MARKING QUEENSLAND FRUIT FLY, *BACTROCERA TRYONI* (FROGGATT) (DIPTERA: TEPHRITIDAE) WITH FLUORESCENT PIGMENTS: PUPAL EMERGENCE, ADULT MORTALITY, AND VISIBILITY AND PERSISTENCE OF MARKS

C. W. Weldon

Fruit Fly Research Centre, School of Biological Sciences A08, University of Sydney, NSW 2006, Australia
Email: cweldon@bio.usyd.edu.au

Summary

A self-marking technique and fluorescent pigments were used to assess the effect of ptilinal pigment marks on pupal emergence, adult mortality, and marker visibility and persistence in Queensland fruit fly, *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae). Marking of *B. tryoni* with fluorescent pigment powders resulted in lower emergence caused by a higher proportion of newly-emerged adults being unable to escape the emergence tray. Mortality of marked adults that emerged successfully was similar to that of unmarked adults. Visibility of pigment marks varied with pigment colour and the wavelength of light used to view the marks. Most pigments were very durable, persisting throughout the experiment. Several fluorescent pigment powders are suggested as candidates for use in mark-release-recapture studies on *B. tryoni*.

Keywords: Sterile insect technique, ptilinum, self-marking

INTRODUCTION

Fluorescent pigments offer a simple, inexpensive marking technique that does not require elaborate protocols or expensive equipment, and enables marking of large numbers of insects (Fleishman *et al.* 1993, Garcia-Salazar and Landis 1997, Reinecke 1990). The pigment can be applied as a dust or suspended in solution and applied directly to the body of the insect, or self-marking may be possible (Turchin 1998). Marking with fluorescent pigments is commonly used to discriminate between insects in dispersal studies employing mark-release-recapture/ resample (MRR) methods. Fluorescent pigment marking has been used to monitor dispersal in grasshoppers (Narisu *et al.* 1999), leafhoppers (Bottenberg and Litsinger 1989), grain borers (Dowdy and McGaughey 1992, Reinecke 1990), mosquitoes (Fryer and Meek 1989, Meek *et al.* 1987, Meek *et al.* 1988, Niebylski and Meek 1989), and moths (Kipp and Lonergan 1992).

Queensland fruit fly, *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) is a serious horticultural pest in eastern Australia. Marking of *B. tryoni* using fluorescent pigments has been used extensively in association with sterile insect technique (SIT) (Dominiak *et al.* 2000a, Dominiak and Webster 1998, Dominiak *et al.* 2003, Horwood and Keenan 1994, James 1996, MacFarlane *et al.* 1987), with the recognition that fluorescent pigment marks provide an efficient method of quickly identifying and separating sterile flies from wild flies caught in monitoring traps (Dominiak *et al.* 2000b). *B. tryoni* and other tephritid fruit flies are marked using a mass-marking method modified from that developed by Norris (1957), and later by Steiner (1965), where pupae are coated in fluorescent pigment which is retained in the ptilinum of the adult after emergence. Flies are examined with a dissecting microscope under an ultra-violet or blue light source for traces of fluorescent pigment (Dominiak *et al.* 2000b, Holbrook *et al.* 1970). Some studies have reported that using this self-marking technique to mark *B. tryoni* leads to decreased rates of adult emergence from the puparium (Dominiak *et al.* 2000b, Dominiak *et al.* 2003), although these trials lacked adequate controls and results were variable.

Numerous fluorescent colours have been assessed and used for marking *B. tryoni* based on the relative visibility of the pigment mark (see Dominiak *et al.* 2000b). In a recent study on dispersal of sterile *B. tryoni* in Sydney (Dominiak *et al.* 2003), recapture rates of flies marked with blue, green and pink fluorescent pigment were lower than that of flies with orange marks in Lynfield traps (Cowley *et al.* 1990) baited with cuelure. One possible explanation for this observation was that blue, green and pink fluorescent pigment colours were toxic to adult *B. tryoni*, resulting in increased mortality. A fundamental requirement for any successful marking technique is that it should not affect the competitiveness, survival, longevity or behaviour of the treated animal (Southwood 1978). Another possibility is that the pigment marks may have disappeared with time. Fluorescent ptilinal marks in the house fly, *Musca domestica* L. (Diptera: Muscidae) decreased in intensity over a period of five days, although no individuals were unmarked (Beck and Turner 1984). Over longer periods of time, it is possible that ptilinal fluorescent marks may fade considerably, although

this theory has not been tested. If increased mortality does result from marking with particular fluorescent pigment colour, then this marking technique would require reassessment for their use in *B. tryoni* release programs, or need to be recognised when analysing recapture data from monitoring traps. Similarly, if permanence after release was shown to be questionable, then this finding would also result in a re-evaluation of these dyes for marking flies.

This study examined the effect of fluorescent pigment marking, using a self-marking technique, on adult emergence of *B. tryoni*. The effect of ptilinal fluorescent pigment marking and fluorescent pigment colour on mortality of adults in the laboratory was observed. Visibility and permanence of ptilinal fluorescent marks of various colours was assessed in the laboratory over a period of five weeks.

MATERIALS AND METHODS

Cultures and pigment application

Bactrocera tryoni were obtained from a laboratory colony maintained at the University of Sydney. The colony was one that had been continuously selected to increase longevity of adult flies by only using eggs from females greater than 16 weeks of age that had been maintained under laboratory conditions for more than 15 generations.

Nine different fluorescent pigments produced by two manufacturers were assessed: Deep Green (JST-31), Chartreuse (RS-10), Orange (RS-13), Cerise (PS-16) and Lilac (PC-98) manufactured by Radglo Color, N.V.; and Aurora Pink (HM-11), Rocket Red (HM-13), Arc Yellow (HM-16) and Strong Corona Magenta (HMS-30) from Dayglo Color Corp.. According to material safety data sheets produced by each pigment manufacturer, Radglo pigments were dye solutions in thermoplastic resins with average particle size of 4 - 5 µm, and Dayglo pigments were solid dye solutions in thermoplastic resins described as 'granules' with particle size greater than 60 µm. For each pigment treatment, approximately 100 pupae were placed in 40 mL plastic pill cups (emergence trays) to which 0.2 g of pigment was added. Pupae were gently agitated by hand for up to 10 seconds to coat the pupae with pigment. Pigment-coated pupae were added to an equal quantity (by volume) of sawdust and agitated gently for approximately 10 seconds. The control consisted of approximately 100 pupae that were agitated and covered with sawdust in the same manner, but with no fluorescent pigment added. Three replicates of each treatment were performed.

Emergence and mortality

Pupae combined with pigment were placed in sleeve cages (45 x 33 x 34 cm), with one pigment treatment per cage. Cages were kept near a window that provided natural light-dark conditions, and maintained at 23 ± 2°C and 41 – 65 % RH. Cages contained a sealed, plastic water container with a cotton dental roll wick, and an open plastic container filled with granulated sucrose to provide emerged flies with water and food *ad libitum*. Four days after adult flies first emerged, emergence trays were removed from the cages. The number of pupae that failed to emerge, partially emerged flies, and emerged flies that failed to escape the emergence tray were recorded. Analysis of variance was performed to identify differences between the results for the pigment treatments. Variables were analysed as proportions to standardise for differences in the number of pupae. No variables conformed to the assumptions of ANOVA, so the arcsine square root transformation, routinely used on proportion data (Underwood 1997), was applied prior to analysis. Tukey multiple comparison tests were conducted to identify homogeneous groups within the fluorescent pigment treatments. Numbers of successfully emerged flies were not analysed due to non-independence of data.

After adult emergence, in addition to water and granulated sucrose already provided, a paste of yeast autolysate paste, sucrose and water was applied to the walls of sleeve cages to provide a source of protein which is required by female *B. tryoni* to become sexually mature (Drew 1987, Drew *et al.* 1983). Cages were inspected at 4, 7, 14, 21, 28 and 35 days after adult emergence for mortalities in each cage, and dead flies were removed. The number and sex of dead flies in each treatment was recorded (total mortality). The cages were then placed in an incubator at 70°C for 30 minutes to kill remaining flies. The total number of successfully emerged flies was recorded. Mortality rate was calculated for each week in each pigment treatment. Two-way ANOVA was used to compare total mortality and mean weekly mortality rates in males and females for different pigment colours. Total mortality did not conform to the assumption of homogeneity of variances and was arcsine square root-transformed prior to analysis.

Marker visibility and persistence

Four days after adults first began emerging, five male and five female flies were chosen at random from each pigment treatment. Flies were examined in glass

vials under four light wavelengths for fluorescent pigment marks on the ptilinal suture: blue (467 nm), green (511 nm), yellow (563 nm), and red (625 nm). Flies were not anaesthetised during observation. The numbers of males and females with marks visible on the ptilinal suture were recorded. If pigment marks were present on the ptilinal suture, but did not fluoresce, they were still counted as visible. After observation, flies were returned to the cage. This procedure was repeated at 7, 14, 21, 28, and 35 days to give an indication of mark persistence.

Marker visibility for each treatment under different light wavelengths over time was analysed using three-way ANOVA. Data did not conform to the assumptions of ANOVA. Square root-transformation of data ($\sqrt{x}+1$) reduced heterogeneity of variances but did not equalise them. In large experiments, ANOVA is robust to departures from the assumption of homogeneity of variances (Underwood 1997) and analysis was continued using square root-transformed data. Significant interactions between pigment treatment and time were of particular interest for assessing mark persistence. Tukey multiple comparison tests were conducted to identify homogeneous groups within light wavelengths and fluorescent pigment treatments.

RESULTS

Emergence and mortality

On average, 14.4 ± 2.9% of pupae failed to emerge during the study. The proportion of pupae that failed to emerge was similar across all pigment treatments (Figure 1). Similarly, the proportion of partially-emerged pupae did not differ between pigment treatments (Figure 1), with an average of 2.9 ± 0.3% during the study. An average of 14.5 ± 2.0% flies emerged from the puparium, but died before escaping from the emergence tray. There was a significant difference ($p < 0.001$) in the proportion of flies that had emerged but failed to escape from the emergence tray. Mortality of emerged flies in the emergence tray was not observed in the control, and was low in Arc Yellow, Aurora Pink and Strong Corona Magenta treatments (Figure 1). The average emergence success of pupae during the experiment was 68.2 ± 3.8%.

Average mortality rate during the study was 2.1 ± 0.1% per week, and total mortality was 11.4 ± 1%.

Figure 1. Emergence success of *B. tryoni* pupae treated with fluorescent pigment powders of different colour (mean ± 1 s.e.). No pigment was applied to control pupae. Pupae that failed to emerge: white bars; partially emerged flies: cross-hatched bars; emerged flies that failed to escape the emergence tray: black bars.

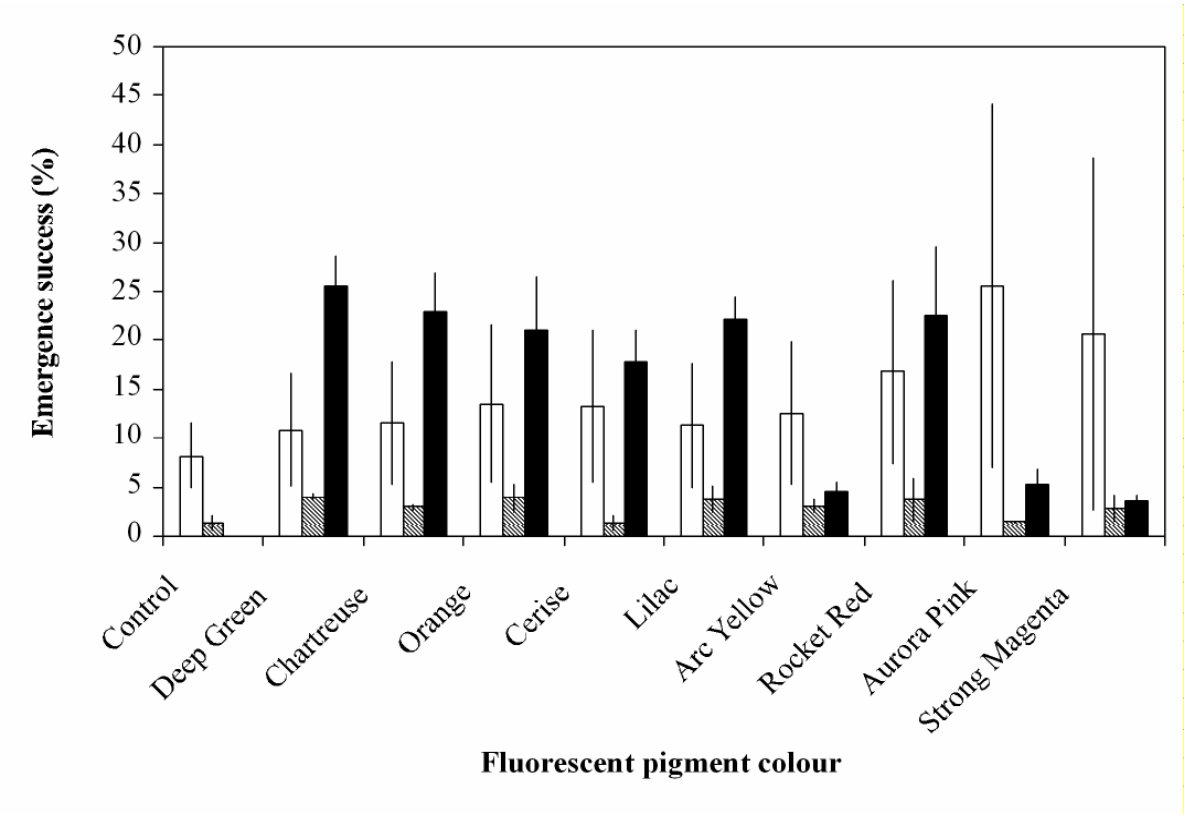
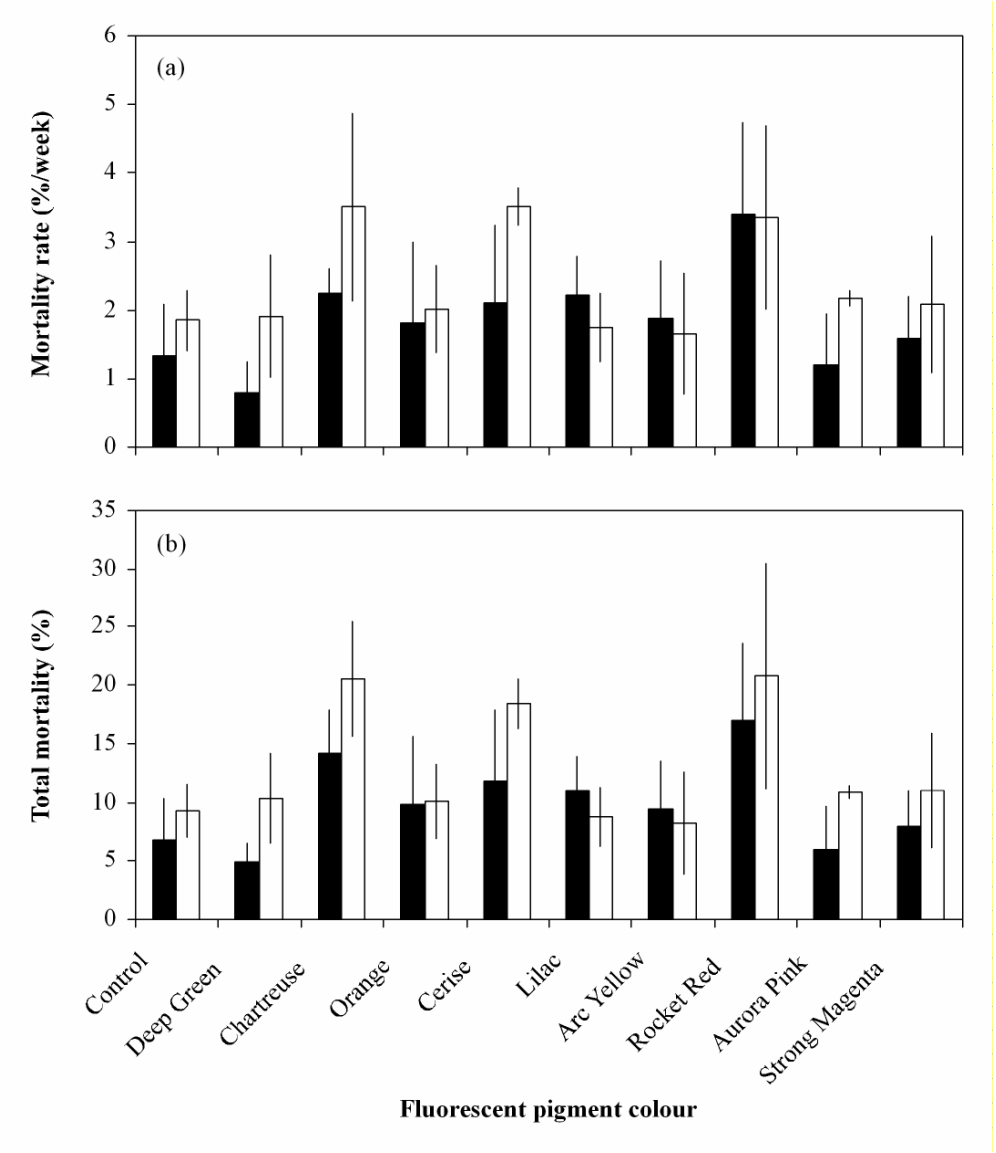


Figure 2. Mortality of *B. tryoni* adults marked with fluorescent pigment powders of different colour (mean ± 1 s.e.). Control flies were not treated with pigment during emergence. (a) Mortality rate. (b) Total mortality. Male: black bars; Female: white bars.



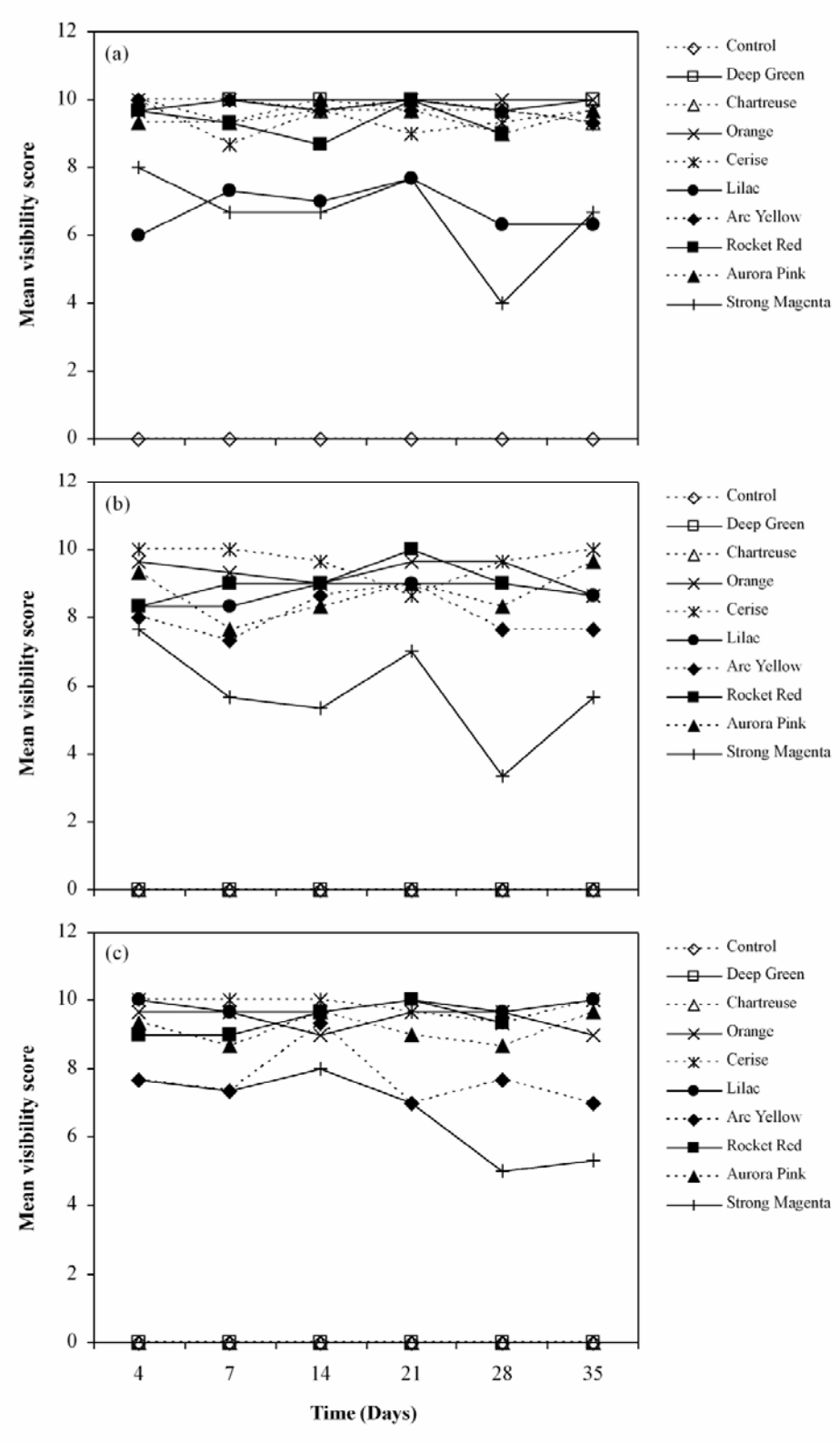
Fluorescent pigment marks had no effect on mortality rate (Figure 2a) or total mortality (Figure 2b) between treatments. Mortality rates and total mortality between males and females were similar, and there was no significant interaction between pigment treatment and sex in either mortality rate or total.

Marker visibility and persistence

Overall, fluorescent pigment mark visibility did not change over a period of 35 days. Mark visibility was significantly different ($p < 0.001$) when observed under different light wavelengths, with fluorescent marks not visible under red light (625 nm) and highest visibility under blue light (467 nm) (Figure 3). There was no interaction between mark visibility over time and light wavelength.

There was a significant difference ($p < 0.001$) between visibility of different fluorescent pigment treatments. Orange, Cerise, Aurora Pink and Rocket Red were the most visible pigments, with Deep Green and Chartreuse being the least visible. However, there was a strong interaction ($p < 0.001$) between fluorescent pigment treatment and light wavelength. Deep Green and Chartreuse fluorescent pigment marks were highly visible under blue light (Figure 3a), but not visible under green light (511 nm; Figure 3b) or yellow light (563 nm; Figure 3c). Lilac was less visible when viewed under blue light than under green or yellow light. Visibility of

Figure 3. Visibility of ptilinal fluorescent pigment marks on adult *B. tryoni* maintained in a laboratory over a period of 35 days. Visibility was scored as the number of flies marked from a random sample of five male and five female flies. Pigment colour visibility was assessed under different light wavelengths. (a) Blue light (467 nm). (b) Green light (511 nm). (c) Yellow light (563 nm).



different pigment treatments also varied with time ($p < 0.05$). Visibility of Strong Corona Magenta decreased during the period of the study (Figure 3).

DISCUSSION

Radglo fluorescent pigment colours Deep Green, Chartreuse, Orange, Cerise and Lilac resulted in lower rates of successful pupal emergence in *B. tryoni*. Particle size of these fluorescent pigment powders was 4 – 5 µm, compared with Arc Yellow, Rocket Red, Aurora Pink and Strong Corona Magenta that were in the form of granules larger than 60 µm. On pupal emergence, flies were completely coated in a layer of fluorescent pigment and there is a possibility that the small particle size of the Radglo pigments may have resulted in blockage of respiratory surfaces. However, this does not explain high mortality of newly-emerged adults marked with Rocket Red. Similarity between pigment treatments for the proportion of pupae that failed to emerge and partially-emerged pupae indicates that differences in emergence are caused by post-eclosion issues.

It has been recommended that an attempt should always be made to ensure that marking techniques do not affect the longevity or behaviour of the animal being studied (Southwood 1978). In this current study, mortality rate and total mortality of *B. tryoni* adults that successfully emerged in the laboratory was not affected by ptilinal marking with fluorescent pigments. This is the first study to demonstrate that survival of male and female *B. tryoni* with fluorescent ptilinal marks does not differ from flies that are unmarked. Earlier studies on the effect of fluorescent pigment marking of various insect taxa have also shown no adverse effects on mortality (Bottenberg and Litsinger 1989, Dowdy and McGaughey 1992, Garcia-Salazar and Landis 1997, Niebylski and Meek 1989, Reinecke 1990). Fluorescent pigment may be non-toxic because the fluorescent chemicals are bound within a stable plastic that is cured and then ground to a fine powder (Reinecke 1990).

Most pigments trialled were highly visible when viewed under at least one of the light wavelengths used in this study. Inspection for fluorescent pigment marks on *B. tryoni* under blue light (467 nm) provided visibility of the greatest range of mark colours, although visibility of Lilac was lower under blue light than under green light (511 nm) or yellow light (563 nm). Inability to detect Deep Green and Chartreuse pigment marks under green and yellow light, as well as lower fluorescence of Lilac under blue light was due to these pigments possessing a peak reflectance wavelength close to that of the transmitted light. The results of the current study may provide an explanation for poor detection of sterile *B. tryoni* marked with Comet Blue 60 (manufacturer unknown) that were observed under blue light (Dominiak *et al.* 1998, Dominiak *et al.* 2003). Current practice for identifying sterile flies released to suppress wild populations of *B. tryoni* is to examine monitoring trap captures using a binocular dissecting microscope under blue light (Dominiak *et al*. 2000a, Dominiak *et al*. 2003), although wavelength of light is not reported. This practice is encouraging, provided that fluorescent pigments used to mark *B. tryoni* have peak reflectance wavelengths that are clearly visible when viewed under blue light.

A majority of fluorescent pigments used to mark *B. tryoni* were persistent, with no change in visibility over the period of the study in the laboratory. The least persistent fluorescent pigment trialled in this study was Strong Corona Magenta, which decreased in visibility over five weeks. It is encouraging that use of this dye colour has not been reported from field releases of *B. tryoni*, even though the majority of fly recaptures are made within 3 – 4 weeks of release (Dominiak and Webster 1998, Dominiak *et al.* 2003, Meats 1998). However, these results do not guarantee durability of the pigment mark in the field (Southwood 1978). There is a theoretical concern that fluorescent pigments may lose their fluorescent properties following extended exposure to sunlight, although this is unlikely to be a problem in studies on dispersal of *B. tryoni* lasting no more than a few weeks. Moreover, sterile flies marked with green fluorescent pigment (manufacturer unknown) have been recaptured and identified with pigment marks up to 26 weeks after release (Dominiak *et al.* 2000a). Despite this, it is advisable that further studies be conducted in the field to examine the persistence of ptilinal pigment marks under natural conditions.

Aurora Pink was the best pigment trialled for fluorescent ptilinal marking due to low mortality immediately after emergence, and high visibility and persistence of the mark. Arc Yellow was also a good candidate for use in mark-release-recapture (MRR) studies with low mortality immediately after emergence, reasonable visibility and high mark persistence. Often it can be useful to be able to distinguish between marker colours when observing trap recaptures. This provides a means to compare two groups of otherwise identical flies that have been released under identical field conditions but have been subjected to different experimental treatments. If sorting flies under blue light, which is the current

practice for identifying recaptured sterile flies released in the field, ptilinal marking with Deep Green and Chartreuse allows for MRR studies with multiple or overlapping releases because they are distinctive from other fluorescent pigments. However, it would be necessary to compensate for lower emergence success when releasing flies marked with these colours. Strong Corona Magenta is the least suitable pigment for ptilinal marking of *B. tryoni*. Despite high emergence in this treatment, low mark visibility and poor persistence of marks reduces the utility of this fluorescent pigment in MRR studies.

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