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Keria Moritsugu-Vandehey
Portland State University

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Genome Editing Using CRISPR-Cas9 in Annual Killifish Species

By

Keria Moritsugu-Vandehey

An undergraduate honors thesis submitted in partial fulfillment of the requirements for the degree of

Bachelor of Science

in

University of Honors

and

Biology

Advisor

Jason Podrabsky

Portland State University

2024

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Acknowledgment

I want to thank my advisor, Dr. Jason Podrabsky, for guiding me through this project and this honors thesis. I would also like to thank the CRISPR team, Dr. Amie Romney, Yakaterina Chmykh, and Isabel Henkes, for all the collaboration and hard work to get this project going in the lab. A big thank you to the National Science Foundation for funding this project and to BUILD EXITO for giving me the opportunity to work in this lab. Lastly, I want to thank the killifish.

Abstract

CRISPR-Cas9 genome editing has been used successfully to knock out genes in model organisms such as zebrafish, turquoise killifish, and cichlid fish. CRISPR-Cas9 genome editing has not been verified in the annual killifish, *Austrofundulus limnaeus*. We hypothesize that targeted editing of the tyrosinase gene in embryos of *A. limnaeus* will lead to fish without the ability to produce black pigment. Embryos at the 1-cell stage were injected with a Cas9 cocktail containing a mix of guide RNA molecules that target the genomic sequence of the tyrosinase gene and either an mRNA coding for the Cas9 protein or Cas9 protein. Guide RNAs were designed using ChopChop, and two guides were selected for injection based on a high predicted percent efficiency for binding with a low probability for off-target effects. Injections with Cas9 mRNA failed to exhibit an edited phenotype. When using Cas-9 protein, many injected embryos developed without expressing black pigment, but some embryos were obviously without black pigment or had lost portions of their black pigment. We found for the first time in this species that CRISPR Cas9 can be successfully used to knockout gene function. In the future, we plan to establish a breeding line of non-pigmented killifish to aid in embryological studies of this species.

Introduction

Austrofundulus limnaeus is a species of annual killifish native to temporary ponds in Venezuela. Annual killifish are unique because they have a complex life history that includes the ability to enter metabolic dormancy called diapause, their embryos are tolerant of environmental extremes, and they have a short adult life span (Podrabsky et al., 2017). Recently, efforts have been underway to develop *A. limnaeus* into a powerful model organism for developmental research (Wagner et al., 2018). The Turquoise killifish, a relative to *A. limnaeus*, has been developed as a model for aging research, and CRISPR-Cas9 genome editing has been successfully used to mutate their genes for studies of aging and disease (Harel & Brunet, 2015). In this thesis, I explore the usefulness of CRISPR/Cas9 genome editing in embryos of *Austrofundulus limnaeus*.

Unique features of Austrofundulus limnaeus as a model for biomedical research

During development, embryos of annual killifish can enter into two fundamental states of dormancy: diapause and quiescence (Martin & Podrabsky, 2017). Quiescence can happen at any time during development and is entered in direct response to unfavorable environmental conditions. When the conditions improve, quiescent embryos immediately resume development. In contrast, diapause is a state of developmental arrest and metabolic depression that embryos enter before they are exposed to unfavorable conditions. Importantly, embryos enter dormancy and can remain dormant even under conditions that are permissive of development. Typically, environmental cues that indicate favorable conditions for development signal an end to the diapause state, and the embryos will begin development again. Diapause is one of the most interesting aspects of this species, and in their life cycle, annual killifish can enter diapause at three different stages of development (Podrabsky & Hand, 2015).

Annual killifish can develop along two alternative developmental trajectories controlled by vitamin D synthesis and signaling (Romney et al., 2018). In *A. limnaeus,* lower incubation temperatures (20°C) induce diapause, while warmer temperatures (30°C) support active development. Gene expression profiling using RNA sequencing revealed that expression of the enzymes that synthesize vitamin D_3, or exposing embryos to exogenous vitamin D_3, supports the continuous development and the skipping of diapause. Further, they found that vitamin D signaling is critical for normal vertebrate development.

Easy assessment of genome editing in fishes

There has been a lot of research on the use of CRISPR-Cas9 genome editing in fishes. Still, this technology has yet to be verified in *A. limnaeus*. *A. limnaeus* has a high environmental stress tolerance and DNA repair capacity (Wagner & Podrabsky, 2015). Thus, because CRISPR/Cas9 genome editing relies on the ability to induce double-stranded breaks in DNA, it is unclear if this technology will work efficiently in this species. CRISPR-Cas9 genome editing has been successfully deployed in the turquoise annual killifish, *Nothobranchius furzeri* (Harel et al., 2016). A useful technique to screen for successful genome editing is to disrupt a gene that will provide a visible change in phenotype and thus make evaluating success easier, quicker, and cheaper. For example, CRISPR-Cas9 was used in zebrafish to target and disrupt the tyrosinase gene, an essential gene in black and eye pigmentation synthesis in vertebrates (Jao et al., 2013). Another study on *N. furzeri* targeted tyrosinase using CRISPR-Cas9 with a subsequent loss of pigmentation (Krug et al.,2023). Knockout of tyrosinase using CRISPR-Cas9 has also successfully blocked the synthesis of black pigment in a cichlid fish (Li et al., 2021). Further, Li et al. (2021) provides a detailed protocol from making needles to hatching edited fish. These results suggest that tyrosinase is an

excellent initial target gene to test for successful genome editing using CRISPR-Cas9 in fishes. I hypothesized that targeted editing of the tyrosinase gene in embryos of *A. limnaeus* will lead to fish without the ability to produce black pigment.

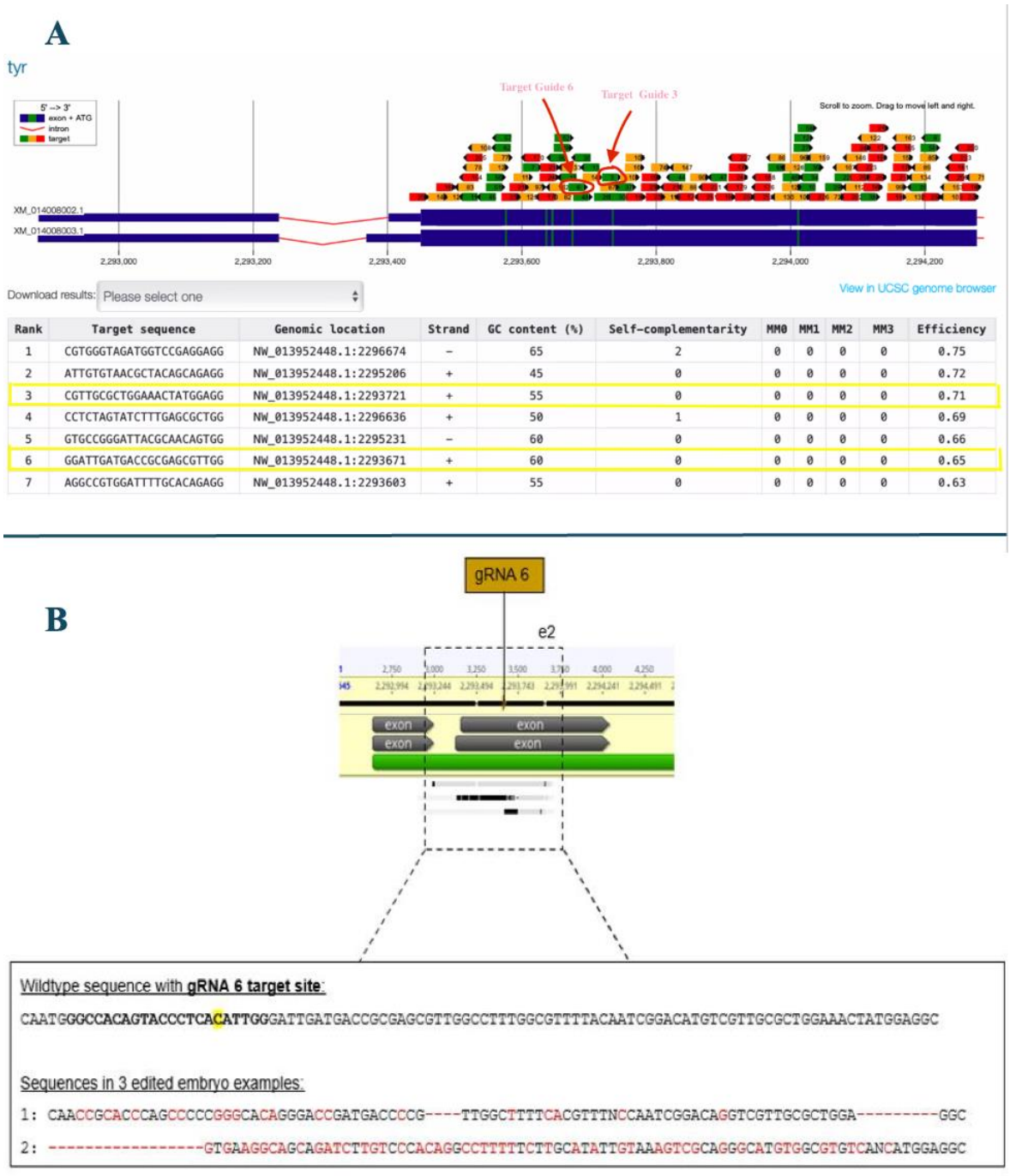
Materials and Methods

Guide RNA selection

Embryos of *A. limnaeus* were injected with a CRISPR-Cas9 cocktail containing a mix of guide RNA molecules that target the genomic sequence of the tyrosinase gene (Figure 1). Guide RNAs were predicted using the program ChopChop (Labun et al., 2019), which can determine high-efficiency targets in the gene of interest while minimizing the chances for off-target editing. Of the many choices provided by ChopChop, guides 3 and 6 were chosen because those guides had high predicted percent efficiency for binding and a position that would result in severe disruption of the gene product. These sequences were used to order RNA oligonucleotides from IDT (Coralville, IA) and are presented in Table 1.

Table 1: *DNA and RNA sequences of PCR primers and guide RNAs used to target the tyrosinase gene in* Austrofundulus limnaeus.

Right Primer	5’ -GGT CGT AGG TGT TGA TGT CTG A- 3’
Left Primer	5’ -GGT CAG GTA GGT TTG AGG AGT G -3’
Guide 3	5’-/AltR1/CGU UGC GCU GGA AAC UAU GGG UUU UAG AGC UAU GCU/AltR/-3’
Guide 6	5’-/AltR1/GGA UUG AUG ACC GCG AGC GUG UUU UAG AGC UAU GCU/AltR2/-3’



Rank	Target sequence	Genomic location	Strand	GC content (%)	Self-complementarity	MM0	MM1	MM2	MM3	Efficiency
1	CGTGGGTAGATGGTCCGAGGAGG	NW_013952448.1:2296674	-	65	2	0	0	0	0	0.75
2	ATTGTGTAACGCTACAGCAGAGG	NW_013952448.1:2295206	+	45	0	0	0	0	0	0.72
3	CGTTGCGCTGGAAACTATGGAGG	NW_013952448.1:2293721	+	55	0	0	0	0	0	0.71
4	CCTCTAGTATCTTTGAGCGCTGG	NW_013952448.1:2296636	+	50	1	0	0	0	0	0.69
5	GTGCCGGGATTACGCAACAGTGG	NW_013952448.1:2295231	-	60	0	0	0	0	0	0.66
6	GGATTGATGACCGCGAGCGTTGG	NW_013952448.1:2293671	+	60	0	0	0	0	0	0.65
7	AGGCCGTGGATTTTGCACAGAGG	NW_013952448.1:2293603	+	55	0	0	0	0	0	0.63

Figure 1. *A) Using the program ChopChop, Target RNA guides were determined for tyrosinase knockouts in the* Austrofundulus limnaeus *genome. Targets 3 and 6 were chosen due to predicted high-efficiency rates and their location within the tyrosinase gene. B) Sanger sequencing alignment results for guide 6. The yellow highlighted C is the spot where cutting should occur based on the design of the guide RNAs. Areas marked in gray show sequence matching between wildtype and potentially edited individuals. Black bars indicate a different DNA sequence between the wild type and edited individuals. The actual sequence differences can be seen in red text at the bottom of the figure.*

Needle Preparation

The needle of choice is pulled using a Narishige thin-walled glass capillary (G-100) or a glass capillary with filament (GD-1) in a Sutter Micropipette Model P-80/PC pipette puller (Sutter Instruments, Novato, CA). The needles should have an open tip with a smooth 30° angle and be no wider than 2µM at the tip.

1. Place capillaries into the micropipette puller. Set to program #7 and pull into micropipette needles. The parameters for program #7:
    a. Loop 1: Heat: 645 Pull: 40 Velocity: 20 Time: 250
2. Handle the capillaries carefully and check the ends to make sure they are not damaged or chipped; this can lead to leaky injections later.
3. The needles best for penetrating killifish chorions have a shorter and more robust tip than a typical microinjection needle (Figure 2).

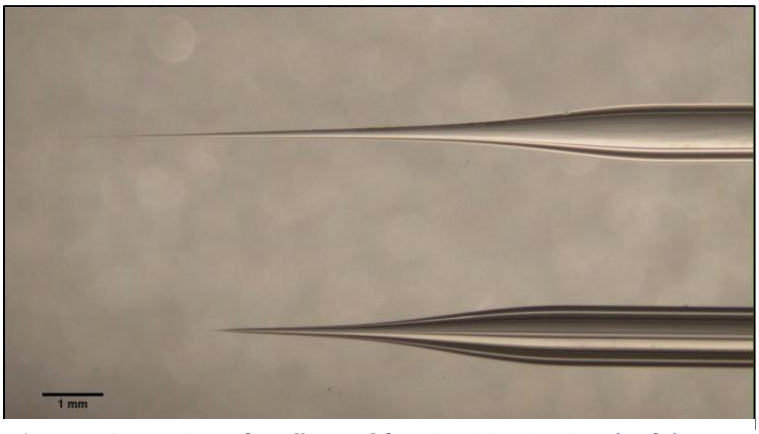


Figure 2. *Comparison of needles used for microinjections in zebrafish (top) and killifish (bottom).*

4. For needles with a filament, the needle will need to be broken before beveling:
    a. Using the Narishige MF2 microforge (Narishige International, Amityville, NY) with the 10X objective lens, mount the needle so the tip is within the field of view, and break the needle where the OD is between 2 tick marks (10µM; at 5X, 1 tick

mark = 20µM). If not using a capillary with a filament, there is no need to break the tip. For tips with a filament, its tip is ground to 2µM.

5. Use the Narishige EG-402 micro grinder (Narishige International, Amityville, NY) to smoothen and bevel the tip. Mount the needle at a 30° angle. Keep the grinding stone wet while grinding (a drop every few seconds). Avoid letting the diameter widen past 2µM. If an excessive amount of debris builds up on the needle from grinding, then wash it using the pipette bulb (95% EtOH and Nano-H_2O).
6. Store pulled needles in a circular jar and beveled needles in a black needle tray or in a Petri dish with modeling clay. The needles are very delicate and need to remain unbroken. Prepare around five needles for each round of microinjections.

Preparing injection solutions

All solutions should be prepared on the day of use, within an hour before performing injections. Guide RNAs (guides 3 and 6, see Table 1), Cas9 enzyme (PNA Bio, Newbury Park, CA), tracer RNA, duplex buffer (IDT), and phenol red are mixed as described in (Table 2). The prepared injection cocktail should be stored at 4°C until used.

Table 2: Example of tyrosinase cocktail for injections.

Guide 3	1.0 µL	Total of 20 µL in one tube. Incubate the tube at 95°C for 5 minutes and 21°C (room temp) for 30 minutes.
Guide 6	1.0 µL	
Tracer RNA	2.0 µL	
Duplex Buffer	16.0 µL	
Add 12.0µL of Cas9 protein (PNA BIO), 80 µL of Nuc-Free water, and 8µL phenol red		

Embedding embryos in agarose

Prepare 70-100mL of 1% low-melting agarose using an embryo medium containing 0.001% methylene blue media. Melt the agarose in methylene blue media mixed in a microwave and then place in a bead bath set to 37°C. Assemble Petri dishes one at a time by pouring 12mL of agarose into each dish and monitor the temperature using a thermocouple until the temperature reaches 30°C. Once the agarose reaches 30°C, pick up 25 embryos onto a spatula and remove liquid surrounding them by blotting on a kimwipe. Tap the spatula of embryos into the agarose and use a dental tool to organize the embryos into a 5 x 5 grid, allowing the agarose to solidify. Label each embryo on the underside of the Petri dish (1-25) with a permanent marker (Sharpie, Chicago, IL). Numbers should be labeled from left to right and written backward so they are straightforward to read when looking down through the microscope.

Micromanipulator and Gas Cylinder Setup

With lighting from below, a stereo-dissecting microscope (Variscope) is best suited for visualizing embryos for microinjections. Accompanying the microscope is an MX130R micromanipulator (Siskiyou Co, Grants Pass, OR) and an MPPI-3 microinjection pressure injector (ASI, Eugene, OR). Airflow to the microinjector is delivered at 20 psi, while injector pressure is set to 12 psi with a pulse duration of 50 ms. The back pressure is set to 0.6 psi. Embryos under 20-40X total magnification can be localized and targeted with a needle.

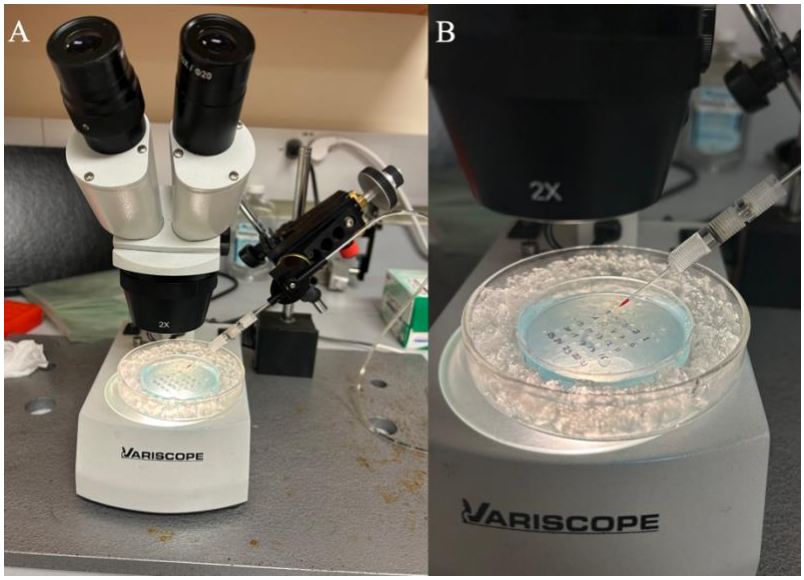


Figure 3. Example of a microscope set up for injections, with needle and embedded embryos. A) a view of the typical setup, and B) a close-up of the labeled embryos and injection needle.

Microinjection of Embryos

Load the Petri dish onto the microscope and keep it cool with a small ice bath to ensure the agarose stays solid during the injections. Load the needle onto the micromanipulator. Identify the first embryo through the microscope and then move the microscope to align the needle with the embryo. Once the needle is in focus, adjust the micromanipulator until the needle pierces the embryo. Inject 1-2 pulses of injection cocktail into each embryo either in the first blastomere cell or in the yolk just under that cell (Figure 4). You can confirm when injection occurs by visualizing a pink bubble leaving the tip of the needle and staying within the embryonic membranes. Record where each embryo is injected. After injections, remove all embryos from the agarose and transfer embryos into fresh Petri dishes containing embryo medium with methylene blue. A control plate of 20-25 non-injected embryos should also be made. Incubate the embryos in the 25°C incubator for four days. The embryo medium should be changed daily. On the fourth day replace the methylene blue media with embryo medium containing 10 mg/l gentamicin sulfate. At this point, embryos may be incubated at either 30°C or 25°C incubators based on the timeline for

development. Freeze some embryos away in liquid nitrogen and store them in a -80°C freezer for later extraction of DNA to check for genomic edits.

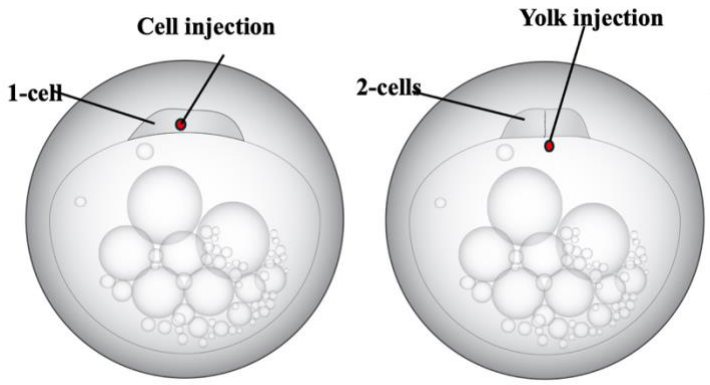


Figure 4. Example of cell injection vs. a yolk injection. Illustrations from Podrabsky et al. (2017). Figure on the left is a one-cell stage embryo with the approx. size of the injectant in the cell. The figure on the right is a two-cell stage embryo, which can be distinguished by the line creating two cells. In the right-hand figure, the injectant is shown to be in the yolk area of the embryo. The circles in the middle of the embryos are lipids, not cells.

DNA Extraction and cleanup

Retrieve frozen samples of the embryos (one embryo/tube) from the -80°C freezer and thaw them on ice; when thawed, they will look clear and yellowish. Add 20µl of 50mM NaOH and crush each embryo with a cleaned, autoclaved pellet pestle. Briefly centrifuge the sample tubes with a quick pulse at full speed in a centrifuge (Spectrafuge 24D, Labnet International Inc.). Transfer the tubes to a heat block to incubate for 10 min at 95°C. Vortex the samples for 10s and briefly centrifuge the tubes again. Add 2.2 µl of 10mM Tris-HCl pH 8 to each sample, then vortex for 10s. Centrifuge at max speed for one minute in a benchtop centrifuge and retain the supernatant in a fresh tube.

DNA was cleaned using a Monarch PCR and DNA Cleanup kit (New England Biolabs Inc, Ipswich, MA). Retrieve the DNA samples from the DNA extraction, dilute each DNA sample with 40µl of DNA Clean, and mix by pipetting up and down. Take a column and collection tube for each sample and label it according to each sample. Insert the column into the collection tube, load the 80µl of total sample onto the collection tube, and spin for 1 minute in the tabletop centrifuge

at max speed. After spinning, discard the flow-through at the bottom of the collection tube and re-insert the column into the collection tube. Add 200µl of the DNA wash buffer to the columns, spin for 1 minute, and discard flow through. Repeat with 200µl of wash buffer spin one more time and discard flow through. Transfer the column to a clean 1.5ml microfuge tube. Add 8-10µl of nuclease-free water heated to 50°C, wait for 1 minute, and then spin in the centrifuge for 1 minute at full speed. Determine the concentration of DNA in the samples by UV spectrophotometry (Thermo Scientific NanoDrop One Spectrophotometer, Waltham, Massachusetts).

Amplification of DNA using polymerase chain reaction (PCR)

The region of interest in the tyrosinase gene is amplified using PCR with primers that flank the potential area of editing as determined by the guide RNA sequences. PCR reactions (20µl total volume) were prepared as outlined in Table 3. Primer sequences are provided in Table 1. For running multiple reactions, a master mix can be made, which consists of the primers and the 5xTaq mix, which can then be added to the DNA and water mixture for each sample. PCR reactions were run using a thermocycler (GeneAmp PCR System 2700, Applied Biosystems) with the following 5x-Taq TYR program setting: 95°C for 3 min followed by 45 cycles of 95°C for 30 s, 52°C for 30 s, 68°C for 75 s, and then a final incubation at 68°C for 5 min before cooling to 12°C until the sample is collected from machine.

Table 3. *PCR reaction mixtures to amplify potentially edited regions of the tyrosinase gene.*

Reagent	Volume, µl
Left primer (10µM)	1
Right primer (10µM)	1
DNA sample + Nuclease-free water	14
5xTaq mix	4
Total	20

Gel Electrophoresis

DNA size and purity were evaluated using 1% agarose (super fine resolution) gel electrophoresis in 0.5 TBE buffer (3g agarose per 30 mL of 0.5 TBE). The gel mixture was microwaved for 1 min or until boiling for two rounds to melt the agarose, and 1 µl of SYBR safe DNA stain solution (ThermoFisher, Waltham, MA) was added for every 10ml of solution. Samples were loaded onto the gel in 6x loading dye (Fermentas 6X DNA loading dye). Gels were run in a Gelato gel electrophoresis rig (miniPCR Bio, Boston, MA) using 5µl of each sample in 6X loading dye. Molecular weight markers were run (6µl each) in the first (GeneRuler 1KB ladder, ThermoFisher, Waltham, MA) and last well (GeneRuler1KB+ ladder, ThermoFisher, Waltham, MA) of each gel. Samples were separated using 100 V for 20 min.

Results

Timing of exogenous mRNA translation

To test for the timing of translation of exogenous mRNAs into protein, we injected embryos with the mRNA for green fluorescent protein (GFP). During the first 10 hours post-injection, observations with a fluorescence microscope (Leica, DMIRB, Nussloch, Germany) revealed that GFP protein was not translated until four hours after injection, at about the 8-16 cell stages in our embryos (Figure 5). Based on this observation, we used Cas9 protein for subsequent injections.

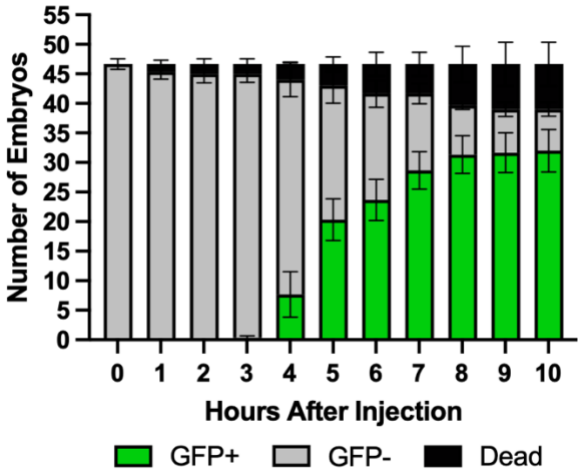


Figure 5. *Time needed to observe green fluorescence in embryos injected with mRNA for green fluorescent protein (GFP). Green bars indicate GFP detection at four hours after injections. Gray bars indicate the total number of embryos that did not express GFP, and black bars indicate dead embryos. Bars are means ± S.D., n=3 different spawning batches.*

Testing site of injection

To test the efficacy of the injection site, we injected embryos with mRNA for GFP directly into the first cell and the yolk right under the first cell. Injection into the first cell appears to be superior for the delivery of reagents compared to injections into the yolk (Figure 6). However, both cell and yolk injection sites illustrated successful delivery of reagents.

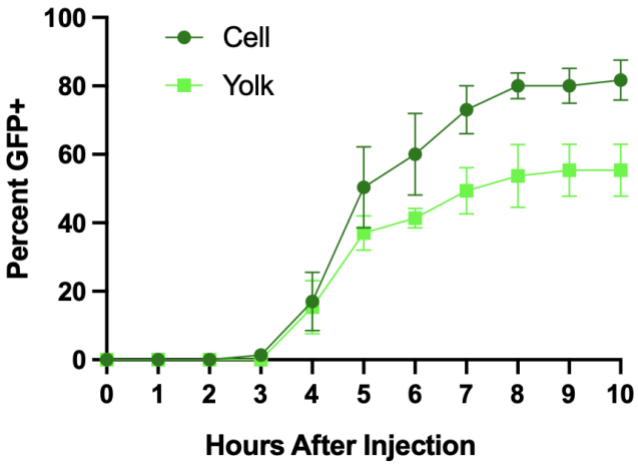


Figure 6*. Time from injection until the appearance of green fluorescence in embryos. Injection into the first cell (the dark green) is superior to injection into the yolk (light green) for reliable delivery of reagents into the blastocytes. However, both injection sites can result in reagent delivery into embryonic cells. The percent of GFP can be seen at four hours post injection. Symbols are means ± S.D., n=70 cell or yolk injections.*

Molecular analysis of genome editing

DNA sequencing of the targeted region of the tyrosinase gene supports effective genome editing. Gene-specific primers were used to PCR amplify the tyrosinase gene (Figure 7). Sanger DNA sequencing revealed large-scale disruption of the DNA sequence directly at the site targeted for disruption (Figure 1).

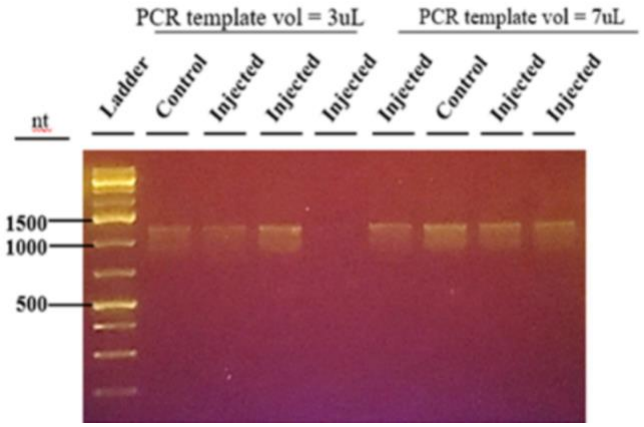


Figure 7. Gel image of PCR product, the size of the expected PCR product is 1113bp. The gel shows DNA amplification of the genomic region in the tyrosinase gene. Sequencing of these PCR products was used to evaluate if the DNA sequence of the genome had been significantly altered.

Tyrosinase knockout phenotypes

Using Cas9 protein and guide RNAs 3 and 6 (Figure 1) resulted in the successful knockout of the tyrosinase gene, as indicated by the lack of black pigment in some embryos. Typical knockout phenotypes compared to control embryos are illustrated in Figures 8 and 9.

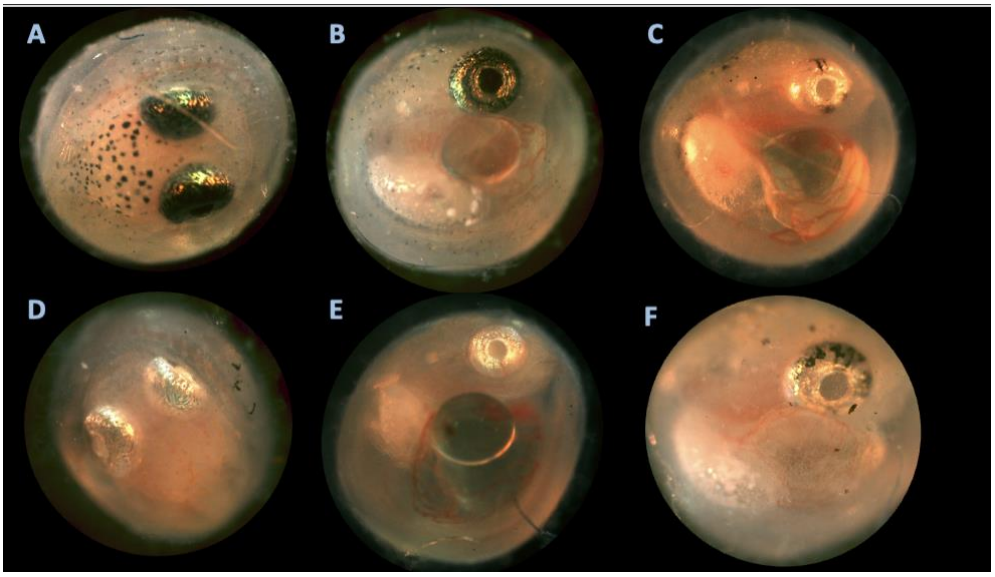


Figure 8. *Tyrosinase knockout embryonic phenotypes. A) Top view of a control embryo with black pigment in the eyes and the melanocytes (black cells/dots on the head and tail). B) Side view of a control embryo with black pigment. C) Side view of a partial knockout embryo. Black streaks in the eye illustrate a mosaic mutant where the gene is knocked out in only some cell lineages. D) Top view of a partial knockout embryo (melanocytes on the tail expressing some pigment). E) Side view of a full knockout embryo with no black pigment in the eye, head, or tail. F) Side view of a partial knockout embryo with some pigment in the eye giving a tiger stripe effect.*

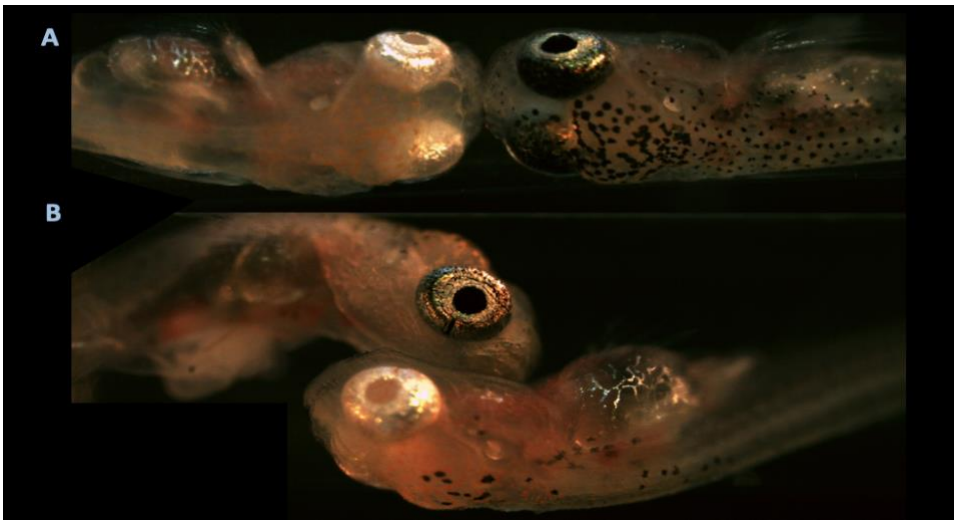


Figure 9*. Tyrosinase knockout larval phenotypes. A) Top view of hatched larvae, the one on the left is a fully knockout out fish compared to the control larva with black pigment on the right. B) Side view of a control larvae on the top and a partial knockout on the bottom. The partial knockout has no pigment in the eye but has pigment-expressing dorsal melanocytes.*

Discussion

Here, I report the first successful gene knockout using CRISPR/Cas9 methodologies in *A. limnaeus.* Evidence supports the induction of DNA editing, which results in a nonfunctional tyrosinase enzyme in the killifish genome. This methodology provides a powerful tool to explore the molecular basis of metabolic dormancy and stress tolerance in this unique model organism.

Cas9 mRNA versus Protein

Initial results using Cas9 mRNA to express Cas9 protein were ineffective at knocking out the tyrosinase gene in early embryos of *A. limnaeus*. We hypothesized that the Cas9 mRNA was not being translated into a protein efficiently, thus preventing genome editing. The 4-hour delay in protein translation from GFP mRNA supports this hypothesis and suggests that early translation of foreign mRNAs is delayed or inefficient in early embryos of *A. limnaeus*. It is highly likely that a delay in Cas9 protein translation until the 8 or 16-cell stage will lead to highly inefficient genome editing. This may be due to multiple factors like slow development (Romney & Podrabsky, 2017) or a species-specific trait that does not allow mRNA to be translated faster. With the Cas9 protein, there is no need for translation to a protein to occur as it is already ready to bind the guide RNAs to produce the gene knockout during the early cell stages of development. This ability to act immediately is likely the reason that editing works with the Cas9 protein, not the Cas9 mRNA.

Site of Injection

Injections into the first embryonic blastomere appear more efficient than yolk injections in delivering reagents into early embryos of *A. limnaeus*. This finding is consistent with previous research on other fish species; injecting into one-cell stage embryos has been shown to be more

efficient because there is a higher chance of generating edits in the first embryonic blastomere stage or one-cell stage (Jao et al., 2013; Krug et al., n.d.; Li et al., 2021). In some fish species, like the African turquoise killifish, injecting into the yolk can lead to little or no genome editing using CRISPR/Cas-9 (Harel et al., 2016). However, in zebrafish Cas-9 will produce edits with both yolk and cell injections (Zhao et al., 2021). My results with *A. limnaeus* are consistent with findings in zebrafish*;* edits are still made with both yolk and cell injections, but there is a higher percentage of edits with cell injections (Figure 6). It is also essential to inject at the one-cell stage because then only one copy of the genome needs to be edited, whereas, at any other stage, there would only be a 50/50 chance of edits, which is also why we prefer to inject in the one-cell stage.

Future plans

In the future, the lab plans to use the edited embryos to establish a line of fish without black/brown pigment. Some future questions to be considered for the following studies would be. What is the effect on the eyes? Does the color change affect sexual selection in this species? Does the color change affect the production of diapausing embryos or vitamin D signaling in the fish and embryos, as observed in (Romney et al., 2018)? These results provide the foundation for future research on this species and will support the use of this unique model organism for mechanistic studies that otherwise would not be possible.

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