Optimal irradiation procedures for sterilization of Queensland fruit flies

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Optimal irradiation procedures for sterilization of Queensland fruit flies

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FINAL REPORT

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Statement of purpose: This report details the research and development undertaken in Project HG06040 to optimize irradiation procedures used to sterilize mass-reared Queensland fruit flies used in sterile insect technique programs in Australia. Laboratory studies were conducted to develop improved quality control procedures, to assess the performance of flies irradiated under current protocols and to identify opportunities to enhance SIT efficacy through optimization of irradiation procedures.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

MEDIA SUMMARY

The sterile insect technique (SIT) is an environmentally and medically benign method used to contain and eradicate outbreaks of Queensland fruit flies, Australia's most damaging insect pest of horticultural crops. Rather than using chemical pesticides, SIT involves the release of sterile flies that corrupt reproduction of wild populations, reducing the number of eggs that hatch into fruit-damaging larvae and reducing the numbers of adult flies in the next generation. Millions of Queensland fruit flies are reared in a factory, irradiated to induce sterility, and then released into the field. Once released, the sterile male flies must succeed in surviving to sexual maturity and in out-competing fertile wild males for copulations with wild females. The sterile males must then succeed in transferring an ejaculate that prevents the females from re-mating. Irradiation is a critical step in SIT, as studies of other fruit flies have reported deficiencies in the performance of sterile flies. Substantial improvements in fly quality, and SIT efficacy, may be achieved through modest modifications to irradiation procedures.

"Optimal irradiation procedures for sterilization of Queensland fruit flies" (HAL project HG06040) was a collaborative project with the central objectives of (1) assessing effects of current irradiation procedures on quality of sterile Queensland fruit flies used in SIT and (2) investigating the scope for safely improving quality of sterile Queensland fruit flies, and hence SIT efficacy, through modified irradiation procedures.

Key outcomes

- Current irradiation procedures have been confirmed as highly effective at inducing sterility, but sub-optimal in terms of sterile fly quality. Several opportunities to improve procedures have been identified.

- By reducing the rate at which an irradiation dose is applied, more accurate dosing can be achieved.

- The quality of sterile flies used in SIT could be improved through a small reduction in irradiation dose without any reduction in sterility.

Recommendations for further research and development

- The findings of these laboratory studies should be followed up with field releases comparing the recapture rates of flies irradiated at the current dose with recapture rates of flies irradiated at lower doses. Such studies would elucidate benefits of reduced irradiation dose for survivorship and dispersal of released Queensland fruit flies in an operational setting.

- Field cage studies should be carried out to compare the sexual competitiveness of flies irradiated at the current dose and flies irradiated at lower doses. Such studies would elucidate the benefits of reduced irradiation dose for ability of released sterile males to compete with wild males for copulations with wild females.

TECHNICAL SUMMARY

The Queensland fruit fly (*Bactrocera tryoni* Froggatt) or 'Q-fly' is Australia's most significant insect pest of horticulture. Outbreaks in NSW, VIC and SA are commonly contained using the sterile insect technique (SIT). We assessed (1) effects of current irradiation procedures on quality and sterility of Q-flies used in SIT and (2) the scope for safely improving quality of sterile Q-flies through modified irradiation procedures.

Flight ability procedures
We investigated variations of flight ability protocols to identify attributes that might affect the flight ability indices and also attributes that might be titrated to vary assay sensitivity. Tube height and colour had strong effects, with fewer flies escaping from taller tubes. Tube height offers a means of titrating flight ability assay sensitivity. Flight tube diameter, clean area at the based of the tube for resting by adult flies, and the container in which pupae were held did not affect flight ability data.

Effects of irradiation dose rate on quality and sterility
Pupae were irradiated at a target dose of 70 - 75 Gy at dose rates of 5, 7, 26, 57, and 80 Gy/min. No effects of dose rate were found on emergence, flight ability or sterility induction. Males irradiated at higher dose rates suffered increased mortality under stress owing to tendency to over-shoot the target dose. For more accurate dosage, the lowest practical dose rate should be used.

Optimising irradiation dose for sterility induction
We assessed the impact of a range of target irradiation doses (60, 65, 70, 75 and 80 Gy) on the quality of mass reared Queensland fruit fly. Sterility induction remained adequate (> 99.5%) across the full range of irradiation doses tested. However there was significant reduction in survivability and competitiveness of irradiated flies as dose increased. The current irradiation dose of 70 - 75 Gy is associated with elevated damage to flies. Our results suggest that adequate sterility and improved fly quality could be achieved through a small reduction in irradiation dose.

Effects of irradiation on adult longevity
Other studies have used the standard 'mortality under stress' assays to demonstrate increased survivorship of crowding and nutritional stress at lower irradiation doses. We investigated whether similar effects are found in the absence of stress. Whereas other studies found a gradual increase in mortality under stress as dose increased, in the absence of such stress the response was much sharper; all irradiation doses were associated with a marked reduction in longevity and this effect was similar over the 60 - 80 Gy range tested. While reduced irradiation dose may enhance survival under stress it is unlikely to increase longevity in the absence of stress.

Low irradiation dose response and fertility resurgence
Other studies have suggested that higher quality flies may be produced without compromising sterility by reducing irradiation dose. This raises questions of safety margin below this dose before sterility induction is compromised. We assessed irradiation dose safety margins at 10 - 15 and 30 - 35 days post emergence. Males required higher doses than females to induce sterility, however all flies were sterile at doses of 60 Gy and above. There was no evidence of fertility resurgence at 30 - 35 days post emergence at any dose. The current irradiation dose of 70 - 75 Gy could be lowered by up to 10 Gy, leaving a 5 - 10 Gy safety margin for males and 20 Gy safety margin for females.

Multiple mating and irradiation-induced sperm depletion: effects on female remating behaviour
We assessed the ability of fertile and sterile (irradiated) male Queensland fruit flies to transfer sperm and to induce sexual inhibition in their mates. Unlike fertile males, sterile males transferred progressively fewer sperm to sequential mates, with only trivial numbers transferred by males that had mated twice previously. Despite the massive deficiency in sperm transfer, sterile males were

undiminished in their ability to induce sexual inhibition in their mates. Induction of sexual inhibition in females is related to products in the ejaculate other than sperm.

Recommendations for further research and development
Laboratory studies are an efficient means of testing systems under precisely controlled conditions. For SIT research, it is essential that substantial laboratory studies be conducted to confirm adequate safety margins of sterility before starting any work that involves release of flies into the environment. This report provides a robust platform for further testing in field conditions, providing evidence that (1) improvements in SIT can be achieved through reduced irradiation dose and (2) that significant safety margins can be retained even at reduced doses.

The findings of these laboratory studies should be followed up with field releases comparing the recapture rates of flies irradiated at the current dose with recapture rates of flies irradiated at lower doses. Such studies would elucidate benefits of reduced irradiation dose for survivorship and dispersal of released Queensland fruit flies in an operational setting.

GENERAL INTRODUCTION

Viability of horticultural production depends on access to markets throughout Australia and internationally. Negotiations over access to new markets and continued access to existing markets commonly hinge on phytosanitary standards. For most Australian fruit producers, and especially citrus producers, Queensland fruit flies (*Bactrocera tryoni*, or 'Q-flies') present one of the most difficult and costly challenges to market access.

The sterile insect technique (SIT) is an important method for containment and eradication of Q-fly outbreaks. In SIT, millions of flies are reared, reproductively sterilized by gamma radiation (Bakri *et al.* 2005), and then released in the field. Released sterile males mate with the wild females and prevent them from bearing viable offspring. Rather than killing the existing flies, SIT instead prevents wild females from reproducing. As the existing wild flies die off there are none to replace them in the next generation (Knipling 1955). SIT has some clear advantages over most alternatives. In particular, SIT releases no toxicants into the production area and is hence environmentally and medically benign. This is an especially important point, as townships are the most common origins of Q-fly outbreaks.

For effective SIT, it is essential that irradiation procedures to ensure an adequate level of sterility in released flies while minimizing the deleterious effects on fly quality and competitiveness. Released flies that are incompetent for survival or mating are of no value to SIT. Deleterious effects of irradiation on fly quality and competitiveness have been reported for numerous fruit fly species, including *Ceratitis capitata* (Barry *et al.* 2003; Lux *et al.* 2002), *Anastrepha obliqua* (Toledo *et al.* 2004), *Anastrepha ludens* (Rull *et al.* 2007), *Anastrepha suspensa* (Walder & Calkins 1993) and *Bactrocera cucumis* (Hooper 1975). Without studies of comparable detail in Q-flies, it has not been possible to ascertain the adequacy of current procedures or to identify opportunities for improvement.

There have been several studies of Q-fly irradiation that have provided guidance for current practises (Monro & Osborne 1967; Bhatti & Shipp 1972; Sproul *et al.* 1992). However, each of these studies is very coarse in its treatments, considers only a very limited range of conditions (many of which are irrelevant to current practises), and uses irradiation equipment quite different from that used today at *Australian Nuclear Science and Technology Organization* (ANSTO, Lucas Heights). Unlike comparable SIT programs for other fruit fly species overseas, irradiation procedures used to sterilize Q-flies for ongoing SIT programs in Australia have not been supported by a strong empirical foundation. The goal of project HG06040 "Optimal irradiation procedures for sterilization of Queensland fruit flies" has been to provide this foundation, thereby (1) providing assurance to trading partners that Q-fly SIT is based on sound scientific principles, (2) assessing adequacy of existing procedures and (3) identifying potential improvements to existing procedures that might further increase the efficacy of SIT in containing and eliminating Q-fly outbreaks.

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1. FLIGHT ABILITY PROCEDURES

Summary

Flight ability is a standard assay used to assess quality of tephritid flies that are mass-reared and irradiated for Sterile Insect Technique (SIT) programs. Several different flight ability assays have been used for Queensland fruit fly SIT with varying results. We here investigate some variations of standard flight ability protocols to identify attributes that might impact the resulting flight ability indices and also attributes that might be titrated to vary assay sensitivity. Specifically, we investigated effects of flight tube height, colour, diameter and resting area for emerging flies, and how pupae are held within the flight tubes. We found that tube height and colour had the strongest effects on flight ability, with progressively fewer flies escaping from taller tubes. There was a significant colour by height interaction, as the effect of increased tube height was more acutely apparent in white tubes than in black tubes. Tube height offers a means of titrating flight ability assay sensitivity. Flight tube diameter, clean area at the based of the tube for resting by adult flies, and the container in which pupae were held did not significantly affect the flight ability index

Introduction

Sterile insect technique (SIT) programs entail the release of sterile males to suppress or eradicate target pest species through the induction of reproductive sterility in wild females (Knipling 1955). Success of SIT relies on mass reared males performing effectively in a number of key challenges, including survival till sexual maturity, finding, courting and mating with wild females, and inducing long refractory periods (reviewed by Hendrichs *et al*. 2002). Effective product quality control is fundamental to any production process, and is a critical component of successful SIT (reviewed by Calkins & Parker 2002). A diverse range of quality control tests are routinely employed in fruit fly SIT programs to ensure quality and competitiveness of mass reared flies, and these are outlined in FAO/IAEA/USDA (2003) "Product quality control manual for sterile mass-reared tephritid flies".

The flight ability test is designed to estimate the percentage of mass reared flies that, when released in the field, are capable of effective dispersal. Flies unable to disperse effectively will most likely be unable to participate in mating and are therefore useless to SIT (Calkins *et al*. 1996). The standard FAO/IAEA/USDA (2003) flight ability test as adapted from the RAPID Quality Control System (Boller *et al*. 1981) also allows for the assessment of percentage of emergence, including un-emerged and partially emerged flies as well as the number flies with deformed wings. This test was initially standardised for Mediterranean fruit fly (*Ceratitis capitata*), and variations have been introduced to accommodate other species.

The RAPID quality control system uses a 9 cm wide 20 cm high tube made of cardboard and painted with FLUON-AD-1 on the inner surface so that flies cannot climb out (Boller *et al*. 1981). This tube is then fitted into a 9 cm Petri dish containing 100 pupae. The current FAO/USDA/IAEA (2003) method uses a 10 cm high flight tube with an outer diameter of 8.9 cm that is painted black on the outer surface. This flight tube is then fitted into a 9 cm Petri dish, also painted black or overlaid by dark porous paper, that contains 100 pupae placed within a 1 cm tall, 6 cm diameter paper ring located in the centre of the Petri dish. The interior of the flight tube is coated with unscented talcum powder to stop flies walking out, with a ring wiped off the lower end of the interior to allow newly emerged flies a place to rest (1 cm for *Ceratitis capitata*, 3 cm for *Anastrepha*). Flight ability index is calculated from the number of emerged flies failing to escape the tube.

Queensland fruit flies were first assessed using 20 cm high tubes from the RAPID system, however flight ability index results were inconsistent (Jessup & Cruickshank 1999). Consequently, flight

ability procedures based on those used for melon flies in Okinawa were adopted. In these procedures, pupae are placed in a 15 cm-wide 12 cm-high tube that is darkened on the outside and coated with talcum powder on the inside. In recent studies, Collins *et al*. (2008a, 2009) have successfully employed methods based on FAO/IAEA/USDA (2003) to assess flies irradiated at various doses and dose rates, reporting flight ability to be consistently 85 - 90% when corrected for emergence. The standard methods might be viable for Queensland fruit fly.

Given the variation in the standard flight ability protocol for differing species and the use of different types of flight ability assays for Queensland fruit fly, the aim of this study is to standardise flight ability assessment for Queensland fruit fly in line with FAO/IAEA/USDA (2003) methods. While identifying what attributes of tube design, specifically colour and height, area wiped off for rest, and how the pupae are held in the tube, affect recorded flight ability, and how they could be used to apply a more sensitive test to the effects of rearing and irradiation on fruit flies in general.

Materials and Methods

General Methods

Queensland fruit fly pupae were obtained from the Industry & Investment NSW Fruit Fly Production Facility at Elizabeth Macarthur Agricultural Institute (EMAI). Pupae were sorted into flight ability assays two days before estimated emergence, with 100 pupae per tube. Flight tubes were made from 3 mm thick transparent plexiglass. Flight tubes were darkened by wrapping the exterior of the tube in black plastic. The interior surface of each flight tube was coated with unscented talcum powder. Upon coating the tube, each was tapped sharply on a bench top three times to remove excess powder. A 2 cm high ring of talcum powder was wiped off at the tube base to allow newly emerged flies an area to rest. Flight tubes were placed on 9 cm Petri dishes that were overlaid with a circle of black paper.

Each flight ability tube with pupae was placed in an individual 325 x 325 x 325 mm cage that was covered in fine white fabric. Cages were set up on shelves each lit by a single 36W fluorescent light tube that was positioned immediately above the cage. A reading of light intensity (lux) was taken on the base of each cage and was included as a covariate in analyses to assess any variation due to light level. An identical 'dummy' tube without pupae was placed in each cage to assess 'fly-back', flies that initially escape from the tube but then return to the tube and fail to escape again. To minimize fly-back, cages were checked daily, and any flies that had escaped the tubes were removed using an aspirator. When all emergence had ceased (two to three days after the first flies emerged) the remaining contents of the tubes were counted.

Following FAO/IAEA/USDA (2003), individual flies were classified as (1) 'not emerged' if still inside un-open pupal case, (2) 'part emerged' if failing to emerge completely (3) 'deformed' if they had completely shed the pupal case but had damaged wings and (4) 'not fliers' if they had completely shed the pupal cases and had morphologically normal wings but failed to escape the tube. Rather than assessing the flies that emerge from the tubes, standard procedures are instead based on an assessment of flies remaining in the tubes. Calculations from Collins *et al*. (2008a) were used for calculating percentage of emergence ((N pupae - (N not emerged + N part emerged) / N pupae) x 100), percentage fliers ((N pupae - (N not emerged + N part emerged + N deformed + N non-fliers) / N pupae)) x 100) and rate of fliers the percentage of fliers corrected for emergence ((Percentage fliers / Percentage emergence) x 100). The calculations used are directly comparable to the corresponding methods of FAO/IAEA/USDA (2003). All analyses were performed using JMP v 5.0 (SAS Corporation, Cary, NC, USA).

Rest area and pupal container set up

To determine how much talcum powder should be wiped off the interior of the tubes to allow newly emerged Queensland fruit flies adequate area to rest, pupae were placed in flight tubes 10 cm high, 10 cm external diameter, that had a 0, 1, 2, or 3 cm high ring of talcum powder wiped off the internal surface at the base. Wearing gloves, the operator uses and index finger to wipe a single ring around the interior at the base of the tube. Accurate height of the area wiped off is measured using a ruler at four perpendicular points within the interior of the tube. Six tubes were set up for each rest area treatment.

The six tubes were further divided into one of three separate pupal container treatments. Within the 9 cm Petri dish that makes up the base of a standard flight ability tube assay, groups of pupae were placed in 1) a 1 cm tall, 6 cm diameter paper ring located in the centre of the Petri dish (following FAO/IAEA/USDA 2003), 2) within a smaller 5.5 cm Petri dish located in the centre of the 9 cm dish (following Collins *et al*. 2008a), or 3) within the 9 cm Petri dish without any other container to hold them (following the RAPID system of Boller *et al*. 1981). For each of the four rest area heights (0, 1, 2, 3 cm) there was two of each dish treatment (none, paper ring or Petri dish). This experiment was repeated three times using different daily batches of pupae.

Tube height and colour

To test the affects of tube height and contrast on flight ability, pupae were placed into one of six treatments of differing tube heights (10 cm external diameter, 5, 8, 10, 12, 15, 20 cm high). Each height treatment was further divided into either a black or white colour. White tubes were produced by wrapping the clear plexiglass in white rather than black plastic, and the 9 cm Petri dish base of white tubes was overlaid by white rather than black paper. Four tubes were set up for each height treatment, two black and two white. This experiment was repeated three times using different daily batches of pupae.

Tube diameter

To test the effect of tube diameter on flight ability, pupae were placed into one of two tube diameter treatments in both cases, 10 cm high, the first being 10 cm outer diameter/9.4 cm inner diameter like those originally used in Collins *et al*. (2008a), the second being 8.9 cm outer diameter/8.6 mm inner diameter as outlined in FAO/IAEA/USDA (2003). This experiment was repeated three times using different daily batches of pupae.

Results

Rest area and pupal container set up

Rest area height wiped off the base of the tube did not influence percentage fliers ($F_{3,74} = 1.89$, $P = 0.14$), with each treatment having an average of 67 - 69% fliers. Rest area height also did not influence rate of fliers ($F_{3,74} = 1.51$, $P = 0.22$) with a average 86 - 87% of fully emerged flies escaping the tubes. There was no evidence that Petri dish set up influenced percentage fliers ($F_{3,74} = 0.96$, $P = 0.39$) or rate of fliers ($F_{3,74} = 0.02$, $P = 0.98$). There was no evidence that light level influenced either percentage fliers or rate of fliers in these experiments (All *P*-values >0.1)

Tube height and colour

Percentage fliers was significantly influenced by both tube height ($F_{5,58} = 54.96$, $P < 0.0001$) and tube colour ($F_{1,58} = 94.96$, $P < 0.0001$), with a highly significant tube height by colour interaction

($F_{5,58} = 6.52$, $P < 0.0001$) (Figure 1.1). Correcting for emergence, rate of fliers was significantly influenced by both tube height ($F_{5,58} = 66.89$, $P < 0.0001$) and tube colour ($F_{1,58} = 107.36$, $P < 0.0001$), with a highly significant tube height by colour interaction ($F_{5,58} = 8.48$, $P < 0.0001$) (Figure 1.2). Black flight tubes display a slow decline in flies that manage to escape as tube height increased, this being evident for both percentage fliers, maintaining 65 - 70% until heights 15 cm and above, and rate of fliers, maintaining 85 - 90% until heights 15 cm and above. White flight tubes displayed a much sharper decline, with very few flies (<50%) escaping from tubes at heights above 10 cm. There was no evidence that light level influenced either percentage fliers or rate of fliers in these experiments (All P-values >0.4)

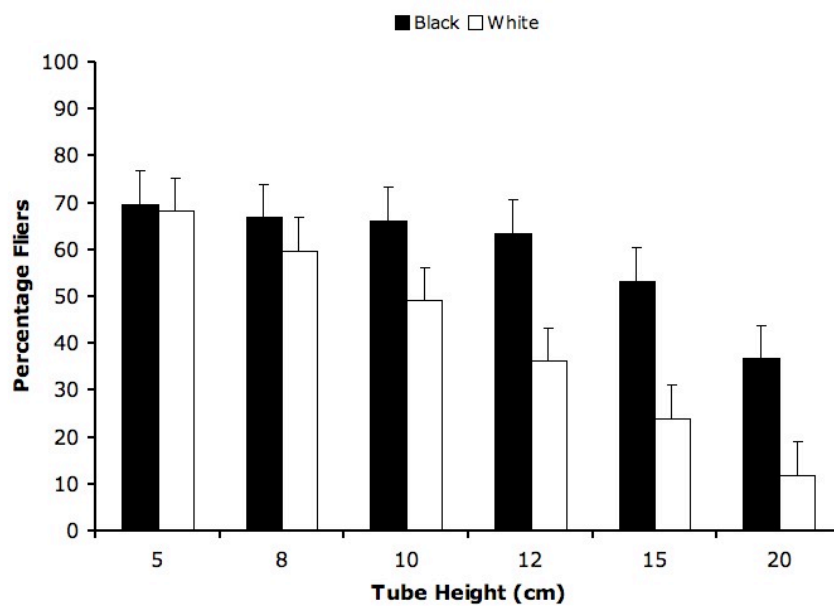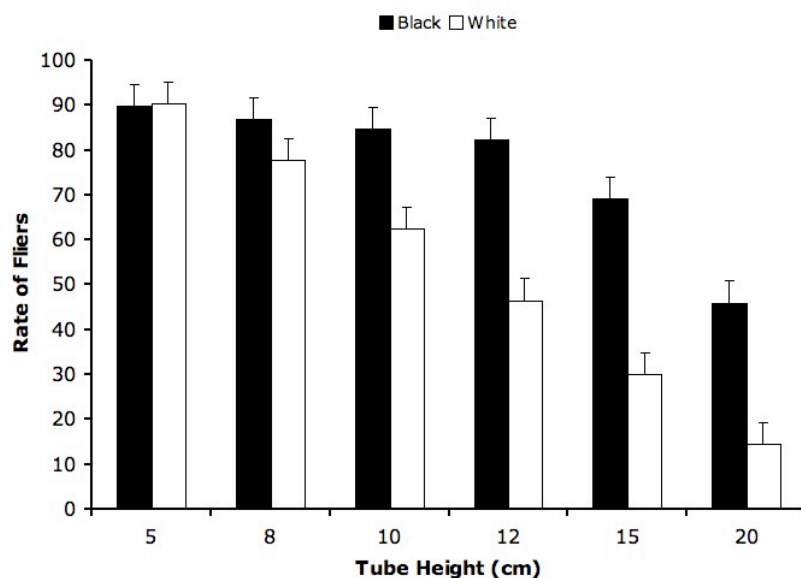**Figure 1.1: Percentage of flies escaping from black and white tubes of varying height.**

Figure 1.2: Rate of fliers, calculated from the percentage of flies corrected for emergence, escaping from black and white tubes of varying height.

Tube Diameter

There was no evidence of difference between the tubes in percentage fliers ($F_{1,40} = 0.04$, $P = 0.84$), with an average of 72% of flies escaping from the tube across both diameter treatments. There was also no evidence of difference in rate of fliers ($F_{1,40} = 0.12$, $P = 0.74$), with an average 80% of emerged flies escaping from the tube across both diameter treatments. There was no evidence that light level influenced either percentage fliers or rate of fliers in these experiments (all *P*-values >0.05).

Discussion

Following the inability of Queensland fruit flies to escape from 20 cm high flight tubes in the initial assessment using the RAPID system, lower and wider tubes were adopted for testing Queensland fruit fly (Jessup & Cruickshank 1999). While consistent with existing melon fly programs in Okinawa, these protocols are quite different from both the RAPID system and the internationally accepted FAO/IAEA/USDA (2003) system. We here confirm that the FAO/IAEA/USDA (2003) method can be successfully applied to Queensland fruit fly and consistently achieve acceptable flight ability index averages (see also Collins *et al*. 2008a, 2009). For Queensland fruit fly, the required flight ability standard (see FAO/IAEA/USDA 2003) is an average 75% with a minimum flight ability of 70% pre irradiation. Post irradiation values should average of 70% (minimum 65%), and post-shipping values should average 65% (60% minimum). Because all pupae tested in this study were unirradiated, the pre irradiation comparison is of greatest relevance. Queensland fruit flies from the SIT mass-rearing facility reliably met the pre-irradiation standards in this study when the standard FAO/IAEA/USDA (2003) protocols were applied precisely.

The FAO/IAEA/USDA (2003) method uses a 10 cm high flight tube. However, our results indicate that flight tube height could be increased to as much as the 20 cm height of the RAPID system with a quite predictable decline in indices with increasing tube height. Although the flight ability index achieved by Queensland fruit fly in this study was below the accepted average when we used a 20 cm high tube, the greater challenge may provide a more sensitive test of post-irradiation and handling effects. Collins *et al*. (2008a, 2009) found irradiation to have strong effects on mortality under stress but no effects of irradiation on flight ability with 10 cm high flight tubes. While this could simply mean that flight ability is unaffected by irradiation at the doses used, it could also reflect an insufficiently sensitive assay.

Aside from the increased challenge presented by increased tube height, tube colour was the most important aspect of tube design affecting flight ability indices. Overall, flight ability indices were much lower when white flight tubes were used and the effects of increased tube height were much more acute for white tubes. It is likely that this effect is related to the greater contrast between the tube interior and exterior. Light contrast is important for Queensland fruit flies in other contexts and has been proposed as a reason why fruit flies move away from trees to field cage walls as afternoon light levels diminish during field cage mating trials (Collins *et al*. 2008b; Koyama *et al*. 1986).

Other modifications to the standard protocol did not have any significant influence on flight ability indices, including the pupal container (none, paper ring, 50 mm Petri dish), size of cleared ring available at the bottom of the flight tube for emerging flies to rest (0, 1, 2, 3 cm), and tube diameter (83 *vs*. 94 mm internal diameter). As in previous studies (Collins *et al*. 2008a, 2009), variation in light levels across cages did not influence flight ability indices. This should not be taken to mean that light levels are not important at all for flight ability assays, but rather that our lighting set up (fluorescent tubes immediately above cages) achieved sufficiently uniform levels. While not studied here, given the strong effects of tube colour/contrast on flight ability, it is quite likely that inadequate overhead lighting would result in reduced flight ability indices.

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2. EFFECTS OF IRRADIATION DOSE RATE ON QUALITY AND STERILITY

Summary

Queensland fruit fly (*Bactrocera tryoni*) pupae are routinely irradiated to induce reproductive sterility in adults released in a Sterile Insect Technique program. While there have been some studies of how total dose influences fly quality, dose rate has not been considered. In the present study, pupae were irradiated at a target dose range of 70 - 75 Gy at dose rates of 5, 7, 26, 57, and 80 Gy/min and were then subjected to routine IAEA/FAO/USDA quality control tests including, emergence, flight ability, mortality under stress and sterility induction. No significant effects of dose rate were found on emergence or flight ability. Sterility induction was also found to be independent of dose rate, a result conforming to a 'one-hit' ionising event hypothesis. While no significant effects were found for females, there was evidence that male flies irradiated at higher dose rates suffered increased mortality under stress. This appears to stem from an increased tendency to over-shoot the target dose when irradiating at high dose rates. We recommend that, to reduce potential error in total target dose, the lowest practical dose rate be used when irradiating Q-fly pupae for use in the Sterile Insect Technique.

Introduction

Queensland fruit fly (*Bactrocera tryoni* Froggatt; 'Q-fly') is among Australia's most costly insect pests of horticultural crops (Sutherst *et al*. 2000). As with some similarly damaging tephritids in other regions, the Sterile Insect Technique (SIT) is a key approach used to contain and eradicate Q-fly outbreaks. SIT has distinct ecological and medical benefits when compared with traditional insecticidal approaches. Rather than using toxins, SIT functions through the induction of reproductive sterility in wild females mating with sterile males that are mass-reared and released in affected regions (Knipling 1955). While females mated by sterile males may produce eggs, these will not yield viable offspring and so the pest population is greatly reduced in the next generation.

Irradiation is the usual method used to sterilise tephritid fruit flies released in SIT programs. For some tephritid species, irradiation has been reported to have deleterious effects on quality and competitiveness of mass reared flies used in SIT (e.g., *Ceratitis capitata* Barry *et al*. 2003; Lux *et al*. 2002; *Anastrepha obliqua* Toledo *et al*. 2004; *Anastrepha ludens* Rull *et al*. 2007). In Q-flies, Dominiak *et al*. (2002) and Dominiak *et al*. (2007a) report reduced emergence and rate of fliers in Q-flies that had been dyed, packed and irradiated for release compared with untreated flies from the same batches. Given the potential for reduced quality and competitiveness of irradiated flies it is imperative that irradiation procedures be fine tuned to both ensure adequate sterility (e.g., for *C. capitata*, 99.5% sterility in crosses between sterile males and fertile females is required (IAEA/FAO/USDA 2003) and at the same time minimize irradiation-induced reduction in fly quality and competitiveness.

Current irradiation practices used to induce sterility in Q-flies used for SIT are based largely on studies of Monro & Osborne (1967), Bhatti & Shipp (1972) and Sproul *et al*. (1992). Each of these studies, however, covered a rather course scale of total doses and used basic Gammacell irradiation equipment that is quite different from the much more flexible Gamma Technology Research Irradiator (GATRI) facility at the Australian Nuclear Science and Technology Organisation (ANSTO) that is used in the current Q-fly SIT program. These previous studies also focus on total dose, and contain no investigation into the potential effects of dose rate.

The relationship between dominant lethal mutations in irradiated fly sperm, believed to be the cause of reproductive sterility, and irradiation dose is linear at low doses but becomes non-linear at higher

doses. This suggests that sterilisation is due to a one-hit ionising event (LaChance 1967), whereby the degree of sterility induced by a given dose should be independent of the dose rate at which it is applied (Hooper 1970, 1975). This was the case for early work conducted on *C. capitata* (Hooper 1970) and *Bactrocera cucumis* (Hooper 1975), in which no evidence of relationship between dose rate and sterility was found. These studies also found no evidence of relationship between competitiveness and dose rate. None the less, there remains conflicting evidence from various other insect species both supporting and opposing the one-hit ionising event hypothesis (Hooper 1975). The present study, the first to consider the effects of irradiation dose rate on quality of sterile Q-flies, is intended both to build evidence for or against the one-hit ionising event hypothesis for this species and to provide guidance for the enhancement of ongoing SIT irradiation practises.

Materials and Methods

Packing and irradiation

Q-fly pupae were obtained from the Industry & Investment NSW Fruit Fly Production Facility at Elizabeth Macarthur Agricultural Institute (EMAI). Pupae from this facility are routinely sent to ANSTO at Lucas Heights for irradiation as part of the Sterile Insect Technique (SIT) control program to suppress outbreak populations of wild Q-flies in and near exclusion zones. The following procedures were carried out for three replicate batches of pupae between January and March of 2007.

Six individual 'zip-lock' plastic bags (100 x 150 mm) containing ca. 8,000 pupae were sealed and packed at EMAI, and transported directly to ANSTO in an air-conditioned vehicle. Five of these were designated test packets for irradiation at various dose rates while the sixth packet was kept as an un-irradiated control. Control and test pupae packets were packed together at all times during transport and holding to ensure that all pupae received similar conditions. To achieve a hypoxic atmosphere prior to irradiation, the sealed packets were held overnight at ANSTO in a temperature-controlled room at approximately 18°C , as is currently done as standard practise with pupae irradiated for SIT. The following day, pupae were irradiated with the standard target sterilising dose for the current SIT program (70 - 75 Gy). Each of the five test packets was exposed to this sterilizing dose of irradiation at a different dose rate.

Four packets of pupae were irradiated at different dose rates using ANSTO's GATRI facility, which is able to accommodate a wide variety of dose rates, and one packet was irradiated in ANSTO's Gammacell 220 (GC220), which generates higher dose rates than are possible in the GATRI facility. Both irradiators contain cobalt-60 sealed sources. Each of the four GATRI-irradiated packets were fixed with tape to the face of separate cardboard boxes. Boxes were then mounted one at a time onto a small metal shelf or 'rig' within the GATRI facility for irradiation. Calibrated Ferrous ammonium sulphate (Fricke) dosimeters (Fricke & Hart 1966) were fixed with tape to the centre of each packet of pupae and were used to later assess total radiation dose.

Within the GATRI facility, the distance of the rig from the cobalt-60 source alters the dose rate. Individual packets were irradiated at one of four rig positions within the GATRI, each resulting in a different distance from the source. Packets were irradiated at 1310 mm, 1000 mm, 310 mm and 0 mm from the source, providing the corresponding dose rates of 5, 7, 26, and 57 Gy/min respectively (calculated from an initial dose-mapping of the irradiator). Each packet of GATRI-irradiated pupae was exposed for a period calculated to achieve the standard target dose range of 70 - 75 Gy.

The remaining packet of irradiated pupae was prepared with Fricke dosimeters in the centre and then exposed using the GC220 irradiator. A 1-L beaker was set up with a small wooden stand inside, the packet with dosimeters attached was placed on the stand so that it rested in the centre of

the beaker. The beaker was then placed into the chamber of the GC220. Again the packet was exposed for a predetermined period to achieve the standard target dose range of 70 - 75 Gy. The GC220 irradiator emits a dose rate of 80 Gy/min, substantially greater than the maximum of 57 Gy /min that could be attained in the GATRI irradiator at 0 mm from the source.

After irradiation, pupae were immediately transported in a closed styrofoam box in an air-conditioned vehicle to a laboratory at Macquarie University, Sydney, where they were set up to emerge in 5 litre plastic cages, each with a large mesh covered ventilation hole in the top. Flies were held, and all experiments run, in a laboratory maintained at $25\pm1°C$, $70\pm5\%$ RH on a 14:10 day night cycle including one hour dawn and dusk periods during which the lights turned on and off in stages. The effect of irradiation dose rate on fly quality was assessed using required routine quality control tests from the FAO/IAEA/USDA (Version 5, May 2003) product quality control manual, measuring emergence, flight ability, mortality under stress and sterility.

Percentage emergence, flight ability and sex ratio

Two days before the adult flies emerged, ca. 100 pupae from each dose rate treatment and the control were placed in separate 55 mm plastic Petri dish lids. The dishes of pupae were then centred on 90 mm Petri dish lids lined with black paper. A black 100 mm tall plexiglass tube (94 mm inner diameter, 3mm thickness) with a fine coat of unscented talcum powder on the interior (to prevent flies from walking out) was placed over the 90 mm Petri dish lid. A 20 mm width of talcum powder was wiped off the base of each tube to provide newly emerged flies an additional vertical surface on which to rest. Each tube with pupae was placed in an individual 325 x 325 x 325 mm mesh cage. An identical 'dummy' tube without pupae was placed in each cage to assess 'fly-back,' flies that initially escape from the tube but then return to the tube and fail to escape again.

Cages were set up on shelves each lit by a single 36W fluorescent light tube that was positioned immediately above the cages. A reading of light intensity was taken in each cage and was included as a covariate in final analysis of emergence and flight ability data to assess any variation due to light level. Five cages of ca. 100 pupae were set up for each dose rate treatment (i.e., a total of 15 cages for each dose rate treatment and the control across the three replicates). To minimize fly-back, flies that escaped from the tube were removed by aspirator daily, and were frozen for later identification of sex. When all emergence had ceased (2 or 3 days after the first flies emerged) the remaining contents of the tubes were counted.

Following FAO/IAEA/USDA (2003), individual flies were classified as (1) 'not emerged' if still inside un-open pupal case, (2) 'part emerged' if failing to emerge completely (3) 'deformed' if they had completely shed the pupal case but had damaged wings and (4) 'not fliers' if they had completely shed the pupal cases and had morphologically normal wings but failed to escape the tube. Rather than assessing the flies that emerge from the tubes, standard procedures are instead based on an assessment of flies remaining in the tubes. We assessed the standard measures of percentage emergence ((N pupae - (N not emerged + N part emerged) / N pupae) x 100), percentage fliers ((N pupae - (N not emerged + N part emerged + N deformed + N non-fliers) / N pupae)) x 100) and rate of fliers ((Percentage fliers / Percentage emergence) x 100).

While it was not possible to check for sex differences in percentage emergence or percentage fliers (because we could not ascertain sex of flies that did not emerge) we did test for effects of treatment on sex ratio by assessing the sex of all flies that emerged, including deformed flies and non-fliers collected from inside the tubes at the end of the experiment as well as fliers collected from outside the tubes (sex ratio = (N males emerged / N total flies emerged)). The standard calculations for percentage emergence, percentage fliers and rate of fliers in FAO/IAEA/USDA (2003) assume that exactly 100 pupae are used in all tests and hence use 100 as the denominator in calculations of

proportion emergence and proportion fliers. While we modified the calculations to accommodate deviation from exactly 100 pupae, using N pupae as the denominator, the results of our calculations are directly comparable with the corresponding calculations in FAO/IAEA/USDA (2003).

Mortality under stress

Within 2 hours after emerging in a 5-L cage without food or water, 50 males and 50 females from each treatment were gently aspirated into a 150 mm diameter plastic Petri dish through a 10 mm diameter hole in the lid, which was then stoppered by a cork to prevent flies escaping. The dishes were placed in a dark cabinet and checked for mortality 24 and 48 hours later. At 24 hours, dead flies were collected by removing the stopper and gently upending the dish to tip the dead flies out, making sure no living flies escaped. Five dishes were set up for each of the dose rate treatments as well as for the non-irradiated control group (i.e., a total of 15 dishes for each dose rate treatment and the control across the three replicates).

Reproductive sterility

Within 3 days after emerging, 50 males and 50 females of each dose rate treatment were placed into 5 litre cages. For each dose rate treatment, one cage was set up for each possible combination of irradiated and non-irradiated flies (irradiated male x irradiated female; non-irradiated male x irradiated female; irradiated male x non-irradiated female) as well as a non-irradiated control (non-irradiated male x non-irradiated female). The flies were allowed to mate *ad libitum*. All cages were provided with water soaked cotton wool and separate dishes of dry granular sucrose and dry yeast hydrolysate enzymatic (MP Biomedicals, Aurora, OH, USA) as food. Q-flies from the source population, maintained under laboratory conditions, start mating between 6 and 8 days after emerging (Perez-Staples *et al*. 2007). No calling, courting or mating was observed prior to sorting.

At 10 days after adult emergence, when at maximum sexual activity (Perez-Staples *et al*. 2007), each cage was provided with an oviposition substrate comprising a parafilm-covered 90mm Petri dish containing water and food-grade lemon essence (Queen Fine Foods Pty Ltd) in a 140:1 ratio. The parafilm was punctured 10-15 times with an entomological pin to release the odour of lemon essence. Oviposition dishes were changed daily for five days. Collected dishes were left for a further four days in the lab to allow fertile eggs to hatch. All unhatched eggs and larvae were counted *in situ* under a dissecting microscope (Leica MZ6) on a matte black stage, illuminated by fibre-optic light (Dolan-Jenner 150W). Fecundity (N eggs + N larvae) and fertility (N larvae / (N eggs + N larvae)) were calculated for each mating combination for each treatment.

Results

Relationship between dose rate and total dose

Total dosage delivered to the pupae (determined post-hoc from Fricke dosimeters) varied among the replicates (least square means ± SE: rep 1 = 71.42 ± 0.20 Gy, rep 2 = 73.24 ± 0.20 Gy, rep 3 = 71.28 ± 0.20 Gy; $F_{2,71} = 28.63$, $P < 0.001$) and was positively related to dose rate (b=0.036±0.004, $F_{1,71} = 82.19$, $P < 0.001$; Figure 2.1). The relationship between dose rate and total dosage requires a cautious approach to data interpretation. If variation in total dosage within or around the target dose range of 70 - 75 Gy (actual dose range recorded was 69.6 - 76.2 Gy, see Figure 2.1) is sufficient to influence fly quality, then apparent differences among tested dose rates could actually be attributed to this dose-rate-dependent variation in total dosage. Hence, where we found significant effects of dose rate we re-ran the analyses including total dose as a covariate. If effects of dose rate are rendered non-significant by the inclusion of total dosage in the analysis, then we conclude that the effects of dose rate had been indirectly driven by associated variation in total dosage.

Figure 2.1: Relationship between irradiation dose rate (Gy / min) and total dosage absorbed by the pupae (Gy).
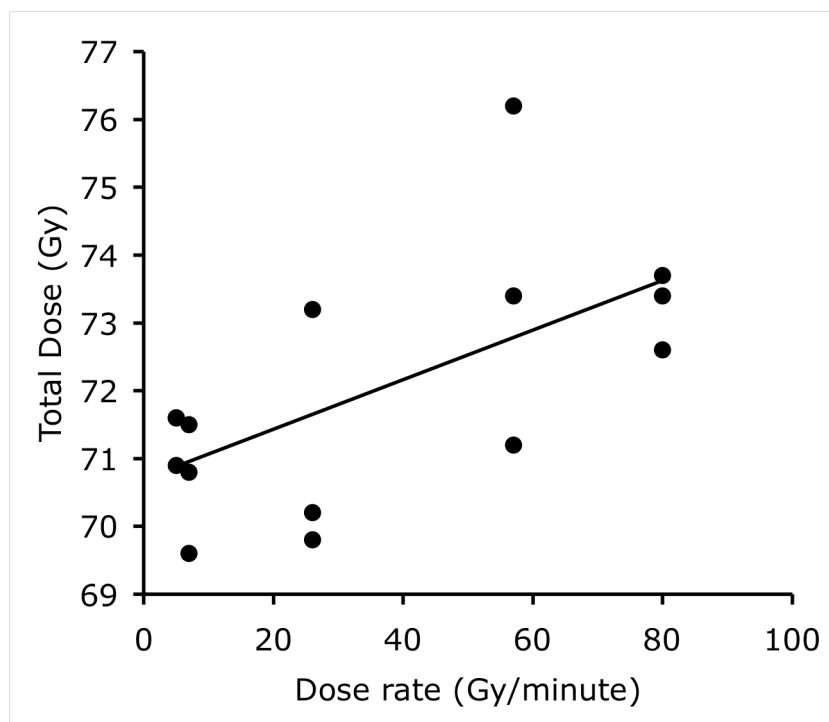
Percentage emergence, flight ability and sex ratio

Percentage emergence. After correcting for variation among the replicates (least square means ± SE: rep 1 = 84.6 ± 0.7 %, rep 2 = 86.8 ± 0.7 %, rep 3 = 89.4 ± 0.7 %; $F_{2,81}$ = 10.47, $P <$ 0.001), there was no evidence that percentage emergence was influenced by dose rate ($F_{5,81}$ = 0.64, P = 0.67; Figure 2.2) or light levels ($F_{1,81}$ = 0.44, P = 0.51).

Percentage fliers. After correcting for variation among the replicates (least square means ± SE: rep 1 = 73.7 ± 0.9 %, rep 2 = 79.5 ± 0.9 %, rep 3 = 80.5 ± 0.9 %; $F_{2,81}$ = 14.83, $P <$ 0.001), there was no evidence that percentage fliers was influenced by dose rate ($F_{5,81}$ = 0.93, P = 0.47; Figure 2.2) or light levels ($F_{1,81}$ = 1.44, P = 0.23).

Rate of fliers. Given that neither proportion emergence nor proportion fliers was influenced by dose rate, it was not surprising that after correcting for variation among the replicates (least square means ± SE: rep 1 = 87.2 ± 0.9 %, rep 2 = 91.6 ± 0.9 %, rep 3 = 90.1 ± 0.9 %; $F_{2,81}$ = 5.71, P = 0.005) there was no evidence that rate of fliers was influenced by dose rate ($F_{5,81}$ = 1.32, P = 0.26; Figure 2.2) or light levels ($F_{1,81}$ = 0.78, P = 0.38).

Sex ratio. After correcting for variation among the replicates (least square means ± SE: rep 1 = 50.8 ± 0.8 %, rep 2 = 52.7 ± 0.8 %, rep 3 = 49.6 ± 0.8 %; $F_{2,81}$ = 3.34, P = 0.04), there was no evidence that sex ratio was influenced by dose rate ($F_{5,81}$ = 1.18, P = 0.33; Figure 2.2) or light levels ($F_{1,81}$ = 1.18, P = 0.28).

Mortality under stress

Females. After correcting for variation among the replicates (least square means ± SE: rep 1 = 78.3 ± 1.8%, rep 2 = 70.9 ± 1.8%, rep 3 = 67.9 ± 1.8%; $F_{2,81}$ = 8.99, $P < 0.001$), the number of females dead after 48 hours under stress was not significantly related to dose rate treatment ($F_{5,81}$ = 1.11, $P = 0.36$; Figure 2.3).

Males. In contrast to the results for females, after correcting for variation among the replicates (least square means ± SE: rep 1 = 83.8 ± 1.4 %, rep 2 = 64.6 ± 1.5 %, rep 3 = 66.8 ± 1.4 %; $F_{2,81}$ = 53.19, $P < 0.001$), the number of males dead after 48 hours under stress was significantly related to dose rate treatment ($F_{5,81}$ = 2.73, $P = 0.03$; Fig. 3). Tukey-Kramer HSD tests revealed that mortality in the highest dose rate treatment was significantly higher than in the unirradiated control, but that there were no other significant differences (Figure 2.3). However, including total dosage absorbed as a covariate negated this effect of dose rate treatment ($F_{5,81}$ = 1.37, $P = 0.24$; Figure 2.3) leading us to conclude that rather than being a direct effect of dose rate, this result is instead a reflection of the unintended correlation between dose rate and total dosage absorbed (see Figure 2.1).

Figure 2.2: Effects of irradiation dose rate on percentage emergence, percentage fliers, rate of fliers, and percentage of males (sex ratio).
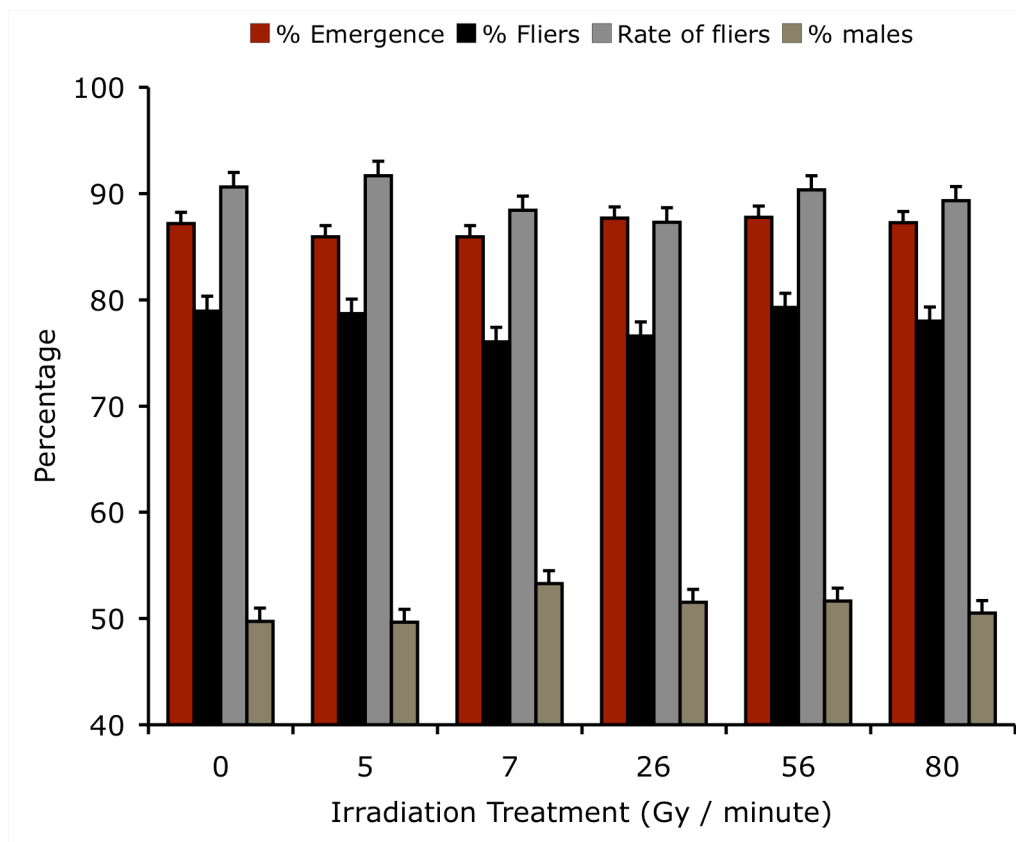
Figure 2.3: Effects of irradiation dose rate on percentage of flies dying within 48 hours of emerging when provided no food or water for sustenance. Letters indicate significant differences across dose rate treatments on the combined mortality results for males and females. The significant difference between control flies and those receiving the highest dose rate is explained by incidental dose rate-associated variation in total dosage absorbed (see Figure 2.1).
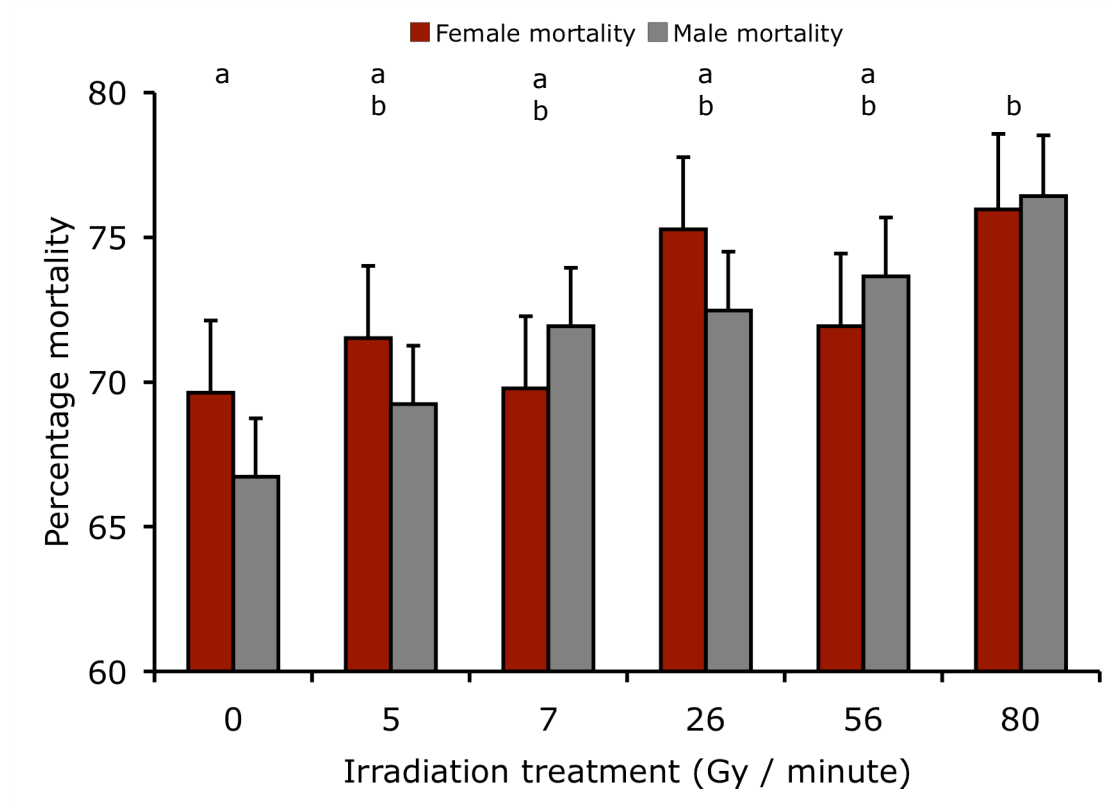
Reproductive sterility

From 3 non-irradiated male x non-irradiated female (control) cages, we collected 14,441 eggs (4,814 / cage), of which 9,480 hatched (65.6 % fertility). From 14 irradiated male x non-irradiated female cages we collected 56,253 eggs (4,018 / cage), of which 44 hatched (0.078% fertility). From 14 non-irradiated male x irradiated female cages we collected just 11 eggs (0.79 / cage), of which none hatched (0 % fertility). From 15 irradiated male x irradiated female cages we collected 3 eggs (0.2 / cage), of which none hatched (0 % fertility). These results confirm that the irradiation dosage used is highly effective in inducing reproductive sterility in both sexes; in males because their fertile mates are normally fecund but have extremely low fertility and in females because very few eggs are oviposited and the few that are oviposited are not viable.

Discussion

Results from the present study of Q-flies are broadly similar to those found previously for two other tephritids, *C. capitata* and *B. cucumis* (Hooper 1970, 1975), in that dose rate had no overall effect on sterility induction at a single target total dose; the level of sterility induced by the target dose range for Q-fly SIT (70 - 75 Gy) did not vary with the dose rate at which it was applied. This result supports the one-hit ionising event hypothesis (LaChance 1967). In applied terms, this result means that it is possible to make use of a wide variety of dose rates as required in irradiating Q-fly pupae without compromising efficacy of the treatment in sterility induction. In particular, for a given total dose, the GATRI and GC220 systems, which operate at very different dose rates, should be equally effective for sterility induction.

Dose rate applied to irradiated pupae had no significant effect on the emergence, flight ability, rate of fliers or sex ratio of mass reared Q-flies in the present study, again indicating that, at a given total dose, the GATRI and GC220 systems can produce flies of similar general quality despite differences in dose rate. Additionally, at a given dose, it is possible to use different dose rates in the GATRI system without any direct consequences for these particular measures of fly quality.

Interestingly, in addition to no significant variation among the dose rate treatments, we found no differences in emergence, flight ability, rate of fliers or sex ratio between any of the irradiated treatments and the unirradiated controls. At first these results appear discordant with the those of Dominiak *et al.* (2002) and Dominiak *et al.* (2007a), who found irradiated Q-flies to suffer significant reductions in emergence and rate of fliers compared with unirradiated flies from the same batch (*nota bene:* Dominiak *et al.* (2002) and Dominiak *et al.* (2007a) refer to 'flightability', but the measure calculated by them was actually rate of fliers, which corrects for number of flies emerged. Flightability as defined in FAO/IAEA/USDA (2003) was not assessed by Dominiak *et al.* (2002) or Dominiak *et al.* (2007a). However it is important to note differences between these studies in factors other than irradiation that might be the source of the apparent differences in effects of irradiation.

Our experiments were specifically designed to consider the effects of irradiation and so we were careful to control for other factors by keeping the irradiated and non-irradiated pupae and flies under close to identical conditions throughout. In our study, the only difference between irradiated and non-irradiated flies was during the brief period in which the actual irradiation was carried out (a few minutes). In contrast, because their studies dealt with production and delivery processes under existing protocols there were many additional differences in the irradiated and non-irradiated flies assessed by Dominiak *et al.* (2002) and Dominiak *et al.* (2007a). Specifically, their studies compared 'irradiated' flies that had been packed in plastic bags, dyed, transported for irradiation, chilled and rendered hypoxic, irradiated, transported back to the factory and returned to ca. 25ºC with 'non-irradiated' flies that had remained at the factory under a constant 25ºC and received little handling. That is, in Dominiak *et al.* (2002) and Dominiak *et al.* (2007a) the term 'irradiated' refers not only to irradiation *per se* but also to the raft of additional conditions that necessarily accompany irradiation treatment in a production setting.

Given that we found no direct effects of irradiation in the present study, we can hence consider these other factors as likely causes of the reduced emergence and rate of fliers in 'irradiated' flies tested by Dominiak *et al.* (2002) and Dominiak *et al.* (2007a). Of these, the ones for which we have direct evidence in Q-flies are the presence of dye and transport. Dominiak *et al.* (2000) reported reduced emergence of Q-flies after dye treatment while Weldon (2005) found that the dyes used to mark Q-flies reduced the ability of adult flies to escape from the trays in which they had emerged. Dominak *et al.* (2007b) report a distinct reduction in emergence of pupae after transport by air and road from the factory to the release zone. We suggest that the differences between

irradiated and non-irradiated Q-flies reported by Dominiak *et al*. (2002) and Dominiak *et al*. (2007a) are likely to have resulted from the presence of dye and other differences in handling rather than from irradiation *per se*.

While our findings for other aspects of quality are quite straightforward, the results for irradiation dose rate effects on mortality under stress present more of a challenge. For females, there was no significant difference among the dose rate treatments or between irradiated and non-irradiated controls. However, for males there was a significant increase in mortality under stress in the highest dose rate treatment compared with the non-irradiated controls. This increase appears to reflect dose-rate variation in total dosage received rather than being a direct effect of dose rate. Specifically, even when a standard total dose (70 - 75 Gy) was targeted, we found a significant positive relationship between dose rate and total dose (see Fig. 1) that appears to have been responsible for increased mortality under stress in the highest dose rate treatment.

As dose rate increases the time needed to reach the target dose decreases markedly. For example, an exposure of 70 Gy takes 14 minutes at 5 Gy/min (= 0.083 Gy/s) but only takes 52.5 s at 80 Gy/min (= 1.333 Gy/s). Accordingly, a momentary delay in terminating exposure has negligible effects on total dose received when using low dose rates but can be sufficient to result in a detectible increase in total dose received when using high dose rates. For example, a delay of 2 s in termination of exposure will result in a total dosage of 70.166 Gy at 5 Gy/min (14 min irradiation time) compared with a total dosage of 72.667 Gy at 80 Gy/min (53 s irradiation time). Essentially 2 s of 14 mins equates to a 0.2% error, while 2 s of 53 s equates to a 3.8% error. These errors would be in addition to the 2% measured uncertainty of Fricke dosimetry.

This study is the first in a series to consider calibration and optimisation of irradiation procedures used in the current Q-fly SIT program. Our results indicate that dose rate does not have a direct impact on basic quality control parameters used to assess fly quality or sterility. To achieve maximum control over total dose, we recommend that whenever possible (providing time allows for the irradiation of the required number of pupae to meet release requirements), the lowest available dose rate be used when irradiating Q-fly pupae for SIT.

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3. OPTIMISING IRRADIATION DOSE FOR STERILITY INDUCTION

Summary

The present study is an important step toward calibrating, validating and improving irradiation methods used for Queensland fruit fly sterile insect technique (SIT). We used routine IAEA/USDA/FAO quality control tests assessing percentage emergence, flight ability, sex ratio, mortality under stress, longevity under nutritional stress, reproductive sterility and sexual competitiveness, to assess the impact of a range of target irradiation doses (60, 65, 70, 75 and 80 Gy) on the product quality of mass reared Queensland fruit fly used in SIT. Sterility induction remained adequate (> 99.5%) across the full range of irradiation doses tested. However there was significant reduction in survivability and competitiveness of irradiated flies as dose increased. The current target sterilizing dose for SIT of 70 - 75 Gy is associated with elevated damage to flies. Our data suggest that adequate sterility and improved fly quality could be achieved through a small reduction in target sterilizing dose.

Introduction

The sterile insect technique (SIT) used to combat tephritid fruit fly pests in many regions of the world depends on the ability of released sterile males to mate with wild females and induce reproductive failure, thereby reducing population numbers in subsequent generations (Knipling 1955). Successful performance by sterile males in the field is dependant on a wide range of key behavioural factors including survival till sexual maturity, successfully finding, courting and mating with wild females, and inducing long refractory periods (reviewed Hendrichs *et al.* 2002).

Gamma irradiation is currently the most common method used to sterilize mass reared flies for SIT (Bakri *et al.* 2005). To ensure that released flies are effective at inducing reproductive failure in their mates, it is important that irradiation procedures achieve an adequate level of sterility; for example, 99.5% sterility from crosses between sterile males and fertile females is usually required in Mediterranean fruit fly, *Ceratitis capitata* (FAO/IAEA/USDA 2003). Tephritids have relatively homogeneous sensitivity to gamma irradiation, with most major pest species requiring less than 100 Gy to achieve suitably high levels of reproductive sterility (Bakri *et al.* 2005).

Assuming that higher levels of sterility will mean greater ability to induce reproductive failure in wild populations, it is tempting to aim for the highest possible levels of sterility induction, basing irradiation protocols on doses well above the minimum required to induce 99.5% sterility. But such practises can diminish ultimate SIT efficacy if higher irradiation doses diminish fly performance. Deleterious effects of gamma irradiation on fly quality and competitiveness have been reported for numerous tephritid species, including *Ceratitis capitata* (Barry *et al.* 2003; Lux *et al.* 2002), *Anastrepha obliqua* (Toledo *et al.* 2004), *Anastrepha ludens* (Rull *et al.* 2007), *Anastrepha suspensa* (Walder & Calkins 1993) and *Bactrocera cucumis* (Hooper 1975).

For effective SIT, it is essential that irradiation procedures be fine-tuned to ensure an adequate level of sterility while minimizing the deleterious effects on fly quality and competitiveness. Overdosing to eliminate a minimal residual fertility (<1%) often produces flies that suffer from substantially reduced competitiveness, resulting in greatly diminished ability to induce reproductive failure in wild populations (Bakri *et al.* 2005; Calkins & Parker 2005). Owing to their superior competitive ability, male flies irradiated at lower doses, allowing some residual fertility, may induce higher levels of reproductive failure in wild populations than males that have been irradiated at high doses to eliminate residual fertility (Hooper 1972; Toledo *et al.* 2004). Released flies that are incompetent for survival or mating are of no value to SIT.

Current gamma irradiation procedures used to sterilize Queensland fruit fly for SIT are based largely on the findings of Monro & Osborn (1967), Bhatti & Shipp (1972) and Sproul *et al.* (1992). Monro & Osborn (1967) discuss the full-scale production of mass reared flies, from rearing techniques to irradiation, transport, and release. Irradiation procedures are only briefly described, reporting a dose rate of 20 Gy/min and a wide target dose range of 60 to 100 Gy with a maximum overdose of 15%. The relative sterility achieved across this dose range was not assessed.

Bhatti & Shipp (1972) tested the competitiveness of flies irradiated at target doses of 30, 50, 75, 90, 100, 120, 150 Gy (reported in Krad, 1Krad = 10 Gy). Mating competitiveness of males irradiated at 50 and 75 Gy was superior to that of males irradiated at 90 Gy and above. Like Monro & Osborn (1967), Bhatti & Shipp (1972) did not assess sterility induction at the various target doses.

Sproul *et al.* (1992) assessed both sterility induction and competitiveness of flies irradiated in air and in nitrogen, as nitrogen induced anoxia can reduce the deleterious effects of ionising irradiation on fly pupae (Bakri *et al.* 2005; Hooper 1976). Sproul *et al.* (1992) tested a range of target doses including 5, 20, 40 and 60 Gy in air and 20, 40, 60 and 100 Gy in nitrogen. While 99% sterility was achieved at a dose of 60 Gy when the pupae were irradiated in air, the sterile males from this treatment were only 38% as successful in obtaining copulations compared against untreated males. The mating competitiveness of males irradiated in air was further diminished as irradiation dose increased. For flies irradiated in nitrogen the mating competitiveness of irradiated males was nearly double that of flies irradiated in air, although a target dose of 100 Gy was needed to induce 99.5% sterility.

While they provided a sound foundation, each of these studies considered only a very coarse scale of target doses and was based on Gammacell irradiation equipment that is quite different from the Gamma Technology Research Irradiator (GATRI) used by the Australian Nuclear Science and Technology Organization (ANSTO) to sterilize pupae for the current Queensland fruit fly SIT programme. In the current SIT program pupae are irradiated under hypoxia at a target dose of 70 - 75 Gy. While this target dose is adequate to achieve >99% sterility, previous study of irradiation dose rate found significant decline in the performance of emerging flies in standard mortality under stress tests (Collins *et al.* 2008). However with increasing dose rates there was increasing chance of overshooting the target dose.

If minor variation across the 70 - 75 Gy target dose range can create significant differences in survivability of released flies, it may be that the current target dose exceeds the threshold where pupae, irradiated under hypoxia, suffer significant damage that could diminish their performance in the field. It may be that a minor reduction of target dose would achieve adequate sterility while also affording greater protection against the deleterious effects of irradiation. The present study provides a detailed investigation of how irradiation dose effects the sterility and quality of Queensland fruit flies, over a much finer scale range of target doses than has been reported in previous studies.

Materials and Methods

Packing and irradiation

Queensland fruit fly pupae were obtained from the Industry & Investment NSW Fruit Fly Production Facility at Elizabeth Macarthur Agricultural Institute (EMAI). Pupae from this facility are routinely sent to ANSTO for irradiation and are then distributed to release zones for the Sterile Insect Technique (SIT) control program that is used to suppress outbreaks of Queensland fruit flies. The following procedures were carried out for six replicate batches of pupae between July 2007 and February 2008.

For each replicate, six individual 'zip-lock' plastic bags (100 x 150 mm) containing ca. 8,000 pupae were packed and sealed at EMAI, and transported directly to ANSTO in an air-conditioned vehicle. The bags of pupae were kept together at all times during transport and holding to ensure that they received similar handling conditions. To achieve a hypoxic atmosphere prior to irradiation, the sealed bags were held overnight at ANSTO in a temperature-controlled room at approximately 18 ^{o}C, as is standard practise in the current SIT program. The following day, five bags were irradiated separately at different target doses (60, 65, 70, 75, 80 Gy) in ANSTO's GATRI facility and the sixth bag was kept aside as an unirradiated control. The plastic bags of pupae were fixed with tape to the face of separate cardboard boxes, which were then mounted in the GATRI facility for irradiation.

The GATRI facility is a Co_{60} source stored in a 5m deep tank of demineralized water (Izard 1988). The source is raised by remote into a 3.5 x 4.0 x 3m irradiation cell. Material to be irradiated is arranged on a metal 'rack' which is placed at a set distance from the source (1310 mm) to achieve a dose rate of 5 Gy/min (Collins *et al.* 2008). Calibrated ferrous ammonium sulphate (Fricke) dosimeters (Fricke & Hart 1966) were fixed with tape to the outside of each bag. The two dosimeters were placed at the centre of each packet of pupae and were used to confirm the target dose received. Packs of pupae were flattened as much as possible to achieve an even packet density, with each packet being no more than 1cm thick across the surface area.

After irradiation, pupae were immediately transported in a closed styrofoam box in an air-conditioned vehicle to a laboratory at Macquarie University, Sydney, where they were set up to emerge in 5 litre plastic cages. The cages had a large mesh-covered opening for ventilation comprising approximately 30% of the total surface area. Flies were held, and all experiments run, in a laboratory maintained at 25±1°C, 70±5% RH on a 14:10 day night cycle including one hour dawn and dusk periods during which the lights turned on and off in stages. Following Collins *et al.* (2008), the effect of irradiation dose on fly quality was assessed using the required routine quality tests from the FAO/IAEA/USDA (Version 5, May 2003) product quality control manual, measuring emergence, sex ratio, flight ability, mortality under stress and sterility. In addition to these required routine tests, flies were also subjected to a test of mating competitiveness and a test of individual longevity under nutritional stress.

Percentage emergence, flight ability and sex ratio

Two days before they emerged, ca. 100 pupae from each dose treatment and the control were placed in separate 55 mm plastic petri dish lids. The dishes of pupae were then centred on 90 mm Petri dish lids that were lined with black paper. A black 100 mm tall plexiglass tube (94 mm inner diameter, 3mm thickness) with a fine coat of unscented talcum powder on the interior (to prevent flies from walking out) was placed over the 90 mm Petri dish lid. A 20 mm width of talcum powder was wiped off the base of each tube to provide newly emerged flies an additional vertical surface on which to rest. Each tube with pupae was placed in an individual 325 x 325 x 325 mm white mesh cage (Australian Entomological Supplies). An identical 'dummy' tube without pupae was placed in each cage to estimate 'fly-back', flies that initially escape from the tube but then return to the tube and fail to escape again, leading to inaccurate counts of flight ability. Flight ability was corrected by subtracting the number of flies in fly-back tubes from the total number of not fliers recorded. Cages were set up on shelves that were lit by a single 36W fluorescent light tube positioned immediately above the cages. A reading of light intensity was taken in each cage and was included as a covariate in final analysis of emergence and flight ability to assess any variation due to light level. In each of the six replicates, five cages of ca. 100 pupae were set up for each dose treatment and the unirradiated control. To minimize fly-back, flies that escaped from the tube were removed

by aspirator daily, and were frozen for later identification of sex. When all emergence had ceased (two to three days after the first flies emerged) the remaining contents of the tubes were counted.

Following FAO/IAEA/USDA (2003), individual flies were classified as (1) 'not emerged' if still inside an un-open pupal case, (2) 'part emerged' if they failed to emerge completely from the pupal case (3) 'deformed' if they had completely shed the pupal case but had damaged wings and (4) 'not fliers' if they had completely shed the pupal cases and had morphologically normal wings but failed to escape the tube. Calculations from Collins *et al.* (2008) were used to assess percentage of emergence, percentage of fliers and rate of fliers (the percentage of fliers corrected for emergence). All flies that emerged (non-fliers and fliers) were sexed to identify effects of irradiation treatment on sex ratio. The calculations used are directly comparable to the corresponding methods of FAO/IAEA/USDA (2003) product quality control manual.

Mortality under stress

Within 2 hours after emerging in a 5-L cage without food or water, 50 males and 50 females from each treatment were gently aspirated into a 150 mm diameter plastic Petri dish through a 10 mm diameter hole in the lid, which was then stoppered by a cork to prevent flies escaping. The dishes were placed in a dark cabinet and checked for mortality 24 and 48 hours later. At 24 hours, the stopper was removed, and dead flies were collected by gently upending the dish, ensuring that no live flies escaped. In each of the six replicates, five dishes were set up for each dose treatment and the unirradiated control.

Longevity under nutritional stress

The facility at EMAI that produces flies for the current Queensland fruit fly SIT program has developed its own test for longevity under nutritional stress. Rather than testing the flies in a crowded Petri dish as in the standard mortality under stress test (see above), the 'longevity under nutritional stress' procedures at EMAI test the flies individually. To compare the results for tests of longevity without nutrition when flies are maintained individually with results when flies are maintained in groups, the current EMAI longevity under nutritional stress test was incorporated into the experimental design alongside the FAO/IAEA/USDA (2003) standard mortality under stress test for the final three replicates between October 2007 and February 2008.

For each replicate, two days before emergence, 100 pupae from each dose treatment and the unirradiated control were individually weighed and placed into individual wells of 12-well microplates. While in the microplate well, each pupa was photographed under a stereomicroscope (Olympus SZX12, Jenoptik Progress C10 camera) for later measurement of size. The microplates were checked for mortality three times each day (09:00, 13:00 & 17:00 respectively). For each pupa, we assessed whether it emerged, the time of emergence, time of adult death, adult sex, and adult size (wing length).

After all flies had died (two to three days after the first flies emerged), the right wing of each fly was removed with a pair of soft forceps and gently placed next to a label on a strip of double sided tape adhered to a microscope slide. A second microscope slide was placed firmly on top of the first slide to secure the wings in place. Each wing was then photographed under a stereomicroscope. Pupal length and wing length were measured using ImageJ v1.37. Wing length (mm) was measured from the intersection of the anal and median band to the margin of the costal band and the R4+5 vein (Perez-Staples *et al.* 2007).

Reproductive sterility

To assess reproductive sterility of male and female flies irradiated at different doses, for each dose treatment we set up 5 L cages containing 50 males and 50 females for each possible combination of irradiated and non-irradiated flies (i.e., irradiated male x irradiated female; non-irradiated male x irradiated female; irradiated male x non-irradiated female) as well as a non-irradiated control (non-irradiated male x non-irradiated female). All cages were provided with water soaked cotton wool and separate dishes of dry granular sucrose and dry yeast hydrolysate enzymatic (MP Biomedicals, Aurora, OH, USA) as food. To ensure that no mating took place before the experiment began, the cages were always set up within three days after the flies emerged. Queensland fruit flies from the source population start mating between 6 and 8 days after emerging in the laboratory (Perez-Staples *et al.* 2007; Prabhu *et al.* 2008). No calling, courting or mating was observed in cages prior to sorting. The flies were allowed to mate ad libitum.

Ten days after adult emergence, when at maximum sexual activity (Perez-Staples *et al.* 2007), each cage was provided an oviposition substrate comprising a parafilm-covered 90-mm Petri dish containing water and food-grade lemon essence (Queen Fine Foods Pty Ltd, Alderly, QLD, Australia), in a 140:1 ratio. The parafilm was punctured 10 - 15 times with an entomological pin to release the odour of lemon essence. Oviposition dishes were changed daily for five days. Collected dishes were left for a further four days in the lab to allow fertile eggs to hatch. Total larvae and un-hatched eggs were counted *in situ* under a stereomicroscope to assess fecundity and fertility.

Sexual competitiveness

To assess mating competitiveness of male and female flies irradiated at different doses, for each dose treatment we ran mating trials in which irradiated and unirradiated males and females competed for matings. When the flies were 10 days of age, separate mesh cages (45.7 x 47.5 x 47.5 cm, BugDorm, MegaView, Taiwan) of virgin flies (maintained in single sex cages from within 3 days after emerging) were set up for each dose treatment at least three hours before onset of simulated dusk (Queensland fruit flies only mate during a short period around dusk; Barton-Browne 1957). Each cage contained 15 irradiated males, 15 irradiated females, 15 unirradiated males, and 15 unirradiated females (60 flies in total per cage, 1:1 sex ratio and 1:1 sterile/fertile ratio). Observations began 30 min before the onset of simulated dusk.

For ready identification, either unirradiated or irradiated flies were dyed in each test (Astral pink, Fiesta FEX series, Swada, UK). Flies were dyed as pupae prior to emergence, by gently mixing pupae and dye in a slowly rotated plastic cup till all pupae were well coated. To account for any effect the dying may have on mating competitiveness, for each replicate the tests were repeated the following day using new virgin flies from the same batch with dye treatments reversed. Mating pairs were removed from cages using glass vials at the onset of copula and the time was recorded. This continued until all of the lights had switched off at the end of simulated dusk. Flies were later identified as irradiated or unirradiated by the presence of dye, and the latency from onset of simulated dusk until copula was calculated for each mating pair.

Sexual competitiveness of flies irradiated at each dose was assessed using the indices of strain sexual compatibility from the required periodic quality control tests (FAO/IAEA/USDA 2003). Relative Isolation Index (*RII*) is a measure of mating compatibility that, when the ratio of sterile to fertile flies is 1:1 *RII* equates to the number of sterile males required to match the mating capability of one fertile male. A *RII* value of 1 represents random mating or sexual compatibility. Values greater than 1 indicate that there is a difference between sterile and fertile flies and that strains are tending to mate assortatively (i.e., sterile x sterile and fertile x fertile).

Stalker's Index (*I*) is similar to *RII* but is less likely to be heavily influenced by small changes in a single type of mating. However this stability also renders Stalker's index less sensitive to a drop in the crucial sterile male x fertile female matings compared with *RII*. The scale for *I* is in the opposite direction for that of *RII*, with results less than 1 indicating increasing sexual isolation, while results of 1 and above indicate increasing sexual compatibility between strains.

The Isolation Index (*ISI*) is a compatibility measure that ranges from -1 (negative assortative mating; i.e., all matings sterile x fertile or fertile x sterile), through 0 (random mating), to 1 (complete sexual isolation). The range from -1 to 1 is used to assess the deviation of a strain away from the expected random mating compatibility of 0. *ISI* is further assessed by separately calculating the Relative Performance Index for males (*MRPI*) and females (*FRPI*). These measures identify from where deviations around 0 in the ISI scale result, by separately identifying the mating propensity of males and females. *MRPI* and *FRPI* values below 0 indicate greater fertile fly mating propensity and values above 0 indicate greater sterile fly mating propensity.

The final measure to assess mating competitiveness is the Relative Sterility Index (*RSI*), a proportional index of sterile male sexual competitiveness. Increasing values of *RSI* indicate increasing ability of sterile males to obtain copulations with fertile females.

Statistical analysis

Results from routine fly quality tests were analysed using standard least squares regression models. Binary data from longevity under nutritional stress tests (i.e., male/female, emergence) was analysed using logistic regression. These analyses were performed using JMP v 5.0 (SAS Corporation, Cary, NC, USA). The various indices calculated from sexual competitiveness tests were each analysed using general linear models and orthogonal polynomial contrasts in SPSS v 16.0 (SPSS Inc, Chicago ILL, USA). To account for variation among production batches, replicate was included as a random effect in each regression model.

Results

Percentage emergence, flight ability and sex ratio

Results reported for flight ability tests are from five replicates, as one replicate emerged unusually early such that it was not possible to set up these tests. There was no evidence that percentage emergence from flight ability tests was influenced by irradiation dose ($F_{5,138} = 1.39$, $P = 0.23$) or light level ($F_{1,138} = 0.03$, $P = 0.86$) (Figure 3.1). There was also no evidence that percentage fliers was influenced by irradiation dose ($F_{5,138} = 0.1.15$, $P = 0.36$) or light level ($F_{1,138} = 1.04$, $P = 0.31$). Given that neither percentage emergence nor percentage fliers was influenced by irradiation dose, it was not surprising that after correcting percentage of fliers for emergence there was no evidence that rate of fliers was influenced by irradiation dose ($F_{5,138} = 1.13$, $P = 0.35$) or light level ($F_{1,138} = 1.56$, $P = 0.22$). There was some evidence that sex ratio (percent males) varied with irradiation dose ($F_{5,138} = 2.69$, $P = 0.024$), with a slightly lower percentage of males in the control and 65 Gy cages (Figure 3.1). This variation was only evident in the sex ratio test from flies emerging in the flight ability tests; sex ratio in the longevity under nutritional stress tests (M/F) did not vary significantly among the irradiation treatments ($G_5 = 2.6$, $P = 0.76$).

Figure 3.1: Effects of irradiation dose on percentage emergence, percentage fliers, rate of fliers, and percentage of males (sex ratio).
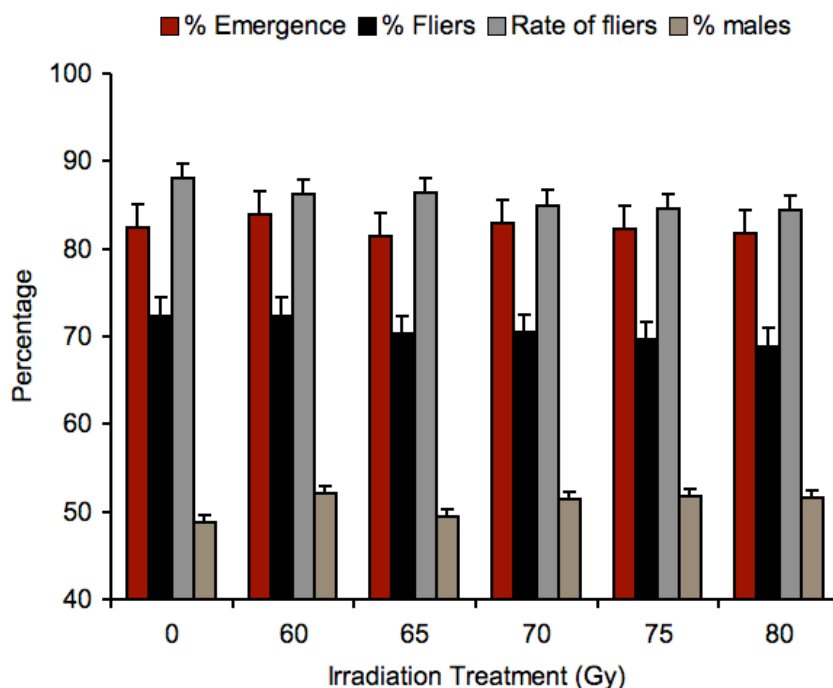
Mortality under stress

The number of dead flies (males and females) after 48 hours under stress was significantly related to irradiation dose and sex (irradiation dose $F_{5,279} = 37.53$, $P < 0.0001$; sex $F_{1,279} = 5.38$, $P = 0.021$; irradiation dose x sex interaction; $F_{5,279} = 2.34$, $P = 0.047$; Figure 3.2). For both sexes there was a distinct increase in mortality as irradiation dose increased, although males suffered greater mortality than females in the 80 Gy treatment (Figure 3.2).

Longevity under nutritional stress

As in the flight ability assays, emergence (y/n) of flies did not vary among the irradiation treatments ($G_5 = 3.01$, $P = 0.70$). However, emergence did vary significantly with pupal weight, as heavier pupae were more likely to emerge than lighter pupae ($G_1 = 26.44$, $P < 0.0001$). Emergence was also related to pupal length; for any given weight, shorter pupae were more likely to emerge ($G_1 = 9.23$, $P = 0.0024$) (Figure 3.3). Pupal weight, pupal length and adult wing length were all significantly correlated: pupal length and weight (Spearman's rho = 0.57, P < 0.0001); wing length and pupal weight (Spearman's rho = 0.67, P < 0.0001); wing length and pupal length (Spearman's rho = 0.42; P < 0.0001).

Due to correlations among pupal weight, pupal length and wing length, each was assessed separately as co-variates in separate linear regression models for longevity. The number of flies dead at 48 hours was not influenced by irradiation dose ($G_5 = 7.05$, $P = 0.2$), a result that differs markedly from the mortality under stress tests in which the flies were maintained in crowded Petri dishes. Longevity (in hours as a continuous outcome rather than a binary dead/alive at 48 hours) was also not influenced by irradiation treatment ($F_{5,1389} = 0.44$, $P = 0.82$). Longevity was influenced strongest by pupal weight ($b = 1050.93$ $F_{1,1389} = 27.59$, $P < 0.0001$), with heavier pupae producing longer living adults. Similar results were recorded for pupal length ($b = 5.58$ $F_{1,1386} = 16.80$, $P < 0.0001$) and wing length ($b = 6.96$ $F_{1,1365} = 18.30$, $P < 0.0001$). Longevity was also different between sexes with males living longer than females ($F_{1,1389} = 15.48$, $P < 0.001$).

Figure 3.2: Effects of irradiation dose on the percentage of flies dying within 48 hours of emerging in standard FAO/IAEA/USDA mortality under stress tests.
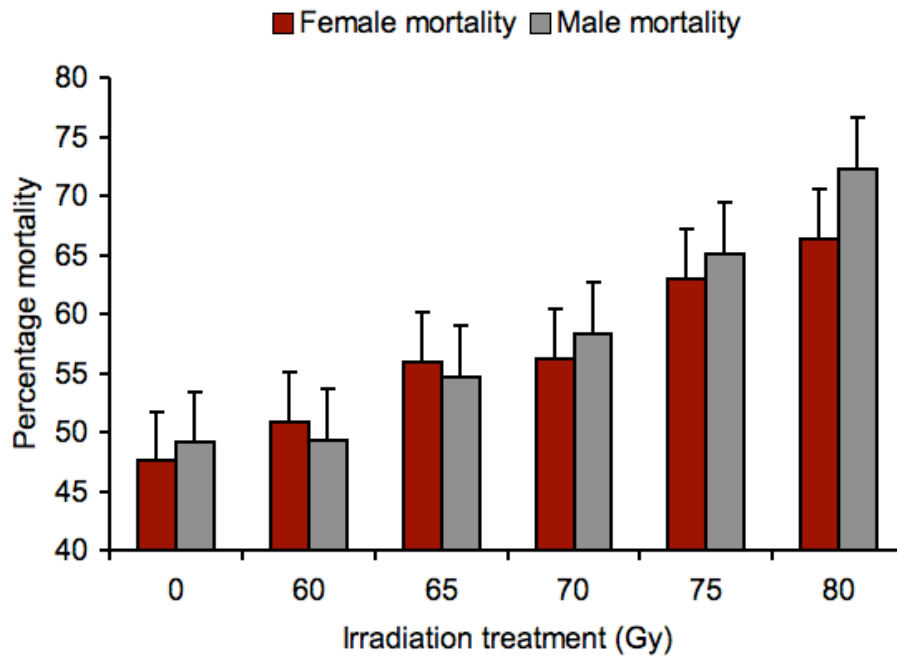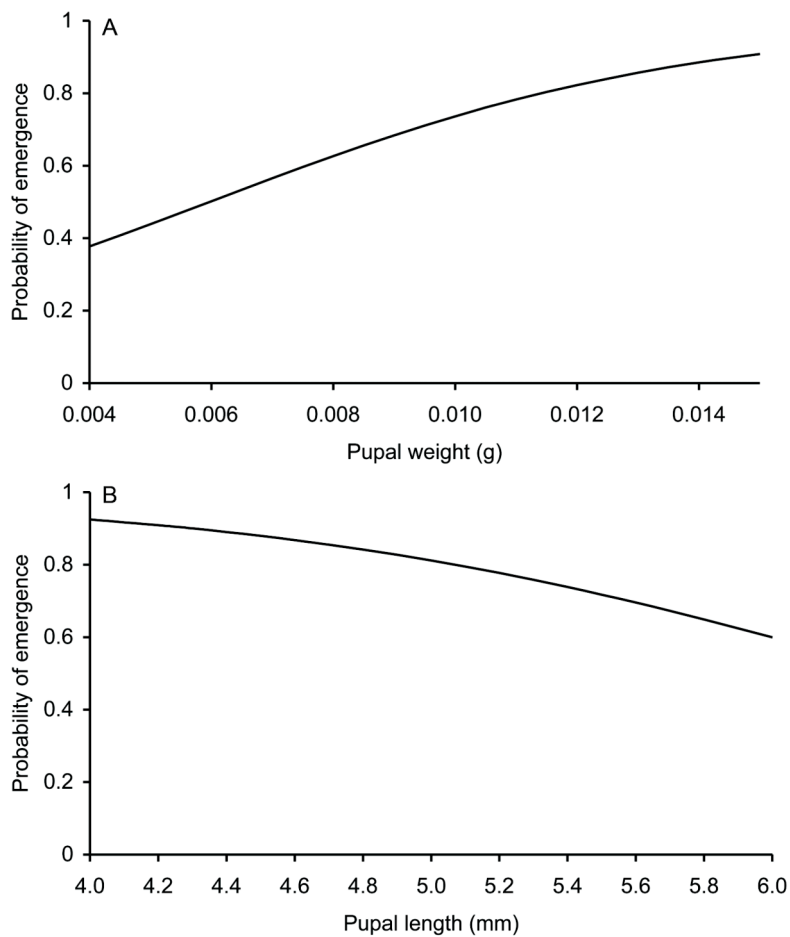

Figure 3.3: Probability of emergence from individual longevity under stress tests for both A) pupae weight and B) pupae length.

Reproductive sterility

From the six unirradiated male x unirradiated female (control) cages a total 14,210 eggs (2,368 eggs per cage) were collected, of which 10,211 hatched (72% fertility). From the 30 irradiated male x unirradiated female cages a total 73,255 (2,441 eggs per cage) were collected, of which 81 eggs hatched (0.11% fertility). From the 30 unirradiated male x irradiated female cages a total of 24 eggs (0.8 eggs per cage) were collected, of which none hatched (0% fertility). From the 30 irradiated male x irradiated female cages just 4 eggs (0.13 eggs per cage) were collected, of which none hatched (0% fertility). These results confirm that irradiation doses of 60 Gy and above are highly effective in inducing reproductive sterility in both sexes, with the average sterility across all irradiation treatments above 99.5% ($F_{4,25} = 0.95$, $P = 0.45$) (Table 3.1).

Table 3.1: Percentage of eggs hatching from crosses between fertile females and males exposed to increasing irradiation doses.

Replicate	Dose (Gy)					
	0	60	65	70	75	80
1	77.33	0.00	0.00	0.00	0.00	0.00
2	64.84	0.00	0.00	0.00	0.00	0.00
3	74.50	0.04	0.09	0.03	0.00	2.72
4	72.54	0.00	0.03	0.00	0.00	0.00
5	71.34	0.00	0.00	0.00	0.00	0.00
6	70.93	0.05	0.03	0.00	0.00	0.00
Mean	71.91	0.02	0.03	0.01	0.00	0.45

Sexual competitiveness

There were no significant differences in *RII* between discrete irradiation doses ($F_{4,24} = 1.80$, $P = 0.16$) from regression models, however the polynomial analysis showed a significant positive linear trend as dose increased ($P = 0.027$) (Figure 3.4a). While the linear trend suggests that *RII* increases with increasing dose, indicating an increase in assortative mating, the lack of significant difference between discrete irradiation doses indicates that the increase in *RII* could also be the result of lower participation in mating by males sterilized at higher does rather than actual sexual isolation. There was also no significant difference in *I* between discrete irradiation doses ($F_{4,24} = 1.57$, $P = 0.22$), and while observed values for *I* did decline with increasing irradiation dose the linear relationship was not significant ($P = 0.063$) (Figure 3.4b). Neither RII nor *I* was influenced by dye treatment (All *P*-values > 0.4).

Similar to the results of *RII*, there was no significant difference in *ISI* between discrete irradiation doses ($F_{4,24} = 2.16$, $P = 0.104$), however the polynomial analysis showed a significant positive linear trend as dose increased ($P = 0.024$) (Figure 3.5a). An increase in *ISI* with increasing irradiation dose again suggests an increasing sexual isolation between sterile and fertile flies. As with the *RII* analysis though the increasing sexual isolation may be the result of lower participation in mating by sterile males irradiated at higher doses.

For the separate indexes of males (*MRPI*) and females (*FRPI*), there was a significant difference between discrete irradiation doses for *MRPI* ($F_{4,24} = 3.47$, $P = 0.022$) and *FRPI* ($F_{4,24} = 2.81$, $P = 0.048$). The polynomial analysis revealed a significant negative linear trend for *MRPI* ($P = 0.002$) indicating poorer sterile male mating propensity as irradiation dose increases (Figure 3.5b). However for *FRPI* the polynomial analysis was only significant at the fourth order (quartic) level ($P = 0.024$). The linear, quadratic and cubic were all non significant (all *P*-values > 0.1), indicating that female mating propensity was highly variable in response to irradiation treatment and no positive or negative trends can be seen with increasing irradiation dose. *ISI*, *MRPI* and *FRPI* were not influenced by dye treatment (All *P*-values > 0.1).

There was significant difference in *RSI* between discrete irradiation doses ($F_{4,24} = 6.85$, $P = 0.001$), the polynomial analysis revealed a significant negative linear trend, with the proportion of sterile males achieving copulations with fertile females declining significantly with increasing irradiation dose ($P < 0.001$) (Figure 3.6). *RSI* was not influenced by dye treatment ($P > 0.3$).

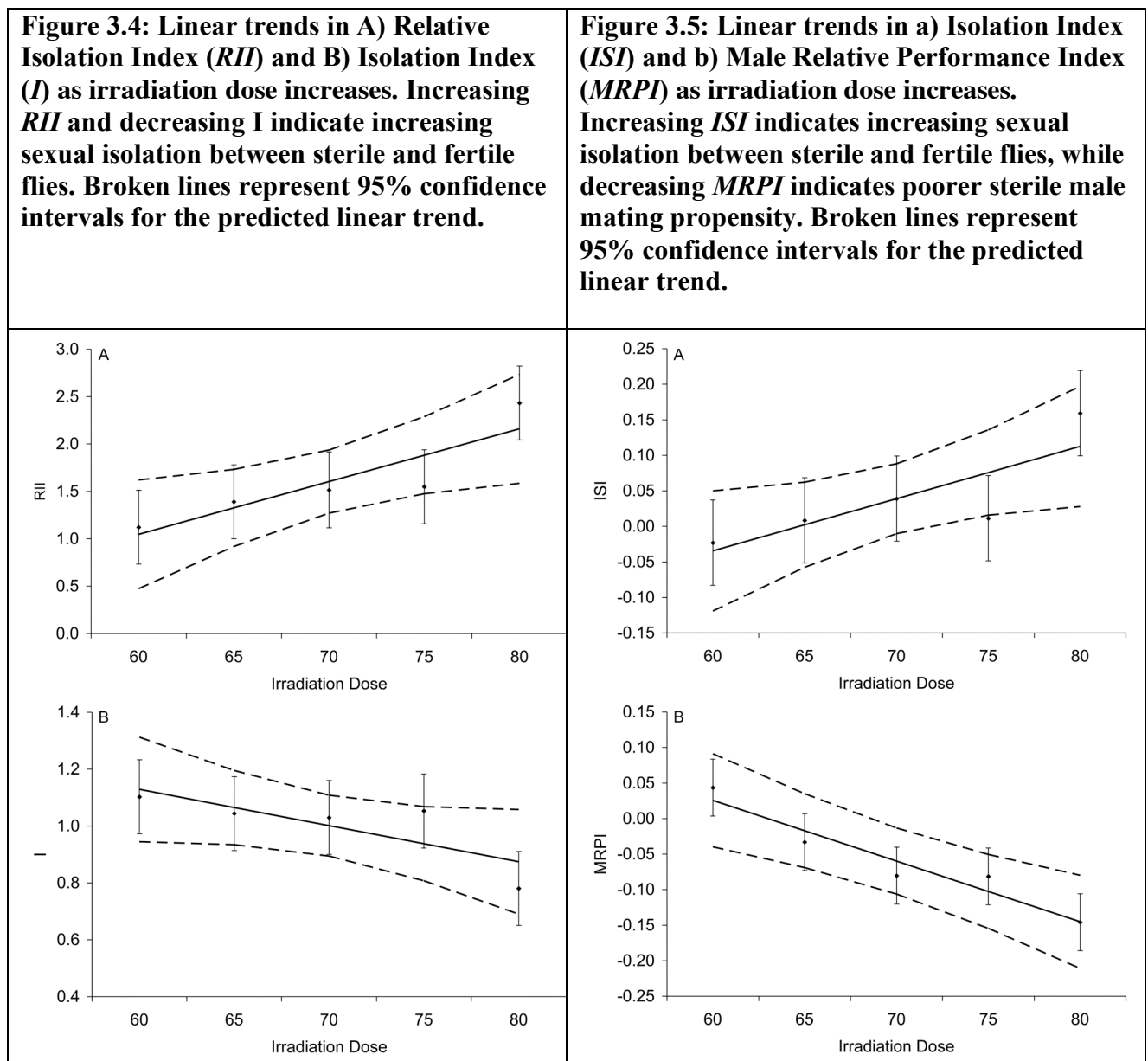| **Figure 3.4: Linear trends in A) Relative Isolation Index (*RII*) and B) Isolation Index (*I*) as irradiation dose increases. Increasing *RII* and decreasing I indicate increasing sexual isolation between sterile and fertile flies. Broken lines represent 95% confidence intervals for the predicted linear trend.**	**Figure 3.5: Linear trends in a) Isolation Index (*ISI*) and b) Male Relative Performance Index (*MRPI*) as irradiation dose increases. Increasing *ISI* indicates increasing sexual isolation between sterile and fertile flies, while decreasing *MRPI* indicates poorer sterile male mating propensity. Broken lines represent 95% confidence intervals for the predicted linear trend.**

Figure 3.6: Linear trends in Relative Sterility Index (*RSI*), the proportion of sterile males to successfully mate as irradiation dose increases. Broken lines represent 95% confidence intervals for the predicted linear trend.
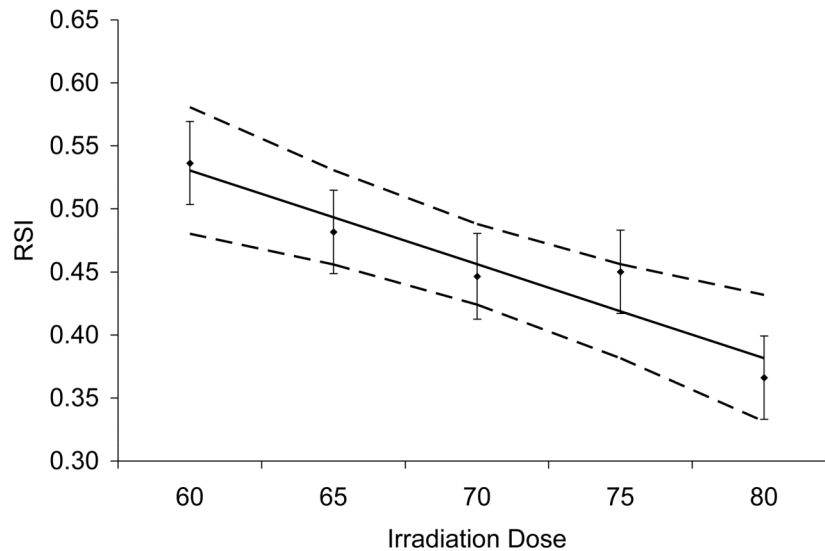
Discussion

Percentage emergence, flight ability and sex ratio

In the present study, like Collins *et al.* (2008), over the range of doses tested we found no evidence that Queensland fruit fly emergence, flight ability or rate of fliers were influenced by irradiation treatment. Our findings contrast those of Dominiak *et al.* (2002) and Dominiak *et al.* (2007a), who found significant reductions in emergence and flight ability (assessed as rate of fliers) in irradiated Queensland fruit flies when compared to unirradiated flies from the same production batch. Differences among these studies highlight the importance of separating effects of handling, dying and transport from effects of irradiation.

In both this study and that of Collins *et al.* (2008), all flies were packed transported and held together throughout the experiment. The only period when treatments and controls were separated was during irradiation (roughly 14 mins). 'Irradiated' flies of Dominiak *et al.* (2002) and Dominiak *et al.* (2007a) had been packed, dyed, shipped to ANSTO, held in hypoxia, irradiated and then shipped back to EMAI for testing, whereas the 'unirradiated' flies they were compared to had been held in the factory at a constant 25°C. Hence, the irradiated and unirradiated flies of Dominiak *et al.* (2002) and Dominiak *et al.* (2007a) differed not just in exposure to irradiation, but also in many aspects of handling. Reduced emergence after transport to release zones has been previously reported by Dominiak *et al.* (2007b), where as Weldon (2005) found that dye treatments reduced the ability of flies to escape from emergence trays. Result of he present study further support the suggestion by Collins *et al.* (2008) that differences reported by Dominiak *et al.* (2002) and Dominiak *et al.* (2007a) are likely to have resulted from the presence of dye and handling rather than from irradiation treatment.

Longevity and mortality under stress

Results for mortality under stress tests (Figure 3.2) are fairly straightforward with a clear positive relationship between dose and mortality. The current target dose of 70 - 75 Gy was associated with a substantial increase in fly mortality in the first 48 hours. Interestingly, there was no evidence that irradiation treatment influenced survivorship in the tests for individual longevity under nutritional stress. While these tests are similar in that they hold flies without nutrition and assess survivorship, there are also distinct differences that are key to understanding the differences in the results and also to understanding the implications for SIT.

While flies in individual longevity under nutritional stress are maintained without any exposure to conspecifics as adults, flies in the standard IAEA mortality under stress tests are held together in petri dishes (50 males and 50 females in each dish) and are hence under intense crowding stress in addition to nutritional stress. Results of the individual longevity under nutritional stress tests suggest that irradiation does not diminish ability to tolerate nutritional deprivation, the IAEA mortality under stress tests do suggest that irradiation at higher doses does diminish an adult fly's ability to tolerate social stress. This has obvious implications for SIT, as large numbers of adults are usually held for 1 - 3 days in plastic bins before release (Dominiak *et al.* 1998; Meats *et al.* 2003). These findings also lead naturally to the related questions of whether irradiation is diminishing ability to tolerate social stress specifically, which would mainly be relevant during pre-release holding, or whether these results point to diminished ability to tolerate stress more generally, such as challenging environmental conditions that might be encountered after release.

Meats (1984) reported that environmental stress (mainly temperature) during larval and pupal development can greatly diminish the survival of adult Queensland fruit flies under both favourable and unfavourable conditions. At a dose of 70 - 75 Gy, the pre-release stress of sterile flies held in release bins and cages in large numbers, may greatly exacerbate the impact of environmental stress after release. This has the potential to reduce the effectiveness of SIT and substantially lower recapture rates of sterile flies in monitoring traps. Indeed, this may contribute towards explaining the low recapture rates of sterile Queensland fruit flies that have been reported in some experimental releases (Dominiak *et al.* 1998, 2003; Meats *et al.* 2003). Meats *et al.* (2003) found that sterile Queensland fruit flies held until sexual maturity (one week after emergence) before release suffered reduced success in suppressing wild population, due mainly to low survival. This low survival may reflect sustained pre-release stress. With their superior stress tolerance, flies sterilized at lower irradiation doses might be better equipped to deal with challenging conditions experienced as adults before and after release.

Although the individual longevity under nutritional stress test did not detect any effects of irradiation, it did detect some interesting effects of pupal weight. The weight of pupae is likely a good representation of the stored water and nutrients, and is a reasonable predictor of adult size. Interestingly, pupal size was a better predictor of adult performance than was adult size, suggesting that there are underlying factors, such as physiology, that extend beyond the pupal stage and into adulthood. Dominiak *et al.* (2002) assessed the quality of factory flies in relation to the average weight of the production batch. While the average batch weight was positively associated with emergence and flight ability, it was negatively related to life span (measured as number of days until 50% mortality with full nutrition available). That is, while our results suggest that larger pupae produce flies that are better able to survive stressful episodes, they have shorter life spans when conditions are favourable.

Reproductive sterility

Sterility induction in the present study is similar to the results reported by Sproul *et al.* (1992), with adequate levels of sterility achieved in irradiated males (> 99.5%) and irradiated females (< 1% fecundity and 0% fertility) at doses of 60 Gy and above. In *C. capitata*, standard IAEA protocols require 99.5% sterility (or < 0.5% hatch) from sterile male x fertile female pairings when released into quarantine areas that are historically pest free (FAO/IAEA/USDA 2003). We found that all tested doses of 60 Gy and above were more than adequate to produce comparable results in Queensland fruit flies with hatches reliably below 0.1 %. While sterility induction is unchanged, mating compatibility and competitiveness declined with increasing irradiation dose, indicating that it may be possible to produce superior flies of uncompromised sterility by adopting lower irradiation doses.

Sexual competitiveness

The competitiveness tests are designed to assess mating compatibility of separate strains of sterile and wild flies in a field cage with a large number of individuals. Given that we are concerned here solely with the effects of irradiation treatment, for mating competitiveness tests we used irradiated flies and unirradiated flies from the same factory batch under laboratory conditions. The tests are applied here to maintain the standardized quality control testing as it is throughout each test of fly quality, however it is possible that differences observed in compatibility are due to variation in the levels of participation in mating, particularly those flies irradiated at higher doses. As the difference in participation in mating between irradiated flies and unirradiated controls increases, the value of the competitive indices will move towards a greater level of sexual isolation. Therefore rather than representing an increase in sexual isolation between strains it is in fact recording lower participation in mating by sterile flies.

Each of the compatibility indices (*RII, I, ISI*) show a trend for reduced competitiveness as irradiation dose increases however the effect is only minimal and driven principally by the drop off in sterile male mating propensity at 80 Gy. This is illustrated further by the significant difference between the discrete doses for *RSI* and the negative linear trend observed as irradiation dose increases. At irradiation doses of 70 Gy and above the number of sterile males obtaining successful copulations greatly diminishes dropping off at 80 Gy.

A stronger result could be expected if sterile flies were to be assessed against wild flies in more natural field cage conditions (Parker & Mehta 2007). As the larger cage size would involve a greater amount of movement and activity to achieve successful matings. However it is likely that differences observed here are due to reduced participation in mating by sterile males as opposed to sexual isolation between strains due solely to irradiation. This result however is still informative for the purposes of testing the effects of irradiation as one would expect reduced participation in mating by sterile males would result in overblown estimations of mating compatibility between sterile and wild strains under field cage conditions.

Negative effects of irradiation on fly quality have been documented for numerous tephritid species (Barry *et al.* 2003; Hooper 1975; Lux *et al.* 2002; Rull *et al.* 2007; Toledo *et al.* 2004; Walder & Calkins 1993). Further, the overdosing of flies to eliminate a minimal amount of fertility (< 1%) often results in poorer sterility induction in wild populations than is achieved by flies irradiated at a reduced dose to increase quality and competitiveness (Bakri *et al.* 2005; Calkins & Parker 2005; Hooper 1972; Toledo *et al.* 2004). Given there was a reduced participation in mating in this study by males sterilised at high doses it is likely that Queensland fruit fly would display similar trends in sterility induction within a wild cohort. The success of SIT however is not reliant on mating competitiveness alone and released flies first need to survive long enough to reach sexual maturity.

This makes the increased mortality under stress with increasing irradiation dose of greater concern, as flies irradiated at higher doses appear to have a reduced capacity to cope with environmental stress, and such factors need to be taken into consideration during releases and monitoring of an SIT program in addition to competitive pressures.

Combined with an improved mortality under stress result, Queensland fruit flies irradiated at 60 Gy display an acceptable level of sterility (<1%) as defined by the FAO/USDA/IAEA (2003) product quality control manual. Working on a fine scale around the current irradiation target dose for Queensland frit fly SIT (70 - 75 Gy), has revealed it may be possible to achieve comparable sterility at irradiation doses lower than 60 Gy. Before making recommendations to lower the target dose it would be necessary to test how much of a "buffer" there was between a reduced target dose of 60 Gy and lower doses that may produce inadequate sterility. However given the reduction in quality associated with higher irradiation doses, it seems that superior fly quality and more effective SIT might be possible through only a small reduction in target dose.

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4. EFFECTS OF IRRADIATION ON ADULT LONGEVITY

Summary

Irradiation procedures used to induce reproductive sterility in tephritid flies for use in Sterile Insect Technique (SIT) programs also induce collateral somatic damage that can impinge on performance and survival. Previous studies of Queensland fruit fly have used the standard 'mortality under stress' assay to demonstrate increased survivorship of these flies at lower irradiation doses when under conditions of crowding and nutritional stress. Here, using the 60 - 80 Gy range of previous studies, we investigate whether similar effects are found when the flies are not subject to such stress. Whereas previous studies found a gradual increase in mortality under stress as dose increased, in the absence of such stress the response was much sharper; all irradiation doses were associated with a marked reduction in longevity and this effect was highly similar over the full range of doses tested. While previous studies suggest that reductions in irradiation dose may enhance the ability of Queensland fruit flies to survive acute periods of stress from crowding and/or nutritional deprivation, results of the present study suggest that reductions in irradiation dose to levels closer to the minimal sterilizing dose are unlikely to bring longevity in the absence of stress much closer to that of unirradiated controls.

Introduction

The sterile insect technique (SIT), used to combat fruit fly pests in many regions of the world, depends on the ability of released sterile males to induce reproductive failure in wild females, reducing population numbers in subsequent generations (Knipling, 1955). Success of sterile males depends on their ability to survive till sexual maturity, to find, court, mate and inseminate wild females, and to induce long refractory periods that reduce the chance of females remating with fertile wild males (Aluja 1994; McInnis *et al*. 1996; Hendrichs *et al*. 2002).

Gamma irradiation is currently the most common method used to sterilize mass reared flies for use in SIT (Bakri *et al*. 2005). Because of its quite general mode of action, in addition to the intended disruption of reproductive tissues, sterilization treatment is also expected to induce collateral damage in somatic tissues. This somatic damage can diminish the ability of sterile flies to survive and mate effectively, and can thereby reduce SIT efficacy. Deleterious effects of gamma irradiation on fly performance have been reported for a numerous tephritid species, including *Ceratitis capitata* (Barry *et al*. 2003; Lux *et al*. 2002), *Anastrepha obliqua* (Toledo *et al*. 2004), *Anastrepha ludens* (Rull *et al*. 2007), *Anastrepha suspensa* (Walder & Calkins 1993), *Bactrocera cucumis* (Hooper 1975) and *Bactrocera tryoni* (Bhatti & Ship 1972; Sproul *et al*. 1992; Collins *et al*. 2008, 2009). Effective SIT requires optimisation of the balance between sterility induction and collateral damage of irradiation to fly quality, as well as ongoing monitoring of performance (Chambers 1977).

The FAO/IAEA/USDA (2003) manual on "Product Quality Control and Shipping Procedures for Sterile Mass Reared Tephritid Fruit Flies", outlines a diverse range of routine quality control tests employed to ensure the quality and competitiveness of mass reared flies for SIT. These tests can also be used to guide the development of optimal irradiation treatments. One such test is the routine assessment of longevity under stress. The longevity under stress test is thought to assess stored nutrient reserves available to an adult fly when it emerges (FAO/IAEA/USDA 2003). The test involves one hundred flies, fifty males and fifty females, placed together in a petri dish without access to food or water. Mortality is recorded daily until the end of the test, the duration of which varies from species to species. Collins *et al*. (2008; 2009) applied this test to Queensland fruit flies (*Bactrocera tryoni*) at various irradiation dose rates and doses, showing that with increasing irradiation dose over the range 60 - 80 Gy there was a substantial increase in mortality within the

first 48 hours. However no effect of irradiation dose was found on longevity in another assay in which individual flies were held without food or water in an individual micro plate well (Collins *et al.* 2009). These results suggest that irradiation does not diminish an adult fly's ability to tolerate nutritional deprivation, but rather the ability to tolerate crowding stress (or stress more generally). Each of the assays used so far to assess effects of irradiation dose on Queensland fruit fly longevity has been in the context of stress, either from nutritional deprivation or from combined nutritional deprivation and crowding. There has not been any investigation into the effects of irradiation dose on longevity in the absence of such stress, and this is the subject of the present study.

The effects of irradiation on longevity are highly variable among tephritid species. In *Ceratitis capitata*, the effect of irradiation on adult survivorship varies with diet regime (Barry *et al.* 2003). When maintained on a diet of sucrose and water the longevity of irradiated flies was always diminished in comparison with unirradiated flies, and when flies were nutritionally deprived survivorship for both irradiated and unirradiated flies reduced quickly to less than 5% after only one-week post emergence. In *Anastrepha suspensa* maintained on a diet of sucrose and autolyzed yeast, females suffer decreased longevity at higher irradiation doses (Walder & Calkins 1993). For *Anastrepha obliqua* maintained on a diet of sucrose and autolyzed yeast, there was a significant decrease in longevity for both sexes as irradiation dose increased (Toledo *et al.* 2004). In contrast, for *Anastrepha ludens* maintained on a diet of sucrose and autolyzed yeast no difference was found in mortality of laboratory flies that were unirradiated, irradiated at 30 or 80 Gy, and unirradiated wild flies after 30 days of observation (Rull *et al.* 2005).

Although the effect of irradiation dose on adult longevity in Queensland fruit fly is largely un-investigated, the effects of current SIT irradiation dose have been studied. Perez-Staples *et al.* (2007) found when comparing unirradiated flies to those irradiated at the standard target dose of 70 - 75 Gy, there was significant interaction between diet and irradiation on longevity. While irradiated and un-irradiated flies had similar longevity when given access to a full diet of autolyzed yeast and sucrose, irradiated flies suffered a much greater reduction in longevity when provided sucrose alone. The present study builds on earlier work investigating the relationship between irradiation dose and quality of Queensland fruit flies. Collins *et al.* (2009) showed that improved performance in mortality under stress tests could be achieved by a small reduction in irradiation dose from the standard 70 - 75 Gy. Here we test whether fly longevity in the absence of nutritional or crowding stress can be similarly improved by small reductions in irradiation dose.

Materials and Methods

Queensland fruit fly pupae were obtained from the Industry & Investment NSW Fruit Fly Production Facility at Elizabeth Macarthur Agricultural Institute (EMAI). Pupae from this facility are routinely sent to Australian Nuclear Science and Technology Organisation (ANSTO) for irradiation (70 - 75 Gy) and are then distributed to release zones for the SIT control program that is used to suppress outbreaks of Queensland fruit flies. The following procedures were carried out for three batches of pupae between July and November 2009.

For each batch, six individual 'zip-lock' plastic bags (100 x 150 mm) containing ca. 8,000 pupae were sealed at EMAI, packed in a closed styrofoam box, and transported directly to ANSTO in an air conditioned vehicle. The bags of pupae were kept together at all times during transport and holding to ensure that they received similar handling conditions. To achieve a hypoxic atmosphere prior to irradiation, the sealed bags were held overnight at the ANSTO in a temperature controlled room at ca. 18 °C, as is standard practice in the current SIT program. The following day, five bags were irradiated separately at different target doses (60, 65, 70, 75, 80 Gy) in ANSTO's Gamma Technology Research Irradiator (GATRI) and the sixth bag was kept aside as an unirradiated control. The plastic bags were fixed with tape to the face of separate cardboard boxes, which were

then mounted in the GATRI facility for irradiation. All bags were placed 1310 mm from the Co_{60} source to achieve a dose rate of 5 Gy/min (Collins *et al*. 2008). Calibrated ferrous ammonium sulphate (Fricke) dosimeters were placed at the centre of each packet of pupae and were used to confirm the dose received. Packs of pupae were flattened as much as possible, without damaging pupae, to achieve an even density with each packet being no more than 1cm thick.

After irradiation, pupae were immediately transported in a closed styrofoam box in an air-conditioned vehicle to a controlled environment laboratory at Macquarie University, Sydney. The laboratory was maintained at $25\pm1°C$, $70\pm5\%$ RH on a 14:10 day night cycle including one hour dawn and dusk periods where the lights turned on and off in stages. The pupae were set up to emerge in 5-L plastic cages that had a mesh-covered opening for ventilation that comprised ca. 30% of the total surface area. These conditions are directly comparable to Collins *et al*. (2009).

Within 2 hours of emergence 25 individual males and 25 individual females for each irradiation dose were transferred, using an aspirator, into individual 70 mL clear plastic cage. For food, each fly was provided with a small dish of sugar and a 1 cm x 1 cm piece of absorbent paper soaked in 1:7 mixture of yeast autolysate enzymatic solution (MP Biomedicals, Aurora, OH, USA) and water. Water was provided in a second 70 mL container from which a cotton dental wick protruded through the base of the maintenance cage. Flies were checked daily, each morning at the end of simulated dawn. Dead flies were removed and longevity was recorded in days since emergence. Longevity data were log transformed to conform to the assumptions of parametric analysis, and all data were analysed using least squares regression in JMP v 5.0 (SAS Corporation, Cary, NC, USA). To account for variation between batches, batch identity was included as a random effect.

Results

Figure 4.1: Log Longevity in days since emergence for each irradiation dose and unirradiated controls. All irradiation doses differed from the control but not from each other.
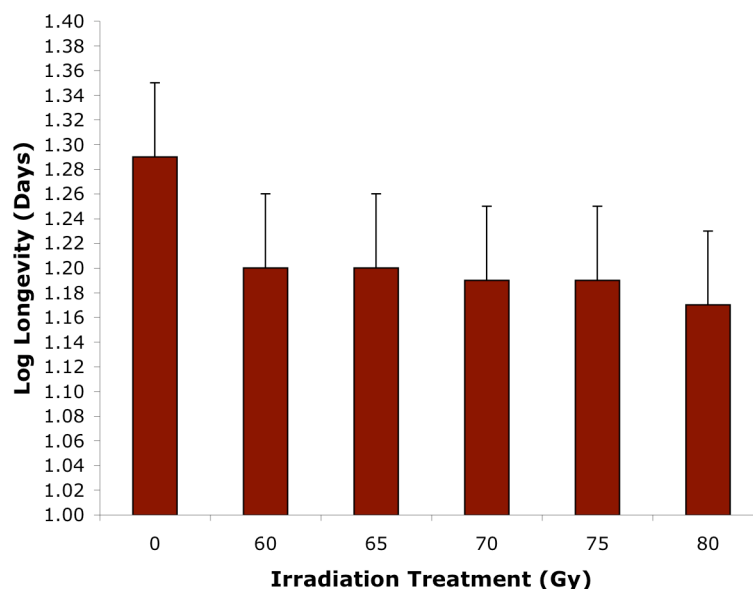
There was a significant overall effect of irradiation treatment on the longevity of flies ($F_{5,799}$ = 3.070, $P = 0.009$). Post-hoc tests revealed that the longevity of unirradiated control flies was significantly greater than that of flies irradiated at 60, 65, 70, 75 or 80 Gy (Figure 4.1).

There was no evidence of significant variation in longevity across the dosage range 60 - 80 Gy. Male flies tended to live longer than female flies ($F_{5,799}$ = 3.893, $P = 0.049$), and the absence of significant interaction between sex and treatment indicates that this tendency did not vary across treatments ($F_{5,799}$ = 1.177, $P = 0.319$).

Discussion

Collins *et al*. (2009) found increased mortality in the first 48 hours under nutritional and crowding stress when Queensland fruit flies were exposed to higher irradiation doses. Doses of 60 Gy yielded results very similar to the unirradiated controls and each 5 Gy increment in dose up to 80 Gy was reflected by increased mortality. These results suggested that by decreasing irradiation dose slightly from the current 70 - 75 Gy standard it may be possible to increase survivorship under crowded pre-release holding conditions and after release into an often-hostile, stressful, environment. Contrasting this, results of the present study suggest a reduced irradiation dose is unlikely to yield much, if any, improvement in Queensland fruit fly longevity in the absence of stress.

All doses tested between 60 and 80 Gy showed a significant reduction in longevity relative to the unirradiated controls, and there was no evidence of relationship between dose and longevity across the irradiation range tested. Presumably there is a dose below the range tested where longevity approaches that of unirradiated flies, but our results indicate that this dose is almost certainly below the range within which acceptable levels of reproductive sterility are induced under current protocols. This raises the question of whether changes to protocols, such as irradiating in a Nitrogen atmosphere or other means of protecting cells from oxidative damage during irradiation, have potential to increase longevity at sterilizing doses.

It is essential for SIT that released flies survive long enough in the field to reach sexual maturity. Low adult recapture rates have been reported in some experimental releases of Queensland fruit fly (Dominiak *et al*. 1998, 2003; Meats *et al*. 2003), and this may reflect both pre-release stress, especially when large numbers of adult flies are held in plastic containers for 1-3 days before release (Dominiak *et al*. 1998; Meats *et al*. 2003), and post-release stress from an inhospitable environment (e.g., heat, desiccation, rain, wind, nutrition). While results of the present study indicate that under ideal conditions flies are unlikely to live longer if sterilized at lower irradiation doses, results of Collins *et al*. (2009) indicate that improved survival might be expected at lower doses if the flies encounter stressful conditions.

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5. LOW IRRADIATION DOSE RESPONSE AND FERTILITY RESURGENCE

Summary

This study is an important step towards calibrating, validating and improving irradiation methods used for *Bactrocera tryoni* (Froggatt) sterile insect technique (SIT). Previous studies have suggested that higher quality flies may be produced without compromising sterility by reducing irradiation dose from 70 - 75 Gy to 60 - 65 Gy. This raises questions of margin remaining below this dose before residual fertility is unacceptable for use in SIT. The following study identifies irradiation dose margins of sterility induction and the potential for residual fertility at lower doses, at 10 - 15 and 30 - 35 days post emergence. There was a significant increase in sterility induction with increasing irradiation dose. Males required higher doses than females to induce complete sterility, however all flies were sterile at doses of 60 Gy and above. There was no evidence of resurgence of fertility at 30 - 35 days post emergence. The current Queensland fruit fly SIT target-sterilizing dose of 70 - 75 Gy could be lowered by up to 10 Gy and still allow for a further 5 - 10 Gy fertility margin for males and 20 Gy margin for females.

Introduction

The Sterile Insect Technique (SIT) has been used widely for the management of major tephritid fruit fly pests around the world (Klassen & Curtis 2005). Millions of flies are reared, sterilized, and then released in the field. Sterile males mate with wild females, inducing reproductive failure and hence reducing wild populations in the next generation. Gamma irradiation is currently the principle method used to induce sterility in mass reared tephritid fruit flies for release in SIT programmes (Bakri *et al*. 2005a,b). The target irradiation dose used to induce sterility has an important bearing on the success of SIT. If irradiation dose is too low, released insects will be insufficiently sterile, which could be disastrous for control programs, especially where SIT is used to eliminate outbreaks in normally pest free areas. With this in mind, it could be seen as sound practice to aim for the highest possible sterility induction through high irradiation doses.

Increasing irradiation dose, however, can cause a significant decline in adult fly performance and competitiveness (Barry *et al*. 2003; Calkins & Parker 2005; Collins *et al*. 2009; Hooper 1975; Lux *et al*. 2002; Toledo *et al*. 2004; Rull *et al*. 2007; Walder & Calkins 1993). Increasing irradiation dose to remove a minimal (< 1%) fertility often produces flies that suffer from significantly reduced competitiveness, resulting in diminished ability to induce reproductive failure in wild populations (Bakri *et al*. 2005a; Calkins & Parker 2005). Sterile males that are incapable of dispersing, surviving, and obtaining successful copulations with wild females are of no use to SIT programmes. To ensure that released flies are effective at inducing reproductive failure in their mates, irradiation procedures must achieve an adequate level of sterility induction, while minimizing the detrimental effects of radiation on adult fly performance.

In Australia, SIT has been used in area wide pest management of Queensland fruit fly, *Bactrocera tryoni* (Froggatt). The current irradiation dose range applied to Queensland fruit fly for use in SIT is 70 - 75 Gy (Jessup & Cruickshank 1999). Doses of 75 Gy and above provide adequate sterility but also cause a significant reduction in performance both standard mortality under stress and mating propensity tests whereas flies irradiated at 60 Gy maintain undiminished sterility and have performance results similar to those of unirradiated flies (Collins *et al*. 2009).

Collins *et al*. (2009) suggested that the current target-sterilizing dose could be lowered to achieve undiminished sterility and improved quality. This suggestion raises questions of what safety margin

remains below 60 Gy before residual fertility increases to unacceptable levels. Furthermore, as we move closer to the margins of sterility inducing doses, increased consideration must be given the possibility that some fertility may be recovered later in life. Assays of sterility induction are usually only carried out soon after flies reach sexual maturity and the possibility of age-dependent sterility induction has received very little consideration in Queensland fruit flies or in other tephritids. Before considering lowering the target-sterilizing dose, it is necessary to ascertain both the doses at which sterility induction becomes inadequate and the potential for increased fertility later in life. The present study addresses these issues to support decisions on optimal target sterilizing dose for Queensland fruit fly SIT.

Materials and Methods

Queensland fruit fly pupae were obtained from the Industry & Investment NSW Fruit Fly Production Facility at Elizabeth Macarthur Agricultural Institute (EMAI). Pupae from this facility are routinely sent to Australian Nuclear Science and Technology Organisation (ANSTO) for irradiation at a target dose of 70 - 75 Gy. The irradiated pupae are then distributed to release zones for the SIT control program that is used to suppress and contain Queensland fruit fly outbreaks.

Approximately 8,000 pupae were placed in each of seven individual 'zip-lock' plastic bags (100 x 150mm). Bags were sealed at EMAI, packed into a styrofoam box, and transported directly to ANSTO in an air-conditioned vehicle. The sealed bags were held overnight at ANSTO in a temperature-controlled room (18 °C) to allow the bags of pupae time to achieve a hypoxic atmosphere. The following day, six bags were irradiated separately at different target doses (20, 30, 40, 50, 60, 70 Gy) in ANSTO's Gamma Technology Research Irradiator (GATRI) and the seventh bag was kept aside as an unirradiated control. The plastic bags were fixed with tape to the face of separate cardboard boxes, which were then mounted in the GATRI facility for irradiation. All bags were placed 1310 mm from the Co_{60} source to achieve a dose rate of 5 Gy/min (Collins et al. 2008). Calibrated ferrous ammonium sulphate (Fricke) dosimeters were placed at the centre of each packet of pupae and were used to confirm the dose received. Packs of pupae were flattened as much as possible, without damaging pupae, to achieve an even density with each packet being no more than 1cm thick.

After irradiation, pupae were immediately transported in a closed styrofoam box in an air-conditioned vehicle to a controlled environment lab at Macquarie University, Sydney. The laboratory was maintained at 25±1°C, 70±5% RH on a 14:10 day night cycle including one hour dawn and dusk periods where the lights turned on and off in stages. The pupae were set up to emerge in 5 L plastic cages that had a mesh-covered opening for ventilation that comprised ca. 30% of the total surface area.

To assess reproductive sterility, 50 males and 50 females for each possible mating combination (i.e., sterile male x sterile female; fertile male x sterile female; sterile male x fertile female) were set up in 12 L plastic cages for each irradiation dose. A non-irradiated control (fertile male x fertile female) was also set up. All cages were provided with water soaked cotton wool and separate dishes of dry granular sucrose and dry yeast hydrolysate enzymatic (MP Biomedicals, Aurora, OH, USA) as food. To ensure that no mating occurred before the experiment began, all flies were sorted into their experimental groups within three days after emerging. Queensland fruit flies from the source population start mating between 6 and 8 days after emerging in the laboratory (Prabhu et al. 2008). No calling, courting or mating was observed in cages prior to sorting. Ten days after adult emergence, when at maximum sexual activity (Perez-Staples et al. 2007a), each cage was provided an oviposition substrate comprising a parafilm-covered 90 mm Petri dish containing water and food grade lemon essence (Queen Fine Foods Pty Ltd, Alderly, QLD, Australia) in a 140:1 ratio. The parafilm was punctured 10-15 times with an entomological pin to release the odour of lemon

essence. Each oviposition substrate was changed daily for five days. Collected oviposition substrates were left for a further four days in the environmentally controlled lab to allow fertile eggs to hatch. Un-hatched eggs and larvae were counted *in situ* under a stereomicroscope to assess effects of irradiation dose on fecundity and fertility at 10 - 15 days of age.

To test for resurgence of fertility, each cage of flies was maintained until thirty days post emergence. Each cage was then again provided an oviposition substrate, which was changed daily for five days, and left for a further four days in the environmentally controlled lab to allow eggs to hatch. Un-hatched eggs and larvae were counted *in situ* under stereomicroscope to assess effects of irradiation dose on fecundity and fertility at 30 - 35 days of age.

Fecundity and fertility data were pooled across the five days of collection for analysis at 10 - 15 and 30 - 35 days of age. The probability of any eggs being present in oviposition substrates was analysed using logistic regression models incorporating irradiation dose, mating group (i.e., Sterile Male x Sterile Female etc.), and age (10 - 15 *vs*. 30 - 35 days) as factors. Where eggs were present, fecundity was analysed using least squares regression. Fertility was recorded as the proportion hatched eggs (larvae) found in dishes. The probability of any eggs hatching was analysed using logistic regression models. For dishes where larvae were present, fertility was analysed using least squares regression. All data were analysed using JMP v5.0 (SAS Corporation, Cary, NC, USA). Initially, all first order interactions were included in models, but non-significant interactions were omitted from final models.

Results

At 10 - 15 days of age an average of 2,109 eggs were collected from fertile male x fertile female control cages and an average of 37% fertility was recorded. At 30 - 35 days of age an average of 2,110 eggs were collected from fertile male x fertile female control cages and an average fertility of 64% was recorded. Perez-Staples *et al*. (2007b) also noted substantially low fertility during the first 5-day oviposition period.
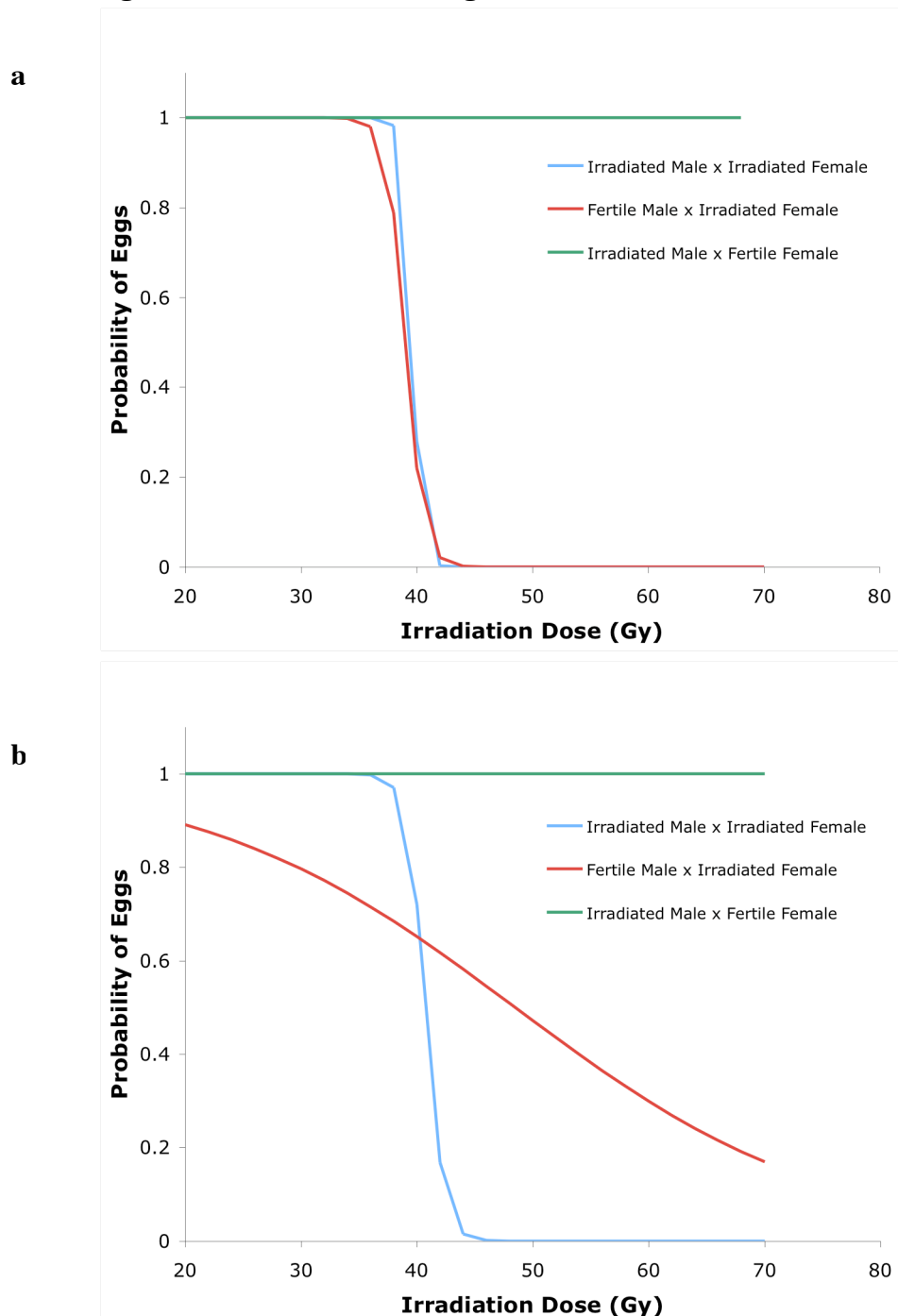
Probability of eggs

Eggs were invariably found in the oviposition substrates provided to cages containing fertile females, irrespective of whether the male was fertile or sterile. Hence the effects of dose, age and mating treatment on presence of eggs were only tested statistically for cages of irradiated females.

The probability of any eggs being present in the oviposition substrate of irradiated treatments was significantly influenced by irradiation dose ($G_1 = 44.53, P < 0.001$), although this effect varied through interactions with mating treatment (mating treatment $G_1 = 1.25, P = 0.264$, dose x mating treatment $G_1 = 4.20, P = 0.404$) and age (age $G_1 = 5.20, P = 0.023$, age x mating treatment $G_1 = 4.20, P = 0.404$) (Figure 5.1a). At 10 - 15 days of age there is a strong effect of irradiation dose, as all cages produced some eggs at 30 Gy but none produced any eggs at 50 Gy (Figure 5.1a). At 30 - 35 days of age a very similar pattern is observed for irradiated females caged with irradiated males, but for irradiated females paired with fertile males there was some evidence of increased oviposition at higher irradiation doses (Figure 5.1b).

Number of eggs

Where eggs were present, the number of eggs (fecundity) declined as irradiation dose increased ($F_{1,44} = 15.73, P < 0.001$). Fecundity also varied significantly among the mating treatments, although the direction of this effect was different at the two ages tested (mating treatment $F_{2,44} = 13.30, P < 0.001$, age $F_{1,44} = 0.20, P = 0.89$, mating treatment x age $F_{2,44} = 9.61, P < 0.001$); fertile females laid fewer eggs at 30 - 35 days then at 10 - 15 days of age, while sterile females laid more eggs at 30 - 35 days than at 10 - 15 days.

Figure 5.1: Predicted probability of finding any eggs in oviposition substrates at a) 10 - 15 days of age and b) 30 - 35 days of age with increasing irradiation dose, for each mating treatment. Lines generated from nominal logistic models.

Probability of fertility

The probability of any eggs hatching decreased significantly with irradiation dose ($G_1 = 77.50$, $P < 0.001$) and mate treatment ($G_2 = 10.70$, $P < 0.01$), but did not change between the two ages tested ($G_1 = 2.12$, $P = 0.15$) (Figure 5.2). Females were more sensitive to the sterilizing effects of irradiation than males, requiring lower doses to induce sterility. For irradiated females the probability of any eggs being fertile drops sharply at 40 Gy with no fertile eggs being found at 60 Gy. For fertile females mated to irradiated males the probability of any eggs hatching declines significantly at a dose of 50 Gy with no fertility at doses of 60 Gy and above.

Figure 5.2: Predicted probability of finding any hatched (Fertile) eggs in oviposition dishes with increasing irradiation dose, for each mating treatment. Lines generated from nominal logistic models.
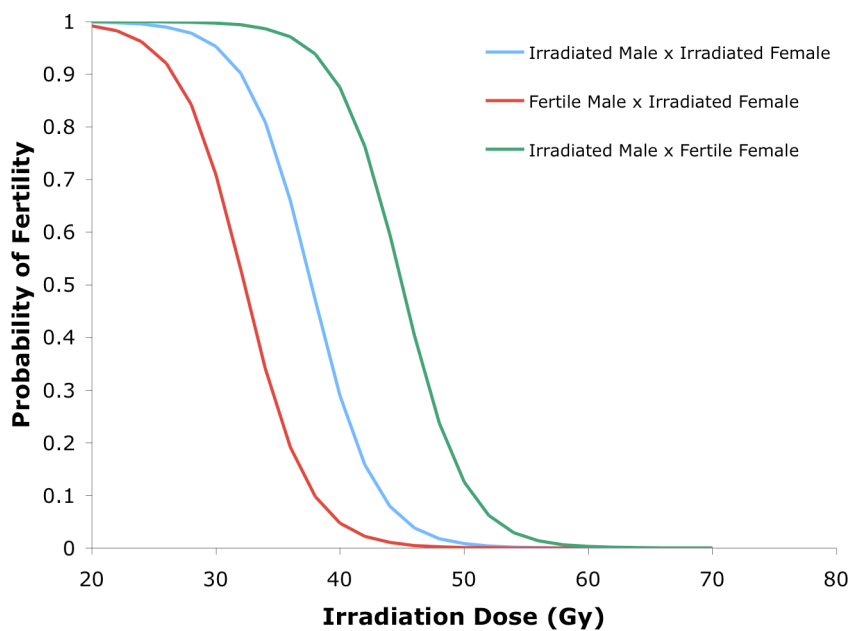
Extent of fertility

Where larvae were recorded, fertility (the proportion of eggs hatching) was significantly influenced by irradiation dose; fertility declined as irradiation dose increased ($F_{1,27} = 6.51, P = 0.01$), with complete sterility achieved at doses of 60 Gy and above (Figure 5.3). Fertility was not significantly different between different mating combinations ($F_{2,27} = 2.28, P = 0.13$) or between the different fly ages ($F_{1,27} = 0.68, P = 0.42$).

Figure 5.3: The proportion of eggs hatching (Fertility) with increasing irradiation dose, for each mating treatment.
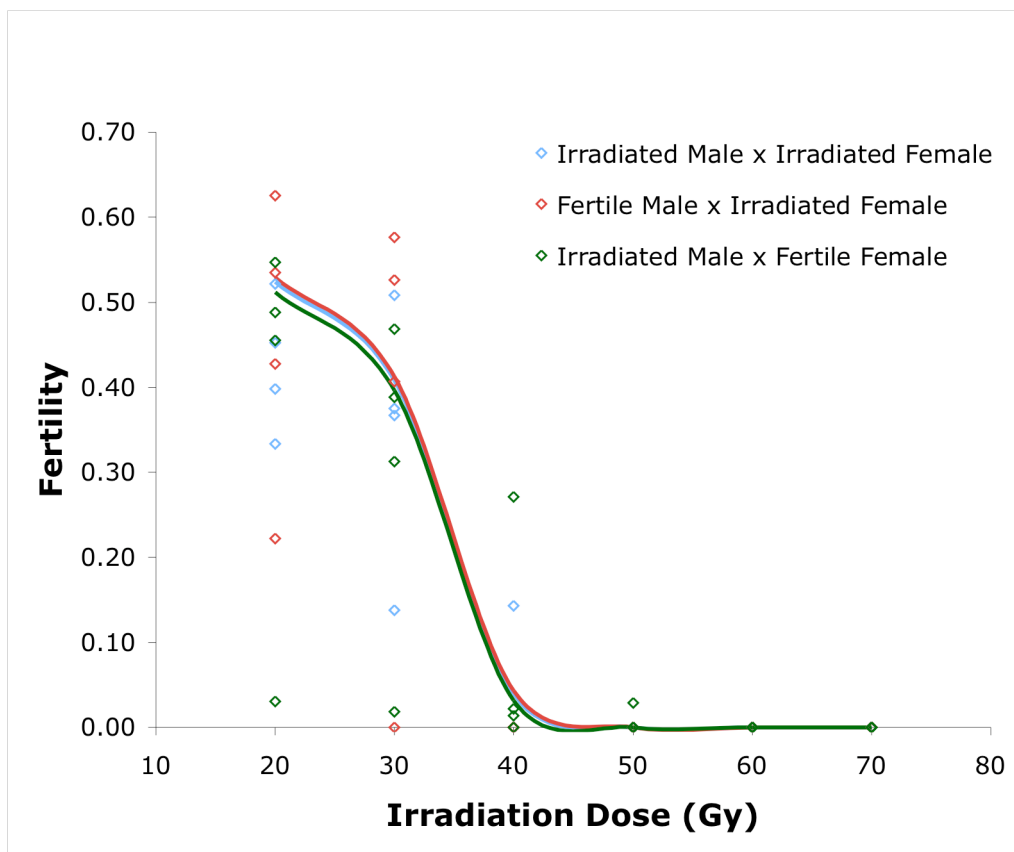
Discussion

Queensland fruit fly show a similar irradiation dose response to other economically important fruit fly species, requiring less than a 100 Gy to achieve high levels of sterility, and complete sterility obtained close to the 63 Gy dose average for tephritids (Bakri *et al*. 2005a). Females were more radiosensitive than males, requiring lower doses to achieve complete sterility induction. Sterility induction induced by gamma radiation results from fragmentation of chromosomes during mitotic cell division (Smith & von Borstel 1972). Female nurse cells undergo chromosome replication without cell division, or endomitosis, at which point they are highly sensitive to chromosome fragmentation caused by gamma radiation, requiring only small doses to cause damage to the cell nucleus (Proverbs 1969). In males, sterility induction occurs due to chromosomal damage during spermatogenesis. Typically the early stages of spermatogenesis are the most radiosensitive as this is when cells are most actively dividing (Proverbs 1969). Differential radiosensitivity between early and later stages of spermatogenesis is thought to underlie potential residual fertility (Trout 1964). It is also likely the reason why males often require higher irradiation doses than females to induce sterility (Bakri *et al*. 2005b).

We found no evidence of resurgence in fertility of irradiated flies after a period of thirty days. No fertility was recorded for male Queensland fruit fly irradiated at doses of 60 Gy and above and this persisted from 10 - 15 until 30 - 35 days of age. Egg production in females declines dramatically at doses above 40 Gy, however at 30 - 35 days of age even some females irradiated at high doses oviposited. Any eggs deposited by irradiated females though were found to be sterile at doses of 50 Gy and above. These results suggest that a small reduction in irradiation dose for the current Queensland fruit fly SIT programme is possible without compromising the level of sterility induction needed for use in containment and eradication.

The accepted level of sterility induction as outlined by the FAO/IAEA/USDA (2003) product quality control manual is $> 99.5\%$ from crosses between irradiated males and fertile females, with assessment of fertility generally carried out soon after sexual maturation. This margin of sterility is required for release of irradiated *C. capitata* males into historically pest free areas. A comparable level of sterility can be achieved in Queensland fruit fly at a dose of 60 Gy. At an irradiation dose of 50 Gy and above the sterility response curve plateaus resulting in larger increases in irradiation dose to achieve proportionately smaller increase in sterility induction. The difference in the probability of any fertility resulting from crosses between males irradiated at doses of 60 and 70 Gy, and fertile females is less than 0.002. That is a probable difference of 2 eggs out of every 10,000 oviposited being fertile.

Previous work has demonstrated how an irradiation dose of 60 or 65 Gy can produce Queensland fruit flies that have survivability under stress and mating propensity similar to that of un-irradiated flies, while higher doses compromise these quality parameters (Collins *et al*. 2009). With an improved ability to survive stresses and an increased participation in mating, Queensland fruit fly irradiated at a reduced dose are likely to be of greater benefit to SIT than those irradiated at the current higher sterilizing dose. At a target dose range of 60 to 65 Gy there is a 5 to 10 Gy margin between sterility induction and a probable fertility in irradiated males, and a 10 to 15 Gy margin between sterility induction and probable fertility in irradiated females.

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6. MULTIPLE MATING AND IRRADIATION-INDUCED SPERM DEPLETION: EFFECTS ON FEMALE REMATING BEHAVIOUR

Summary

Male insects that are unable to replenish sperm supplies between matings can suffer fitness costs either because their mates are more likely to accept subsequent suitors, or because their sperm are outnumbered when females do remate. We assessed the ability of fertile and sterile (irradiated) male Queensland fruit flies, *Bactrocera tryoni* ('Q-flies'), to have sperm stored by five sequential mates as well as the association between sperm depletion and female remating tendency. Sequential mates of fertile males stored similar numbers of sperm, indicating ample ability to replenish and maintain constant supplies between their once-daily mating opportunities. In contrast, sequential mates of sterile males stored progressively fewer sperm, with only trivial numbers of sperm stored by females mated by sterile males that had mated with two or more females previously. Despite the massive reduction in sperm storage by sequential mates of sterile males, fertile and sterile males were similar in their ability to induce sexual inhibition in their mates (to at least 30 days) and neither showed any decline in this ability across sequential matings. The ability of multiple-mated sterile males to induce sexual inhibition in their mates despite near or complete absence of sperm provides compelling evidence that sperm abundance plays no role in the induction of sexual inhibition in this species.

Introduction

Males and females differ fundamentally in constraints on reproductive fitness; males have been known to possess greater reproductive potential than females, but are limited by spermatogenesis and have been known to allocate sperm in order to maximise fitness (Wedell 2003). Females are more commonly constrained by offspring production (Dewsbury, 1982). While number of matings provides a useful first approximation for male reproductive success, post-copulatory processes can have a profound effect on how copulations are translated into fertilizations (Parker 1970; Thornhill & Alcock 1983; Eberhard 1996; Simmons 2001). For males that are able to acquire multiple mates, fertilization success may depend on ability to replenish ejaculate between matings (Dewsbury 1982). Sperm production is often far from trivial, and males of some insects have very limited ability to maintain gametic supplies if they mate in rapid succession, including butterflies *Papilio zelicaon*, *Pieris napae* and *Pieris rapae* (Sims 1979; Bissoondath & Wiklund 1996), parasitoid wasps, *Trichogramma evanescens*, *Spalangia cameroni*, *Dianarmus basalis*, and *Lariophagus distinguendus* (Damiens & Boivin 2005; King 2000; Bressac *et al.* 2007; Steiner *et al.* 2007), yellow fever mosquito, *Aedes aegypti* (Jones 1973) and the Australian sheep-blow fly, *Lucilia cuprina* (Smith *et al.* 1990). Sperm depletion can be permanent for species in which males do not produce sperm during their adult life or temporary for species in which sperm production occurs in adults (Boivin *et al.* 2005). As an apparent response to limited capacity or high costs of sperm replenishment, males of some insects have evolved mechanisms by which they can judiciously allocate a finite number of sperm among multiple mates in order to maximise their reproductive success (Wedell *et al.* 2002; Wedell & Cook 1999; Pérez-Staples & Aluja 2006; Martel *et al.* 2008).

Male post-copulatory success is not limited to the transfer of sperm. In many insects, male fertilization success can be greatly diminished if females later accept additional mates that displace, block, mix, or incapacitate the first mate's sperm (Simmons 2001). This being the case, males are advantaged if they are able to induce sexual inhibition in their mates. In some species, these abilities are closely linked as abundance of stored sperm plays a direct role in the inhibition of sexual receptivity by forming a mating plug (e.g., Lepidoptera, Othoptera, and Diptera) (Gillott 1988), forming a spermatophore and mechanically stimulating the bursa of the female (Sugawara

1979) or serving as 'cheap fillers' to directly induce refraction (Cook & Wedell 1999; Wedell 2001). In these species, female sexual inhibition is inextricably linked to sperm storage.

Males of most tephritid fruit flies are able to mate repeatedly (Kuba & Itô 1993; Aluja *et al*. 2001, Pérez-Staples & Aluja 2004, 2006; Aluja et al. 2008), whereas females typically show a marked and persistent inhibition of sexual activity after their first mating (Cavalloro & Delrio 1970; Aluja *et al*. 2000; Kraaijeveld & Chapman 2004, Pérez-Staples *et al*. 2008b, but see Opp & Prokopy 2000 for an exception). Queensland fruit flies, *Bactrocera tryoni* ('Q-flies'), conform to this general pattern; males can mate repeatedly if given the opportunity (Tychsen & Fletcher 1971; Meats & Fay 2000; Radhakrishnan & Taylor 2008; Radhakrishnan *et al*. 2008) and the majority of females show a marked decline in sexual receptivity after their first mating (Barton Brown 1956, 1957b; Harmer *et al*. 2006; Radhakrishnan & Taylor 2007, 2008; Pérez-Staples *et al*. 2008a).

Even though male tephritids are known to mate repeatedly, and many matings in nature are likely by males that have mated previously, almost all studies of post-copulatory sexual selection in tephritid fruit flies have restricted their focus to virgin males. Other than studies of *Anastrepha striata* by Pérez-Staples & Aluja (2004), studies of *Anastrepha obliqua* by Pérez-Staples *et al*. (2006, 2008b), and studies of *Bactrocera cucurbitae* (melonfly) by Itô & Yamagishi (1989) and Kuba & Itô (1993), little attention has been paid to the ability of male tephritids to maintain sperm transfer or to induce sexual inhibition in sequential mates. The tendency to standardize studies by only considering virgin males belies that we generally expect at least some males, notably those of the highest mate quality, to mate many times (Bateman 1948). In Q-flies, while Radhakrishnan & Taylor (2008) showed that once-mated males have undiminished ability to induce sexual inhibition in their mates, there have been no studies either of whether this ability persists in later matings or whether ability to transfer sperm is maintained across sequential matings. In the present study, we investigate the ability of male Q-flies to remate when offered multiple opportunities, as well as to pass a full complement of sperm and to inhibit sexual receptivity in successive mates.

While focussed principally on postcopulatory performance of males across sequential matings, this study also presents an opportunity to build on earlier work dealing with the question of whether sperm play a role in sexual inhibition of mated females. Harmer *et al*. (2006) found that, despite storing far fewer sperm, mates of virgin sterile (irradiated) male Q-flies are no more prone to remate than are mates of virgin fertile males and that this effect persisted up to at least eight days following their initial mating. These findings suggested that factors other than sperm numbers are important for induction of sexual inhibition in females. By injecting extracts from male accessory glands directly into virgin females we have been able to induce sexual inhibition similar to that of mated females, strongly indicating a direct role for male accessory gland fluids (AGFs) transferred with the ejaculate as mediators of sexual inhibition in Q-flies (Radhakrishnan & Taylor 2008; Radhakrishnan *et al*. 2008, for description of these glands, see Radhakrishnan et al. 2009). However, a role for AGFs does not dispel the possibility that sperm can also play a role. The same injection methods that we used for Q-flies have shown AGFs to be similarly potent mediators of sexual inhibition in female Mediterranean fruit flies (medflies) (Jang 1995, 2002; Jang *et al*. 1998, 1999), but there is none the less evidence that abundance of stored sperm also plays a role in medflies (Miyatake *et al*. 1999; Kraaijeveld & Chapman 2004; Mossinson & Yuval 2003). In some insects it has been suggested that sperm and AGFs influence female sexual inhibition over different time scales. For example, in *Drosophila melanogaster* AGFs are important for short-term sexual inhibition over the hours immediately following mating whereas sperm are important for long-term sexual inhibition over the following 8 - 10 days (Scott 1987). In medflies, the opposite sequence has been suggested; that sperm are important for sexual inhibition over the hours immediately following mating whereas AGFs are important for longer-term sexual inhibition (Mossinson & Yuval 2003).

Sterile flies offer a useful tool to disentangle effects of sperm and other factors. Sterilization by irradiation kills primary spermatocytes along with primary and secondary spermatogonia, but spermatids and spermatozoa survive (Muller 1927; Proverbs 1969; Teruya *et al.* 1985). Because of dysfunctional or absent primary and secondary spermatocytes, sterile males should have little or no capacity for spermatogenesis and should approach aspermy through sperm depletion within a few matings (see Itô & Yamagishi 1989; Kuba & Itô 1993). Compared with the single matings studied by Harmer *et al.* (2006), by studying success of cohorts of fertile and sterile males at having sperm stored and inducing sexual inhibition across a series of mates, we here provide a much stronger test of whether sperm storage plays a role in sexual inhibition of female Q-flies. While fertile males may be able to replenish sperm supplies such that their sequential mates store similar numbers of sperm, we expect that sterile males will not be able to replenish sperm supplies and that their sequential mates will hence store diminishing numbers of sperm. If sperm abundance plays even a minor role in sexual inhibition, we should then find that sequential mates of fertile males are similarly likely to remate and that sequential mates of sterile males are increasingly likely to remate.

Materials and Methods

Fly origin and maintenance

Fertile and sterile (gamma irradiated, 70 - 75 Gy; see Collins et al. 2008, in press) Q-fly pupae were obtained from a mass-reared culture maintained by Industry & Investment NSW at Elizabeth Macarthur Agricultural Institute (EMAI). This culture is replaced with a fresh culture every two years. Adult flies emerged in a laboratory at Macquarie University, Sydney and were initially housed in 5-litre cages containing c. 150 flies. All cages were supplied with water-soaked cotton wool and separate 50 mm-diameter petri dishes containing dry granular sucrose and dry hydrolyzed yeast enzymatic (MP Biomedicals) as food (Perez-Staples *et al.* 2007a; Prabhu *et al.* 2008). All cages were maintained at 24 - 26ºC and 65 - 75 % relative humidity. A LD 12:12 artificial photoperiod was maintained although flies also experienced a simulated dawn and dusk during which the light level ramped up and down through 1 h, respectively. Using an aspirator, adult flies were separated according to sex within 3 days after emerging, several days before sexual maturation (Perez-Staples *et al.* 2007a; Prabhu *et al.* 2008). No calling, courting, or mating was observed in cages prior to separating the sexes. Q-flies only start mating during a c. 30 min window around dusk (Barton Browne 1957a; Tyschen 1977; Pérez-Staples *et al.* 2009), and so all mating experiments took place at this time.

Copulation, sperm and sexual inhibition

Mating trials were conducted to assess mating tendency, mating latency (delay from onset of simulated dusk until mating), and copula duration of fertile and sterile males as well as sperm storage and remating tendency of up to five of their sequential mates.

Experiments and treatments were as follows:

Day 1: Fertile virgin males were paired with fertile virgin females and sterile virgin males were paired with fertile virgin females in 70 mL, mesh topped, cages. All tested flies were 10-12 days old on Day 1. At dusk, when the flies started mating, copulation start and end times were noted for later calculation of mating latency and copula duration. After mating, all flies were fed a mixture of yeast hydrolysate and sucrose solution in water on cotton balls.

Day 2: Fertile and sterile males from Day 1 were paired again with 10-12-day-old fertile virgin females. Fertile females from Day 1 that had been mated by fertile or sterile males were paired

again with 10-12-day-old virgin fertile males to assess remating tendency. During experimental set up, 20 females that had been mated by fertile males and 20 females that had been mated by sterile males were selected at random and were set aside. If these females mated, within one minute of the initiation of copulation they were chilled with an aerosol freeze spray (Dick Smith Electronics/ Electrolube, containing 1, 1 difloroethane) and were separated before sperm was stored from the second mate (see Harmer *et al.* 2006). This procedure allowed us to assess the number of sperm stored from the first mate of females that then accepted a second mate. At the end of simulated dusk, unmated females from the 20 selected were also chilled. The spermathecae and ventral receptacle were dissected out of the selected females to count number of stored sperm using the methods of Perez-Staples *et al.* (2007b). Each storage organ (two spermathecae and the ventral receptacle) was dissected from the female and placed in a 8.5μl drop of deionised water on a glass microscope slide under a stereomicroscope (Olympus SZX12). Each storage organ was then broken apart using entomological pins and the sperm were dispersed by stirring the drop of water vigorously with an entomological pin for *c*. 30s. A 18 ×18 mm glass coverslip was then carefully lowered over the drop and released so that the drop of liquid dispersed to the edges. The corners of the coverslip were secured with a small drop of clear nail polish and the slide was set aside to dry. To maximize accuracy all sperm were counted in all of the samples by slowly working back and forth across each slide under a phase contrast microscope (Leica DME) at 100× magnification.

Day 3-Day 5: Procedures of Day 2 were repeated until Day 5 using the same cohort of male flies. All males were carried over to Day 5 irrespective of how many times they mated. Dead flies were recorded and discarded each day.

On Day 5, a fresh group of virgin fertile males was also paired with 10-12-day-old fertile females (1 Fertiles) and a fresh group of sterile males was paired with 10-12-day-old fertile females (1 Steriles). These males were from the same batch as the males used in days 1 - 5, and were hence 15 - 17 days old when tested. Females mated by males that had mated 4 - 5 times (4-5 Fertiles), and females mated by sterile males that had mated 4 - 5 times (4-5 Steriles), along with 1 Fertile and 1 Sterile females mated on Day 5, were separated into three equal groups and were tested for their remating tendency on Day 6 (one day after mating), Day 16 (15 days after mating) and Day 31 (30 days after mating). Females to be used in these tests were transferred into bigger 250 ml mesh-covered cups with *ad libitum* access to yeast hydrolysate and sucrose as food. Cotton wicks soaked in water were changed every 2 days.

Day 6, Day 16 and Day 31: On days 6, 16 and 31, female flies from the various treatments (1 Fertiles, 1 Steriles, 4-5 Fertiles, 4-5 Steriles) as well as virgin fertile females and virgin fertile males were set up in 70 ml mesh top cages along with a single virgin male per female, 30 minutes before dusk and matings were recorded.

Statistical Analyses

Statistic analyses were performed using SPSS (V. 14.0.2 for Windows, Inc., Chicago, IL, USA) and JMP (V. 5.0, SAS Institute, Cary, NC). Differences in mating latency (delay from the onset of simulated dusk until mating) and copula duration (log-transformed) across the sequential matings of individual males were tested using a mixed model analysis of variance, using fertility (i.e., fertile or sterile) and number of previous matings as fixed factors and individual identity as a random factor.

General linear models (GLM) with binomial errors and logit link function were used to analyse (i) mating probability of males across five days of mating (mated = 1; unmated = 0), (ii) probability of sperm storage (sperm present = 1, sperm absent = 0) and (iii) the ability of males to inhibit female remating across sequential matings (female remated = 1; female did not remate = 0). Fertility was included as a fixed factor with number of previous matings as a covariate. Main models were

constructed and then simplified by removing nonsignificant terms to produce minimum adequate models containing the necessary main effects and interactions (see McGowan et al. 2002). Post hoc pairwise comparisons based on the estimated marginal means were performed using the least significant difference test (LSD, alpha = 0.05).

Individual repeatability (see Falconer 1981; Lessells & Boag 1987; Boake 1989) was determined for mating latency and copula duration. Tests were run separately for fertile and sterile males. Repeatability was calculated as the intraclass correlation coefficients, based on the variance components estimated by a mixed model analysis so that ρ = var (intercept) / var (intercept) + var (residual).

One-way ANOVA was used to compare the number of sperm stored by mates of fertile and sterile males in sequential matings. Contingency analyses were used to determine the mating tendency of females tested on Days 6, 16 and 32.

Results

Mating probability, latency and duration

Rather than suffering a mating disadvantage from the irradiation treatment, sterile males tended to mate even more often than fertile males ($F_{1,330}$ = 112.83, $P < 0.001$, Figure 6.1). Some males died during the five days of testing and so we re-ran this analysis after correcting for the fewer mating opportunities of these flies. After correcting for number of mating opportunities, the tendency for sterile males to mate more often persisted ($F_{1,330}$ = 5.55, $P = 0.019$) (Figure 6.1).

In addition to having higher mating success, sterile males also had significantly shorter mating latency than fertile males (Table 6.1). There were significant differences in mating latency across the sequence of matings with both sterile and fertile males having greater mating latency on the first day of testing. Both fertile and sterile males showed very low repeatability of mating latency (Fertile males: ρ = 0.014; Sterile males: ρ = 0.05).

While there was variation in copula duration across the sequence of matings, this was very similar for fertile and sterile males (Table 6.1). Both fertile and sterile males showed very low repeatability of copula duration (Fertile males: ρ = 0.016; Sterile males: ρ < 0.00).

Figure 6.1: Percentage of males mating one, two, three, four and five times in five mating opportunities with different females over five sequential days. Numbers are sample sizes.
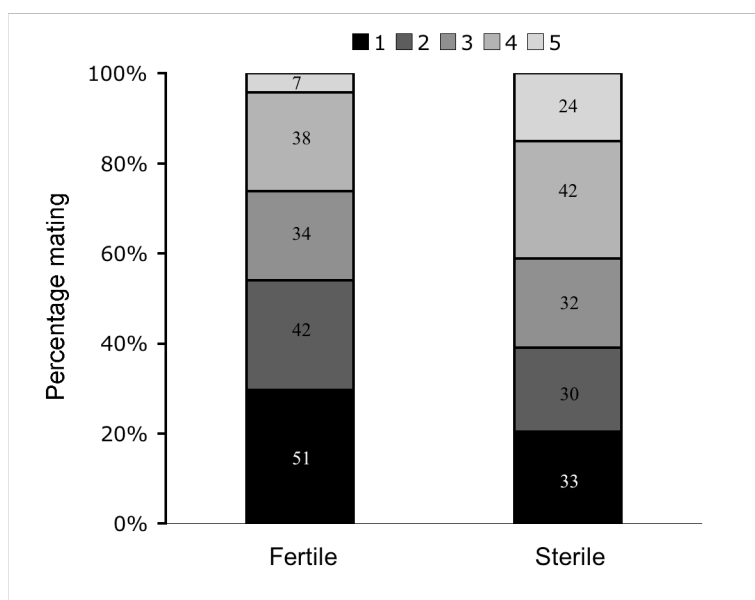
Table 6.1: Linear mixed model for the effects of fertility (fertile *vs.* sterile) and the number of times the male had mated previously on mating latency and copula duration.

|	*df*	*df* error	F	P
Mating Latency				
Intercept	1	392	531.95	0.000
Fertility	1	800	4.63	0.032
Number of times mated	4	740	14.86	0.000
Fertility X Number of times mated	4	737	0.27	0.896
Copula Duration				
Intercept	1	347	1060.10	0.000
Fertility	1	846	0.60	0.438
No. of times mated	4	802	3.22	0.012
Fertility X No. of times mated	4	795	1.98	0.095

Sperm storage by females

Fertile and sterile males differed significantly in trends for sperm storage probability across the sequence of matings (Fertility Wald $\chi^2 = 7.03$, $P = 0.008$, number of previous matings Wald $\chi^2 = 12.58$, $P < 0.001$, interaction Wald $\chi^2 = 5.60$, $P = 0.02$; Figure 6.2). Fertile males were able to maintain highly consistent sperm storage probability across multiple matings whereas sterile males experienced a significant decline in sperm storage probability (Figure 6.2). Whereas most non-virgin fertile males succeeded in having sperm stored by their mates, most non-virgin sterile males failed.

Figure 6.2: Probability of sperm storage by the first four sequential mates of fertile and sterile males. Post-hoc, multiple comparisons by LSD. Different letters indicate significant differences within fertile and sterile male treatments in post-hoc, multiple comparisons by LSD. Numbers inside bars are sample sizes.
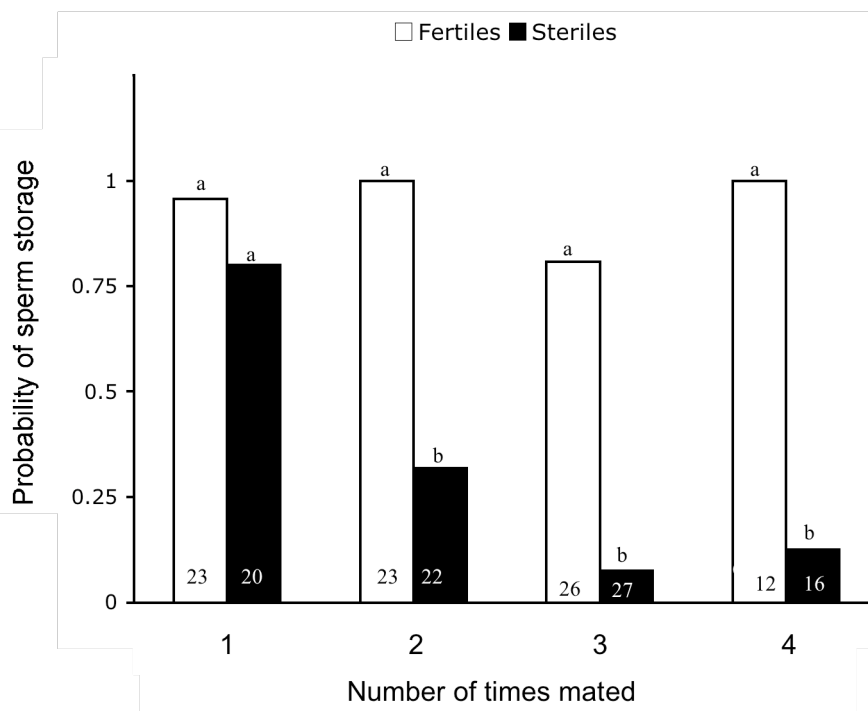
One-way ANOVA was used to compare the number of sperm stored by the sequential mates of fertile and sterile males. Very few males mated five times (see Figure 6.1) and so only the first four matings were assessed. Fertile males showed no significant differences in the number of sperm stored by their first four mates ($F_{3,80} = 0.411, P = 0.75$). In contrast, sterile males did show significant differences in the number of sperm stored by their first four mates ($F_{3,81} = 7.970, P < 0.001$). Tukey's HSD tests showed that significantly more sperm were stored by the first mates of sterile males, with few or no sperm being stored by their third and fourth mates (Figure 6.3). Both fertile and sterile males showed very low repeatability for sperm storage in their mates (Fertile males: $\rho = 0.110$; Sterile males: $\rho < 0.00$). We found no evidence of correlation between copula duration and the number of sperm stored for either fertile males (Spearman $r = 0.11, P = 0.31$) or sterile males (Spearman $r = 0.07, P = 0.53$).

Figure 6.3: Mean number of sperm stored (+SE) by the first four mates of fertile and sterile males. Different letters indicate significant differences within fertile and sterile male treatments in post-hoc, multiple comparisons by LSD. Numbers inside bars are sample sizes.
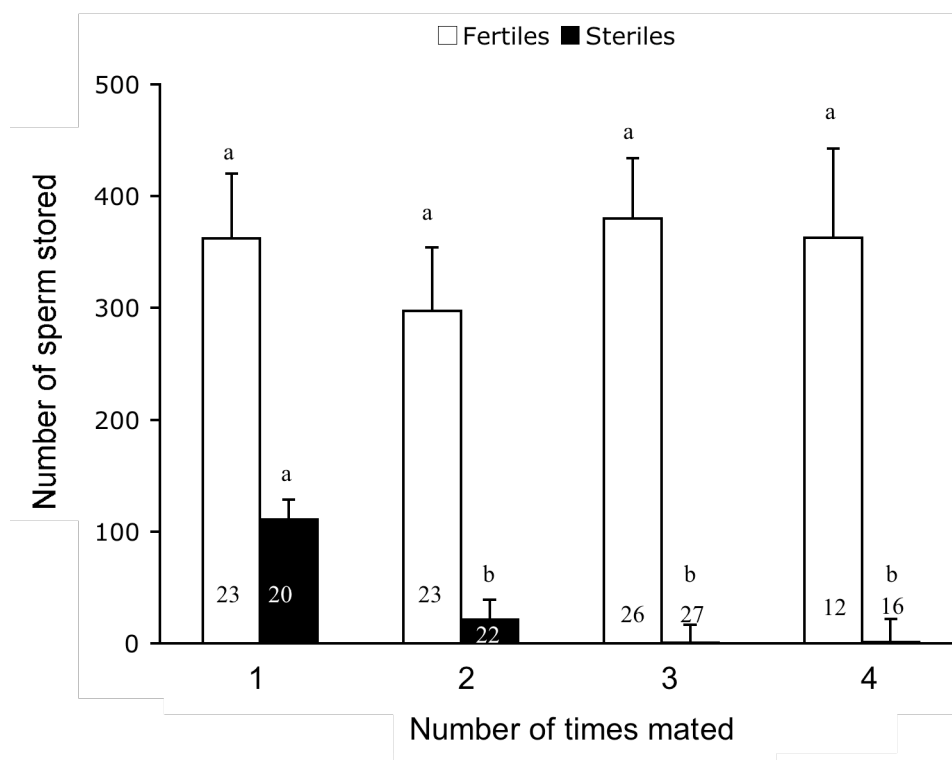

Remating by females on the day after mating

Given the marked differences between fertile and sterile males in sperm storage by sequential mates (Figures 6.2 & 6.3), if female remating tendency is influenced by sperm storage then we should find i) that females mated by fertile males are less likely to remate than females mated by sterile males, ii) that sequential mates of fertile males have similar remating tendency and iii) that sequential mates of sterile males have increasing remating tendency (i.e., significant interaction between effects of fertility and number of previous mates). None of these predictions were upheld.

Opposing predictions of the hypothesis that sperm have a role in sexual inhibition of female Q-flies, mates of sterile males (few or no sperm) proved less likely to remate than mates of fertile males (many sperm) (Wald $\chi^2 = 7.72, P = 0.005$) (Figure 6.4). Remating tendency did not vary across the sequential matings for either fertile or sterile males (number of previous mates Wald $\chi^2 = 2.59, P = 0.11$, interaction between fertility and number of previous mates Wald $\chi^2 < 0.00, P = 0.99$) (Figure 6.4).

Figure 6.4: Proportion of females remating the day after being mating by fertile and sterile males that had already mated one to four times. Numbers inside bars are sample sizes.
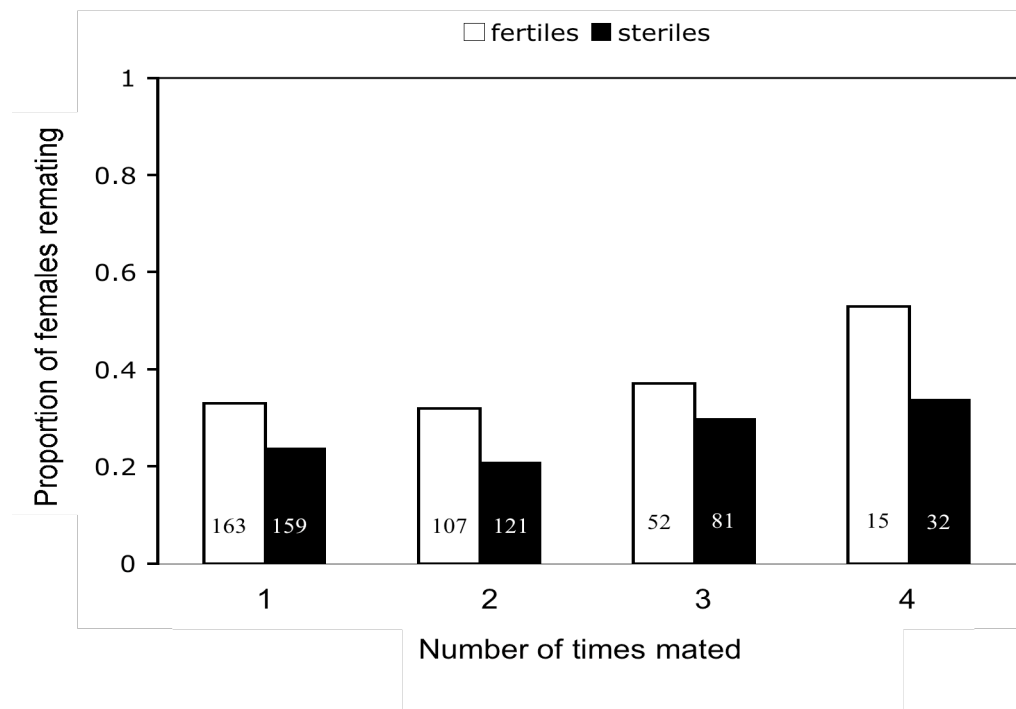

More direct tests also revealed no evidence of relationship between presence of sperm and sexual inhibition of mated females. Separate nominal logistic regression models for each mating day for fertile and sterile males revealed no significant effects of sperm presence on female remating probability on any day for either fertile or sterile males (for all, $P > 0.1$). Further, considering only those pairings that resulted in sperm storage, separate nominal logistic regression models for each mating day for fertile and sterile males revealed no significant effects of sperm abundance on female remating probability on any day for either fertile or sterile males (for all, $P > 0.1$).

Remating by females 6, 16 and 31 days after mating

An initial analysis revealed no differences in remating tendency of females tested at 6, 16 and 31 days after mating and so data were pooled to increase power. Contingency analysis revealed significant differences among the tested groups (Virgins, 1 Fertiles, 1 Steriles, 4-5 Fertiles, 4-5 Steriles) ($G_4 = 22.79$, P < 0.001; Figure 6.5). Pairwise tests revealed that virgin females were more likely to mate than were females in any of the other groups. There were no significant differences in mating tendency among the groups of previously mated females (1 Fertiles, 1 Steriles, 4-5 Fertiles and 4-5 Steriles; Figure 6.5). This indicates that mating-induced sexual inhibition i) lasts at least 30 days, ii) is not influenced by how many times her partner had mated previously and iii) is not influenced by presence or number of sperm stored from previous mates.

Figure 6.5: Proportion of females mating on days 6, 16 and 32 (data pooled). Treatments include virgin males with virgin females (Virgin), females mated on day 5 by virgin fertile males (1-F), females mated on day 5 by virgin sterile males (1-S), fertile females mated on day 5 by fertile males that had already mated 4-5 times (4-5-F), and fertile females mated on day 5 by sterile males that had already mated 4-5 times (4-5-S). Different letters indicate significant differences in post-hoc pairwise comparisons. Numbers inside bars are sample sizes.
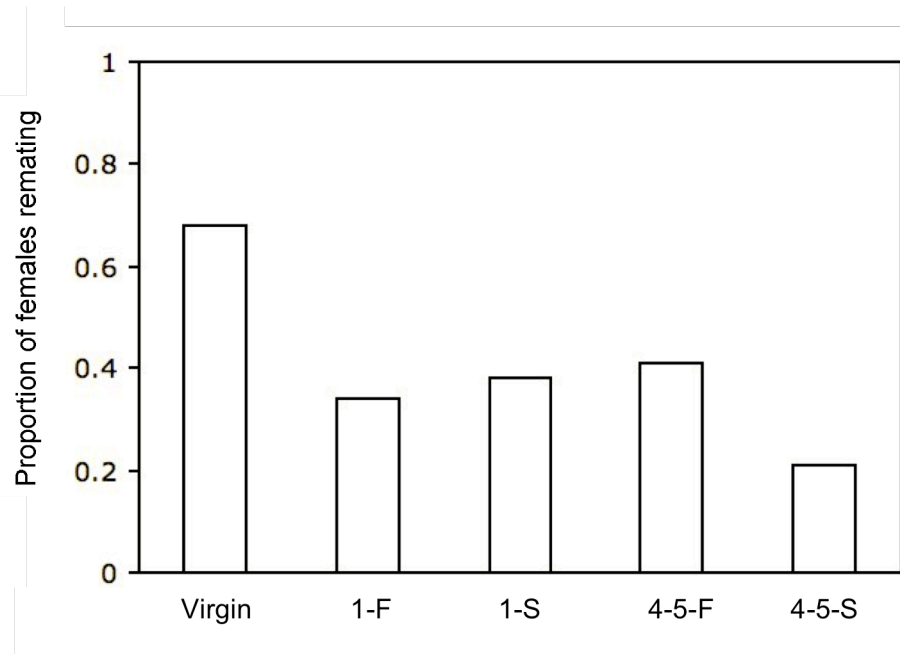
Discussion

Sperm storage

Sequential mates of fertile male Q-flies stored similar numbers of sperm, indicating that males of this tephritid species, like the melonfly (Kuba & Itô 1993) and the West Indies fruit fly, *Anastrepha obliqua* (Pérez-Staples & Aluja, 2006), are able to fully replenish their sperm supplies after each mating and in time for the next mating opportunity. Tephritid fruit flies are able to produce sperm throughout their adult life (Valdez 2001) and appear adapted for the possibility of repeated mating.

It is important to consider the results of our study in an ecological context. Q-fly males are known to mate repeatedly, but with all sexual activity taking place at dusk (Barton-Browne 1956; Barton Browne 1957a; Tychsen & Fletcher 1971; Tychsen 1977; Pérez-Staples *et al.* 2009) and copulations typically lasting several hours (Pérez-Staples *et al.* 2007a,b), these repeated matings each happen one or more days apart. Following their first mating, male Q-flies are able to replenish their accessory gland contents in time for the next mating opportunity at dusk the following day (Radhakrishnan & Taylor 2008). We find in this study that fertile Q-fly males also appear able to replenish sperm reserves between matings in time to ensure the next mate stores a full complement. The ability of male Q-flies to replenish ejaculate between matings, and the schedule of this recovery, naturally reflects the mating system of this species. Because there is always a minimum latency of a day between mating opportunities, the physiology of testes, accessory glands and other reproductive organs are presumably adapted for recovery over this time frame.

Females mated by sterile males showed sharply different sperm storage patterns from those mated by fertile males, reflecting differences in the number of sperm transferred by the copulating male.

First, as has been found previously in Q-flies (Harmer *et al.* 2006), as well as in medflies (Seo *et al.* 1990; Taylor & Yuval 1999; Taylor *et al.* 2000, 2001; Mossinson & Yuval 2003), females in the present study that were mated by virgin sterile males stored fewer sperm than females mated by virgin fertile males. Second, as in melonflies (Kuba & Itô 1993), whereas sequential mates of fertile males stored similar numbers of sperm sequential mates of sterile males stored progressively fewer sperm. Third and fourth mates of sterile males rarely stored sperm at all. This extreme sperm depletion provides a valuable tool to address the question of whether sperm storage plays a role in sexual inhibition. However, this approach requires that sterile males not be deficient in characters other than sperm capacity. Some studies of other tephritids have reported that, compared with their fertile counterparts, sterile males have reduced sexual competitiveness (e.g., Moreno *et al.* 1991; Cayol 2000; Calcagno *et al.* 2002; Lux *et al.* 2002; Kraaijeveld & Chapman 2004; Rull *et al.* 2005). Several recent studies have reported similar mating tendency in sterile and fertile male Q-flies (Harmer *et al.* 2006; Perez-Staples *et al.* 2007a). However, these studies differed from the present study in only considering virgin males. Here, where males were given multiple mating opportunities, we found sterile male Q-flies to mate even more often than fertile males (Fig. 1). Sterile males were also a close match to fertile males in mating latency (Fig. 2) and copula duration (Fig. 3). The higher mating success of sterile males relative to their fertile counterparts may reflect resource budgets. If there is a resource cost to replenishing sperm then fertile males would have a portion of their reserves set aside for this activity and would then have fewer resources available for investment in other traits such as pheromones, or energetic reserves for courtship. Relieved of the expense associated with sperm replenishment, sterile males might be able to redistribute associated resources to other activities that enhance their chances of mating.

The role of sperm in female sexual inhibition

Previous studies have reported female sexual inhibition during the week following their first mating (Barton-Browne 1957b; Harmer *et al.* 2006; Pérez-Staples *et al.* 2008a). We here extended well beyond the time frame of these previous studies, confirming undiminished persistence of sexual inhibition in females up to 30 days after mating. In medflies, the tephritid fly having received the greatest attention in studies of sexual inhibition, females mated by irradiated males store fewer sperm and are more prone to remate than females mated to fertile males and this has been taken as evidence that sperm play a direct role in sexual inhibition (Mossinson & Yuval 2003; Kraaijeveld & Chapman 2004). Additional evidence comes from higher remating tendency of females if they store few sperm from their first fertile mate (Mossinson & Yuval 2003) or if they mate with a castrated male that cannot transfer any sperm (Miyatake *et al.* 1999), and the tendency for female sexual inhibition to diminish as sperm stores decline over time (Nakagawa *et al.* 1971). The approach used in the present study is substantially stronger than previous Q-fly studies in that we considered both the previous comparison between fertile and sterile flies, which are known to transfer different numbers of sperm even when virgin (Harmer *et al.* 2006), and also the effects of declining sperm numbers across matings of the same individual sterile flies. This approach greatly increased our ability to focus on the role of sperm.

Adding to the accumulating evidence (Harmer *et al.* 2006; Radhakrishnan & Taylor 2007), findings of the present study support the hypothesis that sperm play no direct role in sexual inhibition of female Q-flies. First, as for melon flies, *Bactrocera cucurbitae* (Kuba & Itô 1993), we found that the marked reduction in number of sperm stored by successive mates of sterile male Q-flies was not accompanied by any changes in female remating propensity. Second, there was evidence that, despite storing vastly fewer sperm, mates of sterile males were actually less likely to remate than were mates of fertile males. Third, in a more direct test based on the subsample of females for which sperm number was assessed, for mates of both fertile and sterile males we found that females storing no sperm were as likely to exhibit sexual inhibition as were females storing sperm. Mossinson & Yuval (2003) argue that in medflies, which are sexually active throughout daylight

hours and can remate immediately after their first mating, sperm are important for sexual inhibition in the hours following mating and that AGFs then take over as the main inhibitory mechanism thereafter. Assuming a similar time-line for inhibitory mechanisms in Q-flies, the lack of evidence for any role for sperm in sexual inhibition of Q-flies might reflect that the first opportunity for remating occurs only on the day following their first mating, when AGFs would be in full effect and any role for sperm would have passed. Unlike previous studies in which only virgin males were used (Harmer *et al*. 2006), we also found that mates of sterile males were actually less likely to remate than were mates of fertile males. Increased ability of sterile to induce sexual inhibition in their mates may be explained by mechanisms similar to those we have suggested as possibly underpinning their increased mating success. That is, sterile males may be able to redirect resources usually required for sperm production into AGF production or other activities involved in the induction of sexual inhibition.

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TECHNOLOGY TRANSFER

Presentations and meetings

<div align="center">2009</div>

1) Collins, S.R., Weldon, C.W., Banos, C. & Taylor, P.W. (2009) Optimal irradiation dose for sterilization of Queensland fruit fly *Bactrocera tryoni*. (Poster)
    NSW, VIC & SA TriState meeting, Tatura

2) Collins, S.R., Weldon, C.W., Banos, C. & Taylor, P.W. (2009) Optimal irradiation dose for sterilization of Queensland fruit fly *Bactrocera tryoni*. (Poster)
    *IAEA/FAO Coordinated Research Project on development of mass rearing for New World (*Anastrepha*) and Asian (*Bactrocera*) fruit fly pests in support of sterile insect technique (SIT): 4*[th]* Research Coordination Meeting, Pereybere, Mauritius. (invited presentation)*

3) Lemon, M., Harmer, A.M.T. & Taylor, P.W. (2009) Acoustic sexual communication in Queensland fruit flies. (Poster)
    NSW, VIC & SA TriState meeting, Tatura

4) Taylor, P.W., Collins, S.R. & Weldon, C.W. (2009) Progress toward optimised irradiation protocols for Q-fly SIT. (Invited Oral Presentation)
    NSW, VIC & SA TriState meeting, Tatura

<div align="center">2008</div>

5) Collins, S.R., Weldon, C.W., Banos, C. & Taylor, P.W. (2008) Optimal irradiation procedures for sterilization of Queensland fruit flies. (Oral Presentation)
    Australian Entomological Society, Orange

6) Collins, S.R., Weldon, C.W., Banos, C. & Taylor, P.W. (2008) Optimal irradiation dose for sterilization of Queensland fruit fly *Bactrocera tryoni*. (Poster)
    7th* Meeting of the Working Group on Fruit Flies of the Western Hemisphere, Mazatlán, Mexico*

7) Radhakrishnan, P., Perez-Staples, D., Weldon, C.W. & Taylor, P.W. (2008) Sperm-depleted males inhibit female receptivity in Q-flies. (Poster)
    Australasian Society for the Study of Animal Behaviour, Coffs Harbour.

8) Taylor, P.W. (2008) Sexual performance of released flies determines the pest management performance of the sterile insect technique. (Invited Oral Presentation)
    Australian Entomological Society, Orange)

9) Weldon, C.W., Taylor, P.W., Collins, S.R., Harmer, A.M.T., Perez-Staples, D., Prabhu, V. & Radhakrishnan, P. (2008). SIT research at Macquarie University: recent results from the Behavioural Biology Research Group. (Oral Presentation)
    NSW, VIC & SA TriState and Riverina Citrus Fruit Fly Forum (invited presentation)

<div align="center">2007</div>

10) Collins, S.R. & Taylor, P.W. (2007) Optimal irradiation procedures for sterilization of Queensland fruit flies. (Invited Oral Presentation)
    Australian Institute for Nuclear Science & Engineering (AINSE) (invited presentation)

Peer-reviewed Publications

1) Collins, S.R., Weldon, C.W., Banos, C. & Taylor P.W. (2008) Effects of irradiation dose rate on quality and sterility of Queensland fruit flies (*Bactrocera tryoni* Froggatt).
Journal of Applied Entomology 132: 398–405

2) Collins, S.R., Weldon, C.W. & Taylor P.W. (2008) Effects of field cage colour and supplementary shade on mating behaviour of Queensland fruit flies.
Entomologia Experimentalis et Applicata 129: 142-147

3) Collins, S.R. & Taylor, P.W. (in press) Flight ability procedures for mass reared Queensland fruit flies (*Bactrocera tryoni*): an assessment of some variations.
Entomologia Experimentalis et Applicata

4) Collins, S.R., Weldon, C.W., Banos, C. & Taylor P.W. (2009) Optimising irradiation dose for sterility induction and quality of Queensland fruit flies, *Bactrocera tryoni*.
Journal of Economic Entomology 102: 1791-1800

5) Mankin, R.W., Lemon, M. Harmer, A.M.T, Evans, C.S. & Taylor, P.W. (2008) Time-pattern and frequency analyses of sounds produced by irradiated and untreated male *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) during mating behavior.
Annals of the American Entomological Society 101: 664-674

6) Perez-Staples, D., Weldon, C.W. & Taylor, P.W. (2009) Pre-release feeding on yeast hydrolysate enhances sexual competitiveness of sterile male Queensland fruit flies in field cages.
Entomologia Experimentalis et Applicata 131: 159–166

7) Radhakrishnan, P., Perez-Staples, D., Weldon, C.W. & Taylor, P.W. (2009) Multiple mating and sperm depletion in male Queensland fruit flies: effects on female remating behaviour.
Animal Behaviour 78: 839–846

8) Weldon, C.W, Prenter, J. & Taylor, P.W. (2010) Activity patterns of Queensland fruit flies (*Bactrocera tryoni*) are affected by both mass rearing and sterilization.
Physiological Entomology 35: 148-153

Other publications

1) Taylor, P.W. (2007) Fruit fly 'double agents' in the service of fruit growers.
The Vine Technical Annual 3(5): 35

2) Taylor, P.W. (2007) SIT Research and Training at Macquarie University.
Australian SIT Network 2: 1-2

RECOMMENDATIONS

1) Quality control is an important aspect of any production process, and this in certainly true for the mass production of insects for SIT programs. Numerous aspects of rearing, packing and transport can adversely affect quality. By comparing quality at the factory and rearing out facilities it is possible to monitor quality and to identify any changes taking place during transport. There has been substantial variation between facilities in the flightability assays that are a central component of fruit fly quality control procedures. We have confirmed that the internationally accepted standard FAO/IAEA/USDA (2003) protocols are effective for Q-flies and would be straightforward to implement at all facilities.

It is recommended that the internationally accepted standard FAO/IAEA/USDA (2003) protocols for assessment of flightability be introduced in Q-fly SIT both at the factory and at rearing out facilities.

2) The rate at which an irradiation dose is applied has implications for accuracy of total dosage applied to pupae to sterilize them for SIT. Higher dose rates can be more convenient because the irradiation is completed more quickly, allowing higher throughput in the irradiator. However, higher dose rates are associated with an increased tendency to apply total doses that are at the higher end of the currently accepted range. This tendency is associated with reduced quality of emerging flies. Although lower dose rates are less convenient, taking longer to apply the required total dose, they are also associated with a tendency to be more often in the lower end of the currently accepted range and higher quality of emerging flies.

It is recommended that the lowest practical dose rate be adopted for irradiation of Queensland fruit fly pupae for SIT.

3) Total irradiation dose has a substantial effect on quality of emerging flies. Current protocols entail the application of 70 - 75 Gy of gamma radiation to hypoxic pupae. Findings of research contained in this report indicate that quality of emerging flies is higher at total dosage of 65 or even 60 Gy and that sterility induction at these lower doses is comparable to the current 70 - 75 Gy dose. This can be interpreted in two ways. First, these findings offer strong assurance that the current total dose offers a substantial safety margin above doses at which sterility induction is unacceptable. Second, while still retaining a substantial safety margin, it would be possible to improve the quality of flies available for SIT by reducing the total irradiation dose applied.

It is recommended that further consideration be given to the possibility of reduced irradiation dose for Q-fly SIT. This should include (1) Field releases to compare survival and dispersal under operational conditions and (2) Field cage studies to compare mating compatibility with wild type flies.