MICROBIAL SUCCESSION ON THE LAKE STURGEON EGG SURFACE: MECHANISMS SHAPING THE MICROBIAL COMMUNITY ASSEMBLY DURING SUCCESSION AND THE EFFECT OF MICROBIAL SUCCESSIONAL PROCESSES ON HOST LIFE HISTORY TRAITS

By

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ABSTRACT

MICROBIAL SUCCESSION ON THE LAKE STURGEON EGG SURFACE: MECHANISMS SHAPING THE MICROBIAL COMMUNITY ASSEMBLY DURING SUCCESSION AND THE EFFECT OF MICROBIAL SUCCESSIONAL PROCESSES ON HOST LIFE HISTORY TRAITS

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Microbial community assemblages have been studied in a variety of hosts and environments. However, to date, most of the studies conducted on microbial community structure have been observational in nature. As a result, the underlying mechanisms shaping microbial community assembly at a given place and time remain largely unknown. In this study, we were particularly interested in understanding how a microbial community develops on animal hosts. As soon as eggs or neonatal organisms are exposed to environments, microbes colonize surfaces of eggs or the epithelium of host tissues and establish a microbial community. Such a process involving initial colonization and subsequent sequential changes in species composition is called "succession". Microbial succession is a complex process with the number of different factors involved including initial stochastic arrival of microbes at an open space via dispersal, attachment / colonization at the space, subsequent community sorting via adaptation, and continuous dispersal from neighboring spaces. In this study, we examined microbial succession on the egg surface of the Lake Sturgeon, a threatened fish species inhabiting in the Great Lakes. We sought to understand the role of both host factors (e.g. innate immunity, egg chemistry) and various environmental factors (e.g. aquatic microbes, stream flow rate and temperature) in influencing the formation of microbial communities on the egg surfaces over the course of the egg incubation period. We also sought to evaluate the effect of different microbial successional processes on host life history traits, including egg mortality and larval size at hatch. These topics

were important for this system because dams constructed in the Lake Sturgeon's spawning streams can alter environmental factors such as aquatic microbes, stream flow rate, and temperature, which may in turn affect the life history of the sturgeon.

To achieve these objectives, we adopted an integrative approach, which relied on manipulation of environmental factors including aquatic microbial community, aquatic microbial quantity, stream flow rate, and temperature. We also employed a combination of various analytical techniques, including 16S rRNA gene pyrosequencing, 16S rRNA based T-RFLP, 16S rRNA clone library, 16S rRNA based quantitative PCR, light and fluorescence microscopy, and culture methods. We found that egg microbial communities were distinct from source water microbial communities. Host eggs shaped egg-associated microbial communities within 60 minutes of fertilization, selecting for and against certain microbial species. In addition, the egg surface microbial communities were not constant but rather dynamic, as we observed directional changes of microbial communities along with egg developmental stages. Egg-associated microbial communities also varied with the environmental variables they were exposed to during incubation, including temperature, flow rate, and aquatic microbial community. These differences in the egg-associated microbial community composition affected host life history traits including egg mortality and larvae size at hatch. We also identified a key set of microbial species that significantly improved egg survival and could be used for probiotic treatment in this threatened fish species in the future. This study was the first microbial succession study conducted on fish eggs. Our results highlight the complexity of host-microbe-environment interactions. This study has implications for managing threatened host populations such as the Lake Sturgeon inhabiting human-altered rivers, since it demonstrates the potential effect of dams (which alter aquatic microbes and temperature) on downstream host-microbe interactions.

This dissertation is dedicated to my parents and to my friend Wilhelmina Yonkman

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KEY TO SYMBOLS OR ABBREVIATIONS

16 S rRNA…….a component of the 30S small subunit of prokaryotic ribosomes

Ct…….……cycle threshold

OTU……..operational taxonomic unit

PBS…….phosphate buffered saline

PCA……..principal component analysis

PCR……...…….polymerase chain reaction

PT……...…….phylotype

qPCR……..quantitative polymerase chain reaction

RDP……Ribosomal Database Project

RFU…….……relative fluorescence unit

rRNA……..……ribosomal ribonucleic acid

TRFLP……terminal restriction fragment length polymorphism

CHAPTER 1: INTRODUCTION

Global scope of this study

In this present study, we sought to answer fundamental ecological questions, specifically understanding ecological processes shaping microbial community assembly at any given time and place. Microbial community assemblages have been studied in a variety of environments ranging from marine [1-2], lake [3-4], soils [5-6], plants [7-8], animal gut [9], and human gut [10]. The variability that can be observed in the structure of microbial communities across diverse hosts and environments is particularly fascinating and has significant implications for more broadly understanding fundamental ecological roles of microbes. However, to date, most of the studies conducted on microbial community structure have been observational in nature. As a result, the underlying mechanisms shaping microbial community assembly at a given place and time remain largely unknown, as does the role of microbes in the greater ecological system.

One topic of interest in the study of microbial ecology involves understanding how a microbial community develops on animal hosts. Because microbes are ubiquitous [11], their association with a variety of different hosts is inevitable. Microbes utilize host resources and hosts in turn can be benefited or harmed by the presence of microbes [12]. This interaction plays a significant role in both a host's life history and a microbe's life cycle, yet much remains unknown regarding the exact nature of ecological processes governing microbe-host interactions.

Microbe-host interactions start from the beginning of a host's life. Embryos of animals are particularly vulnerable to microbial challenges since their immunity is not fully developed [13-14]. Therefore, both innate immunity [13, 15] and adaptive immunity [14, 16] can be provisioned to embryos by hosts through various different mechanisms. Animals have evolved to protect embryos from microbes via formation of an egg case [13] and a placenta [14]. As soon as

animals are born and exposed to their outside environments, the eggs or neonatal organisms come into contact with microbes [17-18]. Microbes colonize the surfaces of eggs or epithelium of host tissues and establish a microbial community.

The processes of initial colonization of hosts by collections of microbes and subsequent sequential changes in microbial community structure on hosts have been documented [18] and such a process is called "succession". Succession has been more broadly investigated across the field of ecology for many years, mainly using plant systems [19]. However, microbial succession has been recently studied in various host animals [18, 20-21], host plants [7], and natural [22-25] and artificial [26-28] environments. Results from these studies suggest that microbial succession is a complex process [7, 18, 22, 29]. A number of different factors are involved in microbial succession including initial stochastic arrival at an open space via dispersal from neighboring spaces, attachment-colonization at the space, subsequent community sorting via adaptation, and continuous dispersal from neighboring spaces [29]. Microbes serve as ideal models for studying succession because the microbial succession process can be effectively controlled in an experimental setting, thus allowing for novel information to be learned about factors affecting the succession process.

In this study, we examined microbial succession on the egg surface of Lake Sturgeon (*Acipenser fulvescens*), a threatened fish species inhabiting in the Great Lakes. The Lake Sturgeon is one of the 26 sturgeon species inhabiting freshwater [30]. The species is unique in that it has maintained its ancestral morphological form since the Lower Jurassic period [30]. Lake Sturgeon populations have decreased drastically over the past 100 years due to anthropogenic activities such as overfishing and dam construction [31]. One such population is the Black Lake population in Michigan [31-32]. Spawning habitats have been altered since the

construction of Kleber dam in 1949 on the Upper Black River, which is the sole spawning stream for the Black Lake population. Despite recent restoration efforts, natural recruitment is limited, which is likely attributed to high egg mortality [33].

Microbial succession on eggs of the Lake Sturgeon is likely a complex process that is worthy of focused study. Eggs are fertilized in a stream as soon as male and female adults release gametes. A female is usually surrounded by multiple males and releases over 500,000 eggs per spawning season [30]. The water activated eggs develop an adhesive quality [34] so that they can adhere to benthic substrates such as gravel and sand [30]. During this fertilization process, microbes that are drifting in a stream collide with eggs and adhere to the sticky egg surfaces. This process is a stochastic process, since the water microbial community varies temporally and spatially [24, 35-36], and microbes drifting in stream water have no control over their movements. After the initial stochastic collision, the microbial community on egg surfaces are likely selected by local deterministic processes including adhesion [37-38], antimicrobial activities of eggs [15, 39], chemicals that eggs excrete during embryogenesis [40-41], and interspecific competition among microbes [42-46], while microbes in stream water continuously colonize egg surfaces via passive dispersal mediated by water flow.

A number of other factors may potentially affect such successional process such as stream flow rates [25], temperature [24], and structure of the aquatic microbial community [47]. These factors are important to consider in this system because dams in the Lake Sturgeon's spawning streams can alter environmental factors such as aquatic microbes [36], stream flow rate [48] and temperature [49], but the effects of such changes on microbe communities and egg survival are unknown. Microbe-host interactions are long-term in nature and have in many cases coevolved to the point of reaching a fine balance. The perturbation of these interactions as a

result of anthropogenic activities or artificial substances may cause an observable disturbance to the system that is worthy of further study. This is of particular concern for the Lake Sturgeon host when considering the observed high egg mortality rate and the potential for microbe-host interactions to play a role in influencing such mortality, a topic that has not been studied to date.

In this dissertation work, we studied microbial succession on the Lake Sturgeon egg surfaces over the course of their incubation period and under different environmental conditions. The overarching goal of this study was to characterize the egg surface microbial community assembly during succession and to examine how both host factors (e.g. innate immunity, egg chemistry) and environmental factors (e.g. aquatic microbes, stream flow rate and temperature) affect the microbial succession process and subsequently influence host life history traits (e.g. egg mortality and larval size at hatch). It was also our goal to identify putative symbionts for the egg of the Lake Sturgeon to be potentially used in the future for probiotic treatment of eggs in hatcheries. We believed that characterizing microbial community assembly on the egg surface during incubation would help illuminate the potential but as yet unexplored causal relationship between microbes and egg mortality.

To achieve these goals, we took an integrative approach, which relied on experimental manipulation of environmental factors including aquatic microbial community, aquatic microbial quantity, stream flow rate, and temperature while rearing Lake Sturgeon eggs in a streamside hatchery. We then monitored the changes in microbial communities across these treatments and over the course of the egg developmental stages using a combination of various analytical techniques, including 16S rRNA gene pyrosequencing, 16S rRNA based T-RFLP, 16S rRNA clone library, 16S rRNA based quantitative PCR, light and fluorescence microscopy, and culture methods.

Overview of chapters

Chapter 2

The primary objective of this chapter was to document microbial succession (initial colonization and subsequent sequential changes in microbial community assembly) on the surface of the Lake Sturgeon eggs and to characterize the egg associated microbial community assembly using next generation sequencing. We also examined the effect of simulated stream flow rate on the microbial community assembly on the egg surfaces. The effects of natural and dammed stream flow rates on microbial succession were also discussed.

Chapter 3

In this chapter, we analyzed the effect of environmental variables, including temperature and aquatic microbial composition, on microbial succession on the Lake Sturgeon egg surfaces. We also examined the effect of such environmental variables on subsequent life history traits of the Lake Sturgeon egg host, including egg mortality and larvae size at hatch. We analyzed these effects using an experimental design with controlled treatments, including two temperature ranges (warm and cold) and two water types (stream water and UV treated stream water). The changes in egg microbial community during succession and across treatments were analyzed, and the correlation between the microbial community assembly and the egg mortality was discussed.

Chapter 4

In this chapter, Lake Sturgeon eggs were fertilized with a putative symbiont and putative pathogen that were previously isolated from egg surfaces. After initial inoculation, successional

microbial community changes on the egg surfaces were examined. The effect of inoculation of the putative symbiont on egg mortality and other life history traits (including larvae size at hatch) was also studied. The potential application of the putative symbiont isolate for probiotic treatment in the Lake Sturgeon hatchery was also discussed.

Chapter 5

In this chapter, the relative importance of dispersal (the effect of aquatic microbes) and local deterministic effects (host egg effects) in shaping the egg associated microbial community was studied by manipulating the aquatic microbial community composition and quantity throughout embryogenesis. Eggs were fertilized and reared in three different water types (stream water, UV treated stream water, and 0.2 μm filtered stream water) which had different aquatic microbial community compositions and concentrations. We hypothesized that if the dispersal is dominant processes in shaping the egg associated microbial community, egg microbial community would converge with the source water microbial communities. We characterized both the egg surface microbial communities and the aquatic microbial communities across treatments and throughout embryogenesis to elucidate the relative roles of dispersal of aquatic microbes and local egg-related host effects on microbial community assembly.

Chapter 6

In this chapter, 25 microbial strains isolated from Lake Sturgeon egg surfaces were characterized for both antagonistic microbial interactions and biofilm forming capabilities. The soft agar overlay technique and the crystal violet staining technique were used to evaluate antagonistic microbial interactions and biofilm forming capability, respectively. This experiment

was performed to identify a microbe that interacts with fish pathogens antagonistically and thus one that would be a good candidate for probiotic treatment of Lake Sturgeon eggs. We also assessed whether microbial interactions among isolates and biofilm formation of each isolate help explain the changes in the egg microbial community structure observed in previous chapters.

Chapter 7

Findings from previous chapters were summarized. Mechanisms that govern the formation and development of the egg surface microbial communities were discussed based on the findings of the dissertation. Implications of our findings and future suggestions were also provided.

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CHAPTER 2: MICROBIAL COMMUNITY ASSEMBLY AND SUCCESSION ON LAKE STURGEON EGG SURFACES AS A FUNCTION OF SIMULATED SPAWNING STREAM FLOW RATE

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Abstract

We investigated microbial succession on Lake Sturgeon (*Acipenser fulvescens*) egg surfaces over the course of their incubation period as a function of simulated stream flow rate. The primary objective was to characterize the microbial community assembly during succession and to examine how simulated stream flow rate affect the successional process. Sturgeon eggs were reared under three flow regimes; high (0.55 m/s), low (0.18 m/s), and variable (0.35 and 0.11 m/s alternating 12 hr intervals). Eggs were collected from each flow regime at different egg developmental stages. Microbial community DNA was extracted from the egg surface and the communities were examined using 16S rRNA gene-based Terminal Restriction Fragment Length Polymorphism (TRFLP) and 454 pyrosequencing. Analysis of these datasets using Principal Component Analysis (PCA) revealed microbial communities were clustered by egg developmental stages (early, middle, and late) regardless of flow regimes. 454 pyrosequencing data suggested that 90-98% of the microbial communities were composed of phyla Proteobacteria and Bacteroidetes throughout succession. β-Protebacteria was more dominant in the early stage, Bacteroidetes became more dominant in the middle stage, and α-Proteobacteria

became dominant in the late stage. A total of 360 genera and 5826 OTUs at 97% similarity cutoff were associated with the eggs. Midway through egg development, the egg-associated communities of the low flow regime had a higher diversity than those communities developed under high or variable flow regimes. The results suggest that microbial community turnover occurred during embryogenesis, and stream flow rate could influence the microbial succession processes on the sturgeon egg surfaces.

Introduction

The process of succession involves the colonization of open space and subsequent sequential changes in community composition. This process has been studied primarily using plant systems [1]. Microbial succession has been recently studied in conjunction with various host animals [2-4], host plants [5], and natural [6-7] and artificial [8-10] environments. Microbes serve as ideal targets for studying succession because the microbial succession can be effectively controlled and observed in an experimental setting, thus allowing for novel information to be learned about factors affecting the succession process [11].

Results from a number of studies suggest that microbial succession is a complex process [4-6, 11]. For example, Redford and Fierer studied microbial community assembly on the newly synthesized cottonwood leaf surface [5]. The authors found a strong temporal effect whereby microbial community compositions on leaves from different trees sampled on the same day were more similar to each other than those from the same tree sampled on different dates. In contrast, Palmer and colleagues studied microbial succession in the human infant gut, and found that inter-individual variations dominated over temporal variation [4]. Previous studies have also shown that microbial species richness varies with time during succession. Redford and Fierer found a positive linear relationship between microbial species richness on the cottonwood leaf surfaces and time [5]. In contrast, Jackson and colleagues found that species richness of microbial communities on glass slides in aquatic environments fluctuated over time, with high diversity in the beginning due to stochastic colonization, subsequent decline in diversity due to species sorting, followed by an increase due to maturation [6]. These differences across different studies could be attributed to differences in the rate of dispersal, host characteristics, environmental conditions, disturbances, and temporal scales of their studies [11].

Although numerous studies have demonstrated that environmental conditions are key in explaining microbial community assembly [12-15], few studies have examined the effect of environmental conditions on microbial succession [7, 16]. Besemer and colleagues studied the effect of stream flow velocity on microbial succession on ceramic coupon surfaces in streams [16]. They found that midway through the successional process, community assemblages developed under turbulent flow were different from those developed under other flows. Lyautey and colleagues studied the effect of environmental factors such as light and water temperature on microbial succession on pebble surfaces in natural streams and found that both affected the successional process [7]. This small collection of studies suggest that microbial succession is dependent on environmental factors and further research may be needed to better understand such effects.

In addition, despite the solid foundation of research on microbial succession on various hosts, no studies have yet investigated microbial succession on fish eggs. Fish eggs serve as a good model for studying microbial succession, since egg surfaces provide an open niche for aquatic microbes. Aquatic microbes start colonizing the egg surfaces as soon as eggs are deposited in a stream, and the microbial community subsequently develops on the egg surface during embryogenesis. Microbial community turnovers are expected to occur as microbes on the egg surfaces compete for egg nutrients [17], metabolites excreted by eggs change during embryogenesis [18-19], and microbes on the egg surfaces are selected against by host innate immunity including lysozyme secretion [20-21]. Studies about microbial succession on egg surfaces in streams will provide some insights about the effect of live hosts on microbial succession by providing a comparison to previous microbial succession studies performed using natural [7] and artificial substrates [6, 16] in streams.

We present here a study on microbial community succession on the Lake Sturgeon (*Acipenser fulvescens*) egg surface. Lake Sturgeon populations have decreased drastically over the past 100 years due to anthropogenic activities such as overfishing and dam construction [22]. One such population is the Black Lake population, Michigan [23-24]. Spawning habitats have been altered since the construction of Kleber dam in 1949 on the Upper Black River, which is the sole spawning stream for the Black Lake population. Despite recent restoration efforts, natural recruitment is limited, which is likely attributed to high egg mortality [25]. Our primary objective was to understand how microbial community assembly changes throughout the fish embryonic development and to acquire fundamental knowledge about microbial community assembly on the egg surface during succession. Characterizing microbial community assembly on the egg surface during incubation will help illuminate the potential causal relationship between microbes and egg mortality. We were also interested in investigating how changes in stream flow rate affected microbial community assembly during microbial succession. Flow rate is an important environmental factor to consider in this system because construction of dams has altered steam flow rate [26-29], and in turn potentially affected downstream ecosystems in many ways, including by altering the interactions between microbes and fish eggs.

Methods

Experimental Design

This experiment was conducted at a Lake Sturgeon streamside rearing facility (details in [30]) located on the Upper Black River system in Michigan during May 2007 in the midst of the Lake Sturgeon spawning season. Incoming river water was filtered using a sand filtration system to remove large particulate matter before being gravity-fed in a flow-through design to

experimental flumes. We tested the effects of three different flow regimes on microbial community succession on the egg surface over time. A total of six flume channels were used with two replicates for each flow regime. The first flow regime consisted of a constant high flow velocity (0.55 ± 0.01m/s) representing a fast current section of a natural stream which embryos experience in the natural river setting [26]. The second flow regime was a low flow velocity (0.18 ± 0.01m/s) that represented both slower areas in the river and minimum flow that eggs naturally experience during dry spring seasons. A third flow regime was a variable flow. This variable flow regime was set to be high for 12 hours and low for 12 hours (0.35 ± 0.01m/s and 0.11 ± 0.01m/s, respectively), which is typical of many hydro electric dams that operate during periods of peak electrical demand [26]. Gametes used in this study were collected from adult Lake Sturgeon captured in the act of spawning in the Upper Black River, Michigan. Eggs were obtained from one female and were fertilized with milt from two males that were selected randomly from a pool of candidates. Immediately upon fertilization, approximately 100 eggs were placed on plexi-glass plates (7.6cm x 5cm). Eggs were allowed to adhere to plates in standing stream water. Thus, the conditions of initial colonization were standardized across the eggs to be exposed to the three different flow regimes. Plates were then placed within experimental flume channels constructed from a 3" PVC pipe cut lengthwise. All plates were kept at a water depth of 2 cm across treatments. The water source throughout the experiment was exactly the same for all six flumes so infusion of stream microbes was constant across the flumes. Six to ten eggs were randomly collected from each flume channel at five embryonic developmental stages (Day 2, Day 3, Day 5, Day 6, and Day 7) and were immediately preserved in 70% ethanol. Over 90% of all eggs that hatched did so between Day 9 and Day 11. The total number of samples collected in this study was 30 (6 flume channels * 5 developmental stages).

Water temperature (^{o}C) was also measured at hourly intervals throughout the experiment. Dissolved oxygen concentration of the source water was above 9 mg/L throughout the experiment.

DNA extraction and Terminal Restriction Fragment Length Polymorphism (TRFLP)

The thirty microbial community samples were subjected to DNA extraction. Microbial community genomic DNA was extracted from the surfaces of 4 or 5 eggs per sample using the PowerSoil^{TM} Kit (MO BIO Laboratories Inc., CA) following bead beating according to the manufacturer's protocol. 16S rRNA gene based TRFLP [31-32] was performed to compare microbial community assembly across the 30 samples. TRFLP technique has been used to compare microbial community composition across environmental gradients [12, 33-34], locations [12, 35-36], and times [8, 10, 37]. The detailed procedures are as follows. 16S rDNA amplification was performed using the universal bacterial primers 63F (5'-CAG GCC TAA CAC ATG CAA GTC-3') (5'FAM-labelled) and 1389R (5'-ACG GGC GGT GTG TAC AAG-3') (unlabelled) [38-39]. PCR reaction was conducted in a 100 µL reaction volume, containing 20 to 40µL template DNA (1 to 4 ng μL^{-1}), 5.0 U of *Taq* DNA polymerase (Invitrogen Corp., Carlsbad, CA), in a final concentration of 0.2µM of each primer, 0.25mM of each deoxynucleoside triphosphate, 10mM Tris-HCl, 50mM KCl, and 1.5mM $MgCl_2$. PCR was performed under the following cycle conditions: an initial denaturation step at 94°C for 5 min and 30 cycles of denaturation at 94 °C for 30s, annealing at 55°C for 30s, and extension at 72°C for 110s. A final extension step at 72 °C for 7 min was then performed. The PCR product was purified using QIAquick PCR purification kit (Qiagen) according to the manufacturer's protocol.

The purified PCR products were subjected to enzyme digestion with either HhaI or MspI (Gibco BRL). The reaction mixture contained 2.0 µL of 10X reaction buffer (Gibco BRL), 0.3 µL of enzyme (20U/µL, Gibco BRL), about 200ng of purified PCR product and pure water to a final volume of 20 µL. The enzyme digestion was carried out for 2 hours at 37^{o}C. Two technical replicates (10µL each) of the digested DNA samples were sent to Michigan State University's sequencing facility and the DNA fragments were separated on an ABI 3100 Genetic Analyzer automated sequencer (Applied Biosystems Instruments, Foster City, CA) in GeneScan mode. The 5' terminal restriction fragments (TRFs) were detected by excitation of the 6-FAM molecule attached to the forward primer. The sizes and abundance (peak height) of the terminal fragments were calculated using GeneScan 3.7. The resultant peak heights were filtered to eliminate peaks with a height below the background noise threshold (set at 50 fluorescence units). Each terminal fragment corresponds to a phylotype, and peak height indicates relative abundance of a phylotype. In order to align TRF peaks across the 30 samples, the TRFLP profiles were processed with T-Align (http://inismor.ucd.ie/~talign/index.html) and the output of T-Align was used for the microbial community analysis. A total of 123 phylotypes and 130 phylotypes were detected from the 30 samples using endonuclease HhaI and MspI, respectively. On average, 26.6 and 25.6 phylotypes were detected per sample for HhaI and MspI, respectively.

Microbial community analysis using TRFLP data

We employed the Bray-Curtis dissimilarity index [40] to compare microbial community composition among samples from different flow treatments and egg developmental stages. A dissimilarity index of 0 indicates that the community compositions of the two samples are identical, whereas an estimated index of 1.0 indicates that the community compositions of the

two samples are completely different. We employed general linear models with the dissimilarity index as the dependent variable to assess the effect of time and flow treatments on the index. Principal component analysis (PCA) was performed using TRFLP data from the 30 samples to elucidate underlying patterns across samples. The data consisted of 25 columns which represented 25 major phylotypes that had 3% or higher relative abundance for at least one of the 30 samples, and 30 rows which represented relative abundance of each major phylotype in the 30 samples. The scores of principal component 1 (PC1) and 2 (PC2) were used to elucidate the temporal similarity of the microbial community composition, and the loadings of PC1 and PC2 were used to elucidate the distribution patterns of the 25 major phylotypes. Phylotype richness S (S = the number of distinct terminal restriction fragments in each sample) was determined from the TRFLP profiles. All distinct TRFs, including both major and minor phylotypes, were included in this analysis. Prior to analyzing the relationship between phylotype richness and time, extremely high or low total fluorescence signals caused by over or under loading of digested DNA samples were removed to eliminate non-biological effects on phylotype richness. A general linear model was used to investigate the relationship between the microbial phylotype richness and time. A quadratic term for time was also included in the model. We conducted all analyses using both HhaI and MspI, but both showed similar results (data not shown); therefore we present only the HhaI results. The general linear model and PCA were conducted using R version 2.10.0 [41]. The Bray-Curtis dissimilarity matrix was generated in R using the "ecodist" package.

454 Pyrosequencing

To characterize the microbial community at different time points and under different flow regimes, nine samples representing three flow regimes (High, Low and Variable) at three time points (Day 2, Day 5, and Day 7) were assigned for pyrosequencing. Since the TRFLP data suggested that variation among two replicated flows within the same flow regime at the same time point was small, the extracted genomic DNAs of the two replicated flows within the same flow regime were pooled. To evaluate the reproducibility of samples, technical replicates were included for the three Day 5 samples (High, Low and Variable at Day 5). Hypervariable region V3 - V5 in 16S rRNA gene was amplified using a forward primer 357F (5'-CCTACGGGAGGCAGCAG-3') and a reverse primer 926R (5'-CCGTCAATTCMTTTRAGT-3') as previously described [42-44]. 454 'A' adapter and tag sequences were contained in the reverse primer, and 'B' adapter was contained in the forward primer. PCR amplification was performed in 75µL reaction, using 3U of AccuPrime Taq HiFi (Invitrogen, Grand Island, NY), 7.5µL of supplied 10X buffer II, 1.5µL of 10µM primers, and approximately 30ng of template DNA measured by Nanodrop ND1000 (Thermo Scientific Inc). The PCR cycle condition was as follows: denaturation at 95^{o}C for 2 minutes followed by 30 cycles of denaturation at 95^{o}C for 20 seconds, annealing at 50^{o}C for 30 seconds, extension at 72^{o}C for 5 minutes [44]. The PCR amplicons were cleaned using Agencourt AmPure XP Beads (Beckman Coulter, Inc., Brea, CA) and pyrosequencing of the amplicons was performed using 454 GS FLX titanium platform (454 Life Science, Branford, CT) at the research technology support facility at Michigan State University.

Pyrosequencing data processing and analysis

Raw sequence reads were processed using Ribosomal Database Project (RDP) pipeline [45] to sort the data by tag sequence, to trim tag and primer sequences, and to filter out low quality sequences with minimum quality score of 20 (probability threshold of 0.01) and minimum read length of 300bp. The taxonomy of the filtered reads was assigned using RDP Classifier at a bootstrap threshold of 80% [46]. Bray-Curtis dissimilarity among the 9 samples was determined using the classifier output at the genus level. OTUs of the filtered reads at 97% similarity cutoff were determined using RDP complete-linkage clustering algorithms with 0.03 maximum cluster distances. The underlining patterns of the 9 samples were analyzed by PCA using OTUs at the 97% similarity. Jaccard index tree of OTUs at the 97% similarity level was constructed using RDP pipeline. The diversity index including species evenness (E) for each sample was determined by RDP pipeline diversity index estimator using the 97% OTUs. Rarefaction curves of the 97% OTUs for the samples were generated using RDP pipeline. To assess the relationship between the number of OTUs and time, the 97% OTUs were rarefied at 6000 reads per sample.

Results

Analysis on microbial community succession using TRFLP

Using TRFLP analysis, we detected considerable temporal variation in microbial community composition on Lake Sturgeon eggs across sequential embryonic developmental stages (Figure 2.1). The temporal variation in microbial community composition was consistently observed in all six flume channels (two replicates for each of the 3 flow regimes). Microbial community compositions estimated from samples collected from the same developmental stage were more similar to each other than those from different developmental

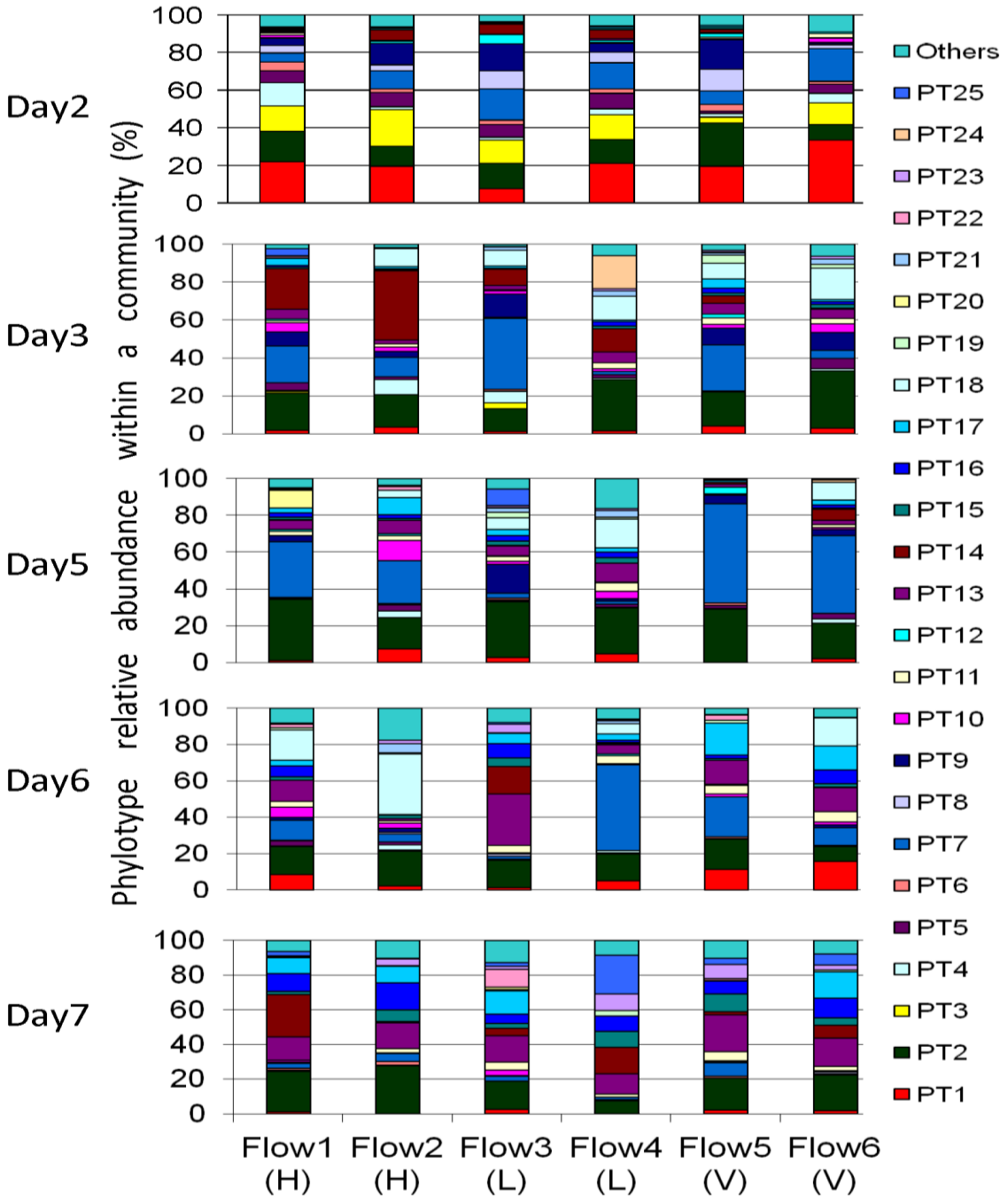


Figure 2.1. Temporal and flow rate effects on microbial community composition during microbial succession.

25 major phylotypes that had 3% or higher relative abundance for at least one sample were included. The rest were grouped into "others". Each color/pattern represents a unique terminal fragment (phylotype, PT). The experiment consists of 3 flow regimes (High: Flows 1 and 2, Low: Flows 3 and 4, and Variable: Flows 5 and 6) sampled at day 2, 3, 5, 6, and 7. HhaI digested TRFLP data were used for the analysis. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

stages, regardless of flow regimes (Table 2.1). For example, the mean Bray-Curtis dissimilarities within Day 2 samples and between Day 2 and Day 3 samples were 0.301 and 0.594, respectively (Table 2.1). These two means were statistically significantly different ($F_{1,49} = 135$, $p < 0.001$). The dissimilarity between microbial community compositions increased during the course of embryogenesis as the community departed from the initial community (Table 2.1, left column).

Table 2.1. Bray-Curtis dissimilarity index matrix using TRFLP data summarized by day

	Day 2	**Day 3**	**Day 5**	**Day 6**	**Day 7**
	Mean[a] (sd)	Mean (sd)	Mean (sd)	Mean (sd)	Mean (sd)
Day 2	0.301 (0.083)				
Day 3	0.594 (0.082)	0.426 (0.082)			
Day 5	0.624 (0.073)	0.443 (0.114)	0.444 (0.104)		
Day 6	0.639 (0.068)	0.510 (0.103)	0.497 (0.141)	0.490 (0.134)	
Day 7	0.747 (0.064)	0.592 (0.092)	0.585 (0.110)	0.518 (0.140)	0.369 (0.095)

a. Mean Bray-Curtis dissimilarity across all flow regimes.

Stream flow rate had a more subtle effect on the composition of the microbial communities than egg developmental stage (Figure 2.1). Microbial communities were similar at the early (Day 2) and late (Day 7) developmental stages, regardless of flow regimes. However, the microbial composition of the low flow regime departed from that in the high and variable flow regimes during mid-developmental stages. This is evident in the diagonal array of the dissimilarity index in Table 2.1 that shows an increasing variance of dissimilarity (which we attribute to differences in flow). At Day 5, the microbial community composition differed

between high (flow 1 and 2) and low (flow 3 and 4) flow regimes (Figure 2.1: Day 5). The mean dissimilarity within the same flow regime (high-high or low-low) was 0.368, whereas the mean dissimilarity between high and low flow regimes was 0.478. Microbial communities reared in the variable flow regime were more similar to that in the high flow regime (dissimilarity = 0.380) than to the low flow regime (dissimilarity = 0.554), suggesting that the high flow portion of the variable flow regime had a more dominant effect on microbial community structure than the low flow.

Principal component plots also showed the strong temporal clustering of microbial communities on the egg surfaces (Figure 2.2). Microbial communities sampled from the same or close developmental stages were clustered together regardless of flow regime. Microbial communities were clustered into three different embryonic developmental stages; the early (Day 2), the middle (Day 3, Day 5, and Day 6) and the late (Day 7), respectively. This PCA plot shows that the temporal effect was more dominant in explaining microbial community assembly than flow rate effects. We also found that certain microbial phylotypes were more strongly associated with certain egg developmental stages (Appendix Figure A.2.1). Out of the 25 dominant microbial phylotypes we detected using HhaI, we found that 8 phylotypes were predominantly associated with the early stage, 7 phylotypes peaked in the middle, and 9 phylotypes were preferentially associated with the late stages of egg development. This trend was also depicted in a loading principal component plot (Figure 2.3).

There was a significant positive linear relationship between microbial phylotype richness on egg surfaces and time (Figure 2.4 solid line: $F_{1,25} = 4.73$, $p = 0.039$). In contrast, there was no significant main effect of flow regime on microbial phylotype richness according to a linear regression model ($F_{2,24} = 0.658$, $p = 0.53$). However, the relationship between microbial

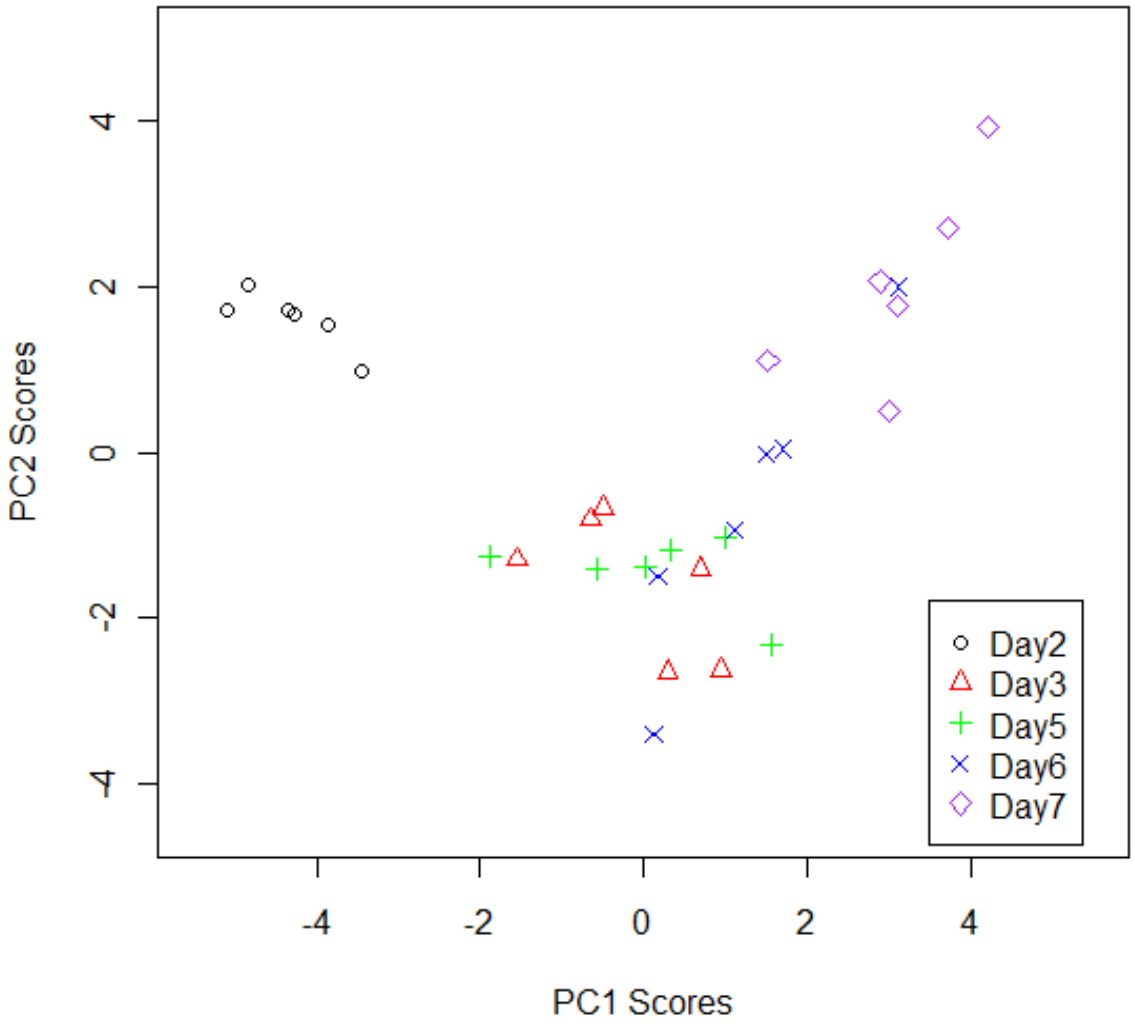


Figure 2.2. Principal component score plots revealing the temporal clustering of microbial community assemblages on egg surfaces.

PC1 and PC2 account for 27.9% and 13.6% of the data variations, respectively. HhaI digested TRFLP data were used for the analysis.

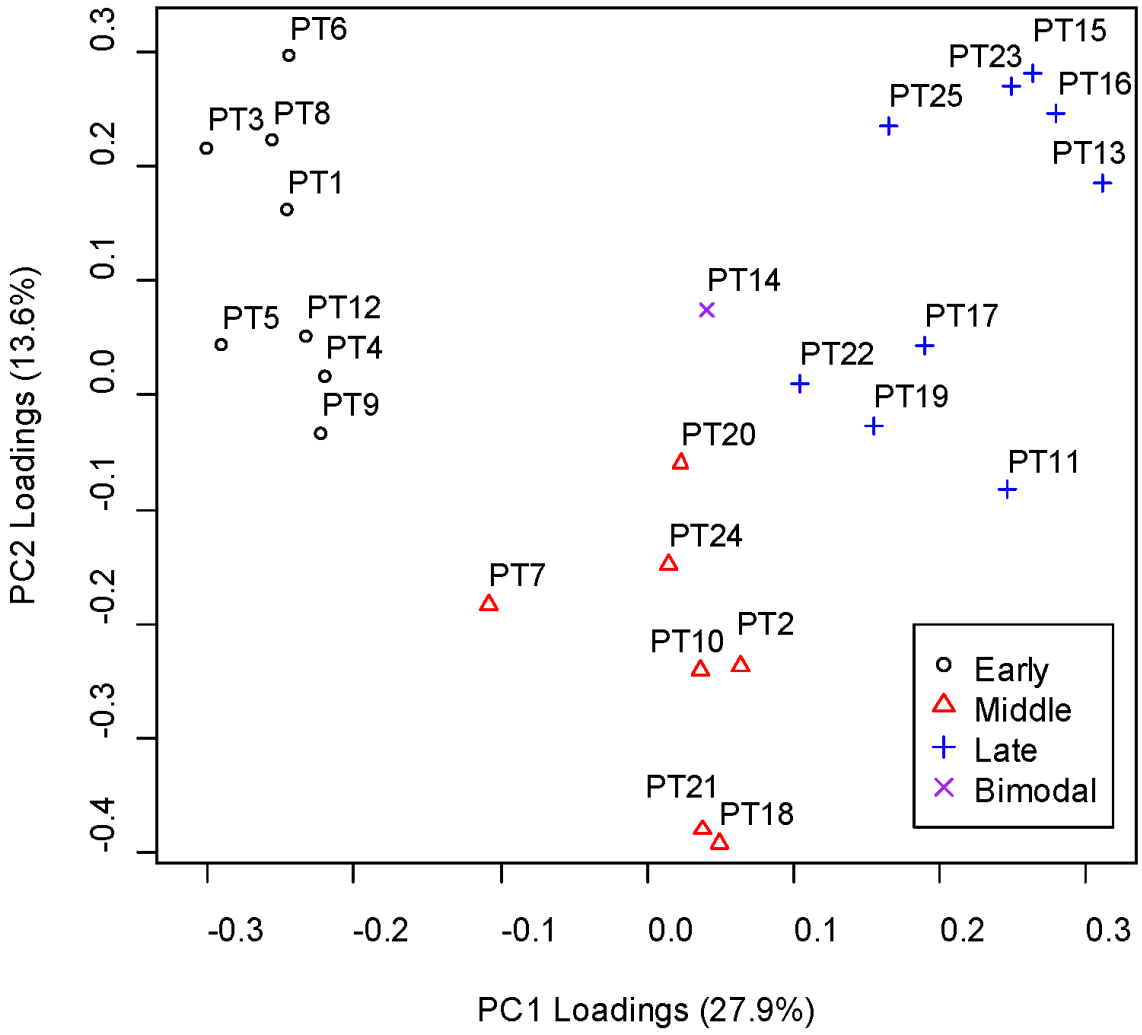


Figure 2.3. Principal component loading plots displaying temporal distributions of 25 major microbial phylotypes (PT) associated with the egg surface.

PC1 and PC2 account for 27.9% and 13.6% of the data variations, respectively. HhaI digested TRFLP data were used for the analysis.

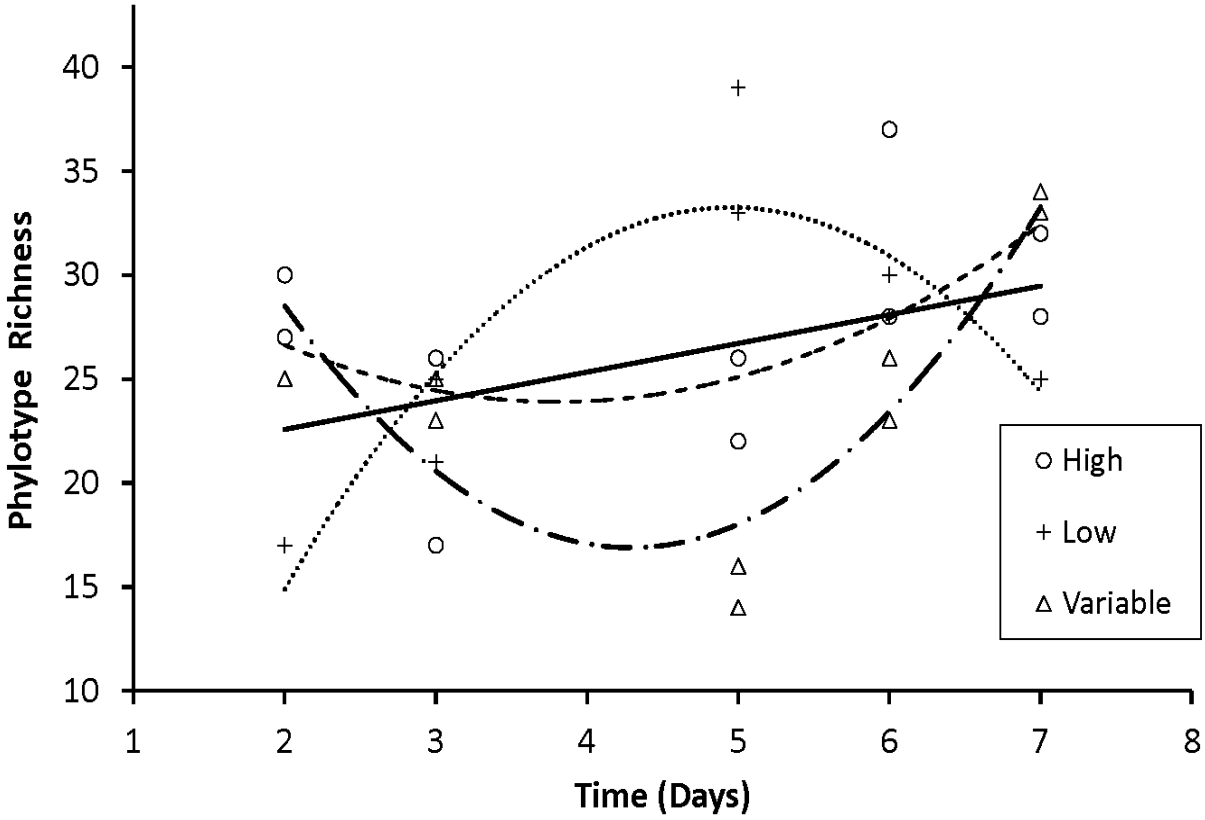


Figure 2.4. Microbial phylotype richness and time relationships during succession across flow regimes (High ○, Low +, and Variable Δ).

The straight solid line is a linear regression line for all data points ($F_{1,25} = 4.73$, $p = 0.039$, $R^2 = 0.16$). The dotted line is a positive quadratic regression for the low flow regime ($F_{2,5} = 10.26$, $p = 0.017$, $R^2 = 0.80$). The breaking line is a negative quadratic regression for the high flow regime ($F_{2,7} = 1.543$, $p = 0.28$, $R^2 = 0.31$). The dotted-dash line is a negative quadratic regression for variable flow regime ($F_{2,6} = 13.1$, $p = 0.006$, $R^2 = 0.81$). The phylotype richness determined by HhaI digested TRFLP was used for this analysis.

phylotype richness and time was dependent on flow regime. At the low flow regime, the relationship between microbial phylotype richness and time was negative quadratic (Figure 2.4 dotted line: $F_{2,5} = 10.26$, $p = 0.017$) with the highest richness at the middle developmental stage. In contrast, under the high and variable flow regimes, there was a moderate positive quadratic relationship between microbial phylotype richness and time (Figure 2.4 breaking line (high): $F_{2,7} = 1.543$, $p = 0.28$; dotted-dash line (variable): $F_{2,6} = 13.1$, $p = 0.006$) with lower phylotype richness at the middle developmental stage. In other words, at Day 5, eggs reared in the low flow regime were associated with a relatively large number of microbial phylotypes, while eggs reared in high or variable flow regimes were associated with a fewer number of phylotypes.

Microbial community analysis using 454 pyrosequencing

To characterize the microbial community assembly at different egg developmental stages and under different flow regimes, nine samples representing three flow regimes (High, Low and Variable) at three time points (Day 2, Day 5, and Day 7) were subjected to pyrosequencing. We obtained an average number of 13468 ± 7779 reads with an average length of 450.08 ± 1.97bp per sample. These reads were classified using RDP classifier into 23 phyla, 45 classes, and 360 genera across the 9 samples. The average numbers of phyla, classes, and genera per sample were 14.5 ± 2.6, 25.3 ± 4.5, and 138.7 ± 20.2, respectively. Two technical replicates for Day 5 samples showed an almost identical community composition at each taxonomic level, indicating that the pyrosequencing data were reproducible.

A temporal compositional trend during microbial succession was detected at the phylum/class level (Figure 2.5). Two phyla, Proteobacteria and Bacteroidetes, comprised 90 to

98% of the egg surface microbial community throughout egg development. Proteobacteria was more dominant in the early (58 to 74%) and late (71 to 82%) egg developmental stages, while Bacteroidetes was dominant in the middle stage (63% for high and 81% for variable flow regime) except under the low flow regime (36%). The dominant classes of the phylum Proteobacteria included β, α, and γ-Proteobacteria. β-Proteobacteria was more dominant in the early egg developmental stage and α-Proteobacteria became more dominant by the late stage.

The temporal trend was also found at the genus level of analysis (Figure 2.6). *Flavobacterium* was one of the most dominant genera in the egg surface microbial community during succession and showed a strong temporal trend (Figure 2.6). Genus *Flavobacterium* accounted for a large proportion of the egg surface microbial community in the early (20 to 40%), middle (25 to 80%), and late (9 to 13%) egg developmental stages. The large variation in

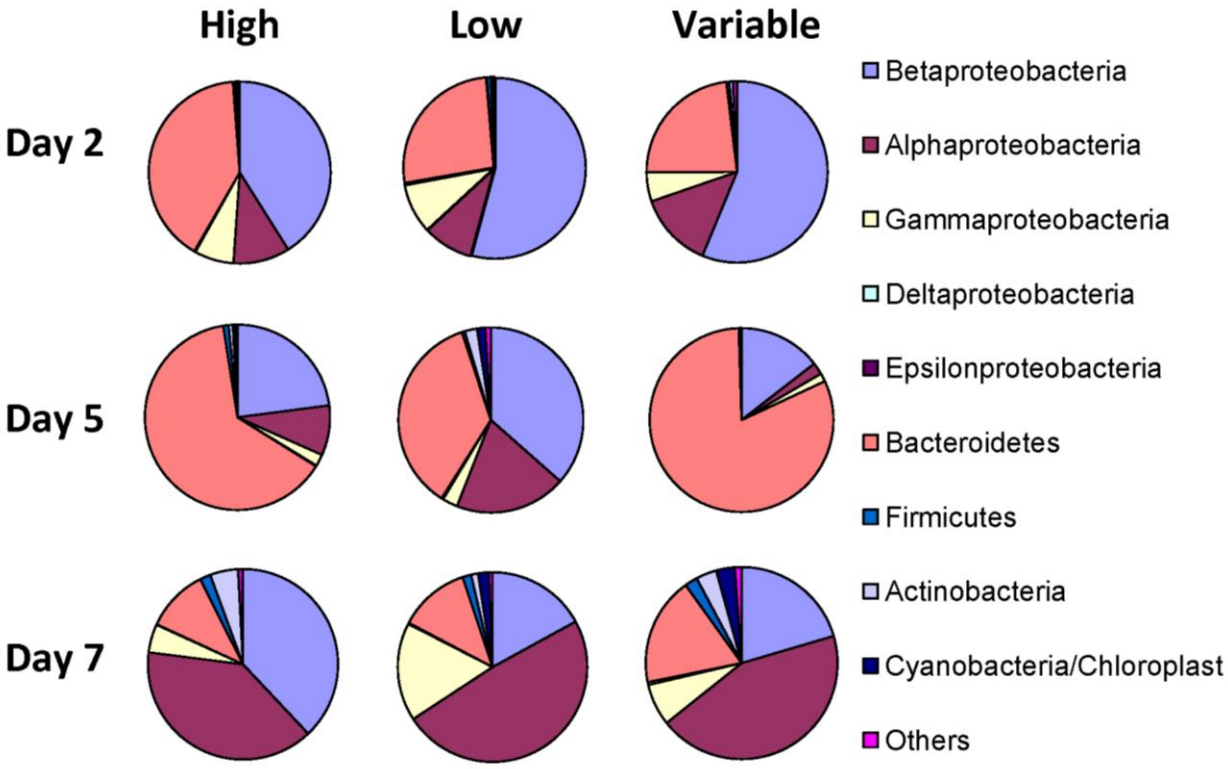


Figure 2.5. Characterization of the egg surface microbial community assembly using the RDP classifier output at the phylum level (and class level for Proteobacteria).

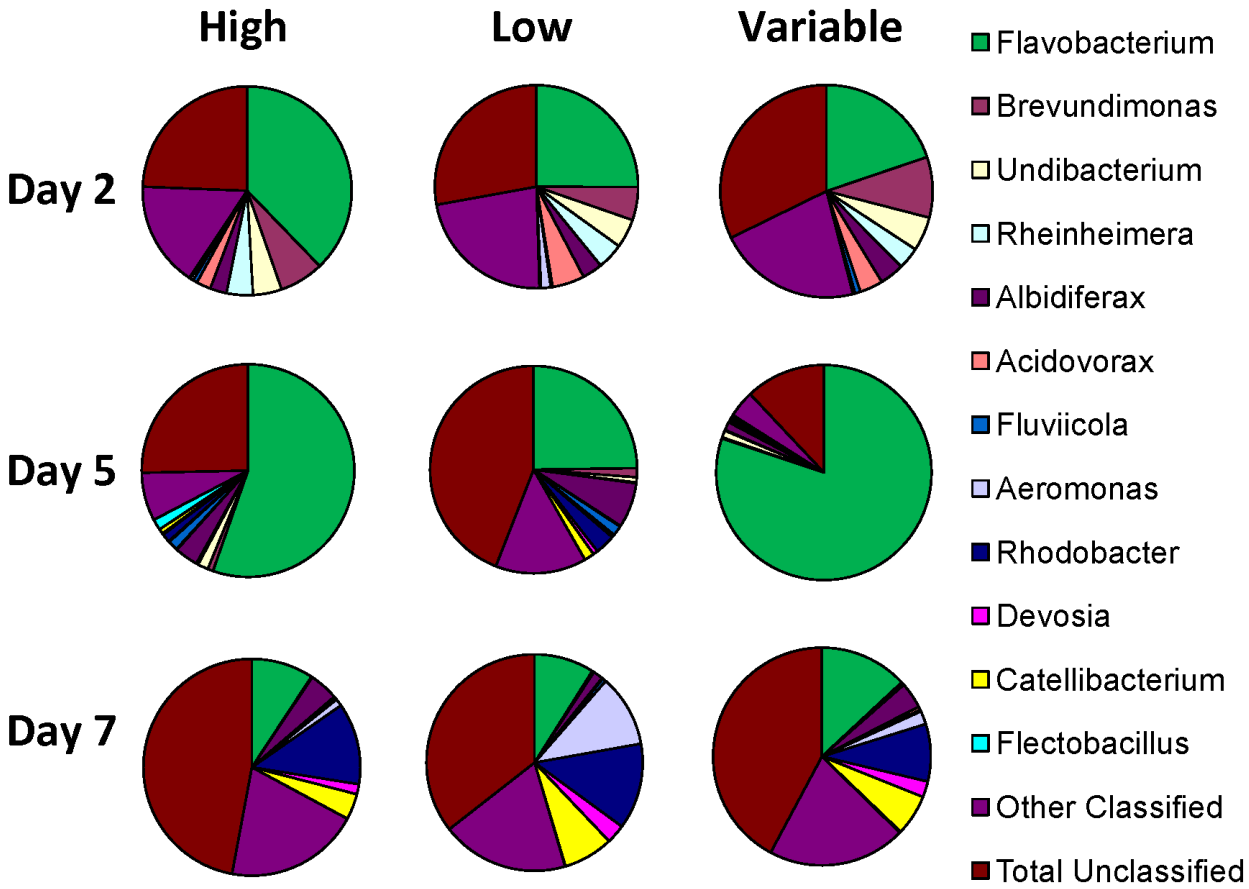


Figure 2.6. Characterization of the egg surface microbial community assembly using the RDP classifier output at the genus level.

Table 2.2. Bray-Curtis dissimilarity matrix using the RDP classifier output at genus level

	High D2	Low D2	Var D2	High D5	Low D5	Var D5	High D7	Low D7
Low D2	0.199							
Var D2	0.261	0.226						
High D5	0.366	0.511	0.561					
Low D5	0.384	0.465	0.512	0.352				
Var D5	0.429	0.581	0.645	0.185	0.481			
High D7	0.684	0.633	0.652	0.688	0.513	0.772		
Low D7	0.754	0.731	0.750	0.740	0.601	0.805	0.323	
Var D7	0.642	0.597	0.606	0.618	0.442	0.712	0.285	0.293

*Unclassified genera were not included in this analysis.

Flavobacterium relative abundance during the middle stage can be attributed to flow treatment. At Day 5, *Flavobacterium* accounted for 55 to 80% of the egg surface microbial community under high and variable flow regimes, but only 25% under the low flow regime. Other genera also exhibited a temporal trend in abundance during succession. Genera such as *Brevundimonas*, *Undibacterium*, *Massilia*, *Acidovorax*, and *Rheinheimera* decreased as egg development progressed, *Flectobacillus* and *Fluviicola* peaked at intermediate periods, and *Rhodobacter*, *Catellibacterium*, and *Devosia* (all α-Proteobacteria) were dominant towards the end of development. Although almost all sequences were classified at phylum/class level, 12 to 47% of the egg surface microbial communities per sample were unclassified at genus level, and the proportion of the unclassified genera increased as egg development progressed. We characterized a total of 360 genera in this analysis, but only two, *Flavobacterium* and *Albidiferax*, maintained a relative abundance over 2% throughout the entire incubation period.

Bray-Curtis dissimilarity analysis conducted at the genus level showed that microbial communities taken from the same time points were more similar to each other than to those from other time points (Table 2.2). The dissimilarity increased during the course of embryogenesis as the later communities departed from the initial community (Table 2.2, left column). At Day 5, the microbial community under low flow regime differed from those under high and variable flow, similar to the results seen with TRFLP.

RDP complete cluster linkage algorithms revealed the existence of 5826 distinct OTUs across the samples at 97% similarity cutoff with the average OTUs 1216.8 ± 201.0 per sample. Rarefaction curves of OTUs at the 97% similarity for the 9 samples including technical replicates are shown in Appendix Figure A.2.2. To assess the underlining patterns across different time points and flow regimes, the data matrix of the 97% OTUs was subjected to PCA. The first two

principal components captured the temporal trend in microbial community structures, while PC3 accounted for differences between low flow regime and both high and variable flow regimes at Day 5 (Figure 2.7). The Jaccard distance matrices using the 97% OTUs also grouped samples collected at the same time point together (Appendix Figure A.2.3), while separating low flow regime from high and variable flow regimes within the Day 5 group. The 97% OTUs rarefied at 6000 reads and time relationship showed a similar trend to what was found using TRFLP (Figure 2.8). The Shannon species evenness (E) calculated using the 97% OTUs revealed that microbial communities developed at Day 5 under high and variable flow were unevenly distributed (Table 2.3), which is congruent with the dominance of genus *Flavobacterium* in these communities.

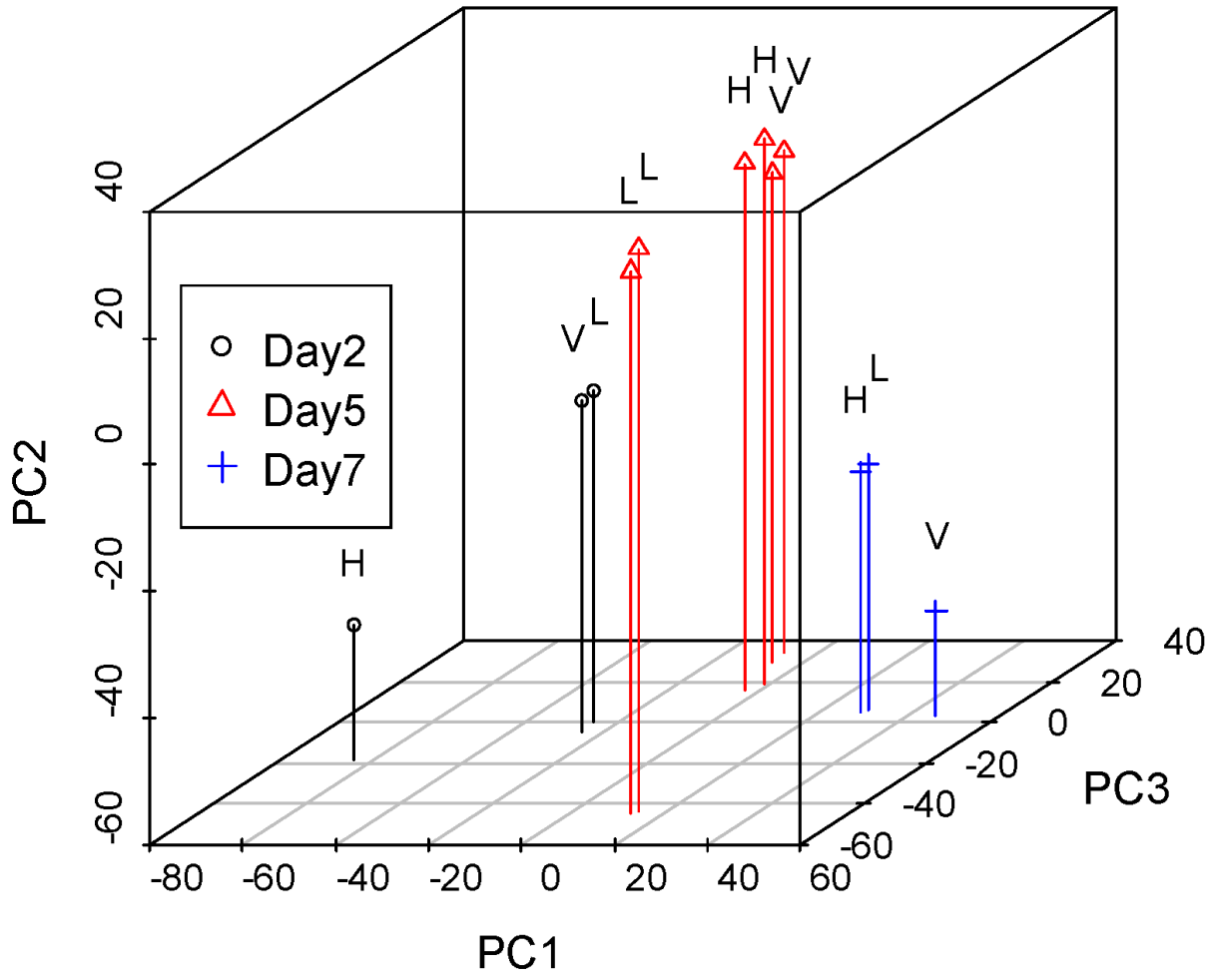


Figure 2.7. Principal component score plots using OTUs defined at 97% similarity cutoff.

PC1, PC2, and PC3 accounted for 15.3%, 12.2%, and 10.8% of the data variations, respectively. H, L, V, in the figure denotes High, Low, and Variable flow, respectively. Two technical replicates of Day 5 samples were included.

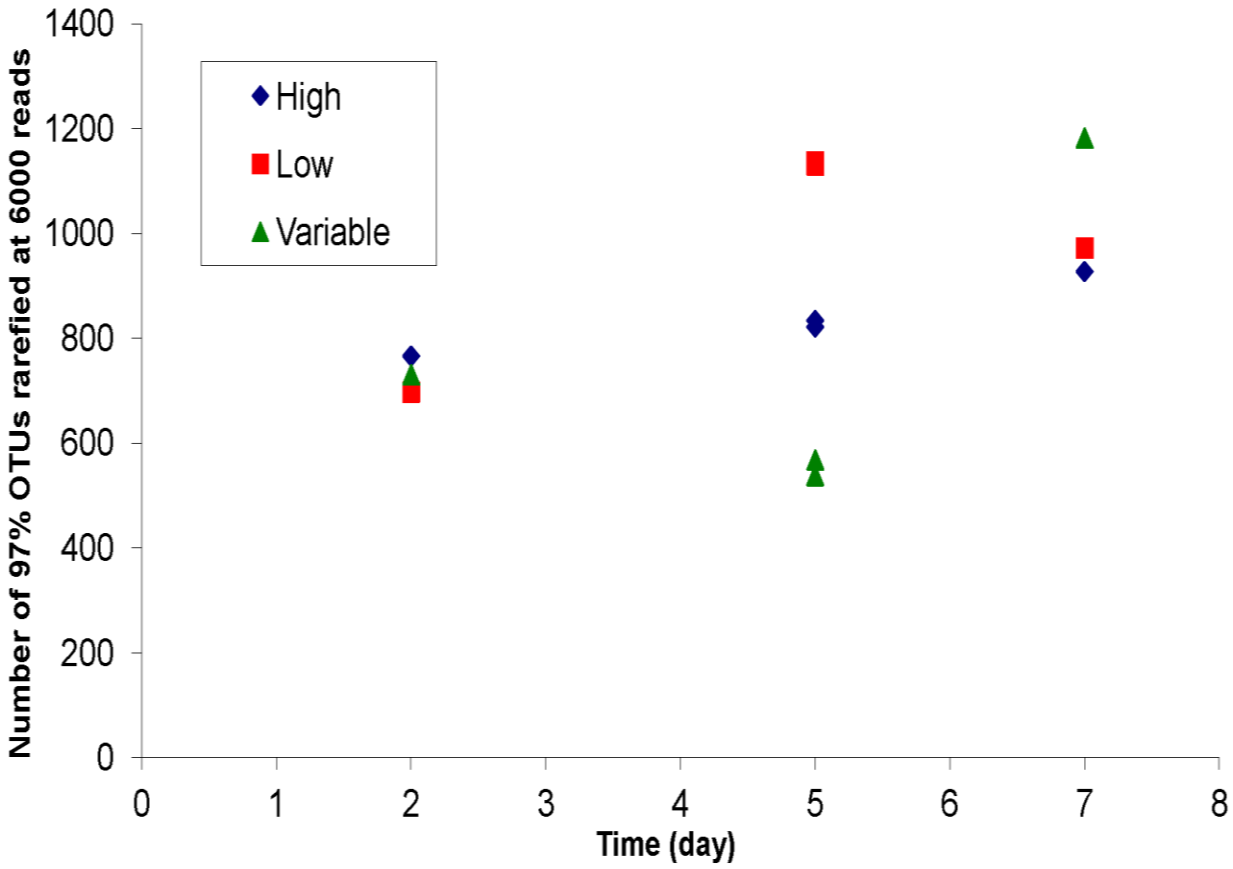


Figure 2.8. Microbial phylotype richness (97% OTUs rarefied at 6000 reads) and time relationships during succession across flow regimes.

Table 2.3. Estimated species evenness (E) using OTUs at 97% similarity cutoff

Flow Regime	Day 2	Day 5	Day 7
High	0.635	0.616	0.754
Low	0.684	0.787	0.731
Variable	0.721	0.501	0.794

Discussion

To our knowledge, this is the first study that documents microbial succession on fish egg surfaces using next generation sequencing. Although we obtained the average of 13,400 reads per sample using the pyrosequencing, our rarefaction curves indicate that microbial community assembly on the fish egg surfaces were not completely covered. However, our integrative approach of combining analysis of microbial community structures using TRFLP and subsequent characterization of microbial community assembly using pyrosequencing was effective in comprehensively identifying microbial community succession patterns. Both our TRFLP and pyrosequencing results clearly demonstrated that microbial species replacement was occurring on fish egg surfaces. The fish egg microbial communities were clustered into three different embryonic developmental stages: the early, the middle, and the late, which is similar to what Besemer and colleagues found with microbial succession on the ceramic coupon in streams [16], although they observed this trend over a longer time frame. The fact that we detected a temporal trend during microbial succession at the phylum/class level suggests that the change in microbial community assembly is not subtle, but rather drastic. This is particularly evident from our assessment that only two genera out of 360 detected genera maintained a relative abundance of over 2% throughout the entire incubation period.

One possible factor that may have contributed to the observed microbial community shifts during embryogenesis is the change in the chemistry of the egg surface. Previous studies suggest that chemistry on fish egg surfaces change during egg development [18-21]. Chadwick and Wright studied the nitrogen excretion patterns from Atlantic Cod eggs and found that the excretion of ammonia increased linearly while urea excretion decreased as the eggs developed [18]. Although nitrogen excretion patterns have never been studied in sturgeon, this type of

metabolite excretion could contribute to a change in the microbial community on the egg surface. Fish eggs are also known to secrete antimicrobial substances such as lysozyme [20-21] thus bactericidal activities might select for or against certain microbial species. One study on a relationship between egg innate immunity of small freshwater metazoan and egg microbial communities demonstrated that microbial communities on egg surfaces were altered when the type of antimicrobial peptides on eggs changed from maternally provisioned to that secreted by eggs [47-48]. Because over 90% of the egg surface microbial communities we found were composed of gram negative phyla Proteobacteria and Bacteroidetes, it is possible that microbial community assembly on the egg surface was shaped by egg-excreted lysozyme, which acts on peptidoglycan layers of gram positive bacteria [49].

The observed microbial community turnover also could be attributed to changes in the microbial community composition of the surrounding water during the experiment. The microbial community in freshwater streams varies temporally and spatially [37, 50-51]. We may have observed a microbial community that stochastically dispersed onto the egg surface from the water column [52-53]. However, the structure of the egg-associated microbial community characterized in this study differed from the structure of the typical aquatic microbial community described previously [54]. Despite having a similar high prevalence of β, α, and γ-Proteobacteria and Bacteroidetes as commonly seen in freshwater communities, our observed egg surface microbial community assemblages had almost undetected levels of the phylum Actinobacteria, a microbe typically dominant in freshwater environments. In fact, we collected water samples from our spawning stream during the spawning season of a different year (Fujimoto et al. unpublished study) and also detected significant numbers of the phylum Actinobacteria in the water column using 454 pyrosequencing, but greatly diminished numbers on the egg surfaces reared in the

stream water. In addition, the dominance of over 50% relative abundance of *Flavobacterium* in the high and variable flow regimes at Day 5 deviated strongly from freshwater microbial communities. These findings are revealing in suggesting that dispersal from water column may not be a strong factor in shaping egg surface microbial community assembly.

The microbial community turnover we observed on fish egg surfaces occurred during a 7 day period, which is significantly shorter than the time frame in which microbial succession is typically observed on non-living substrate surfaces [6-7, 16, 50]. Our results suggest that microbial species sorting on the egg surface can occur in a short time frame, perhaps due to the living egg-related effects such as host innate immunity [21], secretion of metabolites [18], and provision of three dimensional structures, unlike the comparably long time required for such sorting to be observed on abiotic substrate surfaces where species sorting after initial colonization is limited by resource competition and microbe-microbe interaction [50].

Another finding of our study was the effect of flow rate on microbial community assembly. Despite the lack of flow effect on the early and late egg surface microbial communities, the differences in phylotype diversity observed between eggs reared under low versus high and variable flow regimes at Day 5 were revealing. These findings suggest that the microbial community compositions were uniform at the early stage, diverged toward the middle stage dependent on the flow rate experienced by the eggs, and converged at the late stage of embryogenesis. A similar trend was found by Besemer and colleagues [16] who observed a significant effect of the flow velocity on ceramic surface microbial communities midway through the succession process but not in initial and mature microbial communities.

One possible explanation for the flow effect could be that high flow rate selected for certain microbial species that have the ability to adhere to the egg surface under greater shear

force. One previous study tested the effect of flow velocity on microbial community diversity in water pipes found that increasing flow velocity in pipes lowered microbial community diversity [55]. At low flow rate, the selection pressure was presumably relaxed; therefore, many different microbial species with lower adhesive capabilities could stay on the egg surface. The dominance of *Flavobacterium* in the high and variable flow regimes could be related to the fact that it is a particularly adhesive microbe [56-57]. Another possibility is that increased flow velocity increased the concentration of dissolved oxygen, thereby resulting in selection for aerobes that have a high tolerance for oxygen. However, we do not know why the effect of flow on community assembly was observed in the middle of the embryogenesis, but not observed at the early and late time points.

It is important to consider the potential implications of the effect of flow rate on microbe – egg interactions for host life history. Another study by our group [25] showed that over 80% of egg mortality occurs during the first half of the incubation period, which is from Day 0 to Day 5 for our experimental conditions. *Flavobacterium*, the dominant genus in both high and variable flow at Day 5, encompasses ranges of species including known fish pathogens such as *F. columnare* [58], *F. psychrophilum* [59], and *F. branchiophilum* [60]. On the other hand, under low flow regime at Day 5, phylum Proteobacteria, which also includes some fish pathogens such as genus *Aeromonas* [61], was dominant. The net effect of flow velocity on microbe- host interaction is difficult to determine. However, it is important to note that fish eggs deposited in natural streams may not receive the extremely high flow velocity used in this experiment, since the flow at the bottom of streams is lower than that in middle of water column due to the effect of the boundary layer [62].

In this study, we focused on flow rate as a key environmental covariate that could influence microbe – host interactions. However, water temperature is also an important environmental covariate that may affect the microbial community assembly in aquatic systems [7, 63]. In this experiment, all eggs reared in the 6 flume channels were exposed to water from the same source and thus experienced the same water temperature throughout the experiment. However, the daily mean ambient water temperature gradually increased during the course of the experiment (Appendix Figure A.2.4), which may account for the temporal variation of microbial community composition that we observed during embryogenesis.

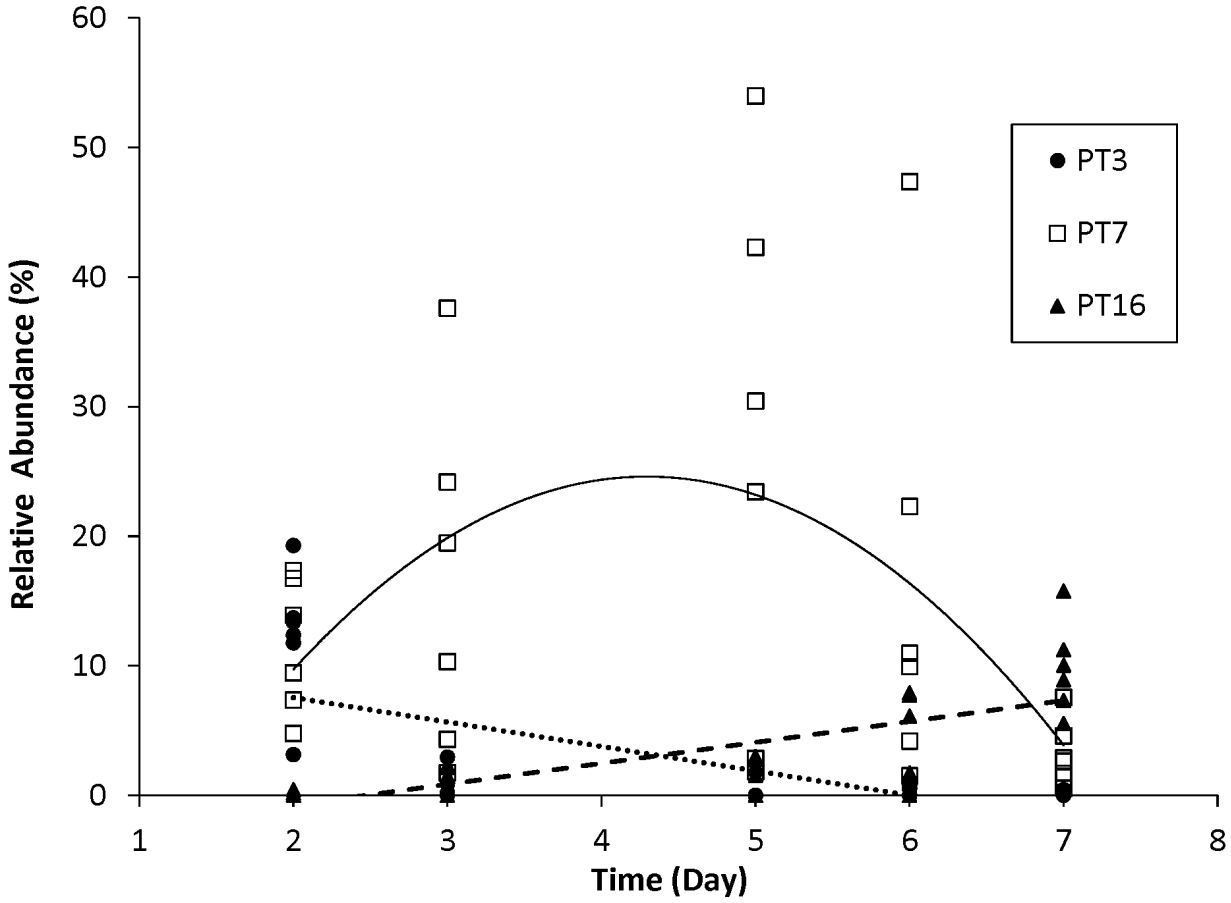
One implication of this study is that natural stream systems could create variation in microbial community succession due to differences in natural flow rate observed in streams during the spawning season. Streams may have significantly less precipitation during one spawning season than other average seasons simply by chance, which could lead to significantly lower stream flow rates during spawning events. Our analysis suggests that we should expect more microbial phylotypes adhering to egg surfaces during the critical period of embryogenesis in such dry spawning seasons.

The variable flow regime, which mimicked dam-manipulated flow, resulted in similar successional patterns to what we observed under the high flow regime. Although the high and low flows were alternated in the variable flow regime, the high flow operation period of the variable flow might have had a dominant effect on microbial community composition. High flow rate of the simulated stream could have acted as a disturbance to microbial community formation on the egg surface. Since naturally occurring eggs potentially experience high flow during embryogenesis, our result implies that dam operations that include periods of high flow would not alter the natural course of successional patterns on egg surfaces. However, not all dams are

operated in the same way as this experiment (alternating 12 hour intervals), thus we cannot generalize our findings to all dam systems. Furthermore, a dam could potentially alter water chemistry, temperature, nutrients, and water microbial community composition in a spawning stream [64], which in turn could influence the microbial communities during succession.

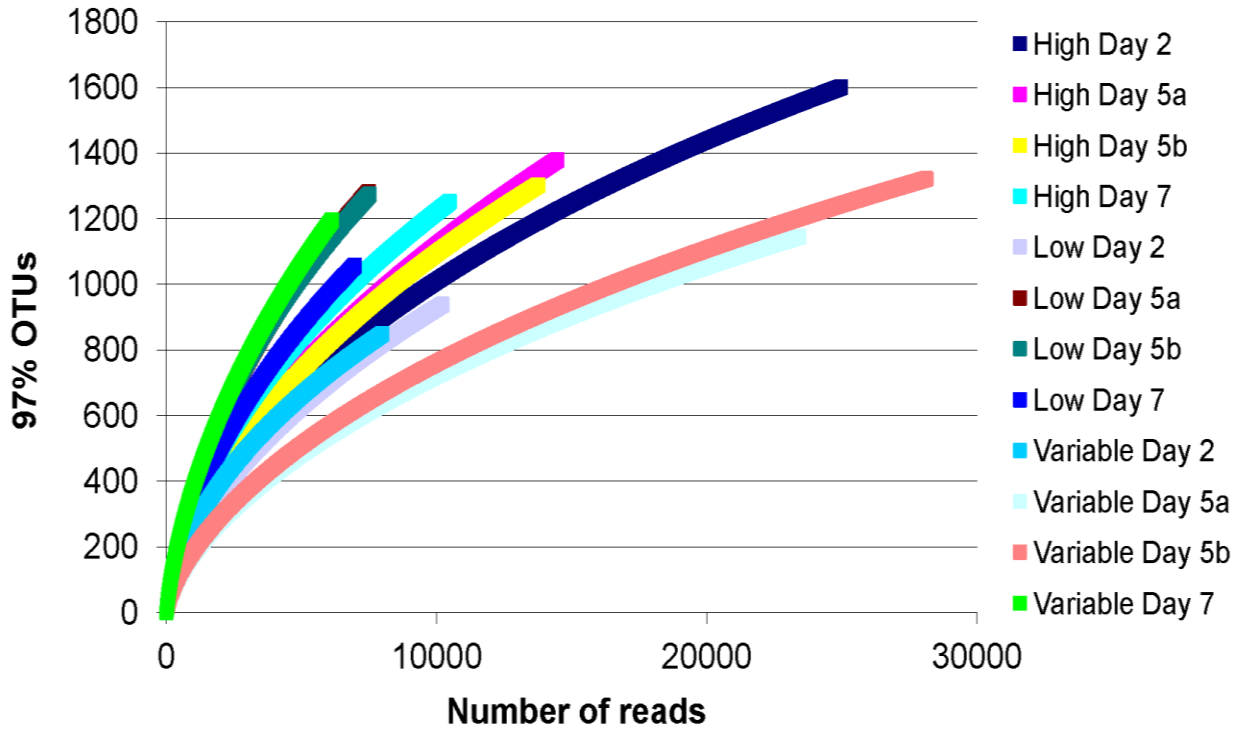
In conclusion, this is the first microbial succession study conducted on fish eggs. Although previous studies have documented succession patterns on abiotic substrates in streams, our study breaks new ground in demonstrating that microbial community shifts can occur in a remarkably short period on a biotic surface. We hope this study will trigger more studies in this area and encourage other researchers to delve deeper into understanding the underlying mechanisms behind the patterns we observed.

Appendix

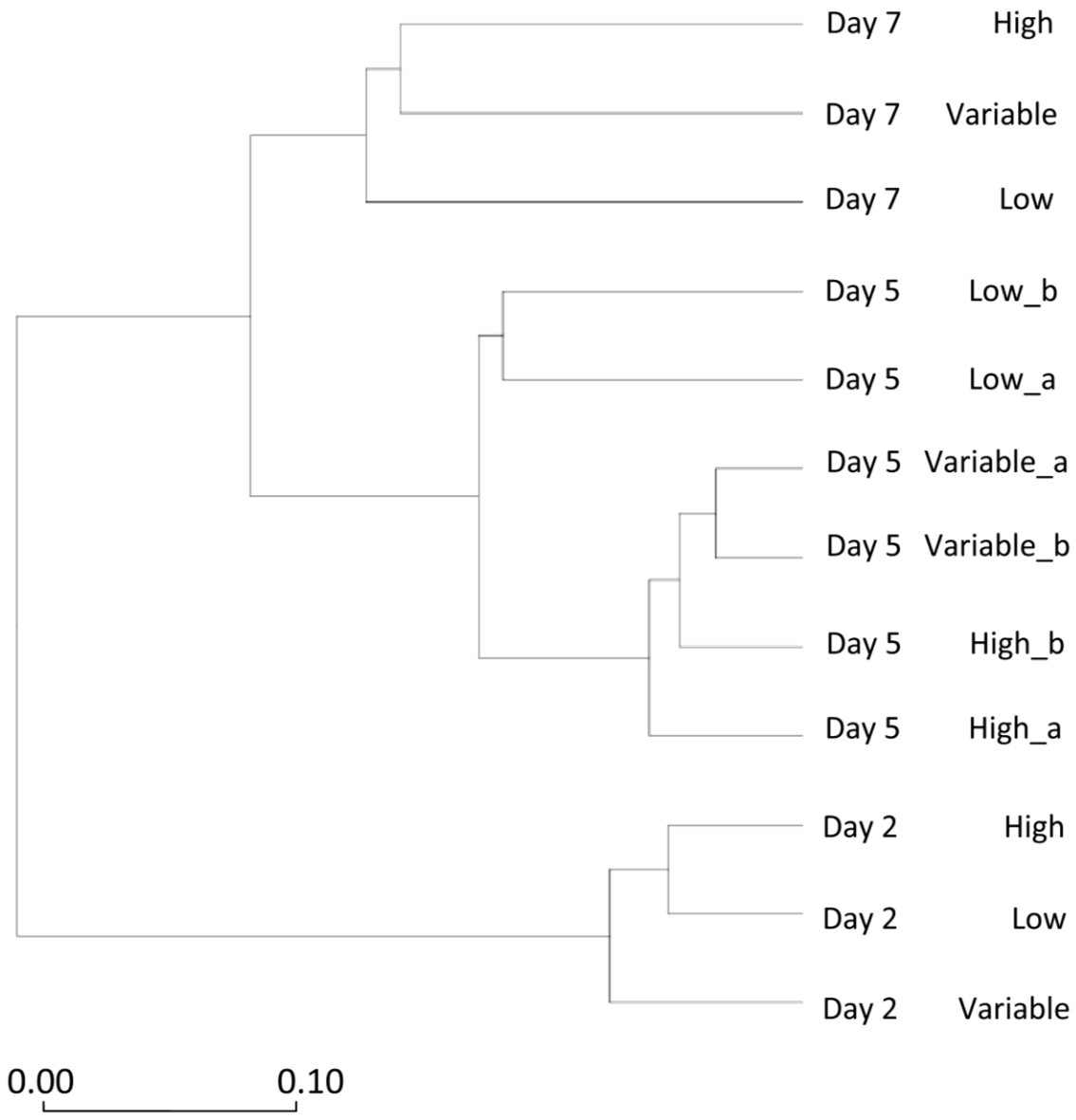


Appendix Figure A.2.1. Examples of the association of certain microbial phylotypes with certain egg developmental stages.

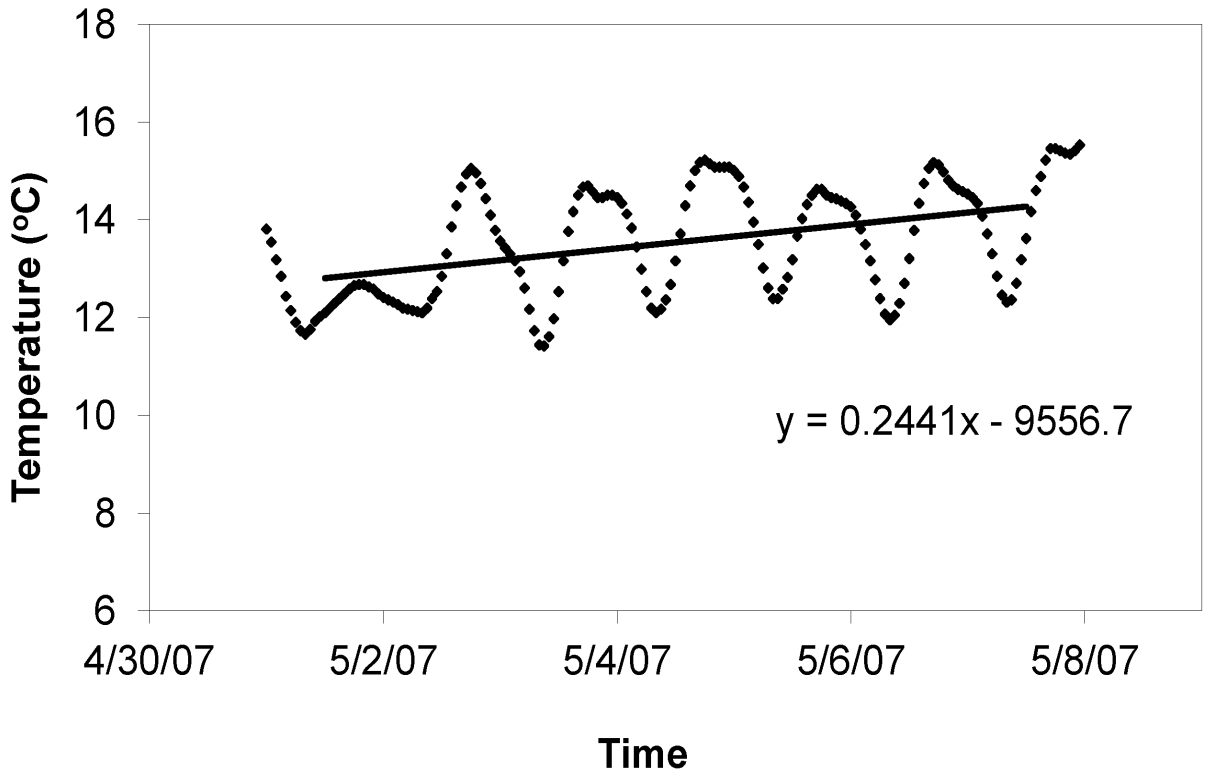
The dotted line (linear coefficient = -1.89, R^2 = 0.44), solid line (quadratic coefficient = -2.83, R^2 = 0.23), and breaking line (linear coefficient = 1.61, R^2 = 0.55) represent regression lines for PT3, PT7, and PT16, respectively. HhaI data were used for the analysis.



Appendix Figure A.2.2. Rarefaction analysis for pyrosequencing data.



Appendix Figure A.2.3. Jaccard index tree constructed using OTUs at 97% similarity cutoff.



Appendix Figure A.2.4. Ambient water temperature throughout the egg incubation periods.

Solid line is a linear regression line for daily mean temperature.

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CHAPTER 3: THE EFFECT OF TEMPERATURE AND WATER TYPE ON THE EGG SURFACE MICROBIAL COMMUNITY AND HOST LIFE HISTORY TRAITS OF THE LAKE STURGEON

Abstract

We investigated the process of microbial colonization on the egg surfaces of the Lake Sturgeon (*Acipenser fulvescens*), a threatened fish species inhabiting the Great Lakes. Our previous studies revealed that microbial community assemblages on the egg surfaces changed over the developmental time of the egg (Chapter 2). To elucidate the factors influencing successional changes, we designed a two factor experiment to analyze the effect of temperature (warm and cold) and the aquatic microbes (filter/UV-treated and non-treated stream water) on the egg surface microbial community assemblages throughout the developmental period. Genomic DNA was extracted from the egg surface and the egg microbial communities were examined using 16S rRNA gene based TRFLP and clone libraries. Microbial quantity on the egg surface was determined by quantitative PCR (qPCR). Principle components analysis of T-RFLP data revealed that the composition of the aquatic microbial community profoundly influenced the egg associated microbial community assemblage during succession while temperature had a lesser influence. The qPCR analysis showed that a significantly higher number of microbes were attached to egg surfaces reared in non-treated water than in UV/filter-treated water ($F_{1,210}$=35.67, $p<0.001$). We also found a significantly higher egg mortality for eggs reared in stream water (77.4 ± 17.6%) compared to those reared in UV/filter-treated water (50.3 ± 14.7%) ($F_{1,17}$=13.42, p=0.002). Eggs reared in stream water also had smaller yolk sac to body area ratio of larvae at

hatch compared to those reared in UV/filter-treated water ($F_{1,156} = 52.39$, $p < 0.001$). The phenomenon may have occurred because embryos used yolk sac resources for defenses against microbes that colonized the egg surfaces. Clone libraries revealed that certain microbes were associated with eggs that had a higher egg survival rate, including *Acidovorax* spp. and *Massilia* spp., making them good candidates for probiotics. The results suggest that egg surface microbial communities vary as a function of environmental factors and influence life history traits of the host. This study also has significant implications for managing threatened host populations such as the Lake Sturgeon inhabiting human-altered rivers, since it demonstrates the potential effect of dams (which alter aquatic microbial community and temperature) on downstream host-microbe interactions.

Introduction

Fish deposit eggs into stream water populated by aquatic microbes. Deposited eggs are rapidly colonized by aquatic microbes, and a microbial community develops on egg surfaces (Chapter 2). The interactions between microbes and host organisms play a significant role in both a host's life history and microbial life cycles. Fish eggs are an important focus of study because the majority of mortality occurs during the egg stage of their life history [1]. Although egg-associated microbes can be pathogenic or mutualistic and even symbiotic, treating eggs against microbes with antibiotic or formalin helps increase the survivability of fish eggs [2-3]. The interaction between microbes and eggs not only affects the mortality of eggs, but also potentially affects other life history traits such as the size of larvae at hatch [4].

Microbe-host interactions are long-term in nature and have in many cases coevolved to the point of reaching a fine balance, and perturbing the environmental covariates will disrupt their mutual relationships. The effect of such disturbances on microbe-host interactions is a particularly important topic to examine with respect to aquatic systems. For example, a previous study found that changes in temperature affected microbial communities associated with frog hosts, which subsequently affected host mortality [5]. Damming streams has been shown to change aquatic microbial community structure [6], but the effect of such disturbance on the downstream host - microbe interactions is unknown.

Investigation into the effects of environmental perturbations such as damming on host-microbe interactions is particularly important for the threatened fish species Lake Sturgeon (*Acipenser fulvescens*). Lake Sturgeon populations have decreased drastically over the past 100 years due to anthropogenic activities such as overfishing and dam construction [7]. One such population is the Black Lake population in Michigan [8-9]. Spawning habitats have degraded in

quality since the construction of a dam in 1949 on the Upper Black River, which is the sole spawning stream for the Black Lake population [9]. Lake sturgeon fish eggs serve as hosts for diverse aquatic microbial communities (Chapter 2). While our previous research revealed that microbial communities develop on the egg surfaces during the incubation period, many questions remain regarding the interaction between microbial communities and their egg hosts.

The objective of this study was to determine the effect of variation in environmental factors on the interaction between microbes and Lake Sturgeon egg hosts. We hypothesized that (i) conditions of the rearing environment (aquatic microbial community composition and temperature) affect the egg surface microbial communities and (ii) microbial communities on the egg surfaces have an effect on egg mortality and other life history traits (larval size at hatch). To test these hypotheses, we designed a two factor experiment to analyze the effect of water type (filter/UV treated and non-treated stream water) and temperature (warm and cold) on the egg associated microbial community assemblages throughout the incubation period, and their subsequent impacts on host life history traits. This study has significant implications for understanding microbe-host-environmental covariates interactions and for managing threatened host populations such as the Lake Sturgeon inhabiting human-altered rivers, since it demonstrates the potential effect of dams (which alter aquatic microbial communities and temperature) on downstream ecosystems.

Methods

Study site

This experiment was conducted in a streamside hatchery located at the riverside of the Upper Black River in Michigan, which is the sole spawning stream for the Black Lake population of the Lake Sturgeon. The experiments were conducted in May 2009 during a part of

the annual spawning season. Stream water was pumped up from the spawning stream and large particulate matter in the stream water was removed using sock filters before the filtered stream water was gravity fed to the hatchery system. Both female and male gametes were collected from spawning adult sturgeon in the Upper Black River. The collected gametes were fertilized and reared in the hatchery under different treatment conditions.

Experimental design

The experiment was composed of two factors, water types and temperature of the egg rearing environment. There were two levels for each factor- filter/UV-treated and non-treated for water types, and cold ($12 \pm 1^{o}C$) and warm ($18 \pm 1^{o}C$) for temperature, respectively. The experiment consisted of a total of four different treatments (warm/treated, warm/non-treated, cold/treated, cold/non-treated). The manipulation of aquatic microbial communities and quantity was conducted using a water treatment system which consisted of a 50µm filter cartridge followed by a UV lamp (25 Watt, Emperor Aquatics, Inc). Under this treatment, microbes associated with particulates larger than 50 µm and sensitive to UV treatment were selectively removed from the stream water microbial community (Chapter 5).

For each treatment, approximately 200 gametes from a single female were fertilized with 1mL of milt from a single male on a sterile polyethylene mesh screen in a heath tray filled with 1L of either non-treated or filter/UV treated water . Gametes were left to sit for 30 to 40 minutes until they were fertilized and adhered to the bottom polyethylene mesh screen, which served as the bottom substrate for embryos throughout incubation. Eggs were then reared in their respective treatments until they hatched. The study was replicated with five different female/male combinations (family codes: BC, CE, DG, EI, and FK).

Egg samples were collected for DNA extraction and subsequent community analysis at six different time points for each treatment. For each egg sampling event, 10 live eggs were collected and placed in a sterile 2mL eppendorf tube that was filled with 80% ethanol and stored at 4^{o}C. For warm temperature treatments (warm/treated, warm/non-treated), egg samples were collected at Day 0 (1 hour post fertilization) and Days 1, 2, 3, 4, and 5 post fertilization. All eggs from warm treatments hatched at Day 6 in this experiment. For cold treatments (cold/treated, cold/non-treated), egg samples were collected at Day 0 (1 hour post fertilization), and Days 2, 4, 6, 8, and 10 post fertilization. All eggs from the cold treatments hatched at Day 11, 12 or 13. The development of the embryo was known to be accelerated at warm temperature (18 ± 1^{o}C) approximately by two-fold relative to the cold water treatment (12 ± 1^{o}C) [10]. With this sampling scheme, the developmental stages of the embryo were expected to be synchronized between the two water treatments.

Mortality assessment

The numbers of dead eggs in each treatment group were recorded on a daily basis for warm treatments and every other day for cold water treatments throughout the incubation period. The arrest of embryonic development was determined by visual observation of developmental stages of embryos relative to reference [11]. All dead eggs were removed from the incubation tray at detection. The number of successful hatches for each treatment was recorded, and egg mortality for each treatment was calculated as follows; egg mortality = total number of dead eggs / (total number of dead eggs + total number of hatches). The effect of water treatment and

temperature on egg mortality was assessed using a general linear model using the "lm" function in the R software version 2.10.0 [12].

DNA extraction and TRFLP analysis

A total of 72 samples (3 families: CE, DG, EI, 4 treatments, 6 time points) were processed for DNA extraction and subsequent community analysis using Terminal Restriction Fragment Polymorphism (TRFLP). Microbial community genomic DNA was extracted from the surfaces of 8 eggs per sample using the PowerSoil™ Kit (MO BIO Laboratories Inc., CA) according to the manufacturer's protocol. 16S rRNA gene based TRFLP was performed to characterize microbial community structure [13-14]. The detailed PCR amplification procedures for TRFLP were described in Chapter 2. The purified PCR products were digested with MspI (Gibco BRL). Two technical replicates of each of the digested DNA samples were sent to Michigan State University's sequencing facility and the DNA fragments were separated on an ABI 3100 Genetic Analyzer automated sequencer (Applied Biosystems Instruments, Foster City, CA) in GeneScan mode. The sizes and abundance (peak height) of the terminal restriction fragments (TRFs) were calculated using GeneScan 3.7. Each terminal fragment corresponds to a phylotype, and peak height indicates relative abundance of a phylotype. In order to align TRFs across egg samples from different treatments, the TRFLP profiles were processed with T-Align software (http://inismor.ucd.ie/~talign/index.html) and the output of T-Align was used for the microbial community analysis. Principle component analysis (PCA) was performed on TRFLP data from the egg samples exposed to different treatments in order to elucidate underlying patterns across samples. PCA was conducted using the "prcomp" function of the R software version 2.10.0 [12].

16S rRNA gene Clone Library analysis

A total of four samples (CE family; warm/treated Day 1 and Day 4, and warm/non-treated Day 1 and Day 4) were subjected to a clone library to identify microbial species present on the egg surface in two different water types at two different time points. 16S rRNA gene of the extracted community DNA was amplified using 27F (5' – AGA GTT TGA TCM TGG CTC AG – 3') and 1389R (5'-ACG GGC GGT GTG TAC AAG-3'). The PCR conditions were the same as those used for TRFLP. PCR amplicons were purified and ligated into a vector plasmid pCR2.1 with kanamicin resistance using a TOPO cloning kit (Invitrogen, Carlsbad, CA) and the vector plasmid was transformed into competent *E.coli* cells. Vector bearing *E.coli* clones were isolated on LB kanamicin plates with X-gal. A total of 96 white colonies per sample were picked and inoculated in LB broth with kanamicin and grown overnight at 37^{o}C. The insertion of the amplicon into the vector plasmid was confirmed using M13 primers. Broth cultures were sent to Michigan State University's sequencing facility and the cloned 16S rRNA gene was sequenced using a 27F primer. The sequences of the clone library were identified using RDP pipeline [15] and the microbial community structures of different samples were compared at the genus level.

Quantitative PCR analysis

Microbial loads of the same 72 samples were determined by performing quantitative PCR (qPCR) with SYBR green. The qPCR was performed using universal bacterial primers 331F (5'-TCCTACGGGAGGCAGCAGT-3') and 519R (5'- CGTATTACCGCGGCTGCT -3') targeting the conserved sequences within the 16S ribosomal RNA gene, as previously described [16]. The qPCR reaction was conducted in a 25µL reaction volume, containing 3µL template DNA, 12.5uL mastermix (2x) (SABIoscience, MD: mixture of DNA polymerase, buffers, and SYBR

green), and 0.16µM of each primer in the final concentration. PCR was performed using the iQ5 (Bio-Rad) thermal cycler according to the following protocols: an initial denaturation at 94°C for 5 min followed by 43 cycles of denaturation at 94 °C for 30 s, annealing at 60^{O}C for 30 s, and extension at 72°C for 30 s. Fluorescence signals were detected at the end of the extension for each cycle.

A standard curve for the relationship between 16S rRNA gene copy number and cycle threshold (Ct) values were constructed using a series of dilutions of the bacterial genomic DNA *Flavobacterium johnsoniae* ATCC 17061 that is known to have six 16S rRNA gene copies in its genome. The standard curves were constructed over 20 times and the PCR efficiency of the standard curves was found to range from 87% to 101%. For determination of microbial quantity of the samples, a series of dilutions were made for each sample, and the triplicates of each dilution were PCR amplified along with the standard. The Ct values for both the standard and samples were determined at 600 relative fluorescence unit (RFU) where the relationship between RFU and Ct was linear in the log-transformed view. The PCR efficiency of each sample was comparable to that of the standard. The quantity of the 16S rRNA gene copy of each sample was determined by substituting the Ct value of one of the sample dilutions into the equation of the standard curve and multiplying it by the dilution factor.

Larval size analysis

Immediately after the hatching of eggs, the larvae were anesthetized using MS-222. The anesthetized larvae were photographed with a ruler as a size standard. The total length, total body area, and yolk sac area of the larvae were determined from the images using ImageJ software. Yolk sac area (YSA) to body area (BA) ratio was chosen for measurement because

they allowed us to examine the effect of microbes on the allocation of yolk resources during embryogenesis. A large YSA to BA ratio indicates that resources in the yolk sac were not used for body area and/or immune responses during embryogenesis and are available for somatic growth following hatch. The effect of water treatment and temperature and their interaction term on the larvae size was assessed using both a general linear model with fixed variables and a linear mixed effect model with family effect as a random variable. The statistical significance of the treatment effect on larvae size was confirmed after accounting for family effect as a random variable using the mixed effect model. The linear mixed effect model was performed using "lme" function in R version 2.10.0 [12].

Results

A total of 146 phylotypes were detected using TRFLP across the 72 samples. On average, 28.0 ± 8.2 phylotypes were detected per sample. With regard to the principal component analysis, principal component 1 (PC1) captured variability due to egg developmental stage, specifically separating immediately post fertilized (1 hour after fertilization) egg samples from the rest of the samples (Figure 3.1). The effect of water type on the egg associated microbial community was captured by PC2, which separated the communities reared in filter/UV-treated water from those reared in non-treated water. PC3 accounted for variation in the egg microbial communities due to temperature (Figure 3.1).

PCA analysis performed on a subset of the data (only the warm treatment) showed that the egg associated microbial communities collected from eggs reared in filter/UV treated water was separated from that reared in non-treated water by PC1 (Figure 3.2). Variability in the egg associated microbial community due to egg developmental stages was captured by PC2 (Figure

3.2). Samples collected immediately after fertilization (Day 0) were also separated from the rest. The temporal trend was not random, but rather directional and corresponding to the egg developmental stages (Figure 3.2).

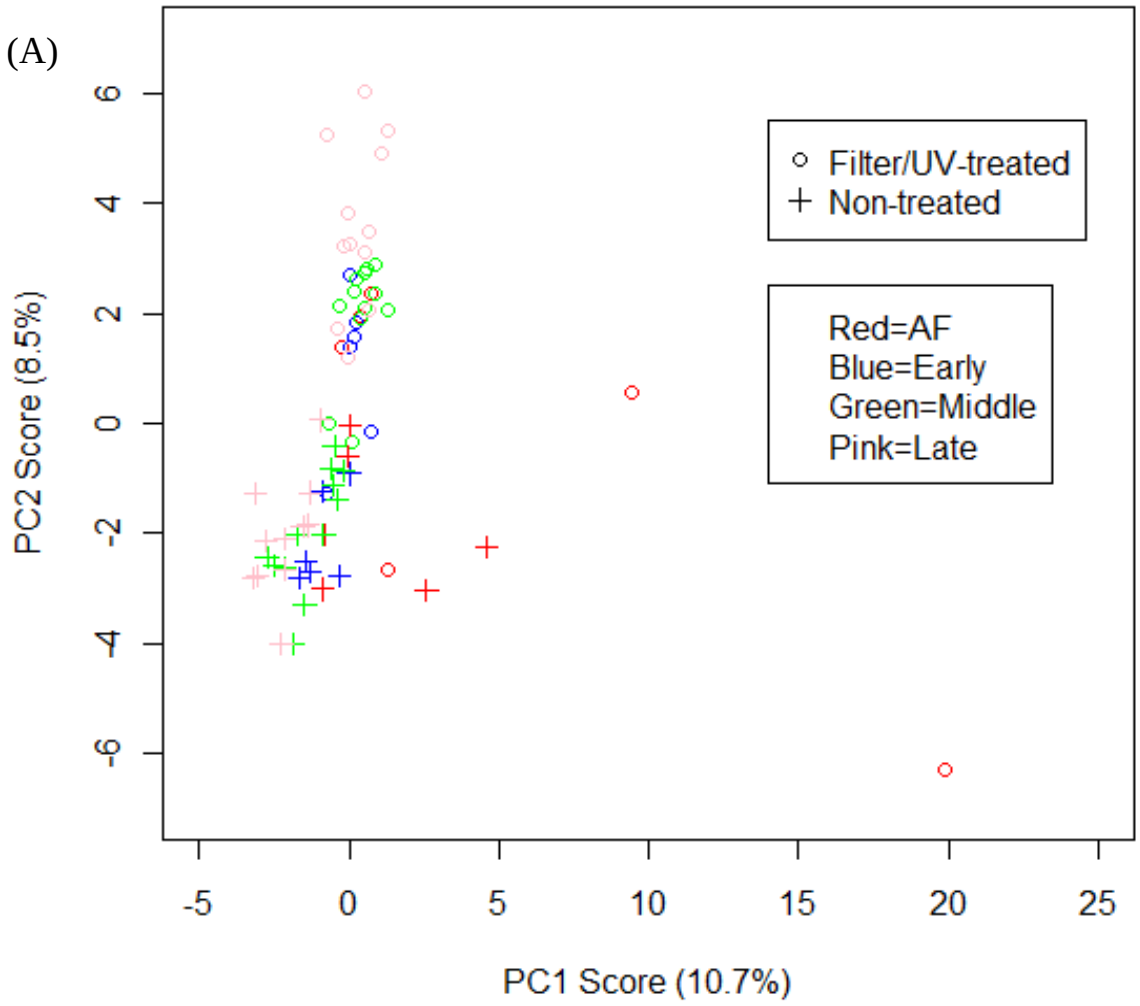

Figure 3.1. Principal component analysis (PCA) plot of TRFLP data of 72 samples from different treatments.

Panel (A) displays clustering due to temporal variation (PC1) and water treatment (PC2). "AF" stands for after fertilization. "Early", "Middle", and "Late" indicate egg developmental stages. "Early" corresponds to Day 1 for warm and Day 2 for cold, "Middle" corresponds to Day 2 and Day 3 for warm and Day 4 and Day 6 for cold, and "Late" corresponds to Day 4 and Day 5 for warm and Day 8 and Day 10 for cold. Panel (B) displays clustering due to water treatment (PC2) and temperature (PC3).

(B)

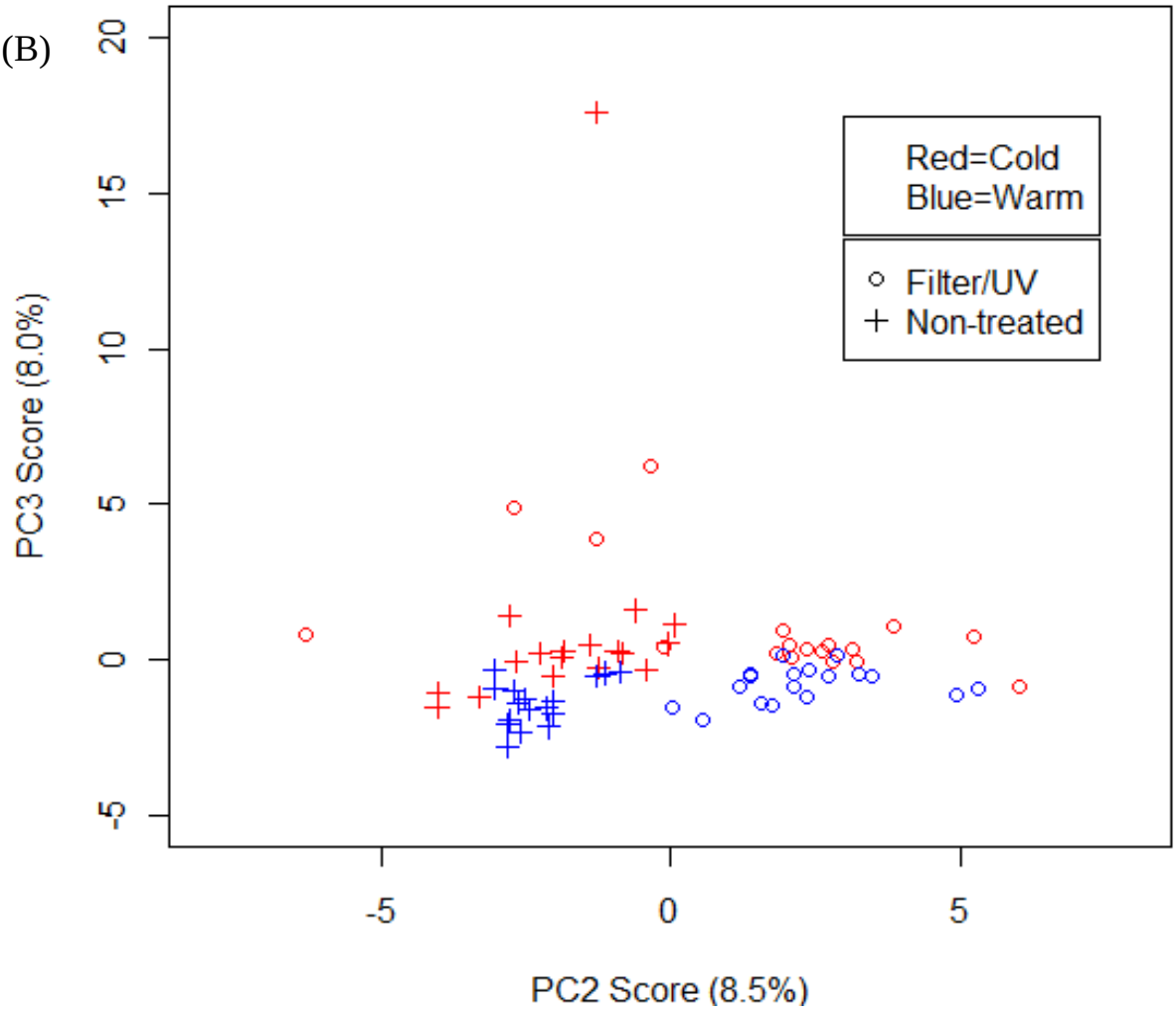

Figure 3.1 (cont'd).

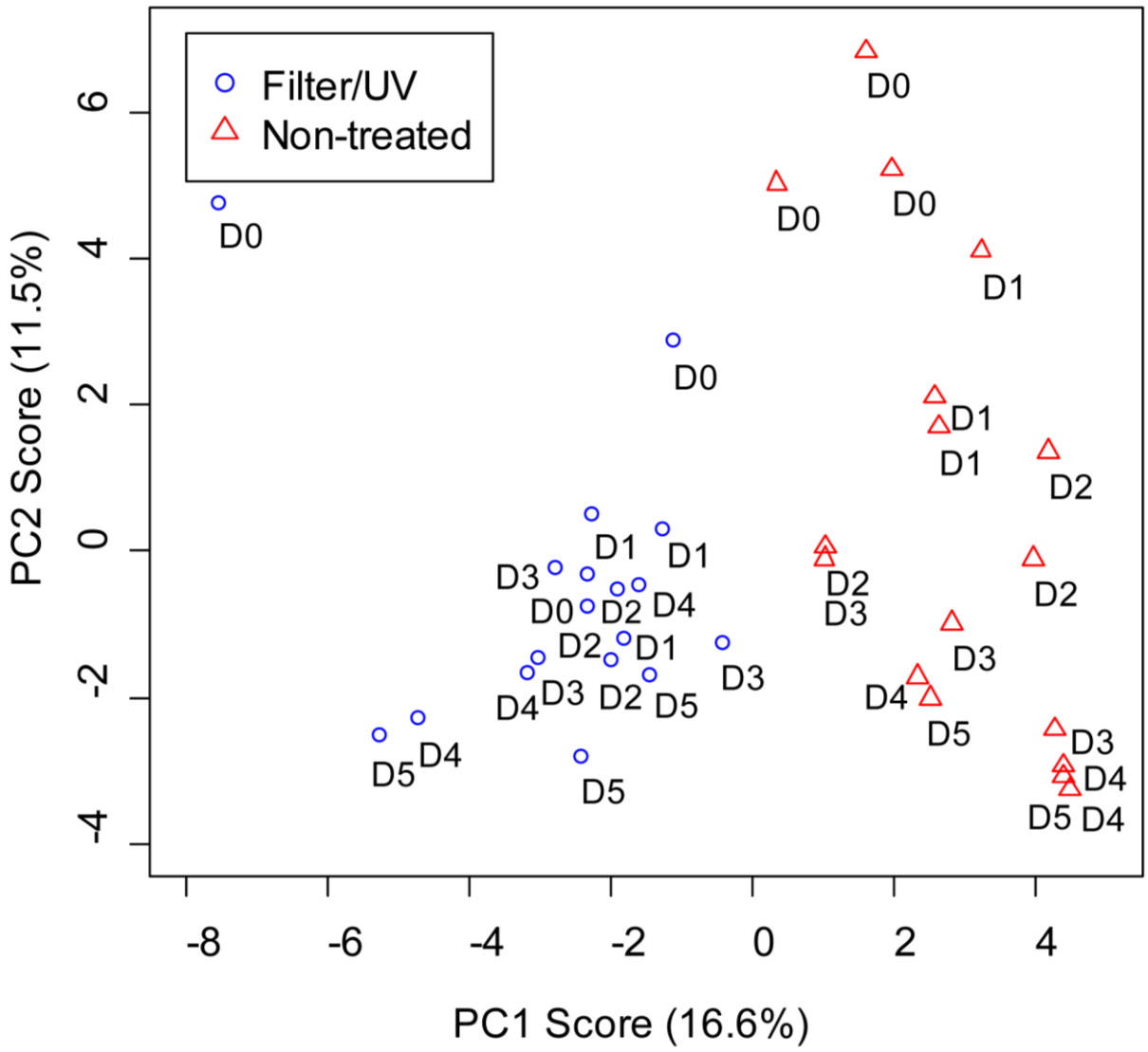

Figure 3.2. Principal component analysis (PCA) plot depicting the effect of water treatment on egg microbial community at warm temperature.

PC1 and PC2 account for 16.6% and 11.5% of all TRFLP data variation, respectively.

The 16S rRNA gene based qPCR revealed that microbial quantity on the egg surfaces increased in a log-linear fashion during the incubation period starting from $10^{5.5}$ and ending at $10^{7.5}$ 16S rRNA gene copies per egg (Figure 3.3). Eggs reared in non-treated water had higher microbial quantities than those reared in filter/UV treated water for all six time points tested (Figure 3.3). The average quantity associated with eggs reared in non-treated water was significantly higher than that reared in filter/UV treated water ($F_{1,210}$=35.67, $p<0.001$, Figure 3.4). There was no significant effect of temperature on microbial quantity on the egg surfaces ($F_{1,210}$=0.178, p=0.674).

We identified a total of 47 genera using the 16S rRNA gene clone library (all except singletons shown in Table 3.1). 8 genera were found only on or were more dominant on eggs reared in non-treated water (*Rhodobacter, Methylotenera, Polynucleobacter, Rhodoferax, Leptothrix, Rheinheimera, Flectobacillus,* and *Flavobacterium*). We also identified 7 genera that were found only on or were more dominant on eggs reared in filter/UV-treated water (*Sphingobium, Massilia, Pseudorhodoferax, Pseudomonas, Acidovorax, Pelomonas*, and *Aquabacterium*).

Manipulation of aquatic microbial community using filter/UV treatment influenced egg mortality. Mortality of eggs reared in filter/UV-treated water (50.3%) was significantly lower than that reared in non-treated water (77.4%) (Figure 3.5, $F_{1,17}$=13.42, p=0.002), whereas the temperature of the rearing environment did not significantly affect egg mortality ($F_{1,17}$ = 0.308, p=0.59). There was also a family effect on egg mortality as evidenced by a moderately different

mortality of family CE from others (p = 0.15, 0.19, 0.09, and 0.20 for CE-DG, CE-EI, CE-FK, and CE-BC, respectively). There was no significant difference in egg mortality

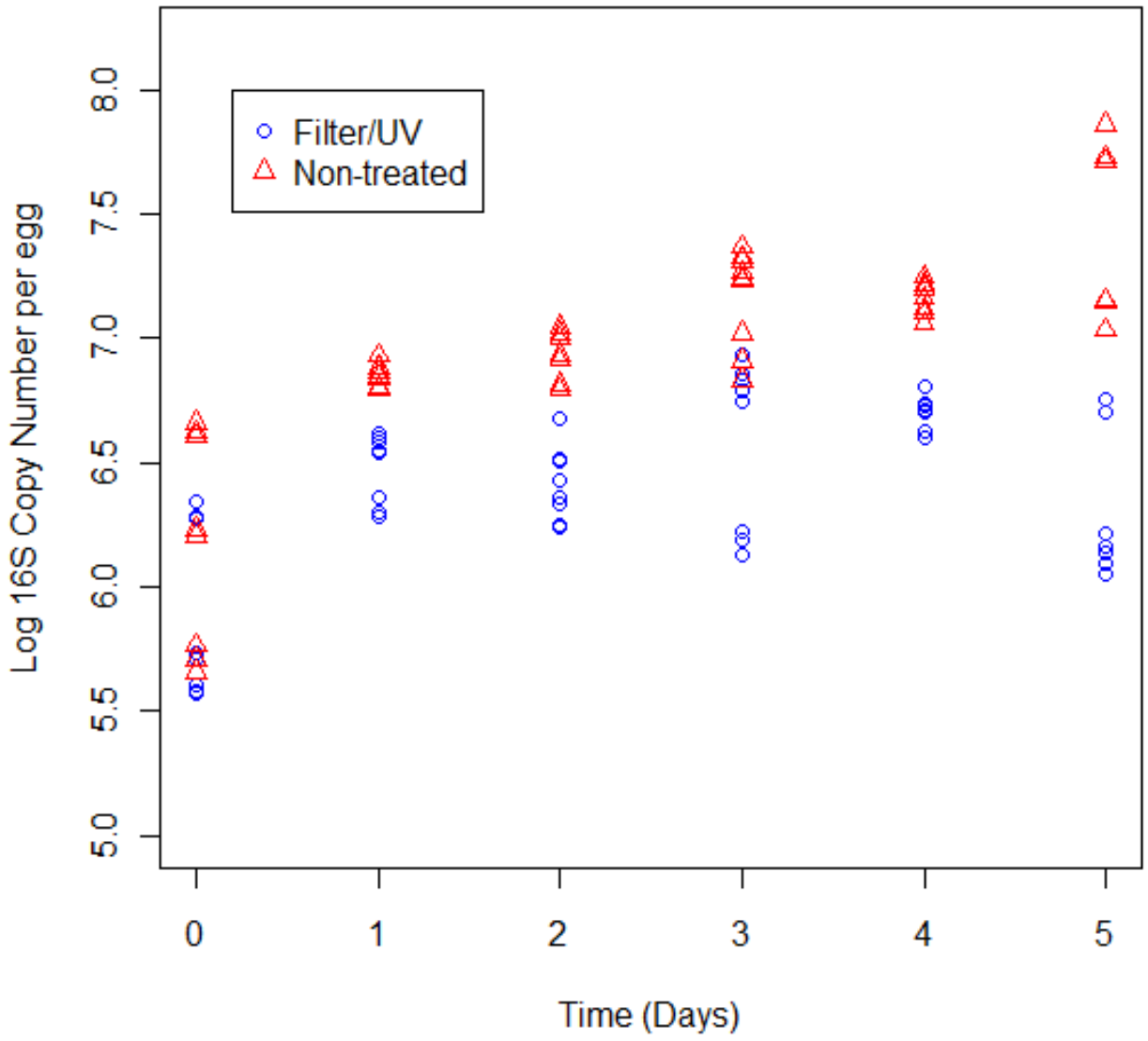

Figure 3.3. Positive linear relationship between time (days post-fertilization) and microbial quantity present on egg surfaces.

Microbial quantity was measured using quantitative PCR (qPCR) on 16S rRNA gene copies. Eggs reared in non-treated water had higher microbial quantity than that reared in treated water at all time points.

Table 3.1. 16S rRNA gene-based clone library for the two different treatments at two different time points for the CE family.

Class	Genera	WN1	WF1	WN4	WF4
Cyanobacteria	unclassified_Chloroplast	0.00	2.08	0.00	0.00
Alpha	Caulobacter	2.20	1.04	0.00	0.00
Alpha	Rhodobacter	2.20	0.00	4.40	0.00
Alpha	Rhizobium	4.40	4.17	2.20	3.26
Alpha	unclassified_Rhizobiales	0.00	1.04	5.49	5.43
Alpha	Sphingomonas	0.00	0.00	3.30	2.17
Alpha	Novosphingobium	6.59	5.21	10.9	3.26
Alpha	Sphingobium	0.00	1.04	1.10	7.61
Alpha	unclassified_Sphingomonadaceae	2.20	2.08	5.49	6.52
Beta	Methylophilus	2.20	0.00	0.00	0.00
Beta	Methylotenera	4.40	0.00	1.10	0.00
Beta	unclassified_Methylophilaceae	4.40	0.00	9.89	0.00
Beta	Polynucleobacter	1.10	0.00	2.20	0.00
Beta	Massilia	1.10	11.4	0.00	3.26
Beta	Hydrogenophaga	1.10	0.00	1.10	1.09
Beta	Acidovorax	1.10	10.4	5.49	8.70
Beta	Curvibacter	4.40	1.04	4.40	4.35
Beta	Rhodoferax	0.00	0.00	5.49	0.00
Beta	Pseudorhodoferax	0.00	0.00	0.00	2.17
Beta	Pelomonas	2.20	7.29	0.00	4.35
Beta	unclassified_Comamonadaceae	3.30	2.08	3.30	4.35
Beta	Aquabacterium	2.20	7.29	1.10	3.26
Beta	Leptothrix	2.20	0.00	2.20	0.00
Beta	Paucibacter	1.10	5.21	1.10	0.00
Beta	unclassified_Burkholderiales_incerta	1.10	9.38	10.9	16.3
Beta	unclassified_Burkholderiales	15.38	25.0	3.30	15.2
Gamma	Rheinheimera	5.49	0.00	1.10	0.00
Gamma	Pseudomonas	1.10	2.08	1.10	5.43
Sphingobacteria	Flectobacillus	8.79	0.00	0.00	0.00
Flavobacteria	Flavobacterium	9.89	0.00	3.30	0.00

Numbers represent relative abundance in each community. Warm (W) rearing temperature, Filter/UV (F) or Non-treated (N) at different developmental stages: Day 1 (1) or Day 4 (4). Genera that showed marked differences in relative abundance between treated and non-treated water are highlighted. Singletons are not included in the table.

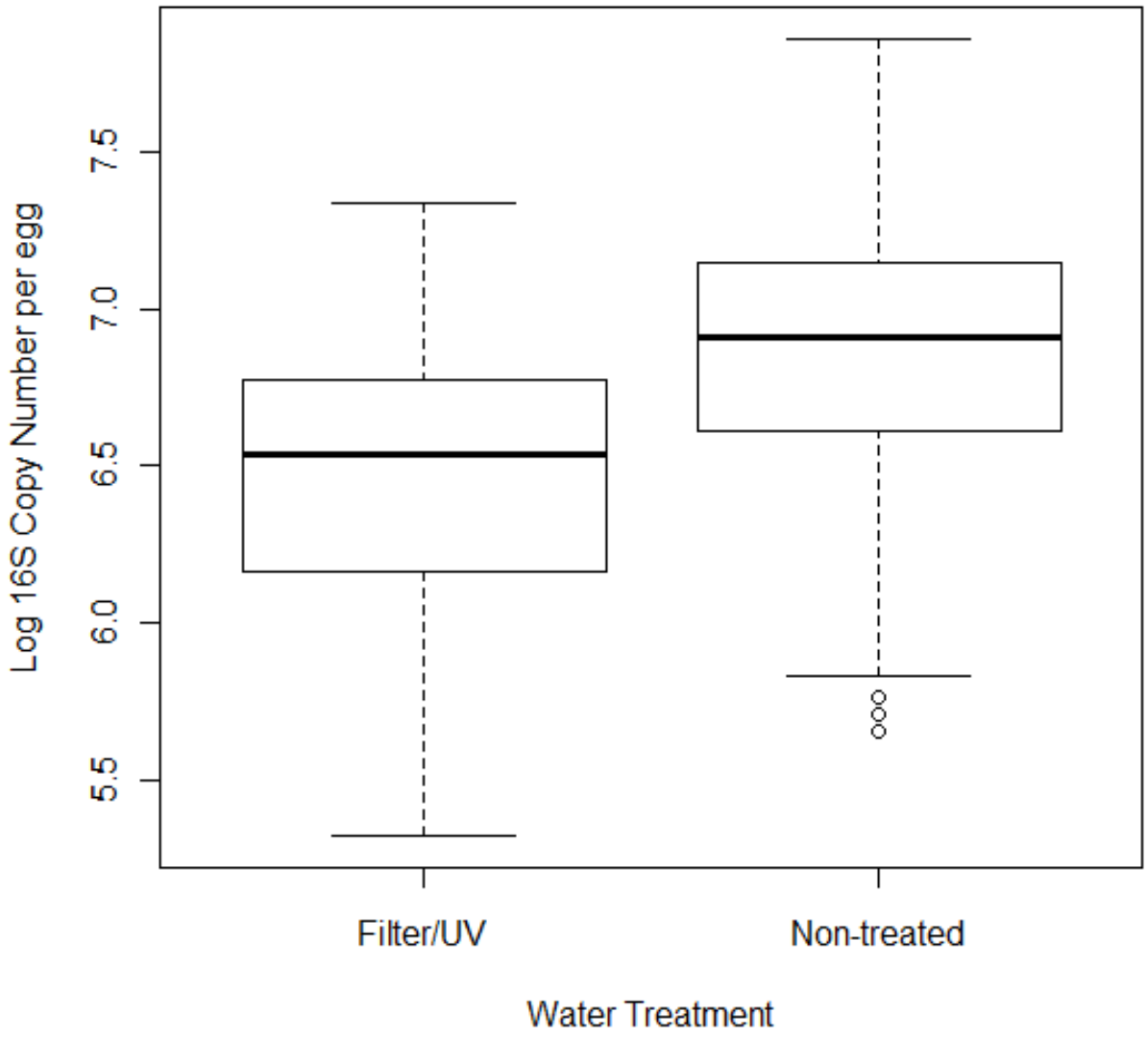

Figure 3.4. The average microbial quantity associated with eggs as measured using qPCR.

Eggs reared in non-treated water had significantly higher microbial load than those reared in filter/UV treated water ($F_{1,210}$=35.67, p<0.001). Egg samples from both cold and warm water treatments at all time points were included for this analysis.

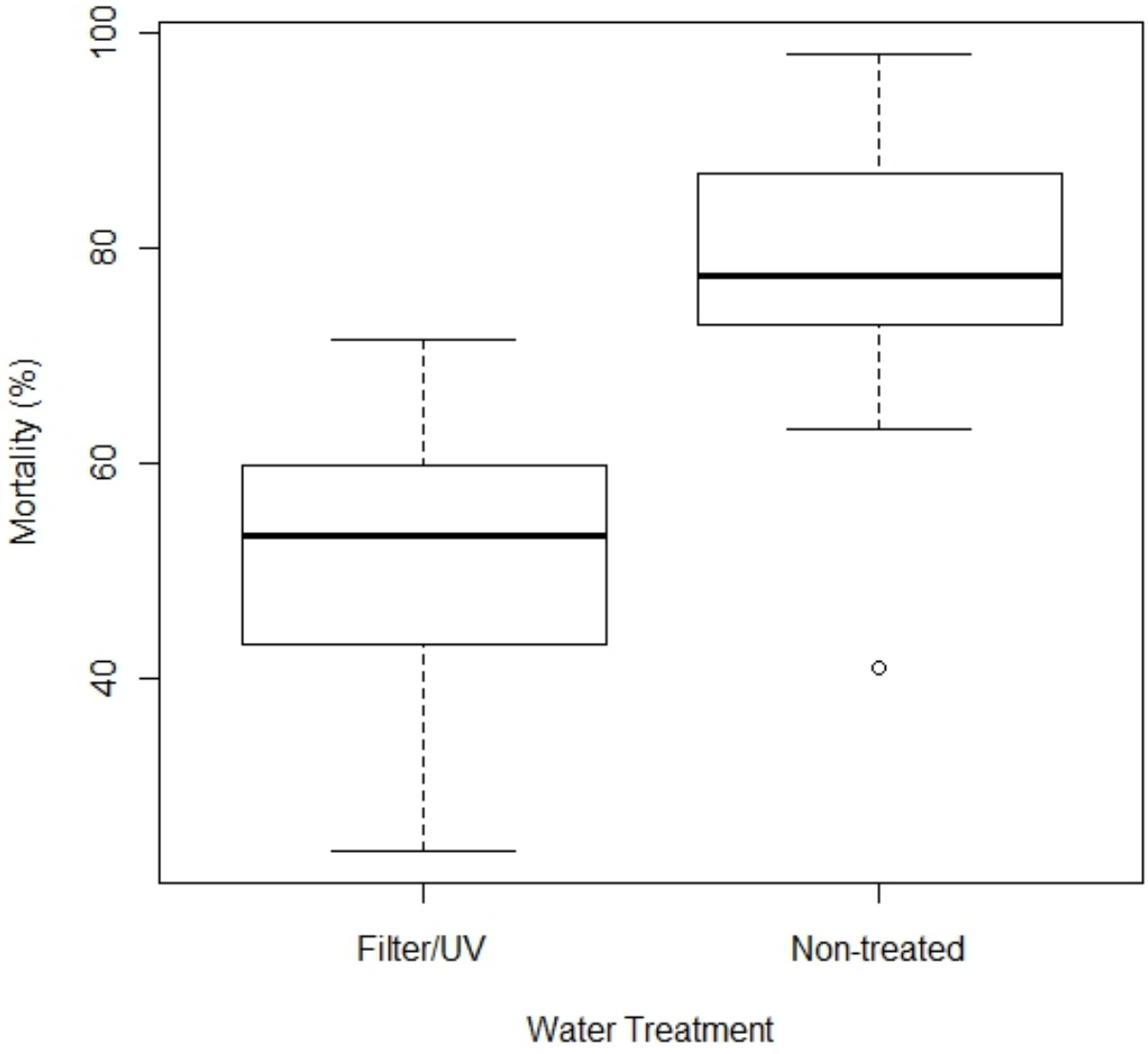

Figure 3.5. The effect of water treatment on egg mortality.

Sturgeon eggs reared in non treated water had significantly higher egg mortality than those reared in treated water ($F_{1,17}$ = 13.42. p = 0.002). The treated group had 10 replicates and the non-treated group had 9 replicates.

among families BC, DG, EI, and FK.

Eggs reared in cold water had significantly smaller yolk sac area to body area ratio than those reared in warm water ($F_{1,332}$ = 448.8, p <0.001, Figure 3.6). The effect of the aquatic microbial communities on larvae size was dependent on temperature. Although we did not see a significant difference in larvae size between the two water types for those eggs reared in cold temperature, we did find a significant difference in larvae size between the two water types at warm temperature ($F_{1,156}$ = 52.39, p <0.001, Figure 3.6). The effect of rearing environment on the larvae size remained significant after accounting for the family effect on the larvae size as a random variable.

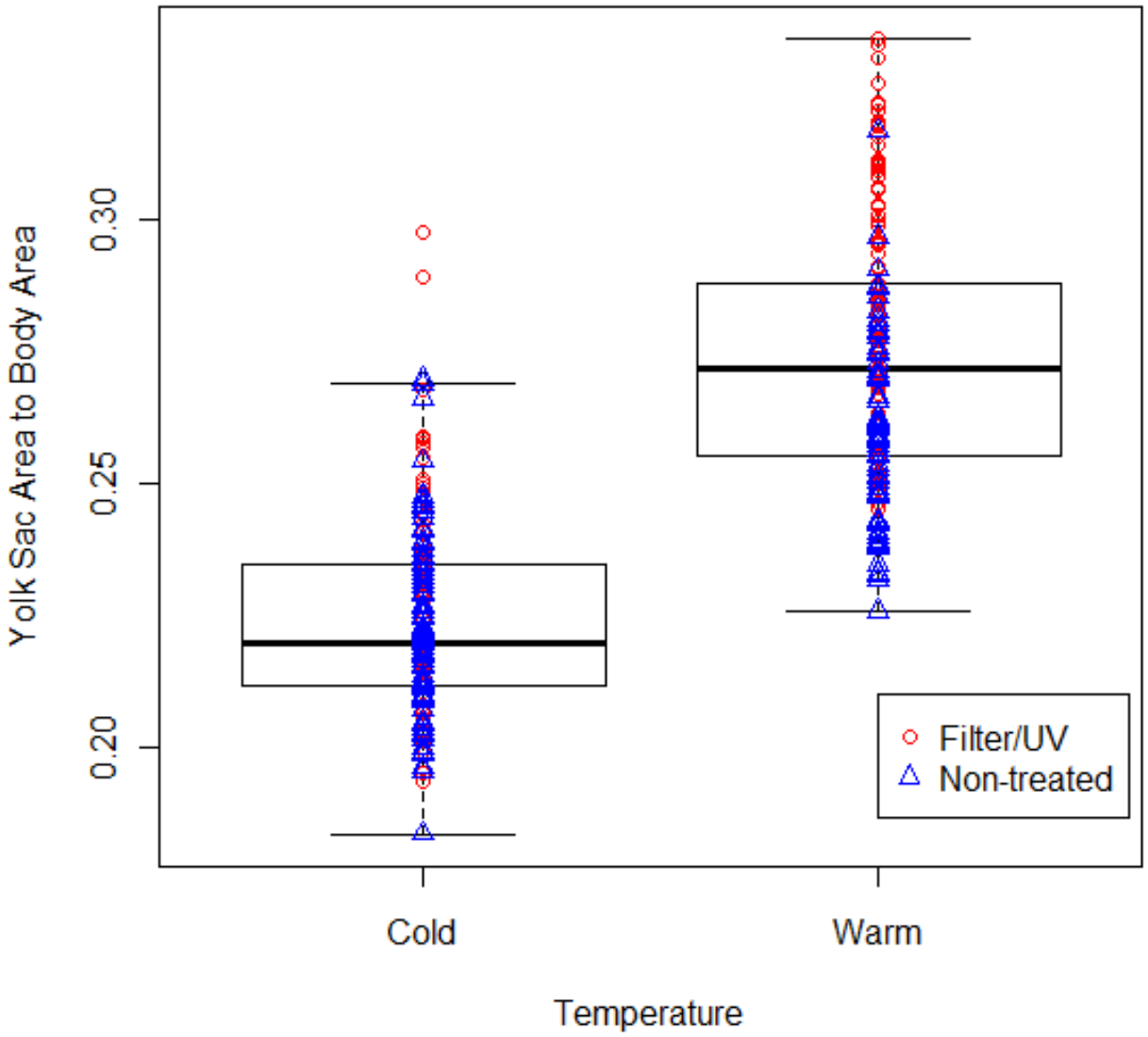

Figure 3.6. The effect of temperature and water treatment on resource allocation.

There was a difference in larvae size at hatch between eggs reared in cold water and warm water ($F_{1,332}$ = 448.8, p <0.001). There was a significant difference in larvae size between the two water types at warm temperature ($F_{1,156}$ = 52.39, p <0.001).

Discussion

This study demonstrated the significance of both aquatic microbial communities (manipulated by water treatment) and temperature on the assembly of egg-associated microbial communities and egg mortality. The study contributes to the literature on this topic by highlighting the complexity of host-microbe interactions and the potential for rearing environments to impact that relationship. To our knowledge this is the first study to examine such effects in the threatened Lake sturgeon. This study also contributes to an understanding of the impact of microbes on the life history of the vulnerable population of this species.

Our study demonstrated that different microbial communities developed on the egg surface when reared in different aquatic microbial community. This result suggests that the type of aquatic microbes that eggs were exposed to during fertilization and the subsequent incubation period was important in determining microbial community composition on the egg surfaces. This in turn suggests that dispersal of microbes from the water column onto the egg surfaces was an important process in explaining microbial community assembly on the egg surfaces.

One of the most significant results from the study was the fact that the egg associated microbial communities sampled immediately after fertilization (Day 0) were significantly different from the rest. The Day 0 microbial communities could have originated from the aquatic microbial community via dispersal during fertilization [17-18] and populated the host eggs through adhesion. The subsequent changes in the egg associated microbial community observed by Day 1 post fertilization could be attributed to local deterministic processes such as the effect maternally provisioned innate immunity on the egg microbial community [19-21]. Alternatively, the Day 0 microbial community on the egg surface could have been mainly attributed to maternally provisioned community structure (if we consider unfertilized eggs to be non-sterile).

These two alternative hypotheses will be further explored using data on both water microbial communities and unfertilized egg communities in forthcoming chapters (Chapter 4 and Chapter 5).

PCA analysis revealed a strong temporal variation within each water type (filter/UV or non-filter). Microbial community on the egg surfaces changed directionally throughout the embryogenesis, suggesting divergence from the initial community throughout embryogenesis. The observed temporal variations could be derived from several factors including changes in metabolites on the egg surfaces [22-23], changes in lysozyme from maternally provisioned to egg secreted [24-25], or microbe-microbe interaction which we investigated in another chapter (Chapter 6). We do not believe that the changes during embryogenesis can be attributed to changes in the surrounding water microbial communities, since our rearing conditions (re-circulating water) should have kept the water microbial communities relatively constant.

In the principal component analysis using the entire TRFLP data set, the first three components accounted for about 30% of the entire variation within the dataset. This suggests that factors other than temporal variation, water type, and temperature which we tested here influenced the microbial community structures on the egg surfaces.

In addition to the temporal trend observed in microbial community composition over the course of embryogenesis, the increase we observed in microbial quantity over the same period was also noteworthy. The fact that over 10^5 16S rRNA gene copies per egg were detected immediately post fertilization (within 1 hour) suggests that microbial colonization on the egg surfaces during fertilization is rapid. There was an effect of water treatment on the quantity of the egg associated microbes. Eggs fertilized and reared in non-treated water had significantly higher microbial quantity on eggs than those fertilized and reared in filter/UV treated throughout

the experiments. This difference in quantity could be attributed to the difference in water microbial quantity (Chapter 5). This significant difference suggests that dispersal of microbes from the water column onto the egg surface is an important process in explaining egg surface microbial population size.

We found that not only quantity but also quality of microbial community on the egg surfaces were different between the samples reared in the two different water types. The significance of this result can best be appreciated by considering the lower egg mortality that we observed in filter/UV treated water compared to non-treated water. Microbes that were dominant in non-treated water including genus *Flavobacterium* may have had negative effect on egg survival. *Flavobacterium* is a genus known to include fish pathogens [26-28]. Microbes that were only prevalent in filter/UV-treated water including genus *Acidovorax* could have had a beneficial effect on egg survival. *Acidovorax* is a genus known to include a symbiont to earthworms [29]. The significance of these putative pathogens and symbionts will be further explored in Chapters 4. However, it is also important to note that the difference in egg mortality between the two treatments could also be attributed to a difference in microbial quantity on egg surfaces between the two treatments. The large microbial load on the egg surface alone could prevent diffusion of oxygen to eggs and suffocate them [30].

Another significant result of this study was that temperature also affected the microbial communities on the egg surfaces. However, our PCA analysis revealed that the effect of temperature on the egg community structure was smaller relative to the effect of water treatment on the egg microbial community. We found that temperature did not affect egg mortality, although water temperature significantly changed the duration of the incubation period, and also the size of larvae at hatch. The significantly smaller yolk sac area to body area ratio observed in

cold water-reared eggs compared to warm water-reared eggs is striking, a phenomenon termed the "temperature-size rule" [31-32]. This result suggests that more resources were allocated to body area for eggs reared under cold temperature at the expense of yolk sac area. The decrease in yolk resources is significant because it may affect post-hatch development and feeding timing of the larvae, which in turn could affect the probability of post-hatch survival. This result also has implications for damming of the Lake Sturgeon habitat, since dams can affect downstream water temperature, dependent on whether the water is released from the surface (epilimnion) or bottom (hypolimnion) [33], which our results suggest may affect the microbial community on the egg surfaces, incubation periods, and size at hatch.

Under cold temperature, the effect of rearing water types on yolk sac area to body area ratio was negligible, since the dominant effect of cold temperature masked the effect of water types. The fact that yolk sac area to body area ratio was smaller in eggs reared in non-treated water compared to treated water at warm temperature suggests that the microbial quantity or composition on the egg surface affected the yolk resource use by embryos.

In conclusion, this study demonstrated that rearing environments can affect both egg associated microbial communities and host life history traits. This study demonstrates for the first time that the effect of water treatment on improving Lake Sturgeon egg survival can be attributed to a decrease in microbial quantity and/or change in the microbial community composition on the egg surfaces. This study also provides evidence that rearing temperature affects the egg microbial community assembly and colder temperatures can result in a smaller yolk sac area to body area ratio. We also identified putative symbionts (e.g. *Acidovorax* spp. and *Massilia*) associated with low egg mortality, which will help guide further studies on identification of microbes for probiotic treatment. Our study provides management implications

for conserving Lake Sturgeon populations by suggesting that damming streams can alter aquatic microbial community and temperature, which in turn can alter the microbial communities on sturgeon eggs and life history of the sturgeon.

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CHAPTER 4: THE EFFECT OF INOCULATION OF PUTATIVE SYMBIONTS ON LAKE STURGEON EGG SURFACE MICROBIAL COMMUNITY ASSEMBLY AND EGG MORTALITY

Abstract

Microbes and their hosts undergo complex interactions and may sometimes form symbiotic relationships, where both the host and microbes benefit from being associated with one another. Few studies have investigated this topic in fish egg hosts, but research is strongly needed in order to identify potential symbionts that could improve egg survivorship for threatened fish species. In this study, the effect of supplementation of putative symbionts during fertilization on the egg microbial community assembly, egg microbial quantity, and egg mortality was examined in the threatened fish species Lake Sturgeon (*Acipenser fulvescens*). The experiment consisted of two treatment groups (eggs fertilized in the presence of a putative symbiont *Acidovorax* sp. F19 and a putative pathogen *Flavobacterium* sp. B17) and two controls (eggs fertilized with 0.2 µm filtered water and eggs fertilized with stream water). Egg samples for community analysis were collected at two time points (immediately after fertilization and day 2 post-fertilization). Microbial community structure was examined using TRFLP and 454 pyrosequencing and microbial quantity was estimated using qPCR. Fertilization in the presence of *Acidovorax* sp. F19 reduced egg mortality by 18% relative to those fertilized in stream water ($t = 1.98$, $p = 0.06$). There was no difference in egg mortality between *Flavobacterium* treatment and stream control. Analysis of microbial community composition immediately after fertilization across the treatments indicated that *Acidovorax* sp. F19 successfully colonized egg surfaces and became dominant in the community, whereas *Flavobacterium* sp. B17 was not as effective at

colonizing eggs. Despite the differences in initial microbial community compositions across the treatments and controls, the egg associated microbial communities of the treatment and control groups converged after two days of incubation in stream water. Microbial quantity on the egg surfaces increased from 10^5 to 10^7 16S rRNA gene copies per egg by day 2, but there was no significant differences in the egg associated microbial quantity between treatments and controls. Early supplementation with *Acidovorax* sp. F19 resulted in a significant increase in yolk sac to body area ratio of eggs ($t_{116} = 2.47$, $p = 0.015$). *Acidovorax* sp. F19 was closely grouped with one of the branches within beta proteobacteria clade found on unfertilized eggs, suggesting the possibility of vertical transmission of this putative symbiont. Although we do not observe any differences in microbial community structure and quantity at day 2, that significant effect of *Acidovorax* supplementation on egg mortality and yolk sac to body area ratio provides strong evidence that this microbe displays a symbiotic relationship with the Lake Sturgeon egg host.

Introduction

The word symbiosis originates from the Greek words “syn” and “biosis”, which mean “living” and “together”. When the word is used to refer to the interaction between microbes and hosts, it usually indicates a long-term mutual relationship between the two. There are no clear criteria to determine a symbiotic relationship between hosts and microbes, unlike the well established criteria for definition of pathogens [1]. However, symbiotic relationships observed between microbes and hosts in nature cover a wide range of spectra, including provision of essential nutrients to the host [2-3], protection of the host from pathogenic organisms [4], and conferral of a certain evasion ability to the host [5], all of which suggest that the association of hosts with symbionts increases fitness of the hosts.

However, the topic of symbiosis with microbes is rarely investigated in fish egg hosts, despite the fact that microbial symbionts may potentially significantly affect egg development and survival. Such research is needed for the Lake Sturgeon (*Acipenser fulvescens*), a threatened fish species inhabiting the Great Lakes, which deposits eggs into stream water populated by aquatic microbes. Deposited eggs rapidly become colonized by aquatic microbes and a microbial community develops on the egg surface. These egg-associated microbes can be pathogenic, commensal, or even symbiotic.

The existence of symbiotic microbes for fish eggs may seem unlikely, considering the fact that the interaction between microbes and eggs could be seen as too short in duration to establish long-term mutual relationship. However, it is possible that some microbial species protect eggs during embryogenesis and subsequently colonize the larvae and establish a long-term relationship thereafter [6]. One way of establishing such relationship is via horizontal transfer [7]. Because adults of the same sturgeon population spawn in the same river for

thousands of years, aquatic microbe–sturgeon interaction can be viewed as long-term and may have coevolved. The sturgeon eggs may have adapted to interact with certain aquatic microbial species in a mutualistic way by controlling their immune response in a way that symbionts can tolerate, a phenomenon which was observed in other hosts [8-11].

Another way of establishing a symbiotic relationship with microbes is via vertical transmission [6]. A long-term relationship between host and microbes can be achieved by directly transmitting symbionts from parents to offspring. For example, *Acidovorax* sp., a symbiont of the earthworm, is transmitted from the parent into the egg capsule during fertilization. *Acidovorax* sp. then colonizes the embryonic duct, excludes other microbes from this area, and migrates to the nephridium (an excretory organ) of the earthworm offspring [6]. Vertical transmission of microbial symbionts from parents (or a parent) to offspring is also documented in the medical leech [12], nematodes [13], insects [14-15], and sponges [16-18]. Since the Lake Sturgeon is a long-lived animal [19], the possible existence of vertical transmission of symbionts to eggs could be a great benefit for both symbionts and eggs.

Previously, we experimentally identified a putative pathogen (*Flavobacterium* sp.) and a symbiont (*Acidovorax* sp.) for Lake Sturgeon eggs based on the correlation between their dominance on the egg surface and egg mortality under different water treatments (Chapter 3). We also isolated these putative pathogen and symbiont from the egg surfaces using R2A media (Chapter 6). In this study, we sought to elucidate the effect(s) of a putative symbiont (*Acidovorax* sp.) and pathogen (*Flavobacterium* sp.) on egg mortality by experimentally supplementing the eggs with the isolate during fertilization. Pathogenesis and symbiosis of these two isolates were assessed based on comparing the difference in egg mortality between a treatment and a control (fertilization with stream water). We also confirmed the association of these strains with the egg

by monitoring changes in microbial community structure with T-RFLP and measured the level of colonization with Q-PCR on the surfaces of eggs exposed to different treatments and controls. We hypothesized that a probiotic would protect eggs by altering microbial community and/or microbial quantity on the egg surfaces. Identifying symbionts to the Lake Sturgeon eggs is urgently needed, since populations of this species are intensively threatened by anthropogenic pressure including overfishing and dam construction [20-22].

Methods

Experimental design

Eggs were fertilized in four different conditions (two treatments and two controls): (1) with a putative symbiont *Acidovorax* sp. F19 (2) with a putative pathogen *Flavobacterium* sp. B17 (3) with 0.2 µm filtered water, and (4) with stream water. Fertilization in 0.2 µm filtered stream water served as a negative control (no microbial impact during fertilization) and the fertilization with stream water served as a control for reference level of mortality at hatchery. For each of the two treatment conditions, one hundred eggs were fertilized in 0.2 µm filter treated stream water using a 0.22 µm disposable 500 mL membrane filter (Corning Inc) but containing the final concentration of 10^6 cfu/mL of either *Acidovorax* sp. F19 or *Flavobacterium* sp. B17. We chose this range of concentration because it corresponds to average microbial concentrations in freshwater [23]. To control the concentration of inocula, a growth curve (the relationship between microbial cell density and absorbance) was constructed for each isolate by measuring optical density of broth culture at 600 µm using a spectrometer and enumerating the viable cell counts of the broth culture on R2A plate medium. The detailed fertilization process was described in Chapter 3, the only difference being that eggs were fertilized in a smaller tray

(500 mL volume) with 0.1 mL milt per 500 mL liquid. After fertilization, *Acidovorax* sp. F19 and *Flavobacterium* sp. B17 coated eggs and the two controls were reared in stream water at 18 to 19^{o}C, which was known to cause about 78% egg mortality from previous years of experiments (Chapter 3). We had a total of seven replicates (gametes from seven different pairs of parents) for this experiment.

Egg samples were collected at two time points (immediately after fertilizations, and day2 post fertilization) from the treatments and controls. The 2 time points were incorporated in order to address our hypothesis that inoculation of putative pathogen or symbiont alters subsequent microbial community structure on egg surfaces. Ten live eggs were collected for after fertilization egg samples, and 5 live eggs were collected for Day 2 post fertilization egg samples. Egg samples were rinsed in filtered stream water and stored in 80% ethanol at 4^{o}C. Unfertilized eggs from each family were also collected and stored in 80% ethanol at 4^{o}C. Stream water samples were collected at two time points (immediately before fertilizations, and Day 2 post fertilization). For each sampling, 100 mL stream water was filtered with 0.22um filter membrane and the filter membrane with microbes was stored in 80% ethanol at 4^{o}C.

Assessment of egg mortality

The death of an egg was defined as the arrest of embryonic development, which was determined by visual observation of developmental stages of embryos [24]. The number of dead eggs was recorded for each treatment and control on a daily basis, and all dead eggs were removed from the incubation tray upon detection. The number of successful hatches for each treatment and control were also recorded. The cumulative egg mortality for each treatment and

control was calculated as follows: Egg mortality = total number of dead eggs / (total number of dead eggs + total number of hatches). The cumulative egg mortality between treatments and controls were then compared. The effect of treatment on egg mortality was assessed using both a general linear model using the "lm" function and a contrast analysis between the treatment and control using "glht" in the R software version 2.10.0 [25].

DNA extraction and TRFLP analysis

A total of 27 egg microbial community samples (3 families, 2 treatments and 2 controls, 2 time points, plus 3 unfertilized eggs) and 2 water microbial community samples (stream water collected at 2 time points) were processed for DNA extraction and subsequent community analysis using Terminal Restriction Fragment Polymorphism (TRFLP). Three aseptically harvested unfertilized eggs and stream water samples were included for comparison with egg-associated communities.

For all samples, microbial community genomic DNA was extracted using the PowerSoil^{TM} Kit (MO BIO Laboratories Inc., CA) according to the manufacturer's protocol. For egg samples, the DNA was extracted from the surfaces of 8 eggs per sample for immediately after fertilization samples and extracted from the surfaces of 4 eggs per sample for Day 2 post fertilization samples. Previous experiments have indicated a substantially denser community on eggs after two days of incubation in stream water. For water samples, genomic DNA was extracted from the filtered material.

16S rRNA gene based TRFLP was performed to characterize microbial community structure [26-27]. The detailed PCR amplification procedures for TRFLP were described in Chapter 2. The purified PCR products were subjected to enzyme digestion with HhaI (Gibco

BRL). Two technical replicates of each of the digested DNA samples were sent to Michigan State University's sequencing facility and the DNA fragments were separated on an ABI 3100 Genetic Analyzer automated sequencer (Applied Biosystems Instruments, Foster City, CA) in GeneScan mode. The sizes and abundance (peak height) of the terminal restriction fragments (TRFs) were calculated using GeneScan 3.7. Each terminal fragment corresponds to a phylotype, and peak height indicates relative abundance of a phylotype. In order to align TRFs across egg samples from different treatments, the TRFLP profiles were processed with T-Align software (http://inismor.ucd.ie/~talign/index.html) and the output of T-Align was used for microbial community analysis. In order to confirm the TRFs of *Acidovorax* sp. F19 and *Flavobacterium* sp. B17, pure cultures of each isolate were subjected to TRFLP analysis. Principal component analysis (PCA) was performed on the TRFLP data in order to elucidate underlying patterns across samples. PCA was conducted using the "prcomp" function of the R software version 2.10.0 [25].

qPCR

Quantification of microbial communities of the same 27 egg microbial community samples and 2 water samples was determined with quantitative PCR (qPCR) using SYBR green. The qPCR was performed using the same protocol described in Chapter 3. A standard curve for the relationship between 16S rRNA gene copy number and cycle threshold (Ct) values was constructed using a series of dilutions of the bacterial genomic DNA *Flavobacterium johnsoniae* ATCC 17061 that is known to have six 16S rRNA gene copies in its genome. The quantity of the 16S rRNA gene copy in each sample was determined by substituting the Ct value of one of the sample dilutions into the equation of the standard curve and multiplying it by the dilution factor.

454 pyrosequencing analysis

A subset of the aforementioned samples including (1) stream water at pre-fertilization, (2) stream water at day 2 post-fertilization, and (3) an egg sample fertilized and reared in stream water at day 2 post-fertilization was submitted to pyrosequencing analysis. This subset was chosen to determine which microbial genera/species occurred naturally in streams and whether their concentrations differed on egg surfaces. The V3-V5 region of the 16S rRNA gene of the extracted DNA (see section above) was sequenced using 454 GS FLX titanium platform (454 Life Science, Branford, CT) at the research facility at Baylor, Texas. Raw sequence reads were processed using Ribosomal Database Project (RDP) pipeline [28] to sort the data by tag sequence, to trim tag and primer sequences, and to filter out low quality sequences with a minimum quality score of 20 (probability threshold of 0.01) and a minimum read length of 300bp. The taxonomy of the filtered reads was assigned using RDP Classifier at a bootstrap threshold of 80% [29]. Microbial communities of the samples were then compared at the genus level.

Vertical transmission analysis using 16S rRNA gene cloning

We investigated possible vertical transmission of symbionts from parent to egg by harvesting unfertilized gametes from a female in the year 2009 during the spawning season. The microbial community of the aseptically harvested gametes was subsequently examined using 16S rRNA clone library. Microbial community genomic DNA was extracted from the surfaces of 10 gametes using the PowerSoil^{TM} Kit (MO BIO Laboratories Inc., CA) according to the manufacturer's protocol. 16S rRNA gene of the extracted community DNA was amplified using 27F (5' – AGA GTT TGA TCM TGG CTC AG – 3') and 1389R (5'-ACG GGC GGT GTG

TAC AAG-3'). The PCR conditions were the same as those used for TRFLP. PCR amplicons were purified and cloned into *E.coli* cells via a vector plasmid pCR2.1 using a TOPO cloning kit (Invitrogen, Carlsbad, CA). The detailed procedure was described in Chapter 3. A total of 48 clones were sequenced at the Michigan State University's sequencing facility using a 27F primer. The sequences of the clone library were identified using RDP pipeline [28]. Sequences of the 48 clones and the putative symbiont *Acidovorax* sp. F19 were aligned using the Ribosomal Database Project (RDP) [28]. The phylogenetic relationships of the aligned sequences were inferred using MEGA version 4.0 [30] by constructing a Neighbor-Joining tree [31] with the Maximum Composite Likelihood method [32].

Larval size analysis

The effect of putative symbiont (*Acidovorax* sp. F19) on the ratio of yolk sac area (YSA) to body area (BA) ratio was measured as well. This measurement permits the evaluation of treatment on the allocation of yolk resources during embryogenesis. A larger YSA to BA ratio indicates that resources in the yolk sac were less used for body area and/or immune responses during embryogenesis and were available for somatic growth following hatch. We hypothesized that if eggs are inoculated with a symbiont, an embryo would use less yolk sac resources and thus have a larger yolk sac area to body area ratio. To test this, we compared the yolk sac area to body area ratio of *Acidovorax* sp. F19 fertilized larvae to that of stream water fertilized.

Immediately after the hatching of eggs, the larvae were anesthetized using MS-222. Twenty individuals per treatment (or control) per family were photographed with a ruler as a size standard. Photos of new hatched larvae from each of 7 families were included for the analysis. For each larvae, the total length, total body area, and yolk sac area were determined from digital

images using ImageJ software. The effect of *Acidovorax* sp. F19 inoculation was assessed using a linear mixed effect model with family effect as a random variable [Model<-lme (YSA.to.BA ~ Treatment, random=~1|Family)]. The statistical significance of the treatment effect on larvae size was determined after accounting for family effect as a random variable using the mixed effect model. The linear mixed effect model was performed using the "lme" function in R version 2.10.0 [25].

Results

Mortality analysis

Eggs fertilized in the presence of *Acidovorax* sp. F19 had an average mortality of 26.3%, which was about 18 percent lower than that fertilized in the stream water (44.6%), and the difference was marginally significant (t_{24}=1.98, p=0.06; box plot shown in Figure 4.1). There was no significant difference in mortality between *Acidovorax* sp. F19 (26.3%) and eggs fertilized with 0.2 µm filtered water (35.5%) (t_{24}=1.00, p=0.33). Treatment with *Flavobacterium* sp. B17 during fertilization did not significantly affect the egg mortality relative to the stream water fertilized control (t_{24}=0.27, p=0.79). Eggs fertilized with *Flavobacterium* sp. B17 had a higher egg mortality than those in 0.2 µm filtered water, although the difference was not statistically significant (t_{24}=0.71, p = 0.48).

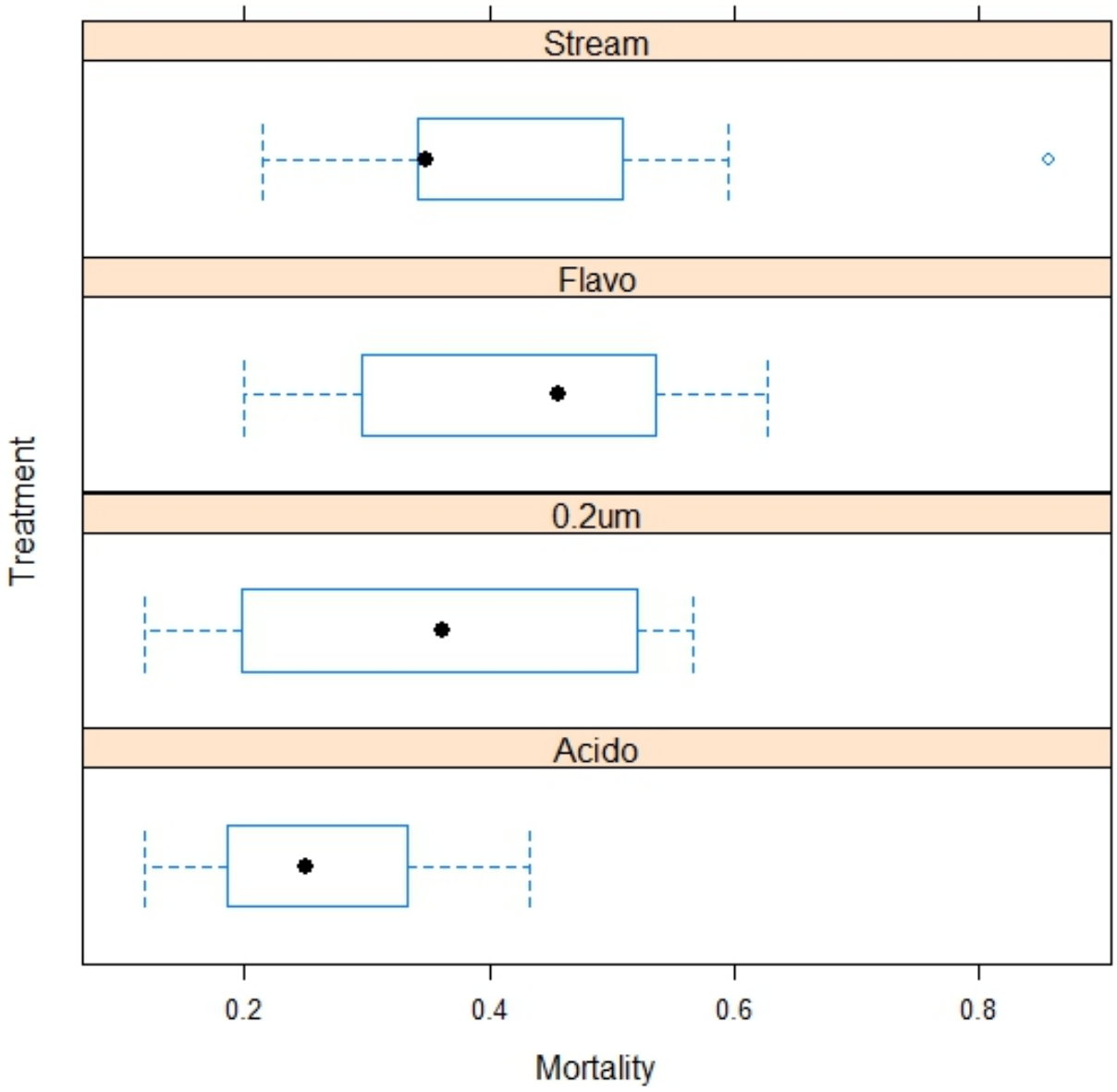

Figure 4.1. A box plot showing the effect of the treatments on egg mortality.

"Stream" corresponds to eggs fertilized in stream water, "Flavo" corresponds to eggs fertilized with *Flavobacterium* sp. B17, "0.2 µm" corresponds to eggs fertilized in 0.2 µm filtered water, and "Acido" corresponds to eggs fertilized with *Acidovorax* sp. F19.

Microbial community analysis using TRFLP

To confirm that treatments during the fertilization changed the egg associated microbial community, we analyzed the egg surface microbial community using TRFLP. TRFLP revealed that there was a difference in microbial community composition immediately after fertilization across the treatments and controls. *Acidovorax* sp. F19 successfully adhered to the egg surfaces and became dominant in the community (accounting for 60 to 65% of the entire community).

Despite being inoculated with the same concentration as *Acidovorax* sp. F19, *Flavobacterium* sp. B17 did not colonize the egg surfaces effectively, accounting for only 10 to 15% of the entire community (Figure 4.2). Microbial communities on the surfaces of eggs fertilized in *Flavobacterium* sp. B17 were similar to that of unfertilized eggs (Figure 4.2). Eggs fertilized in 0.2 µm filtered water had the almost identical community structure to that of unfertilized eggs (Figure 4.2). Microbial communities on eggs fertilized in stream water diverged from the unfertilized egg microbial communities and approached the stream water microbial communities, although they were not identical (Figure 4.2). Although the initial microbial community compositions differed across the treatments and controls, the egg associated microbial communities of the treatments and controls converged by Day 2 post-fertilization (Figure 4.3). The converged microbial community assembly on the egg surface was a subset of the stream water community, but the community structure was significantly different from stream water microbial community (Figure 4.3).

These trends were also detected in a PCA plot constructed using the TRFLP data (Figure 4.4). Microbial communities of unfertilized eggs were clustered with those fertilized in 0.2 µm filtered water and those fertilized in *Flavobacterium* sp. B17 (Figure 4.4). Microbial

communities fertilized in stream water diverged significantly from those of unfertilized eggs and approached the microbial community structure of stream water (Figure 4.4). Day 2 egg samples

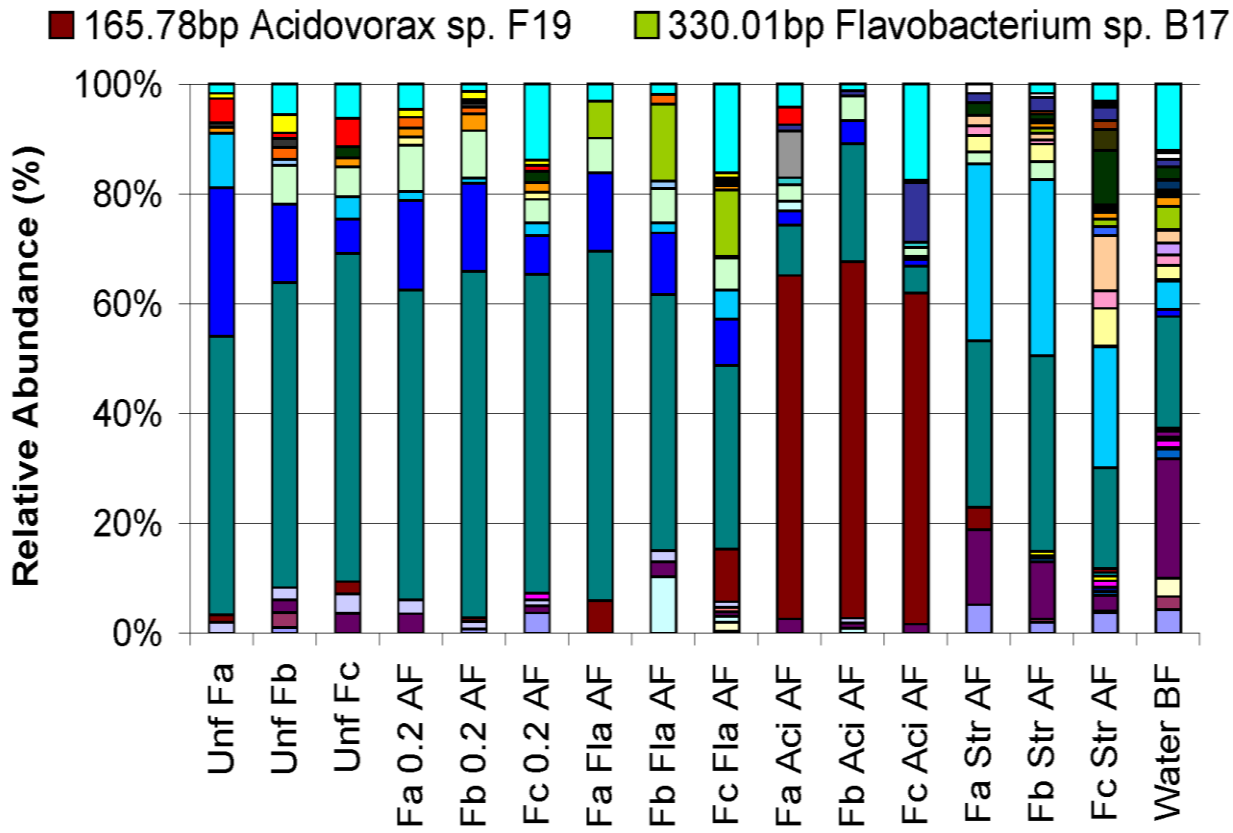

Figure 4.2. Community assembly of unfertilized eggs (Unf) and eggs immediately after being fertilized (AF).

Fa-Fc corresponds to family identities. “Aci”, “0.2”, “Fla” and “Str” stand for *Acidovorax* inoculated, 0.2 µm filtered water (negative control), *Flavobacterium* inoculated, and stream water (control for reference mortality), respectively. Water BF corresponds to stream water microbial community collected immediately before fertilization

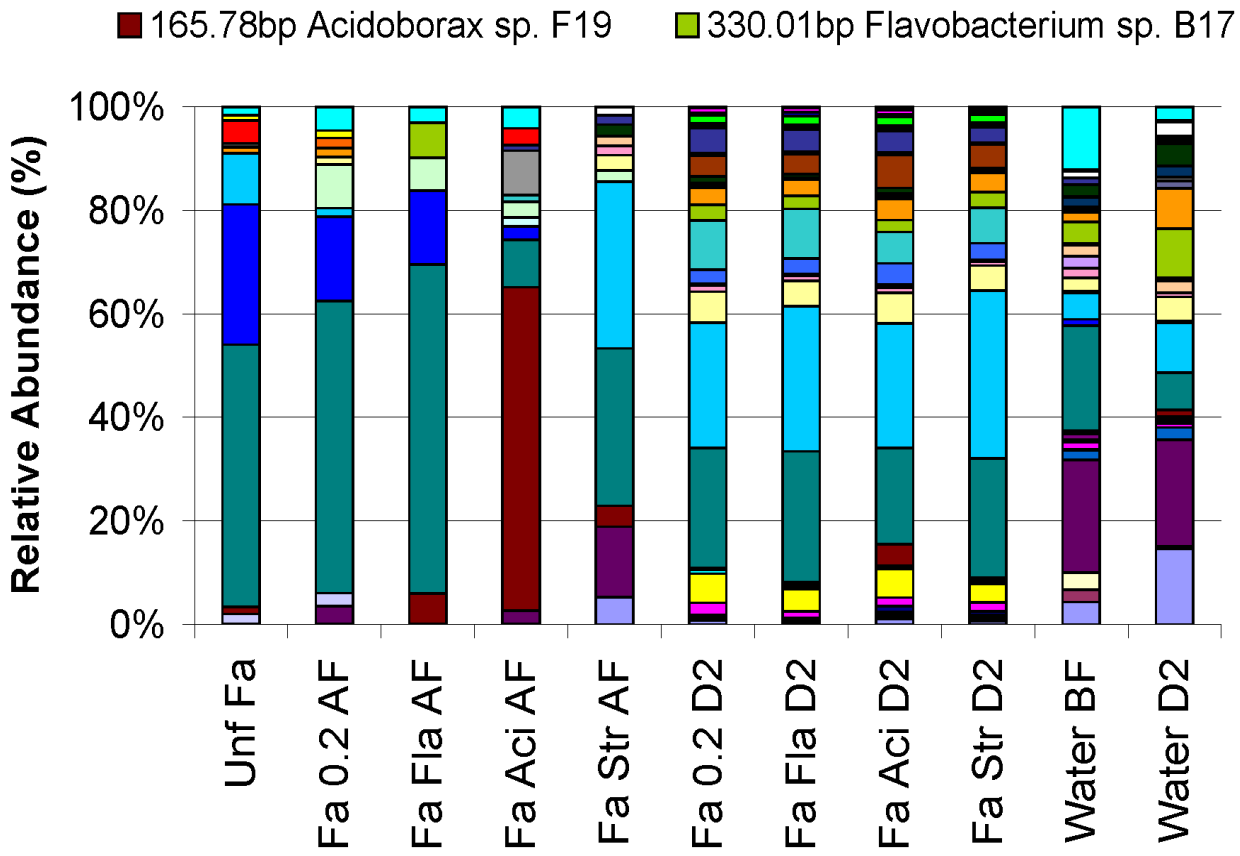

Figure 4.3. Convergence of the egg microbial community structure at Day 2 post-fertilization.

Data were obtained from family "a". Labels on the x axis correspond to those used in Figure 4.2. The letter codes "BF", "AF", and "D2" correspond to before fertilization, after fertilization, and Day 2 post-fertilization, respectively. The egg microbial communities from different treatments and controls converged at Day 2 post-fertilization, but diverged from the stream water microbial community.

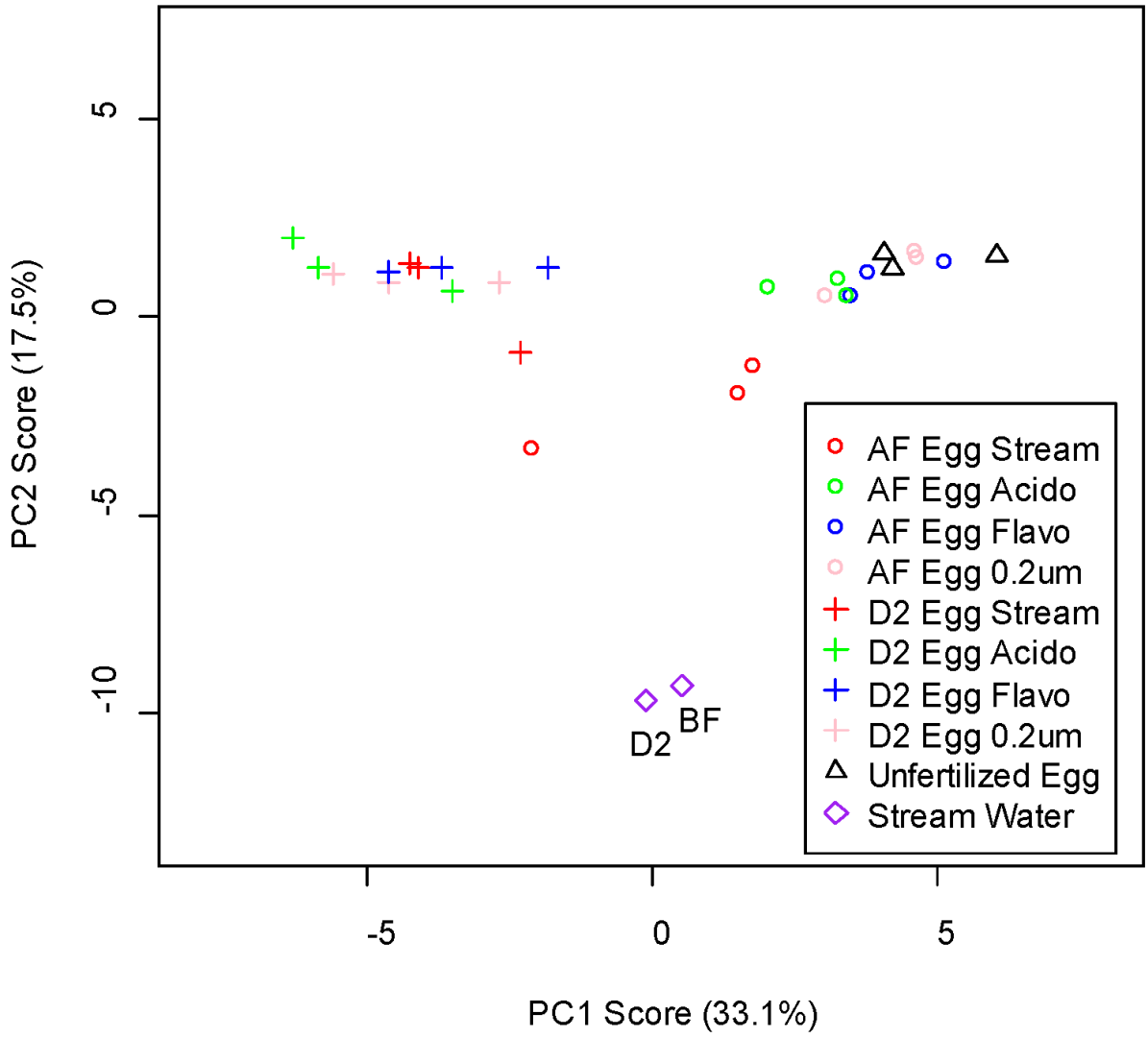

Figure 4.4. Principal component analysis (PCA) analysis using TRFLP data from both egg and water samples.

Microbial communities of unfertilized eggs were clustered with those fertilized in 0.2 µm filtered water and those fertilized with *Flavobacterium* sp. B17 at immediately after fertilization. *Acidovorax* sp. F19 samples departed from unfertilized egg samples. Stream water fertilized egg communities further diverged from unfertilized communities toward the stream water microbial community structure. Day 2 communities were all similar to each other regardless of initial treatment and were distinct from unfertilized communities and stream water communities. Labeling used is the same as that in Figures 4.2 and 4.3.

were all clustered together and diverged from both unfertilized eggs and stream water microbial communities (Figure 4.4). There was variation among the egg microbial communities fertilized in the stream water. One of the stream water fertilized egg samples at immediately after fertilization was similar to the Day 2 microbial community cluster, but the other two fell somewhere in between unfertilized eggs and stream water.

Microbial quantity analysis using qPCR

The microbial quantity on the egg surfaces immediately after fertilization was about 10^5 16S rRNA gene copies per egg for *Acidovorax* sp. F19, *Flavobacterium* sp. B17, and stream water fertilized eggs (Figure 4.5). The microbial quantity on eggs fertilized in 0.2 µm filtered water had a concentration below the detection limit (less than 10^4 16S rRNA gene copies per egg). There was no measurable difference in the quantity of microbes on the egg surfaces at the log scale between the *Acidovorax* fertilized treatment and the stream water fertilized control. Microbial quantity increased from 10^5 to 10^7 16S rRNA gene copies per egg from fertilization to Day 2 post-fertilization for all treatments (Figure 4.5).

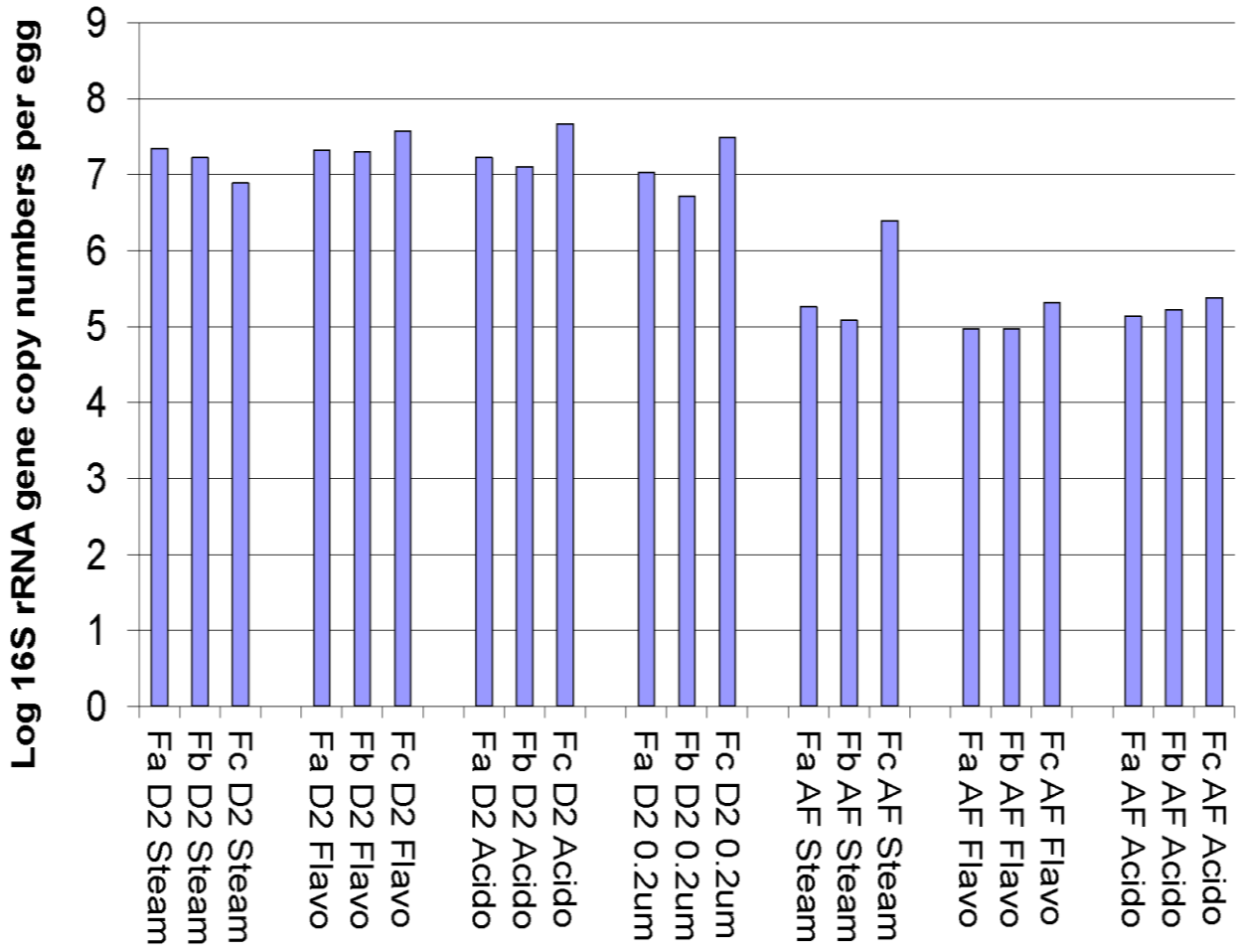

Figure 4.5. Microbial quantity on the egg surfaces of various treatments and controls at different time points estimated using qPCR.

Notation used on the x axis is the same as that used in Figures 4.2 and 4.3.

Microbial community analysis using 454 pyrosequencing

To determine what microbial genus were selected for or against by eggs, we compared stream water microbial community to the eggs surfaces microbial communities fertilized in the same stream water using pyrosequencing data. Approximately 7000 reads per sample were obtained from each sample and average sequence length for the pre-fertilization stream water

sample, Day 2 post-fertilization stream water sample, and Day 2 post-fertilization stream water fertilized egg sample were 509bp, 501bp, and 504bp, respectively.

At the phylum level of analysis, both stream water samples were dominated by phyla Bacteroidetes, Proteobacteria, Actinobacteria, Cyanobacteria, and Firmicutes (Table 4.1).

Table 4.1. Comparison of stream water microbial community and stream water fertilized egg microbial community at the phylum level using 454 pyrosequencing data

Phylum	Water BF	Phylum	Water D2	Phylum	Egg D2
Proteobacteria	3569	Bacteroidetes	3880	Proteobacteria	6492
Bacteroidetes	1736	Proteobacteria	2778	Bacteroidetes	478
Actinobacteria	615	Actinobacteria	586	Actinobacteria	78
Cyanobacteria	184	Verrucomicrobia	28	Cyanobacteria	30
Verrucomicrobia	108	Firmicutes	24	OD1	15
Firmicutes	102	Cyanobacteria	17	Verrucomicrobiai	15
OD1	91	OD1	16	Firmicutes	5
Acidobacteria	62	Armatimonadetes	7	Deinococcus	3
Chloroflexi	23	Deinococcus	6	Acidobacteria	2
Planctomycetes	17	Chlamydiae	5	WS3	1
Chlamydiae	10	TM7	3	Planctomycetes	1
Nitrospira	9	Acidobacteria	1		
Gemmatimonadet	8	Chloroflexi	1		
Chlorobi	6				
TM7	5				
Spirochaetes	4				
WS3	3				
Elusimicrobia	2				
Fusobacteria	1				
Armatimonadetes	1				
Deinococcus	1				
Unclassified	452		76		109
Total reads	7009		7428		7229
Number of phyla	21		13		11

*** “BF” denotes before fertilization and “D2” denotes Day 2 post-fertilization.

However, the Day 2 egg surface microbial communities were noticeably different from the water community. The numbers of reads of Bacteroidetes, Actinobacteria, Firmicutes were reduced by 80%, 88% 91%, respectively, on the egg surfaces compared to the average number in the stream water (Table 4.1). The number of phylum Proteobacteria on the egg surfaces was double that of the stream water (Table 4.1).

At the genus level, the Day 2 egg surface microbial community had a significantly lower number of *Flavobacterium* compared to those in the water samples (approximately 80% lower, Table 4.2). The numbers of *Limnohabitan*, *Fluviicola*, *Polynucleobacter*, *Arcicella* were also lower on the egg surfaces compared to the water samples (Table 4.2). In contrast, the egg surface microbial community had a significantly greater number of *Acidovorax* compared to that in the water samples (approximately 4 times more, Table 4.2). The numbers of *Albidiferax*, *Rheinheimera*, *Novosphingobium*, *Roseateles*, *Caulobacter*, and *Hydrogenophaga* were also higher on the egg surfaces than the water samples (Table 4.2).

Vertical transmission of Acidovorax sp. F19

The clone library showed that the microbial community on eggs harvested aseptically consisted of microbes from four distinct major clades, which corresponded to Bacteroidetes, alpha, beta, and gamma proteobacteria. *Acidovorax* sp. F19 was closely grouped with one of the branches within the beta proteobacteria clade found on the aseptically harvested unfertilized eggs (Figure 4.6).

Table 4.2. Comparison of water and egg microbial communities at the genus level using 454 pyrosequencing data.

Genus	Water BF	Genus	Water D2	Genus	Egg D2
Limnohabitans	1162	Flavobacterium	2376	Albidiferax	819
Flavobacterium	764	Limnohabitans	1221	Rheinheimera	708
Polynucleobacter	259	Arcicella	275	Flavobacterium	267
Pseudomonas	126	Fluviicola	211	Acidovorax	231
Methylophilus	123	Lishizhenia	176	Novosphingobium	212
Bacillariophyta	106	Sediminibacterium	162	Undibacterium	195
Fluviicola	103	Pseudomonas	128	Roseateles	167
OD1_incertae_sedis	91	Polynucleobacter	112	Caulobacter	165
Albidiferax	81	Rheinheimera	88	Pseudomonas	155
Arcicella	66	Sphingobium	80	Brevundimonas	145
Sediminibacterium	54	Acidovorax	79	Hydrogenophaga	123
Opitutus	50	Brevundimonas	74	Massilia	116
Massilia	48	Rhodobacter	62	Sphingobium	115
Lishizhenia	47	Albidiferax	54	Limnohabitans	103
Sphingomonas	46	Rhizobium	40	Rhodobacter	76
Rhodobacter	45	Massilia	34	Sphaerotilus	72
Polaromonas	37	Sphingomonas	29	Pelomonas	69
Ilumatobacter	33	Caulobacter	25	Duganella	68
Acidovorax	32	Novosphingobium	21	Flectobacillus	53
Clostridium sensu	29	OD1_incertae_sedis	16	Aquabacterium	45
Gp6	27	Pedobacter	16	Naxibacter	44
Acinetobacter	26	Hydrogenophaga	14	Vogesella	43
Cryptomonadaceae	24	Achromobacter	12	Rhizobium	42
Ohtaekwangia	23	Undibacterium	12	Ideonella	38
Legionella	23	Aurantimonas	12	Rhizobacter	23
Rhizobacter	19	Prosthecobacter	12	Methylophilus	22
Rheinheimera	18	Arthrobacter	11	Sphingomonas	19
Aquabacterium	17	Algoriphagus	11	Fluviicola	18
Brevundimonas	16	Methylophilus	11	OD1_incertae_sedis	15
Sphaerotilus	15	Bacillariophyta	11	Leptothrix	14
Other classified	628		221		277
Unclassified genus	2871		1822		2770
Total reads	7009		7428		7229
Number of genera	191		108		102

*** Only the top 30 genera in each community are shown. “BF” denotes before fertilization and “D2” denotes Day 2 post-fertilization.

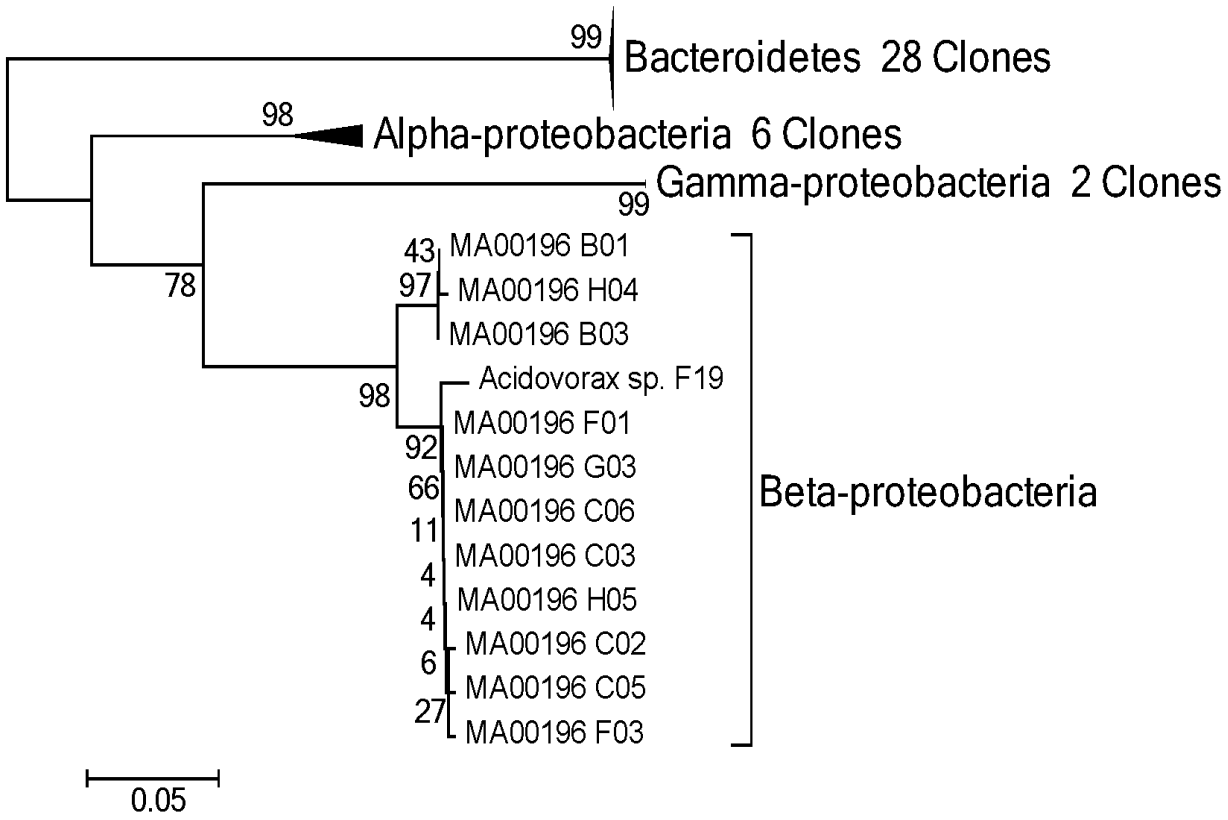

Figure 4.6. Potential vertical transmission of *Acidovorax* sp. F19.

Acidovorax sp. F19 was closely grouped with beta proteobacteria clones identified on aseptically harvested unfertilized eggs. MA00196 represents the plate number of 48 clone library.

Larvae size analysis

We found that larvae treated with *Acidovorax* sp. F19 during the fertilization had a significantly larger yolk sac area to body area ratio than those fertilized in stream water after taking into account the family effect as a random variable in the mixed effect model ($t_{116} = 2.47$, $p = 0.015$, Figure 4.7).

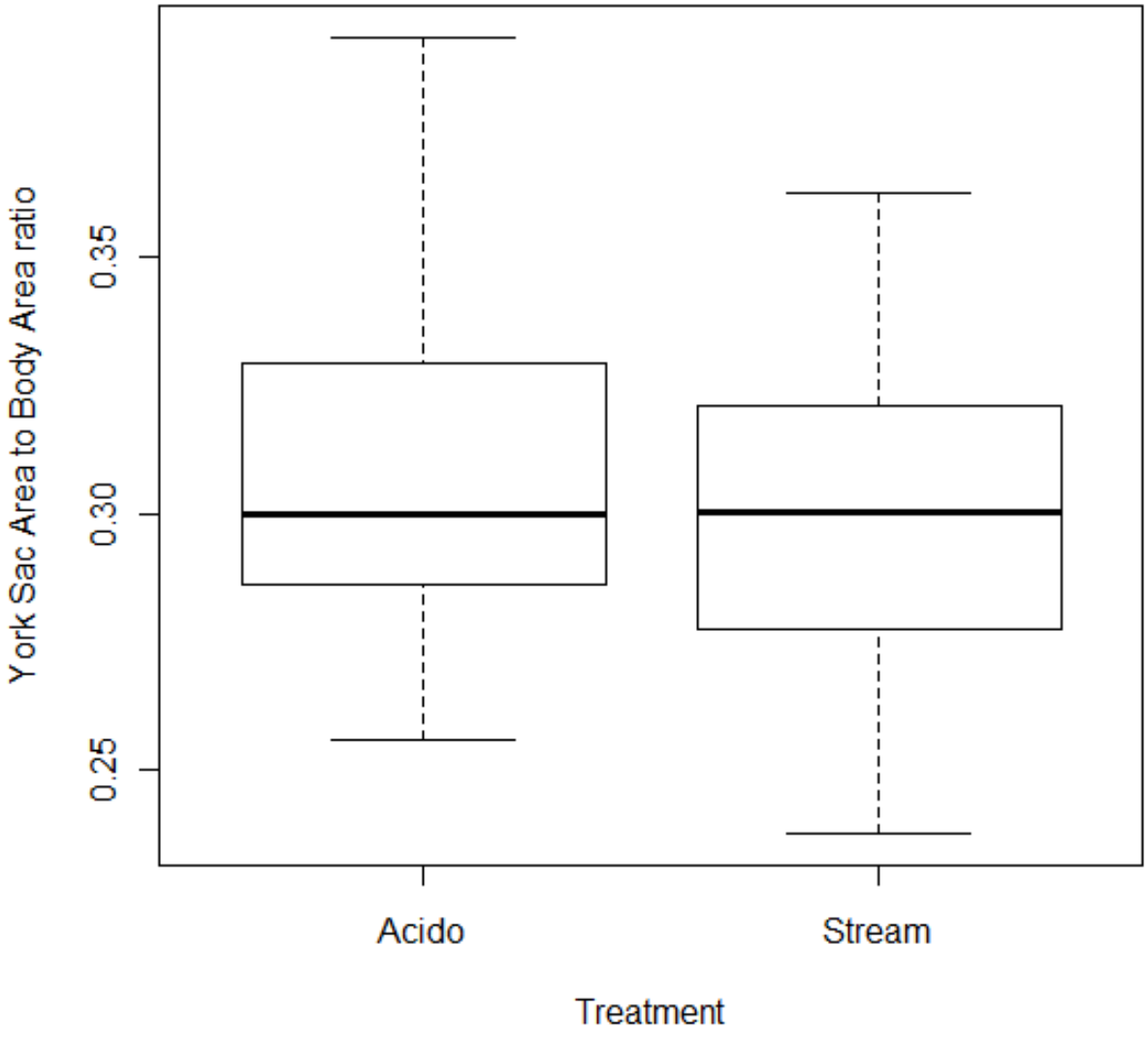

Figure 4.7. Box plot depicting the effect of *Acidovorax* sp. F19 treatment on yolk sac area to body area ratio of eggs.

Discussion

This study adopted an integrative approach to further evaluate microbe-host interactions, specifically those involving a putative symbiont and its Lake Sturgeon egg host. We gathered solid evidence to suggest the existence of a symbiotic relationship between *Acidovorax* sp. F19 and the eggs, in addition to identifying some probable mechanisms to explain the occurrence of this symbiosis. The significant effect of inoculation of *Acidovorax* sp. F19 to eggs during fertilization on reducing the egg mortality and yolk sac resource use suggest that this microbe may be promising for probiotic treatment in this threatened fish species.

One of the surprising results from this study was that the egg associated microbial community of all treatments and controls converged by Day 2 post-fertilization. This result was counter to our hypothesis that we would observe measurable differences in egg surface microbial communities on Day 2 across treatments. This is probably due to the fact that the microbial quantity increased by 100 fold from fertilization to Day 2 post-fertilization, such that a massive amount of dispersal from the stream water onto the egg surfaces effectively masked the effect of any treatment given at fertilization. It was also surprising that we did not detect any difference in microbial quantity on the egg surfaces between eggs fertilized with *Acidovorax* sp. F19 and those fertilized with stream water at Day 2 post-fertilization. These lines of evidence suggest that the mechanism to explain the observed symbiotic relationship between *Acidovorax* sp. F19 and eggs occurred by some pathway other than alteration of the microbial community structure and microbial quantity.

One possible mechanism would be that *Acidovorax* did not induce a host immune response. Eggs may possess a mechanism to recognize the surface molecules of microbes and transduce signals that determine the host responses to the microbes, a phenomenon which was

found in other host tissues [8-11]. It is known that some symbionts do not induce host immune response at attachment [10]. This potential hypothesis is consistent with our finding that the egg yolk resources were not as heavily used upon colonization by *Acidovorax* sp. F19 relative to the stream water control. However, this hypothesis does not explain the lower egg mortality we observed with *Acidovorax* sp. F19 treatment, unless expression of immune response to microbes alone negatively affected the host and increased host mortality.

Another possible explanation could be that the relationship between *Acidovorax* and the host eggs was neutral. In fact, across various host-microbe interactions observed in nature, the majority of microbes are neutral to their hosts [33-34]. This hypothesis is supported by the fact that we did not observe statistically significant differences in egg mortality between eggs fertilized in *Acidovorax* sp. F19 and those fertilized in 0.2 μm filtered water. This line of evidence suggests that *Acidovorax* acted essentially as a no microbe effect. There are numerous micropyles (structures through which a sperm enters into an egg) on each egg of various sturgeon species [35]. It is possible that some microbes could have entered into the inert area of the eggs through the micropyles during fertilization and harmed the eggs, an effect that would have been minimized in the 0.2 μm filter treated group. It is possible that *Acidovorax* may have entered the eggs through the micropyles without harming the egg, unlike the putative pathogen *Flavobacterium* sp. B17 and other pathogenic aquatic microbial species present in the stream water.

Although we cannot definitively determine the mechanism of how fertilization with *Acidovorax* sp. F19 lowered egg mortality and yolk resource use, it is worthy to note that there was a marked difference in colonization success between *Acidovorax* sp. F19 and *Flavobacterium* sp. B17. Our TRFLP revealed that *Acidovorax* sp. F19 successfully colonized

egg surfaces, as opposed to *Flavobacterium* sp. B17 which did not. 454 pyrosequencing also detected that significant numbers of *Acidovorax* spp. were present on the egg surfaces relative to the number found in the water microbial community, whereas the number of *Flavobacterium* was lower on the egg surface relative to the source water microbial community. The rejection of *Flavobacterium* populations by the eggs could be mediated by either host innate immunity or inability of the microbe to adhere to the egg surfaces. The establishment of *Acidovorax* on the egg surfaces could be attributed to either its ability to adhere to the surfaces of eggs [36] and/or evade recognition by the host immune system [10] or its tolerance of the host innate immunity response [11]. This study thus informs other studies by suggesting that the approach of characterization of selection for or selection against certain microbes could help identify potential symbionts and pathogens.

This study also elucidated the mechanisms shaping the egg associated microbial community and the kinetics of the process of community assembly. The fact that the converged microbial community at Day 2 post-fertilization was significantly different from that of the stream water suggests that the egg related local processes (such as host innate immunity) shaped the community structure. Interestingly, one of the stream water fertilized egg samples shaped the egg microbial community much faster than others to the point that its community structure at immediately after fertilization was similar to the community structure found at Day 2. This result is noteworthy in suggesting that a microbial community can be shaped as a result of egg-related effects in as quickly as 60 minutes. This finding suggests that there may be a sense of urgency on the part of the eggs in needing to control the egg associated microbes as rapidly as possible, thereby highlighting that the onset of host-microbe interaction may be critical for eggs in determining the trajectory of their life history.

Our results with respect to yolk sac area to body area ratio were also interesting. Yolk sac area alone was slightly larger in eggs fertilized with *Acidovorax* sp. F19 than that fertilized in stream water, and body area was slightly smaller in eggs fertilized with *Acidovorax* than that fertilized in stream water. Neither of these differences was statistically significant, but a significant difference was found when combining the two measures in a form of a ratio, thereby demonstrating the efficacy of this ratio for this analysis. We also note that the measurement of the yolk sac area alone for assessment of yolk sac resources may not have been a sufficiently sensitive measure because the yolk sac has a three dimensional structure (height, width, and depth) that was not fully captured by our measurement (height and width only). The fact that we also found that body area increased when fertilized in the stream water suggests that embryos could be encouraged to grow faster when pathogenic microbes colonize the egg surfaces (as in the stream condition). However, we did not detect a significant difference in hatch timing among treatments and controls. Alternatively, metabolic activity itself may have been enhanced by the presence of pathogenic microbes on the egg surfaces, which could have resulted in eggs consuming yolk sac resources at a faster rate, leading to an increase in body size.

Another interesting finding of this study was that we showed that *Acidovorax* sp. may be vertically transmitted from parent to egg, since *Acidovorax* sp. F19 was closely grouped with one of the branches within the beta proteobacteria clade found on unfertilized eggs. Furthermore, we found that unfertilized eggs had similar microbial communities across different females using TRFLP. This line of evidences suggests that the egg surfaces of unfertilized eggs may not be sterile, but rather already colonized by certain species of microbes in a non-random manner. Vertical transmission is an efficient way for hosts to select appropriate symbionts for eggs, since only effective symbionts can protect eggs and be successfully transmitted to larvae [37].

The most important implication for future management of this threatened fish species in the hatchery environment is that treatment conducted during the first 60 minutes of an egg's life can cause significant differences in egg mortality and embryonic resource use. This together with the fact that egg can already select certain microbes over others within that same brief 60 minute period suggests that the early interaction between microbes and hosts are important determinants of the trajectory of a sturgeon's life. We can now draw on multiple lines of evidence to recommend *Acidovorax* sp. F19 as a probiotic that can be used to improve survivorship of Lake sturgeon eggs reared in hatcheries to assist in recovery of this threatened species in the future.

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CHAPTER 5: THE RELATIVE IMPORTANCE OF REGIONAL DISPERSAL AND LOCAL DETERMINISTIC PROCESSES IN SHAPING THE MICROBIAL COMMUNITY ASSEMBLY ON THE EGG SURFACES OF THE LAKE STURGEON (*ACIPENSER FULVESCENS*)

Abstract

Research has shown that both dispersal process and local deterministic processes shape microbial community assembly, but few studies have been able to analyze the relative importance of these two processes. We investigated the process of microbial succession on the egg surfaces of the Lake Sturgeon (*Acipenser fulvescens*) with a focus on the relative importance of dispersal and local deterministic processes in shaping the egg microbial community assembly. We experimentally controlled the rate of dispersal of aquatic microbes onto the egg surface by manipulating the concentration of water microbes in order to understand the importance of the rate of dispersal relative to deterministic processes, which in this case was the effect of the egg micro-environment (e.g. lysozyme, metabolites, and microbe-microbe interactions). Eggs were fertilized in different water types (stream water, UV-treated stream water, 0.2 µm filtered stream water) which varied by microbial quantity and community structure, and reared in either the same water type or one of the other two water types. Eggs were collected at different time points during incubation. Genomic DNA was extracted from the egg surface and microbial communities were examined using 16S rDNA-based TRFLP and 454 pyrosequencing. TRFLP-derived microbial community data were subsequently analyzed using principal components analysis (PCA). We hypothesized that dispersal was a dominant process over local deterministic processes in shaping the egg surface microbial community and the effect of dispersal was

dependent on water microbial density. We found that at the rate of dispersal we tested, local deterministic processes were dominant over dispersal. The egg microbial community assembly was significantly different from the source water microbial community. However, dispersal also played a role in explaining the egg surface microbial community. When eggs were reared in water with a high density of microbes, initial inocula on the egg surface were not important in dictating the final community composition. However, as the density of water microbes declined, the final microbial community assemblage on the egg surface became less influenced by the water in which eggs were reared. In this case, the majority of change accrued in the egg micro-environment was nested within the initial inocula and did not appear to be influenced by dispersal. These results suggest that local deterministic processes are dominant over dispersal, and the effect of dispersal on the egg microbial community was dependent on the concentration of aquatic microbes present during microbial succession.

Introduction

Microbial communities vary spatially and temporally. Mechanisms shaping community assemblage at a given place and time remain fundamental questions in ecology [1]. The study of microbial communities has flourished over the last 15 years, partly due to the development of analytical techniques that have allowed us to expose unculturable portions of such communities [2-3]. Microbial community assemblages have been studied in a variety of environments ranging from deep marine sediments [4], marine water [5], lakes [6-7], soils [8-9], plants [10-11], the animal gut [12], and the human gut [13]. However, the underlying mechanisms shaping microbial community assemblage at a given place and time remain largely unknown, as does the role of microbes in the greater ecological system.

In the realm of ecology, there are two competing theories that seek to explain the processes shaping community assemblage: niche theory [14] and neutral theory [15]. Niche theory assumes that organisms are selected by deterministic processes for functional traits that allow them to exist in certain environments. According to this theory, coexistence among different species at a given location is explained by heterogeneity of the local environment (local process) [16]. On the other hand, neutral theory assumes that organisms are functionally neutral in a given environment. Stochastic processes such as dispersal explain presence or absence of organisms in a given environment. In this context, dispersal refers to movement of organisms such as plant seeds, insects, and microbes away from an existing population, a process which is mediated by passive forces such as wind and currents. According to this theory, community assemblage at a given location is shaped by dispersal from neighboring habitats (a regional process) [15].

Several studies have attempted to elucidate the relative importance of these two processes in shaping community assemblage using microbial systems [17-18]. Dumbrell and colleagues studied the relative importance of soil pH and microbial dispersal on soil microbial community structure and found that pH was a more dominant factor over dispersal [17]. Van der Gucht and colleagues studied the relative importance of local processes and regional processes (dispersal) in explaining eleven lake microbial community assemblages, and found that local processes were more dominant [18]. However, these studies are missing one of the key components that may explain the observed microbial community assemblage in a given environment- the history of the community [19].

History can effectively be understood via the study of succession. Succession is, by definition, the colonization of an open space and subsequent sequential changes in species composition. Succession is also a special type of community assembly in which the entire process of community development can be observed. Microbial succession has been recently studied in various host animals [19-21], host plants [10], and natural [22-24] and artificial [25-27] environments. These previous studies found that microbial succession is a complex process affected by a number of factors [10, 19, 22]. Key factors include initial dispersal of microbes from the neighboring communities followed by colonization in or attachment to the open space, and subsequent species sorting via local deterministic processes, while microbes are continuously immigrating from neighboring spaces [28].

Microbes serve as good model systems for studying these processes. This is partly because microbial generation time is relatively short, which allows us to observe the process of adaptation. Microbes also exhibit high dispersal rates, since their small size allows them to disperse freely without geographic barriers [29]. In addition, microbes inhabit various

environments, which allow us to observe the effect of environmental gradients on shaping community assemblage.

One unique host for which these processes have not yet been studied is the egg surface of the threatened fish species Lake sturgeon (*Acipenser fulvescens*). Microbial succession on the Lake sturgeon egg surface is likely a complex process. Eggs are fertilized in a stream as soon as male and female adults release gametes. The fertilized eggs develop stickiness [30] so that they adhere to benthic substrates such as gravel and sand [31]. During this fertilization process, microbes that are drifting in a stream collide with eggs and adhere to the sticky egg surfaces. This process is a stochastic process, since the water microbial community varies temporally and spatially [24, 32-33], and eggs in the stream have no control over their movements. After the initial stochastic collision, the microbial community on egg surfaces is possibly selected by local deterministic processes including adhesion [34-35], antimicrobial activities of eggs [36-37], chemicals that eggs excrete during embryogenesis [38-39], interspecific competition among microbial species [40], while microbes in stream water continuously collide with egg surfaces via passive dispersal mediated by water flow.

In this study, we investigated the relative importance of local deterministic processes and dispersal in shaping microbial community assemblage on the Lake Sturgeon egg surface during microbial succession. The novel contribution of this study is that we can quantitatively assess the relative importance of deterministic and stochastic processes in explaining microbial community assemblage on the egg surface by manipulating the quality, quantity, timing, and duration of microbial dispersal, unlike a field observational study where there is no such control. We hypothesized that dispersal was a dominant process over local deterministic processes in shaping the egg surface microbial community and the effect of dispersal was dependent on water

microbial density. This study has significant implications for understanding mechanisms governing microbial succession and also for the management of the threatened Lake Sturgeon, which is susceptible to high egg mortality as a result of colonization of microbes.

Methods

Study site

This experiment was conducted at a Lake Sturgeon streamside hatchery located on the Upper Black River in Michigan during May 2011 in the midst of the Lake Sturgeon spawning season. Incoming river water was filtered using a sock filter system (100 psi) to remove large particulate matter before being gravity-fed to the hatchery system. Gametes used in this study were collected from spawning adults in the Upper Black Lake and fertilized and reared in the hatchery under different experimental conditions.

Experimental design and sampling

Three different water types (stream water, UV treated stream water, and 0.2 µm filtered stream water) which varied by microbial quantity and community composition were used in this experiment. UV treated water was created using a water treatment system which consisted of a 50µm filter cartridge followed by a UV lamp (Emperor aquatic, Inc). The UV treated water was circulated in the system for 24 hours before being fed to egg samples. The 0.2 µm filtered water was obtained by filtering the hatchery water using a series of filer cartridges- 50 µm, 20 µm, 10 µm, 5um, 1.0 µm (Pentair Ltd) and 0.2 µm (Spectrapure Inc). The 0.2 µm filtered water was stored in a reservoir and filtered again using a series of 1.0um and 0.2 µm filter cartridges before being fed to egg samples.

Approximately, 100 eggs were fertilized in one of the three water types (stream water, UV treated stream water, or 0.2 µm filtered stream water), and continually reared in the same water type for 6 hours at 18-19^{o}C. Six hours after fertilization, eggs were either continually reared in the same water or reared in different water types at 18-19^{o}C. Thus, this experiment consisted of a total of 9 treatments with different fertilization/rearing combinations (Figure 5.1). Rearing water was re-circulated for stream water and UV treated water, while 0.2 µm filtered water was not re-circulated in order to prevent microbial contamination from eggs. Flow rate of this experiment was maintained at 5 L per minute for all water types. The experiment was replicated using 6 families. Egg samples were collected from each treatment at four different time points (AF, Day 1, Day 3, and Day 5) during incubation. Water samples were collected for each water type at the same time points that the eggs were collected. For each water sampling event, 100 mL of water was collected and filtered with a 0.22 µm filter membrane (manufacture) and stored in 20 mL ethanol at 4^{o}C.

Direct microscopic counts

One mL of each of the water microbial samples (stream water, UV treated water, and 0.2 µm filtered water at four different time points) that were stored in 80% ethanol was placed in a 1.5 mL eppendorf tube and centrifuged at 11,000 rpm for 3 minutes. The cell pellets were then re-suspended in 500 uL water. 10 uL of 0.38% Gram Crystal Violet (DIFCO) was added into the cell suspension and was left to sit for 3 minutes to stain the cells. The cell suspension was centrifuged at 11,000 rpm for 3 minutes. The cell pellets were re-suspended in either 100 uL

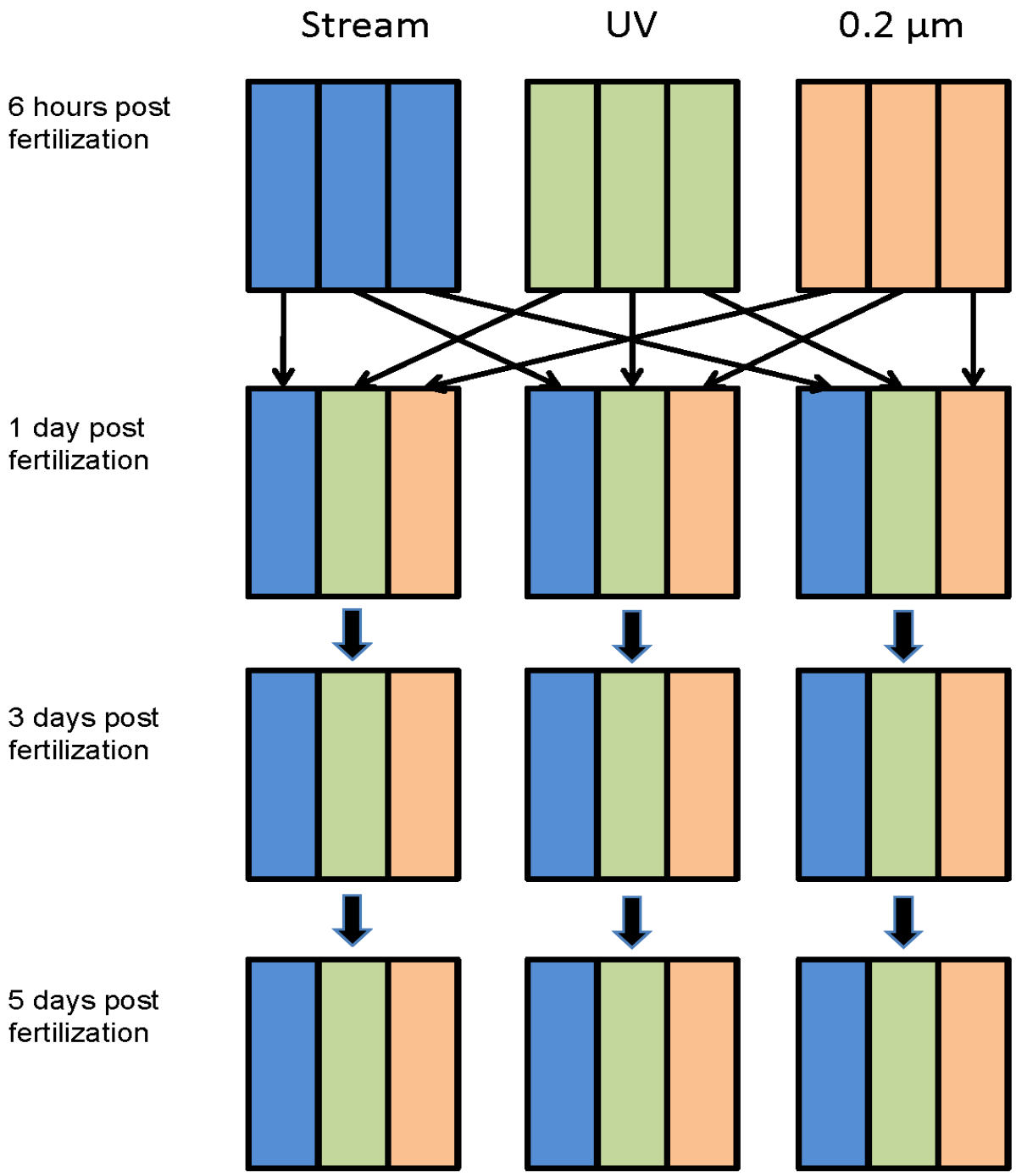

Figure 5.1. Schematic diagram of experimental design to manipulate initial inocula and subsequent rate of dispersal.

Eggs were fertilized in 3 different water types (stream water, UV treated stream water, or 0.2 µm filtered stream water). 6 hours after fertilization, eggs were transferred and reared in the same or different water types. A total of 9 different fertilization/rearing combinations were maintained. Six families have been tested in this manner.

water (for stream water samples) or 10 uL water (for UV treated and 0.2 µm filtered water samples). Three uL of the cell suspension was placed on a Petroff-Hausser chamber. The number of cells on each of 16 out of 25 middle-sized grids was counted using a light microscope with a total magnification of 1000X. The number of cells per mL of original water sample was calculated based on the volume of the middle sized grids and the concentration factor of the samples.

Extraction of DNA

A total of 12 water community samples (3 water types, 4 time points) and 40 egg microbial community samples (2 families, 5 out of 9 treatments, 4 time points) were processed for DNA extraction. For water samples, each water sample was vortexed for 10 minutes with maximum vibration, and 10 mL of the ethanol solution containing suspended bacterial cells were taken into a 15 mL corex tube and centrifuged at 10,000 rpm for 30 minutes at 6^{o}C. Cell pellets from the centrifugation were subjected to DNA extraction using the Power Soil^{TM} Kit (MO BIO Laboratories Inc., CA). For egg samples, genomic DNA was extracted from the surface of 8 eggs per sample using the Power Soil^{TM} Kit according to the manufacture's protocol.

454 pyrosequencing analysis

To characterize both egg associated microbial communities and water microbial communities in each water type, the extracted DNA samples were subjected to 454 pyrosequencing. The V3-V5 region of the 16S rRNA gene of the extracted DNA (see section above) was sequenced using 454 GS FLX titanium platform (454 Life Science, Branford, CT) at

a research facility in Baylor, Texas. Raw sequence reads were processed using Ribosomal Database Project (RDP) pipeline [41] to sort the data by tag sequence, to trim tag and primer sequences, and to filter out low quality sequences with a minimum quality score of 20 (probability threshold of 0.01) and minimum read length of 300bp. The taxonomy of the filtered reads was assigned using RDP Classifier at a bootstrap threshold of 80% [42]. The resultant microbial communities of the samples were compared at both the phylum and genus level.

Community analysis using TRFLP

16S rRNA gene based TRFLP was performed to characterize microbial community structure [43-44]. The detailed PCR amplification procedures for TRFLP were described in Chapter 2. The purified PCR products were subjected to enzyme digestion with HhaI (Gibco BRL). Two technical replicates of each of the digested DNA samples were sent to Michigan State University's sequencing facility and the DNA fragments were separated on an ABI 3100 Genetic Analyzer automated sequencer (Applied Biosystems Instruments, Foster City, CA) in GeneScan mode. The sizes and abundance (peak height) of the terminal restriction fragments (TRFs) were calculated using GeneScan 3.7. Each terminal fragment corresponds to a phylotype, and peak height indicates relative abundance of a phylotype. In order to align TRFs across egg samples from different treatments, the TRFLP profiles were processed with T-Align software (http://inismor.ucd.ie/~talign/index.html) and the output of T-Align was used for the microbial community analysis. Principal component analysis (PCA) was performed on the TRFLP data in order to elucidate underlying patterns across samples. PCA was conducted using the "prcomp" function of the R software version 2.10.0 [45].

Quantitative PCR analysis

To assess the effect of the various treatments on the egg associated microbial quantity, microbial load of egg samples from different treatments were determined by performing quantitative PCR (qPCR) with SYBR green. 2 families were included in the analysis. The qPCR was performed using the same protocol described in Chapter 3. A standard curve for the relationship between 16S rRNA gene copy number and cycle threshold (Ct) values was constructed using a series of dilutions of the bacterial genomic DNA *Flavobacterium johnsoniae* ATCC 17061 that is known to have six 16S rRNA gene copies in its genome. The quantity of the 16S rRNA gene copy of each sample was determined by substituting the Ct value of one of the sample dilutions into the equation of the standard curve and multiplying it by the dilution factor.

Larval size analysis

We were also interested in examining the effect of rearing environments on larval size of eggs at hatch. Immediately after the hatching of eggs, larvae were anesthetized using MS-222. Twenty individuals per treatment per family were photographed with a ruler as a size standard. Larvae from 3 (fertilized/reared in stream water, UV treated, and 0.2 µm filtered) out of 9 treatments were analyzed for the larval size. 6 families were included in the analysis. For each larvae, the total length, total body area, and yolk sac area were determined from the images using ImageJ software. Yolk sac area was chosen for measurement because this allowed us to examine the effect of treatment on the allocation of yolk resources during embryogenesis. The effect of fertilization/incubation in 0.2 µm filtered water on the larvae size was assessed using a general linear model, and the statistical significance of the treatment effect on larvae size was confirmed after accounting for family effect as a random variable using a linear mixed effect model. The

general linear model was performed using the "lm" function and the linear mixed effect model was performed using "lme" function in R version 2.10.0 [45].

Assessment of egg mortality

The death of an egg was defined as the arrest of embryonic development. The arrest of embryonic development was determined by visual observation of developmental stages of embryos relative to a reference [46]. The number of dead eggs was recorded for each treatment and control on a daily basis, and all dead eggs were removed from the incubation tray at detection. The number of successful hatches for each treatment and control was also recorded. The cumulative egg mortality for each treatment and control was calculated as follows: Egg mortality = total number of dead eggs / (total number of dead eggs + total number of hatches). The cumulative egg mortality was compared across treatments using a box plot and the effect of treatments on egg mortality was assessed using a general linear model using the "lm" function in the R software version 2.10.0 [45].

Results

Source water microbial quantity and compositions

Microbial concentrations in each water type were estimated using direct microscopic counts with Petroff-Hauser counting chamber. The average concentrations of microbes for stream water, UV treated water, and 0.2 µm filtered water were found to be $10^{5.28}$, $10^{4.22}$, $10^{3.29}$ 16S rRNA gene copies per 1 mL, respectively (Figure 5.2). The concentration was the highest in stream water, the second highest in UV treated water, and the lowest in 0.2 µm filtered water. There was approximately one order of magnitude difference between stream and UV

treated water, and another one order of magnitude difference between the UV treated and 0.2 µm filtered water (Figure 5.2).

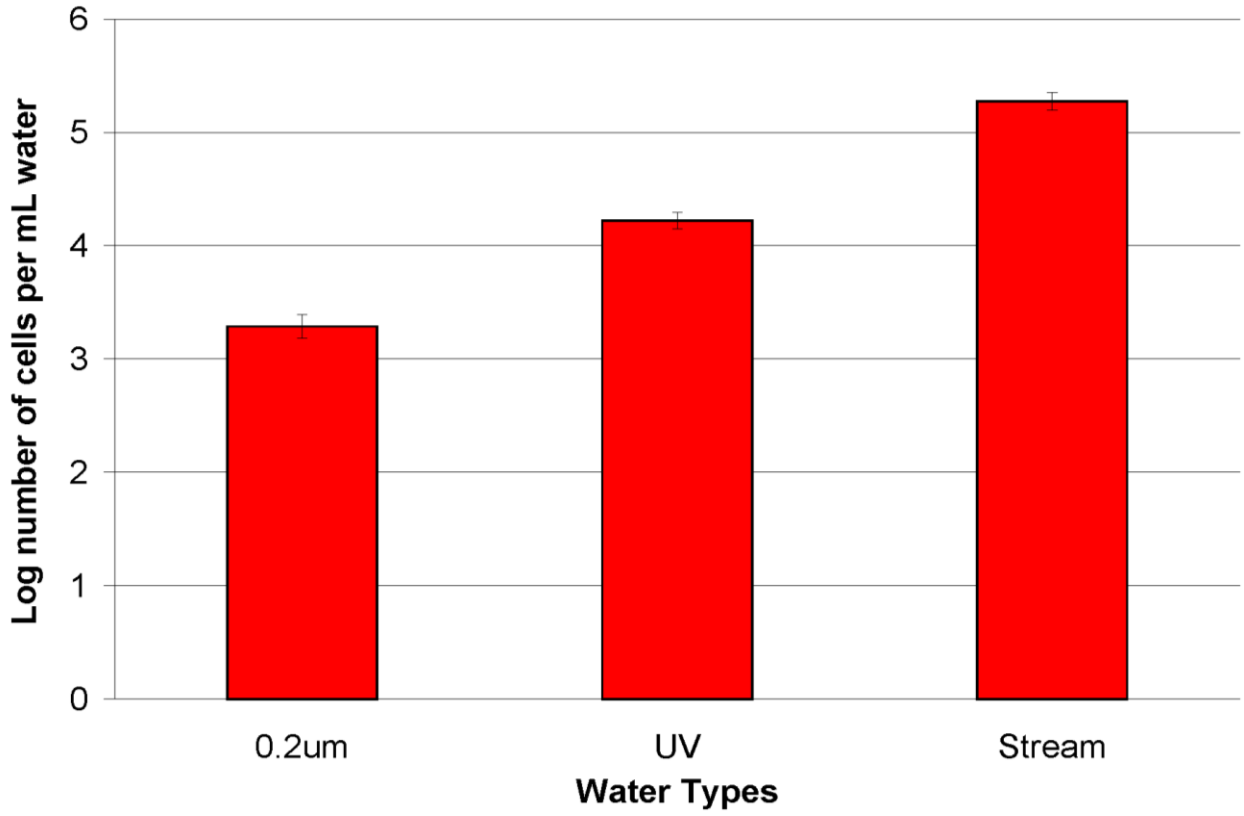

Figure 5.2. Direct microscopic counts for microbial density in each water type.

Microbial composition of each water type was characterized using 454 pyrosequencing at both the phylum/class level (Table 5.1). A significant difference in water microbial community structure among the three water types was detected at the phylum/class level. The stream water microbial community was dominated by beta-Proteobacterium, Bactroidetes, and Actinobacteria. UV treatment decreased the relative abundance of Bacteroidetes and beta-Proteobaccteria, and increased the relative abundance of Actinobacteria, Deinococcus-Thermus, and Firmicutes. The relative abundance of alpha-Proteobacteria and Chloroflexi increased after treatment with 0.2 µm

filtration. The microbial community composition in each water type was also analyzed at the genus level and the results are provided in Table 5.2 for reference.

Microbial community analysis using TRFLP

The effect of water type on the egg surface microbial communities was examined using PCA with TRFLP data. Principal component analysis showed that the microbial communities on the egg surfaces were clustered by water types in which they were fertilized and reared (Figure 5.3). Within each water type, there was a directional temporal trend along with egg developmental stages (Figure 5.3). Temporal patterns were more explicitly observed for eggs fertilized in stream water and 0.2 µm filtered water.

We further tested whether the effect of dispersal is dependent on the concentration of aquatic microbes. PCA showed that when eggs were fertilized in 0.2 µm filtered water and subsequently reared in stream water, the egg surface microbial community assembly converged with the community on eggs fertilized and reared in stream water (arrow in Figure 5.4). However, when eggs were fertilized in stream water and subsequently reared in 0.2 µm filtered water, the egg surface microbial community assembly did not converge with the community on eggs fertilized and reared in 0.2 µm filtered water (Figure 5.4), but rather was clustered with the community on eggs fertilized and reared in stream water.

Table 5.1. Comparison of microbial communities in different water types using 454 pyrosequencing analyzed at the phylum/class level.

Phylum/Class	Stream AF (%)	Phylum/Class	UV AF (%)	Phylum/Class	0.2 AF (%)
Betaproteobacteria	46.3	Actinobacteria	23.8	α-proteobacteria	35.8
Bacteroidetes	29.6	α-proteobacteria	17.9	Chloroflexi	17.9
Actinobacteria	7.4	Deinococcus-Thermus	15.9	β-proteobacteria	12.3
α-proteobacteria	4.4	β-proteobacteria	11.9	Bacteroidetes	9.9
Firmicutes	1.8	Firmicutes	7.3	γ-proteobacteria	4.3
Cyanobacteria	1.3	Chloroflexi	7.3	Actinobacteria	4.3
γ-proteobacteria	1.2	Bacteroidetes	5.3	Deltaproteobacteria	3.1
OD1	1.1	γ-proteobacteria	3.3	Firmicutes	3.1
δ-proteobacteria	0.8	Cyanobacteria	0.7	TM7	0.6
un-proteobacteria	0.8	Chlorobi	0.7	Armatimonadetes	0.6
Verrucomicrobia	0.7	Unclassified phylum	6.0	Acidobacteria	0.6
Acidobacteria	0.2			Unclassified phylum	7.4
Chloroflexi	0.1				
TM7	0.1				
ε-proteobacteria	0.1				
Gemmatimonadetes	0.1				
Planctomycetes	0.1				
Nitrospira	0.1				
Chlamydiae	0.1				
Fusobacteria	0.0				
Armatimonadetes	0.0				
OP11	0.0				
Unclassified phylum	3.6				

Samples were collected from each water type at 6 hours after fertilization. Numbers in the table indicate the relative abundance of each phylum in each water community. Total reads for each sample were 6434, 151, and 162 for stream AF, UV AF, and 0.2 μm AF, respectively. “AF” stands for after fertilization.

Table 5.2. Comparison of microbial communities in different water types using 454 pyrosequencing analyzed at the genus level.

Genus	Stream AF (%)	Genus	UV AF (%)	Genus	0.2 AF (%)
Limnohabitans	22.1	Microbacterium	18.5	Asticcacaulis	16.7
Flavobacterium	13.2	Deinococcus	15.9	Cytophaga	6.2
Polynucleobacter	3.2	Flavobacterium	4.6	Brevundimonas	6.2
OD1_incertae_sedis	1.1	Brevundimonas	4.6	Caulobacter	5.6
Fluviicola	1.0	Caulobacter	3.3	Delftia	1.9
Sphaerotilus	0.9	Novosphingobium	3.3	Limnohabitans	1.9
Acidovorax	0.9	Sphaerotilus	2.0	Pseudomonas	1.9
Methylophilus	0.9	Schlegelella	2.0	Sphaerotilus	1.2
Arcicella	0.9	Asticcacaulis	2.0	Cellvibrio	1.2
Albidiferax	0.8	Pseudomonas	1.3	Bacillus	1.2
Bacillariophyta	0.8	Bacillus	1.3	Thermopolyspora	0.6
Sphingomonas	0.5	Sphaerobacter	1.3	TM7_incertae_sedis	0.6
Aquabacterium	0.4	Aquabacterium	0.7	Fluviicola	0.6
Massilia	0.4	Hydrogenophaga	0.7	Leptothrix	0.6
Clostridium sensu	0.4	Massilia	0.7	Polynucleobacter	0.6
Opitutus	0.4	Naxibacter	0.7	Acidovorax	0.6
Brevundimonas	0.4	Aquamicrobium	0.7	Hydrogenophaga	0.6
Sediminibacterium	0.4	Methylobacterium	0.7	Pelomonas	0.6
Algoriphagus	0.3	Sphingobium	0.7	Methylophilus	0.6
Leptothrix	0.3	Rhodobacter	0.7	Bosea	0.6
Polaromonas	0.3	Acinetobacter	0.7	Rhizobium	0.6
Rhodobacter	0.3	Streptophyta	0.7	Pedomicrobium	0.6
Caulobacter	0.2	Ignavibacterium	0.7	Phenylobacterium	0.6
Other classified	5.6	Paenibacillus	0.7	Other classified	4.3
Unclassified	44.2	Unclassified	31.8	Unclassified	43.8

Samples were collected from each water type at 6 hours after fertilization. Numbers in the table indicate the relative abundance of each genus in each water community. Total reads for each sample were 6434, 151, and 162 for stream AF, UV AF, and 0.2 μm AF, respectively. “AF” stands for after fertilization.

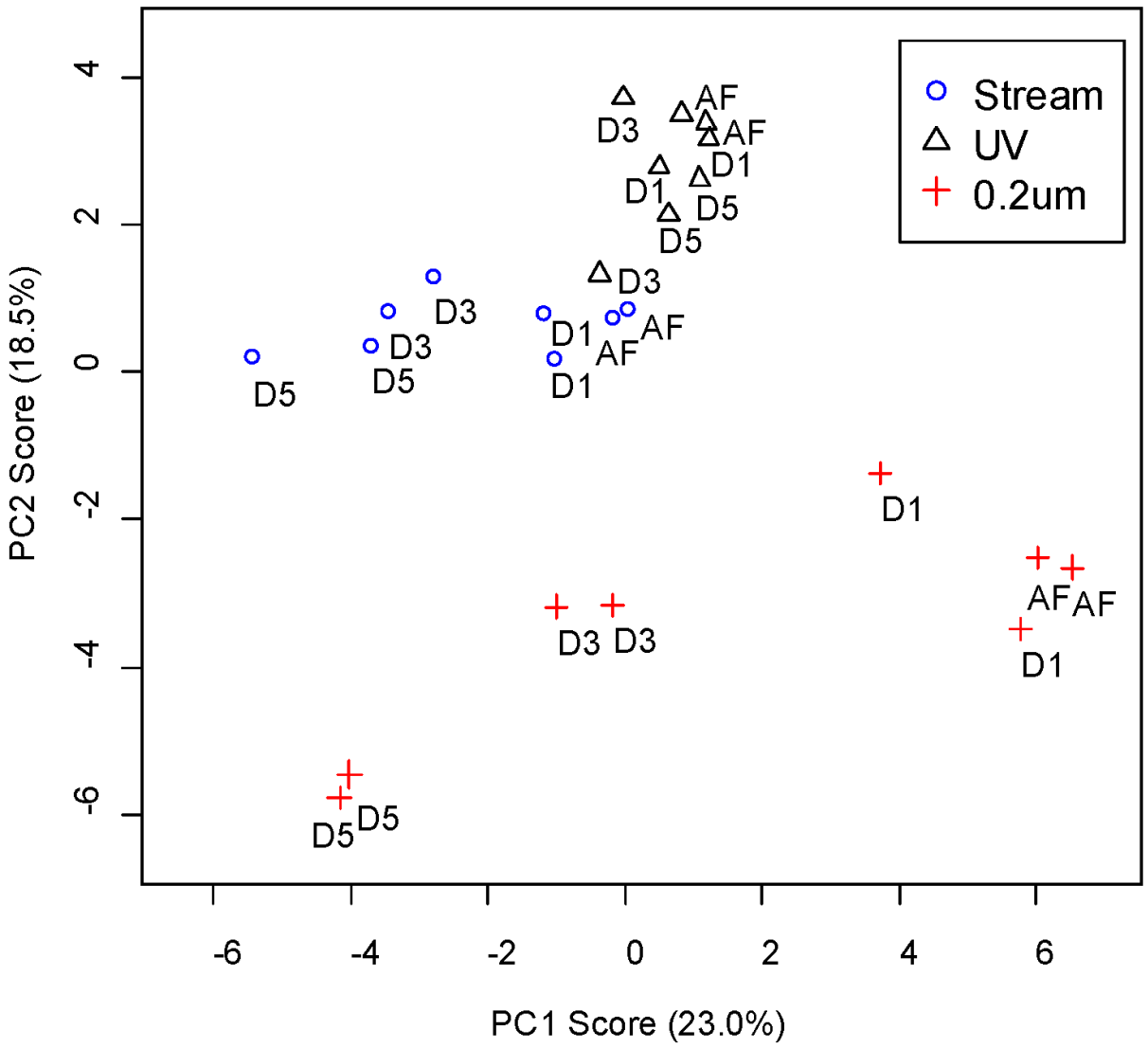

Figure 5.3. The effect of water type (aquatic microbial community) on the egg surface microbial communities as measured using T-RFLP.

PCA shows that eggs that were fertilized and reared in stream water had different egg surface microbial communities from those reared in either UV-treated or 0.2 µm filtered water. “AF”, “D1”, “D3”, and “D5” stand for 6 hours after fertilization, Day 1, Day 3, and Day 5 post-fertilization, respectively.

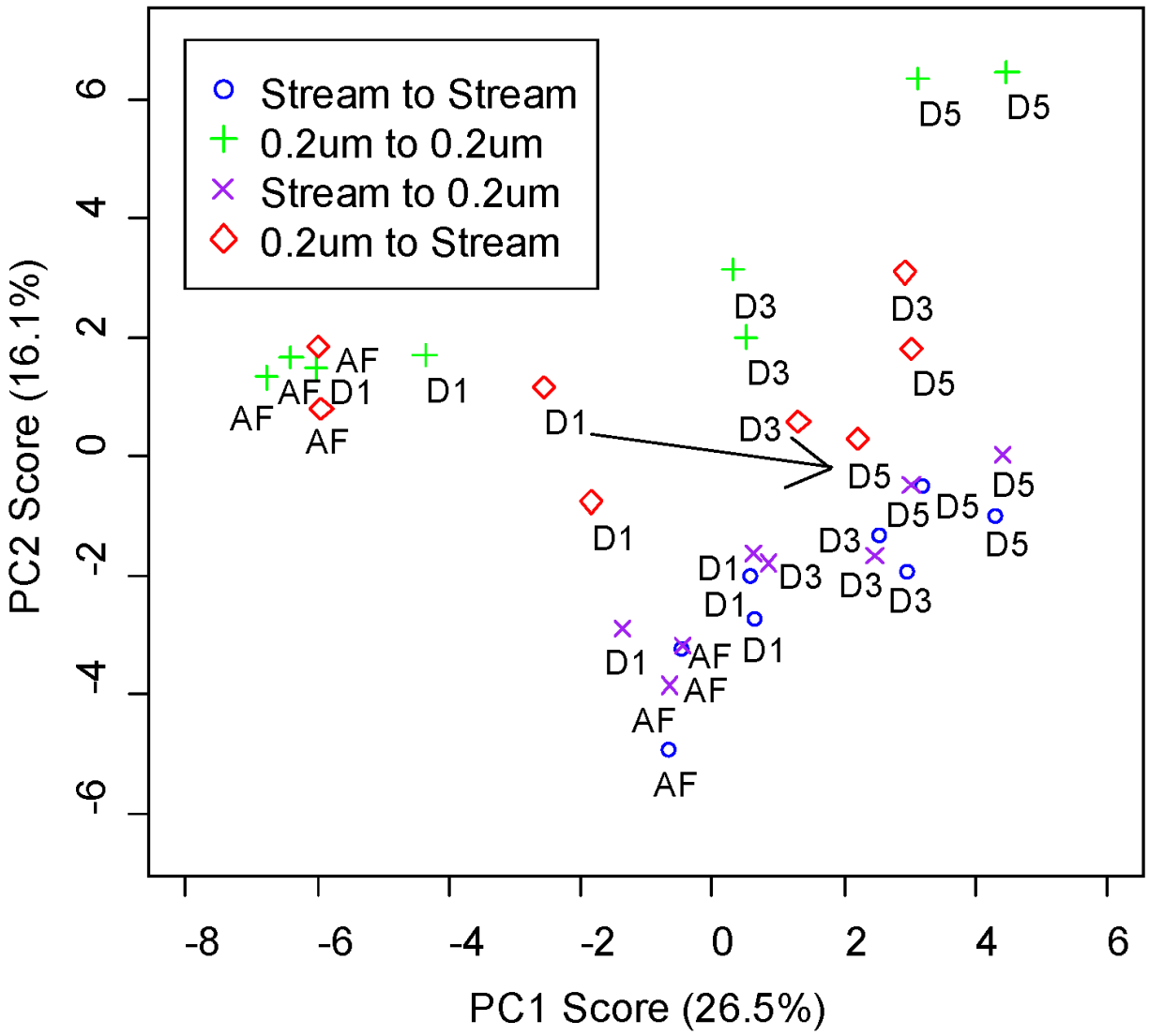

Figure 5.4. PCA plot showing that dispersal was dependent on water microbial density.

When eggs were fertilized in 0.2 µm filtered water and subsequently reared in straight stream water, the egg surface microbial community assembly converged with those on eggs fertilized and reared in stream water. However, when eggs were fertilized in stream water and subsequently reared in 0.2 µm filtered water, the egg surface microbial community assembly did not converge with those of eggs fertilized and reared in 0.2 µm filtered water. “AF”, “D1”, “D3”, and “D5” stand for 6 hours after fertilization, Day 1, Day 3, and Day 5 post fertilization, respectively.

We compared water microbial communities and the egg microbial communities using PCA with TRFLP data. Our hypothesis was that if the dispersal is dominant over deterministic processes, the microbial community assembly of the egg surfaces is similar to that of the source water. PCA revealed that the egg associated microbial communities were not similar to the source aquatic microbial communities (Figure 5.5). Egg surface microbial communities were clustered together and were separated from water microbial communities in the PCA plane. Although the egg microbial communities were clustered tightly (red circle in Figure 5.5), there was an observable effect of aquatic microbial community on the egg microbial community as evident by the clustering by distinct water type within this egg cluster. This effect of the water microbial community on the egg surfaces is small compared to the local egg effect, which separated egg microbial communities from the source water microbial communities. There was also a temporal trend detected in this PCA plane (Figure 5.5). Microbial communities on eggs collected 6 hours after fertilization were closer to the source water microbial communities, and the egg microbial communities diverged from the source water microbial communities as eggs developed.

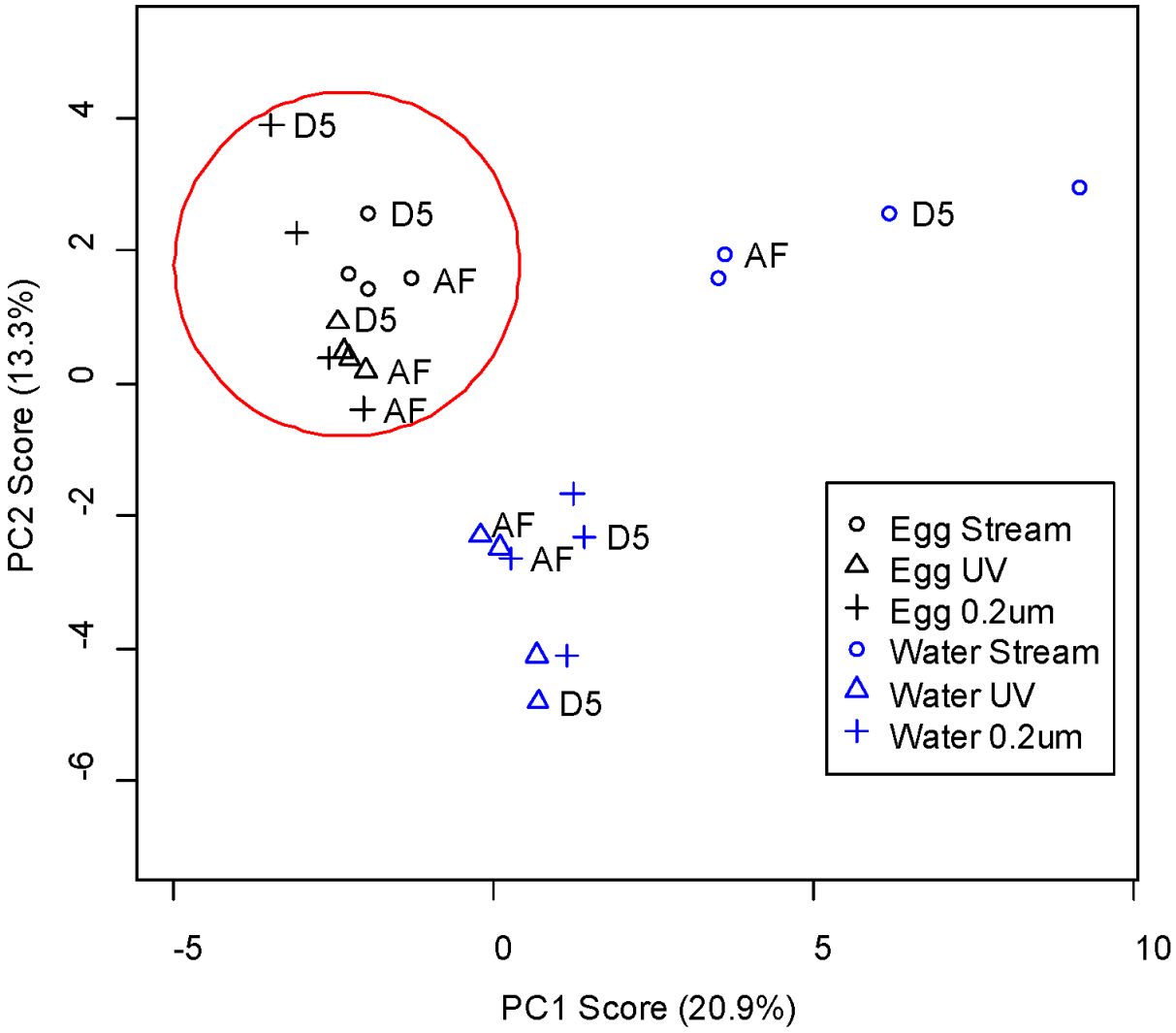

Figure 5.5. PCA analysis on the relative importance of dispersal and deterministic processes in community assembly as measured with T-RFLP.

PC1 and PC2 separated egg surface microbial communities from water microbial communities. The red circle indicates a cluster of egg microbial communities. "AF" denotes samples collected 6 hours after fertilization and "D5" denotes samples collected at Day 5 post-fertilization.

454 pyrosequencing analysis

We performed 454 pyrosequencing for both stream water fertilized egg samples and the source stream water sample to answer a question about what microbes were selected for or against by eggs. Pyrosequencing analysis revealed a difference in the microbial community assembly between the egg surfaces and the source water at the phylum level (Table 5.3). Three phyla including Bacteroidetes, Actinobacteria, and Firmicutes were found to be less abundant on the egg surfaces than the source water. Genus level analysis revealed that genera including *Albidiferax*, *Roseateles*, *Hydrogenophaga*, *Sphaerotilus*, *Aquabacterium*, *Caulobacter*, *Pseudomonas*, and others were found to be more abundant on egg surfaces than in the source water, while genera including *Polynucleobacter*, *Limnohabitans*, and *Flavobacterium* were found to be less abundant on the egg surfaces relative to the source water (Table 5.4).

Table 5.3. Comparison between the egg associated microbial communities and source water microbial communities at 6 hours after fertilization using 454 pyrosequencing analyzed at the phylum level.

Phylum	*QB Egg SS AF	RC Egg SS AF	Stream Water AF
Proteobacteria	3629	4254	3449
Firmicutes	1	2	116
Bacteroidetes	710	768	1905
Chloroflexi	1	2	7
Nitrospira	0	1	4
Actinobacteria	60	114	477
TM7	0	7	6
Acidobacteria	17	3	16
Fusobacteria	0	0	1
Verrucomicrobia	25	40	48
Gemmatimonadetes	1	2	5
Planctomycetes	1	0	5
Armatimonadetes	2	8	1
OD1	6	6	70
Chlamydiae	0	0	4
Cyanobacteria/Chloroplast	109	181	86
Deinococcus-Thermus	2	1	0
OP11	0	1	1
Unclassified phylum	320	175	233
Total reads	4884	5565	6434

*"QB" and "RC" are family codes used for this experiment. "SS" denotes a treatment in which eggs were fertilized in stream water and reared in stream water. "AF" stands for 6 hours after fertilization.

Table 5.4. Comparison of the egg associated microbial communities and source water microbial communities at 6 hours after fertilization using 454 pyrosequencing analyzed at the genus level.

Genus	*QB Egg SS-AF	RC Egg SS-AF	Stream Water AF	Abundance on eggs
Albidiferax	199	201	53	Higher
Roseateles	14	13	0	Higher
Hydrogenophaga	59	57	12	Higher
Sphaerotilus	850	937	61	Higher
Aquabacterium	117	113	27	Higher
Rubrivivax	10	15	0	Higher
Ideonella	366	397	8	Higher
Duganella	44	33	4	Higher
Novosphingobium	71	217	5	Higher
Caulobacter	49	108	16	Higher
Rhodobacter	147	231	17	Higher
Pseudomonas	24	48	2	Higher
Ferruginibacter	16	11	1	Higher
Flectobacillus	172	81	2	Higher
Polynucleobacter	11	28	204	Lower
Limnohabitans	109	101	1419	Lower
Flavobacterium	177	245	850	Lower
Sediminibacterium	2	1	24	Lower
Other classified	510	826	884	
Total unclassified	1937	1902	2845	
Total reads	4884	5565	6434	

* The 18 genera that exhibited a difference in abundance between the egg surface and source water are shown. “QB” and “RC” are family codes used for this experiment. “SS” denotes a treatment in which eggs were fertilized in stream water and reared in stream water. “AF” stands for 6 hours after fertilization.

454 pyrosequencing was also performed to examine the effect of water types on the egg microbial community assembly and the temporal trend within each treatment group (Table 5.5). We found a trend in which some genera were more closely associated with eggs fertilized and reared in certain water types (Table 5.5). Genera including *Acidovorax, Methyloversatilis, Shinella, Bosea, Asticcacaulis, Caulobacter, Pseudomonas, Cellvibrio, Bdellovibrio,* and *Bacteriovorax* were strongly associated with eggs fertilized and reared in 0.2 µm filtered water, while genera including *Naxibacter, Ferribacterium, Brevundimonas,* and *Deinococcus* were strongly associated with eggs fertilized and reared in UV treated water (Table 5.5). Some genera were strongly associated with eggs fertilized and reared in both 0.2 µm filtered water and UV treated water, which were genus *Roseateles* and *Hydrogenophaga*. The abundance of the egg associated microbes, including genus *Hydrogenophaga, Methyloversatilis, Shinella, Methylophilus, Bosea, Bdellovibrio,* and *Bacteriovorax* increased along with the egg development. The abundance of the egg associated microbes including genus *Ideonella, Asticcacaulis, Caulobacter* decreased with egg development. The temporal trend was consistent regardless of rearing water type.

Table 5.5. Water treatment effect on egg microbial community plus temporal trend analyzed using 454 pyrosequencing at genus level (RC family data).

	0.2 um				Stream				UV				Water	Peak
Genus name	AF	D1	D3	D5	AF	D1	D3	D5	AF	D1	D3	D5	Type	Time
Polynucleobacter	1	6	2	6	28	18	36	63	0	0	0	0	Stream	
Acidovorax	33	216	129	118	13	8	8	17	1	8	23	44	0.2um, UV	
Albidiferax	81	481	533	286	201	248	124	97	0	32	49	28	0.2um, Stream	
Roseateles	12	64	45	43	13	7	11	14	5	88	115	72	0.2um, UV	
Limnohabitans	14	16	4	8	101	75	45	23	0	1	0	0	Stream	
Hydrogenophaga	120	657	705	1352	57	53	106	137	14	134	599	838	0.2um, UV	Late
Sphaerotilus	49	334	614	829	937	1382	1446	2324	3	89	41	71	0.2um, Stream	
Aquabacterium	37	140	92	205	113	110	220	209	51	181	242	301	All	
Ideonella	62	139	42	26	397	329	83	36	8	120	33	39	All	Early
Leptothrix	5	20	8	111	58	22	45	63	0	1	0	0	0.2um, Stream	
Undibacterium	10	37	21	17	32	33	5	12	0	7	0	0		
Naxibacter	0	8	4	3	0	2	0	0	0	80	32	2	UV	Middle
Duganella	0	3	1	1	33	47	6	9	2	7	0	0	Stream	Early
Massilia	4	14	8	19	16	13	3	4	0	67	3	2		
Vogesella	0	0	0	0	10	11	4	0	1	6	0	0		
Ferribacterium	0	6	6	19	4	1	1	8	2	17	18	98	UV	Late
Methyloversatilis	16	24	21	111	2	2	0	19	0	2	0	0	0.2um	Late
Shinella	2	7	99	74	0	5	22	23	0	0	2	3	0.2um	Late
Methylophilus	30	81	101	187	66	26	77	111	12	43	126	249	All	Late
Sphingomonas	14	20	14	23	23	6	8	29	10	13	8	5		
Novosphingobium	5	26	21	27	217	43	153	265	33	112	249	633	Stream, UV	

"AF", "D1", "D3", and "D5" stand for 6 hours after fertilization, Day1, 3, and 5 post fertilization, respectively. "Water Type" implies water type affiliation that a genus exhibited on the eggs. "Peak Time" implies the temporal affiliation that a genus exhibited on eggs.

Table 5.5 (cont'd)

Sphingobium	17	1	10	16	8	3	20	22	1	42	52	131		
Bosea	6	2	26	88	1	1	17	10	0	0	4	8	0.2um	Late
Devosia	6	11	21	48	10	2	1	16	0	2	0	2		
Rhizobium	26	71	417	602	13	21	53	76	1	12	17	13		
Asticcacaulis	149	267	28	15	4	0	0	1	0	3	0	0	0.2um	Early
Brevundimonas	44	166	21	46	38	16	0	34	29	203	111	569	UV	
Caulobacter	257	724	72	59	108	41	0	15	8	45	18	18	0.2um	Early
Rhodobacter	19	27	40	33	231	115	17	92	1	18	2	1	Stream	
Rhizobacter	10	17	30	71	8	6	16	16	0	1	1	5		
Pseudomonas	163	445	134	26	48	5	10	2	1	11	2	0	0.2um	
Cellvibrio	178	327	52	24	1	1	0	0	0	0	0	0	0.2um	
Aeromonas	0	3	0	0	1	7	4	3	0	15	3	0		
Rheinheimera	62	206	117	35	25	53	8	1	1	69	2	5		
Bdellovibrio	0	1	41	65	0	0	0	7	0	0	0	0	0.2um	Late
Bacteriovorax	2	22	75	129	0	3	8	7	0	1	0	3	0.2um	Late
Flavobacterium	14	7	10	0	245	62	27	60	1	20	5	10	Stream	
Flectobacillus	0	6	27	31	81	205	78	277	11	46	42	72	Stream	
Bacillariophyta	2	1	0	0	161	60	35	27	0	0	0	0	Stream	
Deinococcus	0	5	0	0	1	1	0	1	113	114	129	290	UV	
Other classified	89	193	160	244	358	119	121	164	37	85	149	132		
Total unclassified	921	3149	3721	3339	1902	1598	2453	1958	720	1847	3305	2777		
Total Reads	2460	7950	7472	8336	5565	4760	5271	6252	1066	3542	5382	6421		

Quantitative PCR analysis

The microbial quantity on the surfaces of eggs fertilized and reared in stream water increased from 10^{6} to $10^{7.5}$ 16S rRNA gene copies per egg (Figure 5.6). Eggs fertilized in UV treated and 0.2 µm water had lower microbial quantities compared to that fertilized in stream water throughout incubation, except at Day 3 when microbial quantity on eggs fertilized in all three water types were similar to each other. The microbial load on the egg surfaces fertilized and reared in UV treated water increased from $10^{5.4}$ to 10^{7} 16S rRNA gene copies per egg during embryogenesis, while eggs fertilized and reared in 0.2 µm filtered increased from $10^{5.5}$ to $10^{6.5}$ 16S rRNA gene copies per egg during embryogenesis (Figure 5.7). The surfaces of eggs fertilized and reared with 0.2 µm filtered water had a slightly greater number of microbes at the beginning of the incubation period relative to those fertilized and reared in UV filtered water, although one of the two replicates had an almost identical quantity in the two treatments.

We were interested in investigating how microbial quantity of the eggs fertilized in stream water and reared in 0.2 µm filtered water changed over time, since we observed that the egg microbial community fertilized in stream water and reared in 0.2 µm thereafter did not converge with the f eggs that were fertilized in stream water and reared in 0.2 µm filtered water followmicrobial community observed on eggs fertilized and reared in 0.2 µm. Microbial quantity oed the trend of eggs fertilized and reared in stream water, which was noticeably different from the trend we observed for eggs fertilized and reared in 0.2 µm filtered water (Figure 5.7).

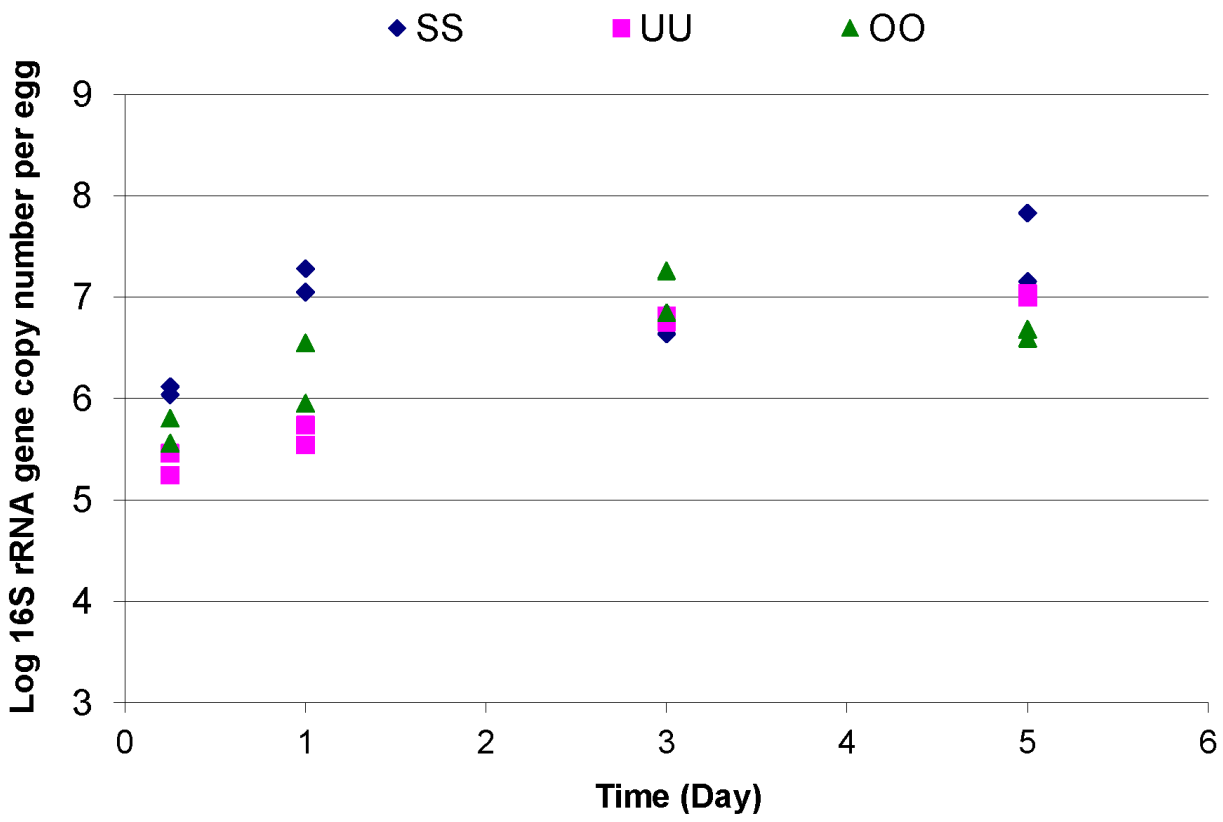

Figure 5.6. Quantification of the egg associated microbes across different treatments using qPCR.

"SS", "UU", and "OO" stand for eggs fertilized and reared in stream water, UV treated water, and 0.2 µm filtered water, respectively.

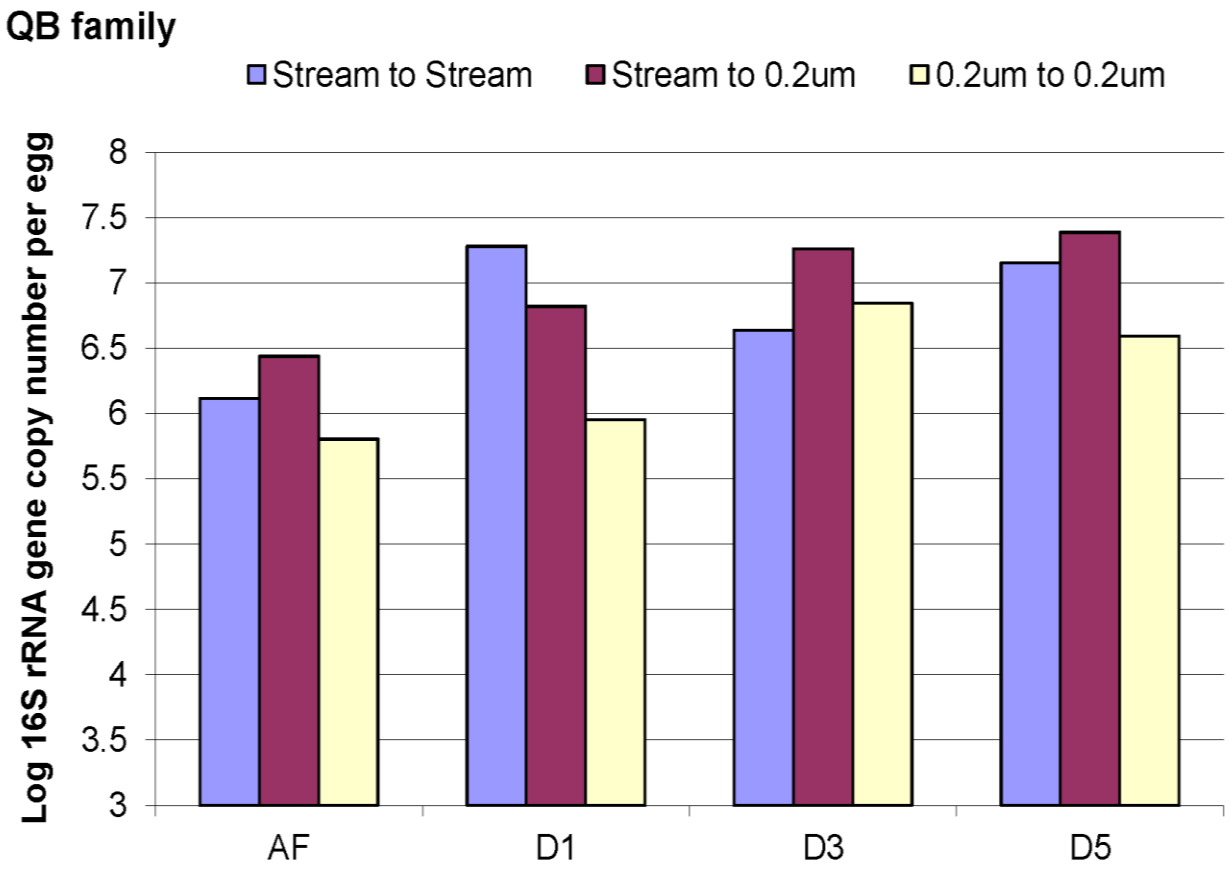

Figure 5.7. The effect of transfer from stream water to 0.2 µm filtered water on the egg surface microbial quantity.

"AF", "D1", "D3", and "D5" stand for 6 hours after fertilization, Day 1, Day 3, and Day 5 post-fertilization, respectively.

Larvae size analysis

The effect of rearing environments on yolk sac area was examined. Larvae that were fertilized and reared in 0.2 µm filtered water had significantly larger yolk sac area (8.35 ± 0.84 mm^2) than those that were fertilized and reared in stream water (7.98 ± 0.65 mm^2) (t_{315}=3.485, p<0.001, Figure 5.8). There was also a statistically significant difference in yolk sac area between larvae fertilized and reared in UV treated water (8.22 ± 0.78 mm^2) and those fertilized and reared in stream water (7.98 ± 0.65 mm^2) (t_{315}=2.247, p=0.03, Figure 8). The effect of water type on yolk sac area was significant after accounting for the family effect on yolk sac area with a family as a random variable using the mixed effect model (data not shown).

Egg mortality

The effect of rearing environments on the egg mortality was studied. Eggs that were exposed to stream water for at least one time point during embryogenesis had higher egg mortality than eggs exposed to all other treatments (Figure 5.9). Both eggs fertilized and reared in 0.2 µm filtered water and eggs fertilized in UV treated water and reared in 0.2 µm filtered water had the lowest egg mortality (33%) of all treatments (Figure 5.9). Eggs fertilized and reared in stream water had the highest egg mortality (58%) (Figure 5.9). The difference in the egg mortality between eggs fertilized and reared in 0.2 µm filtered water and fertilized and reared in stream water was statistically significantly different (t_{43} = 1.76, p=0.086).

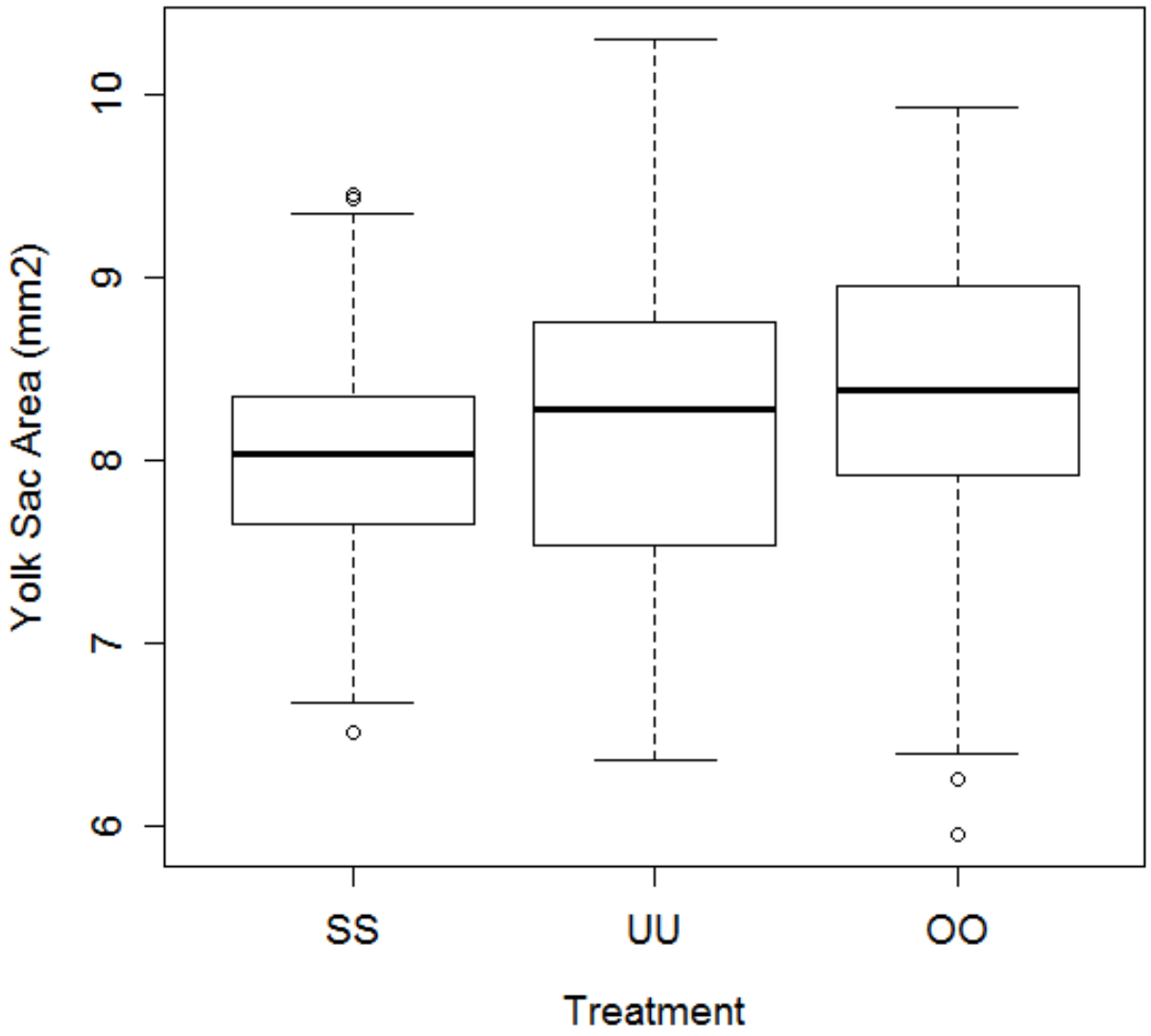

Figure 5.8. The effect of rearing water type on yolk sac resource uses.

"SS", "UU", and "OO" stand for eggs fertilized and reared in stream water, UV treated water, and 0.2 µm filtered water, respectively. The unit of yolk sac area is mm^2.

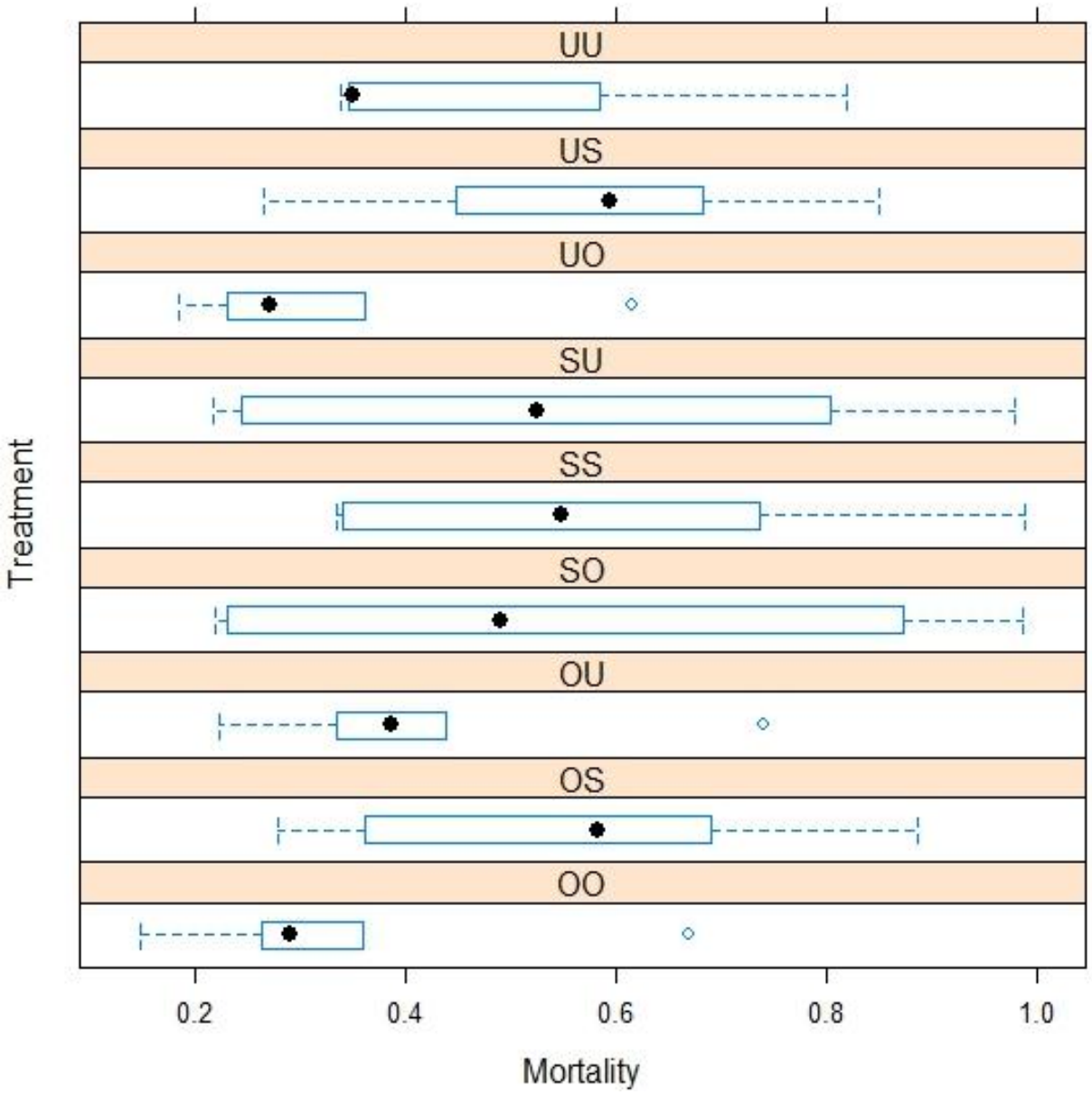

Figure 5.9. A box plot showing the effect of fertilization and rearing environment on egg mortality.

S stands for stream, U stands for UV treated, and O stands for 0.2 µm filtered. The first letter represents the treatment for fertilization and the second letter represents the treatment for rearing environment.

Discussion

In this study, we demonstrated that both egg-related local deterministic processes and the dispersal of aquatic microbes onto egg surfaces are important processes in explaining Lake Sturgeon egg surface microbial communities. At the rate of dispersal we tested, the local deterministic processes appeared to be a dominant process over dispersal, which did not meet my hypothesis. However, dispersal was also an important process in shaping the egg surface microbial communities, and its effect on microbial communities appeared dependent on aquatic microbe density, as we hypothesized.

Our results revealed that 6 hours after fertilization, the egg microbial community assembly was already significantly different from the source water microbial community. This fact suggests that the egg-related deterministic processes acted quickly to shape the community. We believe that this rapid process was mediated by host innate immunity, specifically immunity that was maternally provisioned [36, 47-49]. Phylum Actinobacteria, one of the dominant phyla in water microbial communities, had low representation on the egg surfaces. This is likely because the host innate immunity (including maternally provisioned lysozyme) acted on the peptidoglycan layer of this gram positive phylum. Another possible explanation is that the difference in the community structure between eggs and source water was mediated by the ability of aquatic microbes to adhere to the egg surfaces [34-35], since not all microbes adhered to the egg surfaces with equal affinity. *Sphaerotilus* spp., one of the dominant genera found on the egg surfaces in this study, is known to possess a sheath structure that facilitates adherence to solid surfaces [50].

The temporal trend in microbial community succession that we observed was also driven by deterministic processes. The direction of the community shift observed on the egg surfaces

showed a gradual divergence from the source water microbial community. Furthermore, the egg surface microbial community at Day 5 had significantly diverged from the source water microbial communities, specifically water samples collected at the same time point. This suggests that the changes we observed on the egg surface microbial communities were not derived from the source water microbial communities. In other words, continuous dispersal from the source water did not seem to affect the development of microbial communities on the egg surfaces. The local processes that caused the shift in microbial communities could have been changes in metabolites on the egg surfaces from urea to ammonia [39, 51-52], microbe-microbe interactions (see Chapter 6), biofilm formation (see Chapter 6), or changes in lysozyme type from maternally provisioned to egg secreted [53-54]. *Hydrogenophaga* spp., which are known to be dominant in stream biofilm [55], were found to increase on the egg surfaces in this study.

We believe that the local deterministic processes consisted of two distinct processes. The first process was one that acted quickly within 6 hours post fertilization. This process is representative of factors related to host innate immunity and host surface chemistry that select for attachment of certain microbes and is the most dominant process of all in explaining the egg surface microbial community assembly. The second process had more subtle effect on the egg surface microbial community, but gradually shaped the community over time, a process which is represented by changes in metabolites and microbe-microbe interactions.

We hypothesized that dispersal of aquatic microbes from water column onto the egg surfaces is the most dominant factor and the egg microbial communities converge with the source water microbial community. However, at the rate of dispersal we tested in this study, the hypothesis did not hold true. Instead, egg microbial communities diverged from the source water microbial communities over time. However, dispersal did play some role in shaping the

microbial communities because egg microbial communities fertilized and reared in different water types had different egg microbial community assemblages. One significant finding was that the effect of dispersal on egg microbial community assembly was dependent on the concentration of water microbes. This is evident when considering the fact that initial inocula on the egg surface were not important in explaining the final community composition when eggs were reared in water with a high density of microbes. However, as the density of water microbes declined, the majority of change accrued in the egg micro-environment was nested within the initial inocula and did not appear to be influenced by dispersal.

Dispersal did not play significant role in explaining microbial quantity on the egg surfaces, especially when eggs were transferred from stream water to 0.2 µm filtered water. Microbial quantity continued to grow even after eggs were transferred to aquatic environment with a low level of microbial concentration. The microbial community composition that developed after the transfer from stream water to 0.2 µm filtered water was not similar to that of fertilized and reared in 0.2 µm filtered water, suggesting that the additional microbial load on the egg surface did not come from the 0.2 µm filtered water. These lines of evidence suggest that microbial quantity cannot be directly explained by dispersal and is instead likely attributed to microbial growth among the initial colonizers.

Eggs fertilized and reared with 0.2 µm filtered water had a slightly greater number of microbes at the beginning of incubation relative to those fertilized and reared in UV filtered water, although one of the two replicates showed the almost identical quantity between the two. This difference in microbial quantity on the egg surface could be due to the fact that the compositions of microbes in 0.2 µm filtered water were not selected against by eggs, in other words they successfully colonized eggs. We found that genus *Acidovorax*, which can effectively

colonize the egg surfaces as described in Chapter 4, became dominant at Day 1 on egg surfaces fertilized and reared in 0.2 µm filtered water. This line of evidence suggests that not only the quantity of aquatic microbes, but also composition of the aquatic microbial community may be important in explaining the effect of aquatic microbes on the egg surface microbial quantity.

Our study also demonstrated that history may matter in explaining the future development of a microbial community. Several lines of evidence suggest that the effect of initial colonization on development of subsequent microbial communities was likely dependent on both available space on eggs and/or the concentration of the source water microbes. When eggs were fertilized in 0.2 µm filtered water for 6 hours and were transferred and reared in the stream water with high microbial load, the initial microbial community structure was masked and converged with the community of stream fertilized and reared eggs. However, when the eggs fertilized in stream water (with high microbial load) for 6 hours were transferred and reared in 0.2 µm filtered water, the egg associated microbial community did not converge with that fertilized and reared in 0.2 µm filtered water. This suggested that either available space on egg surface and/or the concentration of the source water microbes plays a role in determining the subsequent microbial community assembly. This suggests that the history of community composition may be important particularly when a large impact (large microbial load) occurred in the initial stage and/or smaller impacts (small microbial dispersal) occurred in the later stages.

Although the effect of dispersal in shaping the microbial community structure was small, the minor differences in community structure affected the life history traits of the host, including egg mortality and yolk sac area at hatch. We identified genera that specifically associated with eggs that had a lower egg mortality and lower yolk sac resource use. We believe that genera associated with eggs fertilized and reared in 0.2 µm filtered water and/or UV treated water

(described in Table 3) are good candidates for probiotic treatment for the fish eggs. In fact, genera *Acidovorax* and *Pseudomonas* which we identified as putative symbionts for the sturgeon eggs in other chapters (Chapter 4 and Chapter 6, respectively) were dominant on the egg surfaces fertilized and reared in 0.2 μm filtered water.

Our study contributes to the broader literature on microbial community assembly and succession by demonstrating that both local deterministic processes and dispersal play roles in shaping the microbial communities assembly on the egg surfaces. PCA separation of egg surface microbial communities from water microbial communities indicates that deterministic processes on the egg surfaces are more dominant than dispersal. These dominant processes occurred fairly quickly within 6 hours. We believe that these processes can be mediated via lysozyme and/or abilities of microbes to adhere to eggs. It is important for us to study sturgeon egg chemistry in the future to further elucidate the mechanisms shaping this early stage of host-microbe interaction.

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CHAPTER 6: CHARACTERIZATION OF BACTERIAL ISOLATES FROM THE EGG SURFACES OF LAKE STURGEON (*ACIPENSER FULVESCENS*) FOR ANTAGONISTIC INTERACTIONS AND BIOFILM FORMING CAPABILITIES

Abstract

Microbes often interact in antagonistic ways, with some microbes displaying aggressive tendencies over other susceptible microbes. Such interactions also relate to biofilm formation by microbes, which is one mechanism that can be used to defend against antimicrobial activity. Analyzing such interactions can help understand microbial community formation and may also assist in identifying potential microbes that can be used as probiotic treatment to protect against aggressors. In this study, we analyzed antagonistic interactions and biofilm forming capability of 25 representative isolates from the egg surface of the threatened Lake Sturgeon (*Acipenser fulvescens*) using soft agar overlay and a crystal violet biofilm assay. Eight isolates exhibited aggression to at least one other isolate. Antagonistic interactions were dependent on temperature and phylogeny. *Pseudomonas* sp. C22 was found to be the most aggressive strain of all, inhibiting growth of 15 out of the 25 isolates. *Flavobacterium* spp. were found to be one of the least aggressive, and one of the most susceptible genera in the community. Four strong aggressors were tested against 6 known fish pathogens. Each pathogen was inhibited by at least one of the 4 aggressors. *Pseudomonas* sp. C22, which displayed the highest aggressiveness in our study, inhibited growth of 5 out of the 6 fish pathogens. Strong biofilm forming capabilities were observed for 11 isolates and they were dependent on environmental and nutritional

conditions. *Hydrogenophaga* sp. and *Caulobacter* sp., two of the most sensitive isolates to antagonists, were among the best biofilm formers as was the strong aggressor *Pseudomonas* sp. C22. Our results revealed the potential for a complex nature of interactions amongst members of a microbial community on the eggs surface. This study was also significant in revealing microbial populations with potential as a probiotic. The eight antimicrobial producing strains isolated from the eggs surface may provide protection from pathogens.

Introduction

One of the central questions in microbial ecology addresses the nature of interactions occurring among microbes in a complex community. These interactions span the range from highly mutualistic to highly antagonistic. Antagonistic microbial interactions have been studied in different microbial communities including communities associated with sponge, coral leaf, and marine water [1-4]. These studies revealed phylogenetic trends in antagonistic interactions, where some genera aggressively inhibited growth of others [1-3, 5]. These antagonistic interactions were not static, but rather dynamic and influenced by environmental variables such as temperature [4, 6], exposure to oxygen [7-8], biofilm formation [7], and nutrient level [8]. One specific type of antagonistic interaction that has been studied extensively in the past involves antagonistic interactions among host associated microbes against host associated pathogens [4, 6, 9-10]. Identifying such interactions has important implications for potential probiotic application in systems in which control of pathogens is a priority [4, 6, 9-10].

There are a number of remaining unanswered questions in the realm of microbial interactions. Although extensive research has been conducted to characterize individual isolates for their biochemical and physiological properties, little is known regarding how properties of individual isolates translate into broader functional or behavioral activities within complex microbial communities that exist in nature. It is therefore important to characterize interactions among microbes as members of broader communities and to approach the study of antimicrobial activities from a community perspective [11-12]. Such an approach could yield new information about fundamental ecological processes within a structurally complex community.

In a complex community frequently these interactions between populations play out within the context of a biofilm. Microbes have been frequently observed to form biofilm on

surfaces of substrates in natural environments [12]. Relevant to interactions between populations are observations that some antimicrobial substances are expressed only in biofilm [7], and a biofilm formation allows some susceptible microbes to escape from antimicrobial activities [13-14]. Biofilm is also formed to avoid host immune systems, which allows microbes to persist in the host environment [15]. Thus, it is important to simultaneously analyze antagonistic interactions and biofilm formation in order to fully understand interactions among microbes and subsequent microbial community assembly.

The study of antimicrobial interactions and biofilm formation is important for the case of the threatened fish species Lake Sturgeon (*Acipenser fulvescens*). Lake Sturgeon have historically been a valuable asset to Michigan fishermen, most notably for their caviar. Declines in the Lake Sturgeon populations due to over harvesting and habitat degradation [16-17] and low natural recruitment [16] have garnered the attention of the scientific community. Previous studies identified the high egg mortality of the sturgeon [18]. Since fish eggs extruded by female sturgeon are rapidly colonized by a diverse collection of aquatic microbes, the role of microbial communities in influencing egg mortality is of particular interest.

Our previous work identified significant associations between egg mortality and both microbial quantity and community structure surrounding the egg surface (Chapter 3). We also found that local deterministic processes such as host innate immunity, metabolite secretion, and microbe-microbe interactions around the egg micro-environment are key processes shaping the egg surface microbial communities (Chapter 4, 5). This current study builds on previous research by investigating the role of microbe-microbe interactions that may potentially alter the microbial community, and in turn affect mortality of the fish embryo.

In this study, we tested antagonistic interactions and biofilm forming capability among 25 representative isolates from the egg surface of the Lake Sturgeon at two temperature regimes, which are relevant to the temperature range they experience during spawning season. We hypothesized that isolates competing for resources and space in the microbial community may exert some antimicrobial activity and demonstrate biofilm forming abilities in order to promote their own persistence. This study is significant for the management of this threatened species in particular because it will identify potential symbionts for probiotic treatment. The study is also significant in contributing to a broader understanding of microbial community formation in elucidating potential mechanisms of microbial community turnover during embryogenesis.

Methods

Study site and sample collection

Microbes were isolated from the surfaces of Lake Sturgeon eggs from the Black Lake Population in Onaway, Michigan. Two types of fertilized eggs were included in the study; those collected directly from spawning stream and those fertilized and reared in a streamside hatchery. Stream eggs were collected on May 7th and May 17th in 2009 from two different sites in the Upper Black River, which is the sole spawning stream for the Black Lake Population of Lake Sturgeon. Eggs were collected from the bottom of the stream in a net with bottom substrates and were subsequently removed using sterile tweezers. Eggs were rinsed with phosphate buffered saline (PBS) and each egg was placed in a 2mL eppendorf tube with sterile PBS. The eppendorf tubes were placed in a cooler box and were transported to the streamside hatchery within 30 minutes.

The hatchery-fertilized eggs were collected in 2010. Streamside hatchery is located at the riverside of the Upper Black River in Onaway, MI. The hatchery water was pumped up from the spawning stream. Large particulate matter in the stream water was removed using sock filters and the filtered stream water was gravity fed to the hatchery system. Gametes collected from spawning adults were fertilized and reared under different conditions in the hatchery for production. The hatchery eggs were collected from fertilized eggs of three different families reared in two water types (UV radiation-treated and untreated) and at two temperature regimes (12^{o}C and 19^{o}C). The collected eggs were rinsed with PBS and placed in a 2mL eppendorf tube with PBS.

Isolation of bacteria

Both stream-collected and hatchery-collected eggs were processed in the streamside hatchery using the following procedures. Eggs in PBS were vortexed for 3 minutes and the supernatant was diluted with PBS at different dilution factors. 100 µL of the diluted supernatant were plated on R2A (0.5 g proteose peptone, 0.5 g casamino acids, 0.5 g yeast extract, 0.5 g dextrose, 0.5 g soluble starch, 0.3 g dipotassium phosphate, 0.3 g sodium pyruvate, 0.05 g Magnesium sulfate, and 15mg agar in 1 L MiliQ Water). The R2A plates were then incubated at two different temperatures; one at 5^{o}C in a refrigerator and the other at ambient temperature in the hatchery (10 – 18^{o}C). The plates with bacterial isolates were transported to a laboratory in Michigan State University and then processed further. Each of the isolated colonies on R2A was re-streaked on a fresh R2A plate, and an isolated colony on the plate was grown overnight in R2B at room temperature in the laboratory (20 – 22^{o}C) and stored at -80^{o}C with a glycerol

concentration of 15%. Throughout the study, stream-collected isolates are identified by ID numbers preceded by the letters A, B, C, D, or E and hatchery-fertilized isolates are identified by ID numbers preceded by the letter F.

16S rRNA gene sequencing and phylogenetic analysis

Isolated strains were grown in 10 mL of R2B overnight and harvested by centrifugation at 10,000 RPM in an SS34 rotor. Genomic DNA was extracted with a MoBio™ Power Soil DNA extraction kit. The 16S rRNA gene was PCR amplified using "universal" primers 27F (5'-AGA GTT TGA TCM TGG CTC AG - 3') and 1389R (5'-ACG GGC GGT GTG TAC AAG - 3') and the resulting amplicons were purified with Qiagen™ PCR Cleanup columns. Purified PCR products were sequenced at the Michigan State University Research Technology Support Facility using an ABI 3730 capillary electrophoresis system with a 27F primer. The phylogenetic relationships among the 92 egg isolates were inferred using MEGA version 4.0 [19] after previously being aligned using Ribosomal Database Project (RDP) [20]. Phylogenetic relationships were inferred using the Neighbor-Joining algorithm [21] with the Maximum Composite Likelihood method [22]. A total of 471 informative sites (with complete deletion option) within 16S rRNA gene were used to construct the Neighbor-Joining tree. At each node, we calculated the frequency that the isolates appeared in the same cluster after conducting a 1000 bootstrap test.

Soft agar overlay technique for screening antagonists

We examined antagonistic interactions between 25 sturgeon egg isolates (Table 6.1). These isolates were chosen from the total collection to best represent the phylogenetic diversity of the egg surface microbial community. The 25 isolates were revived in 10 mL of R2B

overnight at room temperature (20 - 22^{o}C) for 2 to 3 days. Isolates which could not be grown in the broth were grown in R2A media at room temperature for 48 hours.

Table 6.1. List of isolates used for antagonistic interactions.

Assignment	Phylum/Class	Genus/Species	Strain ID
1	Bacteroidetes	Flavobacterium sp.	A20
2	Bacteroidetes	Flavobacterium sp.	D11
3	Bacteroidetes	Flavobacterium sp.	E17
4	Bacteroidetes	Flavobacterium sp.	B30
5	Bacteroidetes	Flavobacterium sp.	C6
6	Bacteroidetes	Flavobacterium sp.	B10
7	Firmicutes	Bacillus sp.	C20
8	Gammaproteobacteria	Aeromonas salmonicida	A12
9	Gammaproteobacteria	Aeromonas sobria	A25
10	Gammaproteobacteria	Aeromonas enchereia	B25
11	Gammaproteobacteria	Pseudomonas sp.	D2
12	Gammaproteobacteria	Pseudomonas sp.	C22
13	Gammaproteobacteria	Serratia sp.	D14
14	Gammaproteobacteria	Rheinheimera sp.	F1
15	Betaproteobacteria	Massilia sp.	B13
16	Betaproteobacteria	Janthinobacterium sp.	F8
17	Betaproteobacteria	Janthinobacterium sp.	F13
18	Betaproteobacteria	Iodobacter sp.	D4
19	Betaproteobacteria	Deefgea sp.	D10
20	Betaproteobacteria	Vogesella sp.	F3
21	Betaproteobacteria	Undibacterium sp.	F9
22	Betaproteobacteria	Hydrogenophaga sp.	F14
23	Betaproteobacteria	Acidovorax sp.	F19
24	Alpha proteobacteria	Caulobacter sp.	F16
25	Deinococcus-Thermus	Deinococcus sp.	F4

Antagonistic interactions among 25 isolates were screened at both 14^{O}C and 20 – 22^{O}C by stamping 23 of the isolates on a soft agar lawn in which one of the 25 isolates was inoculated. Isolates #1 and #5 were removed from the stamp since they exhibited gliding on the soft agar. The soft agar overlay technique was previously described by Mendoza et al 1997 [23]. The detailed procedure is as follows. The preparation of lawn for soft agar overlay was accomplished by pouring 6 mL of soft R2A (one half of the agar concentration relative to the regular R2A media) inoculated at 55 ºC with 100 µL of each broth culture in the equivalent phase of growth for each trial (2-3 days of growth) or with 300 µL of plate culture suspension (3 loopfulls of plate culture suspended in 1 mL of R2B) on top of a 20 mL pre-solidified regular R2A plate. After cooling, the 23 non-gliding stamp strains were stamped in duplicate on the lawn using a 48 spike stamp and one half of a 96 well-plate filled with 200 µL of broth culture of each stamp strain in each well. Three control plates with un-inoculated soft agar (beginning, middle, end) were included for each trial to ensure that the stamp broth cultures were viable and stamped correctly. The stamped plates including the three controls were then incubated at 20 – 22 ºC or 14 ºC for 2 days.

Antagonistic interactions were determined by the presence of zones of inhibition (zone of clearance in soft agar) observed two days after stamping (Figure 6.1). The screening assay was performed thrice with duplicates on each plate for a total of 6 replicates. Stamp strains which exhibited antagonistic interactions with lawn strains in one of the 6 replicates were further examined with a 12 replicate assay on a single plate to confirm the antagonistic interaction on a one-on-one basis and to determine the extent of inhibition (Figure 6.1). The extent of antagonism was quantified by measuring the radial zone of inhibition from the center of the stamped colony to the point where the lawn strain's normal opacity returned. Width of the zone of inhibition was

calculated subtracting the radius of colonies from the radial zone of inhibition. To investigate the reciprocal relationship of antagonistic interactions, the size of colony of all 23 stamp strains were measured for each lawn strain at both 14^{o}C and 20 – 22^{o}C after 4 days of incubation (the timing of measurements were synchronized for both 14^{o}C and 20 – 22^{o}C).

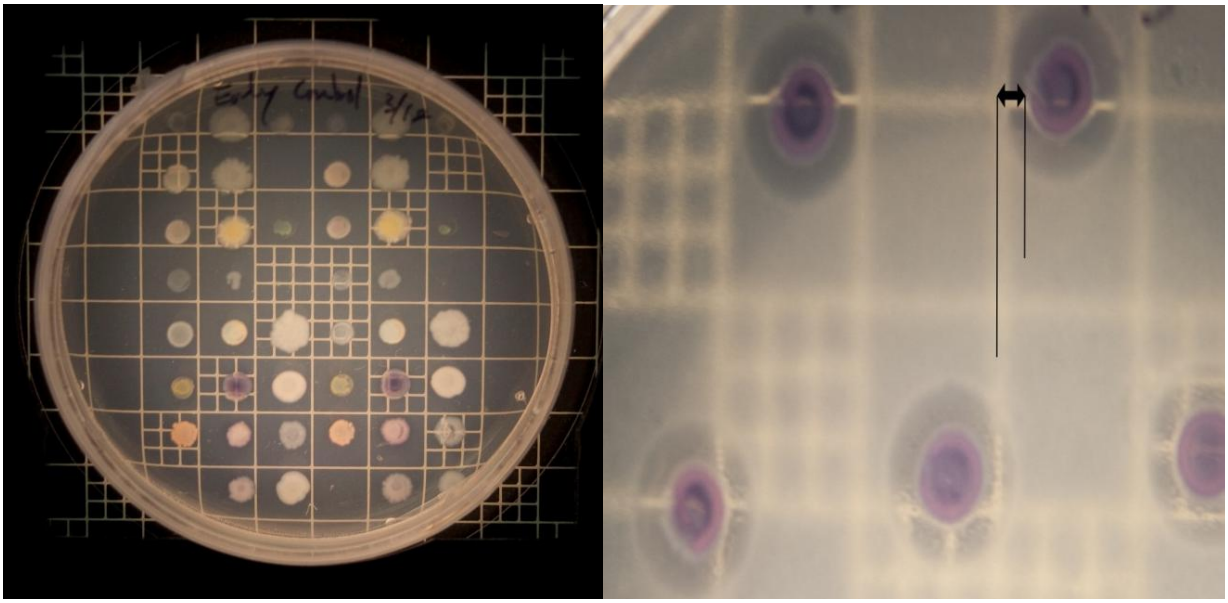

Figure 6.1. Antagonistic interaction screening and confirmation using soft agar overlay assay.

Left: Screening for antagonistic interactions using stamps. Right: Confirmation for antagonistic interaction on a one-on-one basis with 12 replicates.

This procedure was repeated on a select group of the top 4 most aggressive of our 25 isolates with one of 6 known fish pathogens as the lawn strain. Fish pathogens included 3 *Aeromonas* spp. strains and 2 *Flavobacterium* spp. strains isolated from either inland lakes in Michigan or the Great Lakes and *Yersenia ruckeri* ATCC 29473, another known fish pathogen.

Fish pathogens were provided by Dr. Mohamed Faisal and Dr. Tom Loch of Michigan State University.

Biofilm assays

Biofilm forming capability of 23 isolates (excluding isolates #3 and #4 which were not culturable in broth) was investigated through an adaptation of a 96-well plate assay with crystal violet [24]. For each trial, 200 µL of culture broth of each of the 23 isolates were placed in duplicate into one half of a 96-well plate. In addition to 23 isolates, *Pseudomonas aeruginosa* was used as a positive control and a single blank well was used as a negative control. A 48-spike stamp was used to transfer the isolate broth cultures and controls into wells filled with 175 µL of sterile media in triplicate (a total of 6 replicates per isolate). Three nutrient media were tested in the biofim assay: R2B (same as R2A above but without agar): M9+Glucose (12.8g Na_2HPO_4, 3g KH_2PO_4, 0.5g NaCl, 1g NH_4Cl, 2ml of 1M $MgSO_4$, and 4g Glucose per liter**);** and M9+Casamino Acids (M9 + 4g Casamino Acids per liter). The inoculated plates were incubated at 14ºC or 20 – 22 ºC shaking at 100 rpm for 2 days. Post-incubation optical density at 600 nm was measured to quantify growth within the broth. Cells adhering to the surfaces of the plate were stained with 200 µL of 0.1% crystal violet dye for 15 minutes, rinsed in water baths and then inverted to dry completely. The crystal violet stain was then extracted from each well with the addition of 200 µL of 30% acetic acid for 15 minutes. The entire dye solution within each well was transferred into new plates and the absorbance of the dye was measured at 600 nm using a spectrophotometer.

Results

Isolation and phylogenetic affiliation

We collected 92 isolates from the egg surface of Lake Sturgeon (Figure 6.2). Our culture collection covered a range of the egg surface microbial community diversity that was previously characterized using 16S rRNA gene pyrosequencing. Our culture collection contained a broad spectrum of bacteroidetes and beta proteobacteria on the egg surface, but was underrepresented in the α-proteobacteria. 25 isolates out of the 92 that represent the egg surface microbial community assembly were chosen and tested for both antagonistic interactions and biofilm forming capabilities.

Antagonistic interactions between isolates

Among the 25 strains we tested, 8 isolates (30%) were confirmed to be positive for antagonisms to at least one of the other strains at one of the tested temperatures (Table 6.2). The incidence of antagonistic interaction was dependent on temperature. At 21^{o}C, isolates that exhibited aggressiveness to at least one other isolate (aggressor) increased their target range (Figure 6.3) and intensity of antagonism relative to results from 14^{o}C (Table 6.2). The number of aggressors increased by one from the low to high temperature regime, as *Bacillus* sp. showed aggressiveness only at 21^{o}C. Susceptible isolates increased from 12 to 18 as the incubation temperature increased (Table 6.2, Figure 6.3). Six isolates including *Flavobacterium* sp. B10 and *Caulobacter* sp. F16 exhibited susceptibility only at 21^{o}C. Overall, 50 to 70 % (dependent on temperature) of the isolates in the community were sensitive to at least one of the isolates.

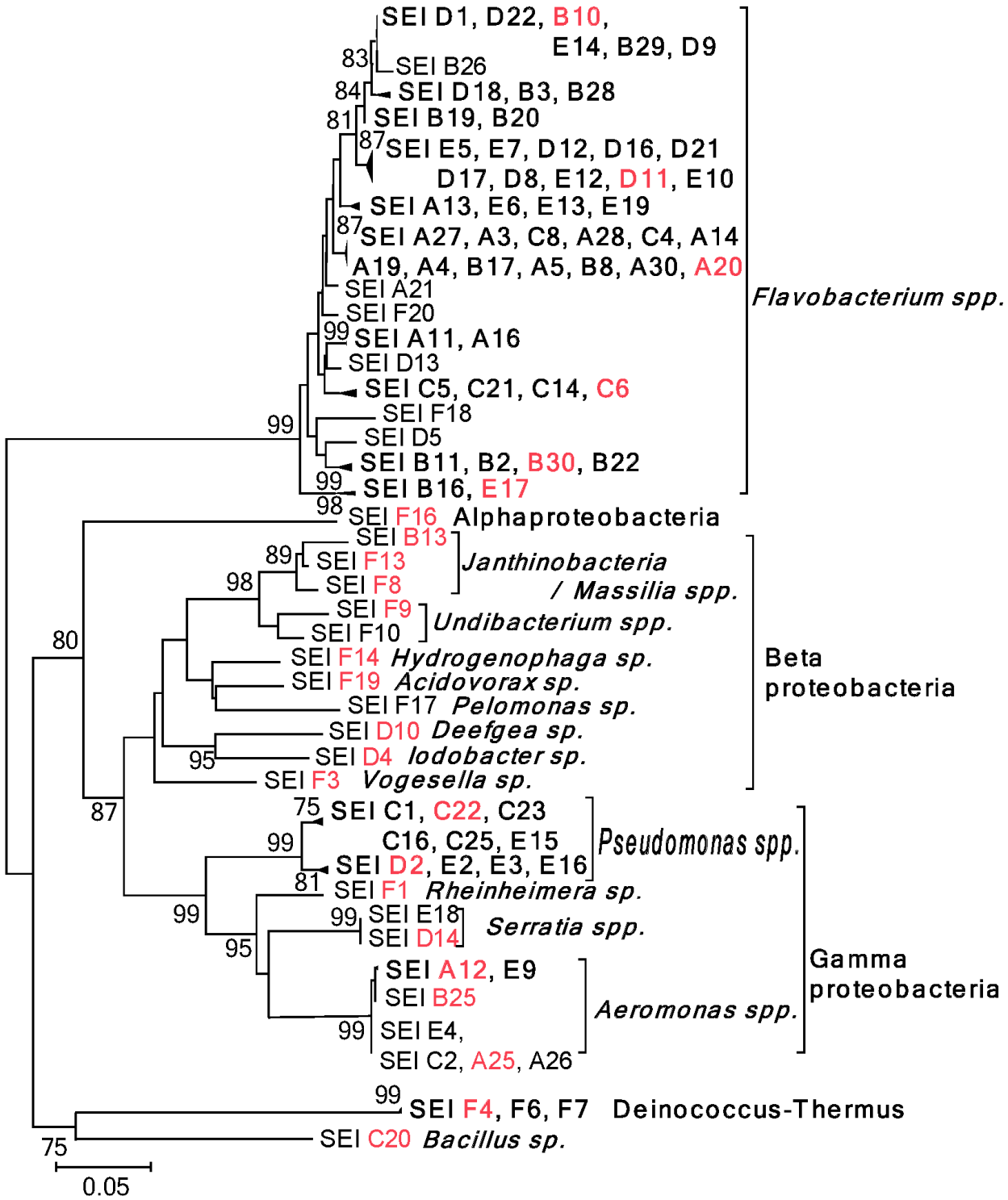

Figure 6.2. Phylogenetic relationships among 92 sturgeon egg isolates inferred using the Neighbor-Joining method.

25 isolates that were used in this study are highlighted red. The bootstrap values above 75 are shown at nodes.

Table 6.2. Antagonistic interactions among 25 egg isolates at two different temperatures

	Temperature 14°C	Stamp Strain																							Lawn
	Lawn Strain	2	3	4	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	Sensitivity
1	Flavobacterium sp. A20																								3
2	Flavobacterium sp. D11																								3
3	Flavobacterium sp. E17																								6
4	Flavobacterium sp. B30																								0
5	Flavobacterium sp. C6																								1
6	Flavobacterium sp. B10																								0
7	Bacillus sp. C20																								0
8	A. salmonicida A12																								1
9	A. sobria A25																								5
10	A. enchereia B25																								1
11	Pseudomonas sp. D2																								0
12	Pseudomonas sp. C22																								0
13	Serratia sp. D14																								0
14	Rheinheimera sp. F1																								1
15	Massilia sp. B13																								0
16	Janthinobacterium sp. F8																								0
17	Janthinobacterium sp. F13																								1
18	Iodobacter sp. D4																								1
19	Deefgea sp. D10																								3
20	Vogesella sp. F3																								0
21	Undibacterium sp. F9																								0
22	Hydrogenophaga sp. F14																								6
23	Acidovorax sp. F19																								0
24	Caulobacter sp. F16																								0
25	Deinococcus sp. F4																								0
	Stamp Aggressiveness	0	0	1	0	0	0	0	0	5	8	4	0	0	5	1	0	0	8	0	0	0	0	0	

Zone of Inhibition (mm) | Zero | Below 2 | 2 - 4 | Over 4

Color code indicates the extent of inhibition as a unit of zone of inhibition from the edge of colonies (mm). The bottom row in the table shows cumulative stamp success of the stamp strains (aggressiveness).The right-most column summarizes the sensitivity of lawn strains to stamp strains (sensitivity). *Flavobacterium* sp. A20 and C6 were removed from the stamp due to gliding ability.

Table 6.2. (cont'd).

	Temperature 22°C	Stamp Strain																							Lawn
	Lawn Strain	2	3	4	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	Sensitivity
1	Flavobacterium sp. A20																								3
2	Flavobacterium sp. D11																								6
3	Flavobacterium sp. E17																								5
4	Flavobacterium sp. B30																								6
5	Flavobacterium sp. C6																								2
6	Flavobacterium sp. B10																								0
7	Bacillus sp. C20																								1
8	A. salmonicida A12																								3
9	A. sobria A25																								2
10	A. enchereia B25																								0
11	Pseudomonas sp. D2																								0
12	Pseudomonas sp. C22																								0
13	Serratia sp. D14																								0
14	Rheinheimera sp. F1																								3
15	Massilia sp. B13																								3
16	Janthinobacterium sp. F8																								0
17	Janthinobacterium sp. F13																								1
18	Iodobacter sp. D4																								3
19	Deefgea sp. D10																								3
20	Vogesella sp. F3																								0
21	Undibacterium sp. F9																								6
22	Hydrogenophaga sp. F14																								7
23	Acidovorax sp. F19																								4
24	Caulobacter sp. F16																								5
25	Deinococcus sp. F4																								3
	Stamp Aggressiveness	0	0	1	0	10	0	0	0	7	15	12	0	0	7	4	0	0	10	0	0	0	0	0	

Zone of Inhibition (mm) | Zero | Below 2 | 2 - 4 | Over 4

The antagonistic interactions displayed a phylogenetic trend (Table 6.2). Genus *Flavobacterium* was found to be the least aggressive genus and one of the most susceptible genera in the community. The majority of β-proteobacteria were also susceptible to antagonistic interactions. *Bacillus* sp. and γ-proteobacteria including genus *Pseudomonas* inhibited growth of *Flavobacterium* isolates and isolates of β-proteobacteria. Seven out of nine β-proteobacteria were susceptible to one of the γ-proteobacteria isolates at 21^{o}C and 5 out of six *Flavobacterium* isolates were susceptible to both *Bacillus* sp. and one of the γ-proteobacteria isolates. Genus *Pseudomonas* and *Bacillus* were found to be strongly aggressive. At a strain level analysis, the most aggressive isolate was found to be *Pseudomonas* sp. C22 which inhibited growth of 15 isolates out of 25. The most susceptible strain was *Hydrogenophaga* sp. F14 which was susceptible to 7 isolates, some with a large zone of inhibition (Table 6.2).

However, Some isolates deviated from the phylogenetic trend. Although about 80% of beta-proteobacteria isolates were sensitive to at least one of the other isolates, strains *Vogesella* sp. F3 and *Janthinobacterium* sp. F8 showed aggressiveness to other isolates and were not susceptible to any aggressors. The strain *Flavobacterium* sp. B10 was also resistant to all aggressors.

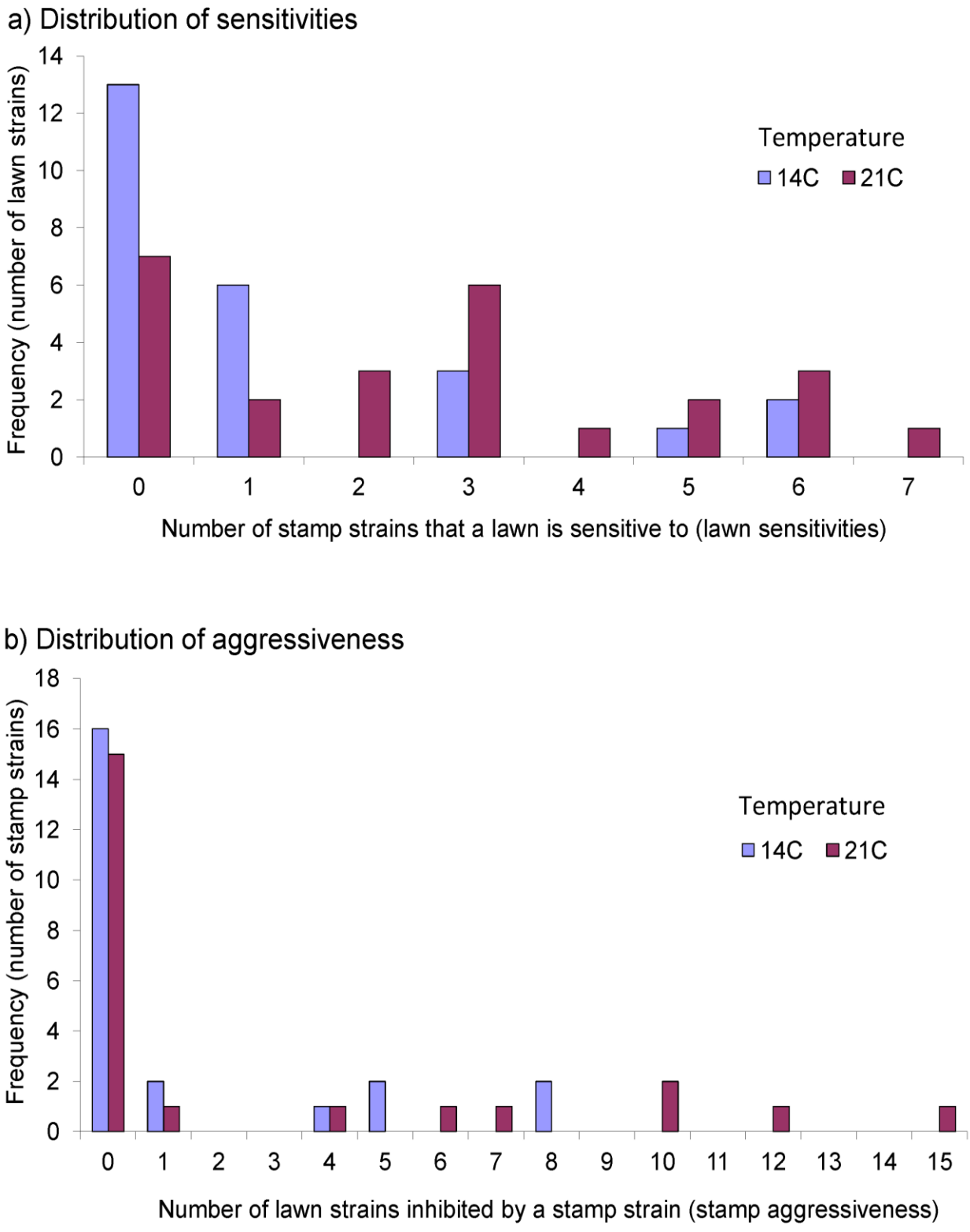

Figure 6.3. Summary of (a) lawn susceptibilities and (b) stamp aggressiveness among 25 sturgeon egg surface isolates at two temperature regimes

The antagonistic interaction held in the reciprocal condition when positions were switched from stamp to lawn. Evidence of aggression was detected when the aggressor was the lawn strain in the soft agar in that the size of the sensitive stamped-strains were diminished (Figure 6.4). This negative effect of lawn isolates on growth of stamp isolates was detected even with non-antagonistic pairs of isolates, suggesting the existence of resource competition among the isolates (Figure 6.5).

The four strong aggressors *Bacillus* sp. C20, *Pseudomonas* sp. C22, *Serratia* sp. D14, *Janthinobacterium* sp. F8 were tested against six known fish pathogens. All four aggressors inhibited at least one of the 6 fish pathogens, and all of the 6 fish pathogens were inhibited by at least one of the four aggressors (Figure 6.6). *Pseudomonas* sp. C22, which displayed the highest aggressiveness in our study, inhibited growth of 5 out of the 6 fish pathogens. The most susceptible fish pathogen was *Flavobacterium* sp. C05, whose growth was inhibited by 3 out of the 4 aggressors we tested. In contrast to *Flavobacterium* sp. C05, *F. columnare* 090702-1 was susceptible to only one aggressor we tested.

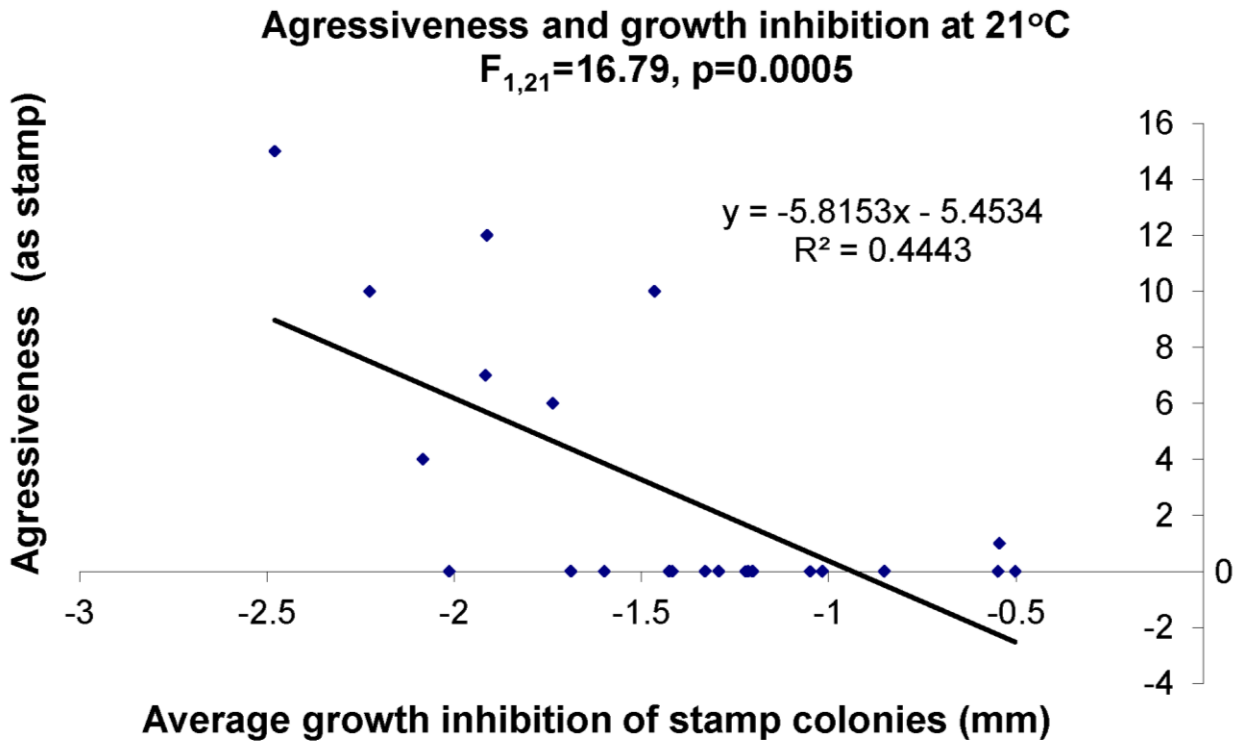

Figure 6.4. Reciprocal antagonistic interactions among 25 egg surface isolates when positions are switched from stamp to lawn.

Isolates that acted as strong aggressors when used as stamps inhibited growth of stamp colonies when used as lawn isolates. The figure depicts the negative correlation between aggressiveness and the average growth inhibition.

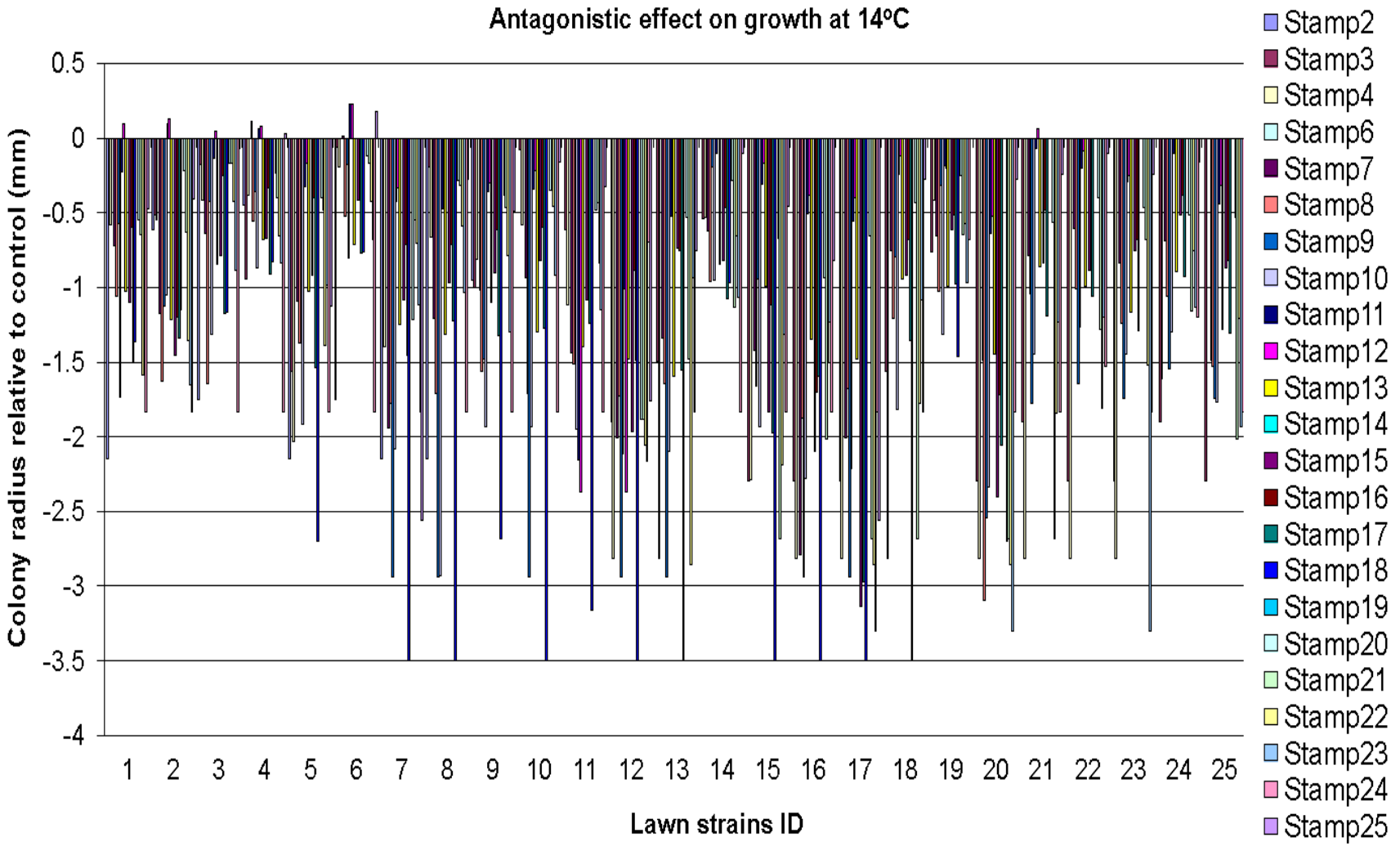

Figure 6.5. Reduction of colony size due to both active growth inhibition by lawn isolate and resource competition under two temperature regimes.

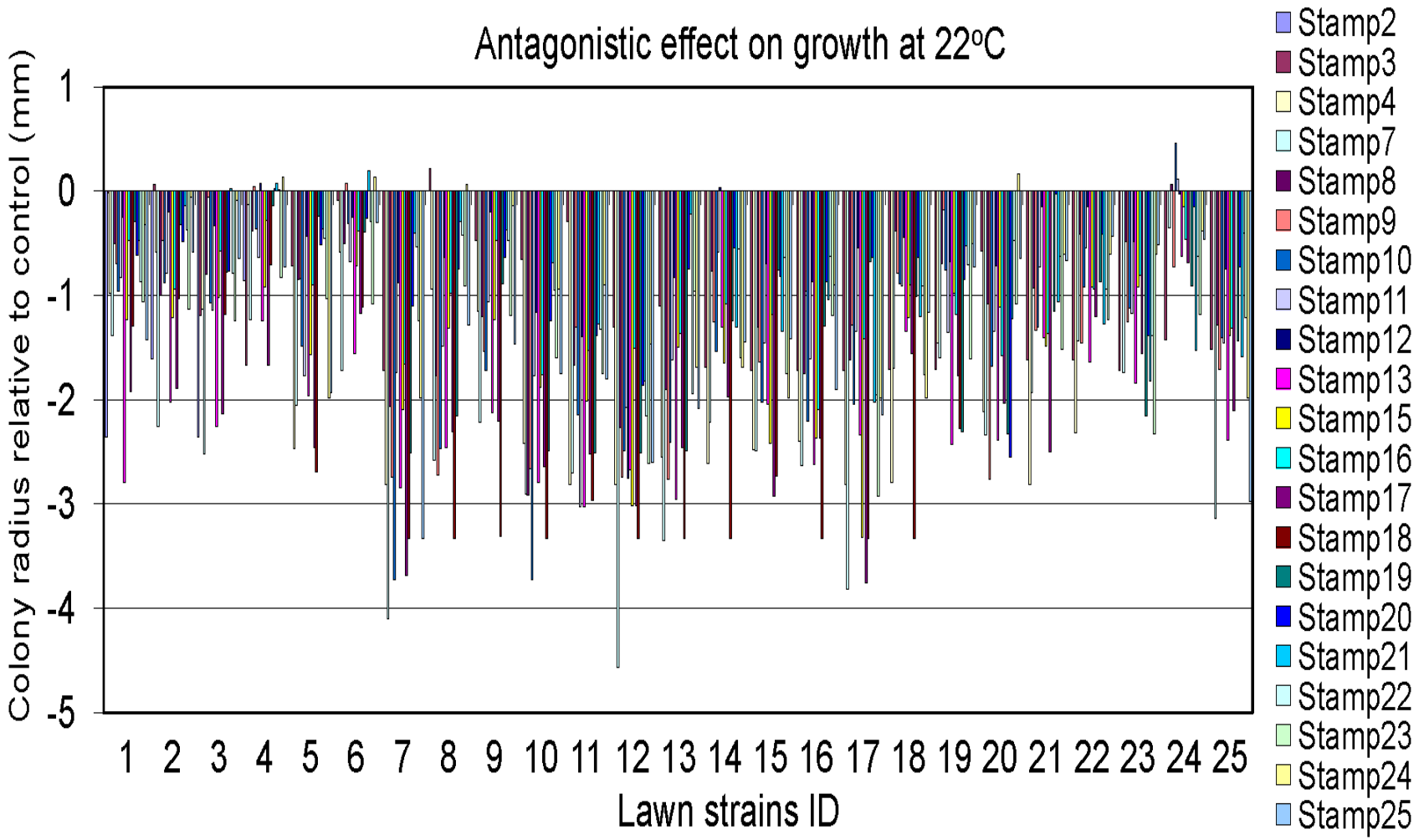

Figure 6.5. (cont'd).

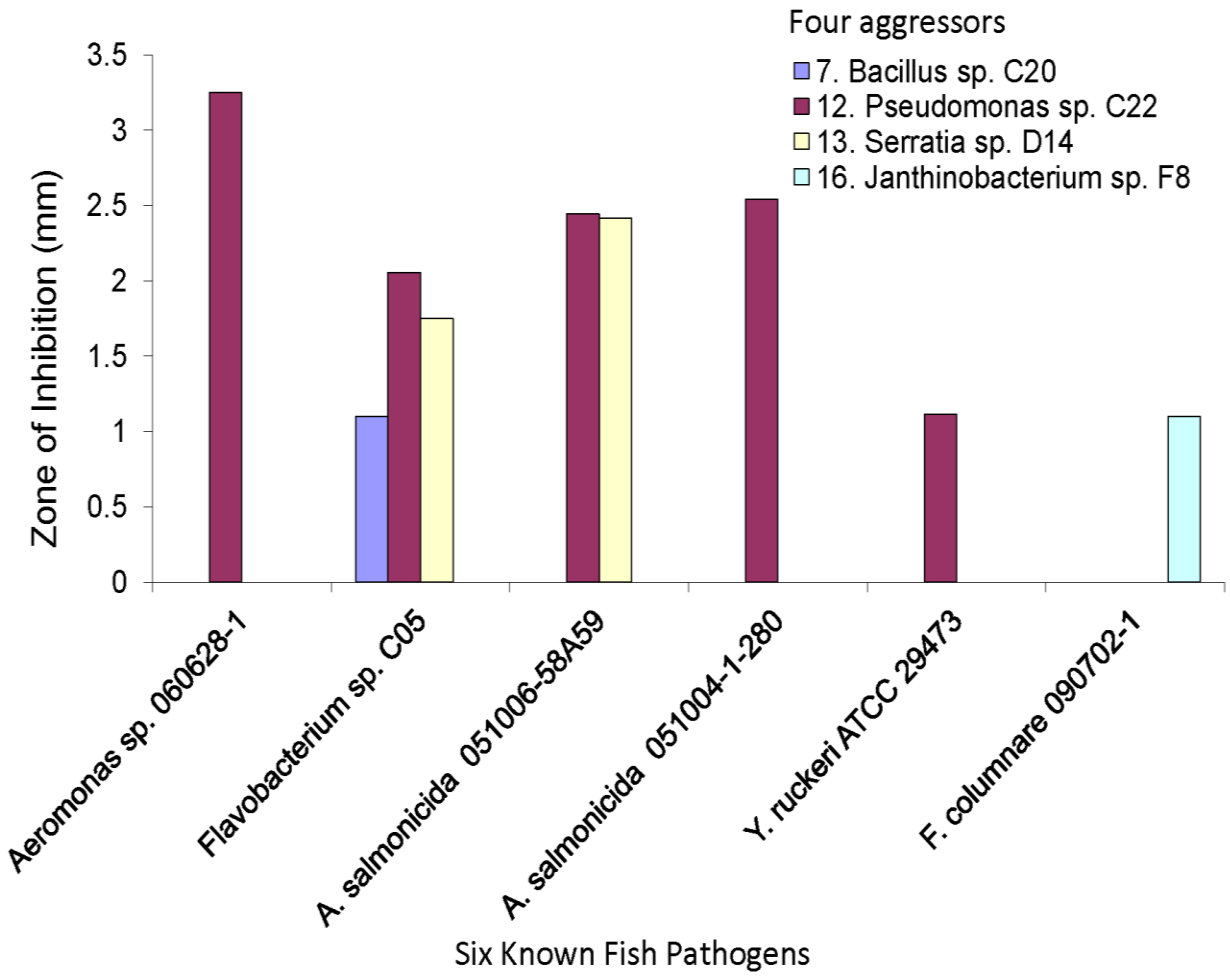

Figure 6.6. Antagonistic interactions against 6 known fish pathogens by the 4 most aggressive isolates identified on the sturgeon egg surface.

Biofilm forming capability

Using the crystal violet assay for biofilm formation, strong biofilm forming capabilities (with average absorbance over 0.5) were observed for 11 isolates (nearly half of the total) and they were both temperature and nutrient medium dependent (Figure 6.7). The pattern of biofilm formation and temperature/medium dependency was strain dependent. *Flavobacterium* sp. A20 and D11 formed biofilm at 21^{o}C when grown on minimum medium plus glucose as did three γ-

proteobacteria isolates (*Pseudomonas* sp. D2, *Pseudomonas* sp. C22, and *Serratia* sp. D14). Two β-proteobacteria isolates (*Hydrogenophaga* sp. F14 and *Acidovorax* sp. F19) formed biofilm when grown on either R2B or minimum medium plus casamino acid at 21^{o}C.

There was also a relationship between planktonic growth in broth medium and biofilm formation (Figure 6.8). However, planktonic growth did not always directly correlate with biofilm forming capability. For instance, *Flavobacterium* sp. A20 and D11 grew well in both R2B and minimum medium plus glucose, but biofilm formation was detected only in the minimum medium plus glucose. In addition, planktonic growth in broth medium was not a necessary condition for biofilm formation. For instance, *Pseudomonas* sp. D2 did not grow planktonically at 14^{o}C in minimum medium plus glucose but displayed the maximum biofilm formation under this same condition.

There was a relationship between biofilm forming capacities and antagonistic interactions. *Hydrogenophaga* sp. F14 and *Caulobacteter* sp. F16 were sensitive to antagonistic interactions in R2A soft agar at 21^{o}C and they were a good biofilm former in R2B at 21^{o}C. On the other hand, *Pseudomonas* sp. C22, the most aggressive isolate, was also found to be a good biofilm former.

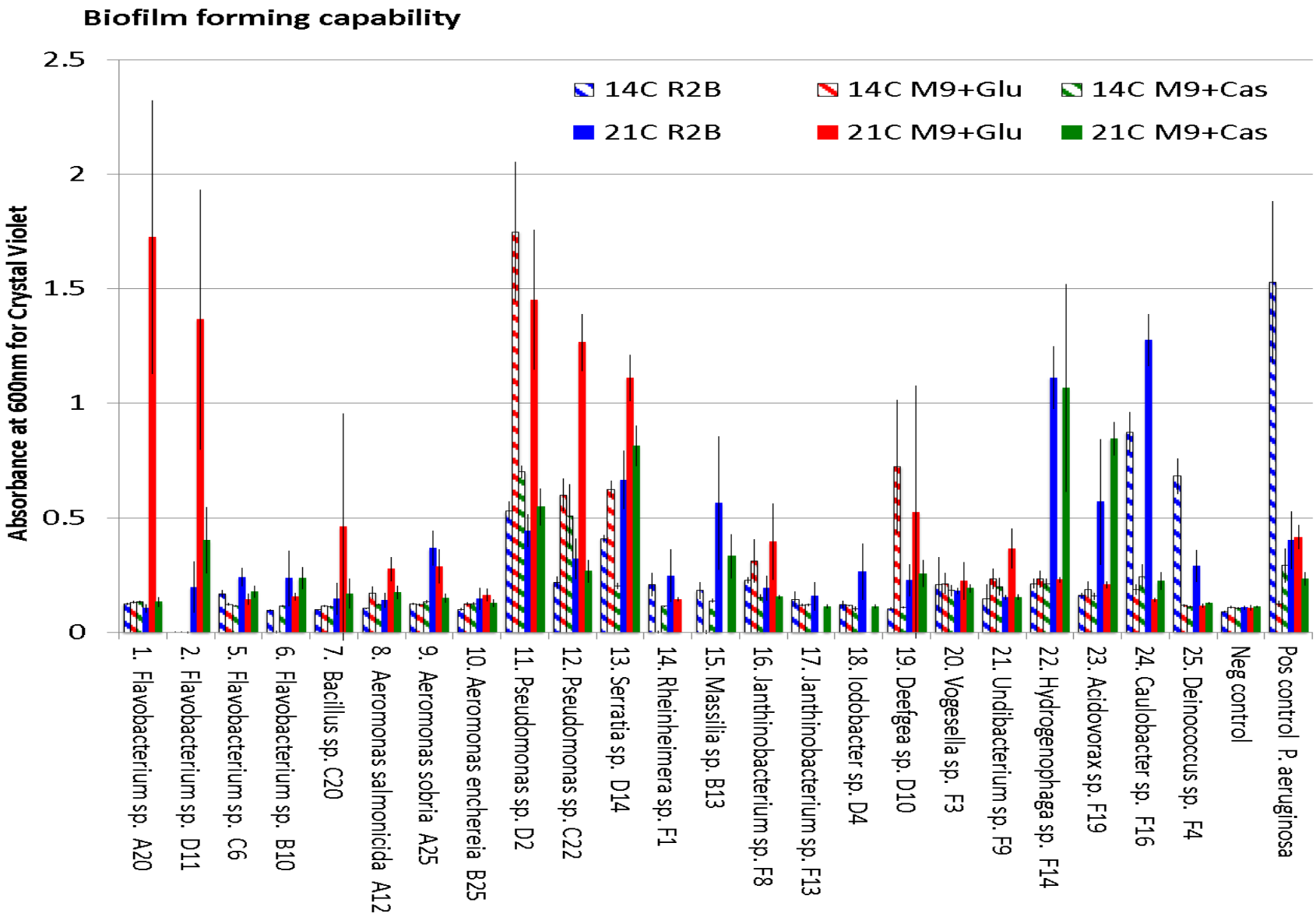

Figure 6.7. Quantification of biofilm formation with crystal violet in a microtiter plate assay under different media

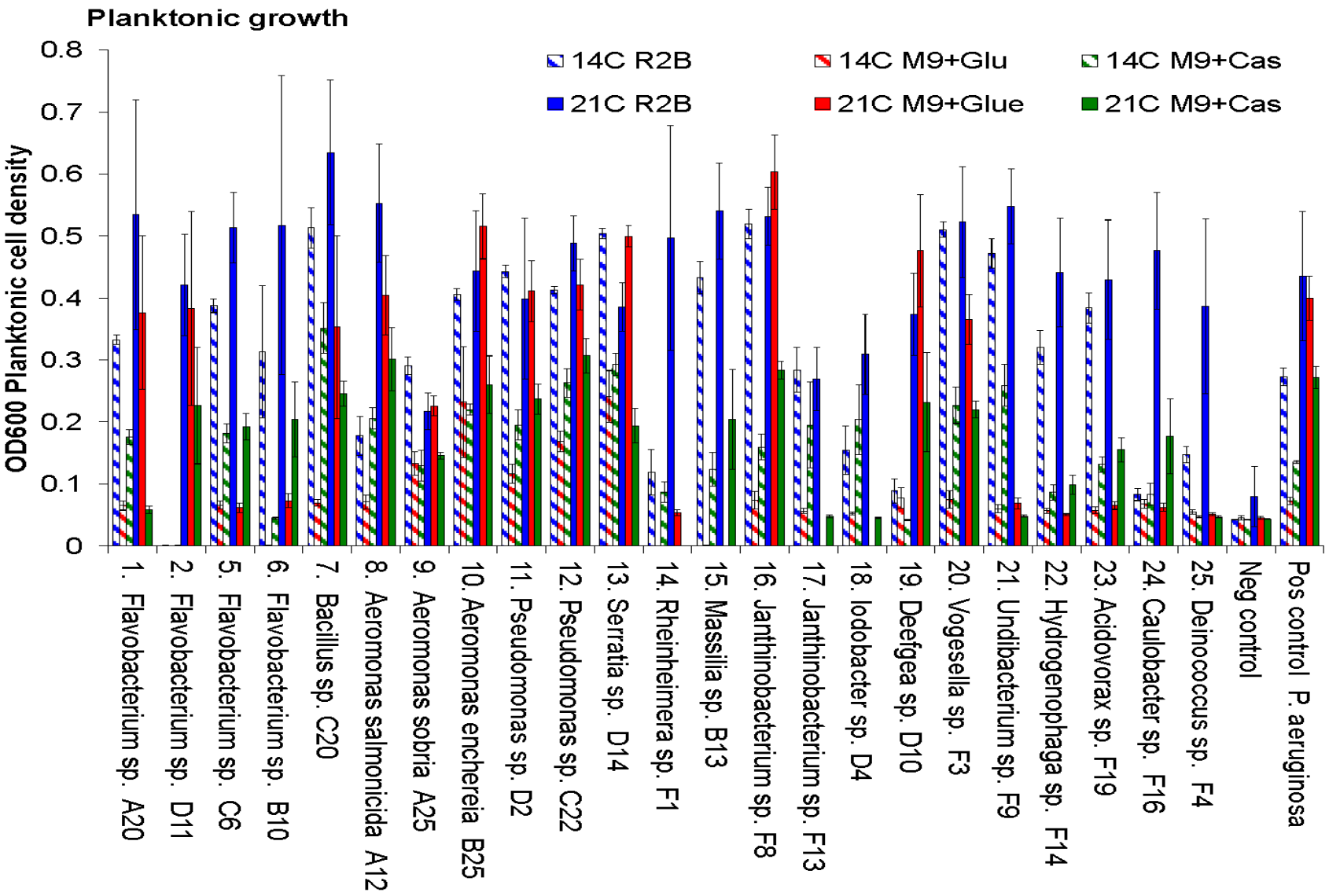

Figure 6.8. Quantification of planktonic growth in different nutrient broth with OD600 in a microtitier plate assay.

Discussion

This study identified antagonistic interactions among isolates from the egg surface of the Lake Sturgeon. The significant effect of temperature on antagonistic interactions reveals the complex nature of microbial interactions on the egg surface microbial community. Temperature dependency of antimicrobial interactions has been previously reported in other systems [4, 6], but has particular relevance to the sturgeon host due to the temperature-dependent spawning behavior in this species. Adult sturgeon spawn at two distinct time points during the spawning season, which normally correspond to stream temperature. Our results suggest that it is possible that different microbial interactions in the communities may be occurring at the different time periods as a result of temperature, which could potentially result in differences in the egg microbial community structure and egg mortality.

The phylogenetic trends we observed in antagonistic interactions were revealing in highlighting the evolutionary origins of microbial interactions. Previous studies by others found a similar phylogenetic trend in antagonistic interactions in other microbial communities [1-3, 5]. Studies on microbial community in marine water found that phylum Bacteroidetes was the least aggressive and the most susceptible to antagonistic interactions [5], and *Flavobacterium* spp. were the least aggressive genus in the community [1]. Strong inhibitory activities by *Bacillus* spp. [1] and γ-proteobacteria including *Pseudomonas* spp. [3] were also reported in the marine organic aggregates community and the sponge associated microbial community, respectively. However, in our study not all strains within the same genus or class follow the phylogenetic trend, suggesting that generalization in genus or class level do not always hold. Other studies also found that some antagonistic interactions were strain specific [2].

Another important finding of this study dealt with the significant effect of resource competition on microbe interactions, irrespective of antagonism. Resource competition is rarely reported in studies on antagonistic interactions, but potentially affects the microbial community structure and functional processes in the community. The negative effect of lawn isolates on growth of all isolates suggests that future studies should also examine this phenomenon of resource competition among members of a community given limited resources.

The significant antagonism displayed by the aggressor isolates against the known fish pathogens suggests that there is potential for microbes to help protect fish eggs against invasion of pathogenic bacteria. *Pseudomonas* sp. C22, was the most aggressive isolate of all of those we tested, since it inhibited 5 out of the 6 known fish pathogens, and should be strongly considered for potential future use in probiotic treatment for this threatened fish species. In fact, *Pseudomonas* spp. have been used as probiotics to protect other fish species and have been found to be effective [25-26].

Another important finding of this study concerned biofilm formation capabilities. Biofilm formation is relevant to microbial interactions since susceptible isolates can persist in a community by escaping from antimicrobial substances in the biofilm [13-14]. The dependence of biofilm formation on both temperature and nutrient medium highlighted the complex nature of biofilm formation, which corroborates findings in other systems [27-28]. Our findings together with observations that egg metabolites can change during embryogenesis [29] suggest that biofilm formation on the egg surfaces may be altered during embryogenesis. Such complexity can also be appreciated through the finding that some isolates showed a small planktonic growth but a large biofilm formation under the same condition, suggesting preference to form biofilm rather than grow planktonically.

It is also interesting to note that we found some correlations between antagonistic interactions and biofilm formation in some strains. For instance, *Hydrogenophaga* sp. F14 and *Caulobacteter* sp. F16 were among the most susceptible strains and also were substantial biofilm formers. They may persist in the egg surface microbial community by evading the antagonistic interactions through biofilm formation as previously reported by other studies [13-14]. On the other hand, we also found a biofilm capability of a strong aggressor, specifically *Pseudomonas* sp. C22. This strain may use biofilm formation as a mechanism to allow persistence in the egg surface microbial community. This finding again highlights the fact that this strain may be a good candidate for probiotic treatment.

The antagonistic interactions we observed could also help explain the community turnover we observed in our earlier studies through 454 pyrosequencing of egg surface microbial community during embryogenesis (Chapter 2, 3, and 5). The pryosequencing analysis revealed that the genera *Acidovoras* and *Caulobacter* persisted in the communities throughout embryogenesis, genus *Hydrogenophaga* increased its relative abundance toward the late egg developmental stage, and genus *Flavobacterium* decreased toward the late egg developmental stage. Our biofilm assays demonstrated that genus *Acidovorax*, *Caulobacter*, and *Hydrogenophaga* were capable of forming a biofilm, which might allow them to persist in the microbial community on the egg surface. The fact that *Flavobacterium* was one of the most susceptible genera to antagonistic interactions could contribute to the decline of this microbe in the community toward the later period of egg development. Our previous work using pyrosequencing also revealed that *Pseudomonas* spp. persisted in the community throughout embryogenesis, but did not become dominant in the community. Although we expected that *Pseudomonas* would increase in relative abundance in the community toward the end of

embryogenesis by utilizing antagonistic interactions and biofilm formation, there may be some unknown mechanisms that inhibit the dominance of *Pseudomonas* in the community.

In this study, we did not investigate the underlying mechanisms behind the antagonistic interactions, but previous studies by others offer insights into potential mechanisms. For instance, mechanisms of active growth inhibition by *Bacillus* spp. [30-31] and *Pseudomonas* spp. [32-33] have been studied including purification of antimicrobial substances and their induction mechanisms. We also found that two strains in beta-proteobacteria *Vogesella* sp. F3 and *Janthinobacterium* sp. F8 exhibited antimicrobial activities, however, the mechanisms of these antagonistic interactions have not been previously characterized. Future research should be directed toward understanding the mechanisms behind these antagonisms, specifically purification of antibacterial substances.

We do not know how our findings translate into the natural setting where microbes interact on the egg surface in a stream. Although we mimicked temperature ranges eggs experienced during the spawning season, the nutrient content, concentrations, and physical structure of the R2A plate are different from the egg surface environment. Our antagonistic interactions represent those between a pure colony and a pure cell aggregate (lawn), which may differ from the situation on the egg surfaces where the density of microbes is more scattered. Furthermore, we tested the antagonistic interactions on a one-on-one basis, but in nature there can be one against many or many against many relationships. We also incubated soft agar plates in the atmosphere, while microbes in the natural setting are submerged in a stream. Previous studies demonstrated that the level of oxygen affect the induction of antimicrobial substances [7-8]. Both nutrient uptake and delivery of antimicrobial substances could be affected by stream flows. Although we tested biofilm forming capability using pure cultures in broth media, the

presence of antagonistic strains or antimicrobial substances can also induce biofilm formation [34]. Future studies should seek to investigate these other avenues of research.

In summary, our results revealed the complex nature of microbial interactions amongst the members of the egg surface microbial community. To our knowledge, this is the first study on any system to simultaneously analyze antagonistic interactions and biofilm formation, an approach which was informative in revealing significant patterns in microbial interactions. Although our findings may not directly translate into microbial interactions observed on the egg surfaces in the natural environment, our results suggest a potential use of *Pseudomonas* sp. C22 for probiotic application as a management tool to decrease egg mortality.

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CHAPTER 7: CONCLUDING REMARKS

Factors affecting microbial community assembly on Lake Sturgeon eggs

Throughout this study, we demonstrated that microbial community assemblages on the egg surfaces of the Lake Sturgeon were affected by a number of different factors. The most important factor was found to be the local egg related factors, probably mediated by maternally provisioned innate immunity, including lysozyme or egg surface chemistry, the latter of which affects adhesion of microbes. This process separated the egg associated microbial communities from the source water microbial communities within 6 hours of fertilization or even sooner (Chapter 5, Chapter 6). The reason we believe that this process is mediated by lysozyme is that phyla Actinobacteria and Firmicutes, both gram positive bacteria, were selected against by the eggs (Chapter 2, Chapter 5, and Chapter 6). One of the lines of evidence that supports the alternative adhesion theory is derived from the experiment in which we inoculated *Acidovorax* and *Flavobacterium* during fertilization. *Acidovorax* successfully colonized the egg surfaces but *Flavobacterium* did not, and the process happened within one hour. Although we cannot exclude the possibility that this low *Flavobacterium* colonization was also mediated by lysozyme (*Flavobacterium* which is gram negative is somehow sensitive to lysozyme), this initial adhesion process is worthy of study in the future.

Our study revealed that another important process explaining microbial community assembly was dispersal of aquatic microbes. Differences in the aquatic microbial community affected the egg-associated microbial community. This effect was more significant in explaining microbial community assembly than that of water temperature (Chapter 3). This process was also dependent on the quantity of aquatic microbes. When the quantity of the aquatic microbial

community was lowered, the effect of aquatic microbial community on the egg surface microbial community was diminished (Chapter 6). This density dependency is thus a fundamental aspect of the nature of the effect of dispersal on microbial community assembly.

Furthermore, directional changes in the egg surface microbial community were observed along with egg developmental stage. These changes were consistent throughout different experiments and treatments (Chapter 2, Chapter 3, Chapter 5). These temporal changes in the microbial community could be mediated by a number of different factors, including changes in metabolites secreted by eggs, changes in lysozyme from types that are maternally provisioned to those secreted by eggs, antagonistic microbial interactions, biofilm formation (Chapter 6) and growth. We do not know the relative contributions of each factor, but this avenue of research is worthy of pursuing in the future.

Temperature was another important factor explaining the egg surface microbial community. The effect of temperature on microbial community assembly is not entirely surprising, since it has been shown that microbes have preferences for certain temperature ranges, with some microbial species growing better in warm temperature and others growing better in cold. There was also an interaction between temperature and antagonistic interactions (Chapter 6). This suggests that antagonistic interactions act differently in their effects on microbial communities at different temperatures. However, while temperature did affect the egg microbial community, the effect was not as significant as that of the aquatic microbial community and the temporal effect in the PCA analysis (Chapter 3). In addition, we did not detect an effect of temperature on microbial quantity or egg mortality in the temperature ranges we studied. However, water temperature did affect the incubation period of embryos and the larvae size at hatch (Chapter 3). In summary, temperature is an important factor to consider when rearing

sturgeon eggs, since it affects not only the egg surface microbial composition, but also the life history traits of the host.

Flow rate was also a factor that significantly affected microbial community composition. High flow rate, as simulated in the flume experiment, lowered the diversity of the microbial community on the egg surfaces, and the effect of flow on the egg associated microbial community was observed only in the middle stage of embryogenesis (Chapter 2). We also found that the lower the flow, the lower the abundance of the genus *Flavobacterium* on the egg surface (Chapter 2). We did not pursue the effect of flow rate on host life history traits including egg mortality and larvae size at hatch, so this effect should be further investigated in the future.

We also studied antagonistic interactions and biofilm forming capabilities among sturgeon egg isolates. We found that both antagonistic interactions and biofilm forming capability of sturgeon egg isolates were complex and dependent on temperature (and nutrient composition for the case of biofilm formation, Chapter 6). However, we do not know how much the microbial interactions or biofilm formation contribute to the observed changes in the egg microbial community structure during incubation, a topic which should be further studied in the future.

Our study also demonstrated that history may matter in explaining the future development of a microbial community. Several lines of evidence suggest that the effect of initial colonization on development of subsequent microbial communities was likely dependent on both available space on eggs and/or the concentration of the source water microbes. When eggs were inoculated with different strains for 60 minutes and were transferred and reared in the stream water with high microbial load, the initial microbial community structure was masked and converged with the community of stream fertilized and reared eggs (Chapter 4). However, when

the eggs fertilized in stream water (with high microbial load) for 6 hours were transferred and reared in 0.2 µm filtered water, the egg associated microbial community did not converge with that fertilized and reared in 0.2 µm filtered water. This suggested that either available space on the egg surface or the concentration of the source water microbes plays a role in determining the subsequent microbial community assembly. This suggests that the history of community composition may be important particularly when a large impact (microbial load) occurred in the initial stage and smaller impacts (microbial load) occurred in the later stages.

Factors affecting egg surface microbial quantity

Egg surface microbial quantity increased to the range of $10^{5.5}$ to 10^{6} 16S rRNA gene copies per egg within 6 hours post-fertilization (Chapter 5). Source water microbial quantity is likely a factor mediating this process, since eggs fertilized in stream water had the highest microbial quantity among those studied in the three water treatment. However, the effect of source water on egg surface microbial quantity is also likely mediated by microbial composition. When source water microbial composition was effectively controlled by eggs, as seen by the lower abundance of phyla such as Actinobacteria, Firmicutes, and Bacteroidetes on egg surfaces, less microbes will likely colonize the egg surface. In contrast, when composition is dominated by microbes that are accepted by host eggs, including *Acidovorax*, the egg microbial community likely increases even if the source water quantity is low.

We also detected potential growth of microbes on the egg surface. This was detected when eggs were fertilized in stream water and transferred to and reared in 0.2 µm filtered water (Chapter 5). The microbial quantity on eggs fertilized in stream water and reared in 0.2 µm filtered water increased relative to the quantity of eggs fertilized and reared in 0.2 µm filtered

water. However, microbial community structure of these eggs (fertilized in stream and reared in 0.2 µm filtered water) did not converge with those fertilized and reared in 0.2 µm, which suggests no effect of dispersal in explaining community structure. We believe that this increase in microbial quantity can be attributed to the growth of microbes nested within the initial colonizers.

The effect of microbes on the host life history traits

Our results regarding effects of microbial successional processes on host life history were also illustrative in contributing to broadly understanding microbe-host interactions. Eggs fertilized and reared in stream water (with high microbial loads) had significantly higher egg mortality and smaller yolk sac areas than those reared in treated water (with lower microbial loads) (Chapter 5). This suggests that yolk resources were being used against microbes that colonized the egg surface. We also demonstrated that eggs fertilized with a putative symbiont had lower egg mortality and less yolk resource use (Chapter 4). The fact that microbes affect host life history traits such as larval size at hatch has significant implications by revealing the extent of complexity in reciprocal interactions between microbes and hosts.

Symbionts and probiotic treatment

We also conducted preliminary work on identifying putative symbiotic microbial species for the Lake Sturgeon eggs for potential future use in probiotic treatment to improve observed high egg mortalities in this species. By rearing eggs in the presence of different compositions of aquatic microbial communities and analyzing the correlation between presence of certain microbes and the egg mortality, we identified a key set of microbial species that significantly

improved egg survival (Chapter 5). We also inoculated a putative symbiont to eggs during fertilization, a treatment which reduced both egg mortality and resources used by treated embryos (Chapter 4). This line of evidence suggests that this putative symbiont indeed improved the fitness of the host, which is the broad definition of a symbiont. We also tested 25 egg isolates representing the egg surface microbial community assembly for antagonistic interactions against known fish pathogens (Chapter 6). Through this experiment, we identified several isolates that are potentially beneficial to eggs by protecting them from known fish pathogens.

Egg microbiome

Prior to starting this project, we were wondering whether an egg microbiome exists. Unlike the animal gut where space is contained and association with microbes can be long-term in nature, eggs are exposed to a large number of aquatic microbes (an average of 10^6 cfu/ mL) throughout incubation, which could be a random collection of microbes and prevent a distinct microbiome from forming. However, our study suggests that it may still be possible for an egg microbiome to form, since we demonstrated that egg microbial communities were distinct from the source water microbial communities. Although we treated eggs with different water types, all microbial communities associated with eggs were clustered together relative to the source water (Chapter 5). It took approximately 6 hours post-fertilization for eggs to shape the communities and form a microbiome, although eggs from one of the families shaped the egg associated microbial community within 1 hour.

However, it is also important to note that there was some variation in the microbiome and a number of factors that affected its formation. In the water experiment, microbial communities were grouped by water type within the egg microbial community cluster (suggesting the effect of

dispersal on the egg microbiome) (Chapter 3 and 5). We also observed directional changes of microbial communities along with the egg developmental stages across a number of experiments (Chapter 2, Chapter 3, and Chapter 5). Temperature and flow rate also affected the microbial communities on the egg surfaces. These findings suggest that egg microbiomes can vary with water type, environmental variables, and egg developmental stages, although they are clustered closely and significantly different from the source water community.

Future work

The findings of this dissertation can be used to develop hypotheses and formulate new research questions for those who may further pursue this topic in the future.

One of the major areas we have not yet explored deals with the chemistry of the sturgeon eggs. One of the largest gaps of information concerns the egg surface chemistry, which is a key to understanding adhesion of microbes, both maternally provisioned and egg secreted lysozymes (which could explain the rejection of certain microbes during the initial colonization process), and metabolite secretion (which could explain the temporal changes in the egg associated microbial communities). Transcriptome analysis of eggs at different time points during embryogenesis would help to elucidate these mechanisms.

Another factor that we have not yet explored deals with fungal infection. While conducting this experiment, we observed that when the development of eggs was arrested, eggs became susceptible to fungal infection. These fungi can spread to other eggs and may cause mortality of other eggs lying in the same incubation tray. It would be interesting to investigate if any microbial species protect eggs from such fungi. In addition, it would be interesting to

investigate the extent to which these fungal populations affect the microbial community on the egg surfaces via predation.

Another topic that we briefly touched on but did not explore fully is the topic of vertical transmission of symbiont strains from maternal to offspring. We found that some microbial communities were present on the unfertilized egg surfaces (as displayed using both TRFLP and clone library in chapter 5). The unfertilized egg surface communities were similar to each other, suggesting a non-random association of microbes among the unfertilized eggs. This together with the fact that *Acidovorax* sp. F19 was clustered with a clade found in clone libraries of aseptically harvested eggs suggests that *Acidovorax* sp. F19 may be vertically transmitted by a female. This hypothesis can be experimentally tested in the future by sequencing microbial genomic samples aseptically collected from different females using pyrosequencing. Using this method, it may be possible to detect that symbiont populations have diverged within each lineage of females if they are indeed vertically transmitted from maternal to offspring.

It would also be worthwhile to explore whether eggs from different Lake Sturgeon populations have different relationships to microbes on their surfaces. We found that the egg related effect was a dominant factor in shaping the microbial community on the egg surfaces, but this egg related process could vary with different hosts and could potentially shape the egg microbial community differently. The effect of host genetic variation on the egg microbial communities would be a particularly interesting avenue to explore in the future. Host variation can be considered across populations (Black lake population versus Wisconsin population) within the same species, or across different species of sturgeon (Lake sturgeon versus white sturgeon). This approach could even be extended to span vastly different fish species (e.g. salmon, walleye, bass).

Although we focused our study on the egg associated microbial community, it would be interesting to extend this approach to different life stages of the Lake Sturgeon. Future research could follow the changes in microbial community assembly from eggs to larvae (including different parts of the body such as the gut), larvae to juveniles, and juveniles to adults. We hypothesize that there are some microbial communities already developed on the skin of larvae immediately after hatching or even immediately before hatching (while moving inside the egg case).

Our study on antagonistic interactions among egg isolates was conducted on a one on one basis, but interactions among more than two microbes can occur (and are probably more likely) within the egg surface microbial communities. It would be important in the future to investigate the interactions among multiple isolates by co-culturing them. We also did not pursue the correlation between antagonistic interaction and biofilm formation, but it is possible that antagonistic interaction induces biofilm formation. These questions can be answered by inoculating multiple strains in a broth culture and examine the relative abundance of each strain in both broth in tubes and biofilm formed on the surfaces of tubes.

We hypothesized that Actinobacteria and Firmicutes, which occupy a significant fraction in water microbial community, are sensitive to lysozyme on the egg surfaces. Others may able to experimentally test this hypothesis in the future by collecting isolates of these two phyla from the water column and testing them against a known chicken egg lysozyme or lysozyme purified from fish eggs, if available.

The degree of adhesiveness of each isolate to the egg surface would be another interesting avenue to take in the future. Although we only inoculated two different isolates on eggs during fertilization, the two we chose showed totally different outcomes. One successfully

colonized egg surfaces, but the other did not. It would be worthwhile in the future to inoculate other 20 egg isolates by controlling concentrations of inocula and subsequently observing the differences in efficiency of colonization.

Implications and significance

This is the first microbial succession study conducted on fish eggs. This study demonstrated that microbial succession can happen even within the short time span of an incubation period. We also demonstrated that egg associated microbial communities can be altered by many different factors, including both host and environmental factors such as stream flow rate, temperature, and aquatic microbial community structure. The host factors are presumably largely mediated by maternally provisioned lysozyme, which is the most dominant factor of all and acts quickly within 6 hours post-fertilization. We also identified a putative symbiont for eggs of the Lake Sturgeon (*Acidovorax* sp.). Our study provides management implications for conserving Lake Sturgeon populations by suggesting that damming streams can alter aquatic microbial community and temperature, which in turn can alter the microbial communities on sturgeon eggs and life history of the sturgeon. Our study also contributes to the broader literature on microbial community assembly and succession by demonstrating that both local deterministic processes and dispersal play roles in shaping the microbial communities assembly on the egg surfaces.