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Wolbachia for *Lucilia cuprina* control

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EXECUTIVE SUMMARY

Improving control strategies for the Australian sheep blowfly ectoparasite, *Lucilia cuprina*, is urgently required to reduce myasis in livestock, improve animal welfare and reduce pest management costs. The use of insecticides, breeding strike resistant sheep, strategic crutching and shearing are all used in integrated programs for flystrike control, but functional control relies primarily on mulesing and insecticide application.

In this project we investigated the potential for a natural bacterium, *Wolbachia*, to control blowflies in area-wide programs. *Wolbachia* are maternally transmitted intracellular bacteria that infect a wide range of insect species and can spread through insect populations by manipulating host reproductive processes. They have many and varied effects on host biology that present potential for use in blowfly control programmes. Their effects can be considered in three main groups, a) Cytoplasmic incompatibility (CI) whereby uninfected females or females that are infected with a different strain of *Wolbachia* are rendered sterile by mating with an infected male. CI can be harnessed for population suppression, population replacement or potentially population elimination, but without the need for irradiation or chemical treatment for sterilisation; b) fitness effects induced by *Wolbachia* infection that can be harnessed to suppress or eliminate populations; and c) transmission blocking of secondary pathogens. Australia has been a world leader in *Wolbachia* research which has culminated in the much-publicised success of use of *Wolbachia* in mosquitoes to suppress dengue in northern Queensland and overseas. *Wolbachia* is also being investigated for use in control of another major livestock pest, tsetse fly, in Africa.

Most research to date has focused on medical and horticultural pests, but in this project, we are seeking to use *Wolbachia* to control a major livestock pest, sheep blowflies. Most current flystrike control programs rely on individual animal treatments, for example mulesing and insecticide application. This approach is expensive and often labour intensive. Overreliance on chemical insecticides risks the development of resistance, and also occupational health and safety exposure, residues in produce and environmental contamination. The approach suggested here, directly targeting pest populations rather than using individual animal-based methods, would provide labour savings and enhance the reputation of Australian wool and sheep meat as clean, safe and ethically produced.

The first stages in such an approach are determination of the current *Wolbachia* infection status of *L. cuprina* and the successful development of *L. cuprina* lines infected with suitable *Wolbachia* strains. Here we surveyed *L. cuprina* populations collected from across Australia for the presence of any *Wolbachia* and performed transinfection of blowflies with strains of *Wolbachia* collected from mosquitoes, *Drosophila* flies and insect parasitoids.

Populations survey: Approximately 500 *Lucilia* flies collected from more than 70 populations across Australia were analysed for the presence of *Wolbachia* using molecular diagnostic screening. *Wolbachia* infection was absent from *Lucilia cuprina dorsalis* across all regions of Australia tested. Samples of the species *Lucilia sericata*, and urban subspecies *Lucilia cuprina cuprina* were also assessed and did not contain *Wolbachia*. We therefore conclude that *Wolbachia* is absent or extremely rare in Australian *Lucilia* species. This result indicates that the release of factory reared *Lucilia cuprina* cultures transinfected with *Wolbachia* are unlikely to encounter wild populations that already carry related strains.

Transinfection: *Wolbachia* strains obtained from ovaries of *Drosophila melanogaster* fruit flies, *Aedes* mosquitoes and fly parasitoids (*Spalangia* sp.) were microinjected into *Lucilia cuprina* embryos, pupae or adult female flies. Injection of each of these stages has enabled successful establishment of stable transinfected lines in other species, but by far the most widely used method has been injection into the cytoplasm of freshly laid eggs. Infection of female reproductive germinal tissues is required to enable transmission to succeeding generations. This has been particularly successful in the case of insects such as mosquitoes. Here we demonstrate successful transmission of *Wolbachia* between generations in a small number of cases.

Embryo microinjections: Embryonic microinjection involves a highly specialized technique that requires a substantial initial outlay of funds for equipment and trained personnel. Thousands of embryo microinjections were performed, which was necessary due to high mortality rates of the injection process. Persistence of *Wolbachia* within single female flies was observed, demonstrating the ability for the bacteria to successfully survive over the course of the fly's lifetime. Females that completed development were crossed with wild type males to determine whether *Wolbachia* could be transmitted to subsequent generations. Transmission to succeeding generations was demonstrated in a number of cases, but sustainably infected strains could not be generated. Females infected with *Wolbachia* were observed to produce relatively few eggs, suggesting the bacteria may significantly impact fecundity.

Pupae microinjections: Introduction of *Wolbachia* during the early pupal stage via microinjection require large quantities of *Wolbachia* but resulted in lower mortality rates compared to embryo injections. Pupal injection provides greater opportunity for the bacteria to grow to higher titres in adult flies and infect the ovaries before egg lay than direct fly injection. *Wolbachia* successfully persisted at low density for multiple generations through this approach. Injections of high *Wolbachia* titres combined with tissue reorganisation during pupae metamorphosis may enhance movement of *Wolbachia* into female germinal tissues.

Female fly microinjections: Injections of *Wolbachia* directly into the thorax of the adult female fly ensures that high numbers of potentially infected individuals can be generated compared to embryo microinjection but means more time needs to be spent on screening progeny for successful transfers.

This study, proposed as a proof-of-concept investigation, has shown a more labour-intensive approach may be required to successfully develop stably infected strains of *Lucilia*. Ultimately the fitness costs of harbouring *Wolbachia* appeared to be high in *L. cuprina*, causing lower oviposition and egg hatch rates, reduced eclosion from pupae and increased adult mortality. Instances of successful transinfection in other insect species often had similar results in the early stages and a long genesis before reaching a successful conclusion. Some transmission of *Wolbachia* across generations was noted in flies resulting from injection of each of the three stages investigated in this study, providing proof of concept that *Wolbachia* can infect *L. cuprina* tissues and can be transferred between generations in this species. However, the resources and ability to maintain much larger numbers of injected *L. cuprina* strains, as has been necessary in other species, may be required to achieve successful establishment of a stably *Wolbachia*-infected strain that is suitable for use in sheep blowfly control programs. Area-wide population control programs such as transgenic expression of *Wolbachia* fertility reducing genes directly in the genome of *L. cuprina* is an alternative approach that may create strains that can be used to eliminate females. The possibility of using different *Wolbachia* strains as well as other potential bacterial endosymbionts should be explored further.

INTRODUCTION

Many diverse *Wolbachia* strains have been reported among dipteran insects, and some have useful attributes for insect pest and disease control. Blowfly population control may be possible via;

- Incompatible insect technique: release *Wolbachia* infected males that mate with wild females and cause embryo mortality. Population suppression or elimination occurs through cytoplasmic incompatibility. *Wolbachia* infected females cannot be released.
- Population replacement strategies: release a *Lucilia cuprina* strain infected with *Wolbachia* into wild populations. *Wolbachia* spread throughout the local population, with a mild deleterious phenotypic effect (e.g. inability to over winter, increased susceptibility to insecticides).

The aims of this project were to determine infection frequencies of *Wolbachia* among *Lucilia cuprina* populations across Australia, sequence a *Wolbachia* genome that is found in a blowfly, transinfect blowflies using *Wolbachia* from other insect sources and assess fitness costs of infected *Lucilia* strains.

Wolbachia infection can cause negative fitness effects on their hosts and provide desirable attributes for pest control. Some of these effects include reduced life span (McMeniman *et al*., 2009), mortality of eggs, slowed larval development (Ross *et al*., 2014), and reduced overall fitness. Infection with *Wolbachia* has also been shown to interfere with blood-feeding efficiency in mosquitoes, and to affect locomotor activity in parasitoid wasps, *Drosophila* species, and some mosquitoes (Fleury *et al*., 2000). In addition, *Aedes* mosquitoes infected with *Wolbachia* strains isolated from *Drosophila* fail to transmit diseases such as dengue fever and Zika virus (LePage *et al*., 2017). Phenotypic effects are often species dependent and require careful assessment.

LITERATURE REVIEW

Wolbachia are endosymbiotic bacteria found in up to 60% of insect species, although infection rates within populations can vary extensively (Werren *et al*., 2008). Insects contain *Wolbachia* in their reproductive tissues and transmission to the next generation occurs maternally via the egg. Many diverse *Wolbachia* strains have been reported among dipteran insects, and some have useful attributes for insect pest control. *Wolbachia* isolates can be successfully transferred between species (e.g. flies and mosquitoes) using embryonic microinjection techniques in the laboratory. The University of Queensland has adapted three strains of *Wolbachia* (*w*Mel, *w*MelPop and *w*AlbB) to *Haematobia* cell lines, which have been reared through more than 50 passages (M. Madhav *et al*., 2020). Transinfection of *Wolbachia* pre-adapted in insect cell lines also represents an exciting potential to increase the success rate of transfer between species (McMeniman *et al*., 2009). As *Haematobia* is closely related to *Lucilia*, the *Wolbachia* pre-adapted in *Haematobia* cell lines may be me more amenable to transinfection than *Wolbachia* from their original hosts (e.g. *Drosophila*). If transinfection is successful between species, *Wolbachia* can potentially be used for two distinct pest management strategies, the incompatible insect technique and insect population replacement strategies.

Incompatible insect technique (IIT)

Wolbachia infected males are released and cause population suppression.

Some Wolbachia species show cytoplasmic incompatibility (CI), preventing fertile mating between infected males and unaffected females. Recent work with infected Drosophila identified two essential Wolbachia genes for CI, cytoplasmic incompatibility factor A (cifA) and cifB (LePage *et al*., 2017) (**Figure 1**). These two Wolbachia proteins act during spermatogenesis to modify histones on the chromosomes carried by sperm. After mating, these modified sperm interfere with cell division post fertilization, thus killing the embryos of Wolbachia-negative females. Females infected with Wolbachia also express CifA and CifB, acting as an antidote which counteracts the modified sperm and rescues the embryo. The Wolbachia bacteria is not transmitted from infected males to uninfected females: only modified sperm. Release of Wolbachia infected males into a population that lack Wolbachia or are infected with dissimilar strains of Wolbachia, can cause population suppression (**Figure 2**).

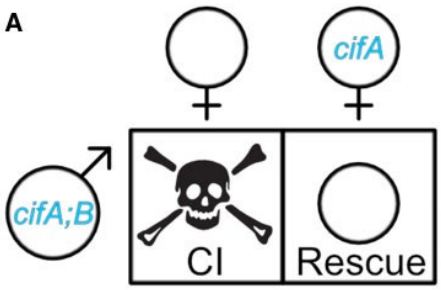

Figure 1. Male Drosophilia expressing cifA and cifb (toxins) during spermatogenesis cause CI and embryo lethality when mated with wild type females. Females expressing cifA (the antidote) rescue this phenotype and do produce fertile progeny when mated to cifA;B expressing males. (Figure adapted from Kaur *et al*. (2021).)

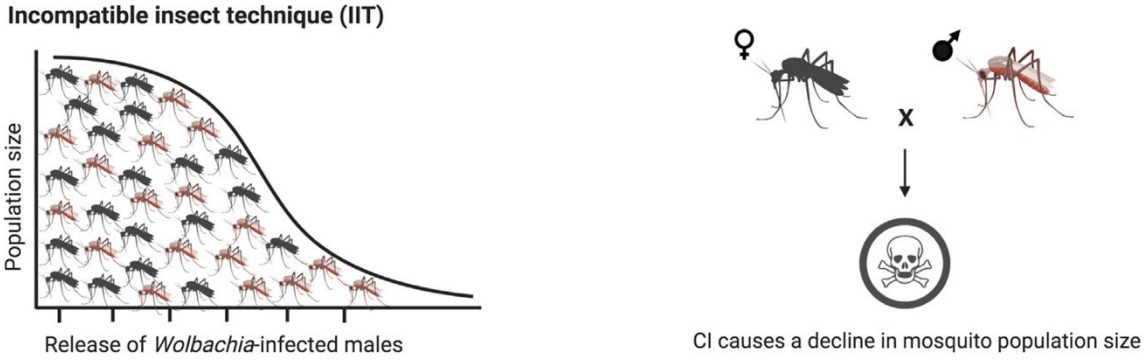

Figure 2. Release of infected male mosquitoes (brown) supresses the wild population (dark grey) over generational time. Wild females do not produce viable progeny when they mate with Wolbachia infected males because of the effects of cytoplasmic incompatibility (CI) (Figure adapted from Kaur *et al*. (2021)).

How could IIT be a used control *Lucilia cuprina*? Male flies infected with a CI-causing Wolbachia strain could be released, causing embryo mortality when mated with wild females. A genetic sexing strain would be required to separate males from females in the factory.

Insect replacement technique

Wolbachia infected insects are released and the endosymbiont spreads through a wild population

Wolbachia infection can cause negative fitness effects on their hosts cause a decline in the pest population. Some of these effects include reduced life span (McMeniman *et al*., 2009), mortality of eggs, slowed larval development (Ross *et al*., 2014), and reduced overall fitness. Infection with Wolbachia has also been shown to interfere with blood-feeding efficiency in mosquitoes, and to affect locomotor activity in parasitoid wasps, Drosophila species, and some mosquitoes (Fleury *et al*., 2000). In addition, Aedes mosquitoes infected with Wolbachia strains isolated from Drosophila fail to transmit diseases such as dengue fever and Zika virus (LePage *et al*., 2017).

The most profound deleterious effects described have been with the 'popcorn' (wMelPop) strain of Wolbachia, initially isolated from laboratory populations of Drosophila melanogaster Meigen (Min & Benzer, 1997). The wMelPop strain replicates in host cells, causing cellular damage, characteristic morphological changes in infected tissues, and a range of physiological effects. These effects reduce life span by approximately one-half in D. melanogaster and transinfected mosquitoes. Reductions of life span of this magnitude, and other fitness characters, can have profound effects on the population dynamics of a species and modelling has demonstrated potential for using fitness reductions induced by Wolbachia to suppress or eliminate mosquito (Aedes aegypti) populations (Rasić *et al*., 2014). Transinfection with wMelPop Wolbachia was shown to be able to collapse overwintering populations in mosquitoes by reducing the resilience of eggs, the overwintering stage (Ritchie *et al*., 2015). Mortality of sheep blowflies is particularly high during the prepupal and pupal phases during overwintering in the soil with survival rates of 1-8%. Inducing further mortality through the introduction of Wolbachia could be used to collapse blowfly populations during the overwintering phase.

Due to cytoplasmic incompatibility, described above, Wolbachia can quickly spread through local populations, as uninfected females cannot be fertilised by infected males. Insect replacement techniques, that involved releasing Wolbachia infected mosquitoes, have shown remarkable success in controlling human diseases in northern Australia and other countries (**Figure 3**) (Walker *et al*., 2011).

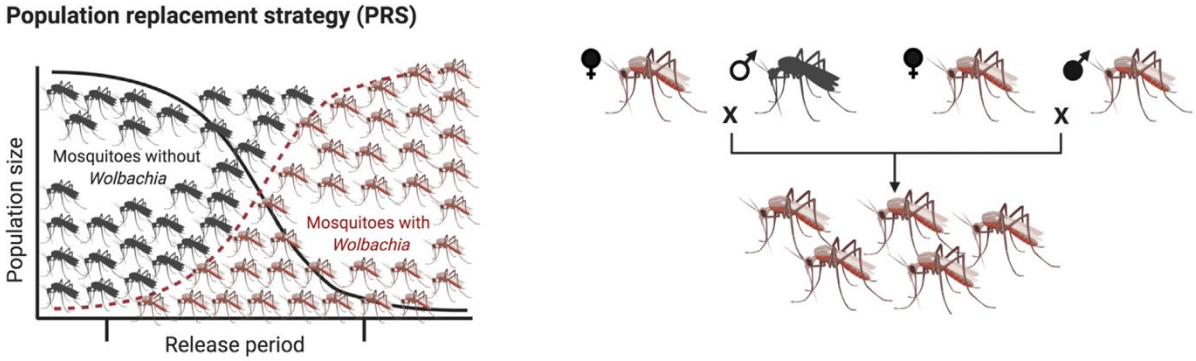

Figure 3. Left: Insects without Wolbachia (dark grey) are gradually replaced with insects infected with a specific Wolbachia strain (brown). **Right:** Wolbachia is transmitted through the eggs of infected females. (Figure adapted from Kaur *et al*. (2021) and populations infected with Wolbachia early death, reduced fertility or increased fitness costs.

How could Wolbachia be used to control *L. cuprina*? *Lucilia cuprina dorsalis* is a subspecies that commonly inflicts flystrike. Replacing L. c. dorsalis with a Wolbachia infected subspecies with lower propensity to strike has potential applications for integrated pest management. Alternatively, identifying fitness effects induced in *Lucilia* flies by different Wolbachia strains could identify traits valuable for pest control. For example, shortened lifespan, reduced reproduction capacity or inability to overwinter.

PROJECT OBJECTIVES

Objective 1. To determine the frequency of *Wolbachia* in field collected populations of *Lucilia cuprina* and *Lucilia sericata*.

Populations of *Lucilia* have previously been collected from diverse regions across Australia to understand population dispersal and movement using molecular genetic approaches (AWI project ON-00624, contract 4500012557). These samples will be repurposed to estimate *Wolbachia* frequency. Samples include *Lucilia cuprina cuprina*, *Lucilia cuprina dorsalis* and *Lucilia sericata*. DNA will be isolated from individual flies, including reproductive tissues which potentially harbour *Wolbachia*. Molecular diagnostic assays using *Wolbachia* specific markers, including the *wsp* gene, will be used to determine the presence or absence of *Wolbachia* in individual flies using classic diagnostic PCR primers.

Outcome and Deliverable 1: Clear understanding of *Wolbachia* diversity and infection rates in blowflies across Australia

Objective 2. To characterise *Wolbachia* strains present in *Lucilia* species

This objective involves performing genome sequencing on a single *Lucilia* fly infected with *Wolbachia* using "long-read" technology to enable assembly of the entire circular genome. Cytoplasmic incompatibility factors of a *Wolbachia* genome can then be identified through gene annotation.

Outcome and Deliverable 2: Whole genome sequencing of a *Wolbachia* isolate

Objective 3. Transinfection of *Lucilia* with different strains of *Wolbachia* using microinjection

Drosophila melanogaster is the model system for functional insect biology, and routinely used for scientific research in the Baxter laboratory. Microinjection methods will be trialled to transfer *Wolbachia* from *Drosophila* to *Lucilia*, and if *Wolbachia* is identified in *Lucilia* species, reciprocal experiments into *Drosophila* will be performed. *Wolbachia* from a range of sources including mosquitoes, parasitoids and cell lines will be isolated in the James laboratory and microinjections into a range of *Lucilia* blowfly developmental stages will be trialled.

Outcome and Deliverable 3: Transfer *Wolbachia* to *Lucilia cuprina dorsalis* using microinjection techniques.

Objective 4. To characterise the fitness effects induced in *Lucilia* by *Wolbachia* infection

Fitness experiments will be performed on a strain of *Lucilia cuprina* that contains *Wolbachia*. Differences in fitness characteristics will be assessed including mating compatibility, fertility and fecundity of flies, morphological or mobility effects and survival of different fly stages induced by infection with different *Wolbachia* strains. The effect of *Wolbachia* on the viability of the larval and pupal stages of *L. cuprina* at low and high temperatures will also be investigated.

Outcome and Deliverable 4: Clear understanding and characterisation of the fitness effects induced in *Lucilia cuprina* by *Wolbachia* infection.

Objective 5. Insect strain maintenance throughout the project.

Outcome and Deliverable 5: Maintenance of transfected blowfly colonies.

SUCCESS IN ACHIEVING OBJECTIVES

Number	Description	Summary
Objective 1	To determine the frequency of *Wolbachia* in field collected populations of *Lucilia cuprina* and *Lucilia sericata*.	
	Screen field collected samples for *Wolbachia*.	446 *Lucilia* flies collected from 67 locations across Australia were screened for *Wolbachia*. No infection was detected. Additional laboratory colonies were also extensively genotypes. Increasing the number of populations analysis to 75.
Objective 2	To characterise *Wolbachia* strains present in *Lucilia* species.	
	Sequencing genomic DNA of individual *Wolbachia* positive *Lucilia* fly.	We have been unable to identify *Wolbachia* in any *Lucilia* population.
Objective 3	Transinfection of *Lucilia* with different strains of *Wolbachia* using microinjection.	
UQ	Microinjection of *Wolbachia* from mosquitos and *Drosophila*.	529 embryonic micronjections performed. 320 pupal injections. 144 Adult female flies. Infection outcomes: **Embryonic injections**: *Wolbachia* was not detected in any of the adult flies tested from injected flies in the same generation (G0). *Wolbachia* was found in 3/8 instances in the subsequent generations (G1). No *Wolbachia* was found in any G2 flies from embryonic injections (0/2) tested. **Pupal injections:** 12/27 groups of flies resulting from pupal injections (G0) were infected with *Wolbachia*. 5/19 groups of G1 flies tested from pupal injections were positive for *Wolbachia* whereas 3/6 groups of G2 flies tested were *Wolbachia* positive. **Adult injections:** No surviving flies from the injections were found positive for *Wolbachia* (0/5 groups) whereas 5/22 groups of G1 flies and 1/8 groups of G2 flies tested were *Wolbachia* positive.
UoM	Microinjection of *Wolbachia* isolated from *Drosophila*.	3,044 embryonic microinjections performed. **Embryonic injections:** *Wolbachia* was successfully detected in approximately 10 female flies. As *Wolbachia* is maternally transmitted via egg, we focussed on analysis of analysis of offspring from infected females. Two G1 individuals show some evidence of transmission, however, stable colonies could not be established.
Objective 4	To characterise the fitness effects induced in *Lucilia* by *Wolbachia* infection.	
UQ		Poor eff lay and fertility rates observed among G2 females infected with *Wolbachia*. While this could be due to the *Wolbachia* infection, it may also have been an artifact of the relatively small number of flies available in mating groups in the G2 generation.

UoM		Transmission of *Wolbachia* between generations was not sufficiently successful. *Wolbachia* positive females showed poor fecundity rates, laying few eggs suggesting a strong fitness cost may occur. Pre-adaptation of *Wolbachia* to *L. cuprina* cell lines may improve success.
Objective 5	Insect strain maintenance.	
UQ/UoM	Maintain *Lucilia* strains.	Multiple *Lucilia cuprina* laboratory cultures were maintained at University of Melbourne and University of Queensland, including those transfected with *Wolbachia*.

METHODOLOGY

Wolbachia in field collected populations of *Lucilia cuprina*

Blowfly populations were collected from across Australia between 2018 and 2021 by University of Melbourne (Dr. Trent Perry) and a network of growers and agronomists (**Appendix Table 1**). This valuable resource was screened for the presence or absence of *Wolbachia* using molecular biology assays. Detailed description of the fly populations collected are outlined in AWI project ON-00624. Five captive laboratory populations were also screened for *Wolbachia*, including KDA, GG, SL, TPC and LS.

At University of Queensland, *Wolbachia* screening was conducted on four captive populations of flies, including the insecticide-susceptible reference strain. This laboratory susceptible strain (LS) was originally isolated as a flystrike strain and is putatively the sub-strain *Lucilia cuprina dorsalis* associated with sheep flystrike. Three additional field strains of the *Lucilia cuprina cuprina* or potentially (*Lucilia cuprina cuprina*) X (*Lucilia cuprina dorsalis*) sub-type were also collected and established in the laboratory. The strains are from Brisbane and from Coochiemudlo Island in Morton Bay, Queensland, were urban breeding strains. It has been suggested that *Wolbachia* is more commonly found in tropically oriented species and that speciation can sometimes be driven by differential *Wolbachia* infection. Previous positive tests for *Wolbachia* have occurred in our laboratory, and it was hypothesised that this may be related to *L. cuprina* subtype or blowfly origin.

DNA Isolation

University of Melbourne: DNA was isolated from abdomen tissue using the DNeasy Blood and Tissue Kit (Qiagen Cat# 69504). Bulk extracts of five flies were performed, again using DNeasy Blood and Tissue kit (Qiagen) from (5 flies per tube (UQ) or 10 flies per tube (UoM)). *Wolbachia* DNA was obtained from *Drosophila melanogaster*, collected from Innisfail, Queensland. These samples are required as positive controls for molecular diagnostic assays, and for sources of transinfection via microinjection.

University of Queensland: A modified Chelex extraction protocol from was used for extraction of DNA (Echeverría-Fonseca *et al*., 2015). Briefly, flies were ground (either bulk or individually) in 2 mL Eppendorf tubes using a sterile tissue grinder with liquid nitrogen, then 600 μL of nucleic lysis buffer (Promega A7941) was added into each tube. The samples were homogenised using a tissue lyser (TIssueLyser II, Qiagen) for 10 min in 2 ml Eppendorf tubes with 1-2 g (1/3 of total volume) of glass beads (1 mm and 5 mm, ratio 1:1) and chilled on ice. DNA was extracted following the instructions of Promega Wizard Genomic DNA Purification Kit (animal tissue part). Samples were then incubated overnight at 55 °C with 17.5 μl of Proteinase K (20 mg/ml), shaking at 200 rpm. On the following day, 3 μL Rnase A was added into each sample (10 mg/ml) and incubated for 30 min at 37 °C and 200 rpm. The remaining steps were performed according to the Promega Wizard Genomic DNA Purification kit to obtain genomic DNA. DNA concentrations were determined using the Qubit 4 fluorometer (Thermo Fisher scientific). Wolbachia DNA was isolated from *Aedes* mosquitoes.

Molecular diagnostic assay (PCR)

Diagnostic assays were performed using 10 microlitre PCR reactions in ABI thermal cyclers. Reactions included 0.1 units of MyTaq polymerase (Bioline), with accompanying 5-times reaction buffer, 1 μM of each PCR primer, 2 μL of DNA and nuclease free water. Products were run on 1.5% agarose gels stained with RedSafe for visualisation under UV light. Primers are listed in **Table 2**. At University of Queensland, DNA samples were tested for presence of *Wolbachia* using *wsp* gene primers (81F and 691R; product size ~610 base pairs) using Phusion plus DNA polymerase (ThermoFisher Scientific) followed by gel electrophoresis.

Wolbachia diagnostic assay (quantitative PCR)

DNA was amplified with *wsp* primers (**Table 1**) using a QuantStudio 5 (Thermo Fisher Scientific). Reactions were run in a total of 10 μL having 5 μL sybr green (Thermo Fisher scientific A25742), 0.5 μL each of 10 mM primer, and 1-3 μL of genomic DNA (approx. 50 μg/μL). Negative and positive PCR controls were run with each batch of the samples. Optimised amplification conditions were 3 min at 95 °C followed by 45 cycles of 10 s at 95 °C, 15 s at 51 °C, and 15 s at 68 °C. Any sample having CT score < 34 was considered positive, <30 are strong positive since positive control always have CT <30. No amplification was recorded if the CT score was >34.

Table 1. PCR primers recommended for screening for *Wolbachia* among insect populations.

Primer Name	Forward and Reverse Primers	Comment	Size, base pairs
Wolbachia *Wsp lau*	F: GCATTTGGTTAYAAAATGGACGA R: GGAGTGATAGGCATATCTTCAAT	(Osborne *et al*., 2009)	139 bp
Wolbachia wPip_ANK16	F: GAGACGAGAATGGAAGAACAGC R: CTCTATTTCTTGCTCCTTGCTTTTAC	(Walker *et al*., 2007)	286 bp
Wolbachia *wsp* 81F, 671R	F: TGGTCCAATAAGTGATGAAGAAAC R: AAAAATTAAACGCTACTCCA	(Zhou *et al*., 1998)	610 bp
Wolbachia IS5	F: GTATCCAACAGATCTAAGC R: ATAACCCTACTCATAGCTAG	(McMeniman *et al*., 2008)	~150 bp
Wolbachia 16S	F: CATACCTATTCGAAGGGATAG R:TTGCGGGACTTAACCCAACA	(Sawasdichai *et al*., 2019)	228 bp
Wolbachia ftsZA	F: CTCAAGCACTAGAAAAGTCG R: TTAGCTCCTTCGCTTACCTG	(Tsai *et al*., 2004) *Wolbachia* A group specific	~1000 bp
Wolbachia ftsZB	F: CCGATGCTCAAGCGTTAGAG R: CCACTTAACTCTTTCGTTTG	(Tsai *et al*., 2004) *Wolbachia* B group specific	~1000 bp
***Lucilia* specific positive control gene MFS**	F: ATCCTTTGGCTACCGGCATT R: TAGCCAATTGCCTTTGCCAC	Positive control gene, unpublished	bp

Transinfection studies

University of Melbourne: *Drosophila melanogaster* collected from Innisfail, QLD, contain *Wolbachia*. DNA sequencing of the *wsp* locus and *FtszA* locus determined the *Wolbachia* strain was indistinguishable from *w*Mel. Ovaries from ten ~3-day old females, which contain *Wolbachia*, were dissected in SPG buffer and transferred to a microfuge tube containing 0.5 mL of SPG buffer (recipe below). A plastic pestle grinder homogenised the tissue in 10 strokes at room temperature and sample centrifuged at 300 G for 5 minutes to remove large debris. Supernatant was transferred to a second tube and centrifuge at 12,000 G for 10 minutes to pellet *Wolbachia* cells. Supernatant was then removed, leaving the pellet in about 50 μL. The pellet was re-suspended through gentle pipetting, centrifuge for 300 G for 3 minutes to clear any debris that might otherwise clog the needle. The supernatant was then transferred to a clean tube and keep at room temperature (25 degrees or so) until injections (less than 5 hours).

Chemical	Name	Molecular Weight	SPG concentration
$C_{12}H_{22}O_{11}$	Sucrose	mw: 342.30 g/mol	218 mM
KH_2P0_4	Potassium phosphate monobasic	mw: 136.09 g/mol)	3.8 mM
K_2HPO_4	Potassium phosphate dibasic	mw: 174.18 g/mol)	7.2 mM
$C_5H_{10}N_2O_3$	L-Glutamate	mw: 146.14 g/mol	4.9 mM

University of Queensland: When attempting across species transinfection the likelihood of success is considered higher when *Wolbachia* is sourced from a closely related insect species. It has also been indicated that pre-adaption in cell lines of a related target species can help to 'preadapt' *Wolbachia* to a new insect context. The prior adaptation of *Wolbachia* in cell lines is thought to have been a critical element in the successful transinfection of *Wolbachia* into populations of the denque-vectoring mosquito *Aedes aegypti* (McMeniman *et al*., 2009). In a previous project (MLA B.AHE.0242) we had established three strains of *Wolbachia* (*w*AlbB, w Mel and *w*MelPop) in buffalo fly cell lines and successfully grown the infected cells through more than 50 passages. These cell lines were subsequently frozen in liquid nitrogen. As buffalo flies are more closely related to *L. cuprina* than mosquitoes or *Drosophila* it was considered prudent to test the cell line-adapted *Wolbachia* strains for infecting *L. cuprina*.

Haematobia cells with two strains – *w*Mel and *w*MelPop were reawakened and grown through a number of passages. The cells from the frozen lines appeared healthy and seemed to have established (**Figure 4).** However, they grew relatively slowly with increasing numbers of irregularly shaped cells becoming apparent.

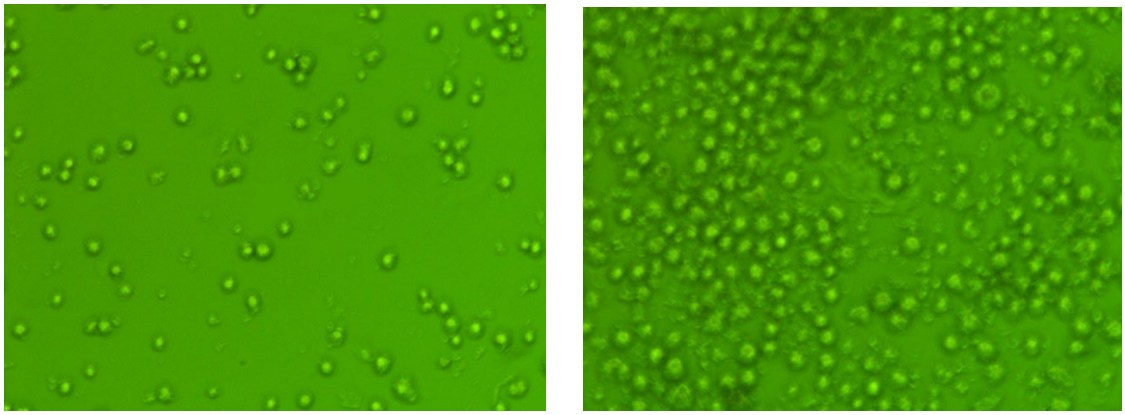

Figure 4. Re-awakened *Haematobia* cell lines containing *Wolbachia*. Left: cells infected with *w*Mel are dividing but not growing very fast. Right: cells infected with *w*MelPop.

The density of *Wolbachia* injected is thought to be important in the likelihood of the bacteria overwhelming host defences and establishing in a new host and the cell lines did not produce enough *Wolbachia* to make this source of cells a viable option for microinjection, particularly in view of the larger volumes required for pupal and adult injections. Ultimately, *Wolbachia* infected insects were able to provide much greater amounts of *Wolbachia* for injection than currently available from the cell line sources and we ultimately used *Wolbachia* isolated from three different live insect sources for microinjection.

Wolbachia (*w*Mel) infected *Aedes aegypti* mosquitoes were used in most of the transinfection attempts. *Drosophila* containing *Wolbachia* (*w*Mel) and *Wolbachia* from (fly) parasitoid wasps were also used as sources for infection. The

parasitoid wasps are available commercially in Queensland for biocontrol of house flies (*Spalangia endius*). These species have been shown to carry *Wolbachia* in a number of studies overseas (Betelman *et al*., 2017; Semiatizki *et al*., 2020) and were shown to be infected in our case using generic *Wolbachia wsp* primers.

Wolbachia injection into *Lucilia* Embryos

The most commonly used method for transinfecting insect species with new strains of *Wolbachia* is via embryonic (egg) microinjection (Hughes & Rasgon, 2014). This approach has been favoured historically because it introduces *Wolbachia* directly into the embryo where it needs to be present for transmission through to the next generation. In addition, much smaller amounts of *Wolbachia* are required with egg injection. However, there are also some disadvantages. It is a painstaking approach, easy to damage the eggs during injection, sometimes resulting in low hatch rates and frequently very large numbers of eggs have been injected before success has been achieved. In addition, the sex of the injected egg cannot be determined until after the eggs have been reared through to adults. Hatching and husbanding of first instar larvae and rearing through to adult flies in injection groups is required to confirm infection.

University of Melbourne: Small pieces of diced beef are placed in a fly cage >1 hour before injections, stimulating females to lay. Fresh meat was added 30 minutes prior to injection experiments. Pressure of the injection equipment was set to, Pc (compensation) 150-250 and Pi (injection) 400-500. Cover slips were coated with rubber cement and collected eggs were rinsed with water transferred to a small agar gel plate, cut in half to produce a straight edge. 50-60 eggs were lined up across the straight edge of the agar, anterior (pointy) end facing outwards. Eggs were then stuck to a pre-prepared cover slip with the posterior (round) end towards the edge. The cover slip was fanned and dried to allow the eggs to stick properly, and the eggs were covered with a drop of paraffin oil. A drop of water was then added to a microscope slide and the cover slip containing eggs was adhered on to using surface tension. Up to 1 µl of injection fluid (*Wolbachia* in cytoplasmic mixture) was loaded into a microinjection needle, and attached to the FemtoJet® microinjector system, which uses an electric pump. Embryos were injected through the posterior end. Following injection, coverslips containing eggs were placed onto an Agar Petri dish, (3 cover slips per plate, lid on) and wrapped in wet paper towel to maintain humidity. Petri dishes were stored in plastic zip-lock bag and incubated at 28°C for 16-24 hours. Larvae that survived were rescued into a 30 mL cup with cat food (Whiskas brand).

University of Queensland: Newly laid eggs (< 2 hour old), collected by adding liver to a cage of protein fed flies, were arranged on double-sided sticky tape attached to a microscope slide using a paintbrush and microinjected at the posterior pole of each egg using a FemtoJet® microinjector system (Eppendorf, Sydney, NSW, Australia). At the completion of injection, the slide was placed in a covered Petri dish on moistened filter paper and sterile sheep serum was added to the slide and alongside to maintain moisture and provide an initial source of protein for hatching first instar larvae. Liver strips were added and supplemented as required and larvae then kept moist reared through to pupation at 26C on sheep liver.

Wolbachia injection into *L. cuprina* pupae and flies.

Injecting female adult insects and pupal with *Wolbachia* has been a successful approach for establishing infections in some cases (Hughes & Rasgon, 2014; Mukund Madhav *et al*., 2020). This is generally a less precise process than embryonic injection, but death rates are generally lower than with eggs. In addition, it is possible to inject only females, as transmission does not rely on infected male flies. This avoids needless rearing of males through all the growth stages. However, although somatic infections are reasonably easy to establish, the difficulty is getting the bacteria to cross the ovarial membranes into the ovaries and into developing embryos and germinal tissue for transmission to the next generation. In addition, injected females or adults with somatic infections can often be used to gain an idea of likely fitness phenotypes. A disadvantage of this approach is that the likelihood of gaining sustaining infections depends on having a high enough concentration of *Wolbachia* to overcome insect immune

response, so much larger amounts of *Wolbachia* are usually required for injection with pupae and adults. Past studies demonstrated reduced longevity, decreased and delayed adult emergence and reduced fecundity in *Wolbachia* infected compared to mock injected flies (Mukund Madhav *et al*., 2020).

Pupae: Newly hatched pupae were aligned on double-sided sticky tape in a similar fashion to eggs and injected in the third last segment at the posterior end close to germinal tissue. Injections were conducted using two methods, the FemtoJet® microinjector system (Eppendorf) with prior penetration of the pupal cuticle and using a Burkhart ® Microdoser and 0.3 ml tuberculin needles. The microinjected pupae were then placed on moist Whatman filter paper and incubated at 27 °C until flies emerged. Freshly emerged flies were separated and placed in a cage with a maximum of five females and five males each. Flies were allowed to oviposit in group oviposition and eggs collected to rear the next generation. Individual flies tested for *Wolbachia* at death and the eggs from groups with the highest number of positive tests retained for breeding.

Female flies: Female flies were collected within 3–4 h of eclosion from the pupae, anaesthetised using CO_2 for 30–40 s and held on a cold stage for injection. All fly injections were conducted using Burkhart ® Microdoser with (0.24 × 33 mm) needles attached for injection. After injection, groups of five female flies were transferred into fly cages with five non-injected males. Flies were provided with liver on day 3 to facilitate ovary development and then again after 1 week for fly oviposition. Freshly deceased flies then tested by quantitative PCR for the presence of *Wolbachia* as described previously. Where flies tested positive the breeding cycle was continued in the next generation. A graphical representation of the general process for injection and testing of flies is given in **Figure 5**.

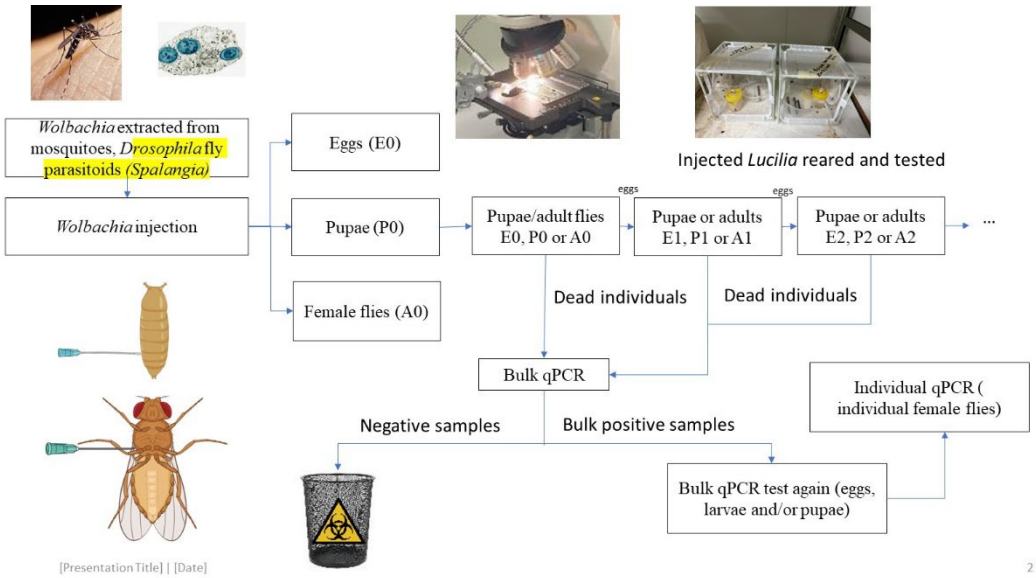

Figure 5. Graphical summary of transfection and testing. *Wolbachia* was injected into eggs pupae or flies, that were reared to sexual maturity and crossed. Screening groups of individuals occurred in each generation using bulk quantitative PCR.

Colony rearing and fly husbandry

University of Melbourne: A laboratory susceptible population (LS) of *L. cuprina dorsalis* was primarily used for microinjection. A field collected strain from NSW (GG) was also occasionally used when the LS strain had poor egg lay. Eggs were collected using raw beef and transferred to plastic cups containing tinned cat food and maintained at 28 degrees. The plastic cups rested on a bed of vermiculite, providing suitable environment for pupation. Flies were maintained at 22 degrees (+/- 2 degrees) with natural light, with wicked water and Sustagen hospital grade nutritional powder. Flies that survived embryo microinjection were combined into a large cage for mating. In some cases, surviving female flies were crossed to un-injected LS males. Individual female flies were placed into 50 mL vials containing a yeast-agar mix plus beef for egg collection. Once sufficient numbers of eggs were collected, flies were sacrificed and assayed for *Wolbachia* using PCR diagnostic assays.

University of Queensland: Four colonies of *L. cuprina* were maintained in our laboratory during the project to produce base stock for our studies. These were an insecticide susceptible strain maintained in colony over many years in our study, originally colonised from sheep strikes of *L. cuprina dorsalis* subtype, and three subtropical strains of *Lucilia cuprina* (*L. cuprina cuprina*) subtypes or potentially hybrids of the two types. In addition, strains of *Lucilia* established from microinjection on different dates were generally maintained until the second generation to allow testing and determine likelihood of ovarial infection. All strains were reared using ovine or bovine liver for adult protein feeding and as an oviposition and larval rearing substrate.

In the later stages of the project, to select for increased infection rates, offspring from fly lines that tested positive for *Wolbachia* by PCR screening were used as parental stock. In each generation, 25 to 50 females from each line were isolated as virgins, placed into individual vials, and in the early stages, mated with male from the same injected batches. This was done as infected males do not give rise to fertile eggs when mated to uninfected females thus potentially providing a reproduction advantage to the *Wolbachia*-infected females in these lines. However, in the later-generations, and in a number of single female matings carried out, males from the laboratory line were used rather than from the same batch. This was because there was some indication that the number and fertility of eggs in later generations flies was decreased. External males were used to guard against potential inbreeding. Lowered fertility, thought to be due to *Wolbachia* infection, has been noted in other studies, particularly in the early stages of a new host-*Wolbachia* associations or when mosquito eggs are stored for long periods of time (Lau *et al*., 2021).

The resulting lines were then monitored by PCR to confirm the infection status These lines were maintained until no infections were detected in qPCR tests, eggs failed to hatch or there were high death rates and the colony collapsed.

RESULTS

Objective 1: Frequency of *Wolbachia* in field collected *Lucilia* populations

Screening for the presence of *Wolbachia* in *L. cuprina* (University of Melbourne)

Tentative evidence for the presence of *Wolbachia* in *L. cuprina* was previously established at University of Melbourne and University of Queensland. Diagnostic analysis of two strains held at University of Melbourne, CB and KDA, showed weak PCR amplification for the *wsp* gene indicating these cultures probably contained a *Wolbachia* isolate. Repetition using DNA isolated from pools of 10 flies from five different strains including KDA, GG, SL, TPC and LS the wild type laboratory susceptible reference strain failed to identify *Wolbachia* (**Figure 6**). This result was unexpected.

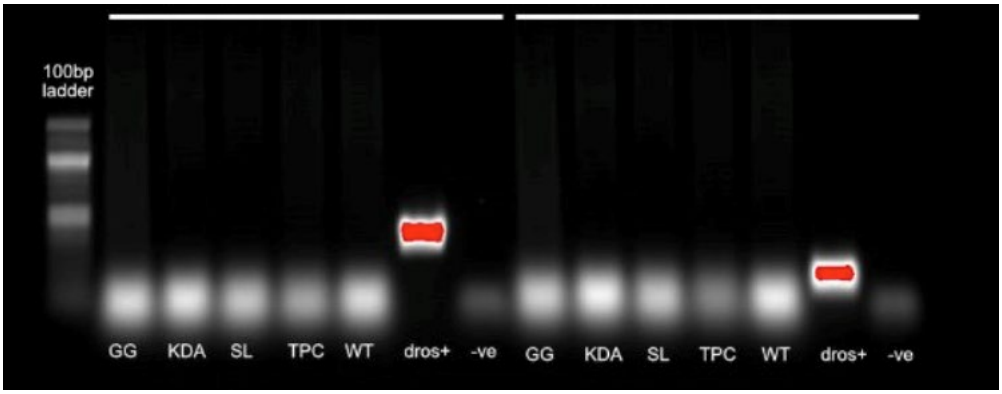

Figure 6. *Wolbachia* is not present in *Lucilia cuprina* strains held at University of Melbourne. Five strains were thoroughly tested using all available primers (**Table 1**). This image shows the results from *Wolbachia*-16S (left group, ~400 bp product expected) and *w*Pip primers (right group, 228 bp product expected), which amplify successfully in *Drosophila* control DNA (dros+). Strains GG, KDA, SL, TPC and WT (also referred to as LS) do not contain *Wolbachia*. The negative control (-ve) does not contain DNA template and shows a faint artifact known as a "primer dimer", which are also seen in other samples. PCR amplification using *Lucilia* genomic DNA were successful (data not shown).

DNA was previously isolated from around 1000 individual blowfly samples using head and thorax tissue (AWI project ON-00624). This readily available source of DNA offered a convenient set of samples for *Wolbachia* screening. We screened 450 individuals, and none were identified as *Wolbachia* positive. At least two different sets of PCR primers have been tested for each individual. Multiple PCR primer sets are required for the screen to maximise the likelihood of a positive detection. As these DNA samples did not contain reproductive tissue, which are expected to have high abundance of *Wolbachia*, these results were not highlighted elsewhere in this report.

In parallel, DNA isolation was performed on abdominal tissue from 446 samples, containing the important reproductive tissue where *Wolbachia* is predominantly found. Isolating DNA from the abdomen is expected to contain reproductive tissue (testes or ovaries) and maximise the chance of identifying *Wolbachia* from field collected samples. In total, 67 populations have been assessed (**Table 2, Table 3**). PCR screening using three sets of PCR diagnostic primers, "wps", "FtsZA" and "FtsZB" were used to screen these samples using standard molecular screens (**Figure 7**). One individual fly from a Western Australian population tested positive for *Wolbachia*, however sequence analysis indicated the sequence was identical to a positive control and was most likely contamination. **Figure 4** highlights the collection sites that have been screened for *Wolbachia* in Australia.

Table 2. Summary of *Lucilia cuprina* sample sites and specimen numbers collected over a four-year period. DNA was isolated from abdominal tissue from 446 individuals collected from 67 populations.

Year	**Number of sample sites**	**Number of *L. cuprina* flies collected**	**Populations used for DNA analysis**	**Number of individuals tested for *Wolbachia***
2018/2019	30	413	10	72
2019/2020	81	1235	14	101
2020/2021	49	1267	35	215
Other	8	58	8	58
Total	**160**	**2915**	**67**	**446**

Table 3. A total of 67 collection sites have been assessed for the presence of *Wolbachia* in *Lucilia* blowfly abdomens via PCR diagnostic assays. The mean number of individuals tested per population was 6.66 (+/- 2.66).

Region	**Populations**	**Individuals**
NSW	23	137
NT	1	6
WA	8	59
QLD	4	17
VIC	13	95
TAS	5	38
SA	5	36
Location NA	8	58
Total	**67**	**446**

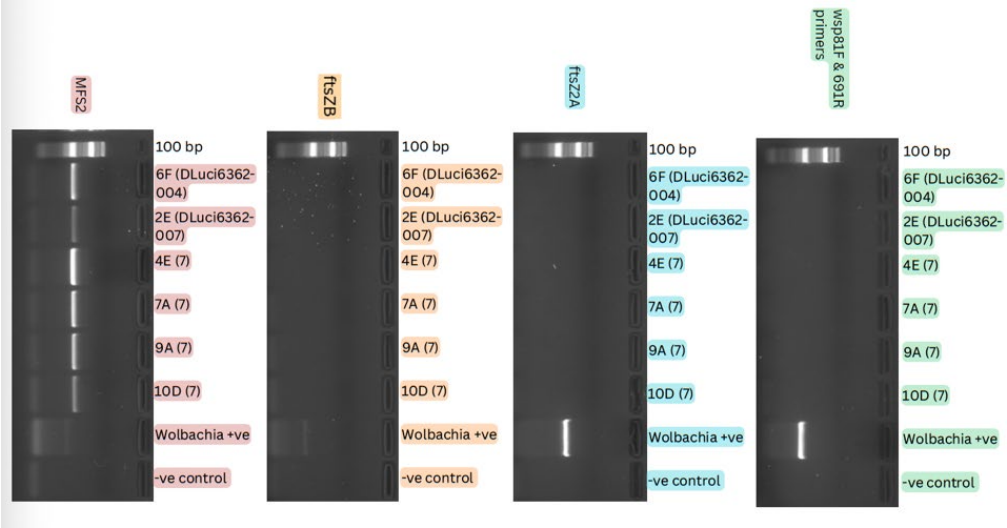

Figure 7. *Wolbachia* PCR amplification assays. First row (pink), *Lucilia cuprina* control genes MFS. Second row (orange), *Wolbachia* FtsZB. Third row (blue), *Wolbachia* FtsZA. Forth row (green), *Wolbachia* surface protein (wsp) primers. *Wolbachia* infected *Drosophila melanogaster* were used as positive controls. The FstZ primers detected *Wolbachia* from supergroups A or B (*w*Mel is supergroup A).

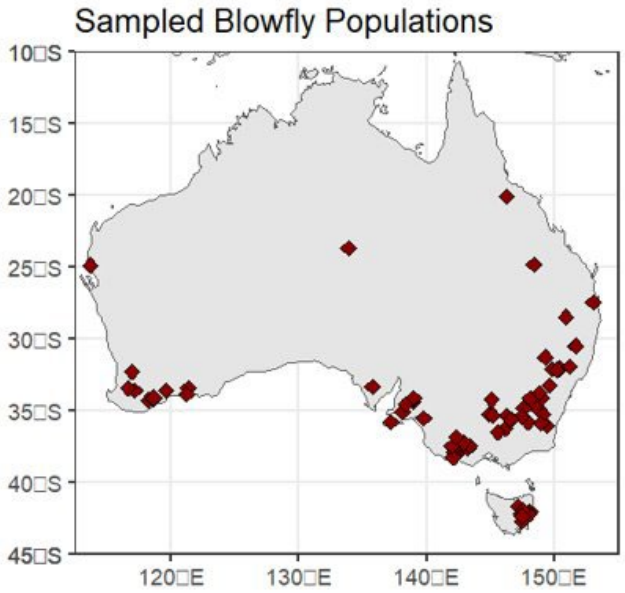

Figure 8. Location of 67 populations tested for presence of *Wolbachia*. Two populations from Tasmania were *Lucilia sericata* specimen (18 individuals screened) and one populations from an unrecorded region was *L. sericata* (6 individuals screened).

Screening Wolbachia in field L. cuprina samples (University of Queensland)

Three additional field strains were collected and established in the laboratory for *Wolbachia* testing and microinjection studies. These strains (two from Brisbane and one from Coochiemudlo Island in Morton Bay) are urban breeding strains, of the *L. cuprina cuprina* or *L.c. cuprina x L. c. dorsalis* subtype (Norris, 1990). This subtype is common in northern areas in Australia, but uncommon in southern areas. It has been suggested that *Wolbachia* is more commonly found in tropically oriented species and it has previously been suggested that *Wolbachia* can drive insect speciation. Thus, it was hypothesised that previous positive tests for *Wolbachia* in our laboratory may be

related to blowfly origin. However no positive detections of *Wolbachia* were recorded in any tests with uninjected flies from the urban lines.

Objective 2. Characterise *Wolbachia* present in *Lucilia* species

The *Wolbachia* genome can vary in size and gene number, however, it generally contains around 1.4-1.8 million DNA base pairs and carries more than 1000 protein coding genes (Vancaester & Blaxter, 2023). Performing genome sequencing on a single fly infected with *Wolbachia* using "long-read" technology, (DNA sequences > 10,000 bases), enables assembly of the entire circular genome. We had anticipated sequencing a *Wolbachia* infected strain held at University of Melbourne, however, subsequent experimentation did not identify the endosymbiont. Extensive molecular analysis of field collected *Lucilia* blowfly samples and laboratory cultures at University of Melbourne or University of Queensland did not find evidence of *Wolbachia* infection. Consequently, this aim could not proceed.

Objective 3. Transinfection of *Lucilia* with different strains of *Wolbachia* using microinjection

University of Melbourne

Ovaries from *Wolbachia* infected female *Drosophila* were gently homogenised and resuspended in specialised injection buffer, generating a bacterial suspension that is directly microinjected into *L. cuprina* embryos. Microinjections were performed into two different strains of *L. cuprina dorsalis*, the Laboratory Susceptible (LS) strain, and insecticide resistant strain GG. Experimentation was performed using different types of microinjection needles that were either produced from borosilicate glass or quartz glass. In total 3044 embryo injections were performed on 10 different occasions, including control buffer injections (**Table 3**).

Survival rates of injected embryos were generally poor. There were 546 larvae that survived the injection process and hatched (~18%). Mortality continued throughout these life stages, and 106 flies were produced in total. Female survivors were placed in cages with either male injection survivors or wild type males and provided with protein source and water for seven days. Individual females were then removed from the cage and placed in individual vials with a piece of beef to stimulate oviposition. Once sufficient eggs were laid, females were sacrificed or collected after death and DNA was isolated to screen for the presence of *Wolbachia*. At least six females were detected with *Wolbachia* and all of these produced very few or no eggs (**Figure 9**). Larvae and flies of G1 progeny were assessed for *Wolbachia* infection *via* the *wsp* PCR diagnostic assay. Transmission of *Wolbachia* to subsequent generations was not detected at University of Melbourne.

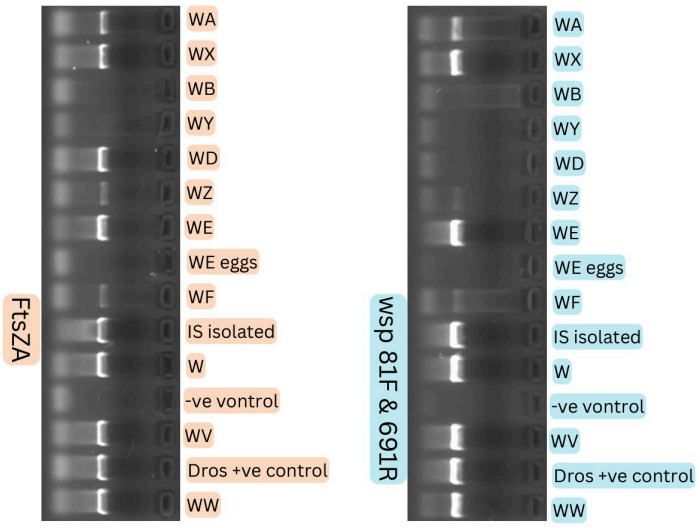

Figure 9. Screening embryo-injected flies for the presence of Drosophila *Wolbachia* isolate *w*Mel. Once embryos were injected with *Wolbachia* they were reared and survivors mated. This representative image shows two different PCR primer pairs that were used to screen five females (3 positive for *Wolbachia*) and six males (six positive for

Wolbachia). Samples labelled WA, WB, WD, WE and WF were female flies that produced limited numbers of G1 embryos and W, WV, WW, WX, WY, WZ were males. The negative control (-ve) does not contain DNA template and samples that were not infected with Wolbachia show similar profiles to the negative control.

Table 4: *Wolbachia* (*w*Mel) isolated from *D. melanogaster* was injected into *Lucilia cuprina* embryos.

Date	Injection mixture	Needle type	Lucilia cuprina strain	Number of eggs injected	Number of hatched larvae	Injection survival rate (%)	Pupae	Flies	Screening for *Wolbachia*
05/05/2023	SPG buffer	Glass	LS	94	17	8.09	N/A	N/A	No Wolbachia detected
17/05/2023	Wolbachia (wMel)	Quartz	GG	258	61	23.64	13	11	No Wolbachia detected
31/05/2023	Wolbachia (wMel)	Quartz	GG	399	43	10.78	1	1	No Wolbachia detected
4/8/2023	Wolbachia (wMel) with food colouring	Glass	GG	173	6	3.47	0	0	No survivors
16/08/2023	Wolbachia (wMel)	Glass	GG	142	5	3.52	1	1	No Wolbachia detected
30/08/2023	Wolbachia (wMel)	Glass	LS	127	17	13.39	3	3	One female Wolbachia positive
11/9/2023	Wolbachia (wMel)	Glass	GG	511	82	16.05	17	0	No survivors
20/09/2023	Wolbachia (wMel)	Glass	GG	647	109	16.85	44	29	5 females Wolbachia positive
21/11/2023	Wolbachia (wMel)	Glass	GG	215	22	10.23	5	4	No Wolbachia detected
08/02/2024	Wolbachia (wMel)	Glass	LS	71	6	8.45	1	1	No Wolbachia detected
08/02/2024	Wolbachia (wMel)	Quartz	LS	407	178	43.73	88	56	No Wolbachia detected (from 12 flies screened)
Total				3044	546	Av. 18%	173	106	6 females Wolbachia positive

By far the majority of studies towards transinfection of new species with *Wolbachia* have used embryonic microinjection as the method of delivery. This technique localises *Wolbachia* directly in the developing germinal tissues. However, despite testing many technique modifications for egg injection in this study, the survival of embryos remained relatively low and variable, generally less than 10%. The maximum survival rate achieved in any injection batch was 21.6%.

As successful transinfection has been achieved in other studies by injecting different insect stages we also tested microinjection of pupal and adult female flies. Pupal injection was initially conducted using the FemtoJet® microinjector system used for embryonic microinjection. However, this method required prior puncturing of the puparium with a larger needle to avoid microinjector needle breakage and was only able to deliver relatively small dosages of *Wolbachia*. As a result we later moved to the use of the Burkhart ® Microdoser with 0.3 ml tuberculin needles attached for injection of pupae and adult female flies. Survival rates were considerably higher using this system (**Table 4**). The injection of female flies has a significant logistic advantage over injection of eggs and pupae because male flies can be visually identified and separated prior to injection.

Table 5: Survival rate following injection of different life stages

Stage injected	Date	Generation	Number injected	Number surviving	Survival %	Average %	SE
Eggs	15/08/2023	P	65	3	4.6		
	23/08/2023	P	55	4	7.3		
	28/08/2023	P	88	19	21.6		
	4/09/2023	P	96	4	4.2		
	11/09/2023	P	96	5	5.2		
	18/09/2023	P	129	4	3.1	7.7	2.8
Pupae	17/08/2023	P	88	51	58.0		
	4/09/2023	P	52	9	17.3		
	18/09/2023	P	20	4	20.0		
	23/10/2023	P	86	51	59.3	38.6	11.6
Adult	31/10/2023	P	53	17	32.1		
	7/11/2023	P	32	10	53.1	42.6	10.5

With injection of eggs and pupae, approximately 50% of injections of these stages will ultimately develop to male flies. In terms of establishing a sustaining *Wolbachia*-infected colony, males are considered dead end hosts when attempting to establish colonies and this represents a substantial wastage of effort. It should be noted, however, that although males are not of significance in the transmission of *Wolbachia* they are important in the population dynamics of host insects, as mating of infected males with an uninfected females or females infected with a different strain of *Wolbachia* confers functional sterility, a property which is important in for the design of *Wolbachia*-based control strategies.

Tables 5, **6** and **7** show the number of test batches in which *Wolbachia* was detected in at least one instance in each generation following microinjection of the three different stages. As these tests were conducted to identify any instances of *Wolbachia* transmission to succeeding generations, flies were tested as bulked samples collected on different dates., with varying numbers of flies in each batch. The bulked results are shown in these tables. The important features of the tables are the results for Generation 1 and Generation 2 where the where the percent positive indicates the percent of test batches in which at least one positive insect was found. The relatively high values for the parent generation are unsurprisingly as these tests represent the occurrence of *Wolbachia* in the

injected generation. The lower total number of tests conducted for the egg injected lines in G2 reflect the higher mortalities observed with egg injection than for the other stages. In addition, as noted the apparently high number of positive results is partially due to the use of bulk sample testing to maximise the probability of detecting rare, infected individuals if they were present. The higher number of positive tests in the pupal injected lines than for fly-injected lines could be due to the longer period for growth of *Wolbachia* and ovarial infection to take place. A similar effect was seen in the studies of Mukund Madhav *et al*. (2020) who examined changes in *Wolbachia* density in buffalo flies following pupal and adult fly injection and observed that there was an initial decline in the density of *Wolbachia* after injection, possibly due to the effects insect immune responses and the time for taken for *Wolbachia* adaptation. However, after a period of time growth rates recovered, presumably as *Wolbachia* adjusted to the new host context. Notably, the density of *Wolbachia* reached higher levels in flies injected as pupae than those injected as adults, presumably because of a longer period for the build-up in *Wolbachia* numbers and potentially provided greater opportunity for ovarial infection to occur.

Table 6. Number of *Wolbachia* -positive injection batches in different generations: Embryo injection

Egg injection	Number of eggs	Number of tests	Percent positive	Fly lines	Percent positive
Parents	629				
Pos		2	25.0%	2	67%
Tot		16		3	
Generation 1					
Pos		5	20.0%	1	33%
Tot		20		3	
Generation 2					
Pos		0	0.0	0	0
Tot		3		2	

*Results for individual flies from the same injection batch/date have been combined so that if at least one fly was positive in an injection batch/test date, this was considered positive

Table 7. Number of *Wolbachia* -positive injection batches in different generations: Injection of pupae

Pupal injection	No. pupae	Number of tests	Percent positive	Fly lines	Percent positive
Parents	318				
Pos		7	23.3%	1	25%
Tot		30		4	
Generation 1					
Pos		4	40.0%	1	50%
Tot		10		2	
Generation 2					
Pos		3	25.0%	2	100%
Tot		12		2	

Table 8. Number of *Wolbachia* -positive injection batches in different generations: Injection of adult female flies

Adult female injection	No. adult females	Number tests	Percent samples positive	Fly lines	Percent positive
Parents	196				
Pos		10	15.9%	8	88.9%
Tot		63		9	
Generation 1					
Pos		6	37.5%	2	100.0%
Tot		16		2	
Generation 2					
Pos		3	27.3%	1	100.0%
Tot		11		1	

The data suggesting differences in transmission rates between flies injected at different stages presented in these tables should be interpreted with caution as it is potentially influenced by the effects of variable sample sizes and difference between injection stages in the length of time between injection and fly testing. However, the results do clearly show that *Wolbachia* was transmitted across generations in some instances, indicating the occurrence of ovarial infections. Vertical transmission across generations was also indicated in the breeding lines which were established from generations from the injected lines described above (**Table 5, 6, 7**).

Table 9. Establishment of *Wolbachia* infected *Lucilia cuprina* colonies

28/08/2023 egg injection batch, colony established 16/11/2023			
Parental generation	Date of test	Numbers positive of number tested*	Percentage
28/08/2023	30/11/2023	1/3	33.3%
	4/12/2023	5/7	71.4%
	5/12/2023	1/2	50.0%
	6/12/2023	5/9	55.5%
	7/12/2023	0/1	0
	11/12/2023	0/8	0
	13/12/2023	0/6	0
	19/12/2023	0/11	0
	21/12/2023	1-/11	9.1%
	22/12/2023	0/2	0
	27/12/2023	0/8	0
	30/12/2023	0/3	0
	31/12/2023	2-/25	8.0%
	2/01/2024	2-/20	10.0%
	30/01/2024	0/24	0.0
	9/02/2024	7-/29	24.1%
	Total	24/126	19.0%
17/08/2023 pupae injection batch, colony established 20/11/2023			
Date pupae collected	Date of test	Number positive of number tested	Percentage
04/12/2023 to 05/04/2024	04/09/2023, 04/12/2023, 05/03/2024	3/88	5.50%
04/09/2023 pupae injection batch, colony established 16/11/2023			
Date flies collected	Date of test	Number positive of number tested	Percentage
30/11/2023 to 04/03/2024	13/12/2023	1/83	1.20%

* For the egg collection colony, percentages are percentage of test batches on each date with at least one positive result. Test batches were dead flies collected in sequential date periods and did not include equal numbers of flies.

Table 8 shows the percent of testing batches where *Wolbachia* was detected in the selection lines. Only dead flies were tested as we needed to retain live individuals in the colony for egg laying. Consequently, the numbers of individuals tested in each batch is variable and the likelihood of detecting at least one *Wolbachia* positive sample is proportional to the numbers tested. Even taking this into account, there is indication that there was significant variation in density over the period of monitoring. This may be reflective of similar large variations in infection density that has been commonly observed during establishment of a new host association following transinfection in other studies (Hughes & Rasgon, 2014; McMeniman *et al*., 2008). There appeared to be an increase infection rate in the 2024 tests in the egg injected line, which represents flies in later generations, but this is more likely to be a sampling effect rather than a real increase. It was also notable that reproduction appeared to decline in the last generation with fewer flies ovipositing, egg batches commonly remaining unhatched failure to properly eclose from

the pupae observed on a number of occasions. As a result, there were relatively few female flies available to establish the next generation. To guard against inbreeding effects, in the later generations uninfected male flies sourced from the main colony were used, but this did not appear to prevent the decline.

In the two colonies established from pupal injected lines infection rates appeared to be lower than for the egg injected lines. Although 8 batches were tested in the case of the 17/8/23 colony only three tests were positive, two in the establishment generation and one in the last test batch. In the 4/9/23-established colony, only one positive was recorded, and that was in the establishment generation. This may suggest that a higher proportion of ovarial infections was achieved with *Wolbachia* following use of egg injection in the in the source fly lines than with pupal infections where a significant proportion of positive of tests are likely to result from somatic infections. Taken overall, this result reaffirms that ovarial infections with *Wolbachia* were achieved with both embryo and pupae injections and that *Wolbachia* was transmitted across generations in a number of instances although at a very low level. Notwithstanding possible fitness effects, this suggests that a much more intensive injection, screening and selection program with the ability to screen and maintain large numbers of individual fly-based lines would be needed to ultimately develop a sustainably *Wolbachia*-transinfected line of *L. cuprina.*

Objective 4: Fitness effects

Wolbachia was isolated from multiple insect hosts and used to microinject thousands of *Lucilia cuprina* individuals at the egg, pupae or fly developmental stage. Extensive effort was invested in caring for injection survivors, and ensuring they had the best possible chance of survival. Mortality rates were high, and vertical transmission of *Wolbachia* via egg cytoplasm to subsequent generations occurred with low efficiency. This made establishing stable lines where all individuals were infected with *Wolbachia* challenging. Females known to be infected with *Wolbachia*, as shown using molecular diagnostics, appeared to lay few eggs with low hatch rates. This result is largely observational, as there have been too few progeny to sacrifice and test whether they do carry the endosymbiont.

Investment in colony maintenance, and the inability to develop strains with 100% *Wolbachia* infection rates meant it was not possible to directly measure fitness effects. Several possible fitness effects were observed.

1. *Injection of Wolbachia may have a fitness cost in embryos*.

   Embryo microinjection for techniques such as CRISPR/Cas9 mutagenesis can see embryo hatch rates as high as 50%. University of Melbourne reported average hatch rates of approximately 18% and University of Queensland at around 7.7%.

2. *Females that carry Wolbachia lay few eggs*

   Collection of embryos from individual females was performed, with those subsequently identified as *Wolbachia* positive found to lay very few or no eggs. Most wild type females can lay large clutches of eggs and this was not observed.

3. Failure of flies to fully eclose from pupae and occurrence of weak or deformed flies

   The occurrence of flies that appeared to die during hatching and did not eclose completely from the pupae, in addition to a higher than usual incidence of small weak flies with wing deformities was noted in batches where pupal injections were conducted was noted. Similar effects have been noted in *Wolbachia* transfection studies with other insect species.

Objective 5: Strain maintenance

The University of Melbourne and University of Queensland currently rear multiple strains, including the Laboratory Susceptible (LS) strain, which has been maintained in culture for >30 years. Extensive care was taken of individuals

injected with *Wolbachia* to give them sufficient opportunity to reach maturity, mate, and produce further generations.

DISCUSSION

This study has demonstrated the proof-of-concept of *Lucilia* transinfection with *Wolbachia*, which has potential to spread itself through populations and could avoid expensive individual animal treatments. Here we have shown that *Wolbachia* infection is extremely rare or absent from *Lucilia cuprina* populations in Australia. Screening hundreds of individuals collected from more than 70 locations failed to identify any of this endosymbiont. Injecting embryos, pupae and flies with *Wolbachia* suspensions produced from *Drosophila*, *Aedes* mosquitoes or parasitoids, demonstrated infection could persist in many cases through the life of the individual. *Wolbachia* could be transmitted from infected females to subsequent generations, although infection was low and inconsistent.

Population survey

More than 70 different populations of *Lucilia* flies were screened for the presence of *Wolbachia*. Multiple sets of diagnostic PCR primers were used in the screening process, including *wsp81/wsp691*, which have been highly successful in detecting *Wolbachia* in an extremely broad range of insect species (Baldo *et al*., 2006; Zhou *et al*., 1998). Detection of the endosymbiont was not recorded in any sample in Australia. Sampling was largely from sheep farms, although some urban collections did occur, including *L. cuprina cuprina* from Queensland. This recognised *Lucilia* subtype is found in northern and coastal areas and is considered a synanthropic fly, as it usually grows in food and animal waste associated with humans and is not generally considered a myiasis causing sheep blowfly. In addition, *L. sericata* samples were also tested but *Wolbachia* was not detected.

Some host species are seemingly resistant to *Wolbachia* infection (Hughes & Rasgon, 2014; McMeniman *et al*., 2009) and testing to date suggests that *Wolbachia* does not appear to be widespread in blowfly (Family Calliphoridae) populations. *Wolbachia* was not found in *L. cuprina* by either Şakİ and Şİmşek (2014) in Türkiye, Mingchay *et al*. (2014) in Thailand or from a single sample tested in the USA and reported on the *Wolbachia* project database (https://wolbachiaprojectdb.org). In addition, no infection was found in closely related species *Lucilia sericata* and *Lucilia porphyrina* (Mingchay *et al*., 2014; Şakİ & Şİmşek, 2014). Many calliphorid species breed in microbe-rich detritus such as human and animal waste and animal carcases. They are likely to have developed very effective immune systems to deal with frequent bacterial challenge and similar immune responses may assist in resisting *Wolbachia* infection. The same may be so in the case of *L. cuprina* which appears to have primarily evolved as a waste breeding species. However, it should be noted that *Wolbachia* was found in all collections of *Chrysomya megacephala* from a number of sites in Thailand, and in nestling-bird infesting blowfly Protocalliphora spp. in a number of studies (Baudry *et al*., 2003; Floate *et al*., 2006). Notably *Wolbachia* of both supergroups A and B were found in both Protocalliphora and *Chrysomya megacephala*. This observation, together with the widespread infection observed in these species would appear to indicate that these closely related calliphorid species are receptive to *Wolbachia* infection.

Wolbachia infection rates can be highly variable between closely related species. For example, the worldwide agricultural crop pest *Plutella xylostella* shows infection rates of *Wolbachia* between <1% to ~5%, (Delgado & Cook, 2009; Perry *et al*., 2018) while a closely related species endemic to Australia, *Plutella australiana*, has shown 100% infection on all samples tested (Perry *et al*., 2018). The *L. cuprina* samples size tested was relatively high, and we predict that if *Wolbachia* infection can occur naturally in this species, it is at a frequency of less than 0.2%. The reasons for previous positive detection of *Wolbachia* at University of Melbourne and University of Queensland remain unclear. They could perhaps be associated with transient infections that later died out, parasitoid infestation, or false positive results caused by amplification of related bacterial endosymbionts such as Spiroplasma (Goto *et al*., 2006).

Transinfection studies

This study has clearly shown that *L. cuprina* can be successfully transinfected with *Wolbachia* and that the bacterium can be subsequently transmitted across succeeding generations of flies. This was regardless of whether initial transinfection was by microinjection of eggs, pupae or adult flies.

Injection directly into eggs has been by far the most commonly used method in other pest species to date and has the advantage that *Wolbachia* is introduced directly into the embryo in the early stages of embryogenesis, providing an opportunity to infect germinal tissues during egg development. Relatively small quantities of *Wolbachia* are required for embryonic injection because of the small size of the eggs. However, this is the most technological challenging method and requires expensive specialist equipment as well as well trained and meticulous staff to obtain good results. In addition, there is usually high mortality with the egg injection method resulting a relatively low proportion of the injected eggs developing to viable female adults, as seen in this project.

Successful transinfection has also been achieved by microinjection of other stages in studies with other insect species (Hughes & Rasgon, 2014) and we have previously had some success with injecting pupae and adults in the James lab (Mukund Madhav *et al*., 2020). Therefore, we investigated the utility and effectiveness with injecting these stages of *L cuprina*. The Femtojet® microinjection system used for egg microinjection was found to be unsuitable for injecting pupae and adults of *L. cuprina* because of excessive needle breakage and of the low volumes of *Wolbachia* delivered. We therefore adapted the Burkhold microdoser® set to deliver 1 µl for injection of these stages. This system was much easier to use and cheaper to purchase and survival rates were much higher than with embryonic injection. In addition, control of the dose rate was much more manageable. Injection of adults had the advantage that females could be selected for injection whereas with egg and pupal injections females could not be identified prior to injection and approximately half of the effort was wasted by injecting resultant males, which are functionally dead-end hosts in terms of colony establishment. With females, injection was into the haemolymph and thoracic tissues and *Wolbachia* had to traverse various membranes and tissues to infect the germinal tissues for transmission to succeeding generations to occur. However, *Wolbachia* have a natural propensity to infect germline cells (Hughes & Rasgon, 2014) and this may partially compensate for difficulties in access. In the case of pupae, germinal tissue infection may be facilitated by the substantial tissue reorganisation that occurs during this stage. In addition, Mukund Madhav *et al*. (2020) indicated that *Wolbachia* built to higher densities in flies that were injected as pupae than in directly injected flies and this may increase the likelihood of ovarial injection and the production of infected eggs.

This study showed that injection of all three stages of *Lucilia* resulted in infections that were transmitted across generations. However, testing for this part of the work was done by bulk sampling and PCR of composite samples, carried out to maximise the numbers tested and the likelihood of detecting infective individuals if they were present. Therefore, even though transmission across generations was demonstrated with all three methods the proportion of flies infected on a per insect basis was low.

The numbers of positive tests for *Wolbachia* in the selection lines remained low and infection was lost completely in a number of instances. Interpretation was also complicated by reductions in fly numbers in the later stages as a result of higher mortality and apparent loss of reproductive efficiency. It has previously been noted that there can be large variability in the density of *Wolbachia* in the early stages of development of new *Wolbachia*-host associations as a result of maladaptation of the introduced bacteria to the new host context (McMeniman *et al*., 2009). Similar variability was noted in our selection lines. This has been previously attributable to incomplete cytoplasmic incompatibility (unlikely in our case because of the low *Wolbachia* prevalence), inefficient vertical transmission, and/or deleterious fitness or pathogenic effects of *Wolbachia* in the host. Hughes and Rasgon (2014) note that because of this initial maladaptation, careful selection plays a critical role in establishing stable transinfected lines. They also note that to overcome diminishing *Wolbachia* levels attributable to the host immune response, more

strenuous approaches can be adopted by injecting more bacteria, which can be more easily accommodated with injection of pupae and adults. They also note an instance with transinfected *Drosophila* lines where relaxed selection pressure led to a drop in infection frequency, but that restatement of a more robust selection regime eventually led to 100% infection which was sustained in subsequent generations. In another instance outcrossing to wild type males was crucial in preventing loss of infection (Suh *et al*., 2009).

Although we did not achieve the development of sustaining infected lines of *L. cuprina*, *Wolbachia* appeared to persist at low levels in the selected colonies. The issue with bulk sampling for testing is that although it maximised the likelihood of detecting infected individuals if they were present in later generations, the positive flies were sacrificed for testing and not available for subsequent breeding. As such we were limited to choosing flies coming from infected batches, but with no knowledge of individual infection status, to establish the breeding lines.

To establish highly infected strains, single female matings would desirably be used to identify infected females. Test females are mated and allowed to oviposit and the eggs collected and either tested directly, or reared through to adult flies with testing then conducted. Testing mated flies after the eggs are laid can facilitate early culling of colonies if they are negative, but when the progeny are positive they must be maintained for subsequent testing to identify flies with somatic, not germinal infection. This is a particularly important consideration when flies are generated from pupal or adult injected stock where initial somatic infection is highly likely. Unfortunately, the ability to establish, manage and test large numbers of single female test batches was beyond the time scale and resources available for this project, which was largely a proof of concept study. Although we did try individual fly matings in a number of instances, or collection of eggs from individual females, none of these flies produce progeny that survived, so the lines were not continued.

Results indicating successful transinfection and the transmission of *Wolbachia* through three generations *L. cuprina* (G0-G2) are significant outcomes from this project but the need to develop sustainably infected strains remains. From our results to date and experience with work in other insect species, this appears an achievable objective. However, a future project to this end will require significant resources to maintain and manage the multiple fly lines required to detect and breed from single fly infections and to conduct the careful selection and outcrossing programs that have been necessary to develop sustainably infected strains in other insect species.

Wolbachia transmission across generations

We found the number and fertility of eggs produced from female flies known or suspected to carry *Wolbachia* decreased, particularly in G2 flies. However, *L. cuprina* has evolved a group ovipositon habit with a putative close-active pheromone to facilitate this behaviour (Barton Browne *et al*., 1969). Mass or multiple ovipositions are thought to facilitate survival of eggs and first instar larvae. The low number of egg masses and failure of eggs to hatch could be an artifact of the low number of G2 flies available. Commencing single female mating with multiple laboratory males will help determine whether lowered fertility is a result of *Wolbachia* infection or pheromone induced oviposition behaviour.

Host immune responses are expected to be high when *Wolbachia* injection occurs into flies, in order to combat and remove bacterial infection. This effect may also limit transfer of *Wolbachia* across generations. To counter host responses, It has been proposed that *Wolbachia* can influence host immunocompetence, to enhance their opportunity for survival and replication (Braquart-Varnier *et al*., 2008). Pre-adaption of *Wolbachia* to *L. cuprina* cell lines, prior to microinjection, may enhance the ability for *Wolbachia* to survive in host reproductive cells and improve transmission rates to subsequent generations. Although *Wolbachia* hasn't been widely detected in the family Calliphoridae, which contains sheep blowfly, it is notable that it has also been found at high frequencies in several species of tsetse flies, an important animal fly parasite attacking livestock in Africa (Doudoumis *et al*., 2013). Identifying and obtaining a range of *Wolbachia* stains from related insect hosts, including tsetse flies, may improve

transinfection rates in *L. cuprina* and transmission across generations. Over time, if colonies can be maintained, then levels of mutualism can be achieved between *Wolbachia* and their host (McGraw *et al*., 2002; Weeks *et al*., 2007; Zug & Hammerstein, 2015).

Finally, Host colonies infected with other bacterial endosymbionts may also have an impact on infection. For example, when *Drosophila* are coinfected with multiple endosymbiotic bacteria can result in *Spiroplasma* dominating ovary infection and outcompeting *Wolbachia* (Goto *et al*., 2006). Curing *L. cuprina* strains of bacterial endosymbionts or mutualists with antibiotics could be performed, in advance of injection of *Wolbachia*. This could potentially reduce competition *Wolbachia* may face with other bacteria in the host.

FUTURE WORK

Results indicating the transmission of *Wolbachia* through three generations of flies (G0-G2) is a significant outcome from the work conducted. The primary objective of the next phase of this project remains to develop a sustainably infected *L. cuprina* line. Experiments to characterise the biological impacts of *Wolbachia* infection on *L. cuprina* would need to be conducted. This would require significant maintenance and management of multiple fly lines during the next period. If it proves that the impacts of *Wolbachia* are a barrier to maintenance of a sustaining fly population, other strains of Wolbachia would be tested, in particular the *w*Mel, *w*MelPop and *w*AlbB that have been reared through more than 60 passages in *Haematobia* cells and which we have shown have very different levels of pathogenicity in buffalo flies (M. Madhav *et al*., 2020) and strains isolated from parasitoid wasps. A *Lucilia cuprina* cell line developed from embryo tissue is now available (Yang *et al*., 2023). Infecting tissue culture with *Wolbachia*, followed by many passages, has the potential for mutualism to occur. Subsequent transfer of adapted *Wolbachia* from cell lines directly into embryos, pupae or adults could then be trialled.

Screening *Lucilia* populations for endosymbionts, including *Spiroplasma* and *Ricketsiella*, and transfer to *Lucilia* from other infection sources should be considered (Montenegro *et al*., 2006).

IMPACT OF WOOL INDUSTRY – NOW & IN 5 YEARS' TIME

An effective area-wide approach directly targeting sheep blowfly populations would address the critical industry issues of blowfly resistance, by reducing the reliance on insecticides and need for ongoing labour-intensive individual animal treatments for control of flystrike. It would help the wool industry in its efforts to transition away from mulesing to protect against sheep blowfly, enhancing the reputation of wool as a natural and ethically produced fibre in the marketplace. While this research has not developed a L. cuprina strain infected with Wolbachia, there is promising potential for the use of endosymbionts in area-wide control of blowflies.

Creation of a Wolbachia infected strain, with the capacity to stability transmit the endosymbiont between generations has potential benefit for the Wool Industry via area-wide pest control. Release of Wolbachia infected L. cuprina could be used for the incompatible insect technique (IIT, Wolbachia infected males are released and cause population suppression) or insect replacement technique (Wolbachia infected insects are released and the endosymbiont spreads through a wild population). These methods could affect fly fecundity or fitness, lowering population numbers.

Different strains of Wolbachia often show a range of success when establishment occurs in a new host, and fitness effects are often unpredictable (Walker *et al*., 2011). For example, transinfection with wMelPop Wolbachia was shown to be able to collapse overwintering populations in mosquitoes by reducing the resilience of eggs during the overwintering stages (Ritchie *et al*., 2015). Spreading Wolbachia through L. cuprina populations may similarly be able to reduce overwintering fitness in blowfly larvae and reduce or eliminate overwintering populations in L. cuprina.

Successful creation of a Wolbachia infected L. cuprina line may require initial infection into a *Lucilia cuprina* cell line, where Wolbachia could adapt to a new host species environment (Yang *et al*., 2023). Following extensive passaging and adaptation, Wolbachia could then be transferred to blowflies. This approach was previously successful and required for transfer from Drosophila to mosquitoes. The Drosophila isolate, Wolbachia wMelPop, was transferred into a cell line derived from the mosquito Aedes albopictus, and then eventually into Aedes aegypti and Anopheles gambiae (McMeniman *et al*., 2008; McMeniman *et al*., 2009).

Here we have shown that L. cuprina can be transinfected with Wolbachia and can be transmitted across generations. Further work towards establishment of a sustainably Wolbachia infected strain, to characterise fitness effects and to develop a control and release strategy is required.

CONCLUSIONS AND RECOMMENDATIONS

Here we have developed methods for transinfection of eggs, pupae and adult L. cuprina with Wolbachia. Infection can be transmitted across generations, however, infection rates were variable and relatively low. Some fitness and reproductive effects apparently related to Wolbachia infection were identified and breeding programs would need to take account of these effects. This may cause difficulty in developing a sustainably infected strain.

Fitness costs can occur among insects following transinfection of Wolbachia. For example, Wolbachia transinfection can cause lethality (Bouchon *et al*., 1998), reduced fecundity (Bian *et al*., 2013), and other pathogenic or fitness effects (Clancy & Hoffmann, 1997; Suh *et al*., 2009). McMeniman *et al*. (2009) managed to eventually get transinfection of mosquito, Aedes aegypti, describes fluctuating and loss of infections from colonies in many instances. They note extensive transinfection and testing was required before eventual success. *Lucilia* flies have a longer life cycle than mosquitos, with more complex and labour-intensive rearing methodologies, which has added to the challenge of generating infected colonies. The concept of cross generation transmission has been demonstrated, however, as shown by (McMeniman *et al*., 2009) a long-term project will be needed to establish infection success.

Recommendations for future research:

- Further microinjection of Wolbachia into young female flies should be considered. Additional resources for managing large numbers of single pair genetic crosses, followed by molecular screening and behavioural assays would be required.
- Introduce three or more different sources of Wolbachia to *Lucilia cuprina* cell lines. Prolonged periods of culturing may enable some strains to adapt from their original host environments to these new hosts. Subsequent injection of pre-adapted Wolbachia into L. cuprina embryos, pupae or flies may then have increased likelihood of survival and transmission between generations.
- Exploration of endosymbionts other than Wolbachia should be considered. Analysis of existing whole genome sequence data would help determine if there are other known endosymbionts in Australian populations.
- Developing transgenic approaches for area-wide management should be considered. These could include systems to kill female progeny, but not male progeny, who could then continue to spread the population-limiting genetic cargo.

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APPENDICES

Appendix 1 – AWI Communication Report

Appendix 2 – Research Capacity Building

Appendix 3 - Tables

Appendix 1 – AWI Communication Report

Name of project
Wolbachia for *Lucilia cuprina* control
Name of research body
University of Melbourne and University of Queensland
Name(s) of any other project co-funding bodies and funding split
N/A
Name(s) of any organisations involved (and specify how they are involved)
University of Melbourne: Objectives 1, 2, 3, 4, 5 University of Queensland: Objectives 1, 2, 3, 4, 5
Project start date
05/04/2023
Project end date
08/04/2024
Other key dates (eg key milestones report(s), events, product launch)
Milestone No: 40070403-0010 “Milestone Report 1” due on 21/04/2023 Milestone No: 4007403-0020 “Draft Report” due on 15/06/2023 Milestone No: 4007403-0040 “Progress Report 2” due on 01/12/2023 Milestone No: 4007403-0030 “Final Report” due on 08/04/2024
Main objectives of the project

The objective of this project was to:

- Determine infection frequencies of *Wolbachia* among *Lucilia cuprina* populations across Australia
- Sequence a *Wolbachia* genome that is found in a blowfly
- Transinfection of blowflies with *Wolbachia* obtained from other insect sources
- Assess fitness costs of *Wolbachia* infected *Lucilia* strains

Project description

This study has demonstrated the proof-of-concept of *Lucilia* transinfection with *Wolbachia*, which has potential to spread itself through populations and could avoid expensive individual animal treatments. Here we have shown that *Wolbachia* infection is extremely rare or absent from *Lucilia cuprina* populations in Australia. Infecting *Lucilia* flies with *Wolbachia* via injecting embryos, pupae and flies demonstrated infection could persist in many cases through the life of the individual. *Wolbachia* could be transmitted from infected females to subsequent generations, although infection rates were inconsistent. Females infected with Wolbachia were observed to have poor egg lay, suggesting Wolbachia needs to adapt to this host environment.

Project (and key milestones) outcomes and outputs

- **Key Milestone No: 4007403-0010** Milestone Report 1, including progress on objectives 1, 2 and 3 (completed)
- **Key Milestone No: 4007403-0020** Draft Final Report 1, updating progress on objectives 1, 2 and 3 (completed)
- **Key Milestone No: 4007403-0040** Draft Final Report 2 (completed)
- **Key Milestone No: 4007403-0030** Final Report. The final report should include an IP register; Recommendations for future R&D activities; A plan for publication of project outcomes in scientific literature.

This project is not yet at the stage requiring and IP register. Recommendations are outlined in section “Conclusions are Recommendations”. This report will form the basis of a peer review publication, including screening of *Lucilia cuprina* populations for the presence of *Wolbachia*, and efforts injecting *Wolbachia*.

Benefits for woolgrowers and wool industry

Area wide management of blowflies involves intentional release of the pest. Release of factory reared males or bisex populations infected with *Wolbachia* have the potential to cause population suppression by introducing fitness costs (poor overwintering or cytoplasmic incompatibility). This proof-of-concept study determined the endosymbiont *Wolbachia* is absent or rare in Australian *L. cuprina* populations and release of *Wolbachia* infected individuals therefore have the potential to spread into naive populations. Further research is required to develop strains with stable *Wolbachia* infections. Reducing reliance on chemical insecticide sprays and mulesing to manage flystrike remains an important goal for the industry and area wide management of this pest should continue to be a future goal.

Is the project related to other AWI-funded or other past/present research

ON-00624 – Informed development of a flystrike vaccine

Potential/real next steps in the research/project
Area wide management strategies for blowfly control will have considerable benefit. This could be achieved using *Wolbachia* or endosymbionts other than *Wolbachia*, or through the use of transgenic insects. Transfer of Wolbachia to *Lucilia* cell lines is a logical next step in this research. Adaptation to a *Lucilia* host cell environment may help the bacteria evade insect immune responses. Furthermore, screening for endosymbionts including *Spiroplasma* and *Rickettsia* should be performed available genomic DNA, or transferred via microinjection from other species into *L. cuprina*. The release of transgenic insects in area wide programmes also have the potential for male flies to carry genes that kill female offspring, for example. This could potentially enable transgenic males to increase in frequency in populations, until female mates are rare, and the population declines or undergoes localised extinction.
Names(s)/roles(s)/contact details of the potential spokesperson/people
Simon Baxter, University of Melbourne, **simon.baxter@unimelb.edu.au** Peter James, University of Queensland, **p.james1@uq.edu.au**
Names(s)/roles(s)/contact details of the key personnel in the project that can be contacted for information for communication purposes (if different from above)
Current images/video assets and potential opportunities

Appendix 2 – Research Capacity Building

Please include the total of the number of Masters & PhD students and post-doctoral fellowships attached to this project.

Number of research personnel attached to the project	Total
Number of research Masters students	1 (Mr Matt Lyons, Masters, Enrolled 2023-2024 at University of Melbourne)
Number of PhD students	1 (Ms Ziyu Shao, PhD, Enrolled 2023-2026 at University of Queensland)
Number of post-doctoral fellowships	0

Appendix 3 – Tables

Population code	Year Sampled	Location	Latitude	Longitude	Number Genotyped
DC_1 C	2020/2021	NSW	-34.808	148.397	6
DEF	2019/2020	NSW	-35.3227	146.3	8
EG-1	2020/2021	NSW	-33.232	149.588	5
GG-1	2020/2021	NSW	-35.824	147.916	8
GL1	2018/2019	NSW	-34.691	148.479	6
HP-1	2020/2021	NSW	-32.169	150.247	8
IAN-1	2020/2021	NSW	-31.933	150.247	6
IM1	2019/2020	NSW	-35.227	144.903	8
JCA-1	2020/2021	NSW	-35.303	145.182	4
KDA	2020/2021	NSW	-34.847	147.566	8
LB	2020/2021	NSW	-34.143	148.103	5
MB	2020/2021	NSW	-29.438	151.846	3
NAH-1	2020/2021	NSW	-35.434	147.394	3
NB2	2020/2021	NSW	-30.485	151.679	6
PMC_1	2020/2021	NSW	-35.627	144.128	8
PRQ_1	2020/2021	NSW	-35.308	148.063	8
RFS1	2019/2020	NSW	-35.594	146.578	4
RL	2020/2021	NSW	-32.155	150.222	6
ST-1	2020/2021	NSW	-33.857	118.608	4
TFR-1	2020/2021	NSW	-32.912	149.189	3
TH-1	2020/2021	NSW	-29.792	151.781	4
TJL_1	2020/2021	NSW	-33.748	148.974	8
TR	2019/2020	NSW	-35.848	148.958	8
AS	2020/2021	NT	-23.705	133.877	6
GB	2018/2019	QLD	-27.47	153.025	7
JCO-2	2019/2020	QLD	-28.47	150.88	3
MTO-2	2020/2021	QLD	-28.674	151.576	3
ND-2	2020/2021	QLD	-28.619	151.157	4
AG	2019/2020	SA	-35.08	138.09	8
ANE1C	2020/2021	SA	-35.5287	139.7685	6
JS	2019/2020	SA	-33.313	135.751	11
NE-1	2020/2021	SA	-36.928	140.246	5
TUR_1	2020/2021	SA	-34.553	138.836	6
G	2018/2019	TAS	-42.037	148.071	8
G1	2018/2019	TAS	-42.037	148.071	6
K1	2018/2019	TAS	-42.195	148.042	6
LAR	2019/2020	TAS	-41.629	147.127	8
SL2	2019/2020	TAS	-41.913	147.515	10
AC2	2018/2019	VIC	-37.395	142.12	5
BL	2019/2020	VIC	-37.444	142.15	8
CA	2018/2019	VIC	-37.887	142.433	2

CB-1 C	2020/2021	VIC	-37.892	142.432	8
DF2	2018/2019	VIC	-37.887	142.337	16
DL1	2019/2019	VIC	-37.887	142.094	11
JC-3	2019/2020	VIC	-36.253	146.132	8
JH-2 C	2020/2021	VIC	-38.262	142.069	8
KL	2018/2019	VIC	-37.329	142.36	5
LK3	2019/2020	VIC	-36.521	145.582	8
RBO-1	2020/2021	VIC	-36.338	146.515	5
TOP	2020/2021	VIC	-37.883	145.053	6
WA-E5	2019/2020	VIC	-36.815	142.286	5
BM(1)	2020/2021	WA	-33.579	117.162	6
BP	2020/2021	WA	-33.579	121.31	8
CAR	2020/2021	WA	-24.881	113.657	16
EL-5	2020/2021	WA	-34.2	118.611	8
KS	2020/2021	WA	-37.214	142.882	5
LH-1	2019/2020	WA	-32.296	116.902	4
NP_2	2020/2021	WA	-33.5521	116.943	8
RH_1	2020/2021	WA	-34.082	118.638	4
EKOS					3
G2					6
GER-6					8
JB					11
JG					6
NN-2					8
PJ					9
TPE-4					7
TOTAL					446