Effects of post-teneral nutrition and ginger root oil exposure on longevity and mortality in bait treatments of sterile male *Ceratitis capitata*

Victoria San Andrés, Jordi Pérez-Panadés[2], Emilio Carbonell[2], Pedro Castañera[3] & Alberto Urbaneja[1,*]

[1]Instituto Valenciano de Investigaciones Agrarias - Protección Vegetal y Biotecnología, Carretera Montcada-Nàquera Km 4.5, Moncada, Valencia 46113, Spain, [2]Instituto Valenciano de Investigaciones Agrarias - Biometría, Carretera Moncada-Náquera Km 4.5, Moncada, Valencia 46113, Spain, and [3]CIB, CSIC – 3 Departamento Biología de Plantas, 28040, Madrid, Spain

Correspondence: Alberto Urbaneja, Instituto Valenciano de Investigaciones Agrarias - Protección Vegetal y Biotecnología, Carretera Montcada-Nàquera Km 4.5, Moncada, Valencia 46113, Spain. E-mail: aurbaneja@ivia.es

Short title: *Effects of pre-release diet and ginger root oil on medfly*

Key words: Sterile Insect Technique, SIT, medfly, sugar, protein, proteinaceous bait, Diptera, Tephritidae

Accepted: 23 June 2009

Abstract

Area-wide Sterile Insect Technique (SIT) programmes against medfly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), are being increasingly implemented worldwide. A key issue for SIT is to release sterile males that are sufficiently competitive with males from the wild population. Post-teneral nutrition and ginger oil (GRO) exposure of sterile males prior to release have been shown to improve male competitiveness or performance. However, few studies are available on the effect of post-teneral nutrition and ginger oil exposure on longevity and mortality in bait treatments by sterile male *C. capitata*. In this study, we found that longevity was increased by the addition of protein to the standard pre-release sugar diet, whereas exposure to GRO did not influence the longevity of sterile males. Mortality in spinosad baits was influenced both by diet and GRO exposure. Sterile males on a protein-deprived diet suffered greater mortality than sterile males fed with both sugar and protein. When sterile males were fed on the protein-deprived diet, GRO exposure increased their mortality. However, no significant differences were found in adults on the sugar-protein diet, whether or not they had been exposed to GRO. These results show, for the first time, a negative effect of GRO exposure in terms of increasing mortality in proteinaceous bait treatments, a common practice in areas where SIT is implemented. Nevertheless, this effect could be reduced by the addition of protein to the standard pre-release diet. The implications of these results for SIT programmes against *C. capitata* are discussed.

Introduction

The Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), is considered a major pest of citrus orchards in Spain, causing huge fruit losses and quarantine restrictions. Current medfly control strategies in Spain have been mainly based on field monitoring and aerial and terrestrial treatments with organophosphate insecticides, especially malathion mixed with proteinaceous baits (Primo et al., 2003). To reduce the environmental impact of these treatments, greater emphasis has been placed on implementing safer environmental measures to control adult medfly in Spain in recent years (Castañera, 2003), such as the Sterile Insect Technique (SIT) and the use of pyrethroids or reduced-risk insecticides, such as spinosad or azadirachtin (Peck & McQuate, 2000; Burns et al. 2001; Raga & Sato, 2005; Chueca et al., 2007; Urbaneja et al., 2009). The success of SIT against medfly depends greatly upon mating performance between released sterilized males and wild females. Moreover, sterile males must survive long enough to apply continuous pressure on wild populations.

Fruit flies need sugars and proteinaceous sources to survive and to reach sexual maturity. In the field, wild populations find these nutrients from various sources, such as bird dung or exuded plant sap and rotting fruits (Christenson & Foote, 1960; Bateman, 1972). Consequently, the composition of the diet supplied to sterile males before release is crucial. Post-teneral nutrition and, in particular, the addition of protein to the standard sugar diet and exposure of sterile males to ginger root oil (GRO) [*Zingiber officinale* Roscoe (Zingiberaceae)] prior to release, have been shown to improve male competitiveness, performance, and longevity (Shelly et al., 2004a,b; Yuval et al., 2007).

On the other hand, medfly males deprived of protein following emergence showed a preference and attraction for odours and sources of protein (Prokopy et al., 1996; Manrakhan & Lux, 2008). Therefore, another beneficial effect of a post-teneral protein-rich diet for sterile males is a reduction of their attraction to protein-based insecticide baits (Barry et al., 2003).

Another approach, which has been shown to improve male competitiveness, is exposure of males to attractants prior to release. In particular, exposure to GRO, which contains the male-biased attractant alpha-copaene (Flath et al., 1994; Nishida et al., 2000), has been recently shown to increase mating success in different experimental settings (Shelly, 2001; Shelly & McInnis, 2001; Shelly et al., 2004a,b, 2006, 2007).

As mentioned above, the influence of dietary protein has been largely studied in terms of longevity, mating competitiveness, as well as the increase of sexual competitiveness with or without exposure to GRO. However, the interaction of these two factors in terms of longevity or attraction to proteinaceous insecticide baits has not yet been described.

Our study was based on the hypothesis that the increase of sexual activity of males exposed to GRO may have an energetic cost that induces them to search for food resources, such as proteins included in bait treatments. This is particularly interesting in those areas where a SIT program is being implemented and chemical treatments are carried out in parallel to maintain orchards unblemished. This could be the case in the Valencia Region (Spain), where the SIT program against *C. capitata* covering 180 000 ha of citrus includes commercial orchards that are treated mainly with bait treatments (Argilés & Tejedo, 2007). Here, we report on the effects of post-teneral nutrition and GRO exposure on longevity and attraction to bait treatments by sterile medfly males.

Materials and methods

Insects

Sterile male flies of the Vienna 8 strain, also named GS1/D53 or T(Y;5D30C) (Franz, 2002) were obtained from the mass rearing facility in Mendoza, Argentina. Vienna 8 is a 'male-only' strain containing a *tsl* (temperature sensitive lethal) mutation that allows elimination of females at the egg stage. Males used in the current study were dyed and irradiated as pupae 2 days before emergence under hypoxia at 90 Gy gamma irradiation. Unless otherwise stated, environmental conditions were 25 ± 4 °C, 75 ± 5% r.h., and L16:D8 photoperiod in a climate chamber.

To obtain a cohort of adults for each experiment, approximately 4 000 pupae were divided into four Perspex cages (20 × 20 × 20 cm) in which adults were allowed to emerge. After emergence, about 1 000 adult males (<24 h old) from each cage were given one of two diet regimes: protein-fed or protein-deprived. Protein-fed flies had ad libitum access to a diet containing a mixture of sugar and hydrolyzed yeast (Biokar Diagnostics, Pantin, France) (4:1 wt/wt) and water, whereas protein-deprived adults only had access to a sugar diet and water. Five-day-old adults were used in all subsequent experiments.

Protein and ginger root oil influence on longevity

Experiment with access to food and water. Four treatments were compared: i) protein-fed flies not exposed to GRO, ii) protein-fed flies exposed to GRO, iii) protein-deprived flies not exposed to GRO, and iv) protein-deprived flies exposed to GRO. The experimental arena consisted of ventilated plastic cylinders (16 cm high × 13 cm in diameter) that were placed in an environmental chamber under the conditions mentioned above. Forty 5-day-old males were confined per container, and maintained on their respective diets (protein-fed or protein-deprived) and water. Six replicates were performed for each treatment. For the GRO treatments, 1 day before starting the assay, flies were exposed for 3 h to GRO (Shelly et al., 2004b) by impregnating a piece of filter paper with 100 µl of ginger root oil (Ginger essential oil; Guinama, Valencia, Spain). Exposure was conducted in separate rooms from those used to hold all other flies, thus avoiding inadvertent exposure of ginger root oil to control and GRO-deprived males. The arena was checked daily and dead males were recorded and removed.

Experiment without access to food and water. For this experiment, the same four treatments were compared as in the previous experiment, except that after adults were introduced into the ventilated plastic cylinders, no food or water was provided.

Mortality in bait treatments

Three factors were studied in this experiment: 1) two diets (protein-fed and protein-deprived), 2) with or without exposure to GRO, and 3) with or without exposure to spinosad bait. The bait treatment assayed was Spintor Cebo® at 10% (Dow AgroSciences, Ibérica, Spain; GF-120® in the Americas), containing spinosad and a mix of sugars, hydrolyzed proteins, water, and attractants.

To assay mortality due to the treatment being tested we applied the extended-laboratory method described by Medina et al. (2004). Clementine leaves [*Citrus clementina* Hort. ex Tan., variety Nules (Rutaceae)] were collected from 2-year-old plants grown in a glasshouse located at the Instituto Valenciano de Investigaciones Agrarias (IVIA) and taken to the laboratory. Five droplets of 5 µl of the corresponding treatment were randomly distributed on each leaf with the use of a micropipette. The petiole of each leaf was placed in an Eppendorf tube containing 3% Triton® (Sigma-Aldrich, St. Louis, MO, USA) to retain leaf turgidity during the experiments. The petiole of the leaf was glued to the tube with plasticine (Plastilina Jovi®; JOVI, Barcelona, Spain). The tube with the treated leaf was fixed with plasticine to the bottom of a plastic cage (15 × 7 cm, 10 cm deep), with a tight-fitting lid that had a gauze-covered aperture of 12 × 8 cm on the upper side for ventilation.

Once droplets were dry, 10 adult flies were introduced into a plastic cage for each replicate, and cages were maintained in a climate chamber at the experimental conditions mentioned above. The flies were fed with a mixture of honey and water in an Eppendorf tube fixed to the bottom of the cage and water was offered ad libitum. Mortality was evaluated daily until the 6th day after introduction of flies. Ten replicates per treatment were made.

Data analysis

Longevity was analysed by two-way ANOVA with 'protein' and 'GRO' as main fixed factors. Mortality in bait treatment data was analysed using a logistic model with random effects (GLIM; Molenberghs & Verbeke, 2005). The aim of this analysis was to obtain a logistic curve fitted for each combination of diet and bait, including random effects in some of the parameters due to the replicates. If Y_{ijkm} denotes the number of dead sterile flies with diet i (i = 1,2,3,4), bait treatment j (j = 1,2), replicate k (k = 1,…,10), and day m (m = 1,…,6), the full model is:

$$\pi_{ijkm}=E[y_{ijkm}|u_k,v_k]=\frac{1}{1+\exp\left\{-(a_{ij}+u_k)-(b_{ij}+v_k)\times day_m\right\}},$$

$$a_{ij}=\alpha_0+\alpha_i\cdot I(diet=i)+\alpha_j\cdot I(bait=j)+\alpha_{ij}\cdot I(diet=i\&bait=j),$$

$$b_{ij}=\beta_0+\beta_i\cdot I(diet=i)+\beta_j\cdot I(bait=j)+\beta_{ij}\cdot I(diet=i\&bait=j),$$

$$y_{ijkm}|u_k,v_k\sim Binomial\left(n_{ijk},\pi_{ijkm}\right), \text{ and}$$

$$[u_k,v_k]\sim N\left([0,0],\begin{bmatrix}\sigma_u^2 & \sigma_{uv}^2\\ \sigma_{uv}^2 & \sigma_v^2\end{bmatrix}\right).$$

I is an indicator variable that equals one if the condition is true, and it equals zero if not true. The last level of each factor (treatment 'diet' or 'bait') was considered the base level and fixed to zero. Therefore, the number of estimated parameters of the full model was 19. From the above full model, the Bayesian information criterion (BIC; Schwarz, 1978), was used to select a reduced model with fewer parameters. Once the model was fitted, the time (days) required to reach 50 and 90% mortality for each treatment was estimated.

Results

Protein and ginger root oil influence in longevity

No interaction was observed between protein and GRO ($F_{1,23}$ = 0.72, P = 0.41). Longevity of 5-day-old sterile protein-fed adult males (Vienna 8 *tsl*) was significantly higher than of protein-derived males, when males had access to food and water (ca. 15 vs. 10 days; $F_{1,23}$ = 103.96, P<0.0001). Moreover, exposure to GRO did not influence male longevity ($F_{1,23}$ = 0.47, P = 0.50) (Figure 1A).

When food and water were not available to 5-day-old males during the experiment, no interaction between factors was observed ($F_{1,23}$ = 0.45, P= 0.51). In this case, longevity was significantly shorter in protein-fed males than in protein-deprived males (1 vs. 1.2 days; $F_{1,23}$ = 33.69, P<0.0001) (Figure 1B), whereas exposure to GRO did not influence male longevity ($F_{1,23}$ = 2.29, P = 0.15).

Mortality in bait treatments

The final selected model included only 14 of the original 19 parameters, corresponding to the fixed effects of "diet" and "bait" (α_0, α_i, α_j), their interaction (α_{ij}), the random effect (u) in the intercept, and only main effects for "day"(β_0, β_i, β_j). Estimates and standard errors for the coefficients of the models are presented in Table 1. Figure 2 shows the data and fitted curves, and in Figure 3 the fitted curves are pooled, for ease of comparison. These curves represent the evolution for an 'average' replicate in each treatment, i.e., a replicate with $u_k = 0$. Estimated values in the curve are close to the mean, indicating a good fit to the model for each treatment. Comparing the parameters of the curve, significant differences were detected between some intercepts (Table 2) but not between slopes (Table 3).

Mortality of sterile male flies was influenced by both diet and GRO exposure. Protein-deprived adults reached 50% mortality before protein-fed adults, but there was no difference found for 90% mortality (Table 4). In the case of protein-deprived adults, GRO exposure increased mortality. Indeed, 50 and 90% mortalities were reached significantly more slowly by protein-deprived adults exposed to GRO than the other treatments (Table 4).

Discussion

Our results showed a positive effect of the addition of protein to the pre-release diet on the longevity of Vienna 8 *tsl* sterile males when flies had ad libitum access to this diet. The effect of protein nutrition on sterile male survival has been shown to be rather complex and straindependent (Kaspi & Yuval, 2000; Chang et al., 2001; Shelly & Kennelly, 2002; Shelly & McInnis, 2003; Shelly et al., 2003; Maor et al., 2004; Levy et al., 2005). Indeed, data relating to the effect of dietary protein on male survival is scant and conflicting (Shelly & McInnis, 2003). In any case, the influence of protein on longevity found under laboratory conditions, whether positive or negative, seems to disappear in the field, where flies have access to various protein sources, such as carcasses or faeces (Shelly & Edu, 2008).

In our study, the ability to withstand a period of starvation was reduced when sterile males had access to protein in the pre-release diet. The same result has been previously discussed by Yuval et al. (2007), who described that a protein-rich diet provided to males before starvation may commit them, metabolically, to reproduction (Carey et al., 2002) by diverting resources to pheromone and accessory glands and energy to sexual advertisement (Levy et al., 2005). This commitment carries higher sexual rewards in some environments, at the cost of survival under starvation conditions. Previous studies have demonstrated that aromatherapy enhances male mating success (Shelly, 2001; Shelly & McInnis, 2001; McInnis et al., 2002; Shelly et al., 2003, 2004a,b, 2006, 2007) even 4-5 days following exposure (Shelly et al., 2007). In our study, we found that longevity of the Vienna 8 *tsl* flies was not affected by GRO exposure or the interaction of GRO and protein diet. These results correspond with those obtained by Shelly et al. (2003) and Levy et al. (2005).

The inclusion of bait treatments resulted in a novel negative effect of GRO exposure on mortality in bait treatments by sterile Vienna 8 *tsl* flies. Exposure to GRO could increase their attraction to the bait treatment. As protein could provide males with an important advantage when copulating (accessory gland secretions are composed of peptides and proteins), an explanation for this attraction to bait treatments for protein-deprived males exposed to GRO could be attributed to intensive male sexual signalling, leading males to search for protein sources. In this context, Yuval et al. (1998) reported that lekking males, which were actively signalling, contained higher levels of protein and sugar than resting males.

In recent years, most of the SIT programs worldwide have adopted a sugar diet as the standard pre-release diet and have used GRO exposure prior to the release of sterile males to enhance sexual behaviour. Here, we have shown that this is the most lethal combination for sterile male survival in the presence of spinosad bait. On the other hand, most effective for sterile male survival was the mixed diet of sugar and protein,. Although it could be concluded that the sugar and protein diet is the best pre-release diet for sterile males, the beneficial effects of GRO exposure would seem to discount this conclusion. Certainly, this detrimental result was tempered with the addition of protein to the diet, as our results have shown. Hence, the addition of protein to the pre-release diet may reduce the need for sterile males to search for proteins under field conditions. Likewise, females that have mated with a sterile male previously exposed to GRO prefer to re-mate with other GRO-exposed sterile males rather than wild males, thus giving sterile males a sexual advantage in sperm competition with wild males (Shelly et al., 2004c)

In conclusion, the findings of this study may have important practical implications for area-wide control strategies for medfly, where both sterile male releases and bait treatments are combined. Therefore, it will be important to ascertain whether similar results are obtained under field conditions.

Acknowledgments

This work was funded by FEOGA Cooperación, the Conselleria d'Agricultura, Pesca i Alimentació de la Generalitat Valenciana and INIA (RTA03-103-C6-01). B. Sabater (IVIA, Spain) provided useful comments on an early draft of the manuscript. We are thankful to R. Argilés and J. García de Oteyza (TRAGSA, Spain) for providing the Vienna-8 pupae and to H. Montón (IVIA, Spain) for technical assistance. V. San Andrés was recipient of a PhD grant from the INIA.

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Figure captions

Figure 1 Effect of diet treatment [sugar or sugar + protein (4:1, wt/wt)] and exposure to ginger root oil on the mortality of 5-day-old sterile Mediterranean fruit flies, (A) with ad libitum access to water and the corresponding diet, or (B) without access to water or diet.

Figure 2 Original data (dots), mean values (x), and mortality fitted curves of 5-day-old sterile Mediterranean fruit flies using the selected logistic model. Each separate graph shows the different combination of diet [sugar or sugar + protein (4:1, wt/wt) with or without exposure to ginger root oil] and bait treatment (spinosad or control).

Figure 3 Comparison of the mortality fitted curves of the different combination of diet [sugar or sugar + protein (4:1, wt/wt) with or without exposure to ginger root oil] for 5-day-old sterile Mediterranean fruit flies using the selected logistic model for each bait treatment (spinosad or control). Dotted horizontal lines denote 50 and 90% mortality.

Table 1 Estimates (± SE) for the coefficients of the logistic curve fitted for each combination of post-teneral diet and bait treatment, where 'a' is related to the intercept and 'b' to the increase in number of sterile male *Ceratitis capitata* dead due to time

	Control		Spinosad	
	a_{ij}	b_{ij}	a_{ij}	b_{ij}
Sugar	-3.032 ± 0.284	0.441 ± 0.062	-0.977 ± 0.207	0.756 ± 0.065
Sugar + GRO	-3.766 ± 0.329	0.510 ± 0.070	-0.570 ± 0.217	0.824 ± 0.075
Sugar + protein	-4.417 ± 0.367	0.549 ± 0.073	-1.630 ± 0.217	0.864 ± 0.069
Sugar + protein + GRO	-3.076 ± 0.276	0.402 ± 0.061	-1.207 ± 0.205	0.717 ± 0.062

Table 2 Estimates (± SE) and P-values to contrast the 'a' parameters of the logistic curve fitted for each combination of post-teneral diet and bait treatment in *Ceratitis capitata* flies

	Control		Spinosad	
	Estimate	P	Estimate	P
Sugar vs. sugar + GRO	0.734 ± 0.406	0.0711	-0.407 ± 0.276	0.1404
Sugar vs. sugar + protein	1.384 ± 0.421	0.0011	0.653 ± 0.281	0.0203
Sugar vs. sugar + protein + GRO	0.044 ± 0.360	0.9026	0.231 ± 0.265	0.3835
Sugar + GRO vs. sugar + protein	0.651 ± 0.455	0.1531	1.060 ± 0.289	0.0003
Sugar + GRO vs. sugar + protein + GRO	-0.690 ± 0.399	0.0842	0.638 ± 0.274	0.0203
Sugar + protein vs. Sugar + protein + GRO	-1.340 ± 0.413	0.0013	-0.422 ± 0.279	0.1310

Table 3 Estimates (± SE) and P-values to contrast the ‘b’ parameters of the logistic curve fitted for each combination of post-teneral diet and bait treatment in *Ceratitis capitata* flies

	Estimate	P
Sugar vs. sugar + GRO	-0.069 ± 0.086	0.4258
Sugar vs. sugar + protein	-0.108 ± 0.086	0.2078
Sugar vs. sugar + protein + GRO	0.039 ± 0.078	0.6162
Sugar + GRO vs. sugar + protein	-0.039 ± 0.092	0.6706
Sugar + GRO vs. sugar + protein + GRO	0.108 ± 0.085	0.2032
Sugar + protein vs. sugar + protein + GRO	0.147 ± 0.084	0.0794

Table 4. Estimates (± SE) (days) and P-values to contrasts at which 50 and 90% of *Ceratitis capitata* mortality was reached

	50% mortality		90% mortality	
	Estimate	P	Estimate	P
Sugar vs. sugar + GRO	0.601 ± 0.278	0.0309	0.844 ± 0.273	0.0021
Sugar vs. sugar + protein	-0.594 ± 0.238	0.0130	-0.230 ± 0.274	0.4013
Sugar vs. sugar + protein + GRO	-0.392 ± 0.257	0.1270	-0.550 ± 0.294	0.0621
Sugar + GRO vs. sugar + protein	-1.196 ± 0.258	<0.0001	-1.074 ± 0.264	<0.0001
Sugar + GRO vs. sugar + protein + GRO	-0.994 ± 0.273	0.0003	-1.394 ± 0.287	<0.0001
Sugar + protein vs. sugar + protein + GRO	0.202 ± 0.232	0.3831	-0.320 ± 0.289	0.2681