

BIOTIC AND ABIOTIC FACTORS INFLUENCE FORMATION AND ONTOGENIC
DYNAMICS OF MOLECULARLY DEFINED GASTRO-INTESTINAL MICROBIAL
COMMUNITIES IN LAKE STURGEON (*Acipenser fulvescens*) AND CHANNEL
CATFISH (*Ictalurus punctatus*).

By

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ABSTRACT

BIOTIC AND ABIOTIC FACTORS INFLUENCE FORMATION AND ONTOGENIC DYNAMICS OF MOLECULARLY DEFINED GASTRO-INTESTINAL MICROBIAL COMMUNITIES IN LAKE STURGEON (*Acipenser fulvescens*) AND CHANNEL CATFISH (*Ictalurus punctatus*).

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Gastrointestinal (GI, gut) microbial communities (microbiota/ microbiomes) play essential roles in host development and physiology. During early life stages, fish gut microbiome composition is shaped by complex interactions of factors including dispersal of bacteria from the surrounding water, age-dependent changes in the gut ecosystem, and changes in dietary regimes. To investigate ecological processes that generate and maintain compositional patterns of gut microbiome diversity, I integrated molecular methods with experimental gut microbiome research and community ecology theory in two important fish species, lake sturgeon (*Acipenser fulvescens*) and channel catfish (*Ictalurus punctatus*) during early larval stages. Sequence reads of 16S rRNA gene were analyzed using multivariate ordination methods based on Bray-Curtis distance matrices followed by hypothesis testing using permutational multivariate analysis of variance (PERMANOVA). In the first chapter, sturgeon larvae were raised in four rearing hatchery treatments representing a combination of two factors: water source (stream vs ground water) and diet (*Artemia* vs *Artemia* supplemented with detritus). As fish grew, microbiota shifted from dominance by phyla *Proteobacteria* to *Firmicutes*. Water possibly served as the primary bacterial inoculant during early (pre-feeding) stages of development. Neutrality tests indicated that neutral processes were not strongly structuring community composition. Sturgeon gut microenvironment appears to have

selected for microbial taxa, regardless of differences in treatments. The second chapter focused on alteration of gut microbiota modulated by diet and nutrient availability. Sturgeon gut microbiota differed among fish raised on different dietary regimes (control vs transition). Gut microbiota of fish exposed to a dietary transition from *Artemia* to frozen *Chironomids* were dominated by the genus *Aeromonas* (phylum *Proteobacteria*) while fish from the control group were dominated by genus *Clostridium_sensu_stricto* (phylum *Firmicutes*) at the end of experiment. Screening for cultured bacteria with extracellular protease activity revealed that fish fed with *Chironomids* harbored protease-positive taxa from phylogenetically distinct and more diverse clades. Next, I documented the impact of prophylactic treatments (Chloromine-T, NaCl followed by hydrogen peroxide; and hydrogen peroxide) on lake sturgeon larvae gut microbiota compared to ambient environmental conditions (control) using hatchery-produced and wild-origin fish. Gut microbiome responses to prophylactic treatments were found to be inconsistent across fish genotypes. The last chapter documented variability in channel catfish larval gut microbiome composition among families and nursery ponds characterized by different rearing water in a pond aquaculture setting. I documented a strong influence of rearing environment (pond water) on gut microbiome colonization. Gut microbiome composition was dominated by different phyla before (*Proteobacteria*) vs after pond stocking (*Firmicutes*). Different predominant genera were also detected over time. Results from my research inform community ecology theory concerning effects of stochastic and deterministic forces affecting microbial community establishment and stability. This research is also relevant for downstream applications incorporating microbial-based management strategies in commercial and conservation aquaculture.

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“Not all those who wander are lost” J. R. R Tolkien

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GENERAL INTRODUCTION

The presence of compositionally diverse microbial communities (also known as microbiota / microbiome) inside gastro-intestinal (GI) tracts has important consequences to proper physiological functioning of the animal hosts. The consortium of microorganisms play crucial roles in many host physiological functions including, but not limited to, nutrition and metabolism functions, development and somatic growth, immunity, and protection against pathogens (Bjorksten, 2006; Hattori & Taylor, 2009; Robinson et al., 2010; Sekirov et al., 2010). Recent expansion and advancement in sequencing technology, in particular the utilization of molecular tools such as metagenomic analyses based on 16S rRNA genes, has led to a growing literature that has characterized the structure and function of the gut microbiota for many taxonomic groups including fish (Ghanbari, Kneifel, & Domig, 2015).

Fish are an important vertebrate group that comprises the vast majority of vertebrate species (nearly 30,000). Taxa show remarkable variation in living environments, ecology, and evolutionary characteristics (Nelson, 2016). Studying gut microbiota in fish is warranted because of the extensive manipulation of microbial communities in aquaculture industries that crucial for food production and economic growth. Interactions of gut microbiota with fish hosts have profound impacts (Nayak, 2010; Romero, Ringø, & Merrifield, 2014; Vadstein et al., 2012), as fish are constantly immersed in aquatic solutions that have high bacterial load (De Schryver & Vadstein, 2014; Skjermo & Vadstein, 1999; Vadstein et al., 2012; Verschueren et al., 2000). In natural and aquaculture settings, wild and cultured fish typically experience a high level of mortality, particularly during early life stages in part due to interactions between the

microbes from surrounding environment and microbes associated with larval fish itself (Hamre et al., 2013; Oliva-Teles, 2012; Vadstein et al., 2012). For threatened fish species like the lake sturgeon (*Acipenser fulvescens*) and important fish for food production like the channel catfish (*Ictalurus punctatus*), the high mortality rates experienced during early ontogenetic stages have been identified as likely bottlenecks to successful recruitment for adult fish populations (Caroffino et al., 2010; Forsythe et al., 2013; Hargreaves & Tomasso, 2004; Hawke & Khoo, 2004).

Colonization of fish GI tracts by microbes occurs during early life stages and these microbial communities can later give rise to relatively stable communities in an adult fish. Yet, extensive spatial, temporal, and inter-individual variation does exist in the composition of gut microbiota which play important roles in homeostatic regulation (Costello et al., 2012; Llewellyn et al., 2014; Yatsunenko et al., 2012; Wong et al., 2015). Ecological processes that underlie the formation and temporal/ontogenetic dynamics of gut microbiota composition are poorly understood (Adair & Douglas, 2017; Christian et al., 2015). Little research has been conducted on how these processes interact in fish hosts (Llewellyn et al., 2014). From an ecological point of view, the GI tract and the microbiota residing within this anatomical structure can be viewed as a distinct ecosystem with its own community. Thus, the general field of community ecology can provide a robust conceptual basis to understand the pattern and diversity of gut microbiota (Adair & Douglas, 2017; Christian et al., 2015; Costello et al., 2012; Vellend, 2010; Zeng et al., 2015).

Due to the importance of gut microbiota to fish hosts, and the complex interaction of fish, microbes, and environmental factors, it is important to investigate the inter-relationships between the fish hosts and their surrounding environment (rearing water and diets) and how these affect microbial community composition and diversity in the fish gut during early life stages. Using gut microbiota as an empirical system, the theoretical foundation and conceptual understanding of processes that generate and maintain compositional patterns and taxonomic gut microbiota diversity will provide insights into the relative importance of ecological and evolutionary processes.

My dissertation is composed of four chapters. In the first chapter, experimental manipulation of gut microbiota colonization in lake sturgeon larvae was performed using full-sib fish produced from a single spawning pair that were raised in environments that differed in the water source (stream vs filtered ground-water) and diet (supplemented vs non-supplemented *Artemia*). Using massively parallel sequencing of a portion of the 16S rRNA gene from the GI tracts of larval lake sturgeon, we quantified the gut microbial composition and taxonomic diversity at three stages (pre-feeding, one, and two weeks after active feeding began). This research addressed two main questions: (1) What factor(s) and/or ecological processes affect the variation in microbial community composition that colonized the GI tracts of lake sturgeon larvae during early ontogenetic stages? (2) How does microbial community composition and diversity vary through early ontogenetic stages as the fish host develops and transitions in different environments? I hypothesized that the colonization and development of gut microbial communities would be influenced by interactions between factors including host ontogenetic stage, diet and rearing environment. Findings showed that the composition of microbial community

varied as the fish ages. Data further suggest that water served as the primary inoculant of the gut communities at early stages of development. As larvae began to feed exogenously, the intestinal habitat of sturgeon guts appears to have selected for specific microbial taxa, regardless of the differences in host rearing environment and feeding regime. Neutral expectation (i.e dispersal) underlying the colonization of these gut microbiota was supported statistically but was not a dominant process.

The second chapter focused on specifically, the taxonomic diversity and gut microbiota composition at the early ontogenetic stages as the consequences for fish with compromised nutritional status associated with dietary transition. Our objectives were to: (i) document the effect of food-transition on the gut microbial composition, (ii) screen and identify the presence of protease-producing bacteria in fish from different feeding treatments. Lake sturgeon that experienced diet switch from brine shrimp to frozen bloodworms performed poorly in terms of growth and survival. Prior to the experiment, fish were expected to perform better as they successfully transitioned to newly offered prey. Results could be due to starvation in this group relative to the control group that was only fed brine shrimp throughout the experiment. Compositional changes were observed throughout the fish developmental stages, but fish in transitioned group exhibited distinct communities compared to the fish in control group at the end of experimental duration. We also observed higher taxonomix diversity of protease-producers in the GI tract of fish, which received bloodworms (transitioned group).

The third chapter investigated the influence of prophylactic treatments using three chemotherapeutants: (i) immersion in antibiotic Chloramine-T, (ii) immersion in salt followed by hydrogen peroxide after 24 hr, and (iii) immersion in hydrogen peroxide; on

the taxonomic composition and diversity of gut microbiome in the lake sturgeon larvae in comparison to a control (no treatment) group. Larvae from two hatchery-produced families and from wild eggs collected at two natural spawning sites in the Black River (Michigan) were exposed to weekly prophylactic treatments. I hypothesized that disturbances in their gut microbiota caused by different treatments would decrease diversity and would increase community compositional differences in taxonomic composition. As fish are treated with chemotherapeuticants, they are believed to experience unfavorable rearing conditions in aquaculture settings that could interfere with their physiology. However, in human and other vertebrates, chemicals might be harmful to some microbes and may affect the composition and stability of their gut microbiomes. We asked the following questions: (1) what impact will the prophylactic chemical treatments (salt (NaCl), antibiotics (Chloromine-T), and hydrogen peroxide) that are commonly used in fish aquaculture have on the gut microbiome of lake sturgeon? (2) do microbiota of fish exposed to prophylactic treatments differ from fish in the control treatment or do communities show resistance following treatment disturbance? Statistical tests of data generated from 16S rRNA gene sequencing indicated that variation in alpha diversity indices of gut microbiomes were greater in fish samples from different origins (hatchery families vs wild caught eggs) relative to variation among fish exposed to different treatments. In one hatchery-produced family, gut communities of fish from the control group were distinct relative to gut communities of fish exposed to all three chemical treatments.

My final dissertation chapter emphasized common themes in microbial community ecology. In this chapter, we focused on commonalities and differences in fish-host microbiome associations in the channel catfish. In contrast to lake sturgeon, the channel catfish is a warm water species, characterized by a different feeding ecology and occupancy of different environments (pond aquaculture) during different ontogenetic stages, which we hypothesized, would influence their gut bacterial community composition and diversity. This study addressed the following questions: (i) what factors influence the formation of channel catfish gut microbiomes when raised in pond aquaculture following common industry practices? (ii) is there variability in microbiome composition across families and ponds characterized by different fish rearing water environment? This chapter involved collaboration with a research group from Mississippi State University (MSU) as part of an effort to identify best management practices to be adopted for catfish rearing in order to combat pathogen outbreaks. Results indicated strong selection occurred within the host where specific taxa proliferated and appeared to have been favored. The gut community also exhibited some resemblance to corresponding water samples. Community characterizations revealed temporal shifts in community composition and the influence of microbes present in the rearing water supply. Implications of modes of gut colonization to proposed probiotic uses in catfish pond aquaculture are discussed.

Results from my research offer empirical evaluation the effects of stochastic and deterministic forces affecting microbial community establishment and stability, which are important aspects community ecology theory. My research is also relevant for downstream application in commercial and conservation aquaculture by explaining

factors that may impact efforts to incorporate microbial-based management strategies such as probiotics usage.

LITERATURE CITED

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CHAPTER 1: LAKE STURGEON GUT MICROBIOTA ASSEMBLY AND SUCCESSIONAL DYNAMICS ALLOW ECOLOGICAL EVALUATIONS OF NEUTRAL VS HOST-SELECTIVE PROCESSES

ABSTRACT

Gastrointestinal (GI) or gut microbiota play essential roles in host development and physiology. These roles are influenced in part by microbial community composition. During early developmental stages, ecological processes underlying the assembly and successional changes in host GI community composition are influenced by numerous factors including dispersal from the surrounding environment, age-dependent changes in the gut environment, and changes in dietary regimes. However, the relative importance of these ecological processes to gut microbial communities are not well understood. We examined effects of environmental and host factors (change in ontogenetic stages and feeding physiology) on compositional changes in gut microbial communities based on massively parallel sequencing of a portion of the 16S rRNA gene from GI tracts of a primitive teleost fish, the lake sturgeon (*Acipenser fulvescens*). Lake sturgeon larvae during early ontogenetic stages were raised in environments that differed in water source (stream vs filtered groundwater) and diet (supplemented vs non-supplemented *Artemia*). We quantified gut microbial composition and taxonomic diversity at three stages (pre-feeding, one, and two weeks after exogenous feeding began). We documented taxonomic compositional divergence between the gut and environmental microbial communities among stages across each water and dietary treatment. Gut microbial community diversity declined and community composition differed significantly among stages. Large percentages of taxa present in the gut were over or under-represented relative to neutral expectations in each sampling period. Findings indicate strong, dynamic relationships

between gut microbiota composition and host gastrointestinal physiology, with comparatively smaller influences associated with rearing environments. Neutral models of community assembly and compositional change could not be rejected, but selectivity associated with microbe-host interactions were evident. Our results have implications for lake sturgeon conservation and aquaculture production specifically, and applications of microbial-based management in teleost fishes generally.

INTRODUCTION

One of the primary goals in community ecology is to understand species compositional diversity and the relative influences of forces underlying patterns of distribution and abundance across spatial and temporal scales (Vellend, 2010). Microbial communities, particularly those associated with the gastrointestinal tract (GI) of vertebrate hosts have coevolved in host-microbial ecosystems to be taxonomically and functionally diverse. Communities have important functions associated with host nutritional, developmental, immunological, and physiological process (Llewellyn et al. 2014).

Host gastrointestinal (GI) tracts are colonized during early ontogenetic stages by subsets of taxa found in the environment. Microbes that occur in the gut during early life stages can exhibit large variation in community composition (Costello, Stagaman, Dethlefsen, Bohannan, & Relman, 2012; Llewellyn, Boutin, Hoseinifar, & Derome, 2014; Yatsunenko et al., 2012). The gut microbiota of an adult may therefore in part reflect the history of past exposure to microbes and other past environmental factors present during earlier ontogenetic stages. Alterations of these early life stage communities have important consequences during later stages (i.e., ontogenetic contingency (Diggle 1994; Orizaola et al. 2010; Huey et al. 2012), including disease susceptibility (Gensollen et al. 2016).

Microbial community interactions with fish hosts have profound impacts because of the high bacterial abundance in aquatic realms (De Schryver & Vadstein, 2014; Vadstein et al., 2013; Verschueren, Rombaut, Sorgeloos, & Verstraete, 2000). Fish rely heavily on gut microbiota for many physiological functions including but not limited to growth, digestion, nutrient production and absorption, protection of animal against

pathogens and regulation of the immune system (Llewellyn et al., 2014; Wu & Wu, 2012; Hooper et al., 2012; Cain & Swan, 2010; Gómez & Balcázar, 2008; Macpherson & Harris, 2004). The initial colonization of microbial communities during early life stages can be influenced by many factors including fish host physiology, nutritional, and surrounding environmental factors (Giatsis et al., 2014; Ingerslev et al., 2014; Llewellyn et al., 2014; Nayak, 2010; Romero, Ringø, & Merrifield, 2014; Stephens et al., 2015).

Although the importance of the gut microbiota to fishes has long been recognized, the compositions of these microbial communities are often highly variable across developmental stages. Therefore researchers are challenged to infer the functional importance of constituent taxa. Understanding processes governing community formation, diversity, and their dynamic compositional changes remain elusive. Understanding the principles associated with bacterial colonization of GI tracts and with compositional changes across life stages will help managers, particularly in aquaculture settings, to manipulate gut communities to promote animal health, performance, and productivity.

Advances in next-generation sequencing technologies have expanded capabilities for the study of complex intestinal microbial community in terms of composition and functions beyond what has historically possible using culture-based methods (Dave et al., 2012; Ghanbari, Kneifel, & Domig, 2015). Microbiomes refer to assemblages of microorganisms existing in or associated with a defined habitats (in this case the gastrointestinal tract); including active and interacting members as well transient or inactive members (Lederberg & McCray, 2001; Shade & Handelsman, 2012). Research on fish gut microbiomes has lagged behind research on other vertebrate hosts until Rawls

and colleagues (2004) characterized the zebrafish gut microbiome and documented the difference between mammalian and teleost microbiota based on gnotobiotic teleost models. Additional studies followed on fish using the same model organism (Burns et al. 2016; Stephens et al., 2016; Yan, van der Gast, & Yu, 2012, Rawls et al. 2011), while a growing gut microbiome literature focused on other important aquaculture species like salmonids, tilapia, carp, and others. (see review in Llewellyn et al. 2014).

The number of studies concerning fish gut microbiota has increased, however, most involved descriptions of microbial community composition. Comparatively less attention has been devoted to studies of ecological processes governing the composition of fish gut microbiota. Examination of host GI tracts and microbiota residing within this anatomical structure and ecosystem would profit from using an ecological conceptual context (Costello et al. 2012). For example, Vellend (2010) describes four processes: selection, drift, dispersal, and speciation that underlie patterns in ecological communities that are also applicable to studies of microbial communities (Nemergut et al. 2013, Costello et al. 2012). In our examination of processes affecting fish GI tract microbiomes, we evaluate alternative predictions from neutrality theory (Hubbell 2001) with niche-based hypotheses (Vellend, 2010).

Gut microbiota assembly typically occurs early in host development, involving factors such as rearing conditions and diet, providing opportunities to study microbial community formation and succession, including exchanges between the environment and gut (Wong et al. 2016). For example, Sloan et al. (2006) applied neutral models to prokaryotic systems. The Sloan Neutral Prokaryotic Models were calibrated using data commonly used by microbial ecologists such as analyses of 16S rRNA gene sequences or

functional gene sequences. The ecological neutrality theory emphasizes the absence of differences in per-capita growth rate, death, and dispersal among species, and assumes equal fitness across species (Hubbell, 2001, Sloan et al. 2006, Vellend 2010, Burns et al. 2016). Deterministic processes include ontogenetic shifts in the gut environment that place certain taxa at a selective advantage over others. Therefore, the pattern of community composition and taxonomic diversity likely are the outcomes of stochastic processes of dispersal and drift (Burns et al. 2016, Venkataraman et al. 2015). By incorporating these conceptual frameworks together with advanced sequencing technologies, an improved understanding of processes governing fish gut microbiota assembly during early life stages is possible and warranted.

Sturgeons belong to one of the oldest groups of the bony fishes (*Osteichthyes*) and many are species of conservation concern. Sturgeons are also an important group in production and conservation aquaculture. Sturgeon species possess a unique valvular hindgut called a spiral valve that is absent in other fish except for *Elasmobranchs* (Buddington & Christofferson, 1985). There have been few studies on the gut microbiota of these primitive fishes. The majority of studies conducted have presented data derived from cultivable intestinal microbes on other sturgeon species (Askarian, Kousha, & Ringø, 2009; Callman & Macy, 1984; Masouleh, Sharifpour, & Arani, 2006; Ghanbari et al. 2009; Geraylou et al. 2012). Of all 27 sturgeon species, lake sturgeon (*Acipenser fulvescens*) is only sturgeon species endemic to the Great Lakes Basin and the only sturgeon in the genus *Acipenser* that spends their life solely in freshwater (potamodromous) in North America (Detlaf, Ginsburg, & Schmanhausen, 1993; Wilson & McKinley, 2004). This species has experienced significant declines in abundance and

distribution due to overfishing and loss and degradation of the habitat (Saffron, 2004). In recent years, sturgeon conservation aquaculture has increased greatly as part of restoration actions.

Successful lake sturgeon production can be limited due to high mortality, especially during early ontogenetic stages. Low survival of larvae is tied to nutritional regimes associated with diet formulation, feeding schedule, food presentation and preference (Mim et al., 2002; Vedrasco et al., 2002). After hatch, yolk-sac larvae gradually develop gastrointestinal (GI) tracts that will eventually resemble adult structures by 10-11 days post hatch (dph). Once the yolk-sac period has completed and the mouth begins to open, fish transition to exogenous feeding, often on brine shrimp (*Artemia*) (Buddington & Christofferson, 1985; Wang, Binkowski, & Doroshov, 1985). During this stage, the early exposure and interaction of the fish gut with microbial colonists from surrounding water is possible, and shifts in intestinal microbiota can be documented as fish continue to develop. Early ontogenetic changes in host diet and physiology can shape gut community dynamics.

Our objectives were to characterize gut microbiota in lake sturgeon during larval stages at the time before they start feeding until 14 days following the onset of exogenous feeding using Illumina Miseq high-throughput sequencing of a portion of the 16S rRNA gene. To our knowledge, this research represents the first experimental evaluation of the ontogenetic changes in community composition and diversity of the intestinal microbiota in sturgeon. This study was also designed to quantify associations of host factors, water supply, and diet on the lake sturgeon gut microbiota. We also quantify whether gut microbiome assembly is consistent with neutral expectations. Our findings have

implications for the management of nutrition, disease, and potential probiotic use in lake sturgeon culture, and characterize dynamic relationships between host ontogeny and environmental epibiota associated with temporal variability of microbiota residing in the gut.

METHODOLOGY

Experimental design and feeding regime

Lake sturgeon larvae were produced from a single mated pair collected during the sturgeon-spawning season on the Upper Black River, Cheboygan, MI in May 2013. Full-sib individuals were used to reducing potential variability in microbiota associated with host genetic background. All individuals were raised under four different rearing conditions. We used a 2x2 treatment factor design associated with water type and feeding regime. Water types included river water from the natal stream and Ultra-violet (UV) treated ground water (GW), reflecting water sources commonly used in traditional hatchery operations. These water types were used throughout the culture process including food (brine shrimp or *Artemia*) preparation. Fish were either fed live *Artemia* nauplii that are commonly used in sturgeon hatcheries (Bauman et al. 2015), or *Artemia* supplemented with organic retentate including detritus and aquatic zooplankton obtained from serial filtration through 100 µ and 50 µ filters used to filter river water entering the hatchery (hereafter referred to as “retentate”). Presence of digestible taxa in retentate was confirmed using massively parallel sequencing of the v9 region of the 18S rRNA gene. All four treatment groups were denoted as **S** (fish raised in natal stream water, fed live *Artemia*); **Sp** (fish raised in natal stream water, fed live *Artemia* fish mixed with retentate); **GW** (fish raised in groundwater, fed live *Artemia*); **GWP** (fish raised in groundwater, fed live *Artemia* mixed with retentate). These acronyms will be used throughout the paper.

Each treatment included six 3.0 L polycarbonate tanks (Aquatic Habitat) that served as biological replicates, each holding 70 individuals to achieve a statistical power

of 0.8. The power analyses were performed based on our preliminary microbial studies (Fujimoto et al., 2013). Fish were exposed to the same water type beginning at the time of egg fertilization and incubation stages. A newly hatched larval group of 70 fish were then distributed into each treatment replicates at 10 days post hatch (dph). The food was offered at 12 dph. However, only at 16 dph, we began to consistently offer food when at least half of the fish were feeding. To ensure fish received consistent amounts of food throughout the experiment, previously established dry-weight feeding rates for sturgeon (Deng et al. 2003) were utilized whereby larvae in all tanks were fed at 26% body weight daily (BWD). Prior to the first feeding each day, retentate were added to the freshly prepared *Artemia* for treatment Sp and GWp. The amount of retentate (in grams) collected daily varied depending on each day collection, yet the food amount was adjusted accordingly to ensure fish in each treatment consumed the same amount of food. Fish were fed to satiation three times daily. Mortality was recorded daily, and the body weight (g) was recorded once in every third day. All experiments were conducted at Sturgeon Streamside Rearing Facility managed by Michigan Department of Natural Resources (MDNR) and Michigan State University (MSU) at Onaway, MI using approved Institutional Animal Care and Use Committee (IACUC) protocols.

Sample collection

We sampled ten sturgeon larvae from each replicate for each treatment at three ontogenetic developmental stages: Time 1 - before active feeding (11-day post hatch, dph), Time 2- after one week of fish actively feeding (22-dph), and Time 3 - after two weeks of active feeding (29-dph). These time points were selected to capture critical phases of GI tract development after fish completely absorbed their yolk sac and

gastrointestinal tract anatomy was completed (approximately at 10 dph). Lake sturgeon started actively feeding between 13-16 dph when food was first offered (Buddington & Christoferson, 1985; Buddington & Doroshov, 1986). Fish were sampled for microbial community interrogation at each time period and were euthanized with an overdose of MS-222 (Sigma-Aldrich, St Louis, MO, USA) at the time of sampling. Each individual larvae was photographed at the time of euthanization and images were analyzed using Image J software to determine total body length (mm). Fish were then transferred and preserved in 50 ml Corning ® centrifuge tubes containing 80% filtered-sterilized ethanol until GI tract dissection and bacterial DNA extraction was performed.

Other environmental samples including water and food were also collected during each sampling period. 250 ml of water samples from stream water and ground water were collected from the hatchery reservoir tank and being filtered through 0.22 um 47 mm filter membrane (Sterlitech®) using a hand-pump to obtain the aquatic microbial communities on 0.45 micron filter paper. These filters were then transferred and preserved in 50 ml Corning ® centrifuge tubes containing 80% ethanol until bacterial DNA extraction is performed. For food samples, approximately 200µl of food were pipetted into 2ml Eppendorf tube and were preserved in the same manner as fish and water samples.

DNA extraction and 16S rRNA amplicon sequencing

Gut microbiota from lake sturgeon larvae were surveyed using high-throughput sequencing of the v4 region of the 16S rRNA gene. The distal gut (spiral valve) of each sturgeon larvae was recovered from fish following aseptic techniques. The distal gut was defined as the section extending from the beginning of the intestine until the spiral valve.

Exterior surfaces were swabbed with 100% ethanol before dissections of the whole digestive tract using sterile instruments. Dissections were performed as previously described by (Milligan-Myhre et al., 2011) with slight modification. The intact GI tracts were cut from the fish body cavities, and the excised GI tract was immediately transferred into filtered-sterilized 80% ethanol for DNA isolation. Due to the small size of the gut, a composite of at least four GI samples of larvae were combined for each tank replicate, within each treatment group, at each time point. Each tube containing GI tract samples was first centrifuged for 15min at 4°C to pellet tissues and bacteria before DNA was extracted. The MoBio PowerSoil® DNA Isolation Kit (Carlsbad, CA, USA) including a bead-beating step was used following protocols for low-biomass samples as suggested by the manufacturer with slight modification. The integrity of each DNA sample was assessed based on the amplification of an approximately 1.4k bp of 16S rRNA gene (27F and 1389R) followed by gel agarose electrophoresis and DNA concentration were quantified using Microplate spectrophotometer (BioTek®, Winooski, VT, USA).

One hundred fifteen DNA samples that have been validated to contain sufficient bacterial DNA (as shown by the presence of 16S rRNA amplicon bands during electrophoresis) were submitted for the sequencing at Michigan State University Research Technology Support Facility, RTSF (East Lansing, MI, USA). All of the sequencing procedures, including the construction of Illumina sequencing library, emulsion PCR, and MiSeq paired-end sequencing v2 platforms of the V4 region (~250bp; primer 515F and 806R) followed standard Illumina (San Diego, CA, USA) protocols. Michigan State's Genomics RTSF (<https://rtsf.natsci.msu.edu/genomics/>)

provided standard Illumina quality control; including base calling by Illumina Real Time Analysis v1.18.61, demultiplexing, adaptor and barcode removal, and RTA conversion to FastQ format by Illumina Bcl2Fastq v1.8.4.

Sequence processing

Sequence data were processed using default sequencing data analyses pipeline and computing workflow. Briefly, paired-end sequence merging, quality filtering, “denoising”, singleton-sequence removal, chimera checking, taxonomic assignments and Operational Taxonomic Unit (OTU) selection was conducted using an open-source workflow based on methods implemented by *mothur* v.1.36.1 (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013). A reference-based OTU clustering and the taxonomic assignment was performed using SILVA-based bacterial reference database file provided in *mothur* to cluster sequences defined with 97% identity. To minimize effect of under sampling while maintaining as broad a dataset as possible, the final OTUs table was rarefied to a depth of 5775 sequences per samples. Five DNA samples with low sequence depth were discarded in downstream analyses. Rarefaction analyses were performed to evaluate the sampling coverage for each samples based on the selected sequence depth.

Analyses of bacterial community profiles and ecological statistics analyses

1.1 Alpha diversity

All measures of community diversity and similarity including Inverse Simpson (1/D) diversity indices and OTUs richness of each sample were calculated from the sequence data within *mothur* to quantify alpha [α] diversity. To test for significant differences in diversity indices among treatment groups (water and diet), and among time periods, a multiple factor ANOVA was performed on the summary files provided by *mothur* using

the programming and statistical software, R (version 0.98.978) base package. The test was followed by Tukey HSD post-hoc tests. P-values below 0.05 indicated significant differences in pairwise mean comparisons.

1.2 Temporal and Differential abundance of OTUs

A custom R code was used to calculate the relative abundance and identify dominant phyla and taxa (OTUs) in all communities across sampling times (gut microbiota, water, and *Artemia*-associated epibiota). Codes were written and implemented using packages *dplyr* and *reshape2*. The relative abundance of all taxa within community samples was calculated, and taxa with (normalized) abundance exceed 0.1% were considered as ‘pre-dominant’ taxa. The most abundant 20 taxa were subset from total number of OTUs based on the 0.1% cut-off, and relative abundances were tabled to show temporal variability in fish gut community composition. The remaining taxa were grouped as ‘Others’.

1.3 Beta diversity

We used several packages implemented in R to perform comparative community compositional analysis of beta [β] diversity and other community ecological statistics using the tabulated OTU dataset of pre-dominant taxa. Briefly, we used the *vegan* function to generate estimates of Bray-Curtis (BC) distances among sample microbial communities (Oksanen, 2015). Subsequently, we used the *cmdscale* function to perform ordination (Principle Coordinate analyses, PCoA) based on BC distance (Bray & Curtis, 1957). The *ggplot* and *ggplots2* packages (Wickham, 2009) were used to create ordination plots to visually compare gut and environmental bacterial community composition among samples collected from different treatments and among sampling

periods based on the two largest eigenvalues. Two multivariate hypothesis tests were implemented using two functions. The *adonis* function was used to perform multivariate hypothesis testing on differences between locations of the centroids of treatment group coordinate ordinations based on Permutational Multivariate Analyses of Variance (PERMANOVA). The *betadisper* function was used to perform a Homogeneity of Multivariate Dispersion (PERMDISP) test on community BC matrices (Anderson, 2001; 2006). These tests were employed because of the non-parametric and skewed nature of microbial community compositional data. OTUs that had the highest correlation with the PCoA x and y component axes were identified based on Pearson correlation coefficients using the *corr* function.

1.4 Influence of water and diet treatments of GI tract microbiota

To analyze treatment effects of water type and diets on fish gut microbiota, PCoA were performed separately on fish gut communities for all four-treatment groups within each developmental stage. Important PCoA axes denoted by comparatively larger eigenvalues to average eigenvalues were selected. Linear regression models were fit, where each axis was a response variable given predictor variables of water type and food type. Under the null hypothesis, we expected gut community composition at each time point to be unaffected by treatment. Axes represent linear correlations of bacterial taxonomic composition present in gut communities. Axes that showed significant effects of treatments or interactions between water and diet treatments were analyzed using the *lsmean* function to determine the effects of each factor (or combination of factor(s) in the interaction) on the bacterial taxonomic composition and relative abundance in larval fish GI tracts.

1.5 Tests evaluating whether GI tract communities were a neutral subset of the environmental (source) communities

We used a neutral community ecological model adapted from Sloan et al. (2006) to explore the relative importance of neutral processes (i.e., dispersal and ecological drift) and selection in gut microbiomes at a given time of sampling (pre-feeding, one week, and two week active feeding). This model also distinguished members of the gut microbiome whose presence was consistent with dispersal from surrounding environmental communities (water as a source) and those that deviated from the neutral model (i.e., were over or under represented in the gut relative to the water source(s)). Using customized R scripts following Venkataraman et al. (2015), we evaluated whether neutral processes associated for example with probabilities of transport from source communities were sufficient to explain the observed composition of the gut microbiomes at each developmental stage. The level of fit of the neutral model served as an adequate representation of gut community composition if the R^2 value was high, whereas the significance of neutral processes was indicated by the p-value.

RESULTS

Sequencing and sample summary

Rarefaction analyses showed that sequencing efforts were consistent across replicate samples and treatments at a depth of 5775 sequences per sample as denoted by total percentage coverage higher than 98%. We were able to sample a large portion of the OTUs and diversity present while still retaining a large number of samples within fish of a given age. After quality filtering, our 16S rRNA amplicon dataset produced 6,034,269 high quality reads. In total, we observed 4137 OTUs (2894 when omitting singleton OTUs) defined at 97% sequence identity. From 118 samples submitted for sequencing, rarefaction at 5775 sequences per sample eliminated five samples below this sequencing coverage.

Morphometric data consisted mean fish weight ($\text{g} \pm \text{SD}$) and length ($\text{mm} \pm \text{SD}$) showing that fish growth in all four treatments was consistent throughout the duration of the study (data not shown). By the end of the trial, fish raised in stream water grew significantly larger compared to fish in UV-treated groundwater. No fish health issues were detected. Survival of fish was nearly 100% in all treatments and developmental stages.

Ontogenetic changes of dominant bacterial taxa in lake sturgeon larvae rearing

We quantified the number of sequences that were represented by each phylum from GI tracts of all fish sampled in each developmental period. We found that gut bacterial communities were comprised of 26 microbial phyla, however the most abundant phyla covering more than 95% of all sequences, in order of abundance included *Proteobacteria*, *unclassified phyla*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Acidobacteria*,

Planctomycetes, *Chloroflexi*, and *Fusobacteria*. General patterns of bacterial phylum-level contributions to gut microbiomes were shown with regards to treatment group and corresponding sampling time (Fig. 1.1). Across all treatment groups, the microbiome composition shifted from communities dominated by *Proteobacteria* and several other phyla prior to initiation of exogenous feeding to *Firmicutes* dominated communities. The relative abundance of all other phyla were reduced after fish began actively feeding.

At the genus level pre-feeding gut communities across all treatment groups were dominated by *Proteobacteria* from the genus *Pseudomonas*, comprising between 13 to 23% of total community composition. This genus was also present in food and water, although at considerably lower levels (~1%). Upon initiation of feeding, several *Firmicutes* genera including *Sarcina*, *Exiguobacterium*, and *Clostridium* emerged as the predominant taxa in the lake sturgeon larval gut microbiota sampled after one week of active feeding. As fish aged, *Sarcina* persisted among predominate taxa comprising ~20-30% of the gut community in all GW, GWP, S, and Sp groups. However, fish raised in stream water (S and Sp) had *Clostridium* as the most predominant taxa after two weeks of active feeding, whereas *Deefgea* in the phylum *Proteobacteria* was as prevalent as *Sarcina* in fish raised in the ground water treatment.

We tested whether changes in larval gut microbiota occurred when fish were raised on experimental diets and rearing water sources across life stages. Over the course of three weeks of development, we found that microbiome diversity varied among age cohorts. Overall, community diversity decreased as fish transitioned to active feeding (Fig. 1.2a and 1.2b). Multiple factor ANOVA analyses quantified sources of variability in alpha diversity indices (Inverse Simpson diversity), indicated statistically significant

differences in diversity as a function of sampling time ($F\text{-value}_{\text{inv}}=11.31$, $\text{df}=2$, $p < 0.001$), whereas OTU richness did not differ across sampling times ($F\text{-value}_{\text{rich}} = 0.91$, $\text{df}=2$, $p = 0.407$) or treatment ($F\text{-value}=1.92$, $\text{df}=3$, $p=0.137$). No significant interaction was found between water and food treatments for Inverse Simpson Diversity or richness ($F\text{-value}_{\text{inv}} = 1.13$, $\text{df}=6$, $p=0.358$; $F\text{-value}_{\text{rich}} = 0.38$, $\text{df}=6$, $p=0.892$, respectively).

Variation in gut bacterial community profiles in association with water, diet, and time

To visualize relationships between gut microbial community composition in the gut and the composition of environmental sources (water and diet), a PCoA was performed to analyze samples in reduced dimensional space using ordination plots. Variation in community membership among environmental microbiota and gut microbiota within each replicates from all treatment groups during each time period indicated age-dependent changes in prevalent microbial taxa (Fig. 1.3). Statistical analyses of beta diversity across sampling periods (fish ages) revealed significant taxonomic compositional divergence of the microbial community (PERMANOVA test pseudo- $F = 4.88$, $R^2 = 0.084$, $p < 0.001$). We reject the null hypothesis of no differences in multivariate centroid location and microbial community or composition across sampling times. In addition, the non-significant results of the PERMDISP ($p = 0.201$) indicated that compositional dispersion within group was homogenous. Therefore PERMANOVA results can be interpreted as true differences in time or age-dependent community composition.

Community composition data were further analyzed to decouple the treatment effects of water source and diet from the pervasive effects of sampling time. Under the null hypotheses, water type and food administered treatments were not expected to

significantly affect gut community taxonomic composition, and there would be no interaction between water and diet treatments within each time point. During the pre-feeding period (sampling period 1), we found significant water treatment effects on the PCoA axis 6 since fish has not officially started feeding yet (Table 1.1). As fish transitioned to active feeding, both water and food treatments interacted significantly to affect gut community composition after one week of feeding (PCoA axis 1, Fig. 1.4a). The effect of water treatment was significant on the PCoA axis 5 (Table 1.1). During sampling period 3 after the second week of active feeding, gut community composition was statistically different between water treatments. There was no effect of diet (PCoA axis 1, Fig. 1.4b). Least-Square means (LS-mean) values for all-important axes across all time are shown in Table 1.1.

Focusing on the first coordinate axis during sampling periods 2 and 3 following initiation of feeding, we investigated whether there was evidence for co-occurrence of taxa in gut communities. Genus *Clostridium* (Otu001) associated with family *Clostridiaceae* (phylum *Firmicutes*) was found to have strong, positive correlation with the first axis during the first week of feeding (Pearson correlation, $r = 0.884$). During the second week of feeding, two other bacterial taxa (*Sarcina*, (Otu002) and Unclassified genera (Otu004), both members of *Clostridiaceae* family) showed a strong correlation (*Sarcina* $r = -0.791$, Unclassified *Clostridiales* $r = 0.743$) with the first coordinate axis. Another taxon identified as genus *Deefgea*, a member of the family *Neisseriaceae* (phylum *Proteobacteria*, order *Gammaproteobacteria*) was also in high abundance and was positively correlated on the same axis ($r = 0.779$).

Neutral processes are not the dominant mechanisms generating and maintaining community composition during early gut microbiome assembly

LS-mean analyses of PCoA axis 1 at sampling point 3 (two weeks after initiation of feeding, 22 dph) suggest that dispersal of bacteria from environmental (water) sources into the gut was important in shaping the community composition in the gut. However, previous analyses did not address the question Is the fish gut community at each developmental stage a neutral subset of the source community? We wished to distinguish between species that are detected in the gut because of neutral processes and deterministic processes that may be associated with age-dependent gut conditions. To answer this question, we applied the neutral model theory based on the Sloan (2006) Neutral Model for Prokaryotes to investigate developmental processes underlying gut microbiome compositional and successional change over time. The neutral model assumes that community composition can be explained by the dispersal of microbial taxa from the surroundings and ecological drift (stochastic change) within the source community (Venkataraman et al. 2016). Based on LS-mean analyses, we found that water was a more important source of gut community members than was diet.

Neutral processes were detected as part of important contributors to microbial community formation during each sampling period (developmental stage) as indicated by significant p-values (Figs. 1.5a-c). These processes are significant, yet not strongly predictive. Using R^2 as a measure of goodness-of-fit, we determined that gut community composition from across all treatments poorly fit a neutral model based on the low R^2 value of model fit.

Across sequential sampling periods, as fish aged, the number of shared microbial OTUs between the gut and water decreased (Table 1.2). At the pre-feeding stage, larval GI tracts contained the most neutrally dispersed taxa (114), but this number declined dramatically as fish began active feeding (45). Two weeks after active feeding, only 38 taxa were shared between gut and water communities, and about half of these taxa were under-represented or over-represented in the gut (Table 1.2). We conclude from these analyses that the OTUs of gut microbiomes are likely under selection. Taxa present in the gut are not a completely neutral subset of the taxa present in the source water communities.

DISCUSSION

Many basic microbial community ecology questions, concerning the dynamics of community composition at the onset of colonization and early ontogenetic changes, are of importance to understand host-microbial relationships in the wild and under domestic conditions. Information pertaining to the source of microbial communities that establish in the fish gut, neutral vs non-neutral dynamics during community assembly and generate inter-individual variability, and the interaction of animal host growth with the establishment of gut communities that collectively affect taxonomic diversity and relative abundance are incompletely known.

In this study, we were interested in characterizing the development of fish gut microbiomes during important early developmental stages. Our experimental system enhanced understanding of the factors affecting initial colonization and development of the lake sturgeon larval gut microbiota prior to and during the critical transition from endogenous to exogenous feeding. Our research contrasts previous studies on other sturgeon species associated with diet and gut microbiota that were either performed on larger/older fish. For example, e.g., Geraylou et al. (2013) and Bacanu & Opera (2011) used from Siberian sturgeon (*Acipenser baerii*) and white sterlet sturgeon (*Acipenser ruthenus*), respectively at the juvenile stage (average weight between 15g-30g and more than 3 months post hatch). Other researchers (e.g., Askarian et al., 2011; Bacanu & Oprea, 2013; Callman & Macy, 1984; Geraylou et al., 2013; Masouleh et al., 2006) used fish that had been exposed to cultivation for extended periods.

To our knowledge, this report provides the first experimental system to evaluate environmental (focusing both on rearing water type and diet administered) and host-

associated factors that affect the compositional dynamics of the intestinal microbiota using high-throughput sequencing based on 16S rRNA specifically in sturgeon, an important aquaculture teleost fish species and a species of conservation concern. These factors have a profound influence on microbial membership and could further impact the metabolic potential of the gut microbiome. Advancing uses of microbial manipulation (pre- and probiotics) is a goal for the aquaculture industry to promote fish growth and health. Accordingly, advancing understandings of compositional dynamics that naturally occur in the gut microbiota of cultured fish species like sturgeon has relevance to commercial and conservation aquaculture. Here, we provide evidence that changes in microbiota in the gut of lake sturgeon occurs early in development. Gut microbiota composition quickly diverged from the environmental communities associated with their respective rearing water and diet conditions.

Lake sturgeon larvae were raised in constant, controlled environments through manipulation of water and diet over three sampling periods from 11 to 29 dph. Our results suggest that major compositional shifts in gut community composition corresponding to different developmental stages. Taxonomic profiles changed across sequential larval stages extending before the onset of exogenous feeding (pre-feeding) through 29 dph when fish were actively feeding, and when GI tract structures resembled adult anatomical structures (Buddington & Christoferson, 1985; Buddington & Doroshov, 1986). Findings suggest that during these early ontogenetic periods, host physiological development likely serves as a strong deterministic force directing the formation of gut communities, regardless of food type or surrounding (water) environmental communities. The temporal shift in bacterial community composition has

also been documented in others studies involving zebrafish and rainbow trout (*Onchorhynchus mykiss*) larvae during periods of constant diet and environmental condition. However, while rainbow trout studies reported the strong influence of diet type and environmental factors to gut microbiota composition, findings from the zebrafish studies differed (Stephens et al., 2015, Wong et al. 2015, Ingerslev et al., 2014).

Pronounced temporal changes in microbial community composition occurred from the pre-feeding to exogenous feeding periods. *Proteobacteria* dominated gut communities during the pre-feeding stage, whereas *Firmicutes* dominated communities after two weeks of feeding. Large decreases in community taxonomic diversity were also documented between pre- and post-feeding periods. Results are concordant with findings for other fish species, that documented *Proteobacteria* and/or *Firmicutes* as among the most abundant phyla in fish gut communities. In zebrafish, studies by Stephens et al. (2015) indicated that *Firmicutes* and *Proteobacteria* are most common in larvae, but adult microbiota were dominated by *Fusobacteria* (Rawl, 2006). Studies on microbiome ontogenetic shifts in rainbow trout fry also indicated the presence of *Proteobacteria* or *Firmicutes* associated with either marine-based or freshwater plant-based diet offered during first feeding (Ingerslev et al., 2014). Geraylou et al. (2013) found that administration of prebiotic to Siberian sturgeon (*Acipenser barii*) shifted gut microbiota primarily in the phylum *Firmicutes*. Comparative analyses of gut microbiota from 8 freshwater fish species encompassing fish with different feeding habits revealed that *Proteobacteria* and *Firmicutes* were the dominant phyla in all fish species (Li et al., 2014a). Another cross-sectional gut microbiome study performed by Bledsoe et al.

(2016) on channel catfish also indicated the prevalence of *Proteobacteria* in fish at 3 dph, and the appearance of *Firmicutes* along with *Proteobacteria* when fish reached 65 dph.

We also found that predominant taxonomic assemblages tended to consist of closely related taxa from the family *Clostridiaceae*. This includes *Sarcina*, *Clostridium*, and unclassified genera from the family. This might indicate that traits underlying assemblage membership were often shared among related organisms. One of these taxa, genus *Clostridium* is enriched following the transition to active feeding. In humans, this genus is part of important commensal microbiota that begin to colonize human intestines of breast fed infants as early as the first month of life, and have been shown to play roles in modulating gut homeostasis over the entire life span (see review by Lopetuso et al. 2013). Studies in mice indicated that commensal *Clostridia* populate specific regions in the intestinal mucosa, thus establishing a close relationship with gut cells and perform critical physiological functions (Lopetuso et al., 2013).

Another taxa, *Sarcina* was also found to be abundant by the time fish reached 22 dph and actively feeding. *Sarcina* are fermenting bacteria that are frequently found in the gastric contents and faeces of human patients with gastro-intestinal disorders. All the strains were obligate anaerobes, fermented cellulose and required a carbohydrate for growth, all produced ethanol but not butyric acid from glucose and can also produce acid and gas from sugar like glucose, fructose, sucrose, maltose, lactose, galactose and raffinose (Crowther, 1971). One species, *S. ventriculi* is widespread in the soil and may be considered to be part of the intestinal flora of human although its significance remains unknown (Crowther, 1971; Smit, 1911). Given the biology of both taxa and feeding nature of sturgeon as a bottom feeder, it is possible to have *Sarcina* and *Clostridium* as

predominant taxon in their distal gut. Sturgeon possess a valvular hindgut (spiral valve) that serves as the primary region of digestion and nutrient absorption, and thus might provide an abundance of nutrients for bacterial like *Sarcina* to flourish (Buddington & Christofferson, 1985; Callman & Macy, 1984) and *Clostridium* to maintain all gut physiological functions. Studies by Callman & Macy (1984) also showed that anaerobic bacterial fermentation takes place in the spiral valve producing volatile fatty acids (VFA's) and hydrogen gas as by-product, supporting the idea that these bacteria may enhance digestive efficiency.

Two important *Proteobacteria* taxa were *Pseudomonas* and *Deefgea*. *Pseudomonas*, was present during all stages, although it was initially present in abundance during the pre-feeding stage and later became less abundant at one and two weeks after active feeding began. The presence of *Pseudomonas* in relatively high abundance has also been reported as part of lake-sturgeon egg-associated community (Fujimoto et al., 2013b). Romero & Navarrete (2006) found *Pseudomonas* sp. present in abundant within gastrointestinal tracts of fish juvenile as well as on eggs, but not in the water nor in food when they studied bacterial communities associated with early life stages in coho salmon (*Onchorynchus kisutch*). This is likely due to vertical transmission of a pioneering strain from eggs to fish GI tracts. *Pseudomonas* is also commonly observed in gut microbiota of mature fish (Hansen & Olafsen, 1999; Llewellyn et al., 2014; Navarrete et al., 2008). A number of diet related studies have reported variability in relative abundance of *Pseudomonas* sp. that were affected by differential food treatment. These taxa are invariably influenced by experimental diet including dietary inulin in surubins (Mourino et al. 2012), dietary mannanoligosaccharides (MOS) in rainbow trout

(Dimitrolou et al. 2009), and dietary yeast culture in both hybrid and Nile tilapia (He et al. 2010)(see review by Ringo et al. 2016).

Little information is available pertaining to *Deefgea* sp. Previous studies by Chen et al. (2010) reported so far only two species found in genus *Deefgea* (Family *Neisseriaceae*, Order *Betaproteobacteria*). Those species were described as *Deefgea rivuli* and *Deefgea chitinilytica* (Chen et al. 2010; Stackebrandt et al. 2007). However, both taxa originated from hard-water and wetland samples, respectively. In another study, Jung & Jung-Schroers (2011) first documented the association of *Deefgea* isolates with fish. They reported six isolates of *D. chitinilytica* were cultured from swabs of skin and internal organs of two freshwater ornamental fish species farm-raised in farm [gold tench (*Tinca tinca*) and goldfish (*Carassius auratus auratus*)]. Several other bacterial taxa from the same family were described as chitin-hydrolyzing species, and *D. chitinilytica* was suspected to have similar function too. Due to this, *Deefgea* could have significance importance listed to be among opportunistic taxa that could play roles in infections of aquatic organism (Jung & Jung-Schroers, 2011).

Throughout the assembly of early gut bacterial communities, stochastic and deterministic factors associated with water and food epibioota could play roles in shaping these ecological communities (Llewellyn et al., 2014; Navarrete et al., 2009; Romero & Navarrete, 2006). As we have shown in ANOVA and multivariate analyses of gut microbiome at each time point, the water rearing environment initially had a strong influence on community composition during the pre-feeding stage, as gut communities reflected aquatic communities of both water treatments. Water appears to serve as primary inoculant before and during the transition stage from endogenous to exogenous

feeding. Early stages of gut microbiota colonization were temporally unstable and stochastic processes such as random recruitment of water epibioota into the gut occurred at the beginning of exposure of the early upper GI tract and gill surface to ambient water (Hansen & Olafsen, 1999). As fish developed further, gut communities changed coincident with initiation of active feeding and diverged in composition from to surrounding communities. Previous studies performed by our lab on egg surface of lake sturgeon also displayed directional changes of bacterial community along with egg developmental stages (Fujimoto et al. 2013). Another study by Giatsis and colleagues (2015) also documented changes in the gut community structure over time with significant contributions of water bacterial communities.

As feeding continued, significant interactions were observed. We observed no significant differences between gut communities of supplemented vs non-supplemented food treatment groups within the stream water environment. In contrast, significant differences were observed between gut microbiota of fish raised in groundwater based on the food treatment administered (supplemented vs non-supplemented diet). Gut communities of fish raised in groundwater differed according to diet, indicating that diet influences gut community membership. However, further analyses showed that the effect of diet on gut community composition after the pre-feeding stage was not evident. Only water treatment was significantly associated with gut community composition at two weeks of active feeding. At this point, gut communities differed between stream and ground water treatments.

Dynamics of community compositional change during the gut assembly processes can be attributed many processes (Costello et al. 2012). First, the composition of the gut

community could be determined by environmental selection. In this study, the environments were associated with either the rearing condition of fish, as fish raised within similar water environments were exposed to similar pools of microbial taxa present in each source. Rearing temperatures of surface water and ground water may also have contributed to gut-selective environments. Sturgeon raised in our hatchery exhibited faster growth rate in surface water with temperature relatively warmer than ground water, supporting previous report that rates of sturgeon during early development are temperature dependent (Wang et al. 1985). The local community could also be under the influence of neutral vs selective processes. In the first situation, taxa present in the gut could be a random draw of species present in water. Subsequently, maturation of the fish gut could impose selective pressures that favor particular subsets of taxa, or inhibit the growth of certain subsets of taxa (Giatsis et al., 2015).

The evaluation of the relative importance of neutral vs deterministic processes was achieved through implementation of a neutral model. Our findings suggested that neutrality (e.g., random dispersal of taxa from water) does occur but is not the pervasive ecological force shaping the gut community during all three developmental stages. Low model R^2 values indicated that neutral processes are not dominant. Deterministic processes are also likely shaping changes in gut community composition associated with development of fish GI tract as evidenced by reductions in the number of taxa between the gut and water (Figs. 1.5a-c), even though the proportional contributions of over- and under-represented taxa remained approximately unchanged across periods.

Morphometric data within sampling period revealed that length and weight of fish larvae differed significantly among water treatment as ambient water temperature

affected growth. Fish in groundwater developed more slowly compared to fish reared in stream water during those ontogenetic stages. As a result, their gut offered different microenvironment that could support certain microbial taxa, which in turn affected microbial relative abundance and taxonomic diversity.

We recommend future studies include experimental designs that sample over a longer duration, including manipulations of the water community. Further studies focusing on taxa that increase in relative abundance over time would be useful to establish ecological functions that could be used for greater efficiency in applications of microbial-based fish management in aquaculture. Such data could also be used to identify suitable release sites for fish from conservation hatchery programs.

Overall, the significant changes in diversity and taxonomic composition of the lake sturgeon gut microbiomes occurred principally associated with early developmental stages in connection with the initiation of first feeding. Microbial communities diverged following initiation of exogenous feeding compared to a community of surrounding water and food epibionts. Our understanding and abilities to control (i.e., application of probiotics) underlying deterministic and stochastic factors associated with the source of microbial inocula appeared to be in part tied to communities in the water when feeding begin. Future studies may profitably explore effects of manipulations of communities in rearing water and food samples to understand the dynamics of microbial community assembly associated with these factors.

APPENDIX

APPENDIX

Table 1.1 showing R²-values, p-values, and Least-square mean values of least-square mean analyses performed on significant PCo axes for each sampling period. Five axes across all three stages that showed significant effect of either water, food, or interaction of both water and food treatments on the microbial community composition were Axis 6 for pre-feeding, Axis 1 and Axis 5 for stage at one-week post-active feeding, Axis 1 at two-week post-active feeding. Interaction plots were shown in Figure 1.4

Least-square means							
Linear regression model : Pco Axes ~ Water type * Food administered				Stream Water		Ground Water	
Important axes	Signif. Treatment	p-value	R2 value	Food - Suppl.	Food - No suppl.	Food - Suppl.	Food - No suppl.
Pre-feeding							
Pco Ax.6	Water type	0.045	0.206	0.044±0.06	-0.137±0.05	0.070±0.05	0.030±0.05
One wk active feeding	Interaction (Water type and food type)	0.004	0.444	-0.079±0.09	-0.048±0.09	0.327±0.09	-0.300±0.12
One wk active feeding							
Pco Ax.5	Water type	0.016	0.233	0.064±0.05	0.079±0.05	-0.049±0.05	-0.142±0.06
Two wk active feeding							
Pco Ax.1	Water type	0.020	0.267	-0.189±0.10	-0.125±0.10	0.086±0.10	0.227±0.10

Table 1.2 Results of neutral models applied to gut microbiota showing the number and proportion of shared OTUs detected in both the gut microbial communities and potential microbial source (water). Over-represented taxa are those that were selected for (i.e., abundance low in water, but detected in higher abundance in the gut) whereas under-represented taxa are present in lower abundance in the gut than in water.

Developmental stages	Pre-feeding	One-week after active feeding	Two-week after active feeding
Total number of shared OTUs between source (rearing water) and target (fish gut) communities	160	45	38
Number of neutrally dispersed OTUs (Proportion, %)	114 (71.25%)	27 (60.00%)	22 (57.89%)
Number of overrepresented OTUs (Proportion, %)	31 (19.38%)	11 (24.44%)	12 (31.58%)
Number of underrepresented OTUs (Proportion, %)	15 (9.37%)	7 (15.56%)	4 (10.53%)

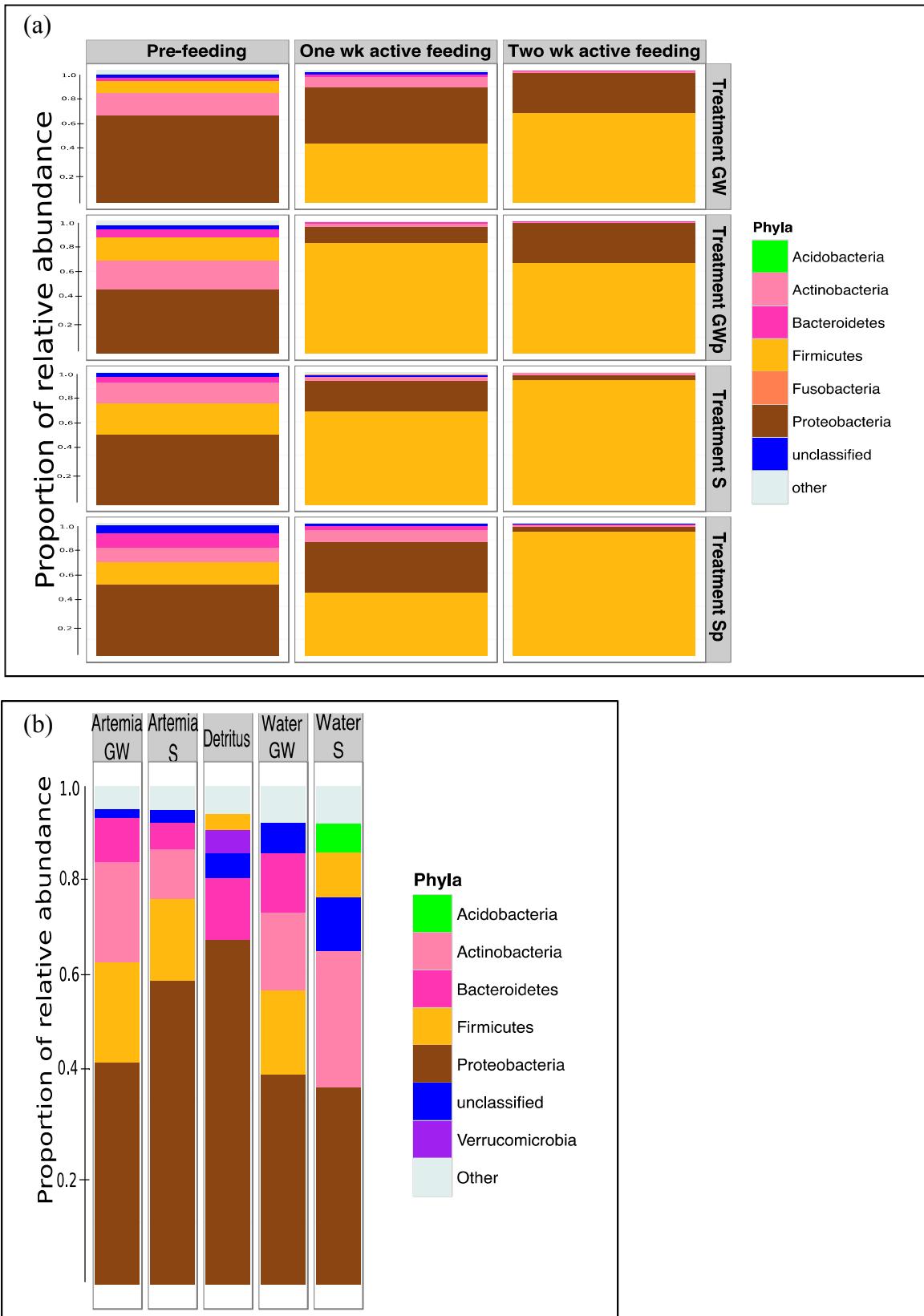


Figure 1.1 Bacterial composition of different communities identified from (a) lake

Figure 1.1 (cont'd)

sturgeon larval gut and (b) environmental samples. (a) Relative abundance of dominant bacterial phyla found in lake sturgeon larval gut microbiota across treatment and during different developmental stages. Only the dominant phyla were shown in the bar chart (*Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Fusobacteria*, *Proteobacteria*) and the rest of taxa were assigned as Others. All four treatment groups were denoted with acronyms **S**, **Sp**, **GW**, **GWp** (b) Relative abundance of dominant bacterial phyla found in **environmental microbiota**. Only the dominant phyla were shown in the bar chart (*Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Verrucomicrobia*) and the rest of taxa were assigned as Others. Classification for environmental communities were denoted with acronym **Artemia-GW**, **Artemia-S**, **Detritus**, **Water-GW**, **Water-S**

S (fish raised in natal stream, fed live *Artemia*);

Sp (fish raised in natal stream, fed live *Artemia* mixed with retentate);

GW (fish raised in groundwater, fed live *Artemia*);

GWp (fish raised in groundwater, fed live *Artemia* mixed with retentate)

Artemia-GW. (food treatment in which live *Artemia* was prepared using ground water);

Artemia-S (food treatment in which live *Artemia* was prepared using stream water);

Detritus. (food treatment in which retentates were collected from sock-filter prior to mixing with live *Artemia*);

Water-GW (water treatment - groundwater that has been UV-filtered);

Water-S (water treatment – stream water)

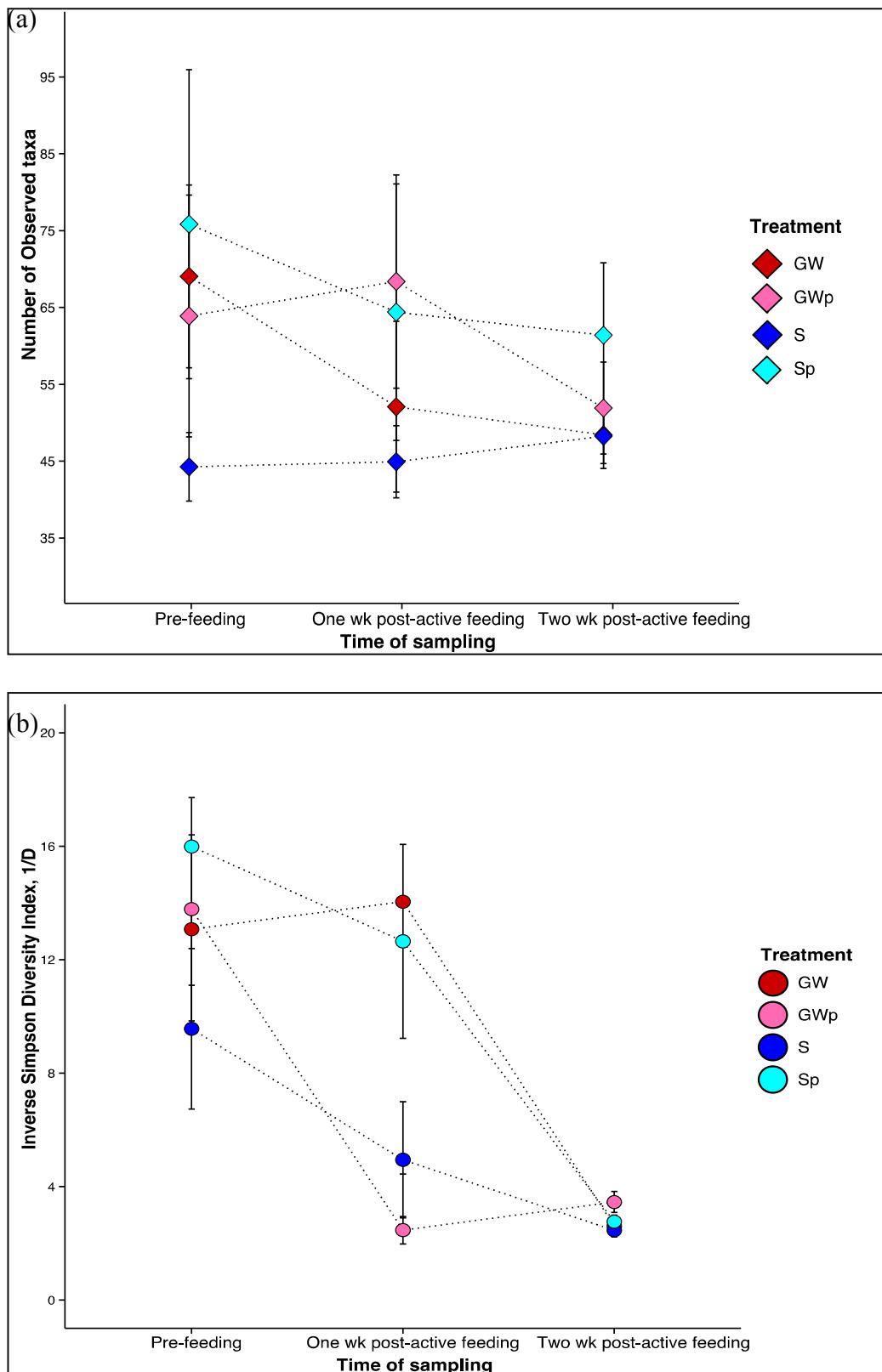


Figure 1.2 Estimates of Alpha diversity for lake sturgeon gut microbial communities

Figure 1.2 (cont'd)
from all treatments across all developmental stages. Statistics comparing gut microbiotas across treatment at different ages were made using a two-way ANOVA. Points indicate mean value of diversity index, colored by different treatments. All four treatment groups were denoted with acronym **S, Sp, GW, GWp**
(a) Alpha diversity in gut microbiota at each time point, as measured by Inverse Simpson indice.
(b) OTU richness based on number of taxa observed in gut microbiota from all treatment and times.

S (fish raised in natal stream, fed live *Artemia*);
Sp (fish raised in natal stream, fed live *Artemia* mixed with retentate);
GW (fish raised in groundwater, fed live *Artemia*);
GWp (fish raised in groundwater, fed live *Artemia* mixed with retentate)

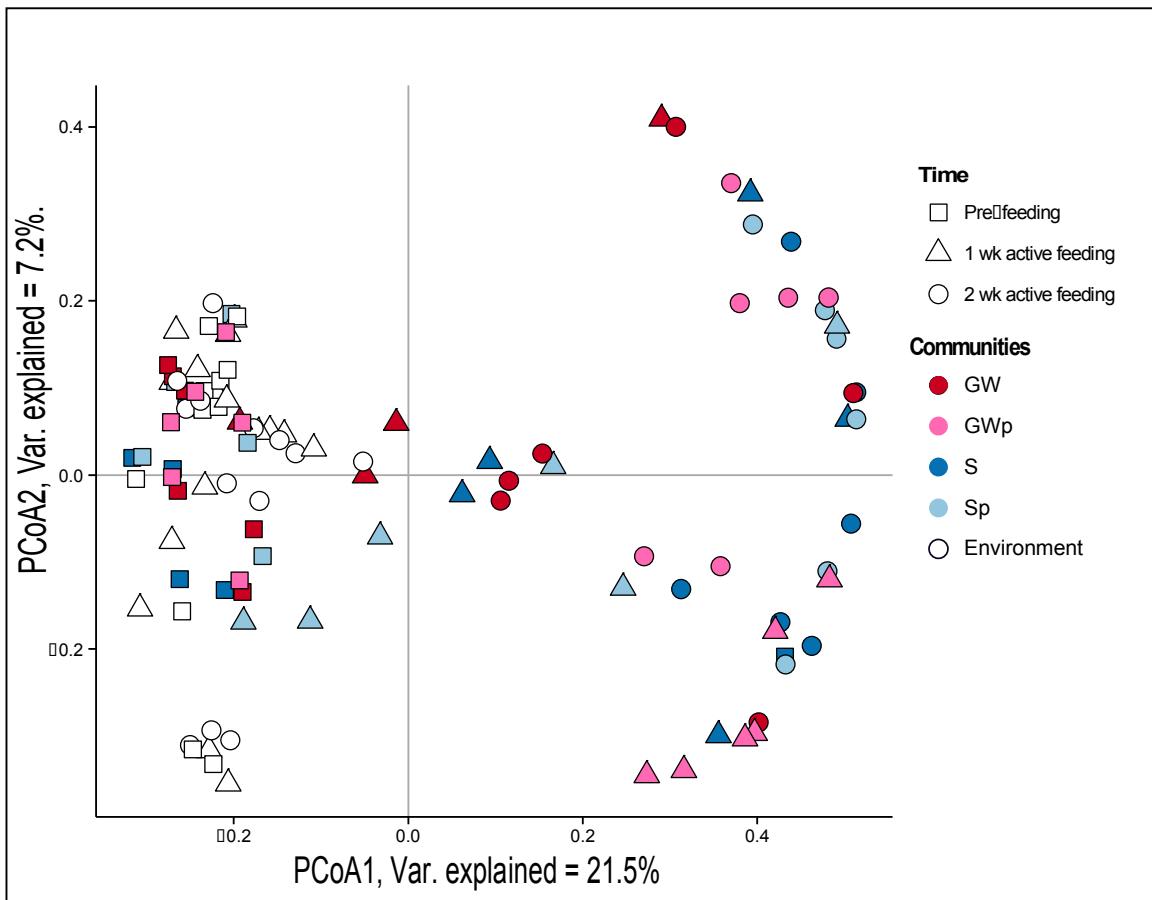


Figure 1.3 Visual representation of ontogenetic changes in lake sturgeon larval gut microbiota based on Bray-Curtis distances. Bray-Curtis dissimilarities between communities originating from gut, water and food samples from all four different treatments at three developmental stages (pre-feeding, a week active feeding, two week active feeding) were visualized using Principal coordinates analyses (PCoA, or also known as Metric Dimensional Scaling MDS) plots. Points represent the samples from each replicate. Fish gut microbial community from four treatment groups were denoted by acronyms S, Sp, GW, GWP, whilst microbial communities from water and food collectively denoted by Environment

S (fish raised in natal stream, fed live *Artemia*);
 Sp (fish raised in natal stream, fed live *Artemia* mixed with retentate);
 GW (fish raised in groundwater, fed live *Artemia*);
 GWP (fish raised in groundwater, fed live *Artemia* mixed with retentate)

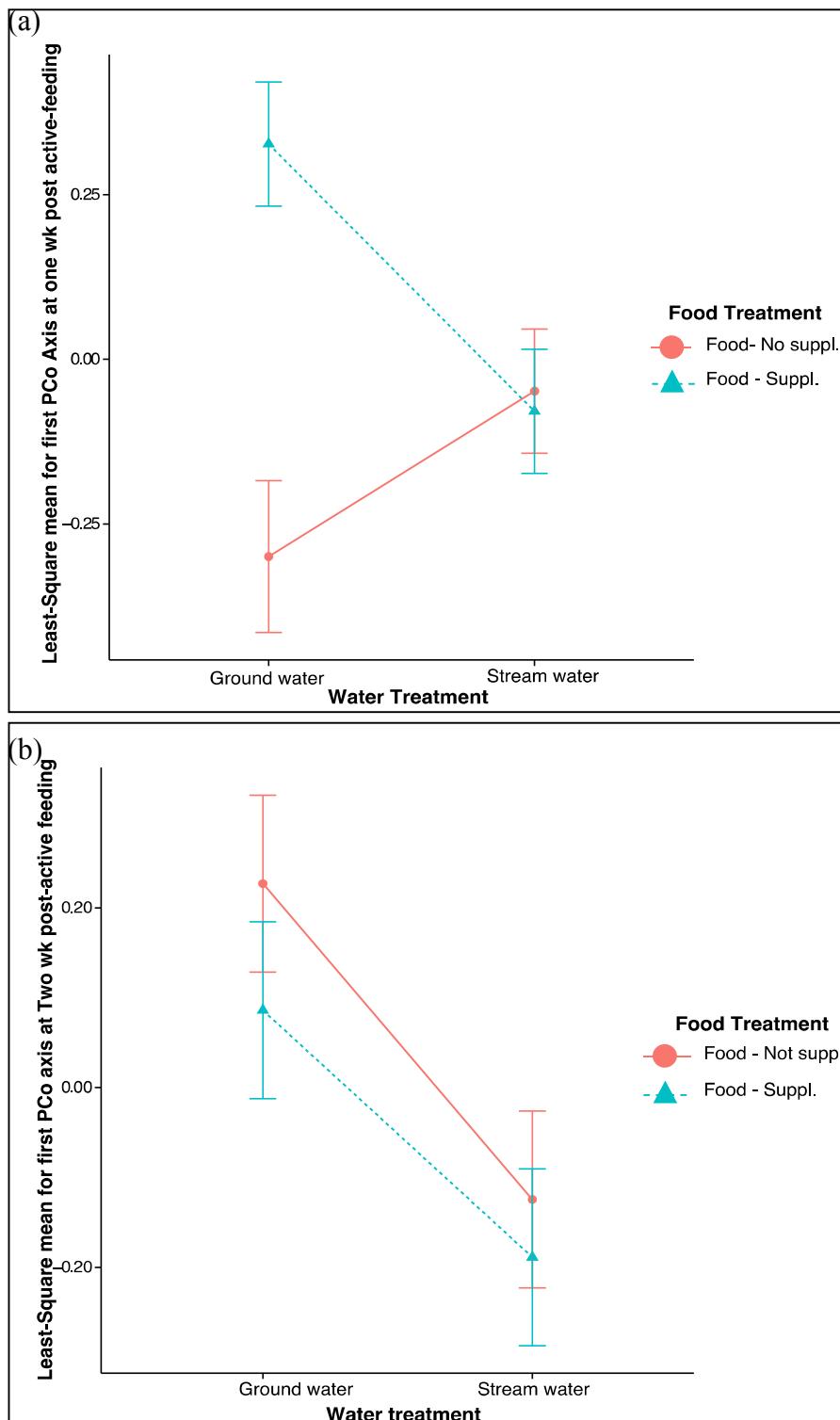


Figure 1.4 Interaction plots of marginal (least-square, LS) means for first PCoA axes (axes that explained largest variation in dataset) at different developmental stages.

Figure 1.4 (cont'd)

Additional information pertaining to the LS means was compiled in Table 1.1. (a) First PCo Axis at one week active feeding; (b) First PCo Axis at two week active feeding.

Figure 1.4(a) indicates significant interactions occurred between food and water treatments. Water and food treatments has influenced on the gut microbial community composition (represented by first PCo axis) during the sampling period at the first week post-active feeding. No significant difference in gut community composition between supplemented vs non-supplemented food treatments within the stream water environment. However, significant difference was observed between gut microbiota of fish raised in groundwater based on food treatment (Fig. 1.4(b)). Significant effects of water treatment on gut composition (represented by first PCo axis) were observed in fish at two weeks after active feeding began. Diet effects were no longer observed.

Food – suppl. (food treatment in which live *Artemia* mixed with retentate was offered to the fish);

Food – no. suppl. (food treatment in which live *Artemia* only was offered to the fish);

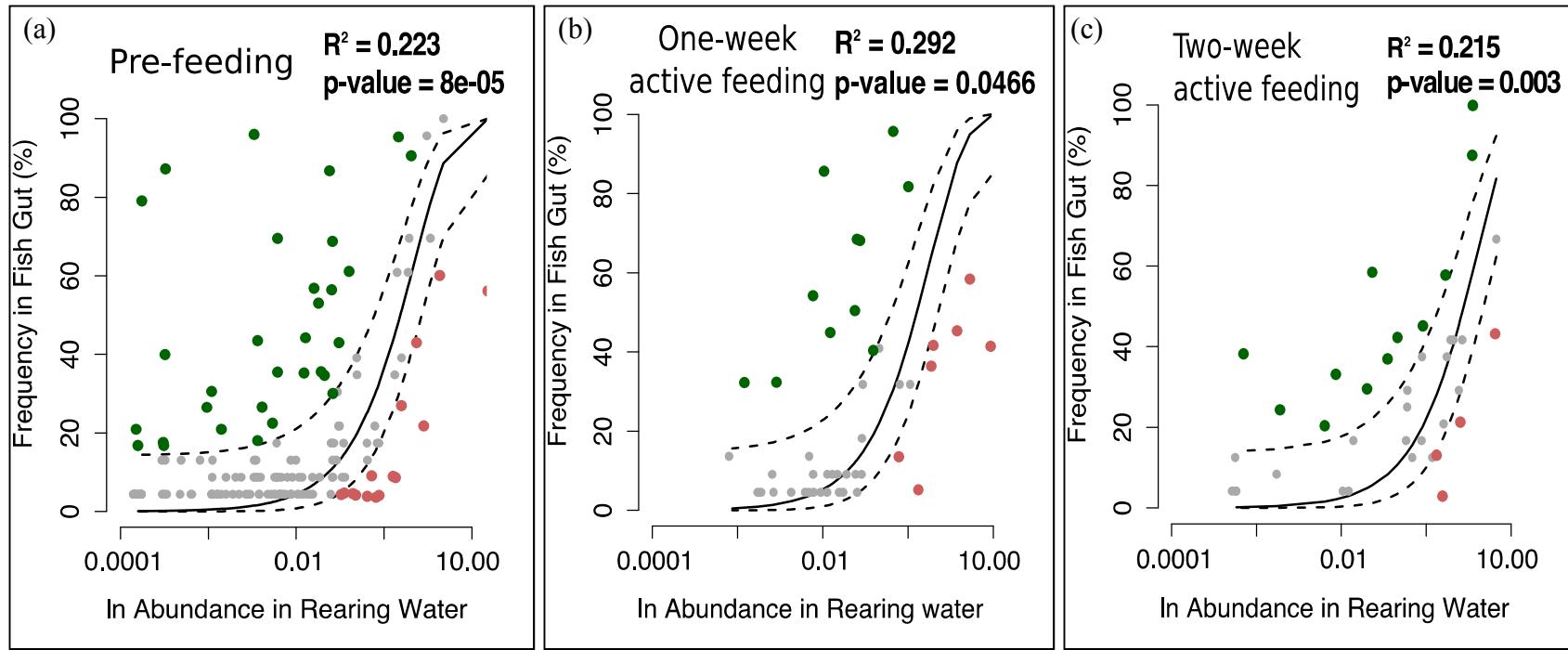


Figure 1.5 Results of neutral model testing with water as the source of gut microbial communities (a) at pre-feeding stage, (b) after one-week active feeding, (c) after two weeks of active feeding. The solid black line represents the best-fit neutral model generated using a beta probability distribution. The model is developed based only on taxa described in both gut and water sources. The dashed lines represent 95% confident intervals around the best-fitting neutral model. Species within the confidence intervals (gray points) are classified as neutrally dispersed taxa, likely present in the gut as a result of neutral processes (such as dispersal, or ecological drift). Species deviating from neutral model (red and green points) were classified as under-represented or over-represented taxa, respectively. These taxa are likely affected by deterministic processes such as selection, or could have differential dispersal ability compared to other taxa in the water. The coefficient of determination (R^2) represents the goodness of fit of relative abundance under the neutral model. The value ranges from ≤ 0 (no fit) to 1 (perfect fit). The P-value indicates that neutral processes that were detected are significant and not by random chance. In general, neutrality could not be rejected during all three developmental stages, but fit of data to neutrality expectations was poor as shown by the relatively low R^2 values.

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CHAPTER 2: COMPOSITIONAL DYNAMICS OF LAKE STURGEON GUT MICROBIOMES ASSOCIATED WITH DIETARY TRANSITION DURING EARLY ONTOGENETIC STAGES

ABSTRACT

Compromised nutritional status associated with dietary transitions in aquaculture operations, especially during early ontogenetic stages can have significant impacts on the growth and survival rates in numerous fish species. In this study, we performed a 36-day feeding experiment using 2 treatments involving control and food-transitioned groups to quantify the impact of dietary switch on both physiological response and gut microbiome composition in lake sturgeon (*Acipenser fulvescens*) larvae. We also investigated differences in taxonomic composition of protease-producing bacteria using culture-based methods and Sanger sequencing. Sturgeon that were transitioned from brine shrimp to frozen bloodworms differed significantly in terms of growth (total length= $p < 0.001$; weight = $p < 0.0001$) and survival rates ($p < 0.01$) based on mixed model ANOVA statistical test. Individuals in the transition group probably experienced starvation relative to fish in the control group that were fed only brine shrimp throughout the experiment. Dietary treatment affected the taxonomic composition and diversity of gut microbial communities as well, although there are successional changes observed in both groups across development periods. Massively parallel sequencing using a region of the 16S rRNA gene revealed that the majority of microbial taxa obtained from the spiral valve of lake sturgeon larvae in the transitioned group were dominated by members of the phyla *Proteobacteria* at 36 days post feeding (dpf), whereas gut microbial communities of fish in the control group were dominated by taxa from the phyla *Firmicutes*. Alpha and beta diversities were significantly different between dietary treatments following introduction

of bloodworms to members of the transitioned group. We identified 98 protease-producing taxa in fish using Sanger sequencing. Analyses revealed that isolates were mostly from genera *Pseudomonas* and *Aeromonas*. However, fish that received bloodworms included bacterial taxa from a greater phylogenetic diversity of genera associated with family *Enterobacteriaceae*. Collectively, data suggest dietary transition affects the composition of intestinal microbiomes of lake sturgeon larvae. Data will be of assistance to hatchery managers when considering feeding practices that are believed to increase growth with establishment of healthy, normal gut microbiota during larval stages. This study demonstrates the utility of using microbiological interrogations of microbial communities when characterizing dietary effects for important aquaculture species.

INTRODUCTION

The growth of vertebrates occurs rapidly during early life stages. Growth and development typically involves phenotypic changes that have a profound impact on ecology, behavior, and physiology. In natural settings, fish experience size-related dietary shifts as a consequence of size-specific changes in foraging abilities that enable individuals to capture and consume progressively larger prey (Olson, 1996). Fish feed either opportunistically or on a diurnal cycle (Wood & Bucking, 2010) in order to meet the nutritional demands for growth.

Fish that were raised in artificial settings also experience shifts in diet as aquaculture professionals attempt to accommodate hatchery management strategies with rapidly changing physiological needs associated with (1) dietary requirements/nutritional needs to maximize fish growth; (2) operational costs, (3) convenience associated with regular food preparations (Hamlin et al. 2006). Ontogenetic food-switching and increases in dietary requirements will directly influence amount of nutrient and food uptake in a stable and constant supply of diet and dietary composition (Hamre et al., 2013).

High mortality during the period of first larval feeding has been reported as one of the major bottlenecks in commercial fish production (Jobling, 2016; Li & Mathias, 1987; M Hixson, 2014). The larval stage is characterized by rapid physiological and ecological changes, and is associated with shifts from use of endogenous to exogenous nutrition sources. At this time, larvae are most sensitive to environmental factors, particularly food type and amount (Li & Mathias, 1987). Studies indicate that larvae receiving inadequate food source or experiencing an imbalance in nutrients are often associated with poor growth and malformation, which may result in decreased survival (Kjørsvik et al., 2011)

The rearing of larval fish of many important aquaculture species (e.g., salmonids, sturgeon) depends on live food such as brine shrimp (*Artemia salina*), rotifers, chironomids, and other zooplanktonic animals (Bjornsdottir et al., 2009; Léger, Bengston, Sorgeloos, Simpson, & Beck, 1987; Ohs, Cassiano, & Rhodes, 2009; Vedrasco, Lobchenko, Pirtu, & Billard, 2002). This dietary regimen must consider not only the cost and labor associated with preparation of food, but also fish nutritional requirements and suitability of diet that can be accepted, ingested, and digested by fish as they grow (Jobling, 2016; Holt, 2011).

Approaches such as co-feeding or food transitioning are often employed to improve larval growth and survival. Co-feeding (offering commercial or starter diets in combination with live food), or transitioning (offering one food source then switching gradually or instantaneously are two alternative feeding regimens that have been used with numerous fish species) are widely used in commercial or conservation aquaculture (Agh et al. 2012; Agh et al. 2013; Hamlin et al. 2006; Hamre et al., 2013). However, previous studies have documented issues associated with providing suitable feed for marine and freshwater larviculture (Agh et al., 2013; Bakke, Glover, & Krogdahl, 2010; Naylor et al., 2009; Rabe & Brown, 2000; Zambonino Infante & Cahu, 2010). Issues are often associated with low survival and growth.

A great deal of interest has focused on development of feeding strategies in fish larviculture based on commercial diet as an economic alternative to live foods (Kolkovski et al., 1997, Agh et al. 2013). However, inert larval feeds may not be a suitable substitute for live food in terms of nutritional composition, palatability, digestibility or physical characteristics of the dry feed, or inability to elicit larval feeding responses (Agh et al.,

2013; Holt, 2011; Jobling, 2016; Langdon & Barrows, 2011; Ohs et al., 2009; Oie et al., 2011; Yufera, 2011; Zambonino Infante & Cahu, 2010). Therefore, approaches using live prey such as brine shrimp, copepods, rotifers, and other aquatic macroinvertebrate like bloodworms (Family: *Chironomidae*) are used to raise larval aquatic species.

The nutrient environment greatly influences the microbial communities (microbiota) inside the gastrointestinal tract (GI) of vertebrates (Ursell et al., 2012). Diet composition is also associated with gut microbiota composition in fish (Ringø et al., 2015). Fish rely heavily on gut microbiota for many physiological functions including but not limited to growth, digestion, nutrient production and absorption, protection of animal against pathogens and immune system function (Llewellyn et al., 2014; Wu & Wu, 2012; Hooper et al., 2012; Cain & Swan, 2010; Gómez & Balcázar, 2008; Macpherson & Harris, 2004). Thus, the presence of a healthy gut microbiota is essential to normal host functioning.

Any disruption in food and nutrient supplies may alter microbiota composition. For instance, fish hosts that experience food deprivation in the wild or cultivated fish that failed to convert during a food transition undergo a phase that represents an ‘energy crisis’ to themselves as well as to gut microbiota due to absence or reduction in the availability of nutrients within the fish host gut ecosystem (Kohl et al., 2014; McCue, 2012). Previous studies indicate alterations of microbial community composition are linked to the quantity and composition of diets. However, questions such as what microbial populations were diminished during the phase or remain unanswered due to lack of studies on microbial community dynamics over the course of prolonged food deprivation.

Sturgeons are important aquaculture species worldwide that are raised for commercial and conservation purposes. Lake Sturgeon *Acipenser fulvescens* is an imperiled species in the Laurentian Great Lakes Region (Peterson et al. 2007), and populations are supplemented through conservation aquaculture programs by stocking hatchery-reared juveniles (Crossman et al. 2011). Hatchery rearing of this species presents difficulties, primarily associated with high mortality during early ontogenetic stages due in part to poor feeding performance (Czeskleba et al., 1985; Hung & Deng, 2002; Klassen & Peake, 2008).

Low larval survival is linked to nutritional regimes associated with diet formulation, feeding schedule, food presentation and preference (Mim, Lazur, Shelton, Gomelsky, & Chapman, 2002; Vedrasco et al., 2002). In the hatchery production of sturgeon, brine shrimp *Artemia* nauplii and *bloodworms* are commonly chosen as live foods for feeding fish larvae and fry due to their high nutritional values (protein content), digestibility and high conversion efficiency (Agh et al., 2012; Agh et al., 2013; Ceskleba & Avelallement, 1985; DiLauro et al., 1998; Vedrasco et al., 2002; Volkman et al., 2004). *Artemia* is easily cultured and harvested on demand from dry and storable dormant cysts (Das et al., 2012; Léger et al., 1987; Oie et al., 2011). Meanwhile, *bloodworms* larvae have been shown to be a major component of lake sturgeon diet in wild and are deemed suitable for hatchery production of bottom feeders (Das et al., 2012; Volkman et al., 2004).

Newly hatched *Artemia* are typically fed to sturgeon at the onset of exogenous feeding, yet the preparation of this food is costly and labor intensive. As fish grow, larvae transition to frozen bloodworms (*Diptera: Chironomidae*). This dietary transition usually

takes place 2– 4 weeks post exogenous feeding (Klassen & Peake, 2007). Transitioning larvae from brine shrimp to bloodworms reduces the harvesting effort associated with *Artemia* preparation, and decreased variability in diet nutritional quality (Klassen & Peake, 2008; Volkman et al., 2004; Léger et al., 1987). In addition, comparative studies conducted by Volkman et al. (2004) showed that fish fed a greater proportion of bloodworms compared to brine shrimp alone experienced higher growth rates. However, experimental data are lacking for lake sturgeon growth performance during periods of dietary transition, specifically effects of the diet transition on gut microbial community composition.

The main objective of this study was to document the composition of gut microbial communities in lake sturgeon during early ontogenetic stages associated with feeding transition and to quantify fish growth. We further compared gut microbial communities as fish were raised using different diet regimes and additionally assessed culturable proteolytic bacteria by culture-based methods to identify associations of diet treatment on the diversity of proteolytic species involved in digestion. We hypothesize that gut microbial community composition of treated fish at times before, during, and after diet transitions will differ relative to control fish (fish fed only *Artemia*). Documentation of gut microbial communities will contribute insight to key features of host-microbes relationships in the larval lake sturgeon gut. A successful dietary transition would lead to increased growth and survivability of cultivated fish.

METHODOLOGY

Fish husbandry and feeding experiment

The feeding experiment was conducted at the Black River Sturgeon Rearing Facility managed by the Michigan Department of Natural Resources (MDNR) and Michigan State University (MSU) in Onaway, MI, USA using approved Institutional Animal Care and Use Committee (IACUC) protocols. Lake sturgeon larvae were produced from a single female and one male adult lake sturgeon from gametes collected during the 2015 sturgeon-spawning season (May 3rd) from the Upper Black River, in Cheboygan County, MI, USA. Gamete collection and eggs fertilization method were described by Crossman et al. (2011) and Bauman et al. (2015), respectively. Full-sib individuals were used to reduce potential variability in microbiota associated with different host genotypes. All individuals were raised using the same conditions until the feeding experiment began. Fertilized eggs were maintained in Aquatic Eco-systems (Pentair) J32 Mini-Egg hatching jars during incubation to simulate stream water flow. Egg mortalities were monitored daily and removed. Upon hatching (May 9, 2015), yolk sac fry were moved to 3.0 L polycarbonate aquaria (Aquatic Habitats). Fry were later assigned randomly to six 3.0 L aquaria of density fifty fish per tank at eight days post hatch (dph; Bauman et al. 2015).

Water supplied to hatching jars and aquaria were taken from the Upper Black River and filtered through 100-micron and 50-micron filters to remove large sediments throughout the experiment. Water temperature was monitored hourly using a YSI ProODO Optical DO-Temp meter. Average daily water temperature ranged from 11.8 °C to 19.7 °C, with the mean (\pm SD) being 16.4 ± 1.8 °C over the duration of this study. The mean (\pm SD) flow rate for each 3.0-L aquaria was approximately 440 mL/min (8.8 aquaria

cycles/hour). A 9 h light, 15 h dark cycle was maintained using fluorescent lights. At nine dph, aquaria were randomly assigned to two treatment groups: Control (**CR**) and Treated (**TR**) with three replicates each ($n = 3$ experimental units). Larvae in the CR treatment were fed live *Artemia* nauplii throughout the duration of the study (five weeks). Meanwhile, larvae in the TR treatment were fed *Artemia* nauplii during the first two weeks post exogenous feeding (pef; or until 14 days post-feeding, dpf). Individuals were gradually transitioned to frozen, commercially grown bloodworms during week three pef. During week four until the end of the experiment, all individuals in the TR treatment were fed only bloodworms. The feeding regime was formulated based on Deng et al., (2003) and Bauman et al. (2016) where the amount of food offered varied by week. In weeks 1 and 2, 26% dry body weight (dbw) was offered; weeks 3 and 4, 13% dbw; week 5, 11% dbw. Each day, fish were fed these amounts based on dbw. On the initial transition day, TR fish were fed 90% dbw brine shrimp and 10% dbw bloodworms. On subsequent days, ratios for *Artemia* and bloodworms were as follows: day 2, 80% and 20%; day 3, 60% and 40%; day 4, 50% and 50%; day 5, 40% and 60%, day 6, 30% and 70%, and day 7, 15% and 85%. Fish were fed an equal amount of food three times daily (0900, 1300, and 1700) while water remained flowing, simulating stream flow.

Brine shrimp cysts, Great Salt Lake strain, were purchased from Brine Shrimp Direct© and were cultured following manufacturer's protocols. Frozen one-inch bloodworms, harvested from ponds in northern China, were purchased from Brine Shrimp Direct©. Bloodworms were rinsed with ground water and mildly chopped using the pulse function on an electric blender before feeding.

Beginning nine dpf, mortality per aquarium was recorded daily. Fish were batch weighed by replicate once a week to measure growth and alter the amount of food allocated to each aquarium in the following week. Each replicate was weighed separately. All fish were collected in a dip net and water was removed by dabbing the net on the dried paper towel several times before weighing to remove excess water. Wet weight of *Artemia* was calculated from dry weight at dry weight = 0.1767 [sieved wet weight] - 0.0541 (Bauman et al. 2016). Wet weight of bloodworms was calculated from dry weight at dry weight = 0.0832 ·[wet weight] + 0.0239 (Scribner, unpublished data).

Sample collection

Sampling occurred at three ontogenetic stages of development: - two weeks (14 dpf), three weeks (21 dpf), and five weeks (36 dpf) post exogenous feeding. Prior to sampling, the food was not administered to the fish for 18 hr. Five fish were collected per replicate and treatment group and anesthetized with MS-222 (Sigma-Aldrich, St Louis, MO, USA) in a petri dish with a ruler. Fish were digitally photographed to obtain total length measurements before being preserved in liquid nitrogen for enzymatic studies. Digital images were later analyzed using Image J software (NIH Image). Another five fish were sampled for microbial community interrogation at each of the three stages. Fish were euthanized with an overdose of MS-222. Individuals were subsequently placed in microcentrifuge tubes and stored at room temperature in 80% ethanol. During the fish sampling, water samples were also collected. 500mL of stream water was filtered using a 0.22 um 47 mm filter membrane (Stereitech®) using a hand-pump. The membrane was placed in a 50 mL tube and preserved with 80% ethanol. All fish and water samples were stored until dissection and bacterial DNA extraction was performed.

Fish dissection

The distal gut (spiral valve) of each sturgeon larval gastrointestinal tract was recovered from fish following aseptic techniques. The distal gut was defined as the section that begins at the intestine until reaching the spiral valve. The spiral valve serves as the primary region of digestion and absorptive function (Buddington & Christofferson, 1985; Callman & Macy, 1984), and thus it is believed to provide an area of abundant nutrients where microbial communities can flourish. Exterior surfaces were swabbed with 100% ethanol before dissections of the whole digestive tract using sterile instruments. Dissections were performed with slight modification as previously described by (Milligan-Myhre et al., 2011). The intact alimentary tracts were removed from the fish body cavities, and the excised gut was immediately transferred into filtered-sterilized 80% ethanol solution for DNA isolation. Due to the small size of each larval gut, a composite of four guts from four individuals was grouped for each replicate (or tank) within CR and TR groups at each of the three-time points.

Isolation of bacterial culture & extracellular protease screening

At the end of the feeding experiment, four fish were sampled from replicate randomly chosen from each of the treatment groups (**CR** and **TR**). Fish were euthanized and immediately dissected following the aseptic dissection procedure previously described. The freshly dissected gut was transferred to a tube containing 10ml of sterile Luria-Bertani (LB) broth and transported to Michigan State University for further processing. To prepare the intestinal homogenate, guts from two larvae were transferred into a separate microcentrifuge tube, containing 250 ul of sterile LB. The guts were subsequently homogenized using a sterile glass rod. Homogenates of guts from another

two larvae, originating from each replicated CR and TR groups, were pooled to create a composite mixture of gut microbial communities for each group.

Homogenate samples were serially diluted in sterile LB up to 1:10⁸. Once the dilution was made, an aliquot of 0.1ml of each dilution was plated on each of three replicate plates of nutrient LB agar using the spread plate technique. Plates were incubated at 25°C for 48-72 hr. Following incubation, all colonies were counted, and their characteristic morphologies were documented. Each isolated colony was subcultured in a microtiter plate well containing 150ul sterile LB media, incubated overnight, and stored at -80°C in 15% glycerol concentration as a stock bacterial culture for further use.

Isolates of pure colonies were subjected to a plate assay to screen for extracellular microbial protease activities (Tetlock et al., 2012; Kazanas, 1978). Milk protein agar plates were prepared in duplicates as follows: 20% (w/v) skim milk solution and 2x purified LB agar was prepared separately in deionized water and autoclaved at 121°C for 15 minutes. The milk solution was then added to agar to give a final volume of 10% (w/v) of milk protein agar. The mixture was kept at 55°C before poured into plates. All isolates were cultured on the agar surface using 96 solid pin multi-blot replicators and incubated at room temperature for the first 24hr and transferred into 30°C for the next 24 hr.

The zone of clearing around an inoculated colony indicated positive protease activity, these colonies were scored as either 0 (negative) or 1 (positive). The clearing phenotype was recorded and photographed. Protease-producing isolates were subjected to Sanger sequencing for the v4 region of the 16S rRNA gene at the Michigan State University Research Technology Support Facility, RTSF (East Lansing, MI, USA) for

further identification. Sequencing of 600bp 16SrRNA via Sanger was preceded to further identify these potential protease producers.

Sanger sequencing data were analyzed using Ribosomal Database Project (RDP, www.rdp.cme.msu.edu) pipeline. Sequences were first converted into FASTA format and manually trim to remove the ambiguous bases. These sequences were first aligned and classified with RDP's aligner and RDP Classifier (Wang et. al, 2007). Sequences that included too many ambiguous positions were not included for the alignment.

Phylogenetic trees were then constructed using RDP's Tree Builder with bootstrap confidence estimates using weighted version of the Neighbor-Joining method (Saitou & Nei, 1987) based on a distance matrix calculated using the Jukes-Cantor model (Jukes & Cantor, 1969). The branch supports were estimated in a bootstrap analysis based on 100 pseudoreplicates for NJ trees bootstrap estimates were generated and displayed at the branches of the majority consensus tree.

DNA extraction and 16S rRNA amplicon sequencing

Gut microbiota from lake sturgeon larvae was surveyed using high-throughput sequencing of the v4 region of the 16S rRNA gene. Each gut sample was first centrifuged for 15 min at 4°C to pellet bacteria before DNA was extracted. The MoBio PowerSoil® DNA Isolation Kit (Carlsbad, CA, USA) including a bead-beating step was used following protocols for low-biomass samples, as suggested by the manufacturer. The integrity of each DNA sample was assessed based on amplification of 1.4k bp of the 16S rRNA gene (amplicon based on 27F and 1389R primers) followed by gel agarose electrophoresis. DNA concentrations were quantified using a Microplate spectrophotometer (BioTek®, Winooski, VT, USA).

Twenty-seven DNA samples (including two control samples) were sequenced at Michigan State University Research Technology Support Facility, RTSF (East Lansing, MI, USA). All sequencing procedures, including the construction of the Illumina sequencing library, emulsion PCR, and MiSeq paired-end sequencing v2 platforms of the V4 region (~250bp; primer 515F and 806R) followed standard Illumina (San Diego, CA, USA) protocols. The Michigan State's Genomics RTSF (<https://rtsf.natsci.msu.edu/genomics/>) provided standard Illumina quality control, including base calling by Illumina Real Time Analysis v1.18.61, demultiplexing, adaptor and barcode removal, and RTA conversion to FastQ format by Illumina Bcl2Fastq v1.8.4.

Sequence processing

Sequence data were processed using default sequencing data analyses pipeline and computing workflow. Briefly, paired-end sequence merging, quality filtering, “denoising”, singleton-sequence removal, chimera checking, taxonomic assignments and Operational Taxonomic Unit (OTU) selection was conducted based on methods implemented by program *mothur* v.1.36.1 (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013). A reference-based OTU clustering and the taxonomic assignment was performed using SILVA-based microbial reference database file provided in *mothur* to cluster sequences defined with 97% identity. To minimize effects of under sampling while maintaining as broad dataset as possible, final OTUs were rarefied to a depth of 9151 sequences per sample. Two DNA samples with low sequence depth were discarded in downstream analyses. Rarefaction analyses were performed to evaluate the sampling coverage for each sample based on selected sequence depth.

Morphometric data and survival analyses

The effects of food transition on larval growth were quantified based on total body length (TL) and batch (replicate/tank) weight (WT). Each of the 3.0-L aquaria represented an experimental unit, and mean weight ($\text{g} \pm \text{SD}$) and length ($\text{mm} \pm \text{SD}$) for three replicates for each treatment group (CR vs TR) at each sampling time are displayed in Table 2.1 and Figure 2.1. Mean TL and mean WT per fish that were repeatedly measured over time were analyzed using a mixed model approach. In this approach, we compared the mean differences of TL and WT between two feeding treatments (CR vs TR; i.e. “feeding” is a “between-subject” factor) over sampling points/stages (i.e., “time” was the “within-subject” factor).

We wished to quantify mean TL and WT per fish as a function of time and as a function of feeding treatment (CR vs TR), as well as to determine potential interactions among stages and treatments. While larval total body length was measured based on three tank replicates at three sampling points, larval body weight was based on three replicates of each feeding treatments measured weekly throughout the experimental duration. Survival was estimated as the mean daily proportion of larvae surviving from week 0 through week 5 pef in R using *survival* package (Therneau & Grambsch, 2000) and survival curve was generated *survfit* function. Proportional survival analyzed using two samples log-rank test using *the survdiff* function. This function was based on a Chi-Square distribution that evaluates whether fish from different feeding treatments are coming from the same distribution or two different distributions. All statistical analyses were conducted using programming and statistical software, R (version 0.98.978). A *p*-value below 0.05 was considered statistically significant.

Analyses of bacterial community profiles and ecological statistical analyses

2.1 Alpha diversity

All measures of microbial community diversity including Inverse Simpson (1/D) diversity indices and OTUs richness of each sample were calculated from the sequence data within program *mothur*. To test for significant differences in diversity indices between CR and TR treatment groups, we first employed a log transformation of Inverse Simpson indices and observed a number of taxa followed by two-way ANOVA using the statistical software, R (version 0.98.978) base package. The test was followed by posthoc Welch two samples t-tests. p-values below 0.05 indicate significant differences in pairwise comparisons.

Relative abundance and identity of all phyla in all fish gut and water-associated microbial communities across sampling times were determined using packages *dplyr* and *reshape2* in program R.

2.2 Beta diversity

We used several packages implemented in R to estimate comparative (beta [β]) diversity measures and ecological statistics at the OTU level. Briefly, we used the *vegan* package (Oksanen, 2015) to generate Bray-Curtis (BC) distance characterizing differences in microbial community composition. We used the *cmdscale* function to perform Principle Coordinate analyses (PCoA) ordination of community composition differences based on BC distance (Bray & Curtis, 1957). The *ggplot* and *ggplots2* packages (Wickham, 2009) were then used to create ordination plots to visually compare gut community composition with aquatic community composition as a function of different treatments and among sampling periods based on the principal coordinate with the three largest eigenvalues.

Multivariate hypotheses testing to quantify differences in community composition among samples collected at different times and from different feeding treatments were performed using the *adonis* function (Oksanen, 2015) in program R. Differences between locations of the centroids of sampling groups for treatments and time periods were based on Permutational Multivariate Analyses of Variance (PERMANOVA) using BC resemblance matrices (2013, 2006; 2001a). Under the null hypotheses, food treatments were not expected to significantly affect fish gut community taxonomic composition. This test was employed because of the non-parametric and skewed nature of microbial ecology data distribution. Then, object scores along the principal coordinate with the three largest eigenvalues (i.e., eigenvectors associated with corresponding eigenvalues) were correlated with object scores along each original variable's axis (i.e., relative proportions of measured taxa/OTU) to measure the OTUs' contribution to a given PCoA axis (Legend & Legendre, 2012) using the *corr* function in program R.

Inferring gut-associated microbial communities function

Metagenomic analyses of 16S rRNA sequencing data and reference genomics databases were used to predict the functional roles of gut microbiota associated with treated fish (TR) and control fish (CR) groups using program PICRUSt (version 1.1.0; Langille et al., 2013) We imputed the putative function of lake sturgeon gut using catalogues of annotated genes within a known sequence database (Kyoto Encyclopaedia of Genes and Genomes; KEGG) based on the abundance of taxa with known function using taxonomic identification established from our 16S rRNA survey. With PICRUSt, we calculated a Nearest Sequenced Taxon Index (NSTI), which measures how closely related the average 16S rRNA sequence in the sample was to a sequenced genome. When this index is low (<

0.05), PICRUSt is likely to perform well in predicting the genomes of the organisms in an environmental sample. High scores (> 0.15) mean few related references are available and prediction will be of low quality (Langille et al. 2013).

Briefly, reference-based OTU clustering and taxonomic assignments were performed using selected marker gene identifiers in the Greengenes database and converted into a *biom* file. The file was transferred into the PICRUSt program, and functional predictions were carried out based on information of the relative abundances of those OTUs across samples using an evolutionary model. The OTU table for each sample with associated Greengenes identifiers was later normalized based on organisms predicted 16S gene copy number using `normalize_by_copy_number.py` script. Functional roles were predicted by searching for pre-calculated genome content for each OTUs using `predict_metagenomes.py` script. Annotations of predicted function were applied and summarized using the KEGG Orthology (KO) classification schemes using the `categorize_by_function.py` script, all included in PICRUSt. The program generated profile tables consisting of annotated gene functions along with abundance for each sample in the OTU table (Langille et al. 2013) to provide a baseline to infer the functional attributes of observed taxa. In addition, PICRUSt calculated the NSTI to quantify dissimilarity between reference genomes and the predicted metagenome presented here. The graphical representation and Welch's t-test were performed both in program STAMP (Parks, Tyson, Hugenholtz, & Beiko, 2014) and R to quantify significant differences in the mean proportion of sequence associated with predicted functions.

RESULTS

Growth performance and survival

The proportion of fish surviving in each diet treatment was statistically different between CR and TR treatments (Fig. 2.1). Larval fish survival among lake sturgeon in the TR treatment from *Artemia* to bloodworms was significantly lower with nearly 50% mortality relative to fish in the CR treatment that mostly survived by the end of 5-week study (Chi square value, $\chi^2 = 85.1$, p-value < 0.01, df=1). Mortality peaked from 30 to 35 dpf.

Analyses of morphometric data (TL and WT; Fig. 2.2a and 2.2b) were achieved by comparing the mean weight of fish ($g \pm SD$) and mean length (mm $\pm SD$) between treatments across time periods. There were significant interactions between sampling time and dietary treatment, influencing the mean length among replicates (df=2, F= 99.62, df = < 0.0001, Table 2.1a). At 14 dpf and 21 dpf, no significant differences were detected between mean lengths from fish in both treatments (14 dpf: CR 30.84 ± 1.00 mm, TR 31.13 ± 1.05 mm, p-value = 0.66; 21 dpf: CR 35.59 ± 1.65 mm, TR 34.63 ± 1.36 mm, p-value = 0.32). However, mean length for TR (33.15 ± 2.36 mm) was recorded to be significantly lower (p-value < 0.001) than CR (52.08 ± 3.64 mm) as fish reach 36 dpf.

Similarly, a strong interaction existed between time (sampling week) and treatment, influencing weight (df=3, F= 111.5, p= < 0.0001, Table 2.1b). Comparison between dietary treatments for each week indicated mean weight for weeks 5 and 6 were significantly different (week 5: CR 0.267 ± 0.04 g, TR 0.101 ± 0.01 g, p-value < 0.05; week 6: CR 0.463 ± 0.05 g, TR 0.104 ± 0.01 g, p-value < 0.01) whereas for week 3 and week 4, they were not different (CR 0.111 ± 0.01 g, TR 0.105 ± 0.01 g, p-value = 0.459;

week 6: CR 0.157 ± 0.02 g, TR 0.121 ± 0.00 g, p-value=0.058). Measurements at the first and second week were not included due to the lack of data from all aquaria replicates.

Characterization of diversity and proportion of microbial phyla in gut community composition of lake sturgeon

At 14 dpf, gut microbial community samples of twenty-four fish from all six replicates in CR and TR treatment groups were collected and sequenced. Comparisons at the level of phyla indicated that six phyla were present in both feeding groups. Although the relative abundance of these phyla differed between feeding groups, all together those phyla were collectively dominate, including more than 90% of total gut microbial taxonomic composition; *Proteobacteria* (mean TR 53.7%, mean CR 65.6%), *Firmicutes* (mean TR 14.5%, mean CR 27.1%), *Actinobacteria* (mean TR 8.8%, mean CR 5.1%), *Acidobacteria* (mean TR 0.05%, mean CR 0.39%), *Bacteroidetes* (mean TR 12.9%, mean CR 0.4%), and *Verrucomicrobia* (mean TR 6.5%, mean CR 0.2%). The differences in mean proportions of these phyla are shown in Figure 2.3a. Each feeding group also had three unique phyla, yet these taxa were present in low proportions (~ 1% or less).

Once bloodworms were added to the TR feeding treatment's regime during the transition week (21 dpf), we observed changes in microbial community composition in samples in the TR group. Greater numbers of phyla were detected in TR fish samples (*Chlorobi*, *Fusobacteria*, *Lentisphaerae*, *Nitrospira*, *OD1*, *OP11*, *SR1*, *Synergistetes*, *TM7*) and these phyla were absent in fish the CR treatment that were fed only *Artemia* (data not shown). We also observed an increased proportions of *Acidobacteria* (mean relative abundance increased from less than 1% to 5.9%) and unclassified phyla (mean relative abundance increased from 2.5% to 8.1%) as fish grew from 14 dpf to 21 dpf in

the TR treatment. Interestingly, in the same feeding group, we documented declines in abundance of *Firmicutes* (14 dpf: 15%, 21 dpf: 6%), while *Firmicutes* proportions remained relatively stable in CR treatment (14 dpf: 27.2%, 21dpf: 20.2%). At 21 dpf, the gut community composition for fish in the CR group was dominated by three major phyla (*Firmicutes*, *Actinobacteria*, *Proteobacteria*) totaling more than 95% of the total number of sequences. At the end of the experiment (36 dpf), the gut microbial communities from fish in both CR and TR groups were distinct. The *Firmicutes* phylum dominated community composition from the CR group (93.5%), while gut communities of TR fish were dominated by *Proteobacteria* (94.3%). Overall patterns of phyla present in the fish gut contrast greatly with phyla detected in water samples during all three sampling periods (Fig. 2.3b).

Measures of gut microbial community diversity including the Inverse Simpson indice and taxa richness (number of taxa detected/observed taxa) indicated fluctuation of index estimates across ontogenetic stages/sampling points (Figs. 2.4a, 2.4b). There was a significant interaction of these stages (dpf) and dietary treatment influencing both indices (\log Inverse Simpson: df = 2, F=4.483, p-value < 0.05; \log Taxa richness: df = 2, F=18.53, p-value <<0.001). Findings suggested that alpha diversity indices (Inverse Simpson and taxa richness) of fish from diet treatments differ significantly during at least at one sampling period.

Welch t-tests for two samples with unequal variances were later performed using log-transformed diversity measures, to test for differences in mean diversity indices for fish samples from different treatment at each sampling time. Statistical tests on both measures of Inverse Simpson's index and taxa richness at 14 dpf failed to reject the null

hypotheses at 0.05 significance levels (\log Inverse Simpson CR = 1.857, \log Inverse Simpson TR = 2.448, p-value = 0.292; \log Taxa rich. CR = 4.748, \log Taxa rich. TR = 4.357, p-value=0.236). However, log mean comparison between treatments at 21 dpf (\log Taxa rich. CR = 5.344, \log Taxa rich. TR = 7.247, p-value < 0.05; \log Inverse Simpson CR = 2.609, \log Inverse Simpson TR = 3.777, p-value < 0.01) and 36 dpf (\log Taxa rich. CR = 4.154, \log Taxa rich. TR = 5.476, p-value < 0.001; \log Inverse Simpson CR = 0.419, \log Inverse Simpson TR = 0.333, p-value < 0.05) were significantly different (TR fish were higher).

Association between gut microbial community composition and feeding treatments, across sampling periods

Principal Coordinate Analyses (PCoA) ordination of differences in taxonomic composition and relative abundance of the gut community was performed to visualize relationships between those community composition associated with different feeding treatments and times of collection. Microbial community composition of fish from both CR and TR treatments were similar when fish were 14 dpf (Figs. 2.5a, 2.5b, 2.5c). Subsequently, communities from fish in the transition group (TR) diverged while gut communities of fish in control group remain relatively similar (Figs. 2.5a, 2.5b). Toward the end of the experiment (36 dpf), gut community compositional differences between feeding treatments resulted in treatments clustering further apart, although considerable community composition overlap in fish and water communities was observed (see Fig. 2.5c). Statistical analyses of beta diversity indicated significant community compositional differences among stages and between treatments (PERMANOVA test, pseudo-F = 2.928, R^2 = 0.151, p < 0.001, df=2; Table 2.2).

We further examined variation in community composition by decomposing treatment effects of diet from pervasive influences of developmental stages using linear regression and least square mean analyses. At 14 dpf, no significant effect of treatment was detected (F -statistics = 1.184, p-value: 0.3377). As diets of fish in the treated group changed from brine shrimp to bloodworms at 21 dpf (F -statistics = 140.2, p-value: < 0.01) and continued through the end of the experiment at 36 dpf (F -statistics = 1.213e+05, p-value: < 0.001), communities differed significantly in taxonomic composition (Table 2.2b). PCoA axes-1 of all three sampling periods explained the most variation in microbial communities representing the linear combination of all taxonomic composition.

Correlation analyses of the relative abundance of observed taxa/OTUs with principal coordinate associated with three largest eigenvalues found the 17 taxa that highly correlated (Pearson-correlation > 0.70) with associated eigenvector (Table 2.3). For example, Otu011 (Unclassified Betaproteobacteria) strongly correlated with first principal coordinate (Pearson-correlation = 0.91), Otu001 (Genus *Clostridium_sensu_stricto*) with second principal coordinate (Pearson-correlation = 0.94), and Otu2 Otu010 (Genus *Aeromonas*) with the third principal coordinate (Pearson-correlation = 0.97).

Predicted functional roles of lake sturgeon gut microbiota

Functional inventories of microbiome genes, such as those involved in the metabolism of macronutrients (carbohydrates, amino acid, lipids) can be predicted from bacterial species assemblages using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States). This study used the KEGG database to match the chosen reference OTUs, focusing at the level 2 (a function defined based on molecular

interactions, protein post-translational modifications). Calculation of NSTI from PICRUSt across all 17 fish gut samples show that this metric fall within the range of 0.048 till 0.258, with an average of 0.22.

The program further inferred 37 gene families from the gut samples of fish from both feeding treatments across sampling points (Figs. 2.6a, 2.6b, 2.6c). Of these 37 gene families, the majority of the genes were associated with membrane transport (mean value across sampling periods $13.58 \pm 0.84\%$ in CR-associated communities and 12.62 ± 0.81 in TR-associated communities, respectively), carbohydrate metabolism ($11.75 \pm 0.03\%$ in CR, $11.56 \pm 0.66\%$ in TR), amino acid ($12.13 \pm 0.45\%$ in CR, $12.01 \pm 0.70\%$ in TR), replication and repair ($8.78 \pm 0.64\%$ in CR, $7.01 \pm 0.29\%$ in TR), and energy metabolism ($6.75 \pm 0.25\%$ in CR, $7.01 \pm 0.29\%$ in TR). The mean relative abundances of these predicted gene families relatively stable across time. Of all 37 genes predicted, none of means relative abundance differs significantly between treatments (data not shown here).

Activity-based screening for protease positive isolates

The screen for microbial taxa possessing genes constitutively expressing proteolytic activity was based on the screens of zones of clearing characterizing pure colonies isolated from gut samples. We collected a total of 288 pure colonies from individuals from TR (160) and CR (128) diets cultured on the LB agar. Out of these pure colonies, we then identified 52 positive-protease isolates for CR and 88 for TR when these microbes cultured on skimmed-milk agar.

Sanger sequencing data generated for all 140 positive-protease isolates detected in the fish gut from both TR and CR group. However, only 54 bacterial sequences from TR and 44 sequences from CR were retained during sequence alignment and taxonomic identification. The remaining taxa were filtered out due to presence of ambiguous base positions in the FastA files. The Neighbor-Joining (NJ) tree generated for isolates from fish in CR group (Fig. 2.7a) indicated the presence of genera *Aeromonas* and *Pseudomonas* that are capable of hydrolyzing protein (casein) that was present in the media. Meanwhile, in TR group, besides genera *Aeromonas* and *Pseudomonas*, six additional genera in the family *Enterobacteriaceae* were also present; genus *Buttiauxella*, *Citrobacter*, *Enterobacter*, *Kluyvera*, *Leclercia*, *Yokenella* (Fig. 2.7b). Species displayed in both trees were based on top matches sequences selected based on S_{ab} index higher than 0.80 and to represent an extensive phylogenetic breadth of all species found during alignment. Bacterial *Thermotoga maritima* was used as an outgroup.

DISCUSSION

Findings of significant differences in survival and low growth rates of TR fish observed in this experiment relative to CR group prompted the feeding trial to be terminated at 36 dpf. Klassen et al. (2007, 2008) demonstrated successful transition of lake sturgeon when they were switched from brine shrimp to black fly larvae several weeks after the fish were first offered with brine shrimp. Nonetheless, transitioned fish in our study failed to transition to bloodworms. Visual inspections on fish stomachs suggested that fish were not eating bloodworm and were starving beginning at 21dpf. Lack of ingestion of bloodworms may explain significant differences in mean total length and average fish weight compared to fish in CR group. We suspect that frozen bloodworms that were used in this study were inadequate to stimulate sensory organs and feeding response in fish. As these inert feeds dissolve in culture water, the taste and smell might be affected, making it no longer desirable to be ingested (Langdon & Barrows, 2011). Studies have shown that live *Artemia* excretes metabolites that serve as strong feeding stimuli, perhaps contribute to greater acceptance to fish larvae (Hamre et al., 2013; Yufera, 2011).

Our study presents comprehensive analyses of lake sturgeon gut microbiomes using 16S rRNA amplicon based sequencing, offering an in-depth characterization of longitudinal changes in microbial taxonomic composition over fish developmental stages in response to diet treatments. We found that these fish exhibited distinct gut microbial community at the end of the experiment (36 dpf) further supporting our hypotheses that gut microbiota was affected by treatment. Phylum *Proteobacteria* dominated gut microbial communities of fish from the TR group whereas fish in the CR group were dominated by taxa in the phyla *Firmicutes*. Statistical analyses on measures of gut

microbial community diversity including the Inverse Simpson index and taxa richness indicated that gut microbial communities in TR fish were significantly more diverse compared to CR fish.

PERMANOVA test followed by linear regression analyses at each sampling periods detected a significant influence of feeding treatment on gut community composition of fish from both treatments occurred at 21 dpf and 36 dpf, but not at 14 dpf. At the beginning of the experiment, gut microbial communities from all samples (CR and TR) were expected to be similar since all fish received the same diet. The compositional divergence in community composition and diversity was observed as soon as bloodworms were introduced to the TR group. This divergence could either be attributed to different diet associated with different microbiota or due to prolonged starvation as a result of poor feed acceptance. We believe the latter is more likely.

Previous studies have indicated an alteration in gut community composition of larvae that experience prolonged fasting or starvation. During the period of fasting, the supply of nutrients to gut symbionts was significantly reduced, causing an “energy crisis” (McCue, 2010, 2012). Fish that were starved also showed some intracellular degradation and reduced endothelium, that subsequently reduce the mass tissue of host digestive system – which in turn affected the taxonomic composition of microbiota (Kjørsvik et al., 2011; McCue, 2012). It has been shown in the large intestine of Burmese python (*Python bivittatus*), fasting was associated with increased abundance of genera *Bacteroides*, *Rikenella*, *Synergistes*, and *Akkermansia*, and reduced overall diversity (Costello et al. 2010). Although our “starved” fish showed higher taxonomic diversity seen in alpha diversity measure (Inverse Simpson and taxa richness), our findings reported the presence

of *Synergistes* in fish fed bloodworms at 21 dpf, and those communities were later highly dominated by *Proteobacteria* at 36 dpf.

Our findings corroborate the results in a study performed by Kohl et al. (2014) that reported a dramatic increase in relative abundance of *Proteobacteria* within prolonged-fasting tilapia (*Oreochromis niloticus*) compared to well-nourished tilapia. Results in another study by Xia et al. (2014) on Asian seabass (*Lates calcarifer*) was in congruent with the Burmese python studies as they documented significant changes in *Bacteroidetes* between control fish that were well-fed vs experimental fish that were starved. In addition, we found a strong, positive correlation of the relative abundance of genus *Aeromonas* with gut community composition of TR fish in our experiment. We believe that though fish were not feeding on bloodworms, the presence of this food in the water might indirectly affect the microbial community of water in the tank (De Schryver & Vadstein, 2014; Skjermo & Vadstein, 1999). Bloodworms feed on detritus as well as microalgae and they could possibly be a reservoir of zoonotic agents of several pathogens such as *Salmonella*, *V. cholera* and could favor the transmission of infectious disease organisms (Rouf & Rigney, 1993; Broza & Halpern, 2001; Sharifian Fard et al., 2014).

Artemia is commonly fed to fish larvae in aquaculture settings. Studies have identified *Artemia* as continuous, non-selective, phagotrophic filter-feeder that can ingest food at a maximum rate. Due to this feature, *Artemia* could accumulate high bacterial loads during the cultivation process that could later be transferred to fish or shellfish larvae as they are eaten by these animals (Oie et al., 2011). It is possible that microbial communities that established inside the gut of fish in the CR treatment were influenced

by microbes presence in *Artemia* as well as the continuous dispersal of communities from surrounding water forming consortium of microbes that distinct from TR group.

Results of the PICRUSt predictive modeling from our study provide insights into effects that realized changes in microbial community composition in nature and in hatcheries may have on microbial community function that could have compensatory or confounding and antagonistic effects to host well being (Langille et al. 2013). Gut microorganisms play a number of functions in host physiology. For example, in mammals, the gut microbiota plays an important role aiding the supply of alternative energy sources, such as ketone bodies, when hosts are faced with fasting and starvation (Crawford et al., 2009, Kohl et al., 2014). However, this prediction of gene family function associated with our dataset has to be applied with caution due to high NSTI metric quantified by PICRUSt. The high NSTI value indicates high compositional discrepancies between our 16S metagenome dataset and reference genomes (Langille et al. 2013). The fact that we are studying novel communities associated with the digestive system of non-model and ancestral fish species that should have distinct taxa compared to human gut microbial communities might attribute to this circumstance.

PICRUSt assignment of predicted metagenome content to Level 2 KO's in our data revealed that functional responses were generally consistent between treatments and across time (Figs. 2.6a, 2.6b, 2.6c). Our study shows that the most abundant functional categories were associated with carbohydrate metabolism, energy metabolism, amino acid metabolism, membrane transport and replication and repair. This is consistent with the general metabolic functions (such as carbohydrate, protein and amino acid metabolism) that are essential for microbial survival (Mao et al. 2015). Studies by Xia et

al. (2014) also reported enriched microbial taxa (and genes) in Asian seabass (*Lates calcarifer*) that have undergone starvation associated with functional categories including carbohydrate transport and metabolism, inorganic ion transport and metabolism, and amino acid transport and metabolism.

It is unknown whether the same functional groups of microorganisms perform similar adaptive functions within other host taxa. While responses at the level of microbial taxa to fasting vary across hosts, there may be certain microbial functions that increase or decrease in abundance in fasted animals as shown by Xia et al. (2014). They documented significant depletion of three categories (transcription, cell division and chromosome partitioning, and replication, recombination and repair) while significant enrichment of other six categories. On the other hand, we found no significant functional differences between TR and CR group after correction for 37 multiple comparisons. As we mentioned earlier, the different in the host taxonomic (sturgeon vs human) might contribute to the non-significance findings. Sullam et al. (2015) also reported the same finding when they performed multiple comparisons between difference ecotypes of Trinidadian guppies. Nonetheless, almost all functional categories differed significantly among when they compared across enterotypes. In their study, Sullam et al. (2015) loosely defined enterotypes as differing community type presence in guppy gut systems that have inherent functional different based on functional predictions.

Protease-producers found in the fish guts of both treatments were dominated by members in genera *Pseudomonas* or *Aeromonas*, however, more genera were detected fish from TR group. While we cannot completely rule out the possibility that culture-based screening methods could bias for identification, the inflated numbers of these two

genera could be attributed to the abundance of these bacterial taxa in the fish-surrounding environment. *Pseudomonas* species are important decomposers of organic matter in soil, water and food products; but several species also known as a pathogen in plants, animals, and human (Palleroni, 1993). *Pseudomonas* also found to be commonly found as part of fish intestinal and fish-egg microbial communities (Romero & Navarrete, 2006). (Hoshino et al., 1997) has described a *Pseudomonas* sp. that exhibit protease activity at low temperature isolated from the fish intestine.

Aeromonas, on the other hand, can be commonly isolated from various aquatic environments and clinical tissue samples from human or animals and were also shown to be present in both foods used in our treatments (Chironomids and brine shrimp) (Laviad & Harpens, 2016; Austin & Allen, 1982). In addition, numbers of *Aeromonas* sp were regarded not only as an important pathogen in poikilothermic animals, but also have been as the etiologic agent causing many intestinal illnesses in human (Janda & Abbott, 2010). Species like *A. hydrophila* ATCC 7966, and *A. salmonicida* can cause great loss in commercially raised salmonids. Production of extracellular protease is one of the virulence factors possessed by these.

Certain microbial taxa, such as *Enterobacteriaceae* are capable of initiating a stringent response to reduce the rates of protein and other macromolecular synthesis by decreasing rRNA synthesis in response to depletion of nutrition. This mechanism helps cells to shut-down energy-draining activities and enter survival mode under poor growth conditions (Yuan, 2006). This could explain the presence of more taxa under family *Enterobacteriaceae* associated with fish from TR group as these fish experienced dietary compromises during the transition, resulting in starvation, which eventually affects the

nutrient content needed by the microbial community in the gut. As a result, numbers of microbial taxa thrived under this condition while others became significantly reduced.

Studies have listed both *Pseudomonas* and *Aeromonas*, as well as species within the *Enterobacteriaceae* family as potential probiotics, and recent findings showed the promising application of these taxa in aquaculture (for review see Balcázar et al., 2006; Hoseinifar et al. 2014; Merrifield et al., 2010). Hence, given the importance of gut microbial communities and potential application of advanced microbial-based strategies in the artificial rearing of sturgeon and fish in general, further in depth studies should be conducted to unravel the genotypic and functional diversity of these communities. The efficiency feeding of live prey should also be evaluated and carefully monitored especially during the first six weeks of exogenous feeding to larvae to ensure the health and maximum growth.

APPENDIX

APPENDIX

Table 2.1: Split-plot ANOVA (mixed design ANOVA) table for (a) total length; (b) average fish individual weight indicate source of variability between-tank replicate associated with feeding treatment and within-tank replicate associated with sampling times. A significant interaction was also observed between treatment and stages.

Table 2.1 (a)

Source	df	SS	MS	F	p-value
Between subject (tank) effect					
Trt group	1	191.94	191.94	64.41	0.001**
Error	4	11.92	2.98		
Within subject (tank) effect					
Time	2	416.90	208.44	119.63	<0.0001***
Time*Trt Group	2	347.10	173.57	99.62	<0.0001***
Residuals	8	13.90	1.74		

Table 2.1 (b)

Source	df	SS	MS	F	p-value
Between subject (tank) effect					
Trt group	1	0.1208	0.1208	121.8	<0.001**
Error	4	0.0040	0.0010		
Within subject (tank) effect					
Week	3	0.1057	0.0352	101.5	<0.0001***
Week*Trt Group	3	0.1161	0.0387	111.5	<0.0001***
Residuals	12	0.0042	0.0004		

Table 2.2 (a) PERMANOVA analysis indicates that variability among fish gut microbiota differed significantly among developmental stages and between treatments. A significant interaction was also observed between treatment and stages (PERMANOVA test pseudo-F = 2.928, R² = 0.087, p < 0.001; permutation=1000). (b) Goodness of fit from linear regression model (R²), p-value, and Least-square means analyses performed on significantly important PCo axes calculated separately for each stages period. PCo Axis 1 for stage 21dpf and PCo Axis 1 for stage 36dpf showed significant different across diet treatment influencing the microbial community composition, but none of PCo Axis for stage 14 dpf was found to be significant.

Table 2.2 (a)

	Df	Sum Sq	Mean Sq	F-value	R ²	Pr (>F)
Stages	3	2.710	0.903	3.518	0.273	< 0.001
Treatment	1	1.106	1.106	4.308	0.111	< 0.001
Stages:Treatment	2	1.504	0.752	2.928	0.151	<0.001
Residuals	18	4.623	0.257		0.465	

Table 2.2 (b)

Linear regression model :			Least square means	
Pco Axes ~ Treatment				
Important axes	Estimates	p-value	Control	Transitioned
PCo1 14dpf (Before transition)	0.321	0.338	-0.160±0.209	0.160±0.209
PCo1 21dpf (Transition week)	-0.754	<0.01*	0.302±0.040	-0.453±0.049
PCo1 36dpf (After transition)	-0.990	<< 0.001**	0.330±0.002	-0.660±0.002

Table 2.3 List of taxa with taxonomic identification that show high correlation with eigenvector of the first three Principal Coordinate axes associated with largest eigenvalues

PCo.	Taxa	Taxonomic identification/	Correlation
Axes	Genera		
2	Otu1	<i>Clostidium_sensu</i> <i>stricto</i>	0.94
3	Otu2	<i>Aeromonas</i>	0.97
2	Otu10	Unclassified <i>Clostridiceae</i>	0.87
1	Otu11	Unclassified <i>Betaproteobacteria</i>	0.92
1	Otu12	Unclassified <i>Microbacteriaceae</i>	0.84
1	Otu15	Unclassified <i>Comamonadeceae</i>	0.88
1	Otu16	Unclassified <i>Actinomycetales</i>	0.86
1	Otu17	Unclassified <i>Comamonadeceae</i>	0.83
1	Otu18	Unclassified <i>Comamonadeceae</i>	0.88
1	Otu19	<i>Polynucleobacter</i>	0.89
1	Otu24	Unclassified <i>Actinomycetales</i>	0.88
1	Otu29	Unclassified <i>Sphingobacteriales</i>	0.88
1	Otu31	Unclassified <i>Cryomorphaceae</i>	0.898
1	Otu41	Unclassified <i>Microbacteriaceae</i>	0.83
1	Otu42	<i>Methylophilus</i>	0.78
1	Otu45	Unclassified <i>Cytophagaceae</i>	0.88
1	Otu48	Unclassified <i>Sphingomonadaceae</i>	0.74

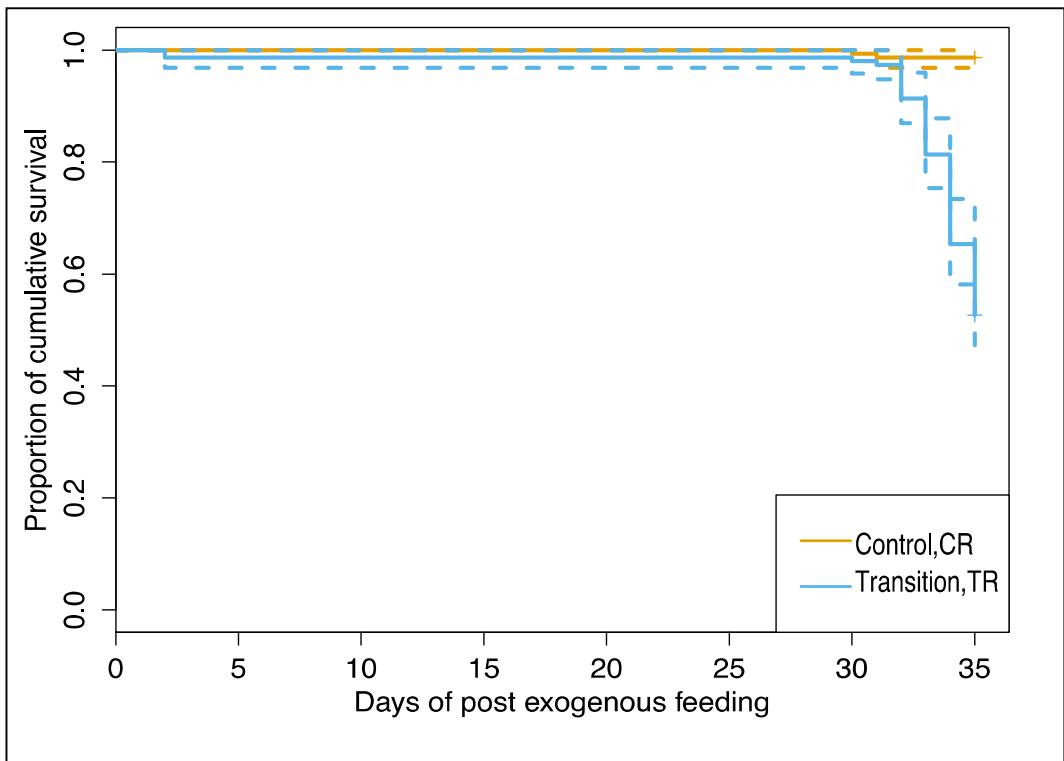


Figure 2.1: Mean proportion of cumulative survival (solid-line) of lake sturgeon from Transition (TR) and Control (CR) treatment group with 95% CI (dashed-line). Survival analyses were based on log-rank tests indicating that survival of fish in the TR treatment (0.527) was significantly lower compared to fish from the CR treatment (0.987) (Chi square value, $\chi^2 = 85.1$, p-value < 0.01, df=1)

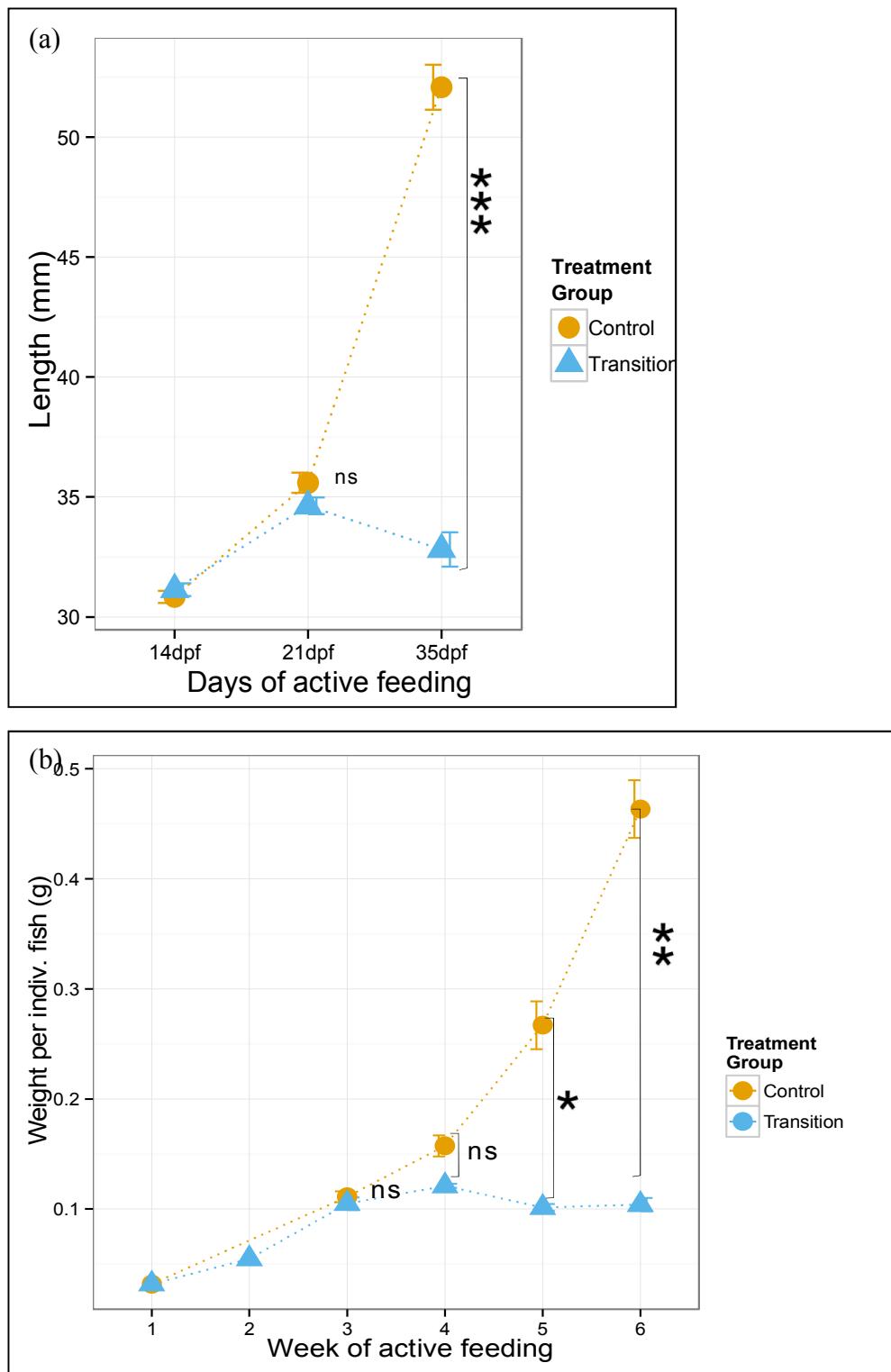


Figure 2.2. Morphometric data with error bar representing standard error mean (a) total length (mm) by treatment group, at each sampling point; (b) average weight

Figure 2.2 (cont'd)

(g) of fish from each treatment group fed with control food vs fish transitioned to *Chironomid* larvae. * indicate significant differences in treatment mean test based on Welch-t test (* p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001)

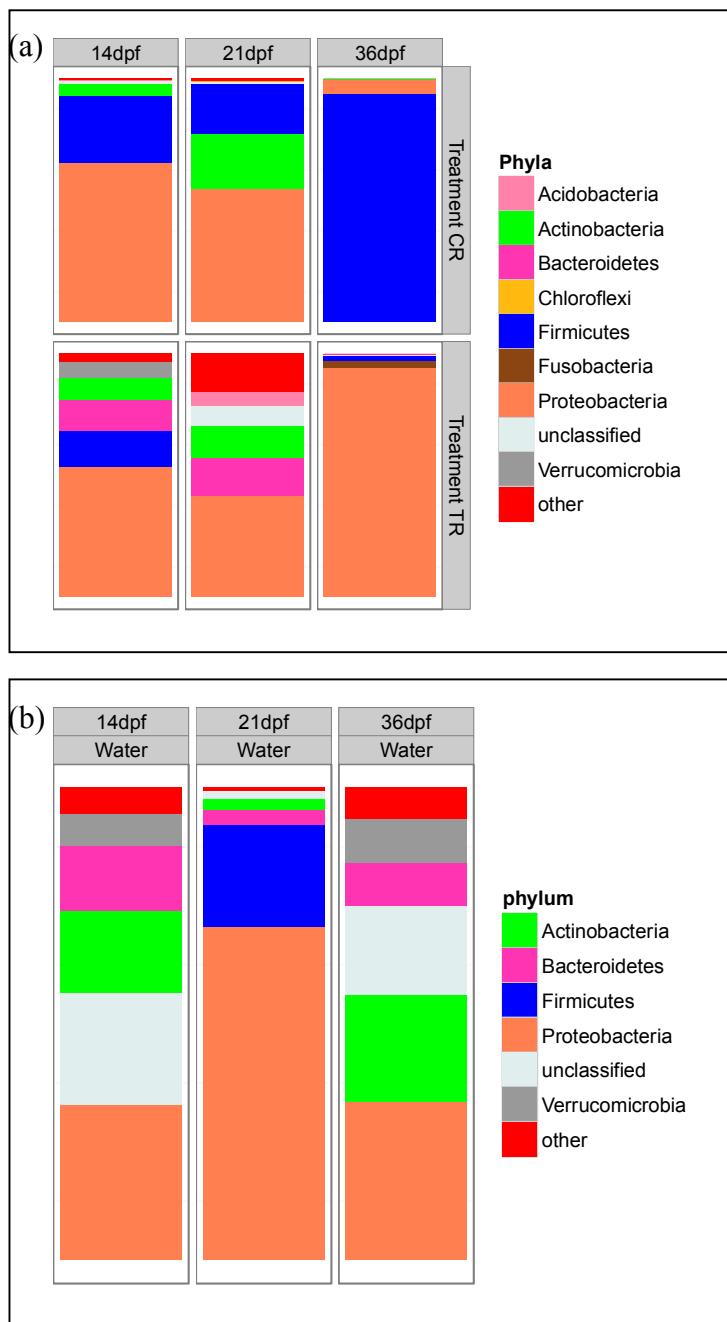


Figure 2.3 (a) Relative abundance (percentage) of six bacterial phyla (*Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Fusobacteria*, *Proteobacteria*) found in gut microbiota of lake sturgeon larvae for each treatment at different times. The remaining taxa were assigned as Others. (b) Relative abundance (percentage) of six bacterial phyla (*Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Verrucomicrobia*) found in aquatic samples. The remaining taxa were assigned as Others.

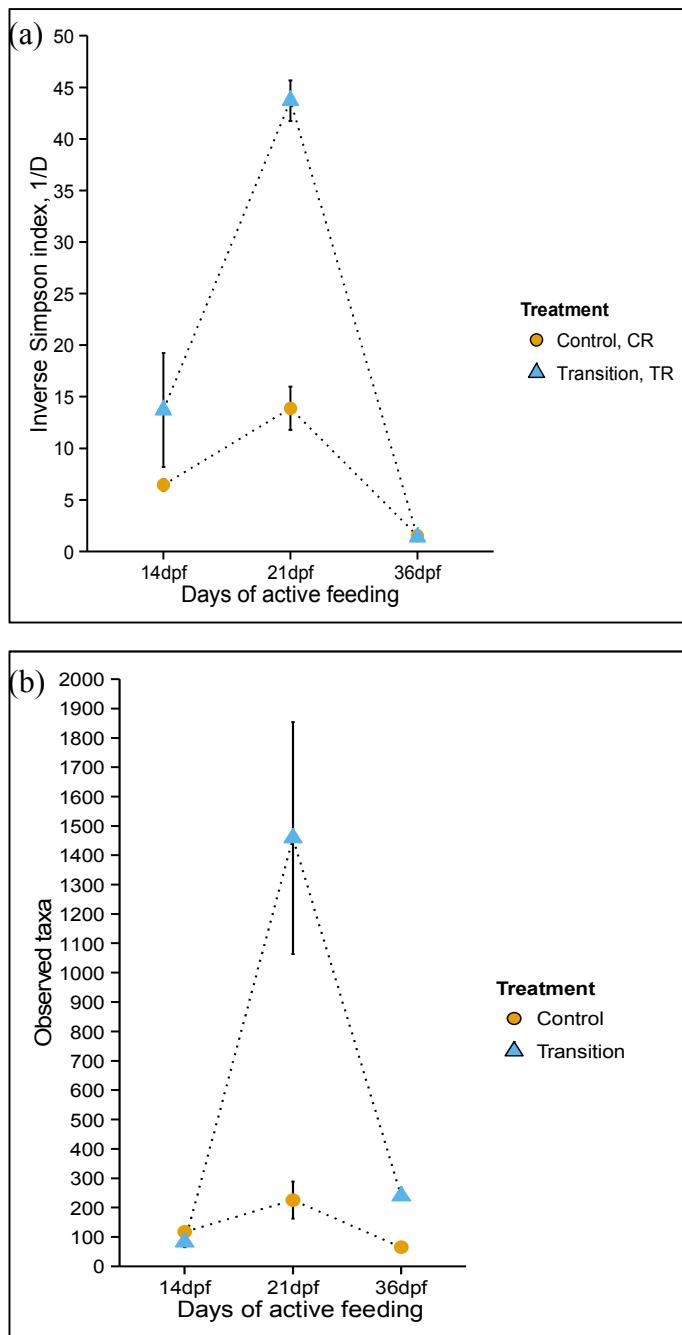


Figure 2.4 (a) Inverse Simpson Diversity index and (b) number of observed OTUs/Taxa richness for both feeding treatment (Control, CR and Transition, TR) at different sampling time

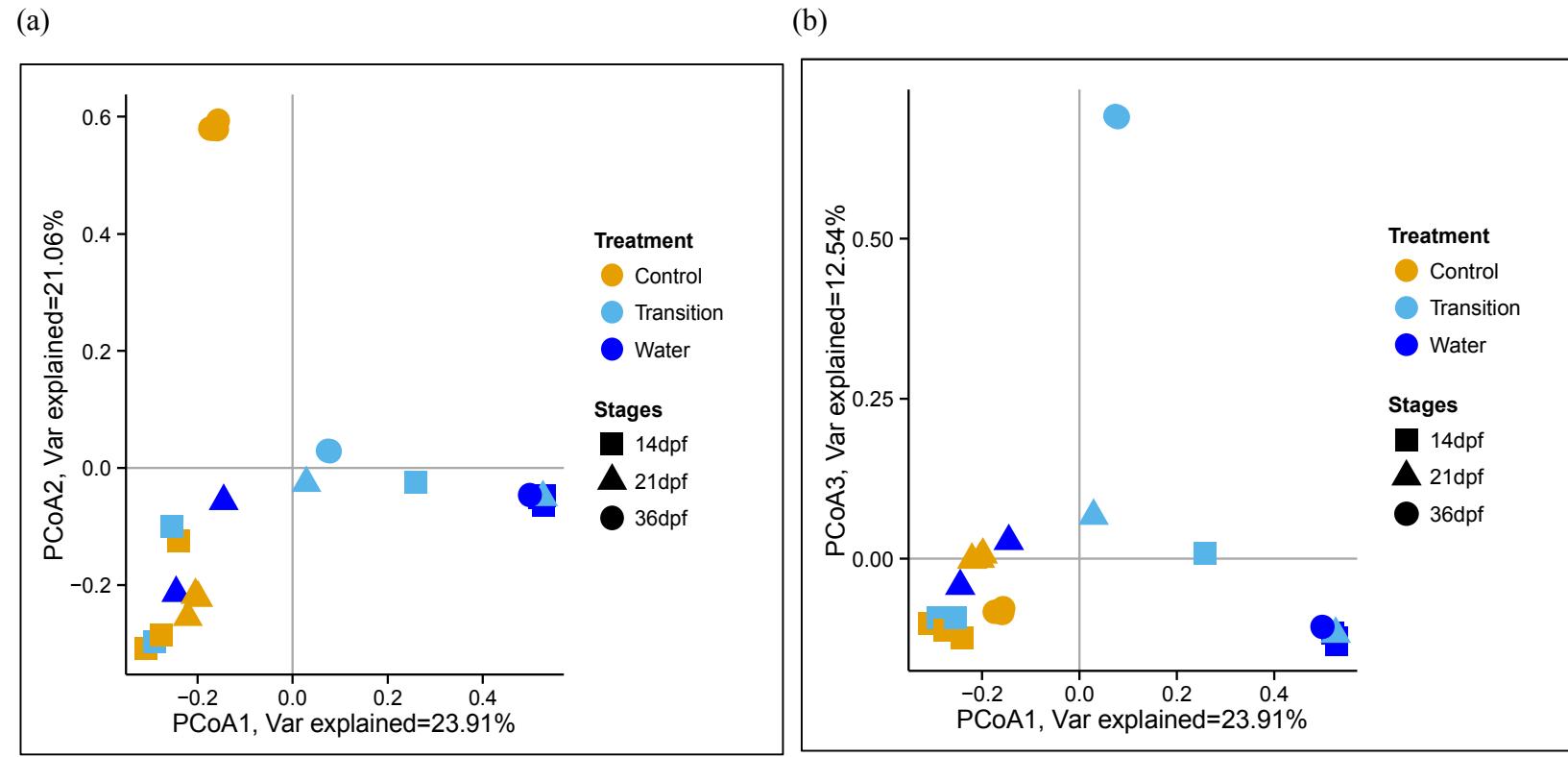


Figure 2.5 Visualization using Multivariate Principal Coordinates Analyses (PCoA) plots of variation in lake sturgeon gut and water microbial community composition among collections made at different times and between food from different treatment

(c)

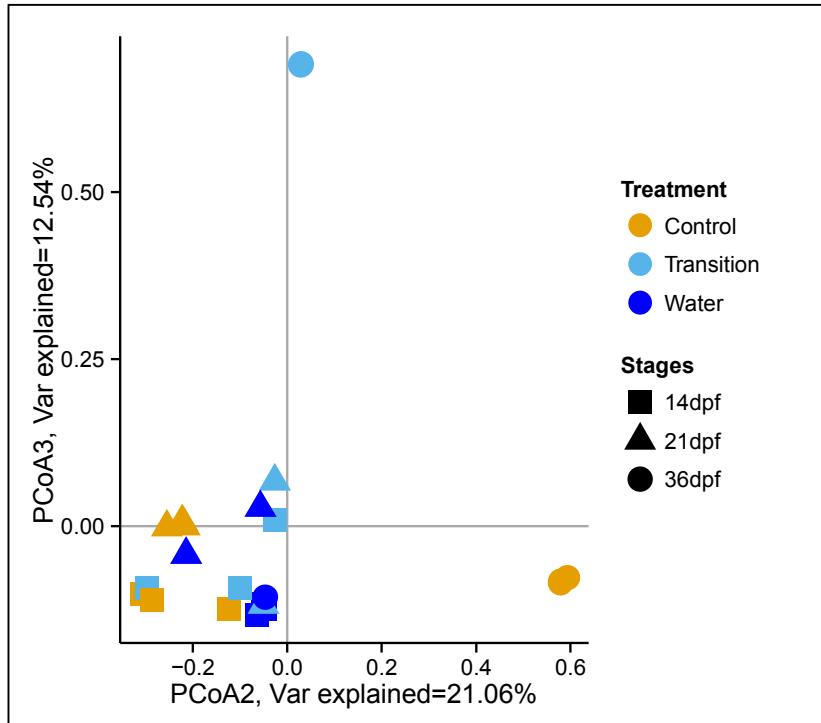
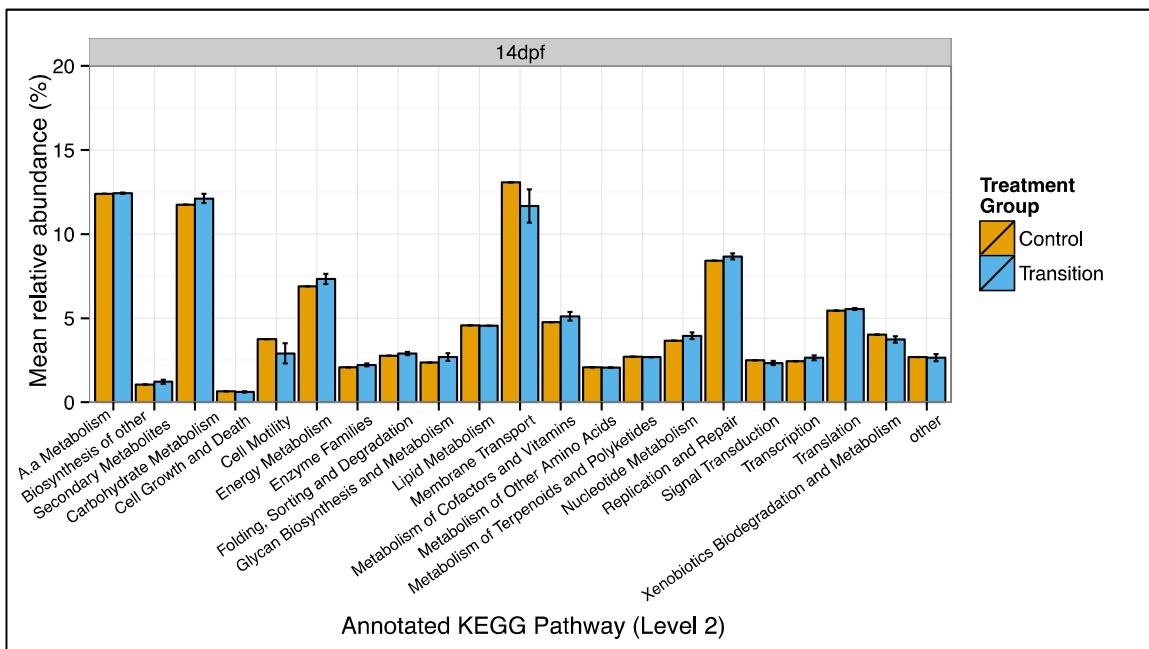


Figure 2.5 (cont'd)

groups using Bray-Curtis distances. Samples were taken from replicates of different treatments at three developmental stages (before transition at 14dpf, during transition week at 21dpf, after transition at 36dpf). (a) Plot based on Bray Curtis distance Axis 1 and 2, (b) Plot based on Bray Curtis distance Axis 1 and 3, (c) Plot based on Bray Curtis distance Axis 2 and 3

(a)



(b)

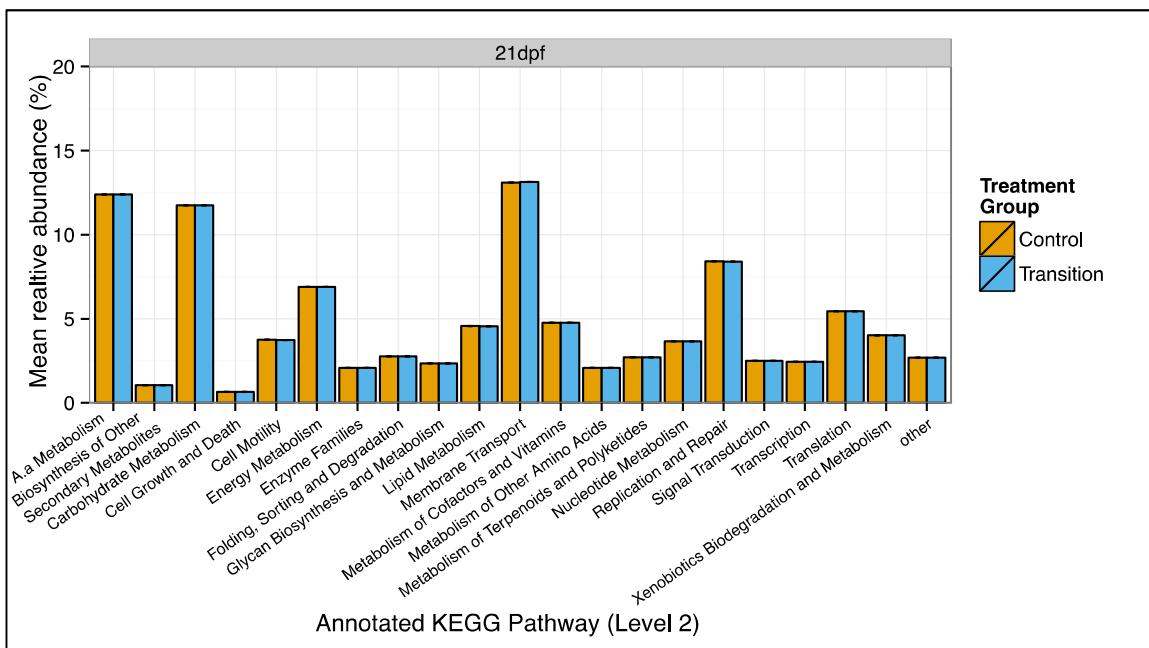


Figure 2.6 Relative abundance of top 20 annotated functional groups identified using KEGG Orthologs pathway categorized at molecular-level 2 associated with

(c)

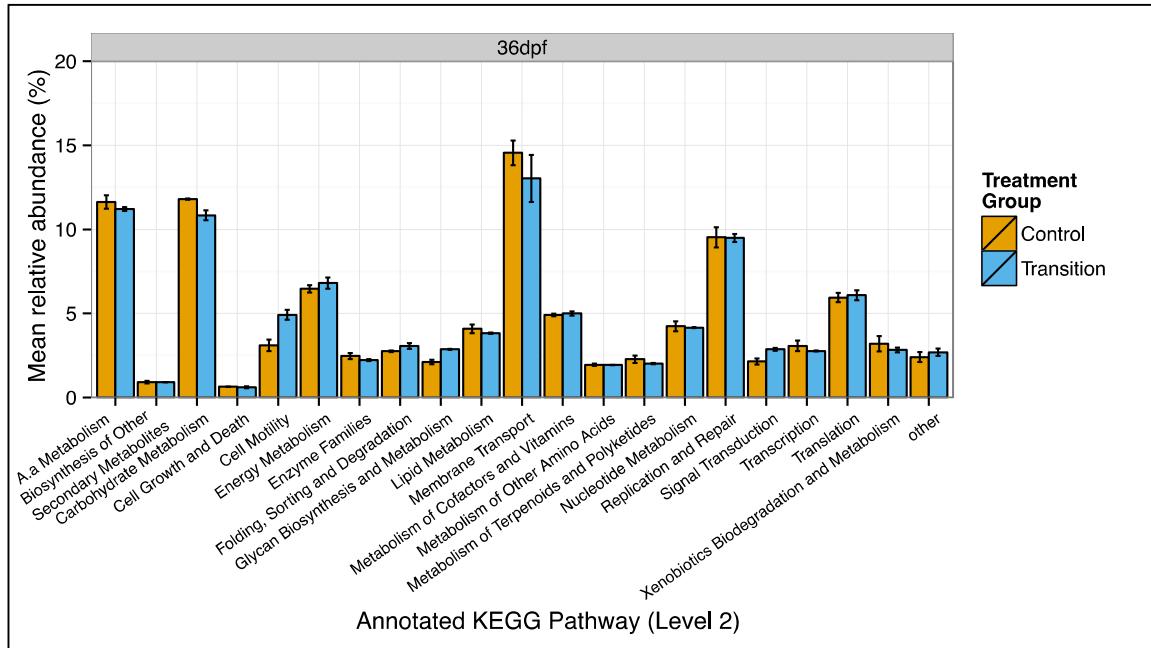


Figure 2.6 (cont'd)

lake sturgeon larval microbiome from two diet treatments, Control (CR) and Transition (TR) at each sampling point. (a) 14 dpf; (b) 21 dpf; (c) 36 dpf

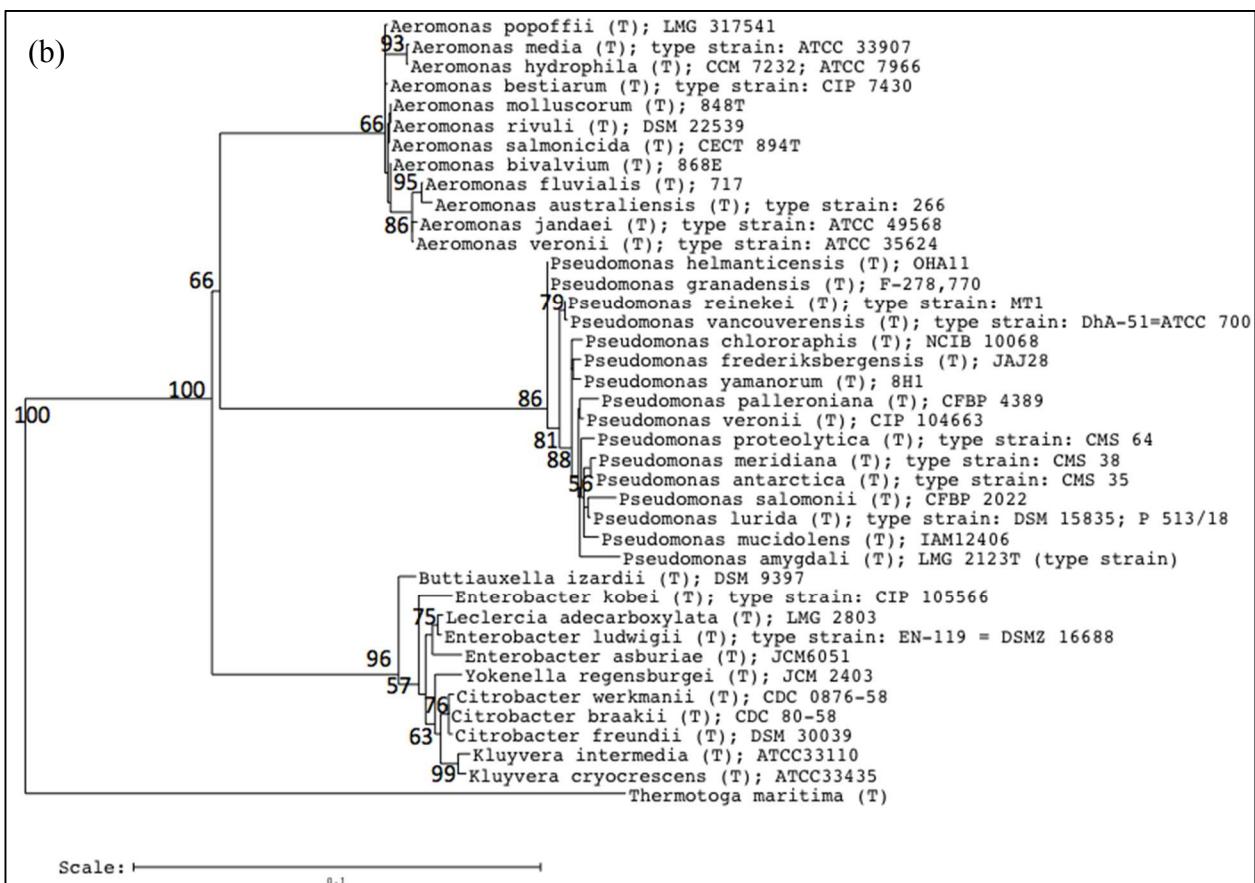
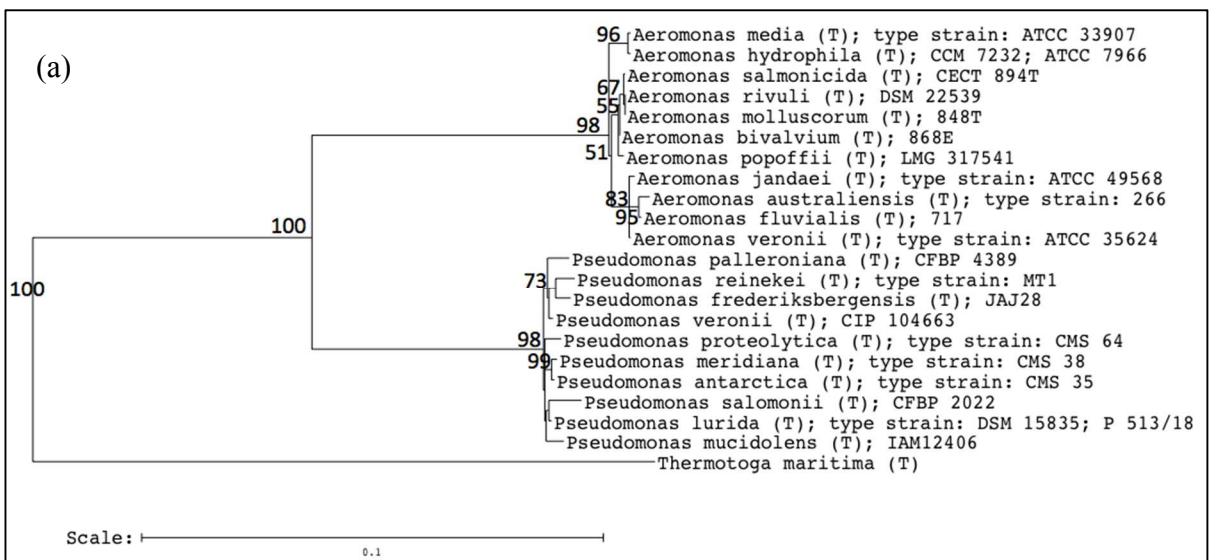


Figure 2.7 Weighted Neighbor-Joining tree showing evolutionary relationship for selected top match taxa with single outgroup species. Branch supports were estimated using bootstrap analysis based on 100 pseudoreplicates for NJ and the

Figure 2.7 (cont'd)
percentage of bootstrap value were displayed next to branches in the inferred phylogeny. (a) NJ tree for isolates from fish in the CR group; (b) NJ tree for isolates from fish in the TR group.

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CHAPTER 3: CHANGES IN LAKE STURGEON GUT MICROBIOMES IN RESPONSE TO CHEMOTHERAPEUTANT TREATMENTS

ABSTRACT

Prophylactic treatments using antibiotic, drugs, or chemical compounds are widely applied in aquaculture to prevent disease outbreaks. Simultaneously, the vital roles of gut microbiota in maintaining host physiological processes and homeostatic regulation have been recognized and a growing number of studies have reported the damaging impacts of antibiotics to gut microbial community composition and function. Here, we characterized the gut microbial composition of an ecologically and economically important fish species, the lake sturgeon (*Acipenser fulvescens*), during early larval stages in response to weekly prophylactic treatments using three different chemotherapeutants commonly used in aquaculture (Chloramine-T, hydrogen peroxide, salt followed by hydrogen peroxide) relative to a control treatment. Gut microbiome composition in treated vs non-treated larval fish from two different hatchery-crosses and multiple groups developed from wild eggs collected at two spawning sites were analyzed using massively parallel next generation sequencing based on the V4 region of the 16S rRNA gene. Results showed that members of the phylum Firmicutes (unclassified *Clostridiales* and *Clostridium_sensu_stricto*) and Proteobacteria were the dominant gut microbiota of all fish samples regardless of treatment. We observed large variation in the diversity of lake sturgeon microbiota between larvae from hatchery and wild origin using Principal Coordinate Analyses (PCoA). PERMANOVA analyses indicated a significant interaction between families/group and treatment. Regression analyses suggested effects of treatments were dependent on the fish origin. The influence of host genotype and the resilience of gut microbiota to prophylactic treatments are discussed.

INTRODUCTION

Widespread use of chemicals, drugs, and antibiotics (chemotherapeutants) is a rising concern in aquaculture. With recent expansion and rapid growth of the aquaculture industry, prophylactic use of chemicals and antibiotics application has been reported in the aquaculture as a means to control disease outbreaks (Cabello, 2006; Mortazavi, 2014). While short-term benefits are often realized, there is great potential for damaging impacts of these practices, as large amounts of veterinary drugs and chemotherapeutants are passed into the soil and aquatic environments. The emergence of antibiotic resistance threatens fish, terrestrial animals and human beings (Cabello, 2006; Fraise, 2002; Samira & Guichard, 2006). In fish, antibiotic resistance poses a more serious problem than for terrestrial animals because water readily supports and spreads bacterial pathogens (Romero, Ringø, & Merrifield, 2014).

Another important aspect of prophylactic treatment practices in aquaculture is the potential impact of drugs and chemotherapeutants on the gut microbiomes. Common treatment strategies include the use of chemotherapeutants to treat infected fish following visual detection of disease or in response to high mortality events. Alternatively, weekly prophylactic chemotherapeutic treatments are used to reduce stress and reduce the probability of pathogen infection (Bowker et al. 2011). Chemotherapeutants and antimicrobial compounds used in prophylactic treatments have been shown to be effective at reducing or preventing mortalities caused by pathogens. However, some compounds are indiscriminant in their effects and may eradicate symbiotic and /commensal gut microbial communities as well (Romero et al., 2014). Downstream effects of antibiotic or chemical treatments on microbiomes are likely to have important

consequences to fish hosts. However, these effects are currently under-studied.

In humans, microbiomes within individual hosts usually varies in composition across anatomical sites, and taxonomic composition can vary over time in response to factors such as diet, physical activities and medication intake (Cho & Blaser, 2012; Dave et al., 2012; The Human Microbiome Project Consortium, 2012). Among these factors, exposure to antibiotics can have profound effects on resident microbial communities inside human guts (Dave et al., 2012; Langdon, Crook, & Dantas, 2016). Several studies reported changes in density or gut microbiome composition, for instance in infants who receive antibiotics (Palmers et al. 2007). Dethlefsen et al. (2008, 2011) documented pervasive effects of orally administered antibiotic to adult gut microbiomes, associated with decreases in taxa richness and evenness.

Antibiotic use can save human lives and the lives of economically important fish species. However, there can be collateral damage to beneficial gut microbes from over-utilization of antibiotics (Becattini, Taur, & Pamer, 2016; Langdon et al., 2016). Antibiotic treatments can threaten indigenous gut microbiota by eliminating certain bacterial taxa, potentially resulting in ecological drift or community alteration that favor the increase in abundance of certain taxa (Costello et al., 2012; Manichanh et al., 2010; Panda et al., 2014). Adverse effects related to antibiotic use include pathogen resistance, suppression of the immune system, increased rates of allergies, autoimmunity, and other immune-inflammatory conditions (see Langdon et al., 2016 for review). We are now just beginning to understand the functions of microbial communities. Therefore it is important to expand studies of the impact of antibiotics to the stability of gut microbiomes in other ecologically and economically important vertebrates, including fishes.

Community ecology places emphasis on studies of patterns in diversity, abundance, and composition of species along with processes underlying these patterns (Vellend, 2010). One core theory in community ecology involves drift, in which community composition changes in response to disturbance (Leibold et al., 2004). Disturbance can be defined as a “single disruptive event or set of events that significantly changes ecological community structure and function” (Christian, Whitaker, & Clay, 2015; Leibold et al., 2004). Ecological communities can respond in one of four ways to disturbance: (i) communities experience no change in composition following the disturbance (resistance); (ii) community composition changes but then returns to its original state (resilience); (iii) community composition changes but the new microbial constituents maintain the same function as the original community (functional redundancy); or (iv) microbial community composition changes, some taxa are extirpated and original community function is lost (perturbed) (Cho & Blaser, 2012; Christian et al., 2015; Francino, 2016; Langdon et al., 2016)

Advances in sequencing technology have allowed researchers to advance studies pertaining to gut microbiomes beyond traditional medical and veterinary applications (Dave et al., 2012; Ghanbari, Kneifel, & Domig, 2015). Complex species interactions across space and over time pose challenges to our understanding of the ecological organization and evolutionary importance of animal-bacterial interactions. From an ecological perspective, the microbial community within an individual host can be viewed as a local community colonized from a regional species pool (Adair & Douglas, 2017). Combining microbiomes studies with the conceptual framework of community ecology

that has developed over the years offers insight to illuminate complexities of host-microbe interactions (Christian, Whitaker, & Clay, 2015; Costello et al., 2012).

Few studies have documented changes in a fish-associated gut microbial community in response to chemical or antibiotic exposure. The effect of orally administered antimicrobial compounds on gut microbiome was reported in several important aquaculture species including rainbow trout (*Oncorhynchus mykiss*) using culture methods (Austin & Al-Zahrani, 1988) and molecular-based methods (Navarrete et al. 2008); hybrid tilapia (*Oreochromis niloticus* X *O.aureus*) (He et al., 2010); and gibel carp (*Carassius auratus gibelito*) (Liu et al., 2012) (for extensive review, see Ringø et al., 2015). Collectively, these studies reported gut microbial communities are negatively impacted by antimicrobial treatments. These studies, however, have focused mainly on describing gut microbiome in fish at the juvenile stage, whereas fish at early life stages are prone to pathogen infection (Vadstein et al., 2012), and thus may often be exposed to antimicrobial compounds and chemotherapeuticants. To evaluate the suitability of prophylactic treatment of fish larvae without compromising fish normal function, more studies are warranted pertaining to the influence of chemotherapeuticants utilized in fish culture on gut microbiota.

We empirically evaluated the response of lake sturgeon larval gut microbial communities raised in a common rearing environment to perturbations associated with the use of four prophylactic chemotherapeuticants using 16S rRNA-based next generation sequencing. We measured the changes in gut community composition of fish produced from either wild origins or from gametes fertilized in the hatchery. We hypothesized that prophylactically treated fishes would show decreased microbial taxonomic diversity and

different community composition relative to fish from the control treatment. We used one-to-all comparison analyses (Segata et al. 2011) to identify biomarkers (taxa) associated with each treatment in contrast to fish from the control group. Our findings provide insight into the consequences of prophylactic treatments and host-microbe interactions.

METHODOLOGY

Fish husbandry

The experiment was performed at the Black River Sturgeon Rearing Facility managed by the Michigan Department of Natural Resources (MDNR) and Michigan State University (MSU) in Onaway, MI, USA using approved Institutional Animal Care and Use Committee (IACUC) protocols. This experiment was conducted from June 26 to July 30, 2013, following the primary spawning period for lake sturgeon in the Upper Black River, Cheboygan County, Michigan.

Different sources of lake sturgeon progeny were utilized in this experiment. First, hatchery-produced, full-sibling lake sturgeon larvae were produced from direct gamete takes from two mated pairs of males and females collected during sturgeon spawning season in the Upper Black River. The fish families were labeled as hatchery A (HA) and hatchery D (HD). Gamete collection and eggs fertilizations followed procedures as described by Crossman et al. (2011) and Bauman et al. (2015), respectively. Secondly, wild, naturally produced larvae from multiple spawning events were raised from wild naturally fertilized eggs that were collected from the stream substrate from two spawning locations at the Upper Black River approximately three days post-fertilization. These eggs were transported to the hatchery, incubated and separated by capture location labeled as Site B and Site C, and fish cohort hatched from these spawns were denoted as wild B (WB) and wild C (WC). Parentage was not determined but previous studies indicated that fertilized eggs are likely from multiple pairs at each site.

Eggs were incubated and raised in similar hatchery rearing environments separately based on their family using Aquatic Eco-systems (Pentair) J32 Mini-Egg

hatching jars. Water was taken directly from the river and filtered through 50 micron and 100 micron filters to remove sediment and biological material. Stream water was used throughout the experiment. Eggs were treated daily with 500 ppm, 15 min immersion in hydrogen peroxide as part of standard incubation procedure until 24 hours prior to hatch. At hatch, free-embryos were transferred into 10L polycarbonate tanks (Aquatic Habitats) consisting of sinking Bio-Balls (Pentair #CBB 1-S) that covered the bottom of each tank. As endogenous yolk resources were depleted and larval fish emerged, Bio-Balls were removed and fish were fed brine shrimp three times daily. Feeding rate was determined following Deng et al. (2003). Ambient mean water temperature during the study was 22.7 °C(range 20.0 to 26.3 °C). Mortalities were recorded daily, and all individuals were raised in the same rearing conditions until the prophylactic treatment experiment began.

Experimental design

We used four treatment groups where fish in each group were exposed to weekly prophylactic treatments using compounds that are commonly used in aquaculture (collectively called chemotherapeutants). Treatments included: 1) 60 min, 15 ppm Chloramine-T (CT) immersion, 2) 15 min, 60 ppm hydrogen peroxide (H_2O_2), 3) 3 parts per thousand (ppt) salt, $NaCl^-$ immersion for 15 min followed 24 hr later by a 15 min, 60 ppm H_2O_2 bath ($NaCl/H_2O_2$), and 4) a control group (no chemical treatment, CTRL). The experimental design associated with how the fish were quartered between treatments is shown in Fig. 3.1.

At twelve days post feeding (dpf), 400 fish from each hatchery family or wild group were transferred and randomly distributed into four 1.2 m diameter tanks which were divided into eight partitions with 50 fish per partition. There were two replicate

groups of 50 fish in each treatment group in each tank (Fig. 3.1). The first prophylactic exposure began at 14 dpf (after two days of tank acclimation). Treatments were conducted at 5 weekly intervals and continued for 35 days (total 5 treatments). Assignment of disinfectant exposure was based on a randomized block designed where disinfectant exposure was randomly assigned to each partition within the tank (Fig. 3.1). Fish mortalities were recorded and total survival was documented at the end of the experiment. Comparisons were made of the effects of different prophylactic treatments on diversity and compositional change in bacterial communities.

During immersion, all fish from each treatment group (including controls) were transferred using a hand net to 10L polycarbonate tanks to expose fish to chemotherapeuticants (or river water for the control group). Following each treatment, fish were briefly rinsed and immediately placed back into their assigned rearing tank and compartment. All exposures were administered on the same day and were repeated once per week except treatment 3 (salt), which included an additional treatment the following day with H₂O₂. Fish in the control group were handled in the same manner as fish from other treatment groups, however, similar to treatment 1 (CT), were held for 60 min in their ‘treatment’ tank before being rinsed and returned to their rearing tanks. Sampling for microbiota analysis took place following the end of the five-week treatment period. From each partition, four fish were randomly collected and were euthanized with an overdose of MS-222 (Sigma-Aldrich, St Louis, MO, USA). All fish were preserved in 80% ethanol and transported to MSU until dissections were performed.

Fish dissection, DNA isolation, PCR validation

The distal gut (spiral valve) of each sturgeon larvae was recovered from fish following aseptic techniques. The distal gut was defined as the section that begins at the intestine until the spiral valve. The spiral valve serves as the primary region of digestion and absorption, and thus may provide an area of abundant nutrients where a microbial community can flourish (Buddington & Christofferson, 1985; Callman & Macy, 1984). Exterior surfaces were swabbed with 100% ethanol before dissections of the whole digestive tract using sterile instruments. Dissections were performed with slight modification as previously described by (Milligan-Myhre et al., 2011). The intact alimentary tracts were cut from the body cavity, and the excised gut was immediately transferred into filtered-sterilized 80% ethanol solution for DNA isolation. Until DNA extraction was performed, all samples were stored in -20°C.

Each gut sample was first centrifuged for 15 min at 4°C to pellet bacteria before DNA was extracted. The MoBio PowerSoil® DNA Isolation Kit (Carlsbad, CA, USA) including a bead-beating step was used following protocols for low-biomass samples, as suggested by the manufacturer. The integrity of each DNA sample was assessed based on amplification of 1.4k bp of the 16S rRNA gene (amplicon based on 27F and 1389R primers) followed by gel agarose electrophoresis. DNA concentrations were quantified using a Microplate spectrophotometer (BioTek®, Winooski, VT, USA).

16S rRNA amplicon sequencing and sequence pipeline analyses

Gut microbiota from lake sturgeon larvae were surveyed using high-throughput sequencing of the 16S rRNA gene. 152 DNA samples (including four positive controls, and 20 technical replicates) that have been validated to contain sufficient bacterial DNA

(as shown by the presence of amplicon bands in electrophoresis) were submitted for sequencing at Michigan State University Research Technology Support Facility, RTSF (East Lansing, MI, USA). All sequencing procedures, including the construction of Illumina sequencing library, emulsion PCR, and MiSeq paired-end sequencing v2 platforms of the V4 region (~250bp; primer 515F and 806R) followed standard Illumina (San Diego, CA, USA) protocols. Michigan State Genomics RTSF (<https://rtsf.natsci.msu.edu/genomics/>) provided standard Illumina quality control, including base calling by Illumina Real Time Analysis v1.18.61, demultiplexing, adaptor and barcode removal, and RTA conversion to FastQ format by Illumina Bcl2Fastq v1.8.4.

Details of the microbial sequence data analyses pipeline and computing workflow were made following default steps. Briefly, paired-end sequence merging, quality filtering, “denoising”, chimera checking, and pre-cluster steps were conducted using an open-source workflow based on methods implemented by program *mothur* v.1.36.1 (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013). Then, sequence pipeline analyses were performed in *mothur* v.1.38.1 to accomplish reference-based OTU clustering (method = opticluster). Taxonomic assignment was performed using the SILVA bacterial reference database by clustering sequences defined with 97% identity. Any sequence singletons that were detected were removed prior to downstream analyses. Rarefaction analyses were performed to evaluate the sampling coverage for each sample based on the selected sequence depth. To minimize effects of under-sampling while maintaining as broad dataset as possible, the final OTUs table was rarefied to a depth of 8000 sequences per sample. Nine DNA samples with low sequence depth were discarded prior to downstream analyses.

Analyses of bacterial community profiles and ecological statistical analyses

3.1 Alpha diversity

All measures of microbial community diversity including Inverse Simpson (1/D) diversity indices and OTUs richness of each sample were calculated from the sequence data within program *mothur*. These indices were first analyzed using linear mixed-effects regression (LMER) with the *lme4* package in R (Bates et al. 2013). In contrast to a more traditional approach to data aggregation and classical ANOVA analysis, LMER controls for the variance associated with random factors without data aggregation (Baayen et al. 2008; Bolker et al. 2009). We started analyses with a null model that included diversity indices as a dependent variable with family and wild groups selected as random factors. We added chemical treatment as the predictor variable (fixed factor), and an interaction between random and fixed effects to quantify whether model fit was improved. Model fit was assessed using chi-square tests on the log-likelihood values to compare different models (Winter, 2013). All analyses were carried out in the R program.

To test for significant differences in diversity indices of gut microbial communities of fish across treatment groups (CT, H₂O₂, NaCl/ H₂O₂, CTRL) taking into account the effect of the random (family) factor, we then used non-parametric Kruskal-Wallis tests for the null hypothesis that diversity for each of fish samples did not differ as a function exposure to a particular treatment. The test was followed by post-hoc Tukey-Kramer pairwise tests. P-values below 0.05 indicated significant differences in pairwise comparisons. Identity and relative abundance and of all bacterial phyla in all fish gut and water-associated microbial communities across sampling times were determined using packages *dplyr* and *reshape2* in program R.

3.2 Beta diversity

We used several packages implemented in program R to estimate comparative (beta [β]) diversity measures and ecological statistics at the OTU level. Briefly, we used the *vegan* package (Oksanen, 2015) to generate Bray-Curtis (BC; Bray & Curtis, 1957) distance characterizing differences in microbial community composition among samples. We used the *cmdscale* function to perform Principle Coordinate analyses (PCoA) ordination of community composition differences based on BC distance. The *ggplot* and *ggplots2* packages (Wickham, 2009) were then used to create ordination plots to visually compare gut community composition with aquatic community composition and as a function of different treatments and among sampling periods based on the principal coordinates with the first and second largest eigenvalues.

Multivariate hypothesis testing to quantify differences in community composition among samples collected from different feeding treatments and across different families were performed using the *adonis* function (Oksanen, 2015) in program R. Differences between the centroids of sampling groups for treatments and family/samples origins were based on Permutational Multivariate Analyses of Variance (PERMANOVA) using BC resemblance matrices (Anderson, 2001; 2006). Under the null hypotheses, the centroids of the groups (fish from either hatchery families and wild groups exposed to different chemotherapeutic treatments) are equivalent for all groups under random allocation of individual sample units to the groups (i.e., based on permutation). This test was employed because of the non-parametric and skewed nature of microbial data.

To further analyze associations between family effects and chemical treatment effects on fish gut microbiota, PCoA were performed separately on fish gut communities

within each family/group. Important PCoA axes denoted by larger eigenvalues in comparison to average eigenvalue were selected, and linear regression models were fitted, where each axis represents response variables given predictor variables of chemotherapeuticants. Under the null hypotheses, prophylactic treatments were not expected to significantly affect fish gut community taxonomic composition within a family. Axes here represent a linear combination of microbial community diversity and composition present in individual fish GI tracts from the same family/group. Axes that showed significant treatment effects were then analyzed using the *lsmean* function to quantify effects of specified factors (or combinations of specified factors for interactions) on the bacterial taxonomic composition and relative abundance in larval fish guts.

3.3 Differential abundance of OTUs and biomarker identification across treatments

To further determine the operational taxonomic units that most likely explained differences in microbial community composition between treatment groups, we next employed Linear Discriminant Analysis (LDA) effect size (LEfSe) methods (Segata et al., 2011). In general, the LEfSe algorithm identifies genomic features (i.e., in the present study – OTUs) that are differentially abundant in different experimental groups (families and treatments), then ranks them based on that abundance differential. The larger the difference in relative abundance between groups, the higher the importance of that OTU.

The algorithm first identifies features (OTUs) that were statistically different among families/groups based on the nonparametric factorial Kruskal-Wallis (KW) sum-rank test. Additional tests assessed consistency of differences using unpaired Wilcoxon rank-sum tests. In the final step, LEfSe uses LDA to rank each differentially abundant taxa in order of the difference in abundance based on an LDA Score (log-scale). Results

represent a scale indicating “importance” of an OTU in group differences in microbiota composition (Segata et al., 2011).

To run LEfSe, the tabular file was generated from a shared file that contained no singletons in program *mothur* v.1.39.5. The tabular file consisted of taxonomic relative abundance in gut community samples from the four different families/groups assigned to four chemotherapeutic treatments. Then, this tabular file was transferred into an online bioinformatics toolkit developed by Huttenhower lab to perform LEfSe analyses (<https://huttenhower.sph.harvard.edu/galaxy/>) and generate LDA figure.

RESULTS

Diversity of gut microbial community composition

A total of 144 samples were retained after quality filtering was performed in the sequence pipeline analyses. Comparisons of gut microbial community composition of sturgeon fry at the level of phyla indicated that three major phyla dominated more than 50% of total community abundance across all fish samples (*Firmicutes* 16%, *Proteobacteria* 36.5%, and *Actinobacteria* 15.1%). Phyla detected in a remainder of the gut community included *Acidobacteria*, *Bacteroidetes*, and *Verrucomicrobia* along with other phyla that collectively comprised 30% of communities.

The relative abundance of the most dominant phylum, *Firmicutes* was fairly consistent across treatments for fish samples from all hatchery families and wild groups (HA, HD and WB, WC, respectively). One exception was WB fish exposed to salt (mean 58%) and WC fish exposed to peroxide (mean 50%) that were relatively low compared to other treatments (Fig. 3.2a). When comparing the abundance of *Firmicutes* across all four families/groups, fish from hatchery family D (HD) had a lower percentage of *Firmicutes* (mean range from 51-66%). *Proteobacteria* relative abundance was likewise relatively uniform across treatments (13% - 28% of total abundance) with the exception of WB fish that were treated with chloramine-T, CT (6%). *Actinobacteria* were present at 1% in fish that were not exposed to any chemotherapeuticant (control) but only in fish from HA and WC groups.

At the genus level, Firmicutes were represented by two genera, *Clostridium_sensu_stricto* & Unclassified genera from family *Clostridiaceae*. We found that *Clostridium_sensu_stricto* were the most dominant genus (mean range: 30 – 51% of

the total community) for all fish of hatchery origin (except for HA fish exposed to peroxide), whereas all fish of wild origin had Unclassified taxa from *Clostridiaceae* family (mean range: 29 – 62%) as the most abundant genus across any treatment (Fig. 3.2b). Genera from phylum *Proteobacteria* including several unclassified taxa from *Alphaproteobacteria*, unclassified taxa from *Betaproteobacteria*, unclassified taxa from *Enterobacteriaceae*, unclassified taxa from *Rhodobacteriaceae*, and *Deefgea* all were present at lower percentages of abundance with more amount of variation across fish groups and treatments (Fig. 3.2b). The only genus in the phylum *Actinobacteria* that was detected among dominant taxa was the genus *Zhihengliuella*, present in HA control fish (mean 2.2%) and WC control fish (mean 1.4%).

To answer question, does treatment, or family or both affect alpha diversity measures, we first generated three alternative models consisting of (i) both fixed (prophylactic treatments) and random (family/group) predictor variables with interaction, (ii) both fixed and random predictor variables without interaction, (iii) random predictor variable only, to best explain variation in Inverse Simpson diversity indices and in the number of observed taxa in fish gut microbial communities. For Inverse Simpson indices, the model that consisted only of the random variable (family/group of origin) had the lowest AIC value (AIC = 917.20, df =3) compared to the other two models that consisted of fixed effects with and without an interaction (Table 3.1a). The log-likelihood test indicated that no models evaluated were statistically different from one another.

The lowest AIC selected model for a number of observed taxa (OTU richness) included both fixed and random variables (AIC= 1893.4, df =6). Comparisons of the best-supported model with other models based on a log-likelihood test indicated that this

model was a significant predictor ($\chi^2 = 17.71$, df = 3, p < 0.001) (Table 3.1b). This indicated a significant relationship between the number of microbial OTUs observed and the family/group of fish samples.

Fig. 3.3 (a) and (b) shows the comparison of Inverse Simpson indices and number of observed taxa for each chemical treatment and families/groups. Fish in the control treatment (CTRL) had less diversity gut communities (both Inverse Simpson and richness) with the exception of fish in family HD. However, Kruskal-Wallis tests for Inverse Simpson for each hatchery family and wild group failed to reject the null hypothesis that diversity does not differ significantly across treatments.

When the same statistical test was applied to fish from wild groups based on observed numbers of taxa (OTU richness), we detected significant p-value therefore rejecting the null hypothesis. Fish from one treatment group were characterized by taxa richness that statistically differed from than other treatment in each wild group (WB, $\chi^2 = 14.38$, df = 3, p < 0.01 and WC, $\chi^2 = 8.54$, df = 3, p < 0.05). Post hoc tests (without adjusted p-values) indicated that fish in the WB group that were exposed to salt treatment were characterized by a significantly higher number of taxa when compared to WB fish in the control treatment (salt-CTRL p < 0.01). Post-hoc comparisons indicated that no differences among groups were detected in other treatments. For WC, we found that fish exposed to peroxide had a significantly greater number of taxa relative to fish from the control group (peroxide-CTRL p < 0.05), but not significantly different when compared to the other two treatments.

Associations between gut microbial community composition among fish groups and prophylactic treatments

Principal Coordinate Analyses (PCoA) ordination of differences in microbial taxonomic composition and relative abundance of gut communities was performed to visualize relationships between community composition associated with fish from different families/groups and fish exposed to different chemotherapeutic treatments. Similarities in community membership across samples of similar origin (either from the wild, or from the hatchery production) were evident regardless of treatment groups as denoted by the ordination pattern (Fig. 3.4). Ordination of sample communities associated with treatments overlap considerably (see Fig. 3.4). Fish from eggs collected from the wild (WB and WC) exhibited considerable variation in community composition relative to variation among fish originating from hatchery crosses (HA and HD).

To test if there was any influence of chemical treatment on the gut communities, PERMANOVA was performed. Statistical analyses of beta diversity across samples showed that microbial communities present in the gut differed significantly based on the significant interaction detected between families/groups (HA, HD, WB, WC) and prophylactic treatments, as indicated by PERMANOVA test (Table 3.2: pseudo-F = 1.675, R² = 0.787, p < 0.001). We reject the null hypothesis of no differences in multivariate centroid location.

We next attempted to explain variation in community composition by disentangling the treatment effect of different chemotherapeutics from the pervasive effect of families/groups using linear regression and least square mean analyses. For fish in family HA and wild group WB, their gut communities differed significantly for at least

one treatment (HA, $p < 0.01$; WB, $p < 0.05$) (See Table 3.3). For Family HA, the interaction plot indicated that community membership of fish in the control group differed from fish exposed to the other three chemotherapeuticants ($p < 0.01$; Fig. 3.5a, Table 3.3). Community membership of fish from WB associated with the control ($p < 0.05$) and salt treatments ($p < 0.01$) differed significantly (Fig. 3.5b, Table2).

Identification of bacterial taxa influenced by chemotherapeuticant treatments

Comparison of taxonomic abundance across all treatments and families/group (all-against-all) in LEfSe did not yield any taxa that differed significantly in relative abundance.

We conducted one-against-all comparisons using the control as a reference group to compareo other treatments. Given the interaction between samples (family/group) and prophylactic treatments influencing the gut community compositions as shown by lsmeans analyses (for both family HA and origin WB), we used LEfSe to identify which taxonomic groups that showed the largest differences in relative abundance when fish from the same origin were exposed to treatments.

We first compared communities from fish from the control exposure group from hatchery family, HA to fish exposed to other treatments at the genus level. We likewise compared communities of fish from the control treatment within WB to other groups. We found taxa associated two genera *Methylocystaceae* and *Loktanella* (both from phylum *Alphaproteobacteria*) differed in abundance (LDA score higher than 2.0 or less than -2.0, $p < 0.05$, data not shown) for comparison between fish communities in the control group (CTRL) and peroxide (H_2O_2) fish (see Fig. 3.6a). Higher *Loktanella* were present in HA

fish samples from the CTRL treatment group (mean = 0.060) while *Methylocystaceae* (mean=0.125) was significantly more abundance when exposed to H₂O₂.

LEfSe analyses performed with fish from the wild WB group detected three differentially abundant taxa associated with genus *Loktanella* (Phylum Alphaproteobacteria), *Lysinibacillus* (Phylum Firmicutes), and Unclassified *Opitutae* (Phylum Verrucomicrobia). However, these three genera were present in high abundant only in the gut of fish exposed to salt treatment with LDA score high than 2.0 ($p < 0.05$) (see Fig. 3.6b). Average relative abundance of these genera that present in WB fish individuals were *Loktanella* (mean = 0.027), *Lysinibacillus* (0.008), and Unclassified *Opitutae* (0.027).

DISCUSSION

The presences of healthy, stable, and resilient gut microbial communities are needed for normal functioning of the intestinal immune system and the general resistance of the fish towards pathogens (Ringo et al. 2016) thus can contribute to sustainable and successful production of cultured fish. However, for decades, animals raised in limited space with high population densities for agricultural and aquaculture purposes are more frequently exposed to chemicals and antibiotics which have been widely administered for disease treatments, disease prevention, and to promote growth (Cabello, 2006). Prophylactic treatments usually refer to antibiotic, drugs, or chemicals given to the animals in order to prevent disease, as opposed to therapeutic treatments, which are administered during pathogen outbreaks to treat the disease (Mortazavi, 2014).

In humans, several lines of evidence have confirmed that antibiotic administration can result in gut microbiota dysbiosis (disturbance in composition and function; Francino, 2016) that could increase host susceptibility to disease infection. While more comprehensive studies on adverse effects of antibiotic use to the gut microbiomes were reported in human and other terrestrial animals used in agriculture (pig - Looft et al., 2012; Schokker et al., 2015; ; chicken –Choi, Lee, & Sul, 2015; Schokker et al., 2017), such data are scarce in fish, especially for important aquaculture species. Thus, the present study was conducted to quantify the effects of prophylactic chemotherapeuticants on the gut microbial community composition of hatchery- and naturally-produced lake sturgeon larvae. Using these findings, we could better inform industry professionals how current practices impact fish health and performance in general.

To our knowledge, relatively few studies have been conducted addressing effects of chemotherapeutics on gut microbial communities in general (not focusing on pathogen), mostly in salmonids or tilapia (Austin & Al-Zahrani, 1988; Navarrete et al. 2010; He et al., 2010) and gibel carp (*Carassius auratus gibelito*) (Liu et al., 2012) focusing on effects of antibiotic or drugs that were orally administered to confer internal protection to fish hosts from harmful pathogenic bacteria. Using ecological theory, De Schryver & Vadstein (2014) suggested that the primary compartment where pathogens could be controlled is the water surrounding animals. We conducted studies focusing on the impact of prophylactic treatments administered in the water on fish microbiota.

Fish and microbes live in close proximity within a shared aquatic environment. Microbes respond rapidly to the changes in their immediate aquatic environment and these changes could be subtle and manifested as activation or inactivation of certain metabolic pathways. These changes could also be seen in changes in community composition and functionality (Bentzon-Tilia, Sonnenschein, & Gram, 2016). However, we were surprised to see that prophylactic treatment administered in our study had little influence on the community composition of lake sturgeon gut microbiomes when compared to gut communities of fish from the control group.

We detected three major phyla *Firmicutes*, *Proteobacteria*, and *Actinobacteria*, that dominated the gut community (Figure 3.2a). Results were consistent with findings from another study conducted to evaluate the effects of orally administered antibiotics to gibel carp (Liu et al., 2012). In contrast to the previous study in our facility (Bauman, 2015 unpublished data), lake sturgeon eggs originated from three hatchery-produced families that were prophylactically treated during incubation had an altered microbial

community on the egg surface. Analyses in our study indicated that the microbial communities of fish across family/groups exposed to prophylactic treatments were influenced to a larger extent by family/group (Fig. 3.2 and Fig. 3.3) than by chemotherapeutic treatments. It is possible that the effect of treatment was not evident due to the short treatment duration (15-60 min bath immersion) and periodicity of treatments. Exposure to chemotherapeutic for such a brief period may not have been strong enough to cause large-scale changes in gut community composition. In addition, fish were returned into their tank partition aftertreatments, possibly allowing rapid recolonization of gut microbiota from the surrounding water.

Major taxa that were detected from phyla *Proteobacteria* (such as *Enterobacteriaceae*, *Rhodobacteriaceae*) are Gram-negative bacteria. Many clinical studies have shown that Gram-negative bacteria are resistant to commercially available antibiotics partly due to their thick cell wall structure compared to Gram-positive bacteria (Slama, 2008; Vasoo, Barreto, & Tosh, 2015). *Enterobacteriaceae* include a group of bacteria known as Extended Spectrum Beta-Lactamase (ESBL) *Enterobacteriaceae* that confer resistance to antibiotics via production of the β-lactamase enzyme which can inactivate certain β-lactam antibiotics (Jacoby & Munoz-Price, 2005).

Another major phylum, *Firmicutes* that were detected in fish guts across all families and treatments was primarily represented by Unclassified *Clostridiaceae1* and taxa *Clostridium sensu stricto*. Although *Clostridia* are Gram-positive, these bacteria have been identified as part of commensal gut microbiota that plays major roles in maintenance the gut homeostasis. Several features possessed by *Clostridium* spp. could explain why this taxon can thrive in the gut and can likewise be resistant to prophylactic

treatments administered in our study. In humans, *Clostridium* spp. are involved in defenses inside intestinal microecosystem along with gut-associated lymphoid tissue (GALT), and confer resistance against pathogen infections. This taxon is thought to have immunological tolerance (Lopetuso et al., 2013). In addition, cultured *Clostridium* spp. exhibit the ability to form endospores, which offers this bacteria with ecological advantages for survival under adverse conditions (Gupta & Gao, 2009; Lopetuso et al., 2013)

The influence of family/groups on gut microbial community composition was most notable when comparisons were made based on alpha diversity indices (Fig. 3.3) and visualization of beta diversity among all samples (Fig 3.4). All chemotherapeuticants used in our study are commonly used for a treatment of external pathogens rather than orally administered to fish. Chloramine-T and peroxide are chosen to control and eliminate infection associated with flavobacteriosis (Bowker et al. 2011). PERMANOVA and least square mean revealed that chemotherapeuticant treatments employed in our study have only a minor effect on intestinal gut microbiome in fish, though effect varied among fish with different genotype background associated with families/their sampling origin. Recently, a study by Navarrete et al., (2012) assessed the relative contributions of a host (genetics) and diet in shaping the gut microbiomes of rainbow trout. Full-sib fish from four non-related families were fed two diet regimes in comparison to control group. Results showed that some relative abundance of some bacterial groups differed among trout families, indicating that the host genotypes may influence gut microbiota composition. In addition, the authors reported that the effect of diet on microbiota composition was dependent on the trout family. Studies on other organisms such as

chickens also showed that under a common diet and husbandry practices, gut microbiota composition differed between two lines (High Weight, HW and Low Weight, LW) of chicken with different quantitative genotypes indicated a prominent influence of host genotype on community composition (Zhao et al., 2013).

Fish from artificially produced family HA experienced greater community alteration compared than fish produced from wild eggs (Fig. 3.5a). Gut microbial communities in wild fish possibly exhibited greater resilience to treatments and maintained their gut compositional similarity. In contrast for fish hatchery, they originated from eggs that have been artificially produced in enclosed facilities; therefore, they had no contact with their respective natural habitat like the wild eggs, except their egg surfaces reflect aquatic communities where their parents spawned. This could also suggest that domestication selection in terms of hatchery gut community establishment occurs in fish that were produced in the hatchery, affecting the community structure of their gut microbiome. Findings from Blekhman et al. (2015) indicate that human gut microbial variation are driven by host genetic variation involving genes that have been previously associated with microbiome-related complex diseases. They also showed that host genomic regions associated with microbiomes have high levels of genetic differentiation among human populations, suggesting that host-genomic adaptation to environment-specific microbiomes. This could be possibly true with fish as well where variation in gut microbiome attributed to their genetic background and populations differences.

In a companion study, Bauman (2015) quantified proportions of fish surviving each of four prophylactic treatments. Fish exposed to salt followed by peroxide 24 hr

later were shown to have higher survival than other groups (Bauman et al. 2015).

Mortalities were observed throughout the course of the experiment. It is possible that gut microbial communities of dead fish may have differed from fish surviving at the end of experiment following the full course of the chemical treatment. However, since the dead fish were not preserved and included in the studies, we have no empirical way to evaluate this effect. Navarrete et al., (2008) reported that gut microbiomes of salmonids exposed to Oxytetracycline (OTC) that were orally receiving antibiotic were characterized by less diversity and were only composed of *Aeromonas*, clustering with *A. sobria* and *A. salmonicida*. *A. salmonicida* has been known as a pathogenic bacteria that could result in huge mortalities during the disease outbreak.

In the LEfSe analyses, only five out of thousands microbial taxa detected that were differentially abundant after fish were exposed to chemical treatments. Those taxa, however, are not among the dominant taxa. Thus it is unclear how treatment differentially affected the relative abundance of these taxa. Results could provide indicate that the gut microbiota were either resistant or exhibited resilience in community composition, where treatment-based changes were short-lived and communities rapidly returned to their original state (Christian et al., 2015). The communities could also have had different compositional taxonomy, yet were still able to maintain function (functional redundancy). Another study by Navarrete et al (2010) focused on determining effects of dietary inclusion of *Thymus vulgaris* essential oil (TVEO) on microbiota composition, compared with a control diet without TVEO over 5 week period. Their study indicated high similarities between gut microbiota in treated and non-treated fish, and TVEO induced negligible changes in gut microbiota profiles. Essential oils include volatile liquid

fractions produced by plants that contain the substances usually responsible for defenses against pathogens and pests due to their antibacterial, antiviral, antifungal, and insecticidal activities (Romero, Feijoó, & Navarrete, 2012). We conclude that gut microbiota composition was persistent and stable throughout the trial, producing temporally consistent molecular profiles. We documented the same outcome. The effect of chemical treatment with regards to LEfSe result (Fig. 3.6a, 3.6b), it is possible that the mode of action of each chemotherapeuticant may explain the detection of biomarker taxa by LEfSe. Salt treatment resulted in “shedding” the mucosal layers, and when followed by peroxide, this prophylactic treatment could potentially remove certain bacterial taxa underneath the mucosal layer.

Overall, our results suggest that treatments during larval stages do not result in large changes in the composition of the intestinal microbiota, at least during the short observation and experimental duration. It is possible that the effects of treatment on gut communities were delayed until fish reach a later stage. Treatment was utilized to control disease outbreaks in the hatchery, hence a prerequisite for developing a strategy for microbial control, is knowledge of aquatic microflora associated with fish larvae, and how interactions between larvae and microflora occur.

Our study serves as a baseline providing information on the indirect effects of chemotherapeuticant intervention that could either positively or negatively affect the normal gut microbiota or facilitate the proliferation of opportunistic pathogens. Further studies should be carried out comparing the effects of treatments before and after chemotherapeuticant are applied, focusing on the prevalence of potentially pathogenic bacteria. Prophylactic effects of chemotherapeuticants could also be performed coupled

with bacterial additives (e.g., probiotics) or challenges to document beneficial or adversarial effects of these treatments on the gut microbiome composition.

APPENDIX

APPENDIX

Table 3.1 Models generated with both fixed and random variables to explain variation among samples of all treatments and families/group (a) for Inverse Simpson diversity indices; (b) for a number of observed taxa (OTU richness).
 The model with the lowest AIC scores was shown in bold.

Table 3.1 (a)

	Residual df	AIC	BIC	Log- likelihood	Residual Deviance	χ^2	p-value
$Y_{inv} \sim (1 Family)$	3	917.20	926.11	-455.60	911.20		
$Y_{inv} \sim Trt + (1 Family)$	6	920.88	938.70	-454.44	908.88	2.315	0.510
$Y_{inv} \sim Trt + (1 Family) + (1 Trt :Family)$	7	922.88	943.67	-454.44	908.88	0.000	1.000

Table 3.1 (b)

	Residual df	AIC	BIC	Log- likelihood	Residual Deviance	χ^2	p-value
$Y_{sobs} \sim (1 Family)$	3	1905.1	1914.0	-949.55	1899.1		
$Y_{sobs} \sim Trt + (1 Family)$	6	1893.4	1911.2	-940.70	1881.4	17.711	0.0005***
$Y_{sobs} \sim Trt + (1 Family) + (1 Trt :Family)$	7	1895.4	1916.2	-940.70	1916.2	0.000	1.000

Table 3.2 PERMANOVA showing variability among fish gut microbiota across all samples. Results revealed a significant interaction of both treatments (D) and family effect (F) influencing gut microbial communities composition for at least one samples across treatments and families/group (PERMANOVA test pseudo-F = 1.675, R^2 = 0.093, $p < 0.001$; permutation=1000).

	Df	Sum Sq	Mean Sq	F-model	R ²	Pr (>F)
Treatment (T)	3	0.870	0.290	1.435	0.026	0.098
Family (F)	3	3.097	1.032	5.109	0.094	P<0.001***
T X F	9	3.046	0.338	1.675	0.093	P<0.001***
Residuals	128	25.866	0.202		0.787	
TOTALS	143	32.880			1.000	

Table 3.3 Goodness of fit from linear regression model (R^2), p-value, and Least-square means analyses performed on significantly important PCoA axes calculated separately for each family/group. (a) PCoA Axis 2 associated with second largest eigenvalue for fish from family HA show that microbial community composition in control group significantly differed than other treatments; (b) PCoA Axis 6 associated with sixth largest eigenvalue for fish from group WB show that microbial community composition in both control and salt fish significantly influence by each respective treatment

Linear regression model : Pco Axes ~ Drug treatments							
Important axes	Signif. Treatment	p-value	R2 value	Least-square means			
				Control	Chloramine-T	Peroxide	Salt
Family HA							
Pco Ax.2	Control (No drug)	0.008	0.138	-0.153±0.07	0.117±0.07	0.034±0.07	0.002±0.07
Family WB	Control (No drug)	0.012	0.2425				
Pco Ax.6	Salt	0.001	0.2425	0.036±0.03	-0.081±0.03	-0.023±0.03	0.077±0.03

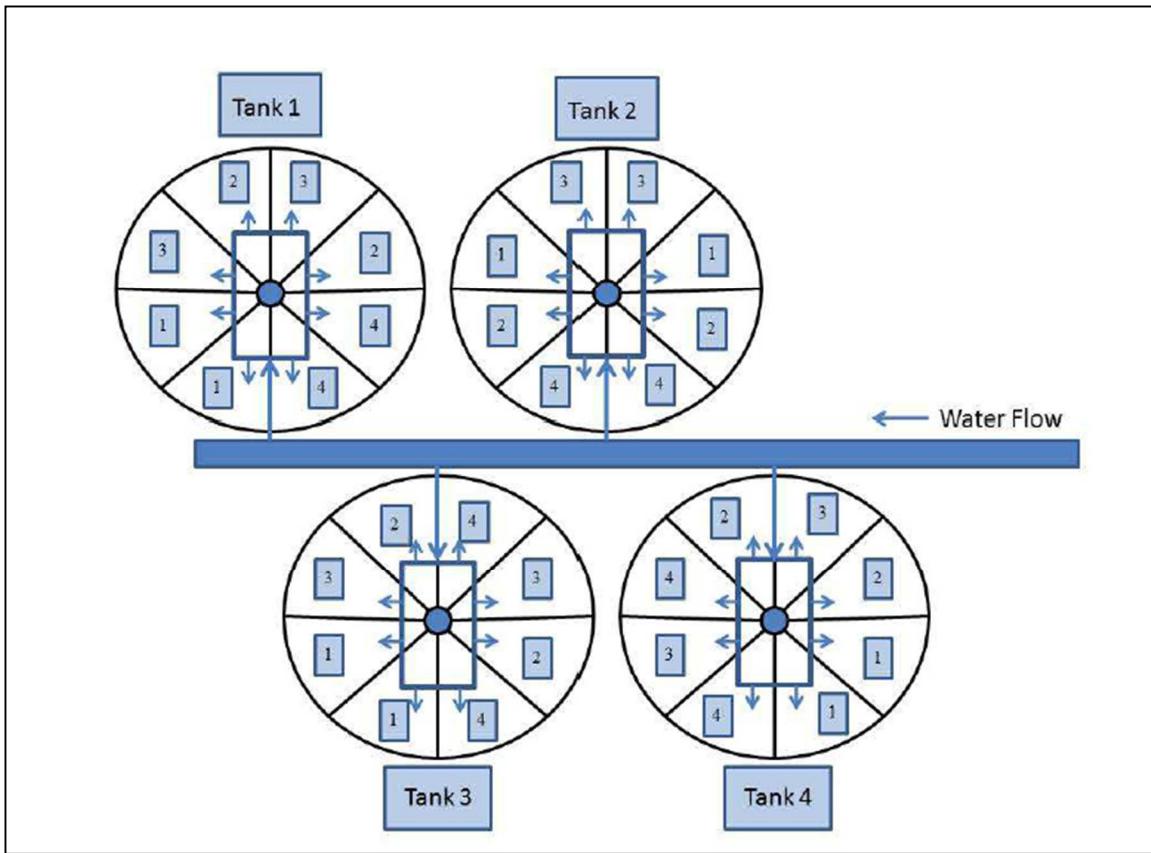


Figure 3.1 Schematic design of the larval chemotherapeutic study. Each 1.2m diameter of tank held 400 fish from hatchery and wild naturally produced fish, which were divided into eight equal sized partitions (50 fish per partition). There was four tanks. Each partition was randomly assigned to one of four weekly treatment types, each with two replicates. Chemotherapeutic treatments included: 1) 60 min, 15 ppm CT bath, 2) 15 min, 60 ppm H₂O₂, 3) 3 parts per thousand (ppt) NaCl-bath for 15 min followed 24 hr later by a 15 min, 60 ppm H₂O₂ bath labeled as NaCl/ H₂O₂, and 4) a control (no chemical treatment) labeled as CTRL. Arrows indicate directions of water flow. The figure was originally from Bauman 2015 and is used here with permission.

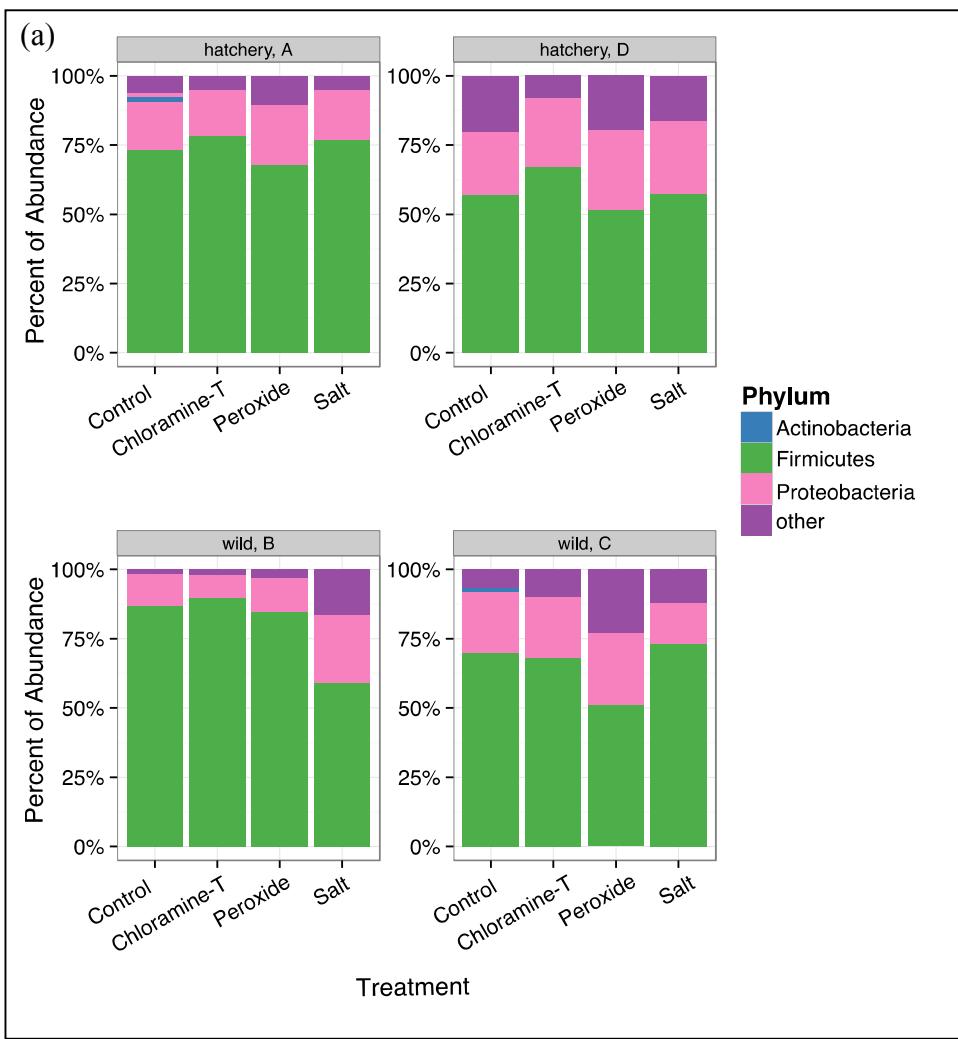


Figure 3.2 Taxonomic composition of bacterial communities identified from the lake sturgeon larval GI tracts (a) at the phyla level and (b) at the genera level.

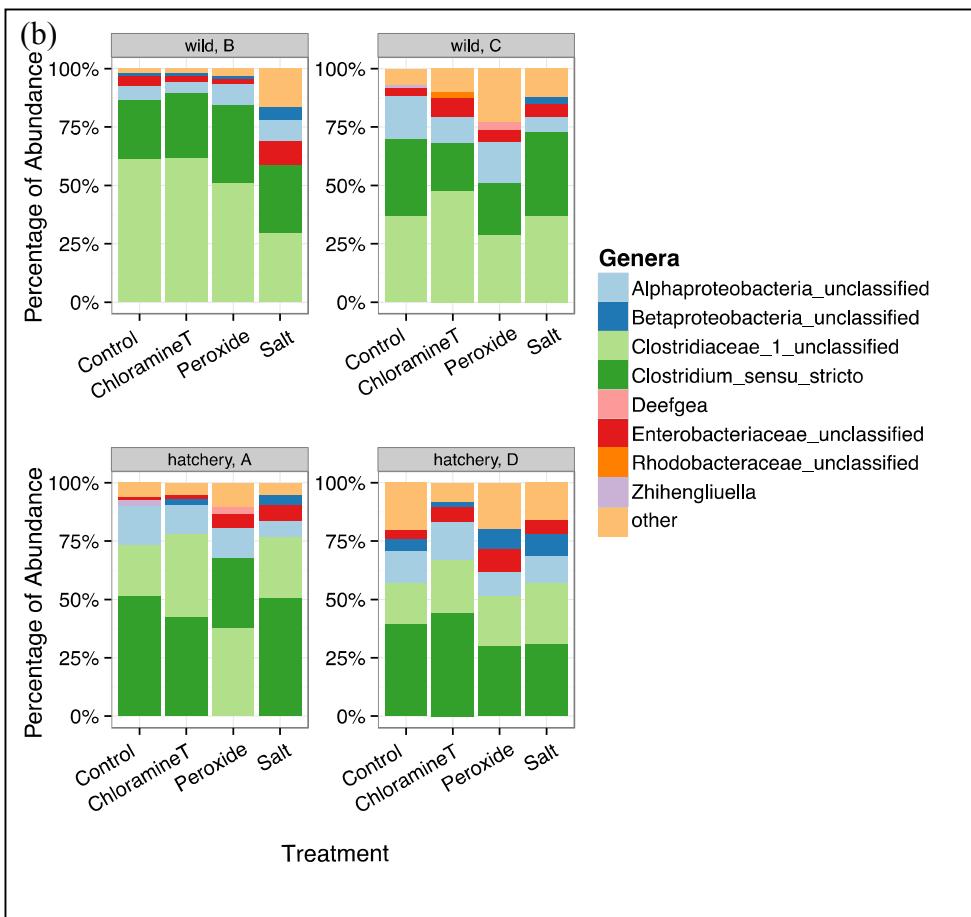


Figure 3.2 (cont'd)

(a) Relative abundance (percentage) of dominant bacterial phyla found in the gut microbiota of lake sturgeon larvae separated based on sample family/group to display variation in communities across prophylactic treatments. Three predominant phyla were present in gut microbial communities (*Firmicutes*, *Proteobacteria*, *Actinobacteria*). The other phyla were characterized as Others. (b) Relative abundance (percentage) of dominant bacterial taxa found in fish gut samples, separated by family/group and treatment. Among the most abundant taxa included *Unclassified Alphaproteobacteria*, *Unclassified Betaproteobacteria*, *Unclassified Clostridiaceae_1*, *Clostridium_sensu_stricto*, and *Unclassified Enterobacteriaceae*.

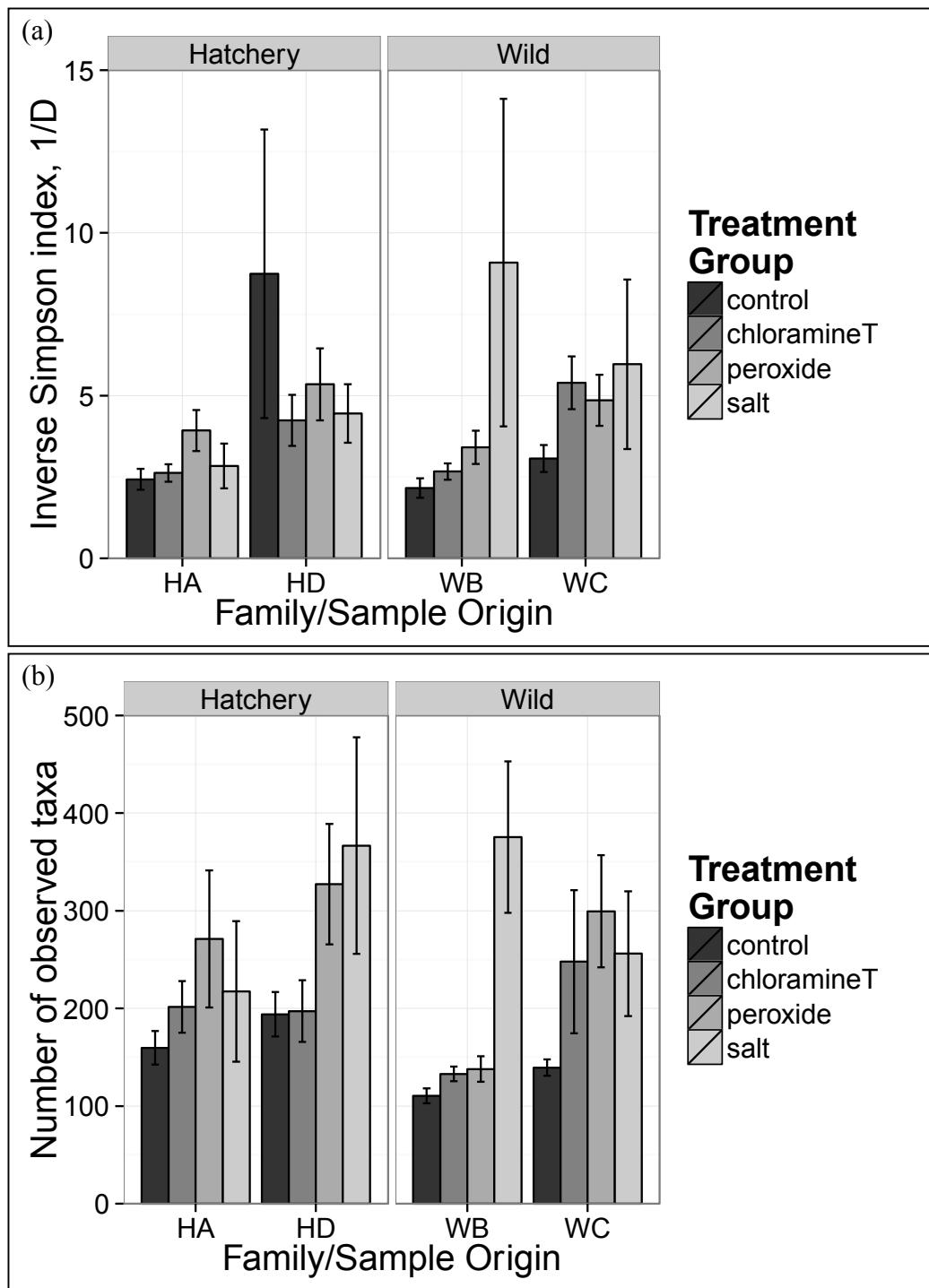


Figure 3.3 Estimates of alpha diversity (a) Inverse Simpson index; (b) number of observed taxa (OTU richness) for lake sturgeon gut microbial communities from all samples of treatments and families/groups. Each bar indicates mean with S.E. for each treatment from each family/group.

Figure 3.3 (cont'd)

Kruskal-Wallis followed by post-hoc test indicate that (i) none of families/treatments differed for Inverse Simpson indices; (ii) for WB, fish that were exposed to salt treatment have a significantly higher richness than fish from control group (salt-CTRL $p < 0.01$); for WC, fish exposed to peroxide have a significantly higher richness to control group (peroxide-CTRL $p < 0.05$).

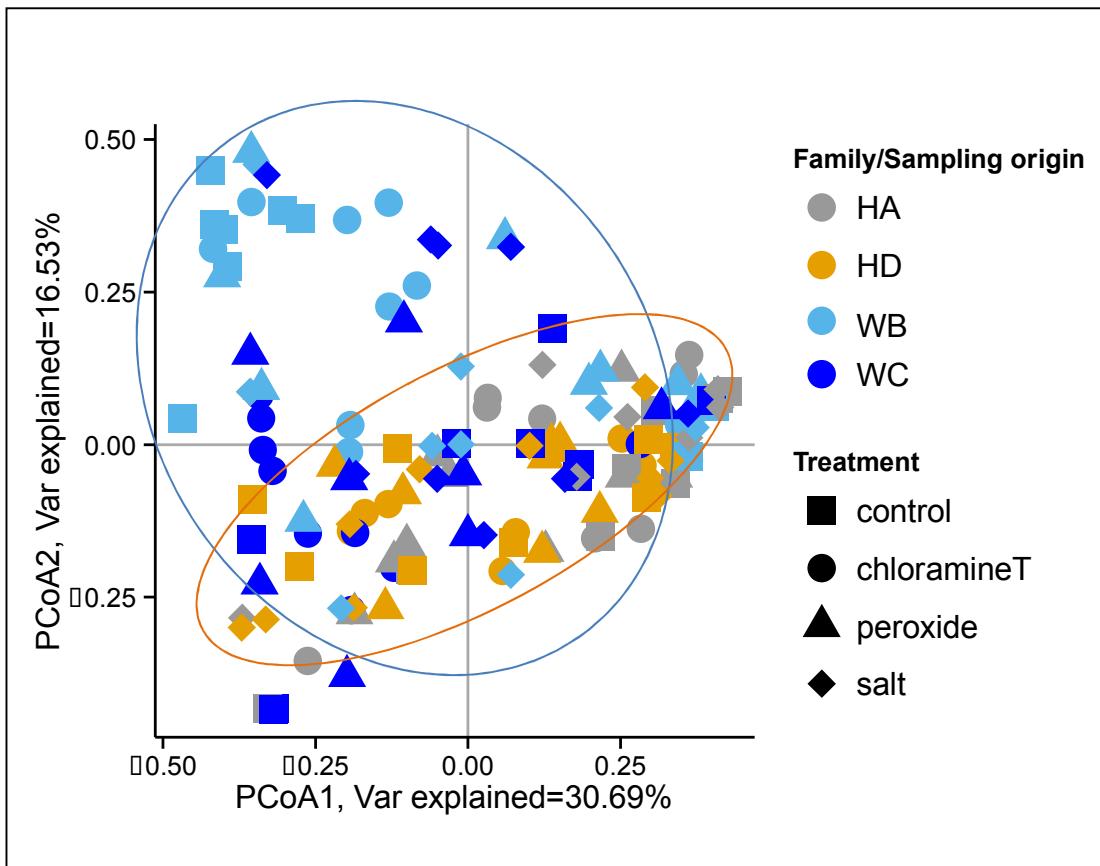


Figure 3.4 Visual representation of differences in the gut microbiota of lake sturgeon larvae (Bray-Curtis distances). Bray-Curtis dissimilarity distances between communities from fish exposed to four chemotherapeuticant across families/group were visualized by Principal Coordinates Analysis (PCoA) plots. Points represent each gut microbial community from each individual fish samples from treatments and families/groups ordinated in multivariate dimensional space using the first and second axis corresponding to the first and second largest eigenvalues.

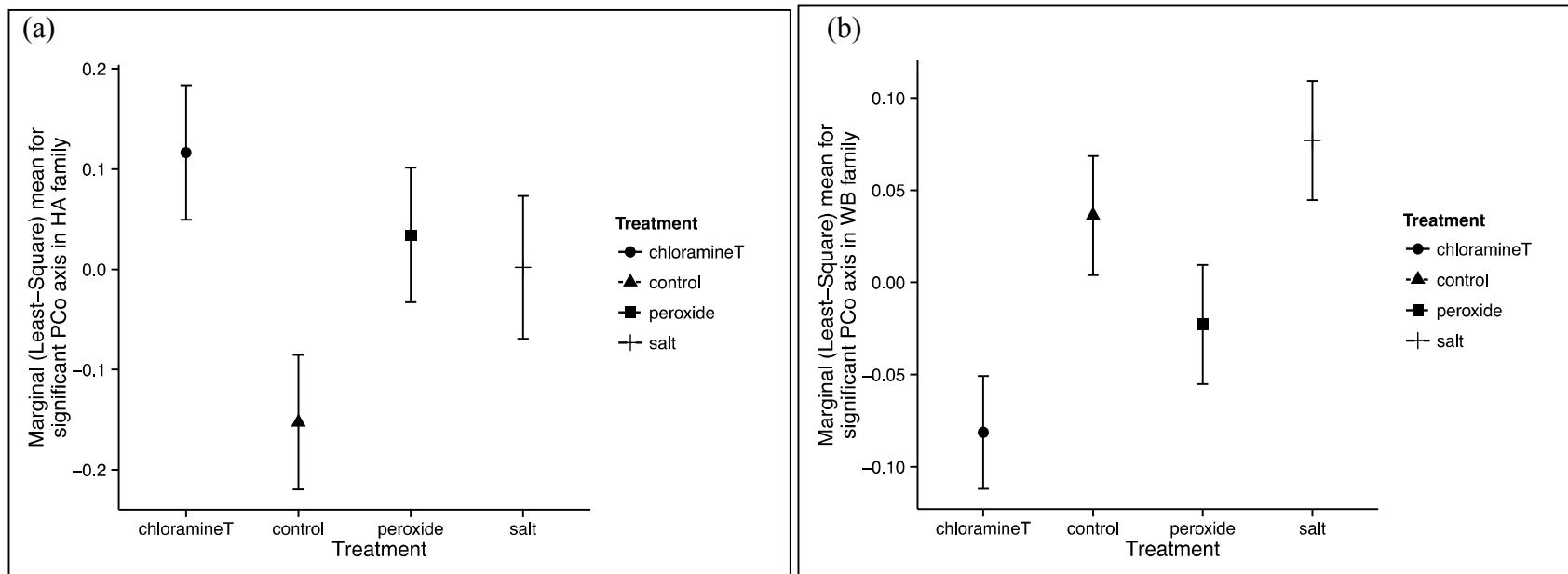


Figure 3.5 Interaction plot of marginal (least-square, LS) means for significant PCoA axes detected from hatchery family (HA) and wild group B (WB). Axes represent variation in taxonomic diversity and relative abundance for a given family/group. (a) A significant difference exists between microbial communities composition of HA fish in control group compared to fish that received other prophylactic treatments; (b) Significant difference exists in the gut microbiota of fish from WB associated with different prophylactic treatments. See details in Table 3.3.

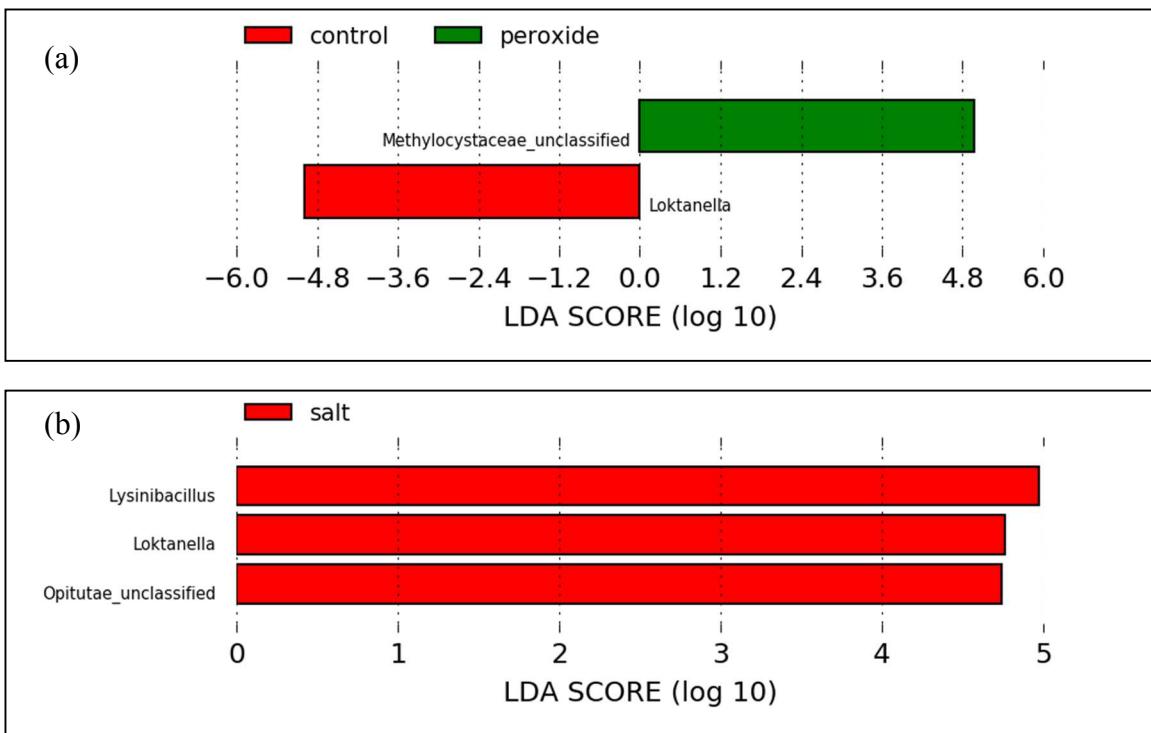


Figure 3.6 LEfSe-detected taxa from one-to-all comparison (control to all other three chemotherapeuticant). (a) Thresholds on the logarithmic LDA scores for discriminative features (taxa) in fish from family HA show that only two taxa were differentially abundant in a comparison between CTRL and hydrogen peroxide treatment. (b) Thresholds on the logarithmic LDA scores for discriminative features (taxa) in fish from group WB detected that salt contain three taxa that were differentially abundant compared to control group

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CHAPTER 4: BIOTIC AND ABIOTIC FACTORS INFLUENCING CHANNEL CATFISH EGGS AND GUT MICROBIOME DYNAMICS DURING EARLY LIFE STAGES

ABSTRACT

This study quantifies the effects of rearing environments associated with pond aquaculture on microbial community composition in larval Channel Catfish (*Ictalurus punctatus*) gastrointestinal (GI) tract across early developmental stages. Larvae were exposed to hatchery water and nursery pond water environments and were sampled at five developmental stages (Egg, Swim-up, Stocked, 24 hr post-stocking in nursery ponds, and 21 d post-nursery pond stocking). Massive-parallel sequencing of a segment of the 16S rRNA gene was using an Illumina MiSeq platform to characterize the diversity and taxonomic composition of gut and water microbial communities. PERMANOVA analyses of differences in egg/gut community composition across stages indicated there was a significant interaction between nursery pond environment, and family ($p < 0.05$) during the last two stages. Several unclassified *Proteobacteria* and *Firmicutes* genera along with other opportunistic pathogens like *Vibrio*, *Aeromonas*, *Flavobacterium* were associated with differences in gut bacterial communities during larval development before and after pond stocking. Our study demonstrate that rearing environment is an important factor influencing the transfer of microbes from water or food into the gut, and provides insight concerning the niche and ecological adaptability of microbes inside the catfish gut. Findings suggest managers may have difficulty adopting probiotic treatments to enhance gut microbiota in channel catfish aquaculture without appropriate pond microbial management during early life stages.

INTRODUCTION

In the United States, channel catfish (*Ictalurus punctatus*; Rafinesque, 1818) and channel x blue (*Ictalurus furcatus*; Valenciennes, 1840) hybrid catfish aquaculture are the largest food fish commodities, listed at 162 million kg production in 2013 with an estimated value of \$354 million dollars (USDA NASS, 2013). In the past decades, catfish farming has intensified to meet consumer demands. As a result, aquaculturists desire more efficient land use and increased production in inland or pond aquaculture settings. These improvements are essential to achieve successful and profitable catfish production (Avery & Steeby, 2004; Hargreaves & Tucker, 2004). Part of these strategies involves research associated with (but not limited to) feed efficiencies (Li, Manning, & Robinson, 2004; Li et al., 2008), increased growth (Silverstein et al., 2000), water quality (Tucker & Hargreaves, 2004) and enhanced disease resistance (Declercq et al., 2013; Park, Aoki, & Jung, 2012).

Features of intensive fish cultivation such as grading, handling, transportation, as well as poor water quality, may create conditions that are stressful to fish but favorable for pathogenic microbes to thrive, thereby increasing the risk of disease (Derome et al. 2016, De Schryver & Vadstein, 2014; Skjermo & Vadstein, 1999). Catfish aquaculture managers have emphasized creation and maintenance of proper living environments for fish, including the design of facilities and operational production methods to minimize the impacts of stress. Proper living environments include high-quality water within tolerance limits of parameters such as temperature, dissolve oxygen; as well as stocking density and feeding rates that is conducive to maximum performance (i.e., best survival growth and feed conversion) (Tucker, Avery, & Heikes, 2004).

However, even in well-designed and properly managed facilities, disease outbreaks still occur, with approximately 45% of inventory losses attributed to infectious disease (Hawke & Khoo, 2004). The complex interactions between fish and microbes (Tucker & Hargreaves, 2004) during disease outbreaks are largely attributed to dysbiosis; a phenomena associated with perturbations and imbalance in commensal microbial communities present on hosts (Llewellyn et al., 2014; Karlsson et al. 2013). Disturbance of natural microbial communities could happen due to acquisition of microbes from shared, poor quality-water environment, or due to any environmental stressor that could trigger negative physiological responses in fish.

In catfish aquaculture, the pond environment is usually characterized by a high level of organic material. Biological activity in catfish ponds is stimulated by nutrient loading derived from feeding (Tucker & Hargreaves, 2004). While this material is a necessary component of catfish husbandry (by encouraging phytoplankton blooms which produce oxygen), materials also support and can accelerate microbial growth. Fish feces and unconsumed feed are important parameters that have to be carefully monitored especially in recirculating water/ pond aquaculture systems. Organics materials can form suspended particles that support growth of heterotrophic bacteria (Martins et al., 2013; Skjermo & Vadstein, 1999). Physiochemical properties of water such as pH, salinity, and dissolved organic carbon concentration can be adversely affected by many factors such as weather, and as a result may trigger the growth pathogenic microorganisms (Martins et al. 2013). The increasing number of harmful microbes could eventually overcome skin or gill protective mechanisms, invading fish internal and vital organs, subsequently causing disease outbreaks.

Next-generation sequencing technologies combined with advanced bioinformatics capabilities have provided opportunities to expand studies of complex microbial communities residing in fish hosts and in aquatic environments, resulting in great potential for downstream applications in aquaculture management. Importantly, recent studies have begun to focus on the importance of gut microbial communities (microbiome/microbiota) in maintaining normal host functions (reviews in Ghanbari, Kneifel, & Domig, 2015; Llewellyn et al., 2014; Nayak, 2010; Ringø et al., 2015). Recognizing the critical roles played by gut microbiota has facilitated development of probiotic applications in efforts to improve feed efficiency, expedite growth, and increase disease resistance (Merrifield et al., 2010; Verschueren et al., 2000) in aquaculture settings. However, an important first step in evaluating the potential of probiotics in catfish aquaculture is to establish the role of genetics (family effects), husbandry practices, and the environment on gut microbial community diversity and composition, especially in pond based aquaculture as employed by the catfish industry in the southeastern United States.

Previous studies have characterized the composition of microbiota associated with several catfish species including Yellow Catfish (*Pelteobagrus fulvidraco*), Vietnamese striped Catfish (*Pangasianodon hypophthalmus* Sauvage), and Amazonian freshwater Catfish (Filhote, *Brachyplatystoma filamentosum* and Dourada (*Brachyplatystoma rousseauxii*) (Damasceno et al., 2016; Tong Thi et al., 2016; Wu et al., 2010, 2012), focusing mostly on descriptive characterizations of microbial community composition. Moreover, these studies were based on culture-based methods, or used molecular-based methods for a specific stage of development.

Catfish producers are continually exploring production practices that will increase feed conversion efficiency and growth. While the administration of pre- and probiotics to beneficially manipulate the fish microbiomes is thought to improve growth and feed efficiency in several fish species, probiotic applications in catfish culture are limited. Moreover, information regarding the gut microbiota of catfish species and the factors influencing them is limited (Carnevali et al. 2014). To maximize the potential of beneficial use of microbes in catfish aquaculture, the dynamics driving catfish gut microbial community development and ontogenetic successional changes needs to be established, especially during early life stages.

To our knowledge, only two studies have used molecular approaches to interrogate channel catfish microbiomes, providing foundational work to molecularly defined-channel catfish microbiota (Bledsoe et al., 2016; Larsen, Mohammed, & Arias, 2014). Production of catfish relies heavily on pond aquaculture. Given the likelihood of compositionally differentiated microbial communities in different catfish production ponds, the stability of the gut microbiome in resident fish populations raised in different ponds needs to be determined. One fundamental but unresolved question is do catfish populations raised in different environments possess the same gut microbial compositions, or do gut microbial communities differ as a function of pond residency? This is especially important when considering the role of probiotics, as catfish gut microbial communities at some life stages and in particular pond environments may be more conducive to probiotic manipulation than others.

In this study, our general objective was to characterize the taxonomic composition and diversity of the gastrointestinal (GI) tract microbial community (microbiota) in

channel catfish across sequential ontogenetic stages from fertilized and incubating eggs until 21 days following stocking into nursery ponds. In addition, comparisons of microbial community composition and diversity were made from water from the hatchery as well as from rearing pond. The study was designed to quantify associations between host genotype (family/brood) and the rearing water supply, focusing specifically on the effects of transitions from the hatchery to the nursery ponds on the gut microbial community composition and diversity. Analyses quantified whether changes in gut microbiota were influenced by family and/or by nursery pond. Our findings have general implications for the management of fish nutrition, disease control, and probiotic use in catfish pond aquaculture. Data will further reveal environmental or deterministic changes during early ontogenetic stages and dynamic relationships between hosts and environmental epibionta to which they are exposed.

METHODOLOGY

Fish husbandry and spawn collection

Channel catfish samples (eggs and fish) in this study originated from mated pairs from spawning events in two outdoor 0.1-acre brood ponds (Ponds 147 and 154) on the same day on May 29th, 2015. Brood ponds had been stocked with 60, three year old channel catfish (1:1 female to male ratio) and maintained according to standard industry practices (Tucker and Robinson 1990). Two discrete spawns/families were collected from each brood pond. Upon collection, individual egg masses from spawns were treated with 100 ppm Povidone-iodine solution prior to transfer into separate tanks in the hatchery. Treated eggs were maintained in discrete hatching tanks, supplied with flow-through well water (~27°C; ~3.5 L/min) and constant aeration.

Upon hatching (June 5th, 2015), yolk sac fry were retained in the same hatching tank and began exogenous feeding approximately 4 days post-hatch. Larvae were fed trout starter diet (Rangen, Buhl, Idaho) until transfer to the nursery ponds at 19 days post-hatch (dph). For each spawn, fry were stocked in net-pens (100 fish/pen), and placed in two 0.1-acre nursery ponds. Nursery ponds were prepared according to standard fertilization and pond preparation protocols (Mischke 2012). The schematic design for this experiment is shown in Figure 4.1. Every two weeks, water samples were collected and analyzed for chlorophyll *a*, ammonia-N, nitrite-N, and pH. Chlorophyll *a* was determined using chloroform-methanol extraction (Lloyd and Tucker 1988). Ammonia (Nesslerization) and nitrite (diazotization) were determined following the methods outlined by the Hach Company (2013). Zooplankton and phytoplankton were assessed as described previously (Tucker et al. 2017). In line with industry practices, feed was

withheld while fish were in net-pens, to encourage foraging on naturally occurring zooplankton communities. The experiment ended 21-days post-stocking (~40 dph). Respective spawns are referenced herein according to the corresponding brood identifiers (147A, 147B and 154A, 154B).

Genomic DNA was isolated from 8 fry from each spawn and 9 microsatellite loci were genotyped according to established methods (Waldbieser and Bosworth, 2103). Parentage analysis using Cervus 3.0 (Kalinowski et al., 2007) revealed all fry from each tank were a full-sib family belonging to one sire and one dam, and no parents were shared between spawns. All fish husbandry was conducted at the Thad Cochran National Warmwater Aquaculture Center in Stoneville, MS.

Sample collection

Fish were collected during five consecutive ontogenetic developmental stages, denoted as *Egg* (fertilized egg samples in brood cans), *Swim-up* (fry collected during swim-up stage in the hatchery, aged 4dph), *Stocking* (fry collected at stage in the hatchery just prior to pond-stocking, aged 19dph), *NP 24hr/Pond 24hr* (fry collected 24hr after stocking into nursery ponds, aged 20dph), and *NP 21d/Pond 21d* (fry collected 21d after stocking into nursery ponds, aged 40 dph). Fish were euthanized using an overdose of MS-222 (250mg/L) and samples were transferred into 50 ml centrifuge tubes containing 95% ethanol.

For water samples, 40 ml of pond water were collected and concentrated via centrifugation (20,000xg) to obtain the pellet, which was resuspended in 40 ml of 95% ethanol. Water samples were first obtained during egg incubation the same day eggs were collected (denoted as *Incubation*), followed by sampling after egg hatch at the sac-fry

stage (*Hatch*). The remaining water samples were collected the same time fish were sampled (designated as *Swim-up*, *Stocking*, *NP 24hr/Pond 24hr*, *NP 21d/Pond 21d*). All fish and water samples were stored at ambient temperatures until dissection and bacterial DNA extraction was performed.

Fish dissection

The intestine of each larval catfish was recovered from individual fish using aseptic techniques. Exterior surfaces were swabbed with 100% ethanol before dissections of the whole digestive tract using sterile instruments. The dissection were performed with slight modification as previously described by (Milligan-Myhre et al., 2011). The intact GI tracts were cut out from the fish body cavities, and the excised gut was immediately transferred into filtered-sterilized 80% ethanol solution for DNA isolation. Due to the small size of the gut, the composite of at least four tissue samples formed each technical replicate, for each spawn within each brood pond (and from particular pond – if applicable) at each time point. In addition to fish collected for molecular analysis, additional fish (n=10) from the *NP 24hr/Pond 24hr* and *NP 21d/Pond 21d* samples were subjected to gut content analysis following previously established protocols (Mischke et al. 2003).

DNA extraction and 16S rRNA amplicon sequencing

Gut microbiota from channel catfish larvae were surveyed using high throughput sequencing of the 16S rRNA gene. Each tube containing gut samples was first centrifuged for 15min at 4°C to pellet tissues and bacteria before DNA was extracted. The MoBio PowerSoil® DNA Isolation Kit (Carlsbad, CA, USA) including a bead-beating step was used following protocols for low-biomass samples as suggested by the

manufacturer with slight modification. The integrity of each DNA sample was assessed based on the amplification of an approximately 1.4k bp of 16S rRNA gene (27F and 1389R) followed by gel agarose electrophoresis and DNA concentration were quantified using Microplate spectrophotometer (BioTek®, Winooski, VT, USA).

Ninety-eight DNA samples (including two control samples) that have been validated to contain sufficient bacterial DNA (as shown by the presence of amplicon bands in electrophoresis) were subsequently submitted for sequencing at the Michigan State University Research Technology Support Facility, RTSF (East Lansing, MI, USA). All sequencing procedures, including the construction of Illumina sequencing libraries, emulsion PCR, and MiSeq paired-end sequencing v2 platforms of the V4 region (~250bp; primer 515F and 806R) followed standard Illumina (San Diego, CA, USA) protocols. Michigan State's Genomics RTSF (<https://rtsf.natsci.msu.edu/genomics/>) provided standard Illumina quality control, including base calling by Illumina Real Time Analysis v1.18.61, demultiplexing, adaptor and barcode removal, and RTA conversion to FastQ format by Illumina Bcl2Fastq v1.8.4.

Sequence processing

Details pertaining to the sequence data analyses pipeline and computing workflows were described as follows. Briefly, paired-end sequence merging, quality filtering, “denoising”, singleton-sequence removal, chimera checking, taxonomic assignments and Operational Taxonomic Unit (OTU) selection was conducted using an open-source workflow based on methods implemented by program *mothur* v.1.36.1 (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013). A reference-based OTU clustering and taxonomic assignment was performed using the SILVA-based bacterial

reference database file provided in *mothur* to cluster sequences defined with 97% identity. To minimize effects of under sampling while maintaining a broad dataset, the final OTU table was rarefied to a depth of 2848 sequences per sample. Rarefaction analyses were performed to equalize sampling coverage for all samples based on the selected sequence depth. Three DNA samples with low sequence depth were discarded in downstream analyses.

Analyses of bacterial community profiles and ecological statistics analyses

4.1 Alpha diversity

All measures of community diversity and similarity including Inverse Simpson (1/D) diversity indices and OTUs richness within samples (alpha [α] diversity) were calculated from the sequence data within *mothur*. To test which predictor variables were important to explain the variation in alpha diversity indices, we fit different regression models tested how the response variables (Inverse Simpson index and OTUs richness) were affected by family, brood pond, and stages depending on the rearing location (brood ponds, hatchery, nursery ponds).

To test for significant differences in diversity indices of egg samples between brood ponds, samples were analyzed using a one-way ANOVA. To Analyze compositional differences in fish gut samples at both swim-up and stocking stages from different brood ponds, nested ANOVA analyses were performed on both Inverse Simpson diversity index and taxa richness (family nested within brood pond). We further tested whether pond effects were associated with variation in fish gut profiles based on a three-way ANOVA. This analysis tested for significant differences in microbial community diversity indices of fish gut samples between spawns from different stocking

ponds across stages, using only samples from the last two stages (after 24 hr and 21 d post nursery pond stocking). The p-values less than 0.05 indicate significant differences between pairwise comparisons. All analyses were performed based on information in the summary files provided by *mothur* using the programming and statistical software, R (version 0.98.978) base package.

4.2 Temporal and Differential abundance of OTUs

Custom R code written and implemented using packages *plyr*, *dplyr* and *reshape2* was used to calculate the relative abundance and determine identity of dominant taxonomic phyla and taxa in all communities across sampling times (fish gut and water-associated microbial communities). The relative abundance of all taxa within community samples was calculated, and abundance at or exceeding 1% were considered dominant taxa present across all OTUs. OTU relative abundances are displayed to show temporal variability in fish gut community structure. Remaining taxa are grouped as ‘Others’.

4.3 Beta diversity

We used several packages implemented in R to perform comparative (beta [β]) diversity analyses and ecological statistics on the OTU dataset. Briefly, we used *vegan* functions to generate Bray-Curtis (BC) resemblance matrices (Oksanen, 2015) followed by the *cmdscale* function to perform Principle Coordinate analyses (PCoA) based on BC distance (Bray & Curtis, 1957). The *ggplot* and *ggplots2* package (Wickham, 2009) were then used to create ordination plots to visually compare gut bacterial community composition and aquatic community composition as a function of different spawns and ponds environment across sampling periods based on the three largest eigenvalues.

Multivariate hypotheses testing were conducted using the *adonis* function. We performed

multivariate analyses on the difference between locations of the centroids of each group based on Permutational Multivariate Analyses of Variance (PERMANOVA) on resemblance matrices (Anderson, 2001; Anderson et al., 2013). Tests were employed because of the non-parametric and skewed nature of microbial community data.

4.4 Influence of spawn, pond effects on intestinal microbiota

To further analyze associations between spawn (families), brood ponds and nursery pond (water) effects with fish egg/ gut microbiota, PERMANOVA analyses were performed separately on Bray-Curtis distance matrices of fish associated microbial communities based on developmental stages and/or rearing location (hatchery vs nursery pond) (Fig. 4.1). At the egg stage, comparisons were made between eggs from different brood ponds using family/spawn as replicate due to lack of biological replication. Under the null hypothesis, we did not expect groupings in egg community composition to differ significantly based on predictor variables (e.g., brood pond).

For fry that were sampled during the next two developmental stages (swim-up and stocking), their gut community compositions were analyzed in PERMANOVA based on a nested analysis (family nested within brood ponds). A nested analysis was conducted because fish that were produced from different mated pairs (spawns or families) but from the same brood ponds were individually maintained in the same tanks inside the hatchery. However, these fish were not exposed to the water from another tank that contained the other two spawns produced in another brood pond. PERMANOVA analyses were performed to test for significant groupings (centroid location) of gut microbial community compositions between fish from different brood ponds (spawns) for each of two developmental stages (swim-up and stocking).

After a brief rearing period in the hatchery, fish from each family were placed in each 0.1 acre nursery pond. We conducted PERMANOVA analyses assuming under the null hypothesis that gut community composition at each time point (after 24 hr and 21 d post stocking), would not differ significantly among families, between brood ponds, and nursery ponds. Whenever analyses revealed a pervasive influence of sampling time on community composition, we performed a PCoA on the fish community for separate stages using predictor variables based on ANOVA analysis to determine significant differences between fish egg/gut communities attributed to each predictor variable.

4.5 Contribution of predominant taxa to sample communities' composition

Contributions of individual taxa to whole-community profiles were represented using a heatmap, constructed using the *heatmap* function from the *Heatplus* library in R. The relative abundance of each taxa present in each sample (fish gut and water) was calculated, and taxa whose relative abundance was less than 1% of at least one sample were removed. The color scheme indicates the relative abundance of each OTU. The dendrogram was added based on average linkage hierarchical (UPGMA) clustering using Bray-Curtis dissimilarity matrices to reveal phylogenetic patterns across samples.

RESULTS

Pond water quality, zooplankton and phytoplankton assessment

Water quality was in the desired ranges for typical of channel catfish nursery ponds (Mischke et al. 2017). Mean water quality variables (SEM) were: nitrite-N 0.04 (0.019) mg/L, ammonia-N 0.75 (0.37) mg/L, pH 8.51 (0.04), and chlorophyll *a* 146 (31.2) µg/L. Zooplankton populations were abundant in the ponds, with desirable zooplankton for channel catfish fry averaging over 450/L (Mischke, 2012). Phytoplankton populations were dominated by Chlorophyta, Chrysophyta, and Cyanophyta.

Channel catfish gut contents and parentage determination

Gut contents revealed the channel catfish fry consumed predominantly copepods, ostracods and cladocerans, depending on availability. This finding is consistent with previous work on channel catfish fry feeding habits (Mischke et al. 2003). Meanwhile, parentage analysis based on microsatellite marker determined that all four spawns were produced from a single pair and all by different dams and sires.

Sequencing and sample summary

After filtering using *mothur*, our 16S rRNA amplicon dataset produced 3,564,729 high quality reads. In total, we observed 4759 OTUs (3066 when omitting singleton OTUs) defined at 97% sequence identity. From a total of 98 samples submitted for sequencing, rarefaction at 2848 sequences per sample eliminated three samples below this sequencing coverage. Rarefaction analyses in *mothur* revealed that sequencing efforts were exhaustive at a sequence depth of 2848 sequences per sample where total percent coverage consistently exceeded 98%. We were able to sample a large portion of the

OTUs and diversity present while still retaining a large number of samples for fish of each age.

Microbial community taxonomic composition

To characterize what phyla were present in the catfish gut community and taxa relative abundance, we quantified the number of sequences represented by each phylum in all fish samples over all developmental stages. We found that gut bacterial communities from all samples were comprised of 29 microbial phyla. However, the most abundant phyla including more than 95% of total sequences, in order of abundance included *Proteobacteria* (28%), *unclassified phylum* (24.9%), *Firmicutes* (13.9%), *Bacteroidetes* (12.4%), *Actinobacteria* (7.1%), *Acidobacteria* (4.2%), *Verrucomicrobia* (3.6%), *Planctomycetes* (1.6%), *Gemmatimonadetes* (1.1%), *Chloroflexi* (0.8%), and *Fusobacteria* (0.7%).

Phylum-level bacterial contributions to the gut microbiomes are shown according to spawn and corresponding sampling time (Fig. 4.2a, 4.2b). Fish were maintained inside the hatchery until they reached the swim-up stage (Fig. 4.2a), and later were stocked into two different nursery ponds (pond 7 and pond 8; Fig. 4.2b). Microbiome composition changed dramatically before nursery pond stocking (in the hatchery) and after nursery pond stocking. *Proteobacteria* and several other phyla dominated gut communities prior to stocking. 24 hrs after being transferred into nursery ponds, *Firmicutes* dominated gut communities. The presence of members of the *Fusobacteria* phylum became apparent at 21 days post-stocking. Bacterial community profiles originating from water samples generally resembled fish gut communities at the same developmental stages, although the relative abundance of *Proteobacteria* fluctuated across sampling times. *Firmicutes* was

the pre-dominant phyla in water samples collected during post-stocking periods (Fig. 4.3a, 4.3b).

At the genus, several taxa from *Proteobacteria*, *Unclassified phyla*, *Bacteroidetes*, *Actinobacteria*, and *Firmicutes* were consistently prominent throughout fish developmental stages (data not shown). *Ralstonia*, *Vibrio*, and *unclassified taxa* from *Unclassified phyla* were most consistent in relative abundance, however the *unclassified Proteobacteria* are documented to occur in the highest percentage across stages, ranging from 11% to 36% of total bacterial abundance. Two genera from the phylum *Fusobacteria* (*Cetobacterium* and *Fusobacterium*) were most prevalent during later stages after being stocked in the nursery pond (24hr and 21d post-release stages).

Microbial community diversity

Regression models using all predictor variables (family, brood pond, stages, pond whenever applicable) did not reveal significant difference in Inverse Simpson index. However, tests for OTUs richness showed significant different between spawns at the egg stage ($df = 1$, $F = 242$, and $p\text{-value} < 0.01$) and between stages (i.e., after 24 hr and 21 d post-stocking) once fish were transferred into nursery ponds ($df = 1$, $F = 6.485$, $p\text{-value} < 0.05$).

Variation in gut bacterial community profiles in association with water and stages

To visualize relationships between bacterial community composition in the gut and environmental (water) sources across fish developmental stages, PCoA was performed to analyze samples in reduced dimensional space using ordination plots. Variation in community membership among all environmental microbiota and fish gut microbiota was influenced by age-dependent changes in the taxonomic composition of

prevalent bacterial in the larval catfish gut. Differences in community composition were particularly prominent for samples collected before and after nursery pond stocking. In contrast, considerable overlap was apparent across time among samples collected during the first three sampling periods (Figure 4.5).

Statistical analyses of beta diversity based on Bray-Curtis distance across all sampling periods testing for significant effects of developmental stage, D, brood pond (B), spawn/family (F), and nursery pond (P) were displayed separately in Table 4.1, 4.2, and 4.3, respectively. Eggs were analyzed separately (Table 4.1). We found no influence of brood pond on egg microbial community composition (pseudo-F = 1.258, R² = 0.386, p = 0.333). We also found no significant difference in gut microbial community composition across brood pond and stages (fish at swim-up and stocking) when fish were reared in hatchery (Table 4.2). However, PERMANOVA comparing the last two stages (after 24 hr and 21 d post stocking) revealed significant interactions (Table 4.3) involved developmental stages, family and pond effects (PERMANOVA test pseudo-F = 1.863, R² = 0.038, p < 0.05). We conclude that at least one pairwise combination of post-stocking stages, family/spawn and pond significantly influenced the composition of fish gut microbial communities.

To disentangling the effect of spawn from the pervasive influence of sampling time on community composition, we performed the PCoA on the fish community for the last two stages using family and pond. Analyses were followed with an ANOVA analysis to compare fish gut communities using spawn as the predictor variable. We included family and pond effects due to the significant interaction previously described. For each stage, we were testing the null hypotheses that gut microbiome variations are not

significant between spawns. No significant effects (either pond or family) were detected based on the independent ANOVA tests for the 24 hr and 21 d post nursery pond stocking periods.

We next identified which genera contributed to gut microbiome variation across sampling times. We performed hierarchical clustering to visualize associations among microbial communities when samples (eggs, fish GI tracts, and water) were grouped together. After rarefaction to remove genera whose relative abundance was less than 1% in at least one sample, a heat map was constructed (Fig. 4.6) from 76 pre-dominant genera. The dendrogram based on the unweighted pair group with arithmetic averaging (UPGMA) clustering method showed that the community-clustering pattern based on Bray-Curtis distance was concordant with PCoA community ordinations (Fig. 4.5). Looking at nodes of dendrogram to the left of the heatmap, bacterial communities from water and fish gut samples collected at the same time cluster on the same branch. In addition, within each sampling time, fish gut communities were more similar to each other than to water samples from the same time.

Several unclassified taxa from *Proteobacteria* (Family *Enterobacteriaceae*) and *Firmicutes* (Family *Peptostreptococcaceae*), including *Clostridium sensu stricto* persisted across all samples and times. Interestingly, about 10 taxa (*Caulobacter*, *Vibrio*, *Unclassified Actinobacteria*, *Unclassified Bacteroidetes*, *Pseudomonas*, *Sphingomonas*, *Staphylococcus*, *Ralstonia*, *Arthrobacter*, *Strenotrophomonas*) can be grouped as representative genera for samples from early time periods (Fig. 4.6). We also detected taxa that usually associated with opportunistic fish pathogen such as *Flavobacterium*, *Vibrio*, and *Aeromonas* to be present in high abundance (more than 1%) in either fish or

water samples. In addition, *Edwardsiella* was also detected in one water sample during egg incubation stage (data not shown).

DISCUSSION

Channel catfish (*Ictalurus punctatus*) is the top pond-aquaculture species in the United States with an estimated commercial value of over \$350 million annually in US alone (USDA NASS, 2013). However, disease outbreaks caused by primary opportunistic pathogen such as *Flavobacterium*, *Vibrio*, *Aeromonas*, *Edwardsiella* often become the limiting factor in this industry. Surveillance and early detection of these pathogens is necessary for disease controls to avoid losses in profits to catfish producers. While farmers usually rely on antibacterial treatments, recent and safer alternatives to combat the disease are currently being widely studied focusing on monitoring healthy gut microbiota and the application of probiotics. To date, only two molecularly defined microbiota studies (Larsen et al., 2014; Bledsoe et al. 2016) have been conducted for channel catfish.

Our findings corroborated the prevalence of *Proteobacteria* as found in the study by Bledsoe et al. (2016) on channel catfish. Bledsoe et al. (2016) indicated *Proteobacteria* was the most prevalent phyla in fish at 3 dph, and *Firmicutes* along with *Proteobacteria* were the prevalent phyla when fish reached 65dph. In addition to exploring changes in microbiome following fish developmental trajectories in control environment, our study also evaluate whether gut microbial communities in fish from different family stocked into different nursery ponds (approach that commonly employed in industrial practice) experience ontogenetic shift in similar direction or being more influenced from consequence of their pond environment. Similarly, we found *Proteobacteria* dominated during early life stages and *Firmicutes* appeared after 24 hr fish were stocked into the ponds. Larsen et al. (2014) conducted studies using three

commercial warmwater fish species (channel catfish, *Ictalurus punctatus*, largemouth bass, *Micropterus salmoides*, and bluegill *Lepomis macrochirus*) that were maintained in a fishing pond for recreational purposes and reported *Fusobacterium* (Genus *Cetobacterium*) was among the most abundant taxa. This is consistent with our results showing the appearance of the same taxa when fish were stocked into the nursery pond (data not shown).

Our study identified putative deterministic (fish developmental trajectories) and stochastic (dispersal of bacteria from surrounding water) factors that influenced catfish intestinal microbiomes. Our results indicate that selection occurred within the host where, specific taxa proliferated and appear to have been favored. However, substantial variability in microbiome composition was documented over time (Fig. 4.2). The gut community also resembled water sample communities that were collected during the same time fish was sampled. Community characterizations revealed temporal shifts in community membership and the possible influence of microbes originated from rearing water supplies. In pond-aquaculture, fish that were stocked into nursery ponds were allowed to feed naturally without artificial feeding (Larsen et al. 2014). Fish stocked into nursery ponds at the time of exogenous feeding on resident pond fauna (including zooplankton and macroinvertebrates available in the pond) appeared to have rapidly acquired microbes from the pond community.

We found that the number of observed taxa (taxa richness) in egg bacterial communities differed significantly between spawns but only during egg stage, suggesting microgeographic variation in pond communities at the time of fertilization and initial colonization of egg surface in containers where eggs were oviposited and fertilized.

Furthermore, the heat map (Fig. 4.6) characterizing clustering of egg communities within the same branch compared to water samples collected during incubation shows that egg communities are similar to water communities at the same time. Previous studies (Fujimoto et al. 2013) have documented distinct egg bacterial communities in lake sturgeon (*Acipenser fulvescens*) that change across early, middle, and late incubation stages regardless of rearing water environment. In contrast, other literature describe egg surface bacterial communities are usually colonized by ambient bacteria in the water as fish spawn axenic eggs (Hansen&Olafsen, 1999; Verschuere et al. 2000).

As fish grow, their gut communities become more similar to other communities from respective sampling point (regardless of family, brood pond origins) indicated by grouping in Figure 4.5(a). We also conclude that environmental (water) communities are important innocula to developing larvae especially during later stages when exogenously feeding in ponds but not as much during early stages (Fig. 4.6). Statistical analyses on the number of taxa also indicate significant differences, yet differences were only detected at the final two stages (24 hr and 21 d following stocking into rearing ponds). Taking pond effects into consideration, PERMANOVA detected a significant interaction between effects of developmental stage, spawns, and ponds (for post-stocking sampling periods, 24 hr and 21d). Further analyses fitting linear regression models (two-way ANOVA) to distinguish which pond had greater influence on variation in community composition revealed neither pond had significantly greater effects in colonization of gut communities. Post-hoc tests could be more conservative, whereas the significance effect of pond was unresolved although significant interaction effects were detected by PERMANOVA.

Some predominant taxa detected in the heatmap (Fig. 4.5) included *Vibrio*, *Aeromonas*, and *Flavobacterium*, which are recognized as opportunistic fish pathogens that have been associated with disease outbreaks. Other fish pathogens, *Edwardsiella ictaluri* and *Edwardsiella tarda* are the causative agents of enteric septicaemia disease and Edwardsiellosis in fish species including catfish and tilapia (Hawke et al., 1998; Griffin et al., 2014, Reichley et al. 2015, Meye & Bullock, 1973). In our data, we have detected the presence of taxa classified as *Edwardsiella* in the water samples during the egg incubation stage (data not shown). However, prevalence in the community was low (less than 1%). Studies have indicated that pathogens can survive in water columns for a long period of time (Austin & Austin, 2012). *F. columnaris* for example can persist for long periods in waters with high organic matter content (Wakabayashi, 1991).

Pathogenic *Edwardsiella sp.* are probably the most significant limiting pathogen associated with first-year channel catfish fingerlings (Wise et al. 2004). The source of this bacterium in catfish populations is unclear. It is thought the bacteria is already present in the environment during early stages in the production cycle and might possibly be part of the resident microbial population. Combinations of asymptotic fish carriers and any environmental trigger later in production (Wise et al. 2004) could lead to disease outbreaks. However, our analyses only identified *Edwardsiella* in the brood pond environment and it was not detected in the fish or environment during the first 21 days of production. This would imply *E. ictaluri* is absent or is present at levels below our limit of detection (sample rarefaction to 2848 sequences). While this suggests *E. ictaluri* may not be present during these early stages of production, more research into the point source of *E. ictaluri* to the resident population is warranted.

Previous studies have documented the applications of some microbial species from genera documented in this study as aquacultural probiotics (Verschuere et al. 2000; Merrifield et al. 2010). Target disease agents are usually bacterial, and fish infected by pathogen have been shown to be successfully treated in aquaculture (Llewellyn et al., 2014). For example, *Aeromonas hydrophila* has been successfully used *in vivo* to treat *A. salmonicida* infection in *Oncorhynchus mykiss* (Irianto and Austin, 2002). In different study involving Vietnamese striped catfish (*Pangasianodon hypophthalmus* Sauvage), strains of *Bacillus* were used as probiotics in in-vitro challenges against *E. ictaluri* and *A. hydrophila* in controlled aquaria environments. These strains are mostly cultured from soil and from intestine of channel catfish (Ran et al., 2012). Authors reported strains conferred significant benefit in reducing stripped catfish mortality when used as probiotics.

We have documented influences of pond environments on catfish gut microbiota. Further investigations and comparisons of these findings relative to other fish species are needed and may help to explain fish resilience and abilities to adapt to different culture systems. Catfish ponds represent unique microcosms, and we have found that in terms of microbial community composition, no two catfish ponds are alike. While some physical and chemical aspects are similar from pond to pond, individual ponds possess unique microbial and eukaryotic micro- and macro-invertebrate communities. In our hatchery system, fish husbandry involved rearing fish in different environments throughout the production cycle. The fact that catfish were artificially raised in different ponds through the production cycle further complicated analyses, as no two catfish ponds are alike in terms of resident microbial communities (Arias et al. 2006).

Initially, catfish were raised as single year classes to harvest in a single pond. To meet demands for year-round products, a multi-batch system was adopted to replace the previously employed “all-in, all-out” strategies. As a result, grow-out ponds are now maintained with multiple year classes of fish and as the larger fish are harvested, they are replaced with smaller fish. As a result, fry are stocked into nursery ponds and raised to fingerlings or stockers. As they grow, individuals are split into separate ponds to be grown to stocker size or are understocked as large fingerlings in grow-out ponds. Catfish can be raised in as many as 3 to four different ponds over the course of the production cycle, exposing them to multiple taxonomically diverse microbial communities.

The catfish in this study were artificially reared in different ponds, which complicated analyses, although this more closely resembles what fish experience over the course of production. Several caveats are also acknowledged in this study. We lacked sufficient replication resulting in large variation in gut microbiomes among samples. Variances decreased our ability to statistically demonstrate differences among treatments (e.g spawn, pond).

Our work suggests the environment influences the gut community composition in individual ponds that may influence levels of inter-pond heterogeneity in resident fish disease resistance, feed conversion, growth, and other important production traits. Data suggests that if the environment could be manipulated in such a way to select for favorable gut communities, managers could potentially improve production efficiency in catfish production. This would be considerably more challenging if the fish biota was less dynamic or developed independent of environmental community organization.

Our work also suggests manipulation of the gut community through feed

administered pre- and probiotics may prove to be challenging, particularly when young fish are feeding exogenously. If pre- or probiotics are going to have a positive effect, intervention should be administered at the hatchery stage where the environment can be more readily altered. However, it's unclear if a gut community developed at the hatchery stage will persist once fish are stocked into ponds. It is difficult to manipulate the environment in 10-acre catfish ponds to promote specific "beneficial" communities if the processes that drive community selection are not well understood.

For future studies, we recommend more biological replicates be taken to quantify the influences of treatments, including network analyses to decipher the microbe-microbe interaction that might give insight to the pathway involved in disease development in fish host. In addition to that, we could also apply community ecology theory to move beyond descriptive towards applied studies (Adair & Douglas, 2017). This result could have implications in future pond-aquaculture management strategies, perhaps involving probiotics and prebiotics applications to combat disease.

APPENDIX

APPENDIX

Table 4.1 PERMANOVA analysis indicating lack of significant differences between centroid location of egg microbial communities from different brood ponds
 (PERMANOVA test pseudo-F = 1.258, $R^2 = 0.386$, $p = 0.333$; permutation=24*)

	Df	Sum Sq	Mean Sq	F-model	R²	Pr (>F)
Brood pond (B)	1	0.276	0.276	1.258	0.386	0.333
Residuals	2	0.439	0.220		0.614	
TOTALS	3	0.716			1.000	

*Maximum number of permutations generated due to small sample size

Table 4.2 PERMANOVA analysis indicating no significant differences between centroid location of gut microbial communities were observed between fish from swim-up and stocking developmental stages (D). Family (F: 147 A, 147 B, 154 A, and 154 B) were nested within brood pond (B: 147 vs 154).

	Df	Sum Sq	Mean Sq	F-model	R²	Pr (>F)
Dev.stages (D)	1	0.286	0.286	0.686	0.031	0.877
Brood pond (B)	1	0.470	0.470	1.129	0.051	0.279
Spawn/Family (F)	1	0.300	0.300	0.720	0.032	0.755
DxB	1	0.330	0.330	0.792	0.035	0.740
Residuals	19	7.916	0.417		0.851	
TOTALS	23	9.302			1.000	

Table 4.3 PERMANOVA analysis indicating summarizing partitioning of variability among fish gut microbiota from samples collected during 24 hr and 21 d post-release into two different nursery ponds significantly influenced by developmental stages (D), family (F), and nursery pond (P). Results revealed a significant interaction of these factors influencing the microbial communities present in the gut (PERMANOVA test pseudo-F = 1.863, R² = 0.038, p < 0.05; permutation =1000)

	Df	Sum Sq	Mean Sq	F-model	R ²	Pr (>F)
Dev.stages (D)	1	1.250	1.250	4.269	0.088	<0.001***
Brood pond (B)	1	0.236	0.236	0.806	0.017	0.737
Family/Spawns (F)	1	0.187	0.187	0.640	0.013	0.932
Rearing pond (P)	1	0.421	0.421	1.437	0.029	0.075
DxB	1	0.227	0.227	0.776	0.016	0.788
DxF	1	0.293	0.293	0.100	0.021	0.437
DxP	1	0.376	0.376	1.282	0.026	0.157
BxF	1	0.222	0.222	0.758	0.016	0.798
BxP	1	0.205	0.205	0.701	0.014	0.861
FxP	1	0.211	0.211	0.719	0.015	0.853
DxBxF	1	0.210	0.210	0.717	0.015	0.866
DxBxP	1	0.264	0.264	0.900	0.018	0.580
DxFxP	1	0.546	0.546	1.863	0.038	0.016*
BxFxP	1	0.230	0.230	0.784	0.016	0.765
DxBxFxP	1	0.315	0.315	1.075	1.075	0.022
Residuals	31	9.078	0.293		0.636	
TOTALS	46	14.269			1.000	

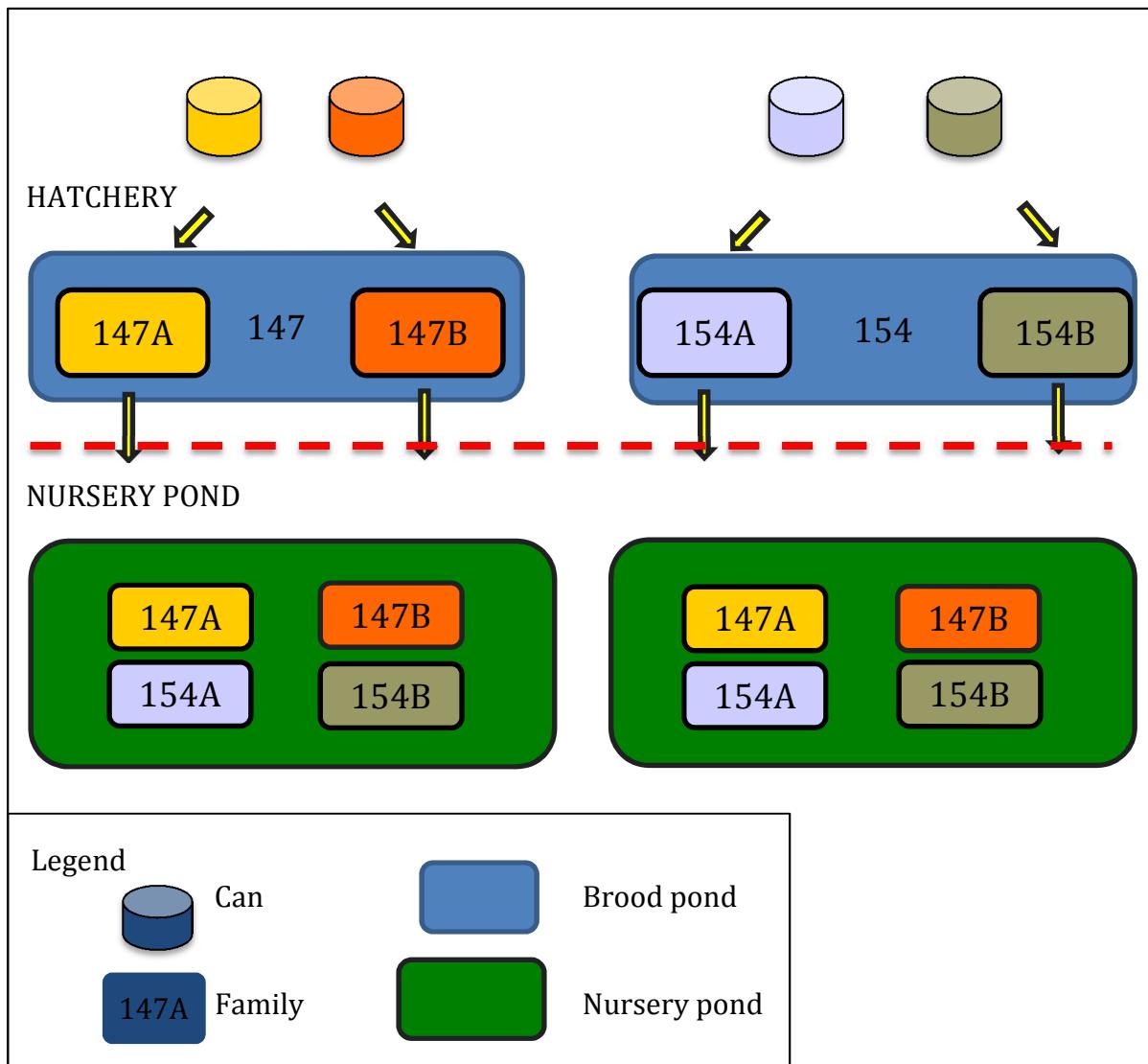


Figure 4.1 Schematic design of the larval catfish microbiome study. Catfish samples (eggs and fish) originated from eggs produced in mated pairs of channel catfish fish from spawning events in two different outdoor 0.1 acre brood ponds (Ponds 147 and 154). Eggs from different spawns were transferred into the hatchery and maintained in discrete hatching tanks (from incubation, hatch, swim-up, and stocked fry stages), supplied with flow-through ground water. Larvae were transfer to the nursery ponds at 19 days post-hatch (dph). For each spawn, fry were stocked in net-pens (100 fish/pen) and placed in two different 0.1 acre nursery ponds. Nursery ponds were prepared according to standard fertilization and pond preparation protocols

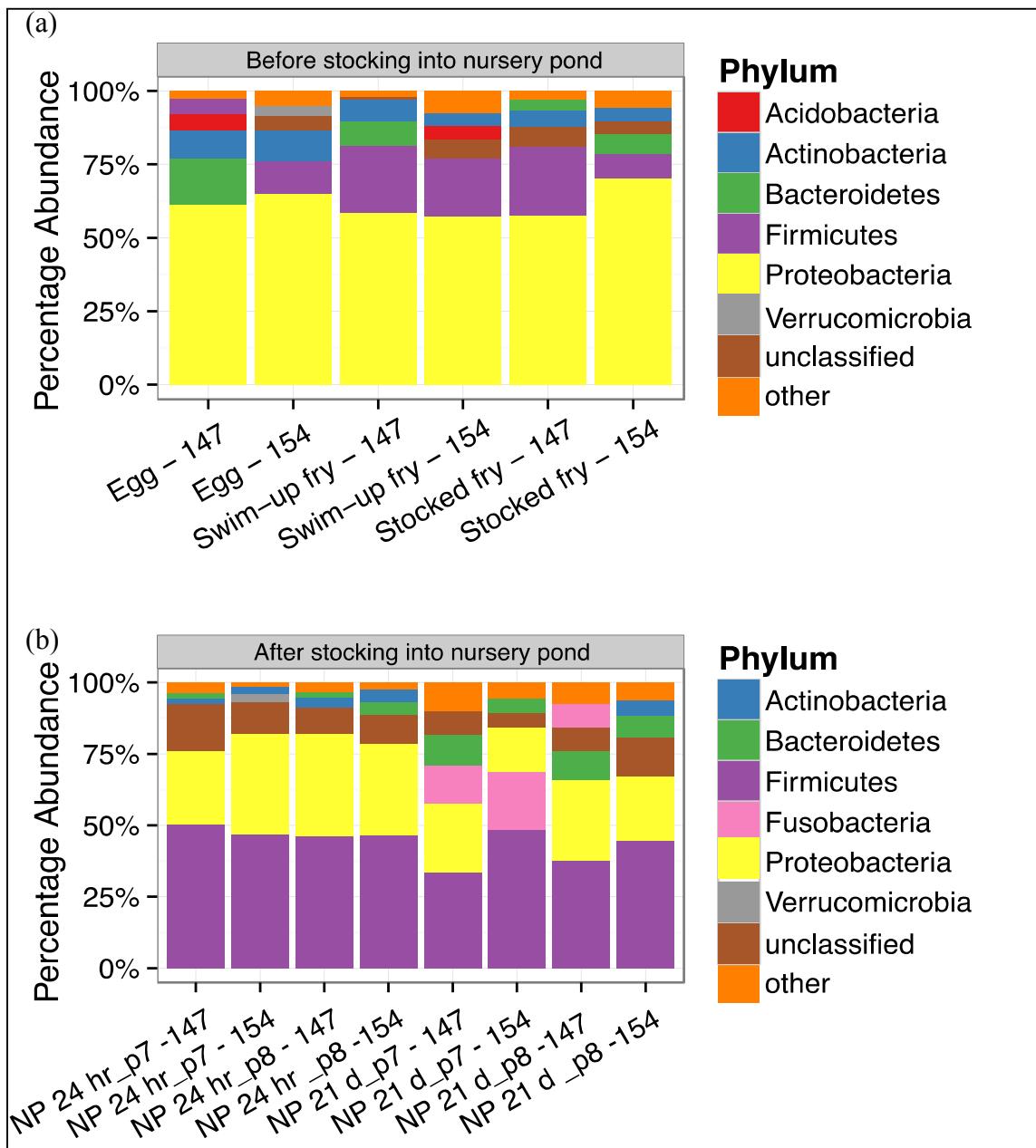


Figure 4.2. Phyla level composition of bacterial communities identified from channel catfish fry GI tracts (a) prior to stocking into nursery ponds (NP) and (b) post stocking into nursery ponds. Relative abundance (percentages) of dominant bacterial phyla found in gut microbiota of channel catfish fry from two spawns (brood ponds 147 and 154) across five different developmental stages. Gut communities collected after 24 hr (NP 24 hr) and 21 days (NP 21d) fish were stocked into nursery pond were displayed for each of two ponds (p7 - pond 7 and p8 - pond 8). The most prevalent six phyla based on relative abundance are shown in the bar chart (*Acidobacteria, Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria,*

Figure 4.2 (cont'd)
Proteobacteria, *Unclassified*, *Verrucomicrobia*) and the remainder of taxa were characterized as Others.

Caption (i)

Egg (fish egg samples);

Swim-up fry (fry collected during swim-up stage);

Stocked fry (fry collected during stage prior to pond-stocking)

NP 24hr/ Pond 24hr (fry collected at stage after 24hr being stocked into nursery pond)

NP 21d/Pond 21d (fry collected at stage after 24hr being stocked into nursery pond)

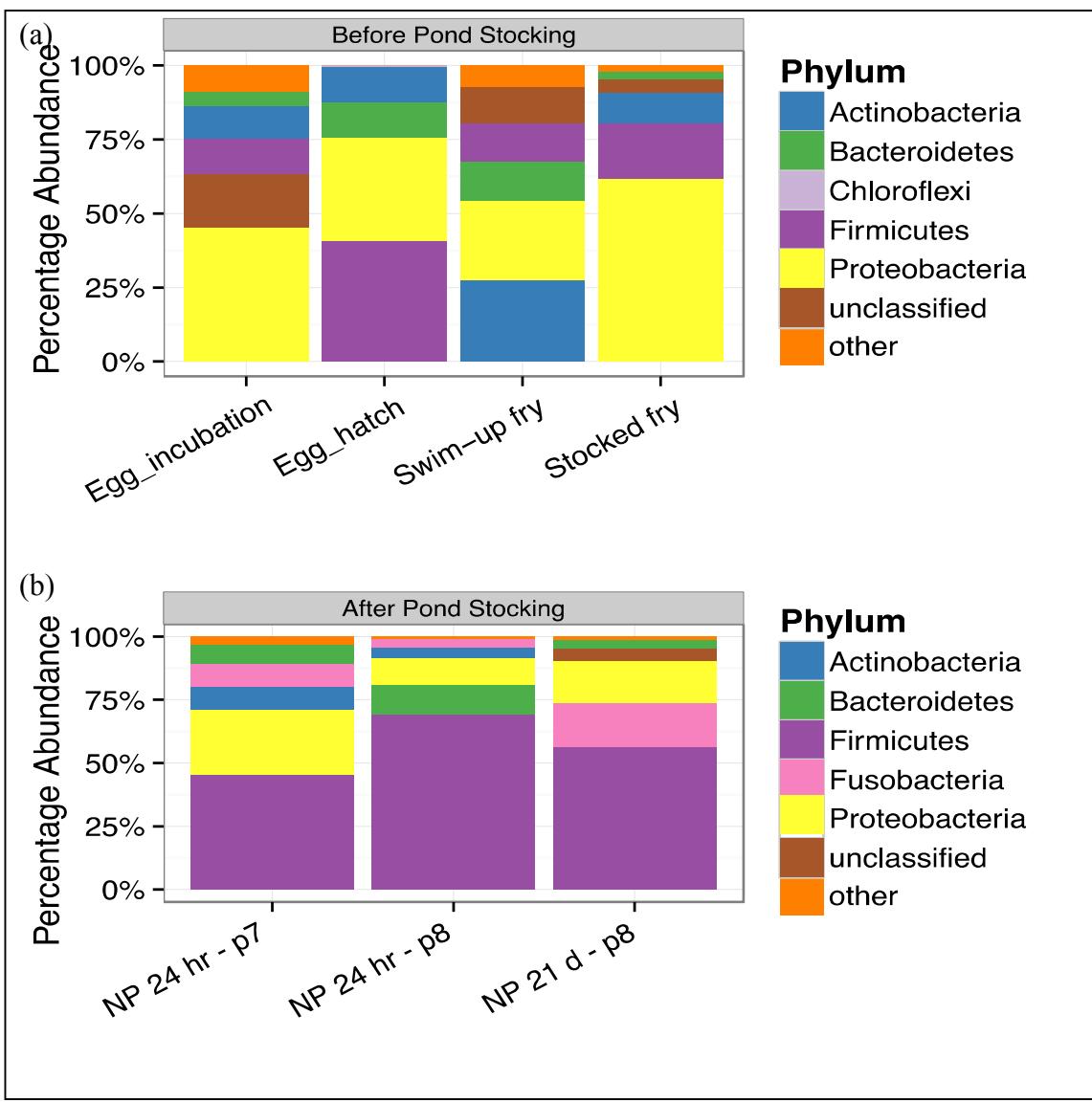


Figure 4.3 Phyla level compositions of bacterial communities identified from water samples (a) prior to fish stocking into nursery ponds (NP) and (b) after fish stocking into nursery ponds. Relative abundance (percentage) of dominant bacterial phyla found in water samples associated with catfish fry husbandry across different developmental stages. Gut communities collected after 24 hr (NP 24 hr) and 21 days (NP 21 d) fish were stocked into nursery pond are displayed for each of two ponds (p7 - pond 7 and p8 - pond 8). Samples from NP 21 d p7 was excluded from analyses due to inadequate number of sequences. Only the top six phyla were shown in the bar chart (*Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Fusobacteria, Proteobacteria, Unclassified*) and the remainder of taxa were characterized as Others.

Figure 4.3 (cont'd)

Caption (ii)

Egg Incubation (water samples collected during egg incubation);

Egg Hatch (water samples collected once egg hatched and went through sac-fry stage);

Swim-up fry (water samples collected during fish swim-up stage);

Stocked fry (water samples collected during fish stage prior to pond-stocking)

NP 24hr/Pond 24hr (water samples collected at 24hr after fish being stocked into nursery pond)

NP 21d/Pond 21d (fry collected at stage at 21day after fish being stocked into nursery pond)

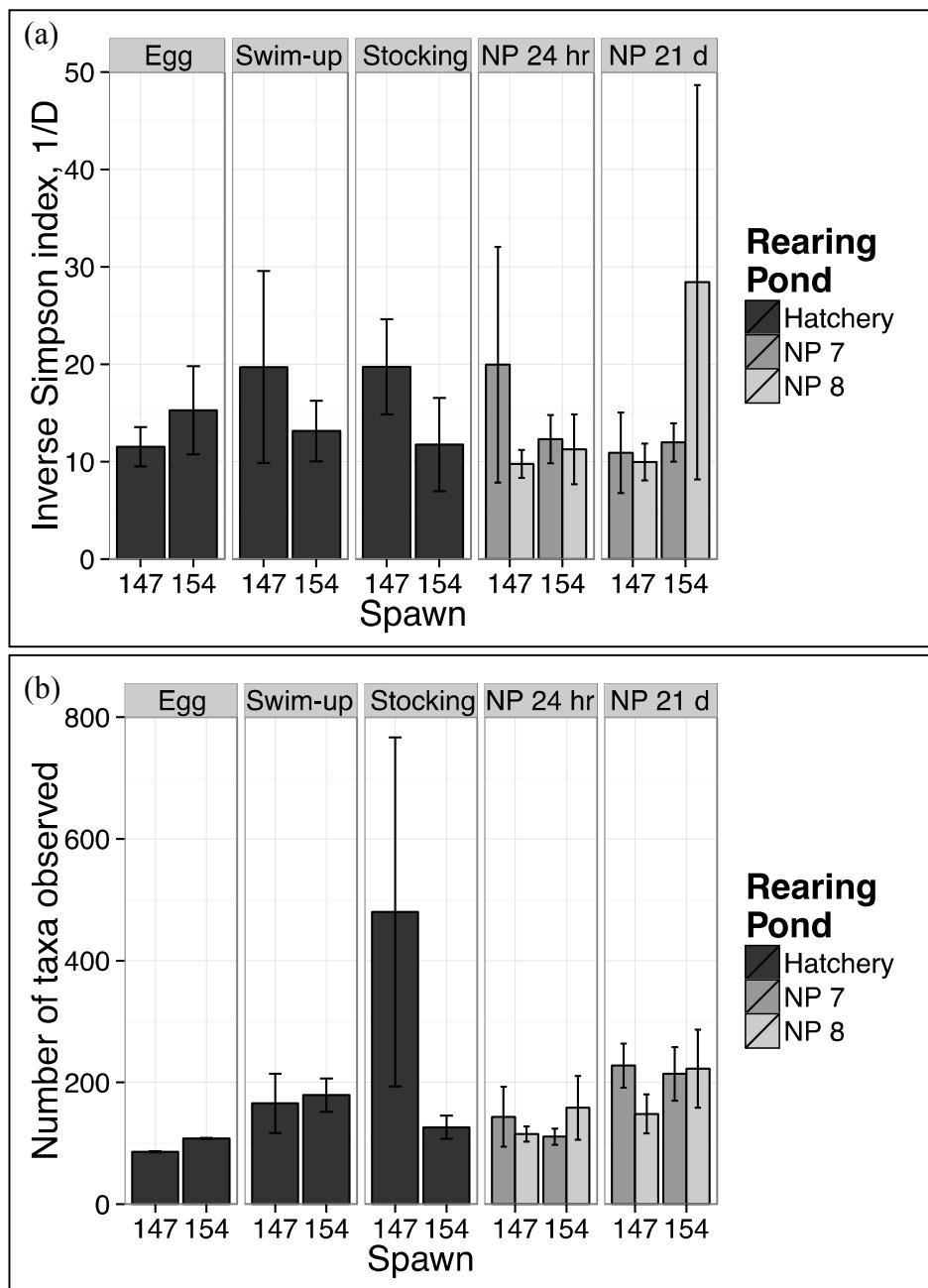


Figure 4.4 Estimates of Alpha diversity (Inverse Simpson index, (a); OTU richness (Number of observed taxa), (b)) for channel catfish gut microbial communities from all treatments across all developmental stages. Statistics comparing gut microbiotas across treatment at different ages were calculated using ANOVA (Simple one-way ANOVA for egg communities; Nested ANOVA –family within brood pond for gut community composition of fish raised in hatchery; ANOVA for randomized design for gut community composition of fish transferred into nursery pond). Each column with error bar indicate mean estimate and standard error of diversity index, colored by rearing pond (Hatchery, Nursery Pond – NP 7, and NP 8).

Figure 4.4 (cont'd)

- (a) Alpha diversity in gut microbiota at each time point, as measured by Inverse Simpson index.
- (b) OTU richness based on number of taxa observed in gut microbiota from all treatment across all time points.

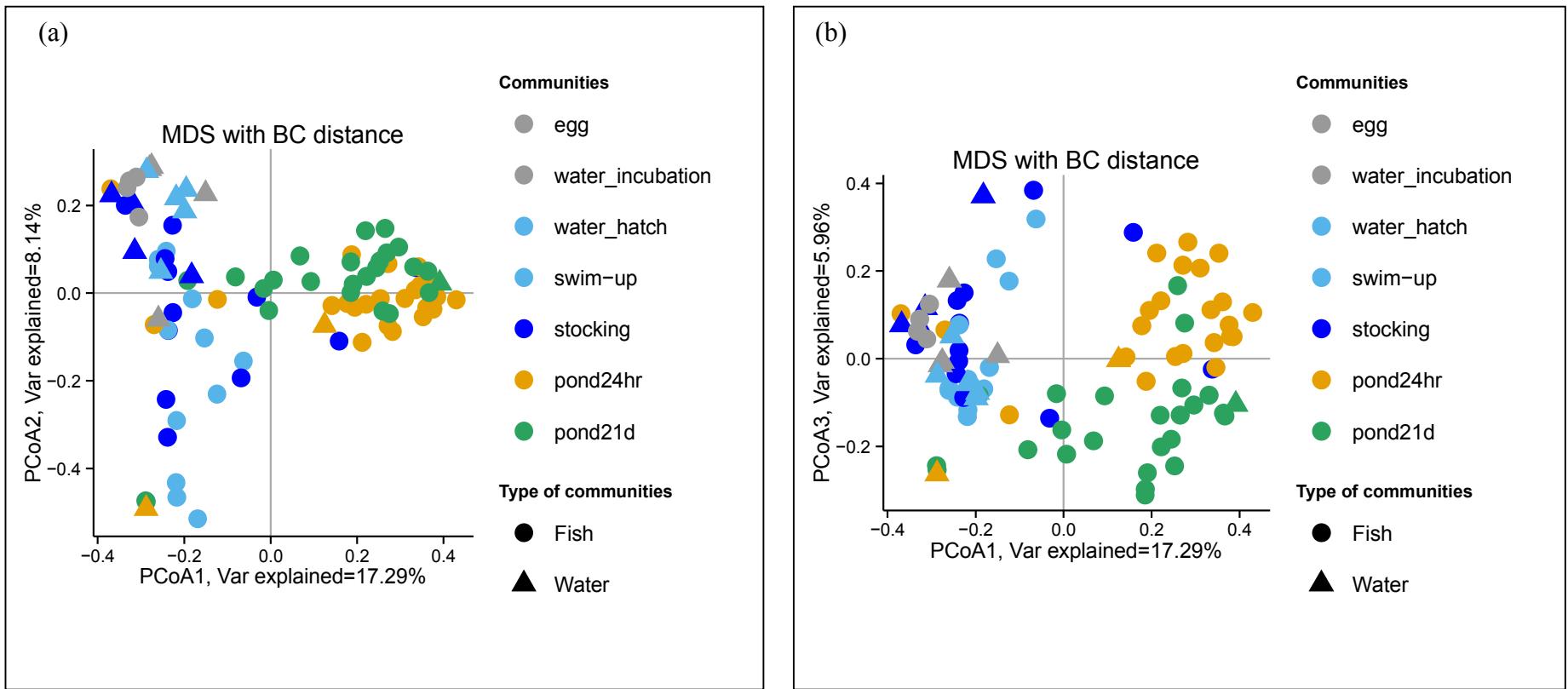


Figure 4.5 Visual representation of differences in gut microbiota of channel catfish fry (Bray-Curtis distances). Bray-Curtis dissimilarities distance between communities originating from fish GI tracts and water samples for all developmental stages

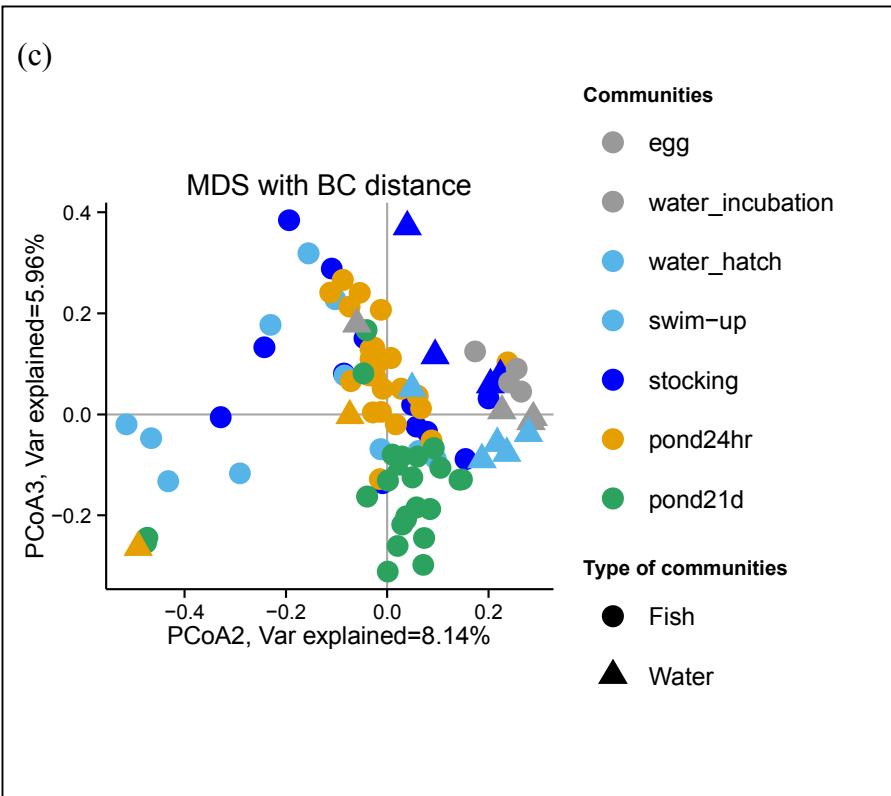


Figure 4.5 (cont'd)
 examined from both spawns are visualized using Principal coordinates analyses (PCoA) plots. Points represent a bacterial community from each sample (a) Two dimensional representation of PCoA using first and second axis; (b) first and third axis; (c) second and third axis.

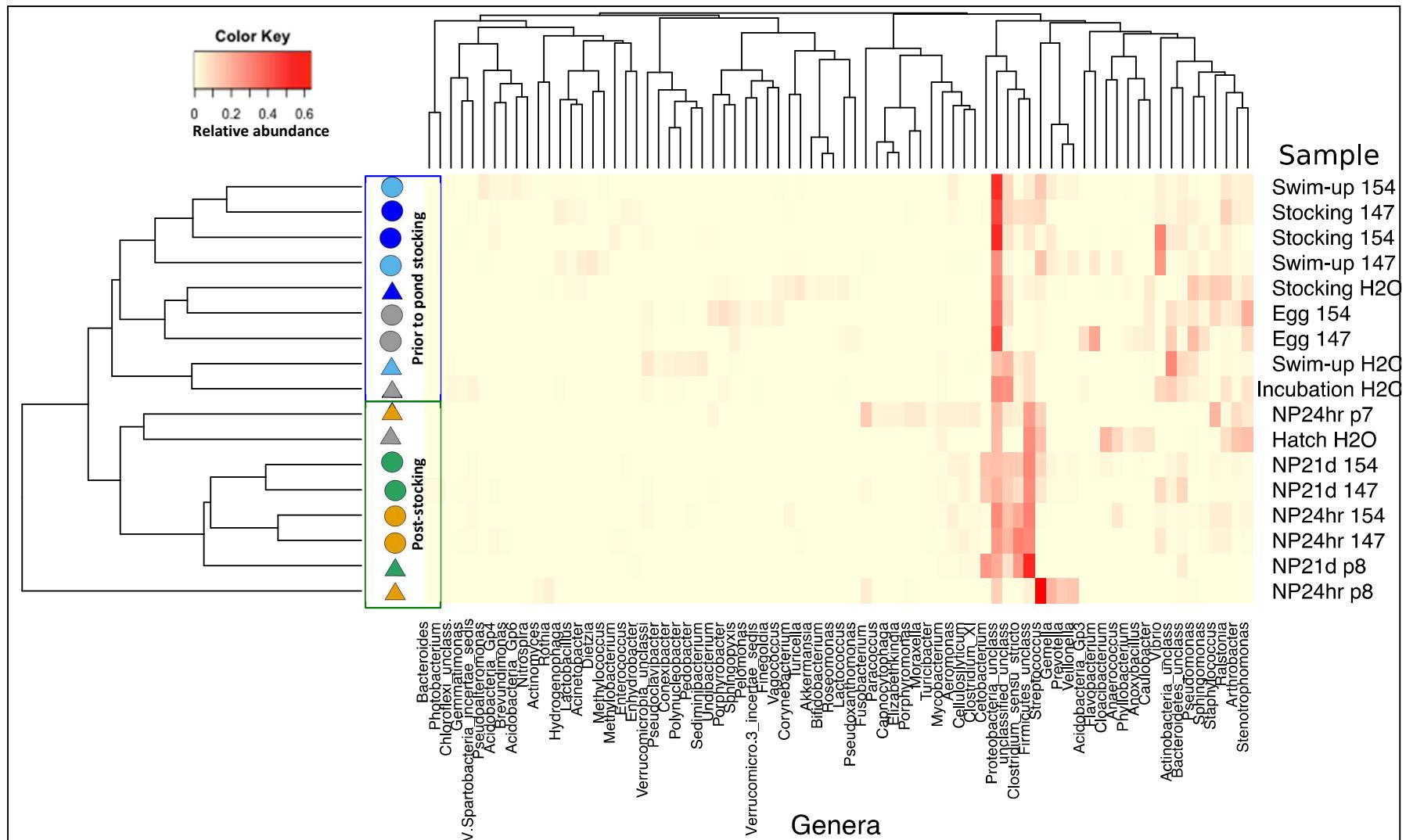


Figure 4.6 Genus-level contribution to gut microbiome community variation. Heat maps show the relative abundance value of

Figure 4.6 (cont'd)
each bacterial genus present in gut microbiota for each sample for all developmental stages. These values are depicted by color intensity with the legend indicated on the top of the figure. A genera that could not be identified during the sequence alignment step was categorized as unclassified, and the respective phylum information is provided. On the left of the heat map, each sample type was distinguished into either fish-origin (denoted by filled circle) or water-origin (denoted by filled triangle). Different colors represent different stages (or sampling point). The keys to each symbol and color is similar to Figure 4.5.

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LITERATURE CITED

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