afin de mettre au point un protocole d'élevage en masse. Nous avons mesuré comme variables principales, la fécondité réalisée, la progéniture des adultes, le sex-ratio et le niveau d'hyperparasitisme. Il n'existe pas de différences significatives entre ces variables dans les élevages qui utilisent comme hôtes des pupes vivantes et ceux faits avec des pupes tuées par le froid; il y a cependant un excès de femelles dans les sex-ratios lorsqu'on utilise des pupes tuées par le froid. En somme, l'utilisation de pupes tuées par le froid représente la meilleure solution pour l'élevage en laboratoire de *S. cameroni*, compte tenu de la facilité d'élevage et de l'évitement de l'émergence d'adultes de la mouche méditerranéenne des fruits.

Introduction

The Mediterranean fruit fly or Medfly, *Ceratitis capitata* (Wiedmann) (Diptera: Tephritidae), is one of the principal pests of Spanish agriculture, mainly affecting the citrus industry. It is a globally widespread, highly polyphagous species (Fimiani 1989; Fletcher 1989; Liquido *et al.* 1991) that exhibits high fecundity (Weems 1981; Fletcher 1989), and is multivoltine (Muñiz and Gil 1984). Moreover, it is one of the most pestiferous of species and is a target of quarantine measures in most countries (European and Mediterranean Plant Protection Organization 2012). This underlines the need for special control measures to be undertaken in the production areas affected by this pest.

Biological control measures are becoming increasingly recognised as a major component of Integrated Pest Management programmes, and the

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use of native species for the control of their natural enemies is gaining interest (Urbaneja and Jacas 2008). Accordingly, studies have been undertaken (Pérez-Hinarejos and Beitia 2008; Tormos *et al.* 2009, 2010; Böckmann *et al.* 2012) to assess the potential of the ectoparasitoid, *Spalangia cameroni* Perkins (Hymenoptera: Pteromalidae), as a biological control agent for *C. capitata*.

Spalangia cameroni is an idiobiont and solitary pupal parasitoid of several Diptera families (Muscidae, Sarcophagidae, Tephritidae), and it is sold commercially as a biological control agent against filth flies, including the stable fly, *Stomoxys calcitrans* (Linnaeus) and the house fly, *Musca domestica* Linnaeus (Diptera: Muscidae) (Birkemoe *et al.* 2009). Following the discovery in Spain that *S. cameroni* is a parasitoid of the Medfly (Falcó *et al.* 2006), the Valencian Institute for Agricultural Research (IVIA) in Spain has explored the possibility of using *S. cameroni* as a biological control agent (Tormos *et al.* 2010; Böckmann *et al.* 2012). Since it is a native parasitoid, there were no previous studies of its impact upon other, nontarget species, or of its interactions with Medfly under natural conditions. Laboratory data on the fecundity, adult progeny, host-induced mortality, and sex ratio of *S. cameroni* grown on *C. capitata* have been reported by Pérez-Hinarejos and Beitia (2008). Although *S. cameroni* does not exhibit higher relative fecundity rates than other tephritid-specific larval parasitoids, such as *Diachasmimorpha longicaudata* (Ashmead) (Hymenoptera: Braconidae) (Martins *et al.* 2010), it could complement the action of these other larval parasitoids.

The initial breeding system, used by Falcó *et al.* (2006) for the laboratory experiments referred to above, relied upon living Medfly pupae. This meant that adult flies could emerge from the non-parasitised pupae. It also caused increases in humidity, leading to the growth of fungi; and the technique presented difficulties in separating the parasitoids (Tormos *et al.* 2010). Furthermore, with this methodology, fly pupae had to pass through the adult-fly emergence stage before being removed, and this presented a risk of Medfly outbreaks in the vicinity of the production facility.

In the past, host pupae (Diptera: Muscidae) killed by cold shock had been proven to be unsuitable for rearing *Spalangia* Latreille species (Morgan *et al.* 1986; Roth *et al.* 1991; Klunker and Fabritius 1992). However, in a recent study we evaluated the use of pupae (of *C. capitata*) killed by "cold shock" as hosts for *S. cameroni* (Tormos *et al.* 2010). This study concluded that parasitism rates obtained with *S. cameroni* using freeze-killed *C. capitata* pupae did not differ significantly from those obtained using live pupae.

The objectives of this study were to examine, within the context of small rearing colonies (between 5000 and 10,000 parasitoid individuals), the extent to which the use of freeze-killed *C. capitata* pupae can affect: (i) parasitoid adult progeny (total number of adult offspring produced per female); (ii) sex ratio; (iii) realised fecundity (total number of eggs laid by a female); and (iv) levels of superparasitism (supernumerary eggs).

Materials and methods

Study centre and insects[1]

Spalangia cameroni and *C. capitata* were obtained from laboratory colonies housed at the Valencian Institute of Agrarian Research (Instituto Valenciano de Investigaciones Agrarias (IVIA), Valencia, Spain). The *C. capitata* colony, named IVIA 2002, was established in 2002 by collecting infested fruits from various locations in the province of Valencia (San Andrés *et al.* 2007; Monzó *et al.* 2010). The *S. cameroni* colony was established in 2003, with specimens obtained from Medfly taken from apples in a town (Bétera) in Valencia province (Spain) (Falcó *et al.* 2004). Since 2010, *S. cameroni* have been maintained at the IVIA by culture on freeze-killed Medfly pupae, as described by Tormos *et al.* (2010). Pupae are freeze-killed at −20 °C for 60 minutes and these freeze-killed pupae can then be stored for up to 30 days at +5 °C to rear *S. cameroni.* However, for the second experiment reported in this work, a culture of the parasitoid on living Medfly pupae was initiated specifically, and maintained for the duration of this study.

All host Medfly pupae (whether freeze-killed or living) that were used for the culture of *S. cameroni* were 3–5 days old, in order to minimise the confounding effects of host age (King 1998). To further minimise the effects of host size upon sex

[1]Experiments comply with Spanish law currently in force.

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Table 1. Experimental protocol to elucidate the effects of freeze-killed Medfly pupae upon adult progeny, realised fecundity, superparasitism, and sex ratio in a laboratory rearing of *Spalangia cameroni.*

S. cameroni developmental pupal type	Medfly pupae type supplied	Number of hosts supplied daily per breeding pair until death of female	Host exposure time (hours)	Parameters observed
Experiment 1				
Freeze-killed pupae	Living pupae	10	24	Adult progeny (a_1); sex ratio Eggs counted (a_2)
	Freeze-killed pupae	10	24	Adult progeny (b_1); sex ratio Eggs counted (b_2)
Experiment 2				
Freeze-killed pupae	Living pupae	20	48	Adult progeny; sex ratio
	Freeze-killed pupae	20	48	Adult progeny; sex ratio
Living pupae	Living pupae	20	48	Adult progeny; sex ratio
	Freeze-killed pupae	20	48	Adult progeny; sex ratio

Three replicates were studied, with five repetitions per replicate: that is, 15 repetitions per experimental parameter. The value for adult progeny was obtained from the total number of adult offspring produced (a_1 and b_1 indicate pupae used to obtain adults). Values for realised fecundity (number of eggs laid) and superparasitism (supernumerary eggs: more than one egg/host) were obtained by dissecting the host pupae (a_2 and b_2 indicate pupae used to observe realised fecundity and superparasitism).

ratio (King and King 1994), only pupae of similar size (l = 3.95–4.68 mm ($\bar{x} \pm \text{SE} = 4.37 \pm 0.01$, $n = 52$), maximum w = 2.1–2.49 mm ($\bar{x} \pm \text{SE} = 2.25 \pm 0.02$, $n = 42$) and colour (brown) were used.

Experimental design

Two experiments were conducted to assess the efficiency of freeze-killed Medfly pupae as hosts for *S. cameroni.* In Experiment 1, parasitism of freeze-killed Medfly pupae was compared to parasitism of living Medfly pupae, using *S. cameroni* that had been reared on freeze-killed pupae. In Experiment 2, parasitism of freeze-killed and living Medfly pupae was again compared, but in this case parasitoids had been reared either on living pupae or on freeze-killed pupae. Experiment 2 was performed to take account of experimental error attributable to the method by which the parasitoids had been reared.

The experiments were performed in a climate cabinet (Sanyo MLR 350; Sartorius, Barcelona, Spain), maintained at a temperature of 24.5 ± 0.5 °C and at 60 ± 10% relative humidity, and under a 16:8 hours (light/dark) cycle. Both experiments were performed within translucent plastic boxes (20 × 15 × 10 cm), which were treated as experimental units.

In Experiment 1, each experimental unit contained one female and one male *S. cameroni* and also held a Petri dish (diameter: 60 mm) with 10 freeze-killed or living Medfly pupae, together with water, and honey to provide supplementary food. The pupae were replaced daily until the death of the female wasp. The parasitoids included in the experiment were newly emerged from freeze-killed *C. capitata* puparia.

There were two different experimental groups: in one group (female mean longevity (± SE): 25.3 ±0.47 days), pupae obtained daily from the experimental units were dissected in order to determine the realised fecundity, including the supernumerary eggs (more than one egg/pupa); and in the other group (female mean longevity (± SE): 25.9 ±0.52 days), pupae were kept until the emergence of adult parasitoids, in order to determine the adult progeny (also % emerging adults/exposed pupae) and the sex ratio. For each experimental group, three replicates (blocks or sets) were undertaken, with five repetitions per replicate; thus, in total, 15 repetitions (experimental units) were performed (Table 1). The results of two-way

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analysis of variance (ANOVA), considering the block effect as a random factor, indicated that the block effect did not provide any additional variability. For this reason, we treated it as if it was one replicate comprising 15 repetitions. This methodology was used for both freeze-killed and living pupae.

In Experiment 2, determinations were made only of adult progeny (also % emerging adults/exposed pupae) and of sex ratio. In each experimental unit, 20 Medfly pupae in a Petri dish were offered to a breeding pair of parasitoids; pupae were replaced every 48 hours. Water and honey were provided as supplementary food, until the death of the female. These wasps came from either freeze-killed or living Medfly pupae.

Four scenarios were analysed: (1) living pupae exposed to parasitoids reared on living pupae; (2) living pupae exposed to parasitoids reared on freeze-killed pupae; (3) freeze-killed pupae exposed to parasitoids reared on living pupae; and (4) freeze-killed pupae exposed to parasitoids reared on freeze-killed pupae. The values for female mean longevity (± SE) for the four scenarios were, respectively: 24.9 ± 0.51 days, 25.7 ± 0.53 days, 26.1 ± 0.49 days, and 25.5 ± 0.54 days. As in the case of Experiment 1, three replicates with five experimental units (repetitions) per replicate and, thus, a total of 15 repetitions (experimental units) were undertaken for each experimental condition (Table 1). The results of two-way ANOVA, considering the block effect as a random factor, indicated that the block effect did not provide any additional variability. For this reason, we treated it as if it was one replicate comprising 15 repetitions.

Statistical analysis

Analysis of variance (one-way (fixed factor: type of pupae: live/freeze-killed pupae) (Experiment 1) and two-way ANOVA (fixed factors: (a) type of pupae: live/freeze killed pupae; (b) parasitoid source: parasitoids reared on living/freeze killed pupae) (Experiment 2)), *a linear regression* (Experiment 1) and χ^2 *analysis* (Experiments 1 and 2) were used to establish the relationships between the different responses being measured (realised fecundity (one-way ANOVA), adult progeny (one-way and two-way ANOVA), sex ratio (χ^2 analysis) and superparasitism (one-way ANOVA)). All variables were normally distributed and were not transformed before analyses. In addition, the distributions of residuals were approximately normal. Values are reported as means ± SE. Analyses were performed using the SPSS statistical software package (IBM, Spain; v15.0; critical *P*-value used, 0.05).

Results

Experiment 1: parasitism of living versus freeze-killed Medfly pupae by *Spalangia cameroni* that had been laboratory-reared on freeze-killed Medfly pupae

In this experiment (Tables 2, 3), the adult progeny were found not to differ significantly between living and freeze-killed hosts and there was no significant effect upon the numbers of females and males produced. There was therefore also no significant difference in the sex ratio according to whether living or freeze-killed pupae were used. The sex ratio, using either living or freeze-killed pupae, was significantly biased towards females (living (18.8% ♂♂, 81.2% ♀♀), $\chi^2 = 70.031$, df 1, $P < 0.001$; freeze-killed (20.8% ♂♂, 79.2% ♀♀), $\chi^2 = 65.873$, df 1, $P < 0.001$).

No significant differences were observed with regard either to realised fecundity or to the incidence of supernumerary eggs (superparasitism) between the two experimental conditions (*i.e.*, freeze-killed or living pupae). Nevertheless, a linear regression analysis revealed a significant positive relationship between realised fecundity and superparasitism, both for living pupae ($F = 55.857$, df 1, 13, $P < 0.001$; $R^2 = 0.811$) and for freeze-killed pupae ($F = 95.957$, df 1, 13, $P < 0.001$; $R^2 = 0.881$). The ratio of emerging adults to exposed pupae (expressed as %) ranged between 12% and 13% (Table 2) in both experimental groups.

These results demonstrate clearly that freeze-killed pupae and living pupae can be used equally well for rearing *S. cameroni*.

Experiment 2: parasitism of living versus freeze-killed Medfly pupae by *Spalangia cameroni* that had been laboratory-reared either on freeze-killed or on living Medfly pupae

In this experiment, the proportion of females obtained was higher with the use of freeze-killed pupae than when living pupae were parasitised (two-way ANOVA: $F(1,19) = 12.452$, $P = 0.026$). Regardless of how the parasitoids had been

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Table 2. Lifetime production of progeny, realised fecundity and superparasitism (mean ± SE) for females ($n = 15$) for *Spalangia cameroni* provided with living or freeze-killed hosts.

	Type of host pupae offered	
	Living	Freeze-killed
Adult progeny		
♀♀ + ♂♂	22.3 ± 0.7	21.7 ± 1.8
♀♀	18.1 ± 0.4	17.4 ± 0.4
♂♂	4.2 ± 0.5	4.2 ± 0.6
Emerging adults/pupae exposed (%)		
♀♀ + ♂♂	12.4 ± 0.4	12.0 ± 0.4
♀♀	10.0 ± 0.2	9.7 ± 0.2
♂♂	2.3 ± 0.3	2.3 ± 0.3
Sex ratio		
♀♀:♂♂	4.3:1	3.8:1
Eggs laid	58.6 ± 2.5	54.5 ± 2.5
Pupae parasitised (%)	32.5 ± 1.3	30.3 ± 1.4
Supernumerary eggs	23.0 ± 2.2	18.3 ± 2.8
Mean number of supernumerary eggs/parasitised pupa	2.9 ± 0.3	2.6 ± 0.2

The *S. cameroni* had been reared upon freeze-killed pupae.

Table 3. Mean number (± SE) of progeny per *Spalangia cameroni* breeding pair.

		Adult progeny			Emerging adults /pupae exposed (%)			Sex ratio
Origin of breeding pair	Type of host pupae offered	♀♀ + ♂♂	♀♀	♂♂	♀♀ + ♂♂	♀♀	♂♂	(♀♀:♂♂)
Living host pupae	Living	21.5 ±2.6	10.5 ± 1.8	10.3 ± 1.3	11.2 ± 1.5	6.1 ± 1.5	5.1 ± 1.0	0.9:1
	Freeze-killed	24.0 ± 1.7	19.2 ± 1.6	4.7 ± 1.0	13.2 ± 1.3	9.1 ± 1.1	4.1 ± 0.8	3:1
Freeze-killed host pupae	Living	27.0 ± 3.8	14.1 ± 1.7	12.7 ± 1.5	15.2 ± 2.1	8.1 ± 5.3	7.0 ± 0.9	1.3:1
	Freeze-killed	25.2 ± 1.6	22.4 ± 1.2	3.0 ± 0.2	14.4 ± 1.2	12.1 ± 2.2	2.8 ± 0.	6.1:1

Breeding pairs had been reared on living or freeze-killed Medfly pupae. They were provided every 48 hours, until the death of the female, with 20 living or freeze-killed pupae.

reared, there were also significant sex-ratio differences between the use of freeze-killed pupae (of the individuals analysed, 19% were males and 81% were females) and living pupae (of the individuals analysed, 47% were males and 53% were females) ($\chi^2 = 66.715$, df 1, $P < 0.0001$). The sex ratio using freeze-killed pupae was biased significantly towards females ($\chi^2 = 46.681$, df 1, $P < 0.001$), whereas when living pupae were used the sex ratio was only slightly biased towards females, and this was not statistically significant (107/255 = 0.42) ($\chi^2 = 3.496$, df 1, $P = 0.075$). As shown in Table 3, however, the two-way ANOVA did not reveal any significant effect of the factors (*parasitoids reared on living pupae versus parasitoids reared on freeze-killed pupae*) upon the proportion of females, nor of the factors [*living pupae versus freeze-killed pupae*; *parasitoids reared on living pupae versus parasitoids reared on freeze-killed pupae*) upon total adult progeny. Furthermore, the two-way ANOVA did not reveal any significant interaction between the factors (*living pupae versus freeze-killed pupae*; *parasitoids reared on living pupae versus parasitoids reared on freeze-killed pupae*) either upon total adult progeny, or upon the proportion of females. The ratio of emerging adults to exposed pupae (expressed as %) ranged between 11% and 15%, in all four experimental scenarios (Table 3).

According to these results, the effects of the hosts being alive or freeze-killed were similar to those observed in Experiment 1, and there was no

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effect of the mode of rearing of the adults upon their adult progeny.

Discussion and conclusions

In a previous study (Tormos *et al.* 2010), we began to evaluate the possibility of using *C. capitata* pupae killed by cold shock in a laboratory-scale *S. cameroni* breeding programme, analogous to the successful use of cold-shock-killed *M. domestica* pupae to rear this parasitoid, reported by Geden and Kaufman (2007), Kaufman and Geden (2009), and Ogawa *et al.* (2012). Freeze-killed *C. capitata* pupae have also recently been used in laboratory assays with *Pachycrepoideus vindemmiae* (Rondani) (Hymenoptera: Pteromalidae) (Wyckhuys *et al.* 2011). Cold shock (defined as exposure to −20 °C for 60 minutes) results in 100% host death, and lengthens the storage life of Diptera pupae (which is otherwise only about 2 or 3 days) to as long as 30 days (maintained at between +4 °C and +5 °C). This reduces production costs while maintaining the degree of host quality required for parasitoid development (Floate 2002; Geden and Kaufman 2007; Tormos *et al.* 2010). Therefore, in this work we have analysed the effects of using freeze-killed *C. capitata* pupae on the following parameters in *S. cameroni*: realised fecundity, adult progeny, sex ratio, and superparasitism. The values we have obtained are in agreement with previous findings relating to superparasitism (Böckmann *et al.* 2012; Tormos *et al.* 2012), natural history (Tormos *et al.* 2009), and breeding-substrate modification (Tormos *et al.* 2010).

Our results show that there are no significant differences in the key indicator that we have analysed (adult progeny) between parasitoids reared on either freeze-killed or living Medfly pupae. However, using freeze-killed pupae the sex ratio favoured females (as shown in Experiment 2); a slight variability in sex ratio was observed when living pupae were used, but this was not statistically significant. In the context of these findings, the argument of behavioural ecology that behaviour is adaptive becomes relevant. Thus, a shift in realised fecundity and in sex ratio could arise in response to the quality of the host. For example, in the present case, the use of freeze-killed pupae is clearly advantageous. Currently, there are companies in South America and Europe, such as Perkins Ltda (perkinsltda.com.co) and Muscidia (www.muscidia.fr) that commercially produce *S. cameroni* for fly control in livestock. In this respect, the results obtained in this study would allow improvements to be made in the mass-rearing of *S. cameroni* on *C. capitata*.

In order to use *S. cameroni* in a biological control programme for fruit flies, the parasitoid must first be mass-produced in rearing facilities. Although the simultaneous production of large numbers of parasitoids and hosts is not problematic, the natural history of *C. capitata* means that high production rates cannot be sustained during periods or seasons when the parasitoid is not being released, except when the pest is used in Sterile Insect Technique programmes (see Tormos *et al.* 2010). However, maintaining low-output rearing programmes for much of the year may mean that it is difficult to increase output rapidly when parasitoid releases are required, given that it usually takes several generations to obtain an adequate number of natural enemies for effective use in the field. This fact, coupled with other drawbacks resulting from the presence of viable hosts in breeding cages (notably increased moisture, fungal growth, and difficulties in separating the parasitoids), makes the use of viable host pupae more difficult and expensive in mass rearing programmes.

Clearly, both the laboratory-scale production and the mass-rearing of *S. cameroni* can be improved by using freeze-killed *C. capitata* pupae, and this system can provide the parasitised host material that is required in order to perform the field trials that are necessary for the real effectiveness of parasitoid releases to be assessed. Indeed, this parasitoid-breeding system, lacking as it does the risk of introducing adult flies, may facilitate the adoption of a strategy involving the release of parasitised pupae in crops before emergence of parasitoids. Such releases might be done with both inundative and inoculative goals in view. Furthermore, the manipulation of parasitised pupae is easier and less damaging to the parasitoids than the handling of adult parasitoids for release, or even than keeping them ready for release in small cages (Cancino and Montoya 2006).

Finally, it is noteworthy that measures of biotic potential, such as realised fecundity, number of parasitised pupae and adult progeny, are slightly higher when freeze-killed pupae of *M. domestica*

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are used as hosts for *S. cameroni* than when freeze-killed pupae of *C. capitata* are used. Moreover, when *M. domestica* is used as the host, the percentage of aborted parasitoids is lower (however, although about 35% of parasitoids abort when *C. capitata* is used as the host, the adult-progeny value is still quite high). Superparasitism is higher with *M. domestica* as host (F.B. and J.T., personal observation), whereas the sex ratio is almost the same for both hosts. In spite of all these considerations, however, it is preferable to rear *S. cameroni* using *C. capitata* as the host, on grounds of economy and convenience.

We conclude that *S. cameroni* may be a promising candidate for use in the biological control of Medfly and other flies that are considered to be pests in Spain. Further studies must now be carried out under field conditions, however, to determine the effectiveness of this parasitoid against these pests.

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