Hort Innovation

Final Report

Development and production optimization of a male only selecting, temperature sensitive lethal, strain of *Bactrocera tryoni* (Queensland fruit fly)

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Project code:

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Development and production optimization of a male only selecting, temperature sensitive lethal, strain of Bactrocera tryoni (Queensland fruit fly) MT13059

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Content

Summary

The aim of this project was to develop a male-selecting, female-temperature sensitive lethal (*tsl*) strain of *Bactrocera tryoni* (*B. tryoni* or Queensland fruit fly - Qfly) for Sterile Insect Technique (SIT) application. The desired *tsl* strain would consist of female flies carrying a recessive mutation that confers sensitivity to high temperatures, resulting in lethality at the embryonic stage upon heat treatment. The male flies would be engineered to carry a normal copy of the gene on their Y chromosome and will be unaffected by the heat treatment. In that way, all female eggs could be eliminated by heat treatment, leaving only male flies that would be made sterile and released into the wild for SIT purposes. A male-only release would increase the effectiveness of SIT as the males would be more likely to mate with wild flies in the outbreak area, unlike a bi-sex release where males tend to mate with the co-released factory-raised females. In addition, a male-only strain would be more cost-efficient as it eliminates the cost of rearing the females past the embryonic stage in the factory.

In order to generate the Qfly *tsl* strain, we focused on *tsl* mutations in the *shibire* gene that are known to cause embryonic lethality and adult paralysis in *Drosophila melanogaster (D. melanogaster)*. The newly established CRISPR-Cas9 genome editing technology was used to manipulate the Qfly genome, with the aim of generating a *tsl* strain. We successfully established CRISPR-Cas9 technology in Qfly and were the first to demonstrate this technology in any tephritid species (Choo *et al.,* 2017). We have also successfully introduced a *shibire ts1* mutation (shi^{ts1}; Choo *et al*., manuscript in preparation) and are working on introducing an alternative *shibire* mutation (shi^{ts2}) into Qfly.

Keywords

Bactrocera tryoni; Queensland fruit fly; Qfly; tephritids; pests; temperature sensitive lethal (tsl) strain; Sterile Insect Technique (SIT); CRISPR-Cas9; genome editing; mass production

Introduction

Fruit flies are the primary pest of fruit and vegetables worldwide. *Bactrocera tryoni*, Queensland Fruit Fly (Qfly) is the major threat to the horticultural industries in Australia (Sutherst *et al.,* 2000; White & Elson-Harris, 1992) and the Asia- pPacific region. Qfly is a polyphagous insect with a wide host plant range of more than 60 native species and most commercially grown fruit and fruiting vegetables (White & Elson-Harris, 1992). The larvae feed on the flesh of fruits and vegetables reducing both quality and quantity of production causing significant economic losses. Qfly is endemic to eastern coastal Australia, from Cape York (Queensland) to East Gippsland (Victoria; Drew, 1989), but recently become common in parts of inland New South Wales and Victoria.

Horticultural regions without Qfly have significant economic and social benefits for production and market access. In Australia, the average annual value of fruit fly susceptible Australian horticulture is $4.8 billion, with roughly 25% traded interstate. Over a five-year period between 2003 and 2008, Australia invested more than $128 million in the management of fruit flies. This amount is excluding management costs to growers in preventing infestation. Fruit flies present a significant phytosanitary threat to horticulture and can have a major impact on Australia's capacity to trade competitively in international horticultural markets. As a result, it is critical that fruit fly species are adequately managed to ensure producers can maintain, enhance and develop access into domestic and international markets.

Sterile Insect Technique (SIT) is the mass rearing, sterilisation and inundated release of insects, ideally male only, to reduce the reproduction and population density of the target pest species. Currently SIT releases of Qfly are two-sex (bi-sex) programs, where sterile males and females are released together. One drawback of this system is that released sterile females may attempt oviposition into fruit causing puncture wounds which degrade fruit and can increase susceptibility to fungal or bacterial infection. Additionally, released sterile females may compete with wild females in mating with released sterile males reducing the efficacy of the SIT program. If females could be removed early in the production phase, desirably at the egg stage through development of a *tsl* strain, then only males would be reared and released thereby improving cost effectiveness and efficacy of the program.

Another desirable sex-linked attribute is where females and males can be segregated by colour of the puparia in which male pupae are brown and female pupae white. Differentiated colour pupae can then be segregated using a modified seed-sorting machine of which several types have been developed and are in use. Pupal colour is used for collection of females for the

next generation and can also be used as a visual cue for the purity of the male pupae to be irradiated and released.

The aim of this program was to develop a temperature-sensitive lethal (*tsl*) strain of Qfly through mutation and traditional breeding. The program also aimed to develop optimized adult and larval diets, heighten post release fitness and mating competitiveness through use of chemical or dietary supplements and develop reliable fitness indicators.

Methodology

Module 1. Male selecting strain of QFly

1.1) A culture of *B. tryoni* was established in the Quarantine insectary at Waite campus from eggs provided by Andrew Jessup from cultures at Ourimbah DPI NSW. The culture was reared on a liquid diet (Khan 2014). The liquid diet was replaced with a gel based formula developed by Macquarie University(HIA Project HG13045), when it became available. Larvae were collected in a sieve and washed to remove excess diet and matured in woven cloth materials in lieu of the more common vermiculite.

1.2) Baseline temperature data for Qfly eggs

We needed to establish the baseline temperature sensitivity of the Waite culture in order to evaluate the initial success of temperature sensitive strains developed as part of the project. To elucidatethis, a heat treatment experiment was conducted in the quarantine laboratory facilities, at Urrbrae. Cages were set up to contain 50 flies (25 males and 25 females) in polyester cubed cages (30 cm x 30cm x 30cm). Flies were fed brewers yeast and supplied with water in 70ml water vials with velida cloth wicks inserts. Cultures were kept in 25 C, 70 +/- relative humidity and 10 hr: 14 hr light:dark conditions.

The test for heat tolerance, consisted of exposing treatment flies to temperatures of 28, 30, 32, 34, 36, 38 and 40 C for 24 hours. Fly Mortality due to heat stress was calculated by factoring in fly death due to natural attrition.

1.3) Development of a *tsl* strain

To generate the tsl strain we used the newly established CRISPR-Cas9 genome editing technology that is being widely used in a broad range of organisms. This technology is a direct, precise and relatively quick form of genome manipulation. CRISPR-Cas9 mutagenesis has been successfully used in a wide range of organisms, including non-model organisms, in which genetic modification has previously been a challenging task.

1.4) Developing colour differentiation of the puparium between male and female Qfly

Male and female pupae can be separated if they differ in colour. In collaboration with IAEA Seibersdorf we generated a hybrid of *B. tryoni* and *B. dorsalis* which

has a naturally occurring white pupae mutation. The hybrids were crossed and white pupae individuals selected and backcrossed with *B. tryoni* 8 times. The sequenced genomes of parental and hybrid strains identified that a 2 megabase section of the *B. dorsalis* genome remained; from this coding sequences were identified and a mutant selected as a candidate white pupae gene

Module 2. Improved QFly Fitness assessment methods

2.1) Validation of laboratory tests for Qfly

Three options for QC validation were developed for fly fitness and irradiation certification. Guideline QC standards have been set by the IAEA based on their long-term research and use of SIT for several species of fruit fly including some *Bactrocera* spp. (IAEA 2014). QC assessments are conducted at the production facility and at rear-out facilities to assess fitness and release rates, these need to be as similar as possible so that fitness loses that result from transport and handling can be monitored without the background variations caused by assessment methods.

2.2) Fitness and Quality assessment of CRISPR generated strains

Study flies and rearing methods

CRISPR generated white eye pupae were obtained from School of Biological Sciences, University of Adelaide. They were reared for two generations at Macquarie University to produce an adequate population for the experiments. The normal Qfly are used from Taylor Lab, Macquarie University the same strain that was used as the parental strain to develop the 'backup white eye' strain. For egg collections, plastic bottles with small puncture holes for the female to oviposit through were used. The bottles were placed inside the cage on 15th days of emergence of the adult in both strains for 24 hours period. Then eggs were transferred in the centrifuge tubes. 150 g of gel-based was put in each plastic tray (17.5 cm long, 12 cm wide, 4 cm deep) and 250 µl of eggs per tray was seeded. The trays were covered with lid until the larva were ready to jump in each colonies and replications. Adult flies were fed with yeast hydrolysate and sugar (1:3) ratio. The experiment is carried out in a laboratory conditions at Macquarie University (25 ± 0.5 °C, 65 ± 5% RH and 11: 1: 11: 1 light/dusk/dark/dawn photoperiod).

Evaluation of quality control parameters

Most of the quality control measurements are based on the standard FAO/IAEA/USDA (2014) procedures. The quality parameters of the white eye strain and normal Qfly were compared by parental egg hatch, developmental period (days) pupal weight (mg) egg to pupa recovery %, eclosion %, sex ratio and fliers %.

Egg hatchability

Eggs were put on the 50 ml centrifuge tube and let to settle and then counted using 100µl pipette stereomicroscope on black filter paper. Ten sets of black filter papers with 100 eggs of each strains were then put over the 90 mm petri dish with gel diet and covered with the lid. Optimum moisture was maintained up to 4 days. On the fifth day, the number of hatched eggs and unhatched were counted. Percentage hatchability was calculated as follows

$$Hatchability\% = \frac{Number\ of\ hatched\ eggs}{Total\ number\ of\ eggs\ seeded} * 100$$

Developmental Period:

Time in days from seeding of the eggs to the start of the emergence. It includes egg incubation period, larval developmental period and pupal developmental period.

Pupa harvesting: 10 sets of the plastic trays of each strain in which eggs were seeded were kept closed with for 3 days of egg seeding. On the fourth day, the lids were opened, and each tray were put in each bucket which were fitted with cone at the bottom.

The plastic rearing trays were then covered with plastic lids until the larvae reached their third instar, when the larvae jumped out from the diet to pupate. The rearing after removing the lids were then placed on the black plastic funnels (funnel 22 cm all purpose; Distributed by Sandleford, Australia) placed inside white buckets (Maxi pail 5 L handy pail; Distributed by Zenexus, Australia). The funnel extended out from a hole on the bottom of the bucket and was inserted into a ventilated 1 L plastic box containing a 1 cm layer of fine vermiculite. The larvae exited the larval tray, rolled down the funnel and landed in the vermiculite where they pupated. Pupae were sifted

from vermiculite by using a fine mesh sieve. Collected pupae were counted and weighed daily until no larvae remained in the diet. On the last day of pupal collection, all pupae of each replicate were pooled for quality control evaluation

Pupal production period: Number of days in which pupa are produced

Peak day of pupation: Time from the egg seeding to maximum pupation occurred

Pupal number: it is estimated by the following formula in each replication of each strain

$$Pupa\ number = \frac{Total\ weight\ of\ Collected\ Pupae}{Mean\ \ weight\ of\ 6\ sets\ of\ 100\ pupae} \times 100$$

Egg to Pupa recovery rate: It was estimated as follows:

$$Egg\ to\ pupa\ recovery\% = \frac{Number\ of\ pupa\ harvested}{Number\ of\ eggs\ seeded\ on\ the\ diet} \times 100$$

Adult Performance:

Five sets of 100 pupa from each were counted and placed in separate 55 mm plastic petri dish. The dishes of pupa were centred on 90mm Petri dishes which were lined with black filter paper. A 100 mm tall black tube (89 mm inner diameter) with a fine coat of talcum powder on the interior (to prevent flies walking out) was placed onto the 90mm petri dish lid. Each tube with pupae were placed in a mesh cage (32.5 × 32.5 × 32.5 cm, Megaview BugDorm-43030F) beneath a 20-watt fluorescent tube positioned approximately 5 cm above the cage. To quantify fly-back (the number of flies that escaped from the tube and later returned and died inside) a second, empty, black tube was placed 6 cm away from the tube containing pupae. Flies that left the flight tube were aspirated daily. All the materials were collected and counted from both tubes after 6th days of emergence. Emergence, partial emergence, deformity and flier's percentage were calculated as following

$$Emergence\% = \frac{\big(N\ pupae - (Non\ emerged + Partially\ emerged)\big) \times 100}{N\ Pupae}$$

$$Partial\ Emergence\% = \frac{Partially\ Emerged \times 100}{N\ Pupae}$$

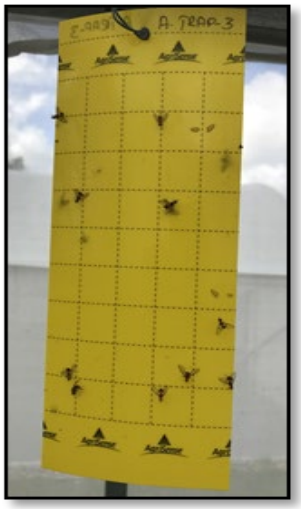
$$Deformity\% = \frac{Deformed\ flies \times 100}{N\ Pupae}$$

$$Fliers\% = \frac{\left(N\ pupae - (Non\ emerged + Partially\ emerged + Deformed\ flies + Non\ fliers)\right) \times 100}{N\ Pupae}$$

Where N= total number of pupae put inside petri dish each 55 mm petri dish to evaluate the flight ability

Field cage release-recapture experiment

For release recapture experiments, 8 sets of 1400 flies of each strain were reared. They were fed with yeast hydrolysate and sugar (1:3) and water was provided with the 100 ml plastic container fitted with sponge on the lid. 4 sets of the flies from each strain were fed fully for five days and inside the large enclosures in Fauna Park at Macquarie University. One set of each strain was released in each of the four enclosures. Five yellow sticky traps were installed in each enclosure after releasing the flies. Five containers with 20 gram sugar mixed with 1 litre water per enclosure was provided. Second release was after 9 days of the emergence in which flies were fed with full diet for 9 days.



Phase 2: Comparison of Backup, Alternative backcrossing and Normal backcrossing strains

We tested the quality of three white eye strains, namely backup, alternative backcrossing and normal backcrossing. Backup strain is the first white eye strain generated and it was not backcrossed. Alternative backcrossing is the backup strain that was backcrossed three times without second intercross. Normal backcrossing is the backup strain that was backcrossed three times including intercrosses.

Virgin wild type and white eye flies from all three strains were setup for mating (2 males x 4 females) in 720ml pint-sized insect pot with donut lid with white nylon (Megaview Bugdorm). The flies were kept at approximately 25^{o}C and 65 - 75% of relative humidity (RH). For each strain including the wild type (control), three replicates were performed. Approximately 10-12 days after the setup of the crosses, we started collecting eggs using egging device with green satin cloth and tart apple (Brambleberry, Handcraft Provisions, Washington, USA), which was changed daily. The eggs were collected for 24 hours during 5 consecutive days. The amount of laid eggs were count daily and the fecundity (eggs/female/day) was calculated by dividing the total amount of eggs by the number of females. Then, the average of the replicates was calculated.

The eggs laid daily were placed on black paper, which was placed onto gel diet to allow access to food to hatched larvae. The amount of hatched eggs were counted after two or three days in order to determine the fertility. The percentage of hatched eggs were calculated based on the total number of eggs hatched and total number of eggs laid per strain. In order to determine pupal recovery, the gel diets with larvae were placed in 720ml pint-sized insect pot with donut lid with white nylon screen (Bugdorm). After pupation, the amount of pupae were count. The percentage of pupal recovery was calculated based on the total amount of pupae and eggs hatched per strain.

In order to determine the survival or longevity under stress, pupae were placed in 720ml pint-sized insect pot with donut lid with white nylon sleeve (Bugdorm) without water and food. The dates of the emergence and death of the first flies were recorded.

Plexiglas tubes (outside diameter 8.9 cm with 3 mm thick walls) were cut into 10 lengths and painted in black so that light enters only at the top when positioned upright. Black paper was cut to fit on the lid of Petri dishes. Porous paper was cut to make rings of 1 cm wide and 6 cm diameter. The inside of the tubes were lightly coated with talcum powder to prevent the flies from walking out. Using a cloth, ca. 1 cm of the talcum powder was wiped off to provide resting places for the newly emerged flies to set their wings. The black paper was placed on the Petri dish lid and them the ring on black paper in the centre of the Petri dish. Then, the tubes were positioned on Petri dishes with the side without talcum powder facing down. Two days before adult fly emergence, 100 pupae were placed within the ring inside the tubes, which was then placed inside a container with a yellow sticky trap attached to the lid. No water and food was provided. After one week, we counted the number of non-emerged pupae, half- or partially- emerged flies, fliers, non-fliers and deformed flies. Due to the limited number of pupae, the 'alternative and normal backcrossing' strains had only one replicate. On the other hand, wild type (laboratory strain) included three replicates.

2.3 Verification and Optimisation of X-ray sterilisation dose

Q fly pupae were obtained from Port Augusta fruit fly facility. Pupae from this facility were irradiated and individual 'zip-lock' plastic bags containing approximately 8,000 pupae were sealed and transported directly to SARDI in an air-conditioned vehicle. All pupae were packed on the day of pupation and all irradiated pupae were treated one-day post the onset of pupation. Flies were irradiated at 40, 50, 60, and 70Gy of Ionizing radiation (IR). Flies used as control flies were not irradiated. Bags of control and test pupae were packed together at all times during transport and storage to ensure that all pupae received similar conditions. Pupae were immediately transported by an air-conditioned vehicle in an esky to a laboratory at SARDI, Waite Campus, where they were housed to emerge. Pupae were held in a laboratory maintained at 25 ± 1°C and 70 ± 5% relative humidity. . The culture cages consisted of 50 males and 50 females. Wild flies (non-irradiated flies) were crossed with flies that had been irradiated at the above-mentioned irradiations doses with all possible combinations. i.e.. WF x IM, IF x WM, IF x IM. (flies were kept in quarantine lab rooms set at 25 ± 1°C and 70 ± 5% relative humidity with day/light periods. Eggs were collected over nine, 24-hour period sessions, in two time periods. Adult (parent) flies had eggs collected when they were 15-19 days of age for the first period. For the later period, adult files were 30 - 35 days old.

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Module 3. Diet optimisation and Implementation

3.1) Ensure regular and reliable supplies of dietary components are available

A minimum of 3 quotes were obtained from suppliers of critical ingredients, information requested with price, reliability of supply and lead times, where possible samples were obtained as evaluated for fly rear-out quality and production parameters such as development time, flies reared per volume of diet and adult fecundity. Preferred supplies were selected and supplies obtained. Regular evaluation are conducted to meet budget and production targets.

3.2) Assess diets developed at Macquarie University

Diet formulations developed at Macquarie University were evaluated to optimize pupal yield per diet volume and minimize waste. All trials were conducted at the Pt Augusta Production Facility using the rearing towers used in on-going production under normal rearing conditions of 25^0-27^0 C at 65% humidity and a 14:10 hour light dark cycle. Trays were loaded with between 1 and 3 litres of diet and seeded with between 20,000 and 50,000 eggs. After larvae completed development recovery rate (larvae/eggs), larval weight and waste diet were measured. Observations were also made to evaluate the proportion of the diet that had dried out prior to consumption by the developing larvae.

3.3) Investigate possible inclusion of enterobacteria in diets

A literature search was carried out to investigate the possible inclusion of bacterial cultures into larval diet to simulate the microflora in fruit stung in the wild. Some of these bacteria have been identified to assist with nitrogen fixation and improving availability of micronutrients. Experimental diets were formulated including strains of bacteria isolated from wild *Dirioxa pornia* (Island Fly) in CT11002. Assessments included development time, pupal weight and adult flightability. Despite production gains the difficulties associated with maintaining the purity of the bacterial culture this process was not trialed at a commercial level.

Module 4. Increased mating propensity- SITplus strain

4.1) Evaluation of volatile compounds to improve field fitness of tsl and GSS Qfly

At this stage neither a *tsl* nor a GSS strain of Qfly has been developed and preliminary testing of yellow body and white eye mutants is to be completed. The evaluation of the volatile compounds identified for this section of the research will be conducted as part of the Post Production Pilot project led by Macquarie University using the Yellow bodied strain, a white pupal strain and the current factory "wild type" strain. These strains will be evaluated for flightability and longevity in the field through use of standard Cuelure and protein traps. Females caught in the protein traps will be assessed for mating and a ratio of wild to sterile mating's will be assessed.

Additional Research

Over the contract period two additional research components were added to ensure the quality of sterile flies released. These additional components were 1) were assessment of different colour dye on fly quality and 2) optimization of the duration of hypoxia prior to irradiation.

After experiencing anomalies in the fitness of some batches of flies which appeared to be related to dye colour, research was undertaken to assess which (if any) of the dyes used for sterile fly identification had negative fitness effects. Methods and results are included in Appendix 3.

During irradiation, oxygen radicals can be generated resulting in reduce fitness of the sterilized flies; to counter this flies are irradiated in a hypoxic state using sealed containers. Hypoxia itself can reduce the fitness of flies if the period of hypoxia is longer than ideal. To address this trials were conducted using flies from Pt Augusta treated in containers used at the facility. Methods and Results are provided in Appendix 4.

Outputs

Module 1. Male selecting strain of Qfly

1) Development of tsl strain of Qfly suitable for release in SIT programs

Identification of Qfly temperature sensitive lethal (tsl) candidate genes

Two strategies were used to identify temperature sensitive lethal (*tsl*) candidate genes in Qfly. The first strategy was to use the *tsl* mutation from the Medfly (*Ceratitis capitata*) *tsl* Vienna-8 strain that is widely used in SIT programs and create an equivalent Qfly *tsl* strain. Collaborators in IAEA, Vienna and USDA, Hawaii made extensive progress towards identification of the Medfly *tsl* mutation prior to commencement of project MT13059. The intention was to obtain the causal Medfly *tsl* mutation from our collaborators to apply the information to this project. However, to date (June 2019) the Medfly *tsl* mutation remains unknown. In order to expedite the identification of this Medfly *tsl* mutation, a new SITplus project (FF17000 – Sex selection genes from fruit fly species for use in SITplus) commenced at the end of 2018. This project is a collaborative effort between Macquarie University, SARDI, CSIRO, IAEA, USDA and Giessen University and the research output from the FF17000 project will be provided to enable us to target the Medfly *tsl* ortholog in Qfly.

As a result of the Medfly *tsl* mutation not being identified prior or during the course of project MT13059, we pursued an alternative strategy for identifying Qfly *tsl* candidate genes. Multiple temperature sensitive embryo lethal mutations have been documented in the model organism *Drosophila melanogaster* (above 29°C). The three most suitable candidates for generating our Qfly *tsl* strain were *shibire* (Grigliatti *et al.,* 1973), *notch* (Neckameyer & Quinn, 1989) and *RNA polymerase II 215* (Mortin & Kaufman, 1982, 1984), and all involve a single nucleotide substitution. Analyses of the Qfly genome identified orthologs of all three genes with 90%, 67% and 93% sequence similarity to *D. melanogaster* proteins, respectively. The *shibire* gene was chosen as the primary candidate since its *tsl* mutation has been reported to give a fully penetrant *tsl* phenotype in *D. melanogaster* (Figure 1), and a Qfly ortholog of *shibire* was identified to have high amino acid conservation with *D. melanogaster,* thus likely to have a similar function (Figure 2). *Shibire* mutations have also been shown in other organisms to be temperature-dependent (Damke *et al*., 1995; Clarke *et al*., 1997); hence it appears that the temperature

sensitive property of *shibire* is conserved across different species.

We first performed heat treatment assays on *D. melanogaster shibire* mutant flies to confirm that it produces the expected *tsl* phenotype previously described in the literature. Different *shibire* mutations have been reported - shi^{ts1} mutation is fully penetrant and results in complete embryonic lethality whilst shi^{ts2} has a milder phenotype. Both mutations involve a single base substitution that changes one amino acid in the Shibire protein. We managed to obtain shi^{ts2} mutants from the Bloomington Stock Centre in the US and replicated the reported *tsl* phenotype. Importantly, we also demonstrated that having a wildtype copy of *shibire* on the Y chromosome was able to produce males that were resistant to the heat treatment – hence only females were killed by heat but the males survived. These results supported that the *shibire tsl* mutation was a promising candidate to introduce into Qfly.

Recreating a *tsl* strain based on the Medfly *tsl* allele was a more appealing strategy as it has already been established as a successful genetic sexing strain used in SIT. The *Drosophila tsl* mutations have not been used in any application work and whilst those mutations appear to be promising candidates, it was unknown if those mutations could be used successfully for a genetic sexing strain. However, as the Medfly *tsl* allele has not yet been identified, a decision was made to proceed with the *Drosophila shibire tsl* mutation(s).

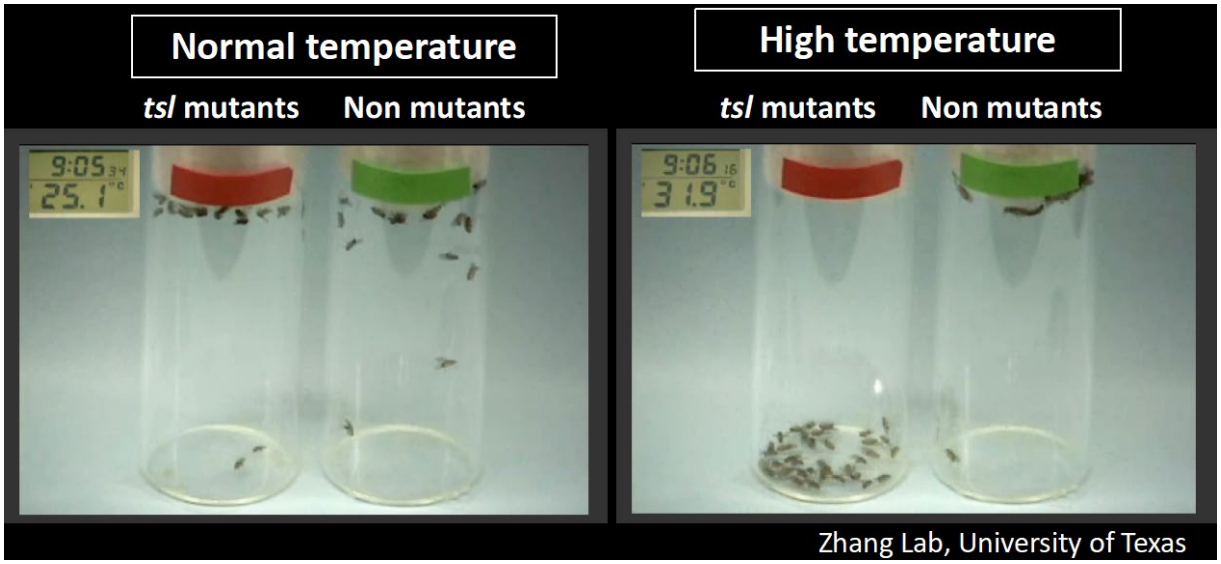


Figure 1: *Drosophila melanogaster* carrying a *shibire temperature sensitive lethal* (*tsl*) mutation are viable at 25.1ºC (left panel) and are paralysed at 31.9ºC (right panel). This mutation is lethal when heat-treated at the embryonic stage. Males with a wild type allele inserted on to the Y chromosome are resistant to heat treatment. (Figure adapted from Zhang

lab, University of Texas).

Figure 2: Comparison of Qfly and *D. melanogaster* Shibire protein. They share 90% amino acid similarity and residues required for *shi* ^{ts1} (glycine to aspartic acid) and *shi* ^{ts2} (glycine to serine) mutants are conserved.

Establishment of CRISPR-Cas9 genome editing technology in *D. melanogaster*

CRISPR-Cas9 genome editing technology was chosen as the method of mutagenesis to introduce the *tsl* mutation into Qfly as it is more precise, less labour intensive and less time consuming compared with irradiation and chemical mutagenesis. As this new method of mutagenesis was not established in tephritid fruit flies prior to our project but successfully used in *D. melanogaster*, we first optimized the CRISPR-Cas9 technique in *D. melanogaster* flies available to us at the University of Adelaide and then transferred that technique to Qfly.

After the successful establishment of the CRISPR-Cas9 technology in *D. melanogaster* at the University of Adelaide Drosophila Facility, a microinjection system was set up in the Qfly quarantine facility (QAP226, University of Adelaide, Waite Campus). We first performed trial microinjections with injection buffer to set up the microinjection protocol in Qfly embryos.

Rigorous optimization steps were then carried out to improve the survival rate of microinjected Qfly embryos.

Establishment of CRISPR-Cas9 genome editing technology in Qfly

A proof-of-principle experiment was first carried out to demonstrate that the CRISPR-Cas9 genome editing technology was applicable to Qfly (Appendix 1). This technology was successfully used to target the *white* gene in Qfly, altering the normal eye colour of the flies from red to white (Figure 3, Table 1). The white-eye phenotype is a clear phenotypic readout of successful CRISPR-Cas9 mutagenesis and a result could be obtained in a relatively short period of time. This result demonstrated that the CRISPR-Cas9 genome editing technology is applicable to Qfly and could thus be used to introduce a *tsl* mutation into its genome. This work has been published in *Journal of Applied Entomology* (Appendix 1).

Initial tests performed by Professor Phil Taylor's group at Macquarie have indicated that the white-eye mutants are blind. Further experiments will be performed to confirm this and we are currently attempting to recover the males' sight (by random mutagenesis using low dose irradiation) to potentially create a functionally male only strain. Extensive research has also been conducted to elucidate possible sites on the Y chromosome for inserting the wild type eye colour gene and subsequently future *tsl* male recovery.

In addition, we have also performed mating experiments to backcross the white-eye mutants to the wildtype lab strain in order to select against any background off-target mutations that may have been introduced during the mutagenesis process. We have observed that the thrice-backcrossed strain has higher fecundity than the original mutant strain, which could suggest that backcrossing may improve the fitness of mutant strains if their fitness had been affected by off-target mutations.

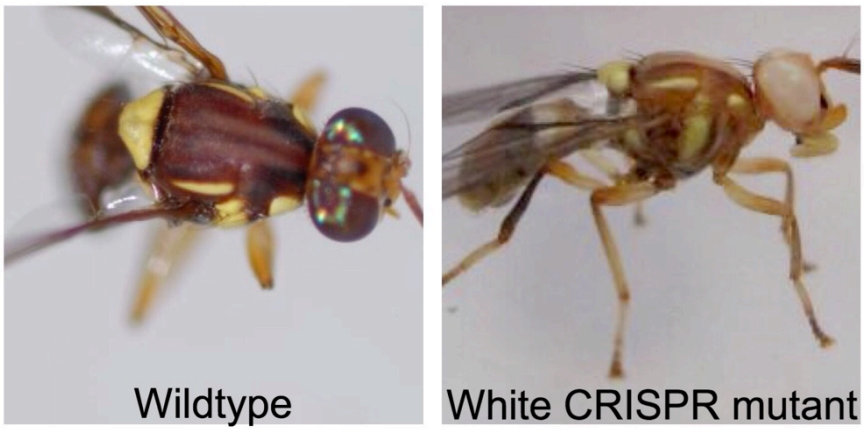


Figure 3: Successful CRISPR-Cas9 mutagenesis of the white gene resulted in a white-eye phenotype in Qfly.

Table 1: Summary of survival rates of injected embryos and number of white mutant flies generated by CRISPR-Cas9 mutagenesis.

No. of embryos injected	No. of larvae	No. of pupae	No. of adults	No. of germline mutants	No. of mutant progeny
187	69 (37%)	16 (23%)	9 (56%)	>1	7/80*

*Nine non-wildtype alleles were detected in mutant progeny.

Introduction of *tsl* mutation into Qfly using CRISPR-Cas9 mutagenesis system

Using our established protocols, we performed CRISPR-Cas9 mutagenesis experiments to introduce the *shibire tsl* mutation into the Qfly genome. We began with the shi^{ts1} mutation as it was the stronger *shibire tsl* phenotype and reported to be fully penetrant. The mutagenesis experiments involved generation of CRISPR-Cas9 components targeting the Qfly *shibire* gene and microinjections of these components into Qfly embryos. Larvae that hatched from the injected embryos were reared to adulthood (G_0 flies) and mated individually to the wild type lab strain flies. Embryos were collected from the mating groups and reared to adults (G_1 flies). Molecular analyses were then performed on individual G_1 flies to identify if any carried the expected mutation.

Initially, we generated *in-vitro* CRISPR-Cas9 components (transcribed sgRNA; Figure 4) to introduce the *shi*^{ts1} mutation in the Qfly genome and microinjected 2254 embryos, from which we had 28 G_0 flies and 21 successful matings yielding G_1 flies (Table 2). From the 21 successful matings, we screened 944 G_1 flies. We identified that the CRISPR-Cas9 components targeted the right genomic location and generated double strand breaks, which led to mutagenesis, but not the correct *shibire tsl* mutation. In order to increase the chances of obtaining our *tsl* mutation of interest, we then tried a modified CRISPR-Cas9 system obtained from Integrated DNA Technologies (IDT; Figure 4). We performed trial experiments with this new system and the results revealed high levels of mutagenesis at the right genome location. We also optimised the injection mix by using different concentrations of single-strand oligonucleotide (ssODN) to alter the efficiency of the specific single base change. Out of 1037 embryos injected, we obtained 29 G_0 flies and subsequently 17 successful matings (Table 2); 2 of which produced shibire mutants, with one of the lines carrying the desired *shi*^{ts1} mutation (Figure 5). This work is currently being written up for publication and may be the first demonstration of homology-directed repair resulting in specific base change mutagenesis in tephritids.

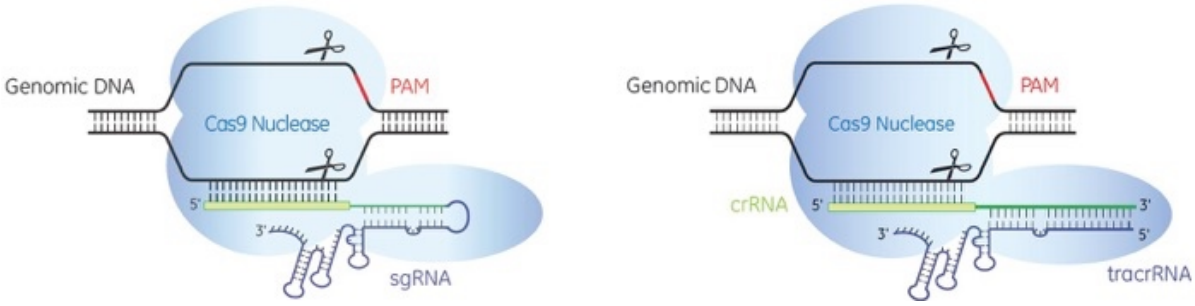


Figure 4: Comparison of the different CRISPR-Cas9 systems used in our study (Figure adapted from dharmacon.horizondiscovery.com). Our *in vitro* transcribed sgRNA CRISPR-Cas 9 system (as depicted in the left) was based on Bassett & Liu (2014) and was successfully used to generate our Qfly *white eye* mutant. Our IDT Alt-R® CRISPR-Cas9 system (similar to the depiction on the right), manufactured by Integrated DNA Technologies, and was successfully used to introduce the shibire *ts*1 mutation in the Qfly genome.

Table 2: Summary of CRISPR-Cas9 mutagenesis experiments that have been performed to introduce the *shi^{ts1}* mutation into Qfly genome.

Injections	ssODN concentration	# embryos injected	# G_0 adults	# successful G_0 matings	# G_1 screened	G_0 germline mutants	
						#	Mutation
Using *in vitro*-transcribed sgRNA[#]	200 -600ng/µL	2254	28 (1.2%)	21	944	1 (3.6%)	16 bp deletion
IDT Alt-R® CRISPR-Cas9 system	200ng/µL	254	14 (5.5%)	7	496	2 (14.3%)	4bp deletion *Shi ts1 mutation
	250ng/µL	473	10 (2.1%)	8	308	0	-
	300ng/µL	310	5 (1.6%)	2	166	0	-
	Overall	1037	29 (2.8%)	17	970	2 (6.9%)	4bp deletion *Shi ts1 mutation

[#] Data shown here is for one particular *in vitro*-transcribed sgRNA, we have also tested multiple other sgRNAs but have not included data here.

* The *shi^{ts1}* mutation is our desired mutation and shows the efficiency of the new CRISPR/Cas system that we have established in Qfly.

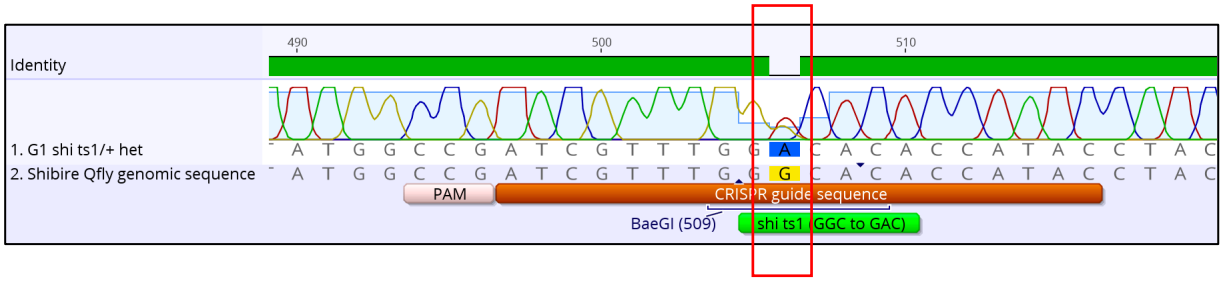


Figure 5: *Shibire temperature sensitive mutation 1* (*shi^{ts1}*) heterozygous genotype (highlighted by the red box). One copy of the *shi^{ts1}* mutation was successfully introduce into Qfly genome using CRISPR-Cas9 mutagenesis. This genotype carries one copy of the *shi^{ts1}* mutation and one normal copy of the *shibire* gene.

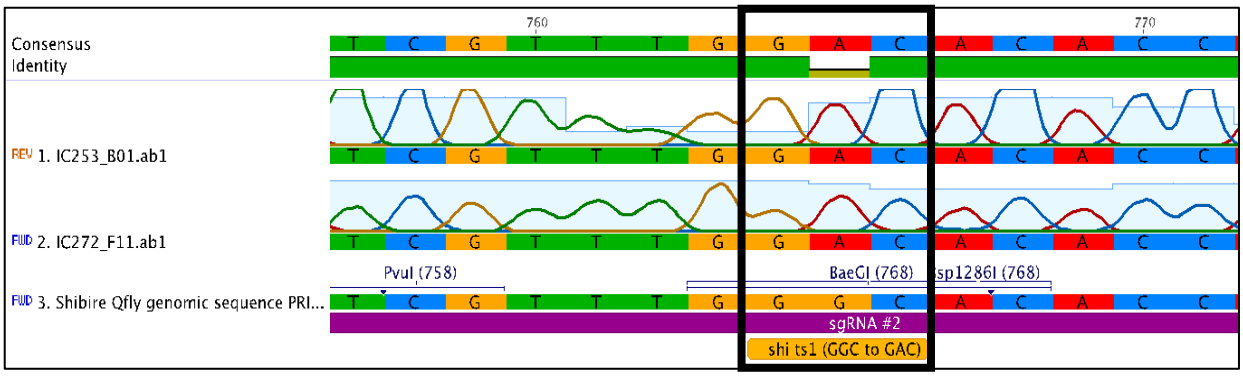


Figure 6: *Shibire temperature sensitive mutation 1* (*shi*^{ts1}) homozygous genotype (highlighted by the black box). Homozygous Qfly flies carries two copies of the *shi*^{ts1} mutation without normal copy of the *shibire* gene. These flies were generated by mass mating of flies carrying one copy of the *shi*^{ts1} mutation (heterozygous) introduced successfully using CRISPR-Cas9 mutagenesis.

In summary, we achieved our target of introducing the *shibire* temperature sensitive mutation 1 (*shi*^{ts1}) into the Qfly genome using the CRISPR-Cas9 genome editing technology. These flies are heterozygous for the mutation – they carry one copy of the *shi*^{ts1} mutation and one normal copy of the *shibire* gene (Figure 5). We then attempted to generate a homozygous strain that carries two copies of the *shi*^{ts1} mutation, which is the desired final genotype for a SIT strain. This can be done by mating heterozygous males to heterozygous females and screening for homozygous progeny by molecular methods. Unfortunately we were only able to identify two individuals with homozygous mutations (Figure 6), out of more than 1000 progeny screened and both individuals died soon after eclosion (emergence for pupae). This suggests that the *shi*^{ts1} mutation in Qfly is more severe than that in *D. melanogaster* and has a negative impact on survival even at ambient room temperatures. Thus it is unlikely that this particular mutation can be used for SIT purposes and an alternative mutation, such as the milder *shibire* mutation, *shi*^{ts2}, will have to be considered.

We have commenced experiments to introduce the *shi*^{ts2} mutation as an alternative mutation into the Qfly genome and had successfully incorporated the mutation on our first experimental attempt (Figure 6); however, we failed to produce a viable stock due to the low number of individuals obtained from that mating. Nonetheless, we have demonstrated that the *shi*^{ts2} mutation can be successfully incorporated into the Qfly genome using our system and we are currently endeavoring to re-introduce this mutation (Table 3). Once we obtain a strain that carries two copies of the mutation (and no normal copy of the *shibire* gene), we will test the

strain to see if embryos can be killed off by heat treatment. Protocols for the heat treatment assays have been established. We will also test the strain at other life stages and determine the range of temperatures at which the flies are affected. This work will be written up for publication.

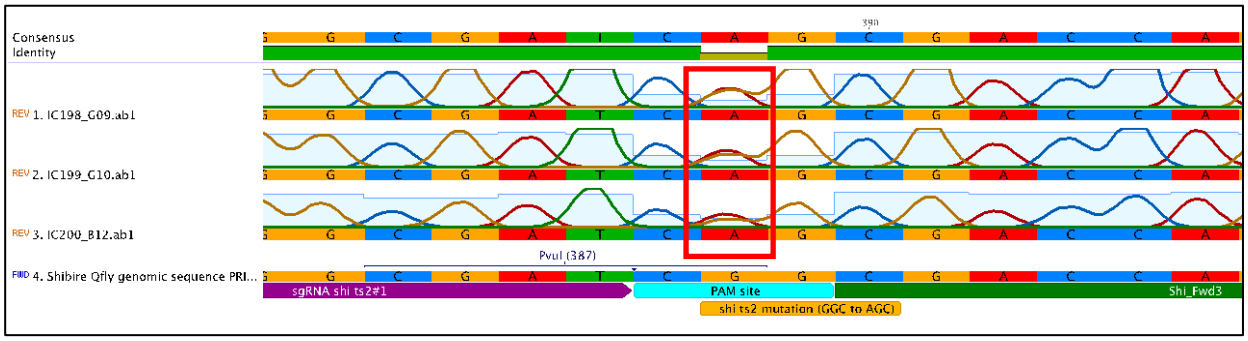


Figure 7: *Shibire temperature sensitive mutation 2* (*shi*^{ts2}) heterozygous genotype (highlighted by the red box). One copy of the *shi*^{ts2} mutation was successfully introduce into *Bactrocera tryoni* (Qfly) genome using CRISPR-Cas9 mutagenesis. This genotype carries one copy of the *shi*^{ts1} mutation and one normal copy of the *shibire* gene.

Table 3: Summary of CRISPR-Cas9 mutagenesis experiments performed thus far to introduce the *shi*^{ts2} mutation into Qfly genome. These experiments are currently on-going.

Injections	ssODN concentration	# embryos injected	# pupae	# adults obtained (G_0)	# successful G_0 matings	# G_1 screened thus far	G_0 germline mutants	
							#	Mutation
IDT Alt-R® CRISPR-Cas9 system	200ng/µL	2 731	58 (~2%)	51 (~88%)	23 (~45%)	869 flies	1	Ts2
							2	deletions
	300ng/µL	814	3	3	1	2	0	0

In addition to developing the *tsl* strain for SIT, we have also been working on alternative male-selecting strains (as described below). This is to increase the probability of obtaining a male-selecting SIT strain and can also be used together with the *tsl* strain to produce a robust genetic sexing strain.

Development of other strains: Identification of *Bactrocera dorsalis* white pupae phenotype

SIT requires mass release of sterilised males, however, separating individuals in rearing facilities according to sex remains a major challenge. Rice sorters have successfully been adapted to separate pupae by sex when they show colour differences, however the genetic basis of pupae colour is unknown. We identified the *Bactrocera dorsalis white pupa* (WP) locus by introgressing the phenotype into Qfly over 12 generations. Whole genome sequencing identified a single introgressed region of ~2.5 Mb that represents the WP locus. This was used to identify the causal WP mutation that could be subsequently used for developing white pupae strains in diverse *Bactrocera* species. We identified a gene mutation that may cause the white pupae phenotype, and currently CRISPR-Cas9 injections are being performed to knock out the candidate gene in Qfly. Identification of the white pupae gene in *Bactrocera* could enable sex-sorting of males and females based on pupae colour. It is hoped this work will enable the FF17000 project (Sex selection genes from fruit fly species for use in SITplus) to advance rapidly.

Development of other strains: Novel body colour phenotypes

Protecting the horticultural industry from Qfly involves careful monitoring with pheromone traps. It is important to accurately discriminate between sterile SIT flies and wild flies collected in these traps, to evaluate the efficiency of the SIT program and prevent mistaken declarations of outbreaks. A detectable fluorescent dye is currently used to label SIT flies, however it can cause dehydration and death, fail to thoroughly coat flies, be washed off or passed on to wild flies through contact. In *D. melanogaster*, disruption of the *yellow-y* gene results in pale body colour. We identified a tandem duplication of *yellow-y* in the Qfly genome and used CRISPR/Cas9 to mutate both copies. Mutation in *yellow-y* generated a phenotype clearly distinguished from the wild type phenotype, as melanisation is restricted in specific tissues including wing veins. Using phenotypic markers instead of fluorescent dyes to identify SIT strains could help improve adult emergence rates, simplifying the SIT release procedure, enable accurate distinction from wild flies, and improve the cost-effectiveness of SIT program. Fitness experiments are due to begin in collaboration with Dr. Phil Taylor at Macquarie University later this year.

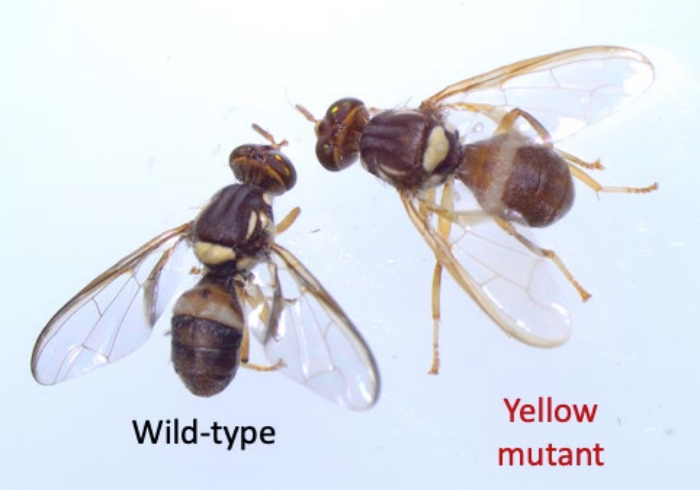


Figure 8: Novel Qfly body marker strain. A yellow body phenotype was successfully developed in Qfly using CRISPR-Cas9 mutagenesis (Zoey Nguyen).

Identification of Y chromosome sequences in Qfly as potential sites for translocation of the wildtype tsl allele

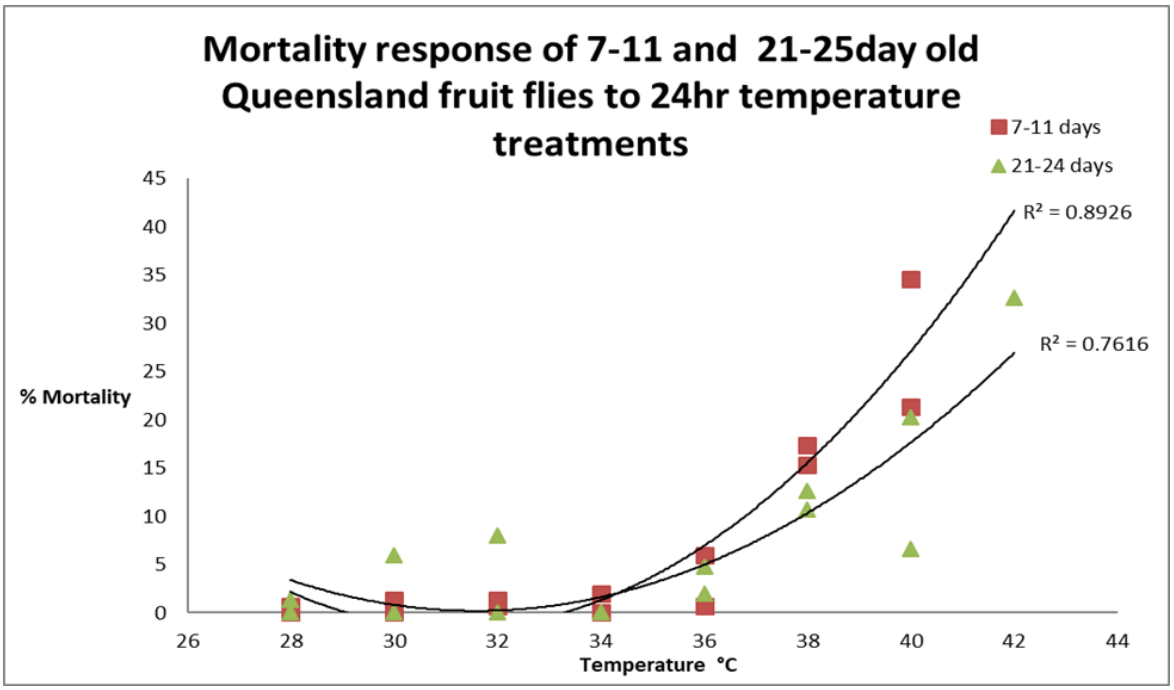
The upcoming FF18002 project will involve translocation of the wild type *tsl* allele to the Y chromosome in males, in order to protect males from the effects of the *tsl* mutation. However, at the start of this project, littlewas known about the Y chromosome in Qfly, other than it is largely composed of repetitive sequences and isheterochromatic. In collaboration with John Sved's group at UNSW, bioinformatic and molecular analyses were performed to identify Y chromosome gene regions and sequences as potential insertion sites for the *shibire*, *white-eye* or *white pupae* wild type alleles (Choo *et al.,* 2019; Appendix 2).

Discovery of the first Y-chromosome protein coding gene common to many *Bactrocera* fruit flies

During the process of identifying Y chromosome gene regions to be used as potential insertion sites for the FF18002 project, we also successfully identified a protein coding gene in Qfly. One of the Y chromosome scaffolds, Btry4096, contained a novel gene with homology to an X-chromosome gene, *gyf*. This Y-gene, which we refer to as *truncated Y-chromosome paralogue of gyf* (*typo-gyf*), encodes five exons and produces a predicted 575 amino acid protein. This is the first full length Y-gene reported from a Tephritid species.

Analysis of thirteen Tephritid transcriptome libraries confirmed *typo-gyf* was also expressed in six other *Bactrocera* species including *B. latifrons*, *B. dorsalis* and *B. zonata.* Molecular dating determined *typo-gyf* evolved within the past 8.02 (CI 10.56-5.52) million years, after the split with *Bactrocera oleae*, but may have been subsequently lost from *B. correcta*. The discovery of *typo-gyf* in different *Bactrocera* species enables it to be used as a diagnostic marker for Y-chromosomes across many *Bactrocera* species. This work has recently been published in Insect Molecular Biology (Choo *et al*., 2019; Appendix 2).

Baseline temperature data for Qfly eggs



From the results above, the temperature in which 10% of the population died was found. The flies which died at this point, 37C are regarded as heat sensitive above this mortality increased, however, it was noted that 7-11 day old flies were increasingly more tolerant as temperatures increasd.. This information was then used in a trial to generate a heat sensitive population by breeding the offspring of heat sensitive parents from mating pairs.

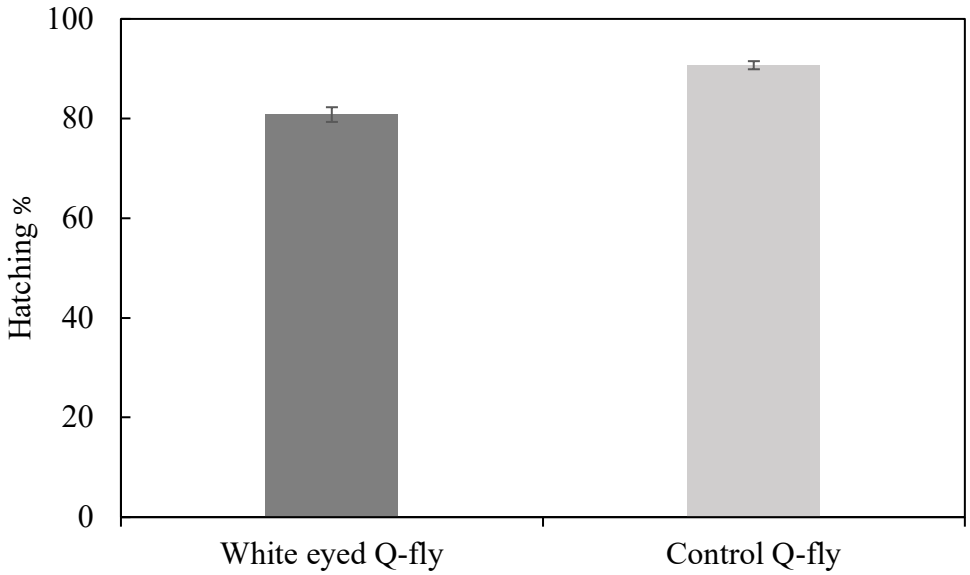
Module 2. Improved QFly Fitness assessment methods

New options and equipment were developed for QC to ensure that identical methods could be used at both the Pt Augusta and regional rear-out centers, minimizing tester error. Equipment has been manufactured and distributed to all facilities, methodological SOP's produced and staff are being trained at Netley in SA as required.

2.2) Fitness and Quality assessment of CRISPr generated strains

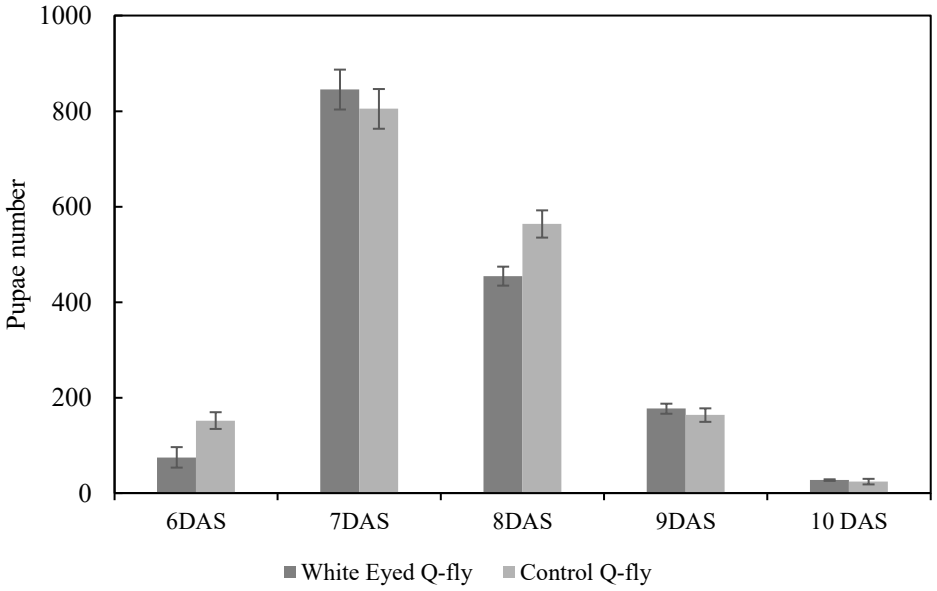
Hatching percentage

Significant difference is found on hatchability between two strains (GLM: $F_{1,18} = 34.62$, P= <.0001). Hatching percentage is higher in control Q-fly than that of White Eyed Q-fly. Hatching percentage is higher than 80 in both strains.



Peak Day of pupation

In both strains the peak day of pupation was 7 days after egg seeding followed by 8 days and 9 days after egg seeding. Pupation started after 6th days and continued up to 10 days after egg seeding. The pattern is exactly similar in both strains.

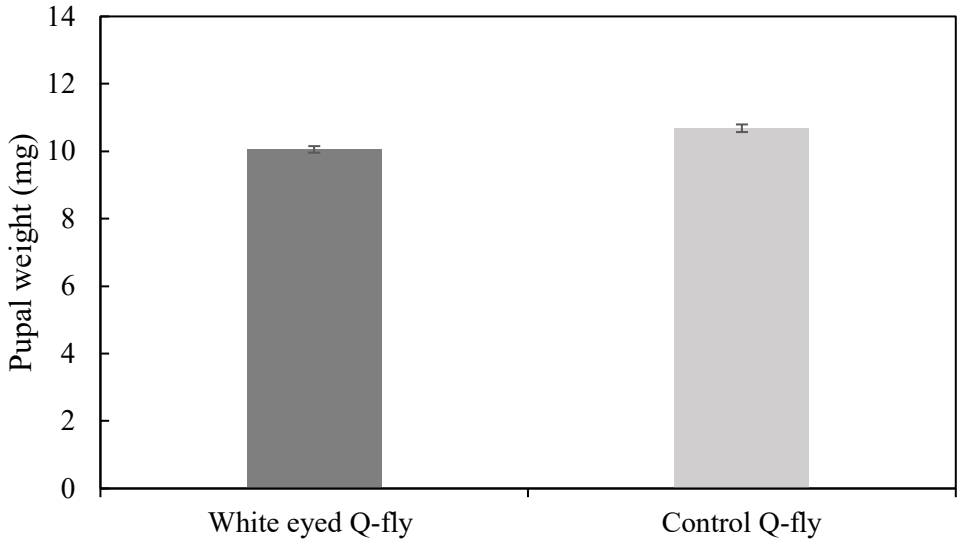


Development period

There is no significant difference between two strains in terms of development period. In both strains the adult emergence started 17 days after egg seeding.

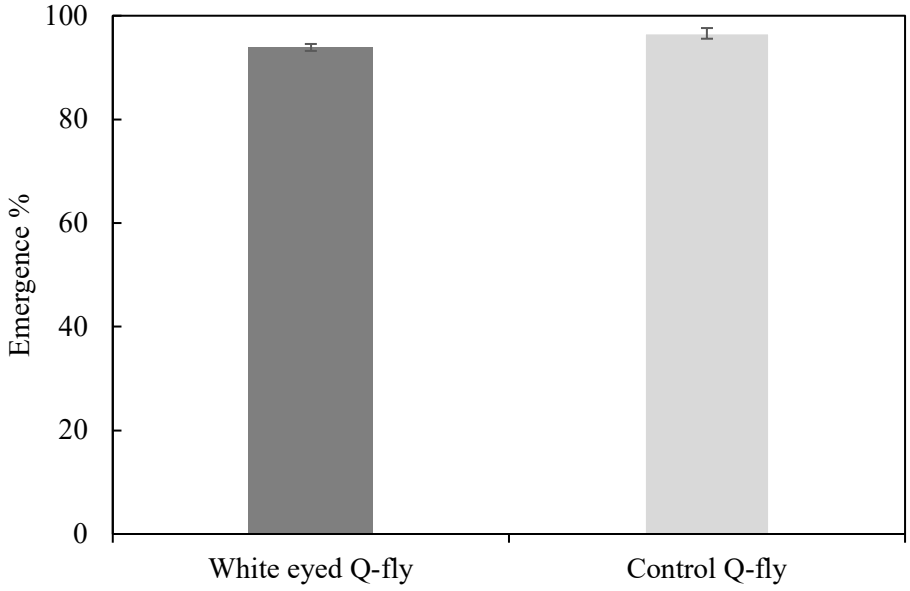
Pupal weight

There is significance difference in pupal weight in between white eyed Q-fly and control Q fly colony (GLM: $F_{1,198}$ = 17.58, P= <.0001). Control Q-fly has higher pupal weight than white eyed Q-fly. However, both strains have pupal weight more than 10 mg.



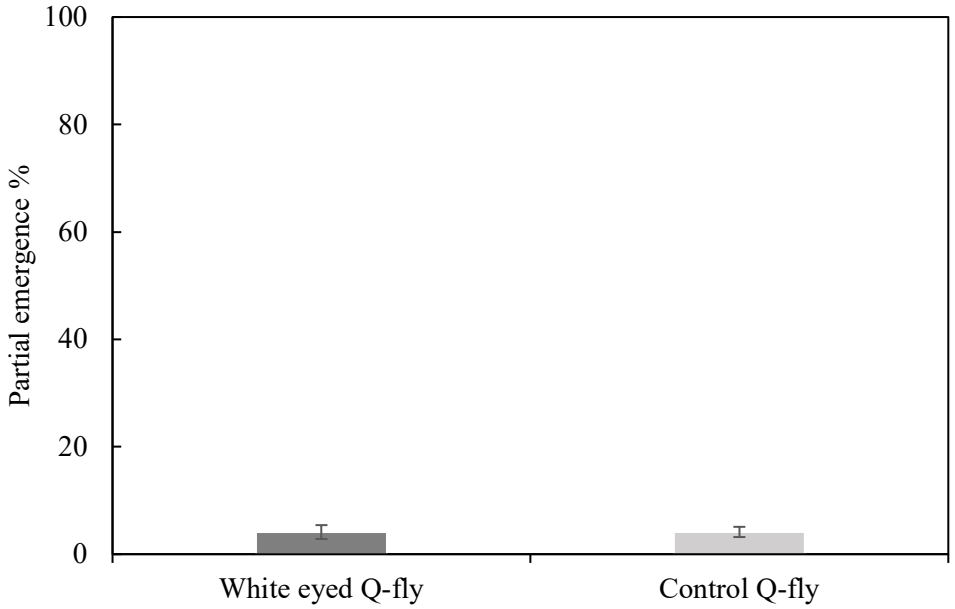
Emergence

White eyed fly emergence is comparable with control flies. There is no significant difference in number of flies emerged from the (GLM: $F_{1,8} = 4.88$, P= 0.0582).



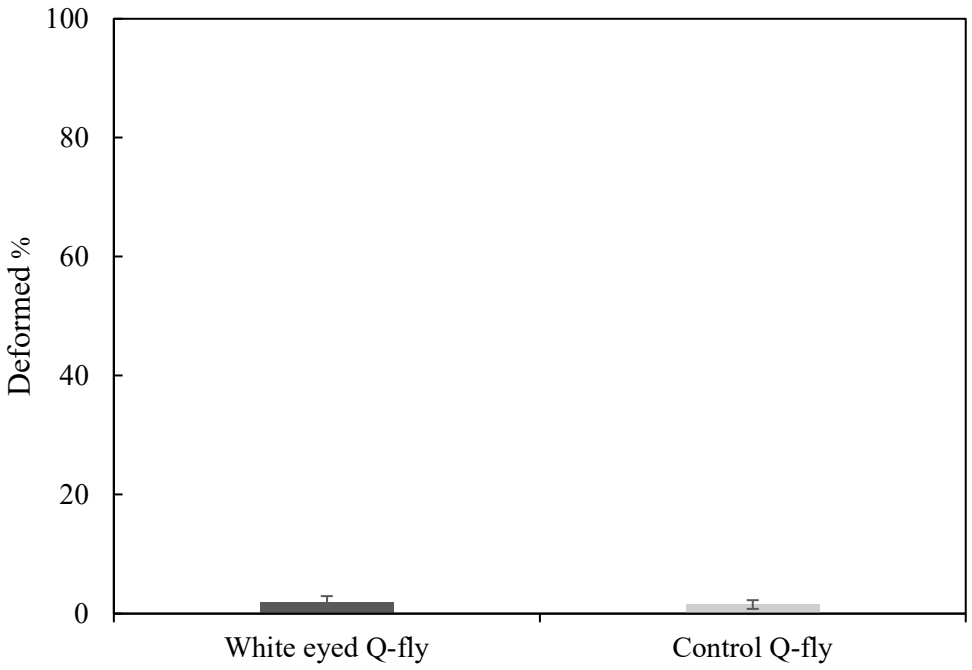
Partial emergence

There is no significant difference between white eyed Q-fly and normal Q-fly in partial emergence percent the (GLM: $F_{1,8} = 0.00$, P= 0.9919).



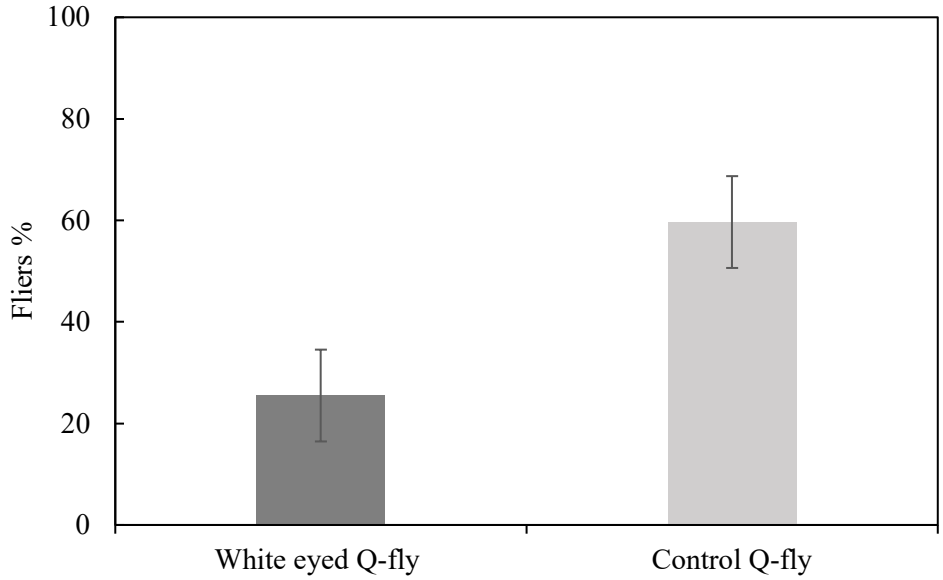
Deformity percentage

No significant difference was found on the deformity percentage between two strains (GLM: $F_{1,8} = 0.4$, P= 0.5467).



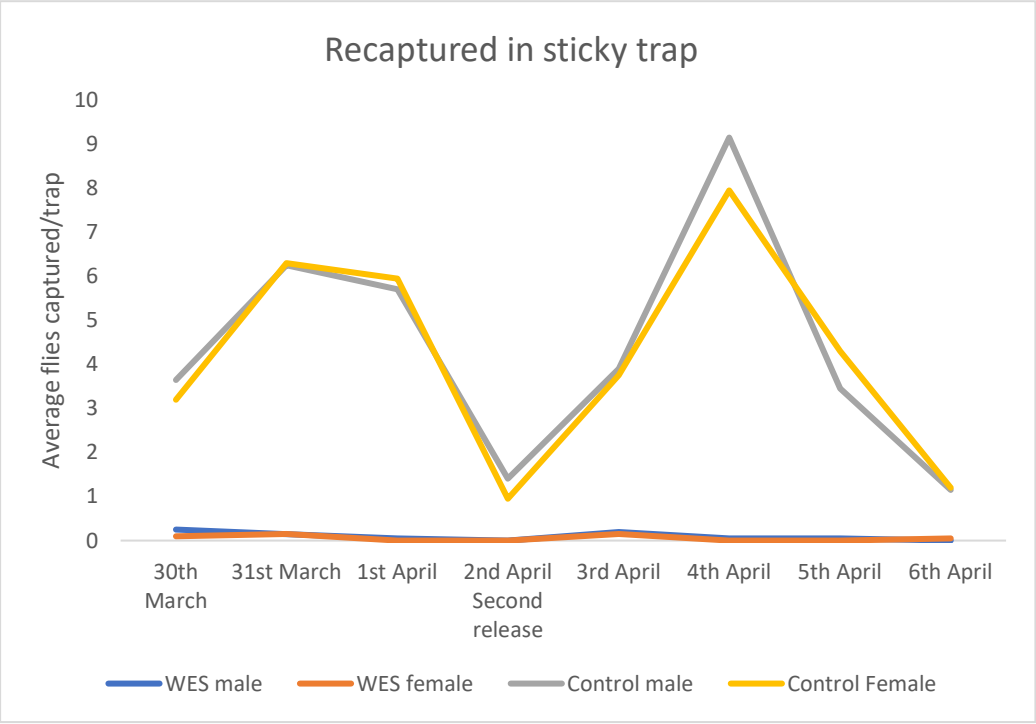
Percentage of fliers

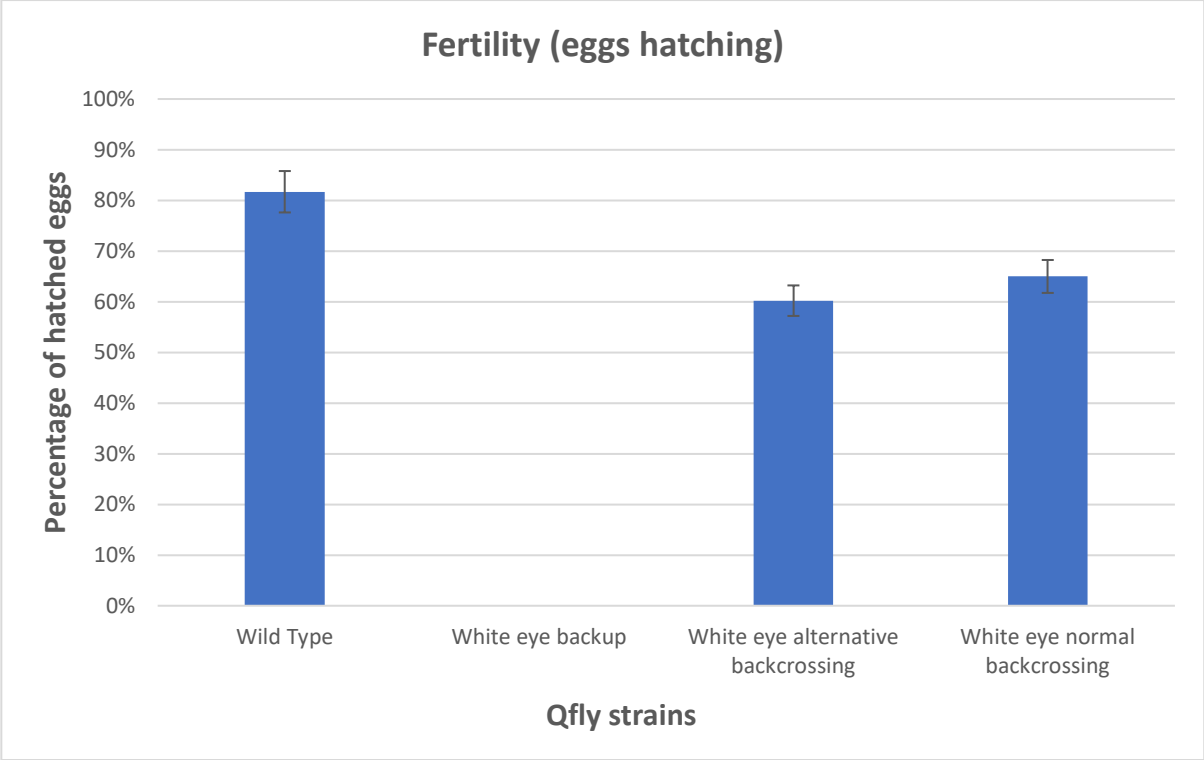
Percentage of fliers is significantly lower in white eyed strain than control strain (GLM: $F_{1,8}$ = 6.35, P= 0.0358). Because light and visual orientation are important aspects of flight-ability assays, these results are consistent with functional blindness of the white eye strain.

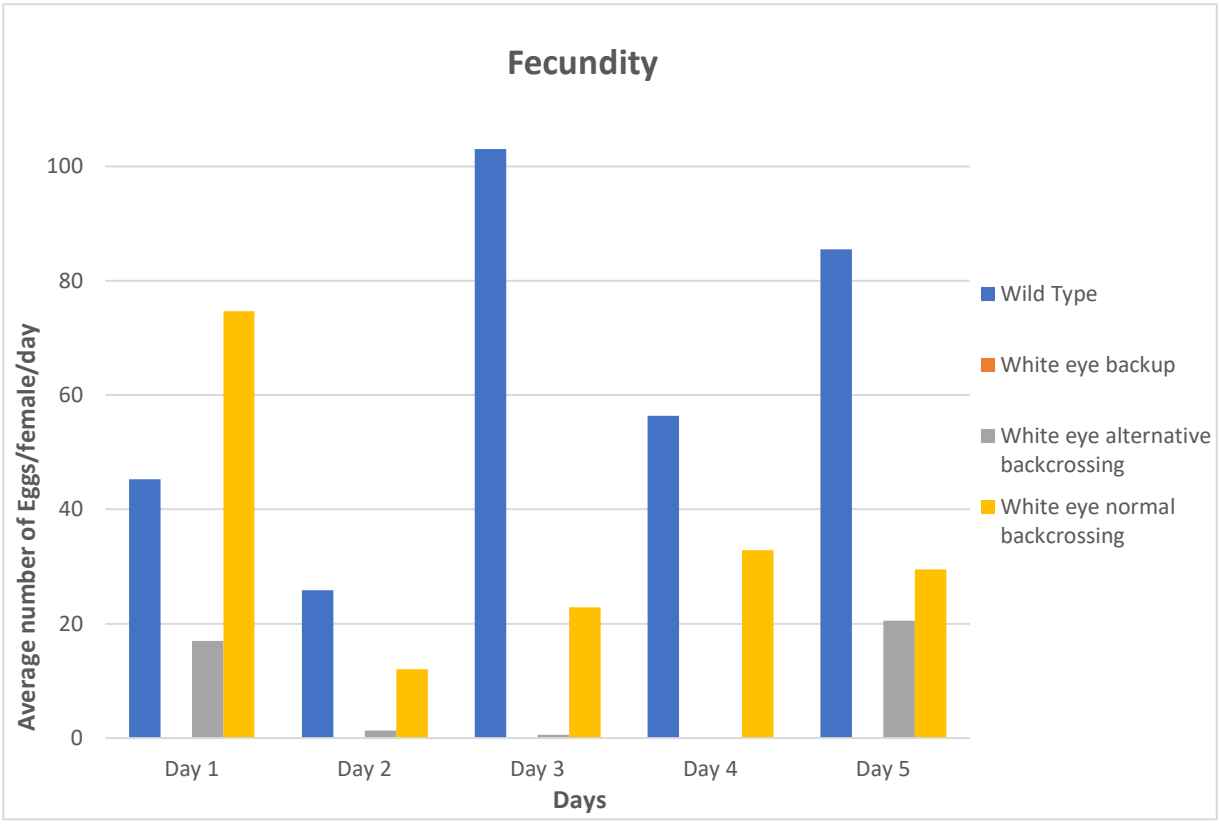


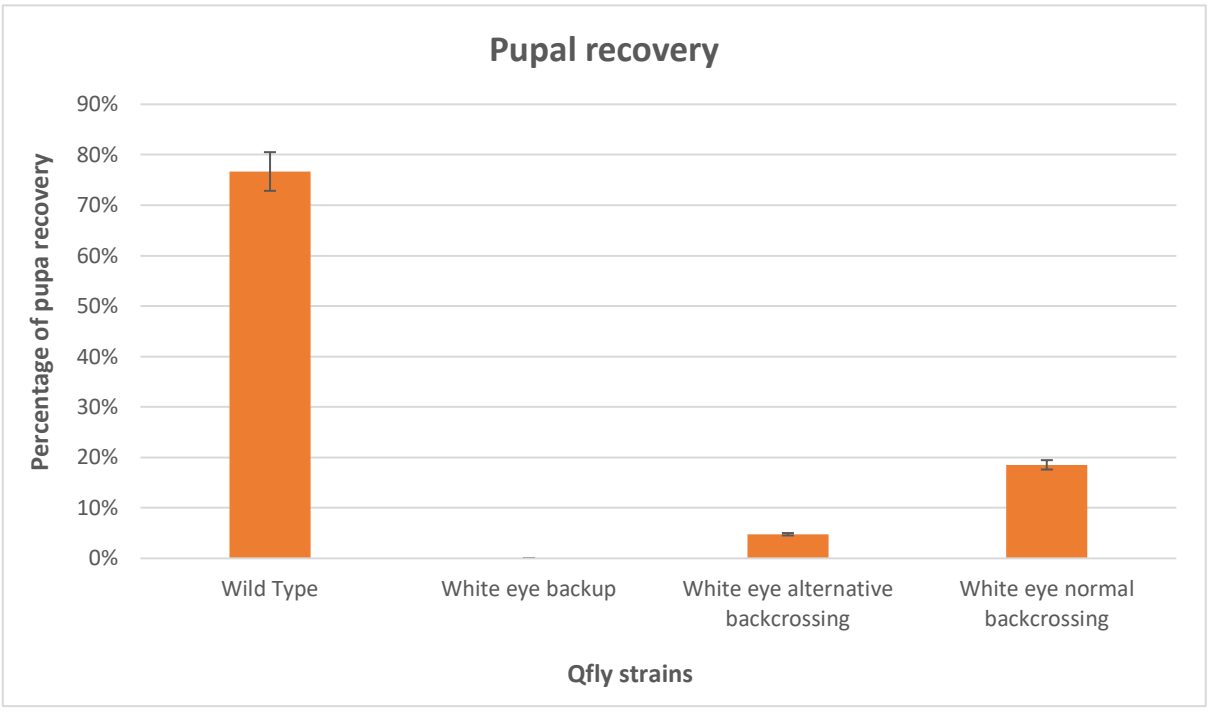
Release recapture

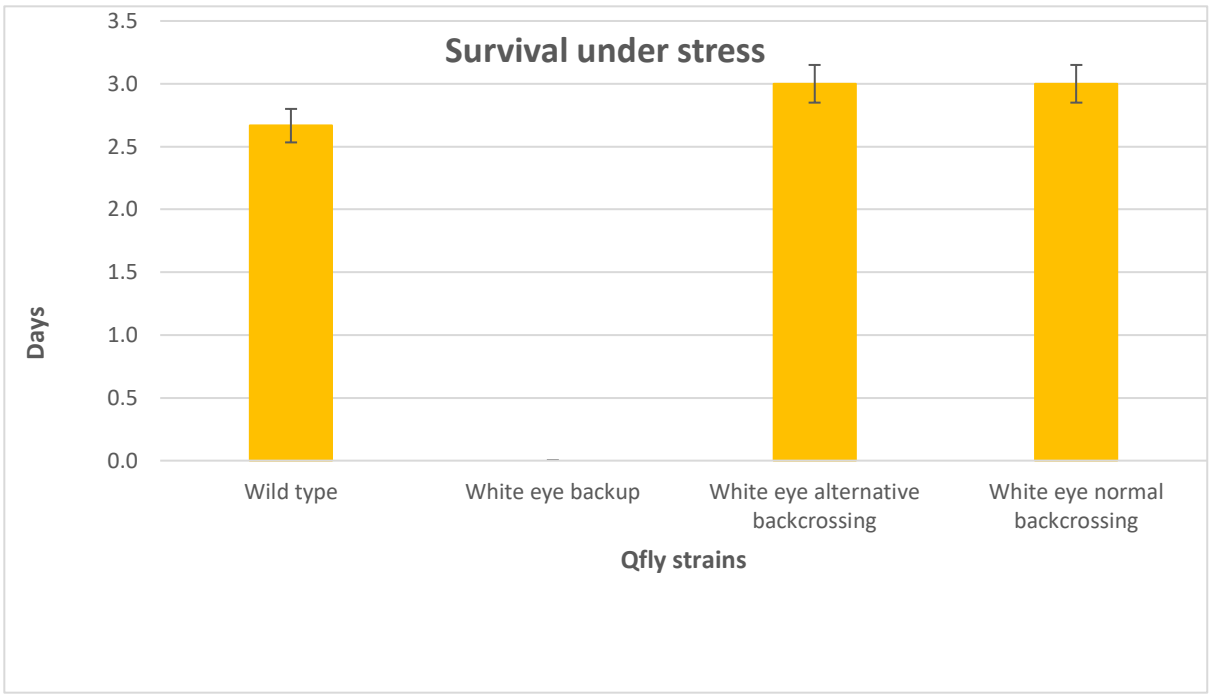
Substantial numbers of males and females of the Parental strain were captured in yellow sticky traps. In contrast, almost no males and females of the White eye strain were captured. This result is consistent with 'functional blindness' of the white eye strain.

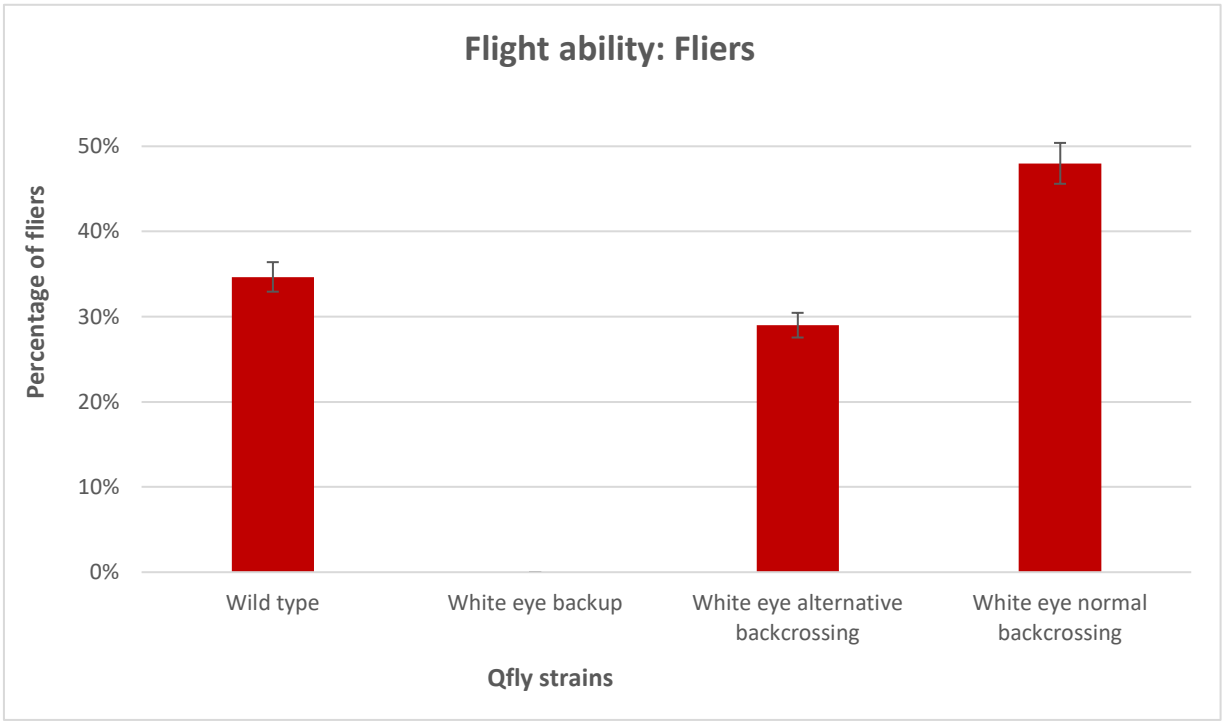

Recaptured in sticky trap
Average flies captured/trap
10
9
8
7
6
5
4
3
2
1
0
30th March
31st March
1st April
2nd April Second release
3rd April
4th April
5th April
6th April
WES male
WES female
Control male
Control Female

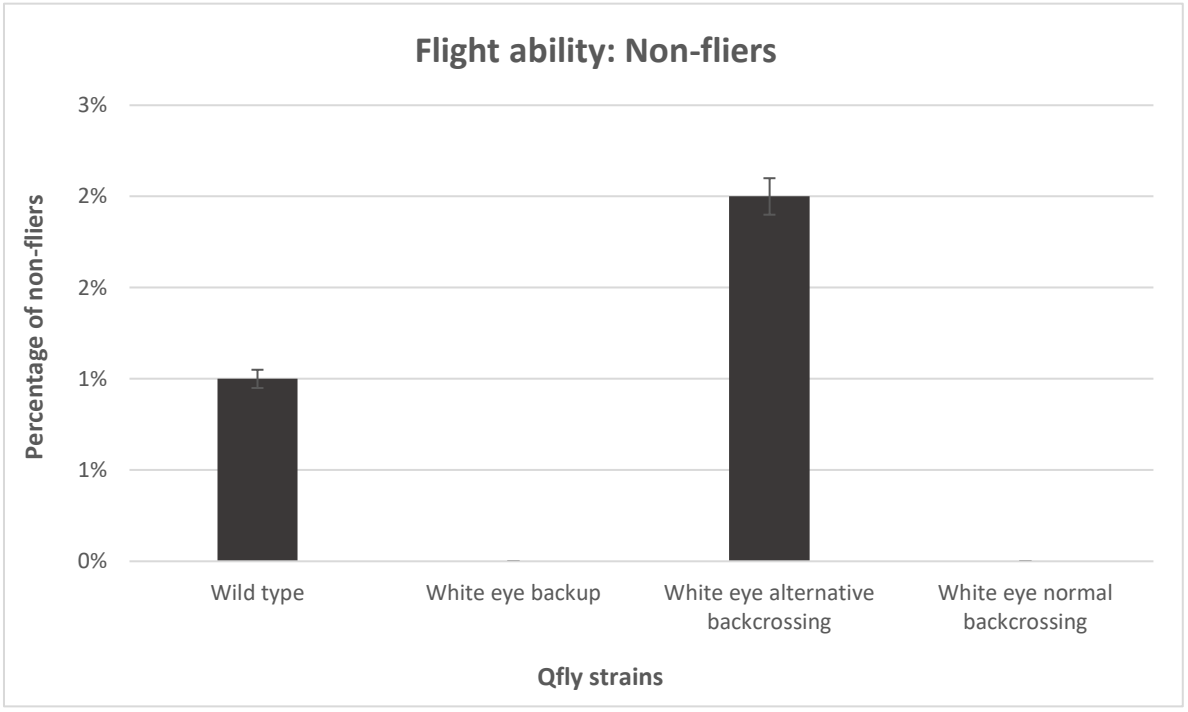

Fertility (eggs hatching)
Percentage of hatched eggs
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Wild Type
White eye backup
White eye alternative backcrossing
White eye normal backcrossing
Qfly strains

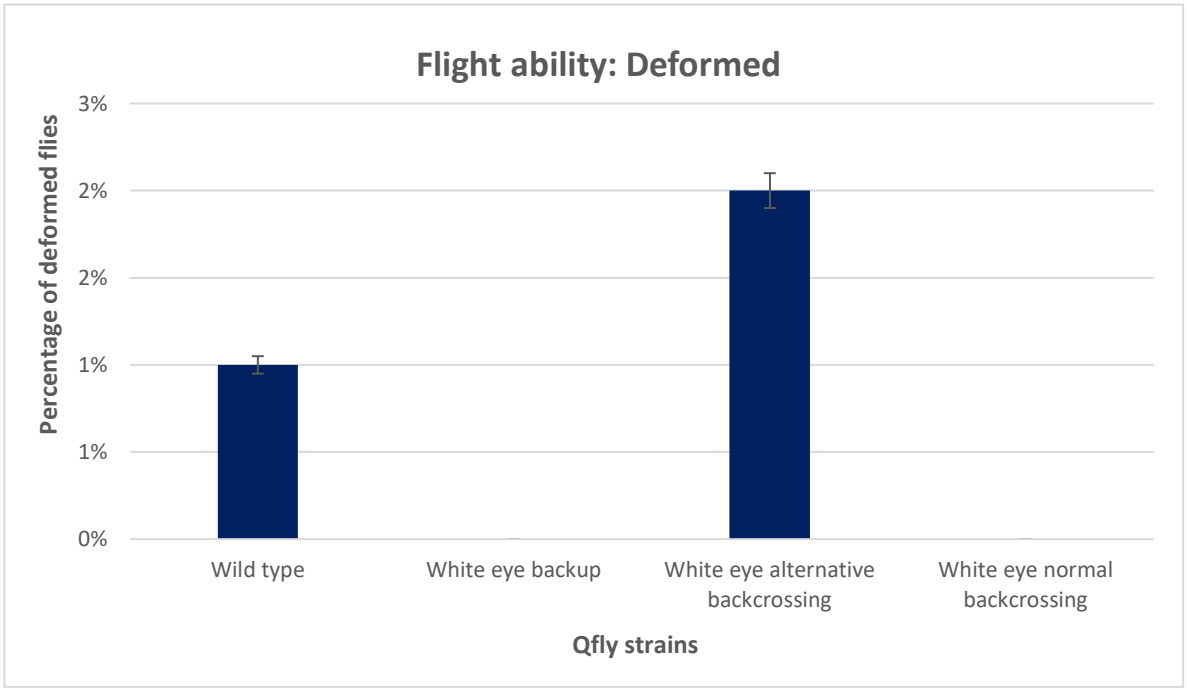

Fecundity
Average number of Eggs/female/day
100
80
60
40
20
0
Day 1
Day 2
Day 3
Day 4
Day 5
Days
Wild Type
White eye backup
White eye alternative backcrossing
White eye normal backcrossing

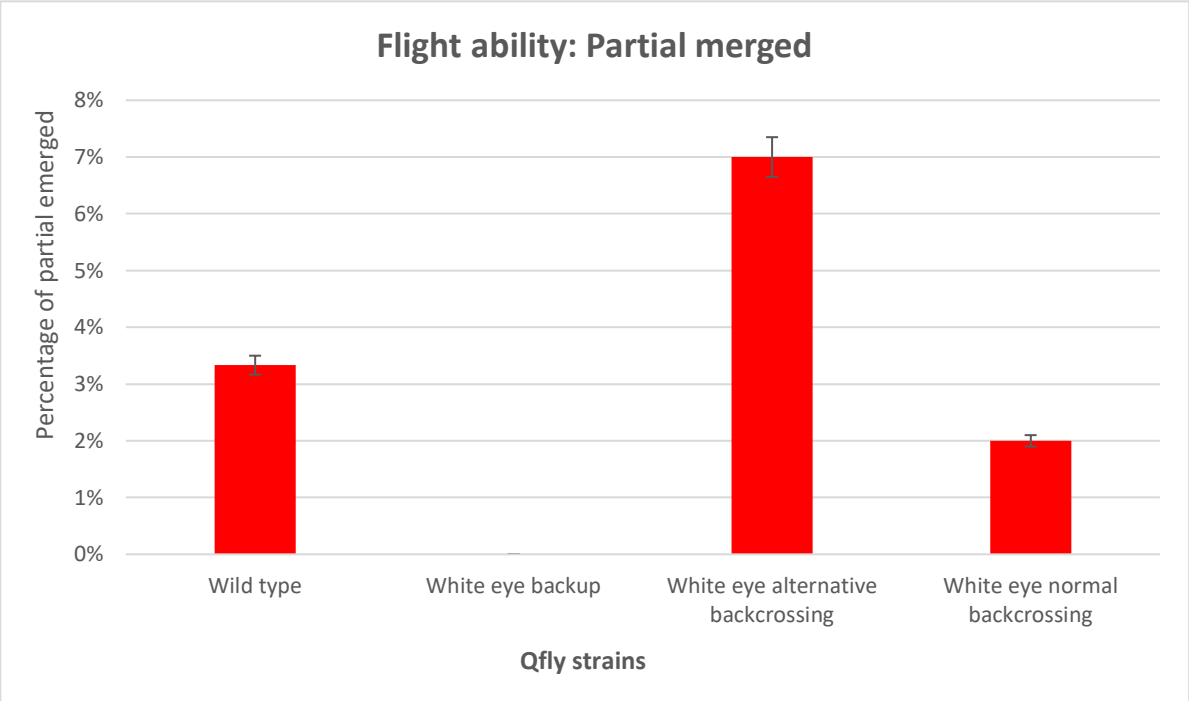

Pupal recovery
Percentage of pupa recovery
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Wild Type
White eye backup
White eye alternative backcrossing
White eye normal backcrossing
Qfly strains

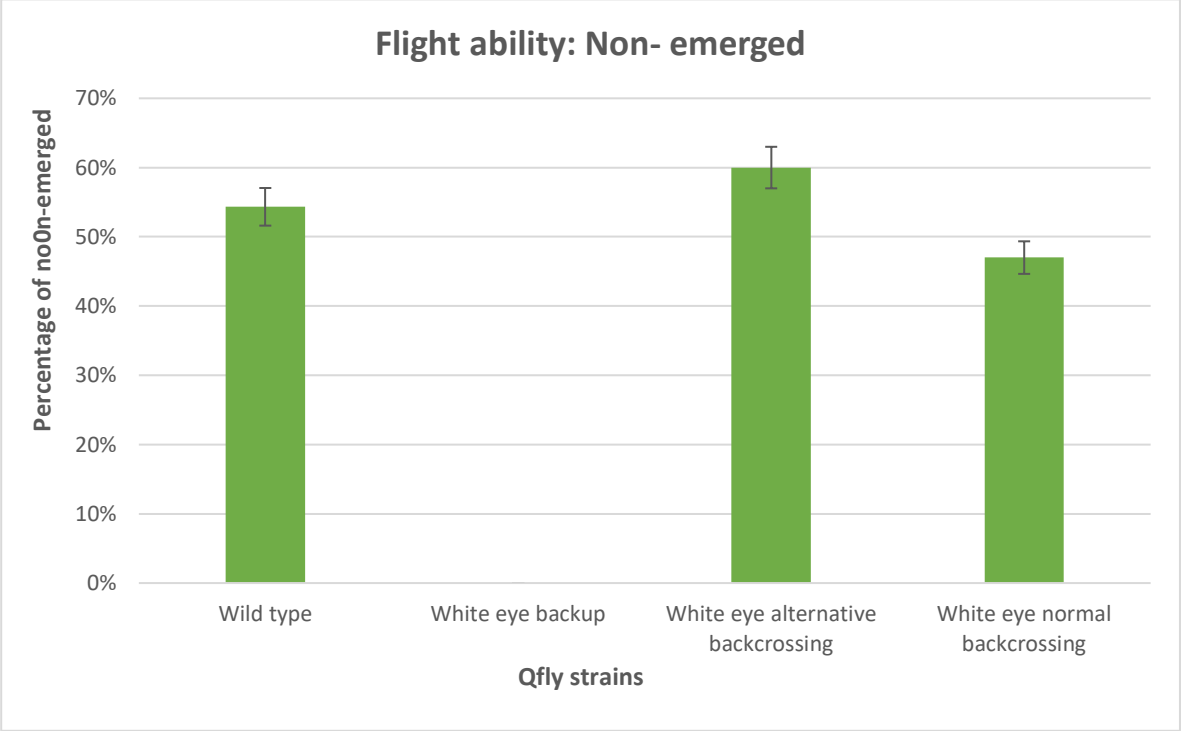

Survival under stress
Days
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Wild type
White eye backup
White eye alternative backcrossing
White eye normal backcrossing
Qfly strains


Flight ability: Fliers
Percentage of fliers
50%
40%
30%
20%
10%
0%
Wild type
White eye backup
White eye alternative backcrossing
White eye normal backcrossing
Qfly strains


Flight ability: Non-fliers
Percentage of non-fliers
3%
2%
2%
1%
1%
0%
Wild type
White eye backup
White eye alternative backcrossing
White eye normal backcrossing
Qfly strains


Flight ability: Deformed
Percentage of deformed flies
3%
2%
2%
1%
1%
0%
Wild type
White eye backup
White eye alternative backcrossing
White eye normal backcrossing
Qfly strains





Module 3. Diet optimisation and Implementation

Diet was optimized at 2.5 kg per rearing tray (45x70cm) to which 4ml or 50,000 eggs are added, this produces pupae of between 0.08 and 0.09 gm which is an optimal size. Higher density egg inoculation results in smaller less fit pupae. Greater volumes of diet does not result in increased production as the critical parameter appears to be surface area rather than depth of diet. Lesser volumes of diet, particularly 1.5 and 1 litre per tray dry and

become unsuitable for larval feeding before larvae can fully develop.

Module 4. Increased mating propensity- SITplus strain

At this stage neither a tsl nor a GSS strain of Qfly has been developed and preliminary testing of yellow body and white eye mutants is yet to be completed. The evaluation of the volatile compounds identified for this section of the research will be conducted as part of the Post production Pilot project led by Macquarie University using the Yellow bodied strain, a white pupal strain and the current factory "wild type" strain. These strains will be evaluated for flightability and longevity in the field through use of standard Cuelure and protein traps. Females caught in the protein traps will be assessed for mating and a ratio of wild to sterile mating's will be assessed.

Outcomes

- We successfully established the new CRISPR-Cas9 genome editing technology in Qfly.
- We were the first to demonstrate CRISPR-Cas9 mutagenesis technology in tephritid species and are continue to make advances with this technology.
- Generation of white eye mutant strain that may potentially be used as a functionally male-only strain.
- Demonstrated that we are able to use the CRISPR-Cas9 technology to introduce precise base changes through successful introduction of the *shibire tsl* mutations into Qfly genome; thus demonstrating it is possible for us to generate a tsl strain using this method in due course.
- Identification of candidate mutations responsible for the *Bactrocera dorsalis* white pupae phenotype and implementation of CRISPR-Cas9 injections to knock out the candidate gene in Qfly.
- Generation of a novel Qfly body colour phenotype which may potentially be used as a marker of sterile flies

- Identification of Y-chromosome sequences in Qfly that can be used as target sites for transgene insertions or translocation of wild type alleles to generate the male-selecting strain.
- Identification of the first Y chromosome protein-coding gene conserved across different *Bactrocera* species.
- Two publications thus far (Choo *et al.,* 2018; Choo *et al.,* 2019) and another manuscript in progress (Choo *et al*., in progress).
- The factory is now capable of producing the 50M flies per week target but is running at a limit of 20M per week which meets current demand
- The X-ray dose required for satisfactory sterilization has been implemented at the Pt Augusta Facility

Monitoring and evaluation

The objectives associated with the Pt Augusta facility, namely diet optimization, X-ray irradiation dose, QC testing, egg production optimization and other support toward the achievement of a weekly production capacity have been achieved. The facility is now capable of producing the target 50M sterile flies per week but it is planned to limit production to up to 20M per week as dictated by demand.

The X-ray irradiation dose has been verified and results are similar to the use of a Gamma radiation source. The 50+ batches of sterile flies shipped for use in South Australia and Victoria have all been tested for DNA damage required for sterility and all have met sterility requirements. This support is expected to continue on an as required basis and will include the provision of improved and genetic sex selecting strains. Rearing equipment has been developed and diet optimized under production conditions and while minor future changes are expected with experience these are largely finalized and have proven reliable. Initial variations in QC measurements, such as pupal weight and flightability are stabilizing and a consistent quality is now being achieved.

While the final *tsl* strain has not yet been developed, the underlying techniques to achieve this have been developed for Quennsland fruit fly. Mutant strains using CRISPR CAS9 have been successfully generated. While the white eye mutant has shown some reduction in fitness in early generations most of this has been recovered through backcross and selection of fitter flies. Early evaluation of the yellow body strain indicates that it may

develop to a mature adult more quickly than wild-type strains and may have higher fecundity. There is also potential that the yellow body strain may be able to be separated from wild flies using artificial intelligence (AI) and machine learning. This could eliminate the need for dye marking flies, reducing costs and eliminating the fitness costs associated with the use of the dye. This will require further research and development but could result in the replacement of the strain currently reared at Pt Augusta.

The hybrid strain developed in collaboration with the IAEA has led to the identification of a candidate white pupal gene that potentially will lead to the development of a genetic sexing strain. Initial trials to knock out the gene using CRISPR techniques have produced a heterozygotic mutant that has a colour pattern not seen in wild type pupae. Before this can be introduced a homozygote strain need to be created through breeding and selection followed by fitness and mating competitiveness trials. If these prove successful the male flies will be recovered to wild type pupae colour through introgression of the wild-type gene onto the Y chromosome. As part of the collaboration with the University of Adelaide on this project the Y chromosome has been characterized and several target locations for introgression of wild-type recovery genes have been identified.

The introgression of wild-type recovery genes onto the Y chromosome can be achieved by 2 means; random mutation or genetic technologies. Random mutation can be achieved through the use of low dose irradiation but is imprecise and other non-target effects are likely and is a matter of luck. To date as part of this project approximately 75,000 male Qfly have been subject to low dose irradiation (20-25 gy) and crossed with White eye females but no recovery of the wild type eye colour in males has been achieved. Molecular methods, such as CRISPR CAS9 are more accurate and there is a lower risk of off target mutations resulting from the process, however, while not transgenic these transformations result in the organism being classed as a Genetically Modified Organism which require permits from Office of the Gene Technology Regulator (OGTR) and Dept for the Environment before they can be released.

Recommendations

- It is recommended that the research and development of a *tsl* or genetic sexing strain of Qfly continue in order that the production and efficiency of the Pt Augusta SITplus Facility be optimized. The development of such a strain will greatly reduce the cost of sterile flies and increase the attractiveness of the technology to growers and governments managing Qfly.
- The development of AI machine learning technology should be considered for identification of colour mutant to facilitate rapid processing of sterile individuals recaptured in monitoring traps after field release.

Refereed scientific publications

- Akter, H., Taylor, P. W., and Crisp, P (2019) Evaluation of fluorescent dyes in relation to visibility and persistence, and impacts on emergence, quality and survival of sterile Queensland fruit fly, *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) (Manuscript prepared for submission).
- Choo, A., Crisp, P., Saint, R., O'Keefe, L. V., Baxter, S. W., 2018. CRISPR/Cas9-mediated mutagenesis of the *white* gene in the tephritid pest *Bactrocera tryoni*. *Journal of Applied Entomology*, **142**:52-58. https://doi.org/10.1111/jen.12411
- Choo, A.*, Nguyen, T. N. M.*, Ward, C. M., Chen, I. Y., Sved, J., Shearman, D., Gilchrist, A. S., Crisp, P., Baxter, S. W., 2019. Identification of Y-chromosome scaffolds of the Queensland fruit fly reveals a duplicated gyf gene paralog common to many *Bactrocera* fruit flies. *Insect Molecular Biology*, DOI: 10.1111/imb.12602
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- Siddiqui, M.S., Taylor, P.W., Nacey, L.C. and Crisp, P. (2019) Chronic hypoxia compromises repair of DNA double-strand breaks in Queensland fruit fly *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) (manuscript in Preparation)

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Appendices

Appendices 3

Evaluation of fluorescent dyes in relation to visibility and persistence, and impacts on emergence, quality and survival of sterile Queensland fruit fly, *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae)

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Abstract

Tephritid flies released in sterile insect technique pest management programs are usually marked with fluorescent dyes so that they can be distinguished from wild flies in monitoring traps. Dyes can have adverse effects on emergence, quality and survival, which is a big concern of SIT success rate. The present study examines the effects of five fluorescent dyes (Fluoro pink, Fluoro Orange, Stella Green, Arc Chrome and Astral Pink) applied at four dose levels (1, 2, 3 and 4 g/L) on Queensland fruit fly (Qfly). Compared with a no dye control, all dyes caused a significant reduction in percentage of fully developed adult flies, and the extent of this effect was similar for all dyes. Incidence of morphological deformity also was affected increased by dye treatment. No effects of dye were found on survival when the flies were held under nutritional stress, without access to food or water. In the absence of nutritional stress, flies receiving low doses of dye lived longer than those that received high doses, and females lived longer than males. Persistence of all dyes was not effected with dye or dose, however, visibility varied with dose. However, within dyes Stella Green had the lowest visibility. A dose of 1g/L dye was less visible than 2, 3 and 4g/L. Considering emergence, deformity, survival and visibility Fluoro Orange is given 1st preference and a dose of 2g/L dye is given 1st priority.

Key words: Emergence, dye marking, dye persistence, sterile insect technique, SIT.

Introduction

Queensland fruit fly (Qfly) *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) is an economically important pest of fruits and vegetables in eastern Australia (Sutherst and Yonow 1998, Sutherst et al. 2000, Clarke et al. 2011), causing significant financial loss through direct damage of fresh produce, restrictions on inter-state and international market access, and management practises (Horticultural Policy Council 1991). As restrictions on the use of insecticides have increased the number of viable options for control of this destructive pest have declined sharply (Dominiak and Ekman 2013), and this has greatly increased the need for alternative, sustainable, control strategies. Sterile insect technique (SIT), in which millions of sterile insects are released to inhibit reproduction in pest populations (Knipling 1955), has been used to manage some of the world's most serious fruit fly pests in many countries such as for *Zeugodacus cucurbitae* (Iwahashi 1976), *Anastrepha ludens* (Rull et al. 2007), *Anastrepha obliqua* (Toledo et al. 2004), *Ceratitis capitata* (Hendrichs et al. 2002,
Holbrook et al. 1970), *Bactrocera philippinensis* (Resilva et al. 2007). SIT involves mass rearing of insects, irradiation to induce sterility, marking, release of sterile insects into the target area, and recapturing a proportion of the released sterile flies to monitor field performance (Meats and Edgerton 2008, Campbell et al. 2009). Following promising early experimental work in the 1960s (Monro and Osborn 1967), SIT was developed into programs to manage outbreaks of Qfly that operated since the early 1990s (Horwood and Keenan 1994, Dominiak et al. 2003, Meats et al. 2003, Worsley et al. 2008, Reynolds et al. 2010, Reynolds and Orchard 2011).

Successful SIT depends on a wide range of biological and physiological attributes of sterile flies, including emergence, flight ability, survival until sexual maturity, courting and mating with wild females, and ability to induce long refractory periods (Hendrichs et al. 2002, Meats and Edgerton 2008). These biological and physiological attributes can be affected by numerous aspects of the production process including, for example, mass rearing conditions, irradiation procedures, dyeing, temperature, transportation (Fay and Meats 1987, Toledo et al. 2004, Dominak et el. 2007ab, 2008, Rull et al. 2007, 2011, Collins et al. 2008, 2009). To maximise the availability of high quality flies for SIT programs, it is important to consider the potential negative impact of each production process.

Marking is an indispensable step of SIT operations to differentiate sterile flies from wild flies in monitoring traps. Sterile flies are marked with dye at the pupal stage prior to emergence, usually 24-48 hours prior to emergence (Norris 1957, Steiner 1965, FAO/IAEA/USDA 2003, 2014, Enkerlin 2008). In early trials oil-soluble dyes were used to coat pupae (Steiner 1965), but these were later replaced with fluorescent dyes which are much more efficient and convenient (Holbrook et al. 1970). Dyed flies accumulate the fluorescent dye in their ptilinum during eclosion (Steiner 1965), which can be seen with microscopic examination under UV light (Holbrook et al. 1970, Enkerlin et al. 1996).

Commonly used fluorescent dyes in marking sterile Qfly in Australia are Arc Chrome (Yeates et al. 1992, Horwood and Keenan 1994, Jackman et al. 1996, Dominiak et al.1998), Fire Orange (Yeates et al. 1992), Orange (James 1992, Reynolds et al. 1995), Red (James 1992, Reynolds et al. 1995), Blaze Orange (Perepelicia et al. 1997), Laser Red (Horwood and Keenan 1994), Nova Red (Jackman et al. 1996, Dominiak et al.1998) and Comet Blue (Jackman et al. 1996, Dominiak et al.1998). Some studies have mentioned the use of dyes to identify released flies, but have not provided details of which dyes were used (MacFarlane et al. 1987, Perepelicia et al. 1993, Perepelicia et al. 1994). Some dyes were difficult to identify over other dyes such as Comet Blue is difficult to identify compared to Nova Red and Arc Chrome (Jackman el al. 1996), Arc Yellow and Signal Green were reported to be more difficult to identify than Blaze Orange and Rocket Red in field condition after 1 week (Holbrook et al. 1970). In addition, some dyes may have lower persistence on flies, for example, Comet Blue has been reported to be more easily removed by fly grooming than Nova Red or Arc Chrome (Dominiak el al. 2000b). In addition to the variation in difficulties in dye discrimination and persistence, fly recapture rates can also vary with dye, for instance, Yeates et al. (1992) found that 0.094% of flies marked with Fire Orange were recaptured in monitoring traps compared with 0.123% of flies marked with Arc Chrome.

In addition to the variation in dyes, different doses of dye were used at different times in sterile release programs. The *Bactrocera philippinensis* Drew and Hancock (currently the species name is replaced with *Bactrocera dorsalis*) program in the Philippines used 1.5 g/L of fluorescent dye per litre of pupae (Resilva et al. 2007). Midgarden et al. (2004) used 4 g/L of Dayglo fluorescent dye on Medfly. In studies of *Dacus cucurbitae* (Coquillett) (currently the species name is replaced with *Zeugodacus cucurbitae*)

Iwahashi (1976) used four different dyes of fluorescent powder at 3 g/L whereas Schroeder and Mitchell (1981) used 5-20g/L. For Qfly, Dominiak et al. (2010b) and Campbell et al. (2009) used 4g/L whereas Reynolds et al. (2010) used 0.8g/100gm pupae (equivalent to 3.2 g/L dye), Weldon (2005) used 0.2g/100 pupae (0.02 g/100 pupae, personal communication, that is equivalent to 8 g/Litre pupae).

Marking with dye can diminish emergence and survival of Qfly (Weldon 2005, Campbell et al. 2009, Dominiak et al. 2000b, 2010ab). Although pink and orange dyes are most commonly used in Qfly trials and suppression programs (Dominiak et al. 2000b), multiple discriminable dyes are sometimes needed to differentiate flies used in overlapping release at the same location. Dominiak et al. (2000b) evaluated the effect of twelve dyes on sterile Qfly at a dose of 4.5g/L of pupae in laboratory condition in three trials. In this experiment they found Nova Red and Orange dyes were best as they had least impact on emergence. Weldon (2005) also tested 9 different fluorescent dyes at a dose of 0.2gm/100 pupae (0.02 gm/100 pupae, personal communication that is equivalent to 8 gm/Litre pupae) and found reduced emergence in marked flies. Dominiak et al. (2010a) tested the effects of dose of Fiesta Astral Pink dye (Swada) and found reduced adult eclosion as doses increased from 1.5 g/L to 4.5 g/L. While there have been studies investigating effects of different dyes on Qfly at a standard doses (Weldon 2005, Campbell et al. 2009, Dominiak et al. 2010b), and effects of dose for one individual dyes (Dominiak et al. 2010a), to more fully evaluate the suitability and use of different dyes there is a need for studies that compare effects of dose of multiple dyes. The present study evaluates the effects of five different fluorescent dyes along with control (no dye) at four doses in relation to adult emergence, survival and persistence of visibility.

Materials and methods

Pupal irradiation and dye treatment. Flies for this experiment were obtained from Queensland fruit fly mass production and X-ray irradiation facility at Port Augusta, South Australia (SA), Australia. Pupae were irradiated 2 days prior emergence under hypoxic condition in a carbon fibre canister at 70 Gy (Dominiak et al. 2008, 2014a, Collins et al. 2009). On the day of irradiation, pupae were immediately transported in a closed Styrofoam box in an air-conditioned vehicle to South Australian Research and Development Institute (SARDI), Waite Campus, University of Adelaide, SA, Australia.

Upon arrival at SARDI, pupae were marked with dyes at a rate of 1, 2, 3 and 4 g/ L of pupae. Five fluorescent dyes were used - Fluoro pink and Fluoro Orange from Tintex dye manufacturers, Australia, and Stella green, Arc Chrome, and Astral Pink from Swada Fiesta Fex series, London, England along with an undyed control group. Dyes from Tintex dye manufacturers have a particle size of 0.5-2µm and dyes from Swada have particle size of 3-4 µm. To obtain each amount of dye for treatment at 1, 2, 3 and 4 g/L, each dye was weighed using a laboratory milligram scale with 0.001 g readability (A&D weighing GX-400, A&D Australasia Pty Ltd., Australia). To evenly coat the pupae, half the volume of pupae was placed in a plastic cylindrical vial (Corning canister ™ 430791, NY, medical grade, sterile) then half the required dye was added, then remaining pupae and dye were added. After adding dyes, the vials were closed with lids and gently swirled to mix dye evenly with the pupae. Undyed control pupae were also transferred to the same vials and swirled to avoid differences in handling.

Adult emergence, Survival, dye visibility and dye persistence study. To study adult emergence, ca. 25 pupae of each dose of each dye were kept in individual 14.8mL plastic cup (No. P050, SOLO cup company, Urbana, Illinois, USA). For each dye, 8 cups were set and each cup was placed separately in each of 8 separate 720 mL Bugdorm with donut lid installed with white nylon screen (BDPS24; Eco plan Korea) to emerge. The cages had a mesh-covered opening for ventilation comprising out approximately 25% of the total surface area. Four days after the first emergence, cups of pupae were removed from the cages and counted to calculate fully developed adults (with expanded wings), deformed flies (either with deformed wing or body or the absence of wings), partially emerged flies (some part of body emerged from the pupal case but remained attached) and unemerged flies (intact pupae).

Fully developed adults were transferred to identical cages to test for survival, half of them were studied under nutritional stress (no food or water) and half of them were studied under no nutritional stress condition (both food and water were provided ad libitum). Cages were observed daily until 5 weeks and any dead flies were removed, dated and stored in freezer to identify the dye and sex at later stage. We chose 5 weeks as few released flies survive longer than this in the field (Dominiak and Webster 1998).

All flies that were died during survival study until 5 weeks were inspected for marker visibility and persistence study. Both male and female were observed for this study, both ptilinum and body parts of flies were inspected in Petri dishes under stereo microscope (Olympus SZX7, Japan) under Royal Blue light (455nm) together with a barrier filter (SFA-LFS-RB, NIGHTSEA, Lexington, MA 02420, USA). If dye particles were observed in the ptilinal suture and body part they were recorded as visible even if no fluorescence was observed (Weldon 2005). All flies were inspected to understand the level of visibility and persistence of different dyes at different doses. The level of visibility was ranked from 0 to 3 where 0=not visible (0% dye in ptilinum and/or body part), 1=poor (1-20% ptilinum and/or body covered), 2=good (21-60% ptilinum and/or body covered), 3=best (61-100% ptilinum and/or body covered).

Eight cages/dye/dose were set up for emergence study which resulted in 192 cages. For survival study, 4 cages /dye/ dose were observed for mortality under nutritional stress (96 cages) and 4 cages /dye/ dose were observed under non-stressed condition (96 cages). The experiment was repeated three times using different production batches of pupae (600 pupae/dye/dose). All experimental procedures were conducted at 65-70% RH, 26±1^{0}C temperature and an artificial photoperiod of 12:12 (L:D) along with natural lights through window in Quarantine facility at SARDI.

Statistical analysis. Data were analyzed with IBM SPSS 25. Data were first analysed to ensure that they reflected the assumptions of normality and homogeneity of variances. Effect of dose, dye treatment, dose*dye treatment interaction on emergence of fully developed adults, deformed adults, partial emergence and no emergence by running MANOVA. Tukey multiple comparison tests were used for comparing within dyes and doses. Cox proportional hazards regression model was performed for regression analysis to see the relationship between survival and dye, dose and sex. Data from flies that remained alive after 5 weeks were censored and the days when flies died during the experimental period were considered as event in the analysis. Survival curve was generated using Cox proportional hazards regression model. Persistence and visibility of dye were assessed by running ANOVA. All statistical analysis were performed in IBM, SPSS 25.

Results

Effect of dye and dose on emergence. Overall dye treatment reduces the emergence of fully developed adults compared with the undyed control. Both dye and dose had significant effect on the emergence of fully developed adults (Table 1, Figure 1). Significant difference in emergence of fully developed adults was observed between control and dyed flies except Fluoro Orange which did not show any significant difference (p=0.003, p=0.248, p=0.041,
p=0.045 and p=0.013 for Fluoro Pink, Fluoro Orange, Stella Green, Arc Chrome and Astral Pink respectively). Within dye colour no difference in emergence of fully developed adults was observed. Dose had effect on the emergence of fully developed adult flies (Table 1). On average, lower the amount of dye higher the percent of fully developed adult's emergence (Figure 1). There was significant difference between doses of 1 and 4g/L ($p<0.001$) and between 2 and 4g/L (p=0.011). No significant differences were detected between doses of 1, 2 and 3g/L.

Deformity was affected by dye treatment and dose (Table 1, Figure 2). Dyeing increased the deformity, undyed flies significantly differed from dyed flies in percent deformity (p<0.001, p =0.039, p =0.05, $p<0.001$, $p<0.001$ for Fluoro Pink, Fluoro Orange, Stella Green, Arc Chrome and Astral Pink respectively). However, deformity percentage did not vary significantly with the colour of dye. Percent deformity showed significant differences within dose rates; 1g/L differed with 3 and 4 g/L (p<0.001), 2 with 3g/L (p =0.013) and 4g/L (p<0.001), 3 with 1 (p<0.001), 2 (p=0.013) and 4 g/L (p=0.032), 4 with 1 (p<0.001), 2 (p<0.001) and 3g/L (p=0.032). Overall lowest deformity found in 1g/L dye treated flies and highest deformity observed in 4g/L dye treated flies.

Effect of dye and dose on survival. In the absence of nutritional stress there were no significant differences between the tested dyes in fly survival, but there were significant effects of both dose and sex (Table 2). A total of 1298, 1258, 1252, 1145 flies were studied for survival at dye doses of 1, 2, 3 and 4 g/L, respectively. Flies survived longer when

lower doses of dye were used; percent survival at 1, 2, 3 and 4 g/L dye treatment was 73.50, 72.02, 71.88 and 67.51 (Figure 3). In total 2348 females and 2605 males were observed for survival study; female survived longer than male, 77.10 and 66.20% survival were found in female and male respectively (Figure 4).

The mean survival of Qfly under nutritional stress was 39 hours (range 24-72 hours). Overall, 46.4%, 46.1% and 7.5% flies survived for 24, 48 and 72 hours respectively. There was no significant effect of dye, dose or sex on the survival of sterile Qfly under nutritional stress (Table 2).

Dye visibility and persistence. Dye-treated flies were inspected for dye visibility; Fluoro Pink (n=255), Fluoro Orange (n=222), Stella Green (n=215), Arc Chrome (n=226) and Astral Pink (n=241). Overall dose significantly affected dye visibility while dye, week following treatment and sex did not (Table 3). Within dyes, pink dye showed better visibility over other dyes and Stella Green showed lower visibility. Stella Green had significant differences with Fluoro Pink, Fluoro Orange and Arc Chrome (p=0.001, p=0.027 and p=0.001 respectively), there were significant variation of visibility between doses as well (Figure 5). Dose of 3 and 4g/L dye were best in terms of visibility over 1 and 2g/L dye (p<0.001); no significant differences were observed between 3 and 4g/L(p=0.171) .

Discussion

Dye treatment reduced the emergence of fliers and among 5 fluorescent dyes only Fluoro Orange treated pupae showed relatively close percentage of adult emergence rate to control. Dyeing caused a significant decline in emergence and this is consistent with findings of other studies (Campbell et al. 2009, Dominiak et al. 2000, 2010b). Dose also had impact on emergence of fliers and deformity, higher the dose of dye lower the emergence and higher the deformity. Dominiak et al. (2010b) found that just washing off the dye after dye treatment prevented 90% of the decrease in emergence rates. However, although dying reduced the emergence, at least 65% flies were emerged as fully developed adults which is the minimum required percentage of emergence for successful sterile *B. tryoni* release (Reynolds et al. 2010). Fluoro Orange is the best dye which showed similar emergence rate to control which is consistent with the finding of

Dominiak et al. (2000b) who showed that Orange and Arc Chrome had similar emergence rate to control in their study. However, in present study similar emergence pattern was found in Arc Chrome, Green (Stella Green) and magenta (Astral Pink and Fluor Pink). Dominak et al. (2010b) found 14% reduction of emergence compare to unirradiated undyed control pupae when they dyed pupae with 4g/L Astral Pink while we found 8% reduction of emergence of fully developed adults compared to undyed but irradiated pupae dyed with Astral pink. However, Dominak et al. (2010b) did not consider deformed flies emerged which in this experiment was calculated separately and deformed flies' emergence was severely affected with the dye treatment; about 133% higher in Astral Pink treated pupae. All dyes increased deformity significantly, however, Fluoro Pink, Arc Chrome and Astral Pink had higher deformity than Fluoro Orange and Stella Green. In *B. dorsalis* dye had significant effect on deformity and partial emergence (Makumbe et al. 2017), however, in present study dye treatment does not have any impact on partial emergence.

Deformity should be a concern in SIT application as deformed flies are less active and cannot fly as actively as fully developed adults, therefore, dispersal rate become lower which constrains success of SIT. Because, dispersion facilitate sterile flies to search for wild females to mate and mating is the key to successful SIT. Unexpectedly, in the present study we found more than 95% deformed flies are wingless (data not included) that is also a big concern which could make huge difference. Therefore, ultimately SIT application would be compromised for unseen reason as deformed flies are also counted as the number of flies' release. Considering fully developed adult's emergence rate and deformity together Fluoro Pink and Astral Pink seemed lower preferred candidates to use for marking.

Dose of dye also affected the emergence of fully developed adults and deformed flies. Pupae that were dyed with 4g/L showed least emergence rate of fully developed adults while deformity was highest in this dose. Similar dye dose-dependent effect was observed in Qfly, with lower doses having little or no effect on emergence rates (Dominiak et al. 2010a). This deformity could be because of the thick layer coverage of dye on pupae that might restrict respiration because of blockage of cuticular pores. However, there is no evidence of impact of dose on partial emergence or unemerged pupae across the treatments which infers it might not be the dye treatment dose rather other factor like post

emergence factor. Not only the dose, the percentage of partial emergence did not even vary significantly due to dyeing in present study which did not match with previous findings of Dominiak et al. (2010b). However, they compared the partial emergence with the pupae that had not been irradiated (Dominiak et al. 2010b) hence, that difference was for irradiation or dye could not be demonstrated. In contrast, present finding is consistent with the study of Weldon (2005) where partial emergence rate and unemergence rate of pupae did not differ significantly across the treatments. Dye dose did not have any effect on adult emergence, partial emergence, deformed adults in *B. dorsalis* (Makumbe et al. 2017).

The persistence and visibility of fluorescent dye is an important factor for discrimination of sterile flies from wild flies which is the main aim to mark flies. Regarding the persistence of fluorescent dyes, all dyes were visible for 5 weeks (5 weeks) regardless of dye types and doses. In previous study also persistence of dye did not vary with dye types over a period of 5 weeks in Qflies (Weldon 2005) that match with our present findings. In contrast, Makumbe et al. (2017), found dyes visible for 14 days but began to fade by 21 days after emergence in *B. dorsalis.* The largest number of sterile flies are recaptured in the field within 3-4 weeks (Dominiak et al. 2003, Meats 1998, Dominiak and Webster 1998) and so persistence of dye until 5 weeks is promising findings in SIT program. Unlike persistence, visibility was significantly affected by dye and dose. Similarly in *B. dorsalis* dye and dose affected visibility (Makumbe et al. 2017). Fluoro Pink, Fluoro Orange and Arc Chrome were better than Stella Green. Dose rates of dyes made significant differences in identifying dyes; 1g/L dyed flies' visibility was poorer than other doses although was not impossible to identify. A dose of 3g and 4g/L dye were the best dose as visibility of these doses were very obvious.

Dye did not show any significant impact on survival which is consistent with the finding in *B. dorsalis* (Makumbe et al. 2017). Females survived longer than males and higher dose treated flies died earlier than lower dose treated one. Unlike Weldon (2005) mortality differed with sex significantly in present study; male's mortality is higher than female in the given period. Like Weldon (2005) mortality did not vary significantly with dye treatment. In the present study dye treatment did not show any adverse effect on the survival of sterile Qfly, however, higher dose showed significant reduction in survival which is similar to the finding of Makumbe et al. (2017) in *B. dorsalis*. As we used 4

doses from which 4g/L which is the highest dose was worst. Therefore, this dose could not be selected for SIT application.

As in field condition sterile Qflies are vulnerable to environmental stresses like temperature, moisture, etc. predators are a great threat that makes the SIT control program challenging as they might kill the sterile flies (Bateman 1972, Meats and Fitt 1987, Vargas et al. 1998, Allwood 1997, Perepelicia et al. 1994, Jackman et al. 1996 and Perepelicia et al. 1997). Because of this adverse situation daily survival rate of sterile Qfly decrement of 58–72% observed in field condition (Dominiak et al. 2000C). Hence, recapture rate of sterile flies is very low, for instance, Dominiak et al. (2000C) released sterile pupae in NSW and recapture rates were 0.88% at Tullibigeal, 0.08% at Ungarie and 0.15% at Lake Cargelligo (mean 0.21%) using cue-lure traps. Marking is the stage when sterile flies' survival could be affected and addition of chances of dying after dyeing is a huge risk that should be cared. Therefore, survival probability of sterile flies is an issue which should be given priority when designing SIT program from the starting point of producing sterile flies and getting ready to release them into the field.

Fluoro Pink and Astral Pink were the least preferred candidate for Qfly dyeing considering emergence although Astral Pink is the standard dye used to mark Qfly in SIT program in Australia (Reynolds et al. 2010). Not only the survival issue in terms of emergence and deformity a dose of 4g/L must not be used as in this dose emergence reduced and deformity increased significantly. Low emergence of flies are of real concern in SIT application as it reduces the cost effectivity of SIT factory as well SIT application as less productivity of active flies compare to huge logistic and financial investment in this sector. Although dye treatment is must in present situation of SIT application as no alternative is available. However, investigating the efficacy of multiple fluorescent dyes and multiple dose rates give us a complete scenario of present Qfly SIT application method. We now will be able to anticipate how much reduction of fly emergence could be after using these dyes. In recent Australian sterile release programs, for suppression of wild flies, pupae have been marked usually with orange or pink dye (Dominiak et al. 2000a). However, as Fluoro Orange showed no significant reduction in emergence, and also less deformity than Fluoro Pink, when only one dye is necessary to use Fluoro Orange could be recommended as best option to apply in current SIT programme in SA.

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Table 1. Effect of multiple dye treatments (Control, Fluoro pink, Fluoro orange, Stella green, Arc chrome and Astral pink) and multiple dose (1, 2, 3 and 4gm dye/L pupae) on percent emergence of fully developed adults (N=575, R^2= 0.090), deformed adults (N=575, R^2= 0.193), partial emergence (N=575, R^2= 0.016) and unemerged (N=575, R^2= 0.029) of sterile Queensland fruit fly.

	Emergence	df	F	*P*
Dye	Fully developed adult	5	3.52	**0.004**
	Deformed adult	5	6.157	**<0.001**
	Partial adult	5	0.531	0.753
	Unemerged	5	1.154	0.331
Dose	Fully developed adult	3	7.751	**<0.001**
	Deformed adult	3	24.146	**<0.001**
	Partial adult	3	0.229	0.876
	Unemerged	3	0.414	0.743
Dye * Dose	Fully developed adult	15	0.911	0.552
	Deformed adult	15	1.923	**0.019**
	Partial adult	15	0.358	0.988
	Unemerged	15	0.626	0.855

Table 2. Effect of multiple dye treatments (Control, Fluoro pink, Fluoro orange, Stella green, Arc chrome and Astral pink), multiple dose (1, 2, 3 and 4gm dye/L pupae) and sex on survival (Cox proportional hazards regression model).

Table 2A. Omnibus Tests of Model Coefficients[a]

-2 Log Likelihood	**Overall (score)**			**Change From Previous Step**			**Change From Previous Block**		
	Chi-square	**df**	***P***	**Chi-square**	**df**	***P***	**Chi-square**	**df**	***p***
23675.23	86.425	9	**<0.001**	86.673	9	**<0.001**	86.673	9	**<0.001**

a. Beginning Block Number 1.

Table 2B. Variables in the Equation

	Wald	**df**	***p***
Dye treatment	8.629	5	0.125
Dose	14.488	3	**0.002**
Sex	61.549	1	**<0.001**

Table 3. Effect of multiple dye (Control, Fluoro pink, Fluoro orange, Stella green, Arc chrome and Astral pink), multiple dose (1, 2, 3 and 4gm dye/L pupae), sex and time duration (week) on visibility (N=1159, R^2= .806) of dye marking of sterile Queensland fruit fly.

	df	**F**	***p***
Dye	4	1.228	0.297
Dose	3	723.762	**<0.001**
Week	4	0.996	0.409
Sex	1	0.06	0.806
Dye * Dose	12	1.684	0.065
Dye * Week	16	0.278	0.998
Dye * Sex	4	0.341	0.85
Dose * Week	12	0.759	0.693
Dose * Sex	3	0.343	0.794
Week * Sex	4	0.07	0.991

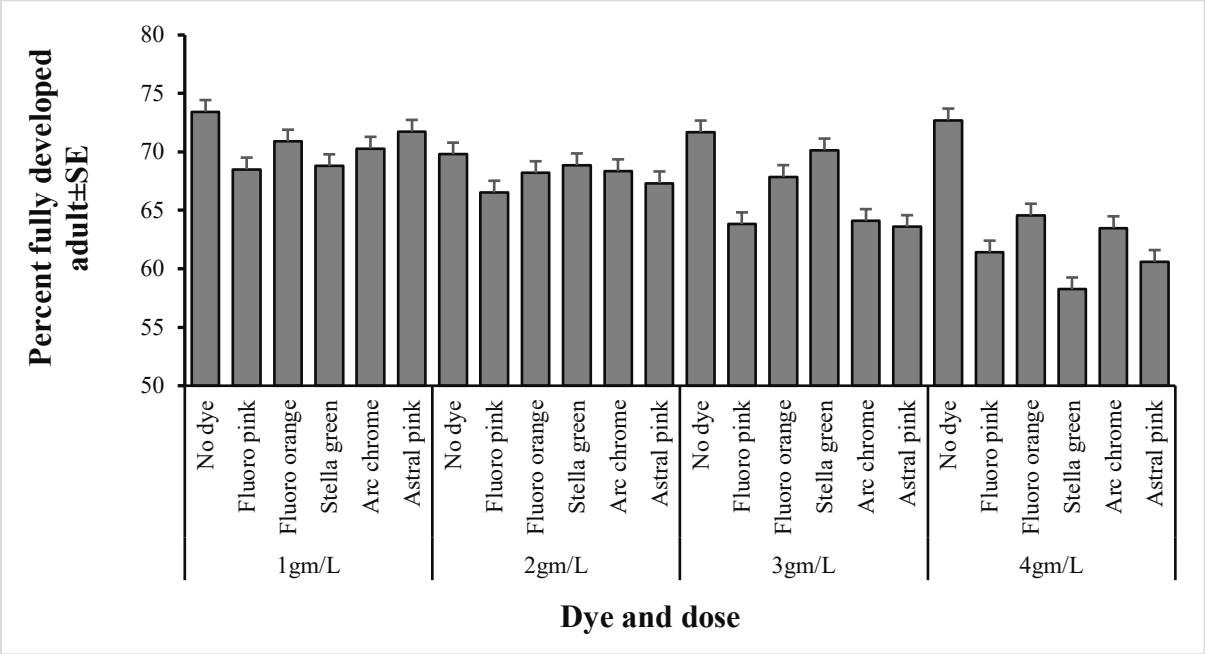

Figure 1. Effect of fluorescent dye and dose on percentage of fully developed adults. Error bars represent standard error (SE).

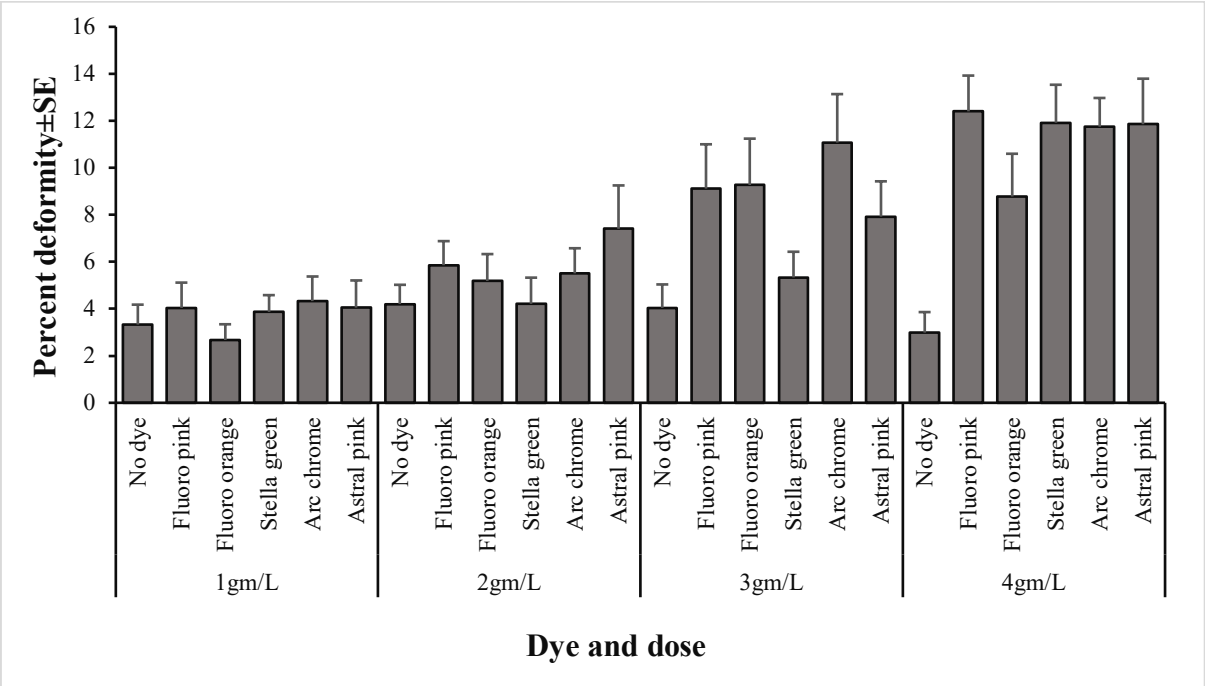

Figure 2. Effect of fluorescent dye and dose on the emergence of deformed adults. Error bars represent standard error (SE).

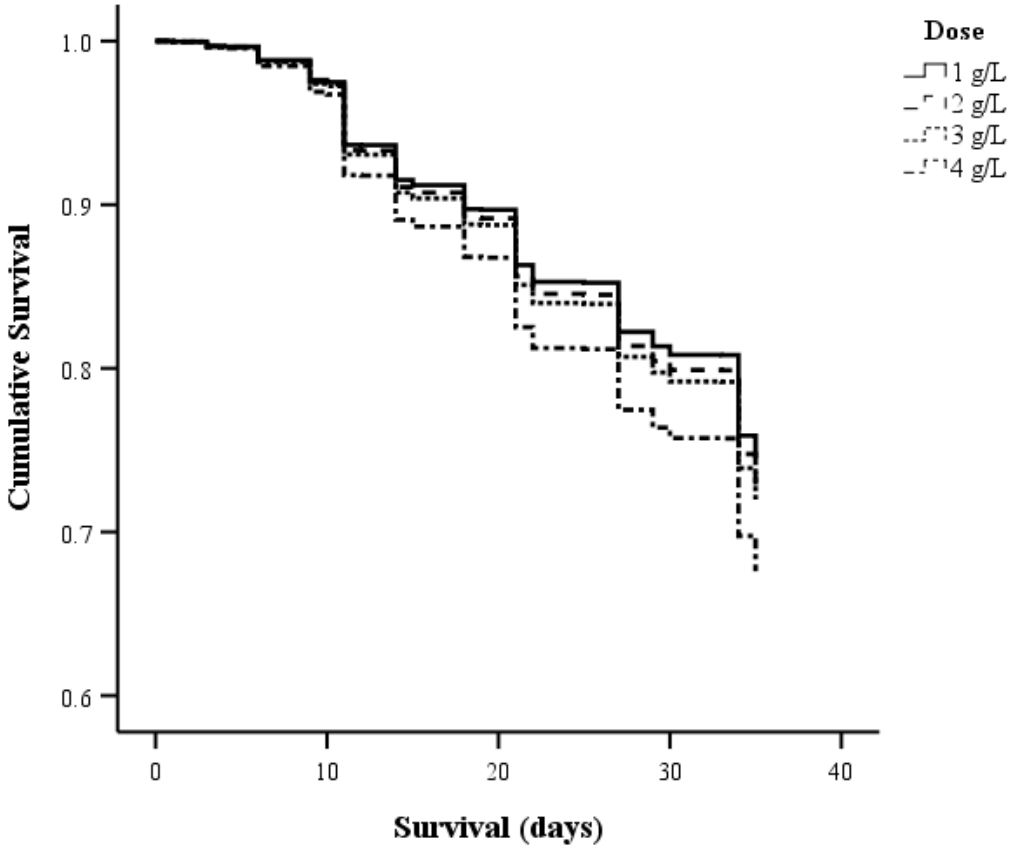

Figure 3. Effect of different dose rates of fluorescent dye on the survival of Queensland fruit fly. Flies that survived longer than 5 weeks were censored.

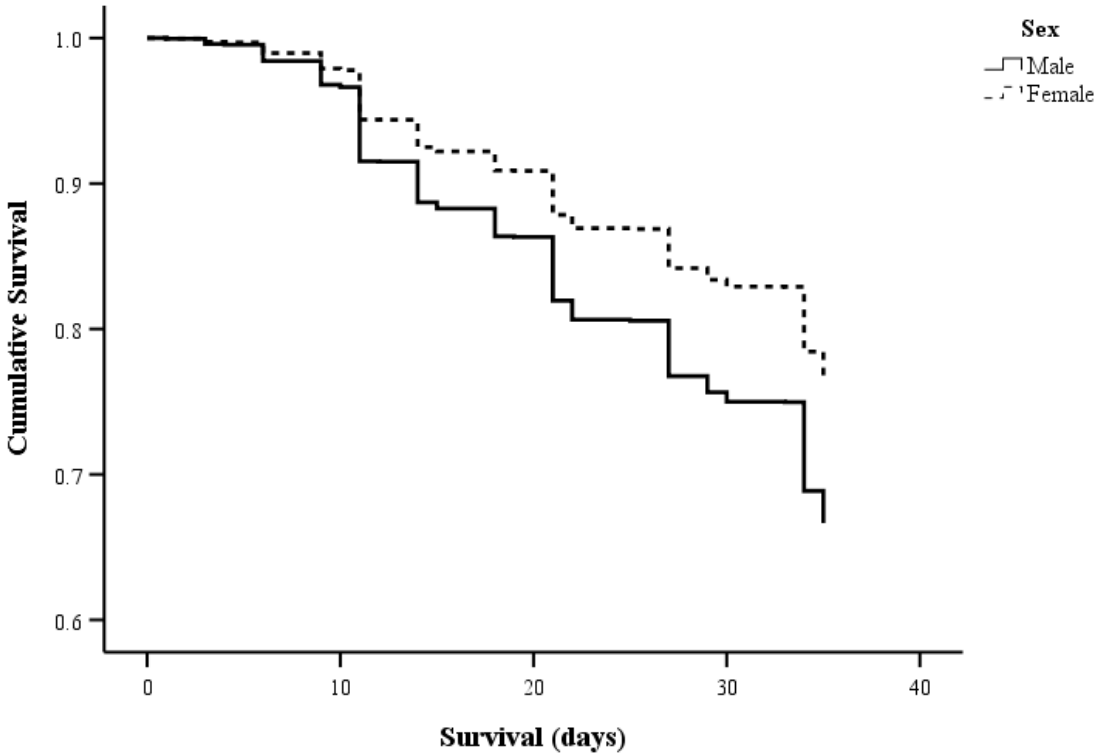

Figure 4. Effect of sex on the survival of Queensland fruit flies. Flies that survived longer than 5 weeks were censored.

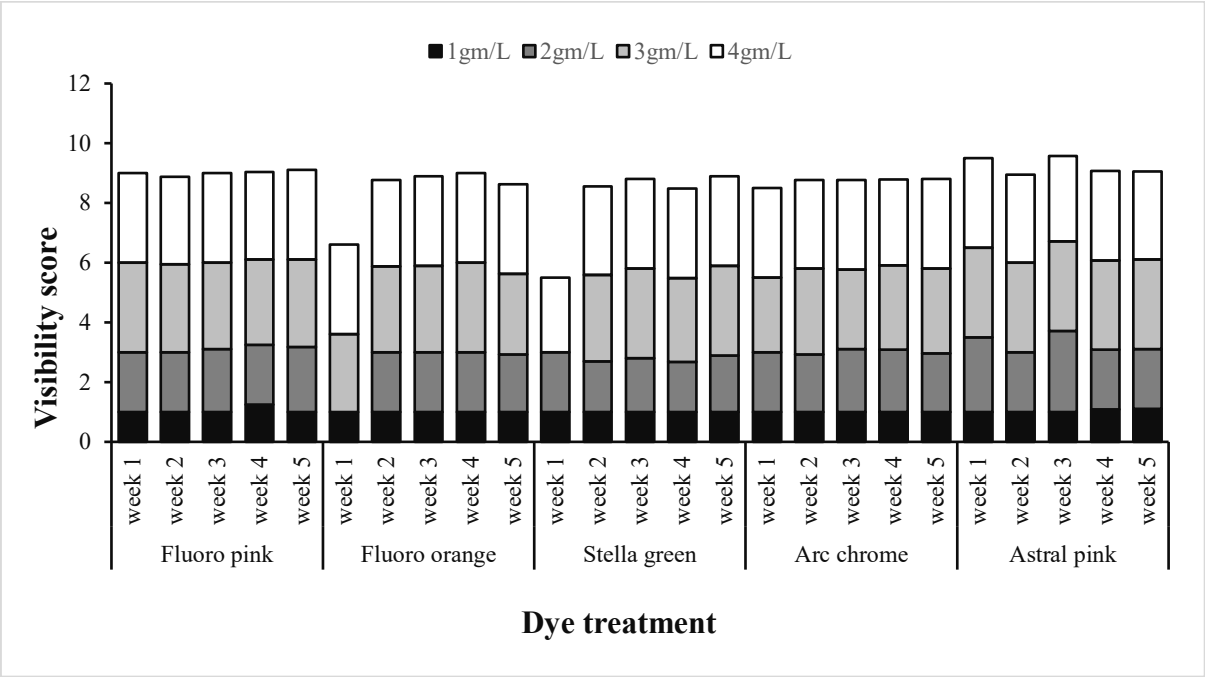

Figure 5. Visibility of dye (flies that died during this period) at different doses and different dyes over 5 weeks. Flies dyed with Fluoro Orange at 2 g/L and flies dyed with Stella Green at 3g/L did not die in week 1.

Appendicies 4:

Chronic hypoxia compromises repair of DNA double-strand breaks in Queensland fruit fly

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DRAFT PAPER EXERT

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Running title: Hypoxia in Queensland fruit fly

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Materials and Methods

Outcome 3: Hypoxia Trial

Pupal collection and Irradiation

Fertile *Bactrocera tryoni* (Queensland fruit fly) pupae was obtained from SIT Plus facility based in Port Augusta. This facility is designed to mass produce sterile Q flies and flies are send to Adelaide where they are released as part of the SIT Plus release program. Two batches of pupae were used (two replicates from each batch). A total of 8.5 kilograms of pupae was harvested and spread out evenly in trays to avoid humidity build up in pupae.

All the pupae were dyed with pink fluorescent powder pigment (TINTEX®) dye at the

rate of 2g per litre of pupae and spread back on the trays. Pupae were kept in a room at temperature of 18 degrees. Six different hours of hypoxic parameters were to be tested with 4 replicates thus approximately 350 grams of pupae was packed in a cylindrical plastic cannister (12.5 cm x 9 cm in diameter) and sealed to subject the pupae into hypoxia and kept in a room at about 18 degrees. When the respective hours of hypoxia (1, 2, 4, 8, 12 and 24 hours) were achieved, the cannister was fed into the x ray unit (Rad Source® RS2400V) and irradiated at 70 Gray dosage. All the 4 replicates of a certain time were irradiated at once. Irradiated pupae were packed in an esky box and transported to SARDI facility for DNA damage, flightibility, emergence, and survival under stress test.

Control Quality Setup

Random sample of 300 pupae (undyed and unirradiated) from each replicate was used to setup baseline quality control. IAEA guidelines for quality control was used to setup this. 4 black cylinders were used, with 100 pupae in each of the 3 cylinders and the forth was left empty in order to get emerged fly fall back data. A plastic container (30 x 25 x 17 cm) was used as the quality control cage for control data. The roof of the quality control cage was attached with two sticky traps to collect all fly emerging. Quality control cages were kept in a room with temperature and humidity of 26.5 degrees and 68-70% Relative humidity.

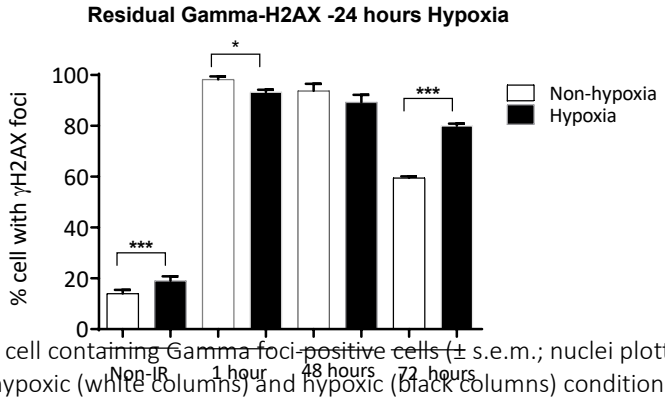
300 Irradiated pupae were randomly selected from each hypoxia treatment and from each replicate. 100 of these pupae were placed in each black quality control tube of 10 cm high, labelled and placed in a large cage. All pipes were similarly placed in the same cage. Multiple yellow sticky traps were hung from the roof of the cage to catch emerged flies.

Survivability under stress

100 non-hypoxic unirradiated pupae (to be treated as control) was placed in a cage (30 x 30 x 30 cm) and allowed to emerge. 100 treated (hypoxic, irradiated and dyed) pupae from each treatment and replicate was placed in a similar sized cage and allowed to emerge. The longevity of flies was measured by the amount of days the flies survived without any survival stimulus.

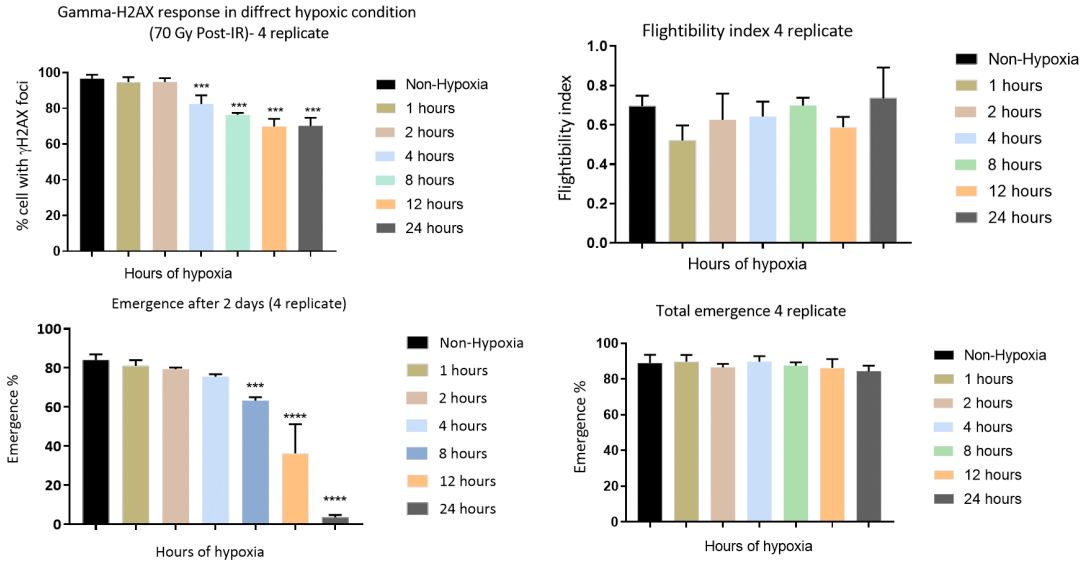
Results:

1. DNA repair under continued hypoxia leads to decreased repair as indicated by increased residual Gamma-H2AX kevel



The mean number of % cell containing Gamma foci-positive cells (± s.e.m.; nuclei plotted as a function of time following 70 Gy of irradiation under Non-hypoxic (white columns) and hypoxic (black columns) conditions. Data are based on 4 replicate experiments. The asterisk denotes a significant difference between non-hypoxic control and hypoxic treatment (*P,0.05).

2. 24 hours hypoxia show optimal level of DNA damage associated with increased level of emergence, eligibility, and survival under stress.



3. urs (1-hour, 2 hours, 4 hours,

Table 1: Summary of correlations between visually scored γH2AX signals and other parameters from the hypoxia trial

Percentage of cells containing γH2AX foci			
Parameters	**Correlation (r)**	**CI**	**p-value**
Flyers	- 0.2679	-0.5828 to 0.1168	0.1681
Fligtability index	-0.3459	-0.6368 to 0.0313	0.0714
Emergence- 2days	**0.5221**	**0.1850 to 0.7492**	**0.0044**
Total Emergence	**0.4041**	**0.0365 to 0.6753**	**0.0330**
Survival under stress	-0.3560	-0.6436 to 0.0197	0.0630

Parameters highlighted in bold text were considered statistically significant. Data were expressed as mean ± SEM