Hort Innovation

Final Report

Breeding a male-only strain of Queensland fruit fly

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FF18002

Project:

Breeding a male-only strain of Queensland fruit fly (FF18002)

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Public summary

Bactrocera tryoni, or Queensland fruit fly (Qfly), is among the most destructive horticultural pests in Australia, threatening hundreds of fruit and vegetable crops. Qfly is established in the eastern states and a constant threat to regions such as South Australia, Western Australia and Tasmania. Outbreaks of Qfly are currently managed by Sterile Insect Technique (SIT) which is akin to a birth control method for pests. SIT involves the release of large number of sterile males into the outbreak areas to mate with wild flies and the sterility of the males then prevent the wild females from producing the next generation of flies. The national Qfly SIT program at present involves the release of both sterile male and females as there is no efficient way of separating and removing the females prior to release, but it is only the sterile males that are useful for the SIT control of outbreak flies. Hence having a sexing strain that allows for the separation of males and females in SIT facilities could reduce the cost of rearing the unwanted factory females for release purposes and at the same time improve SIT efficiency.

This project has produced various genetic strains for consideration of use in the SIT program. These strains were mainly generated using the CRISPR/Cas gene editing technology to induce or introduce mutations but not all strains are considered genetically modified (GM). One of the strains is a pupal colour genetic sexing strain (Greyscale GSS) that consist of males with normal brown pupal colour and females with greyscale (a range of white to grey) pupal colour. Greyscale GSS allows males and females to be separated by colour at the pupal stage, which could be done by mechanical means, thus facilitating the removal of females prior to release – in essence, a male-only strain. This strain is the first pupal GSS and the only GSS in existence for Qfly.

This work has also produced two temperature sensitive strains, with data showing embryonic lethality to varying levels at high heat temperature, such as 33°C and above. Adults were observed to show a paralysis phenotype at high temperatures. These shibire ts strains are currently the only temperature sensitive strains in existence for Qfly and could be used in further development to produce a temperature sensitive GSS (in which females are temperature sensitive and can be eliminated by heat treatment whilst males are similar to wild flies), ideally combined with the pupal colour GSS.

Marker strains have been developed, which can enable easy identification of the flies. The most promising marker strain is the yellow strain, in which flies have yellow body and wing colours that are distinguishable by eye and its identity can also be confirmed by simple molecular methods. If used as a factory strain, the yellow marker can replace or be used in conjunction with the current methods (dye and isotope analyses) employed to identify if flies caught in traps are from the factory or are wild flies, which will subsequently inform area-wide management strategies.

Keywords

Queensland fruit fly, Qfly, Bactrocera tryoni, genetic sexing strain, pupal colour, temperature sensitive, genome editing, male only

Acronyms

CRISPR/Cas9	Clustered regularly interspaced short palindromic repeats/ CRISPR-associated protein 9
D. melanogaster	*Drosophila melanogaster*
GMO	Genetically modified organism
GSS	Genetic sexing strain
IBC	Institutional Biosafety Committee
Medfly	Mediterrenean fruit fly
MFS	Major Facilitator Superfamily
SIT	Sterile Insect Technique
Ts	Temperature sensitive
Tsl	Temperature sensitive lethal
OGTR	Office of the Gene Technology Regulator
Qfly	Queensland fruit fly

Introduction

Queensland fruit fly (Qfly; *Bactrocera tryoni* (Diptera:Tephritidae)) is one of the most devastating horticultural pests in Australia[1], established in the eastern states and a constant threat to South Australia, Western Australia and Tasmania, as highlighted by numerous outbreaks in recent years. It is highly invasive and polyphagous, infesting more than 200 types of cultivated fruits and vegetables[2], with an estimated $300 million cost per year due to losses of produce and market access[3]. In fruit fly free regions, taking for example South Australia, the establishment of fruit flies will threaten the value of crops (approximately $1.3 billion), access to export markets ($70 million in 2019-20) and necessitate post-harvest treatment of produce ($4.2 million per annum for cold and chemical treatment)[4]. In order to prevent this, management of Qfly outbreaks in Qfly-free areas are vital, but can be expensive and time-consuming.

Area-wide management programs involving Sterile Insect Technique (SIT) have been employed to control and/or eradicate Queensland fruit fly in Australia. In SIT, large numbers of sterile males are released to mate with wild females, resulting in the females laying non-viable embryos hence preventing production of the next generation. A SIT facility has been established in Port Augusta, South Australia which breeds up to 20 million flies per week for sterile release. To maximise the effectiveness of SIT for Queensland fruit fly, the sterile flies that are released should ideally be male-only[5]. A male-only release strain is highly desirable as it will not only save significant cost associated with rearing and release but also improve the frequency in which the sterile released males will find a wild female to mate with, thereby making eradication much more likely. Currently, both sterile males and sterile females are being co-released through the national SIT program as there is no cost-effective method of separating millions of flies by sex to allow for a male-only release.

Male-only SIT programs in other tephritid species utilise genetic sexing strains (GSS). Pupal colour GSS have been developed for various species - white pupal (wp) GSS for *Bactrocera dorsalis* (oriental fruit fly)[6] and *Zeugodacus cucurbitae* (melon fly)[7] and black pupal (bp) GSS for *Anastrepha ludens* (Mexican fruit fly, Mexfly)[8] and *Anastrepha fraterculus* (South American fruit fly)[9]. The basis of these GSS is pupal colour dimorphism, in that males and females have different puparium colour which allows differentiation between the sex, and consequently sorting of pupae by puparium colour allows for removal of females prior to SIT release. In Mediterranean fruit fly (Medfly, *Ceratitis capitata*), however, the white pupal (wp) is used as a marker in a temperature sensitive lethal (tsl), wp GSS[10]. Females have white puparium and are temperature sensitive, particularly at the embryonic stage where they can be killed by heat treatment, whilst males have the normal brown pupal stage and are not sensitive to heat treatment. This allows for females to be eliminated by heat treatment early on in the rearing process in SIT facilities, enabling a male-only release. The wp marker which is closely linked to, due to its close proximity to, the tsl mutation in the Medfly GSS allows the pupal colour to be used, in this case, as an indicator of the integrity of the genetic strain. These GSS were all developed (through spontaneous or random mutagenesis) without prior knowledge of the genetic basis of the wp and tsl mutations; lack of such knowledge prevents targeted development of similar GSS in other species. Identification of a pupal colour or temperature sensitive mutation that can be used in a GSS would thus be useful knowledge for transference to other species.

The main objective of this project was to develop a male-selecting strain of Qfly that can be proposed for use in the national Qfly SIT program. The aims of the project included development of a temperature sensitive lethal (tsl) strain and colour differentiation of the puparium between male and female Qfly, using CRISPR/Cas9 gene editing technology and low-dose irradiation. Strain with visible markers were also proposed to be useful. To achieve the desired outcomes of this project, the research would build on knowledge and techniques established during Project MT13059 and knowledge created in the supporting sister project FF17000, including the identification of the Medfly wp and tsl mutations.

The significance of this work would be having novel and/or improved strains that can potentially be adapted for SIT, including a male-only release strain, which use could reduce the overall cost of the program and improve eradication efficiency to facilitate the protection of Australia's horticultural industry. The knowledge produced from this project can potentially be applied to other insect pests that threaten Australia's agri-economy as well as globally.

Methodology

This research project is linked to completed project MT13059 (SITplus: Developing and optimising production of a male-only, temperature-sensitive-lethal, strain of Qfly, B. tryoni) and current sister project FF17000 (Sex selection genes from fruit fly species for use in SITplus). This FF18002 project follows on from the MT13059 project which established the groundwork for this project, including the establishment of CRISPR/Cas9 technology in Qfly, generation of useful information about *Drosophila melanogaster* temperature sensitive candidates as well as the initial work in the development of marker strains. FF17000 sister project allowed coordination with international groups and research groups within Australia to enable parallel research to be conducted whilst avoiding duplication and provided access to relevant information and research for related tephritid species.

This project involved the development of genetic strains using a Qfly laboratory strain that was established from wild flies originating from Qurimbah, New South Wales. Majority of this research was performed in South Australia which maintains a fruit fly free status, hence the work was done in a facility with high level of quarantine containment – the University of Adelaide Waite Biological Insectary, Biosecurity Containment Level 2 (BC2). Research involving genetically modified organisms (GMOs) were performed under an approved GMO dealing from the University of Adelaide Institutional Biosafety Committee and work was done in specific rooms that had been designated Physical Containment Level 2 (PC2) by the Office of the Gene Technology Regulator (OGTR). The research team all received training to be 'Approved Arrangement Accreditation for AA Accredited Persons' as well as PC2 training.

1. Introduction of targeted knockout and precise mutations into the Qfly genome

The CRISPR/Cas9 gene editing technology was used to introduce mutations into the Qfly genome. Loss of function (knockout) mutations were generated by designing a CRISPR guide RNA to target a specific gene, where the CRISPR/Cas9 system will introduce random mutations (insertions or deletions, also known as indels) into that target locus. These indels often result in a frameshift mutation and consequently no functional expression of that gene. Precise base substitutions were generated by introducing a specific donor template (consisting of the desired substituted base) together with the specific CRISPR guide to induce incorporation of the desired template into the Qfly genome. The CRISPR/Cas9 components were introduced into Qfly embryos by embryo microinjections.

Our established protocols for Qfly embryo microinjections and CRISPR/Cas9 mutagenesis have been published as a book chapter in Methods in Molecular Biology (Appendix 1). Details about the mutagenesis of specific strains have been included in the individual publications or manuscripts of the strains (Appendix 2, 3 and 4).

2. Molecular screening and genetic crosses to establish stable strains

After each embryo microinjection, surviving larvae are rescued and reared to adulthood. The adults are designated as G_0 flies. Identification of desired germline mutants among the G_0 flies were done by molecular screening of the next generation (G_1) adult flies. The identified G_1 mutants are heterozygous, in that they will have one copy of the mutation. Homozygous mutants (with two mutant copies and no normal copy of the gene) are obtained by crossing the heterozygous mutants to each other, and then molecular screening of the next generation (G_2) are performed to identify the homozygous mutants which are then used to establish a stable strain.

The protocols for molecular screening and genetic crosses to establish stable Qfly strains were published in the Methods in Molecular Biology book chapter (Appendix 1).

3. Chromosomal translocation using low-dose irradiation

Low-dose X-ray irradiation was used to induce random mutagenesis in Qfly pupae and genetic crosses and pupal colour separation were performed to identify desired flies with a successful translocation of the wild type *MFS* gene region to the Y chromosome. Pupae were irradiated at 30 Gy, 35Gy and 45Gy at the Port Augusta SIT facility, SA. Males from irradiated pupae were mass-crossed to virgin females from the

Greyscale-2 strain, followed by individual F_1 isomale crosses to *Greyscale-2* females. Detection of desired translocation lines involved collecting F_2 eggs individually from the F_1 crosses, rearing them to pupal stage, separating the pupae by colour (brown vs greyscale) and then determining the number of males and females for the different pupal colour groups. Desired translocation line(s) would have no or low number of brown pupal females and no or low number of greyscale pupal males.

In order to assess the efficiency of the successful translocation line (named '*Greyscale-GSS')*, pupae were again separated by colour (brown vs greyscale) and the numbers of males and females from each pupal group were counted This was performed for every generation. A stable translocation line would have brown males (no females) and greyscale pupal females (no males); any brown females or greyscale pupal males would indicate a recombination event has occurred (which is to be expected to a certain degree). The recombination rate can be calculated as (Number of greyscale pupal males + brown pupal females)/Total number of flies x 100%. Each generation is filtered, in that the recombinants are removed immediately after emergence and prior to mating.

4. Fitness assessments

Fitness assessments were performed in the University of Adelaide Waite Biological Insectary and at Macquarie University, NSW to determine the fitness costs associated with the induced mutations. Fitness assessments included the determination of fecundity, egg hatching rates, locomotor activity, mating probability, copula duration, latency until copulation and longevity. The fitness assessments performed for each strain varied - details about the specific assessments performed for the different strains have been included in the individual publications (Appendix 3) or described in the appendices (Appendix 5).

5. Temperature tolerance experiments

For all temperature tolerance experiments, the rearing temperature of 25°C was used as the control temperature and the lab strain were used as the control wild type flies.

For egg hatching experiments, 200 eggs were collected for each replicate (with 3 replicates for each strain) and placed on a gel diet at different temperatures and observed for egg hatching after 3 days.

For shibire ts4, temperature tolerance of adults was also tested. 60 adults of each strain were placed at different temperatures and the flies were observed for 10 minutes. A seizure phenotype is defined as when the flies fall on to the bottom of the vial and demonstrate rapid movement of legs or abdomen, whilst a paralytic phenotype is when they stopped moving. Recovery of flies were tested by placing them back at the control rearing temperature (25°C) and observing them for 10 minutes.

6. Training and capability building

Our research team trained two PhD students (Thu (Zoey) Nguyen and Ngoc Mai Han Nguyen) and two undergraduate students (Phetdalaphone Pathoumthong and Ling Xu). Capability building was also done through training of an early-career postdoctoral researcher (Dr Elisabeth Fung, SARDI), a senior research assistant (Anzu Okada) and a casual technical researcher (Dr Mamoru Okamoto) as part of the collaborative research team to enable the molecular capability in the Qfly species to be retained.

7. Communication

Research findings were communicated through regular presentations at quarterly national Qfly SITPlus consortium meetings, oral and poster presentations at relevant scientific meetings and peer-reviewed scientific publications.

Results and discussion

Greyscale pupal colour strain

Distinct pupal colour variation is a highly valuable trait for a genetic sexing strain (GSS) as it can be adapted as a dimorphic marker to distinguish between the male and unrequired female sexes and be used in a filter rearing system to maintain strain integrity. Examples of this are the white pupae (wp) phenotypes seen for the *Ceratitis capitata* (Mediterranean fruit fly), *Bactrocera dorsalis* and *Zeugodacus cucurbitae* genetic sexing strains. Hence, the first step in generating a GSS is to identify and/or introduce a pupal colour marker into Qfly.

We identified that the wp mutation in *B. dorsalis* was caused by disruption of a *Major Facilitator Superfamily (MFS)* gene (Ward *et al.,* 2021; FF17000 reported in sister project). Different mutations in the same orthologous gene cause the wp phenotype in Medfly and *Z. cucurbitae*. Discovery of the pupal colour causal gene and mutation is highly significant as the genetic nature of the white pupal phenotype had been elusive for over 40 years despite various identification efforts and this discovery has now facilitated work to develop white pupal GSS in various tephritid species.

Using that information, we established two pure breeding Qfly strains with independent mutations in the *MFS* gene generated by targeted CRISPR/Cas9 mutagenesis and found that targeted CRISPR/Cas knock-outs of the Qfly *MFS* gene causes a range of phenotypes, including white pupae, grey pupae and pupae with grey and white stripes – we refer to this broad phenotypic spectrum as "greyscale" and the strains are denoted as "Greyscale" strains (Appendix 6 Figure A1). This range of phenotype was observed even in flies carrying identical MFS mutations. This is unlike what was observed in Medfly, *B. dorsalis* and *Z. cucurbitae* where the phenotype is purely white, suggesting that either the MFS gene behaves slightly differently or there are genetic modifiers in Qfly that affects the pupal phenotype. Although this pupal phenotype in Qfly is different from the other tephritid species, the greyscale pupal colour in the Qfly MFS mutants is still highly distinctive from the normal brown pupal colour of the Qfly species and can be adapted to be a dimorphic marker.

It should be noted that these Greyscale strains are not currently considered GMO as no donor template was used for the CRISPR/CAS mutagenesis – whilst it was a targeted gene knock-out, the mutations introduced into the MFS gene was random. It should however be kept in consideration that regulations may change and advice from the Office of the Gene Technology Regulator (OGTR) should be sought prior to use of any genetic strains for release.

We performed a trial mechanical sorting experiment using a seed sorter system to determine if the greyscale pupae can be mechanically distinguished and sorted from brown pupae. The trial experiment showed promising results in that we were able to achieve removal of *greyscale* pupae at a level similar to those used to discriminate wheat quality (Appendix 6 Figure A2), despite minimal calibration and technical difficulties of having to use dead pupae due to biosecurity regulations in SA. It should be noted, however, that this was just a trial experiment and proper mechanical sorting experiments should be performed with the final GSS to determine suitability and accuracy.

Fitness assessments performed on these strains indicates that there are fitness costs due to the MFS mutations (reported in sister project FF17000), but the strains were sufficiently viable to proceed to the next phase - the development of a GSS (see section below).

Genetic sexing strain (male-only strain)

The main desirable outcome of this FF18002 project is to produce a *B. tryoni* male-only strain. In the male-only strain (pupal colour GSS) used for *B. dorsalis* and *Z. cucurbitae* SIT, males have normal brown pupal colour and females white pupal colour and are separated by mechanical sorting in SIT facilities. Females are removed prior to release, resulting in only male flies released in their SIT programs.

One of the Greyscale pupal colour strains generated in this project was used in further development to produce a Qfly pupal colour GSS, where females can be separated from males by pupal colour. This *Greyscale-GSS* was produced using low dose X-ray irradiation (a random mutagenesis process), in which a normal copy of Chromosome 5 was translocated to the Y chromosome in males (Appendix 7 Table A2). The exact molecular breakpoint and size of the translocated region is unknown. In this strain, males have a copy of the *MFS* mutation but a normal copy of the gene on the Y chromosome, which reverts the pupal colour back to the normal brown colour. As females do not have a Y chromosome, they have the *MFS* mutation and no normal

copy of the MFS gene, thus retaining the greyscale pupal colour. This allows males and females to be separated at the pupal stage by pupal colour. This is the first pupal colour genetic sexing strain produced for Qfly and currently the only Qfly genetic sexing strain in existence. As this translocation process was done through a random mutagenesis process, this pupal GSS/male-only strain is not designated as a GMO by OGTR.

Fitness evaluations of the male-only *Greyscale-GSS* were performed at Waite, South Australia and in Macquarie, New South Wales in collaboration with Prof. Phil Taylor's group at Macquarie University (Appendix 8; reported in sister project FF17000). The Greyscale-GSS strain was observed to have no change in fecundity but have a reduced egg hatching rate (an average of ~40% hatching; usually observed to be a ~25% decrease in hatch rate compared to the lab strain) (Appendix 8 Figure A3). Under our experimental and facility conditions, we do observe variability in results, even for our wild type lab strain, suggesting that the hatch rate could be subject to facility conditions. We recommend that if this GSS strain was to be used in any facility, egg hatching experiments need to be conducted in that facility to determine the hatch rate of the strain under their facility conditions and when reared at a larger scale.

There was no effect on latency until copulation or copula duration (Appendix 8 Figure A4, Table A5) for the GSS strain, suggesting that the observed reduced egg hatch rate is not due to any disruption in copulation. A further experiment looking at different mating pairs, using either males or females from the Greyscale-GSS strain to mate with wild type flies, identified that it was the Greyscale-GSS males that are responsible for the decreased egg hatching of the subsequent progeny (Appendix 8 Figure A5). This was particularly interesting as for SIT strains, the males are sterilised then released to mate with wild females, with the intention to prevent females from laying viable eggs. Males therefore still need to be able to mate with the females but as they are sterilised prior, the eggs will not be fertilised. The Greyscale-GSS males are ideal as there is no effect on copulation but for that strain, the males contribute to a reduced egg hatch rate in the progeny, hence may require a lower sterilising dose than the current SIT strain males – a lower irradiation dose used for sterilisation is beneficial as irradiation can impact on fitness of the males in a dose-dependent manner. Studies looking into how or why the males result in a reduced hatch rate in the subsequent generation could elucidate the biological mechanism into how egg viability can be inhibited by male contribution, which in turn could identify future targets for inducing sterility without irradiation and without affecting mating.

A cost benefit analysis will need to be done prior to recommending the GSS for SIT to determine the cost efficiency of the strains compared to the current bi-sex SIT strain. Recombination rates ranging from 0% - 0.93% were observed with filtering of the GSS colony but found to increase to 1.9% without filtering, indicating that filter colony will be required to maintain this strain (Appendix 7 Table A3 and A4).

Temperature sensitive lethal (tsl) strains

Two approaches have been taken to develop temperature sensitive *B. tryoni* strains: i) identification of tsl candidates from the model organism *Drosophila melanogaster* and introduction of the orthologous mutations into the *B. tryoni* genome using CRISPR/Cas mutagenesis and ii) collaborative efforts with sister project FF17000 to identify the Medfly tsl mutation and introduce the same mutation into *B. tryoni* (results reported in FF17000 final report).

Using the first approach to identify tsl candidates from the model organism *D. melanogaster,* a further literature review search was performed to review point mutations in genes that cause temperature sensitive phenotypes with the potential or ability to cause embryonic lethality (Appendix 9). Amongst the genes identified, shibire tsl mutations were tested in this project due to its close proximity to the *MFS* gene on the same chromosome in Qfly.

A shibire ts1 mutant line (G268D) was previously generated in project MT13059 but found to be homozygous lethal even at rearing temperatures, in that it was not possible to maintain such a strain – this work has now been published (Appendix 10). In this project, two other shibire mutant lines were generated via CRISPR/Cas mutagenesis: shibire ts2 (G141S) and shibire ts4 (P171S) (Appendix 4). Donor templates were used in the generation of both strains, hence both ts strains are currently considered to be GMOs by OGTR.

The shibire ts4 strain was successfully obtained prior to the shibire ts2 strain, hence more experiments have been performed for shibire ts4 (Appendix 11 Figures A6-A9). The shibire ts4 strain showed significantly reduced egg hatch rates at 33°C (with a range from 0% - 17%) but complete embryonic lethality is not always observed (Figure A6). An adult phenotype was also observed – flies are found to fall to the bottom of their vials within 10

minutes or less when treated at high temperatures and show a seizure phenotype or complete paralysis (Figure A7 & A8). If returned to normal rearing temperature of 25°C after 10 minutes of treatment, these flies are able to recover from that paralytic phenotype (Figure A9), which supports that the paralysis is induced by high temperatures.

Initial data shows that the shibire ts2 strain is embryonic lethality when eggs were treated at high temperatures of 33°C and 34°C (Appendix 11 Figure A10). A preliminary observation indicates that a similar adult paralytic phenotype was also observed in the shibire ts2 strain, suggesting that the effect of heat treatment on adult mortality could be further examined. The shibire ts2 strain was only successfully generated at the end of the project and this initial data of temperature tolerance on egg hatch rate has been included, however further assessments will be required to obtain a proper temperature sensitivity profile for this strain.

The observed temperature sensitive phenotype in the shibire ts strains are the first temperature sensitive phenotypes embryonic reported in Qfly. It appears to be an ideal candidate strain for generating a GSS in which females are temperature sensitive embryonic lethal and can be eliminated by heat treatment, enabling a male-only release for SIT. Shibire ts2 seems most promising, as the mutation results in complete embryonic lethality at higher temperatures; it is thus highly recommended that further assessment be performed on this shibire ts2 strain to consider its use for development of a tsl GSS strain. Shibire ts4 should also be kept in consideration – whilst not completely embryonic lethal, it results in low hatch rates at high temperatures, which could still remove most of the females if converted into a GSS. Further studies into the temperature sensitivity profiles of both ts strains as well as optimization of these strains may improve the rate of temperature sensitive lethality beyond than currently observed as improve the overall fitness of the strains. Once an actual tsl GSS strain is developed, the exact temperature sensitive profile of the final GSS will also need to be determined prior to be adapted for use.

In Medfly, having a combined tsl, wp GSS is beneficial as the pupal colour acts as a marker that can be used to track and remove recombinants, resulting in easy filtering of the colony and maintenance of strain integrity. As shibire is located close to the *MFS* gene on Chromosome 5, there is a high possibility that the wild type shibire gene had already been co-translocated along with the *MFS* gene to the Y chromosome in the Greyscale GSS. Experiments could be performed to identify if the translocated region on the Y chromosome includes the shibire loci. If that is the case, experiments should then be performed to incorporate the shibire ts mutation into the existing Greyscale GSS which would produce a Qfly tsl, pupal colour GSS. This removes the need to perform laborious translocation experiments to translocate the shibire gene to generate a tsl strain and the presence of the pupal colour marker in a tsl;Greyscale GSS will make it the ideal GSS.

Marker strains

Strains with a visible and distinctive phenotype can be used as marker strains for SIT release – these strains can be easily identified by eye, hence any release flies caught in traps can be quickly and easily identified as factory flies. At present, SIT release flies are marked with a fluorescent dye, which requires an additional step of adding dye to the pupae prior to release. Identification of these flies require a specific fluorescent microscope to visualize the fluorescence, hence flies cannot be identified on the spot and often have to be transported to a facility with appropriate equipment. The SIT release flies can also be identified through isotope analyses which is a specialized technique that also have to be done in a specific facility and can be costly. Development of marker strains can replace the need for dye and/or isotope analyses or can be used in conjunction to ensure that the accurate identification of release flies in traps, which is important in informing the area wide management of any outbreaks.

Two genetic marker strains were developed in this project – yellow and ebony strains, which were both developed by CRISPR/Cas mutagenesis. These strains were developed by knocking out expression of a particular gene involved in the pigmentation process, resulting in an obvious change in cuticle colour that is identifiable by eye. As these strains contain identified mutations, molecular analyses can also be used to identify these flies if necessary, which ensures that there will be rapid and accurate identification of the flies.

Whilst these strains were produced by targeted CRISPR/Cas mutagenesis, the mutations introduced into those pigmentation genes are random and the process does not include use of a donor template, hence these strains are not considered as GMOs by OGTR.

Yellow strain

Development of the yellow strain was reported in MT13059 with recommendations that fitness assessments should be performed to determine the suitability of the yellow strain as a potential marker strain for SIT use. That yellow strain was generated by CRISPR/Cas mutagenesis but consisted of flies carrying different types of mutations in their two yellow genes. In this project, we report the results of fitness assessments to assess the fitness costs of the overall yellow strain [Appendix 3]. The yellow strain was found to have decreased adult emergence rates, but still above 90%; and shorter longevity of adults, but only approximately 10 days less than control flies. There was also a 16% decrease in the percentage of fliers directly after emergence but greater locomotor activity was observed. No effect was observed for mating probability, copula latency and copula duration. We also went on to perform detailed genetic crosses and molecular genotyping to further produce, from the overall yellow strain, three individual yellow strains each carrying a specific mutation in one or more of their two yellow genes – this may improve the fitness cost of the overall strain and allow for easier molecular identification of the flies.

Ebony strain

The *ebony* mutation was also investigated in this project for its potential as a marker for SIT use. Ebony mutants in *Drosophila melanogaster* have visible changes in pigmentation at the larval, pupal and adult stage, with adults having a darker cuticle colour.[11,12] This darker body cuticle colour presented as a good candidate for a marker in Qfly.

CRISPR/Cas mutagenesis was performed to produce *ebony* knockout mutants and genetic crosses to establish a stable homozygous *ebony* strain. The *ebony* strain has black colouration at the pupal stage as well as the adult stage, which has similarities to the genetic sexing strain (*Tapachula-7*) that is currently being used for another tephritid fruit fly pest *Anastrepha ludens* (Mexican fly) in other countries as well as in a *Anastrepha fraterculus* (South American fruit fly) GSS. The genetic basis of the black pupal phenotype in those Anastrepha GSS is currently not known, hence that information cannot be used to produce a similar GSS in other species. The knowledge that ebony can result in black pupal phenotypes in Qfly can thus be used for development of black pupal GSS in other species.

The Qfly ebony black pupal colour is distinctive from the normal *B. tryoni* brown pupae, however there were initial difficulties in rearing this Qfly strain and fitness assessments have shown that there is a poor hatch rate (~30%, Appendix 12), which is less than ideal for use as a facility strain. The hatch rate of ebony mutants in other species should thus be evaluated to determine the suitability of using ebony in other species to develop a GSS.

Collaboration with other projects

This project is closely aligned with Hort Innovation sister project FF17000: Sex selection genes from fruit fly species for use in SITplus. The work in this project was performed in close collaboration with research groups working on FF17000, which resulted in collaboration between Australian research groups University of Adelaide, SARDI, Melbourne University and Macquarie University as well as at an international level (with groups from the International Atomic Energy Agency (IAEA) and Justus-Liebig-University Giessen). Identification of the MFS mutations as the causal mutation in the Medfly, *Bactrocera dorsalis* and *Zeugodacus cucurbitae* genetic sexing strains were a joint effort and product of this close collaboration. This collaboration also allowed exchange of information and knowledge on mutagenesis and molecular genotyping techniques, development of genetic sexing strains, temperature sensitive assays and rearing of tephritid species and prevented duplication in this field of research.

Outputs

Table 1. Output summary

Output	Description	Detail
Publication of a protocol for CRISPR/Cas9 Mutagenesis to generate novel traits in *Bactrocera tryoni*	Protocol for CRISPR/Cas9 Mutagenesis to generate novel traits in *B. tryoni* for Sterile Insect Technique was published as a book chapter in Methods of Molecular Biology (Springer).	This is the first detailed protocol for embryo microinjection and CRISPR/Cas9 mutagenesis for Queensland fruit fly made available to other Qfly and tephritid fruit fly researchers. Publication available online at: https://doi.org/10.1007/978-1-0716-2301-5_9
Data on temperature sensitive candidate gene mutations	Evaluation of candidate gene mutations from other species such as *Drosophila melanogaster* that demonstrate sensitivity to high temperatures. A review on this work was published in the journal, 'Insects'.	A review was undertaken to identify candidate gene mutations from *Drosophila melanogaster* that demonstrate sensitivity to high temperatures and work was done to investigate these candidates in D. melanogaster to provide supporting evidence to test the mutations in Qfly. Publications available online at: https://doi.org/10.3390/insects12030243
Temperature sensitive strains for Queensland fruit fly	Generation and development of Queensland fruit fly strains that are sensitive to high temperatures Previous work on shibire ts1 (from project MT13059) has now been published	Two temperature sensitive candidate gene mutations (shibire ts2 and shibire ts4) were tested to determine if these mutations were also temperature sensitive in Queensland fruit fly. Temperature sensitivity (to differing degrees) has been observed for both Qfly shibire ts2 and ts4 strains. This knowledge will inform other tephritid fruit fly researchers that are working on developing temperature sensitive strains as to the best candidates to test for their research. A research manuscript on this work is currently being prepared for submission to a peer-reviewed journal. Shibire ts1 publication available online at: https://doi.org/10.1186/s12863-020-00934-3
Publication on the identification of the genetic basis for pupal colour mutations in different tephritid species	Peer-reviewed journal article reporting the identification of the elusive pupal colour gene and mutation in *Ceratitis capitata, Bactrocera dorsalis*, and *Zeugodacus cucurbitae*. This was published in Nature Communications, one of the top 3% journals in the category of General Biochemistry, Genetics and Molecular Biology (ranked #6/204).	Discovery of the causal white pupal MFS gene and its mutations is highly significant as the genetic nature of the white pupal phenotype had been elusive for over 40 years despite various identification efforts; this discovery will now facilitate development of white pupal genetic sexing strains for other fruit fly species. Publication available online at: https://doi.org/10.1038/s41467-020-20680-5
Pupal colour mutant strains	Generation and development of two Queensland fruit fly pupal	Development of the Greyscale pupal strain was an important step in the development to produce

	colour mutant strains (*Greyscale* and *ebony*) that are distinguishable from the normal pupal colour	the Greyscale-GSS strain. The ebony strain is visually identifiable at the larval, pupae and adult stage. The knowledge that ebony can result in black pupal phenotypes in Qfly can be used for development of black pupal GSS in other species. It should be however noted that there are fitness costs associated with ebony mutation in Qfly. Two research manuscripts on this work are currently being prepared for submission to peer-reviewed journals.
Greyscale pupal colour genetic sexing strain (GSS)	This Greyscale GSS consist of males with normal brown pupae and females with Greyscale pupae (a range of white-grey pupae), hence males and females are easily distinguishable at the pupal stage.	This Greyscale GSS can be used to separate males and females at the pupal stage for a male-only SIT release. This strain is not considered a GMO. It should be noted that there is a fitness cost associated with this strain and a cost benefit analysis should be performed by interested parties to determine the suitability of the strain for their SIT use. A research manuscript on this work is currently being prepared for submission to a peer-reviewed journal.
Phenotypic (yellow body/wing) marker strain for Queensland fruit fly	Generation and development of a yellow body and wing marker strain that is visually and molecularly distinguishable from normal flies	This yellow body and wing marker strain can be used to replace the use of dye to identify SIT release flies – it will be a quicker method of visual identification and can be further confirmed by molecular testing if required. Unlike the use of the marker dye, no further treatment will be required with this strain prior to release, which will save cost and labour and reduce any negative effect of treatment to the release flies. This strain is not considered a GMO and there is no obvious fitness cost detected thus far. This work has been published and available online at: https://doi.org/10.1007/s10340-020-01304-9
Building of capacity in Australia for Queensland fruit fly CRISPR research	We provided training for two PhD students (Thu (Zoey) Nguyen - also as an early career researcher- and Ngoc Mai Han Nguyen), an early career researcher (Elisabeth Fung) and senior research assistant (Anzu Okada)	Queensland fruit fly CRISPR research involves specialized embryo microinjection skills and CRISPR mutagenesis knowledge. Training of students and staff ensures that the skills and knowledge are developed, and capacity is retained for future research.
Active communication of results through talks and posters	Our research was communicated to industry stakeholders and researchers through various channels	In addition to peer-reviewed publications in scientific journals, main findings were also communicated through talks or posters at SITPlus Technical Advisory Committee (TAC) and Stakeholder Advisory Committee (SAC) meetings, a joint National Fruit Fly Council (NFFC)-SAC meeting and scientific conferences.

Outcomes

Table 2. Outcome summary

Outcome	Alignment to fund outcome, strategy and KPI	Description	Evidence
Knowledge	Alignment to the Hort Frontiers Fruit Fly involving the SITplus partnership; and the Investment Strategic Intent: Fruit Fly, Investment Theme 4: Novel Control Technologies	Establishment of gene editing technologies and methods in Qfly that will enable genetic manipulation to produce further desired strains	A peer-reviewed paper and a book chapter has been published on the established gene editing methods in Qfly. This information has also been shared with other research groups upon request.
Knowledge	Alignment to the Hort Frontiers Fruit Fly involving the SITplus partnership; and the Investment Strategic Intent: Fruit Fly, Investment Theme 4: Novel Control Technologies	Access to new information – identification of ideal genetic sexing trait mutations that can be applied to various tephritids	The information has been published in three peer-reviewed papers, with four other manuscripts in preparation and to be submitted for publication in near future. The published information has been adopted by various international research groups, as evident from citations of those papers as well as the development of similar mutants in other species.
Availability of new knowledge for next phase project	Alignment to the Hort Frontiers Fruit Fly involving the SITplus partnership; and the Investment Strategic Intent: Fruit Fly, Investment Theme 4: Novel Control Technologies	Access to new information – identification of temperature sensitive mutations that can be used for further development and may potentially be applied to other species as well.	Feedback from Hort Innovation and other partners demonstrated that this information is being considered for next phase projects.
Product	Alignment to the Hort Frontiers Fruit Fly involving the SITplus partnership; and the Investment Strategic Intent: Fruit Fly, Investment Theme 4: Novel Control Technologies	Production of visible marker strains to be presented for use for SIT releases	Feedback from Hort Innovation and other partners demonstrated that our strains are being considered for further development to ascertain effectiveness and adoption by industry.
Product	Alignment to the Hort Frontiers Fruit Fly involving the SITplus partnership; and the	Production of a male-only sexing strain to be presented for use for SIT releases	Feedback from Hort Innovation and other partners demonstrated

	Investment Strategic Intent: Fruit Fly, Investment Theme 4: Novel Control Technologies		that our strains are being considered for further development to ascertain effectiveness and adoption by industry.

Monitoring and evaluation

Table 3. Key Evaluation Questions

Key Evaluation Question	Project performance	Continuous improvement opportunities
Effectiveness	CRISPR/Cas mutagenesis technology has been established in Qfly to generate loss-of-function mutants as well as to introduce specific mutations into the genome. This established technology has been used to produce 6 new types of Qfly strains.	It is important to keep in mind that the Broad Institute is licensing the CRISPR/Cas technology used in this project. No licensing was required for research purposes as per this project, however if the strains are to be offered or used for commercial sale, a written license from the Broad Institute is required.
Relevance	The Qfly yellow mutant strain has a visible yellow body and wing colour that is quite distinctive from wild flies and with no highly significant fitness costs detected thus far. This strain can be used in place of the dye currently used to mark SIT flies prior to release, which could reduce both cost and reduced fitness associated with dye handling. Currently flies caught in traps in SA have to be identified as SIT or wild flies, which will inform the outbreak program – such identification can be labour intensive and costly. This strain will be easily identifiable visually (by eye) and can also be confirmed molecularly, hence will reduce the cost that is usually involved in this identification process. The Greyscale genetic sexing strain consist of males with brown pupae and females with Greyscale pupae (range of grey to white pupae). Males and females can be separated using a sorter at the pupal stage to remove females prior to SIT release, resulting in a male-only release that could result in more males released (at per same release cost) or reduced cost for same level of mating effectiveness, which could overall lead to increased SIT efficiency and	Field trials can be carried out to determine the performance of these strains in the field. An efficient sorting system is required to separate the pupae by pupal colour (and thus sex) effectively in the SIT facility; this system has to be adapted to the current SIT facility practice. A cost-benefit analysis project could be performed to determine the extent of cost savings if these strains are to be adopted by industry.

	efficacy. Further development to incorporate the shibire ts mutations (also generated in this project) into the Greyscale-GSS may lead to production of an ideal tsl, pupal colour GSS.	
Process appropriateness	Project updates and results are regularly conveyed and presented to the Sterile Insect Technique (SIT) Technical Advisory Committee (TAC) and Stakeholder Advisory Committee (SAC) through oral and written reports. Both oral and written reports are presented at quarterly TAC meetings and at SAC meetings when requested. Collaborator Siu Fai Lee (Ronald) reported about the progress of the development of a fit, male-only line of Qfly from the FF18002 and FF17000 projects at the Joint NFFC-SITplus SAC meeting at Canberra on 21 September 2022. Outputs from the projects were also mentioned by Greg Chandler (Research & Development Manager, Biosecurity, HIA at that time & currently Head of Biosecurity, HIA) at the 11th International Symposium on Fruit Flies of Economic Importance on 17 November 2022 in Sydney.	Further engagement with industry to provide information about the genetic strains that have been produced and can be considered for use in the future.
Efficiency	The work was conducted alongside and in close collaboration with sister project FF17000: Sex selection genes from fruit fly species for use in SITplus. This involved collaboration between Australian research groups (SARDI, University of Adelaide, Macquarie University and University of Melbourne) as well as at an international level (with groups from the International Atomic Energy Agency (IAEA), Justus-Liebig-University Giessen and U.S. Department of Agriculture – Agricultural Research Service (USDA-ARS)). This allowed the work to be performed efficiently, ensuring that information was shared for the benefit of the work and prevented duplication of the work among researchers in this field on a global level.	

Recommendations

1. Marker strains

   This work has produced strains with visible phenotypes, in particular the yellow strains that consist of flies with yellow body and wing colour that are distinguishable from flies in the wild. These yellow flies could be considered for use in SIT releases as they can be easily identified as factory flies (through both identification by eye of the body and wing colour as well as a quick and reliable molecular test for the yellow gene(s) mutations); compared to current methods of identification via dye visualization and isotope testing. These flies were generated using CRISPR/Cas gene editing technology (see Point 4) but are not currently considered to be genetically modified organisms (GMO). It would be worth performing further fitness tests and exploring the option of adopting the yellow strains for use as the SIT factory strain.

2. Genetic sexing strain/male-only strain

   The pupal colour genetic sexing strain (Greyscale GSS) allows females and male flies to be separated at the pupal stage as males have the normal brown pupae whilst females have greyscale pupal colour (a range of white to grey pupae). This will allow females to be remove prior to release, which in essence would enable a male-only release. Use of this male-only strain for SIT could reduce the cost of releasing females in SIT release and potentially improve SIT efficiency through a male-only release. Comprehensive large-scale mechanical sorting of pupae should be trialed to optimize effective pupal sorting by colour. A cost benefit analysis could be performed to determine the economic advantage of using this Greyscale genetic sexing strain in SIT release, particularly as this strain has fitness costs, which includes a lower hatch rate than wild flies. There is also the risk of recombination, in which the males can be reverted to greyscale pupae and females to brown pupae, hence a filter colony will be required to maintain this strain.

3. Temperature sensitive strains.

   Temperature sensitive (ts) mutant strains have been developed – the shibire ts4 strain is not completely embryonic lethal but showed significantly reduced hatch rate at 33°C whilst initial data indicates that the shibire ts2 strain is completely embryonic lethal at 33°C. Further studies into temperature sensitivity profiles of both ts strains as well as optimization of these strains should be performed.

   Currently, these ts strains contain males and females that are both temperature sensitive. It would be beneficial to progress this work and develop a temperature sensitive GSS. This could be done by translocating the wildtype shibire gene to the Y chromosome, similar to what was done to obtain the pupal GSS. A potential alternative is the incorporation of the shibire ts2 mutation into the existing Greyscale GSS as the shibire and MFS genes are closely located on the same chromosome and there is a likely possibility that the shibire gene has already been co-translocated to the Y chromosome in the Greyscale GSS. This would produce a desirable tsl male-only strain and the greyscale pupal colour could then be used as a marker to enable easy filtering of the colony as well as to track the integrity of the strain.

   These ts flies were generated using CRISPR/Cas gene editing technology (see Point 4) and are genetically modified organisms (GMO) due to use of a donor template to introduce the specific point mutations; hence the legal and social aspects of releasing GMO flies into the environment also need to be considered. Any further laboratory research involving living shibire ts flies need to be performed in Physical Containment Level 2 (PC2) facility designated by the Office of the Gene Technology Regulator (OGTR) under an authorized GMO dealing from an appropriate Institutional Biosafety Committee (IBC).

4. Licensing required for commercial use of the developed strains

   The strains produced in this work all contain one or more mutations generated using CRISPR/Cas gene editing technology. This CRISPR/Cas technology is licensed by the Broad institute – whilst no licensing is required for research purposes as per this project, however if the strains are to be offered or used for commercial sale, a written license from Broad Institute is required. Any further work with these strains beyond research purposes should include consultation with the Broad Institute to determine if a written license is required and properly applied for.

5. Consideration of genetically modified organisms (GMO)

   Currently, the shibire ts strains are designated to be genetically modified organisms (GMO) due to use of a donor template to introduce the specific point mutations, whilst the other genetic strains produced in this project are not considered to be GMOs. It should however be kept in consideration that regulations may change and advice from the Office of the Gene Technology Regulator (OGTR) should be sought prior to any further research being conducted or use of any the genetic strains for release. For any intentional release of sterile GMO flies, a Dealing involving an intentional release (DIR) license will be required from OGTR for the release of GMO into the environment (with conditions in place) - the organization responsible for the releases will need to apply for and hold this DIR license. Release of any strains, GMO or otherwise, may also require a permit from the Department of Agriculture and Water Resources (DAWE).

Refereed scientific publications

During this project, 4 scientific peer-reviewed articles and 1 book chapter was published as listed below. 4 additional manuscripts are currently in preparation and will be submitted for consideration in peer-reviewed journals. 8 oral updates were given at SITPlus Technical Advisory Committee meetings and 1 at a Joint NFFC-SITPlus SAC meeting, whilst 3 oral and 3 posters were presented at scientific conferences (please see Appendix 13 for details)

1) Choo, A., Fung, E., Chen, I. Y., Saint, R., Crisp, P., & Baxter, S. W., 2020. Precise single base substitution in the shibire gene by CRISPR/Cas9-mediated homology directed repair in Bactrocera tryoni. BMC Genetics, 21(Suppl. 2), 127-1-127-10.

2) Nguyen, T. N. M., Choo, A., & Baxter, S. W., 2021. Lessons from Drosophila: Engineering Genetic Sexing Strains with Temperature-Sensitive Lethality for Sterile Insect Technique Applications. *Insects*, *12*(3), 243.

3) Ward, C. M., Aumann, R. A., Whitehead, M. A., Nikolouli, K., Leveque, G., Gouvi, G., et al., 2021. White pupae phenotype of tephritids is caused by parallel mutations of a MFS transporter. *Nature Communications*, *12*(1), 1-12.

4) Nguyen, T. N. M., Mendez, V., Ward, C., Crisp, P., Papanicolaou, A., Choo, A., et al., 2021. Disruption of duplicated yellow genes in Bactrocera tryoni modifies pigmentation colouration and impacts behaviour. *Journal of Pest Science*, *94*(3), 917-932.

5) Choo, A., Fung, E., Nguyen, T. N. M., Okada, A., & Crisp, P., 2022. CRISPR/Cas9 Mutagenesis to Generate Novel Traits in Bactrocera tryoni for Sterile Insect Technique. In: Verma, P. J., Sumer, H., Liu, J. (Eds.), Methods in Molecular Biology. Springer, US, Vol. 2495, pp. 151-171.

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Intellectual property

No project intellectual property or commercialisation to report.

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- Anzu Okada for contributions towards shibire ts2 and ts4 strain development and temperature sensitive assays, yellow strain establishment and ebony fitness assays
- Dr Thu (Zoey) Nguyen (University of Melbourne PhD student) for yellow strain development and fitness assessments and ebony strain development and ebony fitness data analyses
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Appendices

Appendix 1:

Choo, A., Fung, E., Nguyen, T. N. M., Okada, A., & Crisp, P., 2022. CRISPR/Cas9 Mutagenesis to Generate Novel Traits in Bactrocera tryoni for Sterile Insect Technique. In: Verma, P. J., Sumer, H., Liu, J. (Eds.), Methods in Molecular Biology. Springer, US, Vol. 2495, pp. 151-171.

https://doi.org/10.1007/978-1-0716-2301-5_9

Appendix 2:

Ward, C. M., Aumann, R. A., Whitehead, M. A., Nikolouli, K., Leveque, G., Gouvi, G., et al., 2021. White pupae phenotype of tephritids is caused by parallel mutations of a MFS transporter. *Nature Communications*, *12*(1), 1-12.

https://doi.org/10.1038/s41467-020-20680-5

Appendix 3:

Nguyen, T. N. M., Mendez, V., Ward, C., Crisp, P., Papanicolaou, A., Choo, A., et al., 2021. Disruption of duplicated yellow genes in Bactrocera tryoni modifies pigmentation colouration and impacts behaviour. *Journal of Pest Science*, *94*(3), 917-932.

https://doi.org/10.1007/s10340-020-01304-9

Appendix 4:

Table A1: Sequence of guides and templates (5' →3' orientation) used in CRISPR/Cas mutagenesis and the corresponding mutations introduced in the Qfly genes

	cRNA guide	Donor template	Type of mutation
Greyscale exon1	crRNA#1-TGTGAGTACGGCCAACGCAT crRNA#2-CGATCTACCACAGCAATGTG	N/A	5bp deletion of TATGC (at loci of guide 1); 16bp deletion of GATCTACCACAGCAAT and 1bp insertion of A (at loci of guide 2)
Greyscale exon3	GAGATCAGCATTATAGGCAA	N/A	6bp deletion of TTTGCC and 8bp insertion of AGATGCTG (frameshift)
Shibire ts2	GGTCTAACCAAGGTGGCGAT	TTTAATTTGTTGTTCAATGTCTACGGGCTGGTCGCTGATCGCCACCTTGGTTAGACCAGGCAAATCGATGAGTGTCAGAT	Single amino acid substitution (G141S)
Shibire ts4	TTGGCTAAATCTGTATTAGC	ACGCAAGGAAACTTGTCTCATTTTGGCTGTAACGTCAGCGAATACAGATTTAGCCAATTCGGATGCATTGAAATTAGCTA	Single amino acid substitution (P171S)
Ebony	crRNA#1-AAATGTCAAATGTTGCAGCG crRNA#2-GTAAAGCGTATGTATGCCTT	N/A	2bp deletion (frameshift)

N/A = not applicable as no donor template was used

Appendix 5: Additional Methodology

Fitness assays on *Bactrocera tryoni* ebony mutants

Virgin female flies from the ebony and wild type (Ourimbah reference) strains were sorted by sex within three days of adult emergence, before sexual maturation (Perez-Staples et al., 2007), and maintained with sugar, yeast hydrolysate enzymatic (MP Biomedical, LLC), and water. Crosses were performed using 10 males and 10 females 8-11 days post emergence flies in four possible combinations: (1) wild type males x wild type females, (2) ebony males x wild type females, (3) wild type males x ebony females, and (4) ebony males x ebony females. These assays were performed on three separate occasions to account for environment variations. Each experiment group was set up in a mesh cage (17.5 x 17.5 x 17.5 cm Megaview BugDorm-4M1515), provided with sugar, yeast hydrolysate enzymatic (MP Biomedical, LLC), and water. Each cage was provided with an 'oviposition device' 48 hours after setting up and eggs were collected for 24 hours and counted daily for 5 days to assess fecundity (number of eggs laid). Collected eggs were placed on larval diet and resulting larvae were counted after 3 days to assess embryonic hatching rates. The number of pupae, and adults categorised as fully emerged (flies that completely emerged from puparium), partially emerged (a portion of adult body stuck in puparium) and deformed (fully emerged flies with morphological defects such as twisted or deformed wings) were also recorded. Data were analysis in RStudio (1.4.1717). A one-way ANOVA followed by a Tukey post hoc test was used to compare data from multiple samples.

Fitness assessment on Greyscale-GSS strains

For the egg hatching experiments, two-day-old flies of three strains were separated by sex and reared in separate cages to prevent any mating before the experiments started. Ten-day-old flies (n=10 males and 10 females in each replicate) were mated for three days prior to egg collection. Egg collection was performed daily for five consecutive days with data analyzed for each day separately. Number of eggs laid was recorded for each replicate, followed by counting the number of hatched eggs 48 hours after collecting. For the initial GSS strain assessment, five replicates were set up for each fly strain whilst in the follow up experiment to identify if the reduced egg hatch is from males or females, three replicates were used. Fecundity was calculated as average number of eggs laid by one female per day (unit: average egg number/female/day), whilst fertility = number of hatched eggs*100%/number of eggs laid (unit: %)

For the non-competitive single pair mating assay, single virgin males and single virgin females were paired at the age of 13 days post-emergence. each pair of flies were introduced to each other in a transparent 1.125 L container with a mesh window 4 hours before simulated dusk. Seven possible crosses between wildtype (WT), greyscale exon 3 (GS) and greyscale GSS (GSS) were set up with ten replicates each. (1) wildtype male x wildtype female (WT (M) x WT (F)), (2) greyscale male x greyscale female (GS (M) x GS (F)), (3) greyscale GSS male x greyscale GSS female (GSS (M) x GSS (F)), (4) wildtype male x greyscale female (WT (M) x GS (F)), (5) wildtype male x greyscale GSS female (WT (M) x GSS (F)), (6) greyscale male x wildtype female (GS (M) x WT (F)), and (7) greyscale GSS male x wildtype female (GSS (M) x WT (F)). The time since the flies were introduced until they started mating and finished was recorded. Latency until copulation = latency from the time flies were introduced until they started mating (unit: min); copula duration = the period of mating of each pair (unit: min)

Data analyses were conducted in RStudio (RStudio 2022.07.2 Build 576). For the comparison of fecundity, fertility, latency until copulation and copula duration, generalized linear models (GLMs) with the Gamma family were performed, followed by Tukey post hoc test for pairwise comparison.

Appendix 6: Results for Greyscale strain (phenotypes and pupal colour sorting)

Pupal phenotypes

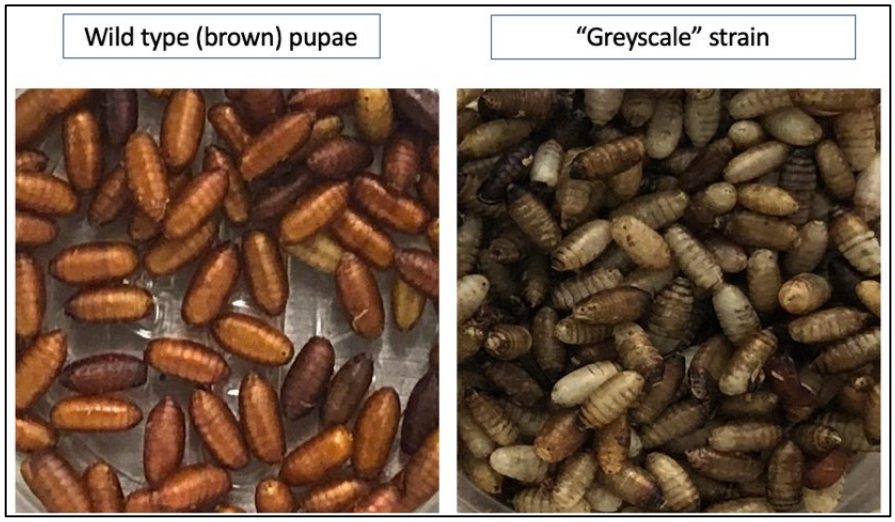

Figure A1. Comparison of pupal colour of the normal Qfly pupae and Greyscale strain

Trial experiment: mechanical sorting by pupal colour using seed sorter

A trial mechanical sorting experiment was performed using a USDA/ARS & National Mfg. seed sorter system (with colour camera and 3 seed channels) at Australian Grain Technologies (AGT), Roseworthy to separate greyscale from brown pupae. The expected accuracy for sorting 20kg/hr of wheat using this sorter is 85-95% per sort-pass depending on the sample contrast and threshold bias (Tom Pearson, 2011, W-751002-A Basic Operations Manual). It is common practice to run two rounds of sorting – *e.g*. the first pass removes ~85% defects and during the second pass, 85% of the remaining 15% defects are removed for a total of 97.7% removal. With each pass, a small percentage of good sample is ejected, hence ejected material will contain a mixture of undesired and good sample. Minimal calibration of the sorter was performed using 201 brown pupae and 201 *greyscale* pupae as required and then three rounds of sorting was performed with >1000 pupae, selecting the brown pupae to be the sorted (non-ejected) sample. After each round of sorting, the sorted (non-ejected) sample was photographed and the percentage of brown and *greyscale* pupae in that sample was calculated to determine the accuracy of each sorting. It is estimated that after the first round of sorting, 82.8% of the sorted pupae were brown (534/645 pupae); after the second round, 88.4% (428/484 pupae) and after the third round, 96.8% (417/431 pupae) of the remaining pupae were brown. Due to biosecurity regulations in SA, live pupae could not be transported out of the biological insectary for this experiment, hence pupae had to be killed by freezing prior to transport and sorting – this had a slight effect on the coloration of pupae, with dead pupae turning black over time. Nevertheless, the pupae separation results from the trial experiment are promising in that removal of *greyscale* pupae was achieved at a level almost similar to that used to discriminate wheat quality despite minimal calibration and less ideal conditions for the pupae.

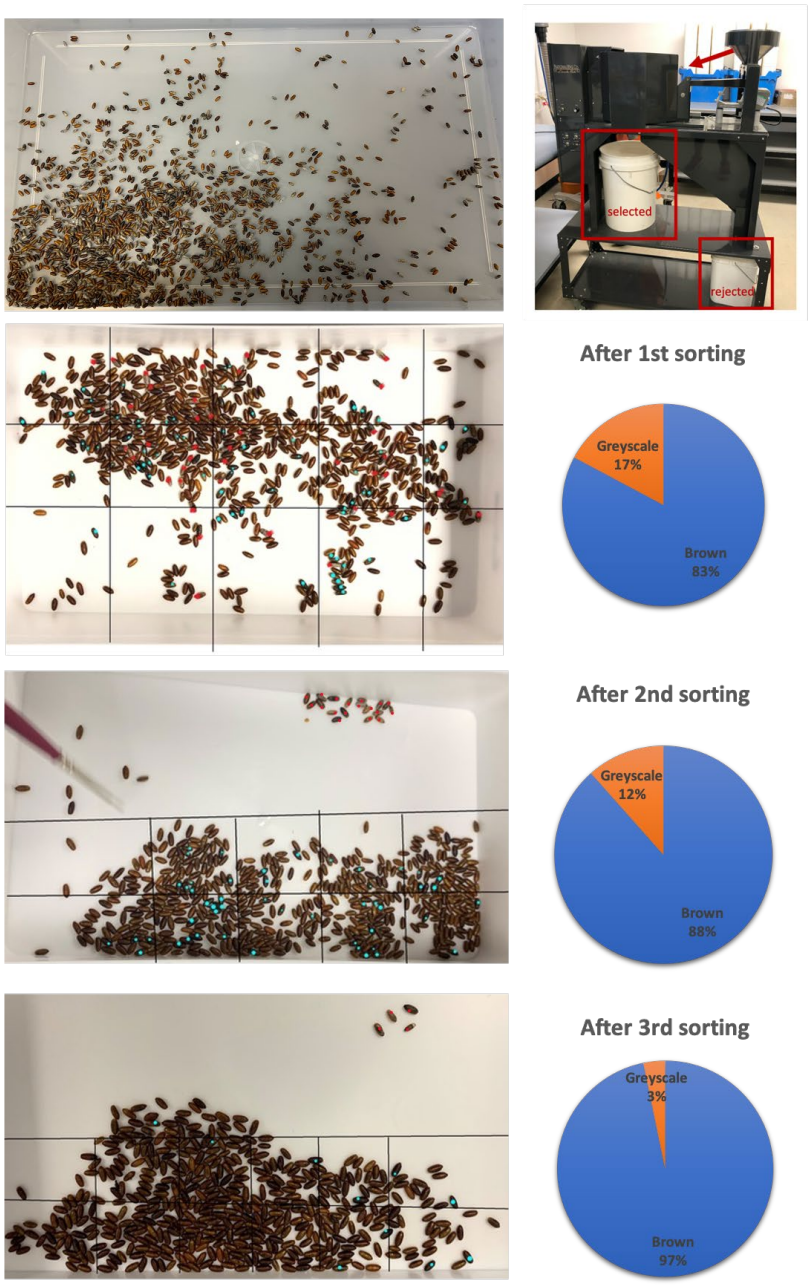

Figure A2. Images of brown and greyscale pupae taken prior to sorting and after 3 subsequent rounds of sorting (only the pupae from the 'selected/non-ejected' bin are shown) using a USDA/ARS & National Mfg. Seed sorter system in a trial experiment. Pupae are loaded into the sorter (where indicated by red arrow) and the system has been assigned to 'select' brown pupae and 'reject' greyscale pupae. Greyscale pupae identified in the non-ejected sample have been marked by red or blue dots in the images.

Appendix 7: Greyscale GSS - translocation experiments and recombination rate

Translocation Experiments

Table A2: Summary of all the experiments performed to obtain a translocation of the MFS gene from Chromosome 5 to the Y chromosome in Qfly

Irradiation Dose	30 Gy	35 Gy	45Gy
Lines set up	200	156	128
Lines screened	101	108	108
Translocation (MFS:Y)	0	**1***	0

* Successful translocation line, named '*Greyscale-GSS*'

Recombination Experiments

In order to assess the efficiency of the successful translocation line (named '*Greyscale-GSS'*), we separated pupae by colour (brown vs greyscale colour) and then counted the numbers of males and females from each pupal group. This was performed for every generation. A stable translocation line would have brown males (no females) and greyscale pupal females (no males); any brown females or greyscale pupal males would indicate a recombination event has occurred (which is to be expected to a certain degree). Each generation is filtered, in that the recombinants are removed from every generation immediately after emergence / prior to mating.

Table A3: Recombination frequency in a filtered Greyscale-GSS colony at Waite facility over 11 generations

Generation	Brown male	Brown female (Recombinant)	GS males (Recombinant)	GS females	Recombinant rate
1	278	0	1	170	0.22%
2	175	0	0	213	0%
3	177	1	1	188	0.55%
4	168	0	0	123	0%
5	142	0	0	124	0%
6	148	0	0	140	0%
7	114	0	2	101	0.93%
8	205	2	1	133	0.89%
9	88	0	0	78	0 %
10	108	0	0	101	0%
11	125	0	0	110	0.%

The observed recombination rate ranged from 0% to 0.93%, with recombinants more likely to be observed when more pupae are counted. The observation of recombination at a low level with filtering suggests that a filter colony is required to maintain the integrity of this strain.

Table A4: Recombination frequency in a non-filtered Greyscale-GSS colony at Macquarie facility after 2 generations

Generation	Brown male	Brown female (Recombinant)	GS males (Recombinant)	GS females	Recombinant rate
2	106	2	2	101	1.9%

Appendix 8: Results for Greyscale-GSS fitness assessment

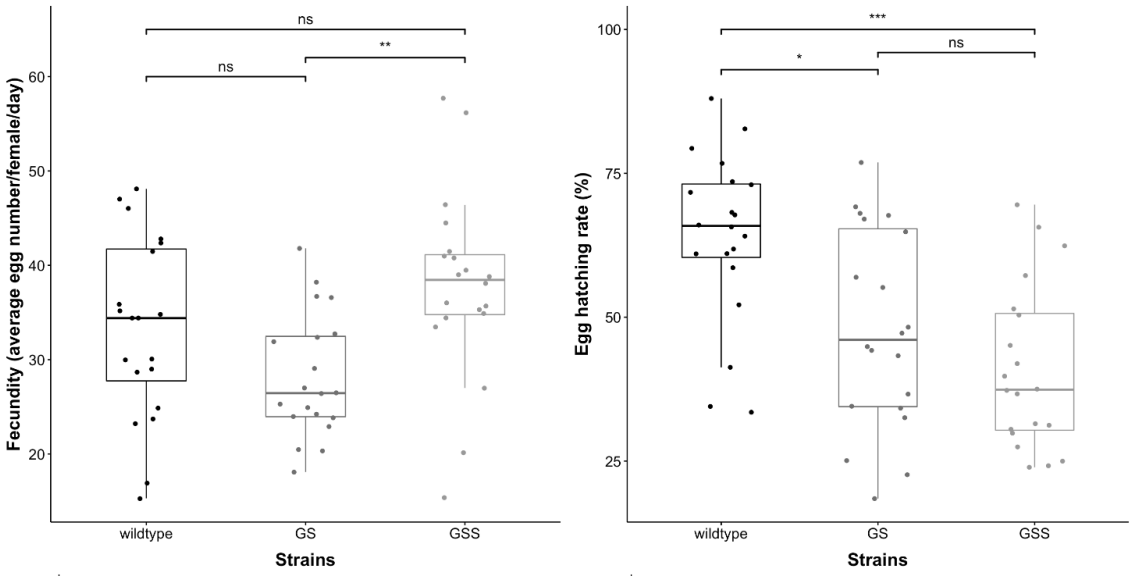

Figure A3: Fecundity and egg hatching of the Greyscale-GSS compared to lab strain (wild type) and Greyscale (GS) strain. Greyscale-GSS showed reduced egg hatching rate but no change in fecundity.

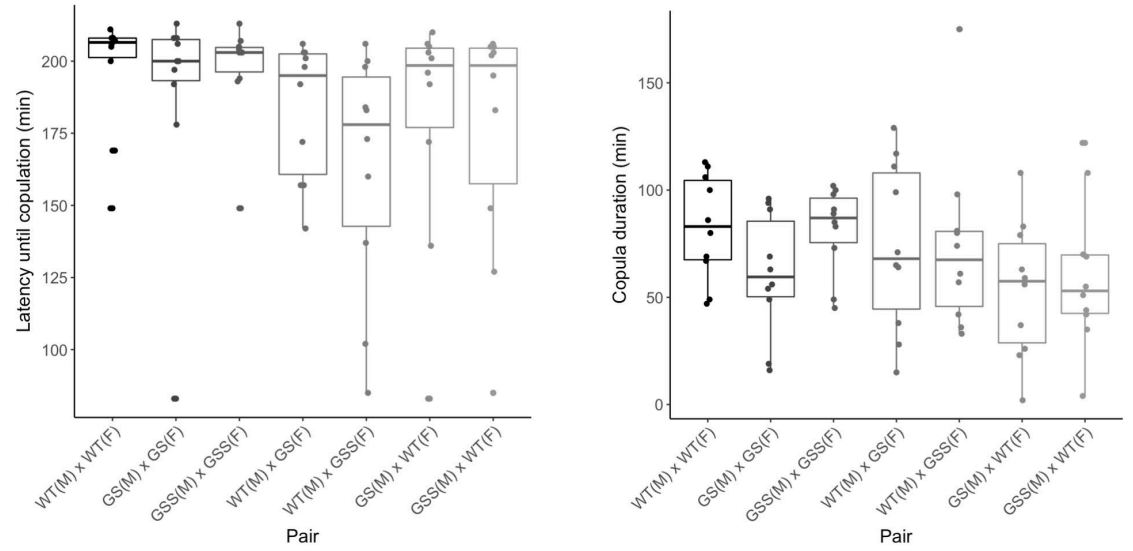

Figure A4: Latency until copulation and copula duration of the Greyscale-GSS compared to lab strain (wild type) and Greyscale (GS) strain. No significant difference was observed.

Table A5: Assessment of copulation between mating pairs of various genotypes

	WT (M) x WT (F)	GS (M) x GS (F)	GSS (M) x GSS (F)	WT (M) x GS (F)	WT (M) x GSS (F)	GS (M) x WT (F)	GSS (M) x WT (F)	Significance*
Latency until copulation	197.1 min	188.5 min	197.4 min	183.1 min	162.8 min	180.4 min	176.0 min	ns
Copula duration	82.8 min	60.7 min	81.5 min	73.7 min	73.7 min	53.6 min	60.0 min	ns

* All pair-wise comparison showed non-significance (ns)

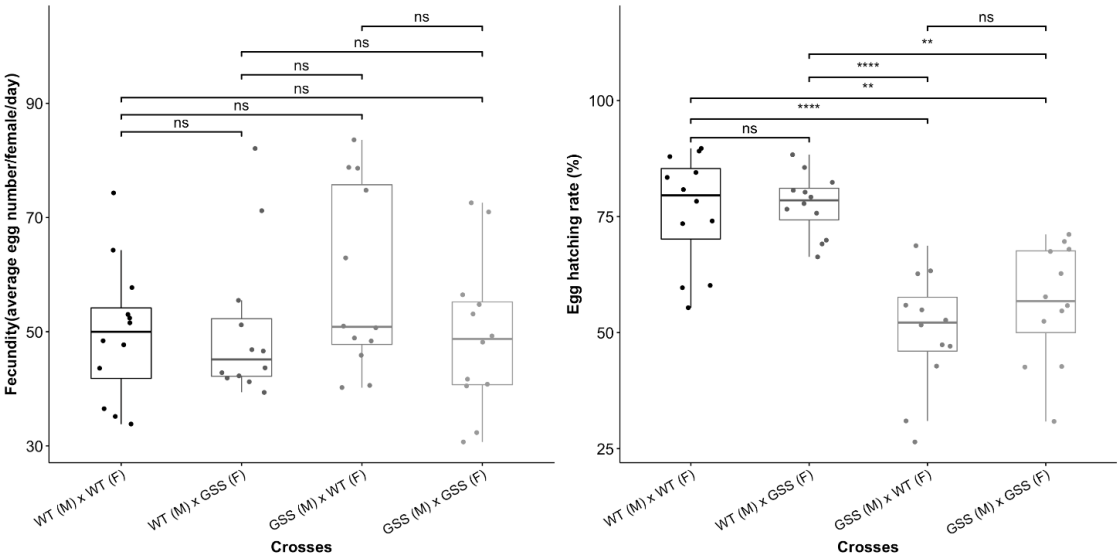

Figure A5: Fecundity and egg hatching rate from mating pairs of either male or female Greyscale GSS flies with wild type flies. No effect on fecundity observed regardless of pairing; reduced egg hatch observed when GSS males are used in the mating but not when GSS females are used. WT = wild type lab strain control, GSS = genetic sexing strain.

Appendix 9:

Nguyen, T. N. M., Choo, A., & Baxter, S. W., 2021. Lessons from Drosophila: Engineering Genetic Sexing Strains with Temperature-Sensitive Lethality for Sterile Insect Technique Applications. *Insects*, *12*(3), 243.

https://doi.org/10.3390/insects12030243

Appendix 10:

Choo, A., Fung, E., Chen, I. Y., Saint, R., Crisp, P., & Baxter, S. W., 2020. Precise single base substitution in the shibire gene by CRISPR/Cas9-mediated homology directed repair in Bactrocera tryoni. BMC Genetics, 21(Suppl. 2), 127-1-127-10.

https://doi.org/10.1186/s12863-020-00934-3

Appendix 11: Generation of shibire ts strains and temperature tolerance experiments

Generation of shibire ts strains

Table A6: Summary of CRISPR/Cas9 mutagenesis experiments performed to introduce the shibire ts2 and ts4 mutations into the Qfly genome

Tsl mutation	# of embryos injected	Surviving larvae	#G_0 pupae	#G_0 adults	#successful G_0 matings	#G_0 lines assessed	#G_0 mutants detected
Shibire ts2	2065	137	34	31	17	17	1 shi ts2 germline mutant
Shibire ts4	1939	265	119	104	81	79	1 shi ts4 germline mutant

Results of heat treatment on shibire ts4 strain:

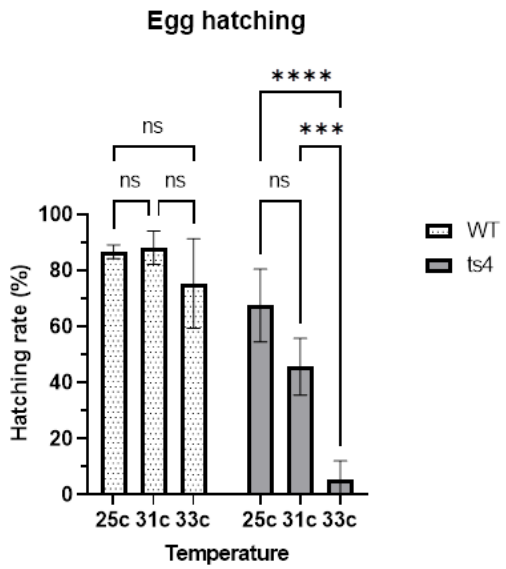

Figure A6: Temperature sensitive effect on egg hatching at 33°C for shibire ts4 strain. A significant decrease in egg hatch rate was observed when shibire ts4 eggs were treated at 33°C but not 31°C. n=3 replicates with 200 tested eggs per replicate.

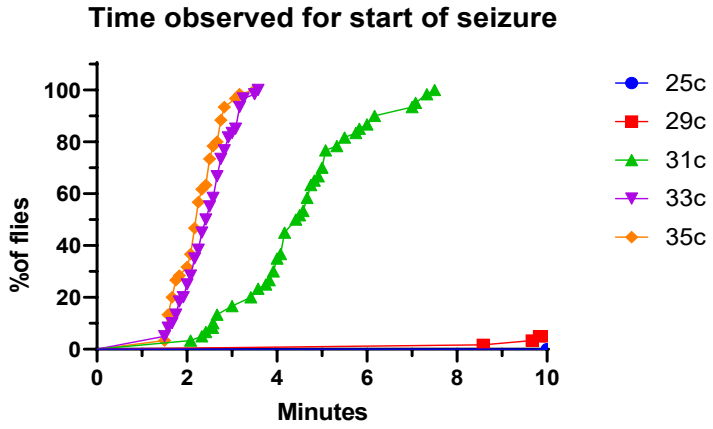

Figure A7: Time observed for flies to start showing a seizure phenotype within 10 minutes of treatment at different temperatures. n= 60 flies per treatment/temperature.

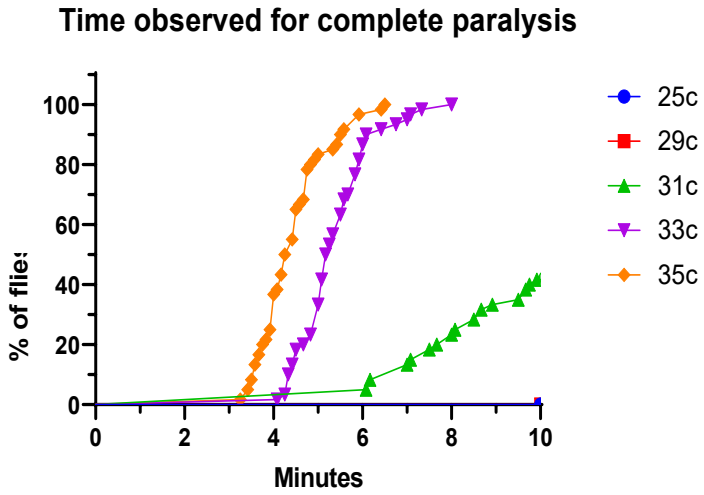

Figure A8: Time observed for flies to show complete paralysis within 10 minutes of treatment at different temperatures. n= 60 flies per treatment/temperature.

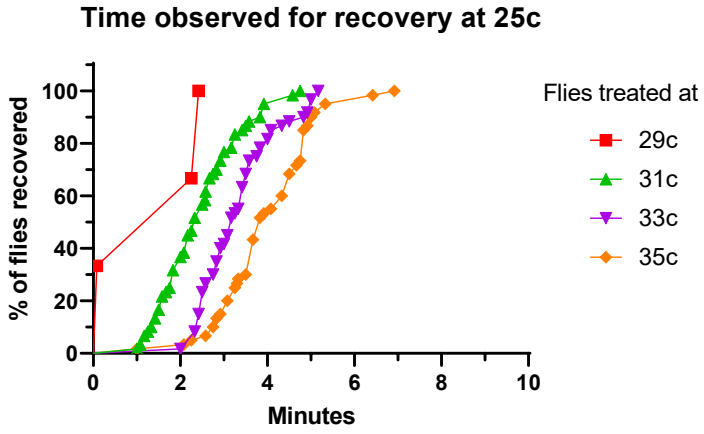

Figure A9: Time observed for flies to show recovery at 25°C after 10 minutes treatment at different temperatures. n= 60 flies per treatment/temperature.

Results of heat treatment on shibire ts2 strain:

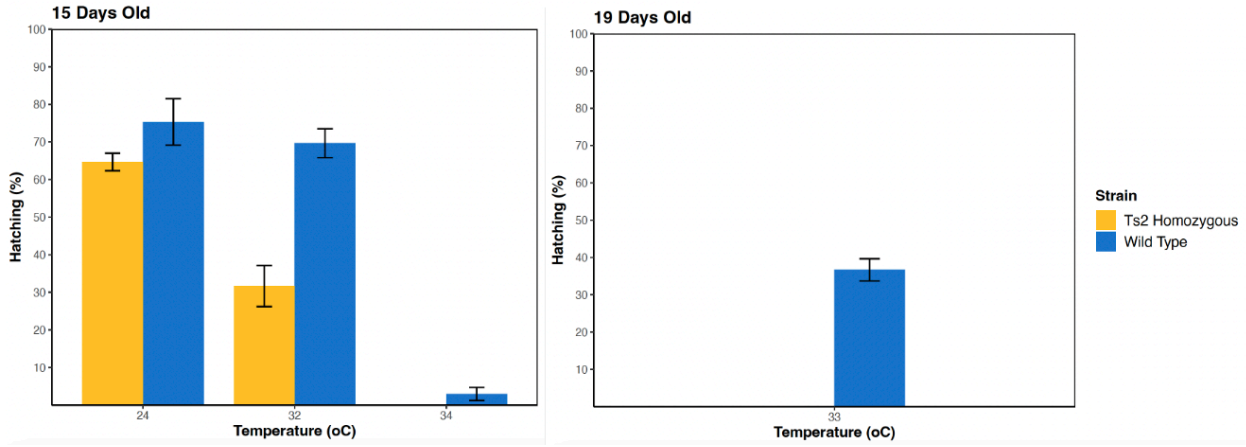

Figure A10: Preliminary experiment showing a temperature sensitive effect on egg hatching for shibire ts2 strain. Embryo lethality for the ts2 strain was first observed at 34°C using eggs collected from 15 day old females as well as in a follow-up experiment when only 33°C was tested using eggs from 19 day old females. 200 tested eggs per replicate; 3 replicates tested per strain.

Appendix 12: Ebony mutant strain: phenotypes and fitness assessment results

Ebony mutant strain phenotypes

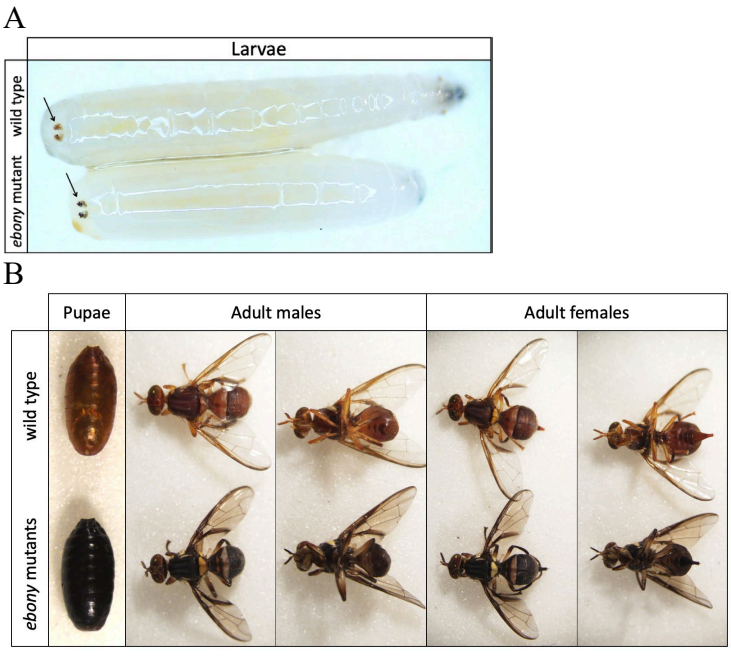

Figure A11: Phenotypic differences in the Qfly ebony mutant strain compared to wild type flies. The mutant strain had (A) black anal lobes in larvae, (B) black pupal colour and black body colour compared to the wild type.

Ebony fitness assessments

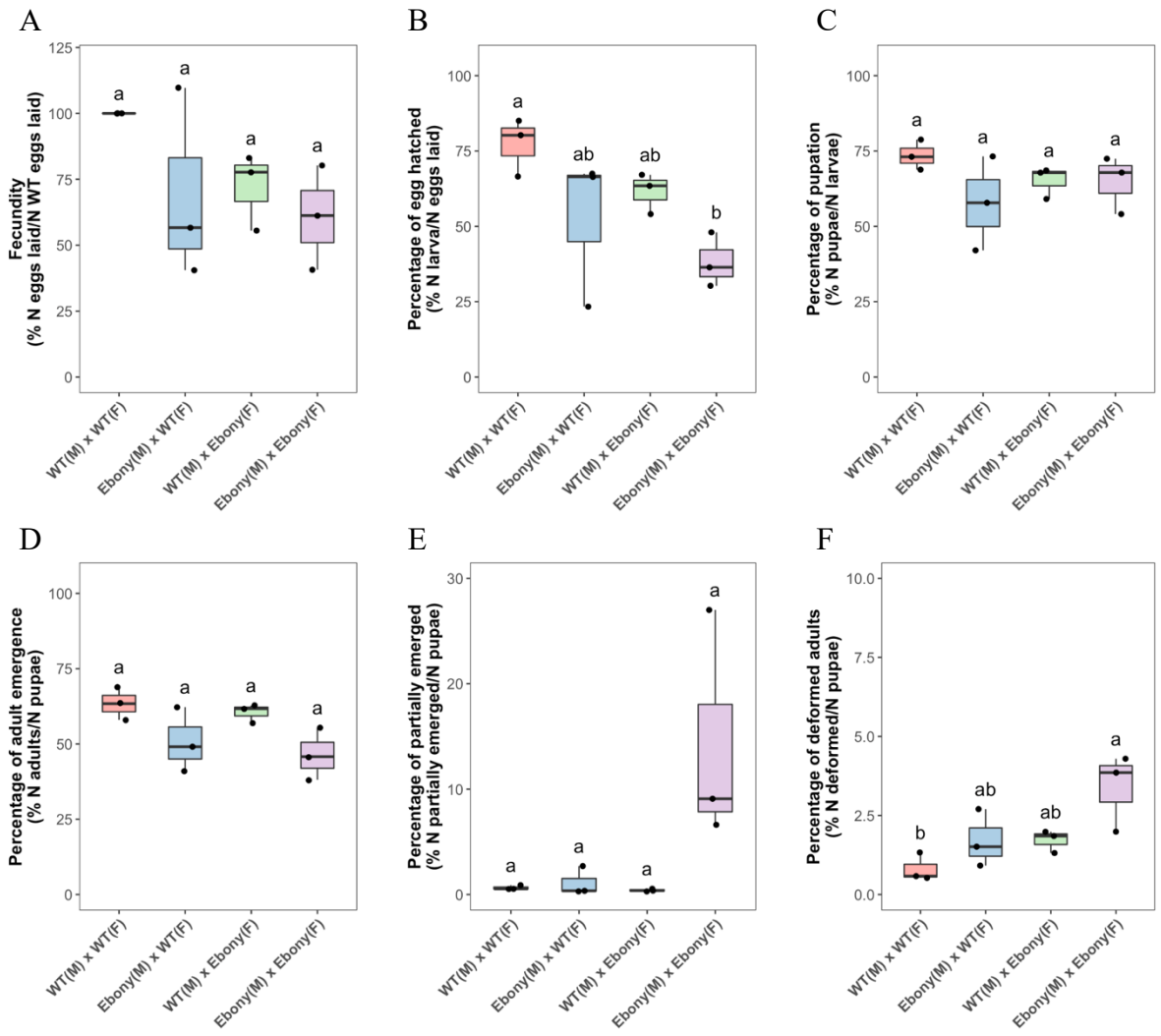

Figure A12. Fitness analysis of Qfly ebony mutants. Four crossing combinations with 10 males and 10 females in each cross were set up and were assessed for (A) Fecundity, (B) Percentage of egg hatched, (C) Percentage of pupation, (D) Percentage of adult emergence, (E) Percentage of pupae that were partially emerged, and (F) Percentage of deformed adults. Three replicates for each combination. Box plots and data points are shown. Box plots represent the interquartile range, and the median value is indicated. One-way ANOVA followed by Tukey post hoc test were used to compare data from multiple combination of crosses. The same letter above each box plot indicates no significant difference ($p > 0.05$).

Appendix 13: List of Presentations

1. Simon Baxter, SIT Plus Technical Advisory Committee (TAC) meeting, Sydney, 25 September 2019 (oral update)
2. Amanda Choo, SIT Plus Technical Advisory Committee (TAC) meeting (virtual), 1 June 2020 (oral update)
3. Amanda Choo, SIT Plus Technical Advisory Committee (TAC) meeting (virtual), 16 Sept 2020 (oral update)
4. Amanda Choo, SIT Plus Technical Advisory Committee (TAC) meeting (virtual), 9 Dec 2020 (oral update)
5. Amanda Choo, SIT Plus Technical Advisory Committee (TAC) meeting (virtual), 2 March 2021 (oral update)
6. Amanda Choo, SIT Plus Technical Advisory Committee (TAC) meeting (virtual), 6 Oct 2021 (oral update)
7. Simon Baxter, Third Research Coordination Meeting (RCM2) on Generic Approach for the Development of Genetic Sexing Strains for SIT Applications, Joint FAO/IAEA Program of Nuclear Techniques in Food and Agriculture, Vienna and online (hybrid meeting, confidential talks), 18 – 22 Oct 2021. Department of Nuclear Applications (oral presentation – 'Developing *Bactrocera tryoni* genetic sexing strains').
8. Amanda Choo, SIT Plus Technical Advisory Committee (TAC) meeting (virtual), 7 Dec 2021 (oral update)
9. Amanda Choo, SIT Plus Technical Advisory Committee (TAC) meeting (virtual), 6 April 2022 (oral update)
10. Simon Baxter, Gordon Research Conference (Genetic Biocontrol), Ventura, California, USA., June 26 - July 1, 2022 (invited oral presentation plus poster – 'Developing Bactrocera tryoni genetic sexing strains for biocontrol').
11. Amanda Choo, SIT Plus Technical Advisory Committee (TAC) meeting (virtual), 5 July 2022 (oral update)
12. Siu Fai Lee (Ronald), Joint NFFC-SITPlus SAC meeting, Canberra, 21 Sept 2022 (oral update)
13. Thu (Zoey) Nguyen, 11th International Symposium on Fruit Flies of Economic Importance on 17 November 2022, Sydney (poster – 'Disruption of the *Bactrocera tryoni ebony* gene produces melanic phenotypes consistent with tephritid *black pupae* genetic sexing strains').
14. Ngoc Mai Han Nguyen, 11th International Symposium on Fruit Flies of Economic Importance on 17 November 2022 in Sydney (poster – 'Targeting *white pupae* gene to develop a phenotypic trait for *Bactrocera tryoni* genetic sexing strain').
15. Simon Baxter, Third Research Coordination Meeting (RCM3) on Generic Approach for the Development of Genetic Sexing Strains for SIT Applications, Joint FAO/IAEA Program of Nuclear Techniques in Food and Agriculture, Vienna and online (hybrid meeting, confidential talks), 24 to 28 April 2023. Department of Nuclear Applications (oral presentation – 'A genetic sexing strain of the Queensland fruit fly, *Bactrocera tryoni'*).