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Intestinal Microbiota Diversity of Pre-Smolt Steelhead (*Oncorhynchus mykiss*) Across Six Oregon and Washington Hatcheries

Christina Carrell Yildirimer
Portland State University

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by

Christina Carrell Yildirimer

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Thesis Committee:
Kim H. Brown, Chair
Rahul Raghavan
Suzanne Estes

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Abstract

The Pacific Northwest is known for its once-abundant wild salmonid populations that have been in decline for more than 50 years due to habitat destruction and commercial overexploitation. To compensate, federal and state agencies annually release hundreds of thousands of hatchery-reared fish into the wild. However, **a**ccumulating data indicate that hatchery fish have lower fitness in natural environments, and that hatchery rearing negatively influences return rates of anadromous salmonids. Recently, mounting evidence revealed that the richness and diversity of intestinal microbial species influence host health. We examined the gut microbiota of pre-migratory hatchery-reared steelhead (*Oncorhynchus mykiss*) to assess microbial community diversity. The Cascade Mountains serve as an allopatric border between two distinct clades of steelhead that show significant differences in genomic and mitochondrial diversity. We identified differences in core microbiota of hatchery-reared fish that correlate with this divergent phylogeographic distribution. Steelhead sampled from hatcheries east of the Cascades had overall greater core gut microbiota diversity. These differences were found despite similarities in diet and rearing conditions.

In addition to taxonomic variation across the geographic divide, we identified significant differences in metabolic pathways using PICRUSt gene

prediction software. Our analysis revealed significant enrichment of genes associated with lipid metabolism in the gut microbiome of western fish. 8 of 19 individual lipid metabolism pathways were more prominent in western populations. Lipids are a vital nutritional component for teleost species involved in migration and subsequent return for spawning in natal environments. We hypothesize that the observed differences in lipid metabolism across this phylogenetic divide results from an increased ability of eastern Cascade (*O. m. gairdneri*) fish to utilize lipids taken in via the diet. This increased absorption and utilization would make lipids less available for the intestinal microbiota of the eastern fish, as evidenced by the lower abundance of lipid metabolism genes in the east. Our research utilizes information from the microbiome to understand the phenotypic implications occurring in segregated populations of hatchery-reared steelhead, further confirming elements of coevolution between an organism and its internal environment.

Dedication

This thesis is dedicated to my family and especially to my partner, Tim. Their love and support during this journey has meant the world to me. I also would like to dedicate this work to my students past and future, you are an inspiration. And to my big sister Leslie, who wanted to be an astronaut and grow plants on the moon.

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Table of Contents

List of Tables

List of Figures

Chapter One

Introduction

1.1 Hatchery Environments and the Salmonid Lifecycle

The Pacific Northwest (PNW) is well known for its populations of anadromous salmonid fish. Their once-abundant wild populations have been in dramatic decline since the 1950's due to habitat destruction and commercial overexploitation (Figure 1). This ecological extinction caused by overfishing has decimated aquatic ecosystems and has preceded all other widespread human disturbance such as pollution, degradation of water quality, and anthropogenic climate change (Jackson et al., 2001). The decline in the PNW population of native anadromous fishes over the last one hundred years is due to this combination of factors.

The Salmonid lifecycle begins in freshwater streams and lakes where juveniles develop and mature until undergoing an arduous downstream journey to the ocean where they will spend 2-5 years as adults. When ready to reproduce, they return to their natal freshwater streams to spawn. These species are significant not only to the economy, but to the cultural identity of the Pacific Northwest, and many efforts have been undertaken to restore dwindling populations (Araki et al., 2007; Schmid and Araki, 2010). To compensate for continued declines, federal and state agencies annually release hundreds of

thousands of hatchery-reared fish into the wild (i.e., hatchery stocking) to enhance fish populations for commercial, recreational, and conservation interests.

To support stockings, the National Fish Hatchery System (NFHS) was established by Congress in 1871 in an effort to conserve fishery resources for future generations and bring to light the anthropogenic threats posed to fisheries. The NFHS advanced a nationwide system of fish culture and propagation, which has evolved to become a network of hatcheries, laboratories and research centers. The mission of this network, which focuses on species listed as threatened or endangered, is to restore dwindling populations before they are listed using science-based fish and aquatic conservation techniques. The NFHS partners with states and federally recognized Native American tribes to help restore depleted native fish stocks lost due to human activity, including federal water development projects which are also called upon to participate in hatchery system funding. For example, the 2013 Strategic Hatchery and Workforce Planning Report put out by U.S. Fish and Wildlife Service indicates there were 291 national propagation programs focused on restoration of listed species, with a cost of nearly 11 million dollars ("National Fish Hatchery System," 2013).

Hatcheries are designed to produce large numbers of big fish, available to release for commercial and recreational use, as well as to aid in maintenance of

wild fish populations (Araki et al., 2007; Brown et al., 2003). While this appears a logical solution to the problem of dwindling populations of wild salmonids, there is growing evidence that results of this practice are mixed. Return rates of hatchery fish are half those of wild fish (Chilcote, 2003; Schmid and Araki, 2010). Accumulating data also indicate that hatchery fish have lower fitness in natural environments compared to wild fish. Despite more than a hundred years of scientific study, the reasons for this discrepancy in return rates remains unclear (Araki et al., 2007), even as hatchery systems are lauded as enviable success stories by federal agencies and related commercial interests.

Hatchery reared salmonids encounter difficulties post-release that may impinge their success and subsequent return rates once time has come to spawn. Given their dependence on time released pellet feedings and lack of prior experience with live prey, hatchery fish have difficulty finding prey in the early stages of life post-release (Brown et al., 2003). These difficulties coincide with a critical period of time in the salmonid lifecycle, smoltification. Smoltification is a physiological transition modulated by the neuroendocrine system that anadromous fishes undergo to prepare for their transition from a relatively unproductive fresh water environment to the highly productive salt water found in the pelagic ecosystem. During smoltification, structural and functional

transformations occur which involve independent yet coordinated developmental changes in the physiology, biochemistry, morphology and behavior of juvenile salmonids. Some examples include the silvering of the body to better shoal in pelagic environments, and improved salinity tolerance as juveniles move through estuaries on their way to the sea (Urke et al., 2010). This is the time when mortality rates for hatchery-raised salmonids are at the highest (Brown et al., 2003). Improving hatchery systems to better prepare juvenile fish for this difficult post-release period is a common goal of both commercial and conservation interests.

A variety factors have been investigated relevant to post-release success for hatchery-reared fish. The length of fish when released has been shown to influence survival and enhance return rates of anadromous hatchery-reared salmonids (Beckman et al., 1999; Tipping, 1998). Return rates appeared to increase when release length was more than 190mm (Tipping, 1998). Yet size is not necessarily the determining factor of success in wild environments. It has also been found that over-summer survival for domesticated, or hatchery-raised, salmonids was consistently lower post-release than for the smaller wild fish of the same species living in the same river system (Schmid and Araki, 2010).

Domestication selection favors certain traits, physiological states, and/or sets of conditions for fish raised in captivity, which are less likely to be

advantageous in the wild. Evidence from a number of studies indicates that genetic diversity and post-release viability is in fact reduced in hatchery stocks (Chilcote, 2003; Schmid and Araki, 2010). The major effects of hatchery rearing include lower survival, reduced genetic diversity and reproductive fitness, although few studies have provided any direct evidence of enhanced traits for wild stock (Schmid and Araki, 2010). Hatchery-raised fish can hinder the success of wild populations through genetic intermixing that occurs when wild and hatchery-raised fish interbreed, indicating a possible genetic component to reduced return rates (Chilcote, 2003; Miller et al., 2004). Genetics, however, is not the sole source of physiological differences between hatchery stock and wild fish living in nearby river systems. Why else might fish raised in hatchery environments have such difficulties during that critical period post-release?

Previous research highlights two significant areas, environment and diet, as sources of influence on the physiology and viability of hatchery stock post-release. Narrowing down environmental factors relevant to success in the wild is difficult when the fish in question spends its life in both freshwater and pelagic ecosystems. A study conducted by The National Oceanic and Atmospheric Association (NOAA) in 2005 investigated the use of models to predict return rates for wild Chinook salmon. Results indicated that environmental conditions

experienced during ocean residency, though difficult to quantify, accounted for only 5% of the variation in return rates, and that incorporating residency in freshwater habitats into the model was critical to understanding the role played by the environment (Greene et al., 2005). This work indicates that the effects of early life in a freshwater habitat is a key factor influencing return rates for Chinook salmon, and likely anadromous salmonids in general.

Hatchery stock reared in aquaculture environments experience vastly different living conditions (i.e., high density and increased competition) during their development than wild fish (Brown et al., 2003). Standard cement raceways, and less commonly gravel-bottomed ponds, are primary hatchery substrates; young hatchery stocks spend their life here before release into river systems. Success in post-release steelhead on the Cowlitz River in Oregon was shown to increase when fish were reared in gravel ponds over cement raceways (Tipping, 1998). Results concluded that ponds grew better quality fish than concrete raceways and that a contributing factor to that success was access to more natural foodstuffs in the pond. This access may have provided both nutritional advantages via ingestion of wild foods like insects and small crustaceans, and offered fish opportunities to learn important foraging behaviors required for a successful life post-release. Incorporation of these types of foodstuffs into the diets of hatchery-

reared fish could assist in overcoming the difficulties experienced during the critical period of life spent in limnic environments.

Fish physiology is heavily influenced by diet. Hatchery systems are successful at growing abundant numbers of large juveniles well suited to a domestic environment. Hatchery stocks are typically fed processed fish meal pellets on a regular schedule, in a crowded environment with increased competition for resources from the thousands of fish that cohabitate in the same run or pond. One downside of this practice is that fishmeal is expensive; therefore much research has been undertaken to identify cost-effective alternatives that have only marginal impacts on fish health and physiology (Mansfield et al., 2010; Wong et al., 2013). For example, in 2005 the cost (U.S.) of one metric ton of fish meal was $600, by 2010 that cost had risen to $2000 (Sánchez-Muros et al., 2014). In addition, fish and soy meal production severely impact the environment. Fish meal production depends on the catch, which is quantitatively and qualitatively variable. It contributes to the "clearly unsustainable" deterioration of the marine environment by stripping fisheries, causing widespread disruption throughout the marine food chain (Barroso et al., 2014). Growing soy for feed necessitates deforestation of areas with high biological value, involves high consumption of water resources and utilizes large amounts of pesticides and fertilizers (Sánchez-

Muros et al., 2014). Soy-based feed is commonly offered as an alternative to fish meal, yet some evidence has shown that high levels of intestinal inflammation occur in both salmon and steelhead when levels of soy in feed were too high (Mansfield et al., 2010). Differences in intestinal microbiota were also detected in fish receiving soymeal-based feed vs. fish meal (Mansfield et al., 2010; Storebakken et al., 2000), with a potential negative impact on development detected. Wild steelhead living in Pacific Northwest waterways forage on a variety of foodstuffs, including salmon eggs, small fish and zooplankton. They also ingest large quantities of arthropods, such as stonefly nymphs, caddis fly pupae, grasshoppers, snails and small crawdads (www.fisheries.noaa.gov, 2016).

1.2 Effects of Diet

It is here that the environment and diet of wild salmonids might converge with hatchery science to offer a unique alternative to the traditional hatchery diet of fishmeal or soymeal pellets; the incorporation of insect meal into hatchery fish feed. Including insect meal in hatchery fish feed might better mimic the diet of their wild counterparts and could alter their gut microbiota, possibly providing an advantage when ingesting wild foodstuffs post-release.

The evaluation of insect meal as an alternative feed source is a new and burgeoning field of research (Makkar et al., 2014; Rumpold and Schlüter, 2013).

Like crustaceans, insects belong to the class of arthropods, and count more than a million species. Insects represent an innovative feed source rich in high quality protein, fats, minerals and vitamins (Rumpold and Schlüter, 2013). There are nearly 2000 species of insects in the world fit for human consumption (Huis et al., 2013), 69 of which are found in the United States. Makkar (2014) investigated five types of insects as an alternative feed source, including mealworms, grasshoppers, silkworm pupae, housefly maggots and black soldier fly larvae; they found that the crude protein content ranged from 42-63%, with lipid content upwards of 36% in some insect species. Soy and fish meals range from 50-73% crude protein and 3-9% fats respectively (Barroso et al., 2014). Insect meal was found to be palatable at replacement levels of 25-100%. From a nutritional standpoint, the best insect candidates are members of the *Orthoptera* family, which includes grasshoppers, crickets and locusts (Barroso et al., 2014), with silkworm pupae also performing well against fish and soy meals with regard to amino acid content and overall nutritional value (Makkar et al., 2014). Fish are a monogastric species that require a high quality and quantity of protein in their diet, high digestibility of foodstuffs and good palatability (Sánchez-Muros et al., 2014). Although scant, current research demonstrates that insect meal is an ideal alternative or supplement to fish and soy meal for teleosts (Makkar et al., 2014), with the added benefit of being

more environmentally responsible. When compared to conventional livestock feed production, insects have higher feed conversion efficiency, require less feed to produce 1 kg of biomass, have higher fecundity, can be raised on organic waste, require less space, and require significantly less water resources (Rumpold and Schlüter, 2013; Sánchez-Muros et al., 2014).

Studies show few, if any, negative effects of using insect meal as a replacement or supplement to traditional fish meal. One area of concern regards the lipid content of terrestrial insects, which is largely comprised of n-6 fatty acids in contrast to fish meal which contains higher concentrations of n-3 fatty acids (Barroso et al., 2014; Sánchez-Muros et al., 2014). However, the poor quality of insect lipids can be modified during the lifecycle of the insects by altering the lipid content of their feed (Sánchez-Muros et al., 2014). One unique aspect that has received little attention is the fact that aquatic insects have a much higher ratio of n-3 fatty acids than do terrestrial insects. This difference is due to the higher proportion of EPA, eicosapentaenoic acid, found in freshwater algae, the main component of aquatic insect diets. EPA is one of several omega-3 fatty acids found in cold-water fatty fish. Aquatic insects may be a superior supplement to traditional fish meal than terrestrial insects due to their high n-3 ratio, and will prove an interesting avenue for future research. Additionally, the lipid content of

wild versus captive-bred insects was also found to differ in regard to fatty acid content, with wild insects having a lower lipid concentration overall. Typically, amino acid content is more determined by insect taxon, whereas fatty acid profiles of insects most likely reflect the lipid components of their food (Barroso et al., 2014). This is further evidence demonstrating that the lipid content of insects can be modified through changes to their diet.

Amino acid content of insect meal is adequate and similar to that of fish meal, but has been shown to be deficient in methionine, lysine, tryptophan, histidine, and threonine (Barroso et al., 2014; Makkar et al., 2014). Fortunately, these amino acids can be easily supplemented to insect meal during production.

With regard to insect meal digestibility, Barroso (2013) and Sanchez-Muros (2014) found a small reduction in growth rate when insect meal comprised greater than 25% of feed, while Makkar (2014) found no such reduction in studies of rainbow trout fed insect meal. Such a reduction in growth might be due to the chitin present in insect meal. Chitin is the second most widespread polysaccharide found in nature, after cellulose. It is a non-toxic, biodegradable and biocompatible polymer found in arthropod exoskeletons and the cell walls of fungi, insects and yeast (Cuesta et al., 2003). Barroso (2013) considers chitin "digestively unavailable" material in the diet of teleosts, and it may be inferred that diminished

growth rates observed when fish are fed insect diets are due to a failure to digest and absorb this material completely (Lindsay et al., 1984). Chitin has poor adhesive properties and may contribute to the friability of pellet feed, aiding in the disintegration of pellets during the flurry of feeding activity, wherein some of the food is rendered unavailable (Lindsay et al., 1984).

Conversely, chitin has also been reported to be an immunostimulant in fishes, though the mechanism remains unclear (Cuesta et al., 2003; Lindsay et al., 1984; Vahedi and Ghodratizadeh, 2011). Immunostimulants have the ability to increase resistance to disease by enhancing nonspecific defense mechanisms (Vahedi and Ghodratizadeh, 2011) and promoting recovery from immunosuppression states caused by stress (Sakai, 1999). Chitin has been shown to increase the activity of the seabream innate immune system (Esteban et al., 2001), and to increase phagocytic activity in fish leukocytes, activating the cellular immune response and enhancing protection against disease (Vahedi and Ghodratizadeh, 2011). Specifically, Sakai (1999) reported that rainbow trout (*Oncorhynchus mykiss*) injected with chitin in the intraperitoneal region showed stimulated macrophage activities and an increased resistance to *Vibrio anguillarum* infection. If chitin is both a nondigestible component of insect meal, yet also serves as an immunostimulant, then the mechanism for such an immune system boost

might be found in the intestinal tract itself: the gut microbiota. Gut microbiota are known to increase immune system function and aid development of the intestinal tract (Bates et al., 2007).

The microbiota of mammalian intestines depends largely on dietary polysaccharides as energy sources (Flint et al., 2008). Fish have also been shown to have resident microbial populations living in their intestinal tracts that utilize undigested materials (Bairagi et al., 2002). This clever evolutionary adaptation of metabolically active microbial societies allows them to salvage energy from nutrients—particularly carbohydrates—that are otherwise nondigestible (Hooper 2002), all while conferring benefits upon the host. These materials serve as *prebiotics*, which are defined as "…nondigestible food ingredients that are selectively utilized by one or more components of the normal microflora" (Hooper et al., 2002). Prebiotics may play a helpful role in the induction and/or restoration of a disturbed microbiota to its normal beneficial composition (Pérez et al., 2010). Chitin may serve as a prebiotic for fish, possibly stimulating the immune systems of teleosts, thereby making insect meal a perfect addition to hatchery fish food. Lindsay (1984) reported that rainbow trout were not extracting much energy directly from chitin, yet were experiencing the positive immunostimulant effects. Rainbow trout may have endogenous chitinolytic enzymes that could help them

utilize chitin in addition to the digestion done by gut microbiota. Chitinase genes have been found and sequenced in some marine teleosts, such as Japanese flounder (*Paralichthys olivaceus*) and striped bass (*Morone saxatilis*) (Sánchez-Muros et al., 2014). And while chitinolytic and cellulolytic bacteria may not use the solubilized products they generate directly as energy sources, those substrates are then made available to other members of the microbial community through cross-feeding (Flint et al., 2008).

Hatchery stock receive or are inoculated with an intestinal microbiota suited to a domestic environment and processed fish meal feed they are given, whereas wild fish develop a microbiota based on their river environment and food acquisition which is based heavily on an arthropod diet. One possible effect of this is that hatchery stock are then un-prepared to digest wild foodstuffs upon release. These differences in foodstuff consumed may influence the richness and diversity of microbial species found in the intestinal tracts of hatchery and wild fish, which in turn would influence the development of the gut and immune system. Developmental differences based on diet have the potential to affect the health and viability of hatchery fish after they are released. This is the question that stirs curiosity: do hatchery salmonids--steelhead specifically--have different gut microbiota than wild fish from the same river systems? And if so, what effects

might this have on their physiology and subsequent success in a post-release environment, prior to migration? What effects might a hatchery-derived gut microbiota have on smoltification—the process of preparing the body for life in a pelagic environment? How are the specifics of diet and hatchery environment connected to the types of gut bacteria found in hatchery fish? Despite the continued progression of knowledge about fish immunology and development, the contribution of the normal endogenous microbiota to overall fish health has been so far underestimated (Gómez and Balcázar, 2008). In addition, the effects of an exclusively fish meal diet on the gut microbiota of hatchery fish has not been given due consideration in current literature. We have investigated the gut microbiota diversity of hatchery-reared steelhead from six hatcheries across Oregon and Southern Washington, just prior to release, hoping to shed light onto this scarcely-researched area of aquaculture science.

1.3 Microbiota Effects on the Host

The effect of intestinal microbiomes on host physiology is a developing field (Bates et al., 2007). Evidence suggests that differences in intestinal microbiomes are associated with measurable physiological traits such as weight, obesity and metabolism (Cotillard et al., 2013; David et al., 2013; Le Chatelier and et al., 2013) and can be altered by diet and environment (David et al., 2013; Moeller

et al., 2013). Metagenomic approaches have allowed researchers to measure microbial populations to a highly specific degree by isolating and sequencing conserved areas of DNA such as the 16s ribosomal gene found across bacterial populations (Caporaso et al., 2011; Claesson et al., 2010; Huntley et al., 2012; Salipante et al., 2013). Modern techniques currently allow scientists to identify microbiota to the level of genus and species. In-depth investigation of possible connections between the intestinal microbiome, physiology and development are more economical and more easily accessed than ever before (Lowe, Beth A., et al. 2012).

Vertebrate microbiomes are initiated through external exposures after leaving the germ-free environment of the womb, or in the case of fish, at the earliest exposure to communal water (Roeselers et al., 2011). This reinforces the notion that environment plays a key role in inoculation of an individual with its specific microbiota, regardless of host provenance or domestication status (Cheesman et al., 2011; Roeselers et al., 2011). In other words, organisms living in the same environment may share microbiota, regardless of whether they are of the same lineage or species. Zebrafish of different lineages were found to share common core gut microbiota when put in the same lab environment together (Roeselers et al., 2011). Additionally, gorillas and chimps living sympatrically in a

shared environment had a more similar core gut microbiota than did chimps of the same species living allopatrically (Moeller et al., 2013).

This has huge implications for hatchery stocks that are expected to thrive in the diametrically opposed environments of hatchery raceways and wild, freshwater river environments where the costly metabolic process of smoltification occurs. How well are hatchery stocks prepared for that metamorphosis? It is known that gut microbiota in fish contribute to important key functions including nutrition, development, immunity and xenobiotic metabolism (Navarrete et al., 2012). Colonization of intestinal mucosal surfaces with normal microbiota has a positive effect on immune regulatory functions of the gut (Pérez et al., 2010) and imbalance may contribute to disease. Host/microbiota interactions are essential to many aspects of normal physiology ranging from metabolic activity to immune homeostasis (Pérez et al., 2010). Karen Guillemin, of the University of Oregon, investigated the link between gut microbiota and epithelial cell development in the digestive tracts of zebrafish and found significant differences between abiotic zebrafish and those with a core gut microbiota. Zebrafish inoculated with a core gut microbiota had increased cell proliferation in intervillus regions of the intestine and a higher percentage of immune cells in the gut than did abiotic fish (Cheesman and Guillemin, 2007).

Perez (2010) reported that intestinal bacteria were essential for normal development of GALT, or gut-associated lymphatic tissue, and that in the absence of luminal bacteria, B and T cells did not localize in the lamina propria layer of the intestine, and subsequently Immunoglobulin A was not secreted.

The idea that hatchery stock might possess a gut microbiota not as suitable to life in the wild fuels my research. My aim is to illuminate a possible developmental impediment caused by the microbiota of hatchery-reared fish that negatively influences return rates. Current information on the importance of the gut microbiome on health and development explores more subtle but integral processes involving host-microbiome interactions. The increased presence of immune cells in the guts of fish with a developed microbiota serves as evidence for a more involved relationship between the host and its microbiome than was previously thought. An increased presence of immune cells in the guts of fish with healthy microbiota indicates a successful host/microbiota relationship (Bairagi et al., 2002).

My research joins that of other scientists and biologists investigating ways to better prepare hatchery stock for life as juveniles in the wild, freshwater environment they will inhabit post-release, enhancing the possibility of a successful migration to the ocean and a consequent return to freshwater spawning

grounds. In comparing hatchery steelhead microbiota to that of wild fish, I can add to the knowledge of what role microbiota may be playing in the return rates of wild steelhead, and generate fresh ideas for prebiotic applications in hatchery environments to better prepare hatchery stock for life in the wild.

Chapter Two

Gut Microbiota Lipid Metabolism Varies across Rainbow Trout (*Oncorhynchus mykiss*) Phylogenetic Divide

2.1 Introduction

Vertebrate animals have a diverse microbial community living on and in their bodies, their microbiota. Microbiota play a fundamental role in their hosts' lives and are critical components of normal physiological processes ranging from immune system development to metabolism (Sullam et al., 2015). Microbiota have been studied in numerous vertebrate systems including humans (Goodman et al., 2011; McHardy et al., 2013; Zhou et al., 2016), pigs (Lowe et al. 2012), gorillas and chimpanzees (Moeller et al., 2013), myrmecophagous mammals (Delsuc et al., 2014), pandas (Zhu et al., 2011) and teleosts (Lowrey, Woodhams, Tacchi, & Salinas, 2015). It is now well documented that the vertebrate gastrointestinal (GI) tract harbors one of the most complex and dynamic microbial ecosystems (Nayak, 2010). Despite this, understanding the effects of these intestinal microbiomes on host physiology is still a developing field (Bates et al., 2007). Evidence suggests that differences in intestinal microbiomes are associated with measurable physiological traits such as weight and obesity (Cotillard et al., 2013) as well as development of the immune system (Montalban-Arques et al., 2015) and defense against environmental pathogens (Lowrey et al., 2015). What would differences in microbiomes potentially indicate? Evidence has shown that a difference of even a single species of bacteria can have profound effects on the host. (Buffington et al.,

2016) explored this question and found that after selective removal, the reintroduction of a single species of bacteria, *L. reuteri*, restored behavioral alterations similar to autism in humans, highlighting the significant influence of microbiota to host health and function.

Terrestrial vertebrates begin to acquire their commensal gut microbiota at birth from maternal bacteria (Dominguez-Bello et al., 2010) and are colonized by a succession of organisms until a stable community structure is achieved (Cox et al., 2014). This contrasts with teleosts where no direct vertical transmission of maternal bacteria to offspring is evident, but they instead acquire their gut microbiota through communal water exposure prior to first feeding (Ingerslev et al., 2014; Roeselers et al., 2011). Water temperature, habitat type, stress levels, feed type, and feeding conditions can all affect the early colonization of the teleost intestinal microbiome (Nayak, 2010). Each generation of fishes is then dependent on the conditions of their specific rearing environment for the inoculation of their initial gut microbiota (Cheesman and Guillemin, 2007), with environment and diet continuing to impact the evolution of gut microbial communities over time (Sullam et al., 2015).

A wide range of analytical tools is now available to identify the contributions of host, environment, and diet on gut microbiota diversity and

richness. Gnotobiotic (germ-free) model systems have proven advantageous in identifying characteristics of gut microbiota-host interactions with regard to physical development and immunity, as well as assessment of the manner in which microbiota are acquired from the environment. Studies of gnotobiotic zebrafish have aided researchers in identifying a core gut microbiome, revealing its relationship to development of the GI tract (Llewellyn et al., 2014; Roeselers et al., 2011) and in elucidating specific interactions of microbiota with intestinal mucosal surfaces that affect development of gut-associated lymphatic tissue (GALT) (Pérez et al., 2010). Gnotobiotic mouse models have been used to scrutinize the effect of diet on gut microbiota in monozygotic twins (Ni et al., 2015) and effects of plant and animal-based diets on microbial community structure (Ingerslev et al., 2014). A study of gnotobiotic cod eggs highlights the role of incubator environment in microbial inoculation of the host (Verschuere and Rombaut, 2000). Aquatic environments have been shown to affect gut microbiota composition and are often rich in detritus, including fecal matter from cohabitants. Fecal matter is commonly used in studies of gut microbiota as it is rich in microbial DNA and can be easy to collect without causing harm (Eichmiller et al., 2016). For teleosts, this aquatic exposure to cohabitant fecal matter could play a significant role in determining the composition of microbiota in the intestinal tract.

Complementing gnotobiotic model systems, high-throughput sequence analyses of 16S ribosomal RNA gene sequences are increasingly accessible and affordable to researchers, and have proved to be invaluable in identifying taxonomic diversity down to the species level, illuminating metagenomic variation and distinguishing functional capabilities of commensal gut microbiomes (Claesson et al., 2010; Ghanbari et al., 2015). These advances have led to a surge in research of host-microbiota relationships with regard to diet, environment and host, as well as identifying effects of gut microbial community structure and composition on host health.

The intersection of diet and environment has a significant effect on both composition of gut microbial communities and co-evolution between the gut microbiota and host. In many cases, a direct link between diet and gut microbiota composition can be seen. In Asian seabass (*Lates calcarifer*) under starvation conditions, gut microbial community structure showed significant differences from a control group (Xia et al., 2014). Diet supplementation with oligosaccharides has been shown to impact gut microbial composition of broiler chickens (Corrigan et al., 2015) as well as supplementation with soybean and insect meal in Atlantic salmon (*Salmo salar*) (Lock et al., 2015; Nordrum et al., 2000). Diet has also been shown to play an important role in nutrient conversion processes of microbiota

that make macromolecules available to the host (Montalban-Arques et al., 2015). Diet and environment can also work in tandem to affect gut microbial composition. Distantly related myrmecophagous mammals develop a shared gut microbial profile due to extreme diet specialization caused by environmental restrictions (Delsuc et al., 2014), while gorillas of the same species living allopatrically developed divergent gut microbial communities similar to the gut microbiota of chimpanzees living in the shared environment (Moeller et al., 2013). The direct cause of divergent gut microbiota profiles within a species is not entirely understood. Chimpanzees and gorillas living sympatrically may well share elements of their diet, leading to a similarity in gut microbial profiles. Another possibility is that regular exposure to fecal material from one species can impact the gut microbial composition of another species living nearby. Gnotobiotic mice co-housed with mice having normal endogenous gut microbiota will develop a similar microbial profile via the presence and ingestion of fecal matter in the habitat (Buffington et al., 2016).

Fishes represent great taxonomic and ecological diversity, yet our understanding of the functional significance of their gut microbiota lags behind that of terrestrial vertebrates (Clements et al., 2014). Furthermore, the direct contribution of endogenous microbiota to fish health has been underestimated

(Gómez and Balcázar, 2008). Recent microbiome research in a variety of teleost species is transforming our understanding of how gut microbiota can affect host development, ecology and evolution (Sullam et al., 2015); however, for important aquaculture species this research has proceeded slowly (Llewellyn et al., 2014). *Oncorhynchus mykiss* (i.e., rainbow trout that exhibit a freshwater resident life history and steelhead that experience an anadromous life history) are native to waterways of the Western United States (Behnke, 2002). They are a vital species from an aquaculture perspective, as their once-abundant wild populations have been in dramatic decline since the 1950's due to habitat destruction and commercial overexploitation. Many efforts have been taken to restore dwindling populations (Araki et al., 2007; Schmid and Araki, 2010), including the annual release of hundreds of thousands of hatchery-reared fish into the wild (i.e., hatchery stocking) to enhance fish populations for commercial, recreational and conservation interests. Hatchery environments provide researchers valuable *in situ* locations to test various perturbations of diet and rearing conditions in a controlled setting, and are designed to produce large numbers of big fish (Araki et al., 2007; Brown et al., 2003). However, results of this practice are mixed, as return rates for hatchery fish are half those of wild fish (Chilcote, 2003; Schmid and Araki, 2010). Hatchery systems are nonetheless lauded as success stories by federal

agencies and related commercial interests. Elucidating reasons for the difference in return rates remains a critical question for members of the aquaculture community as well as conservationists, and research into the gut microbiota of hatchery-reared teleosts is an attractive avenue for exploring the health and subsequent success of fish in the wild across phylogenetic and geographical locations.

Hatchery stocks are often maintained in their geographic region of origin and released at a local level. Released steelhead undergo a process of smoltification (Nichols et al., 2008) during their downstream migration in preparation for life in the marine environment, and eventual return to their natal streams for reproduction. For hatcheries, returning fish are captured as adults and artificially spawned, with the offspring reared in the same local hatchery environment before release and re-initiation of the cycle. In the Pacific Northwest, there are two distinct phylogenetic populations of native rainbow trout/steelhead separated by the Cascade Mountain range (Behnke, 2002; Brown et al., 2004). Across this divide, coastal rainbow trout (*O. m. irideus*) reside west of the Cascades, and the inland rainbow trout (*O. m. gairdneri*) on the east (Figure 2). Hatcheries found east of the Cascade Divide raise fish native to eastern waterways, and western hatcheries follow suit.

In an effort to close the gap between intestinal microbiota physiological effects on rainbow trout we have analyzed hatchery-raised *O. mykiss* from across this phylogenetic divide. Specifically, we identified patterns in gut microbiota composition and function in fish sampled from Northern Oregon and Southern Washington. Significant differences in core microbiota taxonomy and functional pathways related to lipid metabolism were found across the known phylogeographic divide of anadromous rainbow trout.

2.2 Materials and Methods

2.2.1 Sample collection and dissection of intestine

Research fish were collected from six hatcheries across Northern Oregon and Southern Washington (Irrigon, Oak Springs, Round Butte, Skamania, Cowlitz and Cedar Creek) located both east and west of the Cascade Mountain Divide, in order to represent two distinct clades of steelhead found in the Pacific Northwest (Behnke, 2002; Brown et al., 2004). Hatcheries on both sides of the Cascades rear *O. mykiss* in concrete raceways fed with circulating fresh water from local sources nearby the hatchery. Hatchery raceways are a consistent size and contain similar densities of fish regulated by the Department of Fish and Wildlife. Irrigon (east) and Cowlitz (west) hatcheries utilize well water; Cowlitz uses an ozone purification process for well water during the months of May through November

to reduce pathogens associated with higher water temperatures. Skamania and Cedar Creek (west) both use river water from the same systems into which their hatchery fish are released. Oak Springs and Round Butte hatcheries (east) have unique water sources; Oak Springs is fed with local spring water and Round Butte is supplied via seepage tunnels in the rock canyon wall abutting the hatchery that contains an upstream reservoir. Water samples were gathered from each hatchery, but did not constitute a high enough water volume to successfully capture microbial content. Monthly health checks are performed at all six hatcheries and if raceways exhibit disease characteristics, oral antibiotics or vaccines are used when necessary. All sample fish were collected from healthy stock.

Diet across all six hatchery locations is highly consistent. Feed used at Skamania (west) and Round Butte (east) hatcheries had a slightly higher lipid content than the feed used at the remaining hatcheries. Ewos Transfer feed used at Skamania and Round Butte contains 48% protein, 24% lipids, 1.5% fiber and 1.3% phosphorus. BioClark's fry brand of feed used at the remaining four hatcheries contains 47% protein, 18% lipids, 1.5% fiber and 1.3% phosphorus. Each brand of feed was administered to fish at hatcheries east and west of the Cascade Mountains, likely mitigating any direct effect lipid content in feed may have on

gut microbiota identified in these two distinct regions. Hatchery information is listed in Table 1.

Ten fish were harvested from each hatchery location and were dispatched with either a sharp blow to the head or with the anesthetic MS-222 (Wilson et al., 2009). The fish were put immediately on ice and transferred to the lab for dissection within 3-6 hours. Upon arrival at the lab, remaining sample fish were kept on ice while the intestinal tract was removed from one sample at a time and its contents collected. All instruments were autoclaved prior to use and were cleaned of debris, rinsed and then soaked in ethanol for 2 minutes in between each dissection. The ventral surface of each fish was wiped with ethanol before incision along the length of the belly from just behind the pectoral fin back to the anal opening. Each intestine was severed near the pyloric sphincter, distal to the stomach and caecae, and approximately 2-3 centimeters proximal to the anal opening. The intestine was removed to a clean surface, and sterile tweezers were used to hold the intestine over a sterile 2ml tube while the gut contents were gently squeezed from the intestine into the tube.

2.2.2 DNA Extraction

Microbial DNA was extracted from the gut contents of each individual sample fish using the Mobio PowerLyzer™ PowerSoil® DNA isolation Kit (MO

BIO Laboratories, Carlsbad, CA) using the standard DNA extraction protocol, with an additional pre-processing step; approximately 200uL of gut contents was added to a clean 2mL micro centrifuge tube, followed by 60uL of cell lysis solution; tubes were gently mixed by shaking, and placed in a water bath of 65°C for ten minutes, followed by a heat plate at 95°C for ten minutes; after this pre-processing heat treatment, the tube contents were transferred to bead-beating tubes and vortexed at 30 cycles/second for 10 minutes (Wesolowska-Anderson and et al., 2014).

1) After vortexing, bead-beating tubes were centrifuged at 13,000rpm for 30 seconds at room temperature. 2) 400-500uL of supernatant was transferred to a clean 2mL collection tube and 250uL of solution 2 was added, which serves to precipitate out non-DNA organic and inorganic matter. Tubes were then vortexed for 30 seconds and incubated at 4°C for 5 minutes. 3) Tubes were then centrifuged at 13,000rpm for 1 minute. 4) Avoiding the pellet, up to 600uL of supernatant was transferred to a clean collection tube. 200uL of solution 3 was added, to precipitate out remaining non-DNA organic and inorganic materials; tubes were vortexed briefly, and centrifuged at 13,000rpm for 1 minute.

5) Avoiding the pellet, up to 750uL of supernatant was transferred to a clean collection tube, and 1200uL of solution 4 was added; tubes were again vortexed

for 5 seconds. Solution 4 is a high concentration salt solution that facilitates DNA binding to the silica filter membrane, allowing contaminants to pass through the filter to be discarded. 6) 675uL of this supernatant mixture was loaded into a provided spin column and centrifuged at 13,000rpm for 1 minute; this step was repeated until all the supernatant was filtered. 7) 500uL of an ethanol-based wash solution (solution 5) was added and tubes were centrifuged at 13,000rpm for 30 seconds. This step cleans the DNA that is bound to the silica filter membrane of the spin column. 8) After discarding flow through, the tubes were again centrifuged at 13,000rpm for 1 minute. Spin columns were then carefully placed into clean collection tubes, taking care not to splash any remaining supernatant solution on the spin column filter. 9) 100uL of sterile elution buffer (solution 6) was added directly to the center of each spin column filter. Tubes were incubated for 1 minute to allow full saturation of the silica filter and then centrifuged at 13,000rpm for 1 minute. 10) Spin columns were then discarded and eluted DNA was stored at room temperature for 24 hours prior to quantification. Eluted DNA was quantified on a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Following quantification, samples were stored at -20°C until sequencing.

2.2.3 PCR validation of 16S rDNA

Extracted DNA was amplified with polymerase chain reaction (PCR) using standard 503F and 806R primers to validate the presence of the V3/V4 variable region of the 16S ribosomal gene. PCR products were visualized with gel electrophoresis. The expected product length of this region is approximately 300 base pairs, and bands were found of this length using a 1000KB ladder.

2.2.4 Sequencing and Library Preparation

Eluted DNA was normalized to 2-10 ng/uL in preparation for sequencing on the Illumina MiSeq platform at the Genome Sequencing and Analysis Facility (GSAF) at the University of Texas at Austin. 25 uL of DNA at 2-10 ng/uL was dispensed into sterile 1.5mL micro centrifuge tubes for each of 60 samples, packed with dry ice and sent overnight to GSAF. The V4 hypervariable region of the 16S gene was amplified using the following primers: 515F (GTGYCAGCMGCCGCGGTA) and 806R (TAATCTWTGGGVHCATCAGG), generating a product with a length of 292 base pairs (Table 2). Additional barcode primers were affixed to multiplexed DNA sequences in order to bin them by sample in later analysis steps. PCR was performed in triplicate for each sample and re-pooled for sequencing on the MiSeq platform. Library preparation involved a two-step PCR with dual indexes. The V4 region was targeted to minimize contributions of the teleost host.

2.2.5 Initial processing and quality filtering of 16S sequence data

All sequence data received from GSAF was processed using a microbial community sequence analysis pipeline called QIIME (Quantitative Insights into Microbial Ecology) (Caporaso et al., 2010). QIIME was designed to generate lists of Operational Taxonomic Units (OTUs) from extremely large datasets and to perform both phylogenetic and non-phylogenetic analyses based on the OTUs identified. GSAF generated 250 bp paired-end sequences, which were joined prior to quality filtering. Sequence quality checks were then performed with both custom and default parameters, chimeras were removed, and multiplexed reads were assigned to samples based on their nucleotide barcode. QIIME performs quality filtering based on the characteristics of each sequence, removing any low quality or ambiguous reads. A custom Phred score of 30 was used to filter raw sequence data, indicating that the probability of an incorrect base call is 1 in 1000, or that 99.9% were correct. QIIME pre-processing of sequence data removed all singleton and doubleton sequences and performed Sequence, UniFrac and OTU based clustering of samples from which the sequences were obtained, using UCLUST and a 97% similarity threshold. After filtering steps, 2,367,849 total reads were obtained from the 2,411,877 raw reads generated by the GSAF on the Illumina MiSeq platform. The pick_open_reference_otus step in QIIME pipeline

was used to assign sequences to microbial lineages using the Greengenes reference database for known taxa, and *de novo* clustering of taxa not found in the reference database. Both open-reference and closed-reference OTU tables were generated for various analyses carried out in this study. Closed-reference OTU tables contain taxa identified in the Greengenes 13_5 or 13_8 databases.

2.2.6 Statistical analysis of alpha and beta diversity

A suite of alpha and beta diversity analyses was performed on all samples using the QIIME script core_diversity_analysis.py. Alpha diversity, or within-sample diversity, provides estimates of richness and diversity within the study population. QIIME default metrics were used and include Chao1, which assesses microbial species richness, the Observed Species metric, which counts the number of unique OTUs found within each sample, and the Phylogenetic Diversity (PD Whole-Tree metric, which measures the minimum total length of all the phylogenetic branches required to span a given set of taxa rather than comparing "species" designations. Rarefaction analyses assessed sequence coverage and were sampled at a depth of 1000 sequences, with 48 out of 60 total samples containing a minimum of 1000 sequences per sample. Beta-diversity, or between-sample diversity, was calculated to compare gut microbiomes of all samples originating from different hatcheries, and between east and west locations. Weighted UniFrac

is a phylogenetically based qualitative metric that accounts for the abundance of observed organisms and is useful for revealing community differences relative to changes in taxon abundance. Unweighted UniFrac, a quantitative metric based on absolute abundance, measures the presence/absence of taxa between samples.

2.2.7 Identifying a core microbiome

The compute_core_microbiome.py script in QIIME was used to identify a "core" set of microbiota shared among a chosen percentage of samples within a given metadata category. In this study, to be considered a member of the core microbiota, a taxon must have been present in 80% of samples from each hatchery and from locations east or west of the Cascade Mountains. Establishing a "core" microbiome is useful for identifying patterns among gut microbial communities across the most samples possible, while capturing as much variation as is possible within the confines of the membership parameters. Alpha and beta diversity analyses for the core microbiota were conducted with the QIIME script core_diversity_analysis.py. Diversity analyses were conducted on the core microbiota constituents, at a depth of only 20 sequences to accommodate extreme variation in number of microbial DNA sequence reads identified across hatchery samples (WSK07 had 12 sequences, while EOS04 had 171,926 sequences). Non-phylogenetic metrics were used and included Bray Curtis, Observed OTUs and

Chao1, generating a basic picture of core microbiota community structure and diversity. The Bray Curtis dissimilarity index used count data to quantify the compositional differences of bacterial communities between samples from each hatchery site. Input for this analysis included OTU tables representing core gut microbiota constituents identified to the genus level within each individual hatchery environment and within each location category east or west of the Cascades.

2.2.8 Predicting functional profiles

PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) is a software package used to estimate the gene families contributed to a metagenome by bacteria identified through the use of 16S rDNA sequencing (Langille et al., 2013). Gene content between closely related organisms is conserved; therefore, by combining the genes present in a common ancestor, along with the information from a fully sequenced genome, a baseline prediction of the gene content of a modern or unstudied organism is possible. From that list of predicted genes, a detailed list of more than 300 specific functional pathways can be identified, generating a picture of metagenomic function for microbiota of interest. For this study 2166 OTUs, or 63% of all OTUs identified by QIIME that matched the Greengenes 13_8 reference database, were uploaded into the

PICRUSt pipeline. 1266 OTUs that clustered *de novo* were removed from OTU tables before functional analysis. The biom_convert command in QIIME was used to produce files compatible with the PICRUSt program.

The modified files were subsequently uploaded to the Galaxy terminal (https://huttenhower.sph.harvard.edu/galaxy/) for processing and analysis. The first step in the PICRUSt pipeline corrects the input OTU table for known 16S rDNA copy numbers for all taxa identified, to better reflect the true organism abundance. Next, a 'virtual' metagenome of KEGG (Kyoto Encyclopedia of Genes and Genomes) ortholog abundances is produced for each sample in the input file. The final step collapses hierarchical data to a specified level, allowing researchers to examine the extent to which genes are involved in multiple pathways and to highlight metagenomic functional roles played by different populations of microbial organisms in a given environment (Kanehisa et al., 2016). Output files from PICRUSt were then visualized using STAMP (Statistical Analysis of Metagenomic Profiles), a graphical interface allowing easy exploration of statistical results highlighting the biological relevance of features in a metagenomic profile (Parks et al., 2014). Two different input files are required as input for the STAMP software program. The first is a profile file containing DNA

sequence count data and the second is a group metadata file pertaining to categories desired for visualization.

2.3 Results

2.3.1 Microbial diversity analysis

Gut microbial DNA was isolated through collection of gut contents from 60 total sample fish collected in the spring of 2015, just prior to release from hatcheries into native streams for eventual migration. A total of 2,411,877 reads were obtained as raw sequence data from the Genome Sequencing and Analysis Facility (GSAF) at the University of Texas, at Austin. 2, 367, 849 reads remained after paired ends were joined and quality filtering was performed (Table 3). A total of 3432 OTUs were assigned at a 97% identity threshold, with 2166 (63%) mapping to the Greengenes 13_8 database and 1266 (37%) clustered *de novo* as New reference or New Clean Reference OTUs by the QIIME open-reference OTU picking method (See supplemental file S1 for a complete list of 3432 OTUs identified). Rarefaction analysis was conducted to a depth of 1000 reads, with 48 out of 60 total samples containing > 1000 sequences per sample. Results indicate that rarefaction curves neared saturation (Figure 3). Cedar Creek Hatchery (west) fish were fasted prior to release and did not yield large quantities of microbial DNA so only two samples from Cedar Creek are included in alpha and beta diversity analyses. Irrigon Hatchery (east) had 6 out of 10 samples containing

adequate read counts. Alpha and beta diversity analyses were therefore carried out with an $n = 48$. (Tables 5 and 6).

2.3.2 Taxonomic identification and diversity analysis of "core" microbiota

For this study, the "core" microbiota are defined as those identified in a minimum of 80% of samples. Microbial constituents common to eight out of ten fish per hatchery and 80% of samples in eastern and western locations provided a clearer representation of overall patterns in microbial community composition across the phylogenetic and geographical divide created by the Cascade Mountain range. Firmicutes was the most abundant phylum across all samples (48.6% of total in the west and 67.6% in the east). Next in abundance in eastern locations was Proteobacteria (13.6%), Actinobacteria (11.4%), Cyanobacteria, a likely contaminant from ingested water (5.3%) and Bacteroidetes (2.2%). Eastern fish, *O. m. gairdneri*, contained a maximum of 5 phyla, 13 families and 15 genera. In contrast, western fish, *O. m. irideus*, contained only 3 phyla, 5 families and 5 genera. Phyla included Firmicutes (48.6%), Bacteroidetes (27.2%) and Actinobacteria (24.2%) (Figures 4 and 5). A complete list of core taxa can be found in Table 8. (Complete OTU tables can be seen in Files S8 and S9).

Beta diversity analysis of core microbiota indicates a significant difference between east and west hatcheries, with a p-value of 0.004(Chao1) and

0.012(Observed-OTUs) (Table 7). A Principal Component Analysis (PCA) was not directly available in QIIME, but a Principal Coordinates Analysis (PCoA) was carried out using a Euclidean Distance Matrix for the core microbiota. Results show clustering of samples into two distinct groupings based on east or west location. (Figure 6). Historically, some Skamania (west) stock have been reared at Oak Springs hatchery, and beta diversity analysis between individual hatcheries east and west of the Cascade Divide did show that Oak Springs fish (east) follow some of the patterns exhibited by Western Cascade fish. However, overall results of comparison between east and west locations were consistent.

2.3.3 Predicted functional metagenomes of east and west rainbow trout intestinal microbiomes

PICRUSt software was used to identify the functional potential of gut microbiome communities belonging to two distinct phylogenetic populations of hatchery-reared steelhead, east and west of the cascade Mountain range. KEGG orthologs were classified at hierarchical levels that included gene subcategories for metabolic function (level 2) and specific metabolic functional pathways (level 3). 13 out of 24 subcategories of metabolic function were found to be significant between the two groups, and included genes associated with cell growth and death (p=0.025), membrane transport (p=0.035) and genetic information processing (p=0.026). (For a full list of significant KEGG level 2 subcategories, see figure 7).

One of the more significant level 2 categories identified included genes associated with lipid metabolism, having a p-value of 0.00120. Delving further, analysis of the KEGG level 3 functional pathways revealed eight of nineteen functional pathways in the category of lipid metabolism were significantly enriched in the gut microbiota of western fish. These are: linoleic acid metabolism (p=0.000527), sphingolipid metabolism (p=0.00173), lipid biosynthesis proteins (p=0.00829), biosynthesis of unsaturated fatty acids (p=0.013), lipopolysaccharide biosynthesis (p=0.021), lipopolysaccharide biosynthesis proteins (p=0.027), fatty acid metabolism (p=0.033) and arachidonic acid metabolism (p=0.049) (Figure 8).

2.4 Discussion

Salmon are one of the most iconic creatures of the Pacific Northwest and have a great economic, recreational, and cultural significance. This monophyletic group includes several closely-related salmonid species including the anadromous life history form of rainbow trout (*Oncorhynchus mykiss*), commonly known as steelhead (Utter and Allendorf, 1994). Despite their monophyletic grouping, the Cascade Mountain range has been shown to separate many salmon species into distinct subspecies, and native steelhead are no exception (Utter et al., 1980) with distinct subspecies found east and west of the Cascade Divide. *O. m. irideus*, named for the Greek goddess of rainbows (*iris*), occupy the coastal drainages west of the

Cascade Mountains and are the primary source of rainbow trout in worldwide aquaculture. The historical natural distribution of this subspecies extended from the Alaskan Peninsula to the northernmost rivers of Baja Mexico. In the Columbia River basin, *O. m. irideus* is native inland to the Cascade Range and has been observed since the mid to late-19th century when artificial propagation began. On the east side of the Cascade Range, the "redband" or inland rainbow trout (*O. m. gairdneri*) are native residents and have been described since the 1940's as far east as Southern Idaho (Behnke, 2002). The native ranges of these two subspecies can be seen in Figure 2.

The longstanding phylogenetic divide between these two populations of *O. mykiss* provided opportunities for coevolution between the genomes and the microbiomes of individuals. Ample research has been conducted regarding coevolution between animals and their external environments (Palkovacs et al., 2009; Thompson, 1994; Vermeij, 1994), but interactions between an organism and its internal environment (i.e., microbiota) are less clear. It is known that symbiotic bacteria help host organisms acquire nutrients (Sullam et al., 2012). In addition, gut microbiota are able to modulate aspects of host metabolism. For example, gut microbiota have been shown to stimulate fatty acid uptake and lipid droplet formation in the intestinal epithelium and liver increasing bioavailability of lipids

for teleost species (Semova et al., 2012). Additionally, the term "metagenome" describes the combination of host genes and the genes present in trillions of gut microbes that colonize vertebrates, with the vast majority inhabiting the distal gut. This metagenome provides researchers an excellent opportunity to explore the reciprocal adaptations occurring between interacting species that produce physiological changes which can affect metabolic functions in the host (Ley et al., 2008).

Our analysis of intestinal tract 16S rDNA microbial sequence data from hatchery-reared steelhead identified abundant inter-individual taxonomic diversity. Using an 80% minimum occurrence of bacterial taxa among all samples we initially established a "core" microbiota which revealed significant differences among populations east and west of the Cascade Mountain phylogeographic divide. Steelhead sampled from hatcheries east of the Cascades exhibited dramatically higher core gut microbiota diversity than was found in fish from the west side of the mountains (Figures 4 and 5), despite the similarities in diet and rearing conditions revealed in Methods section 2.2.1. Hatcheries on both sides of the Cascades rear *O. mykiss* in similar environments and use fish meal feed with nearly identical components. Historically, introgression of brood stock from Oak Springs (east) with brood stock from Skamania (west) has previously occurred.

Despite this introgression, analysis indicates that Oak Springs hatchery continues to group with the east, suggesting limited levels of genetic transfer.

Looking further at the core microbiota found in 80% of east and west populations, differences emerged as more specific phylogenetic levels were explored. At the family and genus levels, eastern populations contained more than twice the abundance and variety of core microbial taxa as western fish (Tables 8 and 9). Diversity of microbial constituents is often correlated with improved health in humans (Claesson et al., 2012); therefore, identification of microbial community diversity across subspecies of rainbow trout can provide clues as to how to improve the health and success of hatchery fish post-release. Studies of gut microbiota and their relationship to host health is a valuable avenue of research for both aquaculture and conservation interests.

Yet, taxonomic variation is not the only story being told by the commensal gut microbiota of hatchery-reared steelhead. Further analysis of the data using PICRUSt, a gene prediction software that utilizes KEGG ortholog genes and functional pathway information, identified functional differences between the gut microbiota of eastern and western hatchery populations. PICRUSt compiles KEGG ortholog data into three main hierarchical categories. At the most general level, level one, 5 categories are identified: metabolism, genetic information

processing, environmental information processing, cellular processes and human diseases. These are further subdivided into level two which contains 24 sub-categories, and level three containing 330 individual metabolic pathways. At KEGG level two, one of the sub-categories most significantly different between east and west was lipid metabolism, with a p-value of 0.00120 (Figure 7). Gut microbiomes found on the west side of the Cascades showed significant enrichment of lipid metabolism genes, indicating a possible difference at the organismal level regarding how lipids are used by both the host and the microbiota. Lipids are a vital nutritional component for teleost species involved in migration and subsequent return for spawning in natal environments. Lipid content is also an important contribution to species valuable from an aquaculture perspective. For example, wild Copper River Sockeye salmon are highly prized due to their high lipid content derived from their unique geographical range and long inland migration. The Copper River stretches more than 300 miles, and requires the sockeye to rely on large fat reserves to fuel the arduous journey involved in their return to natal streams to spawn (Estabrook, 2010). These fat stores add market value to the sockeye as a commercial species.

Examining lipid metabolism functions of gut microbiota found east and west of the cascades in more depth, analysis of the KEGG level 3 functional

pathways indicates 8 of 19 pathways involved in lipid metabolism are more highly enriched in western populations than in eastern populations (see figure 8), with p-values ranging from 0.000527 for linoleic acid metabolism to 0.049 for arachidonic acid metabolism. Identifying this enrichment of metabolic pathways related to lipid metabolism, our research suggests that western host fish may not require as many lipids from the diet. This reduced competition with their own endogenous gut microbiota for access to those lipids could result in a higher abundance of lipid metabolism genes in the gut microbiota to process lipids unused by the host. Eastern fish, with their longer migration distances, require lipids from the diet for energy storage and may be outcompeting their gut microbiota for access to dietary lipids. Additionally, coevolution over time between these longer-migrating fish and their gut microbiota may have selected for a microbiome less dependent on lipids. The fact that metabolic pathways related to membrane transport of carbohydrates were significantly enriched in eastern fish, but not in the west, supports this conclusion.

We hypothesize that the observed differences in lipid metabolism across this phylogenetic divide results from an increased ability of eastern Cascade (*O. m. gairdneri*) to absorb and utilize lipids taken in via the diet than western Cascade fish, making lipids less available for the intestinal microbiota. Salmonids returning

to spawn do not eat once they return to freshwater environments, depending instead on fat stores to fuel their journey. Thus, the ability to absorb lipids is a vital factor involved in the success of longer-migrating populations (Crossin et al., 2004). West of the Cascades, individuals (*O. m. irideus*) appear not to require as high a lipid content due to their shorter migration distances and may not need large lipid reserves for the return trip to spawn, making dietary lipids more available for utilization by the gut microbiota. Future research is needed to measure differences in lipid content between east and west fish and to further explore how coevolution between host and gut microbiota may affect uptake of dietary components related to hatchery fish health and success post-release. This exploration of interaction between the gut microbiome and the host genome creates a valuable opportunity for aquaculture interests to find fish that have this unique genetic background allowing individuals to retain more lipids from the diet. If it is possible to select fish for this specific genetic and microbial profile, without changing diet requirements or rearing conditions, these fish can serve as source populations with a preferred lipid metabolism pattern that can be selected for artificially. This advance would increase the nutritional profile of commercially reared steelhead and increase the availability of this desired phenotype regardless of hatchery location.

Our research utilizes information from the microbiome to understand the phenotypic implications occurring in segregated populations of hatchery-reared steelhead, further confirming elements of coevolution between an organism and its internal microbiome. This vital piece of the puzzle aids our understanding of how an individual's microbiome helps them succeed differently in divergent geographic and phylogenetic environments and how mechanisms at work in the gut microbial ecosystem truly affect fish health and success.

Chapter Three

Conclusions

Intestinal microbiota impact the health and development of their hosts. In Oregon and Southern Washington, our analysis indicates that the two distinct clades of hatchery-reared *Oncorhynchus mykiss* (*O. m irideus* in the west and *O. m. gairdneri* in the east) exhibit significant variation for gut microbiota composition. The observed differences occur in groups east and west of the Cascade Mountain range, mirroring the phylogenetic divergence between the two populations evidenced in earlier research of both nuclear and mitochondrial genomes. A higher degree of taxonomic diversity and richness was found in the core gut microbiota of hatchery reared fish native to the east side of the mountains.

Determining effects of microbial taxonomic variation on host fish depends on understanding environmental variables that exist between hatchery settings. One significant variable between hatcheries is early exposure to communal water. Future investigation of the bacterial content of hatchery water could clarify when and how host fish are inoculated with their endogenous gut microbiota. Moreover, temporal studies could determine if the core gut microbiota differences identified in this study are stable over time and consistent across phylogeographic environments, irrespective of variable hatchery conditions.

In addition to taxonomic variation, lipid metabolism pathways among the gut microbiome of eastern populations were lower, paralleling an increased need

for host lipids required for longer migration distances to the ocean compared to Western Cascade fish. Future research measuring lipid content of both eastern and western hatchery populations could support this migration distance and lipid requirement hypothesis. To better understand functional differences between eastern and western hatchery salmonids, a metagenomic investigation of proteins and metabolites found in the gut environment could reveal the activity of microbiota with lipid metabolism pathways enriched or reduced in a given population, not just the presence or absence of these genes in the microbiome itself. Understanding the activity of lipid metabolism genes in gut microbiota inhabitants could allow conservationists and aquaculturists to artificially select for steelhead stock with a genetic and microbial profile more conducive to increased lipid uptake by the host. Such stocks would have increased commercial value due to their higher lipid content.

Further investigation of wild steelhead from the same river systems offers future researchers an invaluable source of information into the connection between gut microbiota and return rates of wild and hatchery fish. Identifying taxonomic and functional characteristics endemic to the gut microbiota of wild fish could vastly improve understanding of why wild fish are more successful than hatchery reared fish in wild environments.

Hatchery	Address	Phone	Contact
Irrigon Hatchery, OR	74135 Riverview Lane Irrigon, OR 97844	(541) 922-5732	Marc Garst
Oak Springs Hatchery, OR	85001 Oak Springs Road Maupin, OR 97037	(541) 325-5327	Craig Banner
Round Butte Hatchery, OR	6825 SW Belmont Lane Madras, OR 97741	(541) 395-2546	Craig Banner
Cedar Creek Hatchery, OR	33465 Hwy 22 Hebo, OR 97122	(503) 392-3485	Joe Holbert
Cowlitz Hatchery, WA	165 Osprey Lane Toledo, WA 98591	(360) 864-6121	Clint Fitch
Skamania Hatchery, WA	391 Steelhead Road Washougal, WA 98671	(360) 837-3131	John Aleckson

Hatchery	State	# stocks of summer steelhead	Adults collected/ spawned at the hatchery?	Rivers of origin for hatchery broodstock	Hatchery location (east or west of Cascades)
Irrigon	OR	2	No	Imnaha R. Wallowa R.	East
Oak Springs *	OR	2	No	Deschutes River, and Skamania Hatchery	East
Round Butte	OR	1	Yes	Deschutes River	East
Cedar Creek	OR	2	Yes	Nestucca R. Siltez R.	West
Cowlitz	WA	1	Yes	Cowlitz River sub-basin	West
Skamania	WA	1	Yes	West Fork Washougal River	West

***** Includes some Skamania Hatchery stock, from west side of Cascades

Sources :

http://wdfw.wa.gov/hatcheries/hgmp/pdf/lower_columbia/cowlitz_summer_sthd_2014.pdf
http://wdfw.wa.gov/hatcheries/hgmp/pdf/lower_columbia/ssthd_eflewis_2012_final.pdf

Table 1: Hatchery Information

Primers

Hyb515F_rRNA: 5′- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GTGYCAGCMGCCGCGGTA** -3′
Hyb806R_rRNA: 3′- **TAATCTWTGGGVHCATCAGG**GACAGAGAATATGTGTAGAGGCTCGGGTGCTCTG -5′

rRNA gene-specific sequences in bold and underlined; remainder are Illumina platform-specific sequences.
These primers amplify a 292 base pair region of the 16S gene in *E. coli*.

Table 2: Primers used, V4 region of 16S rDNA for Illumina MiSeq Platform

east hatcheries	sample ID	raw sequence count	processed sequence count	west hatcheries	sample ID	raw sequence count	processed sequence count
Irrigon	EIR01	147,054	147,014	**Cedar Creek***	WCC01	129	124
	EIR02	83,644	83,009		WCC02	258	249
	EIR03	16,747	16,668		WCC03	442	437
	EIR04	428	421		WCC04	1,397	1,485
	EIR05	91,058	90,948		WCC05	244	239
	EIR06	11,620	11,539		WCC06	66	66
	EIR07	525	510		WCC07	621	615
	EIR08	62,016	61,978		WCC08	395	393
	EIR09	903	870		WCC09	1,138	1,084
	EIR10	618	604		WCC10	912	861
Oak Springs	EOS01	11,632	11,498	**Cowlitz**	WCH01	38,421	37,679
	EOS02	10,418	10,318		WCH02	1,849	1,781
	EOS03	147,995	147,902		WCH03	9,660	9,394
	EOS04	177,623	177,395		WCH04	59,161	58,209
	EOS05	94,929	94,851		WCH05	65,450	65,150
	EOS06	101,107	101,076		WCH06	117,452	117,240
	EOS07	80,680	80,648		WCH07	19,501	19,472
	EOS08	67,673	67,646		WCH08	6,167	1,683
	EOS09	110,200	110,103		WCH09	2,024	2,002
	EOS10	49,984	49,456		WCH10	10,653	8,626
Round Butte	ERB01	4,402	4,087	**Skamania**	WSK01	11,326	10,630
	ERB02	22,705	21,346		WSK02	70,001	69,905
	ERB03	23,911	23,448		WSK03	9,534	9,095
	ERB04	7,656	7,215		WSK04	99,370	99,222
	ERB05	6,323	6,145		WSK05	82,677	82,587
	ERB06	9,743	9,038		WSK06	5,961	5,180
	ERB07	6,507	6,402		WSK07	149,476	149,396
	ERB08	23,757	23,053		WSK08	30,438	26,041
	ERB09	44,731	39,943		WSK09	109,665	99,453
	ERB10	3,980	3,716		WSK10	87,160	80,804

***** (Cedar Creek fish were fasted for 2 days in preparation for release prior to sampling)

Total raw sequence count: 2,411,877
Total processed sequence count: 2,367,849

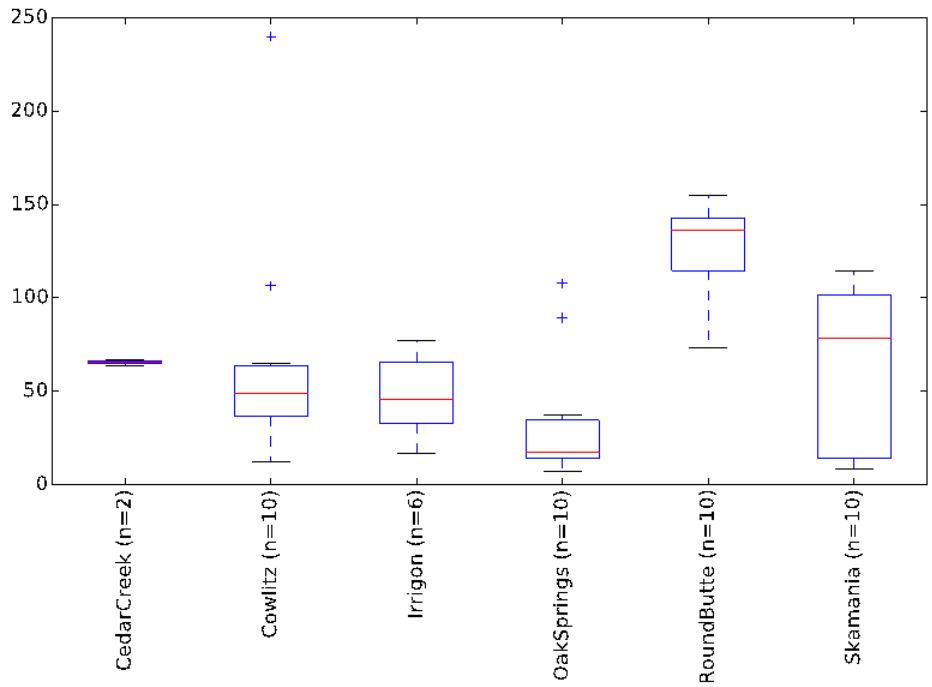
Table 3: Sequence read counts, raw and processed

east hatcheries	sample ID	number of taxa present	west hatcheries	sample ID	number of taxa present
Irrigon	EIR01	342	**Cedar Creek**	WCC01	**20**
	EIR02	437		WCC02	31
	EIR03	230		WCC03	55
	EIR04	56		WCC04	70
	EIR05	280		WCC05	39
	EIR06	169		WCC06	22
	EIR07	87		WCC07	55
	EIR08	150		WCC08	43
	EIR09	127		WCC09	70
	EIR10	88		WCC10	68
Oak Springs	EOS01	270	**Cowlitz**	WCH01	**620**
	EOS02	200		WCH02	81
	EOS03	202		WCH03	418
	EOS04	258		WCH04	226
	EOS05	167		WCH05	315
	EOS06	89		WCH06	160
	EOS07	126		WCH07	111
	EOS08	102		WCH08	61
	EOS09	250		WCH09	69
	EOS10	140		WCH10	134
Round Butte	ERB01	223	**Skamania**	WSK01	172
	ERB02	290		WSK02	133
	ERB03	256		WSK03	130
	ERB04	267		WSK04	151
	ERB05	214		WSK05	119
	ERB06	310		WSK06	187
	ERB07	147		WSK07	117
	ERB08	301		WSK08	397
	ERB09	402		WSK09	607
	ERB10	211		WSK10	532

(Minimum and maximum in **bold**)

Number of samples: 60
Min: 20 Max: 620 Median: 155.5 Standard Deviation: 137.896

Table 4: Counts of unique taxa present, per sample



Group1	Group2	Group1 mean	Group1 std	Group2 mean	Group2 std	t stat	p-value
Cedar Creek	Round Butte	65.45	1.55	126.06	24.657	-3.172	0.075
Oak Springs	Round Butte	34.17	33.485	126.06	24.657	-6.629	0.015
Cowlitz	Oak Springs	68.04	62.437	34.17	33.485	1.434	1
Cowlitz	Skamania	68.04	62.437	63.26	43.130	0.188	1
Cowlitz	Round Butte	68.04	62.437	126.06	24.657	-2.592	0.225
Cedar Creek	Oak Springs	65.45	1.55	34.17	33.485	1.205	1
Oak Springs	Skamania	34.17	33.485	63.26	43.130	-1.598	1
Round Butte	Skamania	126.06	24.657	63.26	43.130	3.792	0.06
Cowlitz	Cedar Creek	68.04	62.437	65.45	1.55	0.0535	1
Oak Springs	Irrigon	34.17	33.485	47.7	21.277	-0.830	1
Cedar Creek	Skamania	65.45	1.55	63.26	43.130	0.065	1
Cowlitz	Irrigon	68.04	62.437	47.7	21.277	0.721	1
Cedar Creek	Irrigon	65.45	1.55	47.7	21.277	1.020	1
Round Butte	Irrigon	126.06	24.657	47.7	21.277	6.053	0.015
Skamania	Irrigon	63.26	43.130	47.7	21.277	0.772	1

Table 5: Alpha diversity statistics, all observed OTUs

	Weighted Unifrac	Unweighted Unifrac
Group 1	all within hatchery	all within hatchery
Group 2	all between hatchery	all between hatchery
t statistic	-9.43430990842	-7.597010624
parametric p-value	2.17436844882e-20	6.35E-14
parametric p-value (Bonferroni-corrected)	5.50115217551e-18	1.61E-11
nonparametric p-value	0.001	0.001
nonparametric p-value (Bonferroni-corrected)	0.253	0.253

Table 6: Beta diversity statistics, all observed OTUs

Location statistics	Chao1	Observed OTUs
Group 1	west	west
Group 2	east	east
Group 1 mean	3.47857142857	3.35
Group 1 std	1.02106636935	0.962326051072
Group 2 mean	6.38088888889	4.873333333333
Group 2 std	4.0589578286	2.73738723766
t stat	-3.61267177551	-2.73976055992
p-value	0.004	0.012

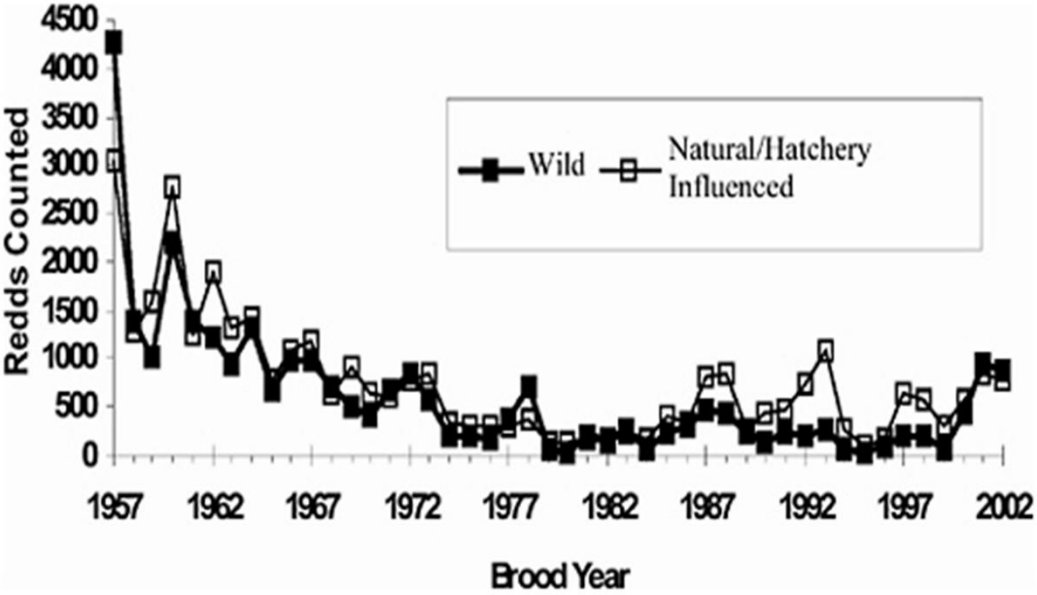
Table 7: Core microbiota non-phylogenetic beta diversity statistics

East Hatcheries: Irrigon, Oak Springs, Round Butte	% abundance phylum level
Firmicutes k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__; g__; s__ k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Enterococcaceae; g__Enterococcus; s__ k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Streptococcaceae; g__Streptococcus; s__ k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Peptostreptococcaceae k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Clostridiaceae; g__Clostridium; s__ k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Peptostreptococcaceae; g__; s__ k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Clostridiaceae; g__Clostridium; s__butyricum k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus; s__ k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Streptococcaceae; g__Streptococcus; s__ k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Staphylococcaceae; g__Staphylococcus k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Streptococcaceae; g__Lactococcus; s__ k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus; s__ k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__; g__; s__	67.6
Proteobacteria k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rickettsiales; f__mitochondria	13.6
Actinobacteria k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Propionibacteriaceae; g__Propionibacterium; s__acnes k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Corynebacteriaceae; g__Corynebacterium; s__	11.4
Cyanobacteria (likely contaminant) k__Bacteria; p__Cyanobacteria; c__Chloroplast; o__Streptophyta; f__; g__; s__	5.3
Bacteroidetes k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Bacteroidaceae; g__Bacteroides; s__fragilis	2.2

Table 8: Table of core microbiota constituents (80%) East

West Hatcheries: Cedar Creek, Cowlitz, Skamania	% abundance phylum level
Firmicutes k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Clostridiaceae; g__Clostridium; s__ k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Staphylococcaceae; g__Staphylococcus k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Streptococcaceae; g__Lactococcus; s__	48.6
Bacteroidetes k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Bacteroidaceae; g__Bacteroides; s__fragilis	27.2
Actinobacteria k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Propionibacteriaceae; g__Propionibacterium; s__acnes	24.2

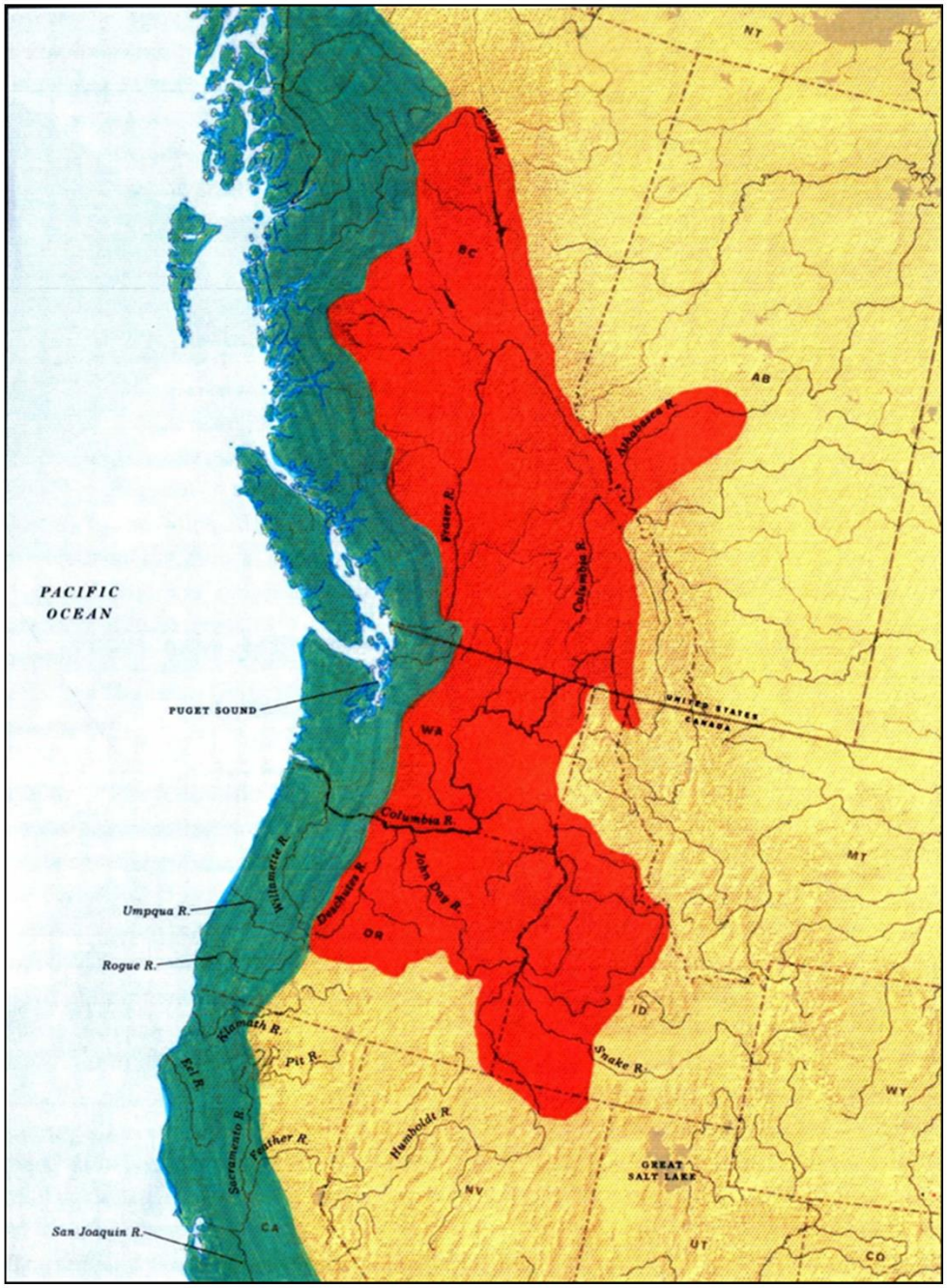
Table 9: Table of core microbiota constituents (80%) West



Number of combined spring and summer Chinook redds (thousands) counted in Salmon River drainage, wild and natural/hatchery-influenced trend areas, 1957-2002.

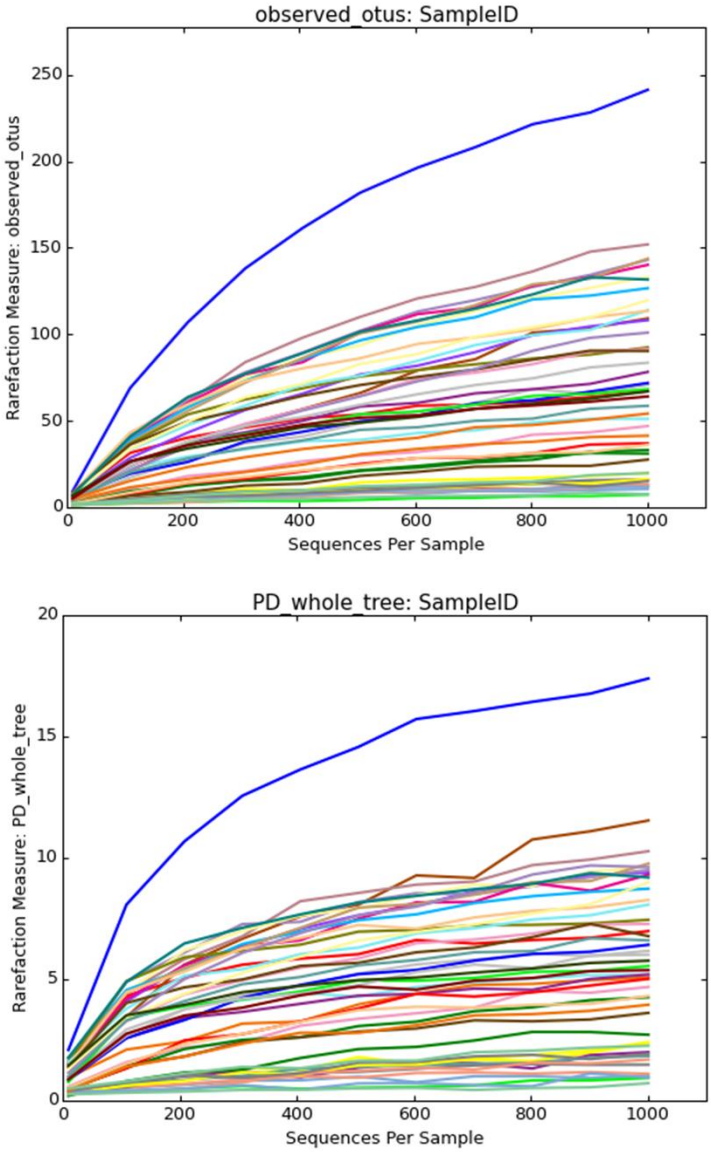
Source : https://www.nap.edu/read/10962/chapter/6#80

Figure 1: Redd Counts



Green: *O. m. irideus* Red: *O. m. gairdneri*

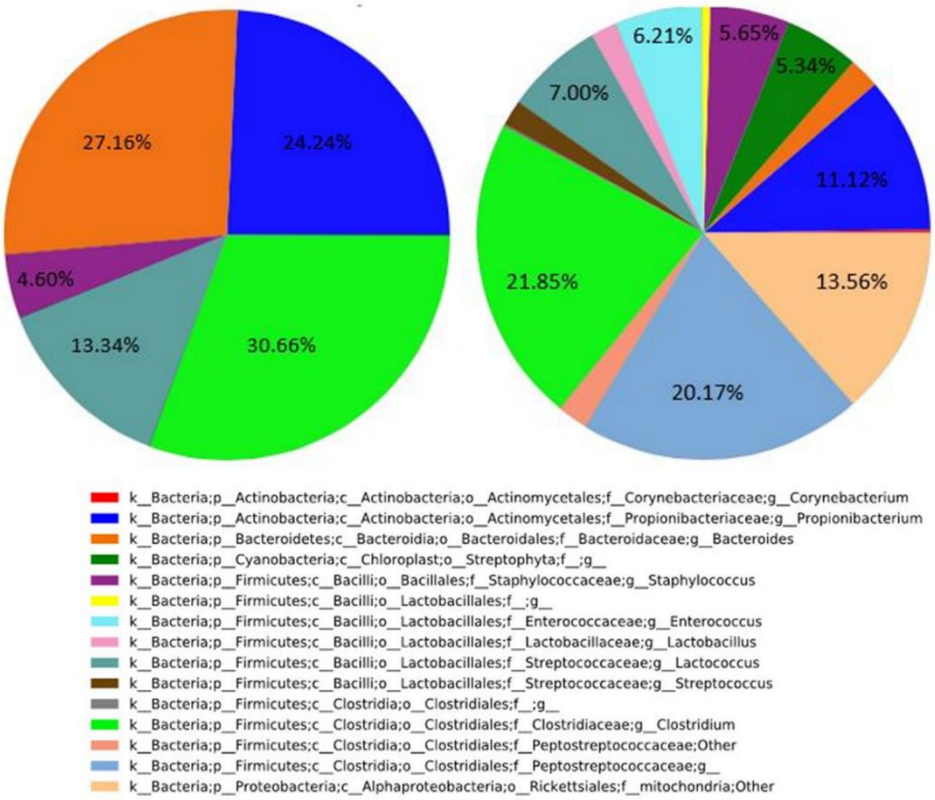
Figure 2: Native range of *Oncorhynchus mykiss* sub-species in Pacific Northwest (modified from Behnke, 2002, page 84)



Figures generated at a sampling depth of 1000 Sequences. Each colored line represents a single sample. n = 48 out of 60 total samples.

Figure 3: Rarefaction curves, all observed OTUs

West Hatcheries: Cedar Creek, Cowlitz, Skamania	East Hatcheries: Irrigon, Oak Springs, Round Butte



(Unannotated regions < 3.0% abundance)

Figure 4. Mean relative % sequence abundance of core microbiota shared between 80% of hatchery samples, genus level

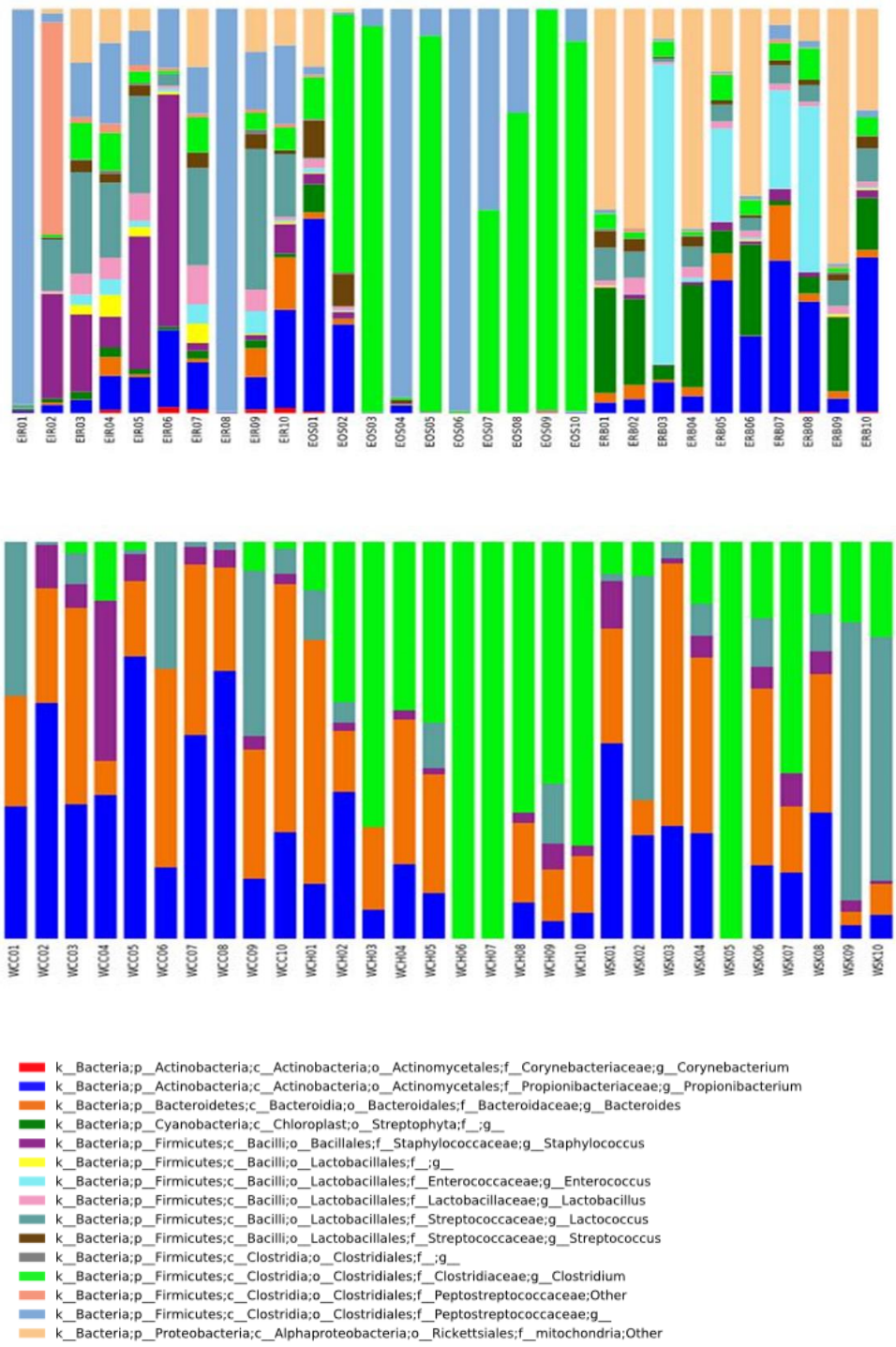
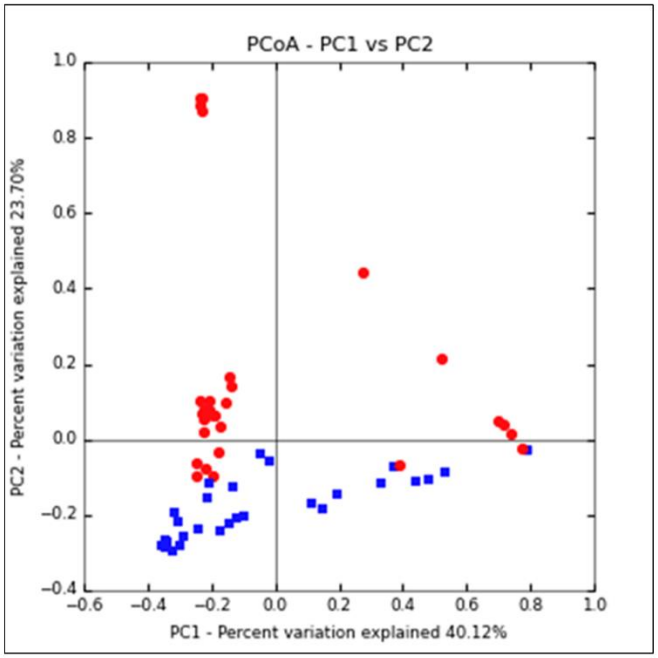
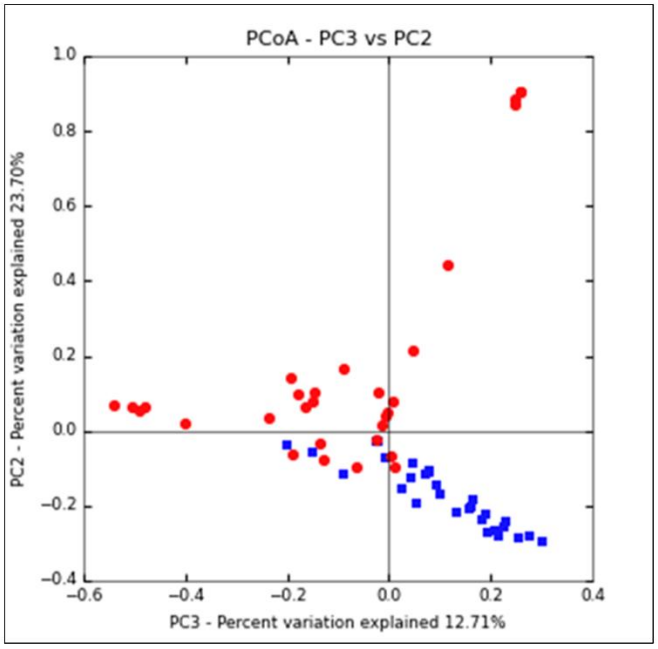


Figure 5: Core microbiota (80%) bar charts, per sample (east: top, west: bottom)





Principal Component Analysis (PCA) done with QIIME using a Principal Coordinates Analysis (PCoA) and a Euclidean distance matrix. Colors signify east and west populations

Figure 6: Principal Component Analysis (PCA) plots, core microbiota

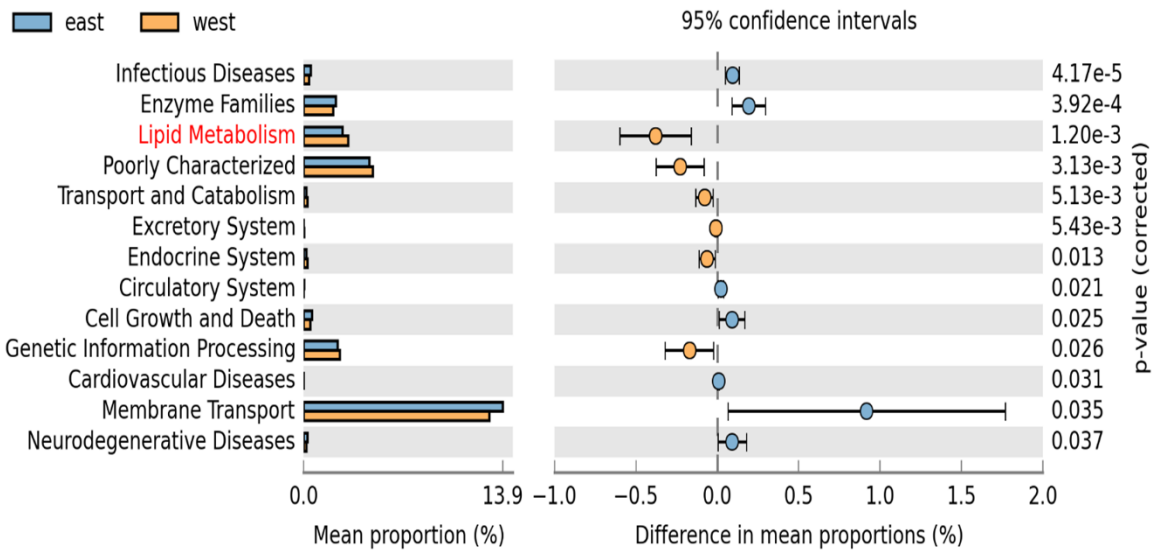
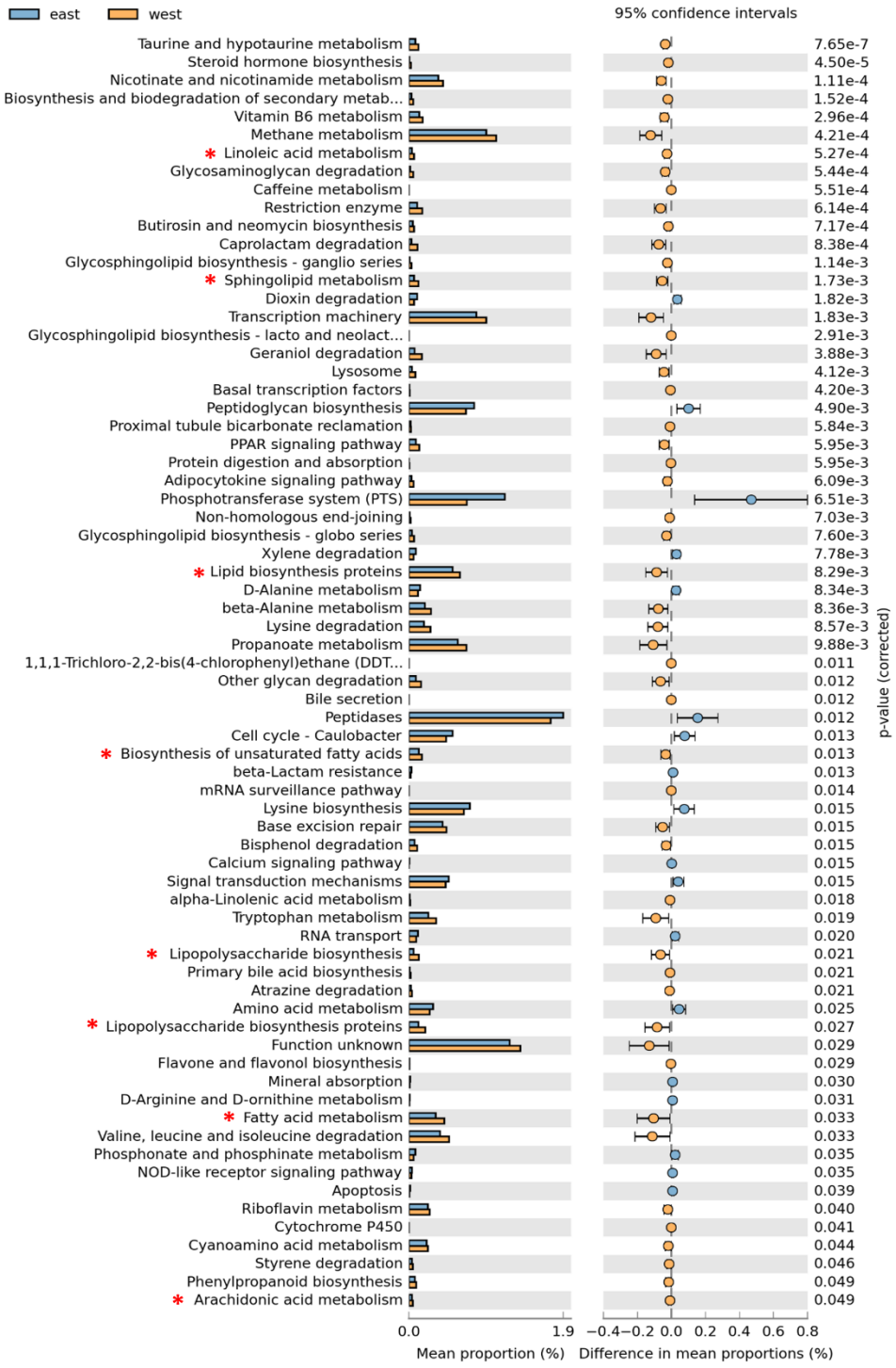


Figure 7: Extended error bar, KEGG level 2 significant sub-categories



(red asterisks indcate lipid metabolism pathways)

Figure 8: Extended error bar, KEGG level 3 significant functional pathways

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Appendix

Supplemental Files

All supplemental files are Excel documents and must be opened through the Excel Program.

S1. 3432_total_OTUs.xml…… 10MB

S2. all_taxa_phylum.xml……... 116KB

S3. all_taxa_class.xml……..... 266KB

S4. all_taxa_order.xml……... 483KB

S5. all_taxa_family.xml…… 933KB

S6. all_taxa_genus.xml…… 1.7MB

S7. all_taxa_species.xml……..… 2MB

S8. core_microbiota_east.xml……..... 38KB

S9. core_microbiota_west.xml……..… 14KB