

USE OF OIL SOLUBLE DYES TO MARK ADULT *ELDANA SACCHARINA* (LEPIDOPTERA: PYRALIDAE)

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Abstract

Behavioural, population and ecological studies of animals and insects commonly employ mark-release-recapture techniques. Dyes incorporated into edible oil have commonly been used to mark many Lepidopteran pests. Two oil soluble dyes, Sudan Red 7B and Calco Red N1700, were incorporated into the diet used to rear *Eldana saccharina* Walker (Lepidoptera: Pyralidae), the most serious pest of sugarcane in South Africa. The dyes, which are taken up in the fat bodies of the target insect, allow laboratory-reared adults to be distinguished from their wild counterparts caught in traps. However, the use of dyes can have detrimental effects on the developmental biology, fecundity and fertility of insects. It was found that Sudan Red reduced *E. saccharina* adult emergence by 38% and fecundity by 70%, and significantly prolonged development time. Development time was measured as % Pupation at the time of sampling. Pupation was reduced by 46% in the Sudan Red treatment compared to the control, although sex ratio and fertility were not significantly affected. Calco Red, in contrast, had no significant effect on the developmental and reproductive biology of this insect, and is therefore more suitable for marking *E. saccharina*.

This breakthrough allows marked adults to be used in mating, field dispersion and population estimation studies. Such information is important for the formulation of sterile insect technology and mating disruption control options for *E. saccharina*, both of which are currently being researched at SASRI for the benefit of the South African sugar industry.

Keywords: *Eldana saccharina*, Calco Red, Sudan Red, marking, reproductive biology

Introduction

For behavioural, population and ecological studies of insects, mark-release-recapture techniques are commonly employed (Southwood, 1966; Hagler and Jackson, 2001). Laboratory-reared insects are marked, released and recaptured in traps that have been set up in the field, which then capture both marked and unmarked wild individuals (Qureshi *et al.*, 2004).

Various methods of marking adult insects have been used. These include dust, paint, mutilation, internal and external dyes, genetic markers and radio-isotopes (Hagler and Jackson, 2001). Ideally, the marker should persist on the insect without affecting its normal biology, and be safe, cost effective and easy to use (Hagler and Jackson, 2001; Qureshi *et al.*, 2004). Ease of marker detection is also an important criterion. However, a marker used with success on one insect species may not be suitable for another, and it is therefore necessary to

test potential markers on the target insect's normal biology (Hagler and Jackson, 2001; Qureshi *et al.*, 2004). Dyes dissolved in edible oil and added to insect diets have been used to mark adults of many Lepidopteran pests (Hagler and Jackson, 2001; Qureshi *et al.*, 2004, Parker, 2005). These dyes accumulate in the insect's fat bodies and can be seen through the integument of developing larvae and pupae, and through the intersegmental membranes in the adult stage. As the dyes do not mark scales, some Lepidoptera adults need to be dissected or crushed to reveal the dye in the internal organs (Hagler and Jackson, 2001; Qureshi *et al.*, 2004). A key advantage in the use of dyes to mark insects internally is that expensive equipment is not needed to identify these insects. The dyes are also easy to administer to the target insect (Parker, 2005).

The dye Calco Red N1700 (Pylam Products) has been used extensively for marking Lepidoptera, e.g. the pink bollworm *Pectinophora gossypiella* Saunders (Lepidoptera: Gelechiidae) and the codling moth *Cydia pomonella* L. (Lepidoptera: Tortricidae) (Bloem *et al.*, 2001; Parker, 2005). Qureshi *et al.* (2004) successfully marked the south-western corn borer, *Diatraea grandiosella* Dyar (Lepidoptera: Crambidae), with Sudan Red 7B and Sudan Blue 670 (Sigma-Aldrich).

The South African Sugarcane Research Institute (SASRI) is currently investigating the use of the Sterile Insect Technique (SIT) for the control of *Eldana saccharina* Walker (Lepidoptera: Pyralidae), a serious pest of sugarcane in South Africa (Conlong, 2007). SIT programmes use ionising radiation to induce sterility in laboratory-reared males. The males are released to mate with wild females and thus induce sterility in the wild population (Bakri *et al.*, 2005; Klassen, 2005; Lance and McInnis, 2005; Robinson, 2005). The mode of action is that ionising radiation, usually from a Cobalt-60 source, produces dominant lethal mutations in male sperm production. Males are able to mate with females and sperm is able to fertilise eggs in the normal manner. The dominant lethal mutations in the sperm cell interfere with embryonic development during cell division and the eggs do not hatch (Klassen, 2005; Robinson, 2005). For a SIT programme to be successful, it is important that released irradiated males are fit enough, and are released in high enough numbers, to compete with wild males for mating success with wild females (Robinson, 2005). To measure the ratio of released males to wild males, and released male flight distances, and to eventually gauge the success of SIT, it is necessary to mark the laboratory-reared sterilised males and monitor their populations through the use of trapping techniques (Parker, 2005).

In preparation for the SIT radiation programme, this study investigated whether *E. saccharina* could be successfully marked with Sudan Red 7B and Calco Red N1700, with no deleterious effects on its developmental and reproductive biology.

Materials and Methods

Colony rearing conditions

Eldana saccharina is routinely reared at SASRI, based on the methods described in Graham and Conlong (1988). The modified *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae) diet described by Graham and Conlong (1988) was modified further by Gillespie (1993). Since then, the components ferric citrate and formaldehyde have been removed. Multicell trays containing artificial diet and developing larvae are held in rearing rooms ($28 \pm 2^\circ\text{C}$; $75 \pm 5\%$ RH; 0:24 L:D photophase) for approximately 619 day degrees (DD), which is the time for peak pupal production (Way, 1995). Pupae are harvested from the artificial diet and

transferred to an adult room ($27 \pm 2^\circ\text{C}$; $75 \pm 5\%$ RH; 8:16 L:D photophase) where adults emerge and are paired for mating. Eggs are laid on paper towelling, which is collected and placed into incubators for 119 DD (Way, 1995).

Sudan Red 7B

Sudan Red 7B (3 g) was dissolved in 30 ml sunflower oil and added to 15 L of the *E. saccharina* artificial diet. This was the concentration used by Qureshi *et al.* (2004) for *D. grandiosella*. Neonate larvae were inoculated onto the diet using a mechanical inoculator, and allowed to develop for 604 DD. Five trays were removed from the larval growth room, and pupae and larvae in the trays were removed and counted to assess for % dead and % pupation. Thirty pupae from each repetition were sexed according to Atkinson (1980) and weighed. Adult emergence and sex ratio were calculated from the emerging adults. Adults were paired at emergence for fecundity and fertility measurements. One repetition comprised 10 pairs of adults emerging from pupae reared on Sudan Red diet. Each pair was placed into a 500 ml paper cup containing a pleated cardboard oviposition substrate (50x10 mm when pleated five times) and a 10 mm dental wick soaked with water for the adults to drink from. The contents were then secured with cup seals supplied by the paper cup manufacturers. Oviposition cards were changed daily until females died. Eggs were counted, as were neonate larvae emerging from the eggs. This process was replicated three times. A routine diet, prepared and inoculated on the same day as the Sudan Red diet, served as a control, and was assessed as described for the Sudan Red reared insects.

Calco Red N1700

The treatments and controls for the Calco Red experiment were sampled at an average of 608 DD which co-incided with the routine colony pupal harvest time of 35 days at the average temperature of 27.5°C . By this time, most *E. saccharina* larvae had pupated. Calco Red N1700 was obtained from the Deciduous Fruit Producers Trust (DFPT) SIT rearing facility in Stellenbosch. The dye (0.62 g) was dissolved in 18 ml sunflower oil and added to 15 L of the *E. saccharina* artificial diet. This concentration is used by DFPT to mark *C. pomonella* (personal communication¹). Neonate larvae were inoculated onto the diet as above, and allowed to develop for approximately 608 DD. Ten trays were removed from the larval growth room, and pupae and larvae in the trays were collected, counted and assessed for % dead and % pupation. Thirty pupae from each repetition were sexed according to Atkinson (1980) and weighed. Adult emergence and sex ratio were calculated from the emerging adults. Adults were paired at emergence for fecundity and fertility measurements. Ten pairs of adults emerging from the pupae reared on the Calco Red diet were placed per pair into ten 500 ml paper cups, with a pleated cardboard oviposition substrate (50x10 mm when pleated five times) and a 10 mm dental wick soaked with water for the adults to drink from. Seals were then placed on the paper cups. Oviposition cards were changed daily until females died. The eggs laid on the cards were counted, as were neonate larvae emerging from the eggs. This process was replicated five times. A routine diet, prepared and inoculated on the same day as the Calco Red diet, served as a control, and was assessed as described for the Calco Red reared insects.

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Statistical analysis

Sigmastat 3.11[®] 2004 was used for statistical analysis.

Sudan Red

Results from the treatments and controls that passed normality were subjected to a *t*-test. No dead larvae were found in these trays, therefore no statistical tests were performed to assess significance. The % survival was calculated from % dead in order to present survival more clearly in Figure 1.

Calco Red

Results from the treatments and controls that passed normality were subjected to a *t*-test. All results passed normality, except the % dead control assessment. The % dead control and treatment were therefore square root transformed to obtain normality and then subjected to a *t*-test. The % survival was calculated from % dead in order to present survival more clearly in Figure 1.

Results

Development time and survival

The day degrees at which the Sudan Red and Calco Red trials were assessed, were not significantly different (Mann Whitney U test: $P=1$). Figure 1 compares the development time, reflected as % pupation, and mortality of the larvae in the control diet, to those in the Sudan Red and Calco Red diets at 604 and 608 DD, respectively. There was no significant difference in % pupation of the larvae reared in the Calco Red and control diets ($t=-0.41$; $df=8$; $P=0.69$). Calco Red had no significant effect on the survival of larvae compared to the controls ($t=0.78$; $df=8$; $P=0.46$). The larvae reared in the Sudan Red diet, however, took significantly longer to develop, as only $42 \pm 21.25\%$ reached the pupal stage (as reflected in their % pupation), compared to those reared in the control diet ($88.2 \pm 0.69\%$) ($t=-3.76$; $df=4$; $P=0.02$) at the time of sampling at 604 DD. In contrast though, no mortality of the slower developing larvae was recorded (Figure 1).

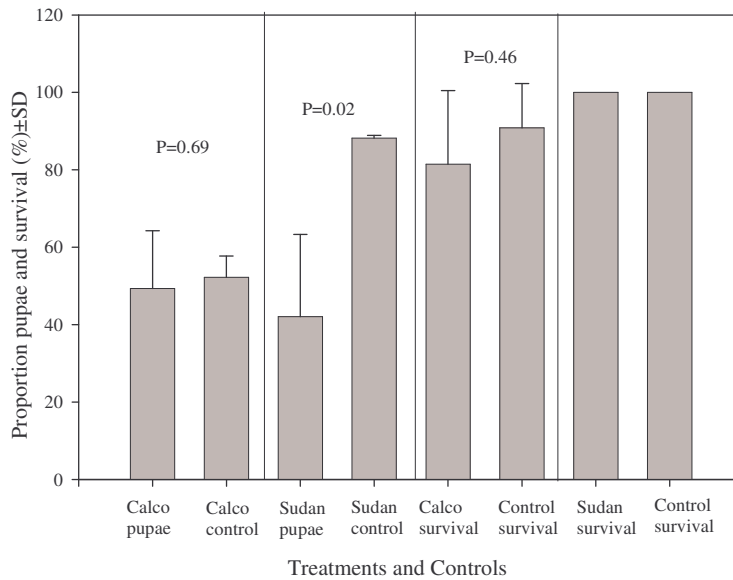


Figure 1. Development of *Eldana saccharina* Walker (Lepidoptera: Pyralidae) larvae, as measured by % pupation, and % survival on control, Calco Red N1700 and Sudan Red 7B diets

Male and female pupal weights

Calco Red had no significant effect on female or male pupal weights (Figure 2: $t=0.38$; $df=8$; $P=0.71$ and $t=0.62$; $df=8$; $P=0.55$, respectively) compared to those reared on control diets. Sudan Red also had no significant effect on the female or male pupal weights (Figure 2: $t=-1.471$; $df=4$; $P=0.22$ and $t=-1.09$; $df=4$; $P=0.34$ respectively), compared to those reared on control diets.

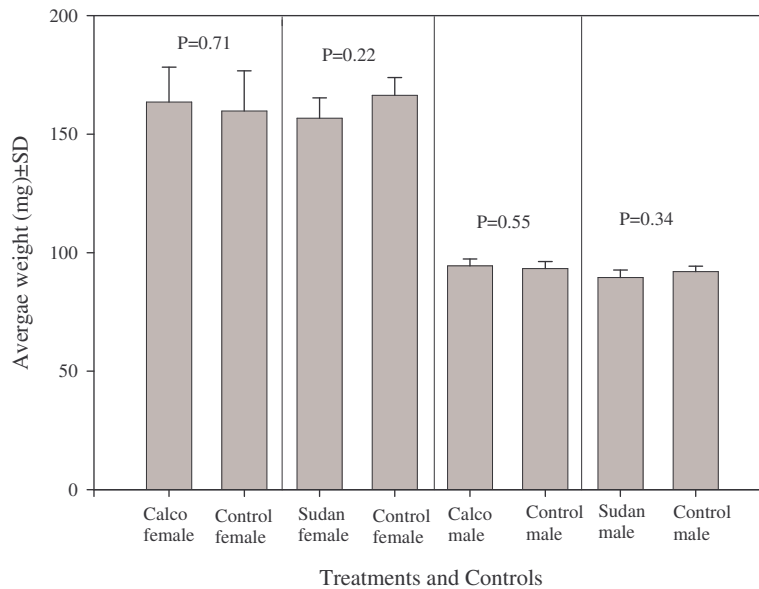


Figure 2. Pupal weights of *Eldana saccharina* Walker (Lepidoptera: Pyralidae) reared on diets containing the oil soluble dyes, Calco Red N1700 and Sudan Red 7B, and on the control diets.

Adult emergence

Adult emergence from pupae collected at 608 DD for Calco Red diet ($87.2 \pm 10.6\%$) and its control diet ($89.2 \pm 6.5\%$), was not significantly different (Figure 3: $t=-0.36$; $df=8$; $P=0.73$). However, adult emergence from pupae obtained from the Sudan Red diet ($17.0 \pm 3.6\%$) at 604.2 DD was significantly reduced compared to those emerging from pupae obtained from the control diet ($55.7 \pm 4.9\%$) (Figure 3: $t=-10.96$; $df=4$; $P<0.001$).

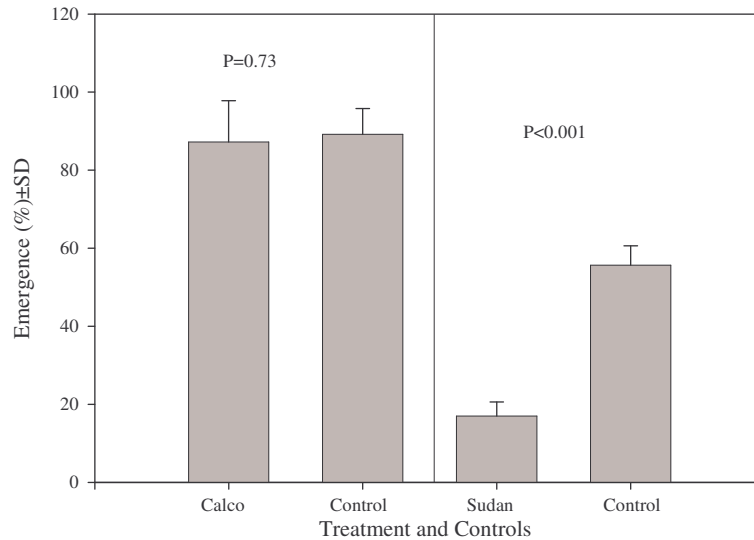


Figure 3. Adult emergence of *Eldana saccharina* Walker (Lepidoptera: Pyralidae) reared on diets containing the oil soluble dyes, Calco Red N1700 and Sudan Red 7B, and on the control diets.

Sex ratio

Calco Red (Figure 4: $t=-1.537$; $df=8$; $P=0.16$) and Sudan Red (Figure 4: $t=-1.26$; $df=4$; $P=0.28$) both had no significant effect on the sex ratio of adults emerging from pupae reared on diets containing these dyes compared to those emerging from pupae reared on their respective control diets.

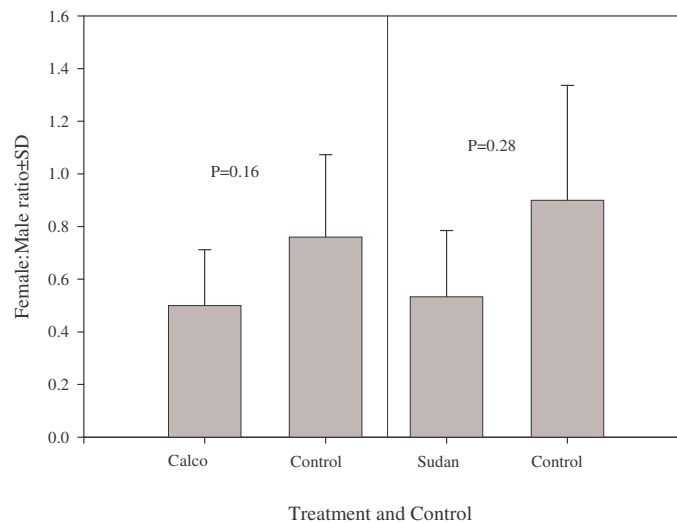


Figure 4. Sex ratio of *Eldana saccharina* Walker (Lepidoptera: Pyralidae) reared on diets containing the oil soluble dyes, Calco Red N1700 and Sudan Red 7B, and on the control diets.

Fecundity

Calco Red did not have a significant effect on the fecundity of *E. saccharina* (Figure 5: $t=-0.74$; $df=8$; $P=0.48$), whereas Sudan Red significantly lowered its fecundity (Figure 5: $t=-8.78$; $df=4$; $P<0.001$), compared to those reared on their respective control diets. The mean number of eggs laid per *E. saccharina* female reared on Sudan Red was 337.67 ± 106.2 compared to a mean of 1141.5 ± 117.1 eggs per *E. saccharina* female on the control diet.

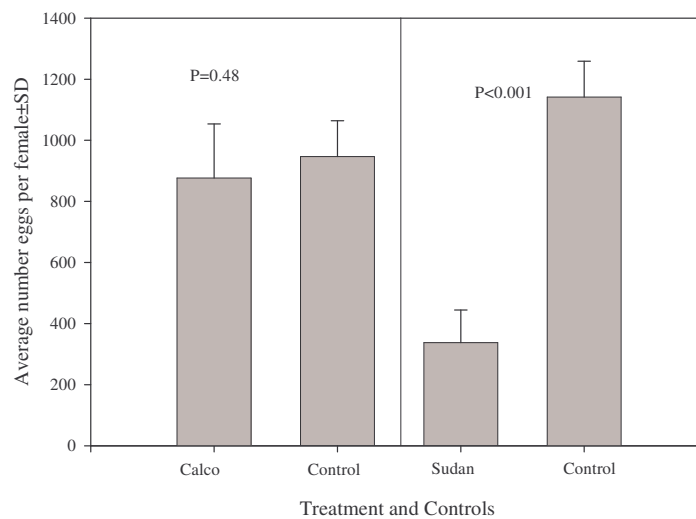


Figure 5. Fecundity of *Eldana saccharina* Walker (Lepidoptera: Pyralidae) reared on diets containing the oil soluble dyes, Calco Red N1700 and Sudan Red 7B, and on the control diets.

Fertility

Calco Red did not significantly affect fertility of *E. saccharina* (Figure 6: $t=-0.480$; $df=8$; $P=0.64$). Sudan Red also did not have a significant effect of fertility of *E. saccharina* (Figure 6: $t=-1.845$; $df=4$; $P=0.14$) compared to that of the *E. saccharina* reared on the respective control diets.

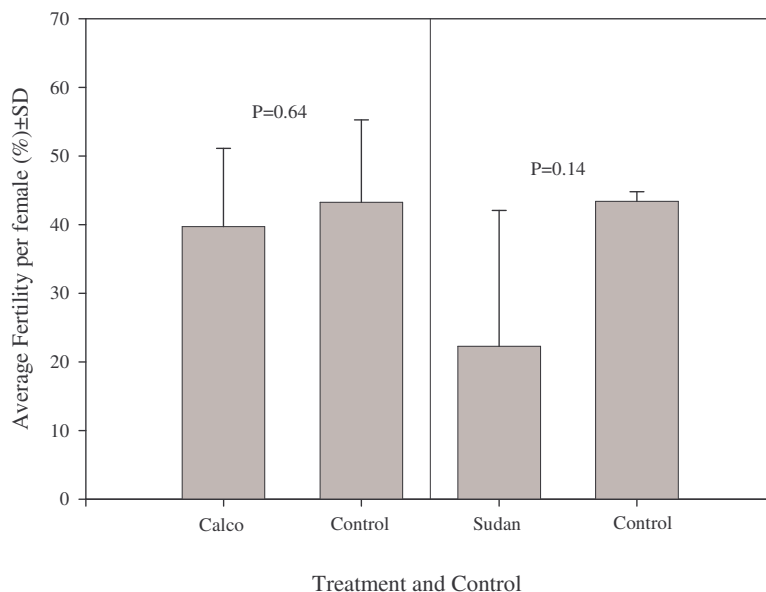


Figure 6. Fertility of *Eldana saccharina* Walker (Lepidoptera: Pyralidae) reared on diets containing the oil soluble dyes, Calco Red N1700 and Sudan Red 7B, and on the control diets.

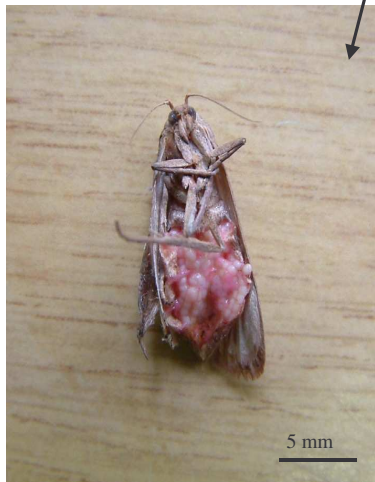
Discussion

Both the Sudan Red 7B and Calco Red N1700 dyes could easily be detected in *E. saccharina* larvae and pupae (Figure 7A). Larvae and pupae which had been fed on diet containing one or the other of these dyes retained a red colour through to adulthood (Figure 7B). This result was similar to those of Gast and Landin (1966) and Daum *et al.* (1969), who tested Calco Red on the adult boll weevil, *Anthonomus grandis* Boheman (Coleoptera: Curculionidae). Daum *et al.* (1969) found it impossible to distinguish between the Sudan Red and Calco Red dyes, when these were used to mark the adult boll weevils. This effect was also found in this study, as both Sudan Red and Calco Red marked *E. saccharina* with a similar red colour. *Eldana saccharina* adults retained both dyes well, which could be seen either by dissection to observe fat bodies or by separation of the intersegmental membranes. The colour effects in females fed on these dyes were carried over to their eggs and to the resulting neonate larvae (Figure 7C). This was similar to results obtained by Qureshi *et al.* (2004) for *D. grandiosella* marked with Sudan Red. However, subsequent feeding on a diet containing no dye reduced the amount of colour that could be observed in the developing larvae. This was also found by Qureshi *et al.* (2004) for *D. grandiosella* marked with Sudan Red.

A



B



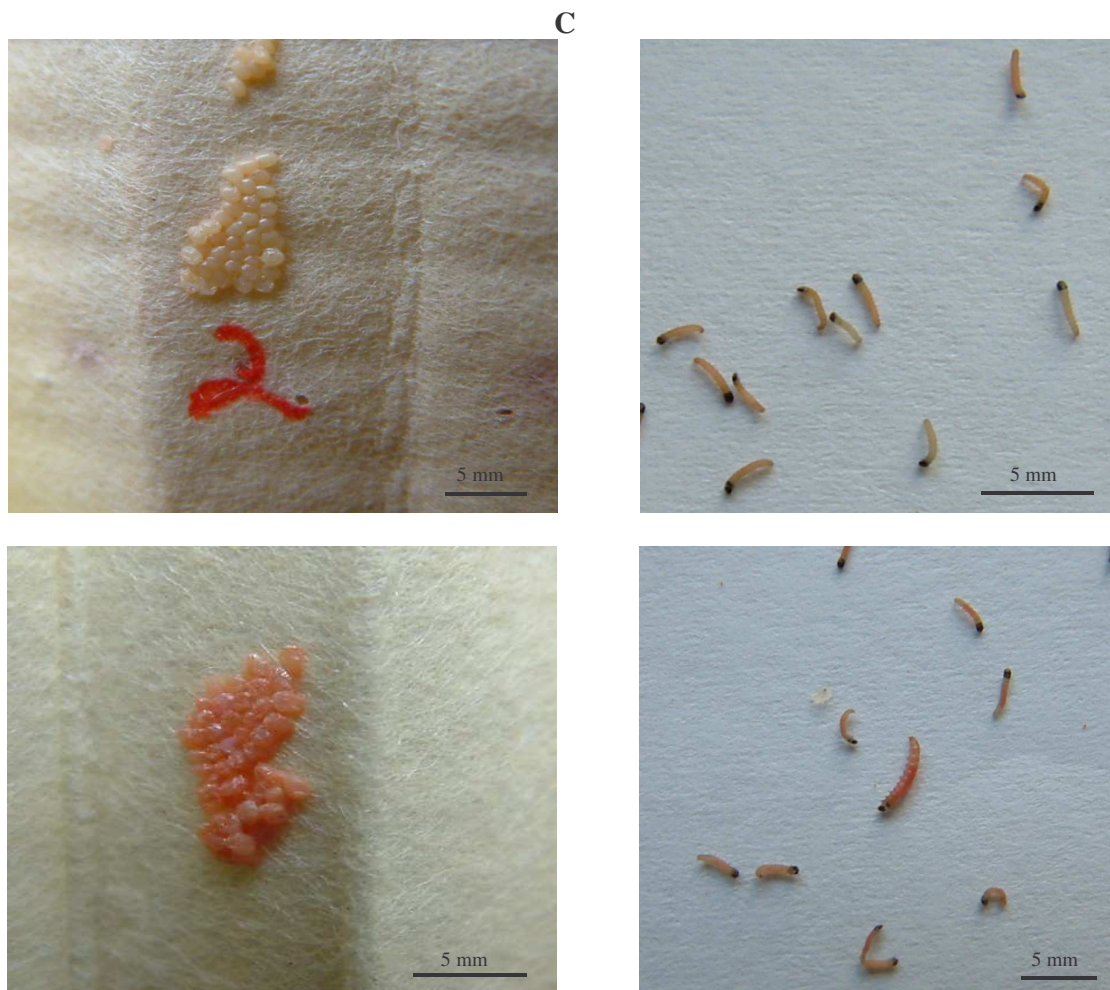


Figure 7. *E. saccharina* Walker (Lepidoptera: Pyralidae) life stages marked with oil soluble dye compared to unmarked individuals of the same stage. (A) Top row: fifth to sixth instar larvae and pupae not marked; Bottom row: fifth to sixth instar larvae and pupae marked with dye. (B) Top: adult marked with dye (left) and adult not marked (right); Bottom left: dissected female marked with dye; Bottom right: dissected female not marked. (C) Top: eggs and neonate larvae not marked; Bottom: eggs and neonate larvae marked with dye.

Sudan Red 7B prolonged development time, and, from the *E. saccharina* pupae produced, adult emergence and fecundity were significantly reduced. Figure 6 shows that mean fertility for *E. saccharina* reared on the Sudan Red diet was $22.30 \pm 19.77SD$, while that for the control was $43.41 \pm 1.4SD$. The mean was skewed in the Sudan Red treatment; however, the difference was not significant ($P=0.14$).

Qureshi *et al.* (2004) found that female larval development time was prolonged and male development time was shortened in *D. grandiosella* when reared on Sudan Red diet. This study did not measure female and male development times separately. However, the adults emerging from the Sudan Red diet had a mean female:male sex ratio of 0.53 ± 0.25 compared to a mean female:male sex ratio in the control of 0.90 ± 0.43 . It can therefore be concluded from the sex ratio of emerging adults, that pupae harvested were male biased in the Sudan Red treatment. This reflects the results of Qureshi *et al.* (2004), in that development times of female *E. saccharina* larvae in this study were also slower in the Sudan Red diet compared to that of the control. There was also a significant reduction in fecundity in *E. saccharina*

females reared on Sudan Red compared to its control. Qureshi *et al.* (2004) found no significant effect on fecundity in *D. grandiosella* reared on Sudan Red diet compared to the control.

Calco Red N1700 had no effect on the developmental and reproductive biology of *E. saccharina*. This was similar to results obtained by Gast and Landin (1966) and Daum *et al.*, (1969) for *A. grandis*. Calco Red is also used extensively in SIT programmes for *P. gossypiella* and *C. pomonella* (Parker, 2005). Because Calco Red had no detrimental effects on *E. saccharina*, it is a suitable marker. In addition, less Calco Red dye than Sudan Red is required per litre of diet, thereby reducing the quantity of dye *E. saccharina* is exposed to while feeding on the artificial diet.

Conclusion

The ability to mark *E. saccharina* with Calco Red N1700 dye without affecting its life cycle parameters and behaviour now allows field population studies to be undertaken using mark-release-recapture techniques. In the SIT programme, the radiated moths released into the field will all be marked, to differentiate them from the naturally occurring moths, so it is important that the marking technique does not further reduce their fitness, as radiation does. The success of a SIT programme is measured by monitoring adults in the release areas. When only marked moths are recovered from the monitoring traps, this will indicate that the wild population has been successfully reduced to negligible levels.

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