Early Life Assembly of Zebrafish (*Danio rerio*) Microbiomes

by

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DISSERTATION ABSTRACT

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Title: Early Life Assembly of Zebrafish (*Danio rerio*) Microbiomes

Microbiomes can influence fish health and development and may play a role in fish evolution. How fish acquire their microbes, especially early in life, remains a fundamental question in microbial ecology. Here, I address this question using the model organism, zebrafish (*Danio rerio*).

Zebrafish are a useful model vertebrate with which to uncover the relationships between a host and its resident microbes, and explore questions related to community assembly of the microbiome. Zebrafish develop quickly, hatching by 3 days post fertilization, and reaching sexual maturity by 75 days post fertilization. Zebrafish eggs can be sterilized to derive axenic larvae. Axenic larvae are a boon for microbiome research, since they allow researchers to study how a lack of microbiome influences larval health, or how individual members of the microbiome modulate different host responses. However, rendering these fish axenic removes the microbes that naturally colonize the eggs surfaces, erasing any contribution of the parents or the environment as source pools of microbes to the offspring. These microbes may be critical for egg survivability and larval development.

In this dissertation, I explore the early-life microbiome assembly of zebrafish, beginning with the egg. In chapter 2, I review current literature on early-life assembly in fishes, drawing from research performed in other species, such as Atlantic salmon (*Salmo salmar*), brown trout (*Salmo trutta*), and lake sturgeon (*Acipenser fulvescens*), to inform my hypotheses regarding how

the egg microbiome might be influenced by parental genetics, transmission, and developmental stage of the embryo. In chapter 3, I characterize the egg microbiome of zebrafish reared in the UO Huestis Zebrafish Facility, and address the role of parentage and embryonic development on microbiome composition. Finally, in chapter 4, I explore the roles of the parental microbiome and the environmental microbiome as sources to the egg and larval gut microbiome.

The egg microbiome of zebrafish is dynamic, and composition rapidly changes between fertilization and hatching. I find that parentage and developmental stage are important drivers for microbiome assembly on zebrafish eggs. Post-hatching, the egg microbiome is also an important source to the larval gut microbiome, therefore its contributions to larval health and development cannot be ignored. I observed that the skin microbiome was a major source of microbes for both eggs and larvae, as was water collected from the crossing tanks in which the eggs were fertilized. This suggests that water may be an important medium of transmission of microbiota from parent to egg.

Overall, this dissertation presents novel research characterizing the egg microbiome of zebrafish. Currently, this is an underexplored life stage with respect to microbiome assembly in this important model organism. These results have implications for future research on host-microbiome assembly and host-microbe interactions using zebrafish as a model.

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I. INTRODUCTION

Host-associated microbiota are important for the healthy development of neonates, in humans as well as other animals. Studies in fish, particularly zebrafish, have revealed the vital roles commensal microbes play in the development of the nervous system, immune system, and intestines, where axenic (or "microbiome-free") individuals experience abnormal development compared to their counterparts raised in contact with microbiota. With their large clutches, quick development time, and ease of sterilization of eggs, zebrafish have been an invaluable study system to uncover the roles and mechanisms of beneficial and deleterious host-microbe interactions. However, a fundamental question remains in the study of host-microbiome interactions: how do individuals acquire their resident microbiota, especially early in life? To my knowledge, no studies have examined the origins of the zebrafish microbiome or described the composition and the assembly of the zebrafish egg microbiome. Additionally, few studies have addressed whether the egg microbiome composition can influence the development of zebrafish, and other fish species, or their microbiota at later life stages.

My doctoral research focuses on understanding the factors that drive assembly of the zebrafish microbiome at early life stages, from fertilization to post-feeding larval fish. I address the roles of development on microbiome composition, and the contributions of parental and environments sources to the egg and larval microbiome.

In chapter 2, in collaboration with Dr. Ingrid Bakke and Dr. Simen Fredricksen at the Norwegian University of Science and Technology (NTNU), I synthesize the current literature concerning early-life assembly of fish microbiomes. We include studies performed in zebrafish, along with economically relevant fish species, such as Atlantic salmon, Atlantic cod, and brown

trout. We discuss the importance of the early-life colonization of fish eggs, the ecological processes that dictate colonization, and the implications of these processes for aquaculture.

In chapter 3, I address the drivers of assembly and microbiome variation on zebrafish eggs. Zebrafish are broadcast spawners, they do not require parental care for successful reproduction, and they develop quickly (embryos hatch into larval fish within 2-3 days of fertilization). This system allowed me to study the role of parental identity and embryo developmental time in driving variation of the egg microbiome. I crossed parents from two stock tanks in the Huestis zebrafish facility and collected the eggs at 0, 1 and 2 days post fertilization. I found that phylogenetic diversity of the microbiome decreases as the embryos age, suggesting that environmental filtering by the developing egg may be an important driver of microbiome assembly between fertilization and hatching. I also found that parental identity significantly explains variation in the egg microbiome, with full-sibling eggs having more similar microbiomes than non-siblings. This finding may be due to host genetics, or transmission of microbiota from parent to offspring, which we address in the next chapter.

In chapter 4, I explore the contributions of various source pools to the egg and larval gut microbiome of zebrafish. I crossed parents from one stock tank and collected eggs (0 and 2 dpf) and larvae before and after feeding (5 and 7 dpf). I also collected gut, skin, crossing tank water, facility water, and rotifers to explore the contributions of these sources to the egg and larval microbiomes. Parental gut and skin microbiomes were identified as sources to the egg and larval microbiomes, although their relative contributions varied over development. I also found evidence suggesting that the egg microbiome is a source to the larval gut microbiome.

This work, taken together, presents novel research uncovering the role of development, parents and the environment as sources to the early-life zebrafish microbiome, and the

importance of understanding the ecological processes of early-life assembly in zebrafish and other fish species. This research can inform future studies in zebrafish by describing the sources to the egg microbiome and how the egg microbiome can influence the larval gut microbiome. By understanding the colonization of egg microbiota of fish, future research can inform microbiome manipulations to improve larval and juvenile fish health in aquaculture.

II: A CURRENT REVIEW OF EARLY LIFE MICROBIOME ASSEMBLY IN ZEBRAFISH (*DANIO RERIO)*

This literature review has been written in collaboration with Dr. Simen Fredricksen (Norwegian University of Science and Technology; NTNU), Dr. Ingrid Bakke (NTNU) and Dr. Brendan Bohannan. Dr. Fredicksen and I have contributed equally to this work, with feedback from Dr. Bakke and Dr. Bohannan.

Introduction

The microbiome can influence the health and development of their hosts, from aiding digestion to modulating the immune system and brain development. Most animals are born or hatched without associated microbes and must acquire them from other animals or their environment. However, the ecological processes driving the acquisition of the microbiome early in life remains a fundamental question in microbiome research. Answering this question is crucial, because early life acquisition of the microbiome has the potential to influence host health and functioning later in life. For example comparisons using the model vertebrate, the zebrafish (*Danio rerio*), between axenic individuals (absent of a microbiome from hatching; aka "germ free") and those with an intact microbiome (i.e. conventionally raised or conventionalized individuals) have shown that a gut microbiome is necessary for proper gastrointestinal (GI)-tract development and immune system development in the larval stage (Bates et al., 2006; Rawls et al., 2004). However, despite the importance of the microbiome, little is known about how it is acquired during the earliest developmental stages, even in a well-studied model vertebrate such as the zebrafish. For example, to my knowledge no studies have addressed how zebrafish eggs might acquire their microbiome from their environment or conspecifics, including their parents.

In this review, I summarize what is known about early life acquisition of the zebrafish microbiome. Given how little research has been done on this topic to date, I also review some research using other teleost fish species, which may be relevant for zebrafish. Much more is known about early life microbiome assembly in commercially important teleost species such as Atlantic cod (*Gadus morhua*) and Atlantic salmon (*Salmo salmar*), and observations in these species may provide insight into the general processes that can drive microbiome assembly in teleost fish. This review focuses on the following questions: 1) how does early life assembly influence zebrafish health? and 2) what is known about the assembly of the egg microbiome? I also explore knowledge gaps in our understanding of early-life microbiome assembly in teleost fish in general. Throughout I use "egg" to refer to all fertilized fish eggs prior to hatching, and "larvae" to refer to fish post-hatching and prior to the development of an adaptive immune system (Figure 1). The embryos within the egg are considered sterile (Bates et al., 2006), with the chorion preventing colonization within the egg by bacteria and fungi (except in the case of disease; ex. (Liu et al., 2014)). Therefore, all microbiomes referred to in this review are either collected from the surface of egg or from larvae post-hatching.

Importance of early microbiome assembly to zebrafish health

The gut microbiome influences development and immunity in larval zebrafish

Prior to hatching, zebrafish embryos are considered sterile, and no commensal bacteria have been detected within the chorion (eggshell) or in the embryo (Bates et al., 2006). As soon as the larvae hatch, usually within two or three days post fertilization, they enter a microbial world, and they are rapidly colonized by bacteria, likely originating from the egg debris and/or from the environment. Within a day of the mouth opening in zebrafish, bacteria are detected in the mouth, esophagus and proximal intestine (Bates et al., 2006). These early microbial members can exert

great influence on the development and later fitness of these larvae. For instance, studies on gnotobiotic and axenic zebrafish have shown that the microbiome greatly impacts the development of a range of traits during early life, such as development of the GI-tract, immune system and neurons (Bates et al., 2006; Bruckner et al., 2022; Galindo-Villegas et al., 2012; Phelps et al., 2017; Rolig et al., 2015). An absence of a microbiome (as when fish are rendered and raised germ-free) negatively impacts development of the GI-tract. For example, in axenic zebrafish gut epithelial cells fail to differentiate and mature (Bates et al., 2006; Rawls et al., 2004). Additionally, axenic fish have reduced ability to absorb nutrients (Bates et al., 2006; Semova et al., 2012; Sheng et al., 2018).

Microbial colonization may be required for proper development of the immune system (Bates et al., 2007; Cheesman et al., 2011; Galindo-Villegas et al., 2012; Kanther et al., 2011; Koch et al., 2018). Additionally, the presence of commensal microbes in the gut can change host gene expression to promote immune tolerance to the microbiome (Koch et al., 2018) and protect the host against damaging inflammation triggered by microbially-derived molecules (Bates et al., 2007). In simple communities (1-3 members), different members of the microbiome can uniquely influence intestinal immunity, such as inflammation and neutrophil recruitment (Rolig et al., 2015). However, it is unclear how these microbes interact in more complex communities.

Colonization by bacteria is also needed for the development of normal behavior in zebrafish larvae. Axenic fish display social behavioral deficits, but inoculation of germ-free larvae with bacteria can alter gene expression in the brain, resulting in improved social behavioral development (Bruckner et al., 2022). Inoculation of axenic larvae with *Aeromonas* and *Vibrio* was also found to prevent the development of anxiety-like behavior in zebrafish larvae, observed as abnormal locomotor behavior (Phelps et al., 2017). Another study found that

bacterial load in the intestine was inversely related to locomotor activity (Weitekamp et al., 2021), indicating that abundance of commensal bacteria is important to consider when studying the effect of the microbiome on certain host traits.

The microbiome may also directly benefit host health, such as through colonization resistance against pathogens. Studies in zebrafish and trout have shown that even small microbiomes (up to 11 strains) or single strains can protect against pathogens (Caruffo et al., 2016; Pérez-Pascual et al., 2021; Rendueles et al., 2012; Stressmann et al., 2020). The microbiome can also enhance host immunity to pathogens (Qi et al., 2023; Willis et al., 2016). For instance, microbially-derived vitamin B_{12} can alter the zebrafish gut microbiome and offer protection against pathogenic *Aeromonas hydrophila* (Qi et al., 2023). Microbiome members can competitively exclude other microbes (Wiles et al., 2016) or predate on potential pathogens (Willis et al., 2016).

The egg microbiome can influence hatching success in other fish species

Relative to what is known about host-microbe interactions in larval zebrafish, little is known about how the egg microbiome interacts with its host and the impact of the egg microbiome on larval development. To my knowledge, no studies have characterized the egg microbiome of zebrafish or the process of microbiome assembly at the egg stage. However, there are studies exploring microbiome composition in other teleost species, especially those relevant to commercial aquauculture, such as Atlantic cod (*Gadus morhua*) and Atlantic salmon (*Salmo salmar*).

A major consequence of egg microbiome composition is its effect on hatching success. In sturgeon (*Acipenser fulvescens*) and Atlantic salmon, the egg microbiome has been shown to prevent potential pathogens from infecting and killing eggs. Members of the egg microbiome of

both of these fish species can exhibit antimicrobial activity against fish pathogens (Fujimoto et al., 2018; Liu et al., 2014). The presence of the bacterium *Frondihabitans* spp. on Atlantic salmon eggs has been correlated with lower disease incidence of the fungal infection Saprolegniosis, likely due to the bacteria's ability to prevent the fungal pathogen from adhering to the eggs (Liu et al., 2014). The egg microbiome could have similar effects on zebrafish hatching success, although this has not yet been demonstrated.

The egg microbiome may influence subsequent larval fitness. For instance, bacterial genera from Atlantic salmon and Coho salmon (*Oncorhynchus kisutch*) egg microbiomes have also been recovered in recently hatched larvae (Lokesh et al., 2019) and in juvenile fish (Romero & Navarrete, 2006) of these species, suggesting that transmission from egg to larvae may occur. If such transmission occurs in zebrafish, the composition of the egg microbiome could directly influence the composition of the zebrafish larval gut microbiome, through transmission of microbes from chorions to the gut during early development.

Less research has examined whether the egg microbiome can influence the developing embryo or vice versa. As stated previously, the embryo inside the chorion is considered sterile (Bates et al., 2006). Additionally, the chorion of fish eggs is likely only permeable to small organic and inorganic molecules (Hansen & Olafsen, 1999), limiting the ability of the embryo and bacteria on the egg surfaces to directly interact. While microbiome diversity is correlated with increased hatching time on brown trout eggs (Wilkins et al., 2016), it is not clear whether this is due to bacteria breaking down the chorion, thus affecting when the larvae can break out of the egg, or the bacteria influencing the speed of embryonic development.

Drivers of early-life microbiome assembly

The egg microbiome of zebrafish is severely understudied. However, egg microbiomes of other fish species, especially those reared in commercial aquaculture, have been studied, and may offer insight into drivers of microbiome composition on zebrafish eggs. Several factors may influence the composition of the zebrafish egg microbiome, including, but not limited to, host genetics (of the parent and the embryo), parent-to-offspring microbial transmission, transfer of parental immunity, and environmental conditions (e.g. temperature, water flow, the environmental microbiome, etc.; Figure 1). The microbiome composition of the eggs may also be influenced by changing conditions on the surface of the egg as the embryo develops. For fish with a relatively long embryonic development time, the egg microbiome may also be influenced by changes in the surrounding environment. Finally, lab-reared and aquaculture reared fish may be subject to disinfection protocols, to either completely sterilize or reduce bacterial loads, which can erase the impact of previous parental or environmental transmission and open new niches for microbial colonization.

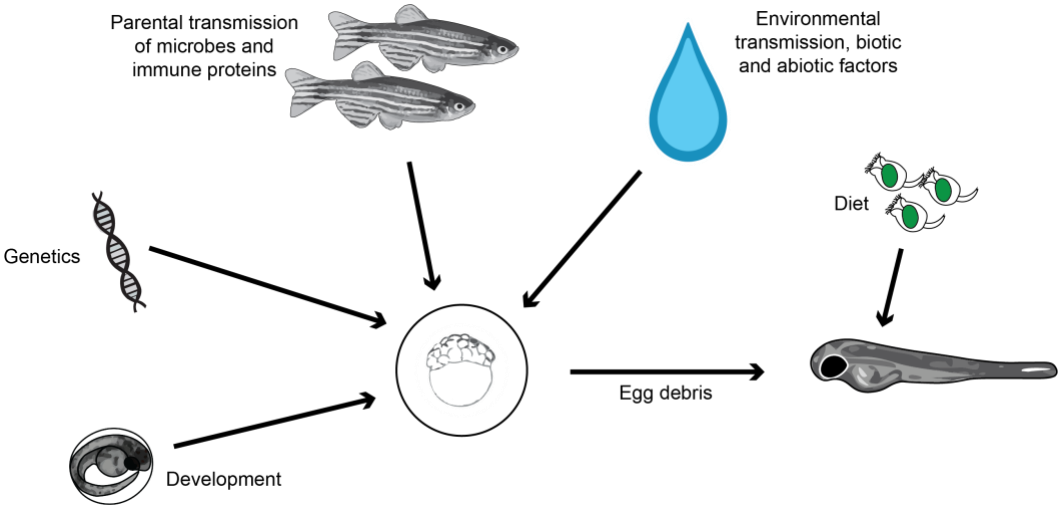

Figure 1: Putative sources of microbes and drivers of variation to the egg microbiome. Adults and environment (e.g. water column) may serve as sources of microbes or influence selection via immunocompetent proteins or biotic/abiotic factors. Host genetics and development may also influence composition of the egg microbiome. Finally, the egg microbiome, along with feed, may be sources to the larval gut microbiome. Adult zebrafish image adapted from Adobe Stock photo by Mirko Rosenau.

Egg microbiome changes over development

A few studies have explored how the diversity and composition of the egg microbiome changes over embryonic development, across several commercially important fish species, and both in nature and in aquaculture facilities (natural environments: whitefish, Wilkins et al., 2015; aquaculture: lake sturgeon, Fujimoto et al., 2013, and Atlantic salmon, Lokesh et al., 2019). Even in zebrafish, which have a very short embryonic development time compared to the previous fish species I mentioned, I have observed significant changes in microbiome composition correlated with embryo age (explored further in Chapter 2).

Why the egg microbiome changes over embryonic development, however, is still unknown. Patterns of succession of these microbiomes may depend on environment and host species. For the fish species mentioned above, the authors of each study found differing patterns of how alpha diversity of the egg microbiome changed over development. One driver of succession is "environmental filtering", by which I mean selection for or against microbes that have particular traits. Environmental filtering of the egg microbiome could be due to changes in the surrounding water column or physiological changes in the egg and could likely influence which bacteria can colonize and persist on the eggs. For example, the microbiome of naturally spawned whitefish eggs was more like the surrounding water at earlier development stages than at the later development stages (Wilkins et al., 2015). This suggests that the aquatic environment may be an important source of microbes, especially early in development, but that selection by the egg and embryo may influence the microbiome as the embryo develops.

How does the egg select for microbes? It is well-documented that there are antimicrobial peptides and immunocompetent factors (both expressed by the embryo and derived from the mother) within the egg (Huttenhuis et al., 2006; Løvoll et al., 2006; Ni et al., 2021; Yousif et al.,

1994). Maternal immunity may also contribute to embryonic immunity (Valero et al., 2023; Zhang et al., 2013). However, the chorion is only permeable to water, gas, and small molecular weight organic and inorganic compounds (Hansen & Olafsen, 1999), so these peptides and proteins are unlikely to interact with microbes on intact eggs. Embryos may exert influence on the microbes colonizing the chorions through excretion of small organic and inorganic compounds. For instance, teleost embryos start excreting urea and ammonia out of the egg shortly after fertilization (Zimmer et al., 2017). A study of Atlantic cod embryos found that the amount of ammonia and urea excreted changes over development and can differ based on the embryo's population of origin (Chadwick & Wright, 1999). Further research is needed to address how excretion of compounds by the embryo may exclude or recruit microbes to the egg surface.

Disinfection affects the microbiome composition on eggs and in larvae

Disinfection is a common practice in aquaculture, with the goal of reducing bacterial load and managing disease, especially at the egg and larval stages of fish. Axenic derivation is a similar practice in host-microbiome research where eggs are sterilized to be raised without a microbiome, or reinoculated with a known microbiome (gnotobiotic). Both practices may erase locally adapted and beneficial parental contributions to the egg microbiota, hampering animal health and efforts to characterize a healthy fish microbiome. These practices may also alter the trajectory of microbiome assembly and stability of the microbiome at later life stages. For example, one study on Atlantic salmon larvae found that the skin and gut microbiome differed depending on whether the eggs were exposed to egg-derived microbes (i.e., conventionally raised) or sterilized and exposed to lake-derived microbes (Fiedler et al., 2023). Differences in the microbiomes of conventionalized (rendered axenic and then reinoculated with a complex microbiome) and conventionally raised larvae have also been observed in zebrafish (Davis et al.,

2016). Such differences in microbiome composition could be due to the elimination of naturally occurring bacteria on the egg by disinfection and germ-free derivation. The impact of axenic derivation on the health and subsequent development of zebrafish larvae is not well understood.

Host genetics and parental transmission may shape the egg microbiome

In some studies, differences in host genetics has been correlated with differences in gut microbiome composition in adult fish (Li et al., 2018; Small et al., 2017; Sylvain et al., 2020). This may also be true for the microbiome composition of fish eggs. For instance, the microbiome composition and diversity of brown trout eggs was influenced by sire and dam identity (Wilkins et al., 2016), suggesting a role for host genetics. In other studies, parent-to-offspring transmission has been shown to influence the gut microbiome composition of larval fish, such as in the discus fish (Sylvain & Derome, 2017) and pipefish (Tanger et al., 2024). To my knowledge, the role of parent-to-offspring transmission in shaping the microbiome of eggs has not been explored in any fish species. However, there have been studies in other egg-laying vertebrates; for example in some bird species the maternal microbiome (from gut, skin and feathers) is an important source of microbes to the microbiome of eggshells, as well as the gut microbiome of chicks (Chen et al., 2020; van Veelen et al., 2018).

Implications for host-microbiome research

The use of axenic and gnotobiotic animals has enhanced our understanding of host-microbiome interactions. However, these studies are limited in their ability to extrapolate relationships between health and the microbiome in humans and wild animals. Captive and laboratory-reared animals have very different microbiomes than their wild counterparts (Bowerman et al., 2021; Lavoie et al., 2018), and the complexity of their microbiomes is erased when rendered axenic and gnotobiotic. These qualities make it difficult to translate these

experiments outside of the laboratory. However, recent studies of 'rewilding' laboratory mice show that diversifying the microbiomes of laboratory mice may result in immune responses more similar to those in wild mice (reviewed in Thomson et al., 2022). Rendering animals axenic also erases any parental or environment transmission of microbes early in life, which can alter early-life assembly of the fish microbiome. As stated in previous sections, the gut microbiome of zebrafish is critical for maturation of the GI-tract and immune system. It is unknown whether the microbes driving development in zebrafish larvae originate from the parents or are acquired through the environment. Understanding how different source pools of microbes contribute to the egg and larvae microbiomes may reveal ecological mechanisms of microbiome assembly and previously unknown members of the microbiome that could have critical roles in the early-life development of zebrafish.

Conclusion

Here I reviewed current research regarding the early-life assembly of microbiomes in zebrafish and other relevant fish species. Gut microbiome composition, especially early in life, is linked to health and proper development of the host, and domains of research from aquaculture to human health have a vested interest in understanding what processes drive microbiome composition. In zebrafish, there is limited information regarding the assembly of the egg microbiome, the factors that drive microbiome assembly on eggs, or the consequences of the egg microbiome on fish health at later stages of life. This is unfortunate, as the egg microbiome might contain the first bacteria that colonize the larval gut, and therefore may influence health.

Bridge

This review summarizes the current research on early-life microbiome assembly in fish, its importance to egg and larval health and the knowledge gaps in the research. The next chapter

will address one of those knowledge gaps: the composition of the zebrafish egg microbiome and the drivers of assembly. Based on research summarized above, I expected parentage and development stage to be major drivers of variation in the egg microbiome. I observed differences in alpha diversity (average richness and abundance of microbial taxa in the egg microbiome) due to development stage and compositional differences due to parentage and development stage, including differences in beta diversity (pairwise similarity in microbiome composition between eggs) and differentially abundant taxa.

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III. DRIVERS OF VARIATION OF THE ZEBRAFISH EGG MICROBIOME

The experimental question and design for these experiments were designed by me, with feedback from Bohannan Lab members. The zebrafish were reared in the University of Oregon's Huestis Zebrafish Facility. Dr. Karen Adair and I collected the samples needed for this study. I performed DNA extractions and data analysis. Dr. Brendan Bohannan, Dr. Karen Adair and Dr. Bob Week were instrumental in the interpretation of the results.

Introduction

The host-associated microbiome is integral to the health and development of animals, especially vertebrates. The gut microbiome is especially important in early life, influencing the development and function of the immune system, nervous system, and GI tract (Banse et al., 2023; Bates et al., 2006; Bruckner et al., 2022; Chung et al., 2012; Mishima et al., 2020; Phelps et al., 2017; Rolig et al., 2015; Thion et al., 2018; Weitekamp et al., 2021). Given the importance of the gut microbiome to the development of young vertebrates, determining the mechanisms behind early-life assembly of the microbiome is crucial for a complete understanding of the interactions between vertebrates and their resident microbes, and the impact of these interactions on development. While the importance of the early-life microbiome has been described in previous studies, relatively little is known about the assembly of microbiomes early in life. Those few studies that address early-life microbiome assembly focus on humans (Duranti et al., 2017; Ferretti et al., 2018), livestock (Furman et al., 2020; Jurburg & Bossers, 2021), some captive and wild birds (Chen et al., 2020; Jurburg et al., 2019; Kreisinger et al., 2017; Martínez-García et al., 2016; van Veelen et al., 2018), and invertebrates (Greer et al., 2020; Hosokawa et al., 2013; Wang et al., 2020). Interpretations of early-life microbiome studies in humans, wild animals, and non-model organisms can be impeded by small sample sizes, lack of environmental control, and

long development time. These limitations highlight the importance of understanding the mechanisms of early-life assembly in model vertebrates, such as zebrafish and mice, reared in controlled environments.

Zebrafish are a powerful model organism with which to understand host-microbe interactions and their impact on host health. They are useful for studying how microbiomes assemble, particularly in the gut. Such studies have revealed the processes that drive variation in the gut microbiome across individuals and through development (Burns et al., 2016, 2017; Stagaman et al., 2017; Stephens et al., 2015, 2016). However, most studies to date have focused on microbiome assembly in larva and adults, rather than in earlier life stages. Although little is known about early-life microbiome assembly in the zebrafish, it has been shown that interactions between the zebrafish and its gut microbiome early in life influence many aspects of zebrafish health and functioning, including social behavior, and tissue and organ development. Experiments on axenic (germ-free), gnotobiotic, and conventionalized/conventionally raised zebrafish larvae have revealed the important roles that specific members of the zebrafish microbiome play in proper neurodevelopment (Bruckner et al., 2022; Davis et al., 2016; Mohanta et al., 2020; Phelps et al., 2017), the onset of social behavior (Bruckner et al., 2022; Mohanta et al., 2020), immune system development (Rolig et al., 2015) and intestinal development (Banse et al., 2023; Bates et al., 2006). However, the sources of these beneficial microbes is not well understood.

Eggs are an especially underexplored life stage in zebrafish microbiome development. The egg microbiome may influence health and survival of early stage fish, as well as the microbiome composition and function in later developmental stages (Lokesh et al., 2019). For instance, the microbiome composition of Atlantic salmon eggs can influence the gut and skin

microbiome composition of the larvae (Fiedler et al., 2023). In zebrafish, axenic derivation of eggs and re-conventionalization of larvae can lead to different microbiome compositions than conventionally-raised larvae (Davis et al., 2016). This study aims to fill this knowledge gap by determining the drivers of variation in the zebrafish egg microbiome.

Parental identity, or clutch identity, may influence microbiome composition on the eggs, through genetic differences (Wilkins et al., 2016) or transmission of microbes. For instance, parental microbiome transmission is an important driver of early life assembly in other teleost fish, such as pipefish (*Sygnathus typhle*; (Tanger et al., 2024)) and discus fish (*Symphysodon aequifasciata*; (Sylvain & Derome, 2017)), but its role in zebrafish is not clear, in particular because in laboratory aquaria zebrafish reproduce via broadcast spawning and are not known to provide parental care (although little is known about the parental care behaviors of wild populations beyond oviposition; Graham et al., 2018; Spence et al., 2007). Transmission may occur between parent and offspring during reproduction (as has been shown for other fish species; Tanger et al., 2024) and through parental care (Sylvain & Derome, 2017), although this has not been previously documented in zebrafish to my knowledge. Parents may also influence the egg microbiome through maternally transmitted immune proteins (De la Paz et al., 2020).

In addition to possible parental influences, the assembly of the egg microbiome may also be influenced by changes to the egg as it develops. As the chorion structure changes prior to hatching, the microbiome of the eggs may also undergo compositional shifts, driven perhaps by changes to the microbial environment provided by the developing egg. This has been observed in other fish species, such as Atlantic salmon (Lokesh et al., 2019), whitefish (Wilkins et al., 2015) and lake sturgeon (Fujimoto et al., 2013). The developing embryo may also introduce selection

pressure on its microbes, as anti-microbial compounds are produced by the embryo prior to hatching (Ni et al., 2021).

In this study, I address whether clutch identity (parentage) and development influence the microbiome of zebrafish eggs from 0 to 2 days post fertilization (dpf). To investigate the roles of parentage and development, I crossed wildtype adult zebrafish and collected fertilized embryos for DNA extraction and 16S rRNA sequencing. Specifically, I address the following questions: 1) does microbial diversity increase or decrease significantly over the course of embryonic development? 2) do clutch identity and development stage significantly explain variation in microbiome composition between eggs? and 3) does the effect of shared parentage on microbiome similarity change over development? For simplicity, development stage and age are used interchangeably to refer to the numbers of days post fertilization of the eggs.

Methods

Ethics Statement

All zebrafish experiments were approved by the University of Oregon Institutional Animal Care and Use Committee (AUP 20-16 and AUP-21-31).

Sample Collection

I bred adult wildtype zebrafish (ABCxTu genotype) according to the protocols for pairwise breeding of natural crosses in false bottom containers as detailed in *The Zebrafish Book* (Westerfield, 2007). Briefly, 20 male/female pairs from two stock tanks (tank "A" and "B") were placed in a crossing tank with a false-bottom insert to separate fertilized eggs from adults. Crossing tanks were set up the afternoon before collection, with clear plastic dividers separating the males and females. Each crossing container had a single male and female pair, from the same tank. The morning of collection, the water in each tank was replaced with fresh facility water and

the dividers were lifted to allow fish to spawn for 30-60 minutes. Four pairs from each stock tank were successful in producing a clutch of fertilized eggs.

I collected fertilized eggs and rinsed them with embryo media. The first clutch from tank A was rinsed and sorted starting at approximately 1 hour post fertilization (hpf), with the last clutch from tank B rinsed and sorted at approximately 8 hpf. Eggs were stored at a density of 50 to 75 eggs per Petri dish in approximately 50 to 100 milliliters of embryo media. I removed non-viable eggs throughout the experiment. Clutches with more than 100 eggs were split across 2 or more Petri dishes. Clutches were not combined, so each dish of eggs only contained eggs deriving from the same pair of parents (Figure 2). Individual eggs were collected at 0 days post fertilization (dpf; 2-8 hours post fertilization), 1 dpf and 2 dpf. The eggs were immediately frozen in liquid nitrogen and stored at -20 C until DNA extraction. For consistency, I collected from the same dishes each day (two dishes per clutch when possible).

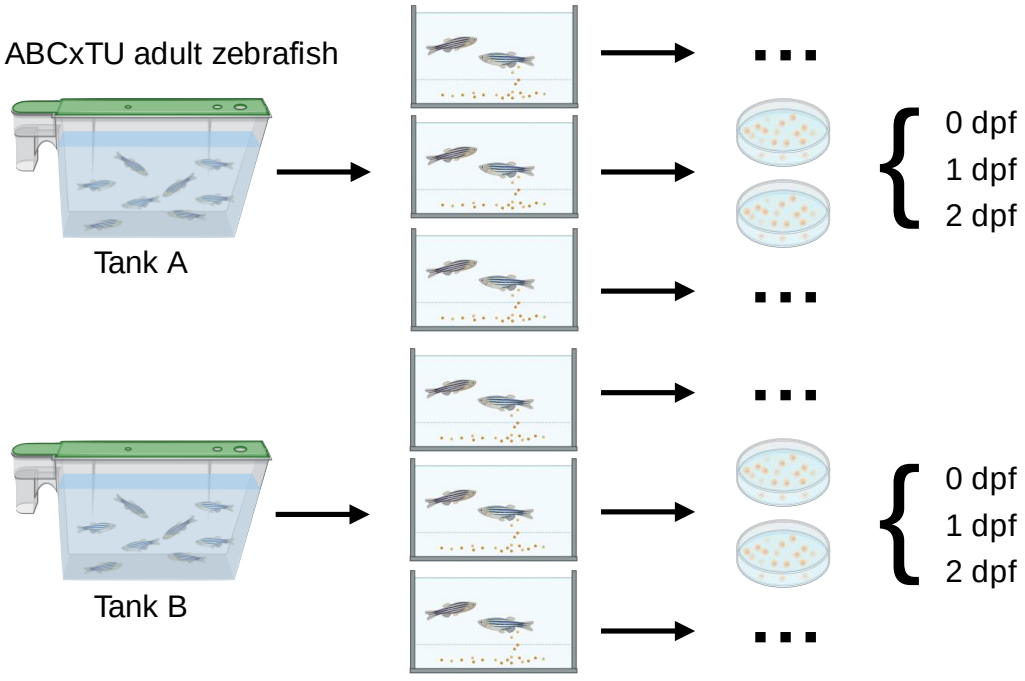

Figure 2: Sampling scheme. Image created with BioRender.com

DNA Extraction and Sequencing

DNA from the whole egg (including chorion and embryo) was extracted using the QIAGEN DNeasy Micro Kit (QIAGEN, Germany) following the manufacturer's protocol for tissue extraction with an overnight lysis step. I amplified the V4 region of the 16S rRNA gene using the following protocol: 12.5 uL NEBNext® Q5® Hot Start HiFi PCR Master Mix (New England Biolabs, USA), 1 uL 515F/806R PCR duel-index primers at 12.5 uM concentration for a final concentration of 0.5 uM (Caporaso et al., 2011; **Error! Reference source not found.**), 10 uL template DNA, and 1 uL Bovine Serum Albumin (Sigma-Aldrich, USA). Amplification was initialized with a 30 second denaturation step at 98° C, followed by 30 cycles of denaturation for 10 seconds at 98° C, primer annealing at 61° C for 20 seconds, and extension at 72° C for 20 seconds, with a final two-minute extension step at 72° C. PCR products were cleaned with Mag-Bind® TotalPure NGS (Omega Bio-Tek, USA) and pooled and sequenced on Illumina MiSeq Micro (Illumina, USA) at the University of Oregon Genomics & Cell Characterization Core Facility (GC3F).

I measured bacterial load using qPCR targeting the V4 region of the 16S rRNA gene using the following protocol: 5 uL PowerTrack SYBR Green Master Mix (Applied Biosystems, USA), 0.8 uL 519F/806R primers at 10 uM concentration for a final concentration of 0.4 uM (Barlow et al., 2020, Eurofins Genomics, Germany, Table 1), 2.95 uL nuclease-free water, 1 uL template, 0.25 uL yellow sample buffer mix Applied Biosystems, USA). Amplification and analysis were performed using QuantStudio 5 and QuantStudio 3/5 Real-Time PCR Software (Applied Biosystems, USA) with the following cycling parameters: an initial 2 minute denaturation step at 95° C, followed by 40 cycles of a 15 second denaturation step at 95° C, a 30 second annealing step at 54° C, and a 30 second extension step at 68° C. Specificity of the protocol was checked via a melting curve analysis, where the qPCR product was subjected to

decreasing temperatures from 95° C to 52° C at 1.6° C/s. I amplified *Escherichia coli* ML35 genomic DNA alongside the egg samples to estimate bacterial 16S copy number per egg. Briefly, I calculated *E. coli* ML35 16S rRNA copy number per uL based on the number of 16S copies per genome, genome size, and GC content (Casale et al., 2018). *E. coli* genomic DNA was serially diluted from 1 to 10^{-6} to calculate a standard curve of threshold cycle (C_t) to number of 16S copies. This standard curve was used to transform mean C_t of each sample to number of 16S copies per uL of sample DNA.

Table 1: Forward (F) and reverse (R) primers. Lower-case and underlined "x" denote index, bold characters denote sequences targeting 16S rRNA gene (Caporaso et al., 2011).

Primer Name	Oligonucleotide Sequence	Use
515F-indexed	AATGATACGGCGACCACCGAGATCTACAC<u>xxxxxxxx</u>TATGGTAATTGT**GTGCCAGCMGCCGCGGTAA**	16S sequencing
806R-index	CAAGCAGAAGACGGCATACGAGAT<u>xxxxxxxx</u>AGTCAGTCAGCC**GGACTACHVGGGTWTCTAAT**	16S sequencing
519F	**CAGCMGCCGCGGTAA**	qPCR
806R	**GGACTACHVGGGTWTCTAAT**	qPCR

Sequence Processing

Raw reads had been demultiplexed and trimmed to remove adapter sequences by the GC3F. I used the *dada2* R package to filter, trim, merge paired end reads, identify amplicon sequence variants (ASVs) and remove chimeric sequences (B. J. Callahan et al., 2016) in R version 4.3.2 (R Core Team, 2023). At position 45 on the reverse reads, there was a drop in quality scores, so reverse reads were truncated at position 46. Both reads were trimmed at 150 bp. Reads with more than 3 expected errors were discarded after trimming. The merged sequence length of each ASV was 207 bp.

I assigned taxonomy using the Silva 138.1 prokaryotic SSU taxonomic data set formatted for DADA2 (McLaren & Callahan, 2021; Quast et al., 2012; Yilmaz et al., 2014). ASV tables, metadata and taxonomy tables were imported into R and stored using the *phyloseq* package (McMurdie & Holmes, 2013) and *decontam* was to identify and remove contaminants using the prevalence-based method with a threshold of 0.05 (B. Callahan & Davis, 2022). After removing contaminant sequences, I used MAFFT 7.525 (Katoh & Standley, 2013) and FastTree 2.1.11 (Price et al., 2010) using the Jukes-Cantor + CAT model to align non-contaminant sequences and infer phylogeny.

Data Analysis

Diversity metrics

All data analysis following sequence processing was performed in R version 4.3.2 (R Core Team, 2023), with *ggplot2* (Wickham, 2016), *cowplot* (Wilke, 2024), and *microshades* (Dahl et al., 2022) used to produce plots. To minimize the effect of library size on variation in alpha and beta diversity, I normalized ASV counts through repeat rarefaction (Cameron et al., 2021; Weiss et al., 2017) and used the *vegan* package to rarefy the ASV table without replacement to a minimum sampling depth of 2,651 reads. I then calculated alpha diversity metrics on the rarefied ASV table using the packages *vegan* (ASV richness, Shannon-Weaver Index/Shannon (Shannon, 1948) , Pielou's Evenness Index (Pielou, 1966)) and *picante* (Faith's Phylogenetic Diversity, Faith, 1992; Kembel et al., 2010). For beta diversity, I calculated weighted and unweighted Unifrac (Lozupone & Knight, 2005) and Jaccard and Bray-Curtis dissimilarity (Bray & Curtis, 1957; Jaccard, 1912) using *phyloseq* and *vegan*. I repeated this rarefaction step and recalculated diversity 10,000 times using the *parallel* package and averaged the alpha and beta diversity metrics across each randomly rarefied community.

Influence of tank, clutch and age on microbiome diversity

Regression models using the package *lmerTest* (Kuznetsova et al., 2017) were used to analyze the effect of development and clutch on mean bacterial 16S copies per egg and alpha diversity. I ran separate models to test how alpha diversity differed by age and between clutches, where sampling depth was provided as a covariate in every test. For regression models exploring the effect of age on alpha diversity, I allowed the intercept to vary between clutches, and when exploring the effect of clutch identity on alpha diversity, I allowed the intercept to vary between ages. I used Tukey tests to conduct multiple comparison tests of mean alpha diversity between groups (i.e. between different ages and between different clutches) with the *multcomp* package (Hothorn et al., 2008). P-values for these comparisons were adjusted using the Holm method.

To investigate phylogenetic clustering or overdispersion in the microbiome, I calculated the Net Relatedness Index (NRI; (Braga & Hérbert, 2022; Webb et al., 2002)) and the Nearest Taxon Index (NTI; (Braga & Hérbert, 2022; Webb et al., 2002)) of each egg microbiome using the package *picante*, calculating mean pairwise distance (MPD) and mean nearest taxon distance (MNTD) and subtracting 1 from the z-score. I tested whether the mean NRI and NTI were significantly different from zero using a Wilcoxon Signed Rank test.

To test for whether the effect of clutch on microbiome composition was confounded by a Petri dish effect (similar to a tank effect observed in cohoused animals; McCafferty et al., 2013; Minich et al., 2020), I performed a nested non-parametric MANOVA using the *BiodiversityR* package (Kindt, 2023). Parents from tank A laid fewer eggs than parents from tank B (9 dishes of approximately 50-75 eggs vs. 16 dishes; Table 2). The only dish from tank A clutch 3 parents (A3) had fungal growth at 2 dpf, so these 2 dpf eggs were not collected. Because I had better representation of eggs across both dishes from the tank B clutches, only these clutches were used in this analysis. I used *vegan* to perform PERMANOVAs (with 999 permutations) and principal

coordinates analyses (PCoA) on the multiple dissimilarity matrices to determine the effect of age, clutch identity, tank, and sampling depth on microbiome composition.

Effect of clutch over development

I then investigated whether the effect of clutch varied across development (i.e. whether eggs with shared parentage remained more similar than eggs without shared parentage across development time). To address this question, I used a Bayesian regression model with the R package *brms* (Bürkner, 2017, 2018, 2021) to model the effect of age and shared parentage (coded as 0 or 1 for different clutch or same clutch, respectively) on pairwise similarity (1-Jaccard Index) between eggs. A Bayesian regression model was ideal to investigate this relationship because it allowed me to control for non-independence in my dataset arising from pairwise measurements and uneven sampling of eggs derived from each stock tank. These methods have also been used to identify drivers of microbiome similarity in wild mice (Raulo et al., 2021). Since Jaccard similarity is bounded by 0 and 1, the models were fit to a beta distribution. Cross-tank pairs (A-B) were excluded from the model, because breeding pairs of fish were always from the same tank. The intercept and slope of this model varied by a multi-membership random effect capturing both eggs in each pairwise comparison (1 | mm[Individual$_X$, Individual$_Y$]), and by a random effect capturing clutch identity of the individuals in the pair. I tested the interaction effect between same clutch status (coded as 1 for pairs of eggs deriving from the same clutch, and 0 for pairs deriving from different clutches) and age pair (whether the pairwise comparison is between a 0 dpf and 0 dpf egg, 0 dpf and 1 dpf egg, for all possible combinations), to determine whether the effect of same clutch status on the microbiome differs depending the development stages of the eggs being compared. The posterior draws were extracted from the model to calculate the posterior means of clutch effect across

different age egg comparisons. I compared posterior means of shared clutch effect between different ages in the model using the "hypothesis" function in *brms*. The package *tidybayes* (Kay, 2023) was used to extract the predicted values of pairwise similarity from the model and calculate the relative effect of shared clutch on Jaccard similarity among eggs of different ages. For Bayesian regression models, if 95% credible intervals (CI) of the posterior distribution do not include 0, the term in question is considered to have a significant effect on the dependent variable.

Differential abundance analysis

To identify taxa driving the differences in composition between developmental stages, I conducted a differential abundance analysis of taxa using the R package brms. Taxa were aggregated at family and genus levels. Using a zero-inflated negative binomial regression model, I tested whether the abundance of individual taxa varied by age, with clutch identity as a random effect. Zero-inflated negative binomial regression models are ideal for microbiome count data, particularly when the data are over-dispersed and sparse. Such models have been applied to differential abundance analyses previously (Sharpton et al., 2021; Zhang & Yi, 2020). Each taxon was tested individually to determine whether the total abundance of said taxon increased or decreased with egg age. A second model included read depth (scaled from 0 to mean abundance of all phyla, families, or genera) as a variable. Both models allowed the intercept to vary with respect to clutch. The function "loo_compare" was used to select the best fitting model for each taxon. All taxon models were run on 4 chains with 4000 iterations. Taxon models with over 10 divergent transitions (out of 8000) and R-hat values greater than 1.03 were discarded. Posterior means were divided by the breadth of the credible intervals to calculate a weighted mean and account for uncertainty in the means. The package *ggtree* was used to plot the estimates for each

taxon alongside its' tip on a phylogenetic tree. To examine whether taxonomic class is associated with differentially abundant genera (i.e. whether genera within each class are associated with increasing abundance, decreasing abundance, or insignificant changes in abundance), I used a Chi-square test and plotted the Pearson residuals with the package *corrplot* (Wei & Simko, 2021).

Results

I collected approximately 450 eggs from tank A, split across a total of 9 dishes, with approximately 50 eggs stored per dish. I collected approximately 800 eggs from tank B, split across 16 dishes. Of those dishes, I sampled eggs from 5 dishes collected from tank A, and 8 dishes collected from tank B. At 1 day post fertilization, I removed 39 dead/nonviable eggs from tank A dishes and 14 from tank B dishes. I also observed what was likely fungal growth in the clutch A3 dish at 2 days post fertilization, so I did not collect 2dpf eggs for DNA extraction from this dish. I collected a total of 124 eggs across the three ages and 8 clutches for subsequent microbial DNA extraction and sequencing.

After filtering using *dada2* and *decontam*, 27 samples had fewer than 2500 reads and were removed. For the subsequent analyses, I used 97 total eggs across 8 clutches and 3 development stages. Tank B parents produced more eggs than tank A, and after filtering, I had microbiome data from more than double the number of tank B eggs than tank A (67 eggs, compared to 30 eggs, Table 2).

Table 2: Egg samples undergone DNA extraction and 16S rRNA sequencing used in analyses. Clutches A1-A4 were derived from adults in stock tank A. Clutches B1-B4 were derived from adults in stock tank B.

Clutch	**All eggs sampled Age (dpf)**			**Eggs > 2500 reads Age (dpf)**			**Number of Dishes**
	0dpf	1dpf	2dpf	0dpf	1dpf	2dpf	
A1	3	3	3	1	3	3	Only 1 dish of viable eggs

A2	4	3	3	0	3	3	3 dishes, 2 sampled
A3	2	3	0	2	3	0	Only 1 dish of viable eggs
A4	6	6	6	6	4	2	2 dishes
B1	6	6	6	5	6	6	2 dishes
B2	6	6	6	4	6	6	2 dishes
B3	7	6	6	5	5	6	2 dishes
B4	6	6	6	6	6	6	2 dishes

The egg microbiome was dominated by members of the phylum *Proteobacteria*, especially at 0 dpf. The most genera within *Proteobacteria* were *Rheinheimera*, *Acidovorax*, *Pseudomonas* and *Aeromonas* (Figure 3a). Log-transformed mean bacterial load had a significant linear relationship with age and increased between ages 0 and 2 dpf (Estimate = 0.500, p = 0.0173, Figure 3b).

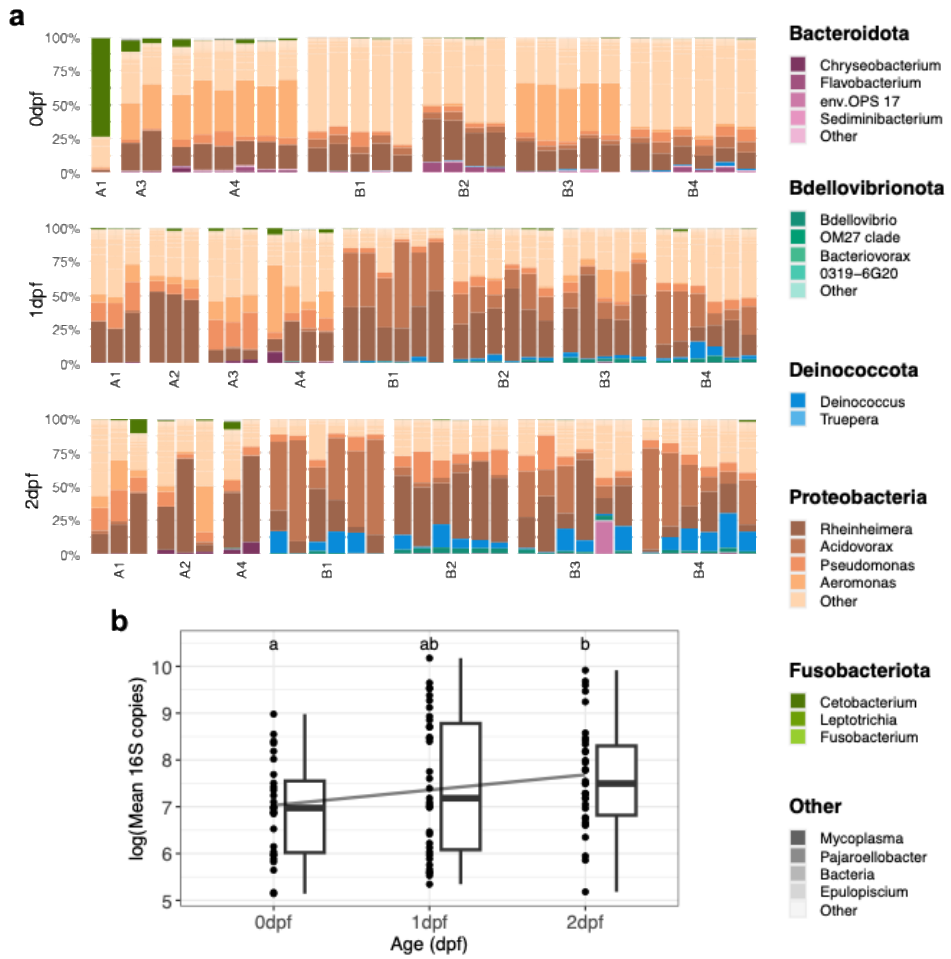

Figure 3: (A) Relative abundance of genera in zebrafish eggs, facetted by age (y-axis) and clutch (x-axis). Genera are colored by phyla to which they belong. (B) Natural log of mean 16S copies per egg. The solid line represents the linear relationship between the log of mean 16S copies and age. Means not sharing any letter are statistically significant according to multiple comparison tests with Tukey contrasts and p-value <= 0.05. P-values were adjusted using the Holm method.

Phylogenetic microbiome diversity decreases during development

Changes in egg microbiome alpha diversity during embryonic development have been observed in lake sturgeon (Fujimoto et al., 2013) and whitefish (Wilkins et al., 2015), but not Atlantic salmon (Lokesh et al., 2019). I measured alpha diversity using ASV richness, Shannon Index, Pielou's evenness and Faith's Phylogenetic diversity of the egg microbiome starting at fertilization (0 dpf) to immediately before hatching (2 dpf) to examine how microbiome diversity changes on zebrafish eggs. There were no significant differences in ASV richness, Shannon's

Diversity of Evenness with respect to age (ASV Richness: F = 0.943, p = 0.393; Shannon: F = 0.879, p = 0.419; Evenness: F = 2.042, p = 0.136). However, there was a significant negative linear relationship between Faith's PD and age (Estimate = -4.188, t-value = -9.045, p < 0.0001; Figure 4: *dashed line*) and a significant, but smaller positive quadratic relationship between Faith's PD and age (Estimate = 1.61, t-value = 3.865, p = 0.0002; Figure 4: *solid line*). This relationship coincides with a significant decrease in mean PD between 0 dpf and 1 dpf eggs (1 dpf - 0 dpf: estimate = -4.94, $p_{Holm} < 0.001$), and no significant difference in mean PD between 1 dpf eggs and 2 dpf eggs (2 dpf - 1 dpf: Estimate = -0.979, $p_{Holm} = 0.093$). Additionally, the standardized effect size of phylogenetic diversity (i.e. PD adjusted for ASV richness of sample) was significantly higher than expected for 0 dpf eggs (V = 475, p = 0.0009) and lower than expected for 1 and 2 dpf eggs (1 dpf: V = 99, p < 0.0001; 2 dpf: V = 0, p < 0.0001), indicating that the differences in PD across age groups were not the result of differences in ASV richness.

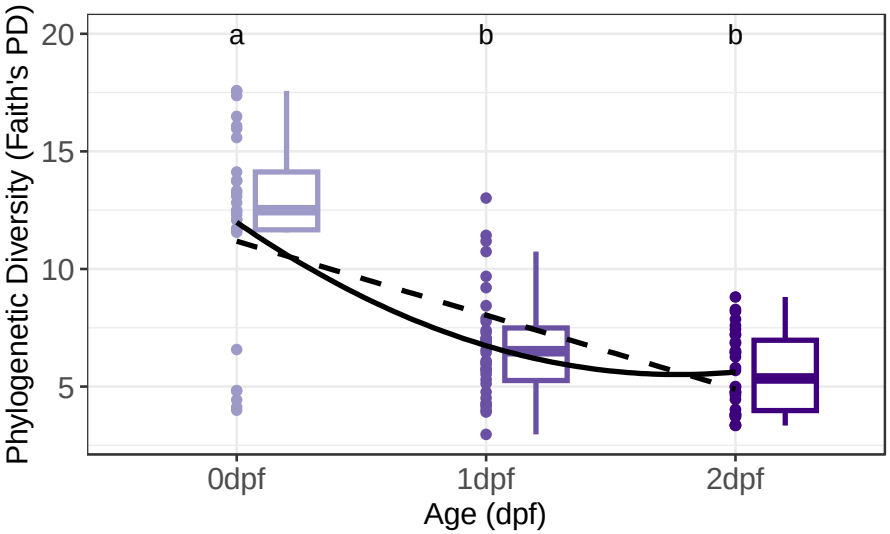

Figure 4: Faith's PD of eggs with respect to age (dpf). The dashed line represents the linear relationship between PD and age, while the solid line represents a quadratic relationship between PD and age. Means not sharing any letter are statistically significant according to multiple comparison tests with Tukey contrasts and p-value <= 0.05. P-values were adjusted using the Holm method.

Since these patterns in phylogenetic diversity could be due to environmental filtering (i.e. "sorting" of specific lineages by the environment of the developing egg), I calculated the net

relatedness index (NRI) and nearest taxon index (NTI) for each microbiome, using the R package *picante*. If mean NRI and NTI are not significantly different from 0, this is an indication of random community assembly with respect to phylogeny. Positive NRI and NTI are indicative of phylogenetic clustering (a signal of environmental filtering), and negative values are indicative of overdispersion (a signal of competition). The mean NRI and NTI of the communities within each age group were significantly different from 0 (Figure 5), so the microbiomes were not randomly assembled with respect to phylogeny. Both NRI and NTI of 0 dpf eggs were less than 0 (NRI: V = 105, p = 0.0003; NTI: V = 192, p = 0.0362). At 1 dpf and 2 dpf, NRI and NTI were significantly greater than 0 (NRI_{1dpf}: V = 1411, p < 0.0001; NTI_{1dpf}: V = 1464, p < 0.0001; NRI_{2dpf}: V = 1378, p < 0.0001; NTI_{2dpf}: V = 1377, p < 0.0001).

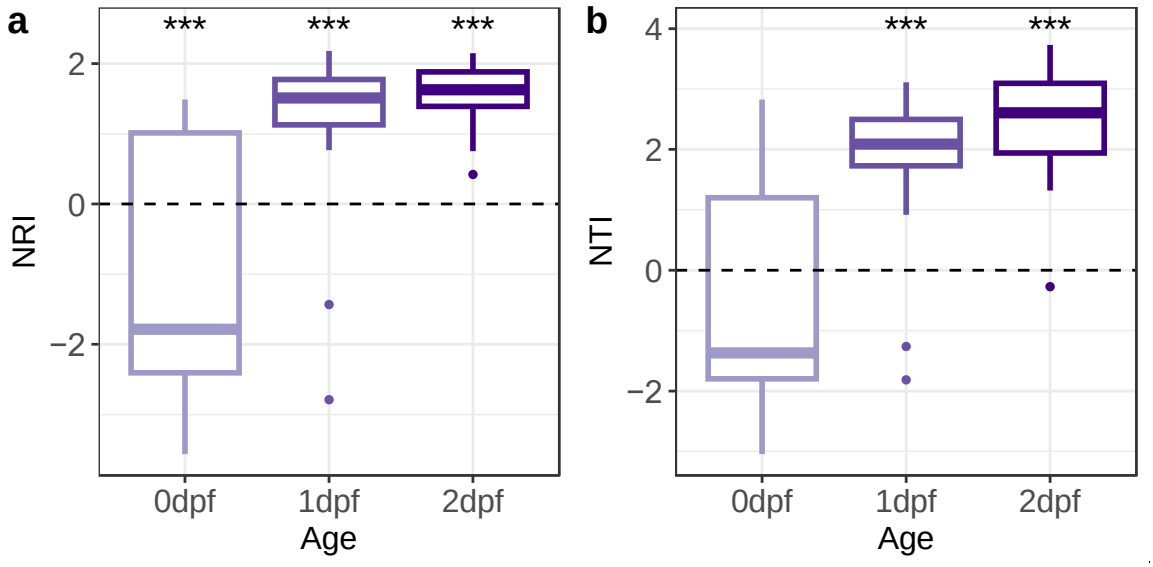

Figure 5: The NRI (a) and NTI (b) of egg microbiomes with respect to age (dpf). Asterisks denote means that are significantly less than 0 (0 dpf) or greater than 0 (1 dpf and 2 dpf) as determined by a one-sample Wilcoxon signed rank test (: p < 0.05, ***: p < 0.0001).*

The values of all alpha diversity metrics significantly varied between clutches (ASV Richness: F = 13.87, df = 7, p-value < 0.001; PD: F = 6.50, df = 7, p-value < 0.001; Shannon: F = 8.49, df = 7, p-value < 0.001; Evenness: F = 7.48, df = 7, p-value < 0.001), with clutches A4 and B4 having significantly greater ASV richness and PD compared to other clutches (Figure 6).

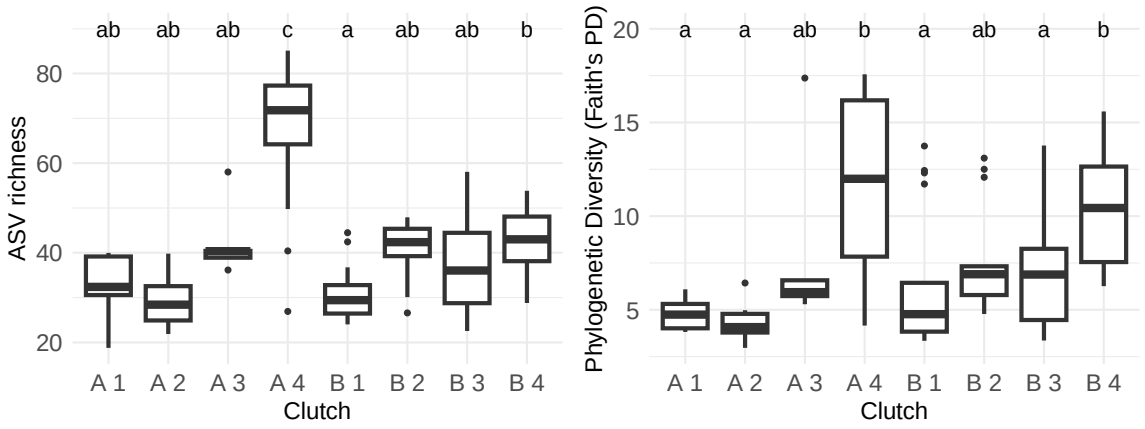

Figure 6: Alpha diversity of eggs within each clutch. Means not sharing any letter are statistically significant according to multiple comparison tests with Tukey contrasts and p-value <= 0.05. P-values were adjusted using the Holm method.

Parentage and Age explain variation in microbiome composition.

At least one study in brown trout eggs has linked dam (female parent) and sire (male parent) identity to variation in microbiome composition of the eggs (Wilkins et al., 2016). Additionally, development stage has also correlated with compositional shifts in the egg microbiome of other fish species (Fujimoto et al., 2013; Lokesh et al., 2019). I investigated whether clutch identity (indicating parentage) and development similarly influence the microbiome composition on zebrafish eggs, measured using Bray-Curtis, Jaccard and Unifrac Dissimilarty indices. Since the eggs from distinct clutches were housed in separate dishes, a nested PERMANOVA with 100 permutations was used to test whether dish was a significant source of variation and a potential cofounder for the effect of clutch identity on microbiome composition. Dish was not a significant source of variation between eggs when nested within clutch for any of the metrics of beta diversity (Bray-Curtis: $F = 1.020$, $p = 0.406$; Jaccard: $F = 1.055$, $p = 0.386$; Unifrac: $F = 0.877$, $p = 0.416$). Therefore, this term was left out of subsequent analyses.

Egg microbiome composition varied by age, clutch identity (proxy for parental identity), and which stock tank the parents originated from (A or B; Figure 7). PERMANOVA tests found that ASV composition and phylogenetic composition varied significantly by age (Bray: $R^2 = 0.209$, $p < 0.001$; Jaccard: $R^2 = 0.170$, $p < 0.001$; Unifrac: $R^2 = 0.226$, $p < 0.001$), parental stock tank (Bray: $R^2 = 0.219$, $p < 0.001$; Jaccard: $R^2 = 0.153$, $p < 0.001$; Unifrac: $R^2 = 0.089$, $p < 0.001$), and clutch identity nested within tank (Bray: $R^2 = 0.157$, $p < 0.001$; Jaccard: $R^2 = 0.152$, $p < 0.001$; Unifrac: $R^2 = 0.104$, $p < 0.001$). A *betadisper* analysis revealed no variation in dispersion between ages when the eggs were not subset by parental stock tank (Bray-Curtis: $F = 1.037$, $p = 0.358$; Jaccard: $F = 1.379$, $p = 0.257$; Unifrac: $F = 1.509$, $p = 0.227$). However, excluding Unifrac dissimilarity, there was variation in dispersion of eggs by age among tank B eggs (Bray-Curtis: $F = 10.252$, $p < 0.001$; Jaccard: $F = 9.913$, $p < 0.001$; Unifrac: $F= 1.508$, $p = 0.229$), but not among tank A eggs (Bray-Curtis: $F = 1.93$, $p = 0.164$; Jaccard: $F = 2.449$, $p = 0.105$, Unifrac: $F = 4.930$, $p = 0.015$). A post-hoc Tukey HSD test on the *betadisper* analysis of tank B eggs found that 2 dpf eggs were more variable than 1 dpf eggs (Bray-Curtis: difference = 0.086, 95% confidence level = 0.032 to 0.140, $p < 0.001$; Jaccard: difference = 0.078, 95% confidence level = 0.026 to 0.129, $p = 0.0016$) and 0 dpf eggs (Bray-Curtis: difference = 0.093, 95% confidence level = 0.037 to 0.149, $p < 0.001$; Jaccard: difference = 0.089, 95% confidence level = 0.035 to 0.142, $p < 0.001$). For Unifrac dissimilarity, the 2 dpf eggs from tank A were less variable than 1 dpf eggs (Unifrac: difference = -0.093, 95% confidence level = -0.190 to 0.005, $p = 0.064$) and 0 dpf eggs (Unifrac: difference = -0.129, 95% confidence level = -0.235 to -0.024, $p = 0.014$). There was no significant variation in dispersion of eggs between clutches (Bray-Curtis, $F = 0.673$, $p = 694$; Jaccard: $F = 0.608$, $p = 0.748$). Scaled read depth also had a small, but

significant effect on microbiome composition (Bray: $R^2 = 0.015$, $p = 0.003$; Jaccard: $R^2 = 0.016$, $p = 0.002$; Unifrac: $R^2 = 0.012$, $p = 0.06$).

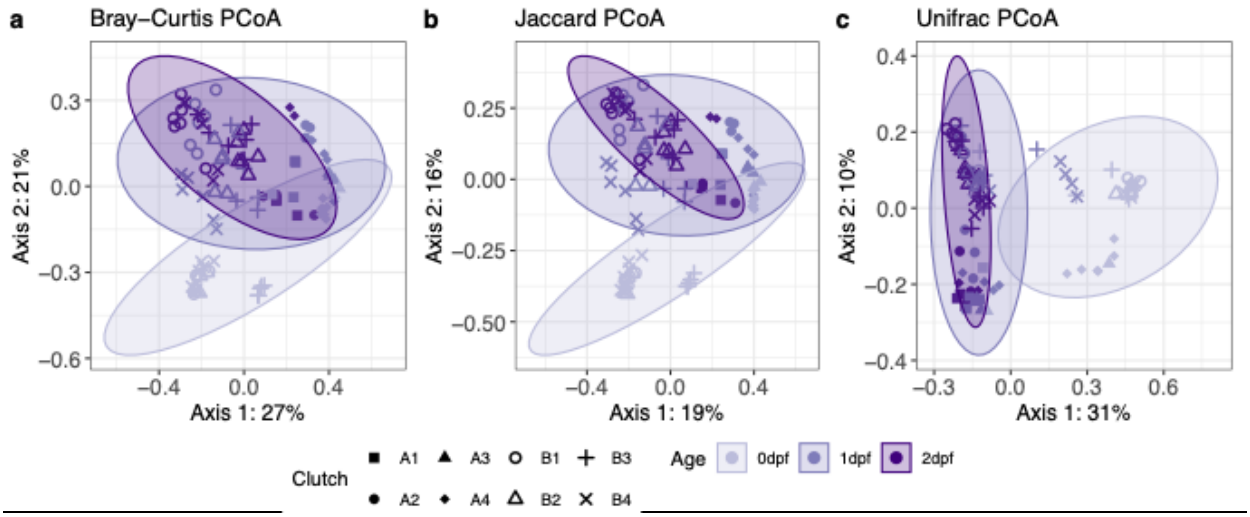

Figure 7: Composition of the zebrafish egg microbiome varies by age, clutch and parental stock tank. The principal coordinate plot (PCoA) show the (a) Bray-Curtis, (b) Jaccard and (c) Unifrac dissimilarity between samples. Ellipses represent the 95% confidence interval around age groups assuming a multivariate t-distribution.

Age and clutch identity have varying effects on pairwise microbiome similarity.

Overall, clutch identity and development stage significantly explained variation in microbiome composition between eggs. I then investigated whether the signal of clutch identity on microbiome composition changed over development. That is, does the signal of shared clutch status (i.e. shared parentage) on the egg microbiome decrease as the embryos approach hatching? I measured pairwise microbiome similarity using Jaccard Index, to calculate the proportion of shared ASVs between pairs of eggs, and then used Bayesian regression modeling (Figure 8) to estimate the effect of clutch on microbiome similarity on pairs of eggs across development (0 dpf-0 dpf, 1 dpf-1 dpf, 2 dpf-2 dpf for all possible age combinations). Tank pair (A-A or B-B) was included as a population level effect. Pairs of eggs from tank B (B-B) were significantly more like each other than pairs of eggs from tank A-A (Jaccard: posterior mean (95% CI) = 0.339 (0.0646, 0.608). In general, eggs originating from the same clutch had higher Jaccard similarity

than eggs originating from different clutches (posterior mean (95% CI) = 1.27 (0.920, 1.64)). However, this effect of shared clutch varied depending on the ages of the eggs (Table 3). The effect of shared clutch was significantly lower for 1 dpf and 2 dpf eggs than 0 dpf eggs (0 dpf - 1 dpf: posterior mean (95% CI) = 0.525 (0.154 to 0.876), posterior probability > 0.95; 0 dpf - 2 dpf: posterior mean (95% CI) = 0.613 (0.009 to 0.121), posterior probability > 0.95). Between 1 dpf and 2 dpf, the effect of shared clutch was not significantly different (1 dpf - 2dpf: posterior mean (95% CI) = 0.088 (-0.532 to 0.720), posterior probability = 0.592).

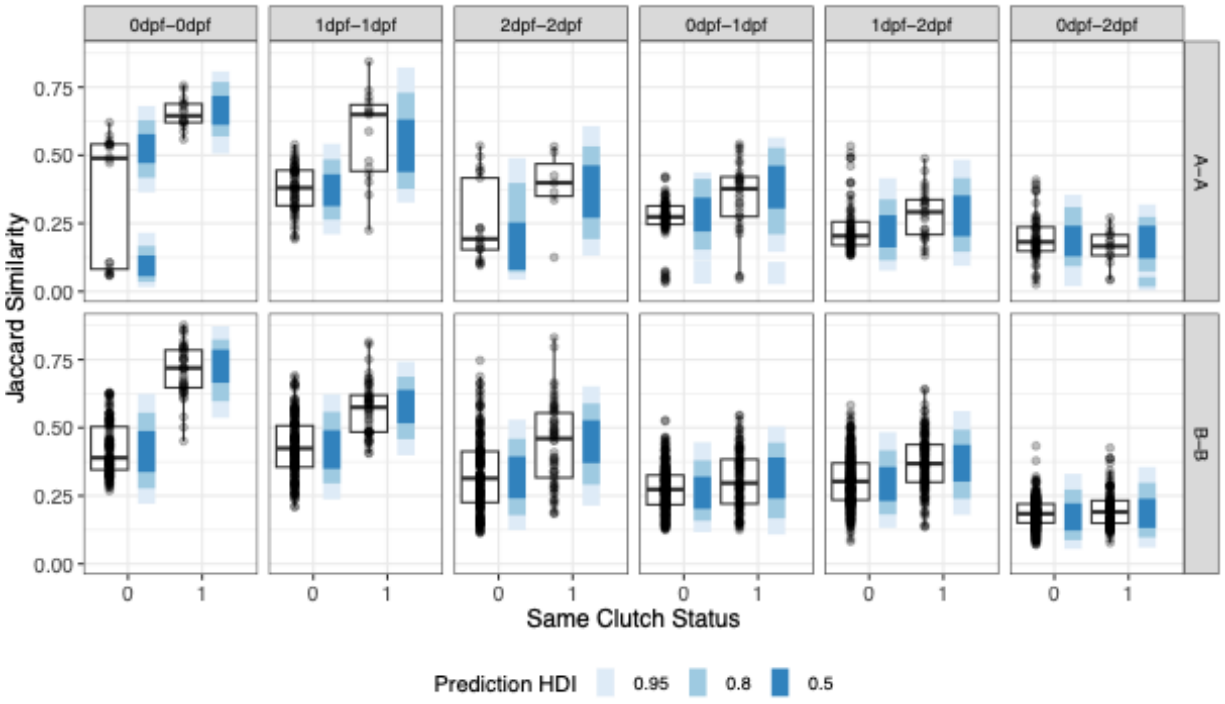

Figure 8: Boxplots of pairwise similarity alongside the posterior distribution of predicted values for Jaccard similarity. Color bar indicates levels of the median highest density interval (0.5, 0.85, and 0.95). '0' denotes pairwise comparisons of eggs from different clutches, while '1' denotes pairwise comparisons of eggs from the same clutches.

Table 3: Posterior mean estimates and 95% quantile credible intervals for fixed effects from Bayesian regression model testing for the effect of shared clutch, age pairing, their interactions, stock Tank, and scaled read depth difference on Jaccard similarity. Bolded values indicate estimates significantly different from 0. '' denotes contrasts from hypothesis testing that have a posterior probability greater than 0.95.*

	Different Clutch (95% CI)	**Shared Clutch (95% CI)**	**Hypothesis: Shared > Different Clutch (95% CI)**

Intercept	**-0.746 (-1.08, -0.422)**	**0.524 (0.143, 0.896)**	1.27 (0.981, 1.58) *
Age pair			
1dpf-1dpf	0.13 (-0.206, 0.481)	**0.875 (0.46, 1.31)**	0.746 (0.449, 1.06) *
2dpf-2dpf	**-0.496 (-1.03, 1.77E-4)**	0.162 (-0.435, 0.745)	0.657 (0.130, 1.19) *
0dpf-1dpf	**-0.553 (-0.844, -0.249)**	-0.344 (-0.771, 0.12)	0.209 (-0.164, 0.596)
1dpf-2dpf	**-0.484 (-0.84, -0.126)**	-0.206 (-0.607, 0.19)	0.279 (0.009, 0.547) *
0dpf-2dpf	**-1.00 (-1.32, -0.664)**	**-1.04 (-1.49, -0.614)**	-0.040 (-0.391, 0.292)
Shared Clutch	**1.27 (0.920, 1.64)**		
Tank B	**0.339 (0.0646, 0.608)**		
Scaled Read Depth	0.155 (-0.0936, 0.413)		

According to the regression model, the predicted Jaccard similarity of eggs at 0 dpf is 0.769 (95% quantile CI = 0.592 to 1.08) times greater when the eggs originate from the same clutch than from different clutches. This is equivalent to an absolute difference of 0.295 (95% quantile CI = 0.247 to 0.358) in Jaccard similarity between eggs from the same clutches versus eggs from different clutches. The strength of the shared clutch effect decreases as the eggs age, with 1 dpf eggs and 2 dpf eggs of the same clutches having 0.408 (95% quantile CI = 0.265 to 0.628) and 0.421 (95% quantile CI = 0.202 to 0.832) times greater Jaccard similarity than eggs from different clutches, and absolute differences in predicted Jaccard similarity of 0.162 (95% quantile CI = 0.114 to 0.230) and 0.117 (95% quantile CI = 0.0547 to 0.192), respectively (Figure 9).

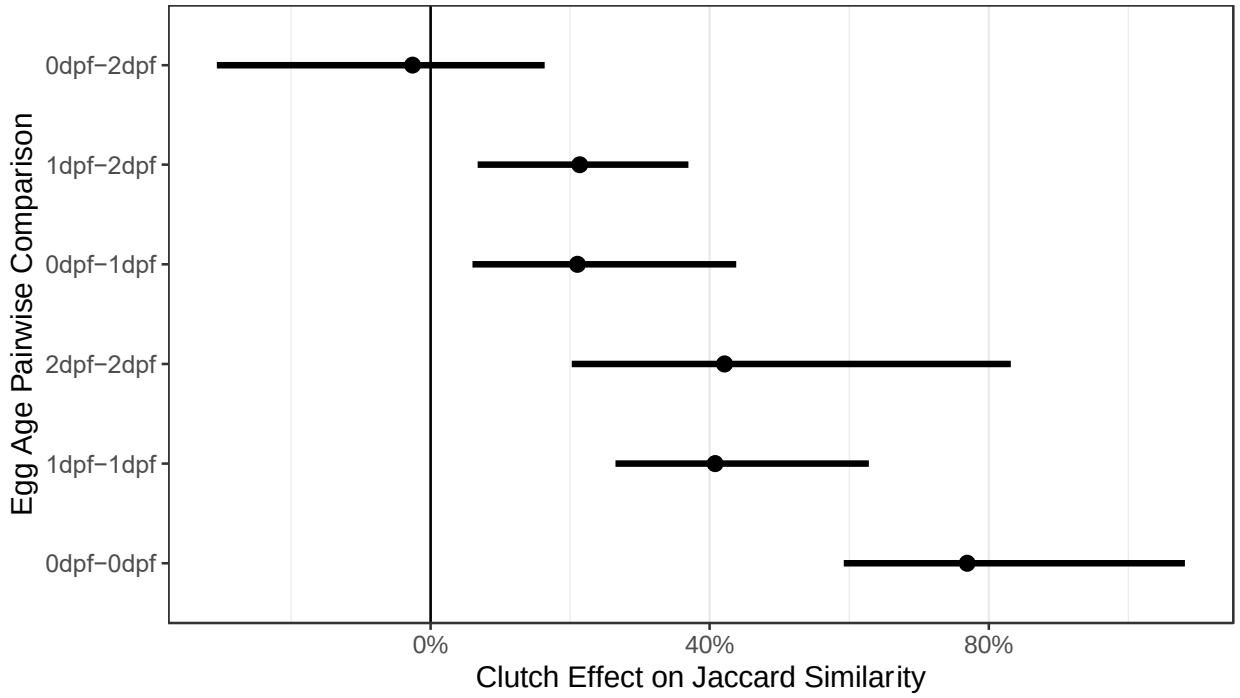

Figure 9: The clutch effect (i.e. the relative difference between pairwise similarity of eggs from the same clutches and different clutches) on Jaccard similarity with respect to the ages (dpf) of eggs in the pairwise comparison.

Alphaproteobacteria are more abundant in 2 dpf eggs than 0 dpf eggs

Using Bayesian regression modeling, the total abundance of 88 out of 216 bacterial genera, and 54 out of 135 bacterial families had a significant linear relationship with respect to age (Figure 10a). A Chi-squared test revealed that genera that are differentially more abundant or less abundant tend to be associated with higher level taxonomic groups ($\chi = 40.923$, $p = 0.032$). For instance, the classes Alphaproteobacteria and Clostridia are positively associated with genera that are increasing in abundance, and negatively associated with genera that are decreasing in abundance, whereas Gammaproteobacteria and Bacteroidia exhibit the reverse pattern (Figure 10b).

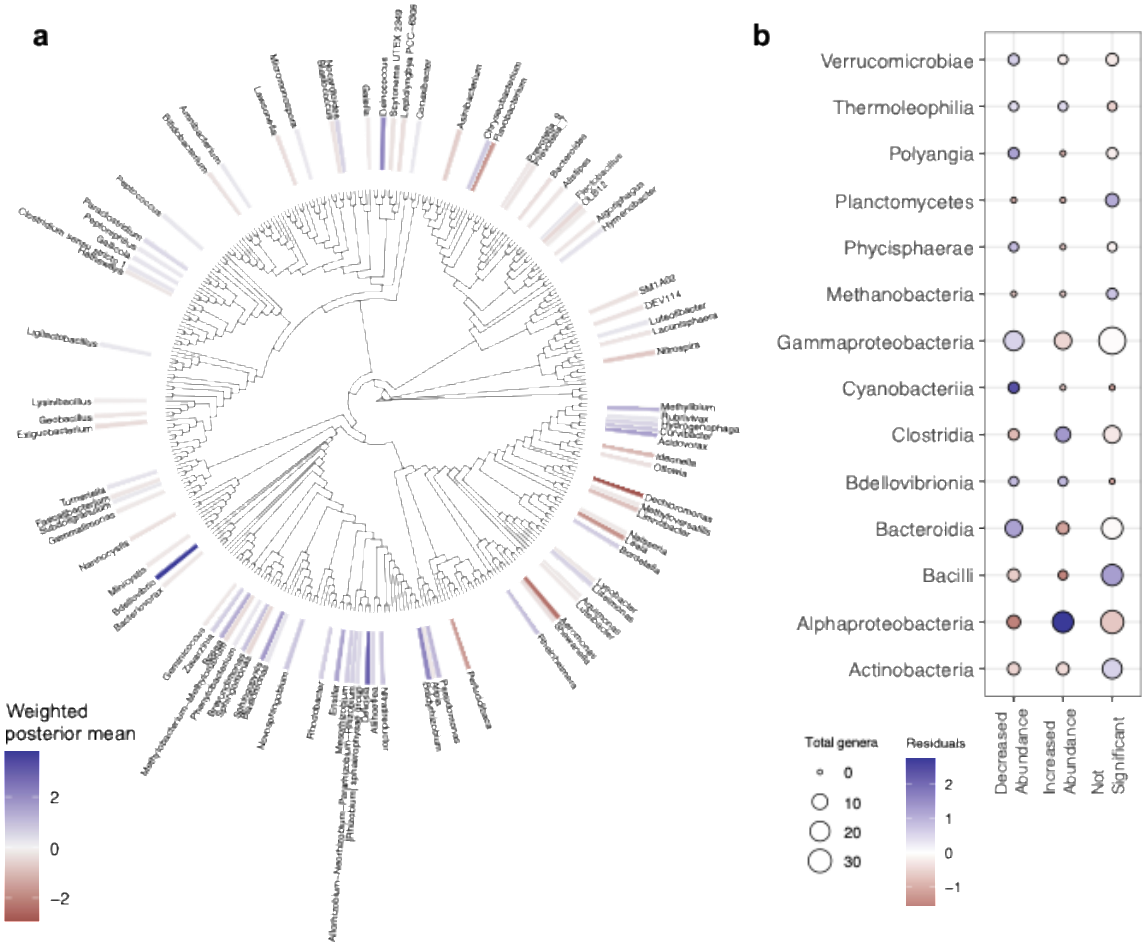

Figure 10: Cladogram (a) of genera with significant linear relationships with age. Bars at the tips of the tree represent the weighted posterior mean calculated from the regression models. Correlation plot (b) of taxonomic classes and differentially abundant genera. Blue represent positive associations, and red represent negative associations. The size of the points represent the total number of genera within that particular class.

Discussion

Early-life assembly of microbiomes can have health and developmental consequences across the life span of a host; however, little is known about the drivers of early-life microbiome assembly in the model vertebrate, zebrafish. Here I present novel research characterizing the egg microbiome of zebrafish, investigating the major drivers of variation of the egg microbiome. In zebrafish-microbe interaction studies, eggs are often sterilized to render them (and the larvae that subsequently develop) axenic. Egg sterilization is also a common practice in aquaculture, where

a major concern is reducing the load of pathogens. However, these practices may eliminate potentially beneficial microbes that could be acquired from the parents or environment in which the eggs were spawned. These bacteria may also persist on the eggs and be later acquired by the larvae. Therefore, understanding the drivers of microbiome assembly on fish eggs may reveal previously unknown commensal microbes, and early members of the larval gut microbiome, which has been shown to be integral for larval health and development (Banse et al., 2023; Bates et al., 2006; Phelps et al., 2017).

In this study, I address the effect of developmental stage and parental identity on microbiome composition of zebrafish eggs. These eggs were derived from wildtype adult zebrafish housed in the University of Oregon's Huestis zebrafish facility. This study focuses on microbiome composition of the eggs during embryonic development, from 0 days post fertilization to 2 days post fertilization and uses 16S rRNA amplification and sequencing to survey the bacterial microbiome on the eggs. I determined how microbiome diversity changes over the course of development and quantified the effect of parentage and development on microbiome composition.

Evidence for environmental filtering of microbes

While there was no observable difference in ASV richness as the eggs aged from 0 dpf to 2 dpf, alpha diversity measured as Faith's Phylogenetic Diversity (PD) significantly decreased (Figure 4). This result could be due to environmental filtering of the microbiome by the developing egg. Environmental filtering, aka species sorting, can lead to phylogenetic clustering (i.e. a pattern where members of a microbiome are more closely related than predicted by random microbiome assembly). I observed that values of the Nearest Taxon Index and Net Relatedness Index were greater than 0 for 1 dpf and 2 dpf eggs, but less than 0 for 0 dpf eggs

(Figure 5). These results indicate that 0 dpf eggs were phylogenetically overdispersed (i.e. microbiome members were more distantly related than predicted by chance). I found that 1 dpf and 2 dpf eggs were phylogenetically clustered. These patterns suggest that competition may be driving assembly at 0 dpf (because competition among related species can result in overdispersion), while environmental filtering may drive assembly on 1 and 2 dpf eggs. These patterns may also arise due to decreasing bacterial abundance. However, using qPCR to quantify 16S copy number, I found that bacterial abundance increased from 0 dpf to 2 dpf (Figure 3b).

I also found that alpha diversity varied significantly between clutches. This may be due to differences in alpha diversity between the parental microbiome, however this was not part of the scope of this study. In Chapter 3, I will explore the relationship between the egg microbiome and the adult zebrafish microbiome.

Age and parentage explain variation in microbiome composition.

In addition to their relationship with alpha diversity, both clutch identity (a proxy for parentage) and age varied significantly with composition (both in terms of ASV identity and phylogeny) of the egg microbiome. Since dish nested within clutch was not a significant source of variation, the similarity of eggs with shared parentage is not due to cohousing of the eggs. Transmission and host genetics may explain why eggs within the same clutch are more like each other in terms of microbiome composition compared to eggs from different clutches. In general, age explained more variation in microbiome composition than clutch identity and is perhaps a more important driver of variation (in terms of effect size) than clutch identity. To explore this possibility, I examined how pairwise similarity between eggs differed within and across clutches (i.e. shared and different clutches) and whether the effect of shared clutch on the microbiome differed depending on the age of the eggs. As egg age increases, egg microbiomes became more

dissimilar (Figure 8, Table 3). Interestingly, the effect of clutch on microbiome similarity persisted from 0 dpf to 2 dpf, although the strength of that effect decreased as the eggs aged, mainly between 0 and 1 dpf (Figure 9, Table 3).These results indicate that the difference in proportions of shared ASVs between eggs of the same clutch versus different clutches is larger when the eggs are first laid, then later in development.

Additionally, I observed a large effect of tank on microbiome composition. In all metrics of beta diversity, the stock tank of the parents left a significant signal on microbiome composition. These results suggest that housing effects may be passed on to subsequent generations of animals. Future studies could address whether this tank effect on the microbiomes persists when clutches derived from separately housed parents are combined.

Overall, our results indicate that age and shared parentage have a significant and substantial impact on egg microbiome composition. The microbiome associated with fish eggs has been shown to change over development in other fish species with longer embryonic stages than zebrafish, such as brown trout and Atlantic salmon (Wilkins et al 2015, Lokesh et al. 2018). In laboratory conditions (i.e. in embryo media dishes with constant temperature and regular photoperiods), I found that the egg microbiome of zebrafish changes rapidly, both in phylogenetic diversity and composition. The rapid changes in the microbiome may be due to the environmental conditions in the embryo media, or species sorting by the egg. Immunocompetent factors are present inside the eggs of several fish species (Huttenhuis et al., 2006; Løvoll et al., 2006), including zebrafish (De la Paz et al., 2020; Ni et al., 2021). However, it is unknown whether these factors can inhibit growth of microbes on the outside of the egg, since the chorion of fish eggs is likely impermeable to large organic molecules (Hansen and Olafsen 1999). Future

research could delve into how physiological changes of the host and chorion during embryonic development might recruit or inhibit growth and attachment of bacteria to the chorion.

The effect of parentage on the egg microbiome may arise from differences in host genetics (which could result in different patterns of species sorting), or transmission of microbes from the parents and/or the specific tank environment at the time of reproduction. Future studies will incorporate 16S rRNA sequencing of parental tissues and environmental sources (in this case, facility water) and use source-sink inference to quantify the contributions of potential sources to the egg microbiome, and whether these contributions change over time (see Chapter 4 of this dissertation).

Differentially abundant taxa

Previous studies found that Gammaproteobacteria are prevalent and abundant in the gut microbiomes of both larval and adult zebrafish (Roeselers et al., 2011; Stephens et al., 2016). Within this class, *Aeromonas* and *Shewanella*—genera that have been identified as prevalent and/or abundant members of zebrafish adult and larval gut microbiomes (Roeselers et al., 2011; Sharpton et al., 2021; Stephens et al., 2016)—were present in the egg microbiomes, and tended to be less abundant in 2 dpf eggs than in 0 dpf eggs. These genera may persist into adulthood or be re-acquired from the environment or via feeding later in life. Stephens et al., (2016) reported that ASVs belonging to the family Comamonadaceae were highly prevalent in zebrafish across development (often found in 85-95% of fish sampled). I observed that two genera within the family Comamonadaceae, *Ideonella* and *Ottawia,* decreased in abundance from 0 dpf to 2 dpf, while *Acidovorax*, *Methylibium*, *Curvibacter*, *Hydrogenophaga* and *Rubrivivax* increased in abundance. *Flavobacterium*, a group that includes fish pathogens of zebrafish and other species (Avendaño-Herrera et al., 2020; Long et al., 2014; Pérez-Pascual et al., 2021; Stressmann et al.,

2021), significantly decreased in abundance on the eggs from 0 dpf to 2 dpf. Since bacterial load increased during development, the decrease in abundance of genera may not be an absolute decrease, and other genera may be increasing in abundance at a greater rate than genera such as *Aeromonas* and *Shewanella*.

Future directions

I focused on the egg microbiome for this study. In fish species such as Atlantic salmon, the egg microbiome can provide defenses against pathogens. I did not investigate the functions of the zebrafish egg microbiome. Future studies could use a combination of culturing, metagenomics, functional predictions from phylogeny, and microbiome manipulation to determine which taxa and/or functions are beneficial for developing zebrafish eggs. Furthermore, I have not investigated whether these members of the egg microbiome are transient or if they can colonize the larval gut post-hatching. If the egg microbiome is an important source of microbes to the larval gut, there could be consequences later in development, revealing an important life stage during which microbiomes could be manipulated to improve survival of larval and juvenile fish.

Combating pathogens is an important consideration for aquaculture, where detrimental host-microbe interactions can lead to high mortality, especially among larval and juvenile fish (Borges et al., 2021). Microbiome manipulation and probiotics are potential alternatives to antibiotic treatment; however research on the effectiveness of probiotics has shown mixed success (Deng et al., 2022; Skjermo et al., 2015; Tarnecki et al., 2019). Understanding the processes of microbiome assembly in different fish species and under different rearing conditions is vital to the development of efficient methods for microbiome manipulation to benefit host

health. This study is a first step toward understanding the processes that shape the early-life microbiome assembly of zebrafish reared in an aquaculture facility.

Bridge

Parentage and developmental stage are major drivers of variation to the egg microbiome of zebrafish. Eggs derived from the same clutch tended to be more similar in microbiome composition than eggs derived from different clutches, although this effect of shared parentage on microbiome similarity decreased in older eggs. The effect of parentage on the egg microbiome may be attributed to genetics of the parents or embryos, or transmission of microbes from parent to offspring. Since zebrafish eggs are commonly derived axenic, the sources of the egg microbiome are not currently known. In the next chapter, I will explore the role of parental tissues and the environment (facility water and crossing tank microbiomes) as sources to the egg microbiome. Additionally, I will examine whether the egg microbiome is a source for the larval microbiome.

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IV. SOURCES TO THE MICROBIOME OF ZEBRAFISH EGGS AND LARVAE

The experimental questions and design were developed by me, with help from members of the Bohannan Lab. All zebrafish were reared in the UO Huestis Zebrafish Facility. Dr. Karen Adair and I collected the samples (eggs, larvae, water, rotifers and adult tissues). I extracted the DNA, performed PCR and qPCR and analyzed the data. Dr. Brendan Bohannan, Dr. Karen Adair and Dr. Bob Week provided feedback on analyses and aided in interpretation of the results.

Introduction

The origin of an animal's microbiome remains a fundamental question in microbial ecology. For oviparous animals, such as teleost fish, development occurs in an environment rich in microbes, and the chorions, or eggshells, are rapidly colonized by a dynamic community of microbes (Hansen & Olafsen, 1989, 1999; Llewellyn et al., 2014). These microbes may influence the health and hatching success of the eggs (Fujimoto et al., 2018; Liu et al., 2014; Wilkins et al., 2016) and be the first to colonize the larval gut upon hatching, affecting the trajectory of future microbiome composition and larval health and development.

Previous results (see Chapter 3 of this dissertation) indicate that parentage and age influence the microbiome composition of zebrafish eggs. The egg microbiome changes significantly in taxonomic composition and in phylogenetic diversity, with increased phylogenetic clustering as embryos develop. Microbiome similarity between full siblings (i.e. those originating from the same clutch) is also evident, with this effect decreasing during development. The previous chapter showed that the egg microbiome of zebrafish is dynamic and changes dramatically between fertilization and hatching, due in part to environmental selection of microbial taxa by the embryo media or chorion during development. Parentage also impacts the microbiome, as clutch identity influenced composition of the microbiome associated with the

egg. These results reveal the influence of development and parentage on the egg microbiome of zebrafish. Taken together with similar studies on other fish species (Fujimoto et al., 2013; Lokesh et al., 2019; Wilkins et al., 2015, 2016), changes in the egg during development and differences in parental identity may be vital driving forces of early-life microbiome assembly across Teleost species.

An effect of parentage on the egg microbiome, indicated by higher similarity in composition between eggs from the same vs different clutches, may result from genetic relatedness leading to similar selection of microbes from the environment or from parental transmission of microbes. Under the standard breeding protocols used for zebrafish husbandry, two (one male and one female) or more (one male and multiple females) are placed in crossing tanks for spawning (Westerfield, 2007). This restricts the number of potential sources to the egg microbiome at the time of spawning to the adults in the tank and the crossing tank water, which may also act as a medium for transmission. Common practice in zebrafish microbiome research, and in general aquaculture whether for research or food production, is disinfection of eggs. In research, this allows for the comparison of axenic (i.e. germ-free) animals and conventionalized or gnotobiotic animals. It reduces the microbial load on eggs, in both research and aquaculture, which can be beneficial for egg survival. However, if parental transmission is a significant source of microbes to the eggs and ultimately the larval zebrafish, then germ-free derivation and disinfection erases this effect. Parentally transmitted microbes may play important roles in zebrafish development. For example, *Shewanella* has anti-inflammatory activity in the larval zebrafish gut (Rolig et al., 2015), and is ubiquitous in zebrafish (Roeselers et al., 2011; Sharpton et al., 2021; Stephens et al., 2016). One possibility for its high prevalence in zebrafish intestines is that it may be transmitted from host-to-host, including from parent to egg. Tracking and

identifying parentally transmitted microbes in embryos and larvae may reveal previously unknown but critical members of the zebrafish microbiome and allow us to better investigate the function of the zebrafish microbiome.

Additionally, the egg microbiome may be an important source of microbes for the larval gut. Feeding by larvae on egg debris is a common behavior in other fish species (such as cod and halibut; Hansen & Olafsen, 1999). Egg debris, such as the chorion, is an early source of nutrients and may also be a source of microbes and a medium for transmission of the parental microbiome to the larvae. The introduction of live food such as rotifers (commonly by 7 days post fertilization, or dpf) may alter the zebrafish larval gut microbiome (Wilkes Walburn et al., 2019), and potentially displace members of the microbiome that successfully colonized earlier.

Here, I investigate the sources of the zebrafish microbiome at egg (0 to 2 dpf) and early larval stages (up to 7 dpf). This range encompasses the earliest stages of embryonic development, from hatching until larvae begin feeding. At 7 dpf, the yolk sac is depleted. For studies on axenic fish, this marks the end of the experiment, as feeding the zebrafish, which introduces microbes, is required to continue rearing the fish. Here, I quantify the contributions of parental and environmental sources to the egg and larval gut microbiome, identify putatively transmitted microbes from these sources to the egg and larvae, and address the role of the chorion as a vehicle for transmission of microbes. I use a source tracking algorithm implemented in the R package *FEAST* (Shenhav et al., 2019) to estimate the proportions of the egg and larval gut microbiome originating from parental tissues and water (Figure 11). Using these estimations, I address the following questions: 1) how do the different source contributions to the egg and larval microbiome change over time? 2) what taxa are likely being transmitted from these sources to the egg and larvae? and 3) does removing the chorions alter microbiome composition

of the larvae and influence the transmission of microbes from egg to larvae? Additionally, I show that the results from the previous chapter are reproducible, and that clutch identity and development are significant drivers of variation in the microbiome composition of eggs and larvae.

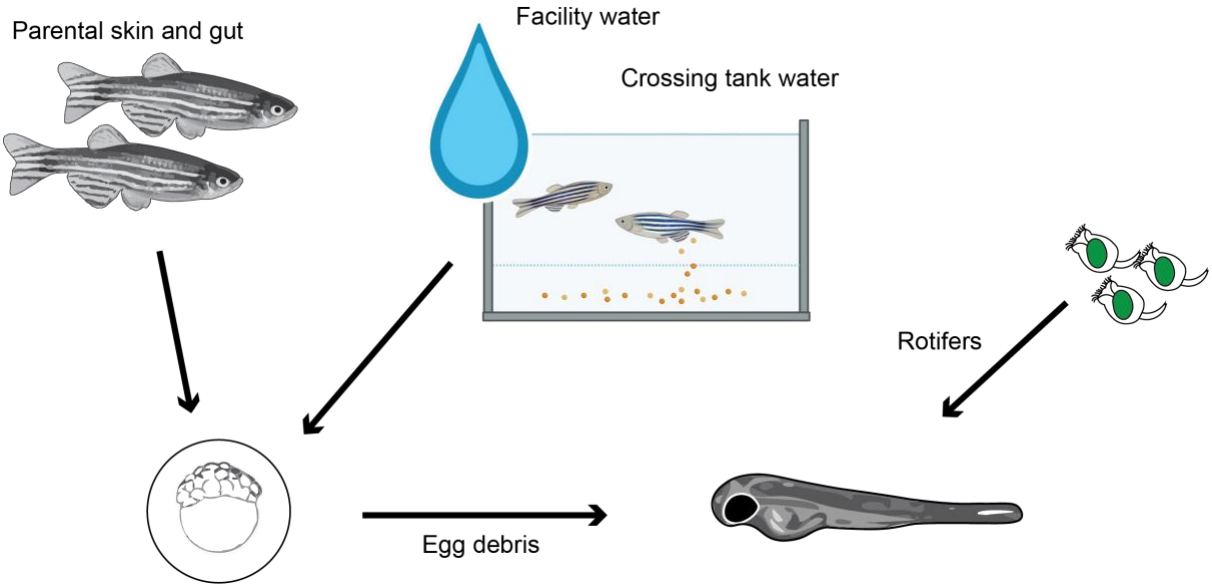

Figure 11: Putative sources to the egg and larval gut microbiome. Eggs are exposed to microbes from the parental skin and gut, facility water and crossing tank water during spawning. Post-hatching, larvae are potentially inoculated by microbes from egg debris and rotifers during feeding.

Methods

Ethics Statement

All zebrafish experiments were approved by the University of Oregon Institutional Animal Care and Use Committee (AUP 20-16 and AUP-21-31).

Sample Collection

I used the same breeding procedures as detailed in the previous chapter. Egg collection methods differed, however, in that I removed the eggs from the crossing tanks within an hour of fertilization to keep the eggs' exposure to potential parent-derived microbes consistent across clutches and closely replicate standard egg collection methods for zebrafish research. I bred adult

wildtype zebrafish (ABCxTu genotype) originating from a single stock tank according to the protocols for pairwise breeding of natural crosses in false bottom containers as detailed in The Zebrafish Book (Westerfield, 2007). I set up 10 male/female pairs in individual crossing tanks. On the day of collection, crossing tank water was replaced with fresh facility water, and fish were allowed to spawn for 30-60 minutes. To investigate the role of water as a source to the egg and larval microbiome, I collected both facility water (i.e. water flowing into the stock tank; "fac.water") and water from the crossing tanks ("x.cage.water") in triplicate in 50 mL falcon tubes. I collected fertilized eggs and rinsed them with embryo media (EM) within an hour and a half of fertilization. Adult fish were euthanized in an ice bath, and I dissected skin and gut tissues. These tissues were stored in a lysis buffer at -20 C for later DNA extraction.

I collected eggs at 0 days post fertilization and 2 days post fertilization and stored these eggs in a DNA lysis buffer at -20°C for later DNA extraction (Figure 12). Zebrafish hatch between 2 and 3 dpf. At 3 days post fertilization, I removed any unhatched eggs. To test for the role of the chorion as a vehicle of transmission of microbes from egg to larva, I transferred half of the larva from each dish to a new dish with fresh EM without their chorions. I collected larvae at 5 dpf, euthanized these larvae in MS-222. I removed the larval gut and transferred them to Eppendorf tubes with lysis buffer and froze them at -20°C for later DNA extraction. After collecting 5 dpf larva, I fed the remaining larva rotifers (day 5 rotifer culture). Larva were fed again at 6 dpf. To maintain the health of the larva during this time, I conducted water quality tests on the (EM) and replaced 75% of the media on day 6 prior to feeding the larva rotifers. Rotifer cultures were collected from the Huestis zebrafish facility on day 5 and day 6 to feed 5 dpf and 6 dpf larvae. All dishes received rotifers from the same culture (Figure 12). DNA was extracted from leftover cultures (keeping the day 5 culture separate from the day 6 culture) using

the same protocols as used for fish to address the role of rotifers a source of microbes to the larval gut. Our last collection was at seven days post fertilization when I dissected the guts from the 7 dpf larvae and euthanized the remaining larvae in the experiment. Prior to all larval dissections, the standard length (SL) of larvae was measured.

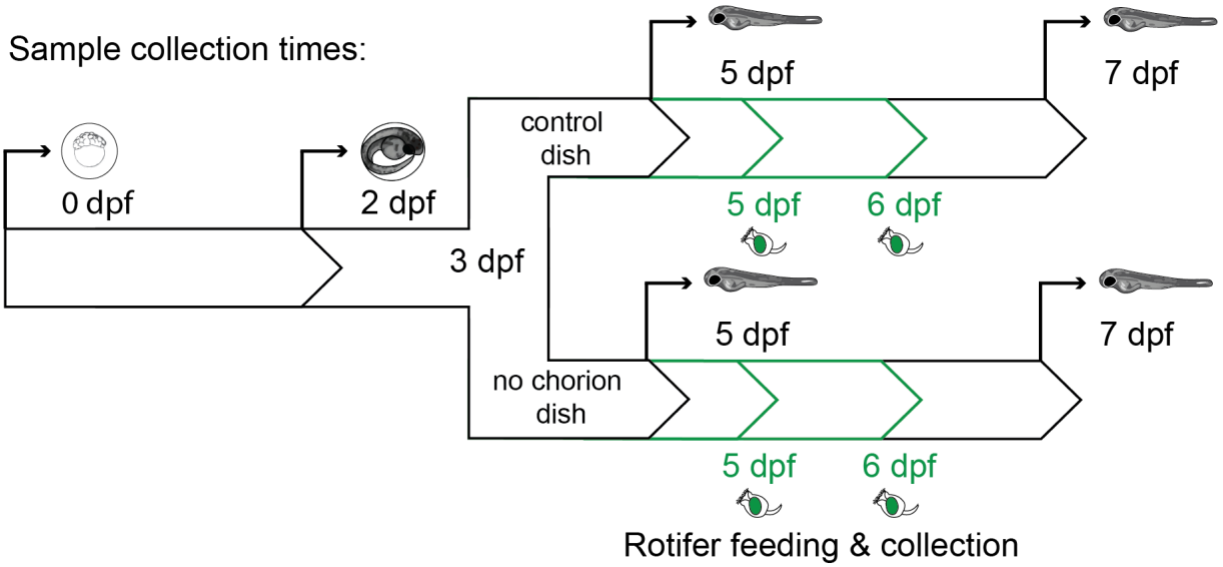

Figure 12: Sampling scheme to estimate contributions of parental tissues, water and food to egg and larval microbiomes. Eggs are collected at 0 and 2 dpf. Dishes are split into "control" dishes and "no chorion" dishes at 3 dpf. Unfed larvae are collected at 5 dpf. Larvae are fed at 5 and 6 dpf. Rotifer cultures are also sampled at these timepoints for DNA extraction. Fed larvae are collected at 7 dpf.

DNA Extraction and Sequencing

I used the DNeasy Blood and Tissue kit (QIAGEN, Germany) with a modified protocol to extract DNA from the adult tissues and rotifers. The protocol is detailed in Stephens et al. (2015). DNA extraction of eggs and larval guts was performed using the DNeasy Micro kit (QIAGEN, Germany) following the tissue extraction protocol with an overnight lysis step. I used Sterivex filters units (MilliporeSigma, USA) to filter each 50 mL water sample and the DNeasy PowerWater Sterivex kit (QIAGEN, Germany) to extract DNA from these filters. I amplified the V4 region of the 16S rRNA gene with 515F/806R barcoded primers. All samples were amplified using the same PCR protocol as the previous chapter. Due to the low biomass of the larval DNA

extractions, I increased the number of cycles from 30 to 35. PCR products were cleaned with omega beads (Omega Bio-Tek, USA) and pooled and sequenced on Illumina MiSeq v2 (Illumina, USA) at the University of Oregon Genomics & Cell Characterization Core Facility (GC3F). Bacterial loads in egg and larval samples were measured by qPCR amplification of the V4 region of the 16S rRNA gene, alongside an *Escherichia coli* ML35 standard, as described in the previous chapter.

Sequence Processing

I used the same bioinformatics pipeline as described in the previous chapter. In brief, I used the *dada2* package (B. J. Callahan et al., 2016) to filter, merge reads, and identify amplicon sequence variants (ASVs), which are used to group and classify taxa. I assigned taxonomy using dada2 and the Silva 138.1 prokaryotic SSU taxonomic data set formatted for *dada2* (McLaren & Callahan, 2021; Quast et al., 2012; Yilmaz et al., 2014). I used *decontam* to remove putative contaminant sequences (B. Callahan & Davis, 2022), and MAFFT 7.525 (Katoh & Standley, 2013) and FastTree 2.1.11 (Price et al., 2010) to align sequences and infer phylogeny.

Data Analysis

Diversity metrics

To confirm that there is an effect of parentage and development on the microbiome of the eggs and larvae in this study (as was reported for the study described in the previous chapter), I use the same data analysis pipeline as described in the previous chapter to address variation in alpha and beta diversity. Briefly, I repeatedly rarefied ASV tables (1000 times) and calculated mean alpha diversity (ASV Richness, Shannon Diversity, Faith's Phylogenetic Diversity, and Pielou's Evenness) and beta diversity (Bray-Curtis Dissimilarity, Jaccard Dissimilarity, and Unifrac Dissimilarity) of the rarefied communities using the packages *parallel* (R Core Team,

2023), *vegan* (Oksanen et al., 2024), *picante* (Kembel et al., 2010), and *phyloseq* (McMurdie & Holmes, 2013). Alpha diversity between age groups and clutches was analyzed using regression models built with the package *lmerTest* (Kuznetsova et al., 2017) and *multcomp* (Hothorn et al., 2008) for multiple pairwise comparisons. Community phylogenetic analyses, such as standard effect size of phylogenetic diversity (SES.PD), net relatedness index (NRI; Braga & Hérbert, 2022; Webb et al., 2002), and nearest taxon index (NTI; Braga & Hérbert, 2022; Webb et al., 2002), were measured using *picante*. The effects of age and clutch identity on microbiome composition were tested using PERMANOVA tests and principal coordinate analyses (PCoA). All plots were built with *ggplot2* (Wickham, 2016), *cowplot* (Wilke, 2024), and *microshades* (Dahl et al., 2022).

Source tracking

I use the package *FEAST* (Shenhav et al., 2019) to identify potential sources to the egg and larvae gut microbiome. *FEAST* uses relative abundance of ASVs in putative sink and source communities to calculate the proportion of the sink community likely derived from a given source community. *FEAST* also calculates the proportions of an unknown source community, representing unsampled sources or contamination. I categorize the egg and larval gut microbiomes as sink communities, and parental tissues, water and rotifers/food as source communities. When identifying sources to the larval gut microbiome, I also include the 0 dpf and 2 dpf egg microbiomes as potential sources to investigate the role of the egg microbiome as a source to the larval gut. I subset the ASV tables and metadata tables by clutch prior to running *FEAST*, so that parents and crossing tank water were only evaluated as sources to the eggs and larvae of the same clutch (ex. clutch 2 parents and water from their respective crossing tank were not evaluated as sources to eggs and larvae from clutch 3). A total of 8 separate *FEAST* models

were run to calculate the mixing proportions of sources to eggs (0 dpf and 2 dpf) and larvae (5 dpf and 7 dpf) from each of the four clutches. Facility water and rotifers were evaluated as sources for all clutches of eggs and larvae. Differences in the mean contributions of each source community were compared across ages using a multivariate Bayesian regression model fit with the R package *brms* (Bürkner, 2017, 2018, 2021). Since the contributions are expressed as proportions of the sink community, I modeled a Dirichlet regression, which is useful for handling compositional data, especially when the data contains compositions of more than 2 groups (Douma & Weedon, 2019). Dirichlet regressions can fail when the data contains 0s or 1s, so source contributions were transformed (Smithson & Verkuilen, 2006). In cases where a source was not evaluated for a given sink (e.g. 0 and 2 dpf eggs were not considered sources to 0 and 2 dpf eggs) and would represent missing values, these values were changed to 1E-300 to force these proportions to be lower than the newly transformed 0 proportions of other evaluated sources to a sink community. To identify taxa associated with groups of sink and source communities (e.g. 0 dpf eggs and parental skin), I performed a Similarity Percentage analysis (SIMPER) with the function "simper" from *vegan* to measure each ASVs contributions to Bray-Curtis dissimilarity between different samples types (egg, larvae, water samples, parental tissues; Clarke, 1993). I also used a multi-level pattern analysis (aka an "indicator species analysis") implemented in *indicspecies* to identify ASVs associated with each sample type and combinations of sample types (De Cáceres et al., 2010; De Cáceres & Legendre, 2009). Indicator species analyses have been used in other studies to identify microbiome sources; for example, such analyses were used to identify taxa present on woodlark and skylark eggs that likely originated from environmental and parental sources (van Veelen et al., 2018). Combined with the

source contributions derived from FEAST, these analyses can identify taxa that are likely transmitted from source to sink communities.

Results

Egg collection and removal details

Five eggs and larvae were collected at each timepoint (0 dpf, 2 dpf, 5 dpf and 7 dpf; Figure 12) for DNA extraction and sequencing, totaling 360 egg and larval samples. Sequences were filtered using *dada2* and *decontam*. Twenty-nine eggs and larvae had fewer than 2500 reads and were removed, leaving 331 eggs and larvae used for the subsequent analysis (Table 4). DNA was extracted from 37 sources, which included 3 samples from water entering the tanks from the facility, 3 crossing tank water samples from each crossing tank, skin and gut tissue from each parent (8 skin and 8 gut total), and 3 samples of each rotifer culture fed to larvae at 5 dpf and 6 dpf (Table 4). The skin sample from the clutch 4 male was removed from analysis, because it had fewer than 2500 reads after *dada2* and *decontam* filtering, so it was not explored as a potential source to the clutch 4 eggs and larvae.

Table 4: Samples with 2500 or more reads were used for data analysis. Samples with fewer than 2500 reads were discarded.

	Clutch			
Age	**2**	**3**	**4**	**5**
0dpf	15	15	13	15
2dpf	15	15	15	15
	Chorions left in dishes			
5dpf	15	14	9	14
7dpf	14	15	12	14
	Chorions removed from dishes			
5dpf	12	11	15	11
7dpf	15	14	15	13
	Source Samples			

Gut	2	2	2	2
Skin	2	2	1	2
x.cage.water	3	3	3	3
fac.water	3			
Rotifers	6 (across 2 days)			

Caption: Samples with 2500 or more reads were used for data analysis. Samples with fewer than 2500 reads were discarded.

The 0 dpf and 2 dpf egg microbiomes were dominated by Proteobacteria, similar to the observations in the previous chapter. Post-hatching, Firmicutes, specifically *Mycoplasma* and *Bacillus*, were prevalent and abundant in the larval gut microbiome (Figure 13a). Additionally, bacterial load, measured via qPCR of the 16S rRNA gene, increased between 0 dpf and 2 dpf (as observed in the previous chapter). Post-hatching, bacterial load decreased, likely due to only sampling the larval gut rather than the entire developing egg. From 5 dpf to 7 dpf, bacterial load increased again (Figure 13b).

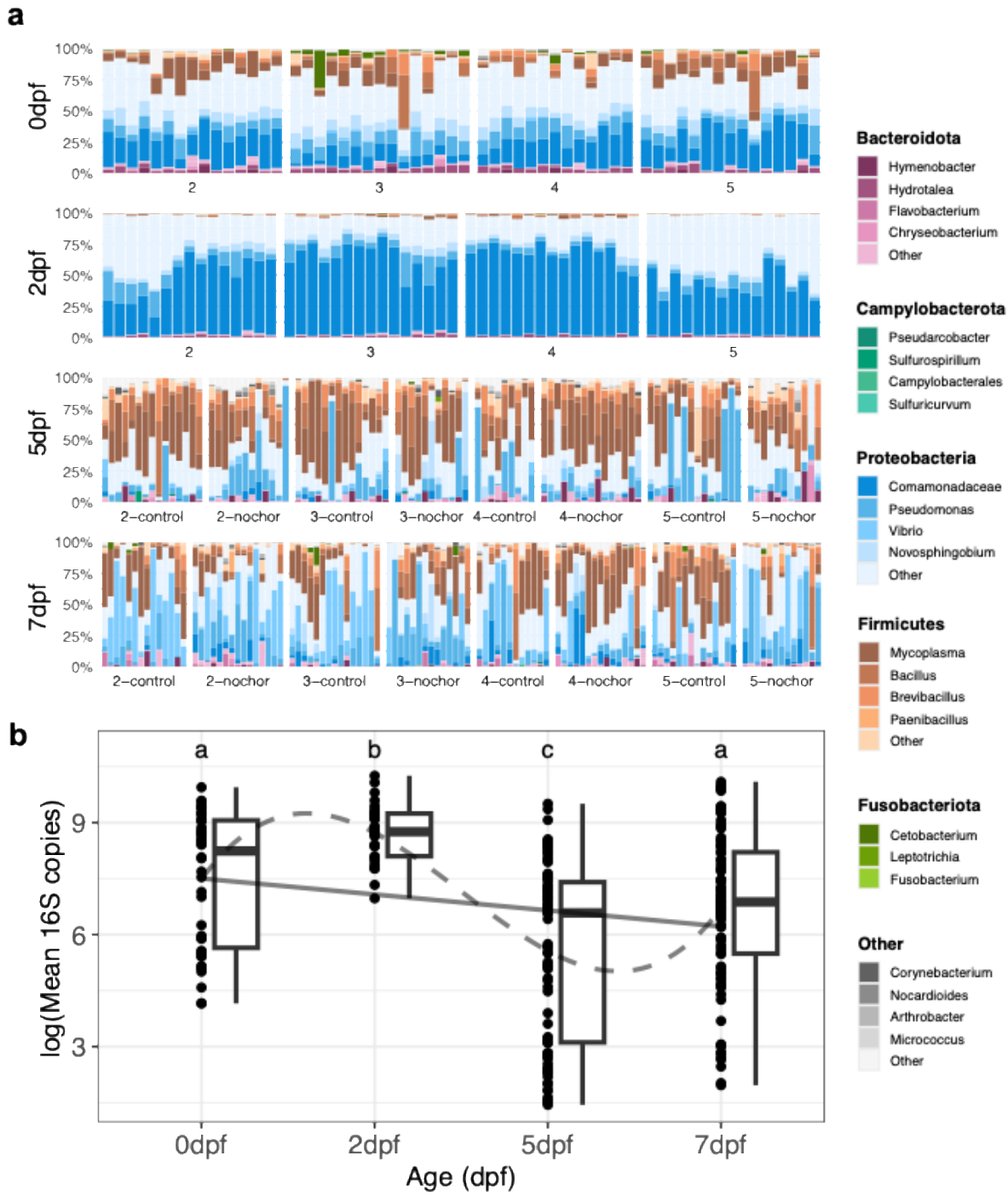

Figure 13: (A) Relative abundance of genera in zebrafish eggs, facetted by age (y-axis) and clutch (x-axis). Genera are colored by phyla to which they belong. (B) Bacterial load, measured by the natural log of mean 16S copies per sample, grouped by age. Means not sharing letters are statistically significant according to Tukey tests and p-values <= 0.05. P-values are adjusted using the Holm method.

Alpha diversity changes over development

To examine changes in alpha diversity over development, I measured ASV richness, phylogenetic diversity (PD), Shannon diversity and Pielou's evenness. In the previous chapter, I did not observe significant differences in ASV richness, Shannon diversity or evenness between 0 dpf and 2 dpf eggs, however, there was a significant decrease in PD over embryonic development. In this study, observed ASV richness and phylogenetic diversity (PD) significantly differed between developmental stages (0 dpf, 2 dpf, 5 dpf and 7 dpf; Richness: $F = 50.46$, $p < 0.0001$; PD: $F = 25.35$, $p < 0.0001$). ASV richness and PD significantly decreased from 0 dpf with age (Figure 14a, Figure 14d). Post-hatching, ASV richness decreased (5 dpf – 2dpf: estimate = -11.25, $p_{Holm} < 0.0001$), but did not significantly change between 5 and 7 dpf (7 dpf – 5 dpf: estimate = -1.34, $p_{Holm} = 0.603$). However, PD did not change significantly post-hatching (5 dpf – 2dpf: estimate = -0.574, $p_{Holm} = 0.096$) or between 5 and 7 dpf (7 dpf – 5 dpf: estimate = -0.269, $p_{Holm} = 0.353$). Shannon diversity and evenness decreased from 0 dpf to 2 dpf, (Shannon: estimate = -1.067, $p < 0.0001$; Evenness: estimate = -0.223, $p < 0.0001$), but rose post-hatching (5 dpf – 2 dpf; Shannon: estimate = 0.398, $p_{Holm} < 0.0001$; Evenness: estimate = 0.154, $p_{Holm} < 0.0001$). From 5 dpf to 7 dpf, these measures of alpha diversity decreased again (7 dpf – 5 dpf Shannon: -0.238, $p_{Holm} = 0.0114$; Evenness: -0.0651, $p_{Holm} = 0.00238$; Figure 14b, Figure 14c).

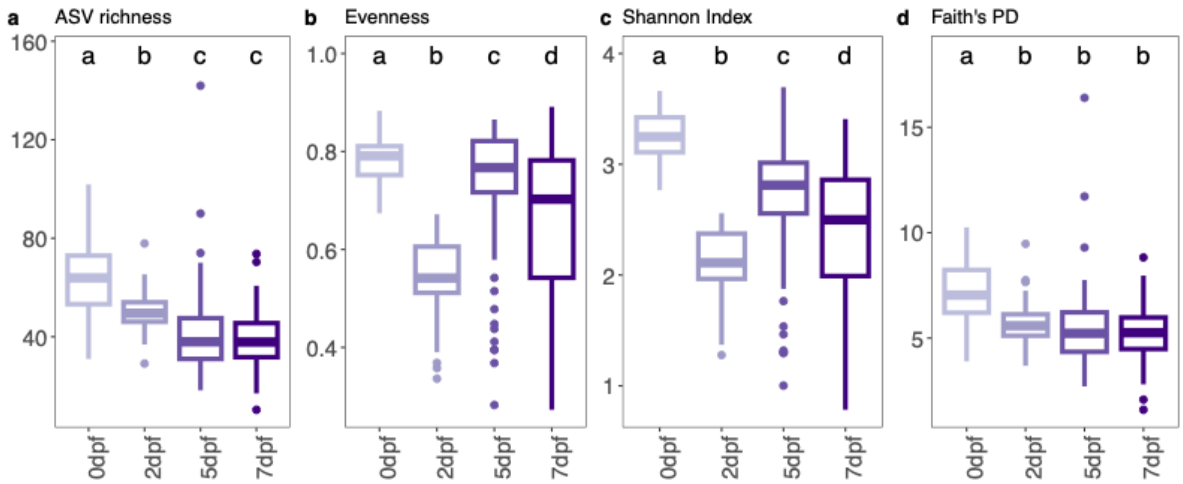

Figure 14: Mean alpha diversity of eggs and larvae at 0, 2, 5 and 7 dpf, measured via ASV richness (a), Pielou's evenness (b), Shannon Index (c) and Faith's Phylogenetic Diversity (d). Means not sharing letters are statistically significant according to Tukey tests and p-values <= 0.05. P-values are adjusted using the Holm method.

To determine if ASV richness explained the decrease in phylogenetic diversity, I calculated the standardized effect size of phylogenetic diversity (SES. PD). For all ages, SES.PD was significantly lower than expected for the ASV richness of the samples (Wilcoxon signed rank tests: 0 dpf, $p < 0.0001$; 2 dpf, $p < 0.0001$; 5 dpf, $p < 0.0001$; 7 dpf, $p < 0.0001$). In the previous study, I found evidence of increased phylogenetic clustering over development, indicating that environmental filtering could be shaping the assembly of the egg microbiome. To address microbiome assembly with respect to phylogeny, I calculated the net relatedness index (NRI) and nearest taxon index (NTI). When NRI and NTI are positive, this is indicative of phylogenetic clustering of microbial taxa, suggesting environmental filtering, whereas negative values indicate overdispersion, suggesting that competition between microbial taxa may be driving microbiome assembly. NRI and NTI values were greater than 0 for all ages, indicating phylogenetic clustering across development (Figure 15). Multiple comparison tests of mean NRI and NTI between age groups revealed that NRI and NTI of larvae were lower than NRI and NTI of eggs. These results suggest that there is less of a signal of phylogenetic clustering in the larval

microbiomes than in the egg microbiomes, perhaps due to other assembly processes shaping these microbiomes.

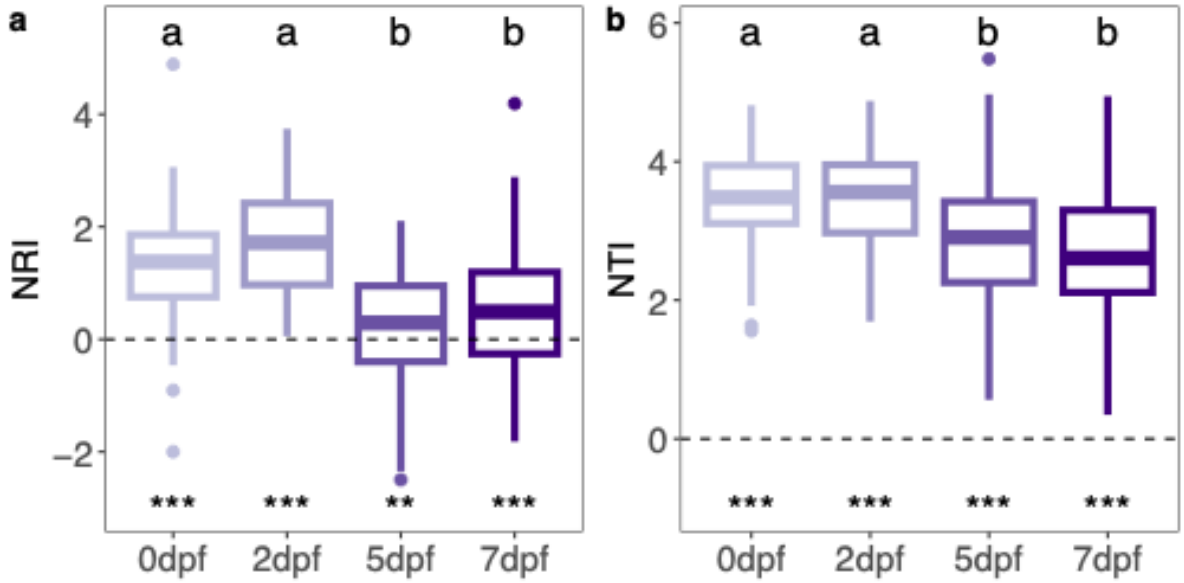

*Figure 15: Mean NRI (a) and NTI (b) of eggs and larvae at 0, 2, 5 and 7 dpf. Indicate p-values of Wilcox test. Means not sharing any letters are statistically significant according to Tukey tests and p-values <= 0.05. P-values are adjusted using the Holm method. Asterisks indicate means significantly greater than 0 according to a Wilcoxon signed rank test: '***', $p < 0.001$; '**', $p < 0.01$.*

Clutch identity and age shape microbiome composition

In the previous chapter and reported in studies on the eggs of other fish species (Fujimoto et al., 2013; Lokesh et al., 2019; Wilkins et al., 2016), both clutch identity, or parentage, and developmental stage were significant sources of variation in microbiome composition between individuals (Figure 16). In this study, age was a significant source of variation in microbiome composition measured via Bray-Curtis, Jaccard and unweighted Unifrac metrics (Bray: $R^2 = 0.371$, $p < 0.001$; Jaccard: $R^2 = 0.269$, $p < 0.001$; Unifrac: $R^2 = 0.153$, $p < 0.001$). Across age groups, there were also difference in microbiome variability (Bray: $F = 152.63$, $p < 0.001$; Jaccard: $F = 143.57$, $p < 0.001$; Unifrac: $F = 18.423$, $p < 0.001$). Clutch also explained a small but significant amount of variation in the egg and larval microbiome (Bray: $R^2 = 0.0286$, $p < 0.001$; Jaccard: $R^2 = 0.0303$, $p < 0.001$; Unifrac: $R^2 = 0.0167$, $p = 0.014$). There were no

differences in variability of the microbiome across clutches, except for microbiome composition measured via Unifrac (Bray: F = 1.046, p = 0.373; Jaccard: F = 1.206, p = 0.309; Unifrac: F = 3.501, p = 0.0163). Additionally, there was a significant effect of dish on Bray-Curtis and Jaccard dissimilarity, and a marginal effect on Unifrac dissimilarity (Bray: R^2 = 0.0356, p = 0.005; Jaccard: R^2 = 0.0363, p = 0.005; Unifrac: R^2 = 0.0352, p = 0.083).

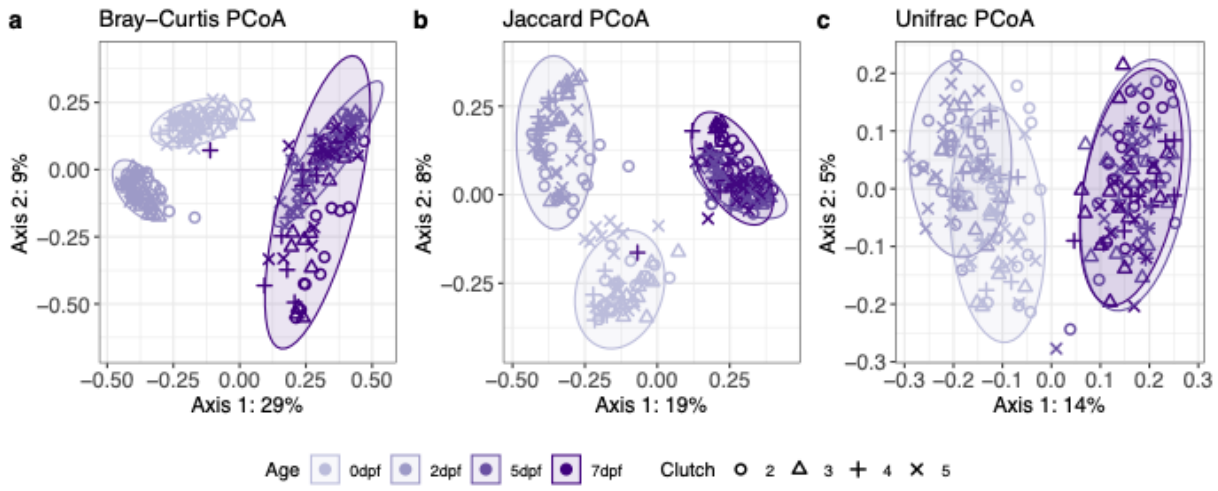

Figure 16: Principal Coordinates Analyses showing microbiome distribution across ages (colors) and clutches (shapes). Beta diversity is measured using Bray-Curtis dissimilarity (a), Jaccard dissimilarity (b) and Unifrac dissimilarity (c). Axes are annotated with the percent variation explained by each axis. Ellipses represent the 95% confidence interval around age groups assuming a multivariate t-distribution.

<u>There are multiple sources of the egg and larval gut microbiomes</u>

Nearly half (49.1%) of the microbiome members of the egg and larval microbiomes could be attributed to a known source, the proportions of which varied between clutches and developmental stages. This percentage is similar to other studies using this approach; for example, a study using *FEAST* to estimate the sources of the little skate (*Leucoraja erinacea*) egg microbiome found that 25-75% of taxa came from known sources (Mika et al., 2021). In my study, *FEAST* detected microbes in the egg microbiomes that likely originated from parental tissues (mainly the skin) and crossing tank. *FEAST* also detected microbes in the larval microbiomes that likely originated from eggs and rotifers (Figure 17). Changes in source

proportions during development coincided with overall changes in microbiome composition over development that I reported in the previous section.

A Dirichlet regression modeled in *brms* allowed for comparisons between overall mean contributions of different sources to the egg and larval microbiomes. The differences between the posterior means of each source contribution (both overall means and means across different age groups) were tested with the *hypothesis* function.

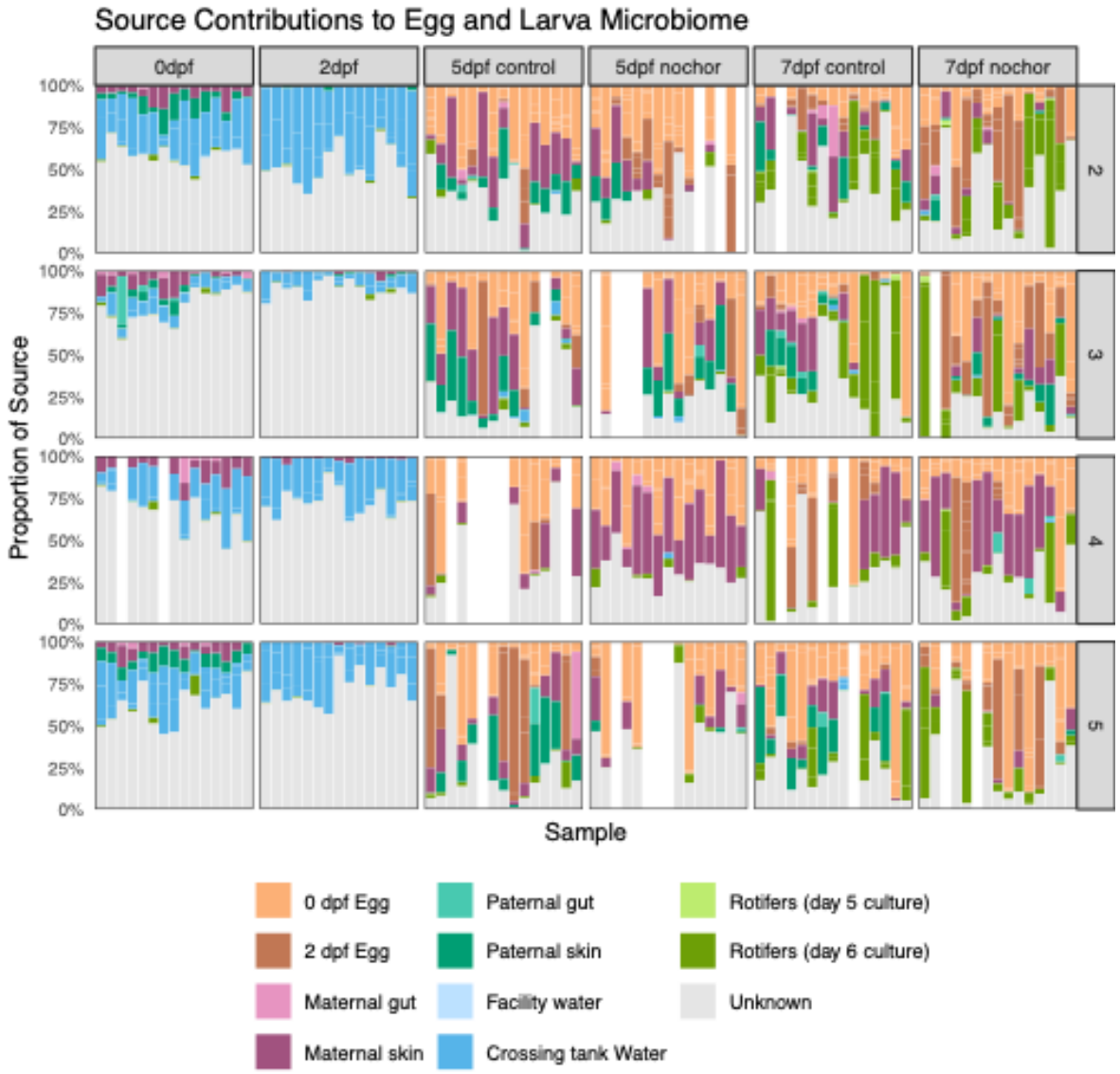

Figure 17: Estimated contributions of source communities (adult skin, gut, water, rotifers and eggs) to the egg and larval gut microbiome. This plot is facetted by clutch (y-axis) and age (x-axis)

Parental tissues as a source

I estimated the mean contributions of parental tissues (gut and skin) to the egg and larval gut microbiomes and tested whether these contributions differed over development. Overall, parental skin microbiomes were estimated to contribute a higher proportion of the egg and larval microbiomes than parental gut microbiomes (Figure 17, Figure 18). Averaged across ages and clutches, the female skin microbiome was a marginally larger contributor to the offspring microbiome than the female gut (estimate (95% CI) = 1.12 (-0.08, 2.45), posterior probability = 0.94). The contributions attributed to the male skin microbiome were not significantly larger than the male gut (estimate (95% CI) = 0.58 (-0.52, 1.64), posterior probability = 0.84). However, the mean contributions of all parental skin samples was larger than the mean contributions of all parental gut samples (estimate (95% CI) = 1.71 (0.04, 3.50), posterior probability = 0.95), indicating that ASVs from the adult skin are more likely to colonize the egg surfaces and the larval gut. Overall, female contributions were significantly larger than male contributions at 5 dpf, and marginally larger at 0, 2 and 7 dpf (Figure 18).

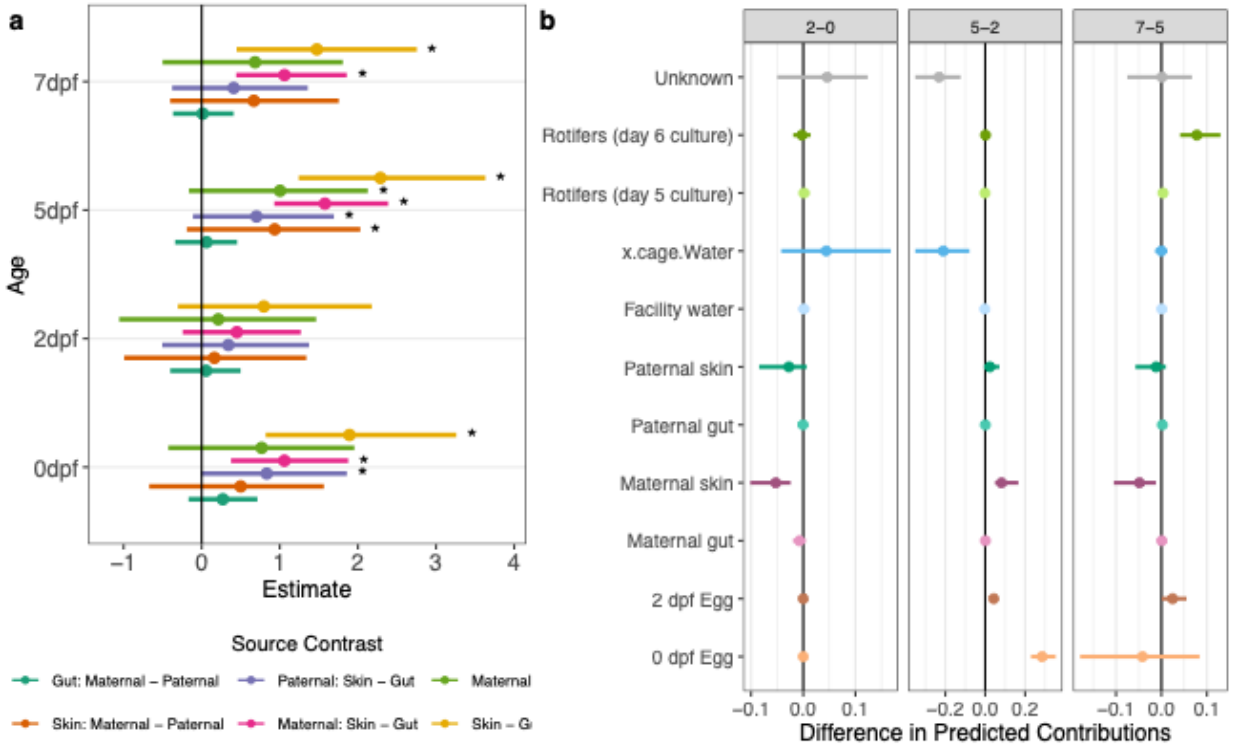

Figure 18: (A) The differences in posterior means between female, male, skin and gut proportions in the egg and larval gut microbiomes across different stages of development, and (B) the estimated differences in predicted contributions of all source communities between development stages. Error bars represent the 95% credible interval. '' denote contrasts with a posterior mean > 0.95.*

It is possible that the parental contributions to egg and larval microbiomes are larger than was reported in this study, because ASV richness of the parental tissues was lower than that reported in previous studies (gut mean: 22.1 ASVs; skin mean: 25.3 ASVs) of adult zebrafish microbiomes (Roeselers et al., 2011; Stephens et al., 2016; Xiao et al., 2021).

Rotifers as a source

Between 5 dpf and 7 dpf larvae, there is an increase in the rotifer microbiome as a source of gut microbiome members, which is to be expected since the 5 dpf larva were not fed prior to sampling. The day 6 rotifers are a significant source, but not the day 5 rotifers. Interestingly, the rotifers are not a source to all 7 dpf larvae; this may be the result of larva varying in their ability to feed from 5 to 6 dpf. From 0 dpf to 5 dpf, there are small proportions of the egg and larval

microbiomes attributed to rotifers as a source, likely due to microbes shared between facility water and rotifers (since facility water was used for both zebrafish husbandry and rotifer culture).

Change in sources across development

The proportion of members of the egg and larval microbiomes attributed to the different sources changed over development. Between 0 dpf and 2 dpf, the proportion of the microbiome attributed to the skin microbiome of both parents decreased (Figure 18). From 2 dpf to 5 dpf, the proportion of parental sources increased. Since 5 dpf and 7 dpf samples were larval guts, this may signal a transition from an "egg" microbiome to a microbiome more typical of the zebrafish gut. There is also a significant decrease in crossing tank water as a source post-hatching, although not a significant decrease in facility water as a source. Overall, facility water was a negligible source of members of the egg and larval gut microbiome (Figure 17, Figure 18b), so this decrease in crossing tank water contributions may be due to bacteria that are transmissible through the water to the eggs, but not able to enter the larval gut or compete with other microbes in the gut. The egg is a substantial source of microbes to the larval gut (Figure 17, Figure 18b) and may include bacteria that are common in adult zebrafish, but not identified by *FEAST* as derived from the adults. Finally, between 5 dpf and 7 dpf, the proportion of gut microbiome members attributed to parental and environmental sources does not change, except for the maternal skin, whose contribution to the larval gut microbiome decreases.

Identification of transmitted ASVs

FEAST is a powerful tool for identify source contributions, but currently does not allow the user to access the ASVs driving its estimations. These ASVs may be potentially transmitted from a source to a sink community. Therefore, to identify putatively transmitted ASVs, I used indicator species analyses (ISA) to identify indicator taxa to offspring samples (eggs and larvae)

and sources (skin, gut, facility water and crossing tank water). This approach allows for the identification of ASVs that are strongly associated with not only individual sample types, but also combinations of sample types, which could include both potential source and sink samples (e.g. ASVs that are associated with the combination of potential sink samples such as 5 dpf and 7 dpf larvae and potential sources such as parental skin). After identifying indicator taxa associated with egg, larvae, and combinations of egg, larvae and source samples, I performed a second indicator species analysis on only source samples to assess how unique these indicator taxa are to different source sample types. If ASVs are identified as indicator taxa in a combined group of sink communities and source communities (e.g. 5 dpf larvae, 7 dpf larvae and parental skin), and are indicator taxa to the same source (e.g. parental skin), I consider these as likely to be transmitted from the source to the sink microbiome.

The ISA identified 5 ASV significantly associated with eggs, larvae, and parental skin (Table 5). *Mycoplasma wenyonii* (ASV6, ASV7, ASV28 and ASV32) were indicator taxa for 0 dpf eggs, 5 dpf larvae, 7 dpf larvae and parental skin (Table 5). When investigating indicator taxa for sources only, these ASVs were identified as indicator taxa for gut, skin and, in the case for ASV6 and ASV28, day 6 rotifers. These ASVs were detected in 60-100% of these samples, with a mean relative abundance of 0.1-16%, with abundance and prevalence tending to be higher in the skin samples, potentially indicating higher fidelity to those sample types. ASV6 and ASV28 while ubiquitous (67-100% of day 6 rotifer samples), were detected in the rotifers at a mean relative abundance of 0.08%. The remaining ASV was identified as *Escherichia-Shigella* (ASV35). This ASV was only identified as an indicator of parental skin in the ISA when comparing only source samples.

Crossing tank water, but not facility water, was also a major contributor to the zebrafish microbiome at 0 and 2 dpf. Three ASVs were identified as indicator taxa of the combined group of eggs and crossing tank water: ASV31 (*Limnobacter thiooxidans*), ASV40 (unclassified *Limnobacter*) and ASV2 (unclassified Comamonadaceae). These ASVs were also indicator taxa of only crossing tank water (when compared to other potential sources), suggesting that they are highly prevalent and abundant in crossing tank water and eggs. ASV8 (unclassified *Undibacterium*) was separately identified as an indicator of water samples and egg and larvae samples. Although only marginally significant, ASV16 (unclassified *Methylibium*) was identified as an indicator of 2 dpf eggs, as well as facility and crossing tank water.

One *Vibrio* ASV (ASV4) was identified as an indicator taxon for 7 dpf larvae and day 6 rotifers ($r_{pb} = 0.589$, $p = 0.03$), and was prevalent in day 5 rotifers. ASV4 was detectable in 0 dpf eggs, 2 dpf eggs and 5 dpf larvae at low frequency (5-20% of samples) and low abundance (0.002-0.02%). It was not detected in any of the water samples or adult fish samples. Since *Vibrio* is a common zebrafish microbiome member, this ASV may have been at low enough abundance in the parents to not have been detected yet still transmitted to the eggs. Rotifer feeding likely reintroduced this ASV to the larvae, hence why it is an indicator taxon in both 7 dpf larvae and rotifers. Day 6 rotifers may be a more important source of this microbe, since ASV4 was higher in abundance (11.9%) in day 6 rotifers than in day 5 rotifers (6.4%). While ASV4 was the only taxon identified as an indicator for 7 dpf larvae and day 6 rotifers, there were 38 ASVs identified as indicator taxa solely for day 5 rotifers, 8 ASVs identified as indicators for day 6 rotifers, and 27 ASVs identified as indicators for both, suggesting that while there are shared ASVs between the two cultures, they each have a distinct microbiome composition.

Table 5: Significant indicator taxa to egg and larval gut microbiomes and their associations to different source microbiomes.

ASV	Taxonomy	Sites	r_{pb}	Sites (Source)	r_{pb} (Source)
ASV31	*Burkholderiaceae; Limnobacter; thiooxidans*	2+X	0.674**	X	0.957**
ASV19	*Pseudomonadaceae; Pseudomonas; stutzeri*	0+2	0.668**	G	0.354
ASV40	*Burkholderiaceae; Limnobacter*	2+X	0.653**	X	0.957**
ASV11	*Sphingomonadaceae; Novosphingobium*	0+2	0.642**	G+X	0.316
ASV17	*Sphingomonadaceae; Novosphingobium; capsulatum*	0+2	0.611**	Rd5	0.577
ASV2	*Comamonadaceae*	2+X	0.605**	X	0.998**
ASV16	*Comamonadaceae; Methylibium*	2	0.600*	F+X	0.683 .
ASV6	*Mycoplasmataceae; Mycoplasma; wenyonii*	0+5+7+S	0.573*	G+Rd6+S	0.997**
ASV1	*Comamonadaceae*	2	0.571*	S+X	0.397
ASV8	*Oxalobacteraceae; Undibacterium*	2	0.568**	X	0.957**
ASV7	*Mycoplasmataceae; Mycoplasma; wenyonii*	0+5+7+S	0.532**	G+S	0.938**
ASV91	*Comamonadaceae; Aquabacterium*	0	0.514*	S	0.378
ASV32	*Mycoplasmataceae; Mycoplasma; wenyonii*	0+5+7+S	0.513*	G+S	0.935**
ASV28	*Mycoplasmataceae; Mycoplasma; wenyonii*	0+5+7+S	0.511*	G+Rd6+S	0.909*
ASV110	*Xanthobacteraceae; Rhodopseudomonas; pseudopalustris*	0	0.486*	F+X	0.447
ASV35	*Enterobacteriaceae; Escherichia-Shigella; coli*	0+5+7+S	0.464*	S	0.808*
ASV56	*Chitinophagaceae; Sediminibacterium*	2	0.435*	X	0.408
ASV4	*Vibrionaceae; Vibrio*	7+Rd6	0.401*	Rd5+Rd6	1.000**

The chorion significantly affects standard length and gut microbiome composition of larvae

To test whether the chorion was a vehicle for transmission of microbes from the egg to the larval gut, I transferred half of the 3 dpf larvae from each dish to a new dish with fresh EM. This treatment prevented larvae from grazing on the chorions and egg debris. There was a significant difference in standard length (SL) between 7 dpf and 5 dpf larvae (estimate = 0.0560, p = 0.0122) and the treatment groups ("no chorion" and "control") at 7 dpf (estimate = -0.0608, p_{Holm} = 0.0211). Chorion removal also significantly correlated with decreased bacterial load (log mean 16S copies, estimate = -1.122, p = 0.0006). Larvae raised in dishes with chorions had significantly lower Faith's PD (estimate = 0.400, p = 0.0497). There was not a significant difference in ASV richness, evenness or Shannon diversity due to treatment (ASV: estimate = 2.196, p = 0.110; Evenness: estimate = 0.0023, p = 0.897; Shannon: estimate = 0.0675, p =

0.369). Lastly, chorion removal also explained a small, but significant amount of variation in microbiome composition (Bray Curtis: R2 = 0.0130, p < 0.001; Jaccard: R2 = 0.0096, p = 0.002; Unifrac: 0.0112, p < 0.001; Figure 19).

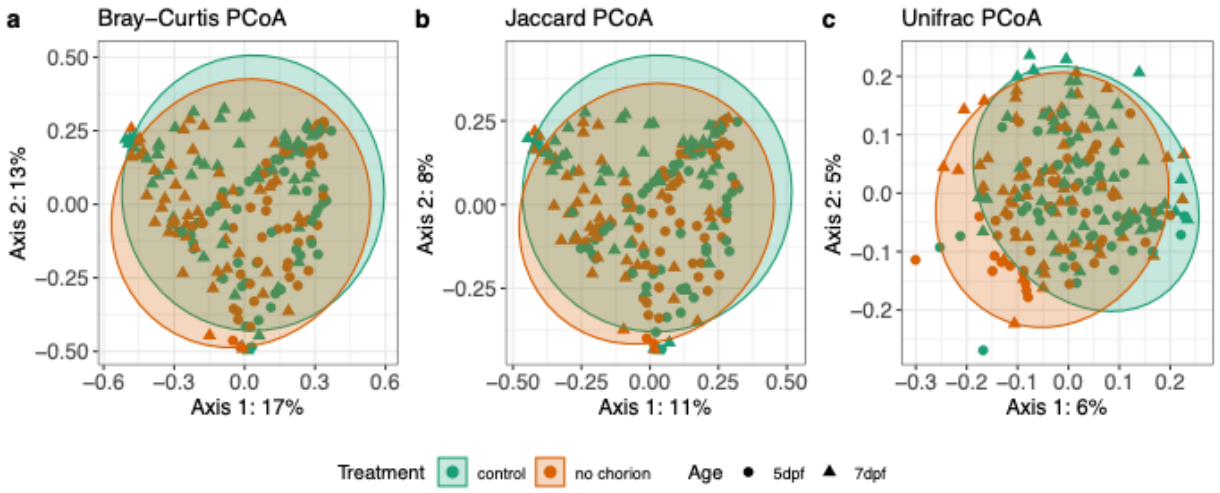

Figure 19: Principal Coordinates Analyses showing microbiome distribution across treatment groups (colors) and larvae age (shapes). Beta diversity is measured using Bray-Curtis dissimilarity (a), Jaccard dissimilarity (b) and Unifrac dissimilarity (c). Axes are annotated with the percent variation explained by each axis. Ellipses represent the 95% confidence interval around age groups assuming a multivariate t-distribution.

Chorion removal slightly decreased the posterior mean of male skin microbiomes as a source and increased the posterior mean of 0 dpf and 2 dpf egg microbiomes as sources but does not otherwise have a significant effect on other source contributions to the larval microbiome (Figure 20). Since chorion treatment explained a small, but significant, difference in Bray-Curtis Dissimilarity, I used a Similarity Percent (SIMPER) analysis to identify ASVs explaining the observed differences between treatment groups. This analysis calculates the contribution of each ASV to the overall Bray-Curtis dissimilarity. To determine whether these ASVs show specificity to either treatment group, I performed an indicator species analysis as well.

Within 5 dpf larvae, ASV7, ASV6, and ASV28, explained significant variation between "control" and "no chorion" groups (2.1-4.6%, p = 0.001-0.014). ASV28 (r_{pb} = 0.250, p = 0.005) was identified as an indicator taxon to control group larvae, and ASV6 (r_{pb} = 0.166, p = 0.04)

was identified as an indicator to both groups at 5 dpf and control larvae at 7 dpf. ASV7 was not identified as an indicator taxon for either group at 5 dpf or 7 dpf.

Within 7 dpf larvae, ASV4, an unclassified *Vibrio*, was the largest contributor of the differences in microbiome composition between treatment groups (12.9%, p = 0.001). ASV4 was also identified as an indicator taxon for 7 dpf larvae in the "control" treatment (r_{pb} = 0.279, p = 0.005). Another *Vibrio* species, *Vibrio navarrensis*, was also identified as a significant contributor to the variation between treatment groups (6.0%, p = 0.001) and an indicator taxon for 7 dpf larvae in the "control" (r_{pb} = 0.282, p = 0.005). Additional taxa identified as indicator taxa and contributors to variation were ASV13 (*Shewanella*, 5.1%, p = 0.001; r_{pb} = 0.266, p = 0.005) and ASV1 (Comamonadaceae, 3.1%, p = 0.001; r_{pb} = 0.281, p = 0.005). ASV13 was an indicator taxon for both treatment groups of 7 dpf larvae. ASV1 was an indicator taxon for 7 dpf taxa in the "no chorion" group.

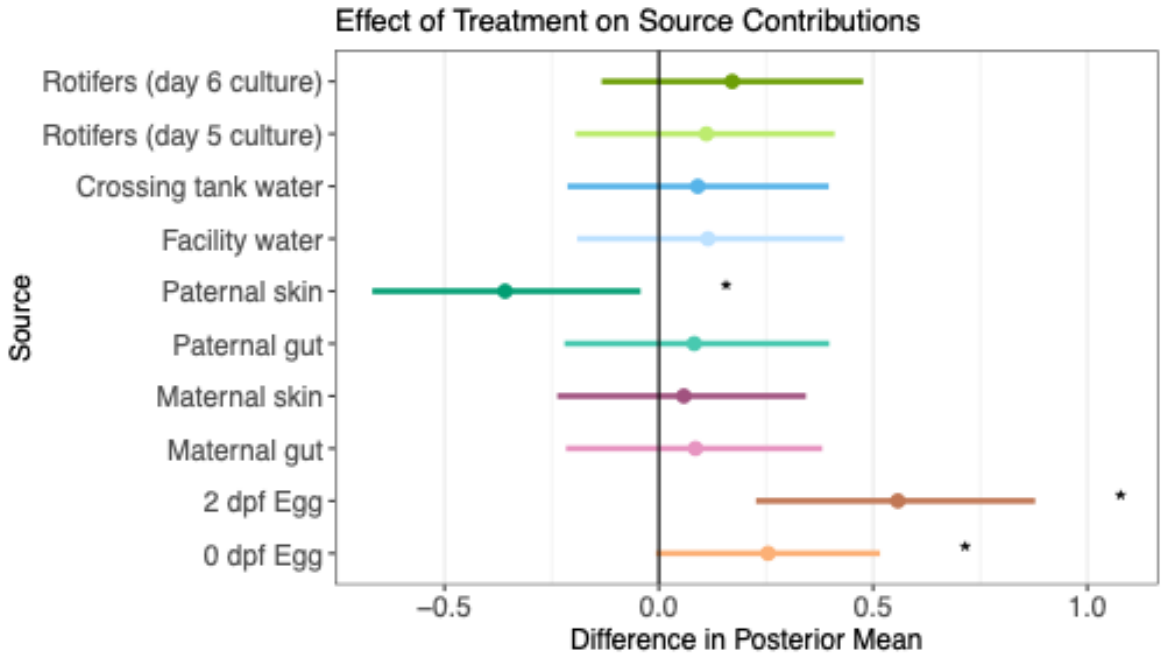

Figure 20: The difference in posterior means of source contributions between "control" larvae and "no chorion" larvae, averaged across 5 dpf and 7 dpf larvae. Error bars represent the 95% credible interval. '' denote contrasts with a posterior probability > 0.95*

Discussion

Zebrafish are a powerful vertebrate model organism for host-microbiome research for many reasons (Stagaman et al., 2020), including that they can be rendered axenic relatively easily through disinfection of eggs. However, axenic derivation erases parental and environmental contributions to microbiome assembly in early life. Microbes that colonize the egg surfaces and later the larval skin and gut may be critical for host health and development, yet little is known about the early-life microbiome of zebrafish, especially at the egg stage. To our knowledge, no studies have documented a relationship between the egg microbiome and larval microbiome of zebrafish, although studies in other fish species have suggested that members of the egg microbiome can colonize larvae (Hansen & Olafsen, 1999; Lokesh et al., 2019; Romero & Navarrete, 2006). In this chapter, I show that the parental skin and gut microbiome are a source to the egg and larvae microbiomes. The egg microbiome was also a major source to the larval gut microbiome, highlighting that its composition may be important for the future health and development of the larvae.

Parental microbiomes are sources to egg and larval microbiomes

Despite the low ASV richness of the adult samples, I observed that parental tissues, particularly the female skin, are sources of microbes to the egg and larval microbiomes (Figure 17). The parental contributions to the egg microbiome marginally decreased from 0 to 2 dpf (Figure 18). There was not a detectable increase in other sources' contributions to the egg microbiome from 0 to 2 dpf. Parentally transmitted microbes may have decreased in abundance and/or been displaced by microbes from an unknown source during embryonic development. Interestingly, post-hatching, I observed that the contributions of parental skin as sources increased and remained stable until the experiment's end at 7 dpf. Since I sampled larval

intestines at 5 dpf and 7 dpf, this may be indicative of the difference in environment (GI-tract vs chorion). The larval intestines may also be selective for "fishy" microbes, explaining the increase in parental contributions in the larval gut compared to the eggs.

The decrease in parental contributions from 0 to 2 dpf cannot be explained through environmental filtering of the egg microbiome over this period. While I did observe a decrease in phylogenetic diversity (Faith's PD) from 0 to 2 dpf on the eggs described in the current chapter, this was not combined with a significant increase in phylogenetic clustering (as measured by the NRI and NTI metrics), suggesting that selection against particular lineages may not be occurring in these eggs.

Feed is a source of microbes to the larval gut

Rotifers, particularly the day 6 rotifers, were a major source of microbes to 7 dpf larvae (Figure 17). This was expected since larval feeding began at 5 dpf, after the collection of 5 dpf larvae for sampling. The higher importance of day 6 rotifers compared to day 5 rotifers as a source may be due to displacement of early rotifer-derived microbes by the later rotifer-derived microbes. Another explanation may be due to differences in feeding. For instance, rotifer microbes were not detected in all 7 dpf larval samples, which may reflect differences in feeding behavior among the larvae. It is possible that, even though exposed to rotifers at 5 dpf, these larvae may not have readily fed on them compared to at 6 dpf. Zebrafish larvae learn to feed through observing conspecifics, and the timing of exposure to prey items such as rotifers may influence when they are able to learn to feed (Lucore & Connaughton, 2021). If larvae were exposed to rotifers at 3 or 4 dpf, and this experiment was extended past 7 dpf with daily feeding, there might be higher rates of feeding, and a higher overall estimation of rotifers as a source to

the larval gut microbiome. I would also be able to determine whether earlier feedings or later feeding have a larger impact on microbiome composition in the larval gut.

Indicator taxa of parental microbiomes are also indicators for eggs and larvae

Using the indicator species analysis, I identified ASVs that are indicators for eggs and larvae, but not significant indicators for any of the parental tissues, water samples or rotifers (e.g. ASV91, ASV11, ASV17, ASV1, etc.; Table 5). While these ASVs are detectable in these samples, they are at low abundance and frequency, so the indicator species analysis is unable to associate them with any of the source sample types. This may be a consequence of the relatively low richness I observed in the parental samples, or because these ASVs may not be as competitive in these environments as other ASVs and are better competitors on the eggs and in the larvae.

ASVs belonging to the genus *Mycolplasma* were identified as indicator taxa for 0 dpf eggs, larvae and parental skin. This genus was detectable in 2 dpf eggs, although at relatively low abundances compared to in the larval samples. Given its high association with these sample types, and the finding that parental skin, particularly maternal skin was a more important contributor to the offspring microbiome at 0, 5 and 7 dpf than at 2 dpf, this genus is likely transmitted from parent to offspring. *Mycoplasma* is common in zebrafish facilities and some species are known pathogens (that, for example, can exacerbate intestinal legions caused by the nematode *Pseudocapillaria tomentosa*; Kent et al., 2021). While I detected *Mycoplasma* predominately in the skin, due to the overall low ASV richness in the parental samples, this finding does not preclude the possibility that *Mycoplasma* may also be transmitted via the gut, or that the parental gut may be a major source of microbes to the offspring in general.

Water as a medium of microbial transmission between parent and offspring

Interestingly, I observed that facility water was a negligible source to the zebrafish microbiome from 0 to 7 dpf, while crossing tank water was a significant source, especially at the egg stages (Figure 17). The crossing tank water came from the same output as the facility water, however it was exposed to adult fish prior to sampling, and was likely seeded by the adult fish. Zebrafish gut communities have been detected as significant sources of microbes to the tank water microbiomes in the Huestis Zebrafish Facility (Evens, 2024). Since contact between adults and eggs is limited by the crossing tank, water may be an important medium for transmission of microbes from parent to egg. This may explain why crossing tank water was identified as a source, but not facility water, which came from the intake and had no previous exposure to the adult fish. Additionally, three of the indicator taxa for both eggs and crossing tank water belonged to the genera *Undibacterium, Limnobacter* and the family Comamonadaceae, all of which have been detected in and/or isolated from zebrafish intestines (Kämpfer et al., 2016; Kayani et al., 2022; Stagaman et al., 2024; Stephens et al., 2016; Stressmann et al., 2020). While it is possible that these ASVs originate in the water and then are acquired by zebrafish, the fact that they were not indicator taxa for facility water, nor was facility water a significant source to the egg microbiome, suggests that these taxa may colonize the adult fish and are then transmitted through the water to the egg during spawning. Post-hatching, crossing tank water was not a significant source to the larval microbiome, and indicator taxa for the larvae are shared with parental tissues and rotifers. As stated previously, this change in the contribution of the crossing tank water may be due to the different tissues sampled (egg vs. larval gut), and the larval gut may select for microbes specialized to live in zebrafish.

The egg microbiome is a source to the larvae microbiome

Notably, I found that a substantial proportion of the egg microbiome was detected in the larval guts. Few studies have shown that the egg is a source of microbes to the larval zebrafish gut. The observation of shared microbial taxa between egg and juvenile fish (Lokesh et al., 2019; Romero & Navarrete, 2006), along with the observed behavior of larvae grazing on egg debris (Hansen & Olafsen, 1999), have implied that the egg microbiome may be an early source to fish larvae. In fact, a study on little skates (*Leucoraja erinacea*), an oviparous cartilaginous fish, found that microbes on the egg case are a source to the microbiome of individuals at later stages of development (Mika et al., 2021). Similarly, the results of this chapter show that the egg microbiome is a source to the larval microbiome of zebrafish. The egg may be an important carrier of microbes for oviparous species and may allow transmission of microbes from parent to offspring in species that exhibit little to no parental care (such as the zebrafish, at least as managed in aquaculture facilities). Additionally, the eggs as a source may include the microbes that were previously attributed to the crossing tank at the previous development stages, which may also explain the decrease in crossing tank water as a source post-hatching.

Chorion removal has a minor effect on microbiome composition

In this study, I found evidence that the presence of the chorion has a significant (but small) effect on standard length of zebrafish larvae and gut microbiome composition (Figure 19). When chorions remained in the dishes, zebrafish larvae grew slightly larger, perhaps due to higher availability of food via the egg debris and had higher bacterial loads. Removal of chorions caused a small shift in microbiome composition, and I identified microbial taxa that may drive this shift. ASV4, an indicator taxon for 7 dpf larvae and rotifers was also an indicator taxon for the "control" treatment, i.e. larvae that remained in dishes with their chorions. However, since

this ASV likely originated from rotifers, it is not clear how removal of the chorions affected its abundance or prevalence in the larvae. Additionally, I did not identify ASVs would be potentially transmitted from egg to larvae via the chorion. If chorions were a vehicle for transmission, the source proportions of the eggs would theoretically decrease in the "no chorion" group. However, the proportion of the egg microbiome in the larvae microbiome increased in the "no chorion" group. It's possible that the chorion plays a very minor role in transmission of microbes, but the length of my experiment may have been too short to detect an appreciable effect of chorion removal on the embryo microbiome. Microbes on the eggs also likely seed the embryo media throughout development and post-hatching, allowing the larvae to ingest them without ingesting the chorions. They may also colonize the larval skin and be transmitted to the embryo media and other larvae, providing more opportunities for those microbes to enter the gut.

Limitations of this study

It is possible that this study underestimates the overall parental contributions to the egg and larval microbiome, considering that fewer microbial ASVs were recovered from the parental skin and gut tissues than what has been reported for adult zebrafish in other studies (Roeselers et al., 2011; Stephens et al., 2016; Xiao et al., 2021). Unfortunately, ASV richness of adult samples was not substantially increased through repeated sequencing attempts. The ASVs that were recovered are those that may be in relatively high abundance in the skin and gut microbiome or are from cells that readily lysed during DNA extraction procedures. However, similar to results found in previous studies (Evens, 2024; Roeselers et al., 2011; Stephens et al., 2016), Proteobacteria, Firmicutes and Fusobacteriota were dominant phyla in the skin and gut microbiomes assayed in my study.

This experiment had standardized exposure times to replicate how zebrafish embryos are likely collected in the UO Huestis Facility (in contrast to the methods used in my previous chapter). However, these limited exposure times could have impacted which bacteria could transfer from the environment to the egg surfaces. This differing treatment may have resulted in the lower effect of the clutch on microbiome composition compared to the previous chapter, since the embryos were removed from the crossing tanks sooner and had less exposure time overall to any parent-derived microbes.

Future directions

This experiment relies on 16S rRNA sequencing to identify and track bacteria from parent and environment to egg. However, identical ASVs may arise from similar but different strains. Consequently, while the results from the *FEAST* analysis are compelling and do imply transmission, this method does not explicitly prove transmission of bacteria from one microbiome to another. Metagenomic sequencing, while more difficult and costly, coupled with strain-level profiling has been used to track transmission of microbes from mother to infant in humans (Ferretti et al., 2018). Another method to track transmission, is to tag putatively transmitted bacterial species either with fluorescent proteins or transposons. Transposon-tracking has been used to track population sizes of *Aeromonas* and *Vibrio* in larvae zebrafish under different colonization regimes (Stephens et al., 2015). These methods could be used to more directly track transmission of microbes from parent to offspring in zebrafish.

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V: DISCUSSION

Zebrafish are a powerful model for studying host-microbiome interactions. The ease at which the larvae can be rendered axenic has allowed researchers to examine how the microbiome influences host immunity, GI-tract maturity and neurodevelopment, and identify critical members of the microbiome that are necessary for proper development of the host. However, the egg microbiome of zebrafish has been severely understudied. This is unfortunate, because axenic derivation erases the egg microbiome and any signal of its potential sources, such the parental microbiome or water microbiome. The egg microbiome may contain previously unidentified microbes that are critical to larval health and development. Furthermore, leveraging the zebrafish as a model to explore the early-life microbiome assembly will inform broader research on the origins of host-associated microbiomes, across many different species of animals.

In chapter 2, I present a brief literature review exploring the current research on early-life microbiome assembly of fishes. While microbiome assembly in zebrafish larvae has been explored, I found no studies exploring microbiome assembly on the eggs, or the consequences of the egg microbiome on later stages of zebrafish development. There is research on other fish species, however, exploring assembly and the role of the egg microbiome to hatching success of the eggs. This research helped inform my questions to explore concerning the zebrafish egg microbiome.

Using wildtype zebrafish, I explored the drivers of microbiome variation at the egg and larval stages. In chapter 3, I characterized the zebrafish egg microbiome and found that parental identity and developmental stage explained significant variation in microbiome composition. For instance, over the course of development, the zebrafish egg microbiome changed significantly, in composition and in phylogenetic diversity. The decrease in phylogenetic diversity was interesting

and further exploration of community assembly with respect to phylogeny revealed that the environmental filtering was an important process in shaping the microbiome from 0 to 2 days post-fertilization.

Parentage was also important in explaining variation in the microbiome. Generally, eggs derived from the same clutch were more similar to each other than eggs derived from different clutches. Since the adult zebrafish used in this chapter came from two different, but neighboring, stock tanks, I also observed a signal of parental stock tank on the egg microbiome. As eggs approached hatching, the signal of parentage decreased. That is, the effect of shared parentage on microbiome similarity between pairs of eggs was lower at 2 days poster fertilization than at 0 days post fertilization. This may be the result of filtering of parentally-derived microbes by the developing egg.

In chapter 4, I explored the roles of different source microbiomes (parents, water, food and eggs) on the microbiome composition of eggs and larvae. Parental skin was an important source to the egg microbiome, as was the crossing tank water. Facility water (i.e. water that had no contact with adult fish) was a negligible source. Since crossing tank water was exposed to adult fish, water may serve as a medium through which microbes are transmitted from parent to offspring. Post-hatching eggs were a significant source of microbes to the larval gut. This finding was especially interesting, because it showed continuity between the egg microbiome and larval gut microbiome. Additionally, using indicator species analyses, I identified putatively transmitted microbes from the different source microbiomes to the egg and larval gut microbiomes. *Mycoplasma* species were major indicator taxa for eggs, larvae and parental skin, and are likely transmitted from the skin to offspring during spawning. Through this method, I also identified *Vibrio* as a larval gut microbe that likely originated from the feed (i.e. rotifers).

My dissertation addresses fundamental questions in the subject of host-microbiome assembly. I present novel research regarding the drivers of variation and sources to the egg microbiome of zebrafish. I also show that the egg microbiome is an important source of microbes to the larval gut. These findings are among the first to address how microbiomes assemble in the first stages of a zebrafish's life. I hope this research can inform future studies on early-life microbiome assembly in zebrafish and other fish species. There are many questions that still remain regarding early-life microbiome assembly, some of which could address the consequences of the egg microbiome on larval health in zebrafish and other fish species, or the efficacy of microbiome manipulation at the egg stage to improve fish health through its early development.