Environment and Natural Resources Trust Fund (ENRTF) M.L. 2019 Minnesota Aquatic Invasive Species Research Center Subproject Work Plan

Today's Date: August 28, 2020

Date of Work Plan Approval:

Subproject Completion Date: December 31, 2022

SUBPROJECT TITLE: MAISRC Subproject 36: RNA-interference screens for zebra mussel biocontrol target genes

Subproject Manager: Daryl M. Gohl, Ph.D.

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Location: University of Minnesota, Twin Cities Campus and collecting sites from area lakes. Project outcomes will impact zebra mussel management throughout Minnesota.

Total Subproject Budget: $260,374

Amount Spent: $0

Balance: $260,374

Legal Citation: M.L. 2019, 1st Special Session, Chp. 4, Art. 2, Sec. 2, Subd. 6(a)

I. SUBPROJECT STATEMENT:

This project will develop methods of RNA-interference (RNAi) for disrupting the expression of genes that are important for the spread and establishment of invasive populations of zebra mussels. The results will reveal genetic weak points, and will establish tools and methods for effective RNAi-based biocontrol technologies that could potentially be scaled up to open water applications in the future. Minnesota has focused its efforts on prevention of zebra mussel spread. State, county and local programs for educating lakeshore property owners and recreational users, for training lake service providers, and for inspecting and decontaminating watercraft improve each year, but still each year more Minnesota lakes and streams are newly infested with zebra mussels. In addition to short-distance spread in clusters of invaded lakes near Detroit Lakes, Brainerd, and Alexandria, long-distance transport is spreading mussels to more pristine regions—most recently, to Red Lake and Lake of the Woods.

Some lakes that are caught soon after they are infested may respond to chemical controls. But about three to five years after a lake is colonized, dense clusters of mussels become so widely distributed within the lake, that the costs and risks to native species of chemical pesticides makes their application impractical. Genetic methods may be effective alternatives in these well-established water bodies. CRISPR/Cas9-induced mutations can be spread to high frequency in pest populations by "gene drives;" this includes mutations that are very harmful (e.g. those that kill male offspring). This theoretical expectation has been borne out by results from laboratory-population experiments (for example in *Aedes aegypti*: the mosquito that carries Zika virus). Yet the risks for spread to native biota are considered to be so high, that environmental release of CRISPR/Cas9 for invasive species control is not a viable option, at present. Moreover, effective methods for culturing genetically modified zebra mussels do not currently exist, making the application of CRISPR/Cas9 less practical in zebra mussels at present.

RNAi technologies are considered safer because the genome of the target species is not altered and in most cases, RNAi effects are not passed to offspring. Instead of mutating an organism's DNA, RNAi works by blocking the expression of genes controlling important biological processes, leading to fitness effects and suppressed population growth of the invasive species. Finally, RNAi-based biocontrol is particularly well suited for zebra mussels since it does not require long-term propagation of the modified mussels. Instead, RNAi reagents are delivered to mussels by feeding them microbes expressing the interfering RNA, which the mussels filter out of the water.

We laid the groundwork for research on RNAi for biocontrol by sequencing and assembling the 16 chromosomes of the *Dreissena polymorpha* genome. This genomic map allowed us identify a long list of target genes involved in critical processes. This is the first step in any genetic biocontrol technology, because the DNA sequence of a target gene must be known so that RNAi (or CRISPR/Cas9) reagents can be designed to recognize that gene. The zebra mussel target genes we chose are involved in processes that are critical for growth and invasive spread—for example, genes controlling growth and calcification of shells, genes that build the "byssal threads" that mussels use to attach to boats or to vegetation on the bottom of lakes, and genes that protect cells from damage under heat stress that occurs every summer in Minnesota. We also identified genes whose knockdown is likely to lead to high mortality, such as genes that control function of the nervous system. RNAi approaches have been applied in many other animals (including other oysters or mussels), but our work represents the first application of this technique in zebra mussels.

This proposal aims to apply RNAi to identify genetic weak points in zebra mussels and to develop the tools to manipulate these critical genes as a stepping-stone towards targeted genetic biocontrol efforts. First, we will create the molecular biology reagents and methods for knocking down dozens of target genes. Then, we will deliver these reagents to live mussels, cultured in the laboratory, and test for a range of molecular and phenotypic effects (described below).

II. SUBPROJECT ACTIVITIES AND OUTCOMES:

ACTIVITY 1 Title: Design, production, and testing of double stranded RNA (dsRNA)-producing bacterial strains.

Description: RNAi is a system for selectively inactivating a gene by targeting the gene's messenger RNA (mRNA) product. The RNAi response is elicited by exposure to dsRNA. In Activity 2, we will deliver the dsRNAs to zebra

mussels by feeding them engineered strains of *E. coli* bacteria. Like other bacteria in their diet, mussels filter the *E. coli* from the water, but these bacteria will express dsRNA fragments corresponding to zebra mussel target genes. Short zebra mussel DNA fragments will be inserted into a plasmid—a circular DNA vector that the *E. coli* maintains and replicates, making many copies of the zebra mussel DNA fragments. The plasmids are engineered such that they synthesize dsRNA molecules targeting zebra mussel genes in response to a chemical inducing agent. Thus, these engineered *E. coli* strains convert DNA fragments from zebra mussels into dsRNA molecules which we will use to silence the expression of these genes in zebra mussels.

Activity 1 tasks are as follows. First is to complete identification of the target genes in the zebra mussel genome. Next is to design the dsRNAs that will block the expression of target genes. The final step is the design of feeder plasmids, and tests to verify that these plasmids can be induced to produce dsRNAs. Feeder plasmids that pass those tests will then be archived in their host *E. coli* strains for use in Activity 2. This whole process will likely need to be repeated throughout the project duration as additional targets are identified or protocols are optimized based on experimental outcomes.

ACTIVITY 1 ENRTF BUDGET: $104,918

Outcome	Completion Date
1. Identify target genes in zebra mussel genome and design dsRNA expression plasmids.	6/30/21
2. Produce dsRNA expression plasmids and create and archive bacterial strains.	12/31/22
3. Verify inducibility of dsRNA constructs.	12/31/22

ACTIVITY 2 Title: Establish phenotypic assays and carry out RNA interference screens for genes affecting zebra mussel feeding success, survival, reproductive function, shell growth, and byssal thread attachment.

Description: We will measure feeding success by adding an *E. coli* strain that is engineered to produce a fluorescent protein to water, and scoring the decline in fluorescence over time. We will screen survival by counting live and dead mussels screened using the shell gape response—dead mussels will open their shells and not close them when their soft tissue is stabbed with a probe. Microscopic examination of dissected gonad will be used to measure tissue growth, and the percent of gonad tissue that is developing into eggs or sperm. Conveniently, zebra mussels can also be induced to spawn using serotonin, so we will run these inductions on animals after treatment with RNAi. Shell growth will be measured with calipers, and shell structural abnormalities will be assayed using electron microscopy (EM). Some of the genes we plan to silence direct the way calcium is deposited as the shell grows and hardens. RNAi silencing of similar genes in pearl oysters results in abnormal layering of the shell that is detectable by EM. Zebra mussels will rapidly grow and reattach byssal threads after they are severed, overnight in healthy animals. We will weigh, measure and count the number of reattached byssal threads in animals treated with RNAi to each of dozens of byssal genes we have identified. In each of these screens of RNAi-treated animals (as well as control, untreated animals) we will also directly test for gene expression levels, either by quantitative RT-PCR amplification or by RNA Sequencing so that we can associate any phenotypic effects to known levels of gene knockdown. In year one, we plan to establish these phenotypic assays and do initial testing and determination of dosing conditions for the RNAi constructs. In year two, we will conduct a large-scale screen of RNAi constructs against dozens of target genes which are likely to have a role in processes critical for zebra mussel growth, shell or attachment fiber formation, survival, or reproduction.

ACTIVITY 2 ENRTF BUDGET: $155,456

Outcome	Completion Date
1. Establish laboratory rearing protocols and tests for feeding, reproductive output, survival, shell growth, and byssal thread attachment).	12/31/21
2. Treat zebra mussels with RNAi reagents developed in activity 1, then run the tests above to determine the phenotypic effects of RNAi treatments.	12/31/22

3. At the same, verify that RNAi treatments block the expression of target genes, using RT-qPCR amplification or direct RNA-Sequencing	12/31/22

III. DISSEMINATION:

Description:

We will report the progress of this project and the results of our research at the annual MAISRC AIS Research and Management Showcase. In addition, we plan to publish the results of our experiments in traditional peer reviewed journals where appropriate and will ensure that the information resulting from these studies remains freely publicly available through posting any resulting papers on bioRxiv (the biology preprint server) and depositing genomic or other data in appropriate repositories.

IV. ADDITIONAL BUDGET INFORMATION:

A. Personnel and Capital Expenditures

PI (Daryl Gohl); Researcher 7; $28,788, 8.3% FTE (1 calendar month effort/year)

Technician (TBD); Researcher 2/3; $62,212, 50% FTE (6 calendar months effort/year).

Work Study Student (TBD); $9,360; 25% FTE (3 calendar months effort/year).

No capital expenditures for equipment are planned for this proposal.

Explanation of Capital Expenditures Greater Than $5,000:

None.

Explanation of Use of Classified Staff:

PI (Daryl Gohl); Will manage the project, advise on technical aspects of synthetic DNA and bacterial strain production, oversee qPCR and RNA-Seq experiments, and will supervise UMGC technician.

Technician (TBD); Will conduct produce RNAi expression strains, test their inducibility, help with the collection, husbandry, and phenotypic analysis of zebra mussels, and do lab experiments to quantify the knock-down of target genes.

Work Study Student (TBD); Will assist with lab and field work during the summer months of years 1 and 2.

Total Number of Full-time Equivalents (FTE) Directly Funded with this ENRTF Appropriation:

Enter Total Estimated Personnel Hours for entire duration of subproject: 1560	Divide total personnel hours by 2,080 hours in 1 yr = TOTAL FTE: 0.75

Total Number of Full-time Equivalents (FTE) Estimated to Be Funded through Contracts with this ENRTF Appropriation:

Enter Total Estimated Contract Personnel Hours for entire duration of subproject: 693.33	Divide total contract hours by 2,080 hours in 1 yr = TOTAL FTE: 0.33

V. SUBPROJECT PARTNERS:

A. Partners outside of subproject manager's organization receiving ENRTF funding

Scott Ballantyne, PhD; $52,428, 16.67% FTE (2 calendar months/year): Will help identify target genes, design experiments to optimize RNAi, oversee the production of dsRNA and GFP expressing bacterial strains, and guide the assays for zebra mussel feeding and survival.

Michael McCartney, PhD; $50,000, 16.67% FTE (2 calendar months/year): Will provide guidance and hands-on effort on the collection, husbandry, and phenotypic analysis of zebra mussels, and will assist in the scientific oversight of the UMGC technician carrying out the in vivo testing.

B. Partners outside of subproject manager's organization NOT receiving ENRTF funding

None.

VI. LONG-TERM- IMPLEMENTATION AND FUNDING:

If this project is successful, we will have identified not only a set of genes that control legitimate weaknesses in the biology of zebra mussels, but also a means for targeting and manipulating these genes. Thus the groundwork for genetic biocontrol will be in place. Ultimately, deploying an RNAi-based gene knock down strategy in lakes and rivers will require additional work to identify a suitable delivery mechanism for RNAi in the wild which is safe, effective, and palatable to the public. There is an existing grant to USGS to identify such a delivery mechanism, and much additional work to do to evaluate these gene knockdowns in natural populations. Pilot testing in impoundments, for example would allow examination of genes whose phenotypes require longer-term evaluation, such as those controlling fecundity, fertility, or sex determination. Ultimately, funding from other sources, such as USBR or the GLRI/Great Lakes Commission, or technology commercialization approaches may also be pursued in order to implement these technologies should they prove effective.

VII. REPORTING REQUIREMENTS:

- Subproject status update reports will be submitted by January 31 and July 31 each year of the project
- A final report and associated products will be submitted between January 1 and February 15, 2023

VIII. SEE ADDITIONAL WORK PLAN COMPONENTS:

A. Budget Spreadsheet
B. Visual Component or Map
C. Parcel List Spreadsheet – N/A
D. Acquisition, Easements, and Restoration Requirements – N/A
E. Research Addendum