Marking *Bactrocera dorsalis* (Diptera: Tephritidae) with fluorescent pigments: effects of pigment colour and concentration

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Daylight fluorescent pigment powders are frequently used to self-mark tephritid flies that are released in sterile insect technique programmes and for studies on their population ecology, movement and behaviour. This study was conducted to determine the effects of pigment colour and dose in marking the Oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae). Six pigment colours (Astral Pink 1, Blaze 5, Stellar Green 8, Lunar Yellow 27, Comet Blue 60 and Invisible Blue 70) were applied to pupae at doses of 0, 2, 4, or 6 g/l. Under laboratory conditions, pigment colour had a small but significant effect on the number of partially emerged and deformed adults; the fewest of these were observed when flies were marked with Astral Pink 1. Pigment concentration, on the other hand, had no effect on adult emergence, partial emergence, deformed adults and mortality on the last day of eclosion. There was no significant effect of pigment colour on adult survival under laboratory and semi-field conditions. Under laboratory conditions, however, there was an effect of pigment concentration on adult survival depending on pigment colour. Visibility under an ultraviolet light and persistence of marks was significantly affected by pigment colour and concentration when observed under laboratory conditions, but not under semi-field conditions. Regardless of colour or dose, pigments used in the study were visible for at least 14 days, but began to fade by 21 days after adult eclosion. To mark *B. dorsalis* under temperate, warm summer African conditions, all pigment colours tested in this study may be applied at 2–4 g/l pupae. Recaptures of marked and released flies may be underestimated as the flies age.

Key words: Oriental fruit fly, mark-release-recapture, sterile insect technique.

INTRODUCTION

Identifying marks are applied to insects for various purposes. They can be used for recognition and recording of released insects during control of pests, for example by the sterile insect technique (SIT) (Weldon *et al.* 2015). They can also be used in studies of insect dispersal and population dynamics using 'mark-release-recapture' (MRR) methods (Plant & Cunningham 1991). The use of MRR to study dispersal involves the collection of insects from either the field or a laboratory culture, and the application of identifying marks before their release into the field. A proportion of the insects are then recaptured from the field after a specified period and/or within a specified geographical range, and their distribution is assumed to be representative of all those released (Hagler & Jackson 2001; Hood-Nowotny & Knols 2007). In MRR studies, the ability to accurately identify released insects is essential. Techniques that have been used to mark insects include the use of mutilation (for example, by wing clipping (Walker & Wineriter 1981), paints (Chen *et al.* 2006), tracers such as inks (Franzen & Nilsson 2007), active or passive transponder tags (Gui *et al.* 2011), radio-labelling (Barnes 1959; Moreau *et al.* 2011), natural mutations (Weldon & Meats 2007), and genetic markers (Lushai & Loxdale 2004).

MRR has been used widely to estimate the dispersal capacity of pest fruit flies (Diptera: Tephritidae), with an almost equally large range of methods used to mark them. Genetic markers like the radiation-induced white-eyed mutant gene, and the recombinant enhanced green fluorescent protein gene, have been used to mark the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Handler 2002) and the Caribbean fruit fly, *Anastrepha suspensa* (Loew) (Handler & Harell 2001), respectively. Visible genetic mutations such as orange eyes, white marks and bent wings (Meats *et al.* 2002; Zhao *et al.* 2003) have been used to identify released Queensland fruit flies, *Bactrocera*

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tryoni (Froggatt) in MRR studies (Weldon & Meats 2007). These techniques provide permanent and visible external markers on a strain of insects that can be mass-reared for studies (Saul & McCombs 1992). However, there are limitations to their application, including the narrow range of species for which they have been developed, the rarity of such mutations, and the ethical considerations of releasing genetically modified insects into the environment. In addition, the development of these markers is expensive and time consuming (Hagler & Jackson 2001). Dyes, inks and paints have also been used to mark tephritid flies. For example, ultra-violet-reflective readmission ink was used to directly mark the Oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Froerer *et al.* 2011). Marking with quick-drying, non-toxic readmission ink was inexpensive and led to marks that were easily identified under a black light with a magnifying glass and persisted for more than 4 weeks (Froerer *et al.* 2011). However, the application of inks and paints is time consuming, laborious, and at times requires that insects be anesthetised through chilling or exposure to carbon dioxide, which may affect the physiology and behaviour of the insects (Walker & Wineriter 1981). Proteins specific to vertebrates have also been used in MRR studies of fruit flies such as the solanum fruit fly, *Bactrocera latifrons* (Hendel) (Peck & McQuate 2004). This technique is ideal for quick marking of small insects internally and externally through ingestion and external application. Examination for the presence of the protein is done using the sandwich enzyme-linked immunosorbent assay (ELISA) (Hagler & Jackson 2001), which is a considerable limitation when large numbers of flies are trapped.

The use of fluorescent pigments in dust or powder form is the most common method for marking tephritid flies (Holbrook *et al.* 1970; Schroeder & Mitchell 1981). Originally developed by Norris (1957), the use of fluorescent pigments has been improved by other researchers (Dominiak *et al.* 2000, 2010). Marking of insects with fluorescent pigments provides a quick and affordable technique to mark a large number of insects (Weldon 2005; Weldon *et al.* 2015). Flies are marked by gently tumbling a known quantity of fluorescent pigment powder with the pupae (Gilchrist & Meats 2012). The dust adheres to the ptilinum of the adult as it emerges from the puparium and is retained within the head when the ptilinum is retracted (Norris 1957). Dust particles that adhere to other body parts are usually disposed of over time by continual grooming by the adult. Flies that have been marked with these pigments can be easily identified by viewing the ptilinal suture and frontalia of the fly under an ultraviolet light (Norris 1957). This method of marking is relatively simple, inexpensive, and may allow for direct field observations under particular conditions.

The use of fluorescent pigments to mark tephritid flies has its shortcomings. An ideal pigment should not affect fly quality, be easily visible, and persist for the duration of an MRR study. However, an increase in the concentration of pigment applied to pupae has been reported to increase the number of partially eclosed pupae and decrease eclosion and flight ability (Campbell *et al.* 2009; Dominiak *et al.* 2010). This may partly result from the blockage of respiratory surfaces by fine dust particles (Weldon 2005). Excessive dye particles on released marked flies may also influence recapture results as unmarked wild flies can be misidentified as released sterile flies when they collect dye particles in traps (Gilchrist *et al.* 2004; Chen *et al.* 2016). International standards set for pigment marking of tephritid flies for SIT have been reduced from a recommended concentration of Day-Glo® powder of 2.0–2.5 g/l pupae in 1999 (FAO/IAEA/USDA 1999) to 1.5 g/l pupae in 2003 (FAO/IAEA/USDA 2003). However, there are still various units of measure of pupae (mass or volumetric) as well as pigment concentrations used to mark fruit flies. Volumetric concentrations of 2 g/l pupae for the olive fly, *Bactrocera oleae* (Gmelin) (Rempoulakis & Nestel 2012), and 5 g/l pupae (Peck *et al.* 2005) or 3 g/l pupae (Shelly & Edu 2010) for the melon fly, *Zeugodacus cucurbitae* (Coquillett), have been used. Concentrations of pigments by weight of pupae such as 0.8 g/100 g pupae (Reynolds *et al.* 2010) and 0.5 g/100 g pupae (Meats & Edgerton 2008) have also been used for marking *B. tryoni*. Comparison of results from different fluorescent pigment marking trials is challenging as the weight and volumes of different cohorts of pupae differ substantially according to strain or rearing system (FAO/IAEA/USDA 2014). The ideal concentration for marking may also vary relative to the particle size of the fluorescent pigment powder. Furthermore, when more than one pigment colour is used for releases of different cohorts of flies, the ability to differentiate between colours is critical (Weldon 2005). This is particularly the case when the reflected

wavelengths of the pigments under UV light are not easy to differentiate, such as orange and pink (Dominiak *et al.* 2003). It has also been suggested (Dominiak *et al.* 2000, 2003) that different colours may have an effect on emergence and mortality of fruit flies. Colours of fluorescent pigment powders within a particular series have different chemical compositions (Safety Datasheets, T-series, Swada, Cheshire, U.K.). Due to these differences in the chemical composition of different pigments, the colours chosen for marking flies could differ in their impact on development and survival of the flies. It will therefore be important that the performance of various colours of pigments for marking of flies be evaluated so that effective pigment colours can be used for MRR studies and for marking of sterile flies during an SIT programme.

Bactrocera dorsalis is an important fruit fly pest that, following its first detection in Africa in 2003, spread rapidly within a short period in the eastern, western and central parts of the continent (De Meyer *et al.* 2010; Drew *et al.* 2005; Mwatawala *et al.* 2004) The pest spread further and has over the years become established in southern African countries including Mozambique, Zambia, Zimbabwe, and some parts of Namibia and South Africa (Chinhanga *et al*. 2013; Correia *et al.* 2008; De Meyer *et al.* 2010; EPPO 2015; Manrakhan *et al.* 2015). Many control strategies have been recommended and used to reduce the population size of this pest in southern Africa, including bait sprays and male annihilation technique, application of bio-pesticides such as *Metarhizium anisopliae*, fruit bagging, and orchard sanitation (Ekesi & Lux 2006; Manrakhan *et al.* 2012; Ogaugwu 2014). The potential for use of SIT against this pest has also been raised. The tendency of this highly polyphagous pest to move into and recolonise treated areas (Weldon *et al.* 2015) makes it critical for knowledge to be gathered on its potential dispersal under southern African conditions. This information could be obtained from MRR studies, but it is first necessary to optimise marking methods for these flies to ensure high emergence and survival of marked flies, as well as high visibility and persistence of marks. In Hawaii, *B. dorsalis* has been marked for dispersal studies with a fluorescent pigment concentration of 3 g/l (Shelly & Edu 2010), but to our knowledge there are no published studies that have determined the best colour(s) and concentration of pigment to mark this species. Studies involving MRR can make use of multiple fluorescent pigment colours in the same experiment. It is therefore important to know of any differential effects (or lack thereof) on the target species by different pigments. This study examined the effects of six daylight fluorescent pigments at different concentrations on adult emergence, survival, and mark visibility and persistence for *B. dorsalis* using a self-marking technique under laboratory and semi-field conditions.

MATERIAL AND METHODS

Insect rearing and maintenance

Bactrocera dorsalis used in this study were from a laboratory colony established from infested fruit (mango, peach and guava) mainly collected from Mashonaland Central Province in Zimbabwe during the period 2012 to 2014. The colony was maintained in the Entomology laboratory of the Plant Quarantine Station in Mazowe (17°33′30.5″S 30°58′54.41″E), Zimbabwe. Wild flies reared from infested fruit or trapped live in methyl eugenol baited Chempac® bucket traps, were periodically added to the laboratory culture to minimise the chance of inbreeding and loss of traits that enable survival in the field. Further generations of the laboratory culture were reared from eggs collected from Granny Smith apples or artificial oviposition substrates that were transferred to a carrot-based larval diet developed and supplied by Citrus Research International (Nelspruit, South Africa). The artificial oviposition substrates consisted of lidded yellow, green and red rectangular plastic containers (500 and 1000 ml) perforated (using a size 3 entomological pin) along all four sides (approximately 15 holes each side). These containers were inverted so that the lids formed the base of the oviposition substrate. Moistened kitchen sponges were inserted into the inverted containers to maintain humidity and prevent egg desiccation. Eggs were collected after 2–3 consecutive days by rinsing the containers and sponge with water from a spray bottle. Excess water was drained away and the eggs were placed on prepared larval diet spread in glass (100 × 15 mm) or plastic disposable (90 × 15 mm) Petri dishes. Eggs were placed on the diet at a ratio of two eggs per 1 g diet (dry mass). The Petri dishes were placed on top of moist sand in 1–2 litre plastic containers covered by voile curtain fabric. Golden to brown coloured pupae were used for the experiments whilst black pupae were excluded as they were presumed dead. For

each batch of pupae, the average weight of one pupa was calculated by counting 100 pupae into a glass Petri dish, weighing them, and dividing their weight by 100.

Experiment 1: Laboratory trials

Laboratory trials with *B. dorsalis* on emergence, mortality and visibility of fluorescent pigment marks were conducted in the above-mentioned facility. To be able to test the effects of pigment colour and concentration on emergence, visibility and persistence of marks, 50 pupae per treatment were dyed with fluorescent pigments in powder form (T-series, Swada, U.K.) 2–3 days prior to adult eclosion. To estimate the correct amount of pigment to apply to 50 pupae, the volume of 200 pupae (~7 ml) from each batch was used to calculate the mass of dye appropriate to give concentrations of 0, 2, 4 and 6 g pigment/l of pupae. Colours tested were Astral Pink 1, Blaze 5, Stellar Green 8, Lunar Yellow 27, Comet Blue 60 and Invisible Blue 70. According to their materials safety data sheets (Safety data sheet, Swada T001, T005, T008, T027, T060, T070, Revision 62, 18-Mar-16, Swada), the pigment powders were dyed melamine, sulphonamide, formaldehyde thermoset copolymers with an average size of ~6 μm and pH 6.0–7.5 at 20 °C. The pigment colours evaluated differed in their chemical composition. Stellar Green 8 and Lunar Yellow 27 contained titanium dioxide. Invisible Blue 70 contained 4-methyl-7-(diethylamino) coumarin. The selected pigments are normally used as pigments for inks, paints, aerosols, and plastisol and organosol coatings.

Pigment concentrations were weighed on weighing paper using a Sartorius BP1105 scale (with max. weight 110 g, d.p = 0.0001 g). The pigment was then placed over the pupae in 10 ml pill cups that ultimately served as emergence containers that were then sealed with laboratory film (Parafilm "M"®, Bemis Flexible Packaging, Neenah, WI 54956, U.S.A.). The pupae were evenly coated with the appropriate pigment by gentling swirling the pigment and pupae in the containers for 30 s. The control pupae (0 g/l pupae) were also swirled as in the other treatments. The emergence containers were opened thereafter and a thin layer of moistened vermiculite was placed over the pupae in each cup. Moistened vermiculite was used to enhance adult eclosion. The vermiculite was moistened prior to use by immersing in hot water at a ratio of 4:1 and cooled overnight (Reynolds *et al.* 2010). Insect cages (24 in total) with dimensions 32.5 × 32.5 × 32.5 cm (BugDorm-43030, MegaView Science, Taichung, Taiwan) were placed along a laboratory bench next to five adjacent windows so that flies experienced a natural light cycle of approximately 12L:12D h. Allocation of treatments to cages was random. Three replicates of each trial were done over time.

Adult emergence in each treatment was recorded daily over four consecutive days until no further eclosion was observed, noting the last day of eclosion (= day 0). Emergence containers were then removed from the cages. Besides adult emergence, the following emergence categories in each treatment were recorded: the number of uneclosed pupae, partly emerged flies with pupal cases still attached and which died before full eclosion, and fully-emerged but alive and deformed adults (*e.g.* misshapen wings, pupal cases still attached, incapable of flying). Live but deformed adults were not included in records of adult mortality. 'Fliers' were in this study described as adult flies with fully expanded wings and with no abnormalities. Adult mortality in each treatment on the day of eclosion (day 0) was recorded as the total number of dead flies with no obvious abnormalities. Mortality of post-teneral flies were then recorded at 7, 14, 21 and 28 days after eclosion to assess the effect of fluorescent pigment colour and concentration on longer-term survival.

To determine the marking effectiveness of each pigment colour and concentration, fliers were observed for ptilinal pigment marks on day 0, 7, 14, and 21 after eclosion before being returned to their respective cages. This was done by placing a random sample of 10 flies from each treatment into clear 15 ml glass or plastic tubes of 7 mm height (1 fly per tube) plugged with cotton wool. The ptilinal suture and other parts of the insect body were inspected for traces of fluorescent pigment under an ausJENA (×50) dissecting microscope illuminated with an ultraviolet (UV) light source (365 nm). Non-destructive sampling was employed at this stage to minimise the chance of changes in density, from the removal of adults, affecting survival. If flies became overactive during the observation, especially when markings were not initially obvious, they were further subdued by placing the tubes between two gel ice blocks wrapped in a paper towel for about 5 min to complete the observation. At 28 days after eclosion, all surviving flies were inspected for pigment marks.

To completely immobilise the flies for the observations, they were placed in a freezer (below 0 °C) for 30 min. At this age, if ptilinal pigment marks were not visible, fly heads were removed from the rest of the body and crushed separately. Each head was placed between a new microscope slide and cover slip and then observed with an ausJENA (×50) dissecting microscope illuminated with an ultraviolet (UV) light source (365 nm) for pigment traces. Each tube was used only once for an observation before being cleaned with 75 % alcohol to prevent pigment contamination between observations. The experiment was replicated three times during the period August 2014 to August 2015 using three generations of *B. dorsalis*.

Experiment 2: Semi-field trials

Semi-field trials were conducted for 28 days (excluding eclosion days) during the periods February to March, March to April, and July to August 2015 to determine fly mortality and visibility of pigment marks associated with different concentrations of the six fluorescent pigments as described above. The trials were performed to verify that laboratory results were also valid for field conditions. Pupae obtained from the laboratory-reared culture of flies were treated with fluorescent pigments in the same way as described above, except that only concentrations of 0, 2, and 4 g/l pupae were used for all pigment colours. The 6 g/l treatment was excluded from the semi-field trials because laboratory results indicated that 2 and 4 g/l were satisfactory for marking flies with high levels of emergence and low mortality. Flies were eclosed in 1-litre labelled glass jars covered by voile curtain fabric for ventilation prior to being transferred to individual sleeve cages (18 in total as described below). Adult emergence for pupae treated with all concentrations of the pigment colours was recorded as described below (Table 1).

One loquat tree (*Eriobotrya japonica* (Thunb.) Lindl.) of approximately 5 m in height, located at Henderson Research Station in Mazowe, Zimbabwe (17°35′37.55″S 30°57′56.93″E), was used for this trial. Eighteen branches were selected for the sleeve cages, which were trimmed to leave four leaves per branch. One sleeve cage of 40 × 40 cm dimensions made of white polyester material and a clear vinyl viewing area of 40 × 10 cm (BugDorm DC3000W-S, MegaView Science, Taichung, Taiwan) was fitted on each branch. Outside the sleeve cage, tree branches were coated with petroleum jelly to prevent entry of ants. Ant management on the tree as a whole was reinforced by application of used engine oil at the base of the tree trunk to keep ants off the tree. The pigment colours and concentrations were randomly assigned to the 18 tree branches. Cages contained water supplied from an absorbent cotton wool swab dipped in water at the start of the trial to supplement the natural condensation of water in the sleeve cage and subsequently replenished weekly during sampling of fruit flies for observations. A sugar and enzymatic yeast hydrolysate (3:1) paste made by mixing a small amount of water to the dry ingredients was applied to the base of one leaf as a food source for the flies. On eclosion (day 0), flies that had emerged into glass jars (Table 1) were released into their respective sleeve cages whist covering the jar with a black cloth.

As in the laboratory trial, visibility of pigment marks on all live flies was assessed at 0, 7, 14, 21, 28 days after adult eclosion by randomly collecting 10 flies from each sleeve cage into labelled, clear glass tubes (1 fly per tube) plugged with cotton wool and then transported approximately 4.2 km to the entomology laboratory in an air-conditioned car for pigment mark observations. The glass tubes

Table 1. Mean (±1 S.E.) percentage of fliers on the day of eclosion of *Bactrocera dorsalis* marked as pupae with fluorescent pigments of six colours at concentrations of 0, 2 and 4 g/l pupae used for semi-field trials.

Name of pigment	Concentration		
	0 g/litre pupae	2 g/litre pupae	4 g/litre pupae
Astral Pink 1	70.6 ± 5.8	68.3 ± 5.4	59.4 ± 6.4
Blaze 5	58.3 ± 7.5	67.8 ± 6.2	62.8 ± 9.3
Stellar Green 8	62.2 ± 8.0	70.0 ± 1.9	71.1 ± 7.7
Lunar Yellow 27	63.3 ± 7.3	58.9 ± 2.4	63.9 ± 7.8
Comet Blue 60	55.0 ± 4.4	63.9 ± 10.3	70.6 ± 5.5
Invisible Blue 70	69.4 ± 8.0	69.4 ± 7.0	66.7 ± 3.3

were kept at ambient room conditions as all samples were assessed for pigment marks using the same methods described under 'Laboratory trials' above. The flies were returned to their respective sleeve cages on completion of assessments. Cumulative mortality of adult flies over 28 days after eclosion was recorded at the end of the assessment period to assess the effect of pigment colour and concentration on survival. On day 28, all live flies were released into a BugDorm cage and the number of dead flies remaining in the sleeve recorded. This experiment was replicated three times using three generations of *B. dorsalis*. Records of the daily maximum and minimum temperature and rainfall were obtained from the Henderson Research Station Meteorology substation (17°35′1.37″S 30°58′0.23″E), which was approximately 1 km from the experiment site.

Data analyses

A two-way multiple analysis of variance (MANOVA) was performed using IBM SPSS Statistics version 23 (IBM Corp., Armonk, NY, U.S.A.) to determine the effect of fluorescent dye colour and concentration on full eclosion, partial eclosion, adult deformities and mortality on day 0. Significant effects from the MANOVA were explored with univariate analyses of variance and *post hoc* tests (Tukey's honestly significant difference). For both the laboratory and semi-field trials, logistic regression was performed using R Studio© (Version 0.99.473, 2009–2015, RStudio, Inc.) running R to compare cumulative mortality by 28 days after adult eclosion in laboratory and semi-field conditions in relation to pigment colour and concentration. The same method was also used to determine the effect of pigment colour and concentration on the presence and absence of ptilinal pigment marks at the end of the 28-day assessment period. Logistic regression models were rerun with the 'relevel' function to vary the reference level so that parameter estimates could be used to identify simple effects. An assessment time of 28 days was selected as it aligned with the duration of planned MRR experiments. It was not possible to incorporate a time effect into this analysis because a repeated measures analysis would be required but the number of replicates was insufficient to allow this. Due to this, and to minimise the chance of type I errors introduced by conducting large numbers of separate analyses at each time period, only data at 28 days were analysed statistically. Patterns up to 21 days are trends shown by the data.

RESULTS

Experiment 1: Laboratory trials

Pigment colour had a significant effect on adult emergence categories (MANOVA: Wilks $\lambda = 0.475$ $F_{20,150} = 1.891$, $P = 0.017$). Neither concentration (Wilks $\lambda = 0.853$, $F_{12,119} = 0.618$, $P = 0.823$) nor the interaction between pigment colour and concentration had an effect on adult emergence categories (Wilks $\lambda = 0.469$ $F_{60,178} = 0.634$ $P = 0.979$). The significant overall effect of pigment colour was due to significant pigment colour effects on the number of partly emerged adults ($F_{5,48} = 3.758$, $P = 0.006$) and the number of deformed adults ($F_{5,48} = 2.529$, $P = 0.041$). Lunar Yellow 27 and Comet Blue 60 resulted in the highest number of partly emerged adults and the highest number of deformed adults, respectively (Fig. 1a, b). The lowest number of partly emerged adults was from the pupae marked with Astral Pink 1 and Invisible Blue 70. The number of partly emerged adults when marked with Comet Blue 60, Stellar Green 8 and Blaze 5 was intermediate between Lunar Yellow 27 and Astral Pink 1 or Invisible Blue 70 but not significantly different from them or each other (Fig. 1a, b). The lowest number of deformed adults was from pupae marked with Astral Pink 1, which were significantly less than those marked with Comet Blue 60. The number of deformed adults when marked with other pigment colours was intermediate between the two extremes but not significantly different from them or each other (Fig. 1 a, b). Pigment colour did not have a significant effect on adult mortality on the day of emergence (Fig. 1c; $F_{5,48} = 0.200$, $P = 0.961$), nor on the number of fliers (Fig. 1d; $F_{5,48} = 0.323$, $P = 0.897$). The crushing of the fly heads between a new microscope slide and cover slip and observing them with the dissecting microscope and an UV light source for pigment traces did not detect pigment marks that had not otherwise been visible without this technique. This verifies that lack of visually-detected marks was not an artefact of the method used.

From 0 to 14 days after emergence in the laboratory, survival of adult flies remained above 90 % regardless of pigment colour or concentration. However, at 21 days survival dropped below 90 % among flies marked with Astral Pink 1, Invisible

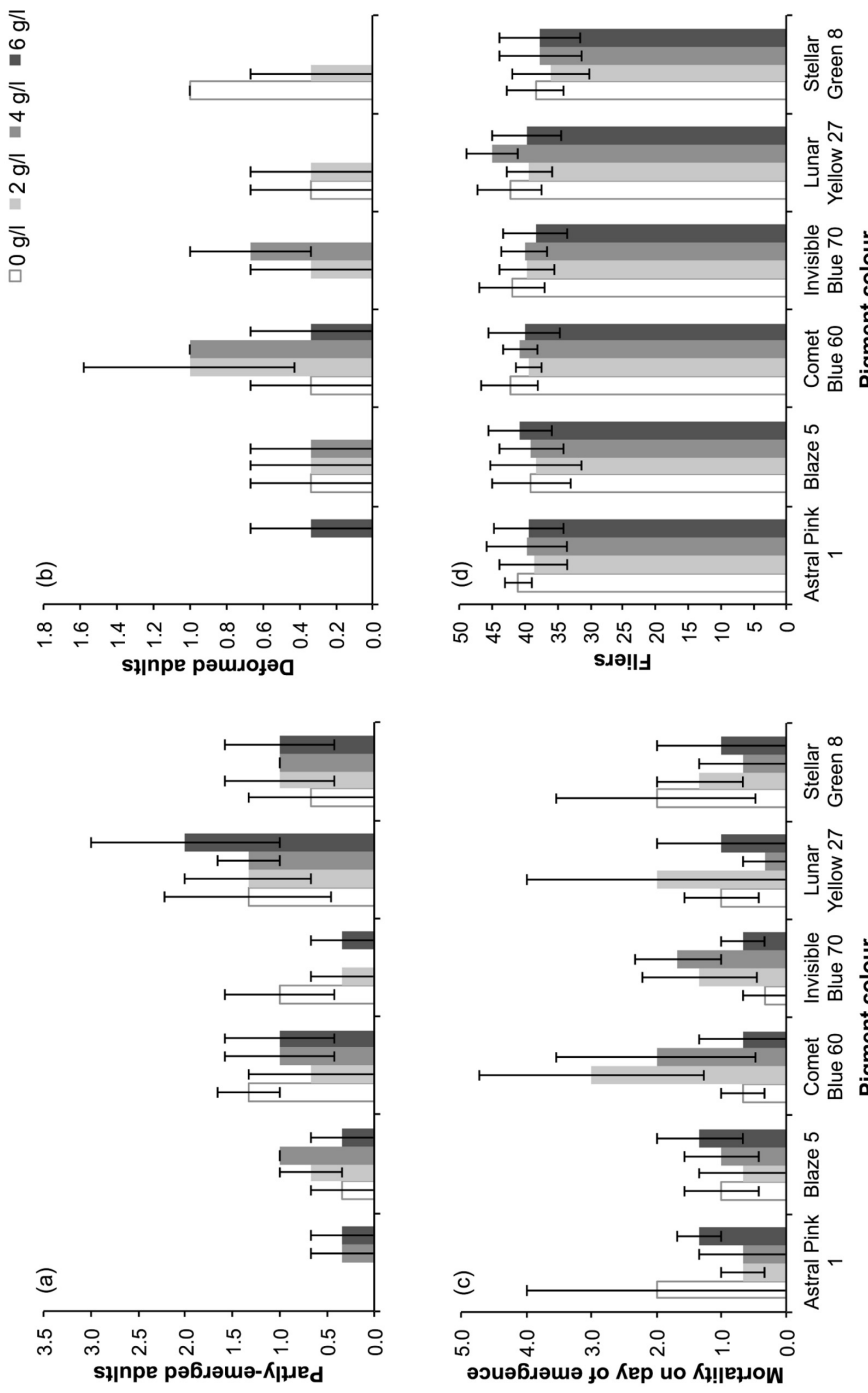

Fig. 1. Effect (mean % ± 1 S.E.) of six fluorescent pigment colours at concentrations of 0, 2, 4, and 6 g/l of *Bactrocera dorsalis* marked as pupae on partial emergence, deformed adults, mortality on day of eclosion, and flight ability. **a**, Partly-emerged adults; **b**, deformed adults; **c**, mortality on day of eclosion; **d**, fliers.

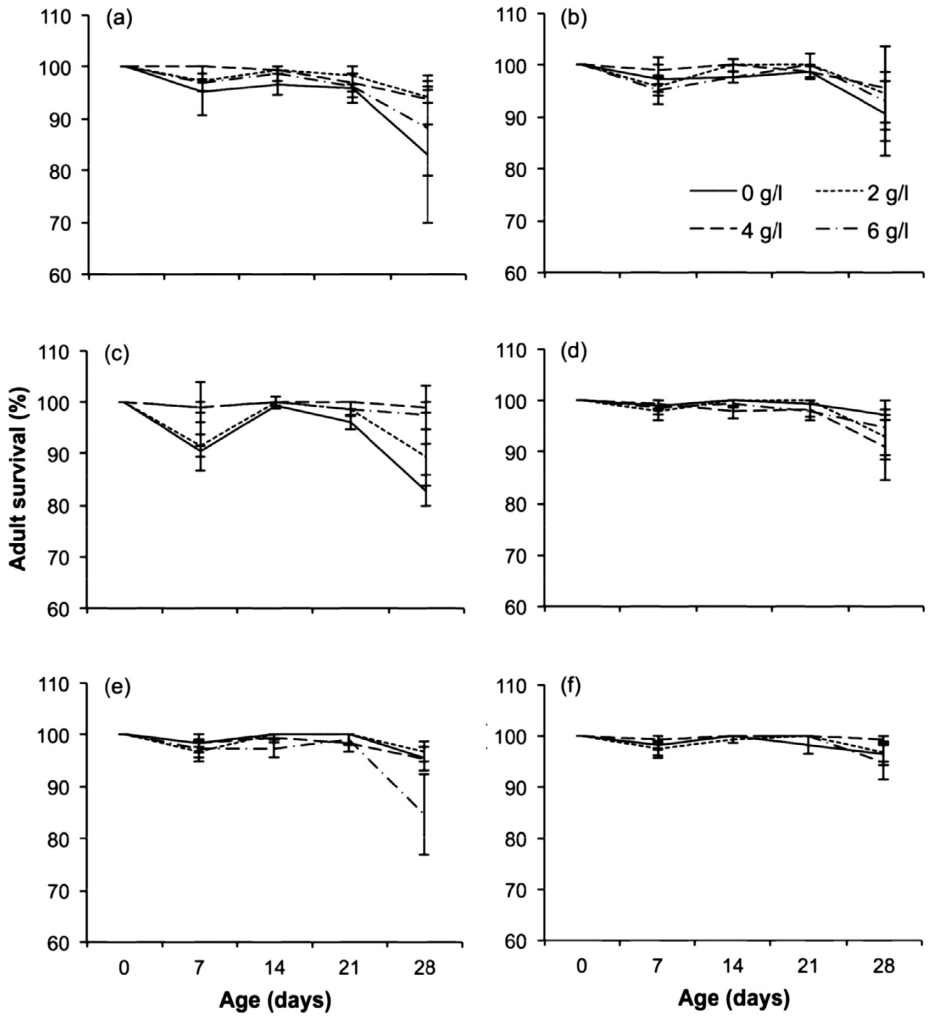

Fig. 2. Survival (mean % ± 1 S.E.) under laboratory conditions of adult *Bactrocera dorsalis* marked as pupae with fluorescent pigments of six colours at concentrations of 0, 2, 4, and 6 g/l pupae. **a**, Astral Pink 1; **b**, Blaze 5; **c**, Stellar Green 8; **d**, Lunar Yellow 27; **e**, Comet Blue 60; **f**, Invisible Blue 70.

Blue 70 and Lunar Yellow 27, whereas survival among flies marked with Blaze 5 and Stellar Green 8 remained above 90 % (Fig. 2). Survival to 28 days after laboratory eclosion was significantly affected by pigment colour ($\chi^2 = 31.900$, d.f. = 5, $P < 0.001$) and concentration ($\chi^2 = 8.249$, d.f. = 3, $P = 0.041$). Overall, cumulative mortality in the laboratory at 28 days after eclosion was significantly higher among flies marked with Astral Pink 1, Stellar Green 8 and Blaze 5 than those marked with Lunar Yellow 27 and Invisible Blue 70. Flies marked with Comet Blue 60 had lower levels of mortality than those marked with Astral Pink 1 and Stellar Green 8, but did not differ significantly from Blaze 5, Lunar Yellow 27 or Invisible Blue 70. Overall, cumulative mortality in the laboratory at 28 days after eclosion when no pigment was applied was significantly higher than when it was applied at 2 g/l pupae. There was however a significant interaction between pigment colour and concentration on adult mortality ($\chi^2 = 49.729$, d.f. = 15, $P < 0.001$). There was no difference in mortality between pigment concentrations within flies marked with Blaze 5 and Invisible Blue 70, whereas flies marked with Lunar Yellow 27 and Comet Blue 60 at 4 g/l pupae suffered higher mortality than their unmarked counterparts (Fig. 2).

Visibility of pigment marks under laboratory

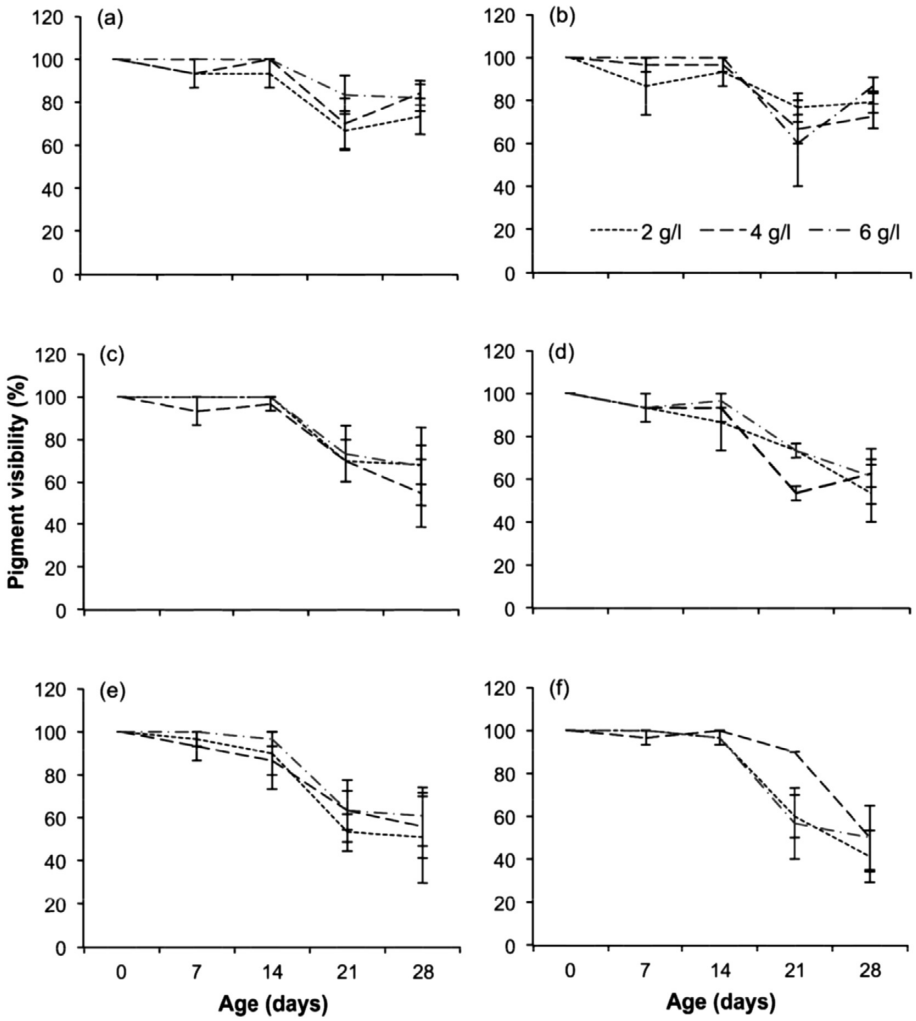

Fig. 3. Mean (±1 S.E.) percentage of adult *Bactrocera dorsalis* with visible marks under ultraviolet light after having emerged from pupae coated with fluorescent pigments of six colours at concentrations of 2, 4 and 6 g/l pupae and held under laboratory conditions. **a**, Astral Pink 1; **b**, Blaze 5; **c**, Stellar Green 8; **d**, Lunar Yellow 27; **e**, Comet Blue 60; **f**, Invisible Blue 70.

conditions remained high from 0–14 days before declining to lower levels between 21 and 28 days after eclosion (Fig. 3). At 28 days there was a significant difference in the visibility of pigment marks between different colours (χ^2 = 11.602, d.f. = 5, P = 0.041). Pigment retention by individuals marked with Astral Pink 1 (80.0 ± 3.8 %) and Blaze 5 (79.6 ± 3.2 %) was significantly greater than pigment retention by individuals marked with Lunar Yellow 27 (59.0 ± 5.8 %) and Comet Blue 60 (55.7 ± 8.5 %), with individuals marked with Stellar Green 8 (63.3 ± 7.8 %) intermediate between those two groups. Retention of Invisible Blue 70 by individuals (47.0 ± 7.3 %) was significantly lower than that for Stellar Green 8, but not different from Lunar Yellow 27 or Comet Blue 60.

Experiment 2: Semi-field trials

Semi-field trials were conducted between February and August 2015. Weather conditions during the months when replicates 1 and 2 were run (February–April) were generally warm, with average maximum and average minimum temperatures ranging from 26–29 °C and 12–16 °C, respectively. This period was relatively dry with the exception of February, when 207 mm rainfall

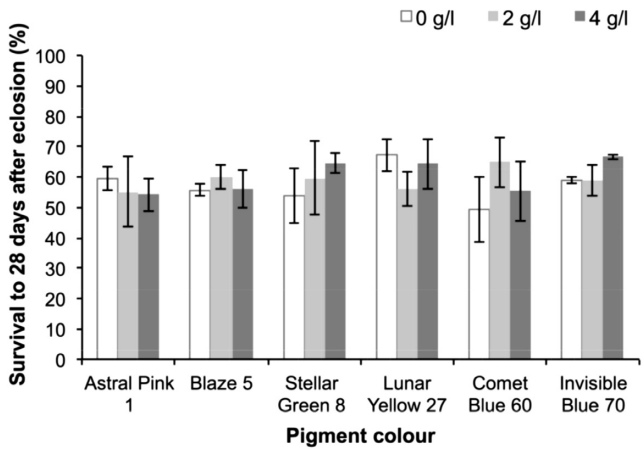

Fig. 4. Survival (mean % ± 1 S.E.) of adult *Bactrocera dorsalis* marked with fluorescent pigment of six colours at concentrations of 0, 2 and 4 g/l pupae after being held under semi-field conditions for 28 days.

was recorded, most of it (~190 mm) in the first 3 weeks before the trials started on 27 February 2015. Although average maximum temperatures were more or less similar during the periods all the three replicates were run, the average minimum temperatures and rainfall was lower in the third replicate of the test. The period July–August was generally less warm with average maximum and average minimum temperatures ranging from 25–28 °C and 0–2 °C, respectively. It was also much drier (0 mm rainfall) during this period than the period February–April. Data on relative humidity and hours of sunshine were not available from the weather station.

At 28 days after adult eclosion, mean mortality across pigment colours and concentrations under semi-field conditions was 46.9 ± 2.6 %. There was no significant effect of pigment colour ($\chi^2 = 4.876$, d.f. = 5, $P = 0.431$), concentration ($\chi^2 = 1.171$, d.f. = 2, $P = 0.557$), or their interaction ($\chi^2 = 7.619$, d.f. = 10, $P = 0.666$) on mortality by 28 days after adult eclosion (Fig. 4). Under these conditions, however, pigment marks remained 100 % externally visible only up to 7 days after adult emergence (Fig. 5). Thereafter, the proportion of flies that retained pigment marks declined over time so that at 28 days after adult eclosion, only 67.2 ± 4.8 % of surviving flies had visible pigment marks when viewed under a dissecting microscope. There was no significant effect of pigment colour ($\chi^2 = 0.392$, d.f. = 5, $P = 0.996$), concentration ($\chi^2 = 0.012$, d.f. = 1, $P = 0.912$), or their interaction ($\chi^2 = 2.590$, d.f. = 10, $P = 0.763$) on retention of pigment marks up to 28 days after adult eclosion.

DISCUSSION

Taking all factors into consideration, all six daylight fluorescent pigments evaluated here were suitable for marking *B. dorsalis*. Astral Pink 1 had a slight advantage compared to the other pigment colours in that it led to fewer partially emerged and deformed flies. Under semi-field conditions, none of the pigments evaluated had an effect on mortality of flies after eclosion or visibility of pigment marks over 4 weeks of observation. These findings on adult survival are similar to those reported by Weldon (2005) on marked *B. tryoni*. It is speculated that poor adult emergence in marked *B. tryoni* reported by Weldon (2005) may have been caused by the small particle size in the pigment powders used (4–5 μm) which may have caused blockage of spiracles. However, there was no evidence of this effect in this study. The T-series of Swada pigments used in this study have an average particle size ~6 μm (Technical datasheet, T-series, Swada, Cheshire, U.K.: http://www.swada.co.uk), which is slightly larger than the FEX series that has been tested in other studies (Campbell *et al.* 2009; Dominiak *et al.* 2010).

Over the range of pigments and concentrations tested, this study did not identify an optimal dose for marking *B. dorsalis*. The different doses of fluorescent pigments in the T series from Swada tested here had no effect on adult eclosion. Flies marked with concentrations of 2 g/l often survived better than the unmarked control flies under laboratory conditions. Considering that an ideal marker is regarded as one that does not negatively

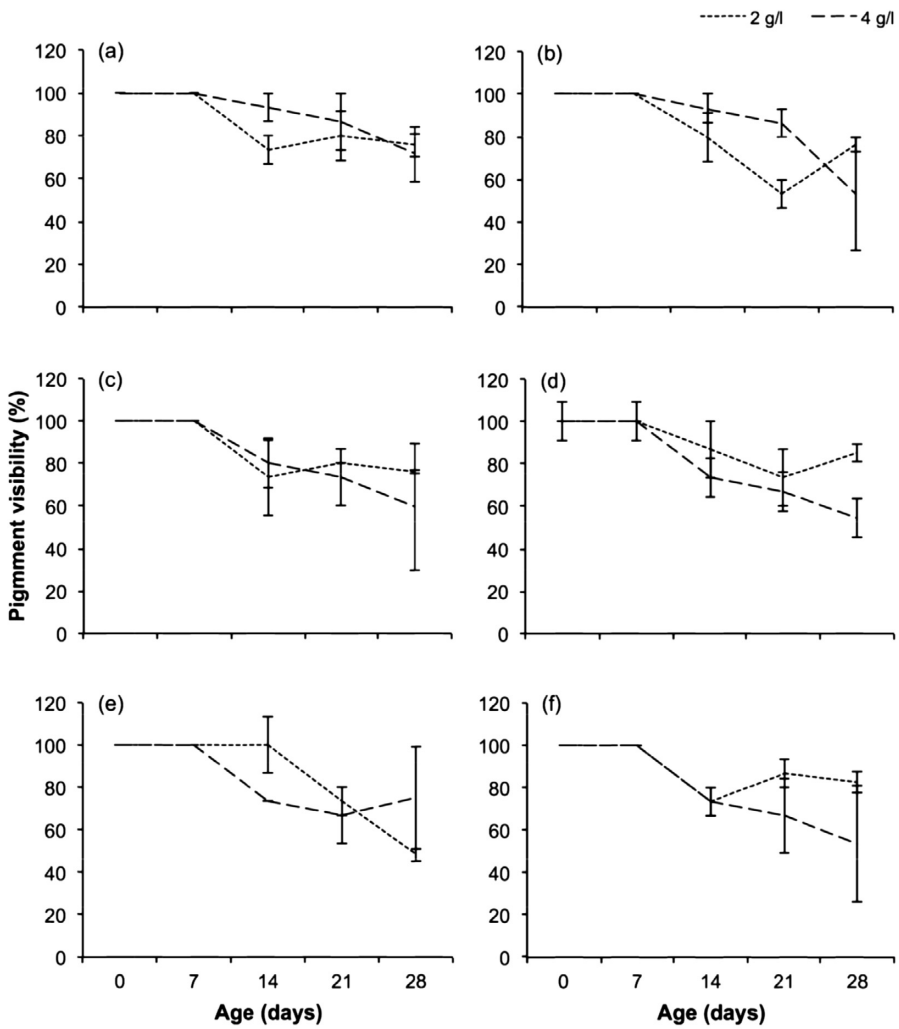

Fig. 5. Mean (±1 S.E.) percentage of adult *Bactrocera dorsalis* with visible marks under ultraviolet light after having emerged from pupae coated with fluorescent pigments of six colours at concentrations of 2 and 4 g/l pupae and held under semi-field conditions. **a**, Astral Pink 1; **b**, Blaze 5; **c**, Stellar Green 8; **d**, Lunar Yellow 27; **e**, Comet Blue 60; **f**, Invisible Blue 70.

affect the normal processes of an insect (Hagler & Jackson 2001), these findings suggest that any dose of the fluorescent pigments in the T series of Swada tested in this study can be used in MRR trials with negligible negative effects. These results contrast with those from other studies and recommendations for marking of tephritid flies. An increase in the concentration of Astral Pink fluorescent pigment of the FEX series from Swada has been reported to cause a decrease in adult eclosion of *B. tryoni*, and an increase in deformed adults and partial eclosion of adult flies of this species (Dominiak *et al.* 2010). Moreover, international standards set for dye marking of flies for SIT have been reduced from a concentration of Day-Glo® powder of 2.0–2.5 g/l pupae in 1999 to 1.5 g/l pupae in 2003 (FAO/IAEA/USDA 2003; FAO/IAEA/USDA 1999). It is likely that effects of pigment concentration on adult development and survival would be different depending on the type of fluorescent pigment used (pigment series) and the species being marked. As such, recommendation of a generic concentration of fluorescent pigment might not be relevant. Concentration of a particular type of pigment for marking of fruit flies should as such be tested before use.

The six pigments exhibited excellent retention of colour for 7–14 days, which gradually decreased thereafter. Under laboratory conditions, Astral Pink 1 had the highest pigment retention after 28 days. Blaze 5, with a characteristic orange colour, also exhibited good visibility for up to 28 days after adult eclosion. Astral Pink 1 in the FEX series produced by Swada (rather than the T-series used here) has been used as a standard colour to mark sterile flies with a concentration of 0.8 g/100 g pupae in SIT programmes against *B. tryoni* (Reynolds *et al.* 2010). Similarly, Blaze 5 matches the DayGlo® colour recommended for use in SIT by the International Atomic Energy Agency (FAO/IAEA/USDA 2014). Under laboratory conditions, the pigment colours with the lowest retention were Comet Blue 60 and Invisible Blue 70, but this difference was not evident under semi-field conditions.

It is unclear why ptilinal pigment marks faded over time in this study. Pigment particles on the surface of the body are removed over time by the grooming exhibited by fruit flies. Fruit flies have been observed to increase the natural action of grooming when minute particles such as conidia from fungi are applied to their bodies (Dimbi *et al.* 2009). Fruit flies have been observed to have increased locomotor activity (including grooming) as the day progressed with a high peak being observed late in the afternoon (Dominiak *et al.* 2014). It is possible that grooming also impacts on retention of pigment on the ptilinum. In the blow-fly *Calliphora vicina* Robineau-Desvoidy, the ptilinal suture does not appear to seal completely when the ptilinum is invaginated within the head (Laing 1935). If this is the case in *B. dorsalis*, continual grooming and exposure to moisture may lead to the gradual displacement of pigment particles from the invaginated ptilinum through the ptilinal suture. In this case, the addition of non-toxic adjuvants such as flour (Nielsen & Nielsen 1953) and gum arabic (Hagler & Jackson 2001) could be explored to improve on the adherence of pigments in order to prolong the visibility of marked flies in MRR experiments. Another possibility is that the membranous cuticle of the invaginated ptilinum loses structural integrity as the adult fruit fly ages. In *C. vicina*, most of the muscles associated with ptilinum function are degraded within two days of adult eclosion, and the ptilinal wall becomes very thin by 14 days after adult eclosion (Laing 1935). If this occurs in *B. dorsalis*, it may be that fluid from the head capsule combined with movements by the fly dislodges pigment particles from the residual ptilinum wall into the head capsule. To test either of these hypotheses for the loss of ptilinal pigment marks, detailed microscopic investigations are required to determine the fate of the ptilinum in *B. dorsalis*.

It is also possible that the colour fastness of the different pigment colours varies with exposure to natural light as exhibited by the slight variation in colour retention of Comet Blue 60 and Invisible Blue 70 under semi-field conditions (Figs 3, 5). The technical data sheets for the tested T-series pigments (Safety Datasheets, T-series, Swada, Cheshire, U.K.), as well as the FEX-series used in other studies (Reynolds *et al.* 2010), state that they have limited lightfastness in exterior applications. The visibility of pigment marks has been shown to differ when viewed under light of different wavelengths. Pigments with peak reflectance wavelengths that are close to that of the transmitted light are usually more difficult to detect (Weldon 2005). This current study used the most commonly used UV (blue) 365 nm light source and confirms this. The better visibility of Astral Pink 1 and Blaze 5 may be attributed to the two colours having peak reflectance wavelengths of ≥600 nm, and hence being more visible than the other pigments. Comet Blue 60 and Invisible Blue 70 have peak reflectance wavelengths of ≤460 nm (Safety Datasheets, T-series, Swada, U.K.), which are difficult to detect with a light source of 365 nm. The visibility of these colours may be improved if light sources of different wavelengths are used (Weldon 2005).

The pigment colours and concentrations tested in this study are effective for use in short term (14 days) MRR studies of *B. dorsalis*. Observations lasting for up to 28 days can still be made using these pigments where a high population of flies is released to increase the chance of recapturing marked flies. However, these longer-term MRR studies and those involving releases of older adults may suffer from underestimated recaptures, which will influence calculations of population density, dispersal capacity, and in the case of sterile insect technique programmes, the sterile:wild over-flooding ratio. We do not believe that our results should be used to adjust for loss of pigment marks over time, which is problematic when no marked flies have been caught in traps, but they do illustrate that recaptures of older *B. dorsalis* in

MRR studies will provide conservative estimates of population parameters.

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