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Development of type 2 and type 3 iodothyronine deiodinase knockout *Xenopus tropicalis* using CRISPR/Cas12a gene editing

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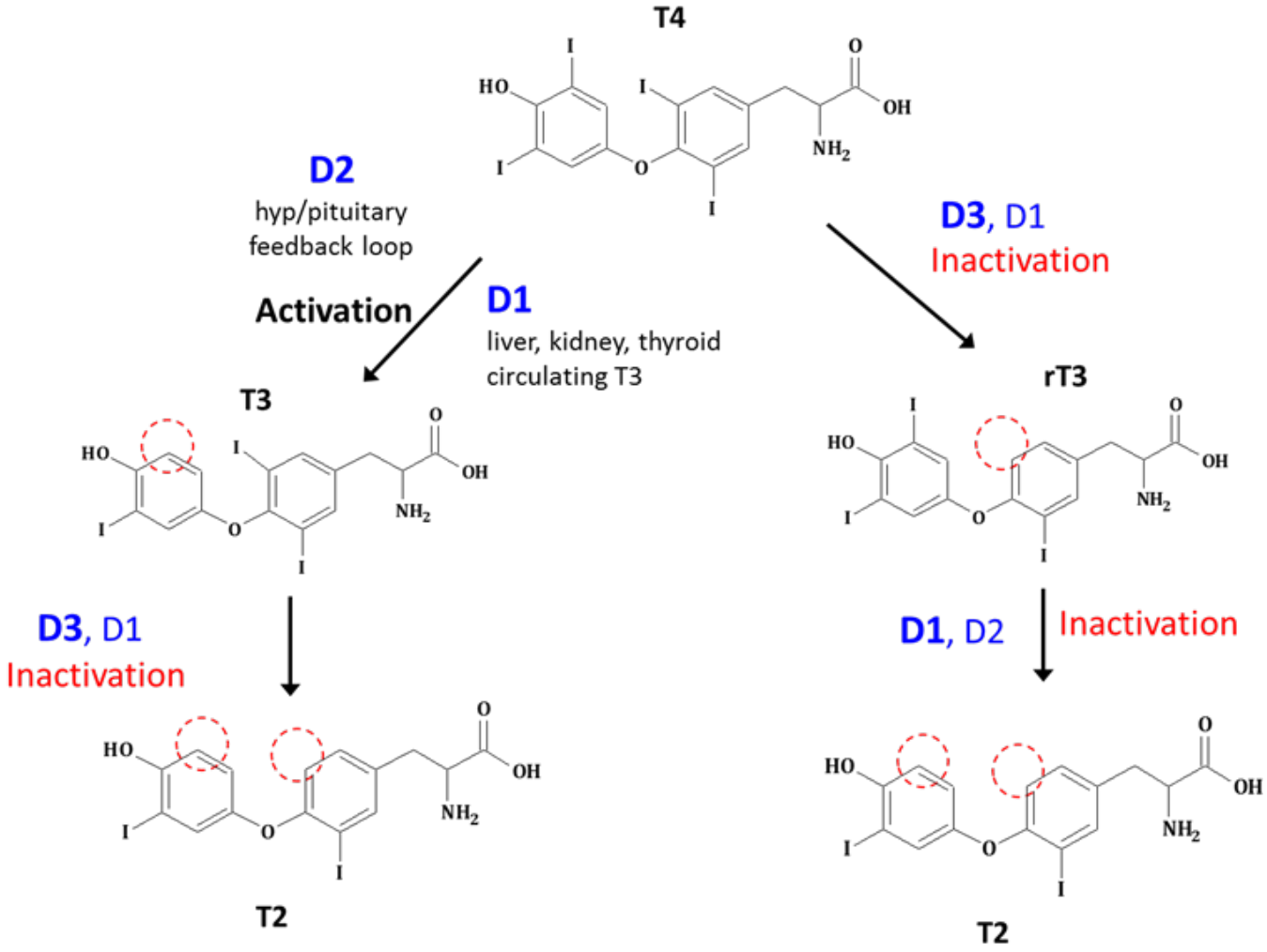
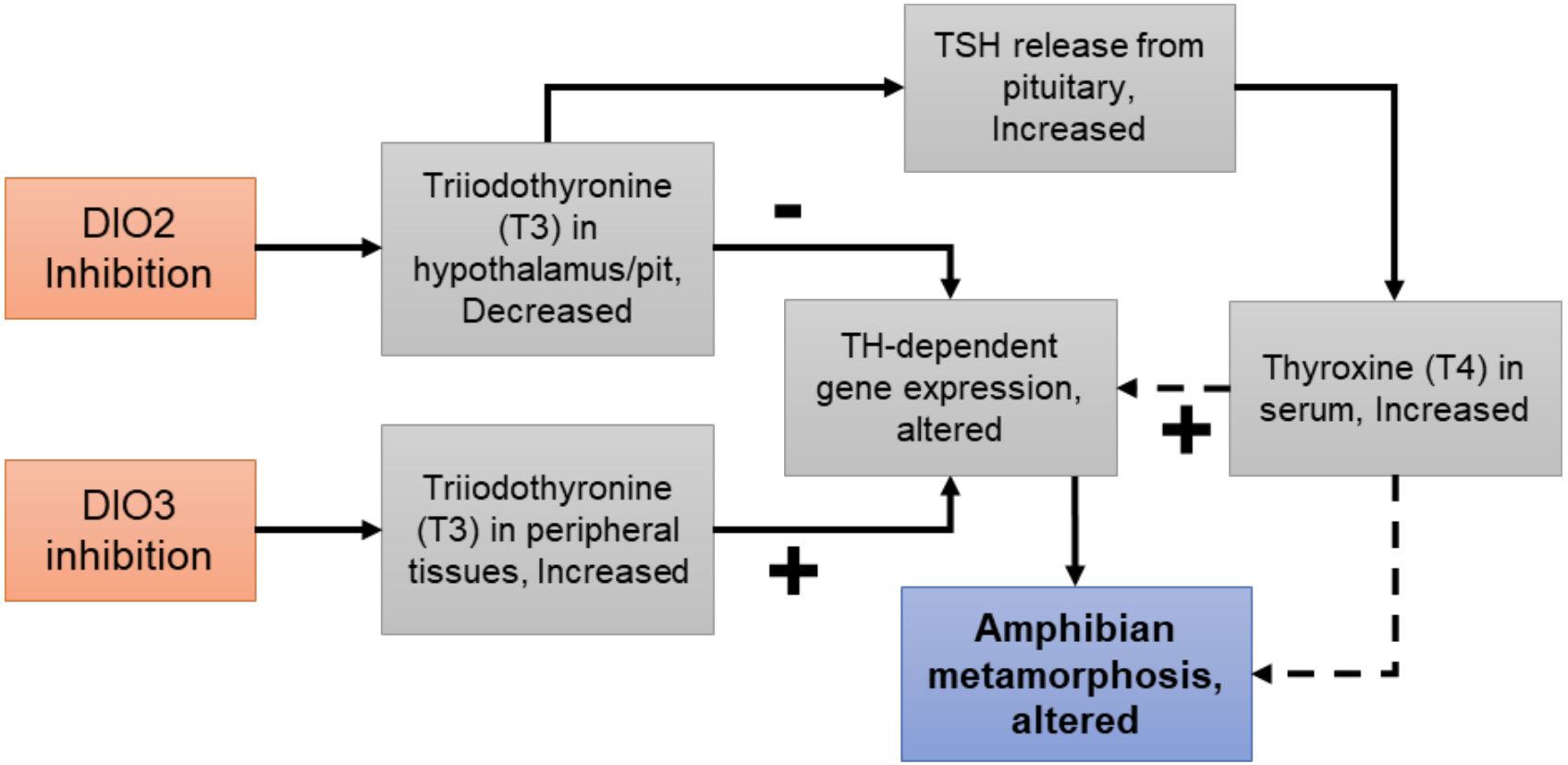
Background

- Iodothyronine deiodinase enzymes (dio1, 2, and 3) catalyze tissue-localized metabolic activation/ deactivation of thyroid hormones (THs)
- We targeted the dio2 (TH activating) and dio3 (TH inactivating) enzymes in this study



Postulated pathway for amphibian deiodinase gene disruption

Purpose of genetic modifications: this work supports the EPA's chemical safety research mission by using an alternative approach for linking molecular mechanisms of thyroid toxicity to apical outcomes relevant to ecological risk assessment



AOP wiki: Adverse Outcome Pathways 190 and 191
aopwiki.org

Overview

- We utilized a CRISPR/Cas12a system to induce DNA cleavage in the dio genes at locations near where the catalytic sites would occur in the translated proteins
  - The Cas12a Protospacer Adjacent Motif (PAM) sites gave us better cutting locations for these genes than those offered by the Cas9 system
  - We also got slightly better efficiency with Cas12a than Cas9 in tests to knock out the tyrosinase gene
  - Cas12a is more convenient; requires only single guide RNA (no tracrRNA)
- Single-cell-stage zygotes were injected with either ***dio2*** or ***dio3*** gene-specific guide-RNA/Cas12a ribonucleo-protein (RNP) complexes

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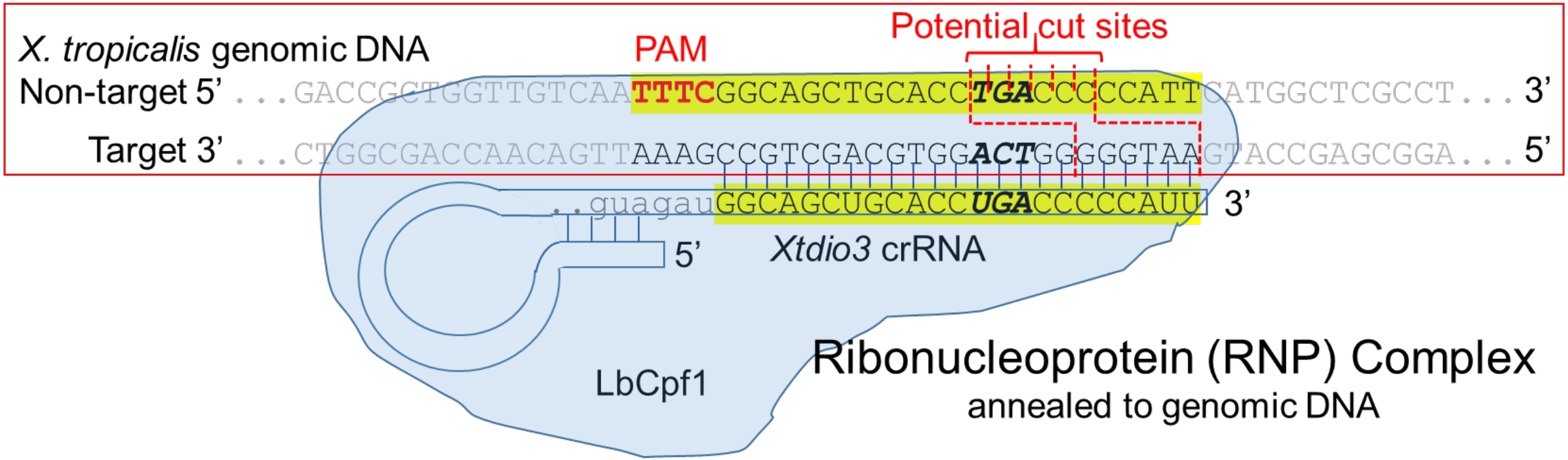
CRISPR/Cas12a (LbCpf1) crRNA design and microinjection methods

- Guide RNA design was aided using CRISPRscan and CRISPR RGEN tools Cas-designer
- The CRISPR guide RNA oligos (crRNA) were synthesized by Integrated DNA Technologies and tested through in vitro cleavage with EnGen Lba Cas12a (LbCpf1) DNA endonuclease (New England BioLabs)
- *X. tropicalis* adults were induced to spawn by hCG injection; eggs were treated with L-cysteine rinse; single-cell zygotes were sorted into 1/9x Modified Ringers with 3% Ficoll
- Zygotes were injected with ~2 nl (20 fmol) of either the dio2 or dio3 RNP complex in Cpf1 working buffer (20mM Hepes pH 7.5, +KCl+MgCl2+TCEP+glycerol) Moreno-Mateos et al. (2017)

https://doi.org/10.1038/s41467-017-01836-2

crRNA-LbCpf1 Ribonucleoprotein complex for dio3



TGA/UGA = Codon for critical selenocysteine amino acid in deiodinase enzyme active site.

PAM = Protospacer adjacent motif

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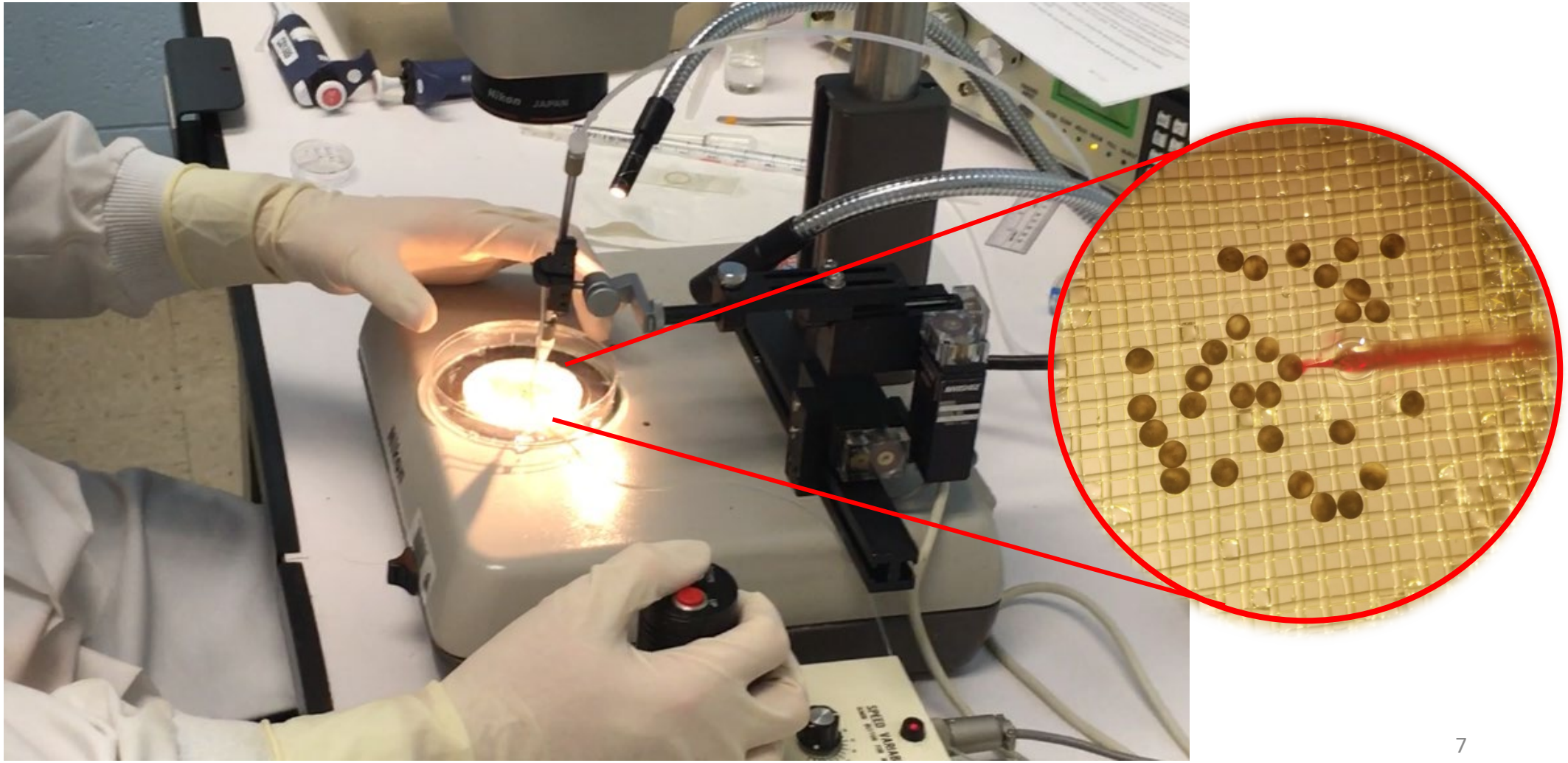
Microinjection of RNP complexes into *X. tropicalis* zygotes



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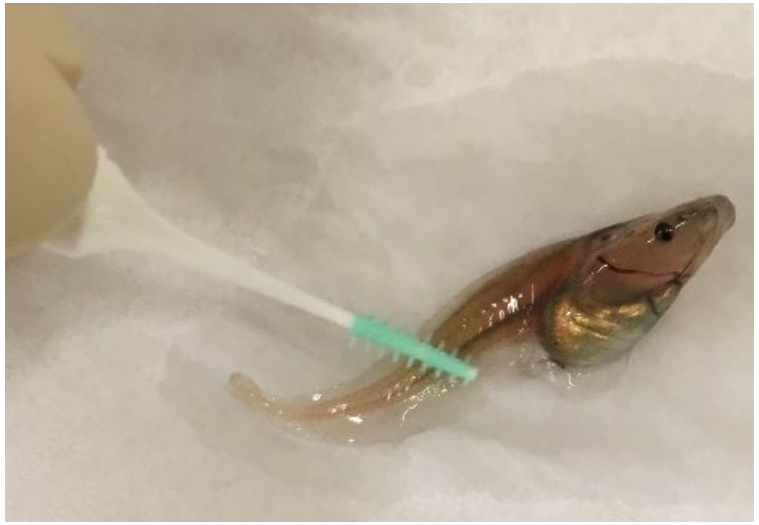
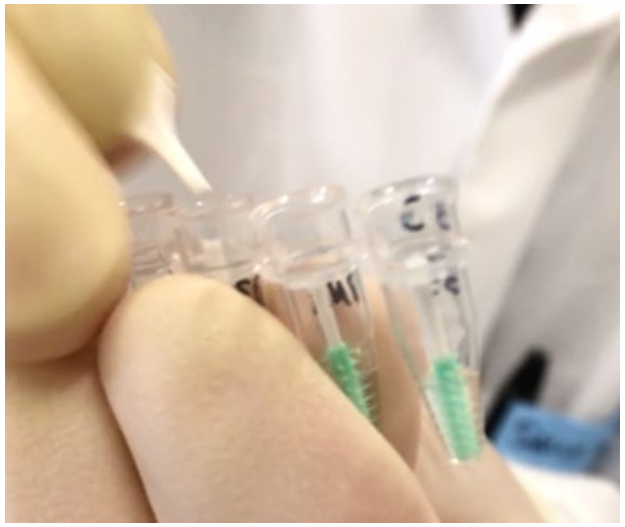
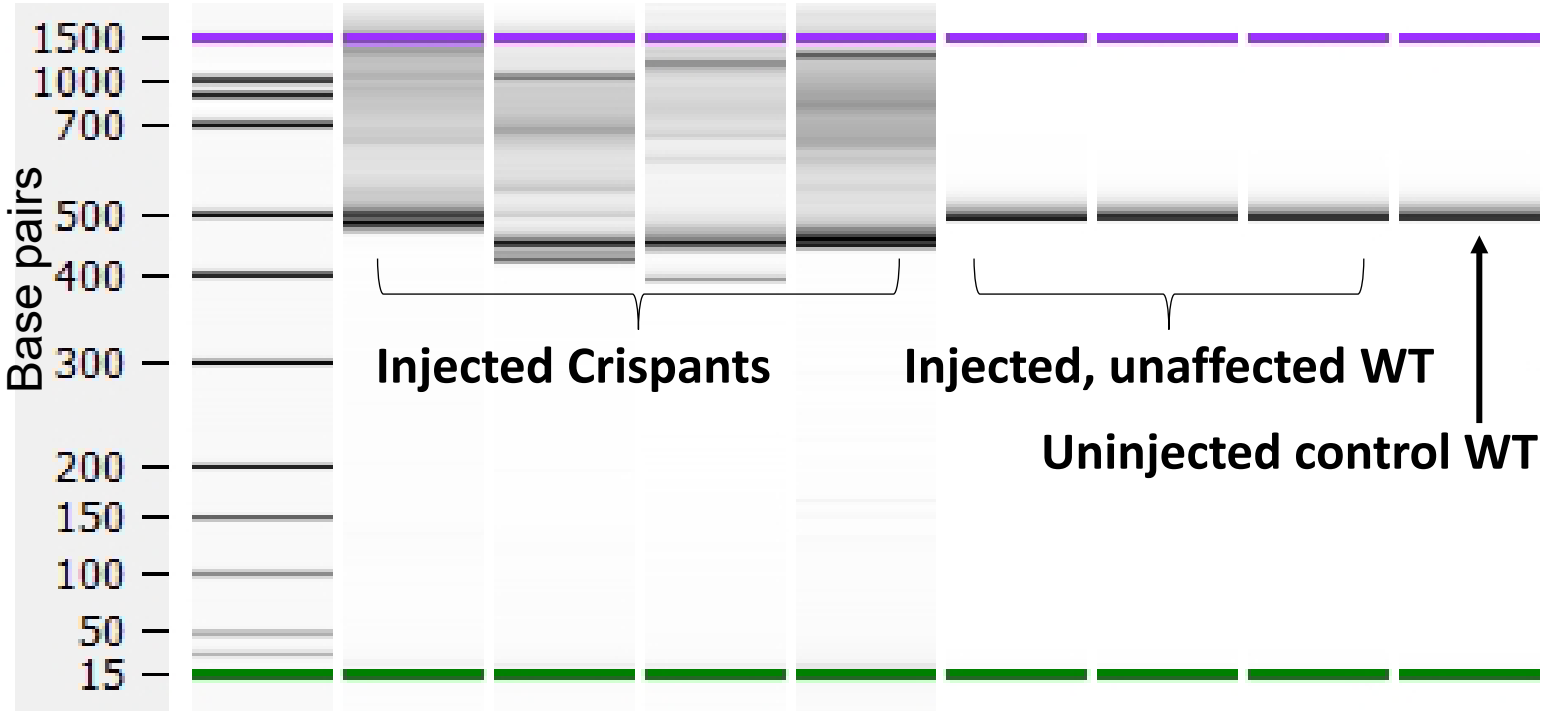
Culture of injected *X. tropicalis* tadpoles

Following 24-30 hours of incubation at 25°C in 1/9x modified Ringers, embryos were transferred to 4.5 L flow-through tanks delivering Lake Superior Water @ ~25 mL/min.

DNA extraction and polymerase chain reaction (PCR) genotype screening







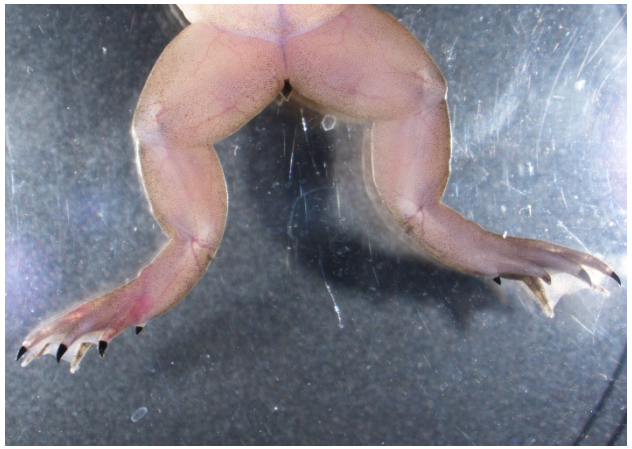
Agilent Bioanalyzer capillary electrophoresis. Crispants (animals with disrupted genes) show multiple bands due to heteroduplex formation during PCR.

Results

- PCR genotyping at 3 weeks post-injection revealed putative mutations at a rate of >60% in dio2-RNP-injected tadpoles, and 50% in dio3-RNP-injected tadpoles
- Apical outcomes included asynchronous metamorphosis in dio2 and high mortality in dio3 crispants

Apical outcomes of deiodinase gene disruption

Injected, unaffected **WT** at 45 days
Nieuwkoop and Faber (NF) stage 66

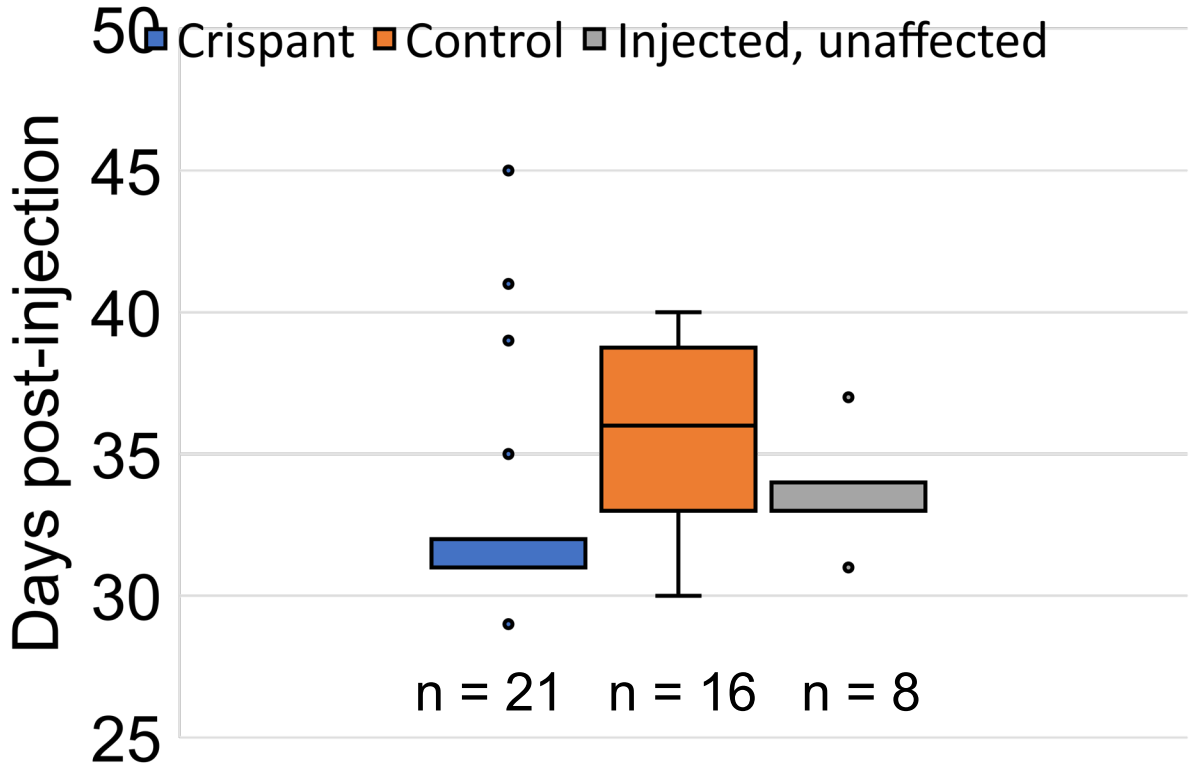


dio2 crispant at 44 days
Asynchronous metamorphosis
(differential limbs)

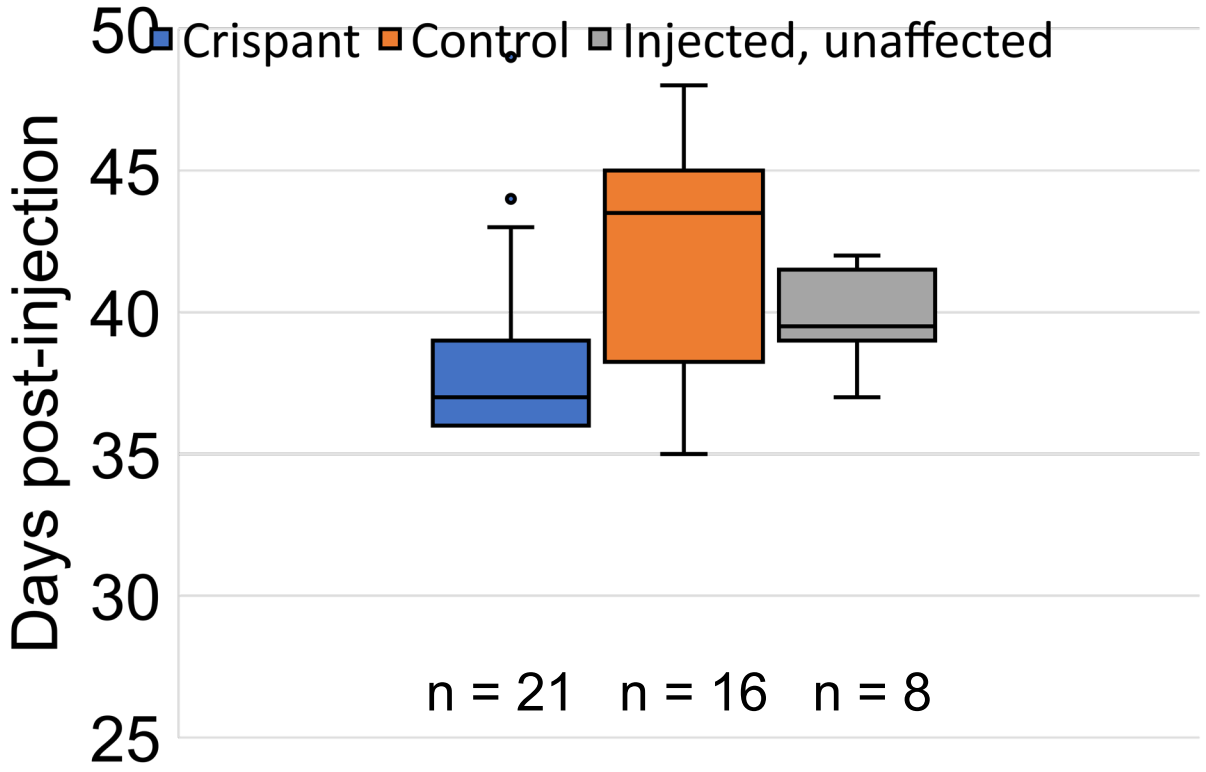
dio3 crispant at 26 days
Precocious metamorphosis/early
tail and gill resorption

Metamorphosis timing in *dio2* crispant and WT phenotypes

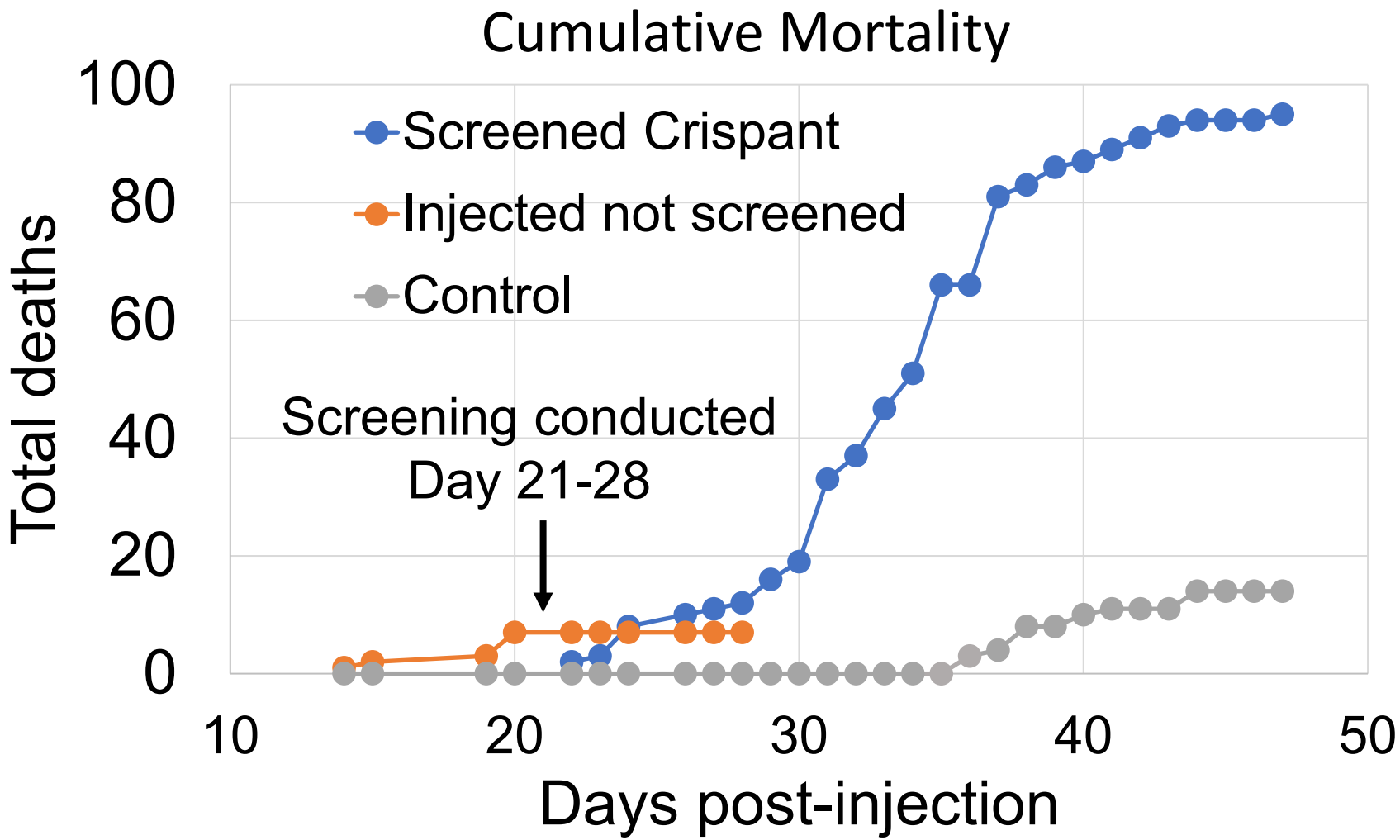
X. tropicalis dio2 CRISPR Days to Stage 58



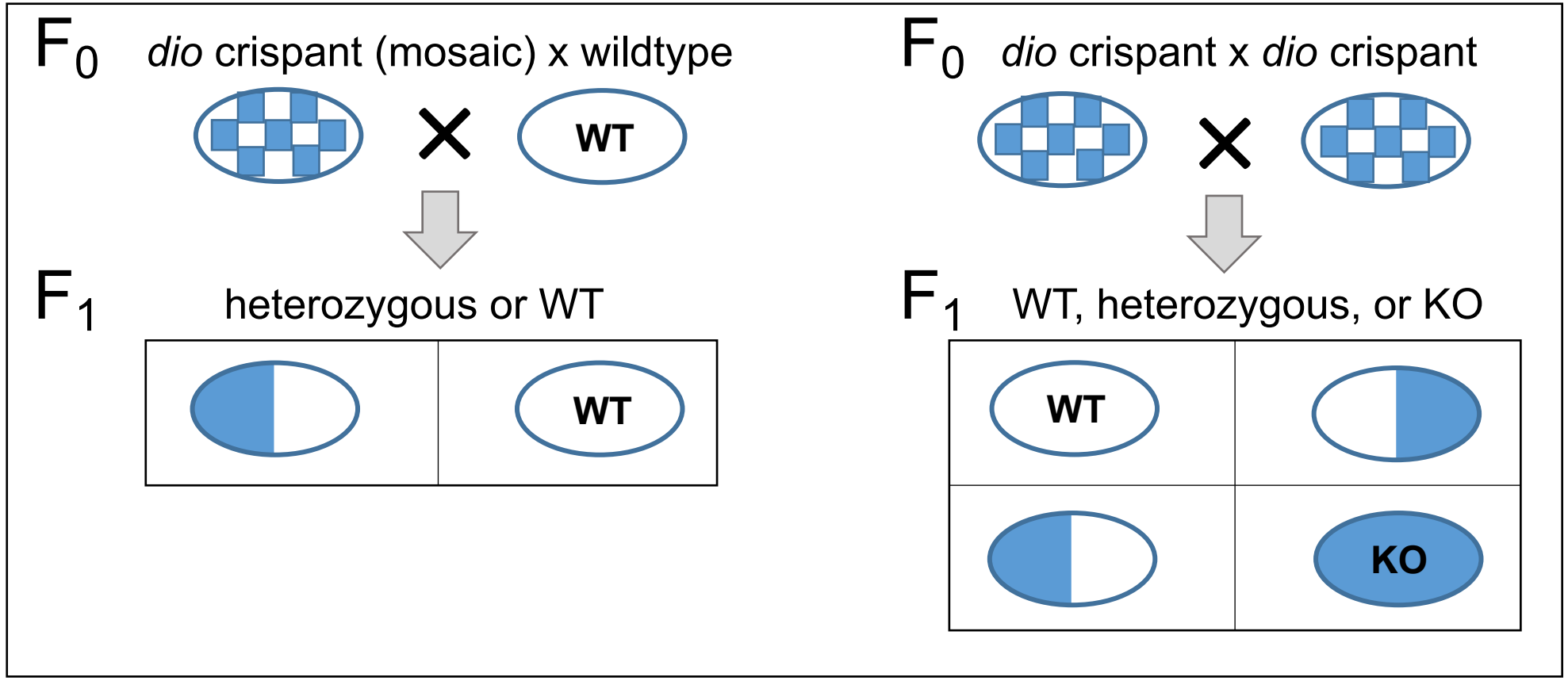
X. tropicalis dio2 CRISPR Days to Stage 62



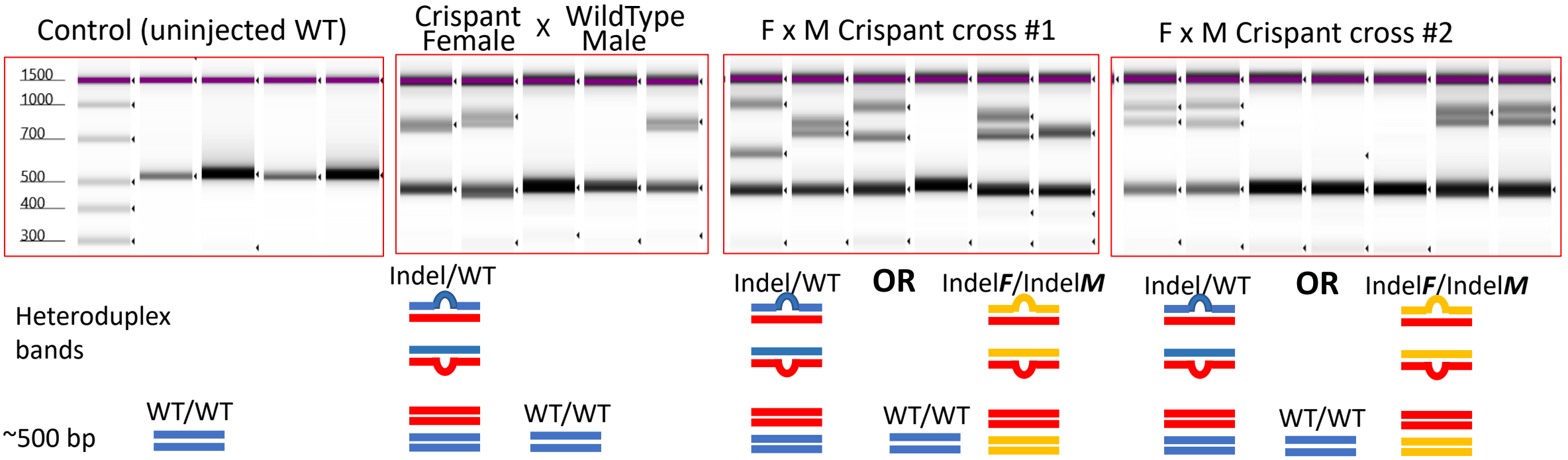
Precocious metamorphosis leads to high mortality in *dio3* crispants



Cross breeding conducted



Example *dio2* crispant cross F_1 genotyping results and interpretation hypothesis



Summary:

- Genotyping results show indel mutations occurred in CRISPR/Cas12a *dio* gene-edited frogs;
- Crispant F0 apical outcomes indicate putative loss of dio activity leading to altered metamorphosis.

Next steps:

- Genotyping of *dio3* crosses and sequencing;
- Evaluation of F1 apical outcomes;
- Multiple *dio* knockouts?

Acknowledgements

EPA/GLTED Thyroid team

Jessica Christensen

Jeff Denny

Thomas Lerdall

Joe O'Flanagan

Jennifer Olker

Badger Technical Services

Stephanie Wisniewski

This work was funded wholly by the U.S. Environmental Protection Agency.

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