UV-C IRRADIATED ROTIFERS AS TOOLS FOR EXPLORING BACTERIAL COLONIZATION IN LARVAL ZEBRAFISH

by

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Studying bacteria-host relationships helps answer many questions regarding the development and health of humans and other animals. In particular, the gut microbiota play a critical part in neurological development, digestion, and overall immune system health. Zebrafish are one of the most commonly used model organisms; they are vertebrates that share a similar genetic structure to that of humans and are fairly transparent during their larval stage, allowing imaging. Additionally, they are capable of being raised devoid of any pre-existing bacteria, serving as an ideal canvas for controlled experiments examining effects of particular species of bacteria. One barrier when working with larval zebrafish is that they must be fed 7 days post fertilization, potentially introducing unwanted bacteria from their preferred living food sources. Little research has been done into feeding larval zebrafish while still controlling their gut microbiome. In this paper, we describe a method to manipulate rotifers, aquatic microorganisms commonly fed to larval zebrafish, by UV-sterilizing them and feeding them to larval zebrafish in combination with fluorescently labeled zebrafish derived bacteria. This enables experiments further into zebrafish development, allowing for a deeper understanding of bacteria-host interactions.

Acknowledgements

I would first like to thank Raghu for welcoming me into the lab in January of 2022. Initially lacking direction and ambition in my academic career, he was the one who molded me into the competent physicist, scientist, and researcher I am today. I also thank the two other members of my committee, Jesse Feddersen and Eric Corwin, for their continued support throughout this project. In particular, after taking Eric's special relativity class, I ultimately realized how much effort it took to excel in physics classes. I thank my family, and my parents in particular, for instilling in me values of diligence, curiosity, and for supporting me in all my pursuits. My friends and sister, Anya, were instrumental during my last few years of college, supporting me through times of isolation and overwhelm. I thank all of my lab mates, both current and past, who taught me what it meant to be a competent researcher and instilled in me the curiosity necessary to be a good researcher. This thesis is dedicated to my grandfather, Igor, and my late great-grandmother Mira, who both taught me from a young age the importance of scientific literacy and hard work.

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Introduction

Jemielita, Matthew, et al. 2014

Fig. 1 & 2: A 4-week old zebrafish and a 6-day post fertilization larval zebrafish, with red dye in the gut (for illustration). Zebrafish stand out as an ideal model organism for studying host-microbe interactions, due to their genetic and physiological similarity to humans (being vertebrates), their ease of cultivation, and their translucency during the larval stage which facilitates real-time imaging (8). Imaging allows for the direct observation of bacterial effects on the organisms, making zebrafish an ideal organism for exploring bacterial interactions, monitoring gut microbes and their spatial organization.

Gnotobiotic experiments.

One of the advantages of working with zebrafish is the potential to conduct gnotobiotic experiments, which involve organisms that have been raised devoid of any pre-existing bacteria (1). In this context, every aspect of the microbial composition of the gnotobiotic organism is controlled. This enables the analysis of the impact of isolated bacterial species on the organism. In the Parthasarathy lab, we conduct gnotobiotic experiments to examine the influence of various bacterial species on gut motility, bacterial colonization, and immune cell response, among other factors.

How are gnotobiotic conditions compromised with the introduction of a food source, more importantly why rotifers

Gnotobiotic conditions are compromised with the introduction of a food source, particularly when rotifers are used. A critical barrier is introduced at the 7-day post-fertilization mark, when the fish must commence feeding. They thrive best on a live food source; hence, we use rotifers, small aquatic microorganisms maintained at the UO (University of Oregon) Fish Facility. The use of rotifers as a food source is optimal due to their size, nutritional content, and ease of cultivation, which aligns with the dietary needs of larval zebrafish. Rotifers are small enough for larval zebrafish to consume and digest efficiently, providing essential nutrients

necessary for their growth and development (9). However, the introduction of rotifers, being live organisms, inevitably carries with it external microbial communities associated with the rotifers. Our objective, therefore, is to develop a practical method for feeding larval zebrafish while maintaining control over their gut microbial community.

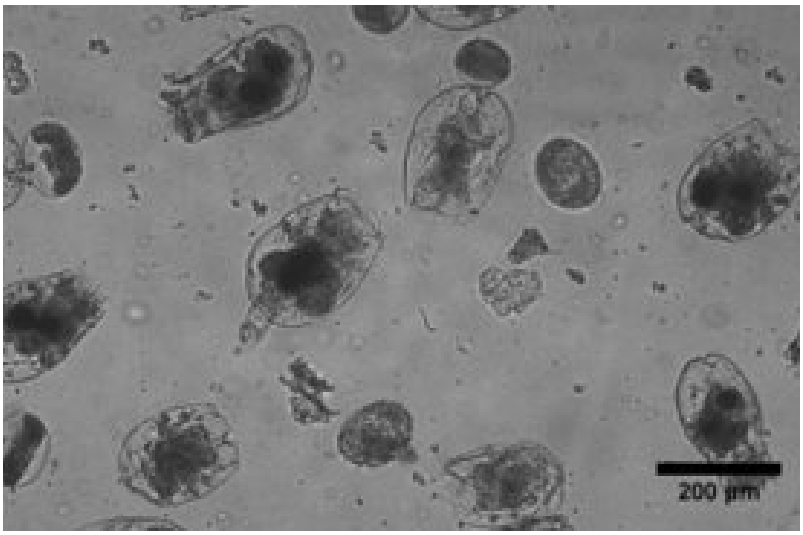

Fig. 3: Brightfield image of rotifers taken with a confocal microscope. For scale, they are approximately 200 microns in length, making them suitable for consumption by larval zebrafish.

What does existing literature say about using UV irradiation to eliminate bacteria, and are there any studies specifically focusing on its application to rotifers?

UV irradiation has been used for the control of pathogenic microorganisms in a variety of applications, as well as disinfection of air, surfaces, and instruments (3). At certain wavelengths, UV light will break the molecular bonds in DNA, thereby destroying the organism.

Specifically, regarding UV-C irradiation (emitted wavelengths of 200-280 nm), the application of UV irradiation for decontaminating rotifers was explored in the study by Munro et al. (1999). This study highlighted a significant advancement in aquaculture practices by addressing the critical challenge of maintaining relatively sterile environments for larval fish. By exposing rotifers to UV-C light, Munro et al. showed that the bacterial load can be reduced by up to 90% without negatively impacting the rotifers' health, nutritional value, or their ability to support larval fish growth and survival (3). The study also observed the potential health benefits of UV-irradiated rotifers as a food source to larval turbot. Apart from this paper, no recently

performed studies exist on the effects of UV exposure to rotifers. Moreover, the principle of conducting long-term gnotobiotic experiments with fish fed UV-irradiated rotifers is a novel, unexplored idea. For the use of rotifers that we employ, the effect of UV-C irradiation is primarily centered around its ability to reduce the bacterial load of the rotifers, ultimately leading to a "germ-free," irradiated yet still living and motile food source for the larval fish.

Materials and Methods

Animal care.

All experiments with zebrafish were performed in accordance with protocols approved by the University of Oregon Institutional Animal Care and Use Committee and by following standard protocols referenced in (5).

Gnotobiotic protocol.

Wild-type (AB X TU line) larval zebrafish (Danio rerio) were derived using protocols described in (1). The larvae at 0 days post-fertilization (dpf) were washed with antibiotic, bleach, and iodine solutions and then moved to tissue culture flasks containing sterile embryo medium solution at a density of approximately 1 mL/larva. The flasks were stored in a temperature-controlled room maintained at 28 C.

UV-C sterilization setup.

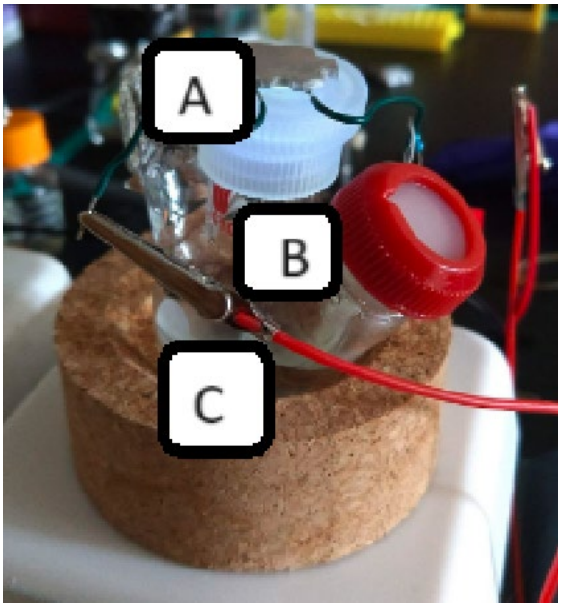

Fig. 4: (A) UV-C LED with a wavelength of approximately 280 nm. (B) 3-neck flask used to hold rotifers that are diluted 1:5 into 4 ppt NaCI solution. (C) A magnetic stir bar suspended on the bottom of the flask. The stir bar rotates at the lowest effective speed to ensure the rotifers are not harmed, but also simultaneously are circulated to ensure complete and uniform UV exposure.

The main goal of the UV sterilization system is to rid rotifers of as much native bacteria as possible, while also ensuring the rotifers are motile post UV irradiation. Prior to the start of the UV treatment, rotifers are collected every morning from the UO Fish Facility and are subsequently diluted by a factor of 1:5 once brought back to the main lab. To ensure that minimal contamination occurs, each component of the UV setup is sterilized with 70% ethanol and immediately rinsed with deionized, ultrapure water. The rotifers are then subjected to four cycles of UV treatment at an intensity level of approximately 0.5 mW/cm^2; one cycle contains a 30-minute period of UV exposure followed by 30 minutes of recovery. To ensure the rotifers are motile post irradiation, their movement speeds can be measured and compared to non-treated rotifers.

Bacterial preparation and fish inoculation.

Bacteria from frozen stocks were grown in lysogeny broth (LB) and shaken overnight for approximately 16 h in a temperature-controlled room at 30 C. One mL of the overnight culture was washed once by centrifuging at 7000 x g for 2 min, and subsequently added to fresh media. 100µl was added to the tissue culture flask on subsequent days throughout the week.

Use of fluorescently labeled zebrafish native bacteria and assessment through plating.

The various bacterial strains used in this study were originally isolated from the zebrafish intestine and have been fluorescently labeled to express green fluorescent proteins (GFP) and red fluorescent proteins (RFP), as explained in (2).

In order to assess how much of the bacteria was intentionally introduced through rotifer feeding and inoculation, fish were euthanized by hypothermal shock, placed in 500 µl of sterile embryo medium, and homogenized with zirconium oxide beads using a bullet blender. The homogenized solution was diluted from ranges of 10^{-1} to 10^{-4}, and 100 µl volumes of these dilutions were spread onto LB plates. Subsequently, the plates were left in a 30C warm room where the colonies were left to grow for upwards of 24hrs. Finally, an Amersham Typhoon Biomolecular Imager was used to image the plates in various fluorescent channels, with excitation wavelengths of approximately 575 nm for RFP and 510 nm for GFP. To briefly describe this process, the fluorescent proteins in the bacteria emit light at specific wavelengths, depending on the wavelength of the incident light that excites them. For example, RFP proteins, when excited by green light of approximately 575 nm, emit red light. Therefore, colonies on the

plates will emit specific wavelengths of light based on the fluorescent proteins they contain.

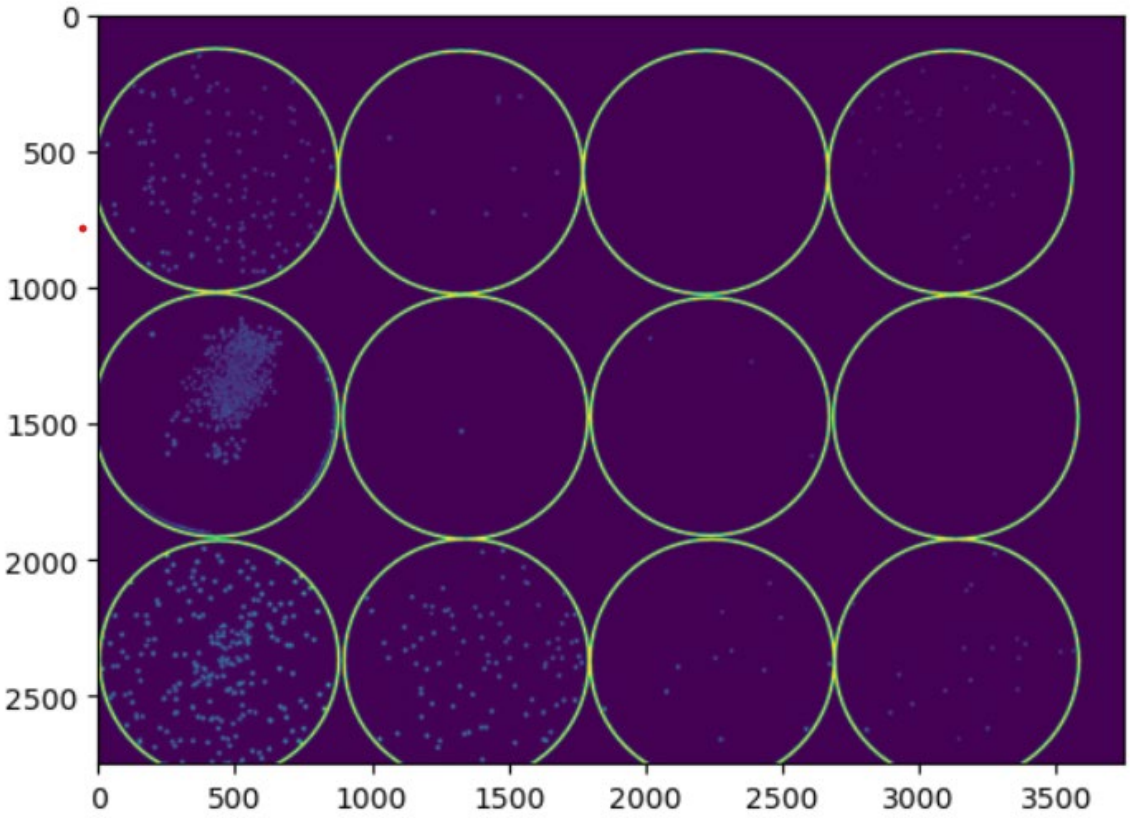

Fig. 5: A scan of plates taken on the Amersham Typhoon Imager in an RFP (Red Fluorescent Protein) channel. Each plate originated from a fish blended at a different dilution. The visible colonies all exhibit traces of red fluorescent proteins, a result of exposure to intentionally introduced bacteria. To establish what fraction of the bacteria was intentionally introduced, the count of these colonies is compared with the total number of colonies, both fluorescent and non-fluorescent. Utilizing a relatively simple object detection algorithm, this process is automated, providing an efficient method for establishing the gut microbial community after feeding and bacterial introduction.

Effect of UV irradiation on bacteria and on the bacterial load of rotifer

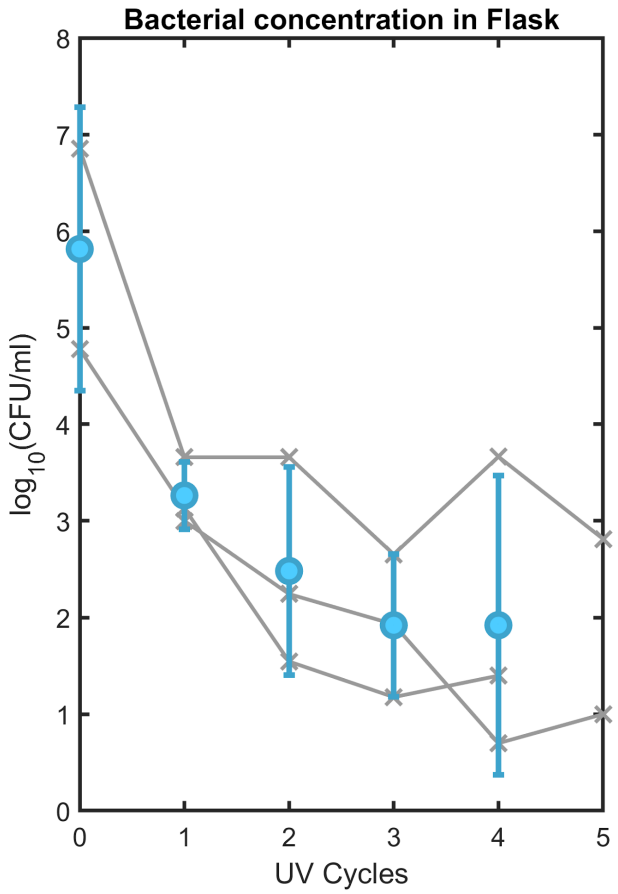

Fig. 6: Bacterial Abundance in Flask Water as a Function of UV Exposure; Credit: Raghuveer Parthasarathy

To determine if UV light is a viable method for reducing total bacterial abundance in rotifers, we must also verify that UV light decreases the total bacterial abundance in the flask water. To assess bacterial abundance in the flask water, samples were diluted to several factors and then plated on LB plates. Fig. 6 demonstrates that overall bacterial abundance, expressed as $\log_{10}$(Colony-Forming Units (CFU) per mL), decreases with increased exposure to UV light by a factor of approximately 10^4.

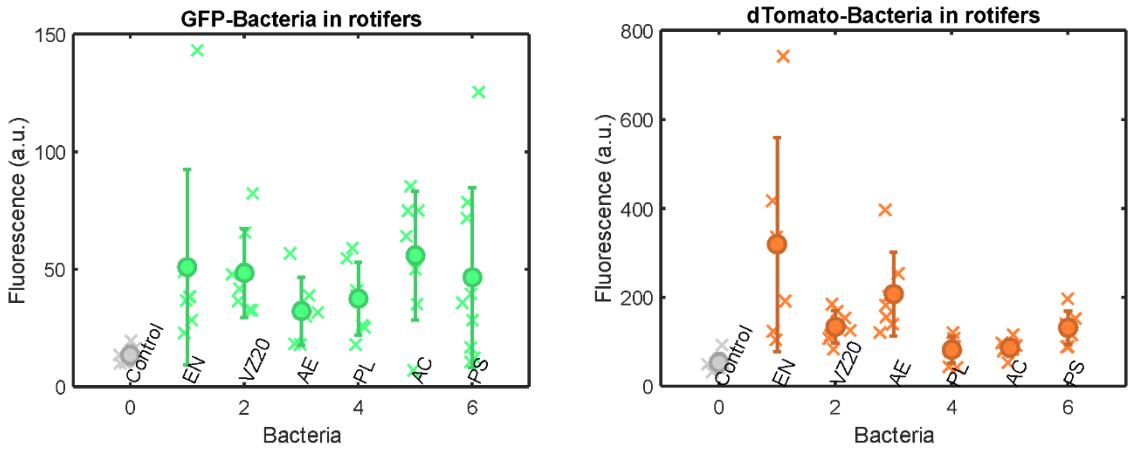

Fig. 7 & 8: Bacterial Abundance Measured by Fluorescence Intensity in Rotifers Inoculated with Six Types of Zebrafish-Derived Bacteria, in GFP and dTomato Fluorescence, After Four Cycles of UV Treatment

To determine the successful colonization of zebrafish-derived bacteria within UV-irradiated rotifers, the rotifers were inoculated with six species of fluorescently labeled zebrafish-derived bacteria and incubated for 4 hours. Subsequently, they were imaged, and the intensities of the gut regions in the rotifers was measured and compared to that of rotifers not inoculated with any bacteria. In Fig. 7, which shows the fluorescence intensity of inoculated rotifers in the GFP channel, every intensity value for the groups introduced with bacteria exceeds that of the control, indicating successful colonization of bacteria within the rotifers. A similar pattern can be observed in Fig. 8, where the bacteria express dTom fluorescent proteins. After confirming that these species of zebrafish-derived bacteria can successfully colonize the rotifers, the next step involved feeding them to zebrafish to attempt reintroducing the bacteria to the fish. It can be observed that all groups, in both fluorescences, have higher fluorescence intensities than the control, signifying successful bacterial colonization within the rotifers.

Effect of UV irradiation on the swimming activity of rotifers

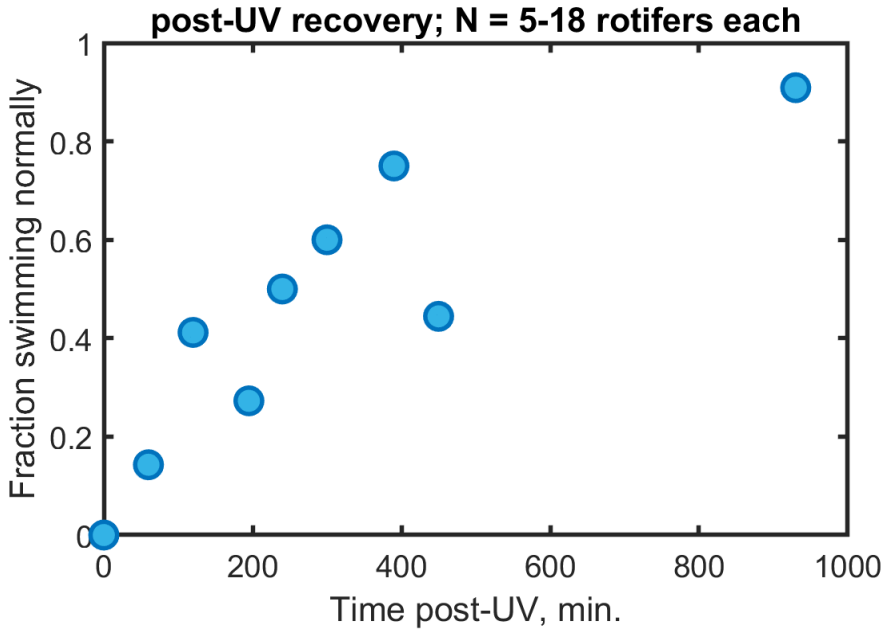

Fig. 9: Fraction of Motile Rotifers Recovered as a Function of UV Exposure Time (Minutes); Credit: Raghuveer Parthasarathy

Fig. 9 is an analysis on the impact of UV exposure on rotifer motility. Here, we measure the fraction of motile rotifers with increased UV exposure. Fig. 9 shows that rotifers (or their descendants) recover from 30 minutes of UV exposure over a timescale of several hours, given an established cutoff speed for “normally” swimming rotifers.

(A) (B)

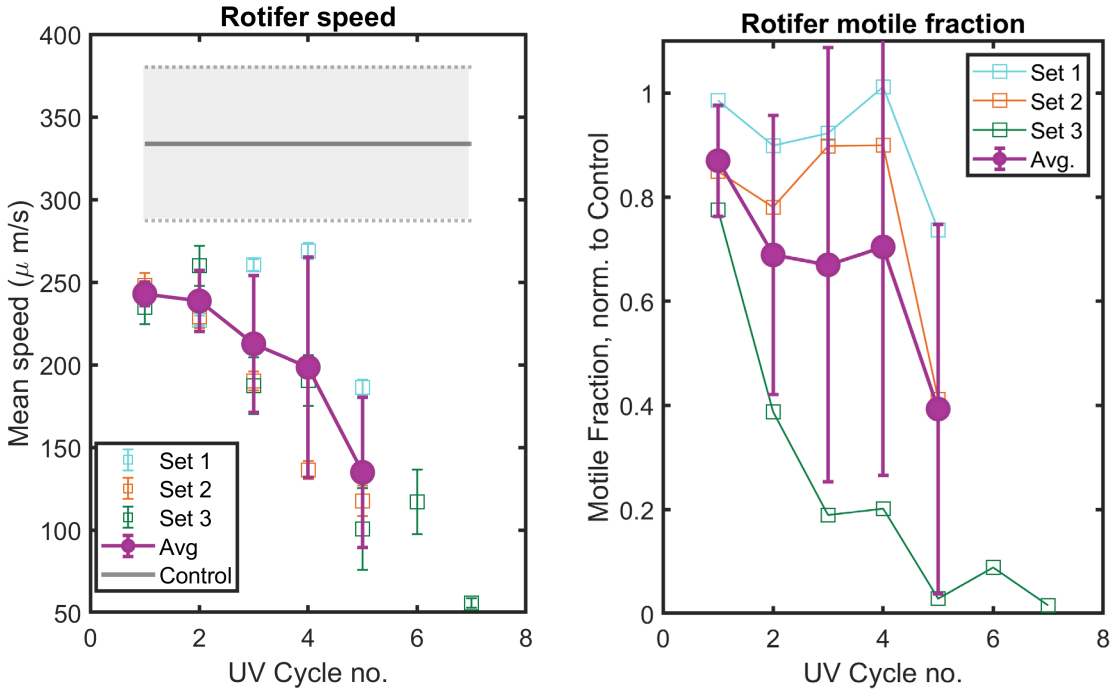

Fig.s 10 & 11: (A) Fig. 10 shows how various amounts of UV cycles affect the mean speed of the treated rotifers (µm/s). (B) Fig. 11 shows what fraction of rotifers are motile as a function of varying numbers of UV cycles. Credit: Raghuveer Parthasarathy

Figure 10 shows several sets of rotifer speeds assessed after various numbers of UV cycles. Around 4-5 cycles is where the most significant drop-off in mean speed occurs, indicating that exceeding this threshold results in unviable rotifers. While there is a direct correlation between bacterial abundance and UV exposure, it is important to note that excessive exposure to UV light will cause the rotifers to become far less motile than desired. For all the feeding experiments involving UV-irradiated rotifers, four cycles of UV treatment were utilized.

Similarly, to establish the optimal number of UV cycles for rotifer irradiation, the motile fraction was assessed in the same sets in Figure 11. It was observed that around 4-5 cycles of UV exposure result in a fraction of motile rotifers that corresponds to a similar drop-off in mean speed as observed earlier. This further verifies that 4 cycles of UV exposure is the ideal amount.

Feeding Data

	4/6 dpf	5/7dpf	6/8dpf	7/9dpf	8/10dpf
Group One	Inoculate EN GFP	Feed Rotifers	Feed Rotifers	Feed Rotifers	Euthanized and plate/count CFU
Group Two	Inoculate EN GFP	Feed Rotifers + EN RFP	Feed Rotifers	Feed Rotifers	Euthanized and plate/count CFU
Group Three	Inoculate EN GFP	Feed Rotifers	Feed Rotifers + EN RFP	Feed Rotifers	Euthanized and plate/count CFU
Group Four	Inoculate EN GFP	Feed Rotifers	Feed Rotifers	Feed Rotifers + EN RFP	Euthanized and plate/count CFU

Fig. 12: An outline of a protocol engineered to quantify the timescale of bacterial stability in the gut of zebrafish after feeding them with gnotobiotic rotifers. The procedure is described in the main text in detail.

Having established that UV irradiation leads to viable rotifers, we can use these rotifers to study whether feeding disrupts resident bacteria in gnotobiotic zebrafish. In brief, we introduce bacteria to initially germ-free zebrafish, and monitor the abundance of the introduced bacterial populations on subsequent days throughout the week.

The following figures, which show total fluorescent bacterial abundance and fraction, were all derived from datasets involving fish fed rotifers over the course of a week, specifically from 4-8 days post-fertilization (dpf) and 6-10 dpf. The structure of these experiments, described in Fig. 12 is as follows: fish were introduced to an initial inoculum on Monday (day 1 of the experiment), and then various groups were introduced to bacteria of the same species but with different fluorescence on staggered days throughout the remainder of the week. Fish were plated on the days following the introduction to the second type of bacteria and on the final day, all groups were plated, except for the group that received the initial inoculum and no other bacteria for the remainder of the week. While there are several aspects to consider from this experiment, particularly the colonization dynamics and bacterial behavior of the types of bacteria, the

primary focus of this paper is to address the amount of bacteria that was intentionally introduced through rotifer feeding and inoculation.

Another important distinction is that all this data was taken from germ-free (GF) fish, which were then introduced to rotifers and bacteria. Trials were also conducted using conventional (CV) fish; however, those figures are not included in this paper. In the context of gnotobiotic fish, 'conventional' refers to fish that are raised in normal environmental conditions, without the specific pathogen-free or germ-free status that gnotobiotic fish maintain.

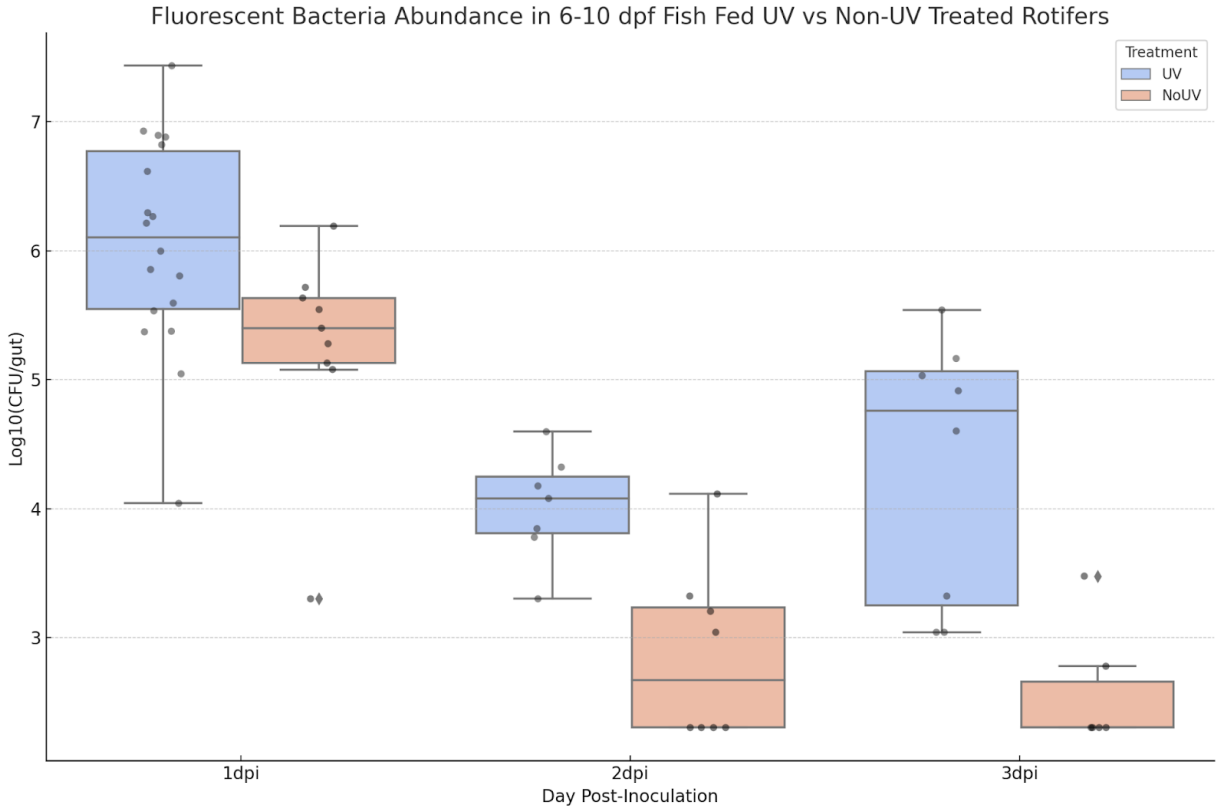

Fig. 13: Fluorescent Bacterial Populations in GF Fish Fed with UV-Treated vs. Non-UV-Treated Rotifers (6-10 dpf) Over Time After Bacterial Introduction

Fig. 13 compares the number of total fluorescent bacterial populations in fish aged 6-10 days post-fertilization (dpf) fed with UV-treated and non-UV-treated rotifers. The fluorescence of the colonies serves as a marker to distinguish the intentionally introduced colonies from those resulting from rotifer bacteria that survived irradiation or other sources. For everyday post inoculation, the number of intentionally introduced bacteria remained consistently higher for the group fed UV-irradiated rotifers. This indicates that UV irradiating rotifers is successful in

generally leading to a higher population of introduced bacteria.

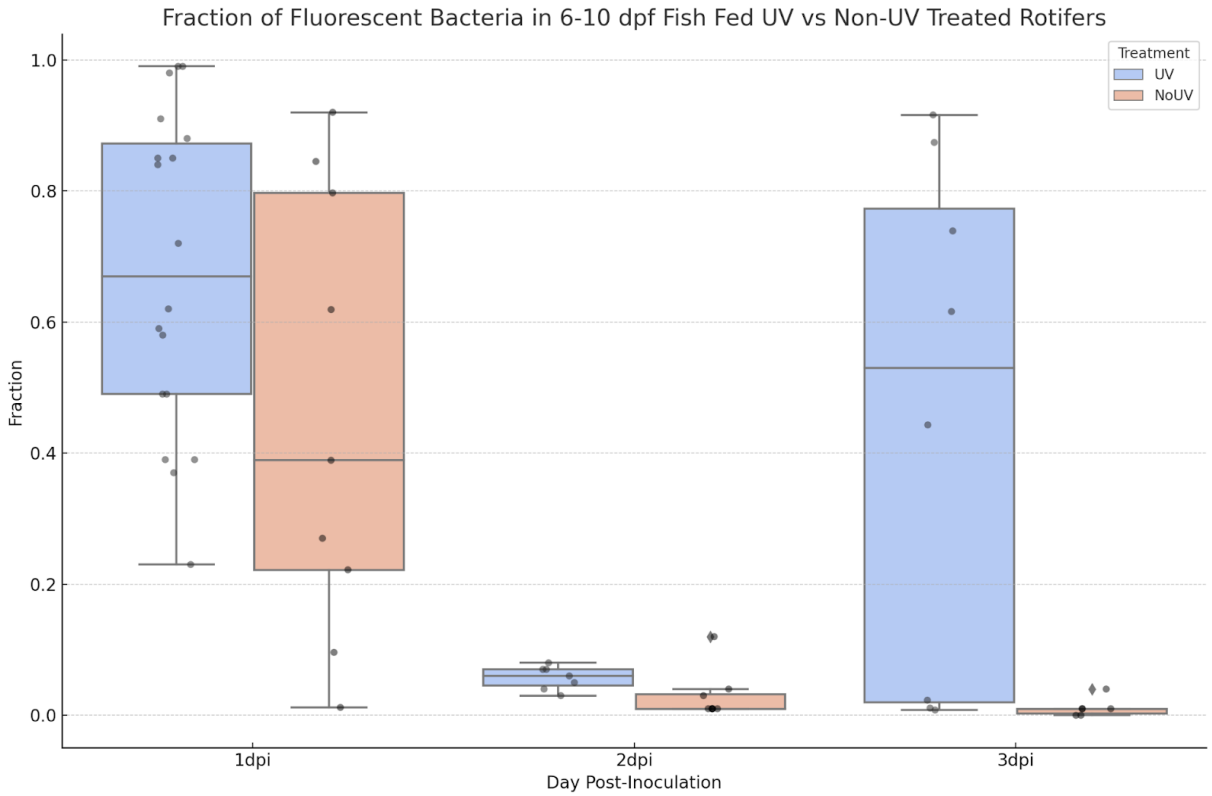

Fig. 14: Fraction of Fluorescent Bacterial Populations in GF Fish Fed with UV-Treated vs. Non-UV-Treated Rotifers (6-10 dpf) Over Time After Bacterial Introduction

Observing the fraction of fluorescent bacteria relative to the total population in fish fed UV-treated and non-UV-treated rotifers, as displayed in Fig. 14, indicates that UV irradiation leads to a higher percentage of the overall microbial composition being intentionally introduced through the introduction of bacteria and UV-irradiated rotifers. It is important to note that the 2 dpi fluorescent fraction for both 4-8 dpf and 6-10 dpf groups is considerably lower than expected; this could be a matter of bacterial stability which extends the scope of this paper. The fluorescent fraction and population seem to normalize at 3 dpi in the group fed UV-treated rotifers for both groups.

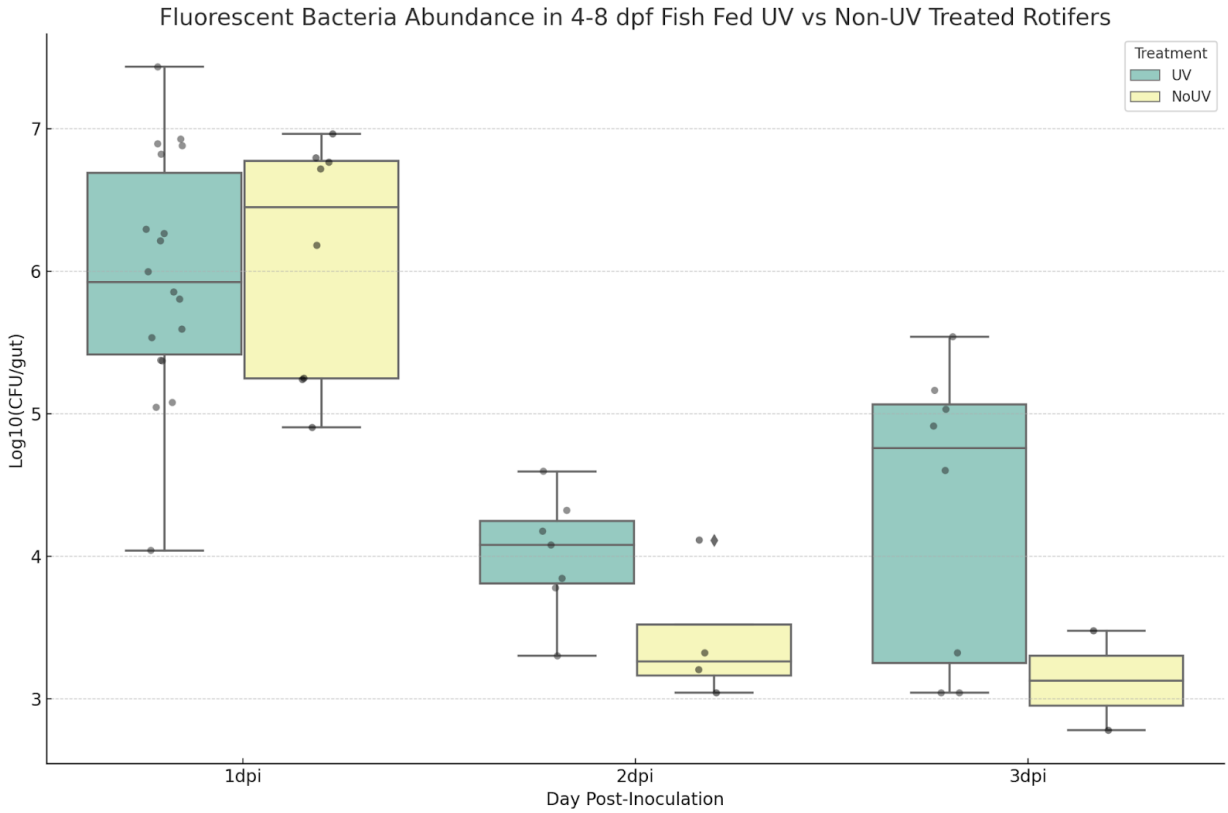

Fig. 15: Fluorescent Bacterial Populations in GF Fish Fed with UV-Treated vs. Non-UV-Treated Rotifers (4-8 dpf) Over Time After Bacterial Introduction

Highlighted in Fig. 15, like the 6-10 dpf group of fish, there was a correlation between UV exposure and abundance of fluorescent and intentionally introduced bacteria in the 4-8 dpf group. While the difference is not as drastic one day post inoculation, the ensuing days show a much more apparent difference in the intentionally introduced bacterial loads in the fish.

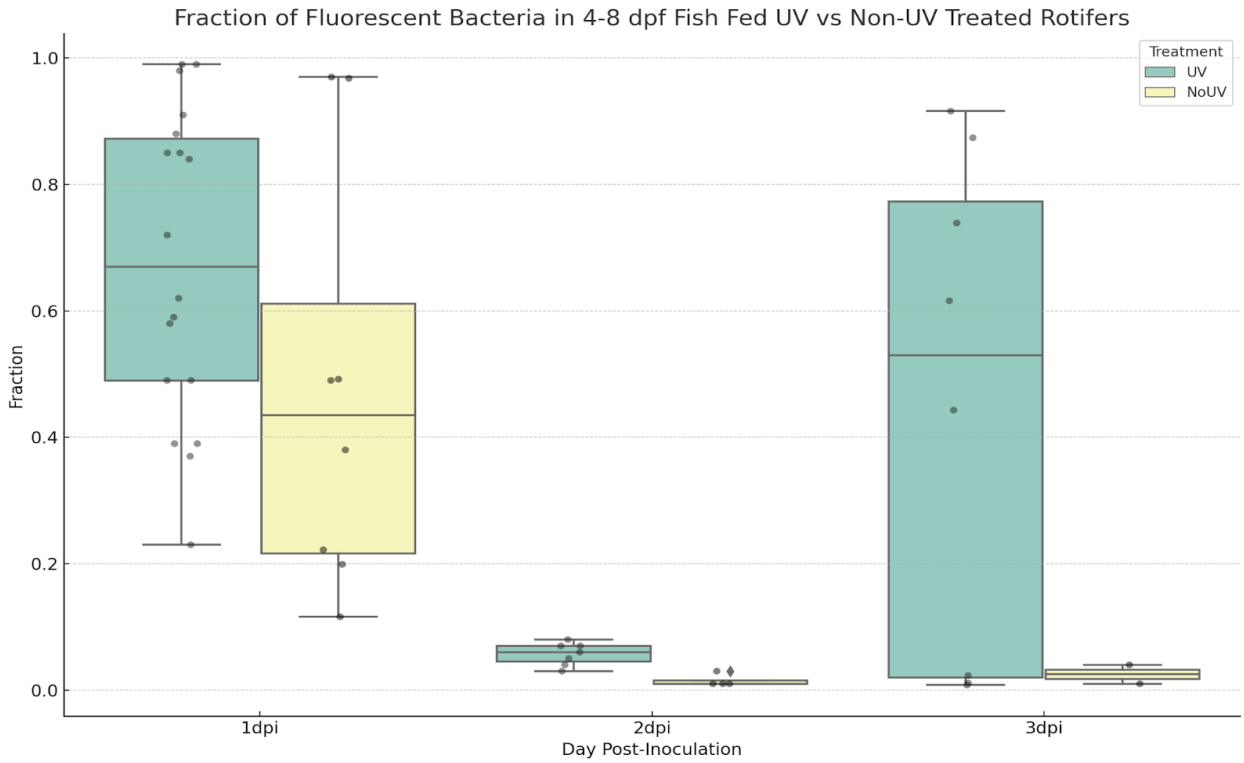

Fig. 16: Fraction of Fluorescent Bacterial Populations in GF Fish Fed with UV-Treated vs. Non-UV-Treated Rotifers (4-8 dpf) Over Time After Bacterial Introduction

Observing the fraction of fluorescent bacteria relative to the total population and comparing the UV-treated and non-UV-treated groups indicates that UV irradiation leads to a higher percentage of the overall microbial composition being intentionally introduced through the introduction of bacteria and UV-irradiated rotifers. As observed previously with the 4-8 dpf fish, this trend appears to be independent of the fish's age, at least during the larval stages.

Future directions.

The cultivation of UV-rotifers and their effects on fed fish brings to light several questions regarding bacterial stability and physical characteristics of fish as a result of different feeding conditions. While this paper is centered around retaining control over the gut microbiome through feeding larval zebrafish UV-irradiated rotifers, we have also simultaneously observed how the initial inoculum and subsequently introduced populations of bacteria behave as a function of the day they are introduced.

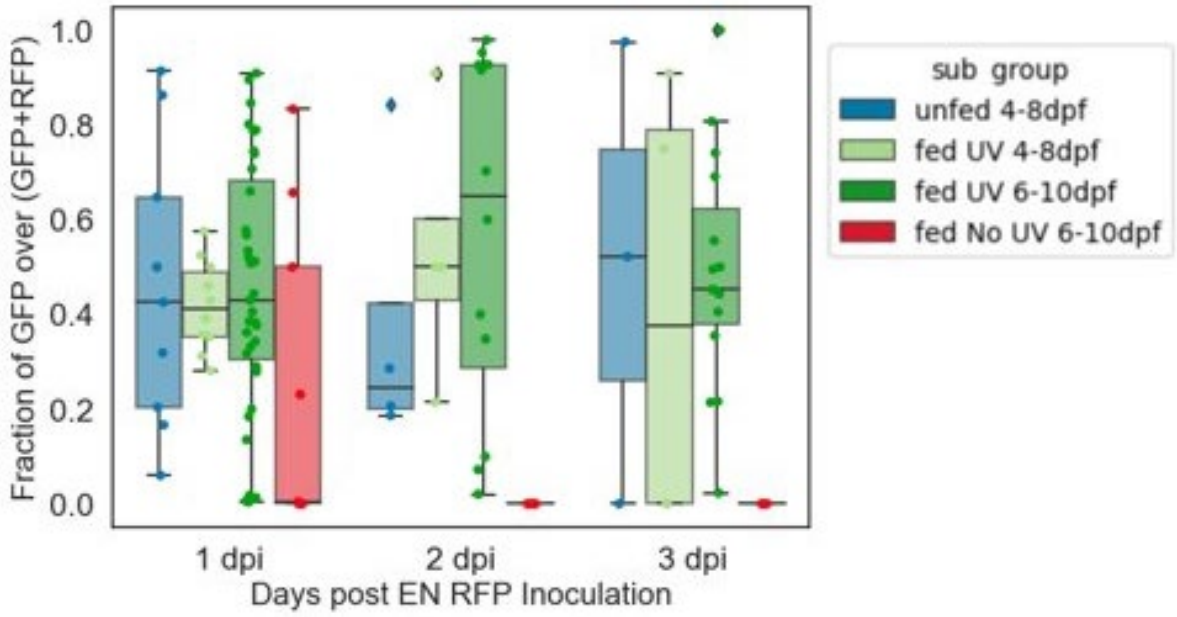

Fig. 17: Relative Abundance of EN GFP to EN RFP in GF Fish Fed with UV-Treated, Non-UV-Treated Rotifers, and Unfed Fish as a Function of Time Post-Bacterial Inoculation

Regarding bacterial stabilization, Fig. 17 suggests that Enterobacter with different fluorescence proteins can coexist with other populations over a few days. Furthermore, approximately 1 day after inoculation, the group fed with non-UV-treated rotifers, on average, has less than 50% of the fluorescent bacteria. After two days, the fluorescent bacteria population becomes undetectable. This is encouraging, suggesting that UV-irradiated rotifers are a favorable food source for conducting these long-term bacterial stability experiments.

Fish respirometry.

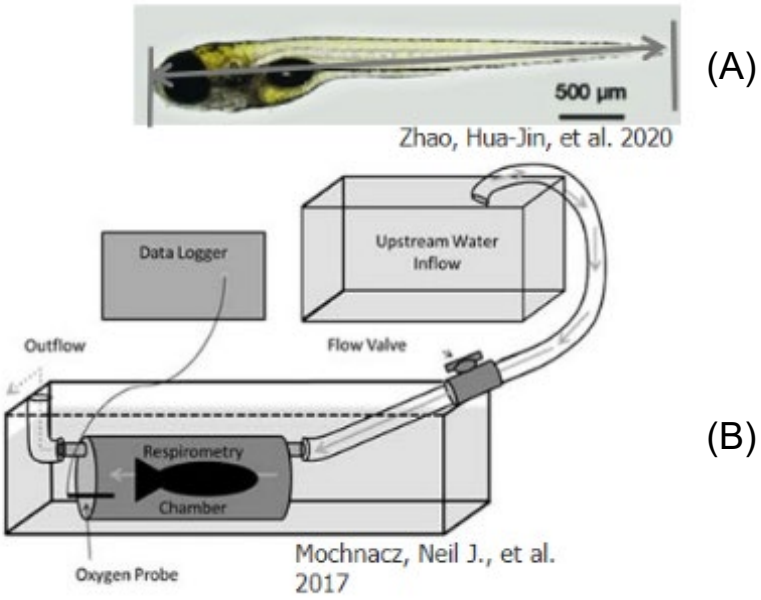

Fig. 18: (A) Example of how a fish length measurement works. The length from the front of the fish to the tip of the tail is referred to as the total length. Image taken from (6). (B) Here is a depiction of how the respirometry apparatus works; the process involves periodically flushing the chamber with fresh oxygen to maintain a suitable environment, while sensors measure the oxygen levels, and a data logger records these measurements. Image taken from (7).

Using UV-irradiated, non-UV-irradiated, and unfed larval fish, we have also begun to measure the respiration rates of the fish under these various conditions. In this experiment, varying numbers of larval zebrafish are placed within a respirometry chamber, where the flux of oxygen in the enclosed area is measured. This method is used to determine the respiration rate of the fish. Preliminary results suggest that respiration rates are noticeably higher in fed fish, indicating that the metabolic health of the fish has been positively affected by the food source. However, the difference between UV and non-UV rotifers in this context is not as clear. Furthermore, by exploring the physical characteristics of the fish under different feeding conditions, we can also measure fish lengths to better understand the relationship between rotifers, bacteria, and their impacts on fish.

Conclusions.

We have developed a protocol to reduce the innate microbiota in the rotifers fed to larval zebrafish. Through four cycles of UV treatment, we are able to eliminate the majority of bacteria from the rotifers while also keeping them motile and able to be fed upon by larval zebrafish, proving them to be a viable food source for larval zebrafish up to at least 10 days post fertilization. Furthermore, we have managed to ensure that approximately 60-70% of the bacterial composition of the gut consists of intentionally introduced microbes, through the introduction of UV-irradiated rotifers and fluorescently labeled zebrafish-derived bacteria. In the observed groups of 4-8 dpf and 6-10 dpf fish, both the total fluorescent bacterial abundance and fraction were higher in fish fed UV-irradiated rotifers, suggesting that the developed UV sterilization system is a viable method to implement a food source in the diet of larval zebrafish that is substantially lower in native bacteria. With the ultimate goal of conducting long-term gnotobiotic experiments, while a purely intentionally introduced bacterial regime is not feasible for representing the entire microbial composition of the gut, we aim to conduct similar feeding experiments over longer timescales with consistent results regarding the bacterial composition retained through the feeding of UV-irradiated rotifers.

References

1. Melancon, E., Canny, S. G. D. L. T., Sichel, S., Kelly, M., Wiles, T., Rawls, J., Eisen, J., & Guillemin, K. (2017). Best practices for germ-free derivation and gnotobiotic zebrafish husbandry. *Methods in cell biology*, *138*, 61-100.

2. Jemielita, M., Taormina, M. J., Burns, A. R., Hampton, J. S., Rolig, A. S., Guillemin, K., & Parthasarathy, R. (2014). Spatial and temporal features of the growth of a bacterial species colonizing the zebrafish gut. *MBio*, *5*(6), 10.1128/mbio. 01751-01714.

3. Munro, P., Henderson, R., Barbour, A., & Birkbeck, T. (1999). Partial decontamination of rotifers with ultraviolet radiation: the effect of changes in the bacterial load and flora of rotifers on mortalities in start-feeding larval turbot. *Aquaculture*, *170*(3-4), 229-244.

4. Sundarraman, D., Hay, E. A., Martins, D. M., Shields, D. S., Pettinari, N. L., & Parthasarathy, R. (2020). Higher-order interactions dampen pairwise competition in the zebrafish gut microbiome. *MBio*, *11*(5), 10.1128/mbio. 01667-01620.

5. Westerfield, M. (2007). *The zebrafish book: A guide for the laboratory use of zebrafish Danio (" Brachydanio Rerio")*. University of Oregon.

6. Zhao, H.-J., Xu, J.-K., Yan, Z.-H., Ren, H.-Q., & Zhang, Y. (2020). Microplastics enhance the developmental toxicity of synthetic phenolic antioxidants by disturbing the thyroid function and metabolism in developing zebrafish. *Environment International*, *140*, 105750.

7. Mochnacz, N. J., Kissinger, B. C., Deslauriers, D., Guzzo, M. M., Enders, E. C., Anderson, W. G., Docker, M. F., Isaak, D. J., Durhack, T. C., & Treberg, J. R. (2017). Development and testing of a simple field-based intermittent-flow respirometry system for riverine fishes. *Conservation Physiology*, *5*(1), cox048.

8. Dooley, K., & Zon, L. I. (2000). Zebrafish: a model system for the study of human disease. *Current opinion in genetics & development*, *10*(3), 252-256.

9. Aoyama, Y., Moriya, N., Tanaka, S., Taniguchi, T., Hosokawa, H., & Maegawa, S. (2015). A novel method for rearing zebrafish by using freshwater rotifers (Brachionus calyciflorus). *Zebrafish*, *12*(4), 288-295.