https://doi.org/10.17221/13/2018-PPS

# Stable isotope labelling of *Ceratitis capitata*

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**Citation**: Al-Khshemawee H., Agarwal M., Ren Y. (2019): Stable isotope labelling *Ceratitis capitata*. Plant Protect. Sci., 55: 54–60.

**Abstract**: The use of stable isotopes to label an insect species, the Mediterranean fruit fly *Ceratitis capitata* (Diptera: Tephidae) (medfly) was investigated. Labelling allows mating and life history characteristics to be investigated experimentally. $^{13}C_6$-glucose was incorporated into the diet of medflies at various stages of development by adding it to larval media or providing adults with sugar water. Data was collected from egg hatching until the death of adults. The results show that stable isotopes successfully labelled medflies in laboratory conditions. There were significant differences between labelled and unlabelled treatments in terms of eggs hatching rates, larval development, pupae emergence, adult survival, and mating behaviour. Labelling during larval development, and combined labelling at the larval and adult stages, resulted in detectable values. Labelling in the larval stage had no effect on mating behaviour, but that in the adult stage did. This study demonstrates that it is possible to label adult medflies and to detect the label after mating.

**Keywords**: $^{13}C_6$-glucose; sterile insect technique (SIT); medfly; mating behaviour; life history

In biological studies, stable isotopes have become very useful as a labelling tool (Misell *et al.* 2006; Hood-Nowoty & Knols 2007) and have been used successfully in insect studies (Heliniski *et al.* 2007). In the past, oil-based dyes (Hendricks *et al.* 1971) and fluorescent dyes (Enkerlin *et al.* 1996) were used to label insects. Oil-based and fluorescent external dyes can have detrimental effects on insects (Schoeder *et al.* 1974) and may be washed off by rain (Logan & Proverbs 1975). To overcome these issues, stable isotope markers can be used, which are incorporated with the tissues of insects (Heliniski *et al.* 2007). Stable isotopes have been used to determine a range of insect characteristics, including dispersal, resource allocation, food preferences, and migration patterns (Hershey *et al.* 1993; Hood-Nowoty *et al.* 2006). In biological systems, stable isotopes interact chemically in a way that is identical to more common isotopes and, thus, they are effective, non-radioactive, and safe. These qualities make them useful natural tracers. Additionally, they are not species-specific, which makes them attractive for use. Potential tracers include isotopes of C, H, O, N, and S (O'Leary 1988; Hayes 2001; De Groot 2004), some of which being present in much greater abundance than others. An isotope of an element has the same atomic number but a different number of neutrons and, thus, a different atomic mass (Macneale *et al.* 2005). The sterile insect technique (SIT) has been used to search for wild pests by labelling males and releasing them into the field (Dyck *et al.* 2006). For efficient monitoring, this method must not affect the male's ability to find a mate (Hood-Nowotny *et al.* 2011). Recently, labelling has been successfully used to mark populations of insects and to conduct genetic research in the laboratory (Markow *et al.* 2000; Helinski *et al.* 2008; Heinrich *et al.* 2012; Mahroof 2013; Hood-Nowotny *et al.* 2016). It can also be used to monitor sterile-to-wild insect ratios by the SIT (Hagler & Jackson 2001). The naturally-occurring levels of stable isotopes have been used in the context of mating by Ponsard *et al.* (2004) and Malausa *et al.* (2005). However, limited research has used stable isotopes to study the life cycle and mating behaviour of the Mediterranean fruit fly *C. capitata* (Wiedemann) (medfly), which is the

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focus of the present study. Medflies pose a serious economic threat worldwide (Thomas *et al.* 2008). They can attack more than 200 hosts in diverse parts of the world (Jessup *et al.* 2007), as they can acclimate to a wide variety of environmental conditions. Recently, medfly control has been conducted using pesticides, which may be harmful to the environment and to humans (Pimentel *et al*. 2005).

In this study, $^{13}C_6$-glucose was chosen as a marker for labelling Mediterranean fruit flies. The study has two objectives and uses two sets of experiments to address them. The first aim was to see whether it is possible to use $^{13}C_6$-glucose in developing insects. We investigated its effects on egg hatching, larval development, the number of pupae, adult emergence, and male survival by using four different treatments. The second objective was to monitor the mating behaviour of labelled medflies.

## MATERIAL AND METHODS

***Fruit fly culture***. A *C. capitata* (Wiedemann) medfly colony was established in 1983 using insects from the Carnarvon area of Western Australia. The collection has been from different areas, including Perth Hills, Dwellingup, Canning Vale, Cannington, Kalamunda, Katanning, Harvey, Maddington, Roleystone, Cloverdale, Bindoon, Chittering, Serpentine, Donnybrook, Nannup, Manjimup, Bridgetown, Mandurah, Carmel, Collie, Stoneville and Belmont,. The colony was renewed with wild fruit flies collected from the Belmont area in 2012. Medflies were obtained from the Department of Agriculture and Food, Western Australia (DAFWA) and reared in the Murdoch University Laboratory in Perth, Australia. All flies were reared at a temperature of 23 ± 2°C, 75 ± 5% relative humidity (RH), and a 12 : 12 h light/dark cycle (Neto *et al*. 2012). Adults were placed in screen cages (40 cm-sided cubes), each containing food of crystalline sugar (Bidvest, Pyrmont, Australia), yeast hydrolysate (4 : 1; Australian Biosearch, Wangarra, Australia), and 50 ml water. Eggs were collected daily and were deposited onto mesh side into water trays. About 10–12 days later, adults emerged from pupae and mating occurred.

***Labelling***. D-Glucose-$^{13}C_6$ 99 atom %$^{13}$C (Sigma-Aldrich, St. Louis, USA) was used as a label. Larvae or adults were exposed to $^{13}C_6$-glucose. In 9 cm sterile Petri dishes (Thomas Scientific, Scoresby, Australia), 100 eggs were added to 25 g of carrot medium on the same day. Then, 0.1 g of D-glucose-$^{13}C_6$ was added when the larvae hatched. For the adult stage, 0.1 g of D-glucose-$^{13}C_6$ was incorporated with 1 g of 99.5% sucrose (Sigma-Aldrich, Castle Hill, Australia) in 15 ml water. For unlabelled treatments, unlabelled glucose D-(+)-Glucose 99.5% (Sigma-Aldrich, USA) was added to 0.1 g of unlabelled glucose and 25 g of carrot medium, and the same number of eggs were added to the Petri dish. Adults were fed a mixture of 1 g sucrose and 0.1 g unlabelled glucose in 15 ml water. For control treatments, 100 eggs were added to 25 g of carrot medium only.

***Experimental design***. Two experiments were designed to evaluate the effects of stable isotopes on insect development and mating behaviour.

The first experiment evaluated of use $^{13}C_6$-glucose on egg hatching, the development of larvae, the number of pupae, adult emergence, male survival, and mating period. All the experiments were maintained until all larvae had pupated and emerged, or died. Eggs were measured by placing 100 eggs, which were laid on the same day, in 25 g of carrot medium at 26 ± 2°C and 75 ± 5% RH, and were checked every 3 h by an Oplenic microscope (PTICS Central, Mitcham, Australia). Larvae were checked every 4 h by the microscope, and the timing of the development of the 1$^{st}$, 2$^{nd}$, and 3$^{rd}$ instars was recorded. The number of pupae was counted and recorded daily. When the majority of pupae had emerged, the pupae were collected and placed in screen cages (40 cm cubes) to monitor adult emergence and male survival.

The second experiment aimed to determine the effect of $^{13}C_6$-glucose on mating behaviour. Males that emerged from trays in the first experiment were divided into treatment groups according to whether they were fed labelled food ($_L$) or unlabelled food ($_U$) in the larval (L) and adult (A) stages. This resulted in four treatment groups: (1) $L_L$-$A_U$, (2) $L_U$-$A_L$, (3) $L_L$-$A_L$, and (4) $L_U$-$A_U$. Twenty males from each treatment were used to monitor mating behaviour. Males were placed into adult cages and fed a labelled or unlabelled sugar solution until mating began. When adults were labelled with $^{13}C_6$-glucose, males were placed in a new cage before mating to prevent cross-pollution of females. In all experiments, the ages of the adult females were the same as those of males.

***Statistical analysis***. All data were analysed by SPSS (2012) software. Significance was tested by univariate analysis of variance (ANOVA) using a threshold of $P < 0.05$. Each factor was tested separately in each experiment. To compare the means, least significant differences (*LSD*, $P \leq 0.05$) were used.

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Table 1. Eggs hatching and number of emerged adults from different treatment

| Treatment | Eggds hatching | | | Number of emerged adults | | |
|---|---|---|---|---|---|---|
| | mean ± SD (%) | 95% confidence interval | | mean ± SD (%) | 95% confidence interval | |
| | | lower bound | upper bound | | lower bound | upper bound |
| Labelled | 79.167 ± 6.675[a] | 75.532 | 82.801 | 50.647 ± 3.932[a] | 45.865 | 55.468 |
| Unlabelled | 85.000 ± 2.366[b] | 81.366 | 88.634 | 57.000 ± 4.690[a] | 52.199 | 61.801 |
| Control | 90.833 ± 1.471[c] | 87.199 | 94.468 | 68.636 ± 7.339[b] | 63.865 | 73.468 |

Values are mean ± Std. deviation (SD); each treatment had 6 replicates; values with different letters are significantly differences at $P \leq 0.05$

## RESULTS

***Egg hatching***. In the first experiment, 100 eggs of each treatment (labelled, unlabelled or control) were used to investigate the effect of glucose labelling on hatching rate (Table 1). There were significant differences between treatments. The highest hatching rate was in the control treatment (90.833%), and the lowest was in the labelled treatment, which was 79.167%.

***Larvae longevity***. Labelling of larvae affected the longevity of fruit flies; there were significant differences between the longevity labelled (4.00 and 3.83 days; $P < 0.05$) and control (3.22 and 3.16 days; $P > 0.05$) treatments in the 2nd and 3rd larval instars (Figure 1). However, there were no significant differences between labelled and control groups of the 2nd instar (Figure 1).

***Larval development and survival***. The rate of pupation was measured by accounting for all larvae from labelled, unlabelled, and control trays. Pupation started at 14–16 days and continued until day 22–23, by which time most of the larvae had pupated. Labelled treatment was 78.16 ± 3.12, while the control treatment was 88.16 ± 1.47. There were no significant differences between labelled and unlabelled treatments, but there were significant differences between labelled and control, and unlabelled and control treatments ($P < 0.05$; Figure 2).

***Number of emerged adults***. There are significant differences between the number of labelled emerged adults (50.932 ± 3.932) and controls (68.636 ± 7.339), but no significant differences between labelled and unlabelled (50.646 ± 3.932 and 57.000 ± 4.690) treatments ($P < 0.05$). These data were with lower and upper bound with 95% confidence interval (Table 1).

***Mating***. Mating durations were measured by choosing pairs of medflies from different treatments, as described in Figure 3A. There were significant differences between $L_L$-$A_L$ (1.98 ± 0.32 h) and controls (3.06 ± 0.47 h), but no significant differences between other treatments ($P \leq 0.05$; Figure 3A). Comparable insemination rates were shown for labelled (9.66 ± 0.88) and control (15.33 ± 1.45) males in all experiments (Figure 3B). $L_L$-$A_L$ males differed significantly from controls ($P \leq 0.05$); however, $L_U$-$A_L$ males did not (Figure 3B).

***Labelling***. After mating, samples were taken from the different treatments, as described below. Samples were taken immediately after mating and three days after mating (Figure 4). There were significant differences between $L_L$-$A_L$ (385.76 ± 17.07) and $L_U$-$A_U$

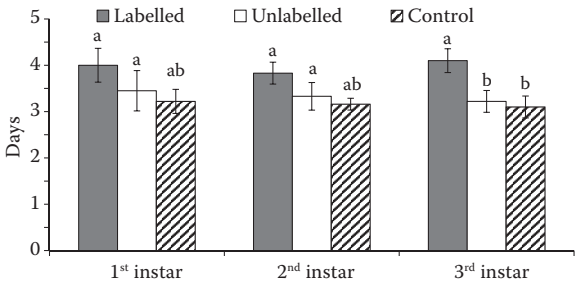


Figure 1. Larval development and longevity between different treatments in three instars ol medfly larvae

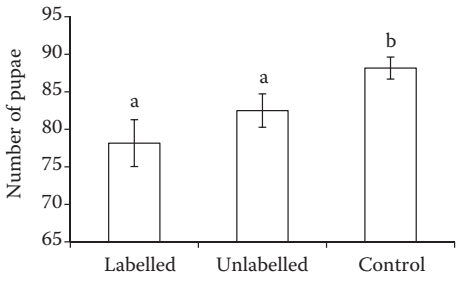


Figure 2. Number of papae survival measured by counting the total number of papae

Bars represented standard error; least significant differeces (*LSD*) at $P \leq 0.05$; different letters mean significant differences

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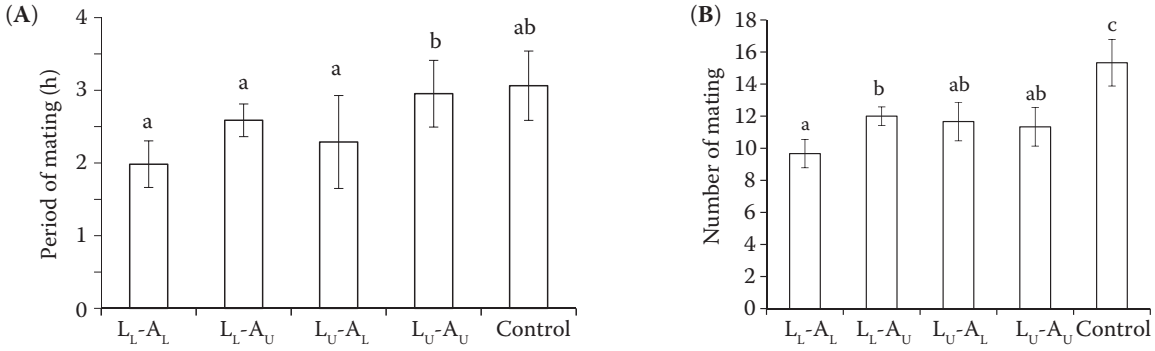


Figure 3. Mating durations (**A**) and number of malting (**B**) of medflies from different treatments

$L_L$-$A_L$ – labelled larvae and labelled adults; $L_U$-$A_L$ – unlabelled larvae and labelled adults; $L_L$-$A_U$ – labelled larvae and unlabelled adults; $L_U$-$A_U$ – unlabelled larave and unlabelled adults; control – without any labelled; different letters mean significant differences

(6.70 ± 4.53) treatments after three days of mating, as there was no for the $L_U$-$A_L$ (377.70 ± 23.33) treatment. The value of labelling that was fixed in the reproductive system of males from this experiment was the same to what we found after mating. Larvae-labelled and adult-labelled flies ($L_L$-$A_L$) had a higher mean $^{13}C_6$-glucose value than the flies labelled only at adult stage. Hence, labelling at both stages was superior to other treatments (Figure 4).

## DISCUSSION

These results demonstrate that it is possible to use $^{13}C_6$-glucose as a labelling tool. Males labelled at both the larval and adult stages had significantly higher levels of $^{13}C$ than males given other treatments. Labelling at the adult stage resulted in an even higher level of $^{13}C_6$-glucose than the labelling of larvae alone (Figure 4). Labelling during the larval stage alone was not sufficient, as low levels of label were detected immediately after mating and three days later. Similar results were found in studies with *Aedes aegypti* L. and the malaria mosquito *Anopheles arabiensis* (Dame *et al.* 1964; Helinski *et al*. 2007). Munro *et al.* (2008) used enriched stable isotopes for marking juvenile golden perch (*Macquaria ambigua*). At the larval stage, exposure did not result in positive labelling, because they were in the early stage of spermatogenesis (Dame *et al.* 1964; Silverman & Selbach 1998). In this research, larvae were labelled successfully but at a low level; therefore, we recommend adult treatment. Although differential labelling may be of benefit at a later stage (i.e. adults), our methods are considered adequate. The highest level of $^{13}C_6$-glucose that was estimated was 385.76 ± 17.06 (three days after mating). Some labels were lost due to the use of larval trays, as not all of the labels added to medfly diet were ingested (Enkerlin *et al*. 1996). This label could be directly through the uptake the label or by micro-organisms to the larvae by add it to the larval diet (Graham & Mangum 1971; Merritt *et al*. 1992; Pauli *et al*. 2009). In the experiments, the labelling persisted after mating. Although the females were not dissected immediately after mating, it was observed that the label was present for up to three days after mating. A higher level of glucose than in controls was also recorded. Three days after mating, males transferred different amounts of the label to younger males. The labelled glucose differed significantly among the treatments, even though the samples were derived and stored in the same conditions. This method can be used to study a variety of problems related to the mating of medflies and other pest species (Merritt *et al.* 1992). Although this method was applied at different developmental stages of medfly and the label was incorporated in adult sugar source, the duration of labelled treatment and the

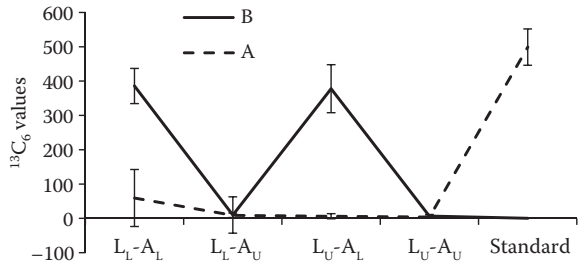


Figure 4. $^{13}C_6$-glucose values: A – values after mating immediately and B – values after three days of mating; bars repsented standard error (SE)

https://doi.org/10.17221/13/2018-PPS

amount of label relative to the sugar source need to be determined. Therefore, this method provides a good potential tool for evaluating pests insects in their natural conditions, and may complement SIT and genetic studies (Scott *et al.* 2002). Also, this study can be used to investigate the dispersal and mating abilities of males released to the field to mate with wild females. It could also be used to determine which males are responsible for mating. This method could be applied in large cages or in the field (Knols *et al*. 2002). This study dealt also with the impact of stable isotope labelling on life history. Labelled glucose affected egg hatching rates, larval longevity, larval development, and emerged adult survival. Mating periods and the number of mating occasions were lower in labelled medflies than in controls, in all replicates. Walker *et al*. (1987), Hagler *et al. (*2001), and Hamer *et al*. (2012) applied this method to mosquitoes at the larvae stage, but with a low level of glucose. They studied the effects of glucose labelling on female size and larval development, and found some variation in males. Similar studies by Young and Downe (1979) and Wilkins *et al.* (2007) investigated the effect of radioactive isotope-glucose labelling on sperm used to inseminate eggs. Thus, stable isotope labels in insects should be durable because they are easily applicable, non-toxic, inexpensive, and clearly identifiable (Silverman & Selbach 1998; Langellotto *et al*. 2005; Macneale *et al*. 2005; Inger & Bearhop 2008; Mastrangelo & Walder 2011).

In general, labelling of adults alone recorded insufficient amount of $^{13}C_6$-glucose from females compared with unlabelled glucose treatment. The labelling amount was higher three days after insemination compared to that immediately after mating. Three days after mating, different amounts of $^{13}C_6$-glucose were transferred to younger males. The glucose label affected egg hatching rates (Table 1), larvae longevity (Figure 1), larval development (Figure 2), and emerged adults (Table 1). Also, the period of mating and the number of mating occasions were different between treatments (Figure 3). Therefore, $^{13}C_6$-glucose is considered an ideal marker for insects labelling. This technique was applied to the Mediterranean fruit fly; however, other pest species are also candidates, such as tsetse flies, mosquitoes, ants, and other species of fruit fly. Thus, stable isotope $^{13}C_6$-glucose labelling is a potential tool for studying mating behaviour and life history in insects. This technique is easy to apply, safe, reliable, and has no effect on the environment. We recommend this technique as part of the SIT for application to other species of insects.

***Acknowledgement.*** We thank the Iraqi Government, High Committee for Educational Development (HCED), and Murdoch University for providing the facilities needed to carry out this study.

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Received: January 19, 2018
Accepted: May 16, 2018
Published online: July 12, 2018