AGRICULTURAL RESEARCH FOUNDATION
INTERIM REPORT
FUNDING CYCLE 2016 – 2018

TITLE: *The Role of the Salmon Gut Microbiome in Resisting Parasitic Infection*

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COOPERATORS: *Aquatic Animal Health Laboratory, Oregon State University*

SUMMARY:

The goal of this project is to clarify the contribution of the gut microbiome to salmonid ecophysiology, with the long-term objective of elucidating the microbiome's specific role in controlling intestinal parasitic infection. In salmonids, water temperature and parasitic infection are connected, as increases in water temperature yield increased susceptibility to infection, and consequently increased mortality. In other vertebrates, the microbiome has been shown to impact the host's physiological response to thermal stress and can also control parasitic infection. We hypothesize that (1) the gut microbiome contributes to salmonid immune homeostasis, (2) increases in water temperature perturb the gut microbiome and its contribution in this regard, and (3) this results in increased susceptibility to parasitic infection, especially by *Ceratomyxa shasta*. To test our hypothesis, we are conducting several laboratory experiments in the model salmonid *Oncorhynchus mykiss* (rainbow trout). Our focus over the past year has been on (1) quantifying how changes in water temperature effect the gut microbiome and (2) establishing a research infrastructure that enables us to determine the microbiome's contribution to salmonid physiology. We recently leveraged these efforts to submit a preliminary proposal to the National Science Foundation. Furthermore, our efforts have resulted in a new collaboration with the Oregon Department of Fish and Wildlife, wherein we seek to determine if the gut microbiome contributes to the defined differences in fitness between wild and hatchery fish. Over the next year, we will use our research infrastructure to conduct two laboratory experiments - one of which is on-going - that collectively determine how increasing water temperature perturbs the gut microbiome to impact salmonid physiology. The results of these studies will be used to obtain federal funds to resolve our long-term objective of how ecological variation and the microbiome interact to influence parasitic infection, and whether manipulations to the microbiome can control or prevent infection. Our ultimate hope is that our efforts will contribute to improved preservation of this important natural resource which faces increasing peril due to habitat destruction and infection.

OBJECTIVES:

Our original proposal focused on the following objectives:

- *Quantify the effect of changes in water temperature on the salmonid gut microbiome.*
- *Quantify the relationship between changes in the microbiome and* C. shasta *infection*.
- *Demonstrate that the gut microbiome protects salmonids from* C. shasta *infections.*

The ultimate goal of the proposal requires resolving the contribution of the gut microbiome to infection, which we proposed to disentangle by producing microbiome-depleted fish that have been exposed to an antibiotic and comparing their infection burden to microbiome-normal fish. We targeted oxytetracycline (OTC) as the antibiotic for this analysis given its wide use in *O. mykiss* aquaculture, and that prior work indicates OTC reduces the cellular abundance of gut

bacteria. However, subsequent discussions with industry experts and scientists prompted us to validate OTC's effect on the microbiome in our facility, as there is growing concern that gut bacteria are developing resistance to the drug. Additionally, we decided that instead of targeting parasitic burden as a singular factor that is influenced by the gut microbiome, we could maximize our ability to acquire federal support for this work by instead evaluating a wide range of physiological markers in microbiome-normal and microbiome-depleted fish across temperatures (note: dropping the parasite exposure cohort made this a cost neutral decision). Consequently, our project is now designed to interrogate the role of the microbiome in a variety of physiological pathways in *O. mykiss*, including immunity, metabolism, and stress. This potentiates a wider array of funding opportunities that are relevant to aquacultural, ecological, and evolutionary problems related to salmonids, including our long-term objective regarding parasitic burden. As a result, we amended our objectives accordingly:

- *Quantify the effect of changes in water temperature on the salmonid gut microbiome [completed]*
- *Measure the impact of OTC-administration on the cellular abundance and biodiversity of the gut-microbiome [on-going]*
- *Quantify how the gut microbiome mediates the physiological response to thermal stress*

PROCEDURES:

Animal experiments. We obtain *O. mykiss* fry from a regional hatchery and grow them at the John L. Fryer Aquatic Animal Health Lab (AAHL). AAHL is capable of precisely modulating each tank's water temperature, a feature that we have capitalized on in our experiments. Influent water is UV-treated to reduce the effect of metacommunity variation over time. Fish are randomly divided into exposure cohorts, where each cohort contains multiple tanks of fish that house multiple fish. This enables us to account for tank and inter-individual variation in our analyses. Fish are given four weeks to microbiomically equilibrate to their tank prior to initiating an experiment. Over the course of each experiment, animals are randomly selected from each tank and sacrificed. Their intestinal contents are collected for microbiome profiling, and tissues are stored for physiological profiling (e.g., immune status). We collect a baseline sample at the start of an experiment (i.e., pre-exposure) and then sample fish on a weekly basis, as well as the final time point. Additionally, we collect weekly fecal and water samples from tanks for microbiome profiling.

Temperature conditions. We modulated tank water between 14°C and 18°C in our initial investigation of the effect of water temperature on the *O. mykiss* gut microbiome. Because the upper thermal limit of *O. mykiss* is 23°C and because we ultimately want to determine the contribution of the gut microbiome to how salmonids adapt to water temperatures near these limits, where they are especially sensitive to infection, we obtained IACUC approval (Aug. 24, 2016) to expose fish to water temperatures over a broader range (7°C to 23°C). Consequently, our third (redefined) objective will evaluate the microbiome's contribution to physiology at several temperatures across this range. Our ACUP also approves the use of OTC administration over the duration of these investigations.

Microbiome-depletion. Germ-free rainbow trout have not been developed, so we will use dietary administration of antibiotics to deplete microbiomes. Microbiome-depleted cohorts will be fed OTC-medicated BioOregon BioTrout feed (Terramycin 200® for fish) with a dosage of 3.0g/100lbs/day per FDA guidelines for 14 days. Control fish will be fed BioOregon BioTrout pellets without OTC. Colony forming units will confirm microbiome depletion.

Physiological profiling. Animal weight and length will be measured before necropsy, which will include collection of intestines, liver, spleen, and blood. Blood will be used to quantify plasma

cortisol and glucose levels as indicators of a stress response. Liver tissue will be used to confirm tissue exposure to OTC. The intestinal and spleen tissue will be subjected to qPCR against a panel of immune, stress, and serotonergic markers, including: TGF-β, TNF-α, and IL-1β, 5HT, HSP70. Δ6–desaturase activity will be quantified in intestinal microsomes to profile fatty acid metabolism.

Microbiome profiling. Evacuated intestinal contents, fecal samples, and water are subject to DNA extraction using MoBio Power Soil kits. DNA then undergoes V4 16S rRNA PCR amplification, sequencing on OSU's Illumina MiSeq, and biodiversity analysis using QIIME. We have also subjected DNA to metagenomic sequencing using NexteraXT library preparation and OSU's Illumina HiSeq3000. Microbial genes and their functions will be predicted from metagenomes using ShotMAP. Beta-diversity will be measured as UniFrac for taxa and Bray-Curtis for genes.

SIGNIFICANT ACCOMPLISHMENTS TO DATE:

We demonstrated that increasing the water temperature that rainbow trout is exposed to results in gut microbiome diversification, but that this effect is reversible. We grew two cohorts of microbiome-normal fish at 14°C for over the equilibration period and then progressively increased the temperature of one cohort to 18°C. These fish were maintained at this temperature for one week before being progressively returned to 14°C. Microbiomic analysis of these fish finds that there is no difference between cohorts prior to temperature elevation, but that 5 days of exposure to 18°C results in a significant alteration to the biodiversity of the microbiome. However, after returning fish to 14°C, there is weak support for a lasting distinction between the two cohorts. These results indicate that the gut microbiome is sensitive to changes in water temperature and that some of the temperature induced changes in salmonid physiology could be due to a perturbation to the gut microbiome. As part of this study, we generated the first shotgun metagenomes from the rainbow trout gut microbiome, which we are currently analyzing to assess the metabolic potential of gut bacteria.

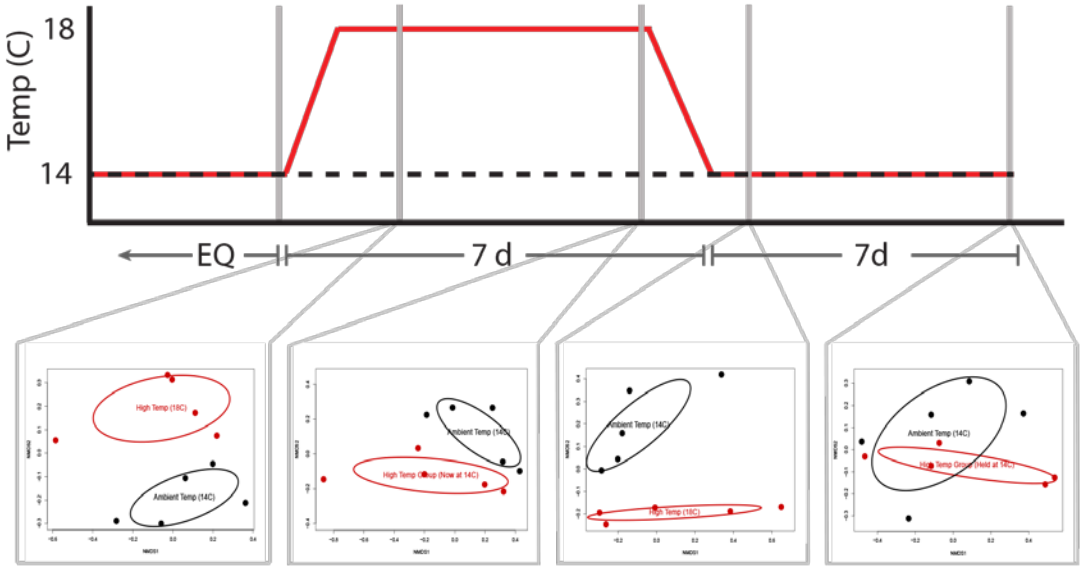

Figure 1 - The results obtained from our first experiment. Top: Two cohorts of fish were exposed to ambient (black) and varying (red) temperatures. Microbiome samples were collected from fish over time (grey bars). Bottom: The results of 16S rRNA biodiversity

ordination (NMDS) analysis for 4 of the 5 sampling points are indicated (the baseline sample had no significant differences between cohorts). Points in these ordinations correspond to samples from the ambient (black) and temperature varying (red) cohorts. Ellipses represent 95% confidence intervals

We also obtained IACUC approval for the two experiments that correspond to remaining objectives. In the first, which is on-going, we are growing rainbow trout using a standard diet or a corresponding diet that is medicated with OTC, and will quantify the effect of OTC administration on gut microbiome abundance and diversity. We anticipate that this study will be completed on March 15, 2017 and that it will determine the effect of OTC exposure on the rainbow trout gut microbiome in our facility. If there is no effect on the gut microbiome, we will explore additional antibiotics before initiating the next experiment, which require microbiome-depleted animals. In the second planned experiment, we will expose microbiome-normal and microbiome-depleted rainbow trout to a variety of water temperatures and quantify the contribution of variation in the gut microbiome to temperature-induced physiological variation in *O. mykiss*. We anticipate observing a significant difference in temperature-induced changes in physiology between microbiome-normal and microbiome-depleted fish. This experiment is scheduled to begin in June 2017. Collectively, these studies serve as the basis for our ultimate objective, which will add the extra variable of parasitic exposure to model how the gut microbiome and temperature interact to influence parasitic infection.

We initiated a collaboration with the Oregon Department of Fish and Wildlife. Our collaborators are collecting wild and hatchery raised fish that are sourced from the same water supply and we will interrogate the differences in their microbiomes. We will use this information to develop a study aimed at determining the impact of aquacultural practices on the gut microbiome and the consequences this has on the fitness of hatchery raised fish that are released into the wild. ARF funds are not being committed to this new line of investigation, but the results of our ARF-funded work served as the basis for launching this investigation.

Additional accomplishments include:

- We have submitted a preliminary proposal based on our initial results to the National Science Foundation (IOS).

- The PhD student leading the described work received 2-quarters of support from the ODFW Fish Health Graduate Research Fellowship.

- As noted in our application, we received additional support from the OSU Department of Microbiology (Pernot Award) and have prioritized spending those funds down given their shorter time horizon. We anticipate exhausting all ARF funds by Oct. 2017.

ADDITIONAL FUNDING RECEIVED DURING PROJECT TERM:

Based in part on the research we have conducted thus far, the Oregon State Department of Fish and Wildlife has committed $10,000 to a new, but related, collaborative line of research. Together, we will determine how the gut microbiome differs between hatchery-raised and wild salmonid broodstocks, and identify associations between riparian land usages and salmonid gut microbiome composition. Ultimately, we hope to build upon this line of investigation to ascertain how aquacultural and land usage practices impact the salmonid gut microbiome to impact fitness.

Additionally, ODFW has provided a 2-quarter fellowship for the PhD student leading the described projects (terminates June 2017).

Finally, we have submitted a preproposal to NSF's Integrative Organismal Systems (Jan. 19, 2017 submission) division that aims to determine the contribution of the salmonid gut microbiome to ecophysiology and thermal stress tolerance.

FUTURE FUNDING POSSIBILITIES:

In addition to the aforementioned NSF funding opportunity, we plan to target a USDA NIFA funding opportunity later in 2017.