Sexual transmitted death of female Queensland fruit flies from horizontal transfer of Amulet Cue-lure Male Annihilation Technique devices.

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Summary

Queensland fruit fly, *Bactrocera tryoni*, is one of Australia's most damaging fruit fly pests. Male Annihilation Technique (MAT) is a method used to manage fruit fly populations by luring and killing male flies, which limits population growth by preventing female flies from mating. MAT devices consist of an attractant and a toxicant, such as maldison or fipronil. Fipronil has a slow knockdown effect, which has been exploited to induce indirect toxicity of a number of insect pests, including cockroaches and termites. This indirect effect, called horizontal transfer, is facilitated through the sharing of food or contact with exposed individuals. Horizontal transfer has been reported in Queensland fruit fly, where exposed males have transferred lethal doses of fipronil to virgin females during courtship and mating. In the present study, we explored three possible modes of horizontal transfer of fipronil against Queensland fruit fly under laboratory conditions. These included: (1) direct contact of females with exposed males (2) contact of males to the regurgitant of exposed males and (3) contact of males with dead exposed males. Our results demonstrated that lethal doses of fipronil were not transferred from males to females during courtship and mating. Male flies that were exposed to fipronil one and six hours prior to dusk were all knocked down, moribund or dead at dusk and therefore did not participate in courtship displays or mating. However, we found that horizontal transfer and subsequent death occurred in males that fed on the regurgitant of exposed males, and in males that were in contact with dead exposed males. Therefore, MAT devices that contain fipronil should not be used as a stand-alone treatment but rather be used in combination with other control methods, such as protein bait sprays to ensure the control of female flies.

Table of contents

Background

Queensland fruit fly, *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae), is the most economically important fruit fly species in Australia (Drew et al., 1982). The impact of this pest on fruit and vegetable production has increased over recent years due to its expanding distribution, as a result of favourable weather conditions (Dominiak and Mapson, 2017). All eastern states of Australia are now deemed to have endemic populations, which imposes significant quarantine and market access barriers (Dominiak and Mapson, 2017). Traditionally, pre-harvest control of Queensland fruit fly relied on the use of broad spectrum organophosphate insecticides (Dominiak and Ekman, 2013). A recent review of dimethoate and fenthion by the Australian Pesticides and Veterinary Medicines Authority has resulted in the restriction and removal of the chemical use patterns for many commodities (APVMA, 2011, APVMA, 2014). Therefore, to achieve adequate protection against fruit flies, management programs require an integrated approach that consists of a combination of control measures (Dominiak and Ekman, 2013). As predicted by Clarke et al. (2011), Male Annihilation Technique (MAT) and protein bait sprays are likely to become more important to manage this pest.

Male Annihilation Technique is a method that is widely used within management programs to control and eradicate fruit fly populations (Clarke et al., 2011). MAT devices consist of a lure, commonly cue-lure or methyl eugenol, combined with an insecticide to kill adult male fruit flies. When sufficient numbers of MAT devices are placed in a given area, high levels of resident males are killed (Cunningham, 1989). This results in a lower ratio of males to females, which limits the success of females locating and mating with males (Cunningham, 1989). Gradually the fruit fly population decreases over time, limiting the risk of infestation occurring in the crop (Lloyd et al., 2010). Amulet Cue-lure stations (Nufarm) are one type of MAT device used for pre-harvest control of fruit flies. These stations contain the active ingredient fipronil to kill adult flies, which is a slow acting insecticide (Holmes and Bull, 2007). Alternative types of MAT devices contain maldison (malathion) and cause males to die rapidly (Lloyd et al., 1998).

The slow acting effect of fipronil has been shown to induce indirect toxicity to a number of insect pests, including cockroaches (Buczkowski and Schal, 2001), termites (Ibrahim et al., 2003) and ants (Soeprono and Rust, 2004). This indirect effect, called horizontal transfer, refers to the passing of a lethal dose to another individual that results in the death of both insects (Buczkowski and Schal, 2001). Horizontal transfer is facilitated through the sharing of food, grooming, or contact with exposed insects (Buczkowski and Schal, 2001, Ibrahim et al., 2003, Soeprono and Rust, 2004). Horizontal transfer has been reported by Bull (2010) in Queensland fruit fly, where lethal doses of fipronil were transferred from exposed males to females. Bull (2010) states that horizontal transmission of fipronil occurred during courtship and mating. According to Bull (2010), several mating pairs were observed during dusk and it was noted that females that died did not contain liquid within their crops. However, further chemical analysis of the female's integument found residues of fipronil present. Based on these observations the patent for Amulet Cue-lure states that it is possible to base fruit fly control on Amulet Cue-lure stations alone (Holmes and Bull, 2007). A similar trial conducted by Spafford et al. (2018) found that horizontal transfer of fipronil occurred in Melon fly, *Zeugodacus cucurbitae* (Coquillett) (Diptera: Tephritidae). However, this research found that fipronil was not transferred from males to females during mating, as all males were significantly impaired or dead within two hours of exposure (Spafford et al., 2018). The authors' findings confirmed that regurgitant deposited by exposed males was the source of horizontal transfer (Spafford et al., 2018).

Project Objectives

In the present study, we explored the claims of Bull (2010) regarding the ability of fipronil in Amulet Cue-lure stations to be horizontally transferred to female Queensland fruit fly. We assessed three possible sources of horizontal transmission independently in order to definitively confirm whether these impacted on fly mortality using methods adapted from Spafford et al. (2018). The sources of transmission that were assessed were: (1) direct contact of females with exposed males (2) contact of males to the regurgitant of exposed males and (3) contact of males with dead exposed males.

If the claims can be substantiated that Amulet Cue-lure stations can control both male and female fruit flies, these MAT devices containing fipronil potentially offer added benefits over alternative MAT devices that contain fast knockdown chemicals such as maldison.

Methodology

Insect rearing

The parental line of the Queensland Fruit Fly (*B. tryoni*) colony was established in 2017 by the Department of Agriculture and Fisheries (Queensland) at the Ecosciences Precinct in Brisbane from fruit collected from the wild. Pupae were placed in a mesh-sided cage (300 × 300 × 450 mm). When adults emerged, females were removed and placed into a mesh cage (300 × 300 × 300 mm) separate to males. Males remained in the initial cage, which contained a mixture of both males and females. Both cages were provided yeast hydrolysate enzymatic (MP Biomedicals, USA) (180 g yeast & 50 mL water), and a yeast-sugar combination (330 g yeast, 33 g sugar & 100 mL water) to enable sexual maturity. All cages were maintained in a controlled environment room (CER) at 26°C (± 1°C) and 70% relative humidity (± 0.5%) and received natural and artificial light (10.5L:13.5D h photoperiod). The artificial lighting system and automatic timer operated to sequentially reduce the level of artificial light to simulate the natural reduction in light at dusk. All artificial light was extinguished by 16:00 h daily, leaving only the natural light associated with dusk. Flies used in all replicates were taken from the main breeding colony and were of the F10 and F11 generation. Experiments were conducted 8 - 10 days after adult emergence.

Laboratory experiments

Direct contact of females with exposed males- six hours prior to dusk

Twelve males were placed in a single 100 mL lidded container (48 mm high × 75 mm diameter) (BS-4; BS-4L, Bonson Industrial Company Limited, NZ) through a 12 mm hole in the container at approximately 1200 hours (h) (6 h prior to dusk). Male flies were placed directly onto one Amulet Cue-lure station (Nufarm, Australia) for four minutes and allowed to freely move and feed. The hole was sealed with a No.3 cotton dental roll (DENTROL3-500, Livingston International Pty Ltd, Australia). Twelve males were placed in a similar 100 mL container without an Amulet Cue-lure, and were provided a cotton dental roll impregnated with 1 mL Cue-lure (FT-Cue-lure, Farma Tech International USA), with no insecticide. These were the control group and flies were also allowed to freely move and feed for four minutes.

Ten virgin female flies were placed in a single 1 L lidded container (173 × 119 × 69 mm) (BS-1000A; BS-LID, Bonson Industrial Company Limited, NZ) with ten males that had been exposed to the Amulet Cue-lure station or the control group. Sugar-water solution (20% sugar) was provided through a 12 mm hole in the side of the container on a cotton dental roll. The number of mating pairs, courtship behaviour and effects of insecticide toxicity were recorded at 0.5 h intervals for 6 h, from approximately 1200 to 1800 h. The effects of insecticide toxicity were knockdown, defined as the state of intoxication resulting in uncontrolled spinning on the cage floor; morbidity, the stage at which flies were immobilised and laying on the cage floor with legs twitching or mouthparts moving; and death, where flies were completely motionless and did not respond to a probing stimulus. Final observations were recorded at 24 h post-commencement. A total of four cages for each of the Amulet Cue-lure station and control group were conducted, which was replicated over three successive weeks.

Direct contact of females with exposed males- one hour prior to dusk

As described above, male flies were exposed to one Amulet Cue-lure station or the control group. Males were fed at approximately 1700 h (1 h prior to dusk). As above, females were paired with males in 1 L lidded containers and provided sugar-water solution (20% sugar). The number of mating pairs, courtship behaviour and effects of insecticide toxicity (knockdown, morbidity, death) were recorded at

five minute intervals for 1 h, from approximately 1700 to 1800 h. Final observations were recorded at 24 h post-commencement. A total of four repetitions for each of the Amulet Cue-lure station and control group were conducted, with three replicates performed over successive weeks.

Contact of males to the regurgitant of exposed males

Ten males were placed in a single 1 L lidded container and provided one Amulet Cue-lure station. In a separate container, ten males were placed in a similar 1 L container without an Amulet Cue-lure, and provided a cotton dental roll impregnated with 1 mL Cue-lure (FT-Cue-lure, Farma Tech International USA). These were the control group. Amulet Cue-lure stations and cotton rolls were placed on a 90 mm Petri dish inside the container to prevent direct contact and contamination of the surrounding container. Male flies were allowed to freely feed for at least 12 h then the containers were placed in a freezer for 3-4 h to kill any alive flies. The containers were removed from the freezer and allowed to reach ambient temperature. The containers were opened and the Petri dish containing the Amulet Cue-lure station or cotton dental roll was removed. Dead male flies were removed and were set aside for the following trial.

The 1 L containers from which the dead flies were removed were checked for the presence of regurgitant on the inside surfaces. The containers were then resealed and 10 untreated males were placed inside and provided sugar-water solution (20% sugar). Effects of insecticide toxicity (knockdown, morbidity, death) were recorded at 0.5 h intervals for 6 h and at 24 h. A total of six repetitions for each of the Amulet Cue-lure station and control groups were conducted, with three replicates performed over successive weeks. A total of three repetitions for each of the Amulet Cue-lure station and control group were conducted, with three replicates performed over successive weeks.

Contact of males with dead exposed males

Ten dead male flies from the feeding containers mentioned in the above trial were transferred to a clean 1 L container. Ten untreated males were placed inside the container with the dead flies of either the Amulet Cue-lure or control group males, and provided sugar-water solution (20% sugar). Effects of insecticide toxicity (knockdown, morbidity, death) and the behaviour of the flies were recorded at 0.5 h intervals for 6 h, and at 24 h. A total of three repetitions for each of the Amulet Cue-lure and control group were conducted, with three replicates performed over successive weeks.

Statistical analysis

A Generalised Linear Mixed Model (GLMM) assuming a binomial distribution and a logit link function was used to compare the proportion of male and female knockdown, morbidity flies and mortality over time. The number of mating pairs was analysed using a GLMM assuming a Poisson distribution and log link function. In all GLMMs replicate was fitted as a random effect and the effect of time was fitted in the fixed effects model. Where under-dispersion was observed the dispersion parameter was fixed at one. Under-dispersion occurs when the amount of variability in the data is less than could be expected in a binomial distribution.

The control groups resulted in a large number of zeroes due to a lack of response. In such cases no measure of variability could be obtained, therefore, statistical analyses of the proportion of flies affected was only performed on the treated groups. Conversely, only the control groups were included in the analyses of the number of mating pairs, due to a lack of mating by the treated groups. All analyses were conducted using Genstat for Windows 19th Edition 2017 (VSN International).

Results

Laboratory experiments

Direct contact of females with exposed males- six hours prior to dusk

Males actively fed on Amulet Cue-lure stations during the initial four minute exposure period and were observed to move off the stations and regurgitate in the treatment containers. Males continued to regurgitate once transferred to the observation cages and both males and females were observed ingesting this regurgitant.

Males were recorded as knocked down and moribund at the first observation time at 0.5 h and male mortality was first observed at 1 h. A significant effect of time ($p<0.001$) was found for moribund male flies, with the proportion of moribund males peaking between 0.5 and 1.5 h before decreasing significantly as they started to die. The proportion of dead male flies significantly increased over time (<0.001). All males exposed to Amulet Cue-lure were recorded as dead at 4 h, which was approximately 2 h before dusk (Figure 1)

Females that were confined with exposed males were knocked down and moribund from 1.5 h and started dying from 2 h. The proportion of dead female flies significantly increased over time ($p<0.001$). At 6 h, the mean proportion of dead females was 0.919 and increased to 0.995 at 24 h.

The control male group regurgitated and interacted with females, similar to the treated group. Mortality in the control group was very low with a proportion of dead males and females of 0.008 at 6 h.

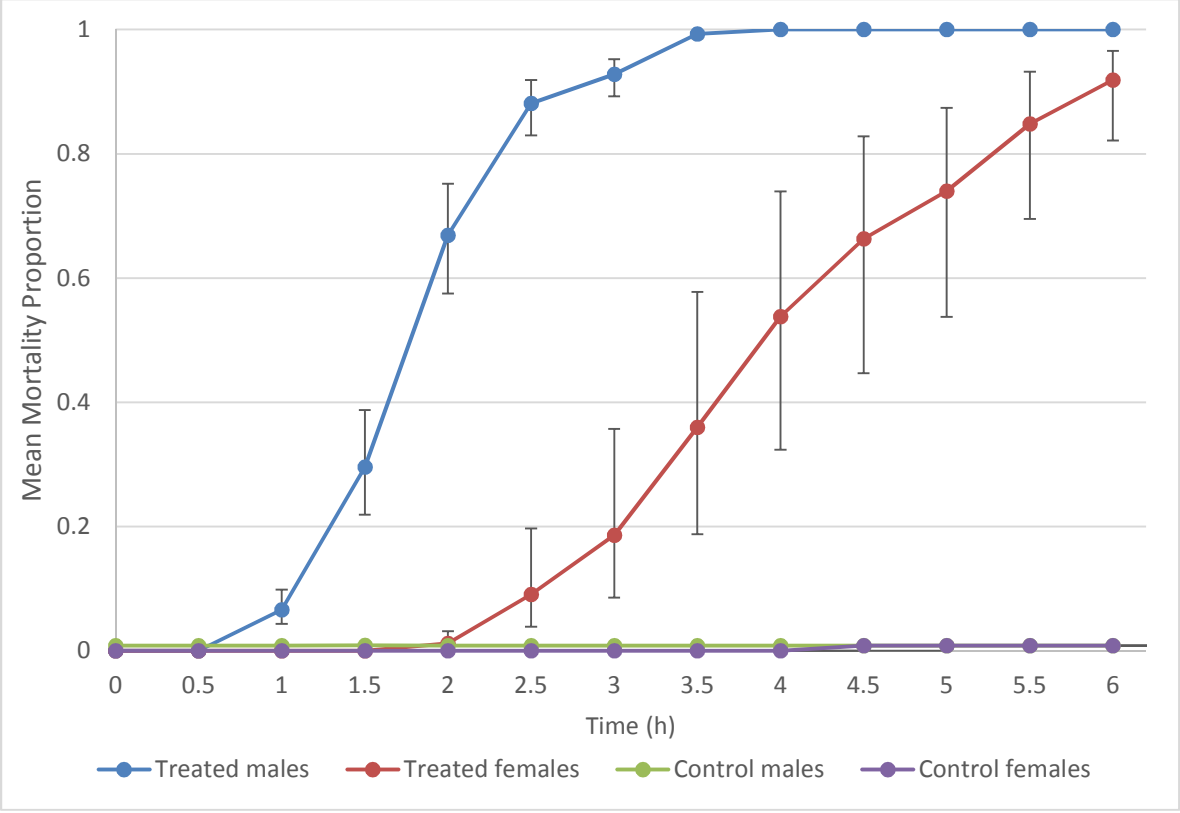

Figure 1 Mean proportion of mortality of female and male flies (± se) exposed to Amulet Cue-lure six hours before dusk.

Observations immediately pre- and post-dusk found that although some female flies were still alive they were not actively engaging in lek-like behaviour consistent with courtship and mating rituals. As a result of the rapid mortality of the males and the absence of alive males at dusk, no mating or courtship behaviours were observed at any time during the experiment in cages where males had been exposed to Amulet Cue-lure (Figure 2).

Courtship behaviour in the untreated control group first consisted of male-male combat observed at 4.5 h. Mating was observed to start at 5 h, as the light levels decreased at dusk. There was no significant effect of time (p=0.241) for mating in the control group. However, male calling and lek-like behaviour was observed to intensify at 5.5 h. Mating reached a peak at 5.5 and 6 h with a mean of 2.1 and 2.4 out of 10 possible matings achieved respectively (Figure 2).

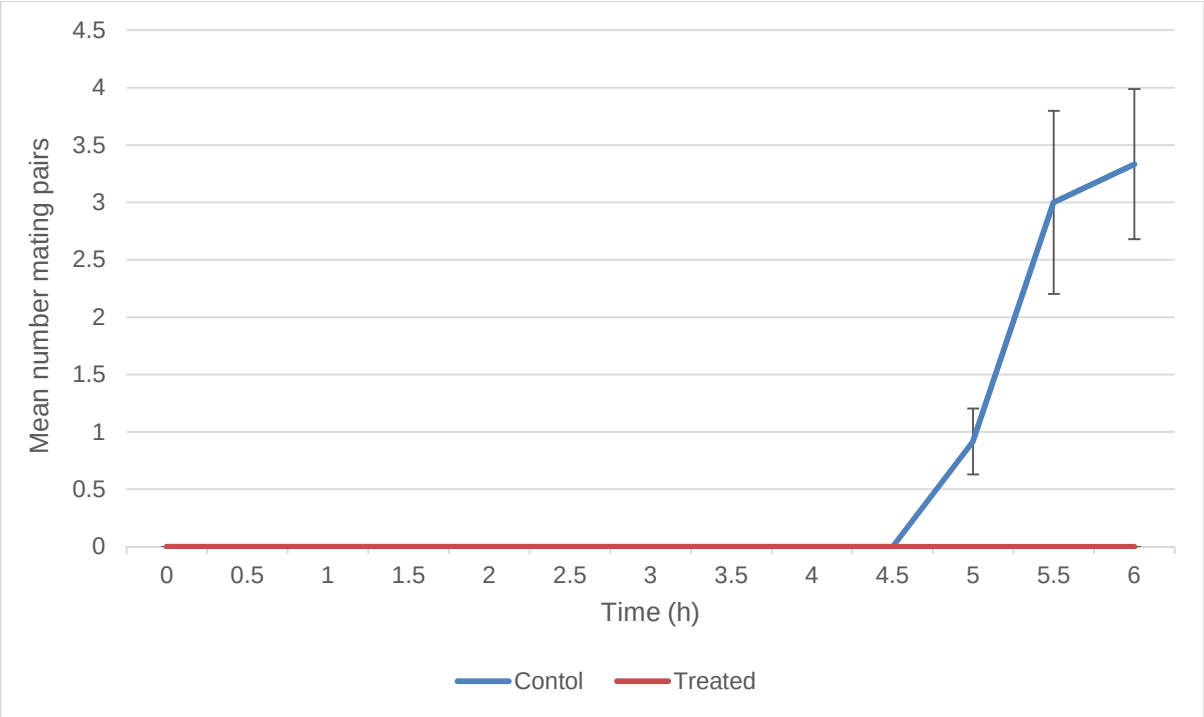

Figure 2 Mean (± se) number of mating pairs formed from a possible number of 10 mating pairs. Male flies were exposed to Amulet Cue-lure (treated) or Cue-lure (control) six hours prior to dusk

Direct contact of females with exposed males- one hour prior to dusk

Males were first recorded as knocked down 15 minutes after exposure to Amulet Cue-lure. A significant effect of time (p=0.026) was found, with the proportion of knocked down males significantly increasing over the duration of the trial. The proportion of knocked down males remained constant and did not

significantly change after 30 minutes. Moribund males were observed at 25 minutes and significantly increased over time ($p<0.001$). A small proportion of 0.008 male flies were dead at 55 and 60 minutes. When measures for knockdown, moribund and mortality were combined, there was a significant increase in the proportion of male flies showing signs of a toxicological affect over time ($p<0.001$). At 60 minutes a mean proportion of 0.86 male flies showed signs of toxicological effects from exposure to Amulet Cue-lure (Figure 3). Over the 60 minute observation period no knocked down, moribund or dead flies were recorded in females paired with exposed males, as well as in males and females from the control groups. At 24 h after exposure, all male flies were dead and a proportion of 0.95 females that were paired with these exposed males were also dead. There was no mortality of females and a proportion 0.025 dead males in the control group.

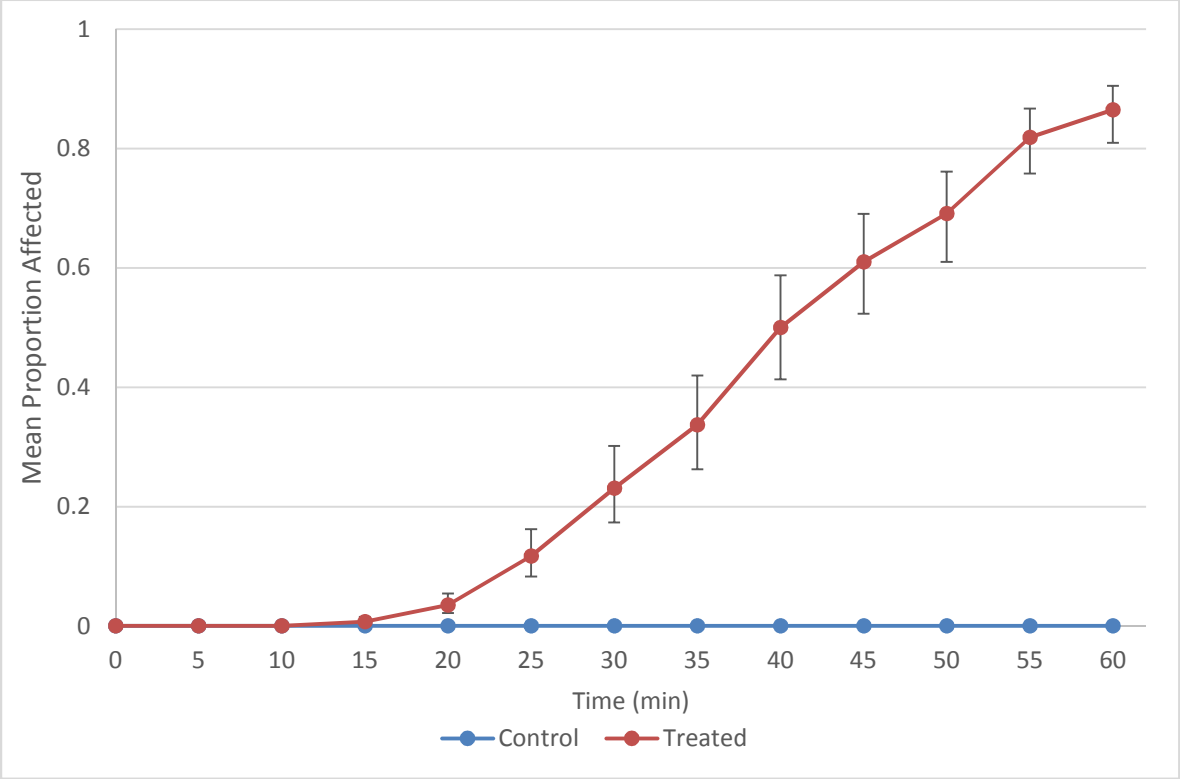

Figure 3 Mean combined proportion of knocked down, moribund and dead males (± se) after exposure to Amulet Cue-lure one hour prior to dusk.

In observation cages containing exposed males a single observation of male-male combat was observed at 15 minutes. Similar combat exchanges were observed between females, especially around dusk. Females further exhibited courtship behaviours that included fast wing beating, rapid walking and small jumps. Although females that were paired with exposed males were engaging in courtship rituals, there was a distinct divide of the sexes. The females congregated at the top of the cage while the males did not move above the bottom one quarter of the cage because they were affected by the insecticide. No mating was observed in observation cages containing exposed males (Figure 4).

In the control cages, mating and courtship behaviours that were consistent for this species were observed. These included male-male combat, fast wing beatings, attempted mounting and successful mounting/copulation. Mating commenced at 10 minutes and significantly increased over time ($p<0.001$). Between 35 to 60 minutes the mean number of mating pairs did not significantly increase and ranged between 1.7 and 2.3 mating pairs (Figure 4).

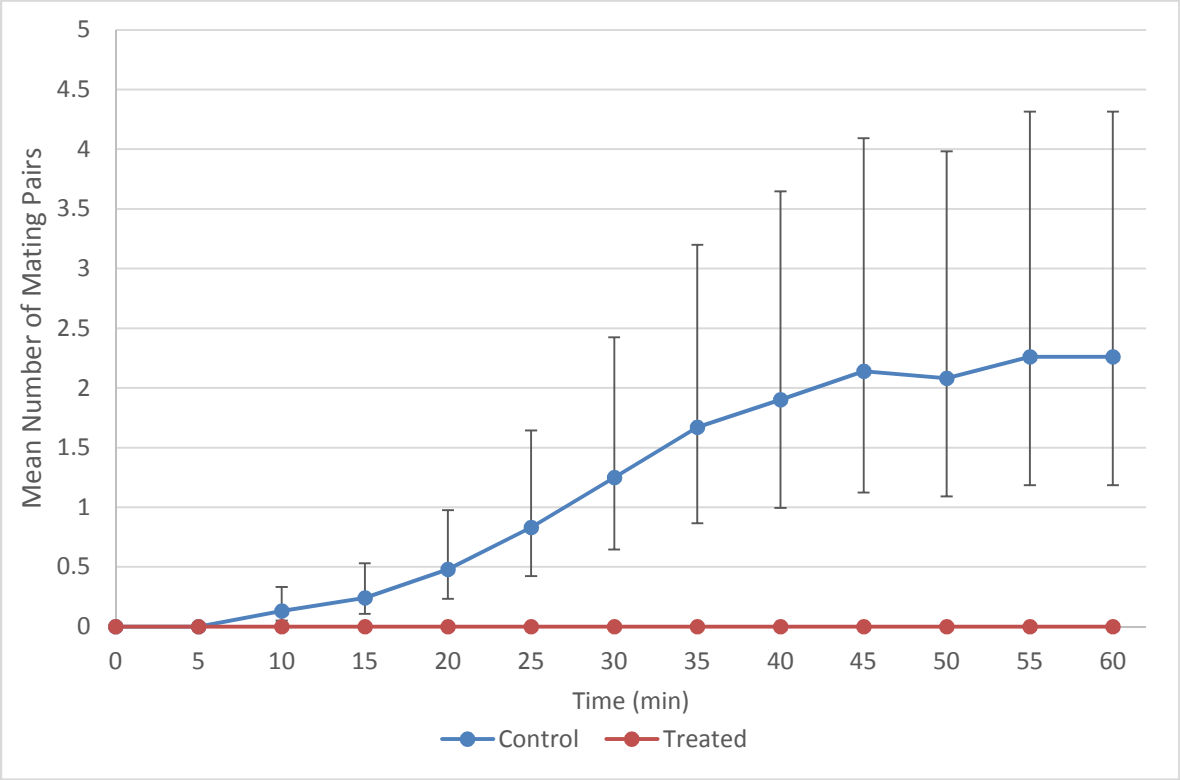

Figure 4 Mean number of mating pairs (± se) formed from a possible number of 10 mating pairs. Male flies were exposed to Amulet Cue-lure one hour prior to dusk.

Contact of males to the regurgitant of exposed males

Males were first recorded as knocked down and moribund 1 h after contact with the regurgitant of Amulet Cue-lure exposed males. The number of knocked down males significantly increased over time ($p=0.023$), as did the number of moribund males ($p=0.002$) (Figure 5). Dead flies were recorded from 1.5 h and significantly increased over time ($p<0.001$). Across all trials and replicates, all males were recorded as dead at 24 h when they were placed in cages containing regurgitate of exposed males. In the control group, a small proportion of 0.011 dead males were observed at 24 h, but due to the general lack of response, this group was not included in the analysis.

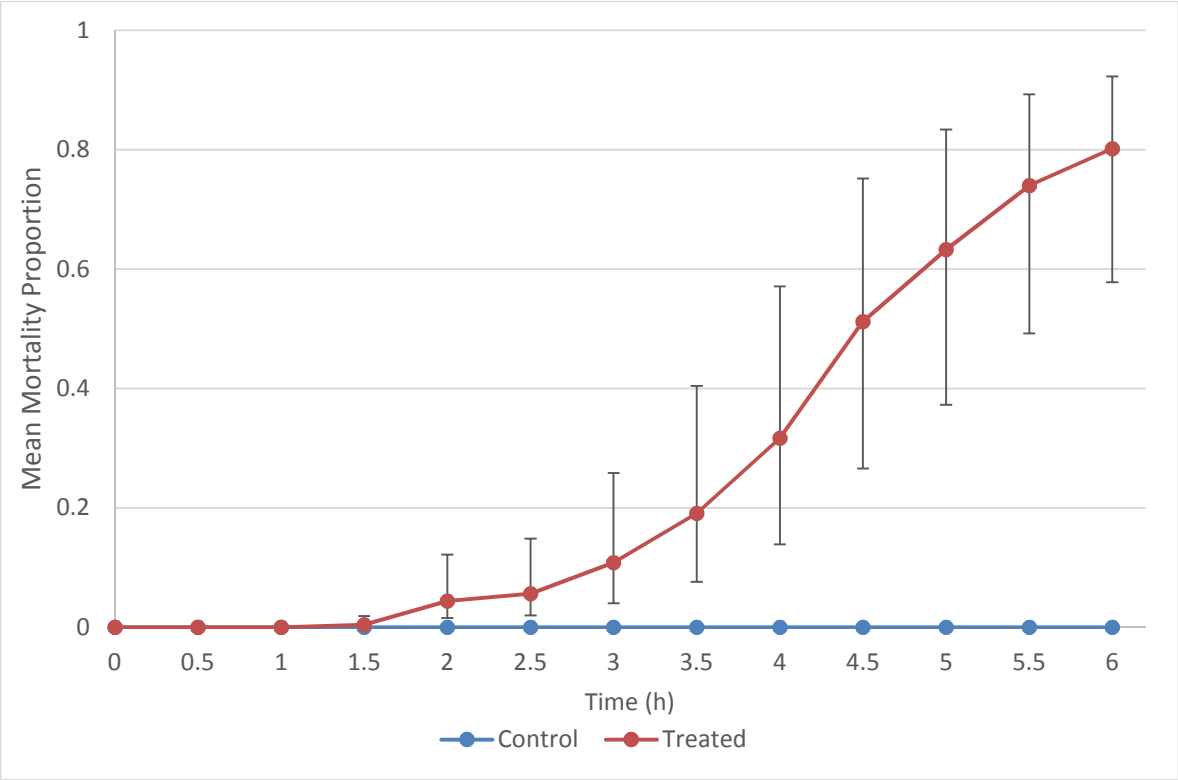

Figure 5 Mean proportion of mortality (± se) after exposure to regurgitate.

Contact of males with dead exposed males

Untreated male flies were placed in observation cages containing dead male flies that were either exposed to Amulet Cue-lure or Cue-lure only. Untreated males flies were observed walking over dead flies and contacting them with their mouthparts. Knocked down and moribund flies were observed at 1 h in cages containing dead males that were exposed to Amulet Cue-lure. A significant effect of time ($p=0.002$) was found for moribund flies, however not for knocked down flies ($p=0.075$). The proportion of moribund flies increased significantly from 1 h to 1.5 h before slowly decreasing after 3.5 h as these flies died. A significant effect of time ($p=0.001$) was recorded for dead flies, with the proportion of dead flies significantly increasing over time. No dead flies were observed before the 1.5 h and at 24 h a proportion of 0.98 flies were dead in cages containing dead males exposed to Amulet Cue-lure.

When measures for knockdown, moribund and mortality were combined, there was a significant increase in the proportion of male flies showing signs of a toxicological affect over time ($p<0.001$). After 6 h a mean proportion of 0.964 male flies showed signs of toxicological effects from exposure to Amulet Cue-lure (Figure 6).

Mortality of the control group was restricted to a proportion of 0.01 up to 6 h and 0.03 at 24 h. No flies in the control group were observed as knocked down or moribund.

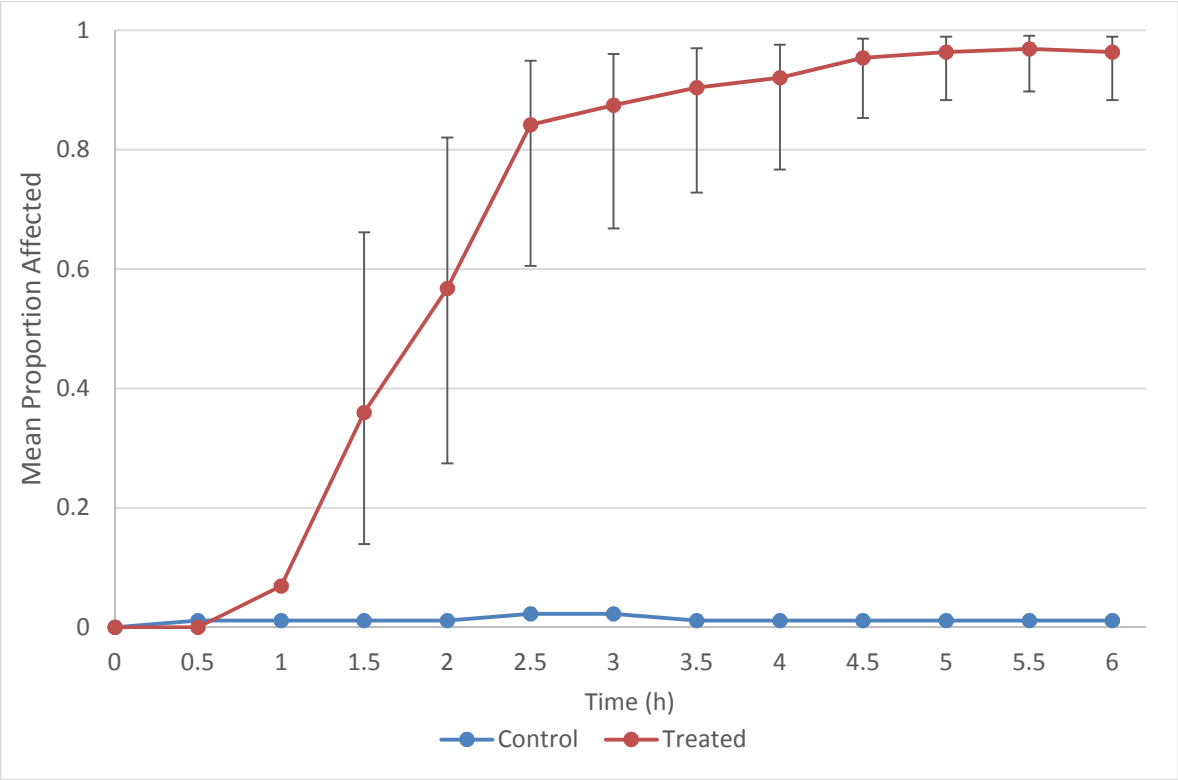

Figure 6 Mean proportion of knocked down, moribund and dead male flies (± se) after exposure to treated flies that had been treated with fipronil.

Conclusions/Significance/Recommendations

Fipronil is a slow acting insecticide that has the ability to indirectly effect insect pests through horizontal transfer. Lethal doses of fipronil can be passed from an exposed individual by sharing of food, grooming and direct contact. Fipronil is the active ingredient in Amulet Cue-lure stations and field trials conducted by Bull (2010) suggested that horizontal transfer occurred between exposed males and females during courtship and mating at dusk.

In trials conducted by Bull (2010), sexually mature virgin males and mature virgin female Queensland fruit flies were released into large field cages. An Amulet Cue-lure station was installed in the cage several hours prior to dusk and remained in the cage for the duration of the experiment. Feeding and mating was observed on the initial day of exposure, then mortality recorded for four days after treatment. During the first evening, low numbers of mating pairs were observed and Bull (2010) concluded that the sex drive of males was unaffected after exposure to fipronil. Chemical analysis was conducted on females that died during the experiment and these results showed that fipronil residues were present on the integument of dead females (Bull, 2010). Bull (2010) suggested that lethal doses of fipronil were transferred from male Queensland fruit flies that fed directly on Amulet Cue-lure to females during courtship displays and mating. In addition, Bull (2010) suggested that transfer of lethal doses of fipronil to females also occurred by females contacting and ingesting fipronil laced residues, regurgitant deposited on leaf surfaces by male flies and direct physical contact by a female with an Amulet Cue-lure station.

These trials conducted by Bull (2010) were significantly flawed because each pathway of horizontal transfer was not separately assessed. The number of males that fed on Amulet Cue-lure stations were not recorded, therefore it cannot be certain that all male flies consumed fipronil prior to mating at dusk. In addition, Amulet Cue-lure stations remained in cages for the duration of the experiment, which could allow females to directly feed on fipronil. Although Cue-lure is a male attractant, there is evidence that female Tephritid fruit flies respond to male para-pheromones. It has been observed that sexually mature virgin female Mediterranean fruit fly (*Ceratitis capitata*) respond to trimedlure (Nakagawa et al., 1970). Similarly, virgin female Northern Territory fruit fly (*B. aquilonis*) are significantly attracted to synthetic male lures (Fitt, 1981). Therefore, the mating pairs observed by Bull (2010) could plausibly have been a result of unexposed males mating with unexposed females. The subsequent mortality of females may therefore have been from oral and/or dermal contact with Amulet Cue-lure stations or contact with dead exposed individuals.

In the present study, we explored the methods of horizontal transfer described by Bull (2010). However, each method was assessed independently in a controlled laboratory environment using methods adapted from Spafford et al. (2018). The sources of transmission that were assessed were: (1) direct contact of females with exposed males (2) contact of males to the regurgitant of exposed males and (3) contact of males with dead exposed males. Results of the laboratory trial in this study found that males that had directly fed on Amulet Cue-lure were affected by the insecticide within 15 minutes, and all died within four hours, which was approximately two hours before dusk (in the 6 hour experiment).

A high proportion of females exposed to the Amulet Cue-lure fed males were also dead at six hours, demonstrating that horizontal transfer of fipronil from exposed males to unexposed females had occurred. However, this transfer was not a result of contact with males during courtship and mating, as all males were dead at dusk. In trials where males were fed Amulet Cue-lure one hour prior to dusk most male flies were either knocked down or moribund. As a result, males did not display courtship behaviours or participate in mating, which suggests that fipronil had a significant effect on the sex drive of males. Similar results were found by Spafford et al. (2018) where all female melon flies (*Z.* cucurbitae) died within six hours of exposure to males that had fed directly on Amulet Cue-lure. In our study we came to the same conclusion as Spafford et al. (2018), which was that the indirect mortality of females was due to direct physical contact with the surfaces contacted by males, the regurgitant deposited by males and/or by feeding on dead exposed males.

Further trials were conducted to independently assess indirect mortality caused by contact with surfaces and the regurgitant of males exposed to Amulet Cue-lure. The indirect effects of contact with dead exposed flies was also investigated. Our results clearly demonstrate that horizontal transfer occurs through both of these processes, as most males died after 24 hours. These results are consistent with the findings of Spafford et al. (2018) where mortality of 100% of males and 75% of females was observed when exposed to regurgitant of Amulet Cue-lure fed males. According to Bull (2010), female flies were observed licking and regurgitating on the leaf surfaces where males were aggregating. This was suggested to be a part of courtship behaviours as males were observed mounting and copulating with these females. This behaviour was not observed in the current study and we suggest that the attraction of females to regurgitant is a result of attraction to Cue-lure, as earlier discussed, rather than being related to courtship and mating. Bull (2010) noted that females that died throughout the trial did not contain liquid within their crops, therefore mortality was not based on oral consumption. However, it is conceivable that a small dose of fipronil could have been orally or dermally collected by females from surfaces and regurgitant and caused indirect mortality.

Key Messages

- Queensland fruit flies are a major pest within Queensland and other eastern states of Australia and cause significant barriers to both domestic and overseas markets. Male Annihilation Technique (MAT) is one component that can be used in pre-harvest management plans for Queensland fruit fly.
- The present study explored the claims that male fruit flies exposed to Amulet Cue-lure stations can transfer lethal doses of fipronil to females during courtship and mating.
- Our results demonstrated that lethal doses of fipronil were not transferred from males to females during courtship and mating.
- Male flies that were exposed to fipronil prior to dusk were all knocked down, moribund or dead and did not participate in courtship displays or mating.
- The present study confirms that horizontal transfer does occur when flies contact regurgitant of exposed males or surfaces they touch.
- Horizontal transfer can occur when flies come in contact with dead flies that have been exposed to Amulet Cue-lure.
- It is suggested that MAT devices that contain fipronil should not be used as a stand-alone treatment but rather be used in combination with other control methods, such as protein bait sprays to ensure the control of female flies.

Where to next

Further research is required to ascertain whether similar effects of horizontal transfer occur when fruit flies feed on fast acting chemicals such as maldison. If similar effects do not occur there would be advantages in using slow acting chemicals in MAT devices to control fruit fly populations.

Budget Summary

Proposed Budget Summary

Base staff (B. Missenden) 0.2 FTE for 12 weeks	$4644.11
Casual staff	$2820.35
Operating	$2295.65
Total	**$9760.11**

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