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Developing field cage tests to measure mating competitiveness of sterile light brown apple moths (Lepidoptera: Tortricidae) in Western Australia

Bill Woods[1,], Donald McInnis[2], Ernie Steiner[1], Alven Soopaya[1], Jeremy Lindsey[1], Ian Lacey[1], Amandip Virdi[1] and Roselia Fogliani[1]*

Abstract

The Australian light brown apple moth (LBAM) (*Epiphyas postvittana*) (Walker) (Lepidoptera: Tortricidae) is a pest in Australia, New Zealand and now California (USA). The use of sterile insects in combination with mating disruption and biological insecticides has the potential to eradicate outbreaks in urban areas. The sexual competitiveness of irradiated insects is an important component of the effectiveness of the sterile insect technique (SIT), but standard techniques to measure the sexual competitiveness have been developed only for irradiated tephritid fruit flies. In particular, field cage trials have been used to measure the compatibility and competitiveness of irradiated fruit flies in comparison with wild fruit flies. Trials were carried out to determine if such tests could be adapted for a moth species. Parameters of quality or competitiveness evaluated were the proportion of the moths that mated, relative sterility index, index of sexual isolation, and mating competitiveness based on the egg hatch in the various crosses. Results showed that with the release of sterile moths of both sexes (bisex) there was little difference in competitiveness—expressed as the Relative Sterility Index (RSI)—between moths irradiated at 200, 250 and 300 Gy (irradiated either in the pupal or adult stages), but if a Fried competitiveness test was used to generate competitive C values then greater competitiveness was found at the lower doses of irradiation, but this difference was not statistically significant. Modified test procedures were developed in which the moths in field cages—after having had sufficient opportunity to mate—were egged individually and dissected to determine the presence of 1 or more spermatophores; then egg sterility and spermatophore presence were used to determine the mating type, e.g., wild female × irradiated laboratory male, etc. Results indicated that sterile-male-only releases have the potential to increase mating competitiveness of the released irradiated moths, but this conclusion requires additional experiments for confirmation.

Key Words: bisex release of *Epiphyas postvittana;* male only; sterile insect technique; irradiation; Tephritidae; fruit flies; spermatophore

Resumen

La polilla tortricid de la manzana de color café claro australiana (PTMA) (*Epiphyas postvittana)* (Walker) (Lepidoptera: Tortricidae) es una plaga en Australia, Nueva Zelanda y ahora en California (EE.UU.). El uso de insectos estériles en combinación con la interrupción del apareamiento e insecticidas biológicos tiene el potencial para erradicar los brotes en las zonas urbanas. La competitividad sexual de los insectos irradiados es un componente importante de la eficacia de la técnica del insecto estéril (TIE), pero las técnicas estándar para medir la competitividad sexual se han desarrollado sólo para las moscas irradiadas tefrítidas de frutales. En particular, los ensayos de campo de la jaula se han utilizado para medir la compatibilidad y la competitividad de las moscas de la fruta irradiadas en comparación con moscas de la fruta silvestres. Se realizaron los ensayos para determinar si tales pruebas podrían adaptarse para una especie de polilla. Los parámetros de calidad o competitividad evaluados fueron la proporción de las polillas que se aparearon, el índice relativo de esterilidad, el índice de aislamiento sexual, y la competitividad de apareamiento en base a la eclosión de los huevos en los diversos cruces. Los resultados mostraron que con la liberación de polillas estériles de ambos sexos (bisexual) había poca diferencia en la competitividad - expresada como el Índice de Esterilidad Relativa (IER) - entre las polillas irradiadas a 200, 250 y 300 Gy (irradiados en el estadio de pupa o adulto), pero si se utilizó una prueba de competitividad "Fried" para generar valores *C* de competitivos entonces una mayor competitividad se encontró a las dosis más bajas de radiación, pero esta diferencia no fue estadísticamente significativa. Se han desarrollado procedimientos de ensayo modificados en los que las polillas en jaulas de campo - después de haber tenido oportunidad suficiente para aparearse – fueron diseccionado individualmente para examinar los huevos para determinar la presencia de 1 o más espermatóforos; luego, la esterilidad de huevo y la presencia de espermatóforo se utilizaron para determinar el tipo de apareamiento, por ejemplo, hembra salvaje × macho irradiado del laboratorio, etc. Los resultados indicaron que la liberación de machos estériles sólo tiene el potencial de aumentar la competitividad de apareamiento de las polillas irradiadas liberadas, pero esta conclusión requiere experimentos adicionales para confirmar.

Palabras Clave: liberación de *Epiphyas postvittana* bisexual; únicamente machos; técnica del insecto estéril; irradiación mosca tehphritida de la fruta; espermatóforo

The competitiveness of sterile insects is critical to their effectiveness in reducing populations of the target pest by means of the sterile insect technique (SIT). This technique involves large scale production, irradiation, and release of large numbers of sterile insects in the target

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area, preferably only males, whose task is to mate with wild females so they lay infertile eggs. Sterile males must compete successfully with wild males for females in the field for the SIT to be successful. The loss of mating competitiveness has been demonstrated in mass-reared fruit flies (Lance et al. 2000; McInnis et al. 1996; Rendon et al. 2004) and remains a constant challenge.

Competitiveness can be assessed in different ways and for fruit flies there are well established protocols (FAO/IAEA/USDA 2003), which are constantly being improved (Cayol et al. 1999). In field cage mating competitiveness tests, irradiated males (for male only strains), or irradiated males and females (for bisex strains) together with wild males and females are released into field cages in a 1:1 ratio, mating pairs are captured, duration of matings is recorded and the proportion of matings of sterile males with wild females is used to calculate the Relative Sterility Index (RSI) (McInnis et al. 1996). Alternatively rather than capturing mating pairs, females may be offered the opportunity to oviposit into egging devices, e. g., artificial fruit, hanging in the field cages. Eggs are collected from the fruit and the percentage hatch is determined. This enables a Fried test to be used to calculate a male competitiveness C value (Fried 1971). Variation among replicates tends to be large, so an adequate number of replicates need to be carried out to obtain valid results.

In area-wide integrated pest management (AW-IPM) programs (Vreysen et al. 2007) with a SIT component there is always a compromise between a dose rate that gives high sterility but less competitive males and one that ensures better competitiveness but lower sterility (Lance & McInnis 2005; Suckling et al. 2011). Moths as a group require relatively large doses to induce sterility. Thus, Bakri et al. (2005) quoted 278 Gy as a mean sterilization dose for Tortricidae. However, one can take advantage of the existence in irradiated moths of the inherited sterility (IS) phenomenon (Carpenter et al. 2005), which is absent in irradiated flies. In most cases female moths are sterilized by a lower dose than male moths and the F_1 progeny resulting from a mating between partially sterile males and wild females are more sterile than their parents and these F_1 progeny are predominantly male. Carpenter et al. (2005) commented "the lower dose of radiation used to induce F_1 sterility increases the quality and competitiveness of released insects as measured by improved dispersal after release, increased mating ability and superior sperm competition".

Saour (2014) noted that to use F_1 sterility, a dose is usually chosen that fully sterilizes females while only partially sterilizes males. He recommended a dose of 150 Gy for F_1 sterility against the European grapevine moth, *Lobesia botrana* (Denis & Schiffermüller) (Lepidoptera, Tortricidae), although males were still partially fertile at 400 Gy. In radiation biology trials with the light brown apple moth (LBAM), *Epiphyas postvittana* Walker (Lepidoptera: Tortricidae) a very small percentage of eggs from female moths irradiated with 200 Gy hatched and developed to the pupal stage, with 250 Gy eggs hatched but no progeny developed, and with 300 Gy no eggs hatched. For males there was 2% egg hatch and larval survival when male parents were irradiated with 300 Gy (Jang et al. 2012). Therefore they recommended a dose of 250–300 Gy for a SIT program against LBAM if parental sterility was the desired outcome but that a lower dose could be used if F_1 hybrid sterility (IS) was desired to obtain "increased competitiveness of moths".

In moths different methods such as flight mills, wind tunnels, flight cylinders and mark-release-recapture trials (Stringer 2013) have been used to measure certain components of sterile insect competiveness. Suckling et al. (2011) used wind tunnels and mark-release-recapture in hedgerows and vineyards to test flight ability and dispersal of LBAM irradiated at various doses. Mark-release- recapture studies and mating tables with virgin females were used to measure field competiveness of diapausing and non-diapausing codling moths, *Cydia pomonella* L. (Lepidoptera: Tortricidae) in Canada (Bloem et al. 2004). A mating table consists of virgin female moths with clipped wings that are confined to small arenas hung in the tree canopy and that allow mating pairs to be collected and identified. Walk-in field cages have been used to demonstrate either the influence of radiation dose or the release ratio on population growth in the cactus moth, *Cactoblastis cactorum* (Berg) (Lepidoptera: Pyralidae) (Hight et al. 2005), the codling moth (Bloem et al. 1999) and the false codling moth, *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae) (Hofmeyer et al. 2005). Likewise, walk-in field cages were used to assess mating compatibility of codling moth populations originating from various geographical areas (Taret et al. 2010). The use of field cages to test mating competitiveness of irradiated moths—as has been done with fruit flies—has not been reported.

Western Australia is fortunate in that 2 key pests of perennial horticulture, the codling moth and the Oriental fruit moth, *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae), have not established in the state, and since 1900 over 20 campaigns have been carried out to eradicate incursions (Woods et al. 2001). Eradication was achieved by tree and fruit removal and tightly scheduled use of insecticides over the entire growing season. These techniques are no longer economically or socially viable and other approaches such as the SIT are needed. The native tortricid species LBAM was chosen as the model species to develop alternative area-wide eradication strategies that include an SIT component.

Initial work identified the irradiation dose required to induce sterility in the species (Soopaya et al. 2011). Thereafter trials were carried out on competiveness of irradiated moths using doses between 200 and 300 Gy, the range most likely to be used in an eradication program. Techniques and walk-in field cages used for assessing Mediterranean fruit fly competitiveness were adapted for use with LBAM and bisex and male-only releases were investigated. LBAM females will lay eggs on any smooth surface with up to 150 eggs per batch (Brown et al. 2010) and up to 600 eggs per female in the laboratory (Bill Woods, unpublished data). The potential for easily separating and counting egg batches from individual females provides options not available for tephritid fruit flies.

The objective of the trials was to investigate the potential use of walk-in field cages to measure mating competitiveness of moths, and to determine whether the release of only sterile males resulted in any competitive advantage as had been shown with tephritid fruit flies. Three trials were carried out initially using the standard techniques as used with fruit flies, but in subsequent trials they were modified to make the techniques more applicable for use with moths.

Materials and Methods

All field cage trials were carried out at the Department of Agriculture and Food in South Perth, Western Australia. Trial 1 took place in Mar 2008, trial 2 in Apr 2009 and trial 3 in Sep and Oct 2009. Mean minimum and maximum temperatures (°C) for these months in Perth were 16.7 and 29.4 (Mar 2008), 13.5 and 27.6 (Apr 2009), 9.8 and 18.6 (Sep 2009), and 12.3 and 22.3 (Oct 2009). Over the period of the trials maximum and minimum temperatures at Perth varied from 26.4 to 32.7 (maximum) and 17.8 to 21.7 (minimum) (3–7 Mar 2008), from 24.9 to 29.7 (maximum) and 11.3 to 15.5 (minimum) (15–19 Apr 2009), from 16.8 to 19.8 (maximum) and 6.2 to 14.6 (minimum) (8–14 Sep 2009), and from 23.0 to 36.9 (maximum) and 9.8 to 13.1 (minimum) (14–17 Oct 2009) (http://www.bom.gov.au/climate/data/). Sunset was near 18.50 h in Mar 2008 and 17.50 h in Apr 2009. In Oct 2009 the light level inside the field cages was reduced from around 400–600 lux at 19.30 h to near zero by 20.30 h. RH varied between 50–80% from 19.30 h to 22.30 h.

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MOTHS

Laboratory moths had been reared in captivity for approximately 20 generations and were collected from a laboratory colony maintained at the South Australian Research and Development Institute (SARDI) in Adelaide. Material was sent to Perth and reared there since Jun 2007. Moths were fed a 10% honey/water solution and larvae were reared on a modified Singh diet (Soopaya et al. 2011) that contained cellulose powder, casein, wheat germ, agar, vitamins and preservatives.

In 2008, no wild moths were available, so unirradiated laboratory moths were used as surrogate "wilds". "Wildish" moths used in 2009 had been reared on the artificial diet only for 4–8 generations. This strain was derived from eggs collected from leaves of grapevines from a table grape (*Vitis vinifera* L.; Vitales: Vitaceae) vineyard in the Swan Valley near Perth.

IRRADIATION

Pupae or adults to be irradiated were placed into a small 30 mL plastic container (Huhtamaki P075) that was positioned by means of a jig in the center of the sample chamber of a Gammacell 220 (Nordion, Ottawa, Canada) ^{60}Co irradiator. Variation in the dose received as measured by Gafchromic dosimetry was ± 10%. After irradiation pupae were placed individually into 30 mL vials for emergence to avoid any possibility of mating. Where adult moths were used they were sexed by size and wing markings after emergence and each sex was irradiated separately. In trial 1 pharate pupae (< 1 day post emergence) and moths (1–3 d old) were irradiated, but in trials 2 and 3 only adult moths (1–3 d old) were irradiated. Dose rate varied between 200 and 300 Gy (Soopaya et al. 2011).

MARKING

Moths (1–3 d old) were immobilized in a refrigerated shipping container at 2–5 °C and given a distinguishing color mark on their thorax using white board markers of varying colors. To avoid bias between irradiated and non-irradiated moths, marking was alternated between wildish and laboratory moths for different replicates.

FIELD CAGES

Walk-in field cages consisted of a cylindrical tent 2 m tall × 3 m diam made with moth proof nylon. Each cage contained in the middle an artificial *Ficus benjamina* tree approximately 1.5 m tall. Moths were released close to dusk. We aimed for 25 ♂ and 25 ♀ moths per moth type in each field cage. However this was not always possible due to moth availability. In these situations, the numbers of irradiated and unirradiated males in a field cage was always equal or only varied by one moth due to mortality during transfer from containers to field cages. Mating pairs were captured in vented 40 mL plastic vials and mating type, time and place of capture were recorded. Temperature and humidity in the field cage were recorded every 30 min. LED headlamps either on red or white settings were used and one person manned each field cage for 4–7 h after moth release, depending on temperature and mating activity.

TREATMENTS

Trial 1

Trial 1 included a control treatment consisting of unirradiated male (U♂) and unirradiated female (U♀) moths. This control treatment is not shown in Table 1.

Treatment 1 consisted of irradiated female (I♀) and male (I♂) moths that had emerged from pupae irradiated with 200 Gy; these irradiated moths were released along with U♀ and U♂ moths.

Treatment 2 was the same as Treatment 1, except that the adult moths rather than pupae were irradiated with 200 Gy.

Treatment 3 was the same as Treatment 1, except that the pupae were irradiated with 300 Gy.

Treatment 4 was the same as Treatment 2, except that the adults were irradiated with 300 Gy (Table 1).

Moth numbers per field cage in Trial 1 varied as follows: U♀s, 10–13 and I♀s, 5–21; U♂s, 10–21 and I♂s, 9–21. Treatments were repeated over 3 nights with rotation of treatments and observers between field cages to avoid bias.

Trial 2

In trial 2, adult moths were irradiated with either 250 Gy or 300 Gy (Table 1), thus generating 2 treatments. Each treatment was repeated 4 times over 2 nights and moth numbers per cage varied as follows: U♀s, 17–20 and I♀s, 15–20; U♂s, 16–20 and I♂s, 16–20.

Trial 3

Trial 3 was carried out over 3 nights and each treatment was replicated 4 times. Treatments consisted of field cages of either a male-only experiment with I♂s, U♂s and U♀, or a bisex experiment with I♂s and I♀s irradiated with 300 Gy plus U♂s and U♀s (Table 1). In this trial, mating pairs were not removed from the field cage at the time of mating but female moths were collected in the morning and egged individually. Mating status was then determined by spermatophore dis-

Table 1. Field cage set up to assess mating competitiveness of the light brown apple moth (LBAM) in trials 1, 2 and 3. To the extent possible, untreated and irradiated moths were released into each field cage in a 1:1 ratio, i.e., 25 ♂ and 25 ♀ moths per moth type in each field cage. However this was not always possible due to moth availability. In these situations, the numbers of irradiated and unirradiated males in a field cage was always equal or only varied by one moth due to mortality during transfer from containers to field cages.

Trial #	Treatment 1	Treatment 2	Treatment 3	Treatment 4
1	U♀, U♂, I♀, I♂ 200 Gy P	U♀, U♂, I♀, I♂ 200 Gy A	U♀, U♂, I♀, I♂ 300 Gy P	U♀, U♂, I♀, I♂ 300 Gy A
2	U♀, U♂, I♀ I♂ 250 Gy A	U♀, U♂, I ♀ I ♂ 300 Gy A		
3	U♀, U♂, I♀ I♂ 300 Gy A, S	U♀,U♂, I♀ 300 Gy A, S		

U = un-irradiated, I = irradiated, P = pupae irradiated, A = adults irradiated, S = presence of spermatophore determined by dissection. Control treatments (not shown) involved only un-irradiated moths.

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section and egg hatch. Moth numbers per field cage varied as follows: U♀s, 12–25 and I♀s, 11–25; U♂s, 12–25 and I♂s, 11–25.

LABORATORY CAGES

Small cages, approximately 20 × 20 × 20 cm, were set up as sterility and control treatments for each experiment with equal numbers of individuals of each sex (25–30) in each cage. The sterility test cages contained irradiated males and untreated colony females while the control test cages had wildish males and females. Irradiated males were from the same batch irradiated on the day of the field cage tests and the eggs derived from the matings in laboratory cages were used to provide data for sterile egg hatch (Hs) and control egg hatch (Hc), respectively, for use in the Fried test formula for calculating moth competitiveness (C value). Eggs were recovered and set up in the same way as described for the eggs derived from the field cage matings.

EGG COLLECTION

Mated females collected in vials the previous night were taken into the laboratory. In trial 1, females were released into 1 of 5 egging cages according to their treatment group. Eggs were oviposited on wax paper inside the cages and cut from the wax paper daily and placed into 100 mL clear plastic cups for hatching. In trials 2 and 3, females captured into plastic vials were placed individually into cups where they oviposited eggs on the inside surface. Before the eggs hatched, the egg masses were circled with a fine marker and the number of eggs counted under a stereo microscope. Then after 8 d at 25 °C and 60–80% RH, the numbers of hatched and unhatched eggs were counted, and the females were removed and held in a freezer until dissected for the presence of spermatophores, as an indication of mating success.

INDICES OF MATING COMPETITIVENESS

The formulas of various indices of mating competitiveness are described in the FAO/IAEA/USDA (2003) Quality Control Manual.

Proportion of Mating (PM)

PM measures the suitability of the tested insects and the field cage environment for mating and represents overall mating activity. For fruit flies a value of 0.5 is considered adequate, whereas a proportion of less than 0.2 will not provide meaningful data. The proportion of moths mating (PM) was defined as:

$$PM = \frac{\textit{No. of pairs collected}}{\textit{No. of females released}}$$

Relative Sterility Index (RSI)

The RSI is the proportion of wild females that mate with sterile males when sterile and wild males are present in a 1:1 ratio. Thus RSI is an indicator of sterile male mating performance (McInnis et al. 1996), and it was defined as:

$$RSI = \frac{SW}{(SW + WW)}$$

where SW is the number of matings between sterile males and wild females and WW is the number of matings between wild males and wild females. The RSI equals the number of sterile matings divided by the number of all matings, and its values can vary between 0 and 1, with 0.5 indicating equal performance of wild and sterile males.

Index of Sexual Isolation (ISI)

The ISI (Cayol et al. 1999) is a measure of sexual compatibility between 2 strains. It measures the preference of insects to mate with individuals of the same versus another strain, and the index takes into account both within strain and between strain matings. This index was defined as:

$$ISI = \frac{(WW + LL) - (WL + LW)}{(LL + WW + LW + WL)}$$

where WW is the number of matings between wild females and wild males, LL is the matings between laboratory females and laboratory males, WL is the matings between wild females and lab males and LW is the matings between laboratory females and wild males. Calculation of the index does not require that both sexes are represented, so it can be used in both bisex and male-only situations. Thus in the case of complete behavioral isolation, the ISI would be 1, and with no isolation (random mating), the ISI would be 0. Hence the smaller the ISI value, the less the behavioral isolation between the 2 strains. Also, positive or negative ISI values indicate positive or negative assortative mating.

Male Mating Competitiveness (C) Based on Egg Hatch

Fried (1971) proposed a method to estimate the competitiveness for sterilized insects that is independent of the ratio of treated to normal insects used, and that can he determined from data collected from a test involving only 1 sterile to fertile ratio, provided that the egg-hatch data are known for matings between untreated insects and matings between sterilized and untreated insects. Thus Fried's formula for calculating male mating competitiveness (C) based on egg hatch data is as follows:

$$C = \left(\frac{W}{S}\right)\left(\frac{Hc - Ht}{Ht - Hs}\right)$$

where W is the number of wild males in the field cage, S is the number of sterile males in the field cage, Hc is the is the egg hatch percentage from wild females in the control cage, Ht is the egg hatch percentage from wild females in the field cage for a particular treatment, and Hs is the egg hatch percentage from females mated with sterile males in the laboratory sterility cage. A Fried test C value of 1 indicates equal competitiveness between sterile and wild males, and values < 1 indicate that the irradiated release strain is less competitive than the wild strain.

In trial 2, the C value was calculated in 2 ways but in all cases the same field cage egg hatch (Ht) was used in all computations with the above formula. Firstly, C was calculated by using hatch data from the laboratory cages as is normal practice to determine Hc and Hs. However we also used egg hatch from individual moths from the field cages, aggregated by mating type, to determine Hc and Hs and thus calculate the C value and we termed this C^1. In trial 3, the C value was also calculated in this way. Mating pairs were not captured but mating type, e.g., sterile male × wild female, was calculated using the egg hatch value from the "wild" females. This was possible because females were collected and egged individually, and in the case of a bisex release treatment either the sterile or wild female was marked. This marking was not necessary for male-only releases. Moths each with 1 or more spermatophores, which indicated that they had mated, and with an egg hatch rate of less than 7% were considered to have mated with an irradiated male. This was based on published data (Jang et al. 2012) which indicated that eggs oviposited by untreated females mated with 300 Gy irradiated male moths had an average hatch of 3.8%. Other unpublished data (Don McInnis, personal communication) indicated that eggs oviposited by untreated females that had mated with males

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that had been irradiated with 300 Gy as pharate pupae had an average hatch of 5.1 ± 0.8%. Therefore egg hatch equal to or below 7% was considered to be from a wild female mated with an irradiated male and above 7% from a wild female mated with a wild male.

SPERMATOPHORE DISSECTION

Spermatophore dissection was carried out to confirm mating. Moths were collected live if possible and quickly killed in a freezer before dissection in 70% alcohol under a binocular microscope. Moths that died before dissection and that were dried out were soaked overnight in a detergent solution to soften them before dissection.

STATISTICAL ANALYSIS

A 2-way ANOVA (trial 1) and Student's t-test (trials 2 and 3) were used where appropriate for multiple and pair-wise comparison testing, respectively, in order to identify significant differences between treatments. Normality and equality of variances were tested and met in all cases and level of significance was tested at $P = 0.05$.

Results

Trial 1

The proportion of mating (PM) in cages during trial 1 was in the range of 0.2 to 0.8 with an average of 0.5 with no difference between treatments (Table 2). The RSI value (0.35–0.44) was not significantly affected by the level of irradiation applied ($F = 0.07$; df = 1,8; $P = 0.792$), nor by the phenological stage (pupa or adult) irradiated ($F = 0.71$; df = 1,8; $P = 0.425$) (Table 2). Similarly for the ISI (0.02–0.46), no significant effects of irradiation dose ($F = 3.13$; df = 1,8; $P = 0.115$) or stage treated ($F = 0.20$; df = 1,8; $P = 0.664$) were detected (Table 2). As the irradiated females also laid eggs, which did not hatch, it was impossible to accurately calculate the Fried C value; therefore these data are not presented.

Trial 2

In trial 2, the PM in the field cages ranged from 0.1 to 0.4 with an average of 0.2 with no difference between treatments (Table 3). Average RSI values for the 250 Gy and 300 Gy treatments were 0.36 and 0.33, respectively, with no significant difference ($t = 0.24$; df = 6; $P = 0.822$,). Mean ISI was 0.31 and 0.14 for the 250 Gy and 300 Gy treatments, respectively, with no significant difference detected ($t = 1.55$; df = 6; $P = 0.171$). There was no difference in the calculated C values when using the standard laboratory protocol or when using egg hatch data from moths in field cages. Irradiation dose had no significant effect on C (Table 3).

Trial 3

In trial 3, the PM was calculated based upon the number of females that had spermatophores, and the average PM was 0.6. Values for RSI and C are presented in Table 4. Though the data for both parameters showed an advantage in favor of male-only releases, that advantage was not statistically significant for the RSI ($t = -1.19$; df = 6; $P = 0.28$) and the C values ($t = -1.75$; df = 6; $P = 0.131$). Individual egg hatch values of the different replicates used to calculate the C value are presented in Table 5.

In the laboratory unirradiated females that had mated with irradiated males contained an average of 1.4 spermatophores/female with a maximum of 2 spermatophores/female, whereas unirradiated females that had mated with unirradiated males contained an average of 1.6 spermatophores/female with a maximum of 3 spermatophores/female. In moths collected from field cages in the morning, the number of spermataphores from mated females varied from 1 to 2 with a mean of 1.03 for females mated with irradiated males and 1.13 for females mated with unirradiated males. Overall 7% of females from field cages had mated twice.

Table 2. Proportion of Mating (PM), Relative Sterility Index (RSI), and Index of Sexual Isolation (ISI) of irradiated male LBAM moths in field cages (Trial 1). Treatments were replicated 3 times.

Treatment	PM ± SE	RSI ± SE	ISI ± SE
200 Gy pupa	0.56 ± 0.11	0.39 ± 0.32	0.02 ± 0.18
200 Gy moth	0.49 ± 0.13	0.44 ± 0.17	0.21 ± 0.18
300 Gy pupa	0.56 ± 0.08	0.35 ± 0.10	0.46 ± 0.11
300 Gy moth	0.50 ± 0.18	0.44 ± 0.06	0.43 ± 0.12

Table 3. Proportion of Mating (PM), Relative Sterility Index (RSI), Index of Sexual Isolation (ISI), and male mating competitiveness (C) of irradiated male LBAM moths in field cages (Trial 2). Treatments were replicated 4 times. Calculation of C involved the use of hatch data from the laboratory cages to determine Hc and Hs—as is normal practice. However the calculation of C^1 involved the use of egg hatch data from individual moths from the field cages, aggregated by mating type, to determine Hc and Hs.

Treatment	PM ± SE	RSI ± SE	ISI ± SE	C ± SE	C^1 ± SE
250 Gy	0.18 ± 0.11	0.36 ± 0.07	0.31 ± 0.03	0.5 ± 0.2	0.5 ± 0.3
300 Gy	0.24 ± 0.14	0.33 ± 0.07	0.14 ± 0.09	0.3 ± 0.2	0.3 ± 0.2

Discussion

Values of the ISI can vary from −1 to +1 with 0 indicating random mating, and positive or negative values indicating positive or negative assortative mating. In trial 1 where irradiated and non-irradiated moths were all from the same laboratory strain—thus, there should have been no mating isolation—values varied from 0.02 to 0.46 but were statistically not significantly different. In the case of the Mediterranean fruit fly, ISI values usually varied between 0.1 and 0.4 and values above 0.5 indicate a moderate to high level of positive assortative mating (FAO/IAEA/FAO 2003). In trial 2, where laboratory moths were of South Australian origin and wild moths were from Western Australia values varied from 0.14 to 0.31 demonstrating sexual compatibility and only a slight tendency for homotypic (i.e., like with like) mating (Cayol et al. 1999).

In our trials when both males and females were irradiated and released there was little difference in competitiveness at different doses when measured as proportion of matings by irradiated males (RSI). Therefore, an advantage of using a dose lower than 300 Gy would not come from an increase in sterile moth competitiveness; rather, an advantage might come from inherited sterility (Kean et al. 2008), and

Table 4. Relative sterility index (RSI), and male mating competitiveness (C) of irradiated male LBAM moths in experiments involving either treated males and females (bisex releases) or treated males-only that were released into walk-in field cages (Trial 3). Treatments were replicated 4 times. The RSI estimates involved the use of mating data based on the presence of spermatophores in the female.. The calculation of C^1 involved the use of egg hatch data from individual moths from the field cages, aggregated by mating type, to determine Hc and Hs.

Treatment	RSI ± SE	C^1 ± SE
300 Gy moth bisex	0.48 ± 0.11	0.85 ± 0.52
300 Gy moth ♂ only	0.64 ± 0.08	2.20 ± 0.57

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Table 5. Egg hatch data for individual replicates in trial 3 from which the C values were calculated.

	Replicate	Hc	Ht	Hs
300 Gy moth bisex	1	42.9	24.1	2.9
	2	24.7	22.3	0.0
	3	38.9	12.1	0.6
	4	68.7	56.1	6.1
300 Gy moth ♂ only	1	41.9	26.5	1.3
	2	45.0	11.8	1.7
	3	44.8	13.1	1.0
	4	42.3	14.3	1.9

Where Ht = egg hatch in treatment cage, Hc = hatch of eggs from matings between wild males and wild females and Hs = hatch from matings between sterile males and wild females.

this would only be possible if the moths were irradiated below 250 Gy (Soopaya et al. 2011; Jang et al. 2012).

The greatest, but not significantly different, RSI values were obtained only when male-only releases were made in the field cages, suggesting there may be an advantage in this approach. It has been assumed that bisex releases of lepidopterans are effective because females attract mates and reduce matings of wild males with wild females, and this would outweigh the disadvantage of sterile females being a sperm sink for sterile males (Stringer et al. 2013). In the case of tephritid fruit fly SIT, the release of only males avoids the fruit damage caused by oviposition attempts by irradiated females (sterile stings), avoids matings between sterile males and sterile females and increases dispersal of sterile males searching for wild females (Rendon et al. 1998). In the present case, until an effective method of sexing pupae of LBAM is developed the advantage of releasing only males can be tested only at a small scale; hence its effectiveness at a large field scale will remain only hypothetical.

The Fried test gives a measure of true mating competitiveness as expressed by egg hatch. In trials 2 and 3, the obtained C values were above 0.3 which is considered acceptable for fruit flies (USDA/FAO/IAEA 2003). In trial 2, there was no difference in C values whether they were calculated using sterility and egg hatch data from untreated insects kept in small laboratory cages or by using egg hatch data of individual moths collected from field cages. The advantage of collecting and egging individual moths and using an aggregate of the values to calculate C means that all values can be obtained from the field cage matings, thus avoiding the need to set up additional laboratory cages. This is advantageous in terms of logistics and in terms of deriving a result that is closer to natural mating behavior in the wild.

In trial 3, a minimum intervention approach was used with both bisex and male-only releases. Not only were the RSI and C values greater than in the other trials, but C values of the male-only treatment were greater than those of the bisex treatment, although not significantly so. This approach probably best mirrors the real world situation as impacts from observers are minimized. However, this approach is dependent on the assignment of the different mating types on the basis of egg hatch. Both sterile ♂ × sterile ♀ and sterile ♂× wildish ♀ matings inevitably will produce some, but consistently low, egg hatch. Therefore, an upper limit on the average egg hatch value below which occur virtually all hatch values from these mating types, must be set—albeit somewhat arbitrarily; and in our studies with LBAM, we set it at 7%.

However, infrequently a mating between a wild male and a wild female is consummated with the transfer of a spermatophore, but without the production of viable eggs. This, combined with the wide variability of egg hatch from wild ♀ × wild ♂ matings (Bill Woods, unpublished data) poses a question as to the validity of this minimal intervention approach. Trials that would compare the egg hatch from females mated to marked male moths with the hatch of eggs of females mated to unmarked male moths would help determine the accuracy of mating assessments based on egg hatch alone. Additionally the conduct of a much larger number of replicates would reduce the standard error, improve the accuracy of mating assessment based on egg hatch, and increase the potential for detecting significant difference among treatments.

In general, male tortricid moths mate more than once (Howell 1991), and LBAM is no exception. Stringer (2013) reported an average of 4.8 matings per laboratory female of non-irradiated males compared to 1.3 matings per female by males irradiated with 300 Gy in laboratory trials. This may impact on the effectiveness of the SIT, and the most effective way to reduce this impact is by use of a larger sterile to wild male over-flooding ratio in the field. Of more concern is whether wild females mated to sterile males are more likely to remate than wild females mated first with wild males. These potential negative impacts on the SIT need to be assessed experimentally and would require that females can actively discriminate between fertile and sterile males and that the sperm from successive matings do not compete in a random manner.

Multiple mating of female moths was observed in this study, especially under laboratory conditions. When provided with multiple mating opportunities, LBAM females mated up to 3 times. In other laboratory trials, the pairing of 1 female with 3 male moths (Soopaya et al. 2011), resulted in the transfer of up to 5 spermataphores per dissected female (Bill Woods, unpublished data). Knight (2007) reported that codling moth females paired at the same 1 to 3 ratio, contained an average 2.2 spermatophores per female upon being dissected, ranging from 1 to 7 per female. Therefore some females of these 2 tortricid species will mate more than once under laboratory conditions when multiple partners are available. However in our trials, the situation in the field cages was very different and only limited multiple matings occurred, irrespective of whether males were irradiated or unirradiated. It could be argued that this was because females were only in the field cage for one night, so a longer mating period may have resulted in more matings. However a result similar to our result was observed in trials performed in Perth (Bill Woods, unpublished data) in which live virgin females were placed in traps in the field for 3 d, and later dissected for evidence of mating. An average of 1.1 spermatophores per female was observed with a maximum of 2 spermataphores per female.

Knight (2007) noted that the codling moth was polyandrous in crowded laboratory conditions and in orchards with high population densities, but Howell (1991) observed that mated codling moth females generally discontinue calling and do not remate. Stringer et al. (2013) used stable carbon isotopic analysis of LBAM spermataphores to determine whether females caged in small field plots had been mated with wild or a small number of released irradiated laboratory males. They showed that 97% of the mated females had mated only once (range 1–3) and 13% of matings were from irradiated males. Use of this technique to separate matings from sterile or wild males has potential to study the impact of multiple mating on the effectiveness of the SIT, but it appears that multiple mating is unlikely to have a major impact especially if the wild population is reduced to low levels before sterile moths are released.

Field cage tests are accepted as the best way to measure competitiveness of sterile fruit flies (Cayol et al. 1999; FAO/IAEA/USDA 2003; Cáceres et al. 2007). As Shelly (2004) commented "the success of the SIT depends, to a large degree, on the ability of released sterile males to attract and obtain matings with wild females. This consideration is especially important for a species, such as *C. capitata,* in which females

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display a high degree of mate discrimination". This is probably not the case with most moth species whose females do not actively choose mates and leks do not occur. Male moths orientate along a pheromone plume to find the female and mate without any obvious courtship behavior (Suckling 2011). In these simple mating systems (Lance & McInnis 2005), males need to fly and respond to females that are calling, and this will usually result in a mating.

Our study assessed the mating competitiveness of LBAM in field cages for the first time. We do not yet know what are acceptable RSI and C values for irradiated moths but it appears that irradiation at the doses tested does not impact significantly on mating performance. The use of a bisex strain in which female as well as male competitiveness are important, requires the development of new mating indices to tease out the relative importance of the competitiveness of each sex. Modifying the male relative performance index (MRPI) and the female relative performance index (FRPI), which measure differences in mating between wild and sterile males and wild and sterile females, may have potential (Cayol et al. 1999). The practical difficulties of dealing with a species which mates after dark will need to be overcome, and light, temperature and wind thresholds for mating need to be investigated. The use of a modified Fried test with minimal human intrusion into the mating environment is an approach that showed promise in this study.

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