Enhancing emergence and release methods of the sterile insect technique (SIT) to improve market access

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Department of Primary Industries

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Enhancing emergence and release methods of the sterile insect technique (SIT) to improve market access

Reynolds *et al.*

EH Graham Centre for Agricultural Innovation (a collaborative alliance between NSW Department of Primary Industries and Charles Sturt University)

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Purpose of report: This project has developed more effective sterile insect emergence and release technologies that will improve the success and cost-effectiveness of sterile *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) releases.

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Media Summary

The Queensland fruit fly, 'Qfly' is Australia's most significant horticultural pest and is a major biosecurity problem. The adults lay their eggs in ripe fruit and the larvae feed on the flesh of the fruit, rendering it unmarketable. The sterile insect technique (SIT) is a form of biological control in which large numbers of sterile flies are used to flood the fly population, reducing the possibility of mating between wild flies and the production of fertile eggs.

In this project, we aimed to develop more effective sterile insect emergence and release technologies to improve the success and cost-effectiveness of sterile Qfly releases. Guidelines for pupal release were developed including optimal pupal loadings and release timings that maximise the emergence and flight activity of sterile adult Qfly. For adult fly releases, we developed a method that maximises emergence success, flight activity, persistence, abundance and mating competitiveness of sterile male flies. Trials using chilled adult flies resulted in the development of a protocol that similarly maximises emergence, flight and longevity. A prototype device for releasing chilled adult flies from a slow moving vehicle was also developed. Overall, our results showed that significant decreases in wild fly populations can be achieved using any one of the three release methods we developed and that a method should be chosen dependent upon the location and resources available. This study also demonstrated that, based on trap recapture rates, a lower numbers of mature sterile flies (i.e. protein fed flies) need to be released to achieve control, relative to releases made using immature flies. Further studies should be completed to scientifically verify and establish the absolute number of mature sterile flies required to suppress and/or eradicate a given wild population, to minimise the requirement for sterile flies and thus further reduce costs.

It is recommended that low-level sterile releases are used in towns surrounding the fruit fly exclusion zone (FFEZ) to suppress wild Qfly populations in these areas, thus minimising the pressure placed on the FFEZ. Sterile fly releases should also be used to eradicate wild Qfly in the NSW FFEZ, using the strategic release plan and the standard operating procedures developed as part of this study. The SIT should also be considered in endemic areas where orchards are geographically isolated, or where control is being attempted on an area-wide basis.

Technical Summary

Sterile Insect Technique (SIT) is the current, internationally approved preventative treatment for fruit fly free areas. In New South Wales, SIT against the Queensland fruit fly, *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) was first implemented in 1991. Since then it has relied on methods adopted from similar release programs or after cursory investigation, rather than developing methods specifically optimised for this program. The methods for adult eclosion (emergence of the adult fly from the pupal case) and pupal and adult release (pupal release involves placing *B. tryoni* pupae in the field allowing the adults to eclose and take flight in the environment while adult release involves rearing and releasing *B. tryoni* as an adult fly) have advanced little, despite being critical areas for the success of SIT programs. Chilled adult release has never been tested for *B. tryoni*, despite potential space and cost savings. The aim of this study was to develop and evaluate more effective emergence and release technologies for *B. tryoni* SIT to improve overall efficiency, effectiveness, and subsequent market access

Several laboratory, field cage and field studies were completed to improve the success and cost-effectiveness of sterile *B. tryoni* for pupal, adult and chilled adult release.

i) In a field study, we tested the seasonal effect of different pupal loadings on eclosion and flight of sterile *B. tryoni*. Pupal eclosion boxes were loaded with either 200, 350, 500, 650 or 800 g (i.e. 80,000 pupae) of pupae during five periods of the fruit fly season (August, October, December, February, and April),

ii) A laboratory study compared whether six different pupal loadings (180 g, 200 g, 230 g, 250 g, 280 g & 300 g) and four mediums (a) wire grid, adults emerged in a b) mesh bag and c) paper bag and adults emerged with d) no medium (control)), affected emergence and flight of sterile *B. tryoni* reared in plastic adult rearing containers (PARCs). A follow-on field study compared the recapture rates of the different treatments in cue-lure baited Lynfield traps,

iii) A field study determined whether release method (PARCs and a bin release method) and trial site (Wagga Wagga, New South Wales (NSW) and Unley, South Australia (SA)) affected recapture rates of sterile *B. tryoni*,

iv) To determine whether dietary yeast hydrolysate (YH) (protein and amino acids) affects the persistence and abundance of sterile male adult *B. tryoni*, several studies were completed. In an outdoor cage study we compared the survivorship of male and female *B. tryoni* continuously provided with either (1) YH and white sugar, (2) YH and brown sugar, (3) white sugar only and (4) brown sugar only after 7, 14 and 21 days. In a small cage and field cage study, sterile *B. tryoni* were held for 48h in the laboratory from eclosion. They were then released in a field cage according to treatment (one treatment per cage). They were fed either i) sugar (continual supply), ii) sugar (48h) then starved, iii) YH (48h) then continual sugar supply, iv) YH (48h) then starved or v) YH (continual supply). Survival of flies was monitored until all were deceased. In a follow-on field study we released on three occasions marked sterile *B. tryoni* fed two diet regimes, YH-supplemented or YH-deprived, and monitored abundance of sexually mature males by recapturing them in Lynfield traps up to 12 weeks after release,

v) Sterile *B. tryoni* were treated with methoprene by dipping the pupae (1:100 dilution of methoprene solution (5µg/µl) for 5 mins) and allowing them to eclose or topical application (1/µl of methoprene dissolved in acetone (5µg/µl)). Test flies were either provided with access to protein for 48 hours post emergence and then maintained on a sugar only diet, or were maintained solely on a sugar diet without any access to protein. A second set of un-irradiated/un-treated control flies were set up in a similar manner but with constant access to a full diet (sugar and protein), to obtain mating partners at peak sexual maturity, 10-14 days old, to be paired with diet and methoprene treated flies

during mating experiments. At 2, 4, 6, 8, 10 & 12 days of age adults were assessed for their sexual activity and copula duration.

vi) A laboratory study tested the recovery, longevity and flight ability of sterile adult flies, chilled for 0-24h and fed either a YH-supplemented diet or a carbohydrate (sucrose) only diet,

vii) A field study compared the recapture rates of sexually mature sterile male *B. tryoni* when released as chilled adults (roving release) and non-chilled adults (stationery release)

viii) Trap capture rates and wild fly larval counts (from picked fruit) were determined in two isolated towns that received either mature sterile adult releases (Uranquinty) or mature sterile chilled adult releases (Lockhart). A control town (Cootamundra) which received no sterile flies was also monitored for wild fly trap captures.

ix) A field study compared sterile and wild trap captures of *B. tryoni* across four treatments: adult release, chilled adult release, pupal release and a control (no sterile release) in separate plots within the same town.

Research findings produced:

i) Guidelines for pupal release including optimal pupal loadings and release timings that maximise the emergence and flight activity of sterile adult *B. tryoni*. SIT based on release of sterile pupae is viable in December and April when ≥500g (equating to 50,000 pupae) is adequate and at all loadings up to 800g during February. Releases from pupae are not recommended from August to October due to low temperatures suppressing adult emergence and flight propensity. For optimal use of pupae in SIT, emergence minimum temperatures should be above 10°C, whilst maxima should not exceed 35°C. Minimum temperature for successful flight should not fall below approximately 6°C while the maximum temperature should exceed 16°C.

ii) PARC loadings and media tested did not affect emergence or flight of sterile *B. tryoni*. The control treatment (no medium) in PARCs gave the best trap recapture rates.

iii) There was no effect of release method (PARC or bin) on the number of *B. tryoni* recaptured, indicating that either method is suitable for release. In the same study, trap recaptures of released sterile *B. tryoni* were significantly and consistently higher in NSW than SA, although why this happened is not clear.

iv) Adult flies fed YH and sugar suffered less mortality after 7, 14 and 21 days compared with flies fed sugar only. In the small cages, both starvation groups suffered high mortality, however YH-supplemented males were more prone to starvation than males fed sugar only. Survival was similar for all other diet groups over a 40 day period. YH supplementation resulted in greater abundance of sterile male *B. tryoni*, with 1.2 YH-fed flies trapped for every YH-deprived fly trapped.

v) The pre-copulatory maturation period of newly emerged *B. tryoni* was reduced when exposed to a combined topical methoprene treatment and access to a 48 hour window of supplementary dietary yeast hydrolysate. Latency till the onset of copula and copula duration was not affected by treatment, although flies given both a topical treatment and access to protein began participating in mating behaviour 2 days prior to untreated flies on a similar diet.

vi) Releases of flies chilled at 4°C allows more efficient handling and was found to have no adverse effect on longevity and flight ability though recovery of *B tryoni* after chilling can take up to 15 min.

vii) In a field comparison, a greater proportion of chilled adult flies were recaptured (4.35±0.54%) compared with non-chilled adult flies (3.27±0.54%).

viii) The urban town that received the chilled adult sterile flies showed a significant decline in wild fly numbers four weeks after sterile release, while the other towns demonstrated increases in the wild fly population at this time. Over the entire fruit collection period,

there was a significant decline in both the number of live larvae and the live larvae rate per fruit in both towns that received sterile fly releases. Resources prevented the control town also being sampled.

x) Wild populations of *B. tryoni* were lower post-sterile fly release for all treated plots (pupal, adult and chilled adult release) but increased in the control plot during this time.

In each study, dye was applied to insects before release and its persistence on the ptilinum (an area of the head that flies are unable to groom) allowed sterile flies and wild flies to be distinguished. This method was considered 'excellent' by surveillance staff at the Orange Agricultural Institute and blind molecular testing at the Elizabeth Macarthur Institute validated its reliability.

The results obtained as part of this project and incorporated into the Strategic Release Plan and Standard Operating Procedures for pupal, adult and chilled adult release should be incorporated into the management plan for wild *B. tryoni.* Overall, this project has led to significant improvements to the efficiency and effectiveness of SIT. All trials completed in this project are in preparation or are published in peer-reviewed journals. Future work should include: i) Studies to scientifically verify and establish the absolute number of mature sterile male flies required to suppress and/or eradicate a given wild population, to minimise the requirement for sterile flies and thus further reduce costs, ii) Continued studies into the use of methoprene and other technologies (e.g. attractants) to enhance mating competitiveness of sterile male flies, iii) The development and incorporation of a male only strain providing economic and biological benefits and iv) Studies on the use of SIT with other compatible environmentally friendly and target-specific control techniques including native parasitoid wasps, entomopathogenic fungi, *Wolbachia*, bait sprays/stations, female lure and kill and male annihilation technique (MAT).

Introduction and Literature Review

The Sterile Insect Technique for Queensland fruit fly (*Bactrocera tryoni*) management: a review of history, current status and opportunities

Phillip W. Taylor, Olivia L. Reynolds, Stuart Gilchrist, Bernard Dominiak, Samuel Collins, Christopher W. Weldon

Introduction

The sterile insect technique (SIT) is widely used in integrated management programs against tephritid fruit fly pests, including the Queensland fruit fly, *Bactrocera tryoni* (Froggatt). The SIT is a form of biological control in which large numbers of sterile flies are used to flood the wild fly population, reducing the possibility of mating between wild flies and the production of viable eggs (Meats, 1996). The SIT for *B. tryoni* was first trialled in New South Wales from 1962 to 1965 (Andrewartha *et al.* 1967), and was then implemented on a broader scale in NSW in 1991. The SIT is one of the current, internationally approved treatments against incursions of *B. tryoni* within the fruit fly exclusion zone (FFEZ) in south-eastern Australia. However, since its inception, the SIT has been based on methods adopted from similar release programs internationally or after cursory investigation, rather than developing methods specifically optimised for the *B. tryoni* program. This review focuses on the history and current state of the SIT program for *B. tryoni* from the domestication and mass-rearing process through to the release of sterile flies and SIT's compatability with other technologies. It aims to highlight aspects of the biology, ecology and behaviour of sterile flies in these areas that are crucial to their performance, while suggesting areas where further work in this area is required.

Domestication and mass rearing

The inescapable principles of population genetics apply as much to insect mass rearing as they do to any other captive or endangered population. Therefore, like any captive population, mass reared fruit flies will inevitably adapt to the mass rearing environment and become increasingly inbred (Frankham 2008). That process is only exacerbated by the particular demands of mass rearing for sterile release. Mass rearing strains are necessarily bred to meet two fundamental requirements; firstly, they must thrive in a controlled rearing environment, and secondly, the strain must produce a sufficient excess of offspring for the planned release program. Without these two attributes, any release program becomes impractical or uneconomical.

However, no wild strain of fruit flies meets these requirements. When wild *Bactrocera*, *Ceratitis* or *Anastrepha* species are bought into the laboratory, all mass rearing programs begin with a period of domestication or establishment. Depending on the species, adaptation to the artificial rearing environment and selection for increased productivity take a varying number of generations (up to 10 or more; e.g. Economopoulos, 1992). Flies must adapt to new food sources, increased crowding, different temperature and humidity regimes and a different reproductive schedule (Leppla, 1989), mortality is high in the initial generations and only a small proportion of females reproduce (e.g. Ekesi *et al.* 2007; Hernandez *et al.* 2009). In response to these selective forces, all species of fruit flies so far studied evolve earlier maturation and higher reproductive rates (e.g. Vargas & Carey, 1989). Field evaluations, undertaken in later generations, commonly show adverse changes in field performance traits, including changes in behaviour and mating competitiveness, time and duration of mating, stress resistance and dispersal ability (Ahrens *et al.* 1976; Miyatake, 1998; Cayol, 2000; McInnis *et al.* 2002; Rull *et al.*, 2005). In general, all mass reared strains become increasingly divergent from their wild counterparts.

Although *B. tryoni* adapts to artificial culture relatively easily, studies show that domestication produces changes similar to those in other tephritids. Meats *et al.* (2004) showed that, within only four generations, domesticated flies matured faster, laid eggs earlier and increased their rate of protein conversion. Mass reared males also begin mating earlier in the day (Weldon, 2005a; Pérez-Staples *et al.* 2009), show reduced overall activity patterns (Weldon *et al.* 2010) and have reduced nutritional requirements for sexual maturation (Weldon & Taylor, 2011). Gilchrist (2010) showed that a mass reared strain of *B. tryoni* had a shorter larval development period (resulting in less feeding time), increased early fecundity and increased early survival at the cost of later age survival. He also showed that changes in maturation, fecundity and survival occurred even if strains were not selected for increased productivity. In terms of field performance, mass reared flies also have lower heat stress resistance (Gilchrist, 2010) and reduced field dispersal ability

compared to outbred domesticated flies (Gilchrist & Meats, 2012). These were the first studies to show an unequivocal link between reduced heterozygosity and field performance in tephritids.

Underlying genetics

For as long as insects have been mass reared, researchers and facility managers have been concerned about the genetic effects of domestication and mass rearing on strain quality (e.g. Mackauer, 1972; Bartlett, 1984). Despite this concern, there has been relatively little detailed research on the genetics of domestication in tephritids. A major reason for this is that many of the largest mass rearing programs use a male-only strain of medfly, *Ceratitis capitata,* that cannot be outcrossed. Their genetic system (a Y-linked translocation linked to a visible genetic marker) prevents any outbreeding of the release strain after distribution.

Nevertheless, various teams have investigated genetic methods of improving mass rearing strains in various species. Zouros *et al.* (1982) studied the initial generations of domestication in the olive fruit fly, *Bactrocera oleae,* measuring extremely strong selection linked to the *Adh* locus. That research on *B. oleae* has been continued in a series of publications (e.g. Cosmides *et al.* 1997; Goulielmos *et al.* 2006). In the melon fly, *Bactrocera cucurbitae*, another series of genetic experiments have quantified the genetic trade-offs between traits such as fecundity and longevity (reviewed in Miyatake, 1996). Subsequent genetic studies involved *B. tryoni.* DNA microsatellite technology was used to investigate the actual *B. tryoni* strain used for releases in Australia. That strain was shown to be inbred compared to wild populations (with a heterozygosity of 0.42 compared to 0.62 for wild populations; Gilchrist, 2010). Despite complete replacement, the new mass rearing strain became as inbred as the old strain within 12 months (Gilchrist & Crisafulli, 2006; Gilchrist, 2010). The similarity between the different mass rearing strains was most likely due to severe genetic bottlenecks in the initial generations, as each version of the mass rearing strain retained only the most common alleles at all loci (Gilchrist, 2010).

The inevitability of these changes was demonstrated by Gilchrist *et al.* (2012). They showed that significant genetic bottlenecks of similar intensity affected eight out of eight lines of newly-established fly colonies. After 10 generations, all replicates had lost similar amounts of genetic variation as a result of both inbreeding and selection. Stochastic modelling of the culturing methods used in mass rearing facilities showed that additional selective pressures arise as productivity increases, meaning that inbreeding continues long after strains enter production.

Strain development

There are three approaches that attempt to counter the deleterious effects of inbreeding and adaptation that occur during mass rearing. The first is complete strain replacement (e.g. Ahrens *et al.* 1976). Although simple, the problem with this approach is that it does not address the causes of the genetic problems it is designed to address. In particular, if most problems arise during the earliest generations of domestication, the strain replacement merely replicates those original problems in the new strain. Most deleterious genetic changes in *B. tryoni* occur within the first 10 generations of domestication. Since the pre-production 'breeding' takes at least 7 generations, the replacement strains are already highly inbred when they enter production (Meats *et al.* 2004; Gilchrist, 2010; Gilchrist *et al.* 2012).

A second approach is to minimise adaptation during domestication by providing a benign or heterogeneous environment. However, results in both tephritids and drosophilids show that supposed 'gentle' or reverse selection is ineffective in preventing inbreeding and adaptation to the mass rearing environment (Leppla, 1983; Gilligan & Frankham, 2003). Individual traits can be improved by field selection (e.g. McInnes *et al.* 2002), but the effects on overall productivity and inbreeding are likely to be offset by the additional inbreeding (Leppla 1983).

The third and most promising approach utilises hybridisation. The potential of outcrossing tephritids was first highlighted by Rossler (1975), who showed that crosses between new and long-term domesticated strains of *C. capitata* increased female productivity, even though one of the strains had been domesticated for 12 years. Shelly (2001) hybridized a mass-reared *C. capitata*

with wild flies and restored near-wild levels of male mating ability. However, performance declined to that of the mass-reared strain after 10 generations. Rull & Barreda-Landa (2007) hybridized a mass-reared strain of *Anastrepha ludens* (Loew) with wild flies, and found that hybrids combined the higher mating success of wild flies with the increased egg production of the mass-reared strain. In *B. tryoni,* hybridisation has been shown to produce highly productive yet robust flies. Gilchrist (2010) and Gilchrist & Meats (2012) showed that instead of using wild flies, hybridisation of independent inbred lines produced strains that combined wild-type longevity and improved dispersal ability and stress resistance with the necessary high levels of productivity. That approach has the advantage of using already-domesticated strains, which due to their high productivity, allows whole strain development and replacement to be completed in as little as three generations (see Gilchrist 2010).

It is important to note that simply adding wild flies to an existing cage of a mass rearing strain does not result in any appreciable hybridisation. Modelling presented in Gilchrist *et al.* (2102) shows that females with low egg production (i.e. wild flies) are unlikely to contribute to the next generation due to the 'pupal sifting' process. Furthermore, mass rearing strains mate earlier than wild strains, meaning wild males have low mating success in a cage of mass reared competitors. Therefore, simply adding wild flies to existing mass rearing colonies will fail. The strain replacement scheme presented in Gilchrist (2010) ensures effective hybridisation at minimal expense.

Apart from reducing the impact of domestication, genetics offer two further avenues of strain development. Marking of released individuals has proved problematic where a high degree of accuracy is required. Most programs rely on fluorescent dusts, however elemental or isotopic identification both also hold promise (see below). Recently, Shearman *et al.* (2010) showed that inter-species hybridisation can be used to introduce easily-detected mitochondrial markers into *B. tryoni*. A final aspect of the genetics of strain development and improvement is the production of genetically engineered male-only strains. One such strain has been produced in *C. capitata* (Morrison *et al.* 2009) but further development is required before that strain is sufficiently competitive to consider field releases.

Sterility induction

Gamma radiation is the principle method used to sterilize mass reared flies for release in SIT programmes (Bakri *et al.* 2005). The goal of radiation treatment for SIT is to damage gonadal cells resulting in fragmentation of chromosomes during cell division (Smith & von Borstel, 1972). Germ cells are particularly sensitive to radiation damage due to the high level of active cell division during the early stages of spermatogenesis (Proverbs, 1969). Chromosomal fragmentation of germ cells creates genetically imbalanced gametes that when paired result in early embryonic death of zygotes (Smith & von Borstel, 1972; Bakri *et al.* 2005).

To ensure that released flies are effective at inducing reproductive failure in their mates it is important that radiation procedures achieve an adequate level of sterility; for example in Mediterranean fruit fly *Ceratitis capitata* (Wiedemann), < 0.5% fertility is required from crosses between sterile males and fertile females for preventative releases into historically pest free areas (FAO/IAEA/USDA, 2003). However, treatment with excessively high radiation doses to remove a minimal level of fertility (< 1%) can often result in flies of compromised competitive quality. Deleterious effects of irradiation on fruit flies has been documented in a number of tephritid species, including *Ceratitis capitata* (Ohinata *et al.* 1977; Zumreoglu *et al.* 1979; Lux *et al.* 2002; Barry *et al.* 2003), *Anastrepha ludens* (Rull *et al.* 2005), *Anastrepha suspensa* (Walder & Calkins, 1993), *Anastrepha obliqua* (Toledo *et al.* 2004), *Bactrocera cucurbitae* (Teruya *et al.* 1975) and *Bactrocera cucumis* (Hooper, 1975a, 1975b, 1976). Typically there is a positive relationship between dose and sterility, which is coupled with a negative relationship between dose and fly competitive quality (Hooper, 1982; Hendrichs *et al.* 2002). Currently there is no defined adequate level of sterility for Queensland fruit fly, rather a general acceptance that the highest possible level should be obtained (Jessup & Cruickshank, 1999).

Irradiation Dose

The current recommended target irradiation dose for application of SIT in Australia is 70 Gy. This target dose is based primarily on the previous irradiation work of Monro (1961), Monro & Osborn (1967), and Bhatti & Shipp (1972). Initial studies investigating the potential for SIT to be used as a successful control for *B. tryoni* found a target dose range of 80 to 120 Gy, applied to pupae at six to seven days of age, successfully sterilized both males and females (Monro, 1961). Males irradiated at eight days of age at a dose of 80 Gy were found to exhibit fertility after six weeks of adult life, while pupae irradiated at a younger age suffered increased mortality. This observation lead to the recommendation that variation in the age of pupae mass reared for SIT be restricted to minimize differences in radiation sensitivity (Monro & Osborn, 1967). Thus the rearing process selected was developed to produce pupae that matured at between seven and eight days, and irradiation was performed within 48 hours of predicted emergence (Jessup & Cruickshank, 1999).

Monro & Osborn (1967) proposed a broad target irradiation dose range for SIT of 60 to 100 Gy. Bhatti & Shipp (1972) demonstrated that males irradiated at doses of 75 Gy and below had superior mating competitiveness to that of males irradiated at doses of 90 Gy and above. Mating competitiveness was assessed as the frequency with which irradiated males obtained copulations with fertile females in direct 1:1 competition with fertile males. While this demonstrated how reduction in irradiation dose could result in improved adult Queensland fruit fly competitive quality, specific sterility induction across the target dose range was not assessed.

A specific sterility inducing target dose for Queensland fruit fly SIT, was identified after preliminary tests into sterility induction were conducted as part of a larger mark-release-recapture programme in northern Victoria (MacFarlane *et al.* 1987). The initial release used pupae irradiated at a target dose of 80 Gy, resulting in 100% sterility of both males and females. In an effort to reduce adult fly mortality, the target dose was lowered for subsequent releases to 70 Gy. This resulted in reduced mortality of pupae while still maintaining 100% sterility of females and 97% sterility of males. Each of these studies forms the basis for a recommended target dose of 70 Gy for use in SIT (Jessup & Cruickshank, 1999). The current Queensland fruit fly SIT programme uses a target dose range of 70-75 Gy based on these recommendations.

More recently, it has been shown that acceptable levels of sterility and improved competitive quality can be obtained through a further small reduction in irradiation dose (Collins *et al*., 2009). The chief goal of SIT is the induction of reproductive failure in wild populations through the release of sterile males. The removal of the last 1% of fertility in released flies however often requires a substantial increase in irradiation dose (Robinson, 2005). This is particularly true at higher irradiation doses, where the predicted probability of fertility approaches zero. As sterility induction approaches 100% at higher irradiation doses, and the typical monotonic sterility induction curve begins to plateau, substantial increases are required in irradiation dose to achieve proportionately smaller (<1%) increases in sterility (LaChance, 1967; Collins & Taylor, submitted). Thus a significant increase in irradiation dose to remove less than 1% residual fertility often results in adult flies with compromised competitive quality due to overdosing. Complete sterility (100%) may therefore be counter productive to the goals of SIT, as flies that are rendered incapable of competing effectively are of no use to release programmes. Flies irradiated at lower sub-sterilizing doses may induce higher rates of reproductive failure in their wild mates due to increased competitive quality (Hooper, 1972; Toledo *et al.*, 2004).

Adequate sterility induction in male Queensland fruit fly can be obtained at an irradiation dose of 60 Gy, 10 to 15 Gy lower than the current SIT programme target-sterilizing dose range. Female *B. tryoni* are rendered completely sterile at a dose of 50 Gy with a 5 to 10 Gy lower margin between sterility induction and the potential for residual fertility. Queensland fruit flies irradiated at a lower dose of 60 Gy have mortality responses similar to that of unirradiated flies when held under nutritional and crowding stress, and an improved sterile male mating propensity (Collins *et al.* 2009). Such a reduction would improve the stress tolerance and mating propensity of released flies, while providing comparably high levels of reproductive sterility in released males and total

reproductive sterility in released females. A reduced irradiation dose of 60 Gy can also be applied without risk of resurgence of fertility up to one-month post emergence (Collins & Taylor, submitted).

Irradiation dose rate

Similar to previous work investigating sterility induction in *C. capitata* (Hooper, 1970), and *Bactrocera cucumis* (Hooper, 1975a), sterility induction in *B. tryoni* can be induced independently of the dose rate at which the chosen irradiation treatment is applied (Collins *et al*., 2008). This irradiation dose response conforms to the one-hit ionising event hypothesis for sterility induction (LaChance, 1967). As long as a target-sterilizing dose is achieved during irradiation treatment then sterility will result regardless of the rate at which that dose is met.

While sterility induction can be achieved independently of irradiation dose rate, there are still consequences of irradiation dose rate for fly quality. Even a minor overdose at the current target irradiation dose of 70 to 75 Gy can cause reduction of fly performance and mortality under stress tests. The higher the irradiation dose rate the greater the variation in total dose received (Collins *et al.* 2008). The current irradiator used to sterilise pupae for use in SIT is reliant on the technician manually initiating raising (initiation) and lowering (termination) of the Co^{60} source from the holding pool. A delay in the termination of irradiation treatment will result in over-shooting of the target irradiation dose. The higher the irradiation dose rate the greater the over-shoot (Collins *et al.* 2008). To minimize additional potential error in target irradiation dose it is recommended that, when possible, the lowest irradiation dose rate available be used to induce sterility. Lower irradiation dose rates will require longer irradiation time. Therefore the chosen dose rate must be low enough to minimise the potential for error while still allowing time for irradiation of enough pupae to meet release requirements.

Irradiation atmosphere

Irradiation in air results in oxidative reactions that generate peroxy-radicals in cells, causing extensive tissue damage. In the absence of oxygen, or in low oxygen atmospheres, peroxy-radicals are neutralized, minimizing the damage caused by radiation treatment (Hooper, 1971, 1976; Ohinata *et al.* 1977; Fisher, 1997; Bakri *et al.* 2005). Studies into the effects of atmosphere followed the successful Queensland fruit fly eradication programme in Western Australia (WA), where flies were irradiated in a nitrogen atmosphere similar to the current WA *C. capitata* SIT programme (Fisher, 1997). The target dose to achieve adequate sterility was 160 Gy, however adult emergence was low and mating propensity of irradiated males was only half that of unirradiated males (Fisher, 1997). Competitiveness of flies irradiated in nitrogen was higher than that of flies irradiated in air. While flies irradiated in nitrogen show increased competitiveness, due to the protective effect nitrogen has on the oxidative damage of radiation, higher irradiation doses are required to achieve an adequate level of sterility (Sproul *et al.* 1992). The current SIT programme does not incorporate nitrogen flushing of pupae, but rather pupae are sealed in plastic bags and held overnight prior to irradiation (Jessup & Cruickshank, 1999). Oxygen in the bag is depleted by respiration resulting in a hypoxic atmosphere providing similar protection against peroxy-radicals as nitrogen flushing (Nestel *et al.* 2007).

Identification of released flies

The success of SIT is contingent on the reliable marking of released sterile flies and the ability of the identification services to quickly and accurately discriminate wild from sterile released flies in subsequent monitoring programs. The critical requirement of any marking procedure for flies is that it should not impact on their normal biology and behaviour. Methods for marking insects were reviewed by Hagler & Jackson (2001), however many of the methods reported are not appropriate for large-scale sterile releases because of cost, handling and detection logistics.

Marking for flies was originally developed for Calliphoridae (Norris, 1957) and subsequently applied to Tephritidae (Steiner, 1965; Holbrook *et al.* 1970; Schroeder & Mitchell,

1981; Enkerlin *et al.* 1996). Oil soluble dye and a head crushing procedure to extract the dye was introduced by Steiner (1965) while Holbrook *et al.* (1970) used fluorescent dust with UV light, but continued to rely on head crushing. The head crushing procedure became obsolete with the introduction of fluorescence microscopy (Enkerlin *et al.* 1996).

To-date only external dust (or powder) marking has been used in Australia. The use of dust has inherent problems; rates of application are critical and it has long been recognised to affect survival and activity in a species-specific manner (Chang, 1946; Reinecke, 1990; Meyerdirk *et al.* 1997; Reid & Reid, 2008). Too much dust has been shown to cause increased mortality, decreased mobility, and it can also interfere with sensory organs (Crumpacker, 1974; Cook & Hain, 1992). Dust can reduce adult eclosion of *B. tryoni* and may affect their dispersal behaviour and recapture rates (Jackman *et al*., 1996; Dominiak *et al*., 2000a, 2003a). Further, the presence of dust pigments on insects may increase time spent in preening (Reinecke, 1990) that may affect in-field performance. The smaller the dust particles the harder they are to remove and the longer the time spent preening. Dust can also be transferred to unmarked insects in the field or within traps used for monitoring (Miller, 1993).

For fruit flies, fluorescent dust is applied to the pupae and some of the dust is retained in the ptilinum of the emerging adult during eclosion. After release and subsequent recapture, identification services look for this fluorescent dust in the ptilinum to positively identify sterile flies. However, a very small proportion of flies from dusted pupae do not accumulate, or fail to retain, dust in their ptilinum, but can be identified using costly time consuming genitalia dissections (Enkerlin *et al.*, 1996) or molecular techniques (Reynolds & van der Rijt 2011). Relying on the detection of dust alone could give rise to the misidentification of a small proportion of flies. These flies could generate false positives in a monitoring program that could initiate costly suppression or eradication programs, cause loss of market access for products, and financially ruin some producers.

The dyed ptilinum method has been used extensively on *C. capitata* during eradication and research programs (Midgarden *et al*.. 2004; Shelly *et al.* 2006) and also on other species (Iwahashi, 1977). The use of external dusts gained favour over internal dyes or tracers (Chambers, 1977; Schroeder & Mitchell, 1981) and the International Atomic Energy Agency developed standards (FAO/IAEA/USDA 1999, 2003) for the production (including marking) and shipping of sterile mass-reared tephritid fruit flies. Data was presented for *B. tryoni* in these documents, but the source of the data is not acknowledged.

Dust colours

Problems occur in distinguishing blue and yellow Dayglo pigments (Dayglo Colour Corp) from naturally occurring particles and natural fluorescence associated with cuticular material of some insects (Miller, 1993). Holbrook *et al.* (1970) found that flies marked with Rocket Red and Blaze Orange were more discernable than Arc Yellow and Signal Green after one week of field exposure. Ito & Iwahashi (1974) reported that the ptilinum of melon flies had a natural green fluorescence which was confusable with blue fluorescent dust. This natural colour was clear under ultra-violet light, the light source commonly used in most programs to separate market flies.

Flies marked with Rocket Red dye exhibited an increased mating frequency compared with those marked in Arc Yellow, Signal Green or Blaze Orange (Holbrook *et al.* 1970). There are no published studies of dust evaluations in early *B. tryoni* SIT trials and programs in Australia, although the work of Holbrook appears to have been recognised. In Western Australia, Yeates *et al.* (1992) used Arc Chrome A6 (orange) and Fire Orange A4 (pink), while in New South Wales Laser Red, Nova Red, Fiesta FEX 1 Arc chrome, Strong magenta, Flame orange and Stellar green have been used (James, 1992; Horwood & Keenan, 1994; Dominiak *et al.* 1998b; Reynolds & Orchard, 2010; Reynolds *et al.* 2012). In South Australia, Nova Red and Arc Chrome were used (Reynolds *et al,*. 1995; Jackman *et al.* 1996). Blue dusts were found difficult to distinguish under UV light (Jackman *et al*.. 1996,) and some pigments are less persistent or more easily removed than others by the flies preening (Jackman *et al*., 1996; Dominiak *et al*., 1998a). Difficulties in

separating pink and orange pigments, after field exposure, have been reported (Dominiak *et al.* 2000).

In other insect studies (Toepfer *et al*., 2005) it has been shown that not all fluorescent dusts could be used concurrently for multiple release trials as some could not be discriminated between under UV light clearly and reliably, and some weather differently in the field. This aspect in relation to *B. tryoni* releases has received little attention but may be critical given sterile *B. tryoni* can survive over 26 weeks in the field (Dominiak *et al*., 2003b) although most are caught within 3 weeks of release, depending on weather conditions.

In Australia, coloured pigments from DayGlo Colour Corporation (Ohio US) or Swada (England) have been predominantly used to mark flies. Since 1999 the Swada Fiesta FEX 1 Series daylight fluorescent pigments have been routinely used. These products are dyed/pigmented melamine, sulphonamide, formaldehyde copolymers with a particle size (laser analysis) of 3.0 – 4.0 microns and contain heavy metals (Product Specifications, FEX Series Daylight Fluorescent Pigments, Swada, Concord House, Stratford, London E15 2PP, England). The pigment properties (formaldehyde content and particle size) have been associated with a reduction in the eclosion and recapture rates of sterile flies (Dominiak *et al*., 2000a, 2003b, 2010; Weldon, 2005b; Campbell *et al*., 2009). No detailed studies are reported in the literature to verify these assumptions and no justification for the change in pigment supplier or comparison of the products, in terms of their effect on fly emergence, behaviour or flight ability, can be cited.

Dust amounts

For most *B. tryoni* releases, the fluorescent pigment is incorporated with the pupae at the Fruit Fly Production facility (FFPF), Menangle, NSW prior to irradiation and mixed with the pupae by gentle rotation in a drum, until the pupae are evenly coated. Pupae for research purposes either have the marker incorporated at the factory or on arrival at the research facility. The FAO/IAEA/USDA (1999) recommendation for marking mass-reared Tephritid pupae (including *B. tryoni*) was 2.0 to 2.5 g of Dayglo® pigment per litre of pupae, and was subsequently reduced to 1.5 g (FAO/IAEA/USDA, 2003). For *B. tryoni* under the current production system 1 litre equates to approximately 40,000 pupae and weighs 400 g.

The weight of pigment used per unit of pupae of *B. tryoni* is still evolving. In early trials in Victoria pupae were dusted at a rate of approximately 3.2 g pigment/L of pupae and then covered with a 2 cm layer of sawdust containing an additional 1 g dye per 14 g of sawdust (MacFarlane *et al.* 1987), while in Western Australia pupae were mixed with 5 g pigment/L of pupae (Fisher, 1996). In South Australia pigment application rates used in earlier *B. tryoni* SIT releases were often not documented (Perepelicia & Bailey, 1993; Perepelicia *et al*., 1994, 1997; Reynolds *et al*., 1995) and in some cases additional amounts of pigment were added, or pupae remixed with a different colour on arrival to overcome difficulties in identification (Jackman *et al*., 1996).

Since 2000 more attention has been given to recording the dusting rates used in release and trial work. The impact of 12 pigment colours (4.5 g /L of pupae) on eclosion was investigated in the laboratory (Dominiak *et al*., 2000c), but not all colours were replicated in each trial, internal replication was low (3) and trends were not consistent. Both the average pupal weight and the emergence rates in the control samples varied between the trials. The lowest eclosion rate (an 18% decrease over the control) is associated with Comet Blue while Nova Red, Arc Chrome and Flame Orange had the least impact. Based on sampling from emergence cages for field releases no significant difference in emergence was found for pupae dusted (4.5 g pigment /L) pink, orange, green or blue (Dominiak *et al*., 2003b). However, pigments were tested under different field conditions and used different cohorts of pupae.

The method of marking and the volume of pigment/L of pupae remains unresolved and two approaches, based on pigment weight used, seem to be employed. Using different pigment sources with different particle size, (60 and 4-5µm respectively) from Dayglo Color Corp and Radglo Color, N. V., Weldon (2005b) applied the equivalent of approximately 80 g pigment in an equal volume of saw dust/L of pupae and found no difference in the proportion of pupae failing to emerge

across all pigment colours. However, Dayglo colours, with the exception of Rocket Red, had lower mortality of emerged flies in the emergence trays. Meats & Edgerton (2008) used pigments from the Fiesta FEX series at rates equivalent to 20g pigment mixed with an equal volume of sawdust/L of pupae. They noted excess pigment could affect adult eclosion and at the rate used > 90% of newly eclosed adults were capable of flight, with no impact on survival rate. No data on the percentage successful eclosion was provided. Weldon & Meats (2009) used the equivalent of 8g pigment/L of pupae in an equal volume of sawdust to mark flies to investigate dispersal of sterile, wild and a laboratory-reared fly with a colour mutation. Again no data on eclosion rates associated with the different colours was provided.

Campbell *et al.* (2009) under laboratory conditions and with no covering medium demonstrated that the equivalent of 4 g Fiesta FEX Astral Pink pigment/L of pupae reduced successful adult eclosion by an additional 16 % over and above the 22% losses incurred due to natural mortality, packaging and irradiation and suggested there was an urgent need to revise the accepted marking protocol. Under the same conditions Dominiak *et al.* (2010) examined 8 rates of the same pigment (1.0 – 4.5 g/L of pupae) and found no significant difference in eclosion and flight ability between the control and 1.0 g of pigment. All other rates (1.5, 2.0, 2.5, 3.0, 3.5, 4.0 & 4.5 g/L pupae) significantly reduced the rate of adult eclosion. For field pupal release, Reynolds *et al.* (2010) adopted the equivalent of 3.2 g Fiesta FEX Astral Pink pigment/L of pupae and covered the pupae with 1-1.5 cm of moistened vermiculite. Samples of emerged flies from different pupal loadings were rinsed in 70% alcohol, dried and examined for residual pigment in the ptilinum. Pigment was detectable in all specimens examined, but sample sizes were extremely low and unlikely to withstand intense statistical scrutiny for quarantine/market access purposes.

It is clear, given the widely divergent dusting rates used for *B. tryoni* (e.g. Meats & Edgerton, 2008; Reynolds *et al.* 2010), that the process of marking pupae is not consistent. Justifications for the rates used are frequently not offered and in these circumstances it can only be concluded that rates are selected for convenience in the identification of the marked flies or are based on unpublished data. No data to justify the adoption of Astral Pink (or any colour) as the preferred dye was cited and no critical assessment of pigment colour, dusting rate, or the value of a cover medium over the marked pupae appears to have been published. Inconsistent trends within and between trials creates confusion and suggests that most of the adverse effects on eclosion occur during the production and marking phase, prior to irradiation (Campbell *et al*., 2009).

A more recent study by Reynolds *et al.* (2012) using the FFPF standard rate of pupal dying (1g dye per 100g pupae) determined whether four fluorescent dye pigments, Fiesta FEX 1 Arc chrome, Strong magenta, Flame orange and Stellar green (Swada, 30–32 Kilkenny Court, Dandenong South, Victoria, Australia), influenced standard quality control parameters and trap recapture rates of *B. tryoni*. Laboratory studies showed that *B. tryoni* pupae dyed different colours did not differ in emergence, flight ability or pupal weight, and while this was satisfactory for the purposes of the trial, comparisons were not made with undyed pupae. In a field release, there was no significant difference in the proportion of total trap recapture of sterile adult *B. tryoni* coloured different pigments over several recapture dates (Reynolds *et al.* 2012).

The impact of the marking medium and volume required for reliable identification while minimising the effects on the basic parameters of adult emergence, flight ability and field performance of the flies needs to be resolved.

Interactions between pupal weight, marking colour and adult eclosion rates have production implications for sterile release programs. When examining the impact of 12 marker colours Dominiak *et al.* (2000c) found the average adult eclosion ranged from 55.6% to 88.0% for pupae ranging from 8.8 mg to 10.0 mg in weight. Subsequently Dominiak *et al.* (2002) reported that pupal weight (average 9.2 mg) was positively linked to eclosion, with significantly higher rates when the pupae had an average weight of 9.36 mgs (Dominiak *et al.* 2007). The effect of pupal weight and dust markers on flight ability and field performance need examining to improve the efficiencies of the FFPF and *B. tryoni* SIT.

Research into the marking process, particularly dusting rates required, cannot be conducted independently of work to optimise pupal size, improve the quality of the pupae produced, mating competitiveness and adult survivorship in the field and the ease and accuracy in discriminating dyed sterile from unmarked wild flies. Ultimately, operational requirements will determine the long term viability of the dust marking protocol.

Alternative marking approaches

For *B. tryoni*, alternatives to dust marking are often mooted but little progress has been made to date. In field cage studies paint marking is reported (Sonleitner, 1973) although is not practical on a large scale, while the use of a visible genetic marker (Meats *et al*., 2002), identified by Zhao *et al*. (2003) was used (Weldon & Meats, 2009) to study dispersal patterns of *B. tryoni*. This genetic mutation has not been incorporated into the FFPF. The lack of progress in this area reflects the size of the current production facility in Australia, the assumption that all species of Tephritidae respond equally, barriers imposed by trade requiring rapid determinations, and the lack of research funding. Methods for marking insects were comprehensively reviewed by Hagler & Jackson (2001) who noted that there was no universal insect marking technique, with the method of choice largely contingent on the purpose of the work. No new marking techniques applicable to large scale sterile releases of Tephritidae have been forthcoming. Alternate marking methods using genetics, isotopes, protein incorporation techniques, etc. are often suggested, but insufficient funding is available to adequately research the proposals to meet the requirements necessary for use in a SIT program.

Regardless of the marking method employed, before any modification or introduction of a new system for marking *B. tryoni* is introduced, extensive investigations are required. Issues such as adverse effects on the behaviour of marked insects must be assessed. Currently in Australia no uniform protocol towards marking sterile *B. tryoni* is in place, except for sterile flies obtained from the FFPF, however justifications for change or the practices adopted are seldom noted.

Rearing out & release methods

Adult *B. tryoni* are known to be strong dispersers (Fletcher, 1974; McFarlane *et al.* 1987). The initial development of an SIT release program in Australia consequently focused on relatively affordable and simple systems, such as stationary point release systems requiring the box release of adults or pupae, as opposed to costly and complex mobile release systems involving aerial or moving vehicle releases. There are currently sterile insect release facilities for *B. tryoni* located in South Australia, Victoria and NSW.

Pupal release

Pupal release is used by few SIT programs worldwide, although it is believed to require fewer resources compared with either adult ground or aerial release (Dominiak *et al.* 2000, 2003). Adults are thought to suffer less crowding stress in a pupal release box, as eclosed adults are free to leave the box upon emergence. This also leads to a steady supply of adult fruit flies to the targeted area, compared with a single large delivery of fruit flies in 1 day using adult ground release. Another advantage of this method is that adults may become partially acclimatised to the local climate as the pupae are exposed to variable temperatures for the days preceding eclosion (Fay & Meats, 1987a). Dominiak *et al. (*2003) believed it to be effective if predation could be kept to a minimum. Several approaches for pupal release have been examined for *B. tryoni*, including a platform release method (Andrewartha *et al.* 1967), the use of 50 L bins (MacFarlane & Betlinski, 1988), the basic 'bed' technique (Dominiak & Webster, 1998), covered trays (Dominiak *et al.* 2000), the use of buckets suspended from trees (Meats *et al.* 2006) and polystyrene foam boxes (Dominiak *et al.* 2003). The latter technique, considered by some the most effective of all those trialled to date, has been used with varying success in Australia (Dominiak *et al.* 2003; Meats & Edgerton 2008). This is probably due to uncertainty about its effectiveness under different climatic conditions and lack of knowledge of adequate pupal loading rates in the boxes (Reynolds *et al*., 2010). Under suitable environmental conditions in Australia, Reynolds *et al.* (2010) considered pupal release a plausible alternative for

use as part of an SIT program against *B. tryoni*. They described a modified polystyrene box with cardboard inserts to provide additional resting space for the flies. In order to maximise emergence and flight, Reynolds *et al.* (2010) demonstrated that pupal release would be most viable from December through to April or when mean minimum temperatures are not lower than 10°C and the maximum no higher than 35°C, with higher pupal loadings (up to 800 g; where on average 10 mg = 1 pupae) ideal. Interestingly, they noted that higher humidity favoured increased emergence, while lower humidity favoured increased flight.

Adult release

Various approaches for the stationary release of adult sterile *B. tryoni* have been trialled with varying degrees of success. *B. tryoni* SIT programs in Australia currently use either the plastic adult rearing container (PARC) (M. Nolan, pers. comm., 2009) or bin techniques (Fisher, 1996; Perepelicia *et al.* 1997) but have included techniques such as cage releases (Dominiak *et al.* 1998; Dominiak *et al.* 2003) and paper bags (Dominiak, pers. comm., 2007) but these have not advanced much beyond experimental use due to various limitations with their designs including practicality, transportability and disposal of refuse. There have been other methods of release trialled, including the use of pizza boxes, but the effectiveness of these have not been documented in the published literature.

Studies have therefore focused on optimising both the bin and PARC techniques, and have shown that loadings of 150 g pupae for bins (Fisher, 1996) and up to 300 g for PARCs (Reynolds & Orchard, 2010), together with the provision of adequate resting space are suitable for favourable eclosion and flight of adult *B. tryoni.* In addition, the use of an eclosion unit within PARCs was not proven necessary in order to obtain adequate adult eclosion, flight or trap recapture rates (Reynolds & Orchard, 2010).

Using the knowledge gained from previous studies for optimizing fly performance when released from bins and PARCs (see above), Reynolds *et al.* (under review), compared both release methods (PARC and bin) simultaneously in NSW and SA. Although release method did not affect the number of *B. tryoni* recaptured, PARCs may be preferred because of the larger number of pupae they are able to hold, better emergence and flight rates and ease of stacking and transporting during sterile *B. tryoni* releases. Interestingly, the authors showed that trap recapture rates of released sterile *B. tryoni* were higher in NSW than SA during the study. Although the authors indicated that this was likely due to higher post-release mortality prior to reaching maturity in SA, the exact reason/s for this remain unclear (Reynolds *et al.* under review). This is an area which needs to be resolved if we wish to better relate trap recaptures from different locations and indeed habitats.

Internationally, the Tower Eclosion system have been used to increase efficiency and reduce space requirements for rearing of *C. capitata,* and are deemed a cost-effective alternative to the PARC system (Salvato *et al.* 2004; Shelly *et al,.* 2006). Such release systems are likely to provide similar advantages with *B. tryoni* and are an area which should attain significant future research attention.

There is evidence to suggest that releasing mature flies as opposed to immature flies increases the flies chances of successful mating as they do not have to find food immediately, are ready to mate upon release, and have greater longevity (Yuval *et al.*, 2002, 2007; also see section on pre-release feeding). Adult nutrition therefore has an important role to play in the improvement of tephritid flies released in SIT programs. However, until recently there was only one published study which had directly compared the field release of both immature and mature sterile *B. tryoni* (Meats *et al.* 2003). The authors released immature flies fed only sugar and water soon after emergence at rates varying from 48000 to 115000 sterile males per km^2 and mature flies fed sugar, water and protein autolysate one week after emergence so that they were, or were close to sexual maturity, at rates varying from 5000-12000 sterile males per km^2. Both releases failed to achieve population suppression. Poor survival, attributed to the holding conditions of pre-released flies, was thought to be the reason for mature flies, while dry stress, failure to apply pre-release insecticidal bait sprays or lure blocks, and immigration of flies into the treatment towns were considered the reasons why

immature flies failed to suppress wild fly populations (Meats *et al.* 2003). Recently, Reynolds *et al.* (in prep.) showed that 1.2 YH-supplemented sterile males were trapped for every YH-deprived fly trapped. The authors concluded that under field conditions, YH supplementation can improve the longevity of sterile male *B. tryoni* and may improve the effectiveness and cost-effectiveness of SIT programs, by lessening the numbers required for sterile male release.

The most opportune time to release sterile insects is believed to be spring when populations are low (Meats & Fay, 1977) and temperatures in the FFEZ are cool and still subject to occasional frosts. Meats & Fay have completed a number of studies on the thermal acclimation of *B. tryoni*. In one of their earliest studies, Meats & Fay (1976) concluded that there was no advantage in acclimating adult *B. tryoni* to the cold as far as mating vigour is concerned. The authors showed that the only enhancement observed was that mating continued to occur in the harshest regime for a greater number of days in the cold-acclimated group than in the warm-acclimated group. However, in all cases cold acclimated flies accumulated a similar or significantly lower total number of matings than the warm-acclimated group, indicating that in any regime cold-acclimated males were never superior in competition to warm-acclimated males. In an experiment the following year, the authors showed that sterile *B. tryoni* (either cold- or warm-acclimated) were equally effective at suppressing the fertility of non-sterile, submature, cold-acclimated *B. tryoni* (Meats & Fay, 1977). Similarly, trials that used sterile tenerals (corresponding to pupal release) also revealed no difference between the two acclimation types, although fertility of the non-sterile *B. tryoni* was significantly depressed. Meats & Fay (1977) suggested this was related to acclimation physiology and the relationship of mating vigour and age, and explains why the warm-acclimated flies were not superior in competitiveness to their cold-acclimated counterparts as might have been expected from the previously reported experiment (Meats & Fay 1976). Notably, a later study sought to test this concept in field cages where natural temperatures fluctuated between extremes, and tested the ability of cold- and warm-acclimated sterile flies to survive and recover fully from frosts in the period between release and maturation. Fay & Meats (1987a), showed that cold-acclimated sterile flies (equal to that of wild flies), survived as well as wild flies and suppressed their fertility such that their mating competitiveness was deemed equivalent to that of the wild flies, if the weather was not too harsh. Conversely, the warm-acclimated sterile flies had poor survival, inferior mating competitiveness and were comparatively ineffective in reducing wild fly fertility. This suggests that cold-acclimating sterile *B.tryoni* for early spring releases could be advantageous in an SIT program. A subsequent study showed that through temperature acclimation, male mating frequency could not be improved for SIT by selection (Meats & Fay, 2000).

Chilled adult release

Chilled sterile adult release is used effectively internationally for the control of wild populations, often as part of an aerial release programme using airplanes (Howell *et al.* 1975; Cunningham *et al.* 1980; Sivinski *et al.* 2000a) or helicopters (Nakamori & Kuba, 1990; Vargas *et al,*. 1995). There have been a number of advances in the use of cryogenics and aerial navigation technologies for delivery of sterile flies (Tween & Rendon, 2007). Ground release programs do not commonly involve chilled adults (Fisher, 1996; Dominiak *et al.* 2000), and there is scant data regarding the efficacy of the roving release of chilled adults (Salvato *et al.* 2003).

In Australia there has been very little work to date on the chilled adult release of sterile *B. tryoni*. Meats & Fitt (1987) developed a model that for a given series of temperature profiles can predict the likely recovery times and the temperatures at which recovery is likely to happen for chilled adult fertile flies. The authors indicated that *B. tryoni* would incur the least stress and have the most rapid recovery if the chill temperature was as close as possible to the cold-torpor threshold, which is approximately 2°C when flies are fully acclimated to cold (Meats, 1976). Meats & Fitt (1987) showed that adult (non-sterile) *B. tryoni* acclimated at 25°C could be chilled for up to 24 h at 1°C and then recover within 1 h at 15^{o}C and 12 min at 25°C. A more recent study demonstrated that recovery of adult sterile *B. tryoni* at chill times of 0.5, 2 and 4 h averaged 14.4 min irrespective of

the length of time spent in chilling at 4°C (Reynolds & Orchard, 2010). Further, recovery took longer at 24 h chill time (14.10min) compared to 8 h chill time (10.72min), although this was not significant. The discrepancy in recovery time between the results of the two studies, taking into consideration the different chill times, is likely because Meats & Fitt (1987) did not use sterile adult *B. tryoni*, but rather fertile adults and sterility is known to lead to decreases in some fruit fly performance variables (Hooper & Katiyer, 1971; Collins *et al.*, 2008). Studies have shown no effect of chilling on longevity of sterile male and female adult *B. tryoni* (Reynolds & Orchard, 2010). However, studies of flight on two different batches of sterile male and female flies obtained from the same source, showed no effect for flies chilled upto 4h for one batch (smaller sized flies). Yet in another batch (larger sized flies) adults fed on a protein and sucrose diet had a decreased tendency for flight as the chilling time increased compared with adults fed a sugar only diet (Reynolds & Orchard, 2010).

Deployment factors

When to deploy?

Sterile male releases are most successful when employed to control wild populations that are isolated and have low numbers (Knipling, 1955, 1959). The first trials of *B. tryoni* SIT were conducted in towns in central New South Wales, Australia, where the natural population usually fell to low numbers towards the end of winter and during spring (Andrewartha *et al.* 1967). While the intention was to swamp the wild males with sterile ones early in the breeding season, it proved logistically difficult to consistently produce and release a sufficient quantity of sterile flies in spring (Andrewartha *et al.* 1967). In a later trial, difficulty in rearing a sufficient quantity of sterile flies in spring was also encountered (James, 1992). In addition to poor production and adult emergence, it was suggested that the experimental trial conducted by James (1992) was implemented too late in the season because a large population had been detected 4-6 weeks prior to the first releases and larval infestations had already been detected by that time.

The potential for SIT to target the low population density of wild flies in spring prompted studies that assessed the effect of cold-acclimated sterile *B. tryoni* to survive and induce reproductive sterility in wild populations (Meats & Fay, 1976, 1977; Fay & Meats 1987a, 1987b; see also Adult release above). Cold-acclimation of sterile male *B. tryoni* has no effect on their mating competitiveness because warm-acclimated sterile males quickly acclimate to early spring temperatures (Meats & Fay, 1976, 1977). However, cold-acclimation of sterile *B. tryoni* significantly enhances their survival when released in early spring, thereby increasing their ability to survive to mating age and compete with wild males (Fay & Meats. 1987a, 1987b). Apart from the need to target wild populations that are at low density, release during the milder temperatures of spring may enhance the survival of sterile *B. tryoni* in the field. Dry stress is considered a key factor with respect to restricting the distribution and abundance of *B. tryoni* (Yonow & Sutherst 1998), suggesting that the species is susceptible to water loss and desiccation. It has been observed that higher average field temperatures (Weldon & Meats, 2010) and dryness (Meats *et al.* 2003) are associated with poor trap catch of released sterile male *B. tryoni*. As will be described later in this review, mass-reared *B. tryoni* have poor resistance to desiccation when compared with their wild counterparts, and their longevity when exposed to water stress declines with age (Weldon & Taylor, 2010; Weldon *et al.* 2010).

The current Code of Practice for Management of Queensland Fruit Fly (Anon, 1997) requires the intensive use of bait sprays and lure blocks to reduce the size of the target population prior to the release of sterile insects. This approach was first suggested by Andrewartha *et al.* (1967) to increase the ratio of sterile:wild males. Earlier work had already established the capacity for a combination of protein bait sprays and cue-lure trapping to effectively suppress wild *B. tryoni* populations in isolated towns in central New South Wales (Bateman *et al.* 1966a, 1966b). Pre-baiting has since been used in many *B. tryoni* eradication campaigns that have included SIT, including successful campaigns in Perth and Tharbogang (Horwood & Keenan, 1994). It has been

noted by Meats (1996) that it is not strictly necessary to undertake pre-baiting unless wild fly density is over 20 per km^2, yet it may lead to faster eradication.

Release rates and over-flooding ratios

Eradication campaigns that use SIT will succeed only when there is sustained and adequate over-flooding of the target population (Steiner, 1969). Rates of release of sterile *B. tryoni* are dependent on the size of the wild population, the area to be treated, the required rate of decline in the wild population, and the dispersal, survival, maturation rate, and mating competitiveness of sterile males (Meats, 1996, 1998; Meats *et al.* 2006). Recaptures of sterile and wild males in traps that are baited with cue-lure are used to monitor the over-flooding ratio that is achieved during *B. tryoni* SIT campaigns. However, empirical evidence to date has not provided data that unequivocally sets the optimal number of sterile flies that need to be recaptured in monitoring traps to indicate effective release rates. Reports of over-flooding ratios are usually based on the ratio of sterile:wild captures from wide arrays of traps, many of which have ceased to catch target flies, and consequently lead to overestimates (Meats *et al.* 2006). The effective ratio of sterile to wild flies should be based on the results of traps where target flies are still being caught, and it has been suggested that an effective over-flooding ratio of 100:1 is desirable (Meats, 1996). The release rates and over-flooding ratios (both area-wide and effective, where available) for reported Q-fly SIT campaigns are summarised in Table 1.

Using a theoretical formula, Meats (1996) estimated that a minimum release rate of 60,000 sterile male *B. tryoni* per km^2 per week should be used when traps spaced at 400 m intervals (6.25 traps per km^2) catch up to 0.2 wild males per km^2 per week. A higher release rate is required when more than 0.2 wild males per km^2 per week are detected, and can be calculated with the same theoretical formula (Meats, 1996).

Some early trials of *B. tryoni* SIT led to suggestions that low-level releases of sterile males could suppress *B. tryoni* populations in areas that are adjacent to the FFEZ (see Meats 1996). Low-level releases would be performed only at an optimal time of the year when the wild population was at a low density, thereby reducing the growth rate of targeted populations while also minimizing the potential for wild flies to develop resistance to sterile males if target populations are exposed to sub-optimal numbers of sterile insects (Andrewartha *et al.* 1967; McInnis *et al.* 1996; Iwahashi *et al.* 1996). Few studies have attempted to empirically test the capacity for low-level releases to suppress *B. tryoni* populations in the absence of quarantine. An exceptional case is a study that was undertaken over three fruit fly seasons in six small towns with generally similar climates in central New South Wales (Meats *et al.* 2003). Sterile males were released at low levels (~6,000 sterile males per km^2 deployed from four points) at weekly intervals in two towns (Trangie and Warren), while high-level releases (~60,000 sterile males per km^2 deployed on a 400 m grid) were performed in two other towns (Gilgandra and Narromine) (Table 1; Meats *et al.* 2003). The wild *B. tryoni* population was monitored in a further three towns to track population growth in the absence of sterile males. Regardless of whether low-level releases commenced in spring or later in the season, they had no effect on wild population size (Meats *et al.* 2003). High-level releases, which represented the release rate typical of conventional SIT campaigns, led to suppression of the wild population in Gilgandra but failed to eradicate it (Meats *et al.* 2003). This was presumably due to immigration of wild flies, either naturally or by human agency (Meats *et al.* 2003).

Spacing and location of release sites

The earliest releases of sterile *B. tryoni*, released as pupae, were from fixed sites that were "evenly" spaced throughout treated towns, with additional releases wherever infested fruit was observed (Andrewartha *et al.* 1967).

The successful eradication of *B. tryoni* from Perth utilised 1,500 points from which newly emerged adults were released into the target area (i.e. ~5.5 points per km^2, or spaced at an average of under 400 m apart) (Sproul *et al.* 1992). Citing the Perth campaign, Meats (1996) noted that a spacing interval of 400 m for fixed release points appeared to be successful and is in accord with the known dispersal characteristics of *B. tryoni*; it has been demonstrated repeatedly that whether wild,

laboratory-adapted or sterile, the average distance of recapture for from a release point is between 100-500 m, and that at least 90% of recaptures are within 0.2-1.5 km (Fletcher, 1974; MacFarlane *et al.* 1987; Dominiak *et al.* 2003; Weldon & Meats 2007, 2010; Meats & Edgerton 2008). All subsequent SIT campaigns to eradicate wild *B. tryoni* populations have sought to release sterile flies from fixed release points on a 400 m grid (Table 1).

The release rate calculated by the formula of Meats (1996) for release points from a 400 m grid (i.e. 60,000 sterile males per km^2 per week) seeks to establish an even distribution of sterile flies, while taking into account the patchy distribution of wild flies in the environment (and wild fly trap captures) due to unexplained abiotic and biotic variables. However, sterile Q-fly recaptures in monitoring traps can also be spatially heterogeneous with no obvious relation to variation in habitat or distance from release points (Horwood & Keenan, 1994). Up to 50% of monitoring traps catch fewer than 50 sterile males per week (Meats *et al.* 2006). This raises concerns that there may be insufficient coverage of the target area to achieve a sufficiently effective sterile:wild ratio due to poor sterile male dispersal. Importantly, there is often a mismatch between the dispersion patterns of wild and sterile flies (Meats, 2007), and a simulation study demonstrated that this mismatch can lead to less effective suppression of target population growth rates (Meats, 2007). Modelling the coefficient a variation (CV = standard deviation/mean catch per trap per week) against mean trap catch for sterile male Q-flies indicated that increasing the release rate from fixed points that are spaced at 400 m intervals would have little effect on improving the evenness of recaptures in traps throughout the trapping array (Meats *et al.* 2006; Meats, 2007). It was suggested that coverage for *B. tryoni* SIT could be improved by the adoption of roving releases (Meats *et al.* 2006), although no trials have been published to date. Roving releases could also be augmented by spot releases at sites where traps consistently perform poorly in terms of catching sterile males, particularly when these traps also catch wild flies (Meats *et al.* 2006).

Table 1. Summary of the release rate, spacing and frequency of sterile male Queensland fruit fly releases, and the sterile:wild over-flooding ratios that were obtained during sterile insect technique campaigns in Australia. Only *B. tryoni* SIT releases that were undertaken to suppress or eradicate a wild population are included. Release rate is given as the mean number (and range) of sterile males deployed per release. Dividing the release rate per week by the area covered will give the release rate per week per km^2. Over-flooding ratios are given as the area-wide estimate, with the effective over-flooding ratio in brackets if reported.

Campaign location and duration	Area covered (km^2)	Total released (× 10,000)	Frequency	Release rate/week (× 10,000)	Distance between release sites	Over-flooding ratio (sterile males/wild/month)
Andrewartha *et al.* (1967)						
Warren, NSW, Australia	5					
Aug 1964-Mar 1965		125.74	Weekly	1.66 (0.17-4.42)	Not reported	Initial = 36, median = 5.9, final = 5.9
James (1992)						
Cowra, NSW, Australia	18				800 m	
10 Oct-5 Dec 1991		39.65	Weekly	4.41		Initial = 1.6*, median = *, final = *
6 Feb-16 Apr 1992		27.49	Weekly	2.50		
23 Apr-7 May 1992		16.22	Weekly	5.41		
Sproul *et al.* (1992)						
Perth, WA, Australia	270				~400 m**	
Jan-Dec 1990		46650.00**	Weekly	918.00**		Initial = 32**, median ≈ 1394**, final > 92,833**
Perepelicia *et al.* (1993)						
Ingle Farm, SA, Australia	7				400 m	
10 Mar-15 May 1993		417.86	Twice/week	41.79 (33.78-48.21)		No wild males caught during campaign
Horwood & Keenan (1994)						
Tharbogang, NSW, Australia	5				320 m	
25 Jan-21 Apr 1994		22.50	Weekly	2.50 (0.62*-10.50*)		No wild males caught during campaign
Perepelicia *et al.* (1994)						
Aldinga Beach, SA, Australia	7	379.20	Twice/week	47.40 (40.36-65.26)	400 m	No wild males caught during campaign
12 Feb-7 Apr 1994						

Campaign location and duration	Area covered (km^2)	Total released (× 10,000)	Frequency	Release rate/week (× 10,000)	Distance between release sites	Over-flooding ratio (sterile males/wild/month)
Reynolds *et al.* (1995)						
Clarence Gardens, SA, Australia	7				400 m	
6 Feb-13 Apr 1995		344.11	Twice/week	34.41 (18.75-44.42)		No wild males caught during campaign
Jackman *et al.* (1996)						
Glenside, SA, Australia	7				400 m	
6 Feb-24 Apr 1996		667.61	Twice/week	55.63 (31.58-75.47)		No wild males caught during campaign
Moana, SA, Australia	7				400 m	
14 Feb-3 Apr 1996		434.35	Twice/week	48.26 (32.86-75.47)		No wild males caught during campaign
Perepelicia *et al.* (1997)						
Linden Park, SA, Australia	7				400 m	
10 Feb-22 Apr 1997		830.51	Twice/week	75.50 (47.60-88.26)		No wild males caught during campaign
Meats *et al.* (2003)						
Trangie, NSW Australia	3				4 points within town	
13 Sep-27 Dec 1996; 21 Feb-25 Apr 1997		24.51; 17.50	Weekly	1.5; 1.8		Final = 2.5 (1.5)
19 Sep-26 Nov 1997; 5 Dec 1997–24 Apr 1998		19.26; 73.53	Weekly	1.8; 3.6		Final = 11.6 (5.1)
Warren, NSW, Australia	3				4 points within town	
23 Feb-26 Apr 1996		15.78	Weekly	1.5		Final = 2.6 (1.1)
13 Sep-27 Dec 1996; 21 Feb-25 Apr 1997		27.03; 16.22	Weekly	1.8; 1.5		Final = 0.8 (0.3)
19 Sep-26 Nov 1997; 5 Dec 1997–24 Apr 1998		18.02; 75.69	Weekly	1.8; 1.6		Final = 10.9 (10.7)
Gilgandra, NSW, Australia	5				400 m	
26 Feb-29Apr 1996		238.05	Weekly	24		Final = 25.8 (16.4)

9 Sep-23 Dec 1996; 24 Feb-28 Apr 1997		386.10; 287.55	Weekly	24; 29		Final = 18.1 (5.9)
15 Sep-24 Nov 1997; 1 Dec 1997–20 Apr 1998		316.31; 1,207.71	Weekly	29; 57.5		Final = 70.6 (39.0)
Narromine, NSW, Australia	5				400 m	
26 Feb-29Apr 1996		267.75	Weekly	27		Final = 10.9 (11.8)
9 Sep-23 Dec 1996; 24 Feb-28 Apr 1997		416.25; 282.60	Weekly	26; 28.5		Final = 15.4 (14.0)
15 Sep-24 Nov 1997; 1 Dec 1997–20 Apr 1998		310.86; 1,186.92	Weekly	28.5; 56.5		Final = 85.9 (26.0)

* Estimated from graphs, ** Values taken from Meats (1996).

Frequency of release

The frequency at which sterile males can be released is limited by the production schedule of the insect rearing facility. Releases at weekly intervals predominate in *B. tryoni* SIT campaigns (Table 1) due to restraints on colony productivity, and the protocols and costs involved in the irradiation and transport of pupae and adults (as described earlier in this review). Within the constraints of the weekly production of sterile pupae, releases in South Australia have been performed twice a week (Table 1) by delaying the development of half of the pupae that are received from production facilities in New South Wales. Holding the pupae at 17°C in an incubator for two days to delay their development has no significant effect on the emergence rate of adults (Perepelicia *et al.* 1993, 1994, 1997; Reynolds *et al.* 1995; Jackman *et al.* 1996). However, it is unclear whether the chilling of pupae has an effect on the performance of sterile adult flies when released in the field. This concern is particularly relevant when considering the generally poor recapture rates for sterile flies that have been released in South Australia: 0.0039% (Perepelicia *et al.* 1993), 0.0089% (Perepelicia *et al.* 1994), 0.0065% (Reynolds *et al.* 1995), 0.0941% (for the Glenside campaign; Jackman *et al.* 1996), 0.0167% (for the Moana campaign; Jackman *et al.* 1996), 0.2239% (Perepelicia *et al.* 1997) and 0.34% (adjusted for emergence and flight; Reynolds *et al*. 2012).

Compatability of SIT with other control techniques

There is little published information on the use of SIT in conjunction with other methods for the control of *B. tryoni*, although there is an increasing interest in this area. As with many control techniques, two or more techniques used simultaneously often produces a far more satisfactory result than one technique alone. With APVMA restrictions now in place on the uses of dimethoate and fenthion, there is increasing interest in SIT and its role as part of an Integrated Pest Management (IPM) program or a systems approach. Here we document what we do know and draw on the international literature for related and other pest species, and indicate where certain techniques may be of use in the *B.tryoni*/SIT system and that would benefit from further research.

Bait sprays & cover sprays

There has been little published evidence that a combination of bait sprays and SIT work together effectively to control wild fruit fly populations, and no published evidence that the combined use is effective for *B. tryoni* control. However what anecdotal and published evidence there is suggests that their combined use is very effective. This seems logical as bait sprays can bring wild populations down to levels that are then controllable with available numbers of sterile flies. In California, the success of the basin-wide programme was attributed to the initial application of malathion bait sprays to all hosts within 200m of all fly infestations and the subsequent release of sterile *C. capitata* (Dowell *et al.* 2000). Similarly, suppression of *C. capitata* populations by SIT in the Mediterranean region is often preceded by the application of insecticide sprays (Papadopoulos & Katsoyannos, 2004). Meats (1996) suggested that pre-baiting should be considered when wild male *B. tryoni* density is over 20 per km^2, which may lead to faster eradication. The successful eradication of a *B. tryoni* incursion in Perth used intensive lure blocking, bait spraying and sterile releases (Sproul *et al.* 1992). In Tharbogang, near Griffith in the Murrumidgee Irrigation Area (MIA), Horwood & Keenan (1994), demonstrated effective eradication of wild *B. tryoni* using bait sprays and SIT. In addition, they showed that the integrated SIT/bait spray strategy was more cost effective than traditional eradication techniques relying mainly on bait sprays ($144 and $323 per hectare respectively). Further studies in this area would certainly serve to assist in looking at enhancing SIT efficacy and cost effectiveness for *B. tryoni* control.

Male annihilation technique

Semiochemical-toxicant combinations commonly referred to as the male annihilation technique (MAT) use cue-lure and insecticide (maldison) impregnated caneite blocks, commonly nailed to trees to attract and kill *B. tryoni*. However, there are no published studies which have trialled the combined use of MAT with SIT for *B. tryoni* control. Notably, the successful eradication of

tephritid flies from a number of Pacific Islands has been achieved with the use of MAT and/or SIT (Messing, 2003), and this is an area that warrants further research for *B. tryoni.*

Biological control

Insecticide sprays have been used with varying degrees of effectiveness to control fruit fly species worldwide (Novelo-Rincón *et al.* 2009; Guillen & Sanchez, 2007), including *B. tryoni* (Jessup *et al.* 2007). However, pesticides are increasingly regarded as a serious threat to the environment and human health (Moreno & Mangan, 2000; Jessup *et al.* 2007), and there is increasing consumer awareness of pesticide residues, with the requirement for residue-free foods further boosting the need for pesticide-free control techniques. In addition, importing countries are increasingly imposing stricter maximum residue limits for pesticides. As a result, many countries are seeking environmentally friendly alternatives as a matter of priority (Montoya *et al.* 2007; Novelo-Rincón *et al.* 2009). It is an area in which Australia has lagged to some degree and below are several areas of research that are currently being pursued, in addition to environmentally-friendly control methods which should be considered for the control of *B. tryoni*.

Parasitoids

There are a number of documented cases where a combination of SIT and the augmentative/inundative release of parasitoids has effectively reduced fruit fly populations, including *Anastrepha* spp. in Mexico (Orozco *et al.* 2004; Montoya *et al.* 2007), *C. capitata* in Hawaii (Wong *et al.* 1992; Kaplan, 2008) and also in Guatemala (Cancino *et al.* 1996; Rendon *et al.* 2006). Population modeling has demonstrated that the combined use of SIT and parasitoids would be more effective than either technique alone for suppressing or eradicating a species (Barclay, 1987). Along the Guatemalan/Mexican border, the combination of SIT and parasitoids was reportedly synergistic in effect in controlling *C. capitata* (Sivinski *et al.* 1996). This synergistic action between SIT and parasitoids is believed to give more rapid and cheaper pest suppression or eradication (Sivinski, 1996). Studies in Australia are currently underway, where a combination of these techniques is likely to provide more economic and effective management of *B. tryoni* outbreaks (Gurr & Kvedaras, 2010). Suburban and native settings, rather than monocultures typical of commercial orchards, are thought to be more conducive to natural enemies in combination with SIT because they provide better availability of alternative food sources, lower pesticide application rates and moderated microclimatic extremes (Wharton, 1989; Sivinski 1996). Indeed, two parasitoid species, *Diachasmimorpha tryoni* and *D. kraussii* have recently been found for the first time, parasitizing *B. tryoni* in the buffer zone (urban towns) surrounding the FFEZ (Spinner *et al.* under review), which may be ideal areas for combined SIT and parasitoid release. A recent study on a related fruit fly species also suggest that the use of two or more parasitoids together with SIT may provide even better control of wild fly populations (Rendon *et al.* 2006). Reasons for this include that not all parasitoid species are equally effective under all possible conditions (Sivinski *et al.* 2000b; Spinner *et al.* 2012). Studies show that parasitoid species display microhabitat preferences, with different species capable of best exploiting different parts of the heterogeneous environments within and surrounding agroecosystems (Rendon *et al.* 2006; Spinner *et al.* under review). Although significant advances in the mass rearing of opiine parasitoids have occurred, particularly in the late 20th century (Purcell, 1998), the development of more efficient and economic rearing techniques will encourage adoption, and this is receiving further attention in a number of countries including Mexico (eg. Sivinski *et al.* 1997; 2000b) and more recently, in Australia.

Entomopathogenic fungi

The use of SIT together with fungal pathogens for the control of fruit flies has gained attention in recent years. Toledo *et al.* (2007) demonstrated that adult male *A. ludens* treated with *B. bassiana* were capable of transmitting the fungus to females during courtship and copulation. Based on this work, Novelo-Rincón *et al.* (2009) suggested the possibility of combining SIT with the use of *B. bassiana* against *A. ludens.* They showed in studies that sterile *A. ludens* males treated with *B.*

bassiana conidia acted as vectors transmitting the fungus to wild female populations during courtship and copulation, thus introducing an additional mortality factor. Therefore, the sterile males were not only able to induce sterility into the wild population, but they were able to induce mortality through the horizontal transmission of *B. bassiana*. This fungus can also cause high levels of infection among other fruit fly species including *C. capitata* (Wiedemann) (Castillo *et al.* 2000) and *B. oleae* (Gmelin) (Konstantopoulou & Mazomenos, 2005). Similarly, the horizontal transmission of *Metarhizium anisopliae* spores from treated to untreated flies in *C. capitata* (Dimbi (2003); Quesada-Moraga *et al.*. 2008), *Ceratitis cosyra* and *Ceratitis fasciventris* (Dimbi 2003) has also been demonstrated. Given the promising work internationally on a number of related fruit fly species, and that treated female flies have also been shown capable of transmitting infection to healthy males resulting in significant mortalities (Dimbi, 2003; Toledo *et al.* 2007; Novelo-Rincón *et al.* 2009), there is certainly potential for this avenue to be explored for *B. tryoni* control.

Semiochemicals and biopathogens

A recently developed form of mating disruption, ExoSex Autoconfusion™, has been developed as an insect control method which involves contaminating the target pest with electrostatically charged powder formulated with a pheromone (Howse & Underwood, 2000; Howse *et al.* 2007). These adhesive particles can be integrated with SIT, using sterile insects as mobile pheromone dispersers which are more efficient than placing static pheromone dispensers in the crop. In this way they would achieve control by mating disruption, reducing the level of mating in the wild population and when matings do occur, they are therefore likely to be between steriles and individuals from the wild population. These adhesive particles may also act as carriers for synthetic or biological pesticides, including fungal entomopathogens, and as a lure-and-kill technique for insect pest control. This technology includes a number of advantages such as reduced environmental contamination, high selectivity (therefore protecting beneficial insects), and a reduction in the number of sterile insects required for release (Howse *et al.* 2007). While this technology remains to be investigated fully in combination with SIT, it is a promising area with various trials showing promise for *C. capitata* management, and one which should be carefully followed for its potential use in controlling *B. tryoni* populations.

Microbial symbionts

Insects are associated with microbial symbionts, and several are known to manipulate mating behaviour and reproduction of their hosts (Crotti *et al.* 2012). By identifying and introducing these symbionts to target populations, they can successfully reduce pest populations and their economic impact. An example is the use of the insect symbiont, *Wolbachia* that was recently identified in several Australian *Bactrocera* species (M. Riegler pers. comm. 2012). *Wolbachia* can induce crossing incompatibilities (sterilities) between infected males and uninfected females (Bourtzis & O'Neill (1998). By infecting pest fruit fly species with *Wolbachia*, female sterility is artificially sustained in the field by repeated releases of cytoplasmically incompatible lab-reared males. Similar to conventional SIT, such repeated releases of *Wolbachia* infected flies can lead to population suppression or eradication. This has been effectively tested under laboratory conditions for two major agricultural pests, *C. capitata* (Ashburner *et al*. 1998; Sarakatsanou *et al.* 2011) and *B. oleae* (Apostolaki *et al.* 2011). Recent work has also shown that *Wolbachia* manipulates the biology of pathogen vectors such as dengue mosquitoes, with the outcome of reduced mosquito fitness and reduced pathogen transmission in *Wolbachia* infected mosquitoes. This technology is being successfully tested in northern Queensland with the aim to eliminate Australia's dengue virus problem (Hoffmann *et al.* 2011). It is timely that these approaches be validated and extended, alone and/or in conjunction with the SIT (i.e. releasing sterile *Wolbachia* infected males), to other target insect pest species, including *B. tryoni*. In addition, preliminary results show that *Wolbachia* is also present in parasitoid wasps of fruit flies (Hymenoptera: Braconidae) (M. Riegler pers. comm. 2012). Based on evidence from other parasitoids (Stouthamer, 1997) it is likely that this *Wolbachia* symbiosis also influences parasitoid reproduction to the advantage of biological control. Further

research is warranted on the potential application of *Wolbachia* technology in optimising parasitoid rearing and field application.

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Technical Report 1

Container loadings and eclosion units for sterile insect technique programs of B*actrocera tryoni* (Froggatt) (Diptera: Tephritidae)

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ABSTRACT
Management of *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) may be achieved through the sterile insect technique (SIT). Plastic adult rearing containers (PARCs) are commonly used to rear and release sterile fruit flies; however these containers have not been optimised for *B. tryoni*. A laboratory study compared whether six different PARC loadings of pupae (180 g, 200 g, 230 g, 250 g, 280 g & 300 g) affected emergence and flight of sterile *B. tryoni*. Four mediums utilised to rear the pupae to adult in the PARCs were compared in a laboratory study of emergence and flight and in a follow-on field study with respect to trap recapture rates of *B. tryoni*. Mediums tested included adults emerged under a i) wire grid, adults emerged in a ii) mesh bag and iii) paper bag and adults emerged with iv) no substrate (control). PARC box loadings and PARC substrates tested did not affect emergence or flight of sterile *B. tryoni*. Trap recapture rates were the highest for control followed by the mesh bag, wire grid and paper bags. The value of these findings for utilising PARCs to release sterile *B .tryoni* are discussed.

Keywords: Queensland fruit fly, plastic adult rearing container (PARC), flight, cue-lure, trap recapture

INTRODUCTION

The Queensland fruit fly *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) is the most significant pest of edible fruit in Australia. In response, a fruit fly exclusion zone (FFEZ), which encompasses some of the most valuable horticultural production areas in New South Wales (NSW), South Australia, and Victoria, was established (Reynolds *et al*. 2010). The sterile insect technique (SIT) is one of several management approaches employed to manage *B. tryoni* in the FFEZ. Plastic adult rearing containers (PARCs) are used for rearing flies for ground release of sterile fruit fly both internationally (Salvato *et al.* 2003) and nationally (M. Nolan pers. comm. 2009) and for chilled adult release internationally (Shelly *et al*. 2006). However, in Australia the use of PARCs has not been optimised for rearing and release of adult *B. tryoni*.

In this study we tested whether PARC loadings of pupae and rearing substrate (covering utilised to rear the pupae to adult in the PARC) affected emergence and flight of sterile *B. tryoni*. We also tested whether rearing substrate influenced trap recapture rates of sterile *B. tryoni*. We discuss the results in terms of the use of modified PARCs for the release of *B. tryoni* as part of a sterile insect release program.

MATERIAL AND METHODS

Study insects

Bactrocera tryoni were obtained as dyed pupae from the Fruit Fly Production Facility at the Elizabeth Macarthur Agricultural Institute, Menangle, New South Wales (NSW), Australia. Eight-day old pupae were irradiated at the Australian Institute of Nuclear Science and Engineering (AINSE) facility, Lucas Heights, to render them sterile before they were transported by road to the Wagga Wagga Agricultural Institute (WWAI) entomology laboratory in NSW. Insects were reared out in a growth room at the WWAI at 26±2°C, 65±15% RH and a light:dark period of 14:10, with a simulated dawn and dusk as the lights ramped up and down at the beginning and end of the light phase. Pupae were dyed (1g dye per 100g pupae) as described by Reynolds *et al*. (2010) with one of four fluorescent pigments; Fiesta FEX Arc chrome. Strong magenta, Flame orange or Stellar green (Swada, 30-32 Kilkenny Court, Dandenong South, Victoria, Australia) to differentiate between treatments. Studies have shown that there is no effect of the dye colours used in this trial on fruit fly performance (Reynolds *et al*. in press), however the four dye colours were rotated through each of the treatments on separate release dates in the trap recapture study.

Rearing protocol

According to treatment, for both trials pupae were placed in a translucent 46L PARC (Silverlock MH 0110, colour "natural", 645mm x 413mm x 275mm high), with a 43cm x 20cm, 1mm mesh on the lid and a 15cm x 10cm mesh on each length (side) of the container for ventilation. Resting space was provided by wedging cardboard dividers (approximately 160 cm in height), two running lengthways and five across the width of the container, to sit just above the pupal bed. Eighteen sugar cubes were placed on the base of each release container and a block of agar containing a mixture of sugar and water (5% sugar) (A. Jessup, unpublished data, 2004) was placed on top of the mesh covered lid. For the pupal load trial (trial 1), the treatments were 180g, 200g, 230g, 250g, 280g and 300g; each replicated three times. For the rearing substrate trial (trial 2), the treatments were i) adults reared under a wire grid (2 x 4mm hole width,), ii) adults reared in a mesh bag (2 x 2 mm hole width), iii) adults reared in paper bags and iv) adults reared with no substrate (control); each replicated four times.

Emergence & flight – pupal load and rearing substrate

The effect of pupal load and rearing substrate on percentage eclosion and flight of adult sterile *B. tryoni* was determined in separate trials, but followed the same general method. After all emerged adult *B. tryoni* had been released (only for rearing substrate was trap recapture also recorded – see below), each PARC was taken back to the lab and sampled by mixing the remaining empty pupal cases, un-emerged pupae, partly emerged adults, deformed and non-deformed dead fruit flies ('*B. tryoni* debris') to ensure even distribution across the box. Each box was then divided into eight equal segments, and each segment was sampled by scooping approximately 30 ml of *B. tryoni* debris into individual vials. For each vial, 0.15 g of *B. tryoni* debris was then weighed, and the number of each component of the debris was recorded.

Emergence

The percentage emergence per release box was estimated based on the summed *B. tryoni* debris totalling 1.8 g for each release method. Emergence was defined as the percentage of *B. tryoni* which fully emerged, irrespective of whether they were deformed or flew, per release method.

Fliers

The percentage fliers were based on the emergence samples described in the previous section. Fliers were defined as the percentage of eclosed *B. tryoni* that flew (i.e. left the rearing container) per PARC. This was calculated as the % fliers = 100% - % non-fliers. Percentage non-fliers was defined as those *B. tryoni* that that eclosed but never flew from the box and was calculated as deformed fruit flies + non-deformed fruit flies.

Trap recapture - rearing substrate

A trapping grid was established in the urban area of Wagga Wagga, NSW (35° 70' S, 147° 22' E), comprising 20 cue-lure and malathion baited Lynfield traps, spaced 400m apart in a 5x4 grid. Four release sites, spaced 400m apart (and 200m from the nearest trap), were located centrally in the grid. At each release site one PARC of each rearing substrate of sterile *B. tryoni* were released when the flies were two days of age. There were four release periods, release 1 (October 2008), 2 (December 2008), 3 (January 2009) and 4 (March 2009) and within each release period there were 6 consecutive weeks of recapture data collected, although some weeks were excluded from the analyses as flies were not trapped during those weeks.

Captured *B. tryoni* were collected, rinsed in 70% alcohol and dried before being sent to the Orange Agricultural Institute, Orange, NSW, Australia where the dye colour observed in the ptilinal fissure (Norris, 1957; Steiner, 1965) was recorded for each fruit fly (see Reynolds *et al.* 2010 for method).

Analysis

ASREML-R release 2 (Butler *et al.* 2007) was used to fit linear mixed models to the logistic transformation (z) of percent emergence and percent fliers using the formulae given by McCullagh & Nelder (1989):

$$z = \ln\left(\frac{y + 0.5}{m - y + 0.5}\right) \text{ where, } v = \text{var}(z) = (y + 0.5)^{-1} + (m - y + 0.5)^{-1}.$$

For percent emergence, y is the number of empty pupal cases and m is the total number of pupal cases while for percent fliers, y is the number of fliers and m is the number emerged.

Treatments, pupal loading (trial 1) or [substrate * release period] (trial 2) were fitted as fixed effects and replicate as a random effect (trial 1 and trial 2 (at each release period)).

Fly count per trap was modelled using a generalised linear mixed model fitted in GenStat (2009) using the method of Schall (1991). Fixed effects included [substrate * release period]. Random effects included trap and trap on a release date. A logarithmic link function was used and an underlying Poisson distribution was assumed. In all models the significance of fixed effects was assessed according to Kenward and Roger (1997).

RESULTS AND DISCUSSION

In both trials, all sterile *B. tryoni* adults had visible dye on their ptilinum (R. Kerslake pers. comm. 2008). In laboratory comparisons (trial 1) of PARC loadings, there was no significant effect of pupal load on emergence (F=0.65, df=5, 11.1, P=0.670) (Table 1) or flight ability (F=1.34, df=5, 7.3, P=0.349) (Table 1). Based on the results of this study, pupal loadings ranging from 180 – 300g per PARC achieved emergence and flight levels well above those recommended for *B. tryoni* used in SIT programs (FAO/ IAEA/USDA 2003).

Table 1. The effect of PARC pupal loadings on emergence and flight of *Bactrocera tryoni*.

Pupal load (g)	Mean emergence ± SE (logit)	Emergence (%) (back-transformed mean)	Mean fliers ± SE (logit)	Flight (%) (back-transformed mean)
180	2.21 ± 0.13a	90.5	1.87 ± 0.12a	87.0
200	2.14 ± 0.13a	89.8	1.91 ± 0.12a	87.5
230	1.99 ± 0.12a	88.4	1.75 ± 0.11a	85.5
250	2.41 ± 0.16a	91.8	2.11 ± 0.12a	89.5
280	2.29 ± 0.15a	91.2	1.86 ± 0.12a	86.9
300	2.21 ±0.14a	90.5	1.92 ±0.12a	87.6

Means in each column followed by the same letter do not differ significantly from each other (P>0.05) using least significant differences.

In a separate study (trial 2), there was no significant effect of rearing substrate (F=2.54, df=3, 37.3, P=0.071; Table 2) or fly batch (F=3.77, df=3, 6.8, P=0.067) on the emergence of sterile *B. tryoni*. Similarly, there was no significant effect of rearing substrate (F=1.64, df=3, 36.8, P=0.197; Table 2) on the number of sterile *B. tryoni* capable of flight. However, there was a significant effect of fly batch on release period (F=13.12, df=3, 24.5, P<0.001), with more fliers reared at release 2 (91.52%) than either release period 1 (86.92%), 3 (78.51%) or 4 (81.98%). Release period 1 had more fliers than release 3. As the flies were reared in controlled temperature and humidity growth rooms we are unable to account for the difference in fliers for the different batches of flies, particularly since there was no difference in emergence between the batches.

Table 2. The effect of PARC rearing substrates on emergence and flight of *Bactrocera tryoni*.

Eclosion substrate	Mean emergence ± SE (logit)	Emergence (%) (back-transformed mean)	Mean fliers ± SE (logit)	Flight (%) (back-transformed mean)
Mesh bag	1.99 ± 0.08a	87.8	1.86 ± 0.15a	86.6
Wire grid	1.90 ± 0.08a	87.0	1.60 ± 0.16a	83.3
No eclosion substrate (control)	1.74 ± 0.09a	85.1	1.90 ± 0.15a	86.9
Paper bag	1.72 ± 0.09a	84.8	1.72 ± 0.17a	84.8

Means in each column followed by the same letter do not differ significantly from each other (P>0.05) using least significant differences.

Trap recapture rates from trial 2 revealed a significant effect of rearing substrate (F= 8.98, df=3, 2.99, P=0.035; Table 3) with higher recaptures rates evident for the control compared to the paper bag and wire grid; the mesh bag did not differ significantly from any of the substrates. Therefore, under controlled conditions *B. tryoni* pupae do not require any covering substrate to optimise trap recapture rates. Paper bags, often used in chilled adult release, recaptured the lowest number of flies, probably as not all flies capable of flight were observed to leave the bags. While this is still a suitable substrate to use for chilled adult release, which requires ease of separation of the pupal debris from the adult flies, there is clearly potential to optimise this method. There was also a significant effect of release period on trap recapture rate (F= 15.06, df=3,5.02, P=0.011; Table 4) with lower recapture rates evident for release 1 compared with release period 2 and 4; release period 3 had lower recaptures than 4 but release 2 and 4 do not differ significantly. This is expected as release environment and climate are known to affect trap recapture rates of fruit flies (Weldon & Meats 2010) and varied for each release period (data not shown).

Table 3. The effect of rearing medium in PARCs on trap recapture rate of *Bactrocera tryoni*.

Rearing medium	Mean trap recapture ± SE (logit)	Proportion (%) trap recapture (back-transformed)
No substrate (control)	1.26 ± 0.44a	29.5
Mesh bag	1.08 ± 0.44ab	24.6
Wire grid	0.97 ± 0.44b	23.8
Paper bag	1.04 ± 0.44b	22.2

Means in each column followed by the same letter do not differ significantly from each other (P>0.05) using least significant differences.

Table 4. The effect of release period on trap recapture rate of *Bactrocera tryoni*.

Release period	Mean trap recapture ± SE (logit)	Proportion (%) trap recapture (back-transformed)
1 (September 2008)	0.37 ± 0.50a	10.6
2 (October 2008)	1.26 ± 0.50bc	28.5
3 (January 2009)	0.81 ± 0.50ab	16.5
4 (March 2009)	1.80 ± 0.50c	44.4

Means in each column followed by the same letter do not differ significantly from each other (P>0.05) using least significant differences.

Collectively, these results indicate that PARCs are suitable as release containers, as described above, at any *B. tryoni* pupal loading in the range of 180 – 300g, with no eclosion substrate required. Paper bags, used commonly for chilled adult release, require improvement to maximise, flight and trap recapture.

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Technical Report 2

Pupal release of the Queensland fruit fly, *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae), in the sterile insect technique: seasonal variation in eclosion and flight

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ABSTRACT
The Queensland fruit fly, *Bactrocera tryoni* (Froggatt), is the most significant pest of edible fruit in Australia. For the control of *B. tryoni* using sterile insect technique (SIT), either pupae or adults may be released. Using pupal release, this study tested the seasonal effect of different pupal loadings on eclosion and the flight of sterile *B. tryoni*. Pupal eclosion boxes were loaded with either 200, 350, 500, 650 or 800 g of pupae during five periods of the fruit fly season (August, October, December, February, and April). Adult flies were allowed to emerge and the remaining pupal debris was sampled to determine the percent emergence and percent fliers. The duration of emergence, dye retention on the ptilinum of the flies, and temperature and relative humidity externally and internally of the eclosion boxes were recorded. The percentage of emergence was influenced by both pupal loading and the period of release. Overall, the percentage of emergence was lower for loadings of 200 and 350 g pupae in August, October and April as compared to the 500 g or higher loadings. This difference was not apparent in December or February. The mean percentage of emergence for each pupal loading in December, February and April was well above 65%, the minimum required emergence parameter for successful sterile *B. tryoni* release. Across all pupal loadings, the percentage of fliers was greater than 99.3% in December, 87.8% in February and 80.8% in April. A high percentage of fliers (> 92.7%) was recorded in October, but the percentage of emergence in August and October was below 65% for all pupal loadings; thus pupal release is a sub-optimal SIT method during this period of time. Dye on the ptilinum was detected on every fruit fly sampled across all pupal loadings and release periods. Minimum temperature for optimal pupal emergence should not fall below 10°C, and the maximum should not exceed 35°C. Minimum temperature for successful flight should not fall below approximately 6°C while the maximum temperature should exceed 16°C. The described pupal release system is considered a possible option for use as part of an SIT program against *B. tryoni* under suitable environmental conditions in Australia.

Key words SIT, emergence, Qfly, temperature, relative humidity, ptilinum

INTRODUCTION

Fruit flies are probably the most destructive pests of edible fruit in Australia. The native fruit fly, *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) is the most economically important, with the national, annual cost estimated at AU$28.5 million (Sutherst *et al.* 2000). Following international protocols, the Fruit Fly Exclusion Zone (FFEZ) in south-eastern Australia is recognized as free from *B. tryoni*, thus allowing the horticultural industry within that zone to export fresh commodities to fruit fly sensitive domestic and international markets. The FFEZ encompasses some of the most valuable horticultural production areas of the country, including Sunraysia, the Mid Murray and the Goulburn Valley in Victoria, the Murrumbidgee Irrigation Area (MIA) of New South Wales and the Riverland of South Australia.

The Sterile Insect Technique (SIT) is used against a wide range of insect pests of plants, animals and humans in more than 50 countries (Krafsur 1998). SIT is one of the current, internationally approved treatments against incursions of *B. tryoni* within the FFEZ. The success of SIT relies on the 'over-flooding' of the released sterile male fruit flies in the wild male fruit fly population and thereby minimising the possibility of wild flies mating to produce viable eggs (Meats 1996). Most SIT programs use fluorescent dyes to coat the sterile pupae, so that upon eclosion, the ptilinum of the fruit fly is marked with dye in order to distinguish between sterile fruit flies and wild fruit flies captured in traps (Enkerlin *et al.* 1996). Adequate retention of dye on the ptilinum of *B. tryoni* is therefore a crucial component of SIT in Australia. In New South Wales, the first experimental work with SIT for the control of *B. tryoni* was carried out from 1962 to 1965, using the methods adopted from similar release programs internationally (Andrewartha *et al.* 1967) as described in Monro & Osborn (1965).

Although few SIT programs have incorporated pupal release, under certain Australian conditions it is believed to be effective if predation can be kept to a minimum (Dominiak *et al.* 2003). High levels of predation of the Oriental fruit fly, *Bactrocera dorsalis* (Hendel), from ant and bird species on Guimaras Island, Philippines, encouraged researchers to change from pupal to adult release (G. Obra pers. comm. 2008). Dominiak *et al.* (2000, 2003) suggested that *B. tryoni* pupal releases should require fewer resources compared with either adult ground or aerial release. Adults are also thought to suffer less crowding stress in a pupal release box, as eclosed adults are free to leave the box upon emergence. This also leads to a steady supply of adult fruit flies to the targeted area, compared with a single large delivery of fruit flies in one day using adult ground release. Another advantage of this method is that adults may become partially acclimatised to the local climate as the pupae are exposed to variable temperatures for the days preceding eclosion (Fay & Meats 1987).

Over the past decade, a method for pupal release as part of the SIT has been developed for *B. tryoni* in Australia; however, it has not become a common practice due primarily to the uncertainty of its effectiveness under different climatic conditions. Various strategies for pupal release have been tested, including a platform release method (Andrewartha *et al.* 1967), the use of 50L bins (MacFarlane & Betlinski 1988), the basic 'bed' technique (Dominiak & Webster 1998), covered trays (Dominiak *et al.* 2000), the use of buckets suspended from trees (Meats *et al.* 2006) and polystyrene foam boxes (Dominiak *et al.* 2003). The latter technique has been used with varying success in Australia (Dominiak *et al.* 2003; Meats & Edgerton 2008). Conducted over the entire *B. tryoni* season (August-April), this study investigates the influence of temperature and relative humidity (RH), as well as the effect of pupal loading (pupal quantity per release box, see below), on the emergence and flight of sterile *B. tryoni* adults from pupal release boxes. The retention of dye on the ptilinum was also examined.

MATERIALS AND METHODS

Release protocol

Pupal releases were conducted at the Wagga Wagga Agricultural Institute (WWAI) (35° 70' S, 147° 22' E) from August 2007 until April 2008. Five stands were placed in a row approximately 2 m apart. Each stand consisted of a single shelf (58 cm wide by 114 cm long) at 75 cm above the ground to allow air to circulate around the pupal release box (see below). The shelf was covered by a 30° insulated sloping roof (150 cm wide by 180 cm long) to allow rain run-off and minimise the effects of direct sun exposure. Each stand held three polystyrene pupal release boxes, spaced approximately 15 cm apart. Stands were held in place with four guide ropes, one on each corner. These were sprayed with a contact insecticide. An area radiating one metre from the stands was sprinkled with Mortein Outdoor Ant Sand ® (Reckitt Benckiser (Australia) Pty Ltd, 44 Wharf Road, West Ryde, NSW 2114) to kill any insectivorous predators.

A modified version of the polystyrene pupal release box described in Dominiak *et al.* (2003) was used to release the pupae. The box was 28 cm high, 57 cm long and 28 cm wide with a wall thickness of 2.3 cm. It had a window cut from each vertical face measuring 3.5 cm high x 20 cm long and 2.5 cm from the top, to allow the fruit flies to leave the box.

Bactrocera tryoni were obtained as pupae from the Fruit Fly Production Facility at the Elizabeth Mcarthur Agricultural Institute, Menangle, New South Wales, Australia, where the larvae were reared on a standard lucerne chaff diet (Prabhu *et al.* 2008). Pupae were dyed with Fiesta FEX 1 Astral Pink fluorescent pigment (Swada, 30-32 Kilkenny Court, Dandenong South, Victoria, Australia) by inverting (mixing) the dye and pupae in a plastic bag to evenly coat the pupae (0.75 g dye per 100 g pupae). This is the standard pigment used to mark sterile *B. tryoni* within SIT programs in Australia. Monthly consignments of dyed *B. tryoni* were sent as the late-stage pupae to the Australian Nuclear Science and Technology Organisation, where they were irradiated at 70.3 – 74.5 Gy to render them sterile.

The dyed, irradiated pupae were then transported by road overnight to the WWAI and weighed the following morning (to 0.01 decimal places; A&D GR-202 Intelligent Analytical Balance, John Morris Scientific, 61-63 Victoria Avenue, Chatswood, NSW 2067 Australia) according to treatment weight (see below) and placed in open plastic bags inside cartons. The weighed pupae were then transported to the release site and spread evenly on the base of a polystyrene box. Vermiculite was moistened with hot tap water (4:1) (A. Jessup, unpublished data 1988; G. Campbell, pers. comm. 2007) and allowed to cool overnight. The cooled vermiculite was spread over the pupal layer in order to just cover the pupae, so that the total depth of pupae and vermiculite was no more than 1-1.5 cm. Additional resting space for adult *B. tryoni* to dry and expand their wings was provided. Cardboard dividers (approximately 160 cm in height) were wedged in each polystyrene box, two running lengthways and five across the width of the box, to sit just below the window cut-outs and just above the pupal bed.

Each box held either 200, 350, 500, 650 or 800 g of pupae. Previous studies examined loadings as large as 1600 g; however, loadings below 800 g were shown to produce better emergence (Dominiak *et al.* 1998). Depending on each consignment, individual pupae weighed 9.2-11.3 mg on average, which was above the acceptable minimum weight (8.5 mg) for *B. tryoni* pupae used in SIT programs (FAO/ IAEA/USDA 2003). There were three replicates for each pupal loading at each release period. A block of agar containing a mixture of white sugar and yeast hydrolysate (MP Biomedicals Australasia Pty Ltd, P.O. Box 187, Seven Hills, NSW 2147, Australia) (3:1 by weight) and water (A. Jessup, unpublished data, 2004) was placed on top of each solid box lid for adult feeding and was replaced as needed. Some predation of pupae and adult *B. tryoni* was noted, but this was not measured and believed minimal.

Emergence

Adult emergence in each pupal release box was checked at 10:00 h every day. Emergence was deemed to have ceased if, after 4 days, no emergence was recorded from any box. The date on which emergence was last observed was recorded as the final emergence date.

Once all adult *B. tryoni* had eclosed and either left the box or died, the remaining empty pupal cases, unemerged adults, partly emerged adults, deformed and non-deformed dead fruit flies ('*B. tryoni* debris') in each box were mixed to ensure even distribution across the box. The *B. tryoni* debris was then divided into eight equal segments. Each segment was sampled by scooping approximately 30 ml of *B. tryoni* debris into individual vials. For each vial, 0.15 g of *B. tryoni* debris was then weighed and the numbers of empty pupal cases, partly emerged adults, unemerged adults, deformed and non-deformed *B. tryoni* were recorded. This weight was chosen as the work was labour intensive and our observation showed that it gave a good representation of the contents of a pupal release box while minimising labour requirements. Emergence per pupal release box could then be estimated based on the summed *B. tryoni* debris of 1.2 g, which was defined as the percentage of *B. tryoni* which fully emerged, irrespective of whether they were deformed or flew.

Fliers

Fliers were defined as the percentage of *B. tryoni* that eclosed per pupal release box and flew or left the box. The percentage of fliers was estimated based on the emergence samples described in the previous section, and calculated as [(total emergence – non-fliers)/total emergence] x 100. Non-fliers were defined as those *B. tryoni* that eclosed but never flew from the box, including both deformed and non-deformed adult fruit flies.

Dye on ptilinum

For each release, on the first day eclosion commenced and every four days thereafter until all *B. tryoni* had eclosed and left the pupal release box, a sample of 15 *B. tryoni* were mechanically aspirated from each box and placed in a labelled vial. If less than 15 *B. tryoni* were available in a box, then all existing fruit flies were sampled. Only completely eclosed fruit flies that had time to dry off were sampled. The sample vials were taken back to the laboratory where the sterile *B. tryoni* were rinsed in 70% alcohol for approximately one minute to remove any excess dye. The adult *B. tryoni* were then left to dry on a paper towel and placed back into their labelled treatment vials. They were then sent to the Orange Agricultural Institute, Orange, NSW, Australia, to ascertain retention of dye on the ptilinum. This was determined under a Leica MZ 8 microscope (Leica Microsystems Pty Ltd, Unit 3, 112 -118 Talavera Road, North Ryde, NSW 2113) with both a UV (Leica Stereo-Fluorescence System 10446271) and blue light source (Leica CLS 150 X light source with a blue filter), as different dyes are more visible under certain light sources (R. Kerslake pers. comm. 2008).

Temperature and relative humidity

At each release, one box was randomly selected for each pupal loading to accommodate one hygrochron data logger (On Solution, PO Box 1007, Baulkham Hills, NSW, Australia 2153) for recording temperature and RH at 10-minute intervals. Two Tinytag Ultra data loggers (Hastings Data Loggers, PO Box 5112, Port Macquarie, NSW, Australia 2444) were also placed external to the boxes to record the external temperature and RH for the duration of the trial. This data was used to determine the effects of environmental temperature and RH on % emergence and % fliers for the period from the time the pupae were placed in the boxes until the last time no more than 15 adult fruit flies were counted inside each box. This period was deemed the most crucial climatic data influencing successful emergence. Mean

maximum/minimum temperature/RH for a release was calculated from the 10 minute logged data. The extreme maximum/minimum temperature/RH (defined as single data points that reflect the highest (maximum) or lowest (minimum) extremes of either temperature or RH) in every 24 hour period from 12 noon was determined, the number of these measures varying with the trial period (2^{nd}-17^{th} August 2007, 3^{rd}-14^{th} October 2007, 5^{th}-11^{th} December 2007, 6^{th}-12^{th} February 2008 and 16^{th}-29^{th} April 2008). The means of these daily values were then taken to be the mean extreme (referred to as 'mean' from herein) maximum/minimum temperature/RH for a trial period and as such were means of between 7 and 16 measurements. If the overall mean were used (i.e. average of all data points for a 24h period), we would be ignoring the extremes of temperature which are believed to impact on mortality rates to a greater extent than those temperatures which generally permit completion of the life cycle (Yonow *et al.* 2004; Meats 1989).

Statistical analysis

Emergence & Fliers

A logistic transformation of both percent emergence and percent fliers was taken. This transformation maps probabilities from the range 0 to 1 to the entire real number line thus permitting modelling using an unrestricted additive linear model. Back transformation from a logistic transformation also ensures that the percentages lie within 0-100%. This transformation is nearly linear for mid range probabilities and has the greatest effect on higher range probabilities and is thus appropriate for the data of percent emergence and percent fliers. For both variables, the data was transformed using an empirical logistic (log odds) transformation (McCullagh & Nelder, 1989):

$$z = \ln\left(\frac{y + 0.5}{m - y + 0.5}\right) \text{ where, } v = \text{var}(z) = (y + 0.5)^{-1} + (m - y + 0.5)^{-1}$$

where in the case of percent emergence y is the number of empty pupal cases and m is the total number of cases, including empty pupal cases, partly emerged adults and un-emerged adults. For percent fliers, y is the number of fliers while m is the number emerged. ASREML-R release 2 (Butler *et al.* 2007) was used to fit a linear mixed model to z. The model included pupal load, release period and their interaction as fixed effects and the blocking structures of stand, stand at a release period and box on a stand at a release period as random effects with an auto-regressive correlation structure fitted to boxes placed in rows. The significance of fixed effects was assessed using approximate F tests with the denominator degrees of freedom calculated according to Kenward and Roger (1997). For percent emergence and percent fliers, least significant differences with P=0.05 were used to test for treatment differences.

Temperature and relative humidity

To explore the relationships between external temperature and RH and percent emergence or percent fliers, a two step process was followed. Initially the relationship between internal temperature and RH and % emergence or % fliers was determined using the cubic spline method detailed below and then the relationship between external and internal measures of temperature and RH were examined. Where a significant linear relationship between external and internal measures of temperature and RH existed, external values could be estimated from the regression equation using internal measures obtained from the cubic smoothing spline analysis. If the regression relationship between external and internal measures was not significant, an indicative range was used. This external temperature or RH range is the lower and upper 95% confidence interval bounds to the nearest 0.5^{o}C estimated at the internal temperature or RH which corresponds to the maximum turning point on the cubic spline

regression. Since the relationship between % emergence and mean minimum RH is linear, a maximum turning point has not been achieved. The maximum % emergence is therefore estimated at the maximum value of mean minimum RH (Fig. 4a).

In order to identify the general relationships between internal mean maximum/minimum temperature/RH and % emergence/fliers, cubic smoothing spline models were fitted to % emergence/fliers assuming the error in the measures of mean temperature/RH to be minimal. Cubic smoothing splines are a smooth (that is, continuous in the first derivative over the domain of definition) function comprising piece-wise cubic polynomials between measurement times. These cubic smoothing splines were fitted as linear mixed models using the approach of Verbyla *et al.* (1999) and the software ASREML (Gilmour *et al.* 2002). Fixed effects included both mean level effects and linear trend effects (over increasing maximum/minimum internal temperature/RH which we refer to as the independent variate), at the overall and pupal load levels. Overall mean level curvature due to the independent variate and curvature over the independent variate specific to pupal load were fitted as random effects in the model. The significance of curvature terms was assessed with doubling the change in the log-likelihood (denoted 2Δlogl) and tested according to the Chi-square methods outlined in Verbyla *et al.* 1999 while the significance of fixed effects was assessed using Wald tests. Only significant terms ($P<0.05$) were retained in the final model. When data loggers were initially placed in the eclosion boxes, humidity was often 100% due to the moistened vermiculite. These data points were therefore excluded from the regression and not presented in Fig 4 a-d.

RESULTS

Effects of release period and loading on emergence

There was a significant effect of release period ($F(4, 51) = 223.6$; $P<0.001$), pupal load ($F(4, 46.2) = 12.8$; $P<0.001$) and a release period x pupal load interaction ($F(16, 49.2) = 3.2$; $P<0.001$) on adult eclosion of *B. tryoni* (Fig. 1). For % emergence, the overall mean (±SE) logit value was 0.364 ± 0.161, indicating high variability. Averaged across pupal loading, the percentage of emergence was comparable for only two releases which coincide with the highest emergence (February and April at 78.1 and 78.2%, respectively). The percentage of emergence was the lowest (18.1%) in August, followed by October (51.9%) and December (67.1%). The percentage of emergence from loadings of 650 g and 800 g were the highest (63.1% and 65.4%, respectively) but did not differ significantly from each other. Lower loadings corresponded to significantly different percentages of emergence (200 g – 49.7%, 350 g – 54.9%, 500 g – 61.7%). There were significantly lower percentages of emergence from lower loadings (200 g and 350 g) in August, October, and April as compared to the 500 g or higher loadings. This difference was not evident during December or February. The emergence period was the longest in August and October (16 and 9 days respectively), followed by April (8 days) and December and February (5 days each).

Effects of release period and loading on fliers

There was a significant effect of release period ($F(4,48)=45.3$; $P<0.001$), pupal load ($F(4,48)=3.4$; $P=0.016$) and a release period x pupal load interaction ($F(16,48)=2.5$; $P=0.007$) on the percentage of fliers of *B. tryoni* (Fig. 2). For % fliers the mean (±SE) logit value was 2.536 ± 0.341, indicating low variability. Averaged across pupal loading, the percentage of fliers in December (99.5%) was significantly higher than those in all other trial periods. The percentage of fliers in February (95.6%) and October (93.5%) did not differ significantly, and that in the latter release period did not differ significantly from that in April (89.9%). August had a significantly lower percentage of fliers compared to all other release periods (59.6%).

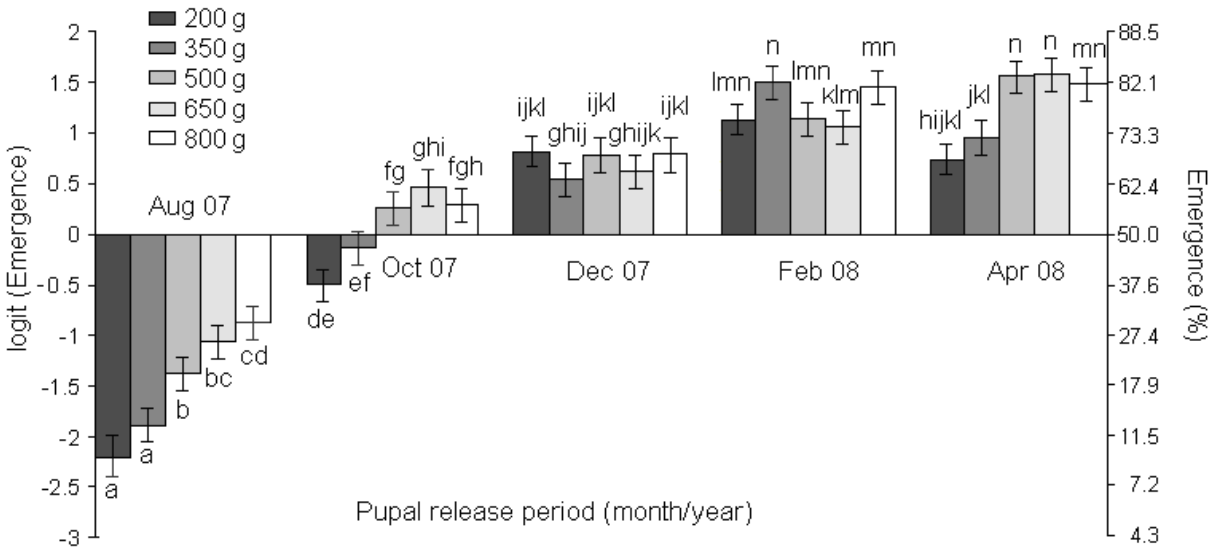

Figure 1. The percentage emergence of *B. tryoni* at different pupal loadings (g), over five different periods during the fruit fly season, from pupal release boxes in inland Australia. The data for emergence and SE is shown on the logit scale with a corresponding percentage emergence scale. Means in each column followed by the same letter do not differ significantly from each other (P>0.05) using least significant differences.

The percentage of fliers for loadings of 350, 500, 650 and 800 g were the highest (93.9%, 94.1%, 93.6% and 93.1%, respectively) without significant differences among them. The lowest loading of 200 g had 90.3% fliers but did not differ significantly from the loadings of 650 g or 800 g. The significant interaction between pupal load and release period reflects significant variation in percentage fliers between the pupal loadings for all periods except October, with no obvious trend consistent for any two periods.

Dye on ptilinum

The total fruit flies sampled from each release for examining the retention of dye on ptilinum varied, depending on the duration of emergence and the pupal load. Therefore, on any single sample period the number of adult *B. tryoni* sampled ranged from 0 to 15 per pupal release box. Dye on the ptilinum was detected on every *B. tryoni* adult sampled across all pupal loadings and periods.

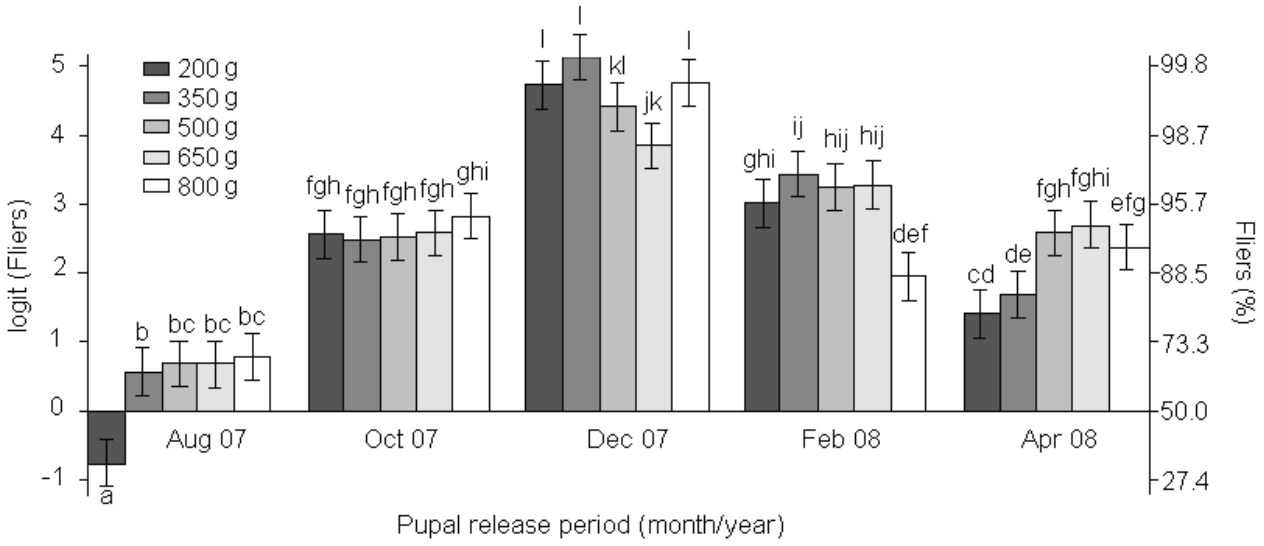

Figure 2. The percentage of *B. tryoni* that emerged and flew from different pupal loadings (g), over five different periods during the fruit fly season, from pupal release boxes in inland Australia. The data for fliers and SE is shown on the logit scale with a corresponding percentage fliers scale. Means in each column followed by the same letter do not differ significantly from each other (P>0.05) using least significant differences.

Temperature relationships

The mean minimum and maximum temperature and RH and standard deviation recorded externally and internally of the pupal release box are shown in Table 1.

The relationship between % emergence and the mean minimum and maximum temperature in the boxes at all release periods is represented by a single curve (Fig. 3a and 3b, respectively). The optimum internal box mean minimum temperature for % emergence as shown by the regression curve (R^2=0.86, 2Δlog*l*=34.82, P<0.001) was 13°C (Fig. 3a). The linear relationship between external and internal box minimum temperatures was significant (F(1,3)=219.0, P<0.01, R^2=98.2%; ext. mean min = -1.498+0.935*int. mean min). Using this equation, the optimal minimum external temperature was calculated as 10.7°C with a 95% confidence interval of 10-11.5°C. The optimum internal box mean maximum temperature for % emergence (R^2=0.69, 2Δlogl=8.75, P<0.001) from the curve is 27°C (Fig. 3b). An optimal external maximum temperature was difficult to determine given that no regression

Table 1. The mean and standard deviation (in brackets) for extreme minimum and maximum temperature (°C) and relative humidity (%RH) recorded externally and internally of the pupal release boxes from August 2007 through to April 2008.

Release period (month/ year)	Mean extreme maximum temperature (°C)		Mean extreme minimum temperature (°C)		Mean extreme maximum relative humidity (%RH)		Mean extreme minimum relative humidity (%RH)	
	External	Internal	External	Internal	External	Internal	External	Internal
Aug-07	19.8 (4.16)	15.1 (0.20)	3.9 (2.66)	5.5 (0.41)	86.1 (8.03)	83.8 (6.11)	64.8 (11.82)	77.1 (4.22)
Oct-07	20.5 (3.90)	23.4 (0.46)	7.0 (4.00)	8.8 (0.26)	49.2 (10.45)	57.2 (3.34)	24.6 (4.38)	44.9 (6.65)
Dec-07	30.8 (2.65)	31.3 (0.60)	14.7 (3.52)	17.7 (0.68)	50.3 (20.36)	60.3 (0.95)	30.8 (11.75)	42.2 (3.55)
Feb-08	37.1 (2.94)	27.6 (0.68)	14.5 (2.62)	16.5 (0.47)	76.0 (12.23)	72.3 (3.98)	47.5 (13.78)	60.8 (4.43)
Apr-08	20.5 (4.47)	21.3 (0.47)	6.7 (2.44)	9.6 (0.40)	85.9 (7.46)	73.1 (1.40)	68.4 (9.37)	61.9 (7.12)

relationship ($F(1,3)=4.25$, $P > 0.05$, $R^2=44.8\%$) could be found between external and internal maximum temperatures. This was due mainly to the recorded variability in mean maximum temperatures external and internal of the box for December and February. Despite mean external temperatures being higher in February (37.1°C) as opposed to December (30.8°C), mean maximum temperatures internal of the boxes were cooler in February (27.6°C) compared with December (31.3°C). Therefore, at best we can say the optimum external maximum temperature is at the uppermost, 35°C.

The relationship between % fliers and the mean minimum temperature in the boxes at all release periods fits to a single curve ($R^2=0.92$, $2\Delta logl=25.46$, $P<0.001$) (Fig. 3c). The % fliers increased quickly when mean internal minimum temperatures were measured at 5-8°C. When internal mean minimum temperatures were higher than 8°C, the % fliers continued to rise but at a much reduced rate. As the relationship between external and internal box minimum temperatures was significant ($F(1,3)=219.0$, $P<0.001$, $R^2=98.2\%$; ext. mean min = -1.498+0.935*int. mean min), an internal box mean minimum temperature of 8°C corresponds to a mean external minimum temperature of 6°C. The relationship between % fliers and the mean maximum temperature in the boxes at all release periods ($R^2=0.97$, $2\Delta logl=16.65$, $P<0.001$) fits to a single curve for pupal loadings of 350 g to 800g (Fig 3d). However, an individual curve for the 200 g loading is indicated as the underlying linear trend for this curve differed significantly from the linear trend underlying the 350-800g combined loadings curve (loadings linear trend $F(4,5)=17.48$, $P=0.004$) (Fig. 3d). This difference is apparent for mean maximum internal box temperatures less than 20°C, where there is a reduction in the percentage fliers for the 200 g pupal loading. As for % emergence, given there was no regression relationship ($F(1,3)=4.25$, $P>0.05$, $R^2=44.8\%$) between external and internal maximum temperatures, the external mean maximum temperature should not exceed 28°C for flight, when the internal mean maximum temperature is 20°C .

Relative humidity relationships

The relationship between % emergence and the mean minimum and maximum RH in the boxes at all release periods fits to a single curve ($R^2=0.78$, $2\Delta logl=18.43$, $P<0.001$, Fig. 4a; $R^2=0.54$, $2\Delta logl=19.91$, $P<0.001$, Fig. 4b). There was a significant positive linear relationship (external mean max RH=-41.768+1.605*internal mean max RH) between external and internal mean maximum RH ($F(1,3)=19.86$, $P = 0.021$, $R^2=82.5\%$). However, the relationship between the external and internal mean minimum was not significant ($F(1,3)=9.53$, $P = 0.054$, $R^2 =68.1\%$). As shown in Fig 4b the % emergence increases with internal box mean maximum RH. When the internal mean maximum RH reached 76.9%, the percentage of emergence was 81.6%. Only data for August 2007 (loading 350 and 650 g) had higher internal box mean maximum RH measures. At this time there was also very low % emergence (and an unusually low internal box temperature) which could singly influence the fitted curve. For this reason these data points were not included when determining the curvilinear relationship, but rather used in the model as a single point (Fig. 4a & b; August 2007 mean).

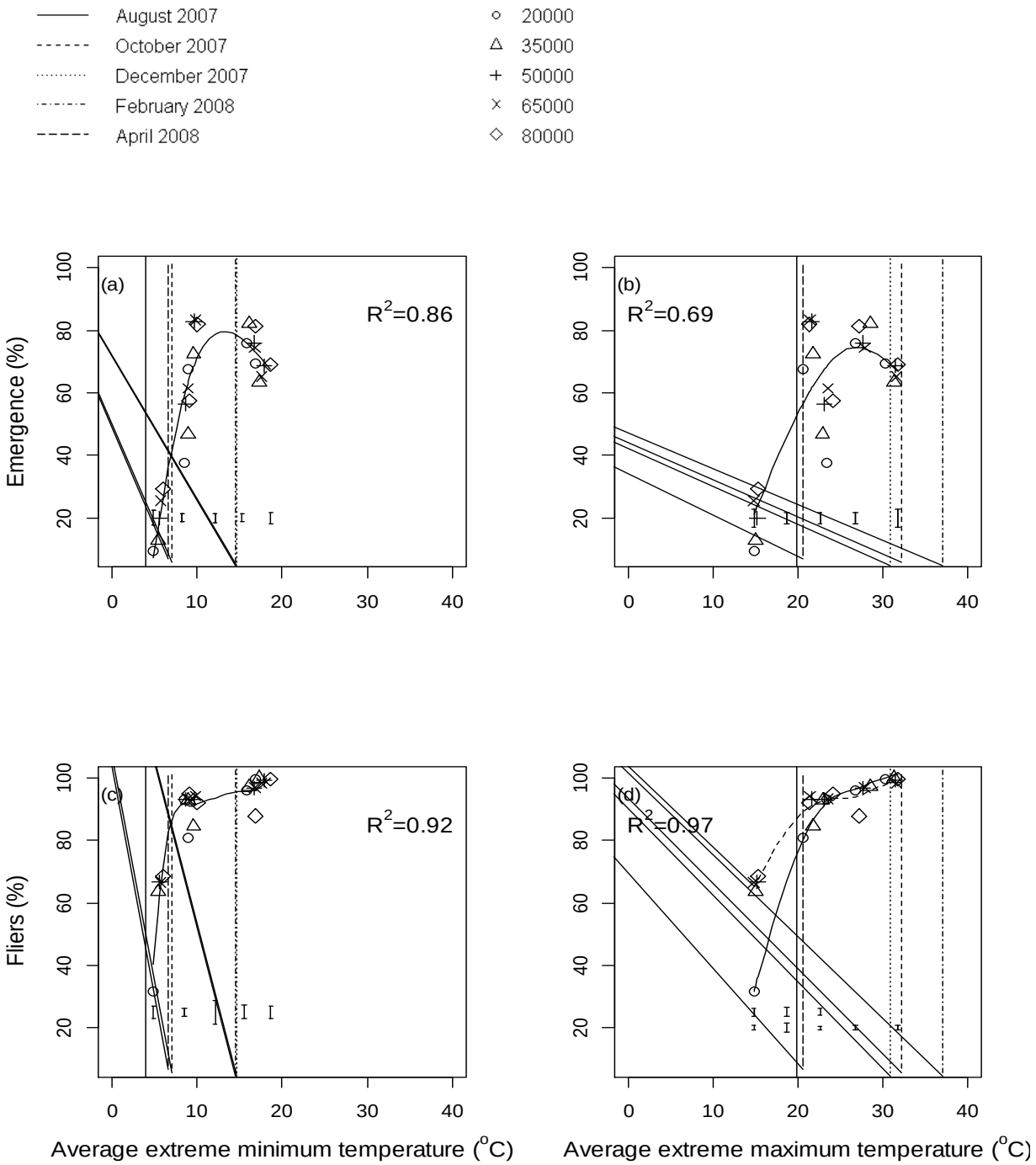

Figure 3a-d. The relationship between percentage emergence and the extreme mean maximum (a) and minimum (b) temperature and percentage fliers and the extreme mean maximum (c) and minimum (d) temperature. The vertical lines represent the extreme mean external box temperature for each release month. Each pupal loading (grams) is represented by a different symbol. The relationship between % fliers and the mean maximum temperature in the boxes at all release periods is represented by a single (non-dashed) curve for pupal loadings of 350 g to 800 g and an individual (dashed) curve for the 200 g loading as the latter loading differed significantly (P>0.05) from the other curve. Bars are indicative SE throughout the domain of the independent variable. Dashed bars are SE for 200g loading.

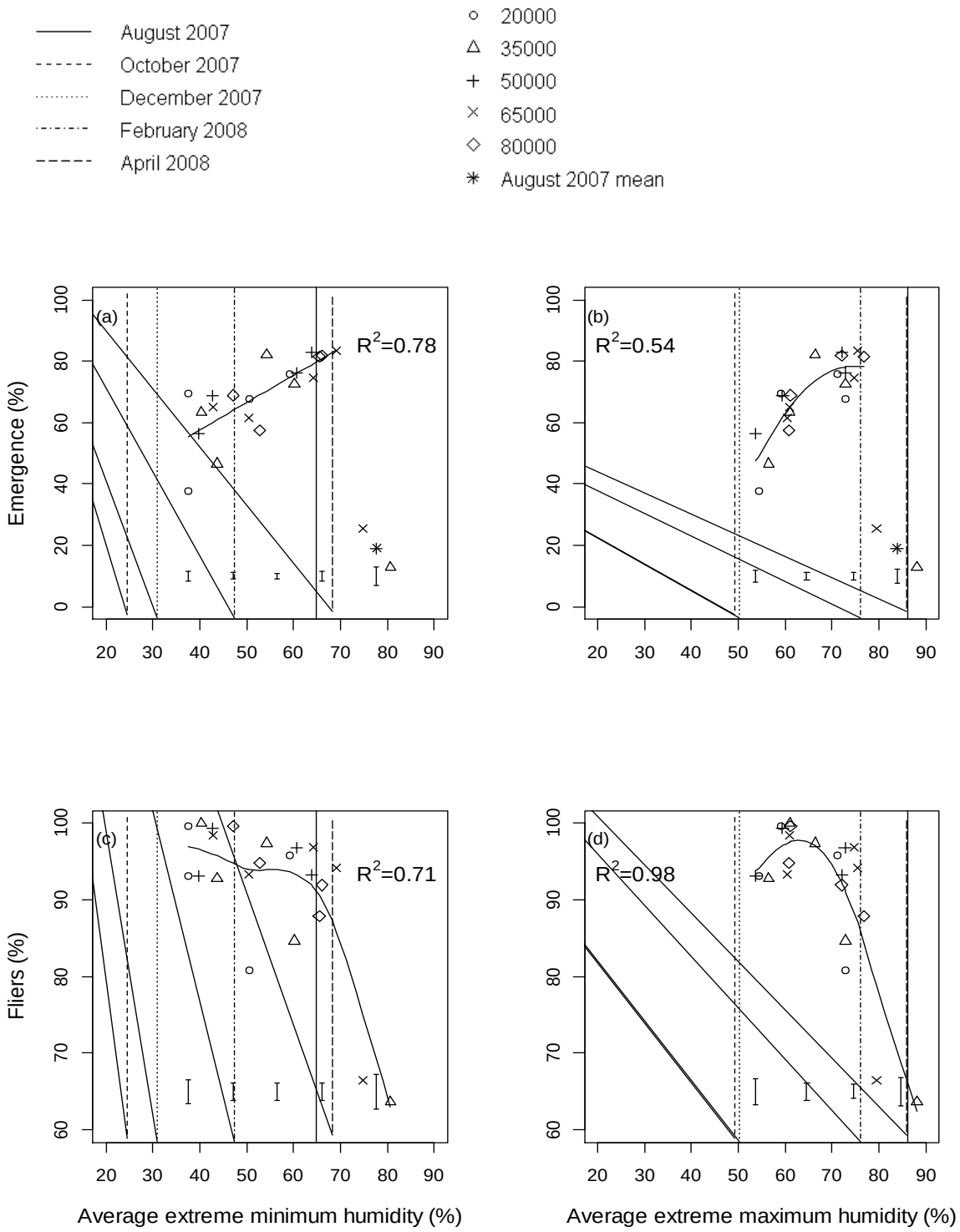

Figure 4a-d. The relationship between percentage emergence and the extreme mean maximum (a) and minimum (b) relative humidity and percentage fliers and the extreme mean maximum (c) and minimum (d) relative humidity. The vertical lines represent the extreme mean external box humidity for each release month. Each pupal loading (grams) is represented by a different symbol. Bars are indicative SE throughout the domain of the independent variable.

The relationship between % fliers and the mean minimum and maximum RH in the boxes at all release periods fits to single curves (Fig. 4c and 4d, respectively) (R^2=0.71, 2Δlogl=7.65, P<0.001, Fig. 4c; R^2=0.98, 2Δlogl=8.71, P<0.001, Fig. 4d). The curve for mean maximum RH shows that the maximum percentage fliers was attained at an internal mean maximum RH of 62.5% (Fig. 4d), which using the regression equation above equates to an external mean maximum RH of 58.5%. With increasing mean minimum RH, a decline in % fliers was evident, with a slight decline between 40-60% RH and a more rapid decline when internal mean minimum RH exceeded 60% (Fig 4c). This internal mean minimum RH of 60% corresponded to an external mean minimum RH of 50%.

DISCUSSION

Both season and pupal loading influenced pupal eclosion, with a lower percentage of adult flies emerged from the loadings of 200 and 350 g pupae in August, October and April compared to the 500 g or higher loadings (Fig. 1). This difference was not apparent during December or February. Similarly, season and pupal loading influenced the percentage of fliers, with significant variation between the pupal loadings for all periods except October (Fig. 2). The *B. tryoni* adults sampled at all release periods displayed adequate dye on the ptilinum and therefore were readily identified as sterile *B. tryoni*, indicating that this method would be adequate for releases within the FFEZ. In general, sterile *B. tryoni* emerged over a longer period of time during the cooler periods, which is consistent with the finding of a previous study (Dominiak *et al.* 2003). However, this is the first time that the seasonal variation in eclosion and flight of *B. tryoni* pupal release has been experimentally demonstrated.

There is a paucity of data on the effects of low and high temperatures on mortality of all life stages of *B. tryoni* (Yonow *et al.* 2004). However, the temperature relationships in this study have allowed an estimation of the approximate external temperature ranges that are suitable for pupal release. For optimal emergence, the minimum temperature should not fall below 10°C and the maximum should not exceed 35°C. This estimated minimum temperature is consistent with the lower (11.2°C) (Yonow *et al.* 2004) and upper (34°C) (Meats 1989) development threshold for *B. tryoni* pupae. Although threshold data is not available for pupal mortality due to either low or high temperatures, several nights at low temperatures (-3 to 5°C) is known to severely reduce adult *B. tryoni* populations (Fletcher 1975) and it is likely that the same applies to pupae. Our study showed that for successful flight, the minimum nightly temperature should not fall below approximately 6°C, and daily maximum temperatures should be above 16°C. This supports the work of Meats and Fay (2000), which showed that *B. tryoni* had an approximate flight threshold of 16°C. In the current study the importance of RH was difficult to define. Emergence could only be associated with the external maximum RH, which was optimal at 81.6% RH. For optimal flight, the minimum external RH was approximately 50% and the maximum external RH 58.5%, suggesting that lower RH levels are more favourable for flight, but emergence is probably better at higher RH.

In the present study, by comparing the mean RH both externally and internally of the pupal release box (Fig. 4; Table 1), when external RH was greater than 68%, internal box RH remained lower. A similar trend was not apparent for temperature (Fig. 3; Table 1). Overall, an increase in temperature (mean max or min) indicated a decrease in RH (mean max or min). Vargas *et al.* (1987) showed that for three species of fruit fly, *Ceratitis capitata* (Wiedemann), *Bactrocera cucurbitae* (Coquillett) and *B. dorsalis* (Hendel), rearing pupae in vermiculite provided better protection from the adverse affects of low RH at 20°C, as compared to no pupal covering. The vermiculite resulted in significantly reduced mortality at both 40 and 60% RH, and the fastest development occurred at 90% RH, [although the reason for this is not clear]. However, in the present study the RH data needs to be treated carefully as for the first few days, RH recordings were as high as 100% due to the layer of moistened vermiculite placed over the pupae.

Pupal release exposes later stage pupae to similar conditions they would experience as wild fruit flies, and therefore are believed to undergo some degree of acclimation to local climatic conditions. Meats and

Fay (1977), indicated that sterile insects are the most easily released at the puparial stage and that the most opportune time would be in spring when the populations are low. Fay and Meats (1987) later showed that cold-conditioned sterile adult *B. tryoni* released in early spring (September – October) survived as well as wild fruit flies and suppressed their fertility to an extent which indicated their mating competitiveness may be equal to that of wild fruit flies, if the weather is not too severe. Given the poor pupal eclosion in the current study during August (late winter) and October (mid-Spring), it is possible that at least during early spring, cold-acclimated sterile adult release may be a better option than pupal release and is an area that merits additional study. In all cases, the relationships between temperature, RH and % emergence or % fliers need to be interpreted carefully as these factors jointly have an effect on successful emergence and fliers. Similarly, temperatures alone do not solely contribute to the mortality of *Bactrocera oleae* (Rossi) pupae (Neuenschwander *et al*. 1981).

Excessive water loss during pupation is a principal cause of mortality in *B. dorsalis* and *C. capitata* (Vargas *et al.* 1987; Jackson *et al.* 1998). *Bactrocera* spp. are known to be less tolerant of dry soils than other tephritid genera (e.g. *Ceratitis*) (Eskafi & Fernandez 1990). Working with *B. tryoni*, Hulthen and Clarke (2006) recorded 85% pupal mortality at 0% soil moisture, 30% mortality at 100% soil moisture, and low (1-10%) mortality at soil moisture levels between 10-90%. In this study, although the vermiculite moisture level was in this range (25%), it is possible that higher or even lower vermiculite moisture levels might have resulted in increased eclosion, most notably during August and October, and should be tested if pupal release is pursued as an option for SIT in Australia.

Despite the use of insect repellents, some predation was noted, particularly by the native ants *Iridomyrmex sp*. and *Ochetellus sp*., where these species were observed carrying off pupae and adult *B. tryoni*. Fluorescent pink footprints on one pupal release box also suggested that birds and other small terrestrial vertebrates may find the pupae and/or adults. In areas where predation may be particularly high, repeated treatment of the ground as well as guide ropes or legs of stands with an insecticide or ant repellent may limit predation. The legs of stands can also be placed in a water bath. Eclosion boxes do not necessarily have to be placed on the stand described. Any form of elevated platform, which allows air to circulate around the pupal release box while minimising predation would be adequate. Likewise, a stand with an overhead shelter is not necessary to provide shade; placing the box under the shade of a tree would be suitable.

Based on the IAEA guidelines (FAO/IAEA/USDA 2003) which state that emergence below 65% post-shipment is not acceptable for *B. tryoni* and that 75% is the desired mean emergence rate, and the results of this study, a recommendation can be made on the optimal pupal load to be used at different times of the season. In August (late winter) and October (mid-spring), pupal release is sub-optimal as emergence is less than 65% for all pupal loadings (Fig. 1) and therefore unlikely to be economically viable despite flight exceeding 92.7% in October for all loadings (Fig. 2). Pupal loadings over 500 g in December and all pupal loadings in February and April produced over 65% emergence with high levels of flight; however in April loadings of 500g and above produced significantly higher emergence (Fig. 2) compared with lower loadings and therefore should be favoured at this time of year.

In an operational situation, the choice of pupal loading is, of course, dependant on a number of factors including available numbers of sterile fruit flies and the required number to be released to provide a suitable 'over flooding' ratio. The best choice, as indicated by the present study in those release periods where pupal release is viable, is the highest loading tested, i.e., 800 g. Although it was previously believed that pupal loadings above 800g led to poor eclosion (Dominiak *et al.* 1998), results of this study suggest that for all release periods, pupal loadings higher than 800 g may be feasible and therefore warrant further study.

The described pupal release system may be practicable under certain environmental conditions, notably from December through to April or when mean minimum temperatures are not lower than 10°C and the maximum no higher than 35°C. Generally, higher pupal loadings (up to 800g) are ideal. This pupal release

system should therefore be considered as a viable option of an SIT program for *B. tryoni*. Pupal release will require the cooperation of landholders and the horticultural industry, as this method does require the placement of polystyrene boxes in backyards or orchards, sometimes for extended periods of time which may hinder harvesting and other activities if not situated carefully.

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Technical Report 3

Field release of adult sterile Queensland fruit fly, *Bactrocera tryoni* (Froggatt): the effect of release method and location on trap recapture rates

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Abstract
The Queensland fruit fly, *Bactrocera tryoni* (Diptera: Tephritidae) is Australia's worst pest of edible fruit. The Fruit Fly Exclusion Zone (FFEZ) in south-eastern Australia encompasses some of the country's most valuable horticultural production areas and is recognized nationally and internationally as free of *B. tryoni*. Outbreaks of *B. tryoni* in the FFEZ are controlled, in part, with the inundative release of sterile *B. tryoni*. To determine whether release method and trial site affected trap recapture rates of sterile *B. tryoni*, four sterile *B. tryoni* releases were conducted simultaneously at two sites; Wagga Wagga, New South Wales (NSW) and Unley, South Australia (SA) from December 07 to April 08, using both a plastic adult rearing container (PARC) and a bin release method. The type of release method (PARC or bin) did not affect the number of *B. tryoni* recaptured. Trap recapture rates of released sterile *B. tryoni* were higher in NSW than SA throughout the trial, and peak recapture rates occurred one week earlier in NSW. Humidity was higher inside the bins compared with the PARCs in three of the four releases; temperature was higher in PARCs compared with bins in two of the releases. For one fly batch tested, emergence and flight were higher in the PARCs compared with the bins. Quality control and recapture studies showed that dye pigment was not a confounding factor in recapture rates of the main studies.

Keywords: sterile insect technique, flight ability, emergence, release techniques

INTRODUCTION

The native Queensland fruit fly, *Bactrocera tryoni* (Froggatt) is a significant risk to the horticultural industries of eastern and southern Australia (Sutherst *et al.* 2000). *Bactrocera tryoni* is polyphagous with a wide host plant range of more than 60 native species and most commercially grown fruit crops (White & Elson-Harris 1992). It is endemic to eastern coastal Australia, from Cape York (Queensland) to East Gippsland (Victoria) (Drew 1989), but is now also common in parts of inland New South Wales (NSW). Although occasional outbreaks of *B. tryoni* occur in South Australia (SA) (Maelzer 1990 a, b) and detections are rare in Western Australia (WA) (Fisher 1996), these two states are normally recognised by horticultural trading partners as free from *B. tryoni*.

The Fruit Fly Exclusion Zone (FFEZ) in south-eastern Australia is recognized nationally and internationally as free from *B. tryoni*, thus allowing the horticulture industry within that zone to export fresh produce to fruit fly-sensitive domestic and international markets. The FFEZ encompasses some of the country's most valuable horticultural production areas, including Sunraysia, the Mid Murray and the Goulburn Valley in Victoria, the Murrumbidgee Irrigation Area (MIA) of NSW, and the Riverland of SA.

The sterile insect technique (SIT) is one of the currently utilised, internationally recognised treatments against incursions of *B. tryoni* within the FFEZ and the pest-free areas of SA and WA. The SIT has been used to effectively eradicate outbreaks of *B. tryoni* in WA (Fisher 1996), NSW (B. Dominiak, pers. comm. 2009) and SA (Jackman *et al.* 1996, Perepelicia *et al.* 1997).

The first experimental SIT campaign to manage *B. tryoni* was carried out from 1962 to 1965 and relied on methods adopted from similar release programs internationally (Andrewartha *et al.* 1967; Monro & Osborn 1967). Mark-recapture experiments have shown *B. tryoni* to be strong dispersers (Fletcher 1974; McFarlane *et al.* 1987). Resultantly, stationary point release systems (box release of adults or pupae) have often been used in preference to more costly and complex mobile release systems (aerial or moving vehicle releases). Several approaches for the stationary release of sterile *B. tryoni* have been trialled, including pupal release (Dominiak *et al.* 2000a; Reynolds *et al.* 2010), cage release (Dominiak *et al.* 1998; Dominiak *et al.* 2003), plastic adult rearing container (PARC) release (Reynolds & Orchard, 2010, this paper) and bin release (James 1992; Sproule *et al.* 1992; Horwood & Keenan 1994) as well as comparisons of releasing immature and mature flies (Meats *et al.* 2003) and laboratory studies comparing chilled and non-chilled flies (Meats & Fitt 1987; Reynolds & Orchard, in press). *Bactrocera tryoni* sterile release programs in Australia currently use either the PARC (M. Nolan, pers. comm. 2009) or bin techniques (Fisher 1996; Perepelicia *et al*. 1997).

Our aim was to determine, using field studies, whether the release method (PARC or bin), trial site (NSW or SA release site) and time from release affected trap recapture rates of adult *B. tryoni*. We also determined whether the dye pigments used influenced standard quality control parameters and trap recapture rates of *B. tryoni*. Internal conditions were monitored for both PARCs and bins, and for one pupal batch, we measured the effect of release method on emergence and flight ability of *B. tryoni*. The results are discussed in relation to sterile insect release programs for *B. tryoni in different parts of Australia.*

MATERIALS AND METHODS

Production & handling

Bactrocera tryoni were obtained as pupae from the Fruit Fly Production Facility at the Elizabeth Macarthur Agricultural Institute, Camden, NSW, Australia, where the larvae are reared on a standard lucerne chaff diet (Dominiak *et al.* 2008). Pupae were dyed (1 g dye per 100 g pupae) with one of each of four fluorescent pigments; Fiesta FEX 1 Arc chrome, Strong magenta, Flame orange and Stellar green (Swada, 30-32 Kilkenny Court, Dandenong South, Victoria, Australia), by inverting the dye and pupae in a plastic bag to evenly coat the pupae. Monthly consignments of dyed *B. tryoni* were sent as late-stage pupae packed

in plastic bags within 1L cardboard boxes to the Australian Nuclear Science and Technology Organisation, where they were irradiated with a ^{60}Co source at 70.3 – 74.5 Gy to render them sterile.

The dyed, irradiated pupae were then transported in a thermally insulated container overnight by road to the Wagga Wagga Agricultural Institute (WWAI), Wagga Wagga, NSW, and overnight by air and road to the Primary Industries and Resources South Australia (PIRSA) Sterile Fruit Fly Rearing Facility, Netley, SA.

Study insects

In NSW, insects were reared in a growth room at 26±2°C, 65±10% relative humidity (RH) and a light:dark cycle of 14:10, with a simulated dawn and dusk at the beginning and end of the light phase. In SA, the pupae were held for 48h at 17°C to delay development to enable flies to be released on a chosen day. The insects were then reared under the conditions described above with the exception of the dawn and dusk periods.

Release protocol

In NSW, the pupae were divided and weighed into 12 x 250g lots for PARCs and 12 x 150g lots for bins and placed in open plastic bags inside 2L cartons. The weighed treatments were then transported to the release facility in NSW (approximately 400m). The same procedure was followed in SA, except that pupae were divided into 10 x 15g lots per PARC in each of ten paper bags within each PARC (150g pupae total).

For PARC-reared flies, in NSW, one lot (250g) of pupae was placed into each translucent 46L plastic lidded container (Silverlock MH 0110, colour "natural", with a 43cm x 20cm, 1mm mesh on the lid and a 15cm x 10cm mesh on two sides of the container for ventilation. In SA, one lot (15g) of pupae was placed in each of ten paper bags within each PARC (150g pupae total). For bin-reared flies at both locations, one lot of pupae was placed directly onto the bottom of each dark green 45L capacity bins with a sandblasted interior (to provide a surface for the flies to grip) and flat wooden lids fitted with a 10cm diameter circle of inlaid 1mm gauge mesh for ventilation. Each bin was supplied with a cone of newspaper for additional resting space.

In NSW, additional resting space was provided by wedging cardboard dividers (approximately 160 cm in height), two running lengthways and five across the width of the container, to sit just above the pupal bed. In SA, additional resting space was provided by the paper bags. Five sugar cubes were placed on the base of each release box. In NSW, a block of agar containing a mixture of white sugar and water (5% sugar) (Jessup A, 2004, unpublished data) was placed on top of the mesh of both the PARC and the bin for adult feeding and was replaced as needed. In SA, a sponge block (8cm^2 x 4cm high) containing approx (83 ml) of 5% sucrose was placed on the mesh in the lid of both release boxes and were replaced on days 3 and 4. PARCs and bins were maintained at 26°C ± 2°C and 65 ± 10 %RH until release. The majority of sterile *B. tryoni* were aged 2-3 days when released. In NSW, temperature and RH within two release boxes were recorded when these variables had stabilised, at 10 minute intervals until just prior to release (approx. 65 hours), with the exception of release 3 when one data logger failed.

Depending on each consignment, individual pupae weighed on average 9.20-11.33 mg. Adult releases were conducted in both the urban area of Wagga Wagga, NSW (35° 70' S, 147° 22' E) and the Adelaide suburb of Unley, SA (34^o 50' S, 138^o 36' E) on four dates (December 2007, January 2008, February 2008 and March 2008). An array of twenty 1 L Lynfield traps baited with cuelure (International Pheromone Systems, Cheshire, UK) and malathion (Meats *et al.* 2002) spaced at 400 m intervals was established in Wagga Wagga, and already existed in Unley. Traps were cleared weekly from the first release until six weeks after the last release, although on different days at each release site (NSW or SA).

The four fluorescent dyes were used to distinguish between treatment and release date, with only two used at any given release. Dye colour was alternated between treatment types, with at least seven

weeks between releases of the same pair of colours. Twelve release sites on a 400m array were established within each trapping array and were located at least 150 m from any trap. During each trial, bins and PARCs containing flies were taken to the release sites in an air-conditioned vehicle in NSW and on an open trailer in SA. One of each release box was opened alongside each other, at each site. Temperature and RH data for both Wagga Wagga, NSW and Kent Town, SA were obtained from the Bureau of Meteorology, Australia. The Kent Town weather station is the main weather station for Adelaide, and is located 3km NE of the Unley trial site.

Fruit flies captured in NSW were collected and rinsed in 70% alcohol for approximately one minute to remove any excess dye. The adult fruit flies were then left to dry on a paper towel before being placed back into a labelled vial. They were then sent to the Orange Agricultural Institute, Orange, NSW, Australia where the dye colour observed in the ptilinal fissure (Norris 1957; Steiner 1965) was recorded for each fruit fly. Fruit flies captured in SA were collected into labelled vials and examined at the South Australian Research and Development Institute.

Fluorescent dye comparisons

In SA, a replicated trial was run to determine whether any of four fluorescent pigments (see above) had an impact on three standard quality control parameters: emergence and fliers (as a percent of the total pupae counted) and pupal weight (FAO/IAEA/USDA, 2003). For each colour in each of three batches of pupae received (different weeks), two or three samples of 100 pupae were weighed, then placed in flight-ability testing conditions to obtain emergence and fliers results 5 days later (FAO/IAEA/USDA, 2003).

In NSW, a release trial was conducted to determine if there was a difference in the trap recapture rate of sterile *B. tryoni* dyed one of the four fluorescent pigments. Pupae treated with each dye pigment were divided and weighed into 6 x 250g lots and then transported to the release facility in NSW where they were reared out as described above for PARCs. A trapping array was established in an abandoned orchard near Borambola, NSW (35°12' S, 147°62' E), containing pome and stone fruit, comprising 12 traps, spaced 400m apart in three rows, each with four traps. Three release sites, spaced 400m apart (and 200m from the nearest trap), were spaced in the middle row of the trapping array. At each release site two PARCs of each dye treatment of sterile *B. tryoni* were released when the flies were two days of age. Traps were inspected for six weeks, although no fruit flies were trapped in the sixth week and this week was therefore excluded from the analysis. Dye retention on the ptilinum for recaptured sterile fruit flies was determined as described above.

Quality control parameters within PARCs and bins

The effect of release method on emergence and rate of adult sterile *B. tryoni fliers* was examined for the cohort of flies that was reared for the March 2008 release date in NSW. After all emerged adult *B. tryoni* had been released, each bin and PARC was taken back to the lab and sampled by mixing the remaining empty pupal cases, un-emerged pupae, partly emerged adults, deformed and non-deformed dead fruit flies ('*B. tryoni* debris') to ensure even distribution across the box. Each box was then divided into eight equal segments, and each segment was sampled by scooping approximately 30 ml of *B. tryoni* debris into individual vials. For each vial, 0.15 g of *B. tryoni* debris was then weighed and counted.

Emergence

The percentage emergence per release box was estimated based on the summed *B. tryoni* debris totalling 1.2 g for each release box. Emergence was defined as the percentage of empty pupal cases of all the pupal cases counted in each sample.

Rate of fliers
Rate of fliers was defined as the percentage of eclosed *B. tryoni* that flew (i.e. left the pupal release box) per box. This was calculated as the count of *B. tryoni* that had eclosed minus the number of dead and deformed flies remaining in release containers, divided by the number of eclosed individuals. These calculations are in essence directly comparable with the "rate of fliers" calculations used in standard quality control tests for SIT programs (FAO/IAEA/USDA, 2003).

Statistical Analysis
Generalized linear models (GLMs) using the logit link function and binomial errors were used to analyse the proportions of counts, and ANOVA was used to analyse temperature and relative humidity. The data were analysed using GLM procedures in R (R Development Core Team, 2006), or with ASREML (Gilmour *et al.* 2007).

Effects of fluorescent dye on quality control parameters and trap recapture rate
Quality control parameters (SA)
The effect of dye colour on emergence and fliers (FAO/IAEA/USDA, 2003) was analysed using a GLM, where the model fitted was:
Emergence (or fliers) = dye treatment + replicate

Recapture rates (NSW)
A Pearson's Chi-squared test for count data was used to test if proportions of total trap recapture of sterile *B. tryoni* differed between coloured dyes. The Chi-squared test was used to test for departure from the expected 1:1:1:1 ratio under differential recapture.

Effect of release method (release box) on emergence and rate of fliers
Counts of emerged flies out of total pupal cases were used to test the effect of box type on adult emergence. Counts of empty pupal cases minus counts of dead flies out of empty pupal cases were used to measure the number of flies that flew (rate of fliers). Counts were summed over samples within boxes, rather than fitting samples within boxes as a random effect, because of evidence flies had migrated between samples within boxes. The effect of release method on emergence and rate of fliers was analysed using a GLM, where the model fitted was:
Emergence (or rate of fliers) = rearing box + replicate

Temperature and humidity inside release boxes
The difference in temperature between each box reading and mean temperature over all 4 boxes (Δtemp), was calculated for each time of observation. The effect of box type on RH and Δtemp was tested using ANOVA, using the model:
Δ temp or RH = (box type + rep) / time + error

where, for each time Δ denotes the difference from the mean (over two treatments and two replicates) for each time of temperature observation and time of observation is nested below box type + replicate.

Field release recapture rate
Recapture rate was analysed for each site with site, box type, release time and time from release as effects. Time from release to recapture (TfR) was made a factor in the analysis, with the same weekly factor intervals across sites. This is an approximating process, as traps were checked on different days at each site (NSW or SA), although at weekly intervals. Time from release to recapture was unbalanced to the overall

design, but balance was restored by considering only the first 4 weeks of recapture data at each site. The field release recapture rate was calculated using the number of *B. tryoni* released adjusted for the number that flew (fliers).
The model used was:
*Qfly recapture / Qfly released = Site/(Box * ReleaseTime)/ Tfr*
where Box * Release Time was nested under Site, and Tfr was nested below all other terms in the model.

Effect of time from release on weekly trap recapture rate
Recapture rates were predicted for each release time * recapture time interaction within sites, to then predict mean recapture rates for release times and recapture times within release times at each site. The effect of time from release on weekly trap recapture rate was calculated using the number of *B. tryoni* released adjusted for fliers.

RESULTS
Effects of fluorescent dye on quality control parameters and trap recapture rate
Quality control parameters (SA)
Adult *B. tryoni* dyed different colours did not differ in emergence (back-transformed mean and standard error (SE): 78.8 ± 2.59 %, 80.5 ± 2.44 %, 81.3 ± 2.44 % and 81.6 ± 2.25 % for Strong magenta, Arc chrome, Flame orange and Stellar green respectively; χ^2 = 1.92, df=3, p=0.59), flight ability (back-transformed mean and SE: 83.1 ± 2.78 %, 84.9 ± 2.53 %, 83.2 ± 2.75 % and 87.1 ± 2.19 % for Strong magenta, Arc chrome, Flame orange and Stellar green respectively; χ^2=4.89, df= 3, p = 0.18) or pupal weight (back-transformed mean and SE: 9.50±0.3 mg, 9.66±0.3 mg, 9.75±0.3 mg, and 9.81±0.3 mg for Strong magenta, Arc chrome, Flame orange and Stellar green respectively; F=0.41, df = 3,8; p=0.75.

Table 1. The proportion of sterile adult *B. tryoni* recaptured on five dates after release.

Recapture date	Proportion of sterile adult *B. tryoni* recaptured across all traps for each dye pigment				χ^2 value	d.f.	P value
	Arc chrome	Stellar green	Strong magenta	Flame orange			
25/02/2008	0.23	0.25	0.27	0.25	1.18	3.00	0.76
3/03/2008	0.24	0.25	0.26	0.25	1.03	3.00	0.79
11/03/2008	0.24	0.23	0.21	0.31	6.52	3.00	0.09
18/03/2008	0.22	0.17	0.17	0.43	4.30	3.00	0.23
25/03/2008	0.17	0.28	0.38	0.17	3.41	3.00	0.33

Recapture rates (NSW)
There was no significant difference in the proportion of total trap recapture of sterile adult *B. tryoni* coloured different pigments at each recapture date (Table 1).

Effect of release method (release box) on emergence and rate of fliers

Emergence of sterile *B. tryoni* was higher for PARCs compared with bins (χ^2 = 73.1, df = 1, P < 0.001) (Fig. 1a). A similar trend was evident for the rate of fliers recorded from PARCs compared to bins (χ^2 = 72.1, df = 1, P < 0.001) (Fig 1b).

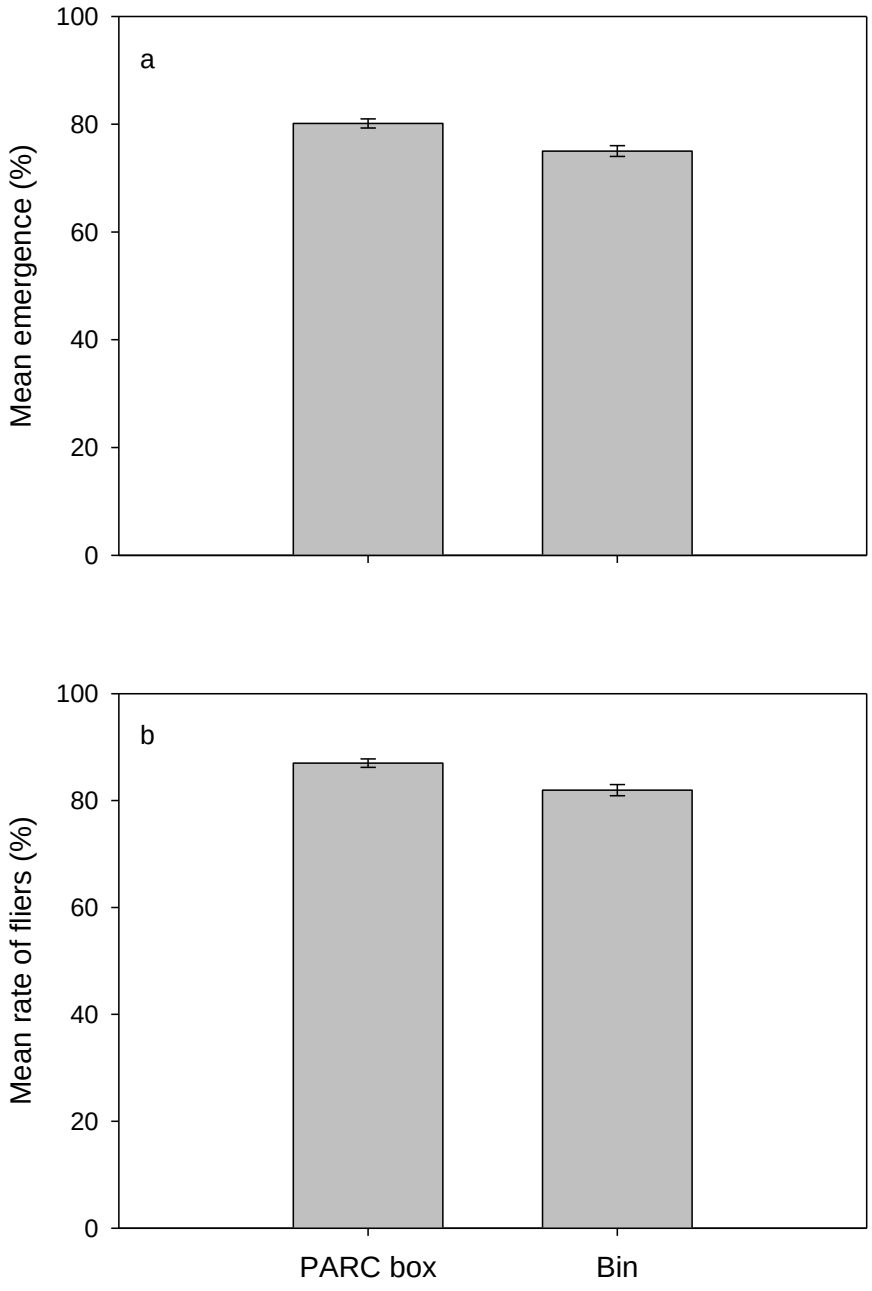
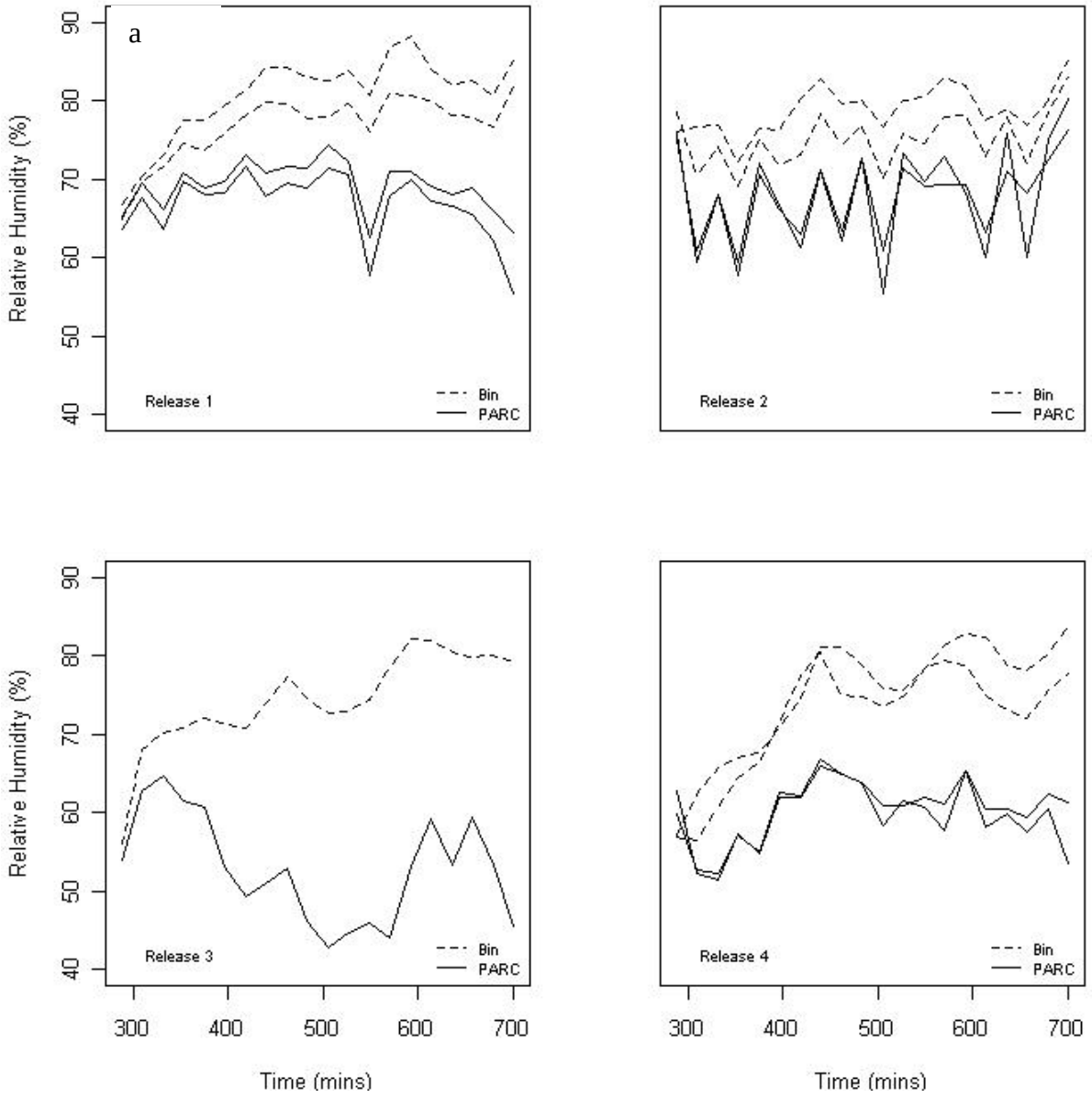


Fig. 1. The effect of release method (release box) on percentage emergence (a) and rate of fliers (b) at Wagga Wagga, New South Wales. Error bars are standard error.

Relative humidity and temperature inside release boxes

Temperature was higher in the PARCS compared with the bins for release 2 (0.5°C higher; F=86.14, df=1,1729, P<0.001) and 4 (0.05°C higher; F=4.92, df=1,1245, P=0.027), but there was no difference in temperature between the release boxes for release 1 (F=1.01, df=1,1833, P=0.32) (Fig. 2b). Relative humidity was higher in the bins compared with PARCs, for release 1 (difference in RH = 13.2%; F=3742, df=1,1833, P<0.001) (Fig 2a), release 2 (difference in RH = 9.3%; F=1245, df=1,1729, P<0.001) and release 4 (difference in RH = 15.8%; F=8572, df=1,1245, P<0.001). For release 3, humidity and temperature appeared higher in bins compared to PARCs but observations were not replicated and therefore could not be statistically analysed.



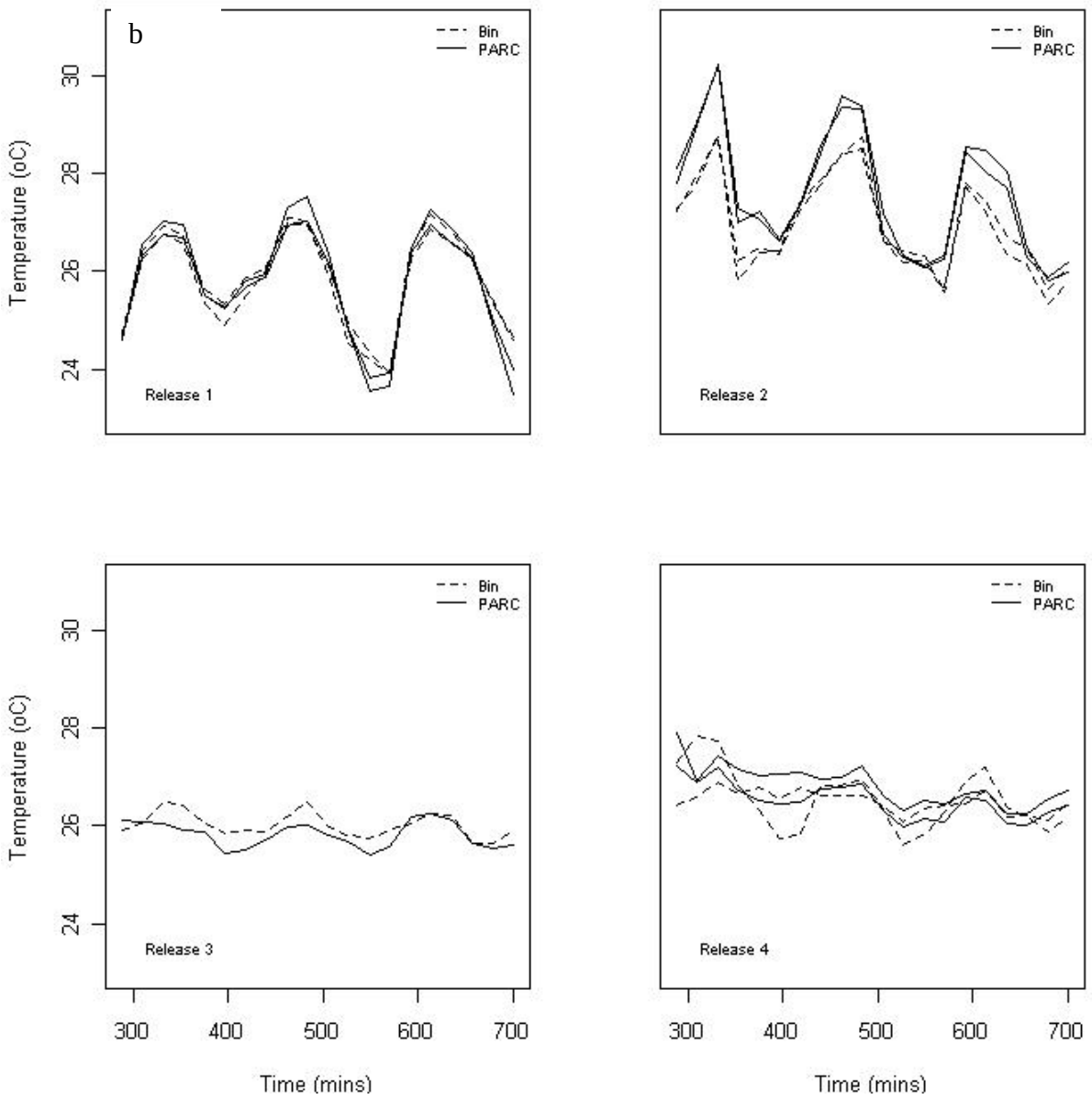


Fig. 2. The (a) relative humidity (%) and (b) temperature (°C) in PARCs and bins while held in growth rooms at Wagga Wagga, New South Wales at 26°C and 65±10% RH, for the four releases; release 1: December 2007, release 2: January 2008, release 3: February 2008, release 4: March 2008.

Field release recapture rate

Site strongly influenced trap recapture rate (χ^2 = 797.9, df = 1, P<0.001) with higher numbers trapped in NSW (1.69%) compared to SA (0.34%). There was a significant site * release time interaction (χ^2 = 608, df = 6, P<0.001), with release 3 (February 2008) recording the highest recapture rate in NSW (though not significant; P>0.05), and release 1 (December 2007) the highest in SA (P<0.05) (Fig. 3). Release box did not significantly affect recapture rate (release box * site * release time interaction: χ^2 = 13.3, df = 8, P>0.05), with recaptures of sterile flies of 1.66% from PARCs and 1.73% from bins in NSW and 0.35% from PARCs and 0.33% from bins in SA.

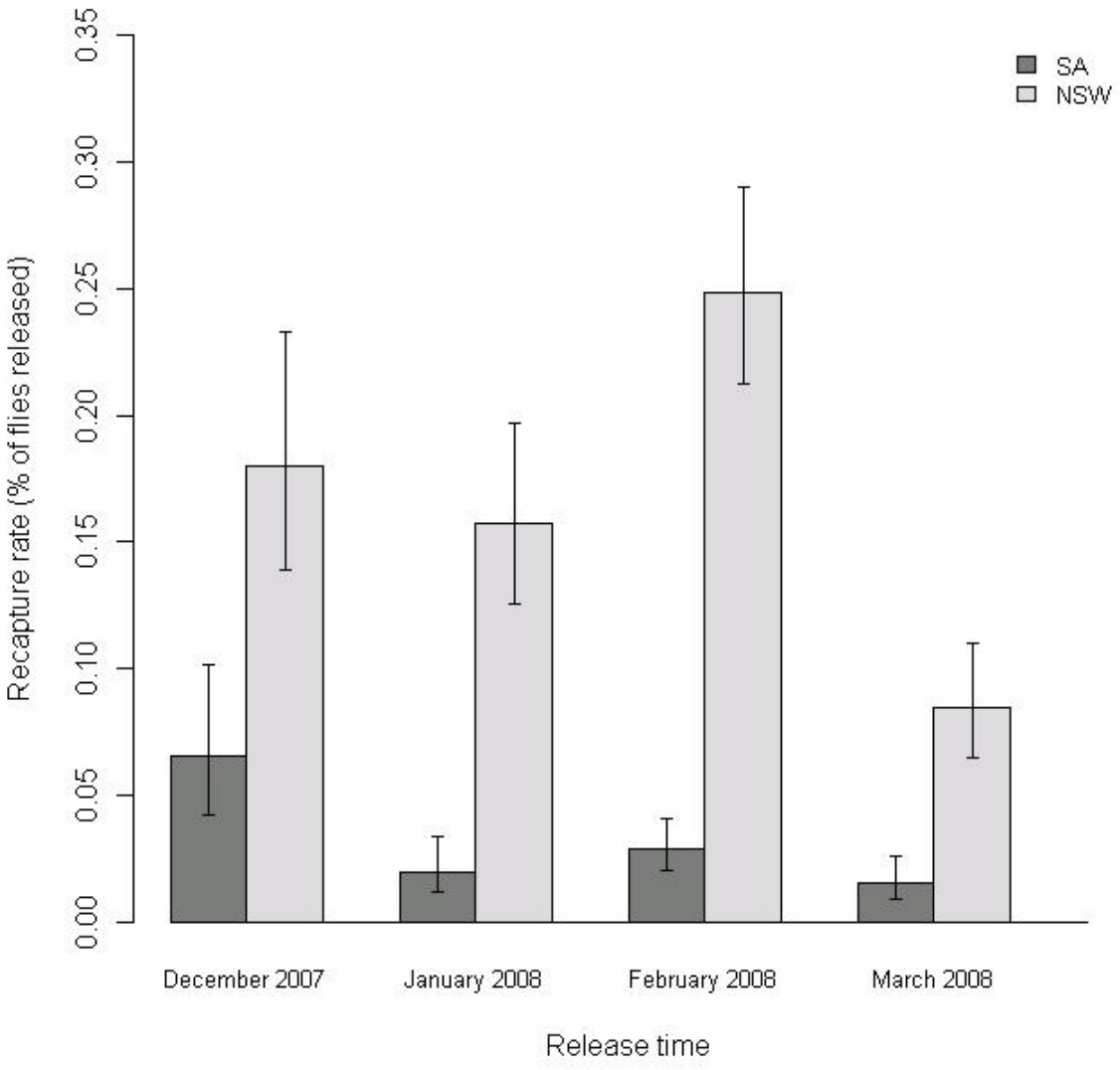


Fig. 3. The percentage recapture rate (corrected for emergence and flight) of sterile *B. tryoni* released concurrently at Wagga Wagga, New South Wales and Unley, South Australia comparing two methods of release (PARCs and bins) at four different release dates. Predicted logit means and their 95% confidence values were back-transformed to the original scale of the data, and the findings plotted with 95% confidence intervals.

Mean daily temperature and relative humidity recorded by the Bureau of Meteorology are presented for information in Figure 4.

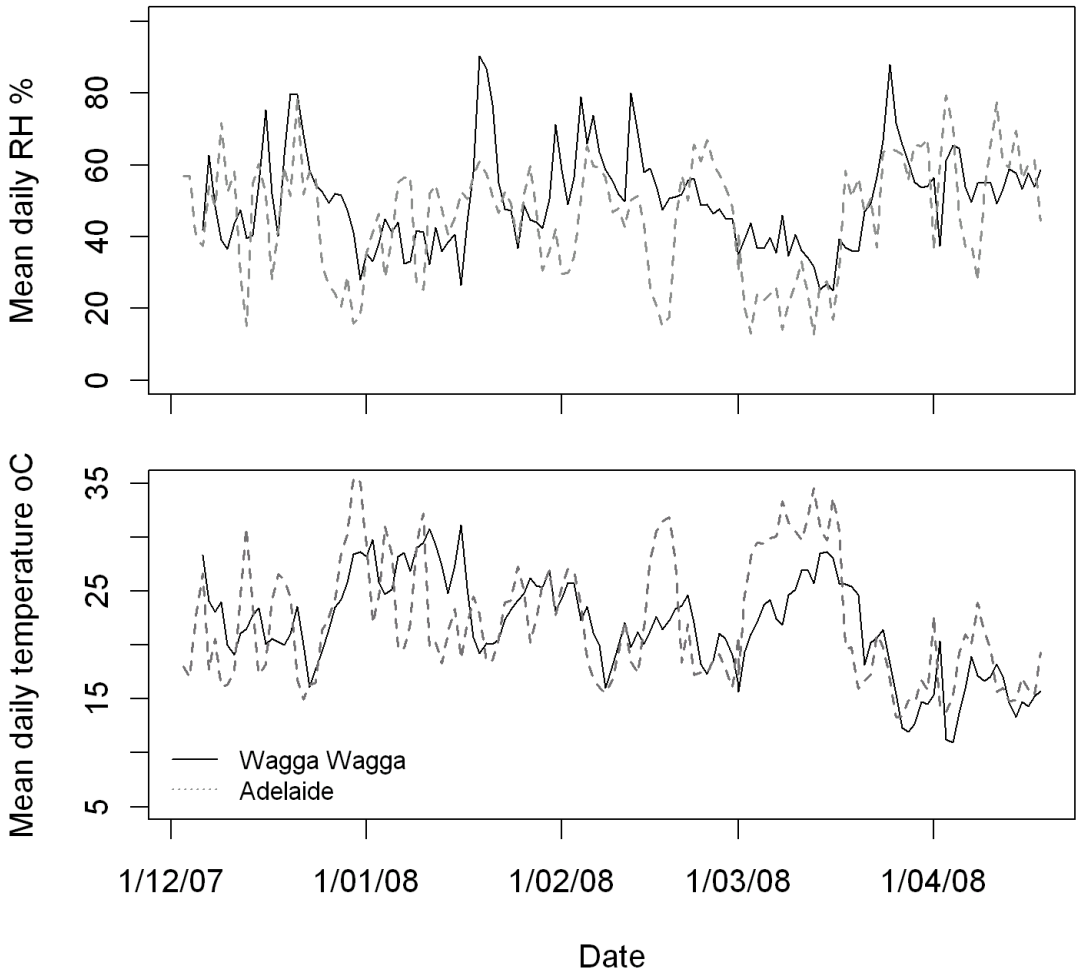


Fig 4. The mean daily relative humidity (RH%) (upper graph) and mean daily temperature (^{O}C) (lower graph) in Wagga Wagga, New South Wales, and Kent Town, Adelaide, South Australia for the duration of the trial period, December 2007 to the end of April 2008.

Effect of time from release on weekly trap recapture rate

Within sites, recapture rates differed strongly between release times (SA: χ^2 = 191.9, df = 3, P<0.001; NSW: χ^2 = 603.4, df = 3, P<0.001), and between TfR within release time (SA: χ^2 = 455.4, df = 16, P<0.001; NSW: χ^2 = 1873, df = 16, P<0.001) (Fig. 5). The peak recapture rate occurred in the second week after release in NSW (P<0.01), and in the third week after release in SA (P<0.001) (Fig. 5).

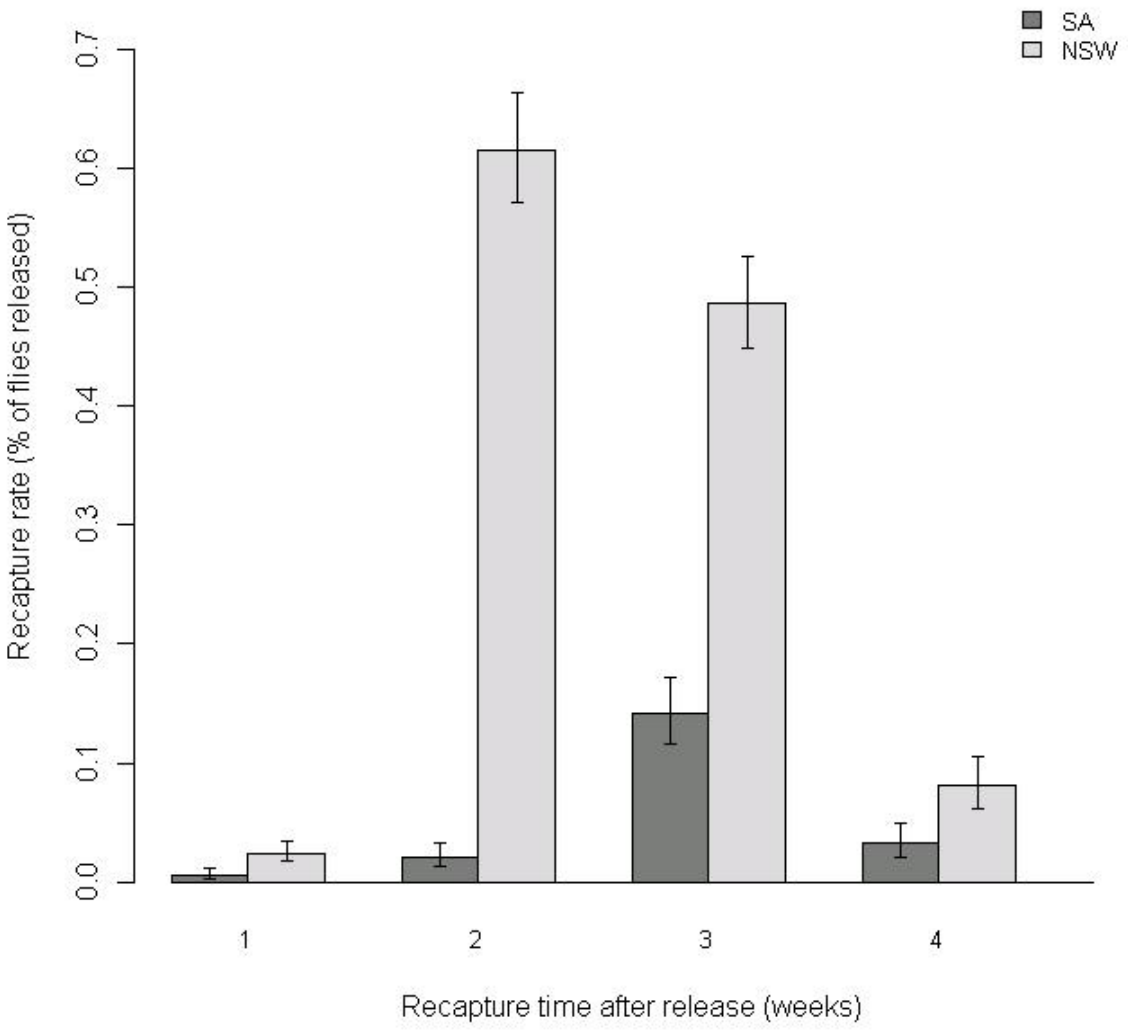


Fig. 5. The effect of time from release on weekly trap recapture rate (corrected for emergence and flight) for New South Wales and South Australia. Predicted logit means and their 95% confidence values were back-transformed to the original scale of the data, and the findings plotted with 95% confidence intervals.

DISCUSSION

Effect of release box type on recapture rates

There was no detectable difference at either location in the recapture rates of flies from PARCs or bins. Previous studies have shown that the upper number of flies and the available resting space in PARCs (Reynolds & Orchard 2010) and bins (Fisher 1996) used in the current trial does not negatively affect emergence or flight ability. However, there are often concerns raised with respect to temperature, RH and carbon dioxide (CO_2) build up in bins, which are less ventilated, compared to PARCS. Preliminary readings of CO_2 levels in PARCs and bins suggest CO_2 builds up more quickly and reaches a higher final concentration in bins compared to PARC boxes (Smallridge *et al*, 2008), although further replicated studies are required. In this trial although temperatures were generally similar between bins and PARCs, RH was

significantly higher in bins compared with PARCs and may account, in part, for the reduced emergence and flight observed for one batch of pupae from bins compared with PARCs. Given the measured differences in emergence and rate of fliers of *B. tryoni*, we might expect higher recapture rates from PARCs. This was not the case, possibly because the flies were able, in some way, to compensate for reduced emergence and flight once released into the environment, or the differences in flies released became insignificant after field mortality variation and trap attractiveness factors came into play to obtain trapped fly numbers. While studies have shown that fly numbers trapped may vary in different areas of a local environment, at different times of the season (see review by Clarke *et al.* in press) or indeed between different locations entirely (this trial), it is not apparent whether this reflects actual changes in population numbers between local or release environments, variation in trap efficiency or a combination of the two. It is also possible that pupal batches vary in their response to the rearing and release environment, and while the batch tested in this trial demonstrated higher emergence and flight ability in PARCs compared to bins, overall this may not be a consistent response.

Effect of trial site on recapture rates

Over the entire trial, there was a large and significant difference in the average trap recapture rate, corrected for the number of fliers released, of sterile *B tryoni* adults in NSW (1.69%) and SA (0.34%). Twelve *B. tryoni* sterile insect technique (SIT) programs run in SA between 1993 and 2004 have achieved an average recapture rate of 0.032%, varying from 0.001% to 0.232%, in the permanent traps located on an approximate 400m grid within and surrounding each 7.5km^2 outbreak release area (Jackman *et al.* 1996; Perepelicia *et al.* 1997; Plant Health Operations PIRSA, unpublished data). By comparison, previous studies have reported recapture rates in NSW greater than 10x that of SA, averaging 0.53%, and ranging from 0.19% to 1.21% using a release rate of 60,000 teneral males per km^2 per week and a similar trapping grid density at Gilgandra and Narromine, NSW (Meats *et al.* 2003). It is worth noting however, that unlike the above studies, the results of the current trial reports recapture rates as the proportion of flies that flew, rather than as the proportion of the number of pupae used (as is the current international standard for determining recapture rates of sterile flies (FAO/IAEA/USDA, 2003). We believe the measure presented here to be a more accurate estimate of recapture rate, as it takes into account batch to batch variations in emergence and flight ability. This study, the first to simultaneously release the same fly production batches in both NSW and SA, shows conclusively that SA recapture rates are significantly lower than NSW. However, the reason/s for this remain unclear. Long term data records have shown that the 17°C incubation period used in SA to delay release has no significant effect on emergence (Jackman *et al.* 1996; Perepelicia *et al.* 1997). One possibility is that the flies suffer a higher post-release mortality prior to reaching maturity in SA. This could result from reduced fly quality due to longer pupal transport periods, reduced ability to locate, or scarcity of, adequate food (carbohydrate and protein) in the release environment, unsuitable environmental conditions, and/or higher predation pressure. In addition, the flies' response to the cuelure traps may be different in each location. These possibilities are discussed in more detail below.

Sterile *B. tryoni* spend longer in transit when transported to SA, compared with NSW. James (1992) reported *B. tryoni* mortality due to transport problems. Another study has also shown a marked decline in emergence between the rearing facility of *B. tryoni* (via the irradiation facility at Lucas Heights) and a release facility located in inland NSW (distance of approximately 500 km) (Dominiak *et al.* 2007). A more recent study has indicated that it is not the vibration of transport that is of concern, but rather the variable temperatures that may be experienced during transport (Campbell *et al.* 2009).

The ability to locate adequate food (carbohydrate and protein) is essential to sterile fly survival. For example Perez-Staples *et al.* (2007, 2008) showed that *B. tryoni* had greater longevity when they had adequate access to both a carbohydrate and a protein source. Whether the environmental conditions in

South Australia result in scarcer or poorer quality food sources is unknown. There is a paucity of knowledge on the habitat use by sterile tephritids in the field, despite its likely consequence for their survival and reproduction (Weldon & Meats 2010).

In the environmental data recorded at Wagga Wagga and Adelaide during the current trial (Figure 4), Adelaide had some of the highest mean daily temperatures and most of the lowest relative humidity records. Although our trial was not designed to directly compare climatic data with recapture rates, Weldon and Meats (2010) have shown that male recapture is significantly negatively correlated with increasing daily maximum, minimum and average temperatures and concluded that this may indicate that high temperatures lead to higher mortality rates for released sterile *B. tryoni*. In addition, irradiation of male *B. tryoni* has been shown to reduce longevity under stress (Collins *et al.* 2009). If the higher temperatures and lower humidity conditions of Adelaide in summer create a more stressful environment, this may contribute to lower survival of the released irradiated *B. tryoni*.

It is a frequently reported problem in many SIT programs that numbers of released sterile flies decrease rapidly in the field for various reasons, including losses to different types of predators. As most operational programs release sterile flies at an age when they are still immature (Hendrichs *et al.* 2007), this is of concern. Field and field-cage tests have established that flies of laboratory strains are less able to evade predators than wild flies (Hendrichs *et al.* 1991; Baker & van der Valk 1992) and that predators are an important cause of adult and immature mortality (Bateman 1972). Delayed responses to traps due to reduced maturation times or other factors may result in increased exposure time to natural enemies and adverse climatic factors, resulting in increased mortality rates.

Variation in environmental conditions may also impact on the response of *B. tryoni* to the trap lure. Cue-lure baited traps are broadly affected by climatic conditions (Monro & Richardson 1969; MacFarlane *et al.* 1987; Weldon & Meats 2010) and this influences their capture efficiency. *Bactrocera tryoni* may shelter in microhabitats, thus reducing the chance of desiccation when temperatures are high, thus reducing the chance that flies move into the range and respond to cue-lure traps (Weldon and Meats 2010). In addition, the attraction of male *B. tryoni*, to cue-lure is strongly associated with sexual maturation (Drew 1987; Weldon *et al.* 2008), and the rate of sexual maturation, generally achieved within 7–10 days after adult eclosion, is dependent on access to protein and carbohydrates (Meats *et al.* 2004; Perez-Staples *et al.* 2007). In the present trial we released immature adult *B. tryoni*, this accounts, at least in part for the delay in response of *B. tryoni* to traps after release. However, it does not explain why in SA, the peak recapture rate was one week later than in NSW. Weldon *et al.* (2008) showed that the attraction of male *B. tryoni* to cue-lure was augmented and occurred at an earlier age when they were fed a protein source. Further, attraction by males denied or provided only 24 h access to protein was always considerably lower than those of males with continuous access. Therefore it is feasible that male *B. tryoni* took longer to respond to traps in SA due to limited access to an adequate diet upon release, given that protein was not provided pre-release in either NSW or SA. It could also explain why trap recaptures are lower in SA than NSW; if there is limited access to dietary protein, male response to cue-lure traps declines. Why there would be reduced protein availability in SA compared to NSW or whether other factors are involved, remains unclear.

Effect of marker dyes on quality control parameters

The fluorescent dye pigments used to distinguish sterile *B. tryoni* treatments in this trial had no effect on emergence, flight ability or pupal weight in laboratory quality control tests, nor did it affect the total trap recapture of sterile adult *B. tryoni* at any recapture date in the field trial. Dominiak *et al.* (2003) also showed that dye colour did not affect emergence, using different pigments than those used in our study. Although they demonstrated a difference in the trap recapture rate of flies, *B. tryoni* coloured different hues were released at least a week apart and therefore other factors are likely to have played a role in influencing trap recapture rate. The results of Dominiak *et al.* (2000b) that compared twelve different dye

pigments were inconclusive, and the dyes tested were not those currently used in SIT programs for *B. tryoni* in Australia. Similarly, Weldon *et al.* (2005) tested nine different fluorescent pigments (Radglo & Dayglo pigments) and although they did show that the pigments lowered the emergence rate of *B. tryoni* when compared with undyed (control) pupae, they did not compare any of the dyes currently used in SIT programs in Australia.

CONCLUSIONS

This study demonstrates that sterile *B. tryoni* releases using the PARCs or the bins were analogous at the loadings tested when comparing trap recapture rates. Trap recapture rates of sterile *B. tryoni* from the same production batches were significantly lower in SA compared with NSW, and the peak recapture period occurred one week earlier in NSW. More research is needed to identify the factors contributing to these effects and their potential impact on the efficacy of SIT in each region.

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Technical Report 4

Yeast hydrolysate supplementation increases sterile Queensland fruit fly (*Bactrocera tryoni* (Froggatt)) field longevity and abundance

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Abstract
The Queensland fruit fly, *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae), is regarded as one of Australia's worst horticultural pests, with quarantine restrictions placed upon the export of most fruit and many vegetables to several domestic and international markets. Inclusion of the sterile insect technique (SIT) in an integrated pest management program is one of the most promising non-chemical approaches for controlling *B. tryoni*. To reach sexual maturity and be competitive with wild populations, *B. tryoni* must obtain adequate nutrition soon after adult eclosion; however, in current SIT programs sterile Queensland fruit flies receive a sub-optimal pre-release diet, and hence must depend on foraging in the field. To improve the effectiveness of the SIT against *B. tryoni*, the chief objective of this study was to determine whether dietary yeast hydrolysate (YH) affects the persistence and abundance of sterile male adult Queensland fruit flies under field conditions. In an outdoor cage study we compared the survivorship of male and female *B. tryoni* provided with (1) YH and white sugar, (2) YH and brown sugar, (3) white sugar only and (4) brown sugar only, after 7, 14 and 21 days. In a small cage study, sterile *B. tryoni* were held for 48h in the laboratory from eclosion. They were then released in cages according to treatment (one treatment per cage). They were fed either i) sugar (continual supply), ii) sugar (48h) then starved, iii) YH (48h) then continual sugar supply, iv) YH (48h) then starved or v) YH (continual supply). Survival of flies was monitored until all were deceased. In a field study we released on three occasions, marked sterile Queensland fruit flies from two diet regimes YH-supplemented or YH-deprived, and monitored abundance of sexually mature males by recapturing them in Lynfield traps up to 12 weeks after release. In the field cage study, mortality was lower after 7, 14 and 21 days for YH-fed flies (52.83%, 74.00% and 85.43% respectively) than YH-deprived flies (76.96%, 95.14% and 99.75%). Females suffered less mortality than males after 7, 14 and 21 days (female: 60.04%, 79.43%, 93.14% and male: 71.07%, 93.51%, 99.43% respectively). In the small cage study, YH-supplemented males were more prone to starvation than males fed sugar only after the food supply was withdrawn. In the field, YH supplementation resulted in higher abundance of sterile male Queensland fruit flies, with 1.2 YH-fed flies trapped for every YH-deprived fly trapped. We conclude that under natural conditions, YH supplementation can improve the longevity of sterile male *B. tryoni* and resultantly may improve the effectiveness of sterile insect technique (SIT) programs.

INTRODUCTION

For the sterile insect technique (SIT) to succeed in suppressing populations or eradicating outbreaks of the Queensland fruit fly, *Bactrocera tryoni* (Froggatt), it is crucial that released sterile males compete successfully against wild males for copulations with wild females, as such copulations result in female reproductive failure. To attain sexual maturity, and therefore be sexually competitive with wild populations, male *B. tryoni* must obtain adequate nutrition soon after adult eclosion (Wheeler, 1996). In current SIT programs, sterile *B. tryoni* are released as immatures, and therefore must forage for protein and carbohydrate sources in order to mature before they can locate and mate with their wild counterparts. Foraging is time-consuming (Prokopy & Roitberg, 1989) and risky for sterile flies as they use up what few nutritional reserves they have searching for food, and many may die before reaching maturity. The post-teneral period during which sterile flies are held prior to release offers opportunities for interventions to enhance male tephritid performance, such as nutritional supplementation (Kaspi & Yuval, 2000; Shelly & Kennelly, 2002). There is no published work on the use of olfactory cues to enhance the performance of sterile *B. tryoni*, however in recent years there has been an increase in studies involving manipulating the adult diet, particularly with the supplementation of yeast hydrolysate (YH). YH provides a rich source of amino acids, minerals, sterols and vitamins (Chang, 2009; Fanson & Taylor, *in press*). For both wild and sterile *B. tryoni*, the provision of YH as a dietary supplement may produce longer copulations (Perez-Staples *et al.* 2007), increased mating probability (Perez-Staples *et al.* 2007; Prabhu *et al.* 2008), increased sperm transfer, higher levels of sexual inhibition in mated females (Perez-Staples *et al.* 2008), quicker reproductive development (Perez-Staples *et al.* 2011; Weldon & Taylor, 2011) and increased longevity (Perez-Staples *et al.* 2008, 2009). Yeast hydrolysate feeding has also proved effective in promoting sterile fly performance in other tephritids including *Anastrepha suspensa* (Pereira *et al.* 2009), *A. ludens, A. obliqua, A. serpentina,* and *A. striata* (Aluja *et al.* 2001), *A. fraterculus* (Segura, 2009), *Bactrocera dorsalis* and *B. correcta* (Orankanok, 2011), *B. cucurbiate* (ul Haq, 2010) and *B. philippinensis* (Obra, 2011).

Despite recent progress in YH supplementation of *B. tryoni*, these studies have all been laboratory based with the exception of Perez-Staples *et al.* (2009) and Weldon *et al.* (2008), which were conducted in field cages, and many have looked at fertile rather than sterile flies. Indeed, there are very few studies that have assessed recaptures of YH-fed and YH-deprived sterile tephritids in field releases (Shelly, 2008). The main objective of this study was to determine whether dietary YH effects abundance of sterile adult *B. tryoni* under field conditions. We compared the provision of two different carbohydrate sources (brown and white sugar) and YH on the longevity of male and female *B. tryoni* in an outdoor cage study. In a small cage study, we determined the effects of starvation after sterile *B. tryoni* were initially provided with carbohydrates and/or YH. In a subsequent field study, we compared trap recapture rates as a measure of field abundance of sexually mature sterile male *B. tryoni* provided with white sugar only and those provided with white sugar and YH. Male *B. tryoni* are only attracted to cue-lure baited traps once they are sexually mature (Weldon *et al.* 2008), so recapture rates indicate the combined influences of survival and sexual maturity of a monitored population. Because only mature males are of value to SIT, this is the ideal metric to assess.

MATERIALS AND METHODS

Study insects

Bactrocera tryoni were obtained as pupae from the Fruit Fly Production Facility (FFPF) at the Elizabeth Macarthur Agricultural Institute, Menangle, New South Wales (NSW), Australia, and marked with either of two distinguishable colours, Pink or Arc chrome (Fiesta FEX 1 fluorescent pigments, Swada, 30-32 Kilkenny Court, Dandenong South, Victoria, Australia). Flies were marked with 1g dye per 100g pupae (which is the standard used in the FFPF). This procedure enabled us to distinguish between treatments. Eight-day old pupae were irradiated with 70-75 Gy of Gamma radiation at the Australian Institute of

Nuclear Science and Engineering (AINSE) facility, Lucas Heights, to render them sterile before they were transported by road to the Wagga Wagga Agricultural Institute (WWAI) entomology laboratory in NSW. This is the standard radiation dose used in *B. tryoni* SIT and induces >99.5% sterility. Insects were reared out in a growth room at the WWAI at 26±2°C, 65±15% RH and a light:dark period of 14:10 with a simulated dawn and dusk as the lights ramped up and down at the beginning and end of the light phase. Depending on each consignment, individual pupae weighed on average 10.5-11.4 mg, which is within the acceptable range produced by the FFPF (Dominiak *et al.* 2008). When tested, sterile flies used in both field cage and field trials were aged 2-3 days.

Release protocol – Outdoor cages

The effect of diet (white sugar and YH, brown sugar and YH, white sugar only and brown sugar only) on longevity was examined for sterile *B. tryoni.* Sterile flies had continuous access to food both before and during testing. All flies were provided with a water soaked cotton wick.

Longevity trials were conducted at the WWAI, New South Wales, Australia. Groups of 100 dyed (pink) sterile flies (males and females) were released at 0830 in outdoor cages (47.5x47.5x138cm; Megaview, Taiwan) that contained two artificial tree branches and were housed in one of four large circular walk-in field cages (3m floor diameter, 2.2 m high). Each field cage held four outdoor cages, one for each diet treatment (ie. four replicates) in a randomised complete block design. Flies from only one diet treatment were released per cage. The cages were monitored daily (0830h) for 57 d, and then weekly for 4 weeks, dead flies collected in vials, and the diet treatment and sex recorded. Individual flies were used for only one trial. Minimum daily temperatures for the duration of the trial ranged from 2.1 to 23.2°C and maximum daily temperatures ranged from 29.7 to 47.1°C.

Release protocol – Small mesh cages

Bactrocera tryoni pupae were obtained from the FFPF as above, although they were not dyed for this trial. To produce sterile flies, pupae were sealed in plastic 'zip-lock' bags ~ 2000 pupae per bag and left overnight to achieve a hypoxic environment. Bag were then exposed to a 70 – 75 Gy dose of gamma radiation from a Cobalt 60 source (dose rate 9 Gy min^{-1}) located at Macquarie University, Sydney, one day before emergence (following Collins *et al.* 2009). To ensure that all test flies were the same age, only those that emerged within 24 hours of irradiation were used for diet experiments.

All flies emerged in a laboratory at Macquarie University in Sydney, and were housed in 5 l cages each containing approximately 110 flies. Flies were sorted on the day of emergence into one of five diet treatment groups. A sugar only diet (Sugar), Full diet comprising of constant access to yeast hydrolysate and sugar (YH Full), 48 hour access to yeast hydrolysate then sugar only diet (YH Sugar), 48 access to yeast hydrolysate then starvation diet comprising of only water (YH Starvation), 48 hours access to sugar only then starvation diet (Sugar Starvation). After being held for 48 hours post emergence with access to varied diets flies were released into small mesh cages (45.7 x 45.7 x 45.7 cm, Bug-Dorm, MegaView, Taichung, Taiwan) and kept undercover in an outdoor environment. Two cages were set up for each diet group. Starvation diet treatments had access only to water once released into the cages, while Sugar and 48 YH sugar maintained access to sugar diets provided in a Petri dish in the centre of the cage. YH full diet had similar access to food but included a dish of YH as well as sugar. Flies were checked daily each morning and any dead flies were collected and their sex recorded and counted. Cages were run for 40 days post release recording daily mortality. Each cage contained 100 flies (50 males and 50 females).

Release protocol – Field

To determine the effect of diet on the abundance of mature male flies in the field, we compared trap recapture rates of released YH-fed and YH-deprived sterile male *B. tryoni*. Sterile pupae were divided into 24 x 250g lots and placed inside translucent lidded plastic adult rearing containers (PARCs) (Silverlock MH 0110, colour "natural", 645mm x 413mm x 275mm high), with a 430mm x 200mm, 1mm mesh on the lid and a 15cm x 10cm mesh on two sides of the container for ventilation. Additional resting space was provided by wedging cardboard dividers (approximately 160 mm in height), two running lengthways and five across the width of the container, to sit just above the pupal bed. Nine sugar cubes were placed on the base of each release container. For YH-fed flies, a block of agar containing a mixture of white sugar, YH and water (5% sugar) (Reynolds, 2011) was placed on top of the mesh of the PARC (12 PARCs) at fly eclosion and was replaced as needed. For YH-deprived flies, an agar block was provided without the added YH (a further 12 PARCs). PARCs were maintained at 26°C ± 1°C and 65 ± 10 %RH until fly release. Because not all adults eclosed successfully, for each replicate of each treatment we determined the proportion of adult flies that eclosed successfully and left each PARC for each treatment, defined as the 'rate of fliers'. To undertake this determination we sampled the '*B. tryoni* debris' from each PARC (comprising empty pupal cases, un-emerged pupae, partly emerged adults, deformed and non-deformed dead fruit flies remaining after emerged adult *B. tryoni* had been released), as described in Reynolds *et al.* (2011), for each of the three releases.

Three adult releases were conducted in the urban area of Wagga Wagga, NSW (35° 70' S, 147° 22' E) from October 2008 until March 2009. Recaptures were made using a 400m spaced trapping grid comprising twenty Lynfield traps baited with Cuelure (International Pheromones, London) and malathion (Meats *et al.* 2002), positioned at 1.5-2m height on trees. Traps were cleared weekly after each release for 12 consecutive weeks or until no more sterile flies were trapped for at least two consecutive weeks. Fluorescent (Pink and Arc chrome) dyes were used, to distinguish between treatments, with dye colour alternated between treatment type for each subsequent release. There was an interval of at least nine weeks between subsequent releases.

Twelve release sites on a 400m grid were established within the 5 by 4 rectangular trapping grid, with release sites located central to four trapping sites, located at least 150 m from any trap. During each release, PARCs containing sterile flies were taken to the release sites in an air-conditioned vehicle and one PARC containing YH supplemented flies and one PARC containing YH deprived flies was opened at each site. The majority of sterile *B. tryoni* were aged 2-3 days when released.

Trapped fruit flies were collected and rinsed in 70% alcohol for approximately one minute to remove any excess dye. The adult fruit flies were then left to dry on a paper towel before being placed in a labelled vial. Flies were then sent to the Orange Agricultural Institute, Orange, NSW, Australia where they were observed and classified by colour (as described in Reynolds *et al.* in press) as YH fed or YH deprived. The total number of flies (for each diet group) caught in the trapping grid was calculated per release, and the mean number of recaptured flies per release was determined. Daily minimum temperature over the monitoring period ranged from 1.1 to 20.0ºC (release period 1), 11.7 to 28.5ºC (release period 2) and 2.6 to 20.0ºC (release period 3) and maximum daily temperatures over the monitoring period ranged from 18.8 to 35.1ºC (release period 1), 30.3 to 43.2ºC (release period 2) and 21.2 to 32.9 (release period 3). Daily relative humidity over the monitoring period ranged from 7 to 98% (release period 1), 5 to 100% (release period 2) and 12 to 97% (release period 3).

STATISTICAL ANALYSIS

Outdoor cage

Proportional hazards (PH) models are a class of survival models that relate the time that passes before some event occurs (in this case, death) to one or more predictors. The Cox Mixed Effects (CME) model fitted using the 'coxme' function in R (Terry Therneau, Mayo Clinic, December 28, 2011) fits the model $\lambda(t) = \lambda_0(t)e^{X\beta + Zb}, b \sim G(0, \Sigma(\theta))$ where $\lambda_0(t)$ is the 'baseline' hazard function (in this instance: Female+YH+white sugar), X and Z are design matrices for fixed effects (gender (M/F), yeast supplementation/deprivation, sugar type (brown/white) and all 2 way and 3 way interactions) and random effects (walk-in field cage and outdoor cage) respectively, β is a vector of fixed effects coefficients and *b* is the vector of random effects coefficients. The random effects distribution G is modelled as Gaussian with mean zero and a variance matrix Σ which depends on a vector of parameters θ. Significance of individual coefficients of fixed effects was assessed by calculating the ratio of the predicted estimate to its standard error and comparing with a standard normal distribution. The effect of treatments on the proportion of flies dead after 7, 14 and 21 days was also examined using a generalised linear model with logit link function and binomial errors in Genstat 11.0. Separate analyses were completed for times 7, 14 and 21 days. These days were selected based on predicted days to 50% survival (5 to 8 days) and 25% survival (6 to 28 days).

Small mesh cages

A Kaplan-meier survival regression was used to determine significance between treatments.

Field release

A generalized linear mixed model (GLMM) for binomial data with weights given by 'total capture per trap per release' and dispersion estimated was fitted in ASReml-R to the proportion of flies which received YH supplementation caught in each trap for each release. Release period was fitted as a fixed effect while trap was fitted as a random effect. The significance of the fixed effect was tested using Wald statistics. Flies per week of release (totalled over traps) as a percentage of 'total fliers' for that release was modelled by fitting a linear mixed model in ASReml–R with release, week of release and treatment and all 2 way interactions as fixed effects and the 3 way interaction considered as the residual variance.

To determine the rate of fliers, an empirical logistic transformation of the form $z = \ln\left(\frac{y + 0.5}{m - y + 0.5}\right)$ with var(z) estimated using $\text{var}(z) = (y + 0.5)^{-1} + (m - y + 0.5)^{-1}$ was taken, where y is the number emerged/fliers, m-y is the number of unemerged/nonfliers respectively and m is thus total pupae/total emerged respectively (McCullagh and Nelder, 1990). $\text{Var}(z)^{-1}$ was used as weights in the linear mixed model with release, treatment and release×treatment as fixed effects and release×replicate as random effects. Residual variance at each release was modelled.

RESULTS

Outdoor cage

Overall, the Cox mixed effects model indicated a significant effect of gender (z=2.75, P=0.006), YH (z=-4.60, P<0.001) and a gender x YH x sugar interaction (z=1.89, P=0.059) on the pattern of mortality (Fig.1). When the 3-way interaction was removed from the model two of the 2-way interactions were significant (YH x gender (z=3.09, P=0.002) and sugar x gender (z=2.35, P=0.019). Yeast hydrolysate supplemented, white sugar fed females had the lowest risk of mortality and are referred to as the baseline control (Fig. 1).

Examining particular days, on day 7 there was a significant effect associated with gender (F=17.6; df=1,24; P=0.003), provision of YH (F=15.7; df=1,8; P=0.0003), and a gender x YH interaction (F=10.69;

df=1,24; P=0.003). Females supplemented with YH had a mortality of 51.39% which did not differ significantly from the mortality of males supplemented with YH (54.25%). Both females and males supplemented with YH had significantly lower mortality than females fed sugar only (68.68%) which was in turn significantly lower than males fed sugar only (83.57%).

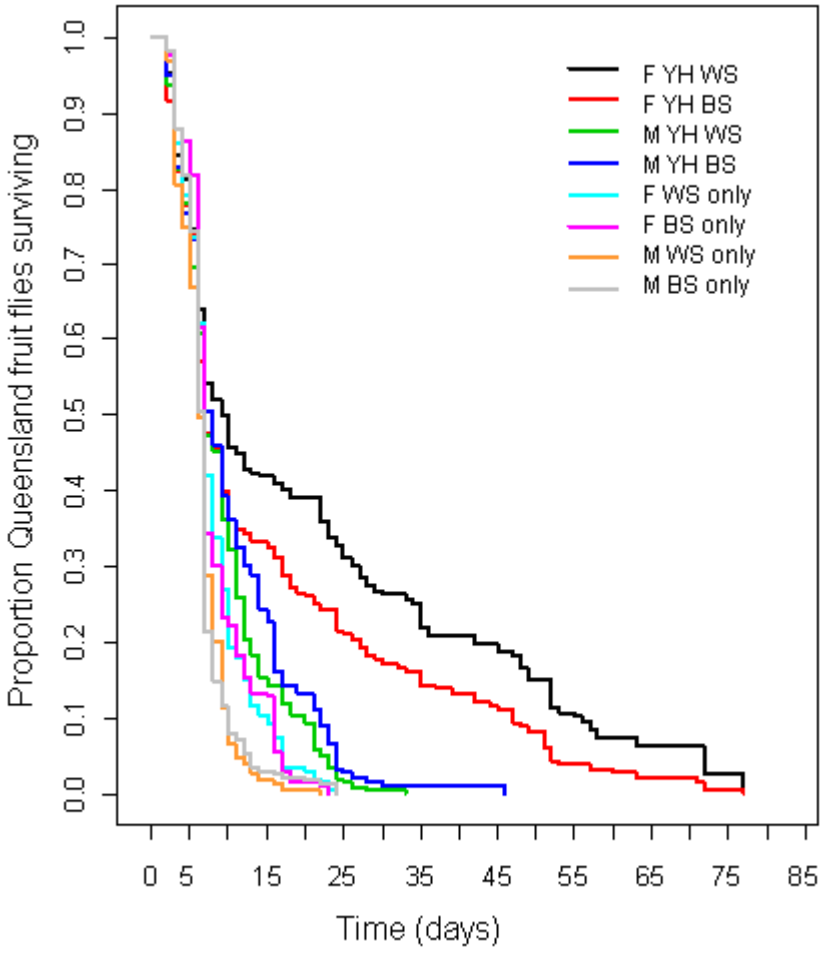

Figure 1. The proportion of sterile male (M) and female (F) *B. tryoni* surviving over the duration of the outdoor cage trial when fed yeast hydrolysate (YH) and either brown sugar (BS) or white sugar (WS), or sugar alone.

At 14 days there was a significant effect of YH (F=27.42; df=1,5; P=0.003), gender (F=58.23; df=1,24; P<0.001), and a gender x sugar interaction (F=4.39; df=1,25; P=0.046) on *B. tryoni* mortality (Fig. 2). Compared with the control on day 14, YH supplemented brown sugar fed females had a risk ratio of 1.14 of mortality. There was a significant effect at 21 days of gender (F=50.59; df=1,24; P<0.001), YH (F=57.54; df=1,6; P<0.001), a gender x YH interaction (F=25.62; df=1,24; P<0.001), and a gender x sugar interaction (F=5.45; df=1,24; P=0.028). At day 21, male flies fed white sugar had higher mortality (98.64%) than females fed either white or brown sugar (mortality 91.53% and 94.51% respectively) while male flies fed brown sugar (mortality 97.02%) differed only from female white sugar fed flies. There was over 98.5% mortality for YH deprived *B. tryoni*, under 75.8% mortality for female YH supplemented *B. tryoni* and under 95.1% mortality for male YH supplemented fruit fly.

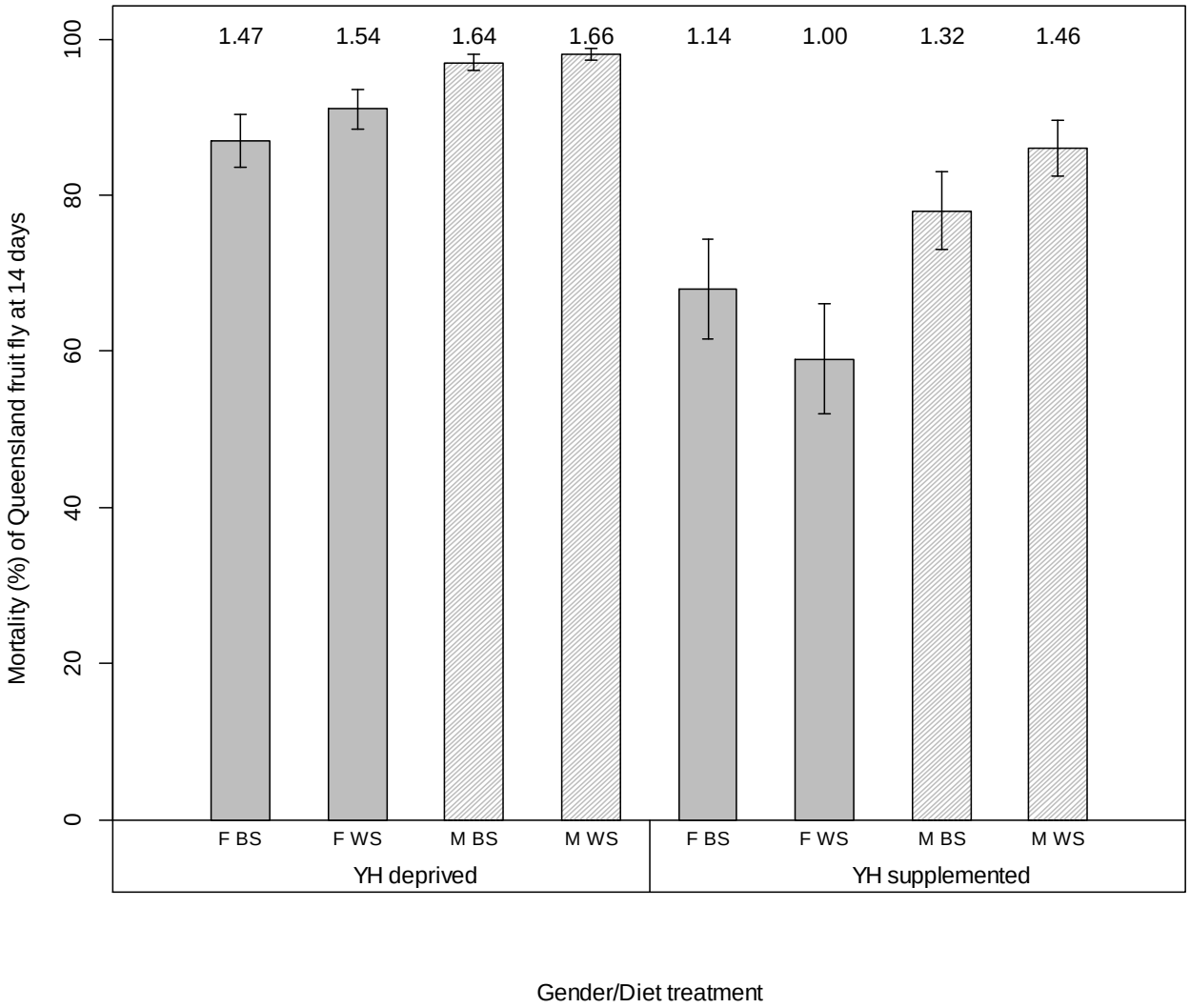

Figure 2. The mean (logit) mortality of sterile male (M) and female (F) *B. tryoni*, fed either a yeast hydrolysate (YH) supplemented or deprived diet, yeast hydrolysate (YH) and either brown sugar (BS) or white sugar (WS), or sugar alone after 14 days in a field cage. The risk of mortality relative to the baseline (ie. Yeast hydrolysate-supplemented, white sugar fed females) is also given above each column.

Small mesh cage

Starvation cages suffered significantly increased mortality over a very short period (~6 days till 100% mortality). Diets overall differed from each other significantly (Whole model survival analysis for males X^2 = 476.18, df = 4, , p = <0.001 Fig. 3; females X^2 = 465.49, df = 4, p = <0.001 Fig. 4). Between non starvation cages (Sugar, YH full, 48 hours YH) there was no significant difference in the decline of flies over the sampling period, for either males or females. For starvation treatments however, males given 48 hours access to YH then starved suffered higher mortality than those given only access to sugar prior to

starvation (df = 1, X^2 = 28.19, p = <0.001; Fig. 5). This effect was only present in males, as no significant difference was detected between starved females from either diet treatment.

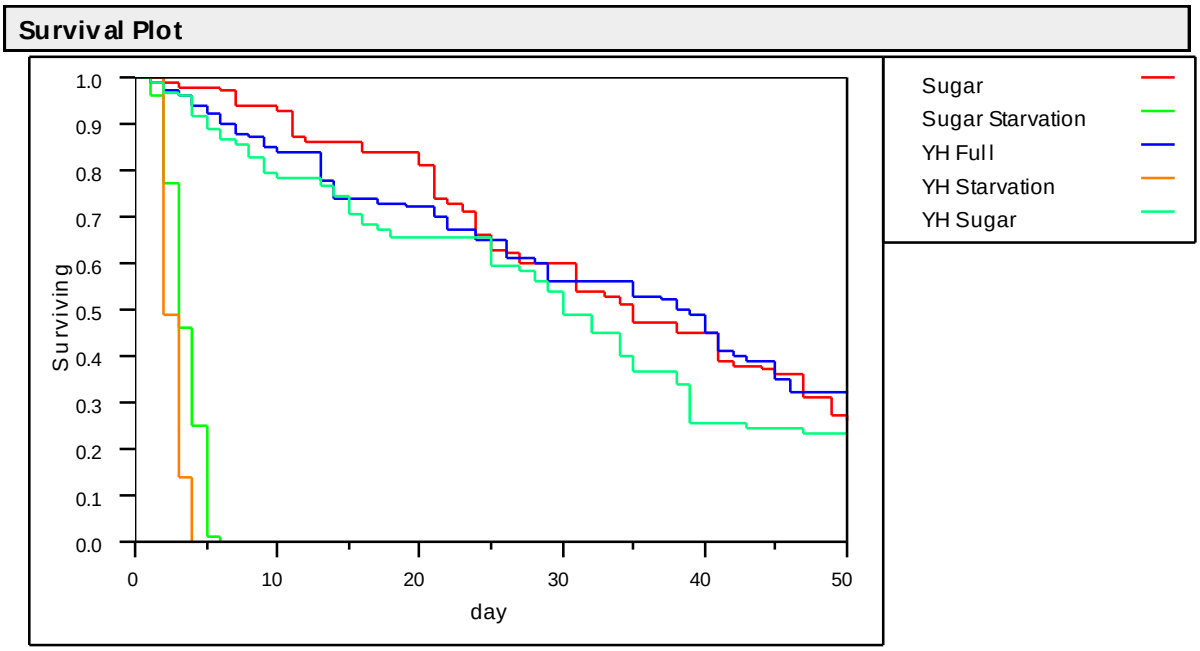

Figure 1. Survival plot of males for each diet treatment. Starvation diets significantly different from all other diets.

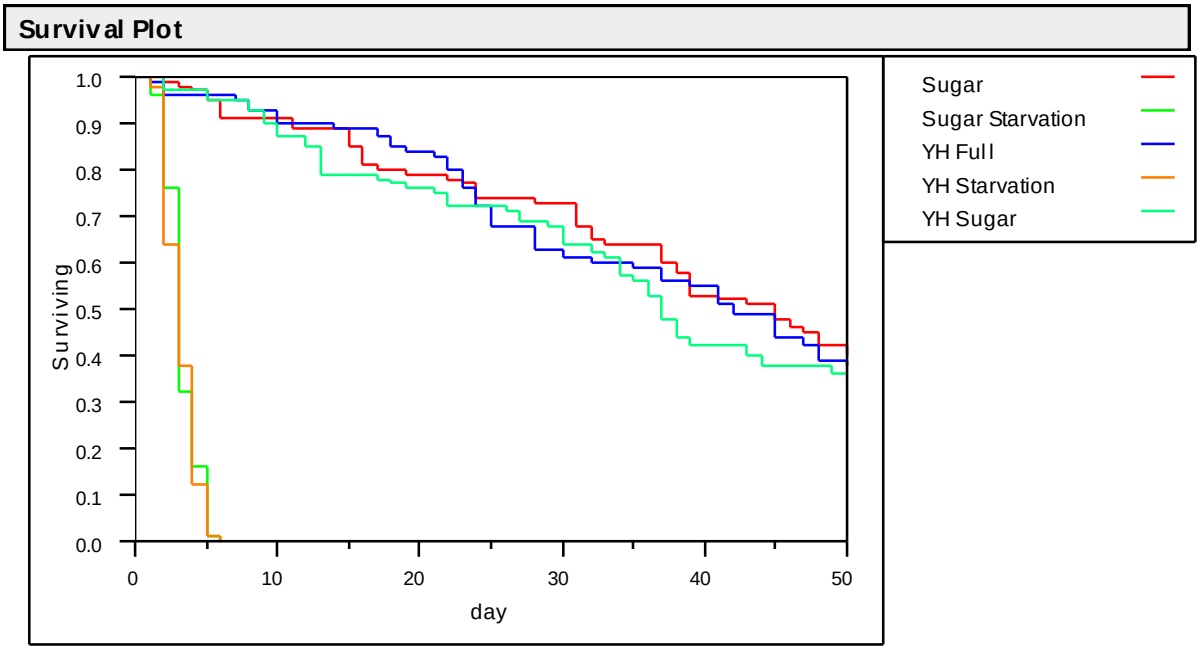

Figure 2. Survival plot of females for each diet treatment. Starvation diets significantly different from all other diets.

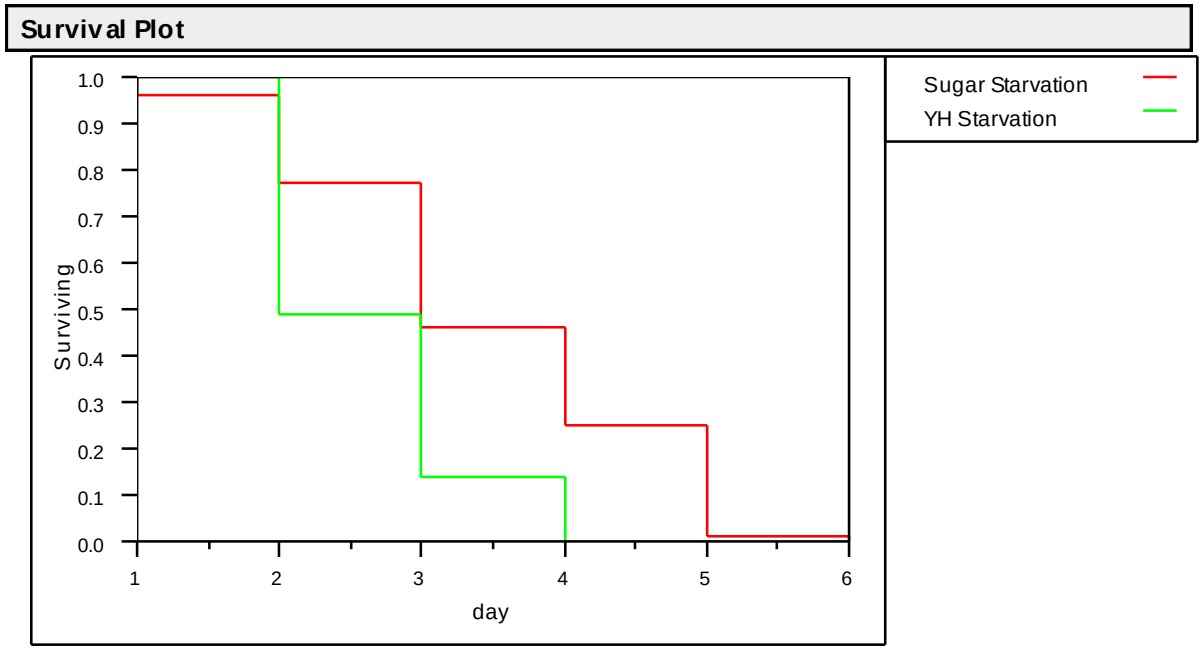

Figure 3. Survival plot of males for starvation diets. Males with 48 hours access to yeast hydrolysate suffered higher mortality than males with access to sugar only before starvation.

Field release

In the open field, cue-lure baited traps had a higher probability of capturing YH-fed flies than YH-deprived flies with 1.2 YH-supplemented flies trapped for every YH-deprived fly trapped (predicted relative probability of capture, 0.54 and 0.46 flies/trap respectively). While release date was not significant the least variable results were from the third release with the probability of trapping YH-fed flies 0.54 ± 0.014 compared to 0.52 ± 0.037 and 0.62 ± 0.047 for release 1 and 2 respectively.

Overall, of the estimated 1.3 million sterile flies released (rate of fliers) at the release sites (0.48 million, 0.37 million and 0.45 million for release 1, 2 and 3 respectively), 1581 YH supplemented and 1431 YH deprived flies were recaptured. In release 1, 102 YH supplemented and 90 YH deprived flies were recaptured with equal numbers of fliers released for both treatments; in release 2, 74 YH supplemented and 46 YH deprived flies were recaptured with 10% more fliers released for YH supplemented than for YH deprived while in release 3, 1405 YH supplemented and 1295 YH deprived flies were recaptured again with equal releases of fliers for both treatments. Recapture of YH supplemented flies was 0.236±0.007 % of all flies released (rate of fliers), comprised of 0.051±0.01 % flies for release 1, 0.043±0.01 % flies for release 2 and 0.61±0.01 % flies for release 3. For YH deprived flies the recapture rate was 0.228±0.007% of all flies released, comprised of 0.029±0.01% flies for release 1, 0.021±0.01 % flies for release 2 and 0.591±0.01 % flies for release 3.

DISCUSSION

The current study demonstrates a strong effect of diet on abundance of sexually mature sterile adult *B. tryoni* under field conditions. In the outdoor cage study, the incorporation of YH in the adult diet was important for longevity; YH-supplemented flies suffered less mortality than their YH-deprived counterparts after 7, 14 and 21 days. Females had greater survival than males when supplemented with YH, after 7, 14 and 21 days. In the small cage study, YH-supplemented males appear more prone to starvation than males

fed sugar only. The higher in-field trap recapture rates of YH-supplemented sterile adult male *B. tryoni*, adds further support to the role of YH in the male sterile adult diet.

The only two prior studies that have determined the effects of YH supplementation on longevity of sterile *B. tryoni* are those by Perez-Staples *et al.* (2007) and Reynolds & Orchard (2011). When fed sucrose and water only, sterile *B. tryoni* of both sexes had reduced longevity when compared with fertile flies, but a greater increase in longevity when provided with YH, such that the longevity of YH supplemented sterile flies was similar to that of YH supplemented fertile flies (Perez-Staples *et al.* 2007). Reynolds & Orchard (2011) compared YH-supplemented chilled adult flies with YH supplemented non-chilled flies, YH deprived chilled, and non-chilled flies and found overall that longevity did not differ based on fly diet, except in one trial where YH supplemented females survived the longest. Taylor *et al.* (in press) postulated that YH supplementation of sterile males might increase survivorship significantly more than YH supplemented sterile females, but this is not apparent in our study. However, given males have a lower dietary requirement for YH than females (Drew, 1987; Perez-Staples *et al.* 2007, 2009, 2011), it may be feasible to reduce the period in which sterile *B. tryoni* are provided with dietary YH to a point where female survival is less than male survival. Male-only release is frequently more effective than dual sex releases at inducing reproductive failure in wild populations (Vreysen *et al.* 2006). This is because in male only releases, there are only wild females to copulate with and no competing sterile females. Thus, if we were able to reduce the quality of the sterile female, then dual sex releases, such as those currently used for *B. tryoni*, may function similarly to those of male only releases. Indeed, a recent study demonstrated that providing yeast hydrolysate for 48 h after adult eclosion led to an increase in reproductive development and sexual performance of male *B. tryoni* (Perez-Staples *et al.* 2011). However, females had poorly developed ovaries and, predominantly at younger ages, were less likely to mate and remate than females with continuous access to yeast hydrolysate (Perez-Staples *et al.* 2011).

In the outdoor cages, it was evident that YH-supplemented flies had lower mortality rates after 7, 14 and 21 days compared with YH-deprived flies, suggesting that YH plays a significant role in long term survival. The type of sugar (brown or white) had no significant bearing on longevity except after 21 days, despite encouraging pilot laboratory studies (unpublished data). Despite females suffering higher mortality when fed brown sugar and males vice versa (ie. higher mortality when fed white sugar), this was not significant. Unlike white sugar, which contains no natural minerals, brown sugar contains molasses, which has traces of protein and minerals (calcium, potassium, iron and magnesium) in addition to a small (2%) water content. Despite the differing nutritional contents, they are possibly too slight to reveal any real nutritional benefits.

In the small cage study, surprisingly, differences in the mortality of flies between the non starvation cages (sugar, YH full, 24h YH) were not evident, suggesting that sugar alone, or only 24h access to YH is sufficient for the flies survival. However, it appears that if males are supplemented with YH pre-release, they may be more prone to starvation than sugar only fed flies. This was, however, not evident in the field trial.

In the present study, we evaluated fly survival by fly recapture in cue-lure baited traps in the field. YH-supplemented flies were trapped at a higher rate than YH-deprived sterile flies. Sexually mature flies are attracted to cue-lure baited traps (Weldon *et al.* 2008) and resultantly, higher trap recapture rates are expected when flies have been supplemented with YH prior to release. However, over time, it might be expected that the YH-deprived sterile flies would find adequate nutrition and mature to a point where the same number as YH-fed flies would be recaptured. Males have a lower nutritional requirement than females (Drew, 1987; Perez-Staples *et al.* 2011), and are therefore more likely to obtain adequate nutrition to complete reproductive development upon release. However, this did not occur in the present study and could mean either of two things. Yeast hydrolysate supplemented sterile flies do indeed have greater longevity and/or YH-deprived flies suffered higher mortality early on before they reached maturity and

were responsive to cue-lure baited traps. This is supported by the outdoor cage study that showed no difference in mortality of sterile flies until 14 days. Meats *et al.* (2003) held sterile *B. tryoni* in large cages supplemented with YH for approximately 1 week, or until they were close to or at maturity. Upon release, opposite to what we found, trap catches revealed very low survival rates, although this was later attributed to the conditions under which they were held in order to mature. In the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) under natural conditions, where food is available, Gavriel *et al.* (2010) concluded that sterile male fly survival is unaffected by YH supplementation pre-release. They showed that the supplementation of YH to the sterile-male diet did not affect survival after 4, 6, and 7 days. Further, Gavriel *et al.* (2010) found no diet-related effect on fly survival, measured by similar numbers of YH fed and YH deprived flies recaptured in trimedlure traps after 4 and 7 days. Earlier, Shelly & McInnis (2003) had also concluded that YH had little effect on the survival of two sterile *C. capitata* strains in a field cage after 4 days. There have been very few field studies that have sought to validate such laboratory and field cage studies comparing the trap recapture of YH fed and YH deprived flies. Shelly & Edu (2008) endeavoured to confirm such laboratory and field cage studies by comparing the short-term dispersal from a central release point of sterile *C. capitata* males. The authors showed no significant difference between sterile males fed a sugar only diet compared to a sugar diet supplemented with YH in either the number or spatial distribution of recaptured individuals; thus demonstrating that pre-release nitrogenous dietary supplements do not improve the field performance of sterile *C. capitata* males in SIT programs (Shelly & Edu, 2008).

A comparison of the overall recapture rates revealed that the first two releases had comparatively low recapture rates, up to 30 times less than the numbers recaptured in the final release. A similar scenario was observed when Reynolds *et al.* (in press) compared recapture rates of sterile flies in NSW and South Australia simultaneously. South Australia consistently recorded much lower recapture rates than NSW (for two different release methods), and although a number of possible factors were highlighted, such as environmental conditions, reduced ability to locate food, and food scarcity, it was not clear what caused these differences. In the present study, the differences were over time, as opposed to location. Temperature and RH data (although not directly compared with recapture rates) did not reveal anything particularly notable between the first two releases and the last release. In addition, quality control (data not shown) and pupal debris sampling did not reveal any differences between the releases (batches of flies) for emergence or flight that may have influenced this observation pre-release. The most likely probability is therefore a scarcity of food in the earlier releases, although why this might have been the case remains unclear.

Although we have only looked at YH as a nutritional supplement for *B. tryoni* to enhance the effectiveness of the SIT, there are other food supplements and treatments that have shown potential for a range of fruit flies, including bacterial (Drew *et al.* 1983) and probiotic diets (Ben Ami *et al.* 2010), raspberry ketone or cue-lure (Tan & Nishida, 1995), juvenile hormone (Faria *et al.* 2008; Smallridge *et al.* 2008), access to fruits (Aluja *et al.* 2001) and aromatherapy (Shelly, 2001; Shelly *et al.* 2004), that may be equally, if not more effective. All of these interventions seek to enhance male sexual performance through accelerated maturation, sexual advantage, increased mate attraction and increased mating success and warrant further investigation.

Although a single previous study on *B. tryoni* showed that sterile male survival is affected by YH in the pre-release diet, this required open field validation. As the inclusion of YH to the adult diet enhances male mating success (Perez-Staples *et al.* 2007, 2009) and as shown here, improves sterile adult persistence and abundance in the field, our results support the supplementation of the fly diet with YH as it may improve the effectiveness of SIT programs. The choice of either white or brown sugar for sterile adults is not important. Our study is relevant to control programs that utilise the sterile insect technique to control wild populations of *B. tryoni*. Such a program is only successful if the sterile flies are able to survive long enough in the open field to find a mate and copulate, thus inducing female reproductive failure in the wild

population. It is therefore evident, that an effective SIT program for *B. tryoni* should include the provision of YH in the pre-release diet.

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Technical Report 5

Accelerating maturation of Queensland fruit fly adults using the juvenile hormone analogue methoprene and a supplementary yeast hydrolysate diet.

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Abstract
A 48 h window of access to dietary protein improved the maturation and mating performance of male Queensland fruit fly, *Bactrocera tryoni* (Froggatt) used for sterile insect technique control programmes. Application of the juvenile hormone analogue methoprene is known to reduce pre-copulatory maturation periods in other species of tephritid flies targeted by SIT. Combining access to dietary protein with methoprene treatment may therefore be a powerful tool increasing the effectiveness of *B. tryoni* SIT to combat outbreaks. In this study we demonstrate that it is possible to reduce the pre-copulatory maturation period of newly emerged fruit fly by exposing them to a combined topical methoprene treatment and access to a 48 h window of supplementary dietary protein. While latency till the onset of copula and copula duration remain unaffected by treatment, flies given both a topical treatment and access to protein began participating in mating behaviour two days prior to untreated flies on a similar diet.

Keywords: Pest control, growth regulation, sexual competitiveness, sterile insect technique, *Bactrocera tryoni*

INTRODUCTION

The sterile insect technique (SIT) has been previously applied to control Queensland fruit fly, *Bactrocera tryoni,* outbreaks within and around the Fruit Fly Exclusion Zone in south-eastern Australia (Jessup *et al.* 2007). In SIT millions of insects are mass reared, rendered reproductively sterile, and then released into the wild. Released sterile males mate with wild females and prevent oviposition of viable eggs, and as a consequence the population declines due to reduced recruitment in successive generations (Knipling, 1955). The factors essential to the success of SIT are the ability of mass reared flies to find, court and successfully mate with wild females. Before this occurs released flies must reach sexual maturity.

The issue of maturation may be addressed by holding newly emerged flies until they reach sexual maturity before release; this, however, is coupled with increased operational costs for fly maintenance and the risk of maintaining adult flies in a controlled indoor environment only to eventually release them into a variable outdoor environment, for which they are not adapted, resulting in increased mortality. On the other hand, releasing newly emerged immature flies into the field requires the assumption that flies can find enough nutrition to allow for full sexual maturation within the short time frame before risks of starvation and/or predation reduce the effectiveness of SIT. Under controlled conditions mass reared adult *B. tryoni* take eight to ten days to reach sexual maturity and typically have a peak sexual performance at ten to twelve days post emergence (Perez-Staples *et al.* 2007, 2008, 2009). The pre-copulatory period until sexual maturation and peak sexual performance may take even longer under variable field conditions where temperature and humidity are not constant (Hendrichs *et al.* 1991).

A number of studies have shown that providing flies with access to a supplementary source of protein can improve their maturation and sexual performance. Adult flies with access to dietary protein tend to experience increased mating propensity and a shorter pre-copulatory period than flies provided with a sugar di*et al*one. (Kaspi and Yuval, 2000; Aluja *et al.* 2001; Yuval *et al.* 2002; Shelly *et al.*, 2002; Perez-Staples *et al.* 2007, 2008). The risk of providing supplementary protein, however, is it may leave adult flies more prone to starvation by setting them on a developmental nutritional trajectory that cannot be maintained if sufficient nutrition cannot be found in the field after release (Yuval *et al.* 2007). Any method that reduces the pre-copulatory period of released flies will, however, be of great benefit to the application of SIT.

Additional developmental acceleration may be achieved by treating flies with a juvenile hormone analogue, methoprene. In fruit flies with long pre-copulatory periods such as *Anastrepha* spp. and *Bactrocera cucurbitae* methoprene treatment has been demonstrated to reduce the pre-copulatory period and increase mating performance (Segura *et al.* 2009; Haq *et al.* 2010). For fruit flies with shorter pre-copulatory periods such as the Mediterranean fruit fly C*eratitis capitata*, there is little to no effect of methoprene treatment on development, probably due to their short pre-copulatory period leaving a very small window for hormonal acceleration of maturation (Faria *et al.* 2008; Shelly *et al.* 2009). Results are also not consistent across *Bactrocera* species, with Oriental fruit fly, *Bactrocera dorsalis* (Hendel), showing no effects of topical methoprene application on pre-copulatory period (Shelly *et al.* 2009). The effect of methoprene application has varied among closely related species, and tests for maturation effects associated with the topical application of methoprene are needed on a species-by-species basis.

The aim of this study was to assess the effects of methoprene on the sexual maturation of Queensland fruit fly, and the combined effects with access to dietary protein supplements. Currently mass reared *B. tryoni* used for release in SIT programmes are given a pre-release diet of sugar only. Newly emerged adult *B. tryoni* display improved maturation and mating performance when provided with a 24-48 h window of supplementary protein (Perez-Staples *et al.* 2008). Combining this access to protein with a topical treatment of methoprene may further enhance the benefits already observed by an improved diet. Newly emerged adult *B. tryoni* were exposed to methoprene using both topical application as utilised in previous studies on other species, and by dipping pupae in a methoprene solution, a method more easily

applied to large scale production for pest management purposes. Enhancing the maturation of *B. tryoni* has potential benefits for the application of SIT. If flies need less time to mature in the field then the likelihood of reaching sexual maturity should be greatly increased. This would result in a greater number of released flies participating in mating and a probable increase in the transfer of sterility to the target wild population, improving both the cost effectiveness and efficiency of SIT.

MATERIALS AND METHODS

General methods and diet treatments

Bactrocera tryoni pupae were obtained from the Fruit Fly Production Facility at Elizabeth Macarthur Agricultural Institute at Camden, New South Wales, Australia. To produce sterile flies, pupae were sealed in plastic 'zip-lock' bags (~ 2000 pupae per bag) and left overnight to achieve a hypoxic environment. Bag were then exposed to a 70 – 75 Gy dose of gamma radiation from a Cobalt 60 source (dose rate 9 Gy min^{-1}) located at Macquarie University, Sydney, one day before emergence (following Collins *et al.* 2009). This is the standard dose used in *B. tryoni* SIT and induces >99.5% sterility. To ensure that all test flies were the same age, only those that emerged within 24 hours of irradiation were used for diet and methoprene treatment experiments. All flies emerged in a laboratory at Macquarie University in Sydney, and were housed in 5 L cages each containing approximately 110 flies. Flies were sorted by sex on the day of emergence, with half of the test flies being provided with access to protein for 48 hours post emergence and then maintained on a sugar only diet, while the other half of the test flies were maintained solely on a sugar diet without any access to protein. Both males and females were assessed for the effects of diet and methoprene treatment on maturation.

A second set of un-irradiated/un-treated control flies were set up in a similar manner, but with constant access to a full diet (sugar and protein combined), a week prior to sterile fly irradiation in order to obtain mating partners at peak sexual maturity, 10-14 days old, to be paired with diet and methoprene treated flies during mating experiments. Mating experiments assessing age until sexual maturity were conducted at 2, 4, 6, 8, 10 and 12 days post emergence of test flies. All flies were maintained in the lab on a 14:10 h light:dark cycle including an artificial dawn and dusk period where the lights turned on and off in stages over the period of an hour. *B. tryoni* mating takes place at dusk so observations began 30 mins prior to the onset of dusk in the lab. No further mating was observed to be initiated after the end of the dusk period once total darkness in the lab was achieved using a red light torch.

Twenty males and twenty females from each treatment group (Topical methoprene / 48 h protein, topical methoprene / sugar only, methoprene dipped pupae / 48h protein, methoprene dipped pupae / sugar only, untreated / 48h protein, untreated / sugar only) were set up on each test day post emergence, resulting in 240 individual mating pairs per test day. At least 4 hours before the onset of dusk the 20 irradiated male and 20 irradiated female test flies from each diet and methoprene treatment combination were individually paired in individual containers with an un-irradiated control fly of the opposite sex that was at least 10-14 days old. The time of onset of copulation was recorded for each mating pair to assess copula latency, defined as the duration from the start of dusk until the onset of mating. Mating was observed past the end of dusk until the time of cessation of copulation to assess copula duration for each mating pair. On each subsequent test day post emergence new flies from each test batch were paired with new unirradiated partners that were at least 10-14 days old. This resulted in 240 paired flies for each test day post emergence.

Methoprene treatment

Flies were treated with methoprene using one of two methods. Topical treatment was delivered by applying 1/µl of methoprene dissolved in acetone (5µg/µl) onto newly emerged males and females. Flies were gently aspirated into a cloth mesh bag and gently held in place by pulling the mesh tight. A micro pipette was used to apply the methoprene by gently placing the pipette tip through a square in the mesh and applying the

required volume onto the back of the thorax of the fly. Flies were then released into a standard holding container and provided with either one of the diet treatments. The dipping treatment was assessed by immersing irradiated pupae in a 1:100 dilution (in water) of methoprene solution (5µg/µl in acetone). Immediately after irradiation treatment one hundred pupae were poured onto a glass Petri dish so that each pupa rested flat on the dish bottom. Pupae were submerged for 5 mins in methoprene solution so that each pupa was covered completely. Pupae where then removed and poured gently onto a paper towel and left to dry for a further 10 mins before being set up to emerge in the standard 5 l emergence boxes. Dipped males and females were sorted by sex on the day of emergence and half given 48 hours access to protein followed by sugar only diet, and half given access to sugar only diets with no protein.

Statistical assessment

The probability of flies mating on each day of testing post emergence was assessed using nominal logistic regression incorporating; age, methoprene treatment and diet into the models. Latency until onset of copula and copula duration were assessed for each treatment using least squares regression incorporating again age, methoprene treatment and diet into full models. All analyses were performed using JMP (v. 5.0.1.2, SAS Institute).

RESULTS

Age at onset of mating

Age at onset of mating was assessed separately for test males and females. For males, participation in mating was significantly influenced by age, diet and methoprene treatment (Table 1). There were also significant interactions between methoprene treatment and access to dietary protein and age. Protein fed methoprene treated males mated at younger ages than their sugar fed counterparts, this effect being strongest for topically treated males. Both topically and dipped methoprene treated males began mating at 4 days of age when provided with 48 hours access to dietary protein. Untreated protein fed males did not begin mating until 6 days of age and did not show a high proportion (>20%) of males participating in mating until after 8 days of age. A greater number of topically treated males mated early (at 2, 4 and 6 days of age) than dipped males. Generally for all treatments males that were only fed sugar had significantly lower participation in mating than males that were given 48 hours access to protein. A small number (<10%) of sugar fed methoprene treated males mated as early as 6 and 8 days of age, however overall mating was low and did not begin to increase till after day 10. Untreated sugar fed males did not participate in mating until days 10 and 12 (Figure 1).

For females the results were more varied. There was still a significant effect of diet, with protein fed females having higher participation in mating than sugar fed females. There were also significant effects of methoprene treatment and age (Table 2), however with the highly varied participation in mating across each day, the significant interaction between age and methoprene, and age and diet were lost. There is still a similar interaction between methoprene and diet, with both topically and dipped methoprene treated females having earlier and higher participation in mating then their sugar fed counterparts. Again this effect is greater in the topically treated females (Figure 2).

Table 1. Regression analysis for age of onset of mating for males.

	df	X^2	*P*
Age post emergence	1	34.74	<0.001
Diet	1	130.46	<0.001
Methoprene treatment	2	18.50	<0.001
Age x Diet	1	3.31	0.068
Age x Methoprene	2	10.60	0.005
Diet x Methoprene	2	6.43	0.040

Table 2. Regression analysis for age of onset of mating for females.

	df	X^2	*P*
Age post emergence	1	18.60	<0.001
Diet	1	89.70	<0.001
Methoprene treatment	2	20.22	<0.001
Age x Diet	1	1.23	0.267
Age x Methoprene	2	0.39	0.823
Diet x Methoprene	2	7.33	0.026

3.2. Copula latency and copula duration

Neither latency until the onset of copula or copula duration was affected by methoprene treatment (copula latency $F_{1,2} = 0.269$, $P = 0.764$; copula duration $F_{1,2} = 0.352$, $P = 0.704$). There was also no effect of diet (copula latency $F_{1,1} = 1.169$, $P = 0.281$; copula duration $F_{1,1} = 0.058$, $P = 0.810$). The only significant effect detected was that latency till the onset of copula tended to get shorter as flies were older, in that older flies took less time to initiate copulation after the start of dusk (copula latency $F_{1,1} = 5.459$, $P = 0.0203$) while age had no effect on copula duration ($F_{1,1} = 0.672$, $P = 0.693$).

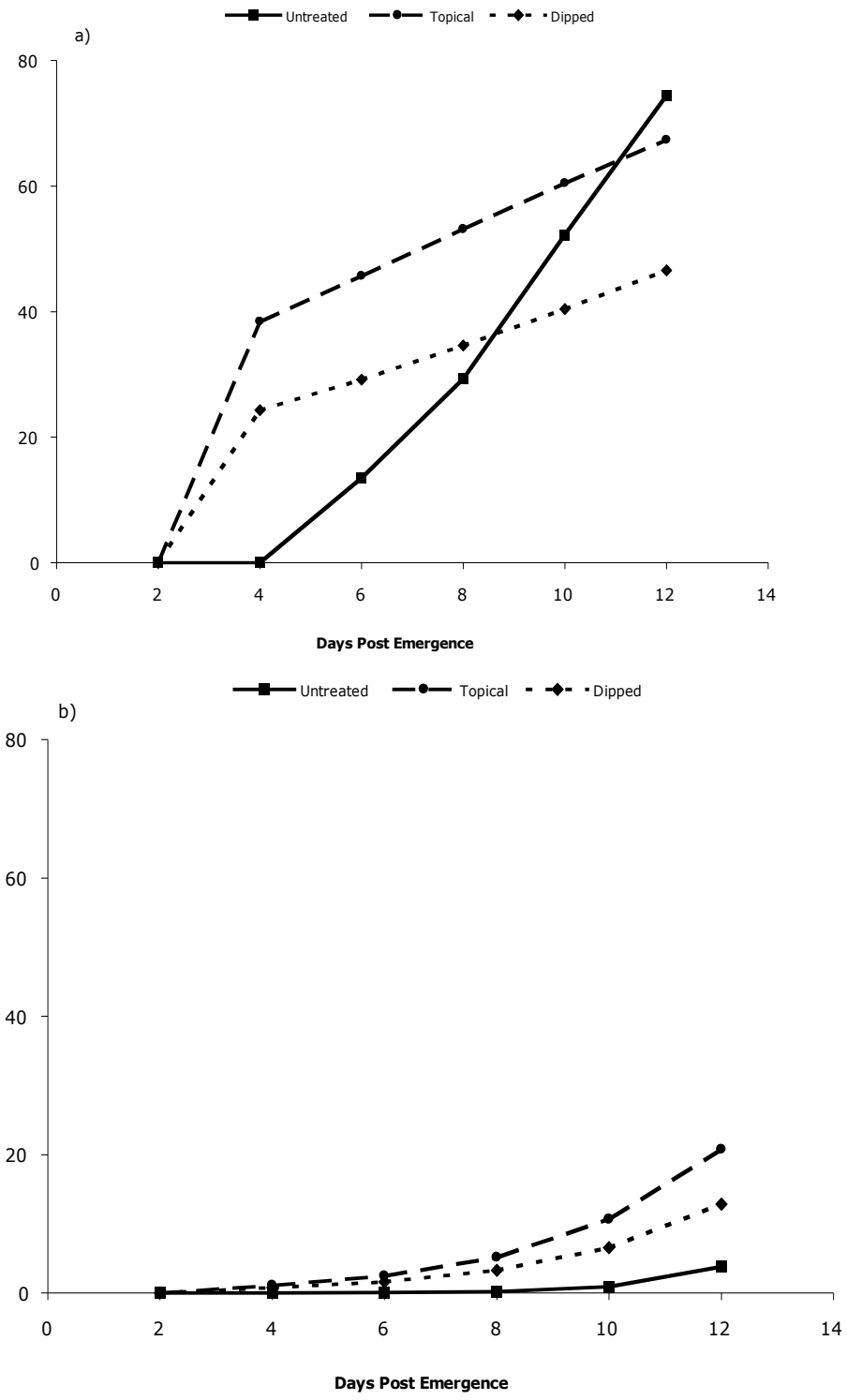

Figure 1. Percentage of methoprene treated and untreated males participating in mating at 2, 4, 6, 8, 10 and 12 days of age for both a) protein fed males and b) sugar fed males.

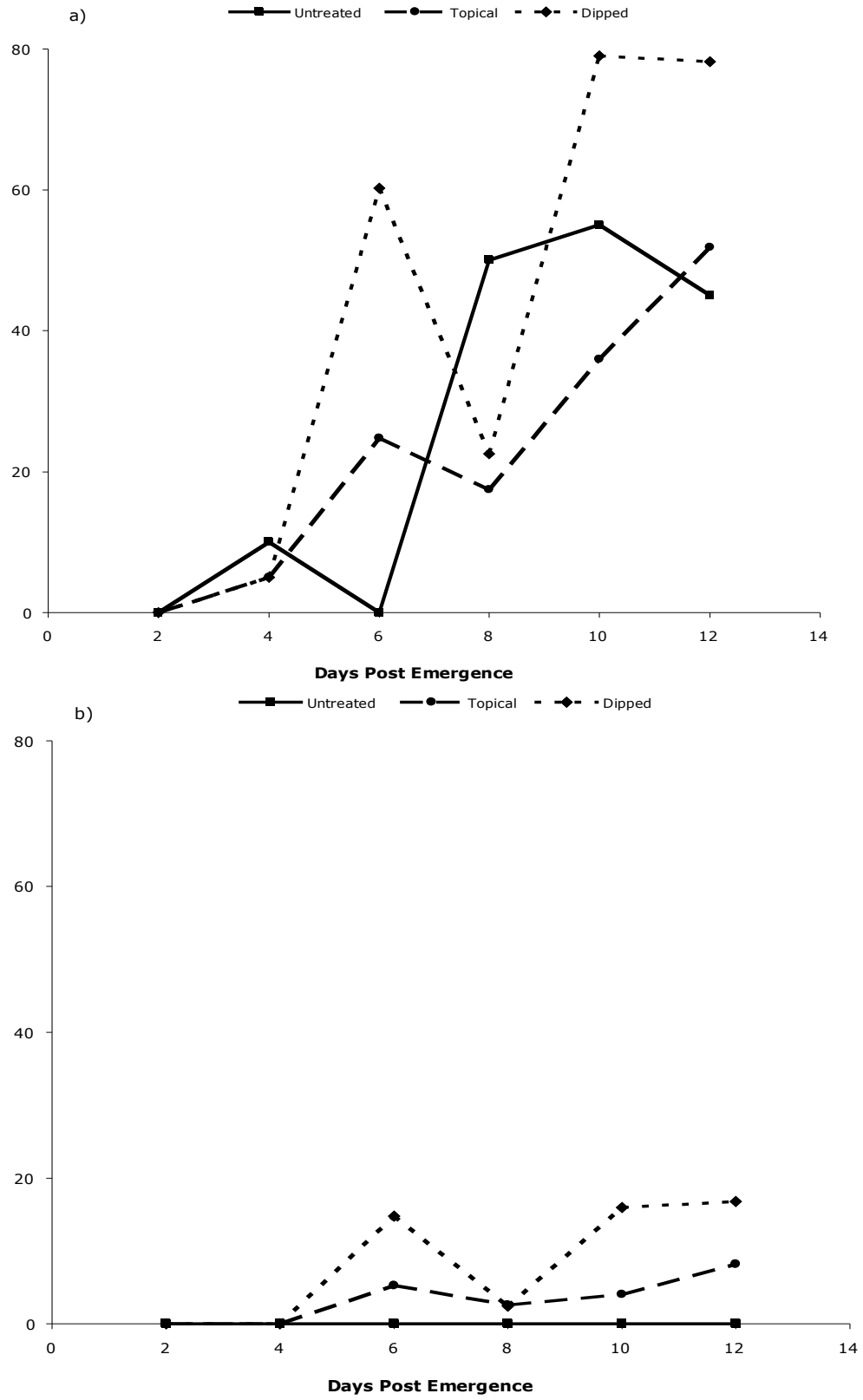

Figure 2. Percentage of methoprene treated and untreated females participating in mating at 2, 4, 6, 8, 10 and 12 days of age for both a) protein fed females and b) sugar fed females.

DISCUSSION

Our results show that methoprene can shorten the pre-copulatory maturation period in *B. tryoni*. This is most evident when flies have access to dietary protein. The response with dietary protein is not unexpected, as *B. tryoni* have already been shown to improve both in maturation rate and sexual performance when given access to dietary protein alone (Perez-Staples *et al.* 2007). The positive effects on sexual performance of dietary protein supplements have also been observed in a number of other tephritid species such as *C. capitata* (Kaspi and Yuval, 2000, Yuval *et al.* 2002), *B. dorsalis* (Shelly *et al.* 2005) and *Anastrepha* (Aluja *et al.* 2001). Combining protein treatment with methoprene appears to be the most effective way to minimise the pre-copulatory period.

Flies fed on a sugar only diet still show a reduced pre-copulatory period when treated with methoprene, with the timing of the onset of copulation being no different to untreated flies that have been provided with a protein diet. It is likely that the effects are the same for both diet groups, however sugar fed flies could lack the nutritional requirements for early gonadal development. It is not yet known whether methoprene treatment only alters the flies behaviourally, or if it speeds up the development of gonad tissue. Perez-Staples *et al.* (2011) demonstrated that males offered a 48 h window of access to dietary protein developed sexual organs at a rate comparable to males that were offered continuous access to protein, however female flies under the same conditions had smaller ovaries than females given continuous protein access. This variable development with diet may explain the variable results between males and females when treated with methoprene. It should be noted however that different responses between males and females treated with methoprene and with access to a full diet including protein have been observed in *Anastrepha,* with accelerated male maturation after treatment, yet no induction of sexual maturation in females observed (Segura *et al.* 2009).

The combined effects of methoprene and protein observed here are similar to results found when treating Melon fly, *Bactrocera cucurbitae* under the same conditions, with protein fed flies showing improved maturation when treated with methoprene, but with no methoprene effects detected in protein deprived flies (Haq *et al.* 2010). For methoprene to be effective in an applied release programme it would have to be combined with access to pre-release dietary protein to maximise effectiveness. It should be noted however that this pilot study ends at 12 days of age, which is often before sugar fed flies begin reaching sexual maturity. Whether there is a pronounced effect of methoprene on sugar fed flies beyond 12 day needs to be investigated. However from an operational sense this is likely to be too long a maturation period for practical use in SIT, which relies on rapid maturation and mating of released flies.

The difference between topical treatment and pupal dipping also warrants further investigation. Dipped flies show a similar trend in early development as topically treated flies, for both protein and sugar only fed males and females. The total number of mating flies, however, was significantly lower than in topically treated flies, and eventually, for protein fed flies, lower than the untreated adults. Exposing pupae to higher levels of acetone in the diluted dip treatment may have other adverse effects on their mating performance. The effects of acetone alone on flies and varying concentrations of methoprene dip need to be assessed before addition of methoprene can be considered as part of a mass rearing programmes.

This pilot study has demonstrated that it is possible to reduce the pre-copulatory period for *B. tryoni* by treatment with methoprene and it has potential as a useful tool for application in SIT programmes.

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Technical Report 6

Effect of adult chill treatments on recovery, longevity and flight ability of Queensland fruit fly, *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae)

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ABSTRACT
Control of Queensland fruit fly, *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) populations or outbreaks may be achieved through the mass-rearing and inundative release of sterile *B. tryoni*. An alternative release method is to release chilled adult sterile fruit flies to decrease packaging and transport requirements and potentially improve release efficiencies. Two trials were conducted to determine the effect of chilling on the performance of two separate batches of adult *B. tryoni* fed either a protein and sucrose diet or sucrose only diet. The first trial compared chill times of 0, 0.5, 2 and 4h; the second trial 0, 2, 4, 8 and 24h. Overall, there was little or no affect of chilling on the recovery, longevity and flight ability of *B. tryoni* chilled at 4°C. Recovery time can take upto 15 min for chilled adult flies. There was no effect of chill time on longevity although females generally had greater longevity on either diet compared with males. Propensity for flight was not adversely affected by chilling at the lower chill times in trial 1, however in trial 2, adults fed on a protein and sucrose diet had a decreased tendency for flight as the chilling time increased. Fly body size did not affect recovery times, although the smaller adult *B. tryoni* in trial 1 had significantly reduced longevity compared to the larger adults in trial 2. Implications of these findings for *B .tryoni* SIT are discussed.

Keywords: sterile insect technique, sterile release, chilled adult release

INTRODUCTION

The Queensland fruit fly *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) poses a significant threat to horticultural crops in eastern and southern Australia, with occasional outbreaks in South Australia and rare incursions in Western Australia. In response, a fruit fly exclusion zone (FFEZ), which encompasses some of the most valuable horticultural production areas in New South Wales (NSW), South Australia, and Victoria, was established (Reynolds *et al*., in press). A bordering Risk Reduction Zone (RRZ) was also established as a buffer zone to minimise movement of wild *B. tryoni* into the FFEZ. The current management protocol for *B. tryoni* in NSW includes extensive monitoring for early detection, bait and cover spraying, and the use of the sterile insect technique (SIT) (M. Nolan pers. comm. 2009). Release of sterile adult *B. tryoni* has proven to be effective as part of an Integrated Pest Management approach in eradicating fruit fly incursions (Fisher 1996) and suppressing wild *B. tryoni* in buffer zones, thereby minimising introductions into pest-free areas (B. Dominiak 2009 pers. comm. and M. Nolan pers. comm. 2009). One form of release, is chilled adult release, which is used to transfer sterile, chilled adult flies from emergence containers to the field for release, to allow more compact packaging and reductions in the space required for transportation (Mangan 1996). Chilled adults are usually released from a moving vehicle (termed roving release), allowing the rapid dispersal of large numbers of sterile flies (Meats and Smallridge 2007). Chilled adult fruit fly release is used effectively around the world, often as part of an aerial release program using airplanes (Cunningham *et al.* 1980, Sivinski *et al*. 2000) or helicopters (Nakamori and Kuba 1990; Vargas *et al.* 1995). Ground release programs do not commonly involve chilled adults (Fisher 1996; Dominiak *et al.* 2000), and there is scant data regarding the efficacy of the roving release of chilled adults (Salvato *et al.* 2003). Due to excessive travel distances between the location of fly production and release along with the large numbers of fruit flies that need to be transported, chilled adult release may be a viable option for *B. tryoni.*

There is concern that chilling sterile adult fruit flies may have negative effects on their performance and resulting SIT efficacy (Serghiou 1977, Mangan 1996). The effects of chilling or chilled adult release on the performance of *B. tryoni* has received very little attention. Based on modelling results, Meats & Fitt (1987) suggested that *B. tryo*ni would incur the least stress and have the most rapid recovery if the chill temperature was as close as possible to the torpor threshold. They showed that adult *B. tryoni* acclimated at 25°C could be chilled for 24h at 1°C and then recover within 12 min at 25°C. Adult diet and sex may also influence the performance (Perez-Staples *et al*. 2007) of chilled fruit flies.

In this study we tested whether chilling and holding sterile adult *B. tryoni* at 4°C for different lengths of time affected their recovery time, flight and longevity. We then determined if a relationship between fly weight and adult wing length exists, the latter which is known to correlate with adult fly size (Prabhu *et al*. 2008). Wing length measurements are tedious and time consuming. Using fly weight to determine fly size, while a much simpler and quicker technique, needs to be examined for its efficiency and reliability. We also determined if fly size had any influence on adult *B. tryoni* recovery and longevity. We discuss the results in terms of the potential to use chilled adults for the sterile release of *B. tryoni* as part of a sterile insect release program.

MATERIAL AND METHODS

Production and handling protocol

Bactrocera tryoni were obtained as pupae from the Fruit Fly Production Facility at the Elizabeth Macarthur Agricultural Institute, Menangle, New South Wales (NSW), Australia. Larvae were reared on a standard lucerne chaff diet (Prabhu *et al.* 2008), and pupae were dyed with Fiesta FEX 1 Astral Pink or Stellar Green

fluorescent pigment (Swada, 30-32 Kilkenny Court, Dandenong South, Victoria, Australia) as described by Reynolds *et al*. (2010). Weekly consignments of dyed *B. tryoni* were sent as 8 day old pupae to the Australian Institute of Nuclear Science and Engineering (AINSE) facility, Lucas Heights, where they were irradiated under hypoxia at 71.3 – 75 Gy of gamma irradiation from a cobalt-60 source. The dyed, irradiated pupae were then transported by road to the Wagga Wagga Agricultural Institute (WWAI) entomology laboratory in NSW.

Study insects

Insects were reared out in a growth room at the WWAI at 26±2°C, 65±10% RH and a light:dark period of 14:10, with a simulated dawn and dusk as the lights ramped up and down at the beginning and end of the light phase. Approximately 200 sterile pupae (emergence 86-91%) were placed in a petri dish on the base of ten, 30 x 30 x 30cm mesh holding cages (Bugdorm, Taiwan). Half the cages were provided with five sugar (sucrose) cubes as a source of carbohydrate and a 30mm diameter plastic petri dish containing yeast hydrolysate enzymatic (MP Biomedical, Auburn, OH, USA; 60% protein) as a source of protein and, the other half were supplied with sucrose only. All cages were supplied with water soaked cotton wicks. Within one day of emergence, adult flies were separated according to sex and assigned immediately to a diet/chill treatment according to trial requirements (see below). Green dyed flies were assigned the protein and sucrose diet, and pink dyed flies the sucrose only diet in the first study and this was reversed in the second study.

Chilling trials

Two chill regimes using split plot designs but different randomisations were carried out on separate dates. For the chilled adults, sterile *B. tryoni* were chilled at 4°C. In trial 1 flies were chilled for 0 (control; no chilling), 0.5, 1, 2 or 4h. In a second trial, flies were chilled for 0, 2, 4, 8 or 24h. At the start of each chill regime, all flies were 48-60 h old, which is the average age at which adult *B. tryoni* are released in an SIT program in NSW.

Recovery and longevity

For each diet/chill treatment (protein, sucrose + no chill; protein, sucrose + chill; sucrose only + no chill; sucrose only + chill), 25 flies of each sex were aspirated from the holding cages and placed individually in 700ml round plastic containers (Centrapak, Australia), containing either a single sugar cube or sucrose and protein, giving a total of 200 flies. Each container was supplied with a water soaked cotton wick changed as needed. A fine mesh (25 filaments per cm) was placed over the top of each container to allow airflow and secured with two rubber bands. For the chill treatments, containers were taken to a separate growth room nearby where they were chilled for different lengths of time, as detailed above. After the designated chill time, flies were placed back under standard conditions and recovery and longevity assessed immediately.

To assess recovery, individual adult *B. tryoni* were observed every 1-3 min, and the time to recover for each individual recorded. Recovery was deemed to have occurred when the fly commenced walking or flying. To assess longevity, the same individuals were observed once daily (08.30 each morning) for 28 days for the first study or until all had died for the second study, and mortality recorded. The mean and standard error are presented. Escaped flies and unnatural death were also recorded. Flies were weighed to 0.001mg immediately upon discovery of death and frozen in individual vials (-20C). At the end of the study, the right wing of each fruit fly was mounted onto a microscope slide following Prabhu *et al*., (2008). Each wing was then photographed using a Leica DFC290 digital camera (Leica Microsystems Pty Ltd, Unit 3, 112-118 Talavera Road, North Ryde, 2113, Australia) through the phototube of a Leica MZ75 stereomicroscope. The wing images were later measured using Leica Application Suite V3.1.0 software. As

a measure of fly size, wing length was measured (mm) from the proximal edge of basal radial cell to the intersection of the costal and R4 + 5 veins on the margin (P. Gillespie pers. comm. 2009).

Flight ability

Trials for flight ability used ten mesh cages, each containing four flight tubes after (IAEA, 2003) setup without food or water in a growth room at 4°C. In each cage, two flight tubes were randomly assigned for protein and sucrose fed flies and two for sucrose only fed flies. Holding cages, containing adult *B. tryoni* were transferred to the chilled growth room. After 3 min, a total of 120 *B. tryoni* were aspirated from each holding cage and thirty randomly selected individuals placed in each individual flight tube according to diet treatment. The chill treatments were the same as described above. Control flies remained in the chilled room for less than 5 min which ensured they were immobile and could be readily aspirated into the flight tubes. Once the flies had undergone their chill treatment, the entire cage holding the flight tubes and chilled *B. tryoni* were placed back under standard rearing conditions in a replicate split plot design. Tests for flight ability were conducted immediately after chilling. Flies that left the flight tube were aspirated four times on the first day and twice a day from then on until all flies had either been aspirated or had died within the flight tube. The mean and standard error are presented. Flies were sent to the Agricultural Scientific Collections Unit, Orange Agricultural Institute, Orange, NSW, Australia, to ascertain dye colour on the ptilinum and counts of 'fliers' and 'non-fliers' were made. This procedure was initially performed under a Leica MZ 8 microscope (Leica Microsystems Pty Ltd, Unit 3, 112 -118 Talavera Road, North Ryde, NSW 2113) under a blue light source (Leica CLS 150 X light source with a blue filter), however where the presence of dye colour was difficult to detect examination of the specimen under a UV light (Leica Stereo-Fluorescence System 10446271 with a blue filter) was performed.

Analysis

All models were fitted using ASReml 2.0 (Gilmour *et al*. 2005). In all linear mixed models the significance of fixed effects was assessed using approximate F tests from the methods of Kenward and Roger (1997) and derived from a scaled Wald statistic together with an estimation of the residual degrees of freedom. Only significant fixed terms ($P<0.05$) were retained in the final model. Experiment design structures including replicate, and cages within replicate were fitted as random effects and retained in all models. Residuals from fitted models were examined for independence, normal distribution and constant variance using residuals versus fitted value plots and normal probability plots for each individual trial.

Recovery

A linear mixed model was used to model recovery time. Chill times between 0-4 h were tested in both trials, but 8 and 24 h chill time were only tested in the second trial. A factor was set up to distinguish between these times so that within and across trial estimates of a regression trend could be obtained for the 0.5-4 h (factor at level 1) chill time. Only trial specific point estimates of recovery time for the longer (8 and 24 h – factor at level 2) chill times were estimated. For the 0.5-4 h chill times, fixed effects included intercept effects of trial, diet, sex and their interactions and linear trends across chill time due to these factors and their interactions. For the 8 and 24 h treatments of trial 2, fixed effects included sex, diet, chill time and their interactions fitted as factors. Replicate and cage within replicate for each trial were estimated as random effects.

Control or 0 chill time flies had zero recovery time with standard error estimates of zero. To ensure that least significant differences (P=0.05) between positive chill times were not underestimated by the inclusion of these zero standard error estimates, control flies were not included in this analysis. Of the 200 flies in the first trial, five flies did not recover from chilling. These comprised two male and two females after 1 h chilling and one female after 2 h chilling. In the analysis, these five flies were identified as outliers

and consequently removed from the analysis. The variable analysed was thus 'minutes taken to recover for flies which survived the chilling process'. In trial 2 all flies recovered from chilling.

Longevity

Linear mixed models were used to model longevity of adult *B. tryoni* in two trials. Trial 1 was terminated at 28 d and the surviving five flies treated as missing values in the analysis. Trial 2 continued until all flies were deceased. The difference in trial management resulted in separate analysis of the trials. Fixed terms in each model included effects of diet, sex and chill time and their interactions. Replicate and cage within replicate were estimated as random effects. Data for longevity or 'days until deceased' was recorded for chilled and control flies including those flies which did not recover from chilling (0 longevity).

Weight and wing length at death

Linear mixed models were also used to model weight and wing length at death. Data from the two trials were analysed in a single model. Trial, chill time, diet and sex and their interactions were fitted as fixed effects, and the blocking structure of replicate and box within replicate were fitted as random effects. A linear mixed regression model was used to determine the relationship between wing length and weight. Since the coefficient of variation for wing length from the previous analysis was only 2.8%, wing length could be used as the independent variable. The covariate wing length plus the design factors of trial, diet, sex and chill time and all interactions of factors and factors with covariates were fitted as fixed effects in a linear mixed model. Random effects included replicate and cage within replicate for each trial.

Effect of fly size (wing length) on recovery and longevity

Bivariate models were fitted to the pairs of 'traits'; wing length and recovery and wing length and longevity. These models included all terms in the univariate analyses of recovery and longevity respectively but interacted with 'trait'. The individual trait residual variances were modelled. The data for the bivariate model relating wing length and longevity consisted of 200 records for both wing length and longevity from trial 2 and 195 records of longevity and wing length from trial 1 as 5 data points were not recorded and treated as missing.

Flight ability

A linear mixed model was used to model flight ability. A factor was set up to distinguish between (0-4 h chill time) and (8 and 24 h chill time). Intercepts and trend effects across chill time of trial, diet, sex and their interactions were estimated as fixed effects, while effects of replicate and cage within replicate for each trial were estimated as random effects.

RESULTS

Recovery

In the first trial, only five flies remained alive after the trial was terminated at 28 days. In trial 2, all flies had died by 66 days. At 0.5 to 4 h chill time, there was a significant effect of trial ($F=18.08$, $df=1, 35.8$, $P<0.001$), chill time ($F=4.54$, $df=1, 40.3$, $P=0.039$), and a trial x chill time interaction ($F=5.60$, $df=1, 70.4$, $P=0.021$) on recovery time of *B. tryoni* (Fig. 1). For trial 1 (Fig 1a), an increase in recovery time with increasing chill time was significant for the chill time range of 0.5 to 4 h ($F=5.60$, $df=1, 70.4$, $P=0.021$). For trial 2 (Fig. 1b), recovery times for 2 h chill time were higher than those for 4 or 8 h chill time. Across both trials, there was a significant difference from 0 for the chill times 0.5-4 h, where the intercept of the line was 14.4 min ($F=308.53$, $df=2,35.7$, $P<0.001$). For trial 2 recovery took longer at 24 h chill time (14.10 min) compared to 8 h chill time (10.72 min) but only at $P=0.060$ ($F=3.66$ $df=1, 70.9$).

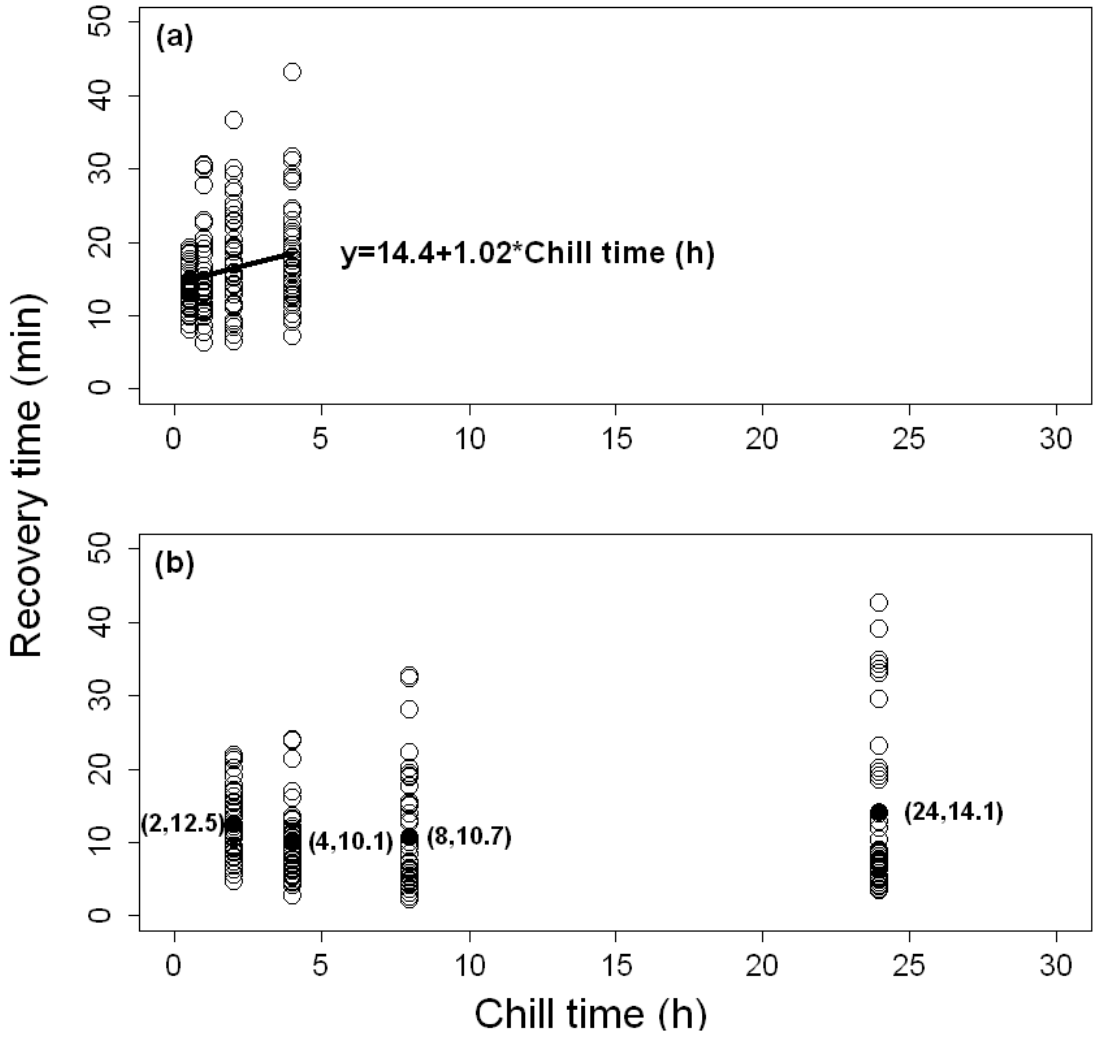

Figure 1. The recovery time for adult sterile *B. tryoni* fed either a protein and sucrose or sucrose only diet chilled at 4°C for (a) 0, 0.5, 1, 2, 4 h (trial 1) and (b) 0, 2, 4, 8, and 24 h (trial 2). Zero chill time (control) was not included in the analysis. x and y values shown in brackets (x,y) are the chill time (h) and recovery time (min) respectively.

Longevity

Trial 1 was terminated at 28 days with only 5 flies still alive at this time. Trial 2 had 66 flies still surviving at 28 days and was monitored until all flies were deceased. In trial 1 for those flies deceased by 28 days, there were no effects of chill time but there was a diet x sex interaction ($F=4.97$, df =1, 191.0, $P=0.027$, Fig. 2). This finding reflected the fact that, among sucrose only fed adults, females (12.22±1.12 days) lived longer than males (8.99±1.29 days), while protein and sucrose fed males and females (11.53±1.23 and 9.67±1.45 days respectively) did not differ in longevity from the sucrose only fed males and females. In trial 2, there was also a significant diet x sex interaction ($F=4.79$, df=1, 175.7, $P=0.030$, Fig 2) with protein and sucrose fed females (30.8±2.3 days) surviving longer than sucrose only fed females (23.37±2.11 days) and males fed either protein and sucrose or sucrose only (25.34±2.14 and 24.98±2.38 days, respectively).

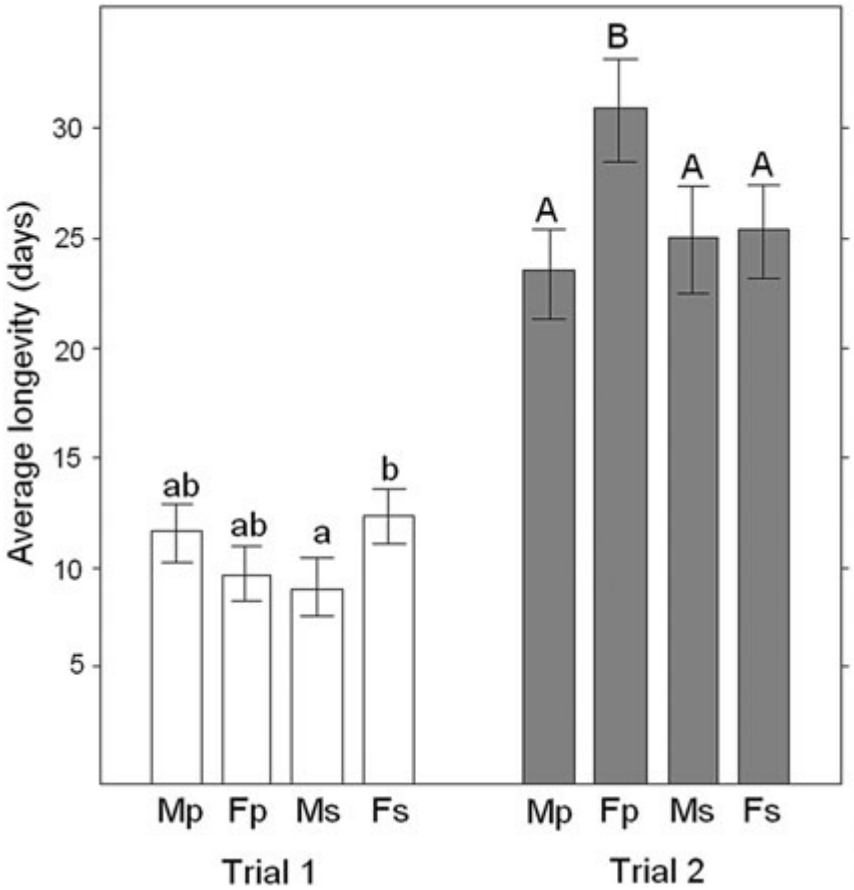

Figure 2. The average longevity of adult sterile male and female *B. tryoni* fed either a protein and sucrose or sucrose only diet for trial 1 (terminated at 28 days) and trial 2. M=male, F=female, p= sucrose and protein diet, s=sucrose only diet. Error bars are SE. Within each trial, means between columns with the same letter are not significantly different from on another (P>0.05) using Fisher's LSD test.

Weight and wing length

There was a significant effect of sex on weight (F=3.99, df=1, 383.8, P=0.047) and a significant trial x diet x sex interaction (F=7.95, df=1, 391.3, P=0.006). In trial 1, the weights of males and females fed on sucrose only or the sucrose and protein diet did not differ (4.6 ± 0.3mg), however in trial 2, males fed on sucrose only (4.1 ± 0.3mg) weighed less than males fed the sucrose and protein diet (5.2±0.3mg) and females on either sucrose and protein or sucrose only (5.0 ± 0.3mg and 5.3 ± 0.3mg respectively).

There was a significant effect of trial (F=9.83, df=1, 18.0, P= 0.006), sex (F=88.35, df= 1,376.7, P<0.001) and a diet x sex (F=6.07, df=1,384.3, P= 0.015) interaction on wing length. Averaged across trials, wing length was lowest for males, with sucrose fed males (4.46 ± 0.016mm) lower than sucrose and protein fed males (4.50 ± 0.015mm), and both of these lower than sucrose or sucrose and protein fed females (4.61 ± 0.015mm and 4.59 ± 0.016mm respectively), of which the winglength of females fed on either diet did not differ significantly.

As wing length had a coefficient of variation of only 2.8 % in the preceding analysis it was a reliable measure to use as the independent variable in a regression analysis examining the nature of the relationship between wing length and weight. Weight was considerably more variable (coefficient of variation 26.4%). In trial 1, wing length and weight were related by a single linear relationship (weight = -0.00649 + 0.00245 * wing length, Fig. 3a), while in trial 2, the relationship between weight and wing length had an identical trend but significant differences in the intercepts for sucrose only fed males (-0.00685) and protein and sucrose fed males (-0.00591, 5% l.s.d. = 0.000854; Fig. 3b). The lower coefficient of variation and the consistency of diet and sex effects in both trials on wing length, make this the preferred measure of *B. tryoni* body size.

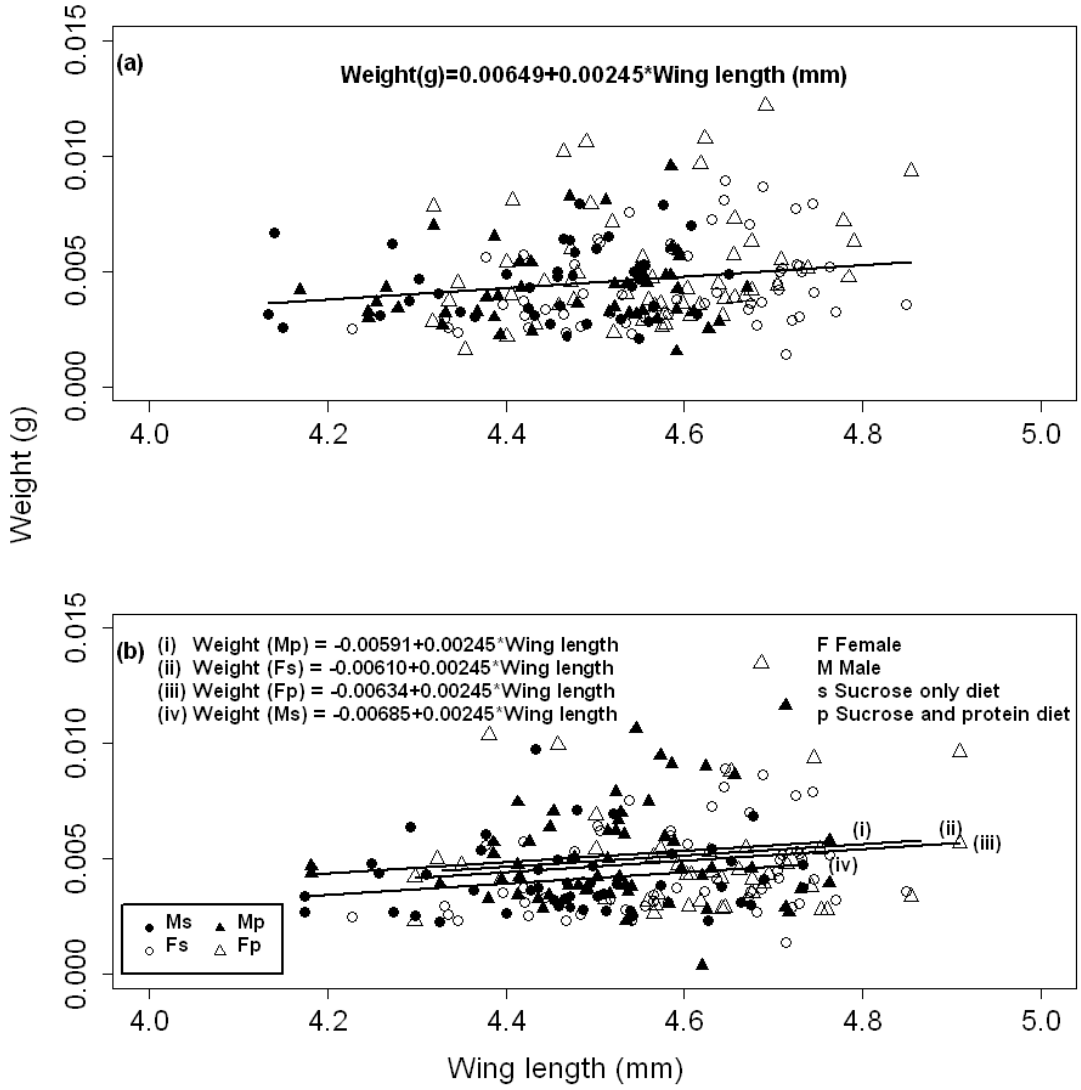

Figure 3. The relationship between weight and wing length of adult *B. tryoni* for trial 1 (a) and trial 2 (b).

Effect of fly size (wing length) on recovery and longevity

As wing length was determined to be the best measure of fly size in the analysis above, this variable was used here to determine if there was a relationship between fly size and recovery and longevity. In the bivariate model relating fly size and recovery, significant interactions included trait (i.e. wing length and

recovery means) x trial (F=18.05, df=2, 100.1, P<0.001), trait x sex (F=40.44, df=2, 392.7, P<0.001) and trait x diet x sex (F=3.20, df=2, 399.7, P=0.033). In trial 1, adult *B. tryoni* were smaller (4.52±0.01 mm) and took longer to recover (16.38±0.58 min) than in trial 2 where flies were larger (4.57±0.01 mm) and recovery was shorter in duration (11.84±0.57 min). While females had longer wing length averaged across trials and diets (4.61±0.01 mm) than males (4.48±0.01 mm), recovery times did not differ between sexes. When averaged across both trials, although there were diet and sex effects on fly size as indicated above, there were no diet and sex effects on recovery time.

In the bivariate model relating fly size and longevity, significant terms included trait x trial (F=111.96, df=2, 45.4, P<0.001) and trait x sex (F=46.78, df=2, 514.9, p<0.001). The smaller adult *B. tryoni* in trial 1 had significantly reduced average longevity (10.5±0.74 days) compared to those in trial 2 (25.9±0.73 days). Averaged across the two trials, males had reduced longevity (17.5±0.72 days) compared to females (19.5±0.72 days). The smaller adult *B. tryoni* in trial 1 (4.52 ±0.01 mm) had reduced longevity, (10.5 ±0.74 days) compared to the larger adults in trial 2, (4.56 ± 0.011 mm), which had greater longevity (25.91 ± 0.73 days). When averaged across trials and diet, the smaller male *B. tryoni* (4.48±0.01) mm also had reduced longevity (16.95 ± 0.72 days) compared with females, (19.5 ±0.72 days) which were larger (4.61±0.01 mm).

Flight ability

For the 0.5 to 4 h chill times, there was a significant effect of chill time (F=4.69, df=1, 15.0, P=0.047) and a trial x diet x chill time interaction (F=6.29, df=1, 50.7, P=0.015) on flight ability (Fig. 4). Adults fed on either diet for the 0.5 to 4 h chill time for trial 1 did not differ in their ability to take flight (mean 96.7%) (Fig. 4a). In trial 2, for the 0.5 to 4h chill time, there was no difference in flight ability among adults fed sucrose only (mean 97.8%) (Fig. 4b). However, for those fed the protein and sucrose diet the propensity for flight decreased with increasing chill time (mean flight ability: 0 chill time - 97.6%, 2 h chill time - 95.7%, 4 h chill time - 93.7%; slope -0.9736±0.29 per h) and differed from those flies fed sucrose only (Fliers (%) = 97.635-0.9736*chill time, slope SE=0.29, P<0.05).

At 8 and 24 h chill times, there was a significant effect of sex (F=6.20, df=1, 21.1, P=0.021) and a significant diet x chill time (F=15.86, df=1, 21.1, P=0.001) and diet x chill time x sex (F=5.80, df=2, 21.1, P=0.010) interaction (Fig. 4b). Females held on a protein and sucrose diet had the greatest propensity for flight after 8 h of chilling (98.39 ±1.53%) and the lowest flight propensity after 24 h of chilling (87.71 ±1.53%). Females held on a sucrose only diet displayed the opposite trend (93.33 ± 1.53% and 97.83 ± 1.53%, respectively). Males did not differ from any treatment combination except females at 24 h chill time fed on a protein and sucrose diet, which had the lowest tendency for flight.

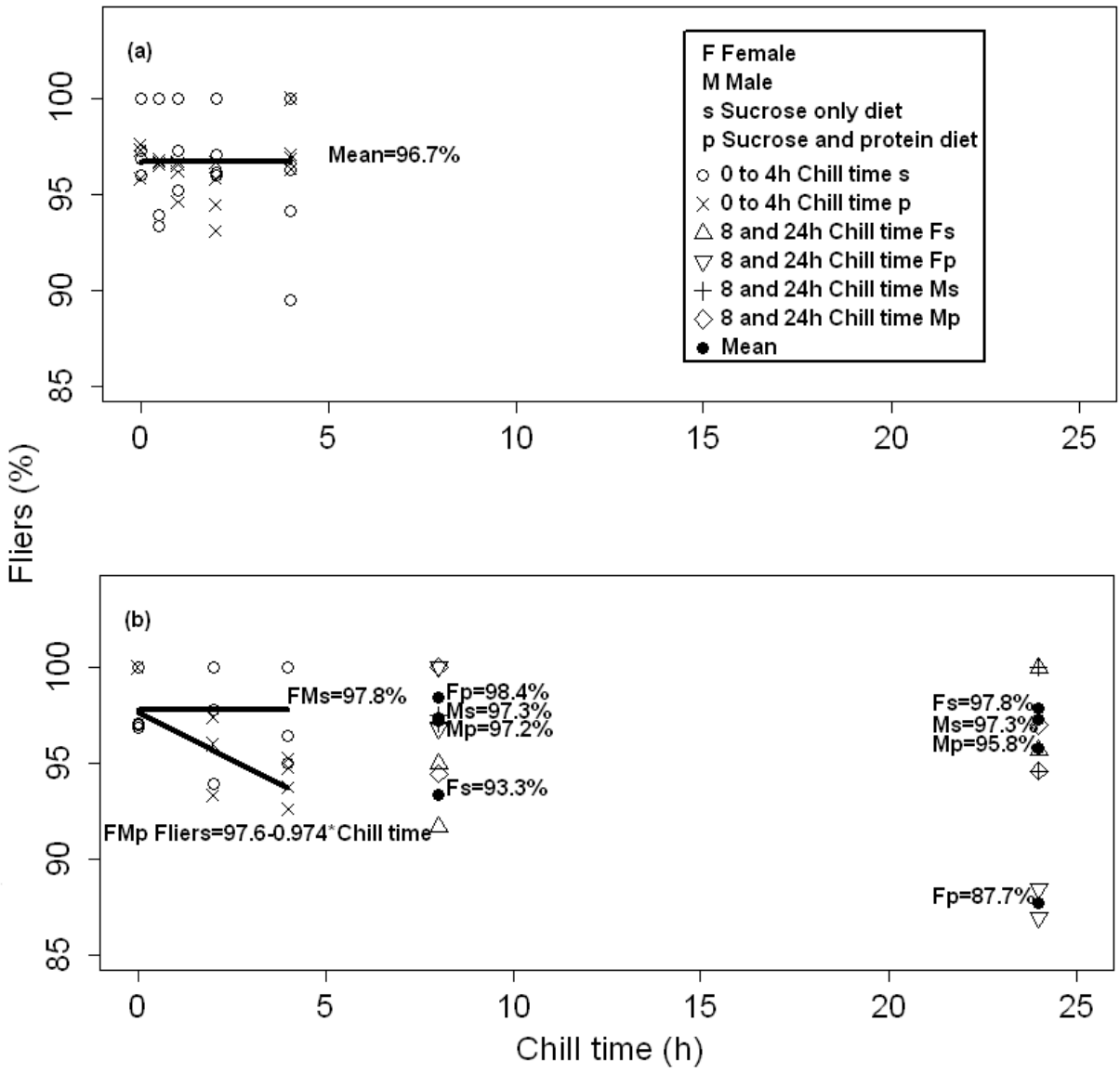

Figure 4. The percentage of sterile adult *B. tryoni* capable of escaping from flight tubes when chilled at 4°C for (a) 0, 0.5, 1, 2, 4 h (trial 1) and (b) 0, 2, 4, 8, and 24 h (trial 2).

DISCUSSION

Adult *B. tryoni* suffered few detrimental effects of chilling under the trial procedures described in this study. Neither adult *B. tryoni* survival nor flight differed substantially between chilled and non-chilled cohorts. Although recovery time increased with chill time in trial 1, our results showed that when recovery time was averaged over both trials there was no effect of chill times of 0.5 to 4 h on the time it took for the fruit flies to recover. However, chilling does introduce a recovery time that is not experienced by non-chilled flies. Nonetheless, what is apparent in all of the variables tested is that there is significant variation between trials, or batches of fruit flies. The general lack of long-term effects of chilling is consistent with the absence of noticeable effects on *Ceratitis capitata* (Wiedemann) mortality (Hooper 1970), sexual efficacy (Taylor *et al.* 2001) and, when aerially released, the propensity to take flight with good recovery

(Vargas *et al*. 1995). In addition, release of chilled sterile males of *C. capitata* results in significant reductions in wild fly infestations (Hendrichs *et al*. 1995; Vargas *et al.* 1995; Dowell *et al*. 1999).

Recovery of adult *B. tryoni* at the lower chill times (0.5 – 4h) averaged 14.4 min irrespective of the length of time spent in chilling at 4˚C (Fig. 1). Modelling by Meats & Fitt (1987) predicted that *B. tryoni* chilled at 1˚C and then warmed at 25˚C would recover within 12 min. However, the authors did not use sterile adult *B. tryoni*, but rather fertile adults, and sterility is known to lead to decreases in some fruit fly performance variables (Hooper & Katiyer 1971; Collins *et al*. 2008). In trial 2, although recovery took longer at 24 h as opposed to 8 h, these lengthy chill times are unlikely to be used in standard release programs within Australia. Most sterile insect release programs aim to chill flies for no longer than 0.75 – 2.5 h at 3-5˚C (Mangan 1996; Tween and Rendon 2007).

Chilling had no effect on adult *B. tryoni* longevity in this study. Similarly, *Ceratitis capitata* chilled at 4˚C for 0, 15, 30 or 60 min had no effect on fecundity, egg hatch (Hooper 1970) or mortality (Hopper 1970; Serghiou 1977). In trial 1, although adult females fed sucrose only diets lived longer on average than males fed sucrose only diets, longevity did not differ to flies fed on protein diets (Fig. 2). However, in trial 2 females fed protein and sugar survived the longest. Perez-Staples *et al.* (2007) also demonstrated that access to protein increased fly longevity, particularly in female *B. tryoni*. Further, the authors showed that access to a source of protein had a greater effect on the longevity of sterile flies compared with fertile flies. These results are consistent with an earlier study by Drew (1987), which showed that female *B. tryoni* require protein to mature and reproduce, while males do not. The requirement of protein for reproduction of female *B. tryoni* was supported by Meats and Leighton (2004). However, recent studies of *B. tryoni* have shown that males experience reductions in mating probability, delayed mating and shorter copulations when deprived of protein as adults (Perez-Staples *et al*. 2007; Prabhu *et al*. 2008). Adult males of the oriental fruit fly, *Bactrocera dorsalis* (Hendel) require protein to obtain matings and additionally the ingestion of methyl eugenol, which acts as a pheromone precursor, increases male attractiveness and mating competitiveness (Shelly *et al.* 2005) and can also counteract a protein lacking diet and enhance male mating success (Shelly *et al.* 2007). Male Mexican fruit flies, *Anastrepha ludens* (Loew), show no benefit from access to yeast hydrolysate as adults (Aluja *et al*. 2001a), although females do (Aluja *et al*. 2001b). While protein may be important in some aspects of sterile male *B. tryoni* performance, it may not be necessary for all performance variables. In our study, although females generally have greater longevity on either diet compared with males, females don't contribute to the overall SIT program and are infertile, however they may still cause damage when they 'sting' or excavate fruit using their ovipositors. This lends further support for the production of a male-only *B. tryoni* strain for sterile release.

In our study, fly weight was affected by both diet and sex although only in trial 2. In this trial, males fed sucrose only weighed less than males fed the protein and sucrose diet and females fed on either diet. A study on the weight of male *C. capitata* showed that males fed sucrose only weighed significantly less than males fed on a full diet of protein and sucrose (Blay & Yuval 1997). As the protein and carbohydrate diet is a more complete diet (Prabhu *et al.* 2008), and protein is particularly important for females to reproduce (Drew 1987), these results are not surprising. Our results also show that male *B. tryoni* generally have shorter wings compared with females. Females of other species of fruit fly have also been shown to be larger than their male counterparts (Jime´nez-Pe´rez & Villa-Ayala, 2006). However, within each sex, our results suggest that protein-fed flies generally have longer wings than sucrose only fed flies, although this was only significant for males and a surprising result as wing length can only be environmentally influenced at the larval stage and not after imaginal moult (Rodruigero *et al.* 2002). Small size (determined as a measure of wing length) resulting from a lack of food at the larval stage, and protein deprivation at the adult stage are both detrimental for male *C. capitata* (Blay & Yuval 1997).

Fly body size has been determined in a number of species using wing length including *C. capitata* (Blay & Yuval 1997) and *B. tryoni* (Prabhu *et al*. 2008), and using weight in *C. capitata* (Whittier *et al.*

1994) and *T. curvicauda* (Jiménez-Pérez & Villa-Ayala 2006). Using fly weight is a much simpler and quicker technique, however for *B. tryoni* the results of our study show that wing length is still the best index of fly body size (Fig. 3). Thus, we used wing length to determine if body size had any impact on both the recovery and longevity of *B. tryoni*. Although chilled adult females were larger in body size when averaged across trials and diets compared with males, recovery times did not differ between the sexes. Similar trials relating fly weight and recovery of other fruit fly species have not been done. The smaller adult *B. tryoni* in trial 1 had significantly reduced longevity compared to those in trial 2. Small size or weight has also been associated with reduced longevity in *C. capitata* (Bloem *et al.* 1994). Male *B. tryoni* were also smaller in size and had reduced longevity compared with females when averaged across both trials. Studies on the papaya fruit fly, *Toxotrypana curvicauda* Gerstaecker showed that females were significantly heavier than their male counterparts (Jiménez-Pérez & Villa-Ayala & 2006). Female weight is generally accepted as an index of potential fecundity, assuming a positive relationship between the number of oocytes in the ovarioles and the weight of the female. Thus, heavy females have more eggs available for fertilization (Tzanakakis 1989; Fay 1989; Sivinski 1993). In our study, the trial differences highlight the variation in the quality of the sterile factory-produced fly and suggests that more careful management of production parameters needs to occur in order to ensure a consistent, high quality sterile fly.

Propensity for flight was not adversely affected by chilling at the chill times in trial 1 (Fig 4a). However, in trial 2, adults fed on a protein and sucrose diet had a decreased tendency for flight as the chilling time increased (Fig. 4b). This suggests that chilling times should be kept to a minimum, particularly when *B. tryoni* are fed a protein and sucrose diet. Although males did not differ across treatments after 8 or 24 h chilling and females fed on protein and sucrose had the lowest tendency for flight overall (Fig. 4b), these lengthy periods of chilling are unlikely to be used in a sterile release program for *B. tryoni*. Studies of chilled *C. capitata* have shown a significant decrease in the flight ability of pre-chilled flies to when immobilised flies were released after 2.5h in an aerial release container (Salvato *et al*. 2003), thus confirming that flight ability is impaired when *C. capitata* remain chilled for extended periods of time.

Overall, our results suggest that chilling does not adversely affect male *B. tryoni* longevity or propensity for flight. Although recovery can take up to 15 min in chilled adults, research has shown that in order to produce anaesthesia, results are more consistent with chilled insects than those that use CO_2 (Harris *et al*. 1965). Although fly size did not affect recovery time, the smaller flies in trial 1 had significantly reduced longevity compared to the larger adults in trial 2. Importantly, though, as evidenced by the differences in *B. tryoni* performance between fly batches and sex, this study highlights the need to produce a more consistent quality factory reared sterile fruit fly and to develop and trial a male-only sterile *B. tryoni* strain. Further research is required to determine the effect of chilling on mating, including mate attractancy and copulation of *B. tryoni* as studies on other related species show varying results including reduced competitiveness of chilled male *C. capitata* (Serghiou 1977, although see Taylor *et al.* 2001), while in male *A. ludens* chilling did not affect female attractancy (Mangan 1966).

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Technical Report 7

Trap captures and larval fruit counts of the Queensland fruit fly using adult and chilled adult sterile insect release techniques

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Keywords: *Bactrocera tryoni*, cue-lure

ABSTRACT
The Queensland fruit fly, *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) is a polyphagous pest whose successful range expansion throughout eastern Australia can be attributed to several factors including climatic suitability and the expansion of its cultivated host range. Sterile releases of *B. tryoni* were carried out in two separate field trials to compare the effectiveness of adult and chilled adult releases of sterile adult Queensland fruit flies under field conditions. The first study in a single urban town, Wagga Wagga, compared the recapture rates of sexually mature sterile male *B. tryoni* in cue-lure baited Lynfield traps when released as chilled adults (roving release) and non-chilled adults (stationery release), each on two release dates. In a subsequent field study, trap capture rates of wild flies and steriles were monitored in three isolated towns that received either sexually mature sterile adult releases (Uranquinty) or sterile chilled adult releases (Lockhart) or no sterile releases, i.e. control (Cootamundra). Wild fly larval counts (from picked fruit) were also obtained in both Uranquinty and Lockhart. In Wagga Wagga, for both release 1 and 2 respectively, significantly more non-chilled flies (52.6% and 61.99%) than chilled flies (47.4% and 38.01%) were recaptured per trap. Over the duration of the trial, recapture of chilled adult flies was 4.35±0.54 % of all flies released (rate of fliers), and of non-chilled adult flies was 3.27±0.54% of all flies released. Overall, in both releases, adult flies designated for chilled adult release had lower recorded adult eclosion (except for release 2 when more chilled adults emerged) and flight compared with non-chilled adults. In trial 2, all three sites, Uranquinty, Lockhart and Cootamundra displayed a significant decline in total trapped wild *B. tryoni* numbers over the duration of the trial. In Lockhart (chilled adult release), wild fly numbers showed a significant decline four weeks after sterile release, despite both Uranquinty and Cootamundra displaying increases in wild fly numbers at this time. No estimable effect of the hotspot releases was identified. Over the entire fruit collection period, there was a significant decline in the number of live larvae and the live larvae rate per fruit in Uranquinty and for seven weeks in Lockhart (25 March – 15 May 2010), which was also reflected in the total number of larvae and the total rate per fruit. The results are encouraging; particularly for the use of chilled adult sterile release for the suppression of wild fly populations. Future studies should encompass consecutive seasons and initially target the overwintering female *B. tryoni* in late winter/early spring. This study lends further support to the use of the sterile insect technique (SIT) in towns surrounding the Fruit fly Exclusion Zone (FFEZ) to reduce the risk of transport of infested fruit into the FFEZ. It also supports the use of the SIT in isolated orchards/towns or on an area-wide management basis, to suppress or potentially eradicate fruit fly populations. Periodic or continual low-level releases of mature sterile *B. tryoni*, particularly in the late winter, spring, summer and autumn (when temperatures are above the *B. tryoni* flight threshold of 16ºC), in these situations would prove invaluable to wild fly management.

INTRODUCTION

The Queensland fruit fly, *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) originally inhabited the tropical rainforests of Australia, before its successful spread to cultivated fruit and vegetables and subsequent rise to pest status. The polyphagous nature of this pest, climatic suitability and the expansion of its cultivated host range has, among other factors, enabled its spread throughout eastern Australia. This pest is currently excluded from valuable horticultural production areas in New South Wales (NSW), SA and Victoria (Reynolds, 2010) by area wide management that relies mainly on surveillance (trapping), public education, bait spraying and the sterile insect technique (SIT) (Dominiak, 2003; Meats, 2003). The purpose of SIT is to flood the wild male fruit fly population with released sterile male fruit flies thereby minimising the possibility of wild flies mating to produce viable eggs (Meats, 1996).

There are two forms of sterile adult release commonly employed, non-chilled or 'adult' release and 'chilled adult' release. Adult release incorporates the mass-rearing of sterile flies within some form of container, provided with food and water, which is then transported to the field (usually when the flies are mature) and opened allowing the flies to freely disperse. This form of release is the most common form of release used for *B. tryoni*, usually using plastic adult rearing containers (PARCs) or bins as the preferred release method (Reynolds, 2012). Chilled adult release involves the rapid chilling of sterile flies usually at 3-5ºC for less than 1h enabling the transportation of greater numbers using more compact packaging and reductions in the space required for transportation (Mangan, 1996). Chilled adults are usually released from a moving vehicle (termed roving release), allowing the rapid dispersal of large numbers of sterile flies (Meats, 2007). Chilled adult fruit fly release is frequently used world-wide as part of an aerial release program using airplanes (Cunningham, 1980; Sivinski, 2000) or helicopters (Nakamori, 1990; Vargas, 1995) and unlike adult release, it is not commonly used in ground release programs (Fisher, 1996; Dominiak, 2000). There is scant published information of the effectiveness of the roving release of chilled adults (Salvato, 2003), and none for *B. tryoni*. Recent studies have shown that chilling does not adversely affect male *B. tryoni* longevity or propensity for flight, and although recovery can take up to 15 min in chilled adults (Reynolds, 2011), due to vast travel distances between the location of fly production and release along with the large numbers of fruit flies that need to be transported, chilled adult release may be a viable option for *B. tryoni*.

The main objective of this study was to determine the effectiveness of adult and chilled adult releases of sterile adult Queensland fruit flies under field conditions. Specifically, we compared the recapture rates of sterile male *B. tryoni* when released as chilled adults (roving release) and non-chilled adults (stationery release). In a subsequent field study, we compared wild and sterile fly abundance of sexually mature sterile male Queensland fruit flies over the peak fruit fly season (i.e. summer), until late autumn in three towns that received either chilled adults, adults or no sterile flies (control). *Bactrocera tryoni* larval counts of infested fruit in the towns that received sterile flies were also compared.

MATERIALS AND METHODS

Study insects

Bactrocera tryoni were obtained as pupae from the Fruit Fly Production Facility (FFPF) at the Elizabeth Macarthur Agricultural Institute, Menangle, New South Wales (NSW), Australia, marked with either of two distinguishable colours, Pink or Arc chrome (Fiesta FEX 1 fluorescent pigments, Swada, 30-32 Kilkenny Court, Dandenong South, Victoria, Australia). Flies were marked with 1g dye per 100g pupae (which is the standard used in the FFPF), enabling us to distinguish between treatments. Eight-day old pupae were irradiated with 70.3 – 74.5 Gy of Gamma radiation at the Australian Institute of Nuclear Science and Engineering (AINSE) facility, Lucas Heights, to render them sterile before they were transported by road to the Wagga Wagga Agricultural Institute (WWAI) entomology laboratory in NSW. Insects were reared out

in a growth room at the WWAI at 26±2°C, 65±15% RH and a light:dark period of 14:10 with a simulated dawn and dusk as the lights ramped up and down at the beginning and end of the light phase until release. Two separate trials were conducted and depending on each consignment, individual pupae weighed on average for trial 1, 11.0 (release 2) and 11.4 (release 1) mg and for trial 2, 9.85 to 11.38mg, which is within the expected range produced by the FFPF (Dominiak, 2008). When tested in all trials, sterile flies were aged 2-3 days.

Emergence and flight

For the Wagga Wagga trial (trial 1), we determined the proportion of adult flies that eclosed successfully and left each PARC for each treatment, defined as the 'rate of fliers'. To do this we sampled the '*B. tryoni* debris' from each PARC (comprising empty pupal cases, un-emerged pupae, partly emerged adults, deformed and non-deformed dead fruit flies remaining after emerged adult *B. tryoni* had been released), as described in Reynolds *et al*. (2011), for each release.

Release protocol

To determine the effect of two release methods on the abundance of mature male flies in the field, we compared trap recapture rates of released adult and chilled adult sterile male Queensland fruit flies (trial 1). In a separate trial (trial 2) to determine the effect of adult and chilled adult release on suppression of wild flies, we compared trap recapture rates of wild fly populations in three towns (Lockhart – Chilled adult sterile release, Uranquinty – Sterile adult release and Cootamundra – Control/no release) . For trial 1, at each release sterile pupae were divided into 16 x 250g lots (eight designated for each adult and chilled adult release) and for trial 2, 22 x 250g lots (11 for each adult and chilled adult release). Additional 'hotspot' releases (see below) used 16 x 250g lots (eight each for adult and chilled adult release).

In both trials, individual lots were placed inside translucent lidded plastic adult rearing containers (PARCs) (Silverlock MH 0110, colour "natural", 645mm x 413mm x 275mm high), with a 430mm x 200mm, 1mm mesh on the lid and a 150mm x 100mm, 1mm mesh on two sides of the container for ventilation. Additional resting space was provided by wedging cardboard dividers (approximately 160 mm in height), two running lengthways and five across the width of the container, to sit just above the pupal bed for adult release. Nine sugar cubes were placed on the base of each release container. For chilled adult release, pupae were set up similar to that described under adult release, except as detailed below. Nine paper bags (base 210mm x 80mm; cut to 160mm height) were placed in the base of each PARC box, to sit comfortably alongside one another, each containing 27.78g of pupae and a single sugar cube. Paper bags were stapled with a single staple at the top centre of the paper bag, with openings either side to permit eclosed adults to escape. A single strap on either end of the PARC was used to secure the lid of the PARC. A block of agar containing a mixture of white sugar, YH and water (Reynolds 2010) was placed on top of the mesh of each PARC at fly eclosion, for both adult and chilled adult treatments. On the day of release, the sterile adults to be chilled were taken from standard rearing conditions in their PARCs and placed into a separate growth room at 4°C for immobilisation for 30-40mins. Once flies were determined to be immobile via a visual inspection, the straps securing the PARC were removed. Food was also removed and discarded. The PARCs were knocked on the floor three to four times to dislodge all of the flies from the sides of the box and paper bags. The lid was then removed and the paper bags shaken to dislodge any remaining flies that were clinging onto the bags, before being discarded. Chilled flies were then packaged into individual 12 oz. paper cups (Uni Cups & Lids (Detpak) Premier Northpak, 13-15 Edinburgh Road, Marrickville NSW 2204) before placement in a polystyrene box lined on the base and top with Techni Ice (Techni Ice Australia P/L, 14 Tooyal Street, Frankston VIC 3199), separated by plastic bubble wrap from the paper cups, to ensure the flies remain motionless until release. Polystyrene boxes containing the chilled flies and adult PARCs were then transported in an air-conditioned vehicle to their respective release locations.

Trial 1 was conducted in the urban area of Wagga Wagga, NSW (35° 70' S, 147° 22' E) and included two releases, on 23 February 2009 and 6 April 2009 over eight release sites respectively. Trial 2 was conducted in three small rural towns, Cootamundra, NSW (34°63'S, 148°04'E), Uranquinty, NSW (35°19'S, 147°25'E) and Lockhart, NSW (35°22'S, 146°71'E). At the latter two towns, there were 18 releases of steriles from 25 January 2010 – 25 May 2010 over 11 release sites. Additional 'hotspot' releases (i.e. Areas (traps) displaying increased fruit fly activity in previous weeks) were conducted in both treatment towns, at eight release sites surrounding the most active traps from 4 – 25 May 2010 (i.e. four releases). The towns were selected based on similar criteria to that of Andrewartha *et al*., (1967): i) presence of wild population of *B. tryoni*, ii) a variety of summer fruits and citrus for sampling from January to June, and iii) evident isolation by distances of more than 4 km from neighbouring towns. Recaptures were made using a 400m spaced trapping grid comprising for trial 1, 20 Lynfield traps and for trial 2, 11 Lynfield traps in the treatment towns and 15 Lynfield traps in the control town (i.e. All towns held the maximum number of traps based on a 400m grid), baited with Cuelure (International Pheromones, London) and malathion (Meats *et al*., 2002), positioned at 1.5-2m height on trees. Traps were cleared weekly after each release for six consecutive weeks for trial 1 and for 22 consecutive weeks after the first release for trial 2 and fluorescent marker dyes used to distinguish between treatment and release date, were alternated between treatment types for each release. In trial 2, wild flies were trapped in Cootamundra, Uranquinty and Lockhart for 5 weeks prior to sterile flies being released and trapped at the latter two sites.

For trial 1, four release sites and for trial 2, 11 release sites were established within each trapping grid and were located at least 150 m from any trap. For trial 1, two PARCs containing adult flies and the equivalent of two PARCs of chilled adult flies (or approximately one and half 12oz. paper cups) were released at each site. For trial 2, one adult PARC was released at each release site in Uranquinty (i.e. Approx. 21,818 flies) and the same number (or approximately one and a half paper cups of chilled adult flies) were released at each site in Lockhart. In Lockhart, a method of roving release was used for the chilled flies, whereby an automated blower using a 12 volt 100mm inline marine bilge blower [6.7 cubic metres per minute capacity (235 Cu Ft Min)] fitted with a PVC pipe 50cm in length modified for *B. tryoni* releases after that of T. Black, (pers. comm. 2008) was used to release the chilled sterile flies 50m either side of the designated release point. Chilled *B. tryoni* were released into roadside vegetation from a moving vehicle at 30km/h by tipping the sterile flies into the funnel of the automated blower from the paper cups in which they were held.

Trapped fruit flies were collected and rinsed in 70% alcohol for approximately one minute to remove any excess dye. The adult fruit flies were then left to dry on a paper towel before being placed in a labelled vial. Flies were then sent to the Orange Agricultural Institute, Orange, NSW, Australia where they were observed and classified by colour (as described in Reynolds *et al*. 2012) as adult or chilled adult. The total number of flies (for each treatment group) caught in the trapping grid was calculated per release, and the mean number of recaptured flies per release was determined. Cootamundra has an average maximum in July (winter) of 12.9^{o}C and maximum in January (summer) of 31.8^{o}C, with an average elevation of 335m and receives 614.2mm rainfall per year, with peaks in both winter and summer. Lockhart has an average maximum in July (winter) of 14^{o}C and maximum in January (summer) of 32.5^{o}C, with an average elevation of 157m and receives 473mm rainfall per year, with a slight winter/spring peak. Uranquinty has an average maximum July (winter) of 12.6^{o}C and maximum January (summer) of 31.5^{o}C, with an average elevation of 199m and receives 569mm rainfall per year, mostly in the winter/spring (May-October).

Larval count (Trial 2)

Mature fruit was collected once per week (when traps were checked) from each of the sterile release study towns (Uranquinty and Lockhart). Fruit was collected within 5-250m of each trap site to ensure an even spatial distribution of fruit sampling, but was dependent on fruit availability at each collection time. At each

weekly sampling period, one to two species of mature fruit were chosen for picking, across both towns (one of which always included citrus) and was consistent across each site. Approximately 3-9 pieces of stung fruit were collected from each trap site (i.e. 33-99 in the study site), held separately in paper bags and placed in insulated cool boxes. The fruit samples were transported back to the laboratory and stored overnight at 15°C.

The fruit was dissected the next day under a dissecting microscope and the number of hatched and unhatched eggs and live/dead larvae recorded per piece of fruit. Unhatched eggs were placed on moistened black filter paper and held at 26°C for 4 days. After incubation, the eggs were examined under a dissecting microscope and the total number of hatched and unhatched eggs was recorded. Although some eggs deemed sterile may have been for some reason unviable fertile eggs, mortality is considered a minor case of non-hatching.

The number of larvae were also recorded and then placed on a carrot medium in a Petri dish inside a round plastic container (Genfac Plastics Pty Ltd, Melbourne, Australia) covered with velo voile (100% polyester) (Spotlight, Australia) and secured with two rubber bands. The fabric allowed ventilation whilst preventing escape by fruit flies. A layer (1-1.5cm) of vermiculite (Uno-Lit Grade 1, L & A Fazzini Mfg P/L, Greenacre, Australia), moistened at a ratio of 4:1 vermiculite: water, was placed in the bottom of the containers to provide a medium for pupation. Once flies had emerged, they were identified to species level.

For each piece of fruit, hatched larvae were defined as the number of larvae found in the fruit, plus the larvae that hatched after the incubation period. Any unhatched eggs after the incubation period were classified as sterile.

STATISTICAL ANALYSIS

Trial 1

Emergence and fliers

To determine the rate of fliers, an empirical logistic transformation of the form $z = \ln\left(\frac{y + 0.5}{m - y + 0.5}\right)$ with var(z) estimated using $\text{var}(z) = (y + 0.5)^{-1} + (m - y + 0.5)^{-1}$ was made, where y is the number emerged/fliers, m-y is the number of unemerged/nonfliers respectively and m is thus total pupae/total emerged respectively (McCullagh and Nelder, 1990). $\text{Var}(z)^{-1}$ was used as weights in the linear mixed model which included release, treatment (adult chilled or adult) and release x treatment as fixed effects and replicate at a release as a random effect. The residual variance at each release was estimated.

Recapture rate of Queensland fruit fly

Generalised linear mixed models (GLMMs) for binomial data with weights given by 'trap recapture total' and estimated dispersion was fitted to the number of recaptured chilled adult chilled and adult flies. The fixed effects in this model included treatment, date and treatment x date while the random effects included trap and trap x date. Since traps were located in fixed locations, tree type, tree and tree maturity effects were also included as random effects to investigate the variance accounted for by these factors but were not significant in any release. All models were fitted using Genstat 11.0 using the method of Schall (1991).

Trial 2

Suppression of Queensland fruit fly in two rural towns

A logarithmic transformation ($\log_e$(total+1)) was taken of the total number (across all traps) of wild flies for each recording date. Cubic smoothing splines were used to describe the relationship between ($\log_e$(total+1)) and days after first trapping, with models fitted as linear mixed models using the methods of Verbyla *et al.* 1999. The model included linear trend over days as a fixed effect and curvature (spl(days)) as

a random effect. Trap level wildfly capture (loge(trap wildflies+1)) over days was also modelled using cubic smoothing splines. Fixed effects included day while random effects included spline curvature over days (spl(day)), trap, trap x day, trap level curvature (trap.spl(day)) and deviations from smooth curvature due to date. For both models significance of fixed effects was assessed using approximate F tests implemented using the techniques of Kenward and Roger (1997) and derived from a scaled Wald statistic together with estimation of the residual degrees of freedom while significance of curvature was assessed by examining 0.5(1-Pr($\chi_1^2 \leq d$)) with the appropriate degrees of freedom, where *d* is the observed value of twice the difference in the log-likelihood for nested models. Traps also corresponded to a combination of treeclass, tree type and fruit maturity, and these factors and their interactions were included as random effects. At Lockhart chilled sterile fly recapture 'totals across traps' and 'trap captures' were also modelled using methods analogous to those for wildflies. Similar models were used for wild flies and sterile but unchilled flies at Uranquinty.

Larval count

Logarithmic transformations of the form $\log_e(x+1)$ were taken of the data were x was in turn the number of live larvae and the number of dead larvae, live larvae per fruit and dead larvae per fruit. Total larvae and total larvae per fruit were derived from survival and mortality models. Cubic smoothing splines were used to describe the relationships between $\log_e(x+1)$ and day of recording. Fixed effects included linear trend over days while random effects included curvature over days (spl(days)). Testing of model terms was as described above.

RESULTS

Trial 1

Emergence and Flight

There was a significant effect of release time (i.e. fly batch) on emergence (F = 53.01, df = 1,14.2, P<0.001) and a significant release time x release method interaction ((F = 9.91, df = 1,17.2, P=0.006). For release 1, more flies designated for adult release (88.69%) emerged than those for chilled adult release (86.08%) and vice versa for release 2 (adult: 92.10%; chilled adult: 93.88%). Emergence was higher for release 2, irrespective of release method. There was a significant effect of release time (F = 5.00, df = 1,19.3, P=0.038) and release method (F = 1264.94, df = 1,8.1, P<0.001) on flight (Table 1).

Table 1. The percentage of chilled and non-chilled adult Queensland fruit flies capable of flight over two releases in Wagga Wagga.

Release	Release method	Mean no. of fliers ± SE (logit)*	Fliers (%)
1	Chilled	0.6023 ± 0.0927a	64.62
1	Non-chilled	2.5851 ± 0.0828b	92.99
2	Chilled	0.3341 ± 0.1004a	58.28
2	Non-chilled	2.3169 ± 0.0920b	91.03

* Within each release , means between rows with the same letter are not significantly different from on another (P > 0.05) using Fisher's LSD test.

Effect of release method on recapture rate of Queensland fruit fly

For release 1, cue-lure baited traps recaptured more non-chilled flies than chilled flies per trap over the duration of the trial (F=248.66, df=1,62.0 P<0.001); with 52.6% non-chilled flies trapped and 47.4% chilled flies trapped. There was also a significant recapture date x release method interaction (F=19.29, df=10, 116.9, P<0.001; Table 2), with more chilled flies than non-chilled flies recaptured in week one and two

after release, and vice versa (i.e. more non-chilled flies than chilled flies recaptured) for weeks three through five. There was no difference in recapture rates of chilled and non-chilled flies for week six.

For release 2, cue-lure baited traps recaptured more non-chilled flies than chilled flies per trap over the duration of the trial ($F=1145.06$, $df=1,148.5$ $P<0.001$); with 61.99% non-chilled flies trapped and 38.01% chilled flies trapped. There was also a significant recapture date x release method interaction ($F=3.96$, $df=10,127.3$, $P<0.001$; Table 3), with more non-chilled flies than chilled flies recaptured every week for six consecutive weeks.

Table 2. The trap recapture rate of chilled and non-chilled adult Queensland fruit flies recaptured over six consecutive weeks.

Recapture week	Release method	Mean trap recapture ± SE (logit)	Trap recapture (%) (back transformed mean)
1	Chilled	0.3069 ± 0.0274a	57.61
	Non-chilled	-0.3069 ± 0.0274b	42.39
2	Chilled	0.5522 ± 0.0686a	63.46
	Non-chilled	-0.5522 ± 0.0686b	36.54
3	Chilled	-0.4503 ± 0.1091a	38.93
	Non-chilled	0.4503 ± 0.1091b	61.07
4	Chilled	-0.6039 ± 0.2099a	35.35
	Non-chilled	0.6039 ± 0.2099b	64.65
5	Chilled	-0.5028 ± 0.1819a	37.69
	Non-chilled	0.5028 ± 0.1819b	62.31
6	Chilled	0.0732 ± 0.1445a	51.83
	Non-chilled	-0.0732 ± 0.1445a	48.17

* Within each recapture week, means between rows with the same letter are not significantly different from on another ($P > 0.05$) using Fisher's LSD test.

Table 3. The trap recapture rate of chilled and non-chilled adult Queensland fruit flies recaptured over six consecutive weeks in Wagga Wagga.

Recapture week	Release method	Mean trap recapture ± SE (logit)*	Trap recapture (%) (back transformed mean)
1	Chilled	-0.3285 ± 0.0343a	41.86
	Non-chilled	0.3285 ± 0.0343b	58.14
2	Chilled	-0.4479 ± 0.0331a	38.99
	Non-chilled	0.4479 ± 0.0331b	61.01
3	Chilled	-0.4957 ± 0.0362a	37.86
	Non-chilled	0.4957 ± 0.0362b	62.14
4	Chilled	-0.5164 ± 0.0756a	37.37
	Non-chilled	0.5164 ± 0.0756b	62.63
5	Chilled	-0.6214 ± 0.0735a	34.95
	Non-chilled	0.6214 ± 0.0735b	65.05
6	Chilled	0.5241 ± 0.1436a	37.19
	Non-chilled	-0.5241 ± 0.1436b	62.81

* Within each recapture week, means between rows with the same letter are not significantly different from on another ($P > 0.05$) using Fisher's LSD test.

Overall, of the estimated 479,467 sterile flies released (rate of fliers) at the release sites (release 1: 143,077 adult and 97626 chilled adult; release 2: 149479 adult and 89285 chilled adult), 8579 chilled adult and 9788 non-chilled adult flies were recaptured. In release 1, 4268 chilled adults and 3156 non-chilled adults were recaptured with lower recorded emergence and flight for chilled adults; in release 2, 4311 chilled adults and 6632 non-chilled adults were recaptured with lower recorded emergence and higher flight for non-chilled adults compared with chilled adults. Recapture of chilled adult flies was 4.35±0.54 % of all flies released (rate of fliers), comprised of 4.37±0.76 % flies for release 1 and 4.3338±0.7602 % flies for release 2 and of non-chilled adult flies was 3.27±0.54% of all flies released, comprised of 2.18±0.76% flies for release 1 and 4.35±0.76% flies for release 2.

Trial 2

Control (Cootamundra)

At Cootamundra there was a significant decline in weekly total wild fly capture (linear trend for loge(total+1): F=28.93, df=1,16.6; P<0.001); curvilinear tend for loge(total+1): *d*= 12.27, P<0.001); Fig. 1 & 2. Trap level underlying linear trends in wild fly capture were present (linear trends at trap level: d=23.568, P<0.001) with traps initially capturing highest numbers declining at the fastest rate. There were also significant deviations from the curvilinear trend due to some dates (*d*=11.48, P<0.001). On 16 February 2010 (22 days after sterile release at Lockhart and Uranquinty) wild fly capture at Cootamundra was significantly below trend
(z = -2.52, P=0.01).

Chilled adult release (Lockhart)

At Lockhart there was a significant decline in weekly total wild fly capture over the duration of the trial (linear trend for loge(total+1): F=18.49; df=1,26; P<0.001; Fig. 1 & 3). While traps were variable, traps initially capturing the highest numbers of wild flies prior to sterile insect release all indicated a significant (P<0.05) linear decline in wild fly numbers over time. Traps low in wildfly numbers prior to sterile insect release remained constantly low. The trend in trap level wildfly numbers (loge(trap wildflies +1)) was linear (F=5.27, df=1,16, P=0.036) with significant deviations from the linear trend due to some dates (*d*=38.99, P<0.001) with flies per trap significantly higher than the overall trend on 12/1/2010, 4/2/2010 and 1/4/2010. However, two weeks after the first sterile release (25/1/2010) the number of wild flies per trap were significantly below the overall trend for three consecutive weeks (i.e. 11/2/2010, 18/2/2010 and 25/2/2010). Only citrus was well represented at trap locations with up to seven traps being located in citrus on some dates. Between 16/12/2009 and 4/2/2010 more wild flies were trapped in citrus that held immature fruit compared with citrus trees that held no fruit.

After the first sterile release, there was a steady rise in trap total sterile fly recapture numbers from 4/02/2010 until the maximum was reached on 4/3/2010 and then a smooth decline until the trial finished (linear trend: F=210.94, df=1,12, P<0.001; spline curvature: *d*=26.30, P<0.001; Fig. 1 & 3). For sterile fly recapture, traps located in citrus with mature fruit for the 3 weeks 29/4/2010 to 13/5/2010 recaptured more sterile flies than citrus with immature fruit.

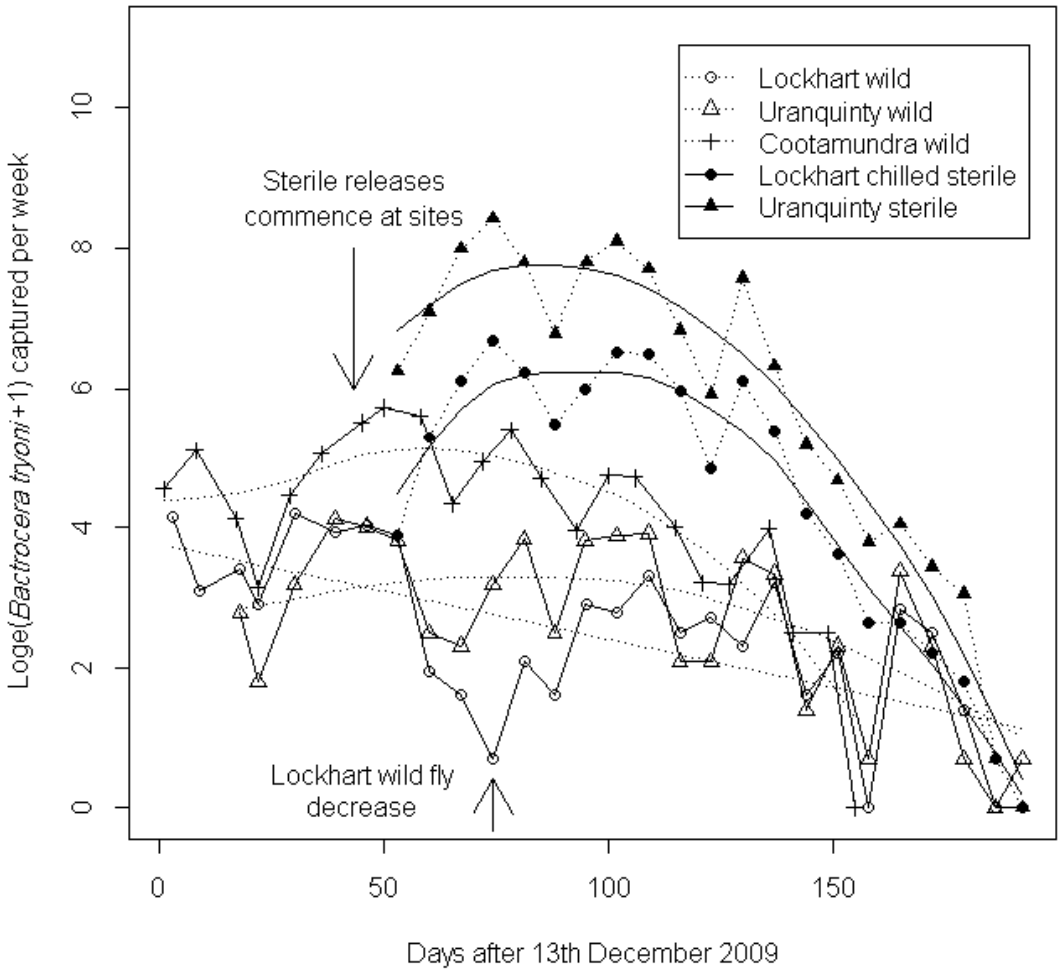

Figure 1.
Wild and sterile (chilled adult / adult) *Bactrocera tryoni* at three sites from 13th December 2009 showing trends fitted to Log_e(total trap capture +1) of over days.

Adult release (Uranquinty)

At Uranquinty there was a significant decline in total trapped wild fly numbers over time (linear trend for log_e(total+1): F=9.14, df=1,23, P=0.006; spline curvature: *d*=4.2122, P<0.001; Fig. 1 & 4). Six of the 11 traps displayed a declining wildfly capture rate (P<0.05) over the trial duration while the remaining displayed a constant capture rate. The trend in trap level wildfly numbers (loge(trap wildlies +1)) was linear (F=6.59, df=1,27, P=0.016) with significant deviations from the linear trend due to some dates (*d*=66.23,P<0.001) with flies per trap significantly higher than the overall trend on 21/1/2010, 28/1/2010 and 18/3/2010-1/4/2010. Two weeks after the first sterile release (25/1/2010) the number of wild flies per trap were significantly below the overall trend for two consecutive weeks (11/2/2010, and 18/2/2010).

Analogous to Lockhart, after the first sterile release, there was a steady rise in sterile trap recapture numbers in Uranquinty from 4/02/2010 until the maximum was reached on 8/3/2010 and then a steady decline until the trial finished (linear trend: F=1835.7, df=1,14, P<0.001; spline curvature: *d* =20.3392, P<0.001; Fig. 1 & 4).

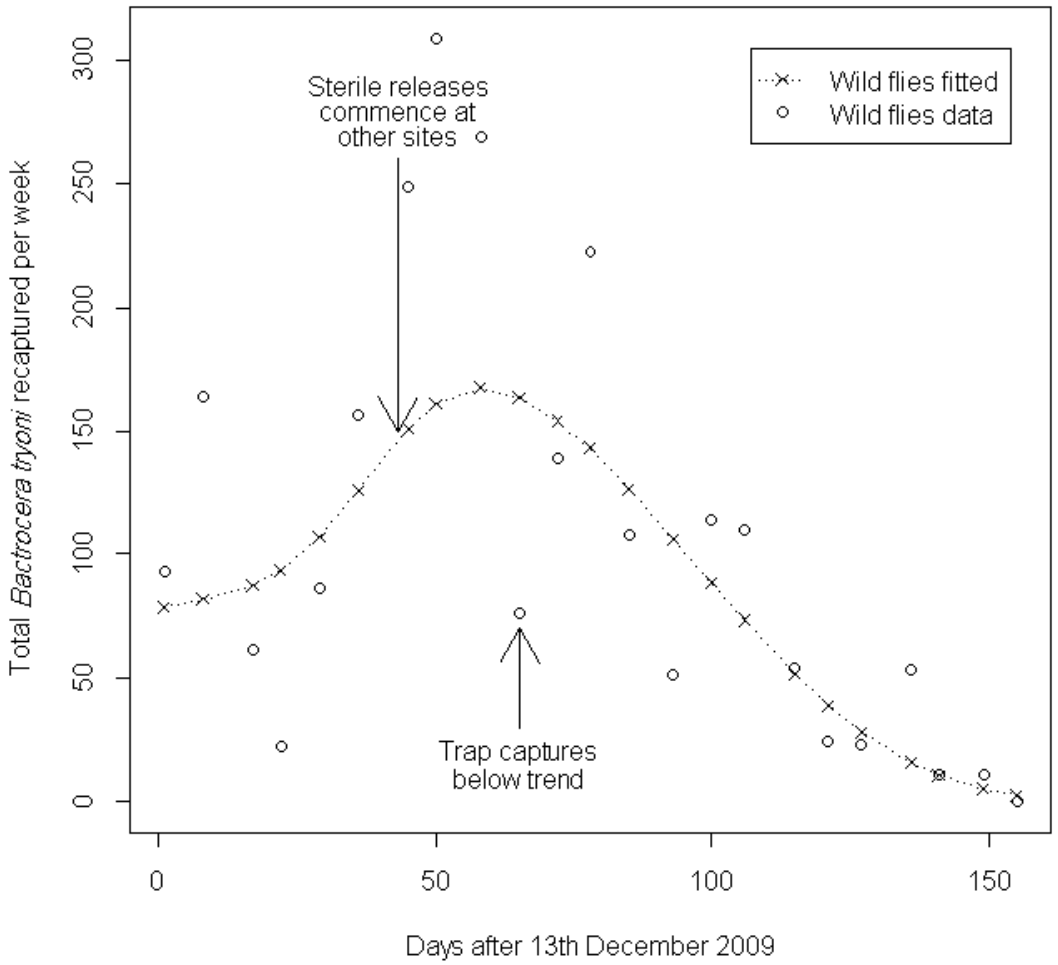

Figure 2. The total trap capture of wild adult *Bactrocera tryoni* at Cootamundra from 14 December 2009 until 17 May 2010.

Hotspot release at Lockhart and Uranquinty

Hotspot releases occurred between 4/5/2010 and 25/5/2010. In Lockhart, the rate of decline in total wildfly numbers in the two weeks prior to the hotspot releases was 0.052 and 0.074 per week and for the weeks of the hotspot release were 0.071, 0.050 and 0.019. In Uranquinty the rate of decline in total wildfly numbers was 0.035 and 0.034 in the two weeks prior to the hotspot release and 0.033, 0.032 and 0.031 in the weeks of the release. No estimable effect of the hotspot releases was identified for either town.

Overall, of the estimated 1,205,298 chilled male sterile (average 57,394 males/week) and 1,865,537 male adult sterile flies (average 88,835 males/week) released at both Lockhart and Uranquinty over the duration of the trial, 5,285 chilled adult and 24,624 non-chilled adult flies were recaptured with estimated recapture rates of 0.44% and 1.32% respectively. A total of 527 (299 in the seven weeks prior to trapping steriles, and 228 in the subsequent 21 weeks) and 567 (157 in the five weeks prior to trapping steriles, and

410 in the subsequent 21 weeks) wild flies were trapped in Lockhart and Uranquinty respectively. Lockhart had an average trap recapture ratio of sterile flies to wild flies of 10:1, while Uranquinty had a ratio of 43:1.

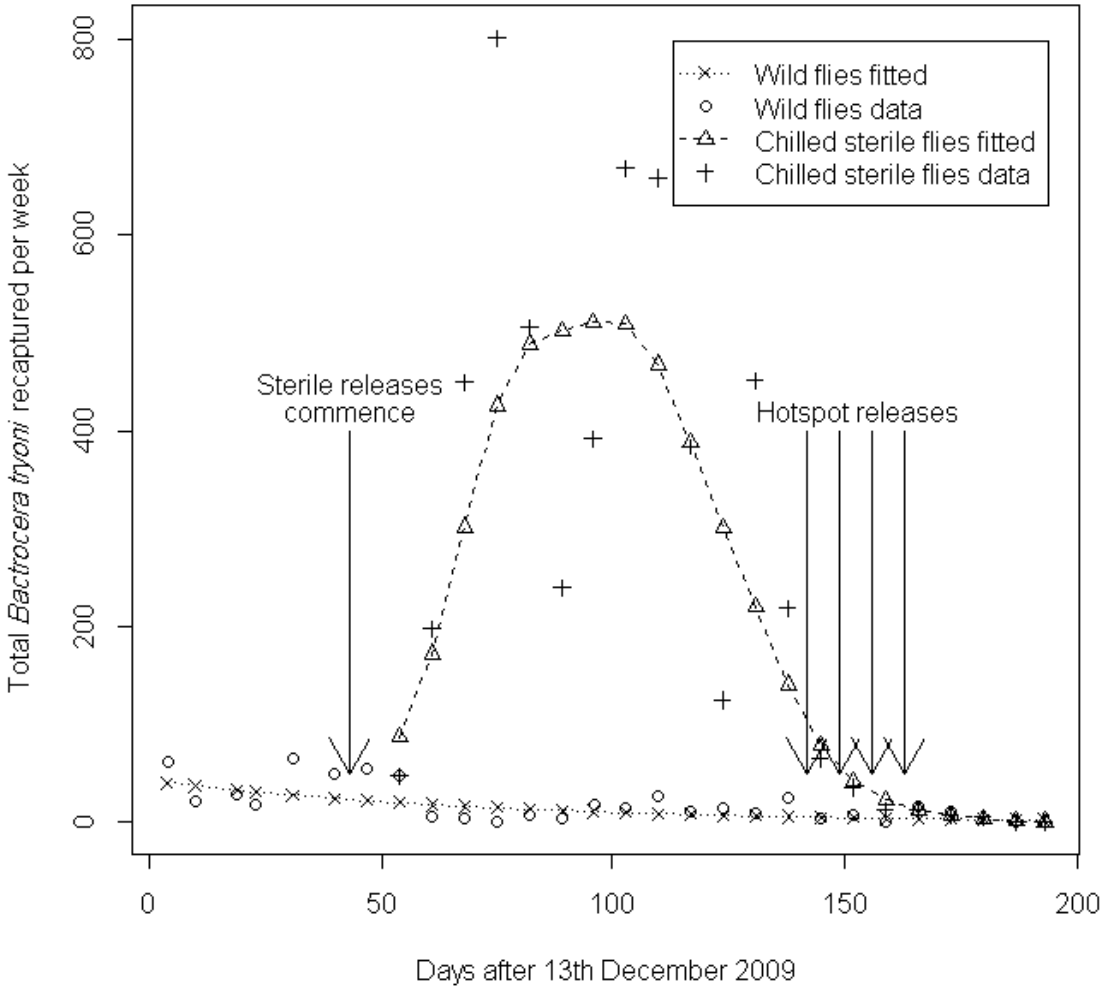

Figure 3. The total trap capture of wild and sterile chilled adult *Bactrocera tryoni* at Lockhart from 16 December 2009 when trapping first commenced until 23 June 2010 when trapping ceased.

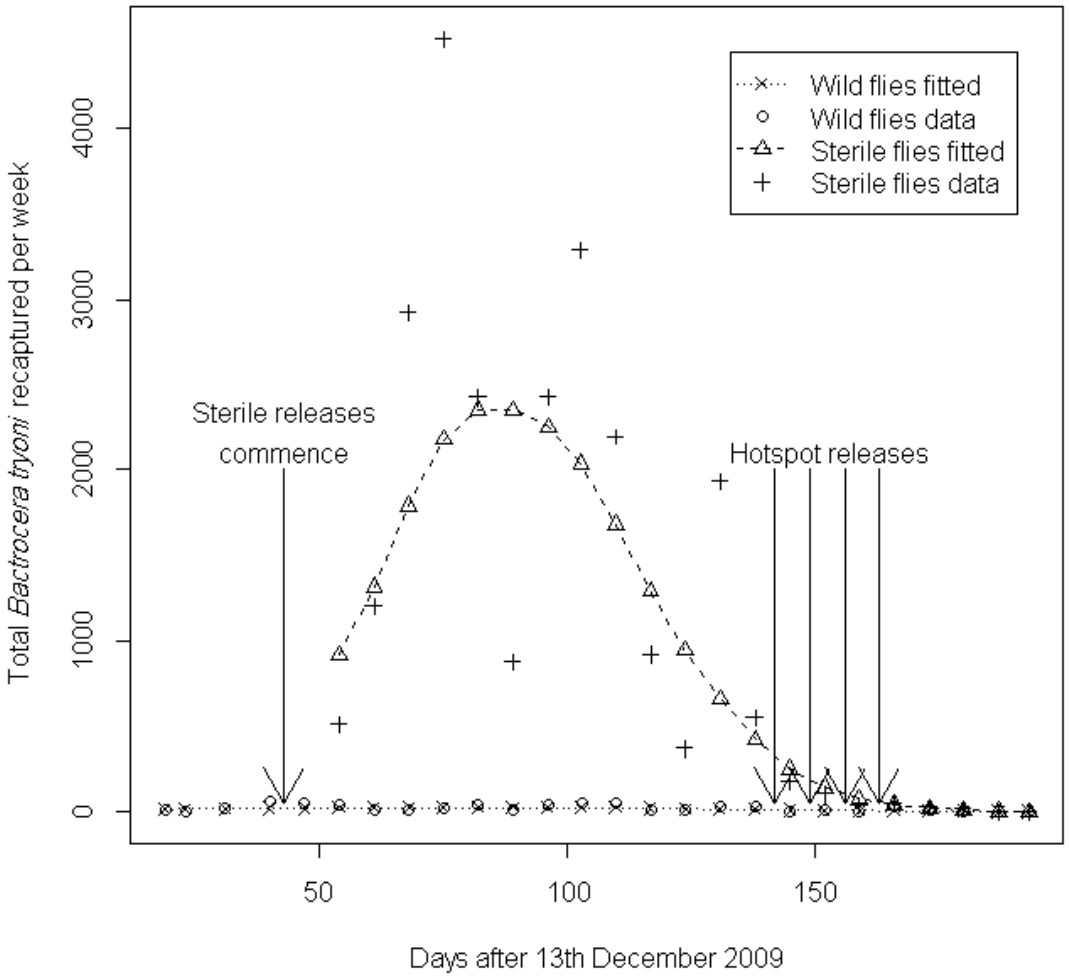

Figure 4. The total trap capture of wild and sterile chilled adult *Bactrocera tryoni* at Uranquinty from 31 December 2009 when trapping first commenced until 23 June 2010 when trapping ceased.

Larval fruit detections

At Lockhart, fruit was collected weekly from 21/1/2010 to 23/6/2010. Total larvae found over the collection period was 280 (265 live,15 dead) from 136 inspected fruit. Only 2 (total) dead larvae were found on the 21/1/2010 and then no further larvae (dead or alive) were found until 25/3/2010. No larvae were found in fruit for 3 consecutive weeks after 27/5/2010. From 25/3/2010 to 15/5/2010 there was a significant decline in the number of live larvae and the live larvae rate per fruit (Table 4a; Fig. 5). This was also reflected in the total number of larvae and the total rate of larvae per fruit. There was no significant trend in the number of dead larvae or the rate of dead larvae per fruit over time (Table 4a; Fig. 5).

Fruit were collected weekly from 24/1/2010 to 23/6/2010 at Uranquinty. Overall, 258 larvae (250 live:8 dead) from 130 whole fruit were detected. No larvae were found until 1/4/2010. All larvae found on every date were alive except for 8 dead larvae on 8/4/2010 from a total of 40 larvae from 12 whole fruit. A significant decline in the number of live larvae and the live larvae rate per fruit was observed (Table 4b; Fig. 6) which was also reflected in the total number of larvae and the total rate per fruit. No trend was found in the number of dead larvae or the rate of dead larvae per fruit (Table 4b; Fig. 6).

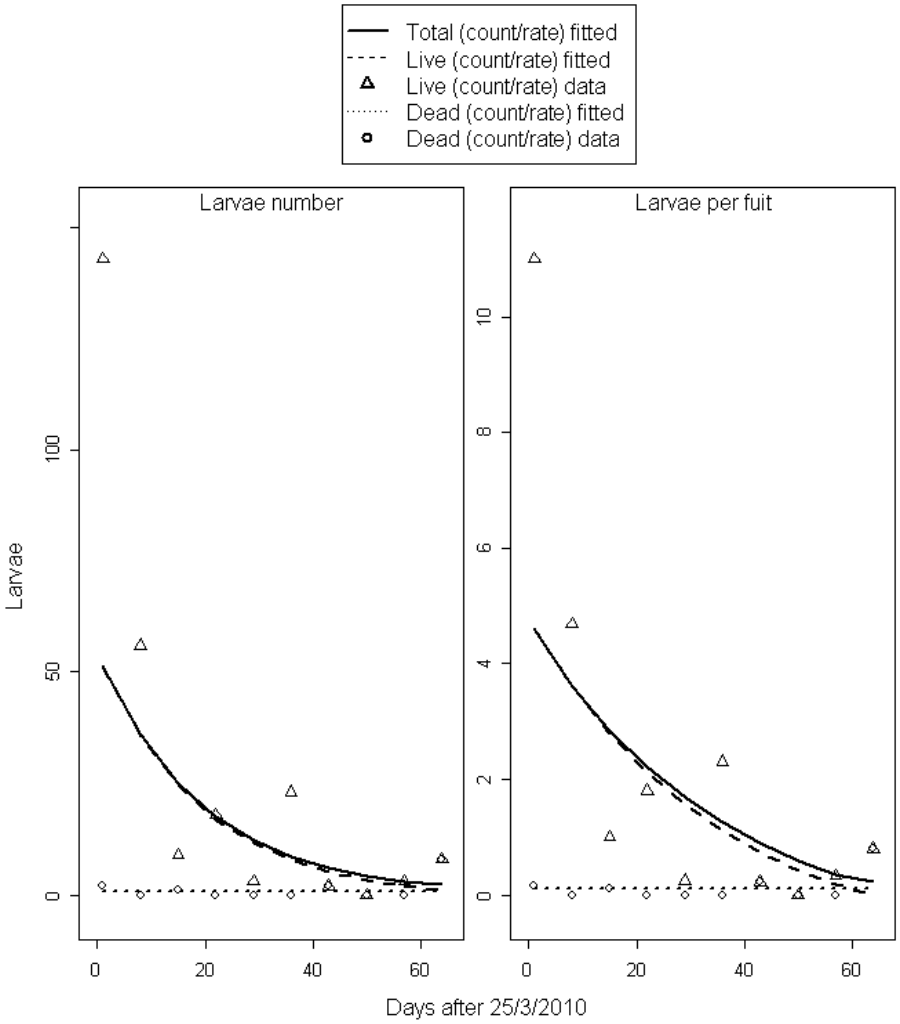

Figure 5. The trend for the number of larvae and rate per fruit for total, live and dead larvae for Lockhart. Trends were derived from back-transforming the linear relationships between $\log_e(x+1)$ and 'days' for live, dead, live rate and dead rate.

Table 4. Significance of linear trend and curvature for the regression of log(x+1) over days where x is in turn larval counts and larval rates per whole piece of fruit for Lockhart and Uranquinty.

(a) Lockhart				
	Counts		**Rates**	
Model terms	**Live larvae**	**Dead larvae**	**Live larvae**	**Dead larvae**
Constant	F=61.24, df=(1,6.2), P<0.001	F=5.80 df=(1,6.3), P=0.053	F=37.12, df=(1,5.8), P<0.001	F=4.91, df=(1,5.8), P=0.069
Linear trend (Days)	F=11.88, df=(1,6.2), P=0.014	F=0.63, df=(1,6.3), P=0.457	F=16.98, df=(1,5.8), P=0.006	F=2.28, df=(1,5.8), P=0.182
Curvature Spl(days)	*d*=0.7498, P>0.05	*d*=0.8566, P>0.05	*d*=1.7274, P>0.05	*d*=1.065, P>0.05
Model fit r^2	47.2%	-	51.1%	-
(b) Uranquinty				
	Counts		**Rates**	
Model terms	**Live larvae**	**Dead larvae**	**Live larvae**	**Dead larvae**
Constant	F=64.65, df=(1,5.4), P<0.001	F=1.48 df=(1,7.0), P=0.263	F=61.72, df=(1,4.6), P<0.001	F=1.48, df=(1,7.0), P=0.263
Linear trend (Days)	F=6.96, df=(1,5.4), P=0.046	F=1.42, df=(1,7.0), P=0.272	F=12.83, df=(1,4.6), P=0.016	F=1.42, df=(1,7.0), P=0.272
Curvature Spl(days)	*d*=0.3658, P>0.05	*d*=0.0, P>0.05	*d*=1.3319, P>0.05	*d*=0, P>0.05
Model fit r^2	37.2%	-	45.8%	-

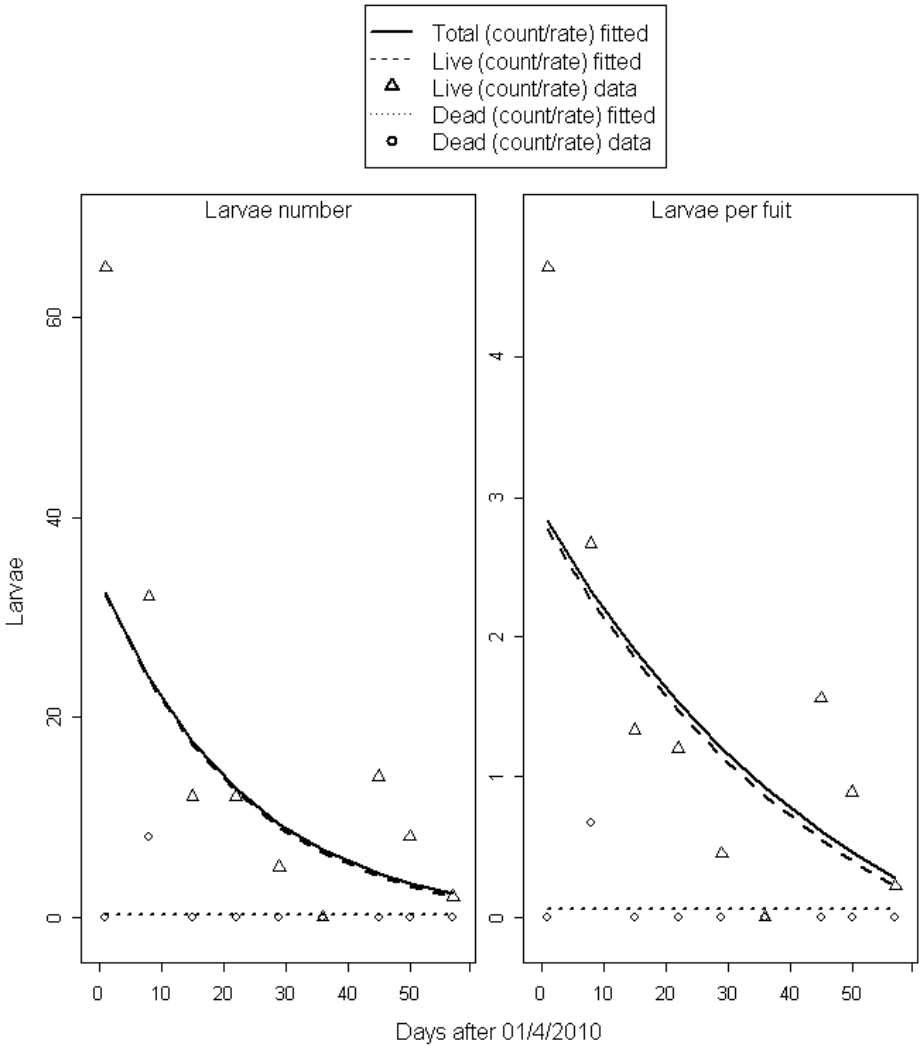

Figure 6. The trend in number of larvae and rate per fruit for total, live and dead larvae for Uranquinty. Trends were derived from back-transforming the linear relationships between $\log_e(x+1)$ and 'days' for live, dead, live rate and dead rate.

DISCUSSION

The current study demonstrates that suppression of wild fly populations in urban towns may be feasible through both adult and chilled adult release. In the single town study (trial 1), trap recapture rates of chilled adult flies were higher than adults. Although declines in wild populations were evident for the treated and the control town in trial 2, after four weeks Lockhart (chilled adult release) showed a significant decline in wild fly numbers that was not evident in the adult release or control town, and coincided with the highest number of sterile recaptures. Although the lack of replication of treatments for trial 2 means that only cautious conclusions can be drawn about the advantages of the distinct treatments, the results are encouraging.

Although overall more adult flies were released compared with chilled adult flies in trial 1, recapture rates of chilled flies was higher. Methods to entice the chilled adult flies out of the paper bags or

other methods to separate the adult flies from the pupal debris should be considered in the future to maximise the release of viable flies. Separation of adult flies from pupal debris is pertinent in fruit fly free areas, where pupal debris must be discarded appropriately after adult release to exclude the chance of poorly dyed or undyed adults eclosing in such areas. There are no published studies of validated methods comparing recapture rates of adult and chilled adult sterile release for any tephritid species.

There have been few studies that have demonstrated successful suppression or eradication, often due at least in part to release rates of sterile flies that were largely inadequate. For example, James (1996) estimated they released 362,433 sterile males per week at Cowra, NSW (approx. 4-5 km^2), and observed wild males per trap rise while the sterile/wild ratios fell lower. The authors indicated that this number would be adequate to suppress 7,200 wild flies with an overflooding ratio of 100:1 (steriles: wild), however the wild fly population was believed to be 10-100 times this size. A couple of exceptions include Warren, NSW and Perth, Western Australia. In Warren (approx. 2 km^2), an average of 0.12 million steriles per week were released in an orchard from October through to April (Andrewartha, 1967). Although it was thought that flies from surrounding orchards may have been a factor contributing to overall poor control, in October 0.3 million were released which appeared effective (Andrewartha, 1967). Similarly, in this study significant suppression of the wild fly population was observed in Lockhart four weeks after chilled adult sterile release commenced, which coincided with the largest number of recaptured sterile flies at this time. The successful eradication of a *B. tryoni* incursion in Perth (100 km^2 before the campaign began which had spread to 270 km^2 before releases began) from 1989-90 used intensive lure blocking, bait spraying and sterile releases at the rate of 26,471 to one (or an *effective* ratio of 125:1; ratio calculated only from those traps catching both wild and sterile males, not those trapping steriles only (see Meats 1996)) to 5,471 to one (or an *effective* ratio of 28:1).

Hotspot releases occurred after several weeks of increases in wild fly captures, although no estimable effect of the hotspot releases was identified. Hotspot releases are often used to target areas where fruit fly numbers have peaked (Harris, 1986), and although a decrease in wild fly numbers was not observed, such releases may have prevented an increase in wild fly numbers.

The decreases observed in detection of live larvae in both Uranquinty and Lockhart are encouraging, although without reciprocal data from the control town it is difficult to say whether this was due to the decline in wild flies observed in each town (and hence expected decreases in larvae), or if it was indeed due to the impact of sterile males. Shelly *et al*. (2007) recognised in their study given the low sterile: wild ratios and similar declines in wild fly numbers in the treated and control plots that the impact of released sterile males would not be evident through a reduction in the wild fly population, but rather from an increase in the occurrence of sterile eggs.

Andrewartha *et al*. (1967) demonstrated that a ratio of 19:1 (steriles: wild) in early spring may ensure that larval populations do not increase during the peak of the fruit fly season (i.e. summer) in isolated towns in NSW. Meats (1996) later indicated that in towns with a wild population of 20 males or lower per km^2, that a minimum release rate of 60,000 sterile males per week would be sufficient to control a wild population. James (1996) also indicated in their studies that starting the releases in mid spring, as opposed to late winter/early spring, thus targeting overwintering females, may have resulted in the sterile releases missing the first and most crucial generation of post-winter offspring. While in this study, our aim was for the rates which Meats (1996) proposed (i.e. 60,000 sterile males/$km^{2)}$, this was not achieved due to lower than expected emergence and flight (particularly for chilled adults) throughout much of the trial. We also only started sterile releases in early summer. Despite this our results are encouraging, particularly for the use of chilled adult release.

While the majority of studies reported to date have used teneral adults, the present study used YH supplemented adults. Meats (1996) postulated that releasing one mature fly is equivalent to at least 10 teneral flies (as mature flies disperse more slowly than immature flies ((Fletcher, 1974 #375)). Teneral flies

can take 1-2 weeks to mature and many are believed to die or leave the area before they mature (Meats 1996). Therefore, releasing a minimum of 6,000 mature sterile male flies per week per km^2 could be feasible and more cost-effective than using immature adults (Meats 1996). However, a recent study by Reynolds *et al*. (see Ch 4), which fed marked sterile Queensland fruit flies on two diet regimes, YH-supplemented or YH-deprived, showed that YH supplementation resulted in higher abundance of sterile male *B. tryoni*, with 1.2 YH-fed flies trapped for every YH-deprived fly trapped. Based on this and the results of the present study which suggest that an average of 28,700 chilled adult males/ km^2 and 44,400 adult males/km^2 , was not adequate to achieve ongoing suppression, perhaps a more conservative number of 50,000 mature sterile male flies per week per km^2 may be viable.

It is feasible that if monetary constraints and lower than expected release rates did not prevent it, that several seasons of sterile releases, commencing in the early spring when overwintering populations start to take flight and reproduce, could provide continual suppression or indeed eradicate wild fly populations in these and similar towns. Such ongoing studies would not only demonstrate long term suppression or even eradication, but towns could readily be maintained in this state, while requiring only small numbers of mature sterile flies when incursions occur, or ongoing low-level releases (Andrewartha, 1967; Bateman, 1991, Reynolds *et al*. unpubl. data).

In this study despite higher recapture totals of adult flies in trial 1 compared with chilled adult flies, a greater proportion of chilled adult flies were recaptured, suggesting that chilled adult release is a viable method of release, although issues pertaining to the number of viable adults released need to be overcome. In trial 2, despite significant declines in trapped wild flies for both adult and chilled adult release in Uranquinty and Lockhart respectively, a similar trend was evident in the control town. Notably however, there was a significant decline in wild fly numbers in Lockhart, four weeks after steriles were released, which coincided with the peak number of sterile males trapped, despite increases in both Uranquinty and the control town. Although we need to be cautious in our conclusions as sterile release started late in the season and only one seasons data is presented, results from chilled adult sterile release are encouraging. It would be valuable to complete further studies over consecutive seasons to determine if these results are consistent, and if levels of suppression can be maintained. Periodic or continual low level releases of mature sterile *B. tryoni*, in isolated orchards/towns or on an area-wide management basis, would prove invaluable to wild fly management in both regions within and bordering pest free zones and also in endemic areas.

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Technical Report 8

Adult, chilled adult and pupal field release for the control of the Queensland fruit fly, *Bactrocera tryoni* (Froggatt) using the sterile insect technique

O.L. Reynolds and B. Orchard

INTRODUCTION

The Queensland fruit fly, *Bactrocera tryoni* (Froggatt) is native to Australia, where it is thought to have originated in subtropical and tropical rainforest habitats along the east coast from Cape York to southern New South Wales (NSW) (Meats, 1981). With the introduction and spread of cultivated horticultural crops, this polyphagous pest soon broadened its host range and now attacks most fruit and fruiting vegetables (Hancock *et al*., 2000). The adults oviposit into mature ripe fruit and the developing larvae feed on the flesh of the fruit rendering it unmarketable. The sterile insect technique (SIT) is a form of biological control whereby a large number of irradiated, sterile insects are released to flood the wild population resulting in non-viable matings, leading to an overall wild population decline (Gurr & Kvedaras, 2009). In NSW, Victoria and South Australia, sterile *B. tryoni* are often used to eradicate outbreaks in pest free areas. There are three established release techniques for *B. tryoni*. These are adult release (Reynolds, 2011; Reynolds & Orchard, 2010; Reynolds *et al*., 2012), chilled adult release (Reynolds, in press; Reynolds & Orchard, 2011) and pupal release (Reynolds *et al*., 2010), however the effectiveness of the techniques have not been compared in the field. This study compared trap recapture rates of all three techniques in an urban town in inland NSW. In addition, egg sterility and larval counts were recorded for each release treatment over the duration of the trial.

MATERIALS AND METHODS

Study site

Field releases were conducted in an urban town, Wagga Wagga, NSW (35° 13' S, 147° 37' E) in inland New South Wales, Australia from August – December 2009. Three separate plots were established, each randomly designated the adult release, chilled adult release or pupal release plot. Each plot was separated by at least 1km, although host plants were present in the areas between the plots. The area has an average winter maximum of 12.7ºC and minimum of 2.7ºC and summer maximum of 31.6ºC and minimum of 16.4ºC, with an average elevation of 240m and receives 578mm rainfall per year, mostly in the winter and early spring (June-October). In addition to the study plots, we also collected fruit from a separate area approximately 1km from the plots to assess natural levels of egg hatch. This area was located in the same town and had similar environmental conditions as the study plots but received no releases of sterile fruit flies.

Production and handling protocol

Bactrocera tryoni were obtained as pupae from the Fruit Fly Production Facility at the Elizabeth Macarthur Agricultural Institute, Camden, New South Wales, Australia. Larvae were reared on a standard lucerne chaff diet (Prabhu *et al.* 2008) and pupae were dyed with Fiesta FEX 1 Astral Pink fluorescent pigment (Swada, 30-32 Kilkenny Court, Dandenong South, Victoria, Australia) (on all but one date; see below) as described in Reynolds *et al*. (2009). Weekly consignments of dyed *B. tryoni* were sent as 8 day old pupae to the Australian Nuclear Science and Technology Organisation, where they were irradiated under hypoxia at 70.2 – 74.8 Gy of gamma irradiation from a ^{60}Co source.

The dyed, irradiated pupae were then transported by road overnight to the Wagga Wagga Agricultural Institute (WWAI) laboratory in NSW. For the pupal release, we weighed 750g of pupae (1g of pupae equals approximately 100 pupae) in open plastic bags inside cartons. The weighed pupae were transported on the same day to the release site (12 release sites) and one carton was emptied in each individual pupal release box and covered with moistened vermiculite following the protocol of Reynolds *et al*. (2009) (i.e. 12 release boxes, each holding 750g pupae = 9000g pupae total per release). For the adult release we weighed and placed 250g of pupae in each individual PARC, containing cardboard inserts to provide resting area for the adult *B. tryoni*. For the chilled adult release, we weighed 27.8g of pupae in paper bags, and placed nine bags in each PARC box (i.e. 250g total). For the adult release, pupae were

placed in a total of 36 PARC boxes, likewise for the chilled adults for each release (i.e. 9000g pupae). For three weeks (weeks 3, 4 & 8) fewer pupae were received and the total number of pupae for each treatment was adjusted (Week 3, adult: 4500g pupae, chilled adult: 4500g pupae, pupal: 4500g pupae; Week 4, adult: 6666.6g pupae, chilled adult: 7406.4g pupae, pupal: 6666 g pupae). Adults and chilled adults were placed in an insect growth room at 26±2°C, 65±10% RH and a light:dark period of 14:10, with a simulated dawn and dusk as the lights ramped up and down at the beginning and end of the light phase until emergence. In the growth room, the majority of adult emergence occurred on days 3 & 4 after pupal placement, and varied depending upon temperature for the pupal release. Emerging sterile fruit flies were fed a block of sugar-yeast- agar (Reynolds *et al*. 2009) placed on top of each solid pupal box lid for the pupal release, and on the mesh opening on top of the PARC box for adult feeding, and was replaced as needed. Eighteen sugar cubes were also placed on the base of each pupal release and PARC box and two in each paper bag for chilled adult release.

Release protocol

Adult *B. tryoni* were released once a week between August and December 2009 (i.e. 8 releases). For pupal release, a single box containing pupae was located at each release site, situated on release platforms similar to those described in Reynolds *et al*. (2009). Adult *B. tryoni* designated for chilled adult release were taken from standard rearing conditions and placed into a separate growth room at 4°C for immobilisation for 30-40 mins. Once flies were determined to be immobile via a visual inspection, the straps securing the PARC were removed and food removed and discarded. The PARCs were then knocked on the floor three to four times to dislodge all of the flies from the sides of the box and paper bags. The lid was then removed and paper bags shaken in the PARC to dislodge any remaining flies that are clinging onto the bags, before discarding the bags. Chilled adult *B. tryoni* were then packaged into 12 oz. paper cups (Uni Cups & Lids (Detpak) Premier Northpak, 13-15 Edinburgh Road, Marrickville NSW 2204) using a funnel, before placing them in a polystyrene box lined on the base and top with Techni Ice (Techni Ice Australia P/L, 14 Tooyal Street, Frankston VIC 3199), separated by plastic bubble wrap from the paper cups, to ensure the flies remain motionless until release. Flies for adult and chilled adult release were transported in an air-conditioned van to their respective release plots, a 10-15 min drive from the WWAI facility.

In each plot we designated 12 evenly spaced release sites, which were used throughout the entire study. Pupal release boxes were placed at each release site weekly and remained there until adults were no longer observed emerging from the release box. Three PARC boxes of sterile *B. tryoni* were released at each release site for adult release and the equivalent for chilled adult release. For adult release, PARCs were placed on the ground and opened and the flies allowed to disperse without coercion. After 10-15 minutes, the PARCs were agitated and a feather duster brushed gently over the box and cardboard inserts to remove the remaining flies. The remaining pupal debris was taken back to the laboratory and discarded. For chilled adult release, roving release was used. Flies were tipped from the paper cups in which they were held into an automated blower using a 12 volt 100mm inline marine bilge blower [6.7 cubic metres per minute capacity (235 Cu Ft Min)] fitted with a PVC pipe 50cm in length modified for *B. tryoni* releases after that of T. Black. This was done from the passenger side of a slow moving vehicle over a 100m distance (i.e. 50m each side of the release point) into roadside vegetation.

Trapping grid

To estimate the relative abundance of sterile and wild *B. tryoni*, we placed 20 Lynfield traps baited with cuelure (International Pheromone Systems, Cheshire, UK) and malathion (Meats *et al.* 2002) spaced at 400 m intervals in a 5x 4 grid layout in each study plot in Wagga Wagga.

In addition to the traps placed within the sterile release sites, we operated 6 traps per week in an area separate to the release plots to assess populations trends of wild flies in an area that did not receive any releases of sterile *B. tryoni*. Estimates of abundance for wild *B. tryoni* in then separate area were made following the procedure described above.

The traps were also used to determine whether the sterile males moved between the study plots, therefore potentially confounding data interpretation. On one release date (week 8) we released the pupal, adult and chilled adult sterile *B. tryoni* dyed with three different fluorescent colours, Fiesta FEX 1 stellar green, arc chrome and flame orange respectively. In subsequent weeks, traps were checked to determine if there had been movement between the treatment plots. Movement was not deemed to have occurred between treatment plots after no incompatibly dyed flies were detected in any plot.

Captured flies were collected weekly. Flies were rinsed in 70% alcohol for approximately one minute to remove any excess dye and left to dry on a paper towel before being placed back into a labelled vial. They were then sent to the Orange Agricultural Institute, Orange, NSW, Australia where dyed and undyed wild *B. tryoni* were identified by observing dye colour in the ptilinal fissure (Norris 1957; Steiner 1965) of each fruit fly.

Egg sterility and larval counts

Mature fruit was collected once per week from each of the treatment plots and the separate site where no sterile *B. tryoni* were released. In each plot, the majority of fruit was collected within 150m of the trap site in an attempt to ensure an even spatial distribution of fruit sampling, however this was influenced by the access to fruit trees in the urban environment. At each weekly sampling period, two species of mature fruit were chosen for picking, across all sites. Approximately 1-20 pieces of infested fruit were collected per site (Table 2), held separately in paper bags and placed in insulated cool boxes. The fruit samples were transported back to the laboratory and stored overnight at 15ºC. The fruit was dissected the next day under a dissecting microscope and the number of hatched and unhathced eggs recorded per piece of fruit. Unhatched eggs were placed on moistened black filter paper and held at 26ºC for 48h. After incubation, the eggs were examined under a dissecting microscope and the total number of hatched and unhatched eggs were recorded. Although some eggs deemed sterile may have been unviable fertile eggs, as demonstrated below the egg hatch from the untreated area was relatively high, suggesting mortality was a minor cause of hatching failure.

For each piece of fruit, hatched larvae were defined as the number of larvae found in the fruit, plus the larvae that hatched after the incubation period. Any eggs unhatched after the incubation period were classified as sterile.

As not all fruit picked had fruit fly larvae, at least one piece of fruit fruit per site was dissected and found to contain at least one egg or concealed larvae on most occasions. Although the aim was five or more pieces of fruit, infested fruit was sometimes very difficult to locate and consequently infested fruit was not detected on all dates at all locations.

Statistical Analysis

A logarithmic transformation of wild fly number (x) per trap per week of the form ln(x+1) was taken. The transformed data was modelled using linear mixed models in ASReml 2.0 with treatment grid, week and treatment grid x week as fixed effects and trap as a random effect. The residual variance for each week was also modelled. The requirement that treatment grids be physically separate meant that only pseudo-replication (at the trap level) was practical and it is further recognised that traps within a grid would be correlated so they are not independent replications. The results are reported within the bounds of these limitations. A similar model was used to model ln(sterile flies +1) for the adult, chilled adult and pupal

release grids for the weeks after release. The control grid caught zero steriles and was not included in the model.

A generalised linear mixed model with logit link function was fitted to the binomial variable presence/absence of fertile eggs. The model included fixed effects of week with random effects of trap, trap x week and tree attributes, including tree family (citrus, stone, etc), species (orange, lemon, plum etc) and a unique tree identification number. Dispersion was estimated. Similar models were used to model presence/absence of sterile eggs, larvae and pupa for each grid. For the weeks when eggs/larvae/pupa were present in a grid, generalised linear mixed models were fitted to counts assuming a Poisson error distribution and using a logarithmic link function. Only those weeks in which non-zero counts were obtained were included in models; results are therefore proportions only and counts were averaged over only those weeks when fertile eggs were present.

RESULTS AND DISCUSSION

Sterile releases

Overall, based on the average pupal weight for each date (data not shown), a total of 17 944 000 pupae were used (2.52 million, 2.43 million, 1.20 million, 1.90 million, 2.48 million, 2.43 million, 2.53 million and 2.45 million for respectively releases 1 to 8). In each treatment plot, adult, chilled adult and pupal release, 5.94 million, 6.03 million and 5.97 million pupae were released respectively (Table 1).

Table 1. The estimated number of pupae, based on the average pupal weight at each release date for sterile *B. tryoni* released from 13th October 2009 to 1st December 2009.

Release	Changes to protocol	Adult plot (pupae)	Chilled adult plot (pupae)	Pupal plot (pupae)
1	As per methods	838 770	839 441	838 770
2	As per methods	810 811	811 459	810 811
3	Half required pupae received. All treatment plots received 4500g pupae	400 356	400 356	400 356
4	Adult : 6660.6g pupae Chilled adult: 7406.4g pupae Pupal: 6660g pupae	609 378	677 002	609 378
5	As per methods	827 206	827 868	827 206
6	As per methods	811 542	812 191	811 542
7	As per methods	843 486	844 161	843 486
8	Adult: 8720g pupae Chilled adult: 8868 g pupae Pupal: As per methods	802 947	816 593	828 729
Totals		5 944 496	6 029 071	5 970 278

Wild and sterile male trap captures

Wild flies were reasonably abundant in all treatment plots (particularly the chilled adult release plot) and the control plot, especially prior to sterile releases (Fig. 1). Over the entire trial period the average number of wild males captured per trap per week was 1.3±0.1 in the adult plot, 3.3±0.1 in the chilled adult plot, 1.8±0.1 in the pupal plot and 2.5±0.1 in the control plot (Fig. 1). For the two periods, pre-release and post release of sterile fly release, average numbers of wild males captures per trap per week respectively, were 1.0±0.1 and 1.6±0.1 in the adult plot, 4.76±0.1 and 2.5±0.1 in the chilled adult plot, 2.5±0.1 and 1.4±0.1 in the pupal plot and 1.8±0.1 and 3.2±0.1 in the control plot. For ln(wild flies +1), there was a significant effect of treatment plot ($F(3,20)=7.05$ $P=0.002$), week ($F(19,380)=16.14$, $P<0.001$) and a significant treatment plot x week interaction ($F(57,380)=1.68$, $P=0.008$) (Fig. 1). Before the release of steriles, the adult grid caught fewer wild flies per trap per week than either the chilled adult grid or the pupal grid for 5 consecutive weeks. For three of these weeks the control plot caught lower wild fly numbers per trap than the chilled adult plot. In week seven, wild fly numbers per trap caught in the pupal grid fell below the number trapped in the chilled adult plot. For the two weeks immediately prior to sterile release, wild fly numbers per trap did not differ between the treatment plots. Post release of steriles, there were significantly more wild flies trapped in week 15 (i.e. 5 weeks after sterile flies were released) in the control plot, than either the adult, chilled adult or pupal treatments. This period followed on from several weeks of the highest sustained sterile fly recaptures across the three treatment plots, suggesting that continued sustained high sterile to wild ratios may have continued to have provided significant suppression.

After sterile release, higher numbers of sterile males were captured in treatment plots than in the untreated control plot except for the pupal grid in the first week after release (Fig. 1). Over the post-release trial period, the average number of sterile males captured per trap per week was 64.6±0.3 in the adult plot, 32.5±0.3 in the chilled adult plot, 10.0±0.3 in the pupal plot and nil in the control plot (Fig. 1).

For ln(steriles +1) there was a significant effect of treatment plot ($F(2,14.9)=14.50$, $P<0.001$), week ($F(10,49)=41.14$, $P<0.001$ and a significant treatment plot x week interaction ($F(20,63.4)=3.73$, $P<0.001$).

Two weeks and three weeks after release more sterile flies were recaptured in both the adult and chilled adult treatment plots than in the pupal treatment plot. In week five and six after sterile release more sterile flies were recaptured in the adult treatment plot then in the pupal treatment plot. In week seven after sterile release sterile flies recaptured in the chilled adult treatment plot exceed those recaptured in the pupal treatment plot. By week 9 after sterile release recapture of sterile flies in the adult treatment plot is again exceeding the recapture in the pupal treatment plot. After week nine no further significant differences in sterile recapture numbers occur.

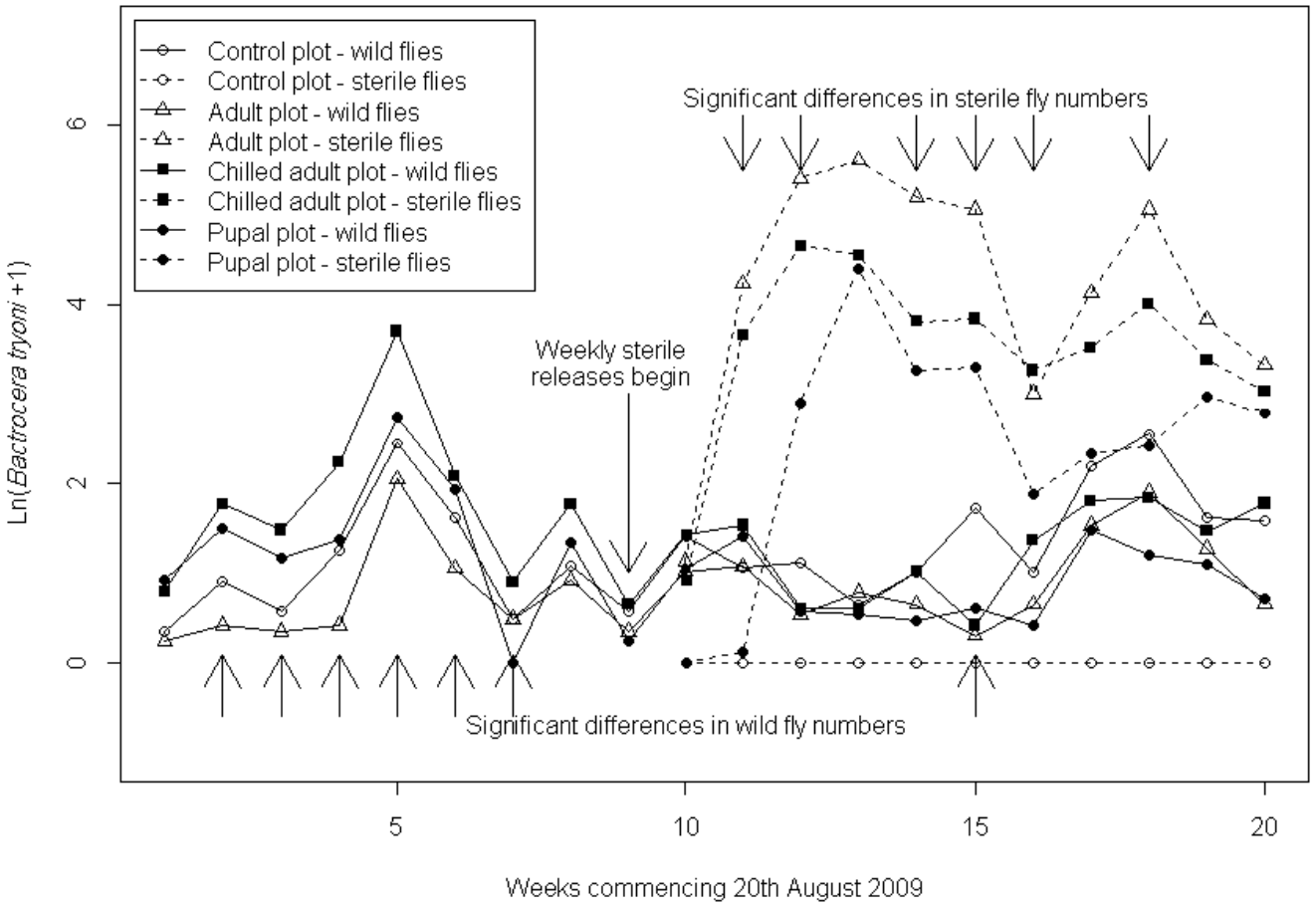

Figure 1. Sterile and wild *B. tryoni* abundance on the logarithmic scale for each treatment plot from 20 August 2009 to 31 December 2010 in Wagga Wagga, NSW.

Although we largely achieved higher sterile fly numbers than wild numbers, we did not achieve ratios of 100: 1 (sterile: wild) males, which is desired for effective suppression. Averaged over weeks, sterile to wild ratios were 40.4:1 for the adult plot, 13.0:1 for the chilled adult plot, 7.1:1 for the pupal plot and 0:1 for the control plot. It is evident that the sterile flies follow the same population cycling that the wild flies do. If we can predict this, it is during this period that sustained levels of sterile flies, or even increased levels of sterile flies may prove beneficial.

Fruit collection yields (eggs, larvae & pupae)

Over the duration of the trial, the total number of fruit examined from each grid was 224 (adult), 248 (chilled adult), 238 (pupal) and 321 (control). Eggs totalled in each plot: adult (67: fruit with eggs 8, 0.30 eggs/fruit, 8.37 eggs/infested fruit), chilled adult (25: fruit with eggs 6, 0.10 eggs/fruit, 4.17 eggs/infested fruit) , pupal (89: fruit with eggs 5, 0.37 eggs/fruit, 17.8 eggs /infested fruit) and control (146: fruit with eggs 9, 0.45 eggs /fruit, 16.22 eggs/infested fruit). The ratio of fertile: sterile eggs for each grid respectively was 1:3.19, 1:2.57, 1:2.87 and 1:4.41. Larvae totals were 328 for adult, 708 for chilled adult, 684 for pupal and 873 for control. Two pupae in total were found in fruit from the adult grid over the 11 weeks (one each over two different weeks) and seven pupae in total were found in fruit from the control grid (over three

separate weeks comprising 4, 2 and 1 pupae). No pupae were found in fruit from the chilled adult or pupal release grids.

Table 2. The number of trees and fruit sampled within each treatment grid over the duration of the trial in Wagga Wagga.

		Adult		Chilled Adult		Pupal		Control	
Week	Date	No. trees sampled	Range of fruit sampled (Median no. fruit)	No. trees sampled	Range of fruit sampled (Median no. fruit)	No. trees sampled	Range of fruit sampled (Median no. fruit)	No. trees sampled	Range of fruit sampled (Median no. fruit)
1	12/10	4	2-5 (5)	6	1-6 (5)	7	2-16 (5)	5	3-5 (4)
2	22/10	5	3-5 (4)	5	2-3 (3)	2	3 (3)	3	3-4 (3)
3	29/10	4	3-4 (3)	4	1-3 (3)	7	1-4 (3)	3	2-4 (3)
4	5/11	3	2-5 (3)	4	3-5 (3)	5	3-4 (3)	4	3-5 (4)
5	12/11	4	3-5 (4.5)	5	1-4 (3)	7	3-6 (4)	7	3-8 (5)
6	19/11	3	4-5 (5)	6	2-10 (3)	6	2-5 (3.5)	7	4-11 (5)
7	26/11	6	1-10 (5)	5	4-9 (8)	7	2-5 (4)	9	3-12 (6)
8	3/12	6	5-19 (6)	4	8-14 (8)	6	3-14 (6)	7	5-20 (6)
9	10/12	5	2-10 (7)	5	4-10 (8)	4	2-9 (5.5)	4	8-10 (8)
10	17/12	2	5 (5)	3	5 (5)	5	1-5 (4)	4	6-12 (8)
11	22/12	2	3-5 (4)	2	4-5 (4.5)	4	1-5 (2)	2	5 (5)
12	Distance range	Up to 150m from trap tree		Up to 150m from trap tree		Up to 80m from trap tree		Up to 200m from trap tree	

Egg sterility

The occurrence of eggs (fertile and sterile) in fruit from each treatment grids was dependent upon the week (Table 3) For the weeks when fruit contained eggs, the probability of the presence of fertile and sterile eggs per fruit were for adult: 0.05 and 0.10, chilled adult: 0.04 and 0.06, pupal: 0.04 and 0.04 and control: 0.01 and 0.10. The expected number of fertile and sterile eggs per fruit for the weeks when eggs were present were for adult: 0.27 and 0.19, chilled adult: 0.07 and 0.21, pupal: 0.25 and 0.57 and control: 0.09 and 1.42 respectively.

The presence of high numbers of sterile eggs in the control plot is difficult to explain. It may be attributed to home owners spraying their trees, as several blocks in the control plot were quite large and some had small backyard orchards (10 or so fruit trees) which the owners maintained to provide fruit for consumption.

Table 3. Presence of fertile and sterile eggs, larvae and pupae of *B. tryoni* for 12 October 2009 to 22 December 2009 and the probability and expected number of each per piece of fruit.

	Week number												
	10 12/10	11 22/10	12 29/10	13 5/11	14 12/11	15 19/11	16 26/11	17 3/12	18 10/12	19 17/12	20 22/12	Probability of presence	Expected no.per fruit (per infested fruit)
Fertile eggs			CA	AD PU	AD PU CO	 CA PU	 CA PU		AD CA	 CO		0.0508 0.0425 0.0439 0.0141	0.2712 (5.33) 0.0660 (1.75) 0.2473(5.75) 0.0904(9.00)
Sterile eggs			CA	AD PU	AD PU CO	 CA PU	 PU		AD CA PU	AD CA CO	 CO	0.1014 0.0575 0.0442 0.0952	0.1909(13.20) 0.2069(3.60) 0.5739(7.29) 1.4167(17.0)
Larvae	AD CA	AD CA PU	AD CA PU	AD CA PU CO	AD PU CO	AD CA PU CO	AD CA PU CO	CA PU CO	AD CA PU CO	AD CA PU CO	 PU CO	0.1425 0.2374 0.2797 0.0980	2.0000(7.33) 3.1749(14.02) 3.5258(11.47) 2.9493(9.08)
Pupae			AD	 CO		 CO	 CO		AD			0.0444 0 0 0.0043	0.0444(1.00) 0 0 0.0070(1.75)

AD=Adult, CA=Chilled adult, PU=Pupal, CO=Control.

Larval presence
The probability of larvae in fruit, for the weeks they were present in a plot and the expected number of larvae were for adult: 0.14 and 2.00, chilled adult: 0.24 and 3.17, pupal: 0.28 and 3.53 and control: 0.10 and 2.95 respectively.

In conclusion, these results are encouraging with suppression evident in all of the treated plots. Future work should involve several consecutive seasons of SIT and trapping to determine if long-term sustained suppression can be maintained in urban centres where *B. tryoni* is considered endemic. Studies incorporating SIT together with other technologies in these areas including parasitoids and the male annihilation technique are also warranted to bring large numbers of *B. tryoni* down to levels where SIT is more economically viable.

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Review

Synergizing biological control: Scope for sterile insect technique, induced plant defences and cultural techniques to enhance natural enemy impact

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ABSTRACT
When used alone, only a minority of biological control programs succeed in bringing the target pest population under sufficient control. Biological control is, therefore, usually employed with chemical, cultural, genetic or other methods in an integrated pest management (IPM) strategy. The interactions between different pest management methods, especially conventional pesticides and host plant resistance, is an area of growing research interest but relatively little consideration is given to novel combinations. This paper reviews the interactions between biological control and other forms of pest management, especially induced plant defences and the novel, non-toxic plant protection compounds that may boost these defences; and sterile insect technique. We also cover the cultural methods that offer scope to support synergies between the aforementioned methodological combinations. We conclude that despite the sometimes negative consequences of other pest management techniques for biological control efficacy, there is great scope for new strategies to be developed that exploit synergies between biological control and various other techniques. Ultimately, however, we propose that future use of biological control will involve integration at a greater conceptual scale such that this important form of pest management is promoted as one of a suite of ecosystem services that can be engineered into farming systems and wider landscapes.

Key words: integrated pest management, induced plant defences, herbivore induced plant volatiles, silicon, induced defenses, landscapes, ecosystem services, ecological engineering.

INTRODUCTION

It is human nature to reduce complexity to simplicity, seek to 'pigeon hole' and categorize; indeed the very process of science is reductionist. Ultimately, however, the real world is complex and attempts to manage a system rarely succeed when an overly simple approach is taken. Biological control has developed into a large and diverse field but remains just one of several pest management methods. According to Way and van Emden (2000), "the IPM toolbox has never been fuller". The global increase in genetically modified crop varieties with insect resistance is one new "tool" and its interactions with biological control – both negative and positive – have been reviewed by Altieri (2004) and continue to be the subject of empirical research (eg Chen *et al*., 2007). But the last decade has also witnessed a high level of interest in conservation biological control, sterile insect technique and induced plant defenses. It is timely, then, to review the nature of interactions between varying pest management approaches and consider their compatibility for IPM. It is appropriate that the compatibility of differing pest management approaches be considered from a biological control perspective because biological control is often considered to be the foundation for pest management systems (Van Driesche and Bellows, 1996) (Figure 1).

Interactions between biological control, host plant resistance and pesticides.

A great deal of research attention has been devoted to the impact of conventional pesticides on natural enemies and this is the topic of Gentz *et al*. (this volume) so reviewed only briefly here in relation to the interactions of pesticides with host plant resistance and biological control. Interactions involving novel plant protection compounds are covered more fully. Host plant resistance may make pests more susceptible to insecticides by slowing their growth such that they are smaller and less well developed or less well nourished at any given time. Such an effect may be especially powerful when penetration of plant tissue by boring or mining pests is delayed (e.g. Kvedaras and Keeping, 2007). An increase in pest susceptibility may also allow the use of an insecticide concentration that is low enough to allow many natural enemies to survive whilst still conferring a high level of mortality of the pest (van Emden, 1990). Such a relationship between host plant resistance, biological control and dose- adjusted pesticide use would allow the application of a product to bring a pest outbreak under control whilst maintaining the within-crop community of natural enemies to persist and provide ongoing protection from future pest establishment. Despite the attraction of this system we are unaware of it being actively practiced in any agricultural system to the extent that pesticide doses are actually reduced. A likely explanation for this is the challenge of reliable data capture from the field with rapid feedback to the farmer of robust management recommendations. If the conceptually ideal pest management system requires complex sampling or lengthy laboratory analyses, the time delays and costs may render the most elegant of theoretical systems impracticable. An illustration of this is provided by recent attempts to rationalize pesticide use for cotton aphid control. Steinkraus (Steinkraus, 2007) report a system whereby crop monitoring involved returning aphids to the laboratory for light microscopy to check for presence of capilliconidia (the infective stage) of the entomopathogenic fungus *Neozygites fresenii* (Nowak). If the proportion of individuals with these minute, lemon-shaped spores on their cuticle was more than 15% a recommendation not to spray was communicated to the farmer. Though the system was technically sound and resulted in effective pest management with lower intensity of insecticide applications and

reduced costs were employed, a constraint to its wider adoption was human in nature. Processing of samples required a moderate level of technical skill and laboratory processing, and the workload was highly concentrated into only a few weeks of each year. Thus human and physical infrastructure were not used for most of the year and struggled to meet demand for the critical time period. Future solutions to such impediments to better use of biological

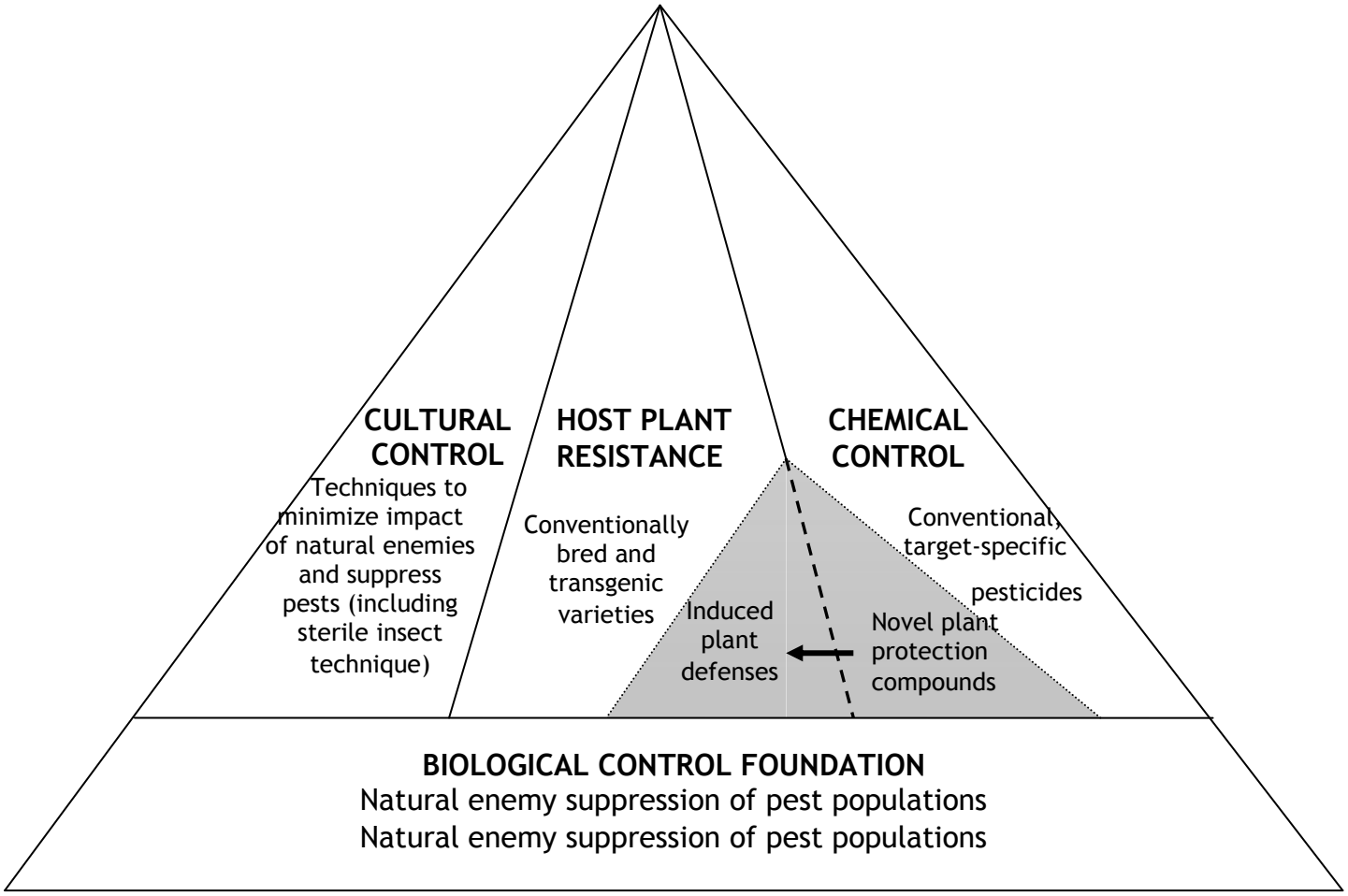

Figure 1. Biological control conceptualized as the foundation of integrated pest management and illustrating some of the positive interactions with other pest management approaches. Adapted from van Driesche & Bellows (1996) *Biological Control*, Chapman and Hall, New York, p. 297.

control may come from technological or human system developments. In the short term, the processing of aphid samples could be carried out by large public organizations such as State departments of agriculture that could use the human and physical capacity for other tasks for the remainder of the year. In the longer term, a bioassay that could provide immediate results in the field such as with the enzyme-

linked immunosorbent assay (ELISA) method or with more advanced forms of DNA barcoding that lead to field test kits would put analysis of samples and decision making in the hands of (quite literally) farmers or crop scouts.

Biological control and host plant resistance

The direct interactions of biological control with host plant resistance have received much research attention and, notwithstanding the many cases of negative interactions (Simmons and Gurr, 2005), synergies are often evident whereby the combination of both methods results in superior pest suppression than when either biological control or host plant resistance are used alone. For example, population growth of the aphid *Schizaphis graminum* (Rondani) has been investigated on susceptible and (conventionally bred) partially resistant varieties of barley with and without the parasitoid *Lysiphlebus testaceipes* (Cresson) (Waterhouse, 1999). In that system, biological control alone resulted in slower population growth of the aphid than occurred on either variety of barley, but the combination of biological control with the partially resistant variety resulted in much lower population growth. Even in cases where detrimental effects of host plant resistance are recorded the combined effect of biological control and resistant crop variety results in fewer pests than does either method alone. For example, in work with *Aphis fabae* (Scopoli), aphids reared on a partially resistant faba bean variety adversely affected the embryonic larval development, pre-oviposition period, fertility and fecundity of the predator *Coccinella septempunctata* (Linnaeus) (Shannag and Obeidat, 2008). Importantly, however, a significant decrease in the number of aphids was achieved compared with any other combination (Figure 2).

Novel plant protection compounds and induced plant defences

The phenomenon of host plant resistance 'breakdown' (actually a change in the pest population rather than any change in the plant) has threatened many traits that originally gave highly effective pest control (e.g. Shen *et al*., 2003). One response to this is exploration of completely novel host plant resistance traits. Amongst the most exciting of these are the induced defense mechanisms of plants. Far from being 'sit there and take it' victims of herbivory, plants have evolved a range of defenses that can be 'switched-on' by herbivore feeding or even oviposition (Khan *et al*., 2008). Induced defenses are widely recognized as an important type of plant defense strategy, particularly in cases where defenses are costly or the threat of herbivore attack is intermittent and predictable from prior exposure (Arimura *et al*., 2005). Amongst these defense mechanisms is the ability to release volatile compounds that recruit predators and parasitoids of pests. These herbivore-induced plant volatiles (HIPVs) are released in response to herbivore damage to aid location by predators and parasitoids of plants where their prey or hosts, respectively, are present. HIPVs and their potential use in IPM have recently been reviewed by Khan *et al*. (2008) so this section focuses chiefly on novel aspects of their use.

Known HIPVs include methyl salicylate, methyl anthranilate, methyl jasmonate, benzaldehyde, cis-3-hexenyl acetate and cis-hexen-1-ol. The qualitative and quantitative characteristics of HIPV blends vary according to the herbivore involved and the plant species (Turlings *et al*., 1993; Takabayashi *et al*., 1994). Work in the USA (Khan *et al*., 2008) has shown that plant-derived or synthetic versions of these chemical cues will attract beneficial insects into treated crops. Current field studies in Australia have shown attraction of parasitoids such as *Trichogramma* spp. to grapevines and brassicas treated with methyl anthranilate and benzaldehyde

(Simpson, M. R., personal communication, 15 November 2008). Thus, HIPVs offer potential for manipulating natural enemy populations in a manner that is far more precise than is the norm in biological control.

Much, however, remains to be resolved before HIPVs can be used commercially to enhance biological control. A key issue that needs to be resolved is the relative importance of direct and indirect effects. Exogenous HIPVs may function as direct attractants, that is, by constituting a signal recognized by natural enemies immediately after application to a plant. HIPVs may also act indirectly on natural enemies by causing plants to emit endogenous volatiles which are then detected by natural enemies. The latter, as well as being a potentially more effective signal, would also be longer lasting than would the influence of artificially applied HIPVs alone, making novel plant protection products based on HIPVs more viable. The likelihood of exogenous compounds triggering production of endogenous compounds is supported by a significant body of recent results. Airborne or topically applied

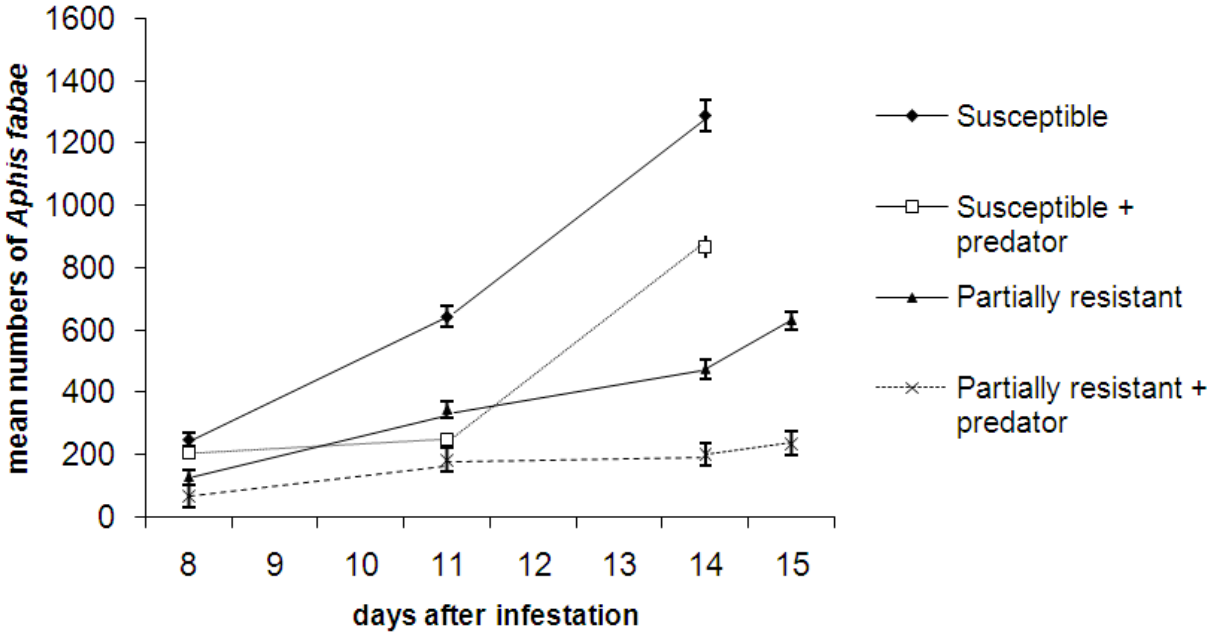

Figure 2. Example of a positive interaction between a host plant resistance and biological control: effect of partial plant resistance faba bean (variety 7954) and a predator (*Coccinella septempunctata*) on numbers of *Aphis fabae*. Drawn using data from Shannag and Oneidat (2008). *Annals of Applied Biology* **152**, p. 334.

methyl jasmonate (MeJA), for example, can cause the emission of volatiles in some plants similar to those produced in response to herbivore damage (Hunter, 2002). There is evidence that methyl salicylate and hexenyl acetate also function as elicitors of plant signaling (Shulaev *et al*., 1997; Ozawa *et al*., 2000; Engelberth *et al*., 2004). Work on rice demonstrated a role of ethylene signaling in induced defenses against arthropod herbivores (Lu *et al*., 2006). Plants attacked by *N. lugens* produced ethylene 2-24hr after infestation along with HIPVs and *Anagrus nilaparvatae* (Pang & Wang), a parasitoid of *N. lugens,* was attracted to emitting plants. Further, exogenous application of ethephon (a compound that breaks down within the plant to produce ethylene) resulted in a similar HIPV profile to that produced by rice brown planthopper-infested plants as well as attraction of its parasitoid. The same authors

also considered it likely that *N. lugens* activates other - most notably the salicylate - signaling pathways. In other work, exogenous applications of jasmonic acid to rice plants have led to dramatically elevated levels of several volatiles including aliphatic aldehydes, alcohols, monoterpenes, sesquiterpenes, methyl salicylate and *n*-heptadecane (Lou *et al*., 2005). The potential for such chemical ecology to be developed into a practical pest management strategy is evident from a doubling of parasitism of *N. lugens* eggs by *A. nilaparvatae* on control rice plants that were surrounded by rice plants to which jasmonic acid had been applied. It is likely that other parasitoids, as well as rice pest predators, make use of such plant-provided chemical cues. The same cues may also affect pest behavior, making treated plants less attractive to planthoppers (Karban and Chen, 2007).

A further complication in the use of HIPVs in practical pest management is the interaction between plant defenses against differing taxa of pests. Crops are often attached simultaneously by pests as differing as arthropods and fungi. Recent work illustrates synergies between the metabolic pathways controlling systemic acquired resistance (SAR) and natural enemies that may result. In a study of pathogens of maize plants and their attraction of parasitoids, application of a salicylic acid mimic led to SAR against the pathogen *Setosphaeria turcica* (Luttr.). Moreover, when benzo-(1,2,3)-thiadiazole-7-carbothionic acid *S*-methyl ester (BTH) was applied to maize seedlings prior to damage by *Spodoptera littoralis* Boisduval caterpillars, treated plants that were under attack from caterpillars were far more attractive to the parasitoid *Microplitis rufiventris* (Kok) than were caterpillar-damaged but untreated plants (Rostás and Turlings, 2008). Thus SAR, whether natural or artificially-induced with compounds such as BTH, may not only be compatible with indirect defenses based on natural enemy attraction but actually enhance biological control.

Other issues that require research before commercial use of exogenously-applied compounds that trigger plants' induced defenses include the possibility that these defenses may be so metabolically costly that yield reductions occur. Such fears are not supported by recent authors (Aharoni *et al*., 2005; Turlings and Ton, 2006). Work on maize by Engelberth *et al*. (2004) suggested that HIPVs at low concentrations have no effect until a treated plant is subsequently attacked by pests (see also Turlings and Ton, 2006). The prior application of an HIPV then gives an augmented level of natural HIPV production.

An additional concern is that artificially-induced production could interfere with the short-range detection of pests by natural enemies and the longevity of their response. That is, the ubiquity of the chemical signal may erode a predator or parasitoid's response to specific prey or host presence either spatially or temporally. Though there is field evidence against such negative consequences from maize intercropping systems (Khan *et al*., 1997), future work will need to use methods such as sentinel baits to determine whether such an effect operates to any significant degree.

A final potential problem with the use of HIPVs to attract natural enemies into a specific crop is that they could starve or leave unless suitable prey or hosts are available. Over time, the response of natural enemies to the chemical cues could diminish unless HIPV applications were well timed. One strategy that would reduce the risk of this potential effect is to employ effective monitoring. Since this is a foundation of IPM and monitoring protocols exist for most major pest species, all that would be required is to develop appropriate thresholds to guide the timing of HIPV application. A further, perhaps complementary, 'attract and reward' strategy has been postulated (Khan *et al*., 2008). The 'reward' component of this approach

aims to maximize the fitness and performance of attracted natural enemies by providing appropriate sources of nectar, pollen and shelter. A still wider scale ecological engineering approach could also be used whereby the use of HIPVs and reward treatments to make crops powerful sinks for natural enemy populations is coupled with manipulation of the nearby non-crop habitat. This should aim to provide refuge areas and either 'corridor' or 'stepping stone' vegetation to facilitate movement of biological control agents into crop sinks.

Of course, HIPVs may not be the only type of compound with scope to enhance biological control. A growth in the level of research interest in the effects of silicon on plants illustrates one research avenue that has barely been explored by biological control researchers. Whilst silicon is the second most abundant element in soil (Ma and Yamaji, 2006), plant available forms of silicon are often deficient, especially in old, leached soils or areas with a long history of cropping. Application of silicon fertilizer may have several agronomic benefits including improved plant growth and increased yield (Epstein, 1994; Ma, 2004). Silicon is taken up by plants as soluble silicic acid ($Si(OH)_4$) and deposited in various parts of the plant as solid amorphous silica ($SiO_2 \cdot nH2O$) (Raven, 1983). McNaughton *et al*. (1985) suggested that silicon could be an important anti-herbivore agent in agricultural systems and its accumulation in plant leaves could increase leaf tissue toughness and thus potentially reduce herbivore damage. Recent work (Kvedaras and Keeping, 2007; Kvedaras *et al*., 2007) showed the application of calcium silicate significantly reduced the growth rate, survival and penetration of the borer, *Eldana saccharina* (Walker) in sugarcane. It is long recognized that silicon can enhance the constitutive plant defenses (i.e. those that are expressed continually even in the absence of biotic or abiotic stressors), but there is now evidence for enhancement of induced chemical defenses (Ma, 2004; Hammerschmidt, 2005). Available studies of the role of silicon in induced resistance are largely confined to plant pathogens, a fact that is surprising given the wealth of evidence from non-silicon-related studies for induced resistance being important in plant defense against arthropod pests (Gatehouse, 2002). Silicon-accumulating plants supplemented with silicon, translocate silicic acid throughout their tissues and, when attacked, produce systemic stress signals such as salicylic acid and jasmonic acid (Fauteux *et al*., 2005) that are key to plant induced defenses (Gatehouse, 2002). Silicon has been postulated to play two important roles in plant chemical defense: (i) enhanced signal transduction at the cellular level leading to an increase in induced systemic resistance and (ii) modulation of the generation of systemic signals (Fauteux *et al*., 2005). Work by Gomes *et al*. (2005) demonstrated the significance of silicon for induced plant defences. Application of calcium silicate to wheat plants that were exposed to the aphid, *S. graminum*, elevated the activity levels for three plant enzymes involved in plant defense and suppressed aphid reproduction. Only one study, however, has tested for the effect of silicon on pests via the activity of predators and parasitoids (Moraes *et al*., 2004). That work showed no effect of silicon on natural enemies, but it employed non-choice conditions in which parasitoid wasps were confined at a small scale on individual plants that were not widely spaced. The experiments that Moraes *et al*. (2004) conducted with predators were still less conducive to detection of induced plant defences involving HIPVs; aphids were removed from the test plants and fed to predators that were not exposed to plants at all. Work recently commenced in Australia by the authors is methodologically and conceptually more advanced in the use of choice tests in which parasitoids and predators range over widely spaced plants (so that effects of HIPVs

are evident). Under these more natural conditions, any effect of silicon on the plants' ability to mount an induced response by attracting natural enemies will be apparent.

The potential for exogenously-applied compounds such as silicon amendments and HIPVs acting as elicitors of induced plant defenses is an exciting possibility for manipulating biological control agents in pest management. Further, if the promising results to date translate to the availability of novel plant protection compounds that promote host plant resistance traits operating via the third trophic level it may prove to be a durable strategy. Conventional pesticides and currently used host plant resistance traits operate directly on pests by mechanisms such as direct toxicity, antifeedant or antixenotic mechanisms. In contrast, HIPV- or silicon-based plant protection compounds that operate via an enhancement of natural enemy activity are likely to be less prone to a diminution of efficacy as a result of genetic adaptation of the target pest population. This is so because pest suppression is likely to be via more than one biological control agent, possibly multiple guilds of agents that would be able to adapt in response to any shift in pest phenotype.

The foregoing sections illustrated ways in which host plant resistance traits may operate in synergistic ways with biological control, especially if integrated with novel plant protection compounds. It is recognized, however, that plant defenses can exert lethal and sub-lethal effects on natural enemies (Simmons *et al*., 2006). One reason for this is because plant breeding to date has strongly favored host plant resistance mechanisms that act directly upon pests with a corresponding neglect of the mechanisms that operate via natural enemy activity. By development of strategies that exploit more fully the subtleties produced from the millions of years of co-evolution by angiosperms and arthropods, both herbivorous and entomophagous, new and more sustainable synergies could be achieved in IPM. In the short term, however, biological control will be best served by adopting approaches that reduce the effects of conventional pesticides. Mitigating the adverse indirect effects of pesticides on natural enemies could employ some of the cultural techniques detailed later in this paper. For example, the preservation of non-crop vegetation near (or connected by suitable corridors) to crops could be important sources of biological control agents (Schellhorn *et al*., 2000; Tscharntke *et al*., 2008) Further, nectar or pollen sources within crops could support natural enemies during periods of prey scarcity following the use of a narrow-spectrum insecticide.

Interactions between biological control and the sterile insect technique (SIT)

Sterile insect technique is an environmentally-friendly option to suppress insect pest populations and even eradicate geographically isolated outbreaks. It uses mass-reared insects that are irradiated before release to render them infertile. The success of SIT relies on sterile releases 'overflooding' the wild population, minimizing the possibility of wild males and wild females mating to produce viable eggs. This biologically-based approach has enjoyed significant success around the world (e.g. Steiner *et al*., 1965; Fisher, 1996; Ito *et al*., 2003). A drawback, however, is that SIT can be expensive, especially when used against dense or widely dispersed pest populations (Parker and Mehta, 2007). Biological control using inundative or augmentative release of parasitoids is an alternative or extension to SIT that has resulted in effective suppression of target species in several regions of the world, for example, opiine braconid wasps to control members of the family Tephritidae in Latin America and parts of the United States (Ovruski *et al*., 2000), several biological control agents for the management of various pest insects in diverse crops in Latin America (see review by Van Lenteren and Bueno (2003) and the native pupal

endoparasitoid, *Chouioia cunea* (Yang) for the control of the fall webworm, *Hyphantria cunea* (Drury), in areas of China (Yang *et al*., 2006). Of greater relevance to this review, however, is that empirical studies and population modeling strongly suggest that a synergistic interaction between released parasitoids and SIT may give more rapid and cheaper pest suppression or eradication (Sivinski, 1996). Here we deal with only those studies that have combined the two methods of control and only those insects that are plant pests.

An augmentative release program is an attractive extension of SIT as the mass-reared sterile pests contribute the supply of hosts for mass-rearing the natural enemies (Thomas, 2007). Unlike SIT which is most economical for small pest populations (that are easily 'overflooded' by released 'steriles'), parasitic wasp releases work best against a high pest population where wasps can readily locate pests. Put simply, combining these two methods together could avoid the limitations of each individual method: parasitoids used to bring a high pest population down to a level where SIT becomes effective. Parasitoids and sterile insects share the advantage of being self-dispersing so give wide coverage including areas where the other techniques, such as chemical spraying, cannot readily be applied.

This logic is supported by theory. Population modeling has demonstrated that the combined use of SIT and parasitoids would be much more efficient than either method alone for suppressing or eradicating a host species (Barclay, 1987). That paper proposed that the greater combined efficiency of SIT and parasitoid release, as opposed to use of either singularly, was an example of a broader principle: that two pest control methods will mutually complement each other if their optimal actions in reducing host numbers are at different host densities. This is the situation for SIT which performs best at low host densities while parasitoid inundation performs better at higher host densities.

Figure 3. Factory scale rearing of the fruit fly parasitoid *Diachasmimorpha longicaudatus* in conjunction with production of sterile fruit fly in Mexico. (Photographs by kind permission of Andrew Jessup, International Atomic Energy Agency, Vienna).

Since the modeling of Barclay (1987), several studies have shown that SIT together with augmentative or inundative release of biological control agents can be effective. An example is the successful eradication from New Zealand of the Australian painted apple moth, *Teia anartoides* Walker. That program employed

widespread spraying of the entomopathogenic bacterium *Bacillus thuringiensis* (Berliner) subsp *kurstaki* to reduce the pest density. In this instance, widespread spraying of the agent compensated for its poor inherent dispersal capacity. Sterile insect technique was implemented in 2003 when trap catches of moths were only 1% of their 2001-2 levels (Suckling *et al*., 2007). Most cases of combined use of SIT and biological control, however, involve dipteran targets and hymenopteran agents.

In Hawaii, augmentative releases of *Diachasmimorpha tryoni* (Cameron) for the control of the Mediterranean fruit fly, *Ceratitis capitata* (Cameron) raised rates of parasitism to 47% compared with 14.2% in the control area, with a significant reduction in both the adult and larval population of *C. capitata* achieved (Wong *et al*., 1991). However, when combined with sterile adult *C. capitata* a significant decrease in the number of male *C. capitata* trapped per day and the mean percentage egg hatch was recorded (Wong *et al*., 1992). Overall, this led to a nine-fold decrease in the number of *C. capitata* recovered from fruit in the region compared with the control area indicating that the two control techniques, when used together, were more effective at reducing fruit fly populations than either alone. The same effect was evident in a study of the leaf miner, *Liriomyza trifoli* Burgess, a pest of ornamental and vegetable crops (Kaspi and Parrella, 2006). In a greenhouse containing chrysanthemums the combined release of sterile adult leafminers and its parasitoid *Diglyphus isaea* (Walker) gave a significant reduction in mine production and the adult leafminer population. Furthermore, a synergistic interaction between these methods was demonstrated such that SIT used with biological control gave better pest control than did either technique alone. A model based on observed data indicated that only the combined use of these methods would effectively eradicate the pest population.

In the early 1990's, as part of an area-wide campaign against *Anastrepha* spp. from Mexico, SIT and augmentative biological control were selected as the main methods of control as they are economically feasible and have minimal or no effect on non-target organisms (Orozco, 2004). Subsequently, a mass-rearing facility to produce 50 million *Anastrepha* spp. and 30 million parasitoids (*Diachasmimorpha longicaudata* (Ashmead)) per week (presently 50 million parasitoids per week; A. Jessup pers. comm. 2007) was built (Figure 3). As part of this integrated management campaign, the states of Senora, Chihuahua and Coahuila are now recognized as fruit fly-free areas. In Costa Rica, combined SIT and parasitoid release is successfully funded by grower groups (Messing, 1996), who clearly see the benefit of a combined release. In a coffee plantation in Guatemala, effective suppression of *C. capitata* was attained using combined releases of the parasitoid *D. tryoni* and sterile adult *C. capitata* (Cancino *et al*., 1996). In another study which involved the aerial release of these same species along the border between Guatamala and Mexico, the combination of tactics was reportedly synergistic in effect (Sivinski *et al*., 1996). However, in neither of these studies, was the degree of control indicated. Another study showed that at various sites in Guatamala, caged F1 *C. capitata* populations were suppressed using a combination of two parasitoids, *Fopius arisanus* (Sonan) and *Diachasmimorpha kraussi* (Fullaway) and sterile male *C. capitata* than the latter technique alone (Rendon *et al*., 2006). The authors suggested that the release of multiple species of parasitoid may be advantageous as they each have clear habitat preferences and therefore differ in their ability to exploit environments within and surrounding agroecosystems.

A recent evaluation in Hawaii showed that SIT and biological control as part of an IPM program over recent years has proven very effective at reducing fruit fly

populations (Kaplan, 2008). In fact, various fruit fly parasitoids are reared for mass release into the field around the world, often in conjunction with SIT. For example, in addition to Mexico and Hawaii, there are mass production facilities for fruit fly parasitoids in Brazil, Peru, and Guatemala (A. Jessup pers. comm. 2007).

There have also been a number of successful studies combining SIT and biological control for lepidopteran targets. The combined use of inheritedly sterile (sterile F_1 adults) potato tuber moth, *Phthorimaea operculella* (Zeller) and *Trichogramma* spp. (oophagous parasitoids) in a laboratory trial was more effective in reducing fertile F_1 *P. operculella* progeny than either technique used alone. Furthermore, the level of suppression attained by the combined releases was thought to be additive in effect (Saour, 2004). The authors predicted that because this reflected a single release, when multiple releases of sterile insects and *Trichogramma* occur, that synergism of treatment effects may be obtained and concluded that further work on the integration of these two control strategies was warranted. Field cage studies of sterile adult codling moth, *Cydia pomonella* (L.) along with the parasitoid *Trichogramma platneri* led to less apple damage than when either tactic was used alone (Bloem *et al*., 1998). In an earlier study, *T. platneri* were released in apple orchards using SIT against codling mothin British Columbia, Canada (Cossentine and Jensen, 2000). Combined use of parasitoids and SIT led to significantly lowercodling moth damage compared with plots where *T. platneri* was not released. A further benefit of this integrated strategy was that the non-viable codling moth eggs produced by released steriles were suitable hosts for *T. platneri* so contributed to persistence of the parasitoid population.

Despite such encouraging findings, indeed factory scale commercialization in several countries, there is still great scope to realize the full utility of the synergies between SIT and biological control. A major, industry-funded project exploring SIT and braconid releases against fruit flies recently commenced in Australia, where a combination of these techniques is likely to provide more economic and effective management of tephritid outbreaks. In surrounding zones, which are usually managed to minimize the pressure that pest populations exert on pest free areas, a combination of both these techniques could provide enhanced suppression of pest populations. It is thought by some (Sivinski, 1996; Wharton, 1989) that natural enemies may be most compatible with SIT in suburban and native settings rather than in the monocultures typical of commercial orchards because the former provide better availability of nectar and other alternative food sources, lower pesticide application intensity and moderated microclimatic extremes. The habitat manipulation approaches described under the following cultural techniques section offer scope to make intensive monocultures more conducive to natural enemies released to complement SIT. Further, switching from disruptive, broad spectrum pesticides to novel compounds such as those in the preceding section could help alleviate mortality of mass released parasitoids and sterile insects.

However, what becomes apparent in a number of these situations is the lack of documentation of the degree of control exerted and the economic benefits achieved from the combined release of sterile insect pests and their parasitoids. Despite studies in this aspect of the integrated use of biological control, spanning more than two decades, relatively little is currently available in the peer-reviewed literature. The majority of studies have focused on Tephritidae, reflecting the economic importance of this taxon. Against these pests especially, there is good scope to make wider use of SIT/biological control synergies. A constraint to expand use to other pest taxa is the generic, biological requirements of SIT; that females can

mate only once and that irradiation can produce sterility without adversely affecting mating success with 'wild' pests. In practical terms, a facility for producing large numbers of pests is also required both to produce sterile pests and hosts for parasitoid rearing. Despite these constraints there is great potential for future applied ecological research in this area.

Interactions between biological control and cultural practices

It has been accepted for many years that cultural methods such as tillage and fire can have negative effects on biological control agents as well as upon pests. Sometimes these can be idiosyncratic and difficult to predict in advance. For example, mass trapping of olive fruit fly led to capture of large numbers of parasitoids of scale insects (Neuenschwander, 1982). But a dominant phenomenon within biological control over the last decade has been a growth in the level of research interest in conservation techniques whereby the release of exotic or mass reared agents is replaced by practices that conserve and make more effective the existing natural enemy fauna of a region. Cover crops of various types have been employed in conservation biological control to provide nectar and pollen (forms of tropic supplementation, *sensu* Daugherty *et al*., 2007), moderate the microclimate and support non-pest herbivores that serve as alternative host/prey (Jonsson *et al*., 2008). If not managed carefully, however, cover crops can also behave as weeds by competing with the crop for water and nutrients (Bugg and Waddington, 1994; Meyer *et al*., 1992; Nyczepir *et al*., 1998). Cover crops can also increase the cost of production, or decrease yields (Brown and Glenn, 1999), as they require extra maintenance, water and/or fertilizer beyond that required by the crop (Horn, 2000). Non-crop plants can also favor at least some pest species, a risk that was identified in very early work on the potential for habitat manipulation in rice (Lim and Hong, 1977). In order to minimize the potential negative consequences of increasing plant diversity in a hit-and-miss manner, "ecological engineering" has been proposed as a framework for use of biodiversity and habitat structure that is characterized by a series of methodical steps aimed at identifying the "right kind of diversity" (Gurr *et al*., 2004).

Cultural practices can favor biological control; for example the use of strip harvesting of alfalfa (*Medicago sativa*) advocated at the dawn of IPM (Stern *et al*., 1964). In more recent years the utility of this method has been assessed in Australian alfalfa hay production (Hossain *et al*., 2002) Releasing paint spot or fluorescent dye marked predators into alfalfa plants immediately before passage of a tractor-mounted mower showed that the majority of predator individuals survived cutting and relocated only a short distance to uncut strips. Subsequently, predators would move from these refuges to re-growing plants in adjacent strips and exert more effective control of *Helicoverpa* spp. pest larvae than in areas where strip harvesting was not used. Still more recently Pearce and Zalucki (2005) have explored the potential for using lucerne to promote natural enemy activity in field crops. Generally, however, the availability of studies demonstrating the importance of shelter to arthropod natural enemies has not resulted in many rigorous studies showing benefits in terms of reduced pest densities and increased crop yield (Griffiths *et al*., 2008).

Crop residue retention too can dramatically influence the performance of natural enemies and shelter is likely to be one mechanism by which this operates. The classic example of this is the sugar cane pest *Pyrilla perpusilla* (Walker) for which markedly improved control was achieved in unburned crop residues compared to those burned (Mohyuddin, 1991). This was attributable to the egg parasitoid,

Parachrysocharis javensis (Girault), the activity of which was enhanced by the moderated microclimate provided by the crop residue. It is surprising that crop residues have not been more thoroughly investigated in more recent years as a means of enhancing biological control of arthropod pests. This is highlighted by the recent work on the benefits of trash retention for biological control of other taxa including weeds, plant parasitic nematodes and plant pathogenic fungi. Field experiments on weed seed predation by arthropods suggested good scope for use of crop residues (Cromar *et al*., 1999). Epigeal invertebrates were found to be the dominant predators of the weeds, common lambsquarters and barnyard grass, responsible for 80-90% of all seeds consumed. Predation was favored by avoidance of tillage and a groundcover of corn residue. The benefits of crop residues in plant disease suppression is also well recognized (Whipps and Davies, 2000). A recent study illustrated potential for this work on the important wheat disease, head blight, caused by *Fusarium graminearum* Schwabe. A study by Perez *et al*. (2008) indicated that green manures (i.e. sorghum–sudan grass hybrid or buckwheat plants tilled into the soil along with wheat residue) promoted the development of indigenous soil microorganisms that were antagonistic to the survival of the fungal pathogen. Other work, on plant parasitic nematodes (Stirling *et al*., 2005), showed that incorporation of sugar cane trash in a field experiment subsequently resulted in a reduction in pest nematode densities of between 71% and 95%, depending on species, compared with an unamended control treatment. Amendment increased readily oxidizable carbon, microbial biomass, microbial activity and numbers of free-living nematodes. Though none of the known predators of nematodes were enhanced, an unidentified predatory fungus was found only in amended soil.

The preceding examples illustrate that the mechanisms by which crop residues may favor predation, parasitism and suppression of pest taxa are likely to me manifold. One that appears a priority for future research is enhancing levels of organic matter, a phenomenon that has been best studied in tropical rice. Since the original work by Settle *et al*. (1996), in which composted cow manure was added to plots of rice and natural enemy densities were increased by the availability of detritivore prey, other workers have sought to measure the benefits of organic matter supplementation. Jiang and Cheng (2004) investigated this approach for enhancing biological control of whitebacked planthopper (*Sogatella furcifera* (Horváth)) in China. Composted barnyard manure was added to plots of rice and synthetic fertilizer added to the control plots at rates equivalent to the nutrients present in the manure. Abundance of collembola was enhanced by the manure treatment and, though no benefits to rates of predation or parasitism were evident for the first 40 days after rice was established, after this time activity of predators especially was enhanced (Figure 4). Ecologically, this strategy infuses the "detrital shunt" of the food webs (Polis and Strong, 1994) (Figure 5) with the allochthonous organic matter constituting a resource subsidy that enhances numbers of detritivores. These additional prey species decouple populations of natural enemies from reliance on pest herbivores, so allowing generalist predators in particular to establish and remain in crop, ready to provide immediate control of immigrating pests. In at least some

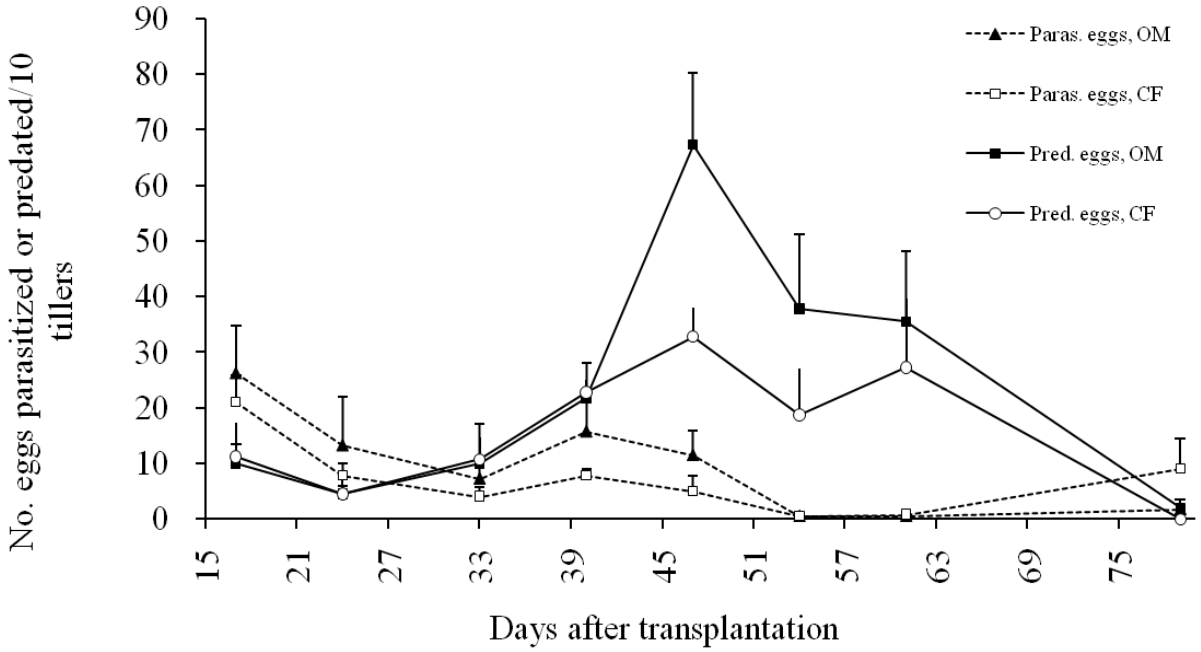

Figure 4. Example of a positive interaction between a cultural treatment and biological control: effect of organic matter (OM) versus chemical fertilizer (CF) on numbers of parasitized or predated whitebacked plant hopper (*Sogatella furcifera*). Redrawn using original data from authors: Jiang and Chen (2004) *Journal of Pest Science* **77**, pp. 185–189.

cases, however, detritivores may play a still more critical function. Feeding studies on *Atypena formosana* (Oi) by Sigsgaard *et al*. (2001) demonstrated that alternative prey is 'an absolute necessity' for the linyphiid spider (*A. formosana*). Spider survival on diets consisting solely of rice brown planthopper *Nilaparvata lugens* (Stål) or the green leafhopper (*Nephotettix virescens* (Distant) was poor. In contrast, a mixed diet of the hemipteran plus collembola and drosophila improved development time and survival of spiders. This illustrates that availability of prey such as collembola is essential for the performance of this linyphiid, rather than being only an early season, alternative food resource. This phenomenon may apply more widely to the use of generalist predators in agriculture whereby the key pest species (when the only prey species present) is not a suitable diet for the development of the potentially efficacious predator.

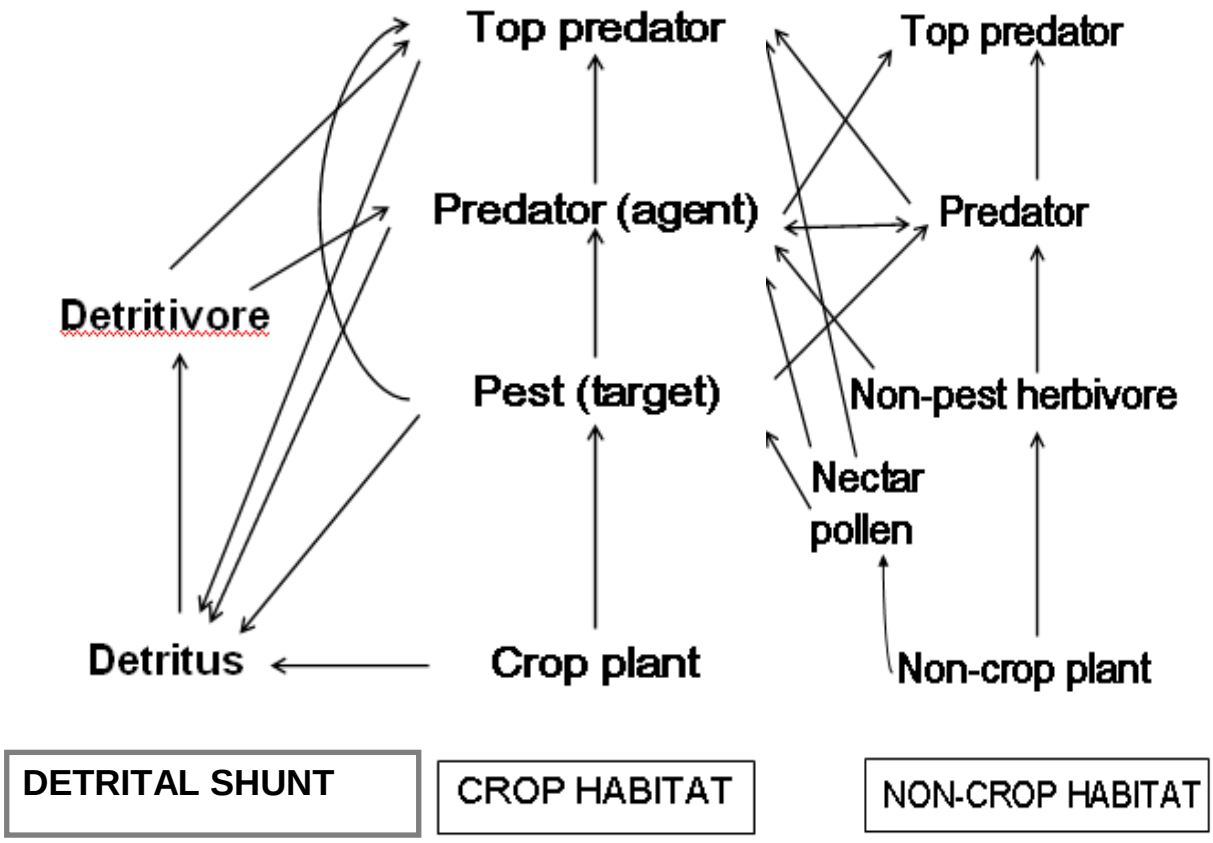

Figure 5. Schematic representation of an agricultural system food web showing the potential importance of non-crop habitat and detritus. The latter can be augmented by application of organic matter to support predators, allowing populations to develop early in the season before pests arrive.

Other than the food-web related effects of organic matter on biological control dealt with above, it has recently been suggested that soil organic matter content may make soils a more favorable structure for burrowing arthropods such as Coleoptera (Pywell *et al*., 2005). Of course not all such burrowing species will be beneficial and this is a further illustration of the need to carefully assess the benefits of any form of habitat manipulation on pests as well as on natural enemies. Ultimately, the use of organic matter enrichment in biological control is likely to be an important future direction. Cultural practices such as crop residue retention, green manures or other amendments will be promoted by those concerned with sequestering atmospheric carbon dioxide. Farmers and land managers are likely to receive 'carbon credits' for adoption of these methods. How best to simultaneously support biological control is an exciting avenue for research.

Conclusion

This review had deliberately emphasized the potential positive interactions between biological control and other forms of pest management. Notwithstanding the potential negative interactions, and the obvious need to avoid them, it is research into the additive and especially synergistic interactions that will yield the pest management strategies required if humanity is to meet the challenges of the future. Our population is expected to expand from the current level of 6.5 billion to 9 billion

by 2050 and agriculture will have to meet the resulting increased demand for food and fiber. This challenge is compounded by loss of agricultural land to urbanization and land degradation (soil acidification, erosion, desertification and salinization), by water scarcity and by increasing use of croplands to produce bio-fuels. Essentially, agriculture is about the management of ecosystem services 'benefits that people obtain from ecosystems' (Millenium Ecosystem Assessment, 2005), to produce food, fuel and fiber. These services include pollination, nutrient cycling, carbon storage, land stabilization, nitrogen fixation and conservation of threatened wildlife as well as biological control. In total, the global value of the ecosystem services has been estimated at US$2.6x10^9 (Costanza *et al*., 1997) . But the sustainability of 'industrial agriculture' (characterized by high inputs of synthetic fertilizers and pesticides, mechanical tillage and other technologies) is increasingly questioned. The Millennium Ecosystem Assessment (2005) highlighted the state of global ecosystems and their role for human well-being. That study examined 24 ecosystem services and found that only four (global climate regulation and production of aquaculture, crops and livestock) had been enhanced over the last 50 years. Fifteen, including biological control of pests had been degraded.

Accordingly, threats to agricultural sustainability such as environmental pollution, pest resistance to pesticides, dependence on fossil fuels and other non-renewable resources have led to research into alternative approaches that aim to promote ecosystem services. The value of biological control of crop pests alone has been estimated at $100 billion worldwide per annum (Costanza *et al*., 1997). Despite the action of biological control, insect pests still destroy an estimated 15% of world food production and lead to annual applications of approximately 3 million tonnes of pesticides. Maybe some of the ideas sketched out in this review will enhance biological control, mitigate these unacceptable levels of pest damage and simultaneously support still broader ecosystem services. An example is provided by the Wetland Integration and Sustainable Expansion into Rice approach. This 'WISER Approach' being developed in Laos offers scope to enhance biological control of rice pests by increasing the numbers of and (especially) early season density of native aquatic and amphibious predators (Jahn, G. pers com., 27 June 2008). In the Mekong basin, rice fields close to the river system flood early in the wet season leading to rapid colonization and breeding in rice paddies of fish and other predators (such as copepods) that can help suppress rice pests and mosquitoes (Fernando, 1993; Teo, 2006). In contrast, rice paddies remote from the river system, though filled with water from local rains, are colonized by larger species, such as fish, one or two months later. This, in turn, results in a long delay in predatory species contributing to pest suppression. At the same time this phenomenon also reduces the extent to which rice paddies contribute to the conservation of aquatic biodiversity on farmland. Scope for rural communities to harvest "wild" foods is also reduced. A solution to these interrelated problems is the construction of deep pits on rice farms that serve as refuges, particularly for fish, during the dry season. By mutual agreement within the human community, harvesting of fish from these is strictly regulated (e.g. on one day of the year only). As soon as the rainy season commences, aquatic predators are able to colonize rice paddies from the pits and rapidly breed to exploit the expanded aquatic habitat. In tropical rice production, the paddies may be connected to, or in very close proximity to, a network of human-made, natural and semi-natural aquatic habitats through which not only vertebrates but many invertebrates and prey of invertebrates (e.g. plankton) may readily move with beneficial consequences for biological control.

More generally, there is significant research interest in the relevance to biological control of connectivity and permeability of terrestrial vegetation features in farmlands (Tscharntke *et al*., 2008). Many agricultural systems other than rice are irrigated or exist in proximity to natural and semi-natural aquatic habitats and the influence of these on natural enemy activity has been little studied. Extending the spatial analysis and metapopulation approaches from terrestrial work to understand and manipulate the aquatic component of agricultural landscapes is an exciting prospect. In the case of the WISER approach, agronomic practices are being explored through collaboration between the International Rice Research Institute and the World Wildlife Fund as a means of reducing harvesting pressure on wild populations and using farms themselves as habitat to complement biodiversity conservation in formal refuges. In the future, measures could be introduced to improve spatial and temporal connectivity of aquatic habitats in agricultural landscapes; a fashion analogous to the use of shelterbelts, hedgerows and 'beetle banks' in temperate farmland. Such features may be as useful to biodiversity conservation as they are to biological control enhancement.

Ultimately, the contribution of biological control to meeting the pest management challenges to sustainable agricultural production will depend not only on its strategic use with other forms of pest management, but promotion of biological control to farmers and policy makers as one of a suite of ecosystem services. These can be enhanced by ecological engineering at scales that transcend individual fields and farms and encompass catchments and provide benefits, including carbon cycling at scales as large as the entire biosphere.

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Technology Transfer

Throughout the life of the project, numerous activities were undertaken to disseminate information and encourage adoption of the sterile insect technique. A list of these is provided below.

Publications

Journals

Reynolds OL & Orchard BA. (in prep.) Adult, chilled adult and pupal field release for the control of the Queensland fruit fly, *Bactrocera tryoni* (Froggatt).

Reynolds OL & Orchard BA. (in prep.). Trap capture and larval fruit counts of the Queensland fruit fly using adult and chilled adult sterile insect technique.

Collins, S, Taylor, P & Reynolds, OL (in prep). Methoprene and yeast hydrolysate increases mating and copulation of male sterile adult Queensland fruit fly, *Bactrocera tryoni* (Froggatt).

Taylor PW, Reynolds OL, Gilchrist S, Dominiak B, Collins S & Weldon CW (in prep.). The Sterile Insect Technique for Queensland fruit fly (*Bactrocera tryoni*): a review of history, current state and opportunities.

Reynolds OL, Orchard BA, Collins, S & Taylor, P. (in prep.). Yeast hydrolysate supplementation increases sterile Queensland fruit fly, *Bactrocera tryoni* (Froggatt) field longevity and abundance.

Reynolds OL, Smallridge, C, Cockington, V & Penrose, LDD (2012). Field release of adult sterile Queensland fruit fly, *Bactrocera tryoni* (Froggatt): the effect of release method and location on trap recapture rates. *Australian Journal of Entomology*, 51: 116-126.

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Gurr GM & Kvedaras OL. (2009). Synergizing biological control: Scope for sterile insect technique, induced plant defences and cultural techniques to enhance natural enemy impact? *Biological Control* 52 (3): 198-207.

Newspaper, magazine, farmer/grower articles

Olivia Reynolds, Geoff Gurr, Andrew Jessup (2010) Biological control enters the fight against 'Qfly' threat. *Australian Citrus News*.

Olivia Reynolds, Geoff Gurr, Andrew Jessup & Jennifer Spinner (2009) Combating the fruit fly threat for Australian fruit and vegetable growers using biological control. *Vegetables Australia and Australian Fruitgrowers.*

Olivia Reynolds, Geoff Gurr, Andrew Jessup & Jennifer Spinner (2008) Combating the fruit fly threat for Australian fruit and vegetable growers using biological control. *The Land.*

Media Release
22 October 2009 'Fruit fly traps set up in backyards across Wagga'.
6 December 2007 **'**Two million sterile flies released to tackle fruit fly'.

Interviews

Radio

ABC Rural radio reporter Dugald Saunders, 28 October 2009

ABC Rural radio reporter Anna Vidot, 8 July 2008, 2009

ABC Rural radio reporter, Bruce Reynolds, 6 December 2007 and other local radio stations.

TV

Prime (7) (7 December 2007, 8 January 2008 & 27 October 2009)

Win (9) TV (7 December 2007 & 8 January 2008)

Field Days

Mudgee Small Farm Field Days. July 2010.

Henty Field Days. September 2008.

Conferences

Kvedaras, OL & Orchard BA (2008) Effect of chilling adult Queensland fruit fly, *Bactrocera tryoni* (Froggatt), on recovery, longevity and flight ability in sterile insect technique. *Australian Entomological Society's 39th Annual General Meeting & Scientific Conference, 28 September – 1 October 2008.*

Meetings
Queensland Fruit Fly, Sterile Insect Technique presentation. November 2007. Riverina Citrus Forum, Griffith, NSW.

Queensland Fruit Fly presentation to growers. October 2007. Leeton Citrus Growers Association AGM, Leeton, NSW.

AQIS Fruit Fly presentation to citrus growers and packers. June 2007. Griffith, NSW.

Guest lecture

Riverina TAFE, Wagga Wagga, NSW. April 2008. 'Queensland Fruit Fly and SIT in Australia'.

Research Study Tours

Florida, Mexico, Guatemala & Hawaii, Central & North America. June 2009. Collaborators and colleagues visited included: Dr 's Tim Holler, John Sivinski, Pedro Rendon, Jorge Cancino, Don Mcinnis and Eric Jang. Observed and studied sterile insect and parasitoid rearing facilities and participated in sterile male fly release.

Griffith, NSW. June 2007. Peter Morrish, Operational Director, Riverina Citrus; toured Pacific Fresh Packing shed (observed an AQIS audit) and citrus grower's properties to discuss fruit fly topics. June 2007.

Adelaide, South Australia. April 2007. Cathy Smallridge and Vanessa Cockington, SARDI.

Perth, Western Australia. April 2007. Bill Woods and team, WA Agriculture to discuss fruit fly research and observe mating trials and other studies.

Stellenbosch, South Africa. November 2006. Meet Brian Barnes, head of the Stellenbosch Medfly Research facility; tour the facilities and discuss the Sterile Insect Technique (SIT).

La Reunion, France. October 2006. Serge Quilici, CIRAD; discussed Pacific fruit fly research. Estelle Roux, FDGDON; discussed fruit fly rearing techniques. Viewed research trials in insect pests of sugarcane and other crops and discussed insect-plant interactions and pest management.

Recommendations

1. That industry and government (commonwealth and state) meet as a matter of urgency to discuss the future implementation of the sterile insect technique for *B.tryoni* control, including infrastructure requirements, cost-sharing and ongoing management of the program. Further refinements of the technique will not flow through to end-users unless there is a commitment to provide adequate operational support for control and eradication efforts.
2. That talks commence with all potentially interested parties including the commonwealth and state governments and other countries (e.g. New Zealand, California, French Polynesia) to contribute towards the SIT for *B. tryoni*.
3. That discussions commence between relevant parties (Plant Health Australia, Horticulture Australia Ltd., Office of the Chief Plant Protection Officer, state governments, industry and other interested and involved parties) to make changes to the Code of Practice to permit the prophylactic release of sterile insects in the Fruit Fly Exclusion Zone.
4. Incorporation of the Strategic Release Plan and Standard Operating Procedures for pupal, adult and chilled adult release developed as part of this project into the management of wild *B. tryoni*
5. Trials looking at the fitness of sterile adult flies reared in tower eclosion systems should be looked at in terms of space and cost savings.
6. Using the technology developed in this study, that surplus flies are used in low-level sterile releases in towns surrounding the fruit fly exclusion zone (FFEZ) to suppress wild Qfly populations in these areas, thus minimising the pressure placed on the FFEZ
7. Mature sterile fly releases should also be used to eradicate outbreaks of wild *B.tryoni* in the NSW FFEZ.
8. That Sterile Insect Technique should be considered in endemic areas where orchards/towns are geographically isolated, or on an area-wide basis.

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Appendix 1

Sterile Insect Technique Strategic Release for the Queensland fruit fly in Australia

A Manual for Operations Personnel

2011

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SCOPE

The aim of this document is two-fold. Firstly, to provide a brief background on the use of the sterile insect technique for Queensland fruit fly in Australia to the present day and secondly to provide a working document that operational groups can follow when rearing out (post-Fruit Fly Production Facility (FFPF)) and releasing sterile fruit flies, based on current knowledge.

INTRODUCTION

In Australia, the native Queensland fruit fly, *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae)(Fig. 1) is the most economically important fruit fly species, with the national, annual cost estimated at AU$28.5 million (Sutherst *et al.*, 2000). The Fruit Fly Exclusion Zone (FFEZ) in south-eastern Australia is under active nationally and internationally approved border control programs to ensure freedom from Queensland fruit fly, thus allowing horticultural industries within that zone to export fresh produce to fruit fly sensitive domestic and international markets. The FFEZ encompasses some of the most valuable horticultural production areas of Australia, including Sunraysia, the Mid Murray and the Goulburn Valley in Victoria, the Murrumbidgee Irrigation Area (MIA) of New South Wales (NSW) and the Riverland of South Australia (SA). The Risk Reduction Zone (RRZ), a buffer zone bordering the FFEZ, was established and is managed to minimise movement of wild Queensland fruit fly into the FFEZ.

Figure 1. An adult sterile Queensland fruit fly, *Bactrocera tryoni* (Froggatt).

The Sterile Insect Technique (SIT) is used against a wide range of insect pests of plants, animals and humans in over 50 countries (Krafsur, 1998). SIT is one of the current, internationally approved treatments against incursions of Queensland fruit fly within the FFEZ. The SIT relies on the released sterile male fruit flies 'over-

flooding' the wild male fruit fly population and thereby minimising the possibility of wild flies mating, to produce viable eggs (Meats, 1996). In Australia, we aim for a ratio of 100:1 (Anonymous, 2011; Hooper, 1982) sterile to wild males. In Australia, like most SIT programs, we use fluorescent dyes to coat the sterile pupae, so that upon eclosion, the ptilinum of the fruit fly is marked with dye (Fig. 1) in order to distinguish between sterile fruit flies and wild fruit flies captured in traps (Enkerlin *et al.*, 1996). Adequate retention of dye on the ptilinum of Queensland fruit fly is therefore a crucial component of SIT in Australia. In NSW, the first experimental work with SIT for the control of Queensland fruit fly was carried out from 1962 to 1965, using the methods adopted from similar release programs internationally (Andrewartha *et al.*, 1967) as described in Monro & Osborn (1967). Recently, these methods have been refined and developed by a number of researchers.

The current management protocol for Queensland fruit fly in NSW, Victoria and SA includes extensive monitoring for early detection, bait and cover spraying and the use of the sterile insect technique (SIT).

GENERAL

1. Receiving sterile fruit fly consignments

a. Background

Queensland fruit fly are obtained as pupae from the Fruit Fly Production Facility at the Elizabeth Macarthur Agricultural Institute, Camden, New South Wales, Australia, where the larvae are reared on a standard lucerne chaff diet (Dominiak *et al.*, 2008), developed by A. Jessup unpubl. data. (circa 1980). Pupae are dyed (Fig. 2) with Fiesta FEX 1 Astral Pink fluorescent pigment (Swada, 30-32 Kilkenny Court, Dandenong South, Victoria, Australia) by inverting (mixing) the dye and pupae in a plastic bag to evenly coat the pupae (1 g dye per 100 g pupae), or with the use of a mixing machine (Jiang pers. comm. 2007). This is the standard pigment used to mark sterile Queensland fruit fly within SIT programs in Australia, although other pigments are commonly used in research to distinguish between treatments (Reynolds & Orchard, 2010; Reynolds *et al.*, in press; Fig. 2) and may be used in sterile release programs if required. Consignments of dyed Queensland fruit fly are sent as late-stage pupae to the Australian Nuclear Science and Technology

Organisation (ANSTO), where they are irradiated under anoxia at 70 – 75 Gy to render them sterile each Tuesday, as required.

Figure 2. Dyed Queensland fruit fly pupal consignments, Astral Pink, Arc chrome, Stellar Green and Flame Orange respectively and the 1L cardboard cartons in which they are delivered.

Consignments usually consist of ten 2-litre cardboard cartons per white polystyrene insulated box. More than one polystyrene box may be in a single consignment. Each 2-litre container usually contains 80,000 pupae, with the pupae contained inside a clear plastic bag. A rubber band keeps the plastic bag air tight (Fig. 2). Although it was thought that long periods on road freight may cause death and/or poor emergence and decreased performance of emerged adults, a recent study has shown that vibration has little effect on adult eclosion of sterile Queensland fruit fly (Campbell *et al.*, 2009). After sealing the plastic bag, there is some removal of oxygen caused by insect respiration which in turn pulls the pupae together and is likely to make them resistant to vibration damage (Jessup pers. comm. 2011). However, there are increasing indications that overheating of consignments causes a decline in fruit fly vigour (Dominiak pers. comm. 2011).

Denying pupae access to fresh air for a long period causes a decline in both the quality of the pupae and emerging adults, particularly at higher temperatures(Dominiak *et al.*, in press). In addition, on exposure to air, the heat generated by recommencement of pupal maturation is believed to be rapid, contributing to heat stress of the emerging flies.

b. Protocol for receiving sterile Queensland fruit fly consignments

Consignments are sent as requested to the fruit fly management group. Sterile Queensland fruit fly pupae are transported from ANSTO, to arrive at the release site by Wednesday morning either by road or air freight. Consignments may be delivered to NSW Department of Primary Industries facilities by courier however it may be preferable for staff to pick up the consignment from the airport or courier office. Air conditioned vehicles should be used, with consignments carried inside vehicles out of direct sunlight.

Upon receipt, check each plastic bag containing pupae, to ensure the fluorescent dye is evenly distributed. If the dye distribution is observed to be poor on removal of the plastic bag from the two litre container, the rubber band should be loosened to allow air into the bag. Reseal the bags and gently invert and rotate the bag several times until the dye evenly covers all the pupae. Do not shake the bag or treat the pupae roughly as this decreases the quality of the emerging adults. This should be done in the field for pupal release. Once the dye is adequately distributed, remove the rubber band and gently place the pupae in the release boxes (PARC for adult, paper bags inside PARC boxes for chilled adult and styrene foam for pupal release) immediately. If pupae are ever received and observed to be poorly dyed (Fig. 3), even after additional inverting as described above, they should not be released, but destroyed and the manager at the FFPF informed. Therefore, pupae should not be left in plastic bags for long periods of time (once opened pupae should be distributed into release containers with minimal delay) after removing the rubber bands.

2. Quality control

In order to determine and monitor the quality of sterile fly batches, a sub-sample of pupae should be reserved for quality control tests at both the factory (both prior to

and after irradiation) and the place of receival for comparison. These tests should include emergence, flight ability (Fig. 4) and pupal weight. At the point of receival, for adult, chilled adult and pupal release, the procedure described by Reynolds (2011a) (http://extranet.dpi.nsw.gov.au/lhpa/plant-directory/fruit-fly/procedures) should be followed. At the factory, quality control should follow the 'Product quality control and shipping procedures for sterile mass-reared tephritid fruit flies' guidelines (FAO/USDA/IAEA, 2003).

Figure 3. An example of poorly dyed Queensland fruit fly pupae. Note lack of dye on a number of pupae.

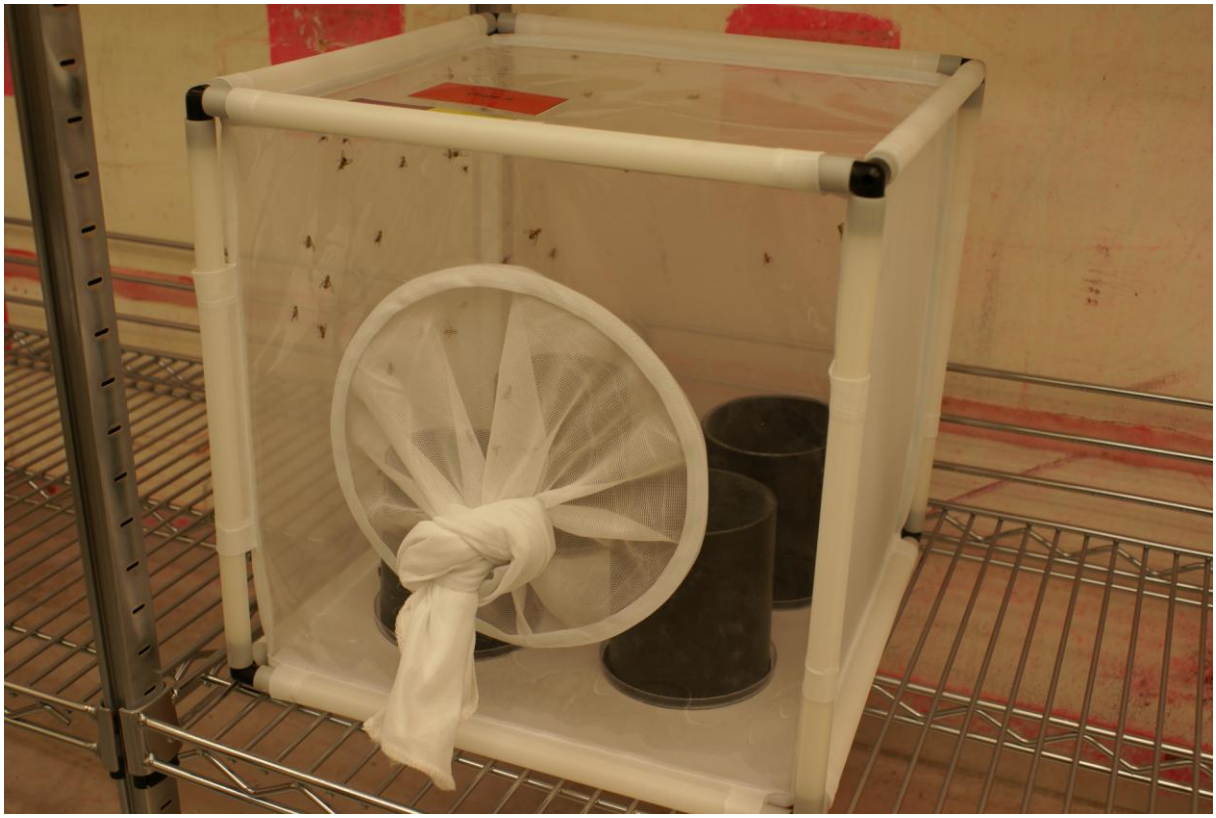

Figure 4. A cage setup demonstrating quality control for emergence and flight ability of the Queensland fruit fly

STERILE FRUIT FLY REQUIREMENTS

1. Dietary protein

a. Background

Taylor *et al.* (in press) have recently reviewed the use of protein supplements for adult Queensland fruit fly. Protein supplements can produce quicker development, increased mating probability and sperm storage by mates, longer copulations, increased ability to induce sexual inhibition in mates, and increased longevity (Taylor *et al*., in press). In addition, recent studies by Reynolds *et al.* (in prep.) have shown that protein fed flies have greater field longevity as evidenced by trap recapture rates. A. Jessup (unpubl. data) has developed a protein and carbohydrate based diet that is readily able to be fed to adult Queensland fruit fly, which is detailed below.

b. Protocol for the provision of dietary protein (pre-release feeding)

Agar containing a mixture of white sugar (carbohydrate) and yeast hydrolysate (protein) (sugar-yeast-agar) (Fig. 5) (MP Biomedicals Australasia Pty Ltd, P.O. Box 187, Seven Hills, NSW 2147, Australia) (3:1 by weight) and water (A. Jessup, unpubl. data) should be made up as per the procedure described by Reynolds (2011b) (http://extranet.dpi.nsw.gov.au/lhpa/plant-directory/fruit-fly/procedures) and should be provided for both adult release (prior to release) and pupal release (during release). Alternatively, a pet drinker or similar plastic container with 2 L of a water/sugar/protein solution may be used. The mixture should be changed weekly. As adults drown in free water, cotton wool should be placed in the base of the container, to hold the solution, thus removing any free solution while still permitting the flies to drink.

Figure 5. Agar block (protein and carbohydrate) with feeding eclosed adult sterile Queensland fruit flies.

i. Pupal release

A block of sugar-yeast-agar (see above) should be placed in a plastic container or directly on the top of each solid release box lid (or the alternate described above)

when flies first start to emerge. This is usually within 2-7 days, dependent upon temperature and RH.

ii. Adult and Chilled-adult release

A block of sugar-yeast-agar (see above) should be placed on the mesh on top of each release box when flies first start to emerge. In a growth room at 26°C and 65% humidity this is usually within 2-3 days of receipt of irradiated pupae. Flies should be permitted to feed from eclosion until release.

STERILE FRUIT FLY REARING OUT AND RELEASE

1. Pupal release

a. Background

Over the past decade, a method for pupal release as part of the SIT has been developed for Queensland fruit fly in Australia; however, it has not become a common practice due primarily to the uncertainty of its effectiveness under different climatic conditions (Reynolds *et al*., 2010) Various strategies for pupal release have been tested, including a platform release method (Andrewartha *et al*., 1967), the use of 50L bins (Macfarlane & Betlinski, 1988), the basic 'bed' technique (Dominiak & Webster, 1998), covered trays (Dominiak *et al*., 2000), the use of buckets suspended from trees (Meats *et al*., 2006) and polystyrene foam boxes (also known as pupal release boxes or eclosion boxes) (Dominiak *et al*., 2003a). The latter technique has been used with varying success in Australia (Dominiak *et al*., 2003a; Meats & Edgerton, 2008).

Although few SIT programs have incorporated pupal release, under certain Australian conditions it is believed to be effective if predation can be kept to a minimum (Dominiak *et al.* 2003). Adults are also thought to suffer less crowding stress in a pupal release box, as eclosed adults are free to leave the box upon emergence. Once eclosed, adults feed and disperse into the environment. This also leads to a steady supply of adult fruit flies into the targeted area, compared with a single large delivery of adult fruit flies in one day using adult ground release. Another advantage of this method is that adults may become partially acclimatised to the

local climate as the pupae are exposed to variable temperatures for the days preceding eclosion (Fay & Meats, 1987).

Based on the results of Reynolds *et al.* (2010) and the IAEA guidelines (FAO/IAEA/USDA 2003) which state that emergence below 65% post-shipment is not acceptable for Queensland fruit fly and that 75% is the desired mean emergence rate, a recommendation can be made on the optimal pupal load to be used at different times of the season. The most appropriate time of year to conduct pupal release and the pupal loadings that should be used are shown in Table 1. Depending upon the temperature and RH, emergence may take several weeks (Reynolds *et al*., 2010) from the time pupae are placed in the environment until emergence ceases, an indication of which is provided in Table 2.

Table 1. The viability of pupal release throughout the Queensland fruit fly season

	Pupal load (g)				
Month	***200***	***350***	***500***	***650***	***800***
August	Not viable	Not viable	Not viable	Not viable	Not viable
October	Not viable	Not viable	Not viable	Not viable	Not viable
December	Not viable	Not viable	Viable	Viable	Viable
February	Viable	Viable	Viable	Viable	Viable
April	Viable, not preferred*	Viable, not preferred*	Viable	Viable	Viable

* Although emergence was above 65% on these dates, higher loadings produced significantly better emergence and are therefore preferred.

Table 2. The total amount of time, from placement of pupae in pupal release boxes in the environment until emergence ceased at different times of the year.

Month	Time (days)
August	16
October	9
December	5
February	5
April	8

b. Release protocol

The modified polystyrene pupal release box described in Reynolds *et al.* 2010; Fig. 6, should be used to release the pupae. The dimensions of the box are 28 cm high, 57 cm long and 28 cm wide with a wall thickness of 2.3 cm. Each box should have a window cut from each vertical face measuring 3.5 cm high x 20 cm long and 2.5 cm from the top, to allow the fruit flies to leave the box. These can be obtained from Woolworths and other supermarkets and are usually what broccoli is packed in.

The dyed, irradiated pupae may be transported in the plastic bags inside 1L cartons (those in which the pupae are sent; Fig. 2) to the release site. The entire contents of one 1L carton (800g) pupae should be spread evenly on the base of the pupal release box. Vermiculite moistened with tap water (4:1) (A. Jessup, unpubl. data) should then be spread over the pupal layer in order to just cover the pupae, so that the total depth of pupae and vermiculite is no more than 1-1.5 cm. Additional resting space for adult Queensland fruit fly to dry and expand their wings should be provided. Cardboard dividers (approximately 160 cm in height) should be wedged in each polystyrene box, two running lengthways and five across the width of the box, to sit just below the window cut-outs and just above the pupal bed (Fig 7).

Pupal release boxes can be placed in the field in a number of ways. Pupal release stands (Reynolds *et al.* 2010; Fig. 6) may be constructed as follows. Each stand comprises a single shelf (58 cm wide by 114 cm long) at 75 cm above the ground to allow air to circulate around the pupal release box. The shelf is covered by a 30° insulated sloping roof (150 cm wide by 180 cm long) to allow rain run-off and minimise the effects of direct sun exposure. Each stand can hold three polystyrene

pupal release boxes, spaced approximately 15 cm apart. Stands can be modified to hold more or less boxes depending upon release requirements.

Figure 6. Pupal release stands, holding pupal release boxes

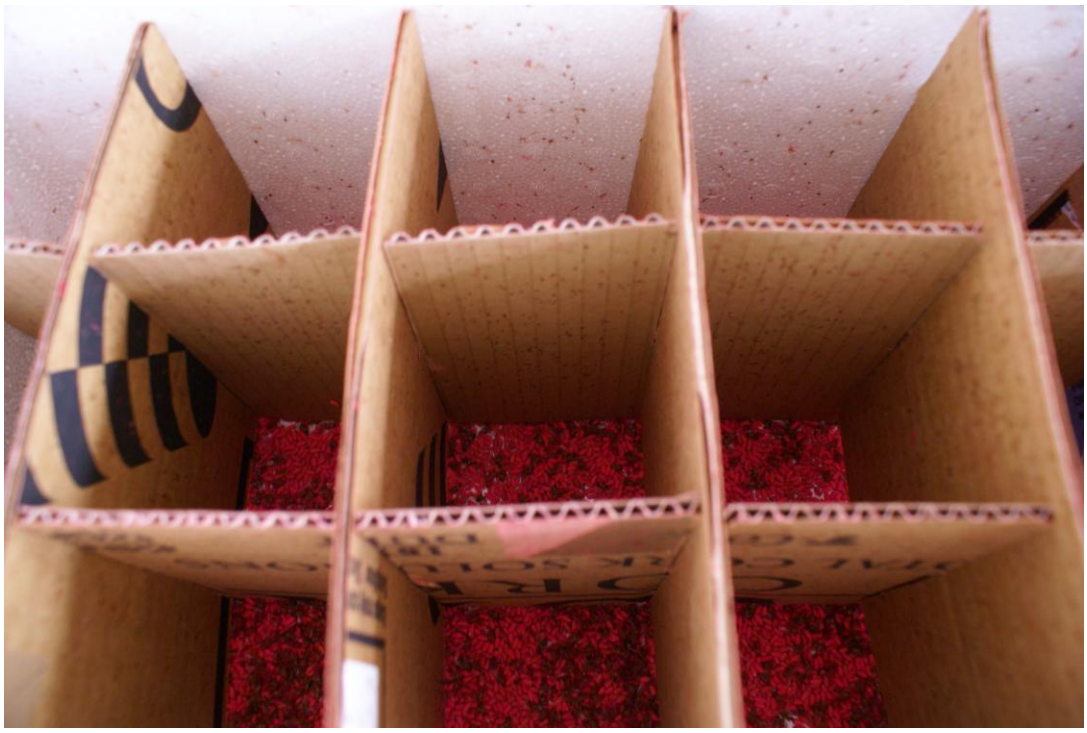

Figure 7. Cardboard dividers used inside polystyrene (pupal) and PARC (adult) release boxes.

Stands are held in place with four guide ropes, one on each corner. These are sprayed with a contact insecticide. An area radiating one metre from the stand should be sprinkled with Mortein Outdoor Ant Sand ® (Reckitt Benckiser (Australia) Pty Ltd, 44 Wharf Road, West Ryde, NSW 2114) or similar, to kill any invertebrate insectivorous predators. In areas where predation may be particularly high, repeated treatment of the ground as well as guide ropes or legs of stands with an insecticide or ant repellent may limit predation. The legs of stands can also be placed in a water bath. Eclosion boxes do not necessarily have to be placed on the stand described. Any form of elevated platform, which allows air to circulate around the pupal release box while minimising predation and providing protection from rain would be adequate. Protection from rain and other forms of free water is essential as exposure to water may wash marker dyes from pupae, resulting in misidentifications of adults in fruit fly traps. For example, placing the pupal release box on a wooden crate (Fig. 7) is also adequate (Gus Campbell, pers. comm. 2009) or directly over water baths with a ply board roof to prevent rain entering the boxes. Likewise, a stand with an overhead shelter is not necessary to provide shade; placing the box under the shade of a tree with some form of overhead protection from the rain would be suitable. In areas of low predation, release boxes may be placed directly on the ground and double-sided adhesive tap can be used around the full circumference at the base of release boxes, in addition to Ant Sand.

Pupal release should only be used from December through to April (Table 1) or when mean minimum temperatures are not lower than 10°C and the maximum no higher than 35°C. Generally, the highest pupal loading tested (800g) is ideal when pupal release is viable (Reynolds *et al.* 2010; Table 1).

Once flies have emerged, pupal debris should be discarded of appropriately, according to the requesting sites requirements.

Figure 8. A version of a pupal release box placed on a wooden crate over water baths to prevent predation.

2. Adult release

a. Background

Since the inception of the SIT in Australia in 1961 by Drs Monro and Osborn, several stationery adult release methods have been attempted including cage release (Dominiak *et al.*, 1998; Dominiak *et al.*, 2003b), plastic adult rearing container (PARC) release (Reynolds *et al.*, in press) and bin release (Horwood & Keenan, 1994; James, 1992; Sproule *et al.*, 1992). However, initially these methods were little researched, and not adapted for the Queensland fruit fly. Recent studies have sought to overcome some of this lack of knowledge. A refined bin technique is routinely used by South Australia (Fisher, 1996; Perepelicia *et al.*, 1997; Reynolds *et al.*, in press) and a refined PARC technique by NSW to control Queensland fruit fly incursions (Reynolds & Orchard, 2010; Reynolds *et al.*, in press). The PARC box

release method has been developed incorporating suitable loadings and cardboard inserts (five the width of the box and two the length of the box, to sit mid-way up the mesh window and just above the pupal bed) as resting/drying space for adult flies which has lead to good retention of dye on the ptilinum and good recapture rates (Reynolds & Orchard, 2010; Reynolds *et al.*, in press; Fig. 7), thus indicating a sound release method.

b. Rearing out protocol

PARC

For adult release, it is optimal to obtain pupae that are from two consecutive hopping dates (as opposed to three or more dates) and should be requested from the factory where possible. This ensures that the majority of adults are of a similar age and minimises death in the rearing out/release boxes.

Upon receipt, pupae from each 1L carton (in which the pupae are transported) containing 800g of pupae should be evenly distributed on the base of three PARC boxes (i.e. approximately 266g per PARC), containing cardboard inserts (Fig. 7). At least nine sugar cubes should also be placed on the base of each PARC box. Once the lid has been positioned, straps on either end of the boxes may be required to minimise insect escape (particularly in old or warped boxes).

Emerging sterile fruit flies should be fed a block of sugar-yeast-agar (see section on 'Dietary protein' above) placed on the mesh opening on top of the PARC box.

The PARC boxes should be placed in an insect growth room at 26±2°C, 65±10% RH and a light: dark period of 14:10, preferably with a simulated dawn and dusk as the lights ramp up and down at the beginning and end of the light phase. They may be stacked in such a way as to allow airflow between the boxes. This prevents ammonia and carbon dioxide (from respiration and excrement) from accumulating in and around the boxes (Fig. 9). In the growth room, the majority of adult emergence occurs on days 3 & 4 after pupal placement (i.e. Receive and setup pupae in release containers on a Wednesday; majority of emergence usually occurs Saturday and Sunday).

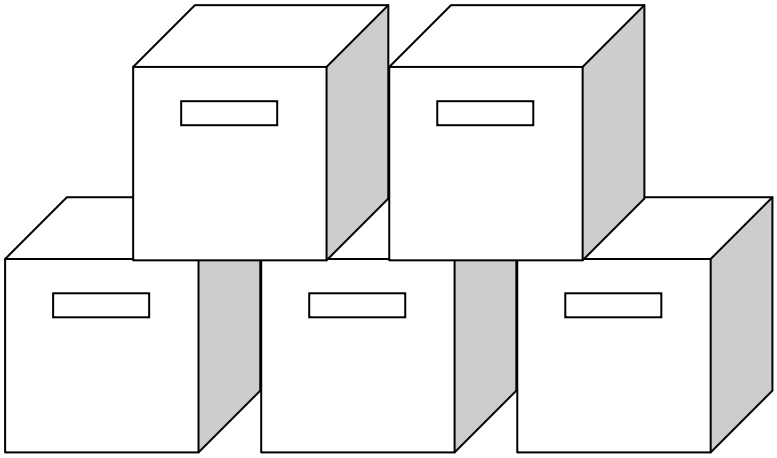

Figure 9. Diagrammatic example of how to stack PARCs to ensure airflow between and around boxes.

c. Release protocol

As a general rule, and in order to allow the adults 1-3 days access to food, particularly protein, the adults should be released when the majority are aged 48-72h.

Flies for adult release should be transported in an air-conditioned vehicle to their required release site. Once at the release site, PARC boxes should be placed on the ground (in the shade if the temperature is 26°C or greater and in the sun if less than 26°C, the straps removed and lids opened to allow the flies to disperse without coercion (Fig. 10). After 5-10 minutes, the boxes can be agitated and a feather duster brushed gently over the box and cardboard inserts to remove any remaining flies. The remaining pupal debris should be taken back to the laboratory and discarded appropriately, according to the site's requirements.

Figure 10. PARCs with cardboard inserts, showing sugar cubes in the base (a) and cardboard inserts (a & b).

2. Chilled adult release (ground)

a. Background

Adult release of sterile adult Queensland fruit fly has proven to be effective as part of an Integrated Pest Management approach in eradicating fruit fly incursions (Fisher, 1996) and suppressing wild Queensland fruit fly in buffer zones thereby minimising introductions into pest-free areas (Dominiak *et al.*, 2003b). However, some of the distances that need to be covered from the rearing out facility to the suppression or eradication site are vast and large numbers of fruit flies need to be transported resulting in high labour costs. In response to this we are considering the use of chilled adult release. This method is used to transfer sterile, adult, mass reared fruit flies from emergence containers to the field for release to allow more compact packaging and reductions in the space required for transportation (Mangan, 1996). Chilled adults are usually released via roving release, whereby sterile insects are released from a moving vehicle, allowing the rapid dispersal of large numbers of sterile flies (Meats & Smallridge, 2007). Chilled adult fruit fly release is used effectively around the world, often as part of an aerial release program using airplanes (Cunningham *et al.*, 1980; Sivinski *et al.*, 2000) or helicopters (Nakamori & Kuba, 1990; Vargas *et al.*, 1995). Ground release programs do not commonly involve chilled adults (Dominiak *et al.*, 2000; Fisher, 1996), with few published studies trialling the release of chilled adults from ground release vehicles (Salvato *et al.*, 2003). The effect of chilling or chilled adult release on Queensland fruit fly performance has received very little attention in Australia. Meats & Fitt (1987), suggested from modelling that for Queensland fruit fly the least stress and most

rapid recovery would result if the chill temperature was as close as possible to the torpor threshold. They showed that adult Queensland fruit fly acclimated at 25°C, could be chilled for 24h at 1°C and would recover within 12 minutes at 25°C. Recently, Reynolds & Orchard (2011) has shown that chilling sterile adult Queensland fruit fly has little or no detrimental effects on recovery time, flight and longevity. Recapture rates of chilled adults are also comparable to those of adult recapture rates (Reynolds *et al.* unpubl. data). Reynolds *et al.* (unpubl. data) has also shown that, following methods used in many overseas SIT programs, the use of paper bags with approximately 28g of pupae in each bag, is an effective way of rearing out adult Queensland fruit fly for chilled adult release. The use of eclosion towers as opposed to PARC boxes for rearing out adults for chilled adult release and the use of automated release systems also needs to be investigated further.

b. Rearing out protocol

Pupae should be set up similar to that described under adult release, except as detailed below. Place nine paper bags (base approximately 21cm x 8cm; cut to required height) in each PARC box, to sit comfortably alongside one another. As approximately 28g of pupae in each paper bag provides good emergence and to ensure efficiency and practicality, it is suggested that a measured volume scoop is used to deliver the approximate required number of pupae to each paper bag. For example, this can be easily done by selecting a small container, weighing the required number and then cutting the container off at the point, or marking a line at the desired volume. Upon receipt of the pupae scoop the required number into each paper bag and add a single sugar cube. Paper bags should be cut so that they sit halfway the height of the window on the side of each PARC box and should be stapled with a single staple (or paper clip), at the centre of the paper bag, with openings either side to permit eclosed adults to escape (Fig. 11).

The PARC boxes should be placed in an insect growth room as described for adult release, and should also be fed the agar-sugar-yeast block.

Figure 11. A PARC with nine paper bags containing sterile, dyed Queensland fruit fly pupae.

c. Release protocol

On the day of release, adult Queensland fruit fly designated for chilled adult release should be taken from standard rearing conditions in their PARC boxes and placed into a separate growth room at 4°C for 30 min. After 30 minutes, the PARC boxes should be banged three to four times on the floor to dislodge all of the flies from the sides of the box and paper bags. The lid should then be removed and the paper bags shaken to dislodge any remaining flies that are clinging onto the bags, before being discarded. Flies should then be packaged into individual 12 oz. paper cups (Uni Cups & Lids (Detpak) Premier Northpak, 13-15 Edinburgh Road, Marrickville NSW 2204) before placement in a polystyrene box lined on the base and top with Techni Ice (Techni Ice Australia P/L, 14 Tooyal Street, Frankston VIC 3199), separated by plastic bubble wrap from the paper cups, to ensure the flies remain motionless until release. Polystyrene boxes containing the chilled flies should then be transported in an air-conditioned vehicle to their respective release locations.

The current prototype developed to release sterile chilled flies is based on that designed by Terry Spithill (WA Agriculture) (Fig.12a&b) and adapted for NSW Queensland fruit fly releases (Fig 12c). Once at the release point, 12 oz. cups holding chilled flies should be taken from the box as needed and tipped into the funnel of the automated blower within 50m each side of the designated release point. This can be done from the passenger side of a slow moving vehicle over a 100m distance (Fig. 13) or as a standing/stationery release between trap sites.

Figure 12. A prototype automated blower (a & c) that is run by a small fan and can be plugged into a car (b) or a battery pack (c) (battery not shown) to release chilled adult fruit flies.

STERILE FRUIT FLY GENERAL RELEASE GUIDELINES

1. Release Guidelines

a. Background

In recent years, we have mad a number of advances in our knowledge of the ecology of Queensland fruit fly with respect to the use of the SIT. This is largely due to the work and publications of Andrew Jessup (NSW DPI), Bernie Dominiak (NSW DPI), Gus Campbell (NSW DPI), Alfie Meats (University of Sydney), Phil Taylor (Macquarie University), Tony Clarke (QUT) and Olivia Reynolds (NSW DPI) and their respective research groups. Although, there are still a number of gaps, and further refinements continue to be made, we have an established and effective release program. This section details the release rates, overflooding ratios (sterile flies to wild flies), frequency of release and spacing/locations for release sites based on our current knowledge.

b. Release protocol

Operations groups should always first refer to the Queensland Fruit fly Code of Practice. Further, operations programs should at the least abide by the following tentative conclusions from Meats (1996):

i) A minimum release rate of 60,000 sterile males per week per km^2 should be maintained until eradication is apparent.

ii) If populations exceed 20 wild males per km^2, a higher release rate is required and can be calculated with a formula provided in Meats (1996). Alternatively, pre-baiting may be considered to reduce wild flies to this level.

iii) Effective intervals for release points seem to be 400m and are in agreement with the known dispersal characteristics of Queensland fruit fly.

Operations programs should also take into account the following:

i) Aim for an over flooding ratio of 100:1 (based on recapture data of sterile males)

ii) Sterile flies should not be released within 100m of a trap

iii) 10 weeks of sterile releases are required as a minimum (as per (Anonymous, 2011). Survival of the released flies means that they should be active in the release area for an average of 4 weeks

iv) For pupal release, a succession of pupal release boxes should be placed at each release point, to meet the above criteria.

v) Any surplus flies should be used to suppress populations in the RRZ towns in order to relieve the pressure on the FFEZ and to minimise factory produced sterile flies going unused.

STERILE FLY IDENTIFICATION

1. Microscopy

a. Background

Most SIT programs use fluorescent dyes to coat the sterile pupae, so that upon eclosion, the ptilinum of the fruit fly is marked with dye in order to distinguish

between sterile fruit flies and wild fruit flies captured in traps (Enkerlin *et al*., 1996). Adequate retention of dye on the ptilinum of Queensland fruit fly is therefore a crucial component of SIT in Australia. A number of studies have shown that when Queensland fruit fly pupae are dyed with the correct amount of dye and are late-stage pupae, adequate dye is retained in the ptilinum (Reynolds *et al*., 2010; Reynolds & Orchard, 2010; Reynolds *et al*., in press).

b. Protocol

Fruit fly trap captures are sent to the Agricultural Scientific Collections Unit, Orange Agricultural Institute, Orange, NSW, Australia, to ascertain retention of dye on the ptilinum. This procedure (OENT 017) is performed under a Leica MZ 8 microscope (Leica Microsystems Pty Ltd, Unit 3, 112 -118 Talavera Road, North Ryde, NSW 2113) initially under a blue light source (Leica CLS 150 X light source with a blue filter). Well dyed flies can be discriminated at this time. Where the presence of dye colour is very low or doubtful further examination of the specimen under a UV (Leica Stereo- Fluorescence System 10446271 with a blue filter) is performed as this will enhance any dye fluorescence. If a fly is observed to have dye on the ptilinum, then the fly is recorded as sterile. If no dye is detected on the ptilinum, the fruit flies will be retained for further testing, including head dissection (internal viewing of ptilinum – under UV light). If dye is still unable to be detected, the fruit flies can be sent for molecular identification (see below).

2. Mitochondrial DNA (mtDNA)

a. Background

In order to confirm if a trapped fly is a wild fly, molecular techniques may be employed. Microsatellite DNA has proved effective in understanding the source of fruit fly incursions (Gilchrist *et al*., 2006). A recent study (Blacket *et al.* unpubl. data) assessed the potential of employing mitochondrial DNA (mtDNA) sequences to characterise outbreak and sterile populations of Queensland fruit fly. This study allowed the extent of gene flow (i.e. dispersal) in different regions to be estimated and detected a number of distinctive populations. Screening of the existing lab colony indicated that much of the genetic variation present appears similar to some wild southern populations (Blacket *pers. comm.,* 2011).

b. Protocol

This mtDNA screening technique is available as a diagnostic service through DPI Vic. If this technique is to be employed, the colony or lab-reared flies for sterile release need to be sourced from a population distinct from the wild southern populations where sterile flies are currently released (therefore are genetically distinctive for mtDNA from the southern populations).

DEFINITIONS AND ABBREVIATIONS

eclose	The emergence of an insect larva from the egg or an adult from the pupal case
Microsatellite DNA	Molecular markers used in genetics; repeating sequences of 1-6 base pairs of DNA
Mitochondrial DNA (mtDNA)	DNA inherited from the mother (maternally inherited)
PARC	Plastic Adult Rearing Container
pupae	The non-feeding stage of development of an insect between the larva and adult, during which it undergoes a complete transformation within a protective cocoon (puparium) or hardened case
puparium	A rigid outer shell of an insect formed from the larval skin that covers the pupa (pupae)
ptilinum	An eversible sac on the head of a fly, above the base of the antenna, used to force off the end of the puparium in order for the adult fly to emerge
SIT	Sterile Insect Technique
SOP (Standard Operating Procedure)	A procedure developed by states and territories that describes in detail fruit fly management
tephritid	Fruit flies belonging to the family Tephritidae

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Appendix 2

A report on the use of Eclosion Towers for rearing sterile Queensland fruit fly in Australia

2011

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NSW DEPARTMENT OF **PRIMARY INDUSTRIES**

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Background

The sterile insect technique (SIT) is used to prevent, suppress or eradicate infestations of a number of fruit fly species, both nationally and internationally. In Australia the SIT is used to suppress or eradicate the Queensland fruit fly, *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae), a pest which attacks most commercial fruit and many vegetables (White & Elson-Harris 1992). The current SIT program involves the production of pupae at the Fruit Fly Factory, located at the Elizabeth Macarthur Agricultural Institute, Menangle, New South Wales (NSW) which is jointly funded by the NSW, Victorian and South Australian governments. Sterilisation of late-stage pupae occurs at the Australian Nuclear Science and Technology Organisation (ANSTO), Lucas Heights, NSW, before they are sent to the requesting adult eclosion facility. Here, after a pre-release holding period of 5-6 days during which pupae mature and adults eclose and feed, ground release of adults into the environment is carried out. As even this brief outline suggests, the SIT for The Queensland fruit fly is a relatively expensive management strategy in terms of materials and labour. As a consequence there is a constant requirement to increase the efficacy of this protocol and reduce overall costs.

SIT operations utilise a number of methods in which to hold pupae and newly emerged adults, including plastic storage (PARC) boxes (Reynolds *et al.* in press; Fig. 1a), plastic bins (Perepelicia *et al.* 1997; Fig. 1b), Clean Fruit Cages (Dantas *et al.* 2006; Fig 1c), paper bags and more recently Eclosion Towers (Salvato *et al.* 2004; Shelly *et al.* 2006; Fig 1d).

Figure 1. Various methods utilised to rear pupae through to adult eclosion including a) PARCs, b) plastic bins, c) Clean Fruit Cages (image from Dantas *et al.* 2006) and d) Eclosion Towers (image from Anon. 2002).

Increasingly, Eclosion Towers are being used by release programs as they save both space and labour compared with PARC systems (Shelly *et al.* 2006) and reduce fly escape (Dowell *et al.* 2005). To hold the same number of flies, the Eclosion Towers require much less space than the PARC system and the eclosion system has considerably reduced labour costs due to the automated pupal loading, puparia separation and disposal and tray washing (Jessup 2003; Shelly *et al.* 2006). They have been used to successfully rear the related Mexican fruit fly, *Anastrepha ludens* (Loew) in southern Texas (Forrester and Worley, 1999, unpublished as cited by Salvato *et al.* 2004) and the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) in Florida (Shelly *et al.* 2006) and Guatemala (Reynolds pers. obs. 2009). The Sarasota, Florida Sterile Insect Release facility (SIRF) was the first SIT operation to fully utilise the Eclosion Towers developed by the Mexican Fruit Fly Facility and Methods Development in Mission, Texas (Anon 2002).

Potential economic benefits of the eclosion system may be outweighed however, if the sterile flies are of poor quality, relative to the PARC or other existing system. Important considerations when rearing flies for sterile release programs are to ensure that, in

addition to economical concerns, any adverse affects of the rearing system on adult weight, flight ability, eclosion (yield), longevity, dispersal ability and mating competitiveness are minimised.

Salvato *et al.* (2004) showed no difference between PARC boxes and Eclosion Towers for yield, weight and flight ability of the Mediterranean fruit fly at the Florida SIT Release facility. While yield was not expected to differ considerably between the two and given that weight did not differ it is suggested that the trays of the eclosion tower system allowed the developing flies ample exposure to the agar food supply. Although the compact conditions of the eclosion tower trays reduce insect movement to walking only, Salvato *et al.* (2004) demonstrated that this did not affect their ability to fly. Further, Shelly *et al.* (2006) showed that sterile Mediterranean fruit fly males held in Eclosion Towers achieved approximately the same mating success as sterile males held in PARC boxes.

Based on these comparative male vigour trials, Salvato *et al.* (2004) and Shelly *et al.* (2006) concluded that the tower eclosion system is an efficient alternative to the PARC system.

Plastic adult rearing containers (PARCs)

Plastic bins and PARCs are the most commonly used forms of sterile adult release in Australia (Reynolds *et al.* in press). Recently, PARCs were adapted under Australian conditions to ensure optimal emergence, flight, longevity and trap recapture rates for Queensland fruit fly sterile adult release (Reynolds 2010; Reynolds & Orchard 2010, Reynolds *et al.* in press) and chilled adult release (Reynolds & Orchard 2011, Reynolds unpubl. data). NSW utilises PARCs routinely at their Yanco Sterile Fruit Fly Release Facility (Yanco SFFRF). PARCs comprise a translucent 46L (64cm x 41cm x 26 cm) plastic lidded container (Silverlock MH 0110, colour "natural"), with a 43cm x 20cm, 1mm mesh on the lid and a 15cm x 10cm mesh on two sides of the box for ventilation. Up to 300g pupae are placed on the base of the PARC with a cardboard structure provided for additional resting space (Reynolds & Orchard 2010). On top of the mesh on the lid, a block of Agar containing a mixture of white sugar (carbohydrate) and yeast hydrolysate (protein) (3:1 by weight) and water (A. Jessup, unpublished data; Reynolds & van der Rijt 2011) is provided as food, in addition to sugar cubes placed on the base of the PARC. PARCs are stacked in a staggered format to ensure airflow in and out of the PARCs, which is assisted by several rotary fans at the Yanco Release facility.

In Australia, as in any sterile insect release program, the most efficient and economical use of staff and space with minimal fly escape are important considerations. Yanco has the capacity to hold 400 PARCs (Jessup 2003), each with up to 300g pupae (Reynolds & Orchard 2010) i.e. 30,000 (10mg pupae) to 37,500 (8mg pupae). This is a total of 12 million (10mg pupae) to 15 million (8mg pupae) at a given time.

Eclosion towers

The Eclosion Towers utilised by SIRF, Florida for Medfly and Texas for Mexican fruit fly consist of interlocking screen-panelled aluminium frames (trays) stacked on a trolley base (Dowell *et al.* 2005). The pupae are placed into a channel around the inside perimeter of each tray (Dowell *et al.*2005), together with an agar/sugar-based gelatin as food and water (Dowell *et al.*2005; Anon 2002, 2004). The floor of the tray is comprised of fly mesh (Jessup 2003). When a frame is placed on top, there is no gap between the outside edge

of the lower and upper tray but there is a slight gap on the inside (Dowell *et al.* 2005). Each tray is 25mm high and 760mm square (Jessup 2003). A small, direct current axial exhaust fan is placed on top of each eclosion tower, providing forced upward ventilation (Dowell *et al.* 2005). Eclosed adults move from the outside channel to the mesh screen of a tray, leaving the empty puparia in the channel. The flies remain on the screen and feed for 4-6 days allowing them to attain sexual maturity prior to release (Anon. 2002; Dowell *et al.* 2005).

Each eclosion tower may hold up to 80 trays (Dowell *et al.* 2005), with each tray holding approximately 25,000 Medfly (Anon. 2002) which are slightly smaller than The Queensland fruit fly, or 400ml pupae (Jessup 2003). Each tower holds approximately 1.67 million pupae.

On the day of release, the towers are moved into a cold-room where high-volume exhaust fans suck cold air into each tower to accelerate fly immobilisation (Dowell *et al.* 2005). Following a brief period of exposure to the cold, pupae are vacuumed from the tray channels and the screen trays holding chilled flies, are emptied into a hopper, with a pan beneath for collection. Flies are then transferred to the release container, ready for chilled-adult release (Dowell *et al.* 2005), a method whereby adult flies are released while 'chilled'. This method is used to transfer sterile chilled adult flies from emergence containers to the field for release and allows more compact packaging and reductions in space required for transportation (Mangan 1996).

Eclosion towers for the Queensland fruit fly

In Australia, considerable travel distances between the locations of fly production and release, along with the large number of flies that need to be transported in an incursion situation, result in high labour and travel costs (Reynolds & Orchard *2011*). Chilled adult release utilising Eclosion Towers is an option which will reduce overall costs and space requirements.

Reynolds *et al.* (2010) determined that chilling adult Queensland fruit flies to 4ºC had no adverse effect on longevity and flight ability, although recovery may take up to 15 minutes for chilled flies. Field studies have also shown that trap recapture rates of chilled flies are similar to those of non-chilled flies (Reynolds unpub. data). Yanco has a suitable room to chill the flies (Fay 2003). This suggests that adult Queensland fruit flies would be a suitable candidate for chilled adult release, and therefore for Eclosion Towers. Indeed Fay (2003), recommended that the use of Eclosion Towers be pursued at Yanco as they allow the flies to escape the pupal mass and are a more practical means for collecting and transporting emerged flies for release. The excessive travel distances between the locations of fly rearing and release, together with the large number of flies that need to be transported currently results in high labour and travel costs (Reynolds & Orchard *2011*).

In order to compare the space requirements of both PARCs and Eclosion Towers at Yanco for an equivalent number of pupae, the following calculation was developed (after Jessup 2003). If a single tray of an Eclosion Tower holds 400ml of flies, a tower of 73 trays (i.e. 72 filled with flies and an empty one on top as the lid) would hold 28.8L of pupae. If this size eclosion tower was used at the Yanco SFFRF, each tower would hold 1.25 million (10mg) to 1.56 million pupae (8mg). Therefore, approximately 1.25 million pupae per tower equates to 42 PARCs (ie. 30,000 pupae per PARC).

Thus, the floor area required, including a 20cm space between each PARC (stacked four high, 13 on the base) and towers for aeration, used by the two systems to produce 1.25 million flies would be:

Yanco (PARCs): 4.82m^2
Texas (Eclosion Tower): 0.92m^2

Therefore, 12 Eclosion Towers would house 15 million flies and take up 11m^2, including 20 cm between each tower; considerably less than PARCs.

Cost considerations **(after Jessup 2003)**
An eclosion tower comprising 73 frames, with a trolley base and a 24 Volt DC fan for ventilation is approximately $US 1,750 (approximately $US 1,030 for material and $US 720 for labour at $US 9.00 per hour).

With the current exchange rate (US$1 = AU$1.03), 12 Eclosion Towers would cost approximately AU$20,390, not including freight. However they can be made locally and may offer further price reductions. A tray loading system, dump hopper and tray washing/drying system would also be required in the initial set-up but costs for these should not exceed AU$3000.

Andrew Jessup is able to obtain the manufacturing specifications if we wish to proceed with building the Eclosion Towers.

Conclusions
These results are encouraging for the Queensland fruit fly and suggest that economic considerations, rather than problems with fly quality, are likely to be the main influence in operational decisions to change from PARC or other similar space-craving and labour-intensive operation systems to the Eclosion Tower system.

Recommendations
A study is funded by HAL and industry to trial the Eclosion Towers to include a comparison of the quality of flies produced from both PARC boxes and Eclosion Towers and a field release trial comparing the two methods.

Acknowledegments
This project has been funded by HAL using the R&D funds from the citrus industry and vegetable industry levies and matched funds from the Australian Government.

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Appendix 3

Ref: INT10/63305

Procedure
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QUEENSLAND FRUIT FLY – ADULT STERILE INSECT RELEASE

NUMBER PRO 2010/XX	VERSION 1
AUTHORISED BY [click and type]	
AUTHORISED DATE [click and type]	EFFECTIVE DATE [click and type]
ISSUED BY Primary Industries, Biosecurity	

REVISION HISTORY

VERSION	DATE	AMENDMENTS SECTION	AMENDMENTS DETAILS
1	09/06/2010		

NEXT REVIEW DATE: 30/06/2012

Purpose

The purpose of this procedure is to detail how to conduct adult sterile Queensland Fruit Fly (Qfly), *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) releases using plastic adult rearing container (PARC) boxes.

Qfly are sterilised prior to release. Sterilisation is achieved by irradiation of pupae at the Australian Nuclear Science and Technology Organisation (ANSTO) at Lucas Heights in Sydney. After irradiation, there is no residual radiation emanating from the pupae or their packaging.

Scope

This procedure applies to staff of Industry & Investment NSW.
This document provides a mandatory framework for AQIS audit requirements.

Definitions and acronyms

Qfly - Queensland Fruit Fly.

Pupae – an insect stage after larvae and before adult.

PARC - plastic adult rearing container. Modified versions are used to rear pupae through to adults and release them into the environment.

Warnings

Users are reminded of the need to follow safe work practice when applying any techniques described in this procedure. This includes identifying, assessing and managing any occupational health and safety risks.

Safe Work Method Statements that refer to activities included in this procedure must be used in assessing and managing risks.

PROCEDURE

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Appendix A 7
Appendix B 10

Receival of Queensland Fruit Fly Consignments

1.1 Equipment

Operators will ensure all the required equipment is on hand before deliveries of sterile Qfly commence.

Equipment includes:

1. Weighing scales (accurate to 0.1g)
2. Plastic bags
3. White sugar cubes (from cane sugar)
4. Adult diet ingredients
5. Cardboard partitions
6. PARC boxes and straps
7. Global Positioning System (GPS) units
8. Insect growth room under standard conditions (ie. 26±2°C, 65±10% RH and a light:dark period of 14:10, with a simulated dawn and dusk as the lights are ramped up and down at the beginning and end of the light phase)
9. Quality assurance spreadsheet.

Relevant information from the consignment will be recorded on a standardised spreadsheet saved as a paper and an electronic copy which documents:

Shipping Date
Pupal Batch Number (weeks)
Collection Date
Level of radiation exposure (ANSTO details)

Quality assurance will check each weekly batch for emergence and flight ability using the procedures detailed in the FAO/IAEA/USDA (2003) manual (Appendix A).

1.2 Consignment Receival

Sterilised Qfly pupae are delivered from ANSTO on a Wednesday morning, by air (preferably) or road freight. Long periods at variable temperatures on road freight may cause death and/or poor emergence and decreased performance of emerged adults. Staff should pick up consignments from the airport to avoid delivery delays. Air conditioned vehicles with soft suspension should be used, with consignments located out of the sun.

Consignments of pupae are sent in white foam polystyrene insulated boxes. More than one white polystyrene box may be in a single consignment. Pupae are contained inside clear plastic bags within 2L cardboard boxes. A rubber band always keeps the plastic bag air tight. On exposure to air, the heat generated by recommencement of pupal respiration can be rapid, contributing to heat stress to the emerging flies. Therefore once the bag is opened, pupae should be placed in modified plastic adult rearing containers (PARC) in a timely manner. For a description of a PARC see section 2 below.

Plastic Adult Rearing Container (PARC) construction

PARCs should comprise a translucent 46L plastic lidded container (Silverlock MH 0110, colour "natural", 645mm x 413mm x 275mm high), with a 43cm x 20cm, 1mm mesh on the lid and a 15cm x 10cm 1mm mesh on two sides of the container for ventilation (Fig. 1). Resting space should be provided by wedging cardboard dividers (approximately 160 cm in height), two running lengthways and five across the width of the container, to sit just above the pupal bed. Dividers can be reused. PARCs should be washed after each release with detergent and rinsed thoroughly to ensure that they do not harbour any harmful bacteria or fungi. PARCs should also be dried thoroughly (preferably inverted upside down in the sun until dry) as free water inside the containers may interfere with dye retention of released adults and adults may also drown in any free water.

Fig. 1. A plastic adult rearing container showing the mesh lid and sides, cardboard insert and dyed irradiated pupae on the floor of the container.

PARC Pupal loadings

The weight of pupae per PARC should range between 180g and 300g (1g is approximately equivalent to 100 pupae) to maximize emergence and flight. Emergence of flies from the boxes should be monitored to determine the peak day of emergence (usually 3 days after receipt and setup). This will enable ready determination of the optimal day for release to allow sufficient access to a balanced diet (see Nutrition for adult Qfly below).

Pupae should be weighed out or measured by volume for each release box. If pupae are to be weighed the following method should be followed. Cardboard 2L cardboard boxes received in the consignment are lined with new plastic bags, and are tared on a top loading balance. Pupae are gently poured into the plastic bag-lined box to the predetermined weight to within 2g. The pupae-containing plastic bags can then be distributed immediately into the PARC boxes. This weighing should ideally occur within the same facility as the growth room.

Pupae should be gently poured onto the bottom of the release box and spread evenly. Do not drop pupae from more than 5 cm into the release boxes as this may damage the pupae and/or the resulting adult fly.

PARC SETUP

To minimise fly escape, particularly in older and/or warped boxes the lid should be secured with two tie down straps placed at either end of the PARC box.

Once the PARC boxes are setup they should be placed in an insect growth room under standard conditions, i.e. 26±2°C, 65±10% RH and a light:dark period of 14:10, with a simulated dawn and dusk as the lights are ramped up and down at the beginning and end of the light phase. Fans should also be used in the growth room to ensure airflow that will aid in keeping PARCs free from build-up of excessive ammonia, CO_2 and heat.
PARCs should be stacked in such a way as to allow airflow. Under standard conditions in a growth room, the majority of adult emergence usually occurs on day 3 after pupal placement, but this should be monitored.

Nutrition for Adult Qfly

Adult flies survive better and are more competitive if provided with nutrition and water. Nine cubes of white sugar should be placed randomly on the floor of each PARC box. In addition, on the day of expected fly emergence, a block of agar (15 x 15cm) comprising a mixture of white sugar and yeast hydrolysate (3:1 by weight) and water (A Jessup, unpublished data) (see Appendix B) should be placed on top of the mesh of each box lid for adult feeding and replaced as needed.

Releasing adult Qfly

As a general rule, and in order to allow the adults 1-3 days access to food, particularly protein (Perez-Staples *et al.* 2007), the adults should be released when the majority are aged 48-72h (ie 2-3 days after the majority emerge).
Flies for adult release should be transported in an air-conditioned vehicle (cooled to under 30˚C) to their required release site. Release sites should be spaced at a uniform distance between cue-lure baited traps in a trapping grid, but no closer than 100m to any trap site. Operators will use a GPS unit (or similar equipment) to precisely locate the release site (see Procedure BC&MS 2007/044 and Procedure BC&MS 2007/045 for correct use of GPS). They will not rely on a visual estimation of distance. The GPS coordinates must be obtained for all release and monitoring sites and recorded on data sheets electronically (paper may be used in the field, but all data should be written and saved to an electronic spreadsheet; both hard and soft copies should be kept).
Once at the release site, PARC boxes should be placed on the ground (in the shade of a tree or similar if the temperature is greater than 27°C and in the sun if less than 26°C (but still close to a tree where possible), the straps removed and lids opened to allow the flies to disperse without coercion. After 5-10 minutes, the boxes can be carefully agitated and a feather duster brushed gently over the box and cardboard inserts to remove any remaining flies. The remaining pupal debris should be taken back to the laboratory and discarded appropriately.
Flies should not be released when the temperature is below 16˚C as this is the flight threshold for Qfly. Rather, wait for the day to warm up. If temperatures are not predicted to rise above 16˚C and a release is required, then PARCs may be transported in a heated vehicle to maximise the chances of the flies taking flight upon release.
Following this procedure carefully will ensure that adult flies retain sufficient dye in their ptilinum for ready identification as sterile flies by experts trained in fruit fly identification at the Orange Agricultural Institute.

Supplier details

Yeast hydrolysate: MP Biomedicals Australasia Pty Ltd, PO Box 187, Seven Hills, NSW 2147, Australia.
Silverlock crate MH 0110 (PARCs): Blackwoods, http://www2.blackwoods.com.au/Default.aspx or phone 137323 for your nearest store/supplier.

References

Food and Agriculture Organisation of the United Nations (FAO), International Atomic Energy Agency (IAEA) & United States Department of Agriculture (USDA). 2003. *Product Quality Control and Shipping Procedures for Sterile Mass-Reared Tephritid Fruit Flies*, Version 5. IAEA, Vienna, Austria.

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Prabhu, V., Perez-Staples, D. & Taylor, P.W. (2008) Protein: carbohydrate ratios promoting sexual activity and longevity of male Queensland fruit flies. *Journal of Applied Entomology,* **132,** 575-582.

Reynolds, O.L. & Orchard, B.A. (2010). Container loadings and eclosion units for sterile insect technique programs of *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae). *General and Applied Entomology*, **39**, 19-22.

Legislation

- Nil

Related Policies and Procedures

- Procedure BC&MS 2007/044 – Monitoring and surveillance: Setting Garmin 72XL GPS receivers to the correct position format map and datum
- Procedure BC&MS 2007/045 - Monitoring and surveillance: Setting Garmin 72/76 GPS receivers to the correct position format map and datum
- Appendix A
- Appendix B

Contact

Position Dr Olivia Reynolds, Research Scientist
Contact no. 02 4640 6426

NSW GOVERNMENT | Industry & Investment

Quality Assurance
Pupal weight, Emergence and, Flight Ability

1. Introduction

The joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture and the US Department of Agriculture developed an internationally accepted set of procedures for the quality assurance of Sterile Insect Technique (SIT) reared and released flies. This includes quality assurance measures to determine the emergence, flight ability and pupal weight of each batch of sterile pupae received that is used for research or release purposes.

2. Principle of Procedure

To measure the emergence, flight ability and pupal weight of SIT mass-reared Queensland fruit flies using a standardized method.

3. Scope and Application

This document is a slightly modified version of the internationally accepted procedure for Quality Control (QC) of mass reared Queensland Fruit Flies used for the Sterile Insect Technique (SIT)

4. Safety Procedures

Personal Protection
HANDS/FEET
- Safety footwear

OTHER
- Protective clothing (lab coat).

Material Safety Data Sheet (MSDS) – Read relevant MSDS before handling any chemicals.
Safe Work Method Statements – Read relevant assessments (Setup and release of SIT fruit flies; Receival and allocation of sterile dyed Queensland fruit fly pupae; Qfly quality assurance, emergence and flight ability) before completing tasks and handling any chemicals (available on the Risk Management Database).

5. Apparatus

- Plexiglass tubes: outside diameter 8.9 cm with 3mm thick walls; spray painted black; 10 cm high (care must be taken to not scratch the inner surface of the tubes)
- Plexiglass chamber: 30 x 40 x 30 cm (w x d x h) with a mesh covered ventilation hole in the top (similar sized mesh cages may also be used)
- Petri dish lids: 100 x 15 mm, overlaid with black paper disks.
- Strips of black porous paper 1 cm wide stapled at the ends to form a ring 6 cm in diameter.
- Analytical Balance (accurate to 0.001 gm)
- Miscellaneous equipment: unscented talcum powder, forceps, spoons, spatula
- Aspirator and vials
- Stereo microscope or magnification lamp
- Insect growth room

- Temperature 26 C ±2 C
- Humidity 65 %RH ± 10%
- Photoperiod 14:10 hours light: dark

6. Procedure

Each batch of fruit flies received is identified by the larval week and larval hopping date (the date the larvae commence pupation). The shipment date and radiation dose are also included on a sticky label on the shipment box. This label should be peeled off and stuck onto the data form '*Fruit Fly QC Emergence & flight ability template*'.

- When received, for each batch of pupae dyed a different hue, five replicates of 100 pupae are counted and placed within a black paper ring on a black paper lined petri dish lid.
- The total weight of each of three replicates are weighed to 0.001 gram accuracy and recorded on the excel spreadsheet – '*Fruit fly QC Average weight pupae template*' to determine the average pupal weight for each batch.
- The inner surface of the black plexiglass tubes are coated with unscented talcum powder (excess removed by tapping tubes on a firm surface) before talc is removed from the bottom 2 cm with a damp soft cloth.
- Petri dish lids holding pupae are placed within a clear & colourless plexiglass chamber and the black plexiglass tubes are placed over the top. The chamber is then placed in the growth room.
- Fruit flies that exit the tubes are removed from the chamber using an aspirator. This is carried out daily or more often during peak emergence.
- When the fruit flies cease to emerge for two consecutive days, the QC is deemed finished.
- The apparatus is disassembled, cleaned and the fruit fly debris are separated into four categories (not emerged, part emerged, deformed adult flies and non-fliers (non-deformed adult flies)), counted and recorded on the excel spreadsheet '*Fruit Fly QC Emergence & flight ability template*'.

7. Calculations and Reporting

Average pupal weight results are entered into the excel spreadsheet – '*Fruit Fly QC Average weight pupae template*'. This is an Excel spreadsheet which calculates average pupal weight in the format shown below**.** The results are saved as hard copies as well as electronically.

Replicate	Number of pupae	Weight (to 0.001g)
1	100	
2	100	
3	100	
Mean		

Emergence and flight ability results are entered onto the excel spreadsheet – '*Fruit Fly QC Emergence & flight ability template*'. This is Spreadsheet calculates % emergence and flight ability in the format shown below. The results are to be saved as hard copies as well as electronically.

Setup Date: _____

	Shipment date	**Batch #**	**Collection date**	**Test date**	**Tester**
Pre Release					

		Replicate					
Elements		**1**	**2**	**3**	**4**	**5**	**Mean**
T	Number of pupae	100	100	100	100	100	100
A	Not emerged						
B	Part emerged						
C	Deformed						
D	Not fliers						

Calculations

			Value
E	**T-(A+B)**	% Emergence	
F	**T-(A+B+C+D)**	% Fliers	
	(F/E)*100	Rate of fliers	

If the percentage emergence and/or fliers falls below 75%, the Officer who conducted the test should contact the SIT Program Leader who should in turn inform the Fruit Fly Breeding Facility at the Elizabeth Macarthur Agricultural Institute (EMAI), Menangle.

8. Quality Control

Analytical balance used is calibrated in accordance with I&I NSW laboratory procedures.

9. Method Precision

Numbers of each count category are in whole numbers but when averaged between replicates, results are reported to two decimal places. Accuracy of average pupa weight is reported to three decimal places.

10. References

FAO/IAEA/USDA. 2003. Manual for product quality control and shipping procedures for sterile mass-reared Tephritid fruit flies, Version 5.0. International Atomic Energy Agency, Vienna, Austria. 85pp.

Adult Queensland Fruit Fly Diet

1. Safety Procedures

PERSONAL PROTECTION

EYES:
- Safety glasses (hot liquids).

HANDS/FEET
- Gloves (hot liquids).
- Safety footwear

OTHER
- Protective clothing (lab coat)
- Dust mask when using yeast hydrolysate
- Eyewash unit.

Material Safety Data Sheet (MSDS) – Read relevant MSDS before handling any chemicals.
Safe Work Method Statements – Read relevant assessments (Making adult Queensland fruit fly media) before completing tasks and handling any chemicals (available on the Risk Management Database).

2. Apparatus and Reagents

Mixture A: (for one batch)
500 ml water
4.5g agar
50g white sugar

Mixture B: (for one batch)
0.06g calcium propionate
20g Yeast hydrolysate

3. Procedure

- Boil Mixture A then allow to cool to about 40°C or for about 1h 30 minutes
- Stir in Mixture B
- Pour into clean dish and cover. Mixture when poured should be approximately 1.5 cm in depth
- When set cut into desired size. Each block should measure approximately 15cm x 15cm.
- Blocks are distributed to the insect rearing facility outside of the lab

Appendix 4

Procedure
PRIMARY INDUSTRIES
Biosecurity
Locked Bag 21, Orange NSW 2800
Tel: 02 6391 3100 Fax: :02 6361 9976

QUEENSLAND FRUIT FLY – CHILLED ADULT STERILE RELEASE

NUMBER PRO- XXX	VERSION 1
AUTHORISED BY	
AUTHORISED DATE	EFFECTIVE DATE
ISSUED BY Primary Industries, Biosecurity	

REVISION HISTORY

VERSION	DATE	AMENDMENTS	
		SECTION	DETAILS
##	[dd/mm/yyyy]		

NEXT REVIEW DATE: 01/08/2012

Purpose

The purpose of this procedure is to detail how to conduct chilled adult sterile Queensland Fruit Fly, *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) releases using plastic adult rearing containers (PARC).

Bactrocera tryoni are sterilised prior to release. Sterilisation is achieved by irradiation of pupae at the Australian Nuclear Science and Technology Organisation (ANSTO) at Lucas Heights in Sydney. After irradiation, there is no residual radiation emanating from the pupae or their packaging.

Scope

This policy applies to all staff of NSW DPI.
This procedure applies to staff of NSW DPI.

This document provides a mandatory framework for AQIS audit requirements. Definitions and acronyms

Pupae – an insect stage after larvae and before adult.

PARC - plastic adult rearing container. Modified versions are used to rear pupae through to adults and release them into the environment.

Warnings

Users are reminded of the need to follow safe work practice when applying any techniques described in this publication. This includes identifying, assessing and managing any occupational health and safety risks. Safe Work Method Statements that refer to activities included in this procedure must be used in assessing and managing risks.

PROCEDURE

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1. RECEIVAL OF QUEENSLAND FRUIT FLY CONSIGNMENTS

1.1 Equipment

Operators will ensure all the required equipment is on hand before deliveries of sterile *B. tryoni* commence.

Equipment includes:

Weighing scales (accurate to 0.1g)

1. Plastic bags
2. White sugar cubes (from cane sugar)
3. Adult diet ingredients
4. Cardboard partitions
5. PARC boxes and straps
6. Global Positioning System (GPS) units
7. Insect growth room under standard conditions (i.e. 26±2°C, 65±10% RH and a light:dark period of 14:10, with a simulated dawn and dusk as the lights are ramped up and down at the beginning and end of the light phase)
8. Quality assurance spreadsheet.

Relevant information from the consignment will be recorded on a standardised spreadsheet saved as a paper and an electronic copy which documents:

Shipping Date
Pupal Batch Number (weeks)
Collection Date
Level of radiation exposure (ANSTO details)

Quality assurance will check each weekly batch for emergence and flight ability using the procedures detailed in the FAO/IAEA/USDA (2003) manual (Appendix A).

1.2 Consignment Receival

Sterilised *B. tryoni* pupae are delivered from ANSTO on a Wednesday morning, by air (preferably) or road freight. Long periods at variable temperatures on road freight may cause death and/or poor emergence and decreased performance of emerged adults. Staff should pick up consignments from the airport to avoid delivery delays. Air-conditioned vehicles with soft suspension should be used, with consignments located out of the sun.

Consignments of pupae are sent in white foam polystyrene insulated boxes. More than one white polystyrene box may be in a single consignment. Pupae are contained inside clear plastic bags within 2L cardboard boxes. A rubber band always keeps the plastic bag airtight. On exposure to air, the heat generated by recommencement of pupal respiration can be rapid, contributing to heat stress to the emerging flies. Therefore, once the bag is opened, pupae should be placed in modified plastic adult rearing containers (PARC) in a timely manner. For a description of a PARC, see section 2 below.

2. PLASTIC ADULT REARING CONTAINER (PARC) CONSTRUCTION

Each PARC should comprise a translucent 46L plastic lidded container (Silverlock MH 0110, colour “natural”, 645mm x 413mm x 275mm high), with a 43cm x 20cm, 1mm mesh on the lid and a 15cm x 10cm 1mm mesh on two sides of the container for ventilation (Fig. 1). Nine paper bags (base 210mm x 80mm; cut to 160mm height) should be placed in the base of each PARC box, to sit comfortably alongside one another, each containing the required number of pupae (see PARC Pupal Loadings below) PARCs should be washed after each release with detergent and rinsed thoroughly to ensure that they do not harbour any harmful bacteria or fungi. PARCs should also be dried thoroughly (preferably inverted upside down in the sun until dry) as free water inside the containers may interfere with dye retention of released adults and adults may drown in any free water, before re-use.

Fig. 1. A plastic adult rearing container showing the dyed irradiated pupae contained within stapled paper bags on the floor of the container.

3. PARC PUPAL LOADINGS

The weight of pupae per PARC should range between 180g and 300g (1g is approximately equivalent to 100 pupae) i.e. 20g – 33.33g per paper bag, to maximize emergence and flight. Pupae should be weighed out or measured by volume for each paper bag. If pupae are to be weighed the following method should be followed. Cardboard 2L boxes received in the

consignment are lined with new plastic bags, and are tared on a top loading balance. Pupae are gently poured into the plastic bag-lined box to the predetermined weight to within 0.5g. The pupae-containing plastic bags can then be distributed immediately into the PARC boxes. This weighing should ideally occur within the same facility as the growth room.
Pupae should be gently poured onto the bottom of paper bag. Do not drop pupae from more than 5 cm into the bag as this may damage the pupae and/or the resulting adult fly.
Each paper bag should also include a single sugar cube. Once pupae and sugar have been added, each paper bags should be stapled with a single staple at the top centre of the paper bag, with openings either side to permit eclosed adults to escape.
Emergence of flies from the boxes should be monitored to determine the peak day of emergence (usually 3 days after receipt and setup). This will enable ready determination of the optimal day for release to allow sufficient access to a balanced diet (see Nutrition for adult *B. tryoni* below).

4. PARC SETUP

To minimise fly escape, particularly in older and/or warped boxes a single strap on either end of the PARC should be used to secure the lid of the PARC.

Once the PARC boxes are setup they should be placed in an insect growth room under standard conditions, i.e. 26±2°C, 65±10% RH and a light:dark period of 14:10, with a simulated dawn and dusk as the lights are ramped up and down at the beginning and end of the light phase. Fans should also be used in the growth room to ensure airflow that will aid in keeping PARCs free from build-up of excessive ammonia, CO_2 and heat.
PARCs should be stacked in such a way as to allow airflow. Under standard conditions in a growth room, the majority of adult emergence usually occurs on day 3 after pupal placement, but this should be monitored.

5. NUTRITION FOR ADULT *B. TRYONI*

Adult flies survive better and are more competitive if provided with nutrition and water. In addition to the sugar cubes placed in each paper bag, on the day of expected fly emergence, a block of agar (15 x 15cm) comprising a mixture of white sugar and yeast hydrolysate (3:1 by weight) and water (Reynolds 2010) (see Appendix B) should be placed on top of the mesh of each box lid for adult feeding and replaced as needed.

6. RELEASING CHILLED ADULT *B. TRYONI*

As a rule, and in order to allow the adults 1-3 days access to food, particularly protein (Perez-Staples *et al.* 2007), the adults should be released when the majority are aged 48-72h (i.e. 2-3 days after the majority emerge).

On the day of release, the sterile adults to be chilled should be taken from standard rearing conditions in their PARCs and placed into a separate growth room at 4°C for immobilisation for 30-40mins. Once flies are determined to be immobile via a visual inspection, the straps securing the PARC should be removed. Food should also be removed and discarded. The PARCs should then be knocked on the floor three to four times to dislodge all of the flies from the sides of the box and paper bags. Remove the lid and shake the paper bags in the PARC to dislodge any remaining flies that are clinging onto the bags, before discarding the bags. Package chilled flies (by tipping them from the PARC) into individual 12 oz. paper cups (Uni Cups & Lids (Detpak) Premier Northpak, 13-15 Edinburgh Road, Marrickville NSW 2204) using a funnel, before placing them in a polystyrene box lined on the base and top with Techni Ice (Techni Ice Australia P/L, 14 Tooyal Street, Frankston VIC 3199), separated by plastic bubble wrap from the paper cups, to ensure the flies remain motionless until release.

Transport the polystyrene boxes containing the chilled flies in an air-conditioned vehicle to their respective release locations in an air-conditioned vehicle (cooled to under 30˚C). Release sites should be spaced at a uniform distance between cue-lure baited traps in a trapping grid, but no closer than 100m to any trap site. Operators will use a GPS unit (or similar equipment) to precisely locate the release site (see Procedure BC&MS 2007/044 and Procedure BC&MS 2007/045 for

correct use of GPS). They will not rely on a visual estimation of distance. The GPS coordinates must be obtained for all release and monitoring sites and recorded on data sheets electronically (paper may be used in the field, but all data should be written and saved to an electronic spreadsheet; both hard and soft copies should be kept).
Once at the release site, either roving or stationery release should be used. Both forms of release include the use of an automated blower using a 12 volt 100mm inline marine bilge blower [6.7 cubic metres per minute capacity (235 Cu Ft Min)] fitted with a PVC pipe 50cm in length modified for *B. tryoni* releases after that of T. Black, (pers. comm. 2008). This can be done from the passenger side of a slow moving vehicle over a 100m distance (i.e. 50m each side of the release point) into roadside vegetation from a moving vehicle at 30km/h by tipping the sterile flies into the funnel of the automated blower from the paper cups in which they were held (Fig 2a) or as a standing/stationery release between trap sites (Fig. 2b).

Figure 2. An automated blower (a & b) that is run by a small fan (c) and can be plugged into a car (c) or a battery pack (battery not shown) to release chilled adult fruit flies via roving (a) or stationery (b) release.

Flies should not be released when the temperature is below 16˚C as this is the flight threshold for *B. tryoni*. Rather, wait for the day to warm up if possible to maximise the chances of the flies warming up and taking flight soon after release.
Following this procedure carefully will ensure that adult flies retain sufficient dye in their ptilinum for ready identification as sterile flies by experts trained in fruit fly identification at the Orange Agricultural Institute.

7. SUPPLIER DETAILS

Yeast hydrolysate: MP Biomedicals Australasia Pty Ltd, PO Box 187, Seven Hills, NSW 2147, Australia.
Silverlock crate MH 0110 (PARCs): Blackwoods, http://www2.blackwoods.com.au/Default.aspx or phone 137323 for your nearest store/supplier.

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Reynolds OL (2010) Queensland Fruit Fly – Adult Sterile Insect Release. Standard Operating Procedure. NSW Department of Primary Industries, Australia.

Reynolds, O.L. & Orchard, B.A. (2010). Container loadings and eclosion units for sterile insect technique programs of *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae). *General and Applied Entomology*, **39**, 19-22.

Legislation

- Nil

Related Policies and Procedures

- Procedure PRO 2011/01 Queensland Fruit Fly - Adult sterile insect technique
- Procedure BC&MS 2007/044 – Monitoring and surveillance: Setting Garmin 72XL GPS receivers to the correct position format map and datum
- Procedure BC&MS 2007/045 - Monitoring and surveillance: Setting Garmin 72/76 GPS receivers to the correct position format map and datum
- Appendix A
- Appendix B

Contact

Position Dr Olivia Reynolds, Research Scientist
Contact no. 02 4640 6426

Appendix A

Quality Assurance
Pupal weight, Emergence and, Flight Ability

1. Introduction
The joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture and the US Department of Agriculture developed an internationally accepted set of procedures for the quality assurance of Sterile Insect Technique (SIT) reared and released flies. This includes quality assurance measures to determine the emergence, flight ability and pupal weight of each batch of sterile pupae received that is used for research or release purposes.

2. Principle of Procedure
To measure the emergence, flight ability and pupal weight of SIT mass-reared Queensland fruit flies using a standardized method.

3. Scope and Application
This document is a slightly modified version of the internationally accepted procedure for Quality Control (QC) of mass reared Queensland Fruit Flies used for the Sterile Insect Technique (SIT)

4. Safety Procedures

Personal Protection
HANDS/FEET
- Safety footwear
- Gloves for warmth when in 4ºC growth room

OTHER

- Protective clothing (lab coat)
- Warm outerwear (suitable for conditions of 4ºC).

Material Safety Data Sheet (MSDS) – Read relevant MSDS before handling any chemicals.
Safe Work Method Statements – Read relevant assessments (Setup and release of SIT fruit flies; Receival and allocation of sterile dyed Queensland fruit fly pupae; *B. tryoni* quality assurance, emergence and flight ability) before completing tasks and handling any chemicals (available on the Risk Management Database).

5. Apparatus
- Plexiglass tubes: outside diameter 8.9 cm with 3mm thick walls; spray painted black; 10 cm high (care must be taken to not scratch the inner surface of the tubes)

- Plexiglass chamber: 30 x 40 x 30 cm (w x d x h) with a mesh covered ventilation hole in the top (similar sized mesh cages may also be used)
- Petri dish lids: 100 x 15 mm, overlaid with black paper disks.
- Strips of black porous paper 1 cm wide stapled at the ends to form a ring 6 cm in diameter.
- Analytical Balance (accurate to 0.001 gm)
- Miscellaneous equipment: unscented talcum powder, forceps, spoons, spatula
- Aspirator and vials
- Stereo microscope or magnification lamp
- Insect growth room
    - Temperature 26 C ±2 C
    - Humidity 65 %RH ± 10%
    - Photoperiod 14:10 hours light: dark

6. Procedure

Each batch of fruit flies received is identified by the larval week and larval hopping date (the date the larvae commence pupation). The shipment date and radiation dose are also included on a sticky label on the shipment box. This label should be peeled off and stuck onto the data form '*Fruit Fly QC Emergence & flight ability template*'.

- When received, for each batch of pupae dyed a different hue, five replicates of 100 pupae are counted and placed within a black paper ring on a black paper lined Petri dish lid.
- The total weight of each of three replicates are weighed to 0.001 gram accuracy and recorded on the excel spreadsheet – '*Fruit fly QC Average weight pupae template*' to determine the average pupal weight for each batch.
- The inner surface of the black plexiglass tubes are coated with unscented talcum powder (excess removed by tapping tubes on a firm surface) before talc is removed from the bottom 2 cm with a damp soft cloth.
- Petri dish lids holding pupae are placed within a clear & colourless plexiglass chamber and the black plexiglass tubes are placed over the top. The chamber is then placed in the growth room.
- Fruit flies that exit the tubes are removed from the chamber using an aspirator. This is carried out daily or more often during peak emergence.
- When the fruit flies cease to emerge for two consecutive days, the QC is deemed finished.
- The apparatus is disassembled, cleaned and the fruit fly debris are separated into four categories (not emerged, part emerged, deformed adult flies and non-fliers (non-deformed adult flies)), counted and recorded on the excel spreadsheet '*Fruit Fly QC Emergence & flight ability template*'.
- The above should be repeated for adult flies, after they have been chilled for 30-40mins, by placing the chilled adult fly within the black paper ring on a black paper lined Petri dish lid. This should be done in the 4ºC growth room and once complete the flies can then be transported within the plexiglass chamber to the 26 C growth room.

6. Calculations and Reporting

Average pupal weight results are entered into the excel spreadsheet – '*Fruit Fly QC Average weight pupae template*'. This is an Excel spreadsheet, which calculates average pupal weight in the format shown below**.** The results are saved as hard copies as well as electronically.

Replicate	Number of pupae	Weight (to 0.001g)
1	100	
2	100	
3	100	
Mean		

Emergence and flight ability results are entered onto the excel spreadsheet – '*Fruit Fly QC Emergence & flight ability template*'. This is Spreadsheet calculates % emergence and flight ability in the format shown below. The results are to be saved as hard copies as well as electronically.

Setup Date: _____

	Shipment date	Batch #	Collection date	Test date	Tester
Pre Release					

Elements		Replicate 1	2	3	4	5	Mean
T	Number of pupae	100	100	100	100	100	100
A	Not emerged						
B	Part emerged						
C	Deformed						
D	Not fliers						

Calculations

			Value
E	**T-(A+B)**	% Emergence	
F	**T-(A+B+C+D)**	% Fliers	
	(F/E)*100	Rate of fliers	

If the percentage emergence and/or fliers falls below 75%, the Officer who conducted the test should contact the SIT Program Leader who should in turn inform the Fruit Fly Breeding Facility at the Elizabeth Macarthur Agricultural Institute (EMAI), Menangle.

8. Quality Control

Analytical balance used is calibrated in accordance with I&I NSW laboratory procedures.

9. Method Precision

Numbers of each count category are in whole numbers but when averaged between replicates, results are reported to two decimal places. Accuracy of average pupa weight is reported to three decimal places.

10. References

FAO/IAEA/USDA. 2003. Manual for product quality control and shipping procedures for sterile mass-reared Tephritid fruit flies, Version 5.0. International Atomic Energy Agency, Vienna, Austria. 85pp.

Appendix B

Adult Queensland Fruit Fly Diet

1. Safety Procedures

PERSONAL PROTECTION

EYES:
- Safety glasses (hot liquids).

HANDS/FEET
- Gloves (hot liquids).
- Safety footwear

OTHER
- Protective clothing (lab coat)
- Dust mask when using yeast hydrolysate
- Eyewash unit.

Material Safety Data Sheet (MSDS) – Read relevant MSDS before handling any chemicals.
Safe Work Method Statements – Read relevant assessments (Making adult Queensland fruit fly media) before completing tasks and handling any chemicals (available on the Risk Management Database).

2. Apparatus and Reagents

Mixture A: (for one batch)
500 ml water
4.5g agar
50g white sugar

Mixture B: (for one batch)
0.06g calcium propionate
20g Yeast hydrolysate

3. Procedure

- Boil Mixture A then allow to cool to about 40°C or for about 1h 30 minutes
- Stir in Mixture B
- Pour into clean dish and cover. Mixture when poured should be approximately 1.5 cm in depth
- When set cut into desired size. Each block should measure approximately 15cm x 15cm.
- Blocks are distributed to the insect rearing facility outside of the lab.