CENTRE FOR
INVASIVE SPECIES SOLUTIONS

PROOF OF CONCEPT FOR GENETIC BIOCONTROL IN VERTEBRATES

FINAL REPORT FOR PROJECT P01-B-005

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and Chandran Pfitzner

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PROOF OF CONCEPT FOR GENETIC BIOCONTROL IN A VERTEBRATE

FINAL PROJECT REPORT FOR P01-B-005

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EXECUTIVE SUMMARY

This project describes the development of a novel genetic biocontrol system and its assessment as a population suppression method for invasive vertebrate pests; this is an alternative to the gene-drive strategies proposed a few years ago.

Gene drives, based on the clustered, regularly interspaced, short palindromic repeats (CRISPR)/Cas9 genome editing system, have been hotly debated in recent years for their potential to control invasive pest species in counterpoint to their hypothetical, perpetual, uncontainable global spread through a target species (National Academies of Sciences, Engineering, and Medicine 2016; Hoffman et al. 2017).

More recently, novel genetic strategies have been conceived that build in safety and controllability, along with efficacy. A new concept has emerged known as the self-stocking incompatible male system (SSIMS) – genetically modified male and female fish are engineered and released and they only generate male offspring (they are ***self-stocking***); however, when they mate with wild-type pest fish in the waterway, the offspring are not viable (they have an ***incompatible*** reproductive characteristic). These ***incompatible males*** continue to be generated from the SSIMS females (the females are also incompatible with wild-type males) until they are too old to reproduce, which delivers an enduring effect in the waterway, as they compete for wild-type females, the wild-type pest population is driven into decline. Once the wild-type fish are below a threshold level and the SSIMS females are too old or die, only SSIMS males remain and they grow old and die.

The outcome is a system that delivers genetic biocontrol, through controlled reproductive suppression, of a target species without the perpetual spread of a classical gene drive as it employs CRISPR/Cas9-based genetic incompatibility with wild-type fish to collapse reproductive potential with a built-in, self-limiting mechanism in wild populations, such as fish in lakes or waterways.

Table 1. Comparison of the functional characteristics of CRISPR-type gene drive systems and the new SSIMS system in development in this project

Characteristic of genetic biocontrol system	Synthetic gene drive	SSIMS
Uses CRISPR/Cas9	✓	✓
Normal Mendelian inheritance	×	✓
Dominant inheritance	✓	×
Local contained spread of genetic cargo	×	✓
Broadscale/global spread of genetic cargo	✓	×
Self-limiting/self-removing genetic system	×	✓

- SSIMS fish (male and female) are added to a lake or waterway and only produce male offspring – every breeding event with wild-type carp[1] leads to no viable offspring.
- Wild-type pest fish are sequentially excluded from the system.
- SSIMS fish only produce males and eventually die out and are removed from the system.

[1] or gambusia

The data generated and the outcomes of this project have validated the two critical components:

1. engineered incompatibility system (EIS)
2. female-lethality system (FLS).

This work facilitates the next step, which is to bring these two components together to generate and assess the functional efficacy of the SSIMS hypothesis in a model species and vertebrate pest species. These data provide a solid basis for key stakeholder engagement to try to secure support for the next level of development of genetic biocontrol strategies for vertebrate pests.

INTRODUCTION

CONTROL OF INVASIVE VERTEBRATE PESTS

Invasive vertebrate pests remain a significant threat to both threatened and endangered wildlife species and to agricultural land and major waterways. Currently, trapping, poisoning and culling are the predominant means of control. This is a major logistical effort that is repetitive, enduring and costly because it is likely to be only partially effective. Biological control systems have proven effective for a period of time (e.g. myxomatosis and calicivirus in rabbits) and have the advantage that they are self-spreading, making them easier to manage logistically and from a cost perspective.

However, they are lethal methods that cause concern for some sectors of the public, and eventually lose effectiveness if disease resistance arises in the pest population. A new possibility for biocontrol has emerged in the past five years with the advent of precision genome engineering (CRISPR) based on the Nobel Prize winning technology developed by Doudna and Charpentier. This technology has revolutionised biotechnology and medical research, but more specifically it has enabled the precise engineering of the genomic DNA of many animals, including those that are important as model systems in agriculture, and those that constitute invasive threats.

This project takes those latest developments to build and assess the components of a hypothetical genetic biocontrol strategy (SSIMS) that, if proven functional and effective, could be adapted to a wide range of invasive vertebrate pests.

PROJECT OBJECTIVES

Specifically, this project will use zebra fish (*Danio rerio*) to design, build and test the genetic components necessary to successfully apply SSIMS to pest fish populations. This approach will involve engineering a speciation event in zebra fish so that mating between the engineered strain and the wild-type results in non-viable offspring. Genetic approaches to create synthetic barriers to sexual reproduction have been shown to function in yeast (Maselko et al. 2017). More recently, a modification of this approach was shown to be effective in creating multiple species-like genetic incompatibilities in a non-vertebrate animal: the insect Drosophila (Maselko et al. 2020). These results support the approach of optimising the equivalent genetic systems in vertebrate animals. This approach will include establishing a repressible female-lethal genetic circuit that results in embryonic death of females outside of laboratory conditions.

Although the European carp (*Cyprinus carpio*) and gambusia (*Gambusia holbrooki*) are the ultimate target species for the technology, the assessment will take place in the zebra fish due to the need for a tightly defined model system with short reproductive intervals of 10 weeks; that is, in achievable timescales compared to carp at more than 12-month reproductive intervals. Direct transfer of the technology to carp and gambusia is not an objective of this project due to the longer timescales and diverse resources required for husbandry and genetic/reproductive technology. However, pest fish species are an ideal platform for the first wild testing of a genetic biocontrol strategy due to their ecological confinement to Australian waterways. In the case of carp, the potential for a viral biocontrol system to first reduce the population with a genetic biocontrol to ‘mop up‘ the resurgent surviving population, is an attractive opportunity. The project will generate valuable knowledge in the emerging field of genetic biocontrol for invasive and pest species across the globe. By contributing knowledge on safety and efficacy, Australia could take a global leadership position on informing public debate around the use of genetic biocontrol for conservation and environmental protection.

The objective of the project is to develop and assess the two functional genetic components that will then be brought together to demonstrate the proof of concept of this genetic biocontrol technology. This assessment will be performed in the zebra fish, as a biologically relevant model of the invasive pest, carp. The project will develop a female-targeted conditional lethal system, which has value in its own right, enabling a ‘synthetic species’ system that will be self-stocking and self-limiting. Both stages in this process have proof of principle already demonstrated in yeast (Maselko et al. 2017) and also in

insects (Maselko et al. 2020). In addition, the necessary targets have been identified for these regulatory systems in vertebrate animals. The combination of these two functional elements will be used to assess the concept proposal of an SSIMS, as summarised in Figure 1 and Figure 2. This is a population suppression system with similarities to the high-profile gene drive system, but it deals with one the major concerns with gene drive, specifically their self-perpetuating characteristic (NASEM 2016; Hoffmann, et al. 2017) by incorporating a self-limiting component that follows an effective suppression event. Outcomes of this laboratory-based research and development have the potential to be rapidly transferred to the target pest species: carp and gambusia. The data produced will be prepared for two peer-reviewed publications. These publications will be used to engage with key regulators to define conditions required for a deployment (wild release). The findings will be complied into a summary whitepaper for the Centre for Invasive Species Solutions (the Centre) and key stakeholders.

NET BENEFIT OF INVESTMENT: An evaluated genetic biocontrol system that can be assessed for transfer to the target species, which is carp. Alternatively, the net benefit will be a decision not to make any further investment in gene-drive development because gaps or barriers have been identified that cannot be overcome within acceptable time frames. This will either validate the necessary next stage investments or preserve significant resources to contribute to traditional control measures or to explore new innovations.

CHANGES IN PRACTICE AND BEHAVIOUR (QUALITATIVE AND QUANTITATIVE OUTCOMES): The outputs from this project will provide a balanced analysis of the benefits and risks and all relevant dimensions of genetic biocontrol systems, which will be used to engage with key stakeholders to support the technical development of genetic biocontrol system, should this approach be agreed. Alternatively, this project will enable the Centre's stakeholders to shelve the technology, move forward and direct their investment to other technologies.

IMPACTS ACROSS FIVE DIMENSIONS (DEMAND, SUPPLY, RISK, ENVIRONMENT, SOCIAL): Demand for new tools is clear across a range of pest species. This project will provide data for decision-making and prioritisation to determine the potential for genetic biocontrol technologies as a control tool for pest species, particularly fish. The project will provide data for the scoping of risk, environmental benefit and the social dimensions that these technologies could deliver.

THE TESTABLE HYPOTHESIS AND RESEARCH QUESTIONS

The testable hypothesis for this project is that a genetic biocontrol mechanism, including female-lethal and synthetic-species approaches, which has been demonstrated to work in yeast and insects, can function effectively in vertebrate species.

This project used the same approach that successfully developed the genetic biocontrol system in lower organisms. The specific research questions were: (1) identify promoter regions of genes that can be targeted with programmable transcriptional activators to result in lethality of female embryos only; (2) identify mutations in these regions that are well tolerated; (3) engineer the promoter mutants to express a programmable transcriptional activator targeted to the wild-type sequence; and (4) characterise the fitness and genetics of the engineered strains and their inability to reproduce with wild-type fish.

The design of female-lethal genetic strategies will also be informed by success in insects, as well as other work conducted in zebra fish. This involved: (1) linking promotors known to be specifically active in females during early development to various lethal effector constructs under the control of a small molecule repressor (e.g. tetracycline); and (2) determining the efficiency of female-specific lethality and male-fitness effects.

PROJECT MILESTONES

Table 2. Milestones that were originally set for the project

Milestone	Description
Contract execution	Fully executed project detail
Development of project monitoring, evaluation, reporting and improvement plans	Extension strategy and project monitoring, evaluation, reporting and improvement agreed
Project plan execution	Animal ethics committee approvals and OGTR permits obtained
Capability established and project initiated	Postdoctoral fellow recruited, inducted and commenced construction of genetic systems
CISS progress report	Progress report approved by CISS
Construction of optimized programmable transcription activator	Promoter identified and constructs assembled to assess optimal level of dCas9-VPR in *D. rerio*
Fish constructed with PTA function identified	At least one zebra fish line constructed with demonstrated dCas9-VPR activity
CISS progress report	Progress report approved by CISS
Demonstration of PTA-controlled female lethality	Repressible/inducible PTA system demonstrated to prevent female embryo development
Manuscript preparation and submission for peer review	Data on female lethality system published (target *PLOS ONE*)
CISS progress report	Progress report approved by CISS
Synthetic species construction	Zebra fish lines generated with modified promoter to evade PTA induction of lethality – generating a 'synthetic species' line of zebra fish
Construction of incompatibility system	Crossing of synthetic species with wild type zebra fish to demonstrate incompatibility (embryo development failure in crosses)
CISS progress report	Progress report approved by CISS
Population assessment of SSIMS	Small-scale population study of crossing synthetic species with wildtype zebra fish to assess self-stocking of the synthetic species
Manuscript preparation and submission for peer review	Data on synthetic species genetic system submitted (*PNAS*/*Nature Biotechnology*)
CISS final report	Final report submitted – outcomes complied for summary white paper and submitted to CISS. Final report accepted by CISS management

METHODS AND RESULTS

CONSTRUCTION OF OPTIMISED PROGRAMMABLE TRANSCRIPTIONAL ACTIVATOR

Milestone 6

Initially, a *doublesex and mab-3 related transcription factor 2a* (*dmrt2a*) promoter fragment was used to control expression of the programmable transcriptional activator (PTA) (dCas9-VPR protein). The promoter fragment comprises the 5' region, exon 1, intron 1 and part of exon 2; the promoter has been characterised and shown to express at reasonable levels between the blastula and pharyngula stages of development and in a variety of adult tissues (Zhou et al. 2008).

Initial suitability of the promoter was assessed by creating transgenic zebra fish with the *dmrt2a*:dCas9-VPR construct and assessing the survival over the first seven days post-fertilisation (dpf). The pNJ5 plasmid shown in Figure 3 was generated, which also included a reporter gene, and was injected into 0 dpf zebra fish embryos along with Tol2 mRNA to generate transgenic embryos. Survival from 0–7 dpf is shown in Figure 4. Other than an expected higher death rate from the physical damage caused by microinjection, survivability of transgenic zebra fish embryos containing the *dmrt2a*:dCas9-VPR construct was good.

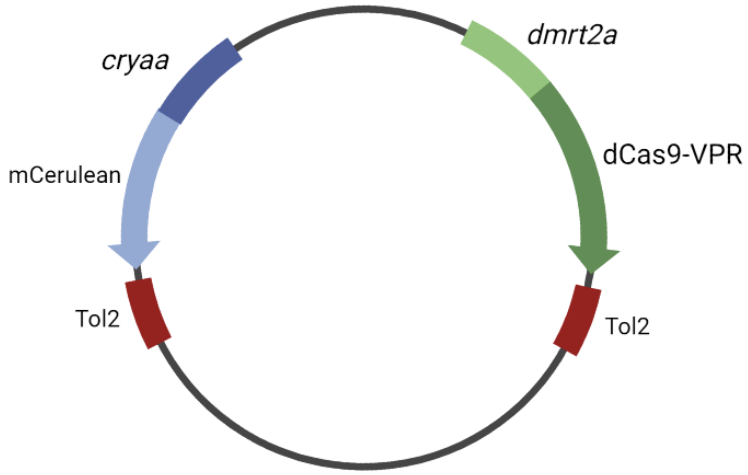

Figure 1. Map of pNJ5 plasmid containing Tol2 integration sites (red) surrounding the transgene construct for integration. Includes mCerulean reporter gene (blue) under the control of an eye-only promoter (cryaa) and dCas9-VPR (green) under the control of dmrt2a.

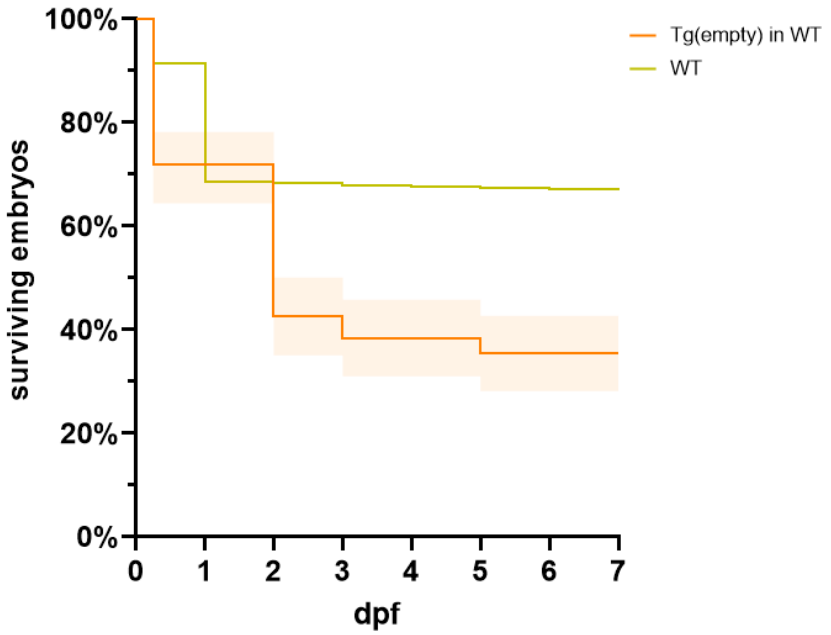

Figure 2. Survival of zebra fish embryos with 95% CI after being injected with pNJ5 plasmid containing dmrt2a:dCas9VPR shown as the 'empty' transgenic Tg(empty) vs survival of uninjected controls. n=738 and n=3,020 respectively.

CONSTRUCTION OF FISH WITH PROGRAMMABLE TRANSCRIPTIONAL ACTIVATOR FUNCTION, SYNTHETIC SPECIES CONSTRUCTION, AND CONSTRUCTION OF INCOMPATIBILITY SYSTEM

Milestones 7, 12 and 13

After ensuring the viability of fish containing the *dmrt2a*:dCas9-VPR construct, the next step was to identify genes that could be targeted by the PTA to induce lethal overexpression. Table 2 shows the five different genes that were chosen as targets. Multiple factors went into this decision, which included whether the genes were controlled or were involved in important aspects of development and if they were expressed in critical organs, such as the heart or brain. Data in the literature on overexpression of these genes via non-PTA methods were also strongly considered.

Table 3. List of genes targets for overexpression for dCas9-VPR

Gene name	Gene information
ern1	Multiple functions including endoribonuclease and kinase roles, expressed in the brain
fgf8a	A fibroblast growth factor involved in neural and heart development
foxl1	Transcriptional repressor responsible for regulating central nervous system development
gata5	A DNA-binding transcription factor involved in organogenesis
wnt1	A regulator for neurogenesis

For each of the genes listed in Table 1, guide RNA (gRNA) targets were identified within the promoter regions to allow dCas9-VPR to localise to the gene to induce overexpression. Two to three targets were chosen for each gene and they are shown in Figure 5.

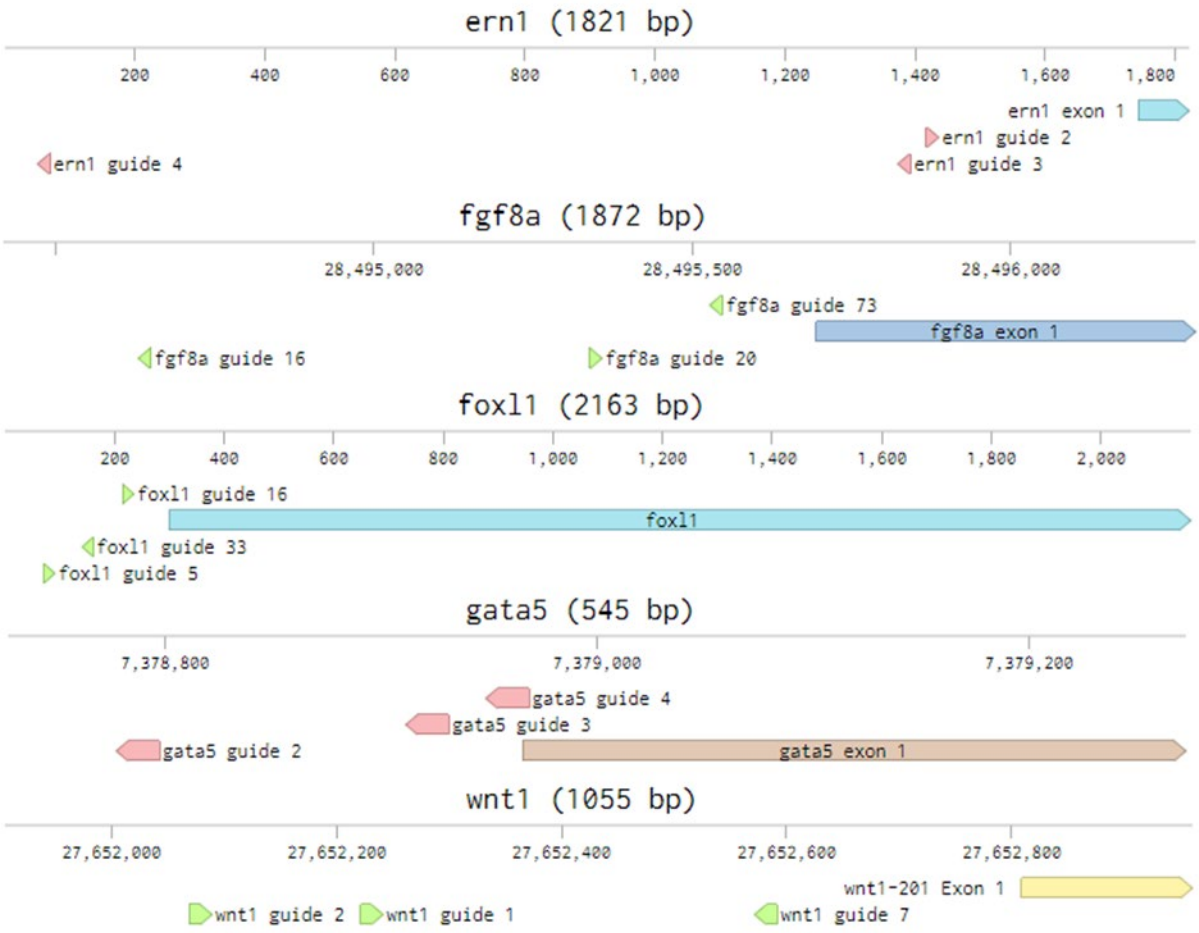

Figure 3. Schematics of each of the five target genes, showing the first exon and the associated 5' upstream region. gRNA target sites are listed as the gene 'guides'.

Each of the individual gRNA target sites for all of the target genes were required to be capable of fulfilling two functions. Firstly, they needed to be efficiently targetable by dCas9-VPR using an associated gRNA so that a high level of overexpression occurs – enough to be lethal. Secondly, they needed to be able to be mutagenised as a result of cleavage by Cas9 so that they are 'rescued' from being targeted by dCas9-VPR. This means the gRNA target sites would need to both be efficiently cleaved, and for the zebra fish to survive and not suffer any fitness effects from the mutant sites that would be generated.

Rescue lines for each of the target genes were created by injected zebra fish embryos with Cas9 protein complexed with gRNAs targeting each of the gRNA target sites. All embryos, except the ones with gRNAs targeting ern1, had good survival rates, whereas ern1 could not be created at all; disruption of the ern1 promoter was likely causing lethality so this gene was dropped as a target altogether. gRNA target sites were analysed with Sanger sequencing to confirm mutations were being generated in the G0 injected mutants before performing intercrosses to generate homozygous mutants. Figure 6 shows representative Sanger sequencing traces.

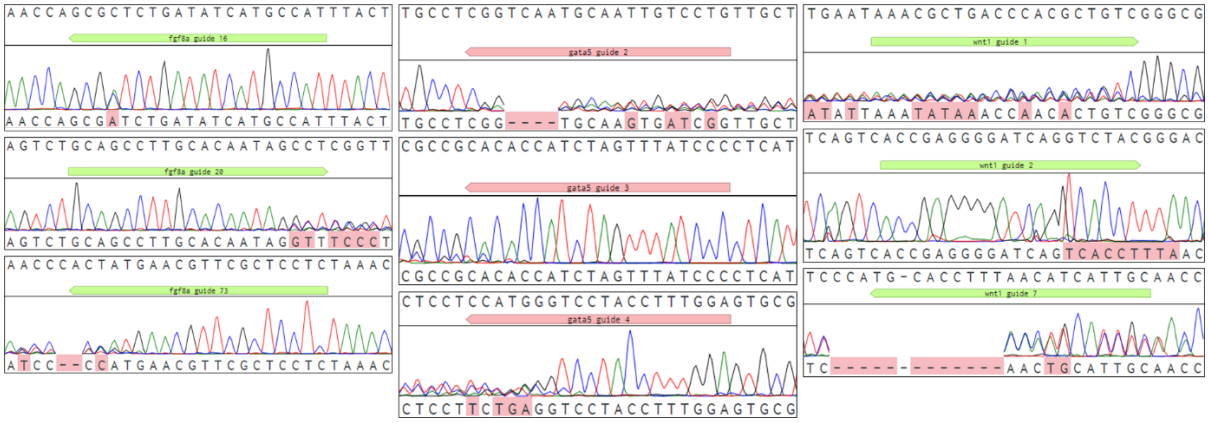

Figure 4. Representative Sanger sequencing traces of rescue mutations at the gRNA target sites for fgf8a (left), gata5 (middle), and wnt1 (right). DNA was extracted and sequenced from G0 mutants.

The next step was to assess the other requirement for the gRNA target sites, which is testing the lethality when targeted by dCas9-VPR. To achieve this, plasmids were constructed similarly to pNJ5 (Figure 3) with the addition of a triple-gRNA expression cassette, as shown in Figure 7, where the three gRNAs are expressed under ubiquitous U6 promoters. Each plasmid targeted a single gene with the three gRNAs for that individual gene as shown in Figure 3.

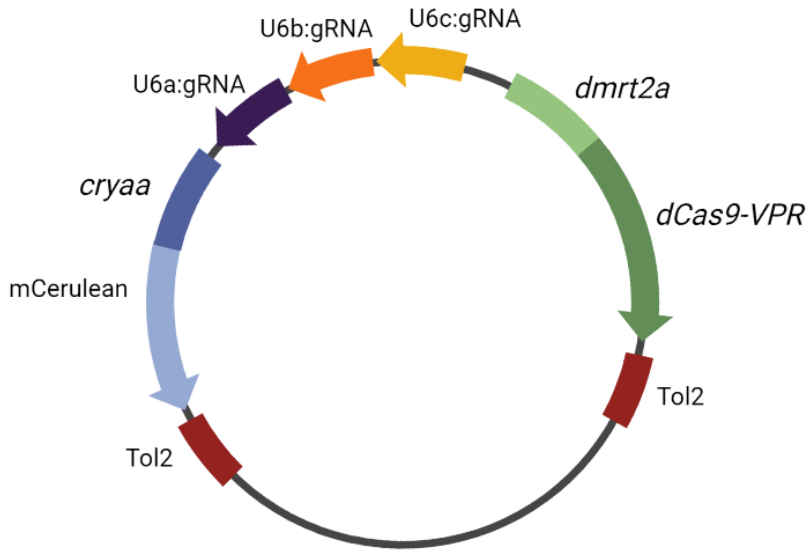

Figure 5. Map of plasmid containing Tol2 integration sites (red) surrounding the transgene construct for integration. The map includes mCerulean reporter gene (blue) under the control of an eye-only promoter (cryaa), dCas9-VPR (green) under the control of dmrt2a, and three different gRNAs under the control of U6a (purple), U6b (orange), and U6c (yellow) promoters.

After construction of the plasmids, in order to test the lethality of each individual construct, the associated plasmid was injected into 0 dpf zebra fish embryos along with Tol2 mRNA to generate transgenic embryos. Survival of those embryos from 0–7 dpf is shown in Figure 8. Two of the target genes (*fgf8a* and *wnt1*) showed a significantly higher death rate than both the uninjected control and the injected control lacking gRNAs. *fgf8a* was the best performing.

These data are an underestimation of the true lethality of the construct for three primary reasons. Firstly, because we are directly testing F0 embryos, they will not all be transgenic. Secondly, of those

that are transgenic, some will be mosaic where the transgene will have integrated at the 2-cell or later stage and, therefore, will not be expressed in all the expected tissues. Finally, for all of the transgenics, regardless of mosaicism or any other consideration, expression levels of the transgene may vary greatly due to position effect as the transgene is integrating in random locations in the genomes.

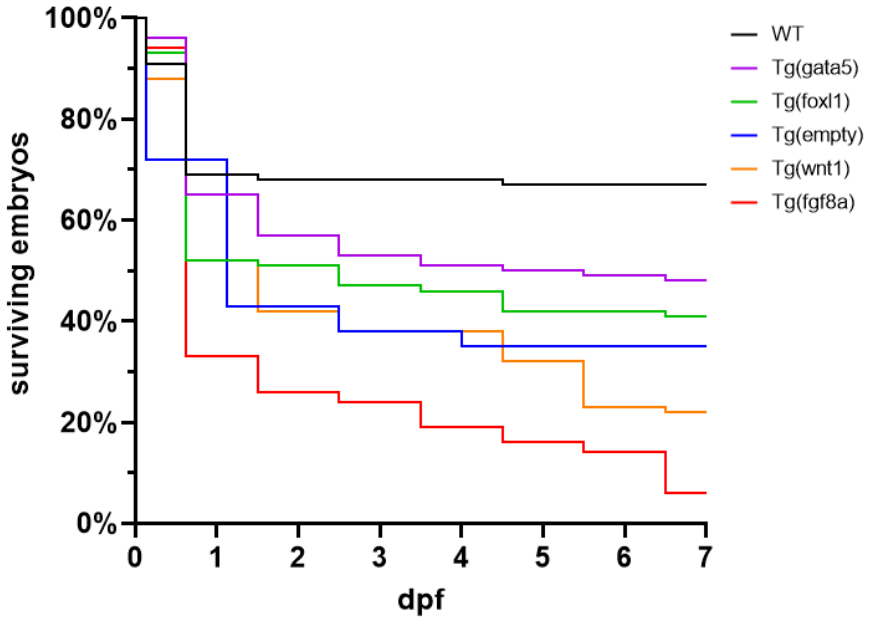

Figure 6. Survival of zebra fish embryos after being injected with plasmid containing dmrt2a:dCas9VPR and triple-U6:gRNA cassette targeting an individual gene. Individual genes targeted as shown, where Tg(empty) targets no genes and wild type are uninjected controls. From top to bottom respectively in the key, n=3,020, n=356, n=475, n=738, n=329, and n=438.

Due to the long generation time of zebra fish (three months from zygote to breeding age, or six months/two generations to obtain homozygotes after intercrossing F0s) it was prudent to test the effectiveness of the rescue mutations to actually rescue the lethality, as demonstrated in Figure 8. Another injection/survival test was performed for *fgf8a*, where zygotes were injected with the same components (dCas9-VPR/triple-U6:gRNA plasmid and the Tol2 mRNA) but, in addition, they were also injected with Cas9 protein complexed with gRNAs targeting the three sites in the *fgf8a* promoter. The Cas9 would act immediately after injection, causing cleavage at the gRNA target sites and generate some rescue mutations that would protect *fgf8a* from the transgene that was being integrated and expressed at the same time. These data are shown in Figure 9 and demonstrate that the mutations were rescuing the lethality.

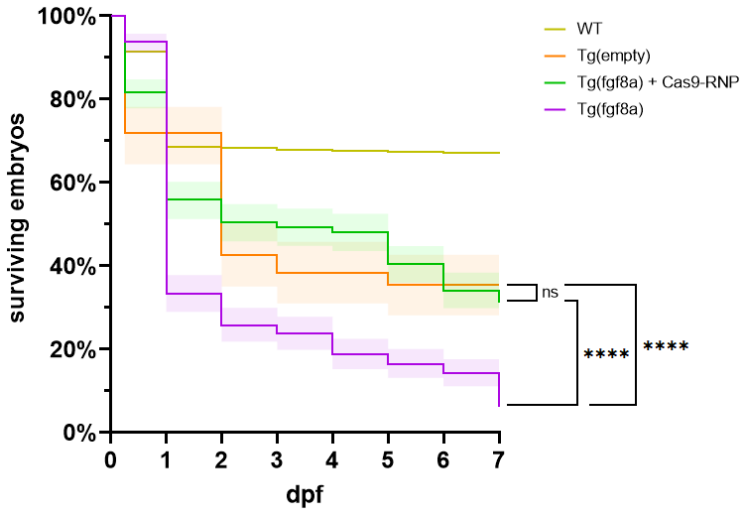

*Figure 7. Survival of zebra fish embryos with 95% CI after being injected with plasmid containing dmrt2a:dCas9VPR and triple-U6:gRNA cassette targeting fgf8a, listed as Tg(fgf8a). WT are the uninjected controls (n=3,020). Tg(empty) is the injection control that lacks gRNAs (n=738). Tg(fgf8a) + Cas9-RNP contains the addition of the Cas9 protein complexed with the same 3 gRNAs expressed in Tg(fgf8a) (n=478). Log-rank (Mantel–Cox) test was performed for comparison of survival curves. **** signifies p<0.0001; ns signifies no significance.*

This same rescue test was performed for the *wnt1* gene, with injections of dCas9-VPR/triple-U6:gRNA plasmid, the Tol2 mRNA, and Cas9 protein complexed with gRNAs targeting the three sites in the *wnt1* promoter. These data are shown in Figure 10 and do not show the same promising protective effect. Instead, they show an increase in lethality, which suggests that when the *wnt1* promoter is mutated at the target gRNA sites, it may be negatively affecting the survivability of the embryos. A line that contains homozygous mutations at all three target gRNA sites is currently being bred to investigate this outcome further and more effectively test the rescue mutations.

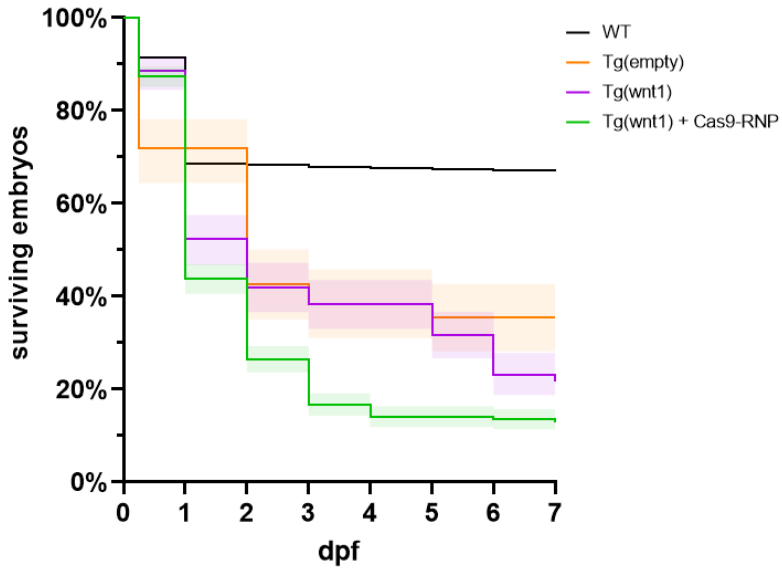

Figure 8. Survival of zebra fish embryos with 95% CI after being injected with plasmid containing dmrt2a:dCas9VPR and triple-U6:gRNA cassette targeting wnt1, listed as Tg(wnt1). WT are the uninjected controls (n=3,020). Tg(empty) is the injection control that lacks gRNAs (n=738). Tg(wnt1) + Cas9-RNP contains the addition of the Cas9 protein complexed with the same three gRNAs expressed in Tg(wnt1) (n=932).

Figure 8 and Figure 9 show the transgenic line containing the *dmrt2a*:dCas9-VPR and the triple-U6:gRNA cassette targeting *fgf8a* functions, as well as a lethal construct and as is able to be rescued by mutations to the *fgf8a* promoter. A line containing homozygous mutations at all three gRNA target sites in the *fgf8a* promoter has been bred and F2 adult fish are currently being genotyped via Sanger sequencing for selection. This line will be used to more thoroughly test the rescue mutations by crossing F1 fish from a stable transgenic line containing *dmrt2a*:dCas9-VPR and the triple-U6:gRNA-*fgf8a*. In addition, *fgf8a* is a good target for lethal overexpression and, therefore, the *fgf8a* rescue line will be used to further optimise the promoter of dCas9.

The selection of new promoters for dCas9-VPR is shown in Table 3. Two highly characterised promoter fragments for ubiquitous expression have been chosen – *actb2* and *ubb* – as well as an early embryonic developmental gene promoter (*wnt8a*) that has also been characterised. A well-characterised, heart-specific promoter that is often used for reporter genes, *myl7,* was also chosen to complement the role *fgf8a* plays in heart development. The remaining genes (*cnbpa*, *drl*, and *tal1*) were all chosen for their roles in heart and brain development, as a complement to *fgf8*'s role.

Table 4. List of gene promoters for optimisation of expression of dCas9-VPR.

Gene name	Gene information
actb2	Ubiquitous promoter
cnbpa	Highly active in early embryo development, involved in neural development
drl	Transcription factor, active in cardiovascular system
myl7	Heart-specific expression
tal1	Expressed in cardiovascular system and central nervous system
ubb	Ubiquitous promoter
wnt8a	Active in early development, responsible for many early patterning events

Of the dCas9-VPR promoters listed in Table 3, *wnt8a* has already been tested, but was shown to be ineffective at inducing a high rate of lethality, as shown in Figure 11. Plasmids, identical to that shown in Figure 7 with gRNAs targeting *fgf8a* and the dCas9-VPR, driven instead by each of the different promoters in Table 3, have been constructed (*tal1* is still pending) and are undergoing testing. *actb2*- and *ubb*-driven dCas9-VPR plasmids targeting *wnt1* are also undergoing testing.

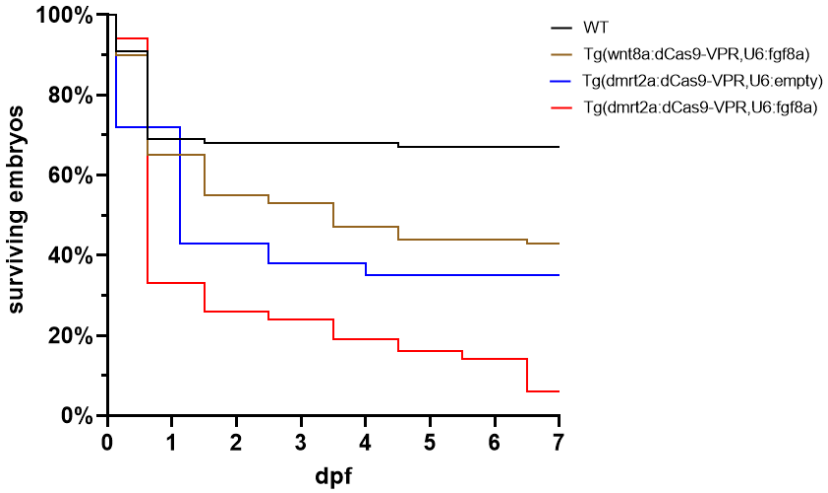

Figure 9. Survival of zebra fish embryos after being injected with plasmid containing triple-U6:gRNA cassette targeting fgf8a, with dCas9-VPR under the control of either dmrt2a or wnt8a as indicated. WT are the uninjected controls (n=3,020). Tg(empty) is the injection control that lacks gRNAs (n=738). Tg(fgf8a) + Cas9-RNP contains the addition of the Cas9 protein complexed with the same 3 gRNAs expressed in Tg(fgf8a) (n=478). From top to bottom respectively in the key, n=3,020, n=1,192, n=738, and n=478.

DEMONSTRATION OF FEMALE LETHALITY

Milestone 9

Initial plans to develop a method of female lethality were superseded when a literature search revealed the more powerful outcome of male-to-female sex reversal. The *fancl* gene, when knocked out, has been shown to induce 100% sex reversal in multiple papers (Rodriguez-Mari et al. 2010; Ramanagoudr-Bhojappa et al. 2018). *fancl* knockouts have been analysed at all stages of development and found to be normal, healthy males, with intact gonads and are fertile. Further to this, the sex-reversal effect is entirely dependent on a process of *tp53*-mediated germ cell apoptosis in the early embryo and knockout or knockdown of *tp53* rescues the sex-reversal phenotype. All of this makes an ideal strategy for SSIMS; the self-stocking aspect will be boosted by the addition of more males due to sex reversal and the stock lines can be made by knocking down *tp53*.

To prototype this idea, a transgenic Cas9 knockout of *fancl* was decided as the knockout would need to be induced in any of the offspring of the SSIMS fish; expressing Cas9 or other variants in the germline of the SSIMS fish would achieve this. The plasmid shown in Figure 12 was constructed for integration into the zebra fish genome. It includes a ubiquitously expressed *SpCas9* gene under the control of the *ubb* promoter, along with a triple cassette of ubiquitously expressed gRNAs targeting *fancl*.

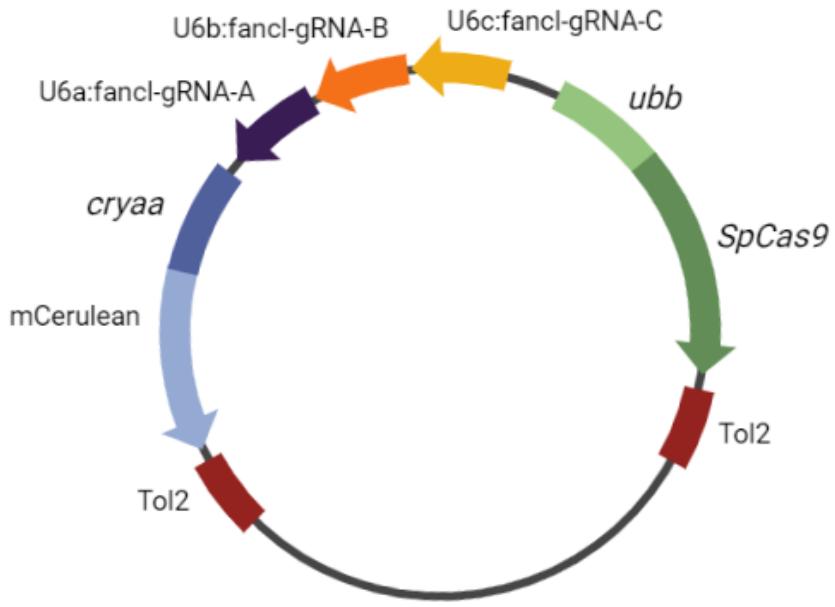

Figure 10. Map of plasmid containing Tol2 integration sites (red) surrounding the transgene construct for integration. The map includes mCerulean reporter gene (blue) under the control of an eye-only promoter (cryaa), SpCas9 (green) under the control of the ubiquitous promoter ubb, and three different gRNAs targeting fancl under the control of U6a (purple), U6b (orange), and U6c (yellow) promoters.

The gRNA target sites for *fancl* were chosen for predicted guide efficiency with the intention of causing multiple missense/nonsense mutations or deletions between multiple target sites. The five chosen sites are shown in Figure 13.

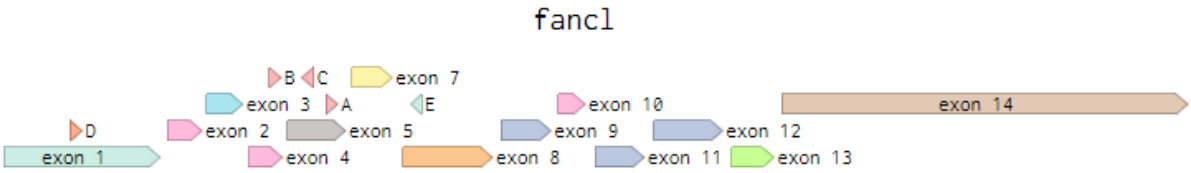

Figure 11. Schematic of zebra fish fancl gene showing position of exons and gRNA targets A, B, C, D and E. Introns are not to scale.

Initial gRNAs were only targeting sites D and E, and after generating transgenic zebra fish they were outcrossed to WT and gRNA cleavage efficiency was assayed by polymerase chain reaction (PCR) and Sanger sequencing. There was no detectable cleavage in these lines, so a second transgenic line was created that targeted sites A, B and C. Again, these transgenic lines were made, outcrossed to WT and assayed via PCR/Sanger sequencing. Representative traces are shown in Figure 14, which demonstrate highly efficient cleavage at both sites B and C, inducing both deletions between the two sites and at the individual sites.

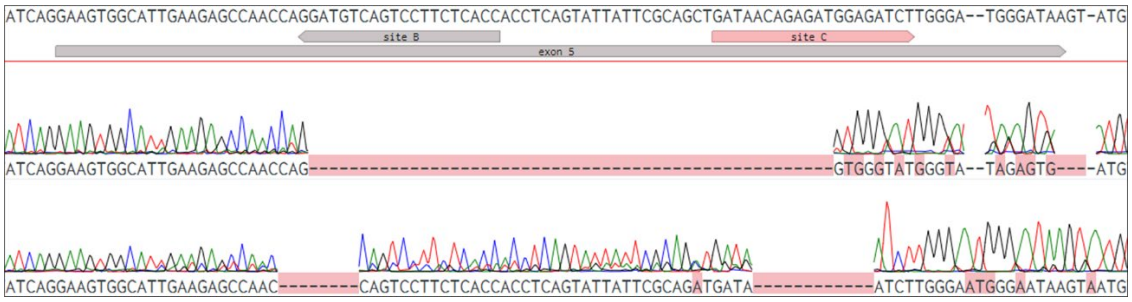

Figure 12. Representative Sanger sequencing traces of fancl exon 5 after outcrossing transgenic zebra fish. The topmost line shows reference sequence, the middle shows a deletion between sites B and C, and the bottom shows individual deletions at the same sites. DNA was extracted and sequenced from outcrossed F1 zebra fish larvae.

Deletions of the size shown in Figure 14 are very likely to be knocking out the function of *fancl* and leading to sex reversal. The sequencing traces were collected from sacrificed larvae, zebra fish from the same egg clutches, along with WT of the same age that are currently being grown. At ~2.5 months they will be able to be sexed to determine the effectiveness of these mutations at inducing sex reversal.

Although sex reversal is going to be our preferred strategy over female lethality, we continued to work on the latter as an alternative and fallback option if it was deemed necessary and useful. The first step was to ensure we could efficiently induce lethality from a heterozygous transgene, an essential requirement of SSIMS. Based on a literature search, the gene encoding thiaminase 1 (*thi1*) from *Bacillus thiaminolyticus* was chosen as it has been shown to be highly effective at inducing lethality via mRNA injections into zebra fish zygotes (Noble et al. 2017).

The first step was to recapitulate this lethality using a transgenic approach. The plasmid shown in Figure 15 was created for ubiquitous expression of *thi1*. To test the lethality, WT zebra fish zygotes were injected with a mixture containing that plasmid and Tol2 mRNA to generate transgenics, those zebra fish embryos were then monitored from 0–7 dpf to assess lethality of the construct. This data is shown in Figure 16 and, as can be seen, was nearly 100% effective at causing lethality within the first seven days, not accounting for mosaicism, position effect or lack of integration of the transgene.

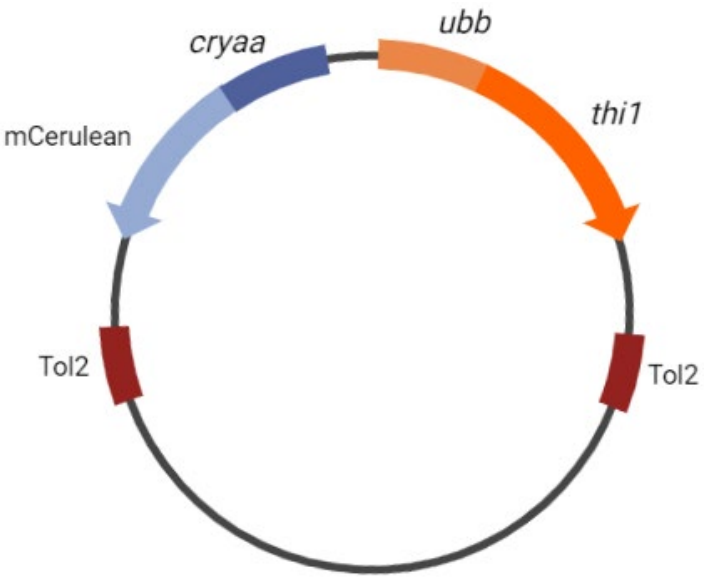

Figure 13. Map of plasmid containing Tol2 integration sites (red) surrounding the transgene construct for integration. The map includes mCerulean reporter gene (blue) under the control of an eye-only promoter (cryaa) and thi1 (orange) under the control of the ubiquitous promoter ubb.

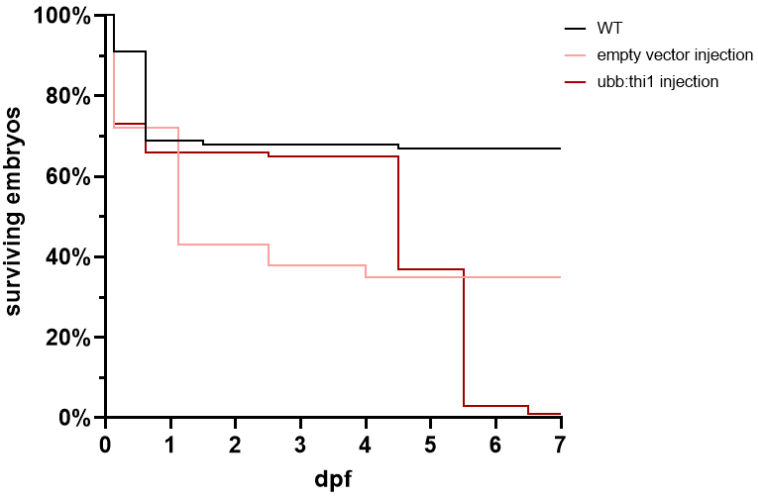

Figure 14. Survival of zebra fish embryos after being injected with plasmid containing the ubb:thi1 transgene (n=613) with empty vector injection (n=574) and WT as controls (n=193).

After confirmation of the lethality under a ubiquitous promoter, we moved on to testing the lethality under a female-specific promoter. The *vtg1* promoter was chosen and multiple plasmids were made similar to Figure 15 with *ubb* replaced and with different reporters. These plasmids included *vtg1:thi1*; *myl7*:*FusionRed*, HS4-*vtg1:thi1*-HS4; *myl7*:*FusionRed*, and HS4-*vtg1:thi1*-HS4; *cryaa*:*FusionRed*. The latter two transgenics were created due to the high lethality seen when injected with the *vtg1:thi1*; *myl7*:*FusionRed* construct as shown in Figure 17. The initial hypothesis was that heart-specific *myl7* promoter, used for a reporter, was causing ectopic expression of *thi1*in the heart. The initial step was to place insulators on either side of the *vtg1*:*thi1* cassette to prevent this; however as shown in Figure 17 this approach did not help. Further testing and complete removal of the *myl7* and replacement with an eye-specific promoter *cryaa* greatly increased the survival of the injected, transgenic embryos.

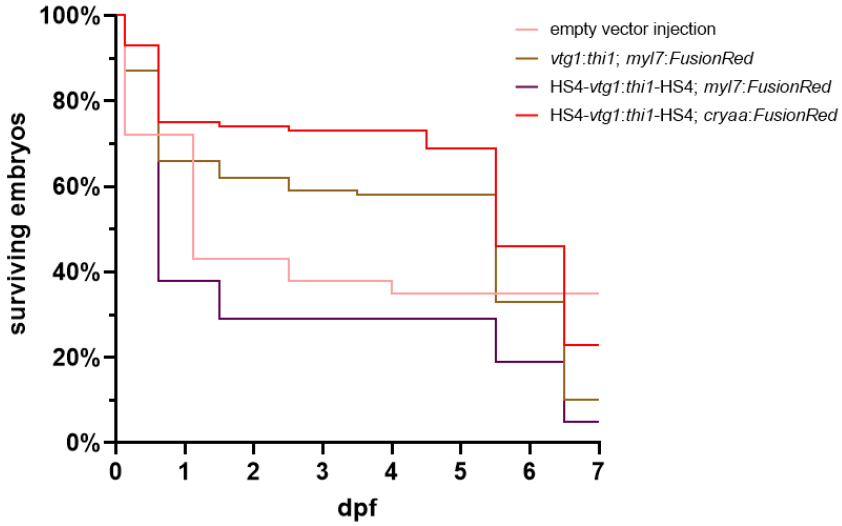

Figure 15. Survival of zebra fish embryos after being injected with various plasmid constructs to generate transgenic lines as indicated in the key. From top to bottom in the key, n=574, n=546, n=221, and n=342.

F0 zebra fish containing HS4-*vtg1:thi1*-HS4; *cryaa:FusionRed* have since been outcrossed to WT to assess whether they are inducing female-only lethality in comparison to WT controls.

DISCUSSION

The project has successfully shown that an EIS can be constructed. The CRISPR dCas9-VPR system was tailored to the promoter region of several genes for which overexpression was hypothesised to lead to lethality. In parallel, the target regions adjacent to the promotors for these genes were edited to remove the dCas9-VPR target sites so that the resulting animals could carry the dCas9-VPR + sgRNA (the activating complex) without self-lethality. Crossed with a wild-type fish, the outcome is lethality for the offspring as the activating complex provided by the genome of the engineered incompatible (EI) fish caused lethal overexpression. The strongest and clearest effect was with the EIS targeting the *fgf8a* gene (see Figure 8). The rescue mutations selected show protection of the EI fish from the lethal effect of the dCas9-VPR-*fgf8a* construct. Another promising gene was wnt1. Additional work is being conducted to understand its potential as an EIS effector. Together, these data validate the first required component of the SSIMS approach.

The second part of the project investigated mechanisms to create a single-sex population in the EIS, with male only offspring being the initial plan. In parallel, the zebra fish model was used to demonstrate the functionality of the *fancl* gene as a target that induces female-only offspring, via sex reversal. This work has identified the necessary components and assembled the appropriate genetic cassette. This approach functions in sacrificed embryos, which are now being grown out to assess functional sex reversal during development to adulthood. The priority of the initial project focus was on induced female lethality during development, which has been investigated and a function genetic cassette targeting the *thi1* gene has been demonstrated to have the required lethality effect. This genetic cassette is also now under functional assessment in zebra fish embryos.

Completion of this work, which is currently funded by CSIRO, will establish the FLS, is the second required component of a functional SSIMS. The final steps for the current objectives will be to assemble a fish that brings together EIS and FLS to generate a full SSIMS animal. Development of this animal will enable the population studies to fully validate the functionality of SSIMS in a vertebrate animal.

The key implication of this study is that the two key components necessary for the SSIMS hypothesis to be realised in a vertebrate animal system are now in place. This data should help inform discussions with key stakeholders and decision-makers as they consider the future of vertebrate pest control and the tools required to advance the long-term aims for more effective control systems.

PEST CONTROL BENEFITS FROM THE STUDY

Due to the nature of this research project and the emerging nature of this field of study, the outcomes of the work do not provide any immediate benefit in terms of tools that can be deployed in the field. However, the urgent need for new tools was the driver behind the investigation of an entirely novel approach to pest control. The research outcomes have validated the hypothesis and positively address the research question in relation to the viability of a genetic biocontrol approach to vertebrate pest management. The project used a model species, the zebra fish, that is closely related to carp, one of the major invasive pests in the Murray–Darling Basin, which ensures these outcomes are relevant and ready for translation into a functional tool to empower environmental remediation.

A key tangible benefit of this project is new strategic knowledge that relates to the Technology Readiness Level (TRL) framework, outlined in Australia's Biosecurity Futures: Unlocking the next decade of resilience report (CSIRO 2020). The results of this project have taken a formulated concept for an application of technology (TRL 2 – details from the CSIRO report are shown Table 4) through to TRL 3 (a proof of concept) and close to TRL 4 where it can be validated in a laboratory environment. The components of the system (engineered genetic incompatibility and female lethality) have clearly reached TRL 3. Bringing them together is the next step and this will enable TRL 4 to be achieved. The data look promising at this stage.

Table 5. Applying TRL 2 technology through to deployment for invasive fish species control

Concept validation Prototyping and incubation of emerging technology ideas and developing knowledge to support industry development				Development and demonstration De-risking and demonstrating promising technologies opportunities and understanding scale-up requirements			Commercial deployment Delivering continuous improvement in mature technologies and supporting deployment and trouble shooting	
Basic principles observed	Formulation of concept or application	Proof of concept	Validation in lab environment	Validation in relevant environment	Pilot scale validated in relevant environment	Full scale demo. in relevant environment	System complete and qualified and hypothetical commercial proposition	Actual system operated full range conditions (commercial trial, small scale)
TRL 1	TRL 2	TRL 3	TRL 4	TRL 5	TRL 6	TRL 7	TRL 8	TRL 9

Source: Table 8, CSIRO 2020.

The project was very specifically designed to use the zebra fish model species to enable rapid translation of outcomes to one of the key pests identified as high priority by an earlier study funded by the Centre (Ruscoe et al. 2021). The findings of the current study, developing a functional genetic biocontrol tool, will also be highly relevant to plans to develop genetic biocontrol for the cane toad, another key pest identified in that report. The group conducting the current study at Macquarie University have received funding from the Australian Research Council, in association with the Minderoo Foundation, to develop new work in cane toads.

The relevance of the technology described in this project for genetic biocontrol of carp pathways relates very closely to work undertaken by the National Carp Control Program (Wedekind 2019). This work highlights the potential use of the koi-herpes virus (cyprinid herpesvirus 3/KHV-3) as a conventional viral biocontrol agent to reduce carp numbers, but recognises the possible emergence of a virus resistant population and a bounce back of the invasive pest, as has been seen with viral biocontrol of rabbits. Studies by Thresher et al. (2014) highlight that the best approach is to combine a viral biocontrol of carp and follow with genetic biocontrol on the reduced population for forceful decline and to avoid the emergence of resistant populations.

This project provides a strong dataset that supports the possibility of development of genetic biocontrol tools that are not based on the gene drive model, and are free of some of the controversy and public concern associated with debates involving gene drive. This dataset will enable stakeholders to have clearer discussions about the merits of the technology as they consider future investment in research and development in this area.

ADVANCES MADE AND NEXT STEPS

Since its inception and initiation by funding from the Centre, this project has moved the field forward significantly, particularly by demonstrating the genetic biocontrol system that is not a traditional gene drive. Gene drive systems attract additional scrutiny from regulators and require specific control systems built in. The SSIMS is conceptually self-limiting and is programmed for self-removal once the invasive population has been removed.

This project has validated the two key functional elements that need to be brought together to enable the development of the SSIMS genetic biocontrol strategy. This is a significant advance for the field of vertebrate biocontrol as it demonstrates that there is potential and justification for the next level of research and development investment.

For the model system, the next steps will be to bring together the EIS and FLS to assemble a functional SSIMS. This will enable pilot population studies to demonstrate the SSIMS in suppressing a native population with an SSIMS population and the programmed self-removal of the SSIMS

population. These are the key functional elements and data that will be required by regulating bodies such as the Office of the Gene Technology Regulator and the Department of Environment.

For the target species, the next steps will be to characterise the homologous genes in the invasive carp population and customise the genetic vectors. Generating a laboratory-based breeding colony will be essential to engineering an SSIMS population.

This project has provided a solid grounding in data to engage key stakeholders at the state and federal level to discuss coordinated investment to build on these findings. For the next calendar year (2024), the CSIRO has undertaken to support a postdoctoral fellow at Macquarie University who is conducting this research, through its Health and Biosecurity Business Unit and its biosecurity research program. This support is to ensure continuity of project workflow and to maintain critical capability in the growing field of genetic biocontrol for vertebrate pests.

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