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Transgenerational effects on the microbiome of Chinook salmon (Oncorhynchus tshawytscha)

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Transgenerational effects on the microbiome of Chinook salmon (*Oncorhynchus tshawytscha*)

By

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DECLARATION OF CO-AUTHORSHIP / PREVIOUS PUBLICATION

I. Co-Authorship

I hereby declare that this thesis incorporates material that is result of joint research, as follows: This thesis incorporates the outcome of a joint research effort undertaken in collaboration with, and under the supervision of, Dr. Daniel Heath and Dr. Subba Rao Chaganti. Details of submission title and authorship are covered in Chapter 2 of this thesis. In all cases, the primary contributions, including experimental input, data analysis, interpretation and writing were performed by the main author, and co-authors contributed to this thesis solely in an advisory capacity and in securing funds to perform the research.

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Thesis Chapter	Publication title	Publication Status
Chapter 2	The effects of host genetic architecture on the gut microbiome composition of Chinook salmon (Oncorhynchus tshawytscha)	Submitted to Molecular Ecology, April 27, 2020

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ABSTRACT

A critical emerging factor in the fitness of individuals is their microbiome, defined as the community of microorganisms found in and on the body of an individual. Despite the rapidly accumulating evidence of the significant role of the microbiome to host health and disease, there is a lack of studies partitioning microbiome variation into explanatory source components in fish, especially those relating to host genetics. To address this knowledge gap, this thesis made several contributions to estimate the transgenerational effects on the microbiome of an ecologically, economically and culturally important salmonid – Chinook salmon (*Oncorhynchus tshawytscha*). To achieve this goal, breeding designs were utilized to estimate various genetic architecture components, including additive among-population variance, additive genetic variance and maternal effects. DNA was extracted from hindgut contents of saltwater juveniles, the surface of eyed eggs, and maternally sourced gut content and ovarian fluids. Polymerase chain reactions (PCRs) were conducted to amplify and metabarcode the 16S rRNA encoding gene, and high throughput sequencing was then used to generate millions of sequences based on amplified PCR products. Taxonomic operational units (OTUs) were generated to measure microbiome diversity and allow for microbial community profiling. Using a combination of parametric and non-parametric modelling, significant hybrid-cross and sire were found on the gut microbiome at the juvenile saltwater stage, respectively indicative of population and additive genetic effects. Further, significant maternal effects were found on the surface of eyed eggs. Although no correlations were found between the ovarian fluid and the eyed eggs, a surprising and significant similarity was found between the microbiomes of the dam-sourced ovarian fluid and hindgut samples. Together, the findings presented in this thesis contribute to the characterization of the genetic architecture underlying microbiome variation in Chinook salmon and to its adaptive potential. The results presented in this thesis will have critical consequences for fisheries and conservation efforts and lead the way to exciting microbiome research with the ultimate goal of selecting for microbiomes associated with improved survivability and performance.

DEDICATION

To my mother, Ghada Ghadban, and my father, Ghassan Diab,
for their everlasting love, kindness, and support.

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CHAPTER ONE
General Introduction

The host-associated microbiome, and its role in fish

Multicellular organisms host diverse resident microbial communities, together known as their microbiome (Lederberg & McCray, 2001; Mandel, 2010). These microbiomes were first described as ecological frameworks characterized by their microbial community composition and functional role, found within a specific habitat defined by physiochemical parameters (Burge, 1988). Rapidly accumulating evidence gained from high throughput sequencing is revealing the universality of the potential benefits conferred by resident microbiomes –primarily bacteria– found externally or internally in numerous host species, ranging from vertebrate animals (Colston & Jackson, 2016) to invertebrates (Nyholm & Graf, 2012) and plants (Vandenkoornhuyse *et al.*, 2015). Although vertebrates' microbiomes vary by body site (e.g. in humans, Spor *et al.*, 2015; in fish, Zhang *et al.*, 2019), the majority of the microbial diversity and abundance is found within the gut, where a diverse community of microbes maintain and improve host health by playing critical roles in nutrition, immunity, behaviour, development and reproduction (reviewed in Nayak, 2010; Ghanbari *et al.*, 2015; Colston & Jackson, 2016). Curiously, although fish comprise half of the known vertebrate species (Nelson *et al.*, 2016), most gut microbiome studies have focused on mammals (Tarnecki *et al.*, 2017) rather than fish (Llewellyn *et al.*, 2016; Tarnecki *et al.*, 2017), with mammals making up less than 10% of all described vertebrate species (Table 3a "Summary statistics", IUCN, 2020). Nonetheless, studies across a wide range of fish taxa have shown evidence of the benefits provided by the microbial communities harbored within the gut (Nayak *et al.*, 2010; Ghanbari *et al.*, 2015), similar to those reported for mammals.

In recent years, interest has grown in characterizing the factors influencing the gut microbiome to quantify their effects on host fitness and the partition the differences observed among and within hierarchical levels of organismal organization across various habitats (Bahrndorff *et al.*, 2016). These factors are classified as either host- or environment-driven (Figure 1.1), and sometimes require controlled experiments to partition the contribution of each factor driving the microbiome (Goodrich *et al.*, 2014). Collectively, studies have consistently shown the significant roles of the host and the environment in determining the microbiome composition.

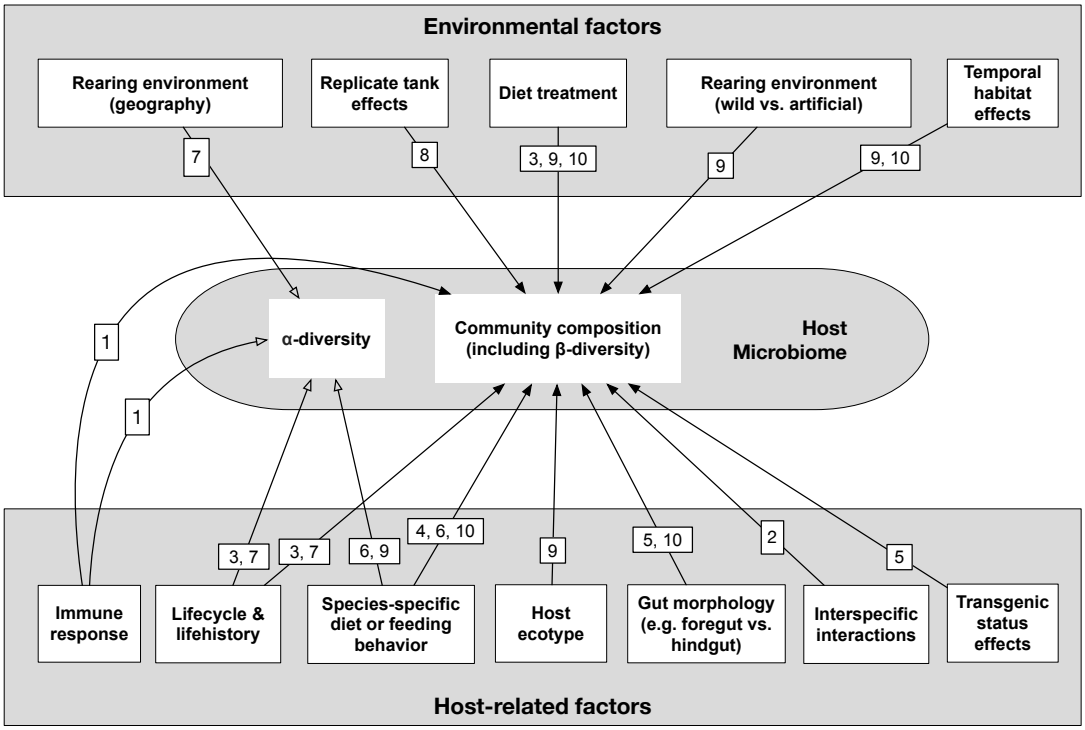

Figure 1.1. Schematic of the various environmental and host-mediated factors (EF and HF, respectively) and their effects on the gut microbiome in fish. Numbers in boxes represent studies that used high-throughput sequencing techniques to study the effects of those factors on the bacterial microbiome: 1) Bolnick *et al.*, (2014); 2) He *et al.*, (2019); 3) Ingerslev *et al.*, (2014); 4) Larsen *et al.*, (2014); 5) Li *et al.*, (2013); 6) Li *et al.*, (2015); 7) Llewellyn *et al.*, (2016); 8) Schmidt *et al.*, (2016); 9) Sullam *et al.*, (2015); 10) Ye *et al.*, (2014).

The association of the microbiome with the host encompasses diverse forms of ecological interactions, such as host-parasite, host-symbiont, and microbe-microbe interactions (Foster *et al.*, 2017; O'Brien *et al.*, 2019). The observed complexity in host-microbe interactions led to the development of various ecological and evolutionary theories to describe how they might evolve in parallel (Foster *et al.*, 2017; Koskella *et al.*, 2017; O'Brien *et al.*, 2019). In some cases, adaptations arise to increase the fitness of the microbe, while being costly to the host; this has been seen in host-parasite/pathogen interactions, which results in selection acting on the host (Paterson *et al.*, 2011). Such interactions may select for increased host genetic diversity to effectively compete with the microbe, resulting in an "arms race" (Kaltz *et al.*, 1998). For example, *Caenorhabditis elegans* infected with the bacterial pathogen *Serratia marcescens* were selected for outbreeding, leading to the replacement of selfing as a mode of reproduction (Morran *et al.*, 2011). In that study, *C. elegans* evolved greater outcrossing rates as a means to reduce infection (Mallo *et al.*, 2002), and in response, *S. marcescens* co-evolved greater infectivity rates (Morran *et al.*, 2011). However, since the microbiome mediates a diverse array of processes in the gut, it is expected that selection would favor associations that lead to positive impacts on host's fitness (Koskella *et al.*, 2017). This is the case in mutualism, where reciprocal adaptations evolve to benefit both the host and the microbiome (Herre *et al.*, 1999). A classic example of these mutual symbioses in host-microbe systems is found in squids, which have evolved specialized light organs that host a monospecific culture of the light-producing *Vibrio fischeri* bacterium (Ruby & McFall-Ngai, 1992). Another example is found in pea aphids (*Acyrthosiphon pisum*), which have evolved specialized cells to host bacterial symbionts (*Buchnera aphidicola*) that

synthesize nutrients essential to the host (Baumann *et al.*, 1997). Mutualism is also seen within the microbiome: Adaptive interactions among microbes arise to increase their overall survival, as exemplified by bacterial cell-to-cell communication in quorum sensing, the process of responding to cell population density through gene regulation (Darch *et al.*, 2012). Finally, microbiome-host interactions may be intergenerational, as observed in maternal vertical transmission in pea aphids, where microbes transferred through a maternal line provide the potential for them to co-evolve with their host (Baumann *et al.*, 1997; O'Brien *et al.*, 2019). Therefore, the microbiome and its host are competing in a constant arms race, developing adaptations against each other as they continue to coevolve (Kaltz *et al.*, 1998). Characterizing these complex interactions within the microbiome and between the microbiome and its host are important to determining the evolutionary mechanisms that promote the coevolution of the microbiome with its host (Koskella *et al.*, 2017).

Quantitative Genetics

Quantitative genetics is the study of traits that vary continuously due to differences in gene contributions and interactions across many loci (Falconer & Mackay, 1996; Connor & Hartl, 2004). The history of quantitative genetics begins in the twentieth century, when the genetic basis for evolution consisting of genetic elements following Mendelian inheritance were cemented into the original Darwinian theory of evolution and incorporated into a mathematical framework, known as the modern synthesis (Fisher, 1930). The modern synthesis (or evolutionary synthesis) relied on decades of advancements in the fields of Mendelian genetics, evolution, and population ecology (Fisher, 1930), and gave rise to population genetics and quantitative genetics. This

perspective stated that phenotypic variation is dictated by various allelic combinations in individuals, and that higher degrees of individual survivability within a population are attributable to the possession of certain combinations of alleles, which allowed them to be phenotypically better adapted to their environment, and to attain higher reproductive success, i.e. fitness (Fisher, 1930 Huxley, 1942). The goal of quantitative genetics is to use frequency distributions of phenotypic variation among related individuals to partition the variance into main explanatory source components, such as the environment and host genetics (Falconer & Mackay, 1996; Connor & Hartl, 2004). To this end, quantitative genetics utilizes breeding designs involving parents and offspring in parent-offspring regression or sibling analyses (among others) to quantify the mean and variance of traits, and statistically determine the significant sources of variation (e.g. Connor & Hartl, 2004). Thus, it is possible to design experiments to partition the effects of a common (or unique) environment factor and host genetics on the total phenotypic variance of a trait. Examples of studies partitioning phenotypic variance into genetic and environmental sources of variance are common for traits related to fitness (reviewed for Atlantic salmon (*Salmo salar*) in Garcia de Leaniz *et al.*, 2017; and for salmonids, in general, in Carlson & Seamons, 2008). Examples of similar breeding designs are also used to study microbiomes in fish are scarce (e.g. Wilkins *et al.*, 2016), with studies more often utilizing natural populations to experimentally partition microbiome variation into explanatory components (e.g. Bolnick *et al.*, 2014; Chiarello *et al.*, 2018).

The total observed variation in a trait is referred to as phenotypic variance (V_P), and is attributable to genetic, environmental, and gene-by-environment interactions (Falconer & Mackay, 1996; Connor & Hartl, 2004; Visscher *et al.*, 2008). The total genetic

variance can be attributed to various subcomponents such as among-population variance (additive population effects), additive genetic variance, non-additive genetic variance, and maternal effects (Falconer & Mackay, 1996; Connor & Hartl, 2004; Visscher *et al.*, 2008; Aykanat *et al.*, 2012a; Aykanat *et al.*, 2012b), together comprising the genetic architecture of a trait (Aykanat *et al.*, 2012a).

Additive genetic variance (V_A) refers to the deviation from the mean phenotypic trait due to inheriting various combinations of alleles from either parent (Falconer & Mackay, 1996; Visscher *et al.*, 2008). Estimates of additive genetic variance are population-specific and are known to be sensitive to changes in the environment or evolutionary forces acting upon a population (Visscher *et al.*, 2008). Because selection directly acts on additive genetic variance (Falconer & Mackay, 1996; Visscher *et al.*, 2008; Garcia de Leaniz *et al.*, 2017), estimates of additive genetic variance are important to predicting the population's response to natural and artificial selection (Clayton *et al.*, 1957; Falconer & Mackay, 1996; Visscher *et al.*, 2008; Hill 2010; Garcia de Leaniz *et al.*, 2017). Additive genetic variance has been estimated for many traits in salmonids. This is reviewed by Garcia de Leaniz *et al.* (2007) for Atlantic salmon, where V_A estimates are given for size and growth rates (e.g. body size), life history traits (e.g. egg survival), disease resistance and health conditions (e.g. red blood cell count); and in Chinook salmon (*Oncorhynchus tshawytscha),* where additive genetic effects were found in various studies for similar traits, including body length and weight (Winkelman and Peterson, 1994), jacking rates (Heath *et al.*, 2002), flesh color (Withler, 1986) and plasma lysozyme activity (Johnson *et al.*, 2003). The development of high-throughput sequencing has made it easier to measure various components of the microbiome such as

its diversity or composition, allowing us to partition genetic and environmental variance components for the microbiome. Thus, many microbiome studies have used next generation sequencing to characterize the microbiome to test for additive genetic variance effects on the microbiome, and examples of studies investigating the extent of these effects are scarce in fish (but see Navarrete *et al.* (2012) and Kokou *et al.* (2018) for family effects; Wilkins *et al.* (2016) for additive effects). Studies investigating the presence of additive genetic variance (or lack thereof) outside humans remain scarce and present an exciting opportunity to study the role of host genetics on the composition and diversity of the microbiome.

Maternal effects represent another critical subcomponent of additive genetic variance and can lead to unpredictable responses to selection (Kirkpatrick & Lande, 1989; Falconer & Mackay, 1996). Two sources of variation may give rise to maternal effects. First, the maternal phenotype may influence the offspring phenotype for the same trait by altering the environmental conditions that affect the offspring's phenotype (Falconer & Mackay, 1996; Conner & Hartl, 2004; Freeman & Herron, 2007; Wolf & Wade, 2009). This is seen in mice, for instance, where maternal body size positively correlates with milk yield and thus offspring growth and size (El Oksh *et al.*, 1967; Falconer & Mackay, 1996). Second, maternal effects may arise among offspring of the same dam, but not between the offspring and the dam herself (Falconer & Mackay, 1996). In these instances, the correlation is not due to environmental factors, but rather due to a maternal genetic (i.e. additive) component (Falconer and Mackay, 1996; Wolf & Wade, 2009). Increasing evidence suggests that maternal effects may have been shaped by natural selection and have evolved as a mechanism for adaptive phenotypic responses

to environmental heterogeneity in the offspring's environment (Mousseau & Fox, 1998). This is supported in Chinook salmon studies, where maternal effects have been shown for traits related to fitness such as immune response (Aykanat *et al.*, 2012a) and for numerous early life-history traits such as egg size (Heath *et al.*, 1999) and survival (Aykanat *et al.*, 2012b). Evidence of maternal effects on the microbiome is growing rapidly, and the current body of literature shows their universality across various animal taxa, including marine animals (Funkhouser & Bordenstein, 2013). Currently, there are very few studies utilizing NGS to investigate maternal effects on the microbiome in fish (e.g. Wilkins *et al.*, 2016). Despite this, maternal effects on the microbiome have been characterized across vertebrate taxa such as rabbits (Kovács *et al.*, 2006), squirrels (Ren *et al.*, 2017) birds (van Dongen *et al.*, 2013), apes (Ochman *et al.*, 2010) and humans (Faith *et al.*, 2013); and in marine invertebrates such as corals (Sharp *et al.*, 2012). Furthermore, studies in humans have shown the influence of various maternal traits on the microbiome, including maternal diet (Chu *et al.*, 2016), breastfeeding (Gregory *et al.*, 2016), and maternal health condition such as obesity (Garcia-Mantrana & Collado, 2016) or HIV infection (Bender *et al.*, 2016). The potential for maternal effects to impact traits related to fitness in terms of selection pressures (e.g. in Atlantic salmon, Houde *et al.*, 2015) and estimates of genetic contributions (e.g. in Chinook salmon, Aykanat *et al.*, 2012a) presents exciting opportunities to explore their contribution to microbiome variation and its evolutionary trajectory.

When a population becomes more phenotypically suited to its environment than other populations of the same species and exhibits higher fitness (i.e. reproductive success and survival), it is said be 'locally adapted' to that environment (Kawecki &

Ebert, 2004; Garcia de Leaniz *et al.*, 2007; Fraser *et al.*, 2011; Savolainen *et al.*, 2013). Local adaptation arises due to selection pressures mediated by spatial environmental heterogeneity (Kawecki & Ebert, 2004; Garcia de Leaniz *et al.*, 2007; Fraser *et al.*, 2011; Savolainen *et al.*, 2013). Local adaptation is often reported across fish taxa, and the extent of its occurrence and the mechanisms leading to its formation are extensively discussed, especially in salmonids (reviewed in Garcia de Leaniz *et al.* (2007), Fraser *et al.* (2011), and Savolainen *et al.* (2013)). The idea that the gut microbiome is locally adapted to host populations has been discussed extensively in humans for many bacterial species (reviewed in Walter & Ley, 2011; discussed in Alberdi *et al.*, 2016). In fish, local adaptation of the microbiome has also been proposed by Webster *et al.* (2019), who tested interpopulation differences in the microbiome among Atlantic salmon populations originating from wild or hatchery environments (Webster *et al.*, 2019). Among-populations differences in the microbiome indicate patterns of co-divergence in host-microbiome systems, possibly reflecting their co-evolution (O'Brien *et al.*, 2019). Detecting patterns of local adaptation for various traits (including the microbiome) is critical for conservation and restoration efforts across species of salmon (Hendry *et al.*, 2003; Taylor *et al.*, 2011; Kawecki & Ebert, 2011).

Microbiome 16S metabarcoding

Until the 1990s, culture techniques were the only approach used to advance the field of microbiology and contributed to our knowledge of the microorganisms in the gut (Fraher *et al.*, 2012). Although culture techniques have become more sophisticated (Fraher *et al.*, 2012), they result in incomplete descriptions of microbial communities, since many microbes require special culturing conditions, many of which remain unknown (Asfie *et*

al., 2003; Nayak *et al.*, 2010). However, the field of microbiology was revolutionized with the use of the 16S rRNA gene sequence in phylogenetic characterization of microbes (Olsen *et al.*, 1986; Woese *et al.*, 1987; Woese *et al.*, 1990). For example, in the human microbiome, cloned 16S rRNA gene sequencing showed that only 20% to 30% of the gut microbiome had been identified by culture (Wilson & Blitchington. 1996; Suau *et al.*, 1999; Eckburg *et al.*, 2005). Therefore, culture techniques were replaced with molecular genetic techniques that capitalized on sequence variation observed in the variable regions of the 16S rRNA encoding gene to profile microbial communities. Therefore, many techniques were developed to accomplish this goal, including: denaturing gradient gel electrophoresis (DGGE; Liu *et al.*, 1997); temperature gradient gel electrophoresis (TGGE; Muyzer *et al.*, 1998); terminal restriction fragment length polymorphism (T-RFLP; Marsh *et al.*, 1999); DNA microarrays (Amann *et al.*, 1992); fluorescence in-situ-hybridization (FISH; Cummings & Relman, 2000)). However, those microbiome characterization techniques were replaced by high throughput sequencing techniques (Fraher *et al.*, 2012; Bordenstein & Funkhouser, 2013; Ghanbari *et al.*, 2015; Koskella *et al.*, 2017), which now represent the majority of studies of gut microbiome composition (Ghanbari *et al.*, 2015). Also referred to as next generation sequencing (NGS), high throughput sequencing has facilitated the collection of sequence data from mixed microbial communities (Nayak *et al.*, 2010; Foster *et al.*, 2012; Fraher *et al.*, 2012; Ghanbari *et al.*, 2015). NGS can target either whole bacterial genomes ("metagenomics") or 16S rRNA gene amplicons ("metabarcoding"; Foster *et al.*, 2012; Fraher *et al.*, 2012; Ghanbari *et al.*, 2015). NGS sequencing platforms are distinguished by their speed, large data generation capacity, and their ability to provide taxonomic information for

uncharacterized bacteria (Fraher *et al.*, 2012; Ghanbari *et al.*, 2015). Overall, advancements in sequencing technology have allowed us to characterize the microbiome to levels that were previously unattainable.

Although the generation of millions of sequences allows better characterization of the microbiome, it also represents many bioinformatic and statistical challenges. First, to improve diversity estimates, sequences undergo quality control, removing sequences that show mismatches from expected sequences, yielding high quality usable sequences for processing (Bokulich *et al.*, 2013). In microbial sequence analysis, sequences are initially used to cluster sequences based on a pre-defined percent similarity —usually 97%— of sequence composition amongst them, generating operational taxonomic units (OTUs) – the 97% threshold was originally proposed as proxy for species-level variation in bacteria (Stackebrandt *et al.*, 1994). Various methods have been proposed to forming OTU clusters (Navas-Molina *et al.*, 2013), and alternatives to the 97% threshold have been proposed, with some studies suggesting the use of 'zero-radius' OTUs (Edgar, 2018), or amplicon sequence variants (ASVs; Callahan *et al.*, 2017) in place the 97% threshold OTUs (Callahan *et al.*, 2017; Edgar, 2018). Second, the generation of sequences with NGS rarely occurs uniformly across samples, resulting in a biased representation of sampling depth (i.e. the number of sequences per sample), and the subsequent normalization (rarefaction) methods to remedy this issue is another area of debate (see opposing views in McMurdie & Holmes (2014), and Weiss *et al.* (2017)). Third, differential sequence read number abundance used as a proxy for differences in the relative frequencies of OTUs or ASVs is often used with disregard of the underlying data structure and characteristics, furthering the necessity for development of mathematical

models to accurately test microbiome hypotheses (Xu *et al.*, 2015; Weiss *et al.*, 2017). The inconsistency in these bioinformatic and statistical approaches across NGS generated microbiome data analyses has generally resulted in studies choosing to rarefy their data and apply non-parametric models (e.g. Llewellyn *et al.*, 2017) or to normalize their sequence read counts and analyzing it with parametric models (e.g. Ingerslev *et al.*, 2014). The application of NGS to characterize the microbiome, and the development of bioinformatic and statistical modelling tools to study it, allows us to quantify the microbiome semi-quantitively and test microbiome-specific ecological and evolutionary hypotheses (e.g. Bolnick *et al.*, 2014; Ye *et al.*, 2014; Llewellyn *et al.*, 2017).

Study system: Chinook salmon

Chinook salmon (*Oncorhynchus tshawytscha*) is the largest of the Pacific salmonid species, and is thought to have evolved around 500,000 to 1,000,000 years ago with the other Pacific salmonids. (Neave, 1958).

Chinook salmon have evolved a complex life history (Quinn, 2005). It begins in freshwater streams, where they hatch in gravel nests and later become free-swimming fry (Groot & Margolis, 1991). As fry, the individuals adapt physiologically to transitioning to the saltwater environment ("smolting"), where they then spend the majority of their life cycle until they are ready to return to their natal streams to spawn (Groot & Margolis, 1991). Chinook salmon are anadromous and semelparous, meaning they ascend up natal rivers from sea water to spawn and die (Quinn, 1990; Quinn, 2005; Hasler, 2012). In this thesis, the microbiome is characterized for Chinook salmon reared in saltwater net pens (Chapter 2), and for the surface of fertilized eggs from freshwater incubators (Chapter 3). Microbiome diversity (Chao1) and microbiome composition (Unifrac) is known to be

differentiated between the freshwater and marine environments in Atlantic salmon (Llewellyn *et al.*, 2016); thus, the life history patterns of Chinook salmon (and salmon, in general) involves a wide range of environmental selective pressures and necessitates the characterization of the microbiome at various life stages of salmon.

Chinook salmon are ecologically, economically and culturally important through their range (Ruckelshaus *et al.*, 2002; Quinn, 2005; Heard *et al.*, 2007). First and foremost, the significance of salmon for the economy is rooted in aquaculture production and various fishing activities (Ruckelshaus *et al.*, 2002; Heard *et al.*, 2007; Kendall & Quinn, 2011). In 2017, Canadian aquaculture produced 191,416 tones of finfish and shellfish, of which 63% was accounted for by salmon species (Statistics Canada, 2017a). This production output was valued at $1 billion (out of $1.4 billion total for aquaculture), with British Columbia being the biggest contributor to production value (CAIA, 2018). Currently, British Columbia is the only province to use Chinook salmon in aquaculture, where it has been farmed since the 1970s – making up a fifth of all farmed salmon stocks in the province (Kim *et al.*, 2004; Bryden *et al.*, 2004; CAIA, 2018). Chinook salmon support important commercial fisheries, which occur in Oregon, Washington, British Columbia, Alaska and Bristol Bay (Heard *et al.*, 2007). In addition, sports, subsistence and recreational fisheries all target Chinook salmon and contribute to the economy (Heard *et al.*, 2007; Kendall & Quinn, 2011). Second, salmon are important to maintaining regional biodiversity, as they themselves are considered important prey for various vertebrate predators in fresh water (Willson & Halupka, 1995). Third, Chinook salmon are coveted species among the First Nations people in Canada, and to the states of Alaska and Oregon in the United States of America. Every year, “First-salmon

ceremonies" ensue to celebrate the first Chinook salmon catch in the Spring (Jackson, 1978). In Alaska and Oregon, Chinook salmon is known as "king salmon" for its large body size, where it is used as the state's fish symbol. In summary, Chinook salmon is indispensable to the ecology, economy, and culture of many Pacific regions.

Pacific salmon face many challenges throughout their range that threaten their survival and reproduction. These threats include increasing ocean temperatures (Richter & Kolmes, 2005), pathogens (Fryer & Pilcher, 1974), predation, competition, negative interactions between wild and hatchery salmon, and anthropogenic stressors such as hydropower projects (Keefer *et al.*, 2004) and harvesting (reviewed in Ruckelshaus *et al.*, 2002, and Weber & Fausch, 2005). Due to their immense value to the economy, human culture and ecological biodiversity, it is critical to preserve populations of salmonids (Willson & Halupka, 1995; Ruckelshaus *et al.*, 2002). Using genetic techniques, many populations of Chinook salmon have been shown to be reproductively isolated, providing evidence of local adaptation, and thus constituting distinct ESUs that require separate management efforts (Beacham *et al.*, 2006; Davis *et al.*, 2008). Studies have shown patterns of inherited adaptive traits in salmon, but this evidence remains incomplete and challenged (Garcia de Leaniz *et al.*, 2006). Of the many traits studied in fish, the microbiome has gained increasing interest in the past decade, coinciding with the advent of high-throughput sequencing technology (Ghanbari *et al.*, 2015). Although studies have determined the effects of various environmental and host drivers on the microbiome in salmonid species (e.g. Ingerslev *et al.*, 2014; Schmidt *et al.*, 2016; Llewellyn *et al.*, 2016), evidence of host-microbiome codivergence in salmonids, and to a broader extent,

in fish, is lacking. Characterizing the microbiome will have important implications for the conservation and management efforts of animal species (Bahrndorff *et al.*, 2016).

Compared to other salmonid species the gut microbiome of Chinook salmon has received less attention, and this may present a hurdle in conservation efforts for this species. For instance, the gut microbiome has only been recently described in Chinook salmon using small sample sizes of farmed fish (n = 4, Booman *et al.*, 2018; n = 30, Ciric *et al.*, 2018; n = 30, Ciric *et al.*, 2019). On the other hand, the gut microbiome of Atlantic salmon has been more extensively studied, including factors such as the effect of gut morphology (Gajardo *et al.*, 2016) and biogeography (Llewellyn *et al.*, 2016) on the composition of the gut microbiome bacterial community. Utilizing Chinook salmon as an animal model to characterize the microbiome and its interactions with the host will allow us to achieve a more holistic view of the microbiome composition across salmon species and, ultimately, their co-evolutionary history.

Thesis objectives

The main goal of this thesis is to characterize the role of transgenerational effects in driving the composition and diversity of the microbiome in Chinook salmon. Here, we define transgenerational microbiome effects as processes that drive microbiome effects in offspring resulting from genetic and non-genetic signals from the parents, including multiple generation effects. As discussed, Chinook salmon were selected as my study species due to their cultural, economic and ecological importance in the Pacific Northwest as well as logistical aspects of their life history that makes quantitative genetic analyses straightforward (e.g. many large eggs and no parental care). The specific objectives that address my main goal comprise two data chapters:

In Chapter 2, I investigate the nature of inheritance acting among and within populations in determining the diversity and composition of the gut microbiome. More specifically, eggs from 12 highly inbred females were mixed and subsets of the mixed eggs were fertilized by 10 sires from each of one domestically farmed and seven wild populations of Chinook salmon to produce 80 full- and half-sib families. By dividing offspring from each family between replicate pens and using an inbred dam, this breeding design allowed me to partition microbiome variance into population-of-origin (hydrid-cross) and additive genetic (sire) effects while controlling for environmental (pen) and maternal (dam) effects.

In Chapter 3, the presence and mechanisms of maternal effects on offspring microbiome composition and diversity are studied, with an emphasis on possible maternal vertical transmission of microbiome components on the surface of fertilized (eyed) eggs. To achieve these goals, milt from 6 domestically farmed males were mixed, and a subset of the mixture was used to fertilize a set of eggs from each of 39 females of a domestically farmed population of Chinook salmon. The maternal effect on the resulting eyed eggs' microbiome were examined using surface egg material, along with maternally sourced gut and ovarian fluid samples.

In both chapters, I apply quantitative genetics and evolutionary theory to determine the nature and extent of the forces driving gut microbiome composition among and within hybrid-crosses of Chinook salmon using microbiome meta-barcode 16S rRNA sequence data. By testing for population effects in a common garden experiment, I was able to determine the extent of host-microbiome co-diversification. Multiple sires used within each hybrid-cross allowed me to determine the magnitude of additive genetic

variance within the observed variation in the gut microbiome (Chapter 2), and the utilization of various dams (Chapter 3) allowed me to explore maternal effects and the possibility of maternal vertical transmission. Highlighting the roles of population, additive, and maternal effects is crucial to characterizing how the microbiome may evolve in parallel with its host, and to determining the role of host genetics in mediating differences observed among the microbiomes.

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CHAPTER TWO
The effects of host genetic architecture on the gut microbiome composition of Chinook salmon (*Oncorhynchus tshawytscha*)

Introduction

The microbiome is a community of microbes that live in or on a multicellular organism, and is most commonly studied in the gastrointestinal tract ("*gut microbiome*"), where it plays important roles both in the health and development of the host (reviewed in Nayak, 2010; Romero, Ringø & Merrifield, 2014; Ghanbari, Kneifel & Domig, 2015). In fish, the gut microbiome has been shown to be symbiotically associated with the host, and it plays many beneficial roles, such as aiding in metabolism (Semova *et al.*, 2012; Tremaroli & Bäckhed, 2012), immunity (Galindo-Villegas*,* García-Moreno, de Oliveira, Meseguer, & Mulero, 2012; Milligan-Myhre *et al.*, 2016), and development (Bates *et al.*, 2006). Additionally, the gut microbiome generally reflects host species (Ye, Amberg, Chapman, Gaikowski & Liu, 2014), life stage (Llewellyn *et al.*, 2016), diet (Bolnick *et al.*, 2014a; Bolnick *et al.*, 2014b; Webster, Consuegra, Hitchings & de Leaniz, 2018), physiology (Bolnick *et al.*, 2014b; Ye *et al.*, 2014), geographical isolation (Ye *et al.*, 2014; Webster *et al.*, 2018), and genetic divergence (Sullam *et al.*, 2015; Webster *et al.*, 2018). While the gut microbiome for many fish species has been characterized, variation in diversity, establishment mechanisms, and role in host phenotype/genotype requires additional study (Nayak, 2010; Llewellyn, Boutin, Hoseinifar & Derome, 2014; Ghanbari *et al.*, 2015; Sullam *et al.*, 2015).

In addressing the factors that shape the host's gut microbiome (hereafter "microbiome") composition, it is important to consider the broad effects of the environment, host genetics, and gene-by-environment interactions. With over 32,000

described species (Eschmeyer & Fong, 2015), fish comprise more than half of the known vertebrate species and encompass a wide range of phenotypes, life histories and ecologies (Neslon, Grande & Wilson, 2016). Thus, even with a wealth of published fish microbiome studies, the effects of host genetics, the environment, and their interactions on the microbiome composition remain poorly understood (Wong & Rawls, 2012; Bolnick *et al.*, 2014b; Ghanbari *et al.*, 2015). Host genome variation is expected to play a role in shaping the microbiome, as it is responsible for encoding intestinal mucosa and immune factors that play essential roles in the establishment and maintenance of the microbiome (Roeselers *et al.*, 2011; Spor, Koren & Ley, 2011; Romero *et al.*, 2014; Ghanbari *et al.*, 2015). Using high throughput sequencing technology, the effects of host genetics on the microbiome have been investigated at various levels, ranging from species-level effects (Roeselers *et al.*, 2011; Ye *et al.*, 2014; Larsen, Mohammed & Arias, 2014; Li *et al.*, 2015) to among-population (Roeselers *et al.*, 2011; Webster *et al.*, 2018) and within-population effects (Webster *et al.*, 2018). For example, at the species-level, individuals from three species of carp (*Ctenopharyngodon idella*, *Carassius carassius* and *Hypophthalmichthys nobilis*) reared in a common environment showed strong microbiome compositional differences, despite their taxonomic relatedness and common environment (Li et. al 2014). Gut microbiome composition and diversity differences have also been found at the population level, and have been attributed to genetic drift and bottleneck effects using zebrafish (*Danio rerio*; Roeselers *et al.*, 2011) or to environmental variation and genetic divergence using zebrafish (Roeselers *et al.*, 2011) and Atlantic salmon (*Salmo salar*; Webster *et al.*, 2018). Finally, within-population effects were shown to explain less variation than among-population effects for

microbiome composition in Atlantic salmon (Webster *et al.*, 2018) but explained less variation than diet in shaping the microbiome of various rainbow trout families (*Oncorhynchus mykiss*; Navarrete *et al.*, 2012). Differences among families within a population may be indicative of heritable components in the microbiome, defined as the proportion of phenotypic variance in a population attributable to additive genetic variance (Visscher, Hill & Wray, 2008). Thus, host-based drivers of the gut microbiome often have a strong underlying genetic architecture, which may include among-species, among-population (within a species) or among-family (within a population) variance components; however, population effects are perhaps the least understood (Ghanbari *et al.*, 2015). More importantly, there are currently no studies on interpopulation effects on microbiome composition in fish using controlled environmental conditions. Collectively, the literature shows that the host genome plays a pivotal role in determining the composition of the microbiome across various fish species, but there is still a gap in our understanding of the variance components of host genetic effects on the microbiome in fish.

In addition to host genetics, it is known that the host environment affects gut microbiome establishment during various ontogenetic stages of fish development (Llewellyn *et al.*, 2016; Bledsoe, Peterson, Swanson & Small, 2016). Fish ingest water and particulate matter directly from their aquatic environment, which unquestionably affects their gut microbiome (Llewellyn *et al.*, 2014; Ghanbari *et al.*, 2015). Various environmental conditions such as diet (Naverrete *et al.*, 2012; Wong *et al.*, 2013; Webster *et al.*, 2018), or fish rearing environments (Roeselers *et al.*, 2011; Wong *et al.*, 2013; Webster et. al 2018; Parshukov *et al.*, 2019) also contribute strongly to microbiome

variation. Thus, it is critical to consider the potential environmental factors and control for them while investigating non-environmental drivers of the microbiome (Goodrich *et al.*, 2014a; Ghanbari *et al.*, 2015).

Selection pressures are known to act on the host-microbiome interactions, leading to co-evolved microbiomes across host species over evolutionary timescales (O'Brien, Webster, Miller & Bourne, 2019). These co-evolutionary dynamics may be demonstrated, for example, as genetic co-divergence of the host and their associated microbiome, as evident between ecotypes of Trinidadian guppies (*Poecilia reticulata*; Sullam *et al.*, 2015) and among Atlantic salmon populations (Webster *et al.*, 2018). In addition, these patterns may be reinforced by the presence of strong metabolic complementarity between the microbiome and its host (O'Brien *et al.*, 2019), as reported in diet and microbiome correlations across mammals (Ley *et al.*, 2008), and in fish (Sullam *et al.*, 2015). Therefore, the microbiome itself reflects evolutionary selection pressures acting at the host level and the microbial cell level (Ley *et al.*, 2006). Despite this, evidence for reciprocal adaptation in host-microbiome systems as a result of bi-directional selection is weak, and more empirical work is needed to better understand co-evolutionary dynamics (Foster, Schulter, Coyte & Rakoff-Nahoum, 2017; Koskella, Hall & Metcalf, 2017). While host-microbiome co-evolution in fish has not been explicitly characterized, salmonids are known to show strong patterns of population divergence, consistent with local adaptation (Garcia de Leaniz *et al.*, 2017). Putative locally adapted traits are exhibited across many populations of Atlantic and Pacific salmon, ranging from trophic ecology and feeding behavior to immune and metabolic function (reviewed in Fraser, Weir, Bernatchez, Hansen & Taylor, 2011). Since the host itself exerts selection

pressures on its microbial community through host related factors (Ley *et al.*, 2006), it is intuitive that if host populations were locally adapted to their environments, that microbiome differences would be observed among-populations as a consequence of variation in host-related factors. However, the role of host genetic architecture in determining fish microbiome composition is under-studied, especially at the among-population and among family (within-population) levels. Demonstrating among-population microbiome variation in a controlled environmental setting may therefore indicate the microbiome's co-divergence with the host genome.

Perhaps among the best studied genetic architectures of non-model animals are those of salmonids', including the Pacific salmon (Waples, Naish & Primmer, 2019). In this study, we focus on the economically and ecologically important Chinook salmon (*Oncorhychus tshawytscha*), which are native to the North Pacific Ocean and grow to be the largest of the Pacific salmon species (Quinn, 2018; Ohlberger, Ward, Schindler & Lewis, 2018). Chinook salmon are anadromous and semelparous, and many populations remain in coastal waters until they return their natal streams to spawn (Rounsefell, 1958; Quinn, 2018). Underpinning the ecological significance of salmon is their importance in nutrient cycling, freshwater and saltwater trophic ecology, community behavioural interactions, and evolutionary relationships (reviewed in Hilderbrand, Farley, Schwartz & Robbins, 2004). Salmon production contributes significantly to the Canadian economy: Over 123,000 tonnes of salmon were farmed in 2018 alone, accounting for over 1.1 billion Canadian dollars (DFO, 2018). While there are limited recent statistics to partition the economic contribution of Chinook salmon from that of all salmon species, they are the largest cultured species of salmon in BC and have domesticated in the province since

the 1970s, accounting for a fifth of all farmed salmon stocks in the province (Kim, Withler, Ritland & Cheng 2004). The mid- and distal- gut microbiome of farmed Chinook salmon from New Zealand was recently sequenced and *Vibrionaceae* was dominant in both (Ciric *et al.*, 2018; Ciric *et al.*, 2019). Furthermore, the diversity and composition of the gut microbiome in Chinook salmon is known not react to a soybean (plant) based diet (Booman *et al.*, 2018). However, other than those studies, Chinook salmon gut microbiome dynamics research has not been reported.

This study aims to address two main questions: 1) do evolutionary forces reflective of genetic divergence among natural populations affect the microbiome composition in controlled hybrid crosses of Chinook salmon?, and 2) are there within-population sire effects that act on the microbiome differentially among populations, reflective of additive genetic variance (heritability) effects? Salmonids are known to lend themselves to traditional breeding designs, permitting us to partition genetic and environmental sources of variance (Lynch & Wash, 1998). Here, we reared half-sib families from a single fully-domesticated and seven wild-domestic hybrid crosses of Chinook salmon in replicated pens to test for population and within-population additive genetic effects on gut microbiome diversity and composition. Measuring the extent of gut microbiome variation among and within populations is important in efforts pertaining to the management and conservation of salmonids (Garcia de Leaniz *et al.*, 2007).

Materials and Methods

Field collections, breeding design and rearing environment

All fieldwork was carried out at Yellow Island Aquaculture Ltd. (YIAL), a Chinook salmon hatchery and organic-based farm located east of Campbell River on Quadra

Island, Vancouver Island, British Columbia, Canada (Figure 2.1). Wild sourced eggs from Robertson Creek and milt from Big Qualicum were used to produce the seven-generation, fully-domesticated, stock of Yellow Island (YIAL), which has been in production since 1985. Eggs from 17 highly inbred female (offspring from a self-crossed hermaphrodite) were mixed, and subsets of the mixed eggs were individually fertilized using 10 sires from each of the domestic wild stocks of Chinook salmon (details of breeding in Semeniuk *et al.*, 2019). This produced 80 full- and half-sib families belonging to seven outcrossed hybrid stocks (YIAL x Wild) and a fully inbred domesticated stock (YIAL x YIAL; Figure 2.1). Consequently, the maternal line is identical for all crosses, but the paternal line for those crosses varies depending on the geographical origin of the paternal line. The aim of this breeding design was to minimize maternal effects (Heath, Fox & Heath, 1999; Semeniuk *et al.*, 2019), while allowing the characterization of the genetic architecture underlying the microbiome variation due to sire (additive genetic variation) effects, pen (environmental) effects, and hybrid cross or 'population' effects. Husbandry conditions are detailed in Semeniuk *et al.* (2019).

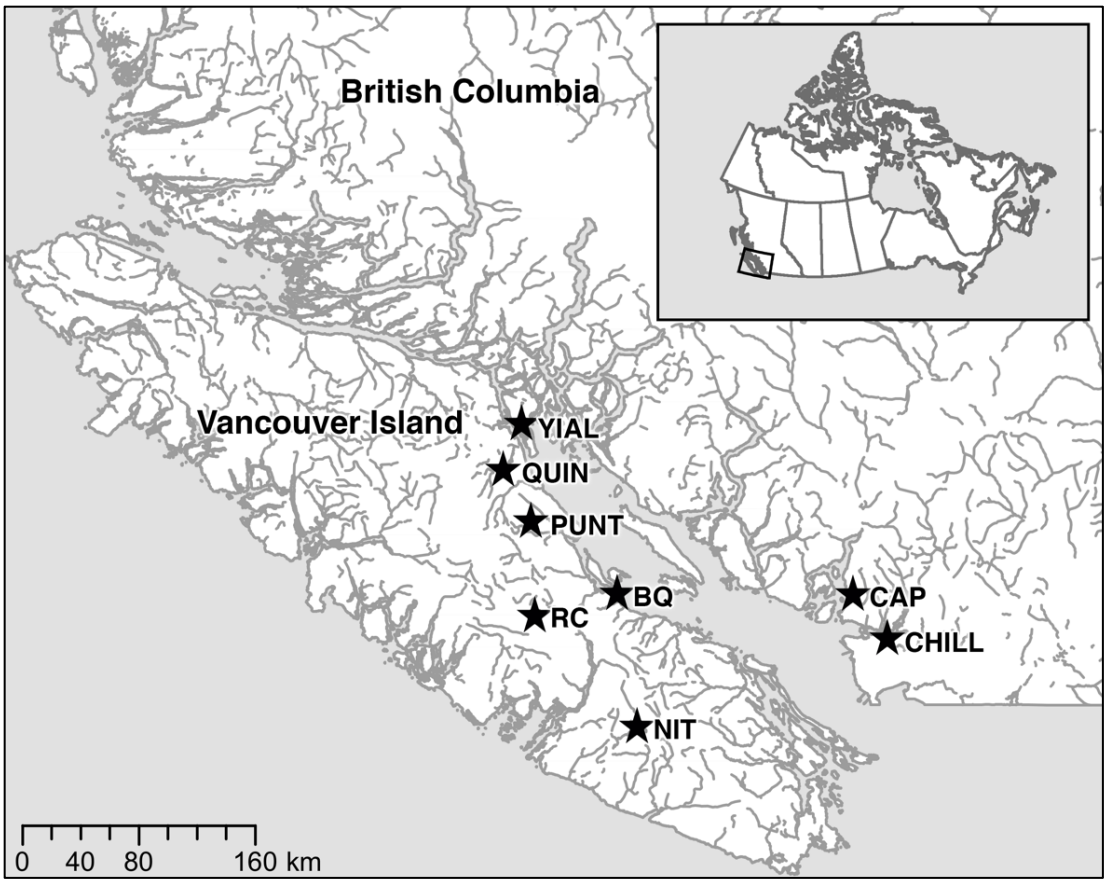

Figure 2.1. Map of stock sources of the male Chinook salmon used for fertilization of eight pure and hybrid crosses used in this study. Crosses included pure (YIAL) and hybrid (CAP, CHILL, NIT, PUNT, RC, BQ, QUIN) crosses of Chinook salmon. **Abbreviations:** Robertson Creek "RC", Big Qualicum River "BQ", Capilano River "CAP", Chilliwack River "CHILL", Nitinat River "NIT", Puntledge River "PUNT", Quinsam River "QUIN".

Sample collection, DNA extraction and next generation sequencing

A subset of 2 year-old fish ranging from 10 to 25 (mean size = 182g) was randomly selected from each pen (Sampling distribution in Appendix A1), and the fish were humanely euthanized and sacrificed to sample the gut contents. To obtain gut content samples for DNA extraction, the body cavity was cut open with a sterile scalpel and the distal gut of each offspring was collected. We chose to study the distal gut as opposed to other compartments due to the lower alpha diversity associated with that part of that gut

(McDonald, Schreier & Watts, 2012 ; Gajardo *et al.*, 2016), reflective of a specialized (McDonald, Schreier & Watts, 2012) or well-adapted microbial community (Gajardo *et al.*, 2016) in that region. The gut samples were immediately stored in RNAlater™ for transport to the research facility, where it was stored in the freezer at -20ºC until DNA extraction.

We extracted DNA from gut content (digesta) of the distal intestine using commercially available E.Z.N.A Stool DNA Kit (OMEGA Bio-tek) following the manufacturer's protocol. Next generation sequencing library construction was completed in two steps as previously described (He *et al.*, 2017). Briefly, the universal primer set of 787F (V5F; ATTAGATACCCNGGTAG) and 1046R (V6R; CGACAGCCATGCANCACCT) was first used to PCR amplify the 16S rRNA encoding gene sequences containing the V5-V6 hypervariable regions. A short, Ion Torrent adaptor sequence was added to the 5' end of the forward (acctgcctgccg) and reverse (acgccaccgagc) primers. The PCR product was visualized for amplification success on a 2% agarose gel, and PCR product purification was then carried out using Agencourt AMPure XP beads (Beckman Coulter Genomics GmbH, Mississauga, ON, Canada). A second short-cycle of PCR was conducted to ligate adaptor and the barcode sequences to the amplicon using purified PCR product from the first round PCR. The second round of PCR used: forward primer UniA (CCATCTCATCCCTGCGTGTCTCCGACTCAG<u>XXXXXXXXXX</u>GATacctgcctgccg), and reverse primer UniB (CCTCTCTATGGGCAGTCGGTGATacgccaccgagc), where the underlined sequence in UniA consisted of unique 10-12 bp barcode sequences necessary for the sample demultiplexing in sequence analysis and the lower-case

sequence were the reverse compliment of the added sequence in the first primer set. Barcoded samples were combined based on PCR band intensity and a commercially available kit (GenCatch™, Epoch Life Science, Inc., Sugar Land, TX., USA) was used to purify the PCR product from incomplete amplicons and primer dimers. The final library was sequenced with an Ion Torrent™ Personalized Genome Machine (Thermo Fisher Scientific, Inc., Mississauga, Canada).

Sequence processing and data analysis

Sequence quality checks were initially conducted using personal genome machine (PGM) software (Torrent Suite™, v5.6) using default parameters to conduct the following tasks: 1) removal of mixed clonal libraries on Ion Sphere Particles (ISPs) known as polyclonals, 2) removal of low-quality sequences, and 3) removal of sequences with low quality data at the 3' end of the read.

Unless otherwise stated, all sequence processing was performed using the Quantitative Insights into Microbial Ecology (QIIME) pipeline, v1.9.1 with default parameters (Caporaso *et al.*, 2010). Briefly, raw sequences were processed with a Phred quality score cut-off of 25 and then demultiplexed. Any raw sequence with one or more mismatches in the primer sequence were detected and excluded. In addition, forward and reverse primer sequences, and barcode and adapter sequences were removed. Chimeric sequences were detected using USEARCH v6.1 (Edgar, 2010) and excluded from analysis. To perform operational taxonomic unit (OTU) clustering, the open reference approach in QIIME was used with default parameters and a 97% sequence identity threshold. In this approach, clustering is completed with the UCLUST algorithm (Edgar, 2010), wherein a reference database was used to determine a cluster of sequences, and

unassigned sequences were allowed to cluster *de novo* (Caporaso *et al.*, 2010). To assign taxonomy to OTUs, alignment of candidate OTU sequences was completed in PyNAST (Caporaso *et al.*, 2009) against the GreenGenes database (v13.8) at 90% sequence identity using UCLUST (Edgar, 2010).

After sequence processing in QIIME, 6,411,635 high quality sequences (out of 6,602,610 usable sequences) remained for analyses. After filtering unassigned taxa OTUs, and OTUs from Archaea, mitochondria and Chloroplasts, a total of 8,038 unique OTUs were identified, with 6,317,692 working sequences (out of 6,411,635) and used for all subsequent statistical analyses. After sequence filtering and alignment, any samples with 3,000 reads or less were dropped from all statistical analyses. The mean sample depth across all samples was 22,871, and the range was 3,061 to 175,783 reads per gut sample. In the final analysis, a total of 278 gut samples were used (Appendix A1).

Alpha Diversity analyses

To estimate diversity and richness of the microbiomes, the Shannon and Chao1 indices were computed for all samples in QIIME by rarefaction with 999 iterations using a 3000-sequence cutoff, and the average was calculated across all bootstrap runs. The purpose of this analysis was to ensure comparable estimates of alpha diversity across samples with non-uniform sequencing depth. To test for differences from the overall means in alpha diversity, linear mixed effects models were fit for Shannon diversity and Chao1 indices, and likelihood ratio tests (LRTs) were used to test for the significance of cross, sire (nested within stock) and pen (nested within stock) effects on each index. To test for specific differences between pairs of hybrid-crosses outside of the grand mean, multiple t-tests were conducted in the *emmeans* package (v1.3.5; Lenth, Singmann, Love,

Buerkner & Herve, 2018) in R (v3.6.0; R Core Team, 2016), and corrections for multiple tests were made using false discovery rate (BH; Benjamini & Hochberg, 1995).

To explore differences in the microbiome community structure, OTUs with 3 reads or less across all samples were discarded from the analysis. Then, the number of sequences across all samples was normalized to relative frequencies using the cumulative sum scaling (CSS) technique (Paulson, Stine, Bravo & Pop, 2013), and Bray-Curtis dissimilarity matrices were generated using *adonis* in the R (v3.6.0; R Core Team, 2016) package *vegan* (v2.5-5) for beta diversity analysis (Oksanen *et al.*, 2013). The CSS normalization method was chosen as it considerably enhances PCoA clustering (Paulson *et al.*, 2013) and outperforms other normalization techniques in clustering accuracy, especially for libraries with high variation in the pair-wise distance measures, such as observed in this study (Bray-Curtis; Weiss *et al.*, 2017).

To visualize patterns of differences in the microbiome composition among population crosses, principal coordinate analyses (PCoA) were performed on the Bray-Curtis dissimilarity matrix in PAST (v3.25; Hammer, Harper & Ryan 2001). To simplify the visualization of a large number of samples (n = 278) on the PCoA plots, the averages and 95% confidence intervals of the first two principle coordinates were calculated across crosses and used to construct a representative coordinate of each cross on the plots.

To characterize the microbiome composition among crosses, a list of all OTUs present in each stock was created and an intersection plot was created using the *UpSetR* package (v1.4.0; Lex, Gehenborh, Strobelt, Vuillemot & Pfister, 2014) in R (v3.6.0; R Core Team, 2016). The intersection plot shows the OTUs found exclusively in certain stocks, and OTUs found commonly among multiple stocks.

To test for cross, sire and pen effects on the microbiome community structure, a nested form of the permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001) was used in the *vegan* package (v2.5-5) using the *adonis* function on the Bray-Curtis dissimilarity matrices with 9999 permutations. The model was used to test for among-cross effects, among sires (within cross) effects, and between pens (within cross) effects, with the strata argument specified at the pen-level. The mean sum square values were subsequently used to partition the variance explained due to all factors. Separate models were also constructed for each stock to partition the variance explained due to sire and pen effects within each stock separately, and to test for their effects. To further explore specific patterns in community composition differences in the microbiome among populations, *ad-hoc* test comparisons were conducted using 9999 permutations to quantitatively assess the differences among stocks using *adonis* in the R (v3.6.0; R Core Team, 2016) *vegan* package (v2.5-5). Corrections for multiple simultaneous *ad hoc* tests were adjusted using BH (Benjamini & Hochberg 1995).

OTU and bacterial family-level abundance analysis

To explore variation in the microbiome at the level of taxonomic groups, unique OTUs that were most abundant, and bacterial families (consisting of OTUs that were identified and collapsed to the family level), were used for differential abundance analyses. Unique OTUs and taxonomic families that were most abundant with 3000 and 1000 sequences or more, respectively, were selected for analysis. In total, 110 OTUs and 45 taxonomic families were used, accounting for 92.3% and 99.5% of all sequences and all sequences with assigned taxonomy, respectively. Cumulative sum scaling was used to normalize OTU and family-level data to account for differences in read-depths and allow for a

meaningful differential abundance analysis (Paulson *et al.*, 2013). The OTUs and taxonomic families of interest were fitted to zero-inflated linear mixed effects models (LMM), with population cross as a fixed factor, and sire and pen as random factors nested within cross using the *glmmTMB* package (v.0.2.3; Magnusson *et al.,* 2017) in R (v3.6.0; Brooks *et al.*, 2017). The Akaike information criterion (AIC; Akaike, 1973) was calculated for models with competing zero-inflation structures (absent, constant, or population cross-specific; Brooks *et al.,* 2017) and used to select the best model in the analysis (as suggested in Xu, Paterson, Turpin & Xu, 2015). To test the statistical significance of fixed and random terms in the model, a reduced model for each term was used, and the change in the log likelihood between the models was compared against a χ^2 distribution using the likelihood ratio test (LRT). Estimated marginal means were calculated using the selected model and pairwise comparisons were computed in the *emmeans* package (v1.3.5; (Lenth *et al.*, 2018). Multiple comparisons were adjusted for each factor (in LRTs) and within each factor (for pairwise comparisons) using false discovery rate (Benjamini & Hochberg 1995). Barplots were created using the *ggplots2* (Wickham, 2016) in R (v3.6.0; R Core Development Team, 2016).

Results

Factors driving microbiome alpha diversity

The microbiome community mean observed Chao1 index ranged from 240 (NIT) to 311 (CHILL), and Shannon's H diversity ranged from 3.4 (NIT) to 3.9 (YIAL; see Figure 2.2) within crosses. Large standard errors in the alpha diversity measures were observed across all crosses in the study for Shannon and Chao1 indices. Using LRTs, no significant differences were found among crosses, among sires within crosses, or between pens

within crosses (Table 2.1). Pairwise t-tests analyses showed no statistically significant differences in the means between the pairs of crosses for Chao1 and Shannon indices (Table 2.1), corroborating the overall analysis results. Finally, the no significant population cross, sire or pen factors effects were found for either of the diversity indices (Table 2.1).

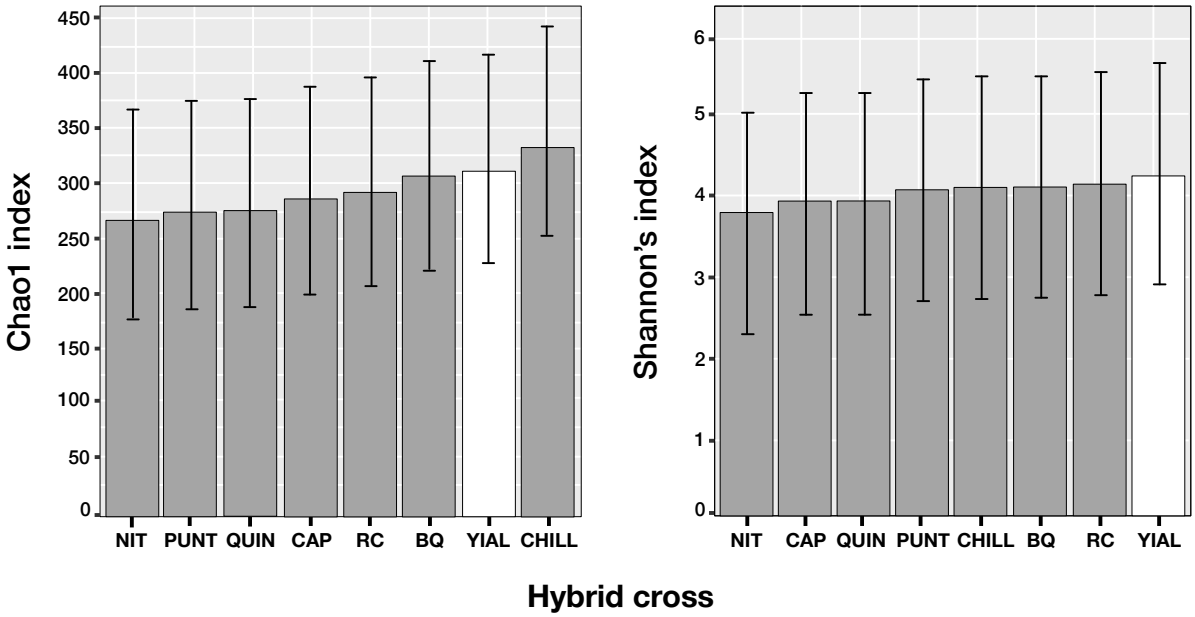

Figure 2.2. Mean (± 1 SEM) Shannon's index and Chao1 across all breeding crosses. Significant cross differences ($P < 0.05$) from the grand mean were not found for Chao1 or Shannon's index. No statistically significant pairwise differences between stocks were found for either alpha diversity metric. Hybrid cross abbreviations are defined in Figure 2.1.

Table 2.1. Alpha diversity analysis results using LMMs. The likelihood ratio test was used to calculate the significance of the differences for each alpha diversity metric.

α-diversity index	Factor	χ^2	Df	P-value
Shannon	Cross	7.45	7	0.38
	Pen	0	2	1
	Sire	0.73	2	0.70
Chao1	Cross	14.44	7	0.065
	Pen	0	2	1
	Sire	0.33	2	0.88

Factors driving global microbiome composition: Beta diversity

Overall, the first two PCs accounted for almost 40% of all variance in the Bray-Curtis distances across all samples. The clustering patterns revealed YIAL as an outlier cross, CHILL as intermediate, and the remaining stocks clustering more closely together on the axes (Figure 2.3). Using the overall PERMANOVA model, significant cross effects were found in the overall microbial community structure using Bray-Curtis distances (p = 0.001, R^2 = 0.05; Table 2.2). Pairwise PERMANOVA tests using Bray-Curtis distances showed that YIAL was statistically different from BQ (p = 0.0028), CAP (p = 0.005), NIT (p = 0.005), PUNT (p = 0.005), QUIN (p = 0.0065), and RC (p = 0.005) but not from CHILL ($p > 0.05$), and all other pairwise PERMANOVA comparisons showed a lack of significant pairwise differences (Table 2.3).

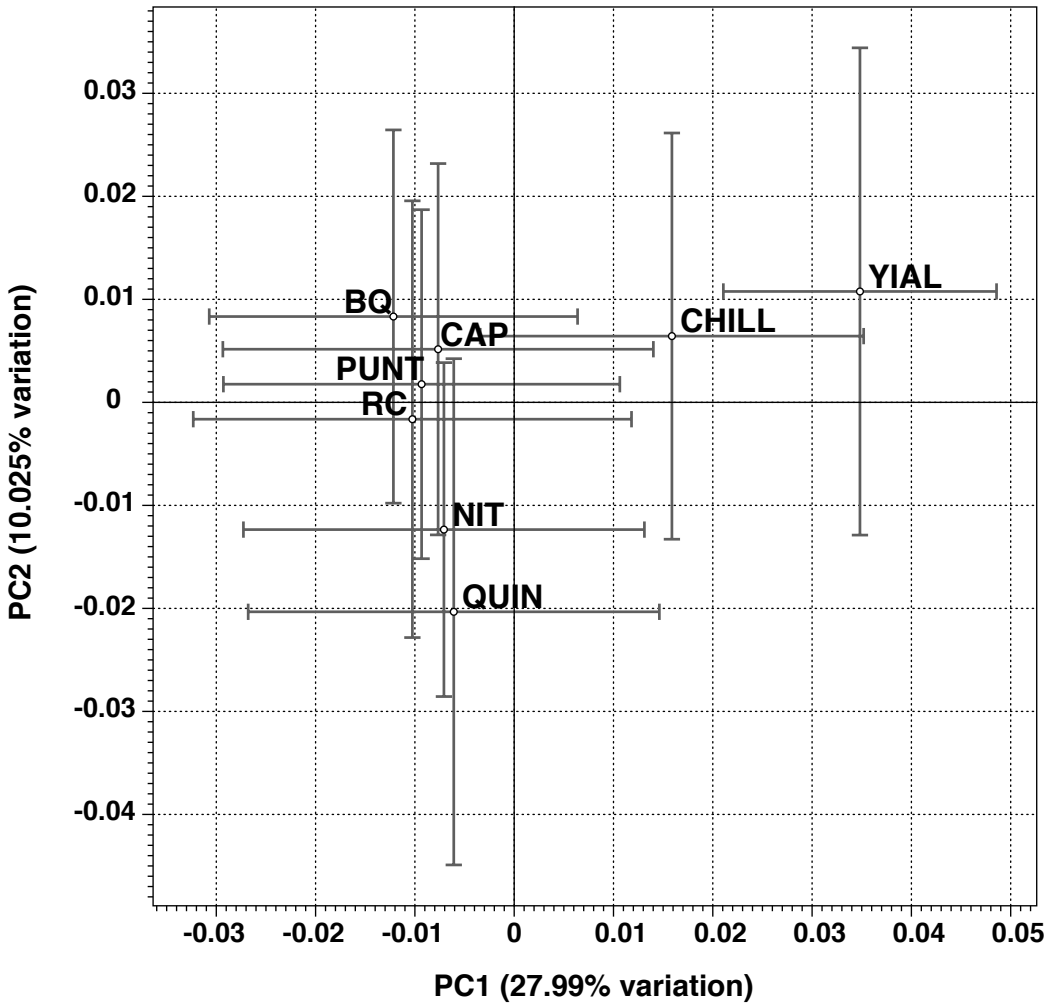

Figure 2.3. Principal coordinate analysis (PCoA) plot with the first two principal coordinate (PC) values. PCoA used pairwise Bray-Curtis distances across all gut microbiome samples from Chinook salmon hybrid offspring with sires from each identified source hybrid cross (see Figure 2.1). Each open circle represents the average PC coordinates for a breeding cross, and error bars represent 95% confidence intervals (CI). Hybrid cross abbreviations are defined in Figure 2.1.

Table 2.2. Overall PERMANOVA analysis using Bray-Curtis dissimilarity distances. Sire and Pen factors are nested within Cross. (Significance codes: '***' 0.001 '**' 0.01 '*' 0.05)

Factor	Df	Sums of Squares	Mean Squares	F-Model	R^2	P-value
Cross	7	3.72	0.53	1.97	0.05	0.001^{***}
Sire	59	19.91	0.34	1.25	0.25	0.002^{**}
Pen	8	2.17	0.27	1.01	0.03	0.443
Residuals	203	54.68	0.27		0.68	
Total	277	80.47	1.41		1.00	

Table 2.3. Pairwise PERMANOVA comparisons (BH-corrected p-values) based on Bray-Curtis. The number of permutations used in the analysis was 9999. P-values are corrected to two significant figures. (Significance codes: '***' 0.001 '**' 0.01 '*' 0.05)

	BQ	CAP	CHILL	NIT	PUNT	QUIN	RC
CAP	0.75						
CHILL	0.067	0.08					
NIT	0.52	0.79	0.067				
PUNT	0.71	0.76	0.067	0.75			
QUIN	0.31	0.54	0.076	0.79	0.50		
RC	0.75	0.87	0.11	0.76	0.70	0.75	
YIAL	0.0028^{**}	0.005^{**}	0.097	0.005^{**}	0.005^{**}	0.0065^{**}	0.005^{**}

In the overall PERMANOVA model, sire effects were found to have significant effects on the microbial community ($p = 0.002$, $R^2 = 0.25$; Table 2.2). Furthermore, using a unique PERMANOVA model for each cross, sire effects on Bray-Curtis distances were found to be significant within CHILL and NIT ($p = 0.001$, $R^2 = 0.434$ and $p = 0.006$, $R^2 = 0.308$, respectively), but no significant sire effects were found within other crosses (Table 2.4). Finally, while pen effects did not contribute significantly to differences in overall microbiome community composition for Bray-Curtis distances, a significant difference was found between replicate pens for RC ($p = 0.021$ and $R^2 = 0.262$, Table 2.4).

Table 2.4. Population cross-specific results of nested-PERMANOVA analysis using the Bray-Curtis distance. Sire and pen factors are nested within population crosses. Significance codes: '***' 0.001 '**' 0.01 '*' 0.05)

Population Cross	Factor	Df	Sum of Squares	Mean Squares	F-Model	R^2	P(>F)
BQ	Sire	7	2.26	0.32	1.16	0.20	0.20
	Pen	7	1.96	0.28	1.00	0.18	0.48
	Residuals	25	6.98	0.28		0.62	
	Total	39	11.21	0.88		1.00	
CAP	Sire	7	2.24	0.32	1.05	0.24	0.37
	Pen	6	1.13	0.19	0.61	0.12	1.00
	Residuals	19	5.80	0.31		0.63	
	Total	32	9.17	0.81		1.00	
CHILL	Sire	7	3.15	0.45	2.01	0.43	0.001***
	Pen	6	1.21	0.20	0.90	0.17	0.73
	Residuals	13	2.91	0.22		0.40	
	Total	26	7.27	0.87		1.00	
NIT	Sire	8	3.10	0.39	1.65	0.31	0.006**
	Pen	6	1.80	0.30	1.27	0.18	0.11
	Residuals	22	5.18	0.24		0.51	
	Total	36	10.07	0.92		1.00	
PUNT	Sire	8	2.45	0.31	1.14	0.25	0.21
	Pen	7	2.16	0.31	1.15	0.22	0.21
	Residuals	19	5.11	0.27		0.53	
	Total	34	9.73	0.88		1.00	
QUIN	Sire	8	2.71	0.34	1.22	0.30	0.14
	Pen	7	1.69	0.24	0.87	0.19	0.75
	Residuals	17	4.71	0.28		0.52	
	Total	32	9.11	0.86		1.00	
RC	Sire	7	2.07	0.30	1.21	0.24	0.18
	Pen	6	2.30	0.38	1.56	0.26	0.021*
	Residuals	18	4.41	0.25		0.50	
	Total	31	8.78	0.92		1.00	
YIAL	Sire	7	1.91	0.27	0.97	0.17	0.55
	Pen	8	2.41	0.30	1.06	0.21	0.32
	Residuals	25	7.07	0.28		0.62	
	Total	40	11.38	0.86		1.00	

Factors driving microbiome composition: OTU and family-level differential abundance

Overall, 1239 OTUs were found to be commo90o78n to all crosses, and the largest number of unique OTUs per cross (tripletons removed) were found to be in CHILL (220), YIAL (118) and BQ (105; Figure 2.4).

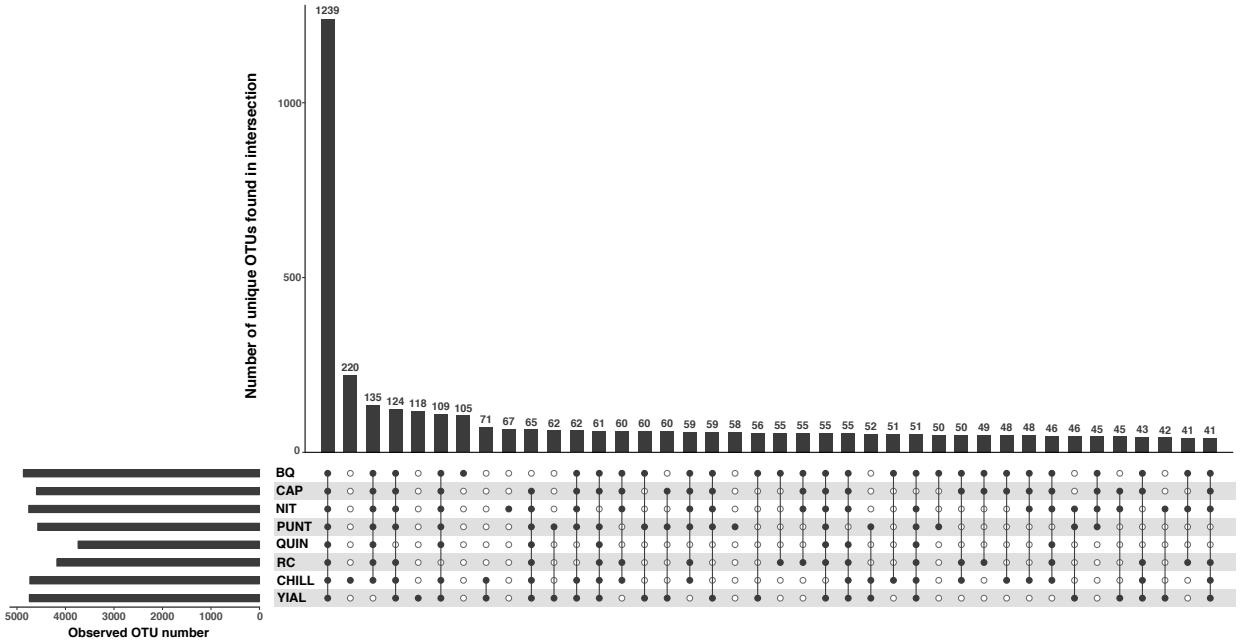

Figure 2.4. Unique OTU analysis presenting the largest overlap sizes and the corresponding hybrid crosses of Chinook salmon used in this study. OTUs occurring less than 3 times (tripletons) in the dataset were removed prior to the analysis. Overall, 1,239 OTUs commonly occurred in all crosses. CHILL, YIAL, and BQ were the stocks with the greatest number of unique OTUs. Conversely, YIAL, QUIN, and RC showed the greatest number of missing OTUs commonly found in other crosses. Hybrid cross abbreviations are defined in Figure 2.1.

After correcting for multiple tests (FDR), significant cross effects were found for 13 of the 110 tested microbial OTUs in the differential abundance analysis using linear mixed effects models (Appendix B1), and sire and pen effects were found for a single OTU (OTU.315506; Order: *Lactobacillales*). YIAL and CHILL crosses showed the most substantial divergence at the OTU level (Figure 2.5). Specifically, YIAL was significantly different from at least one stock for 11 of the 13 OTUs showing significant

cross effects, while additionally showing the highest or second highest relative abundance in 8 of them, after normalizing for uniform sequencing depth (Figure 2.5). YIAL exclusively accounted for all the pairwise differences in 3 OTUs (OTU.1061429, family: *Comamonadaceae*; OTU.1085832, family: *Streptococcus*; OTU.567840, family: *Bradyrhizobiaceae*), and CHILL in a similar way for a single OTU (OTU.315506, family: *Lactobacillales*). Interestingly, each of RC and NIT additionally showed significant pairwise comparison differences for a single OTU (OTU.145914, family: *Mycoplasmataceae*; OTU.NR.92, family: *Sphingomonadaceae*). A single OTU did not occur in CAP: OTU.315506 (Order: *Lactobacillales*).

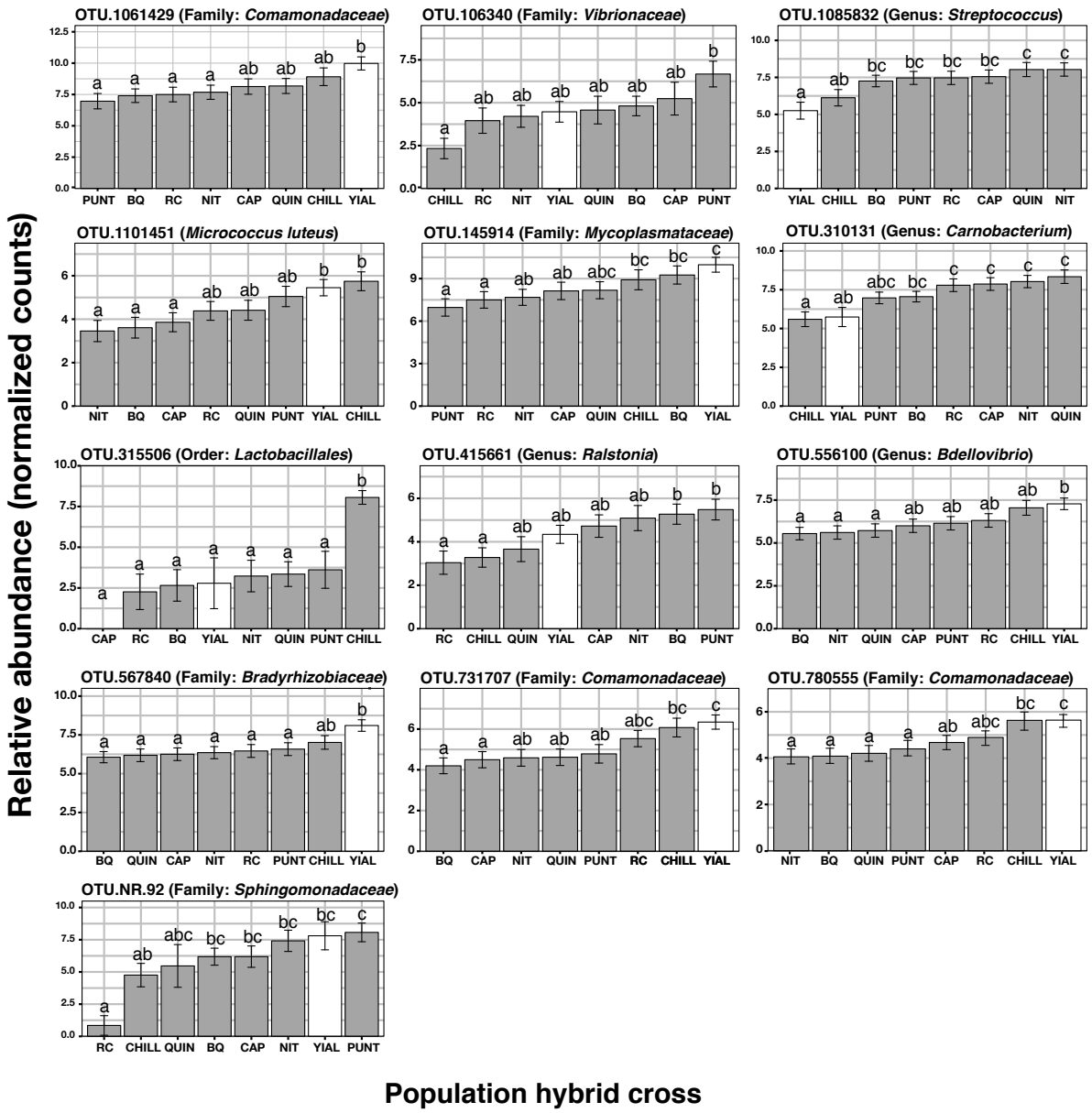

Figure 2.5. Histograms showing relative frequencies of candidate gut microbiome OTUs across all eight Chinook salmon hybrid crosses. Shown are 13 OTUs that showed significant differences among the crosses. Error bars represent standard error of the mean. Letters above the error bars represent post-hoc pairwise statistical differences among crosses, based on multiple student T tests of the mean ($P < 0.05$), adjusted for multiple comparisons with BH. Hybrid cross abbreviations are defined in Figure 2.1.

Using linear mixed effects model to test for hybrid cross, sire and pen effects, significant cross differences were found for seven (out of 44) tested taxonomic families (Appendix B2), pen effects were found for 3 taxonomic families (*Streptococcaceae*,

Carnobacteriaceae, and *Lactobacillaceae*) and sire effects were found for a single taxonomic family (*Bacillaceae*). Similar to OTU-level differences, YIAL and CHILL showed the greatest divergence in pairwise comparisons across all taxonomic families (Figure 2.6). Substantial divergence was exhibited by YIAL in 4 taxonomic families (*Bacillaceae*, *Streptococcaceae*, *Chitinophagaceae*, and *Mycobacteriaceae*) and by CHILL in 2 taxonomic families (*Leuconostocaceae*, *Lactobacillaceae*), and by both crosses in *Carnobacteriaceae* (Figure 2.6).

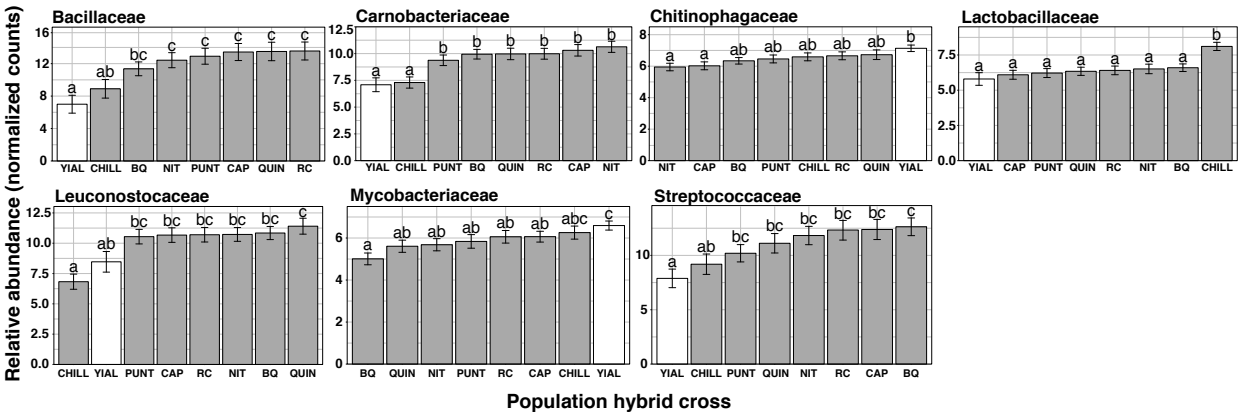

Figure 2.6. Histograms showing relative frequencies of candidate gut microbiome taxa families across all eight Chinook salmon hybrid crosses. Shown are seven taxonomic families that showed significant differences among the crosses. Error bars represent standard error of the mean. Letters above the error bars represent post-hoc pairwise statistical differences among crosses, based on multiple student T tests of the mean ($P < 0.05$), adjusted for multiple comparisons with BH. Hybrid cross abbreviations are defined in Figure 2.1.

Discussion

Host effects on the microbiome are less commonly reported at the among-population than within-population level, since many studies are based on single populations, albeit often involving various treatments (see Tables 2 & 3, Ghanbari *et al.*, 2015). Here, we provide evidence for significant cross effects reflective of strong inter-population genetic divergence effects, and significant sire effects indicative of additive genetics effects

acting within populations on the composition of the microbiome at the community (alpha- and beta-diversity) and finer (OTUs and taxonomic families) levels of the microbiome. In addition, we also found significant, but small and rare, pen effects that reflect environmental effects. The pen effects were not expected as the replicate pens were designed to be as similar as possible (size, water quality, feeding regime, etc.); however, these differences are likely due to the generally reported high magnitude of environmental drivers on the microbiome (Wu *et al.*, 2013; Goodrich *et al.*, 2014a; Sullam *et al.*, 2015; Rothschild *et al.*, 2018). Based on the published literature for fish, we expected to find among-population gut microbiome differences, indicative of previously reported host genetic divergence effects and the known role of the microbiome in assisting the hosts to cope with their environment (Sullam *et al.*, 2015; Webster *et al.*, 2018). As for sire effects, the differences observed in the microbiome phenotypic outcome were not expected due to the generally low heritability reported for humans (Yatsunenko *et al.*, 2012; Kurilshikov, Wijmenga, Fu & Zhernakova, 2017; Rothschild *et al.*, 2018; but see Goodrich *et al.*, 2014b), although this is the first report of additive genetic variation studies in fish microbiome composition. Given that all of the offspring in this study were reared in a common environment, from a common dam, two evolutionary processes may explain the among-stock differences found among the microbiomes: genetic drift and natural selection. Overall, the pattern of observed microbiome-based phenotypic differences among population crosses are consistent with patterns of host-microbiome co-divergence (Sullam *et al.*, 2015), while the pattern of within-population additive genetic variance may have been shaped by population-specific

selection pressures resulting from local stressors experienced in their native habitats (Savolainen, Lascoux & Merilä, 2013).

We found significant and consistent population cross effects on the composition of the microbiome, which reflect, primarily, among-population effects. Interpopulation differences and effects of origin (wild vs. hatchery) on the gut microbiome were recently reported using three hatchery-reared and four wild Atlantic salmon populations, showing substantial differences in the overall microbiome composition (beta diversity) and the core microbiome (Webster *et al.*, 2018). While the study did find larger differences among the genetically more divergent populations (based on microsatellite marker genotypes), it lacked the power to control for environmental effects, and diet was suspected to be a major factor driving the variation in composition of the microbiome among the studied populations (Webster *et al.*, 2018). It is critical to note in the breeding design used in this study, emphasis was placed on studying the contribution of cross and sire effects while controlling for maternal effects on growth (Semeniuk *et al.*, 2019). In our breeding design, we were able to estimate additive effects while virtually eliminating the potential for maternal effects. Maternal effects are composed of environmental and dam effects (Aykanat, Bryden & Heath, 2012a) and are known to contribute substantially to among-population phenotypic variation in Chinook salmon for life history and fitness-related traits (Aykanat, Heath, Dixon & Heath, 2012b). By using a common dam in our breeding design, we eliminated the potential for these maternal effects, thereby potentially limiting the detection of the maternal adaptive microbiome variance, such as those relating to immunity in Chinook salmon (Aykanat *et al.*, 2012a).

While consistent beta diversity population effects were observed, alpha diversity effects were not, despite other studies that did report them (Dehler, Secombes & Martin, 2017; Reveco *et al.*, 2014; Webster *et al.*, 2018). This might be due to the common and controlled pens. In this study, the magnitude of population cross effects on the microbiome varied in a consistent manner across analytical approaches: beta diversity and variation at the individual OTU or taxonomic family levels showed that the fully-domesticated cross, YIAL, exhibited the most divergent microbiome characteristics relative to all the hybrid crosses. It is likely that these differences derive from strong domestication selective pressures experienced within the YIAL production stock, perhaps driving rapid divergence in both the host and gut microbiome community. Interestingly, YIAL was not found to be significantly different in microbial community structure from CHILL, indicating that CHILL possess an intermediate microbiome structure. Although we found no differences in pairwise comparisons of microbial community structure level for CHILL versus the other hybrid cross stocks, they did approach significance for some populations (BQ, NIT, and PUNT), which suggests that functional differences may be present, but were undetectable given the statistical power of this study. Using the same study system and hybrid crosses, the CHILL hybrid cross was shown to vary from the other crosses in related studies. For example, in a study designed to detect gene expression differences among and within the hybrid crosses, CHILL exhibited a marked difference in gene transcription profile relative to the other hybrid cross stocks (including YIAL) consistent with the observe divergence pattern in this study (Toews, Wellband, Dixon & Heath, 2019). Furthermore, over the entire production period, CHILL was found to exhibit the lowest survival relative to the other crosses (Semeniuk *et al.*, 2019). Local

adaptation occurs when there is strong selection and limited drift effects (Yeaman and Otto, 2012). While large floods were experienced in the Chilliwack River (CHILL) between the years of 1952 and 1980 (Ham, 1996), conclusions about possible drift genetic (e.g. bottleneck events) are difficult to make, as the impact of those floods on Chilliwack River Chinook salmon stocks is unknown (Bradford, 1995). While we cannot rule out genetic drift acting to differentiate our study populations for gut microbiome composition (e.g., Whitehead, 2012), the patterns we observed at the OTU and bacterial family levels —which may involve crucial symbiotic roles— suggest divergent selection effects (Kawecki and Ebert, 2004). Therefore, our individual taxon analyses (at the OTU and taxonomic family levels) allowed us to explore potentially functional patterns of differences among the hybrid cross stocks. With some exceptions, YIAL and CHILL harboured significantly lower counts of several lactic acid bacteria (LABs), thought to contribute favourably to host health in fish (Ingerslev *et al.*, 2014; He, Chaganti & Heath, 2018). If higher LAB abundance is indeed adaptive, this suggests that lower LAB levels may account for the low survival observed in CHILL (Semeniuk *et al.*, 2019). However, it also leaves the higher levels of *Lactobacillales* and *Lactobacillaceae* in CHILL unexplained. Interestingly, CHILL also showed high levels of the pathogen *Micrococcus luteus*, which has been associated with health disorders in rainbow trout (Austin and Stobie, 1992; Pękala *et al.*, 2018) and brown trout (*Salmo trutta*; Pękala *et al.*, 2018). Together, these patterns suggest that individuals from our hybrid population crosses may experience reduced resistance, potentially due to *hybrid breakdown*, wherein a farmed population outbreeding with a wild counterpart results in reduced performance (Edmands, 1999; Lehnert, Love, Pitcher, Higgs & Heath, 2014). Furthermore, given that

YIAL exhibited intermediate cumulative survival (Semeniuk *et al.*, 2019), this begs the question of whether YIAL has adapted to survive in this environment through means other than accumulating beneficial bacteria through its multi-generational domesticated rearing. Finally, YIAL and CHILL harboured higher counts of OTUs classified as Comamonadaceae or Bradyrhizobiaceae, which commonly exhibit biochemical and ecological versatility (de Souza, Carrareto Alves, de Mello Varani & de Macedo Lemos, 2014; Willems, 2014). While not conclusive, these results point towards non-neutral co-divergence in host genetic architecture and microbiome community structure. Further work is needed to characterize the effects culminating in the formation of divergent microbiome community compositions among population crosses.

Significant within-population family differences, or sire effects, allowed us to estimate additive genetic variation effects. Additive genetic variation is a critical component of the overall genetic architecture for any trait, and it defines the scope for traditional evolutionary response to selection (Gjedrem, 1983; Garcia de Leaniz *et al.*, 2007; Visscher *et al.*, 2008; van Open, Oliver, Putnam & Gates, 2015). Within-population microbiome variation was found among unrelated families of rainbow trout (Naverrete *et al.*, 2012), but estimates of additive genetic variation in fish gut microbiome studies are lacking. This study presents the first report of additive genetic variance effects on the microbiome composition in fish. While the contribution of additive genetic effects to the composition of the gut microbiome in fish has not been studied, efforts have been made to quantify it in human studies, mainly showing that the microbiome generally exhibits low heritability (Yatsunenko *et al.*, 2012; Kurilshikov *et al.*, 2017; Rothschild *et al.*, 2018). In the breeding design used in this study, variation among sires within stocks

were estimated using half-sibling families as a measure of additive genetic variance, since a common egg source (i.e. highly inbred females combined) was used for all stocks. Given this breeding design and previous reports of low additive genetic variance, we expected that no additive genetic effects would be observed in this study. Despite that, we found consistent additive genetic effects on the on the microbiome composition at the beta-diversity level of the microbiome. Interestingly, significant additive genetic effects were found to be stock-specific (e.g., CHILL and NIT), perhaps reflecting lower selection pressures on the microbiome experienced in those populations. A previous study showed that microbial quantitative trait loci (mbQTLs) interact with host immunity to shape the gut microbiome in humans (Kurilshikov *et al.*, 2017). Additionally, MHC class II complex genotypes contribute to the regulation of the microbiome composition among hosts in a sex dependent manner in three-spine stickleback (*Gasterosteus aculeatus*; Bolnick *et al.*, 2014b). Although maternal vertical transmission is unlikely to be contributing to our additive genetic variation estimates, the role of paternal vertical transmission or other epigenetic effects on offspring microbiome composition have not yet been investigated. Variation in underlying genetic architecture (specifically additive genetic variance) among populations is critical to predict a population's response to selection and are a requisite for selective commercial (e.g. aquaculture) and non-commercial (e.g. conservation and restoration) breeding applications (Gjedrem, 1983; Visscher *et al.*, 2008; van Open *et al.*, 2015).

Pairs of replicate net pens for each population were included to allow the partitioning of possible environmental effects; however, our use of common rearing environments and matched net pens made strong environmental effects on gut

microbiome unlikely. Nevertheless, replicate pens effects were found to be significant for microbial community composition for the RC hybrid-cross, and in the differential abundance analysis at a single OTU (OTU.315506, order *Lactobacillales*) and three taxonomic families (*Carnobacteriaceae*, *Lactobacillaceae* and *Leuconostocaceae*), suggesting that some form of environmental effects across pens contributed to microbiome variation at the specific taxon level. These environmental effects may be explained by fine-scale environmental heterogeneity. Such effects can drive subtle phenotypic differences, often complicating the study of local adaptation, or genetics, in host-microbe systems (Kaltz & Shykoff, 1998; Savolainen *et al.*, 2013). Furthermore, we suspect that uncontrollable variation in social interactions among individuals may exist within pens (Gilmour *et al.*, 2005), and drive microbiome differences between replicates. This emphasizes the challenge in minimizing the effect of the environmental factors driving the gut microbiome, which have been shown to dominate host-related factors in humans (Wu *et al.*, 2013; Rothschild *et al.*, 2018).

In conclusion, our study shows a rarely reported pattern of population-level variation in the gut microbiome community in fish. Such a pattern is consistent with local adaptation, perhaps due to strong selection associated with seven generations of domestication combined with local selection forces acting to create divergent microbiome community compositions. Inter-population effects were the largest and most consistent drivers of gut microbiome variation among the hybrid cross stocks. Additive genetic variance and environmental effects contributed to variation at different hierarchical levels of the microbiome, with additive genetic contributing at the overall microbiome composition more strongly than pen effects. Microbiome OTU and taxonomic family

effects were found to be population-specific, further supporting the role of local population effects driving microbiome structure, despite rearing in a common environment with a common dam. Our results highlight the importance of preserving genetic variation in Chinook salmon to respond to environmental heterogeneity especially in the face of oceanic climate changes and habitat degradation from urban development and anthropogenic practices.

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CHAPTER THREE
Maternal effects on the egg-surface microbiome of Chinook salmon (*Oncorhynchus tshawytscha*)

Introduction

Organisms host complex microbial communities ("microbiomes"; McFall-Ngai *et al.,* 2013) across diverse organs, and these microbiomes are known to play important roles in host health and performance (de Bruijn *et al.,* 2017). Previous work has shown that the ontogenesis of the microbiome is generally a multi-step process involving interactions with the environment, host-specific selective immune factors, feeding-mediated microbial diversification (reviewed in Llewellyn *et al.,* 2014), and maternal vertical transmission (reviewed in Funkhouser & Bordenstein, 2013, and in Rosenberg & Rosenberg, 2013). Despite recent reports of ontogenetic effects on microbiome community composition and diversity in early and late fish life history stages (Bledsoe *et al.,* 2016; Llewellyn *et al.,* 2016), few studies have characterized the composition and function of the "egg-associated microbiome" (de Bruijn *et al.,* 2017). The egg-associated microbiome is used to describe microbial communities from either whole-egg homogenates or those that are found on external surfaces of eggs (Wilkins *et al.,* 2015a; Wilkins *et al.,* 2015b; Wilkins *et al.,* 2016). Historically, studies on egg-associated microbes focused on infective pathogenic strains during early egg development in salmonids, or "vertical transmission" (Evelyn *et al.,* 1986a; Cipriano 2005; Thoen *et al.,* 2011). The outer layer of fish eggs enables surface cell adhesion and colonization by microorganisms from the environment (Hansen *et al.,* 1989; Ringo and Birkbeck 1999), where bacteria colonize eggs as soon as they are laid (Yoshimizu *et al.,* 1980). Pathogenic bacteria capitalize on these growth-promoting conditions, proliferating on the surfaces of embryos prior to hatching, and

rapidly infect larvae shortly after hatching occurs (Olafsen, 1984; Olafsen & Hansen, 1992; Bergh & Hansen, 1992). In spite of the overall negative effects observed in single-strain infection studies, the majority of bacteria isolated from fish hatcheries are not found to be harmful to their hosts (Verner-Jeffreys *et al.,* 2003). For example, the microbiome bacteria play a critical role in egg health, forming the first line of defense against potential invading opportunistic pathogens (Boutin *et al.,* 2012; Liu *et al.,* 2014) — quite different from the pathogen-focused studies. The microbiome continues to develop once the fish start feeding, and numerous studies have shown evidence of diet-mediated gut microbiome establishment at this stage (Korsnes *et al.*, 2006; Reid *et al.*, 2009; Lauzon *et al.*, 2010; Bledsoe *et al.,* 2012; Ingreslev *et al.,* 2014). However, in catfish (*Ictalurus punctatus*) the gut microbiome community structure stabilizes around 4 months post-hatch (Bledsoe *et al.,* 2012). Therefore, due to the presence of egg-associated pathogens and the known role of the microbiome in host health and performance, more studies should focus on characterizing the microbiome development in early life stages in fish (Llewellyn *et al.,* 2014).

Despite the early work focusing on vertical transmission of pathogens in eggs, there is limited published research on the impact of environmental and host-related factors driving egg-associated microbiome diversity and composition (Wilkins *et al.,* 2015a; de Bruijn *et al.,* 2017). The microbiome of fish during their early-life stages was initially thought to be determined primarily by the surrounding aquatic environment (Llewellyn *et al.,* 2014; Ghanbari *et al.,* 2015). With the advent of next generation sequencing (NGS) technology and hence microbial community meta-barcoding, the estimation of various environmental and host effects on microbial communities became feasible (Funkhouser

& Bordenstein, 2013). Studies using NGS showed that fish egg-associated microbiomes are driven by variation in pathogenic stress (Liu *et al.,* 2014), water temperature (Wilkins *et al.,* 2015a), host development stage (Wilkins *et al.,* 2015b), nutrient-availability, and parental effects (i.e. sire and dam effects; Wilkins *et al.,* 2016). Furthermore, culture-based studies show species-specific differences in microbial communities enveloping the chorion (outer layer of eggs), suggesting a host receptor-mediated selective process in recruiting microbial communities to developing eggs (Hansen & Olfasen, 1989; Hansen & Olfasen, 1999). Host genome effects on microbiome community structure may be supplemented by maternal effects, which are mediated by maternal-environmental or maternal-genome contributions (Falconer, 1960; Heath & Blouw, 1998; Aykanat *et al.,* 2012a). Although maternal effects have been demonstrated for the eyed-egg associated microbiome of brown trout (*Salmo trutta*; Wilkins *et al.,* 2016), the potential mechanisms driving those reported effects have not been explored. Overall, despite recent studies that partition the role of genetics and the environment on eyed egg microbiome composition (Wilkins *et al.,* 2016), there is still a lack of certainty on what controls the egg surface microbiome, but it is expected that the aquatic environmental microbial community would be the major factor.

Chinook salmon are a species of Pacific salmonid occurring from northern Asia through North America (Beacham *et al.,* 2016). Female Chinook salmon prepare nests (redds) in the gravel and guard the redds from disturbance from other salmon (Quinn 2018). Maternal effects have been widely documented in Chinook salmon, and include, for example, immune response transcription variation (Aykanat *et al.,* 2012a), offspring size (Aykanat *et al.,* 2012b; Heath *et al.*, 1999), and flesh pigmentation (McCallum *et al.,*

1987). Maternal vertical transmission of the causative agent of enteric redmouth disease (ERM), *Yersinia ruckeri*, has been documented on unfertilized egg, eyed eggs, and fry surfaces (Glenn *et al.,* 2014). Further, intra-ovum infections with *Renibacterium salmoninarum* have been reported in Chinook salmon when exposed to ovarian fluid with higher concentrations of this bacterium agent (Lee & Evelyn, 1980). While there are no published reports of the egg-associated microbiome in Chinook salmon, a few studies have reported the gut microbiome in Chinook salmon (Booman *et al.,* 2018; Ciric *et al.,* 2018; Ciric *et al.,* 2019; Chapter 2, this thesis). Detailed study of Chinook salmon microbiome composition and function is particularly relevant as Chinook salmon have important economic and ecological significance. In 2017, the aquaculture production of Chinook salmon was in excess of 14,800 tonnes, valued at over $195 million USD, while the global Chinook salmon capture amounted to 5,751 tonnes (FAO, 2017). Chinook salmon are key to ecosystem ecology through nutrient cycling (Helfield *et al.*, 2001), trophic ecology (Koehler *et al.*, 2006), behavioural (Bernatchez & Dodson, 1987), and evolutionary relationships (Waples *et al.,* 2004).

While gut and skin microbiome dynamics have been widely studied in fish (Ghanbari *et al.,* 2014), the mechanisms of host-associated microbial community development are not well understood (Llewellyn *et al.,* 2014). To address this knowledge gap, we quantify maternal effects on bacterial egg surface microbiome diversity and composition in domesticated Chinook salmon (*Oncorhynchus tshawytscha)* from British Columbia (BC). Here, we measured dam effects, combining all genetic and environmental components of maternal inheritance, and tested for vertical transmission of microbial operational taxonomic units (OTUs) from the ovarian fluid to the egg surface

microbiome. Specifically, we estimate the maternal microbiome diversity and composition variance among individuals then tested for maternal vertical transmission by determining shared OTUs between ovarian fluid and eyed egg surface microbiomes, and tested for correlations between the alpha- and beta-diversity of the ovarian fluid and eyed eggs surface microbiomes. Estimating maternal effects is an essential component of characterizing the role of host genetic architecture on egg-associated microbiome diversity and function. Furthermore, determining the composition and mechanisms of egg-associated microbiome development is important to a more holistic characterization of the factors determining the microbiome composition in early life history stages of Chinook salmon, and may guide future conservation efforts of wild populations.

Materials and Methods

Experimental fish, and offspring maintenance and collection

Fieldwork was completed at Yellow Island Aquaculture Ltd. (YIAL), a commercial Pacific salmon hatchery and farm, located on Quadra Island, British Columbia, Canada. Production fish at YIAL have been domesticated for 11 generations, and were used to create all breeding crosses in this study. Briefly, milt from six Chinook salmon sires were mixed, and a subset of the mixture was used to fertilize eggs from each of 39 dams. The adult fish were humanely euthanized, and ovarian fluid (4 mL) and distal gut samples were collected from each dam to study maternal microbiome effects. The ovarian fluid (4 mL) was sampled from unfertilized eggs by immersing a sterile Falcon tube into bucket containing the eggs immediately after expressing the eggs, and subsequently stored in 11mL of high salt solution (Recipe: 25 mM sodium citrate, 10 mM EDTA, 70.3 g ammonium sulfate/100 ml solution, pH 5.31) in sterile 15 mL Flacon tubes for later DNA

extraction. The body cavity was cut open with a sterile scalpel, and the hindgut (1 to 1.5 inches long) was aseptically removed and stored in high salt buffer for later DNA extraction. Fertilized eggs from each full-sib family were reared in replicate cells in vertical incubation trays at 10°C with dissolved oxygen levels above 90% saturation. Accumulated thermal units (ATUs) were used to quantify the development stage of fertilized eggs, and at 280 ATUs, the "eyed" eggs were subsampled (15 eggs per tray cell) in duplicate. All eggs were stored in high salt buffer (see above) and held at -80°C for later DNA extraction.

Sample processing and DNA Extraction

Eyed egg surface DNA was collected as described by Liu *et al.,* (2014), with modifications to the protocol. Briefly, eyed eggs (n = 15 eggs per sample tube) were first rocked for 4 hours in the high salt buffer solution at a low speed using a Nutating 3D Platform Mixer (Thermo Scientific) at room temperature. The eggs were removed from the supernatant, which was then centrifuged at 20°C for 20 minutes, which precipitated a pellet in all tubes. The high salt buffer was removed without disturbing the pellet, and 400 µL of ddH_2O was added to each tube to resuspend the pellet for DNA extraction. The ovarian fluid samples were centrifuged at room temperature for 30 mins, then the supernatant was removed without disturbing the pellet, and the pellet (yellowish orange) was resuspended in 400 uL of ddH_2O. DNA from the resuspended pellet (400 uL) from the centrifuged ovarian fluid and eyed egg supernatant was extracted using the sucrose lysis buffer method as described in Shahraki *et al.,* (2018). DNA from hindgut content samples was extracted using a commercially available DNA extraction kit (SKU: D4015-01, Omega Bio-tek).

Library preparation and next generation sequencing

Library preparation of the PCR amplicons for next generation sequencing (NGS) was completed using two rounds of PCR. First, the V5-V6 region 16 rRNA gene (~220bp) was amplified from all extracted DNA samples in a 25 µL reaction mixture. The mixture contained 15.9 µL of ddH_2O; 2.5 µL of 10 × buffer (including Mg^{2+}); 3.5 µL of $MgSO_4$ (2 µM); 0.5 µL dNTPs (10 mM), 0.5 µL of V5F forward primer (10 µM, acctgcctgccgATTAGATACCCNGGTAG) and 0.5 µL V6R reverse primer (10 µM, acgccaccgagcCGACAGCCATGCANCACCT), 0.1 µL Taq polymerase (5 units/ µL, BioBasic, SKU: D0089) and 2 µL of DNA. The thermal cycler protocol consisted of an initial denaturation step (95°C for 60s), followed by 28 cycles of repeated denaturation, annealing and elongation (95°C for 15s, 55°C for 30s, and 72°C for 30s, respectively) and a final elongation stage (72°C for 7 min). The PCR product was visualised on a 2% agarose gel to determine amplification success. AMPure XP SPRI paramagnetic beads (Beckman Coulter Genomics GmbH, Mississauga, ON, Canada) were used to purify the PCR products. A second round of PCR was conducted to ligate adaptor and barcode sequences to the first-round amplicon for sample multiplexing in NGS (see Wellband *et al.,* 2019). The PCR reaction mixtures (total volume of 20 µL) consisted of 2.3 µL of ddH_2O, 2.5 µL of 10 × Buffer (including Mg^{2+}), 3.5 µL of $MgSO_4$ (2 µM); 0.5 µL dNTPs (0.10 mM), 0.5 µL UniA forward primer (10 µM, CCATCTCATCCCTGCGTGTCTCCGACTCAGXXXXXXXXXXGATacctgcctgccg), 0.5 µL UniB reverse primer (10 mM, CCTCTCTATGGGCAGTCGGTGATacgccaccgagc), 0.2 µL Taq polymerase (5 units/ µL) and 10 µL of purified first PCR product. In this round of PCR, the forward primer

consisted of a string of unique sequences (barcodes) for each sample which were used later to assign sequences to each sample based on the unique sequence (denoted above by XXXXXXXXXX) used for that sample. The thermal cycler protocol consisted of an initial denaturation step (95°C for 60s), followed by 7 cycles of repeated denaturation, annealing and elongation (95°C for 15s, 60°C for 30s, and 72°C for 30s) and a final elongation stage (72°C for 7 min). Amplicons were run on an agarose gel to visualize band intensity, and pooling of the amplicons was performed based on relative PCR band intensity. A commercially available gel extraction kit (GenCatch™ Gel Extraction Kit, Epoch Life Science, Inc., Sugar Land, Texas, USA) was used to purify the barcoded PCR product mix. We used the Agilent 2100 Bioanalyzer (Agilent Technologies, CA, U.S.A) to measure DNA concentration and verify amplicon size of the sample pool, followed by dilution appropriately for NGS (50 pM). The Ion Torrent™ Personalized Genome Machine (PGM; Thermo Fisher Scientific, Inc., Streetsville, Canada) was used for sequencing using a 318™ chip.

Sequence filtering and processing

Initial sequences quality check using default parameters was performed in the PGM software (Torrent Suite™ v5.7) to remove polyclonal and low-quality sequences. Further quality control and processing was performed on the sequences generated from the PGM in Quantitative Insights into Microbial Ecology (QIIME) pipeline, version 1.9.1 (Caporaso *et al.,* 2010). Raw sequences were demultiplexed, barcodes and adapter sequences were removed, and a quality sequence score threshold of 25 was maintained across all sequences. We identified and removed chimeric sequences using the USEARCH 6.1 algorithm (Edgar *et al.,* 2011). Clustering of sequences into operational

taxonomic units (OTUs) was performed and taxonomy assigned with UCLUST (Edgar 2010) at 97% similarity level. We used a 0.9 consensus threshold against the GreenGenes database for taxonomic identification (v13.8; DeSantis *et al.,* 2006). Non-bacterial OTUs were identified and removed. After sequence quality filtering, all samples with fewer than 3,000 reads were removed from further analysis.

Ordination analysis and community composition across all samples

The OTU table containing all generated OTUs (n = 12,280; singletons, doubletons, and tripletons removed) for eyed egg, dam gut and ovarian fluid samples was normalized using cumulative sum scaling (CSS), and then used to generate the Bray-Curtis based distance matrices. To visualize relationships among microbiome community composition across all samples (eyed egg surface, ovarian fluid, dam gut content), a principal coordinates analysis plot was generated in the PAST (v3.25) software package (Hammer *et al.,* 2001) based on a Bray-Curtis dissimilarity distance matrix. To test for beta-diversity level differences among microbiome types (ovarian fluid, dam gut content, and eyed egg samples) a permutational multivariate analysis of variance (PERMANOVA) with 9,999 permutations was run on the Bray-Curtis distance matrix in PAST (v3.25; Hammer *et al.,* 2001).

Testing maternal effects on eyed egg surface microbiome

Nested PERMANOVA models were conducted using the *adonis* function in the *vegan* package (v2.5-6; Oksanen *et al.,* 2013) to test for relative pairwise divergence of the surface bacterial community composition within versus among maternal family eyed egg samples. The PERMANOVA model (9,999 permutations) for eyed egg surface microbiome samples (n = 116) included the 39 dams, with incubation replicates nested

within dams. The mean (± 1.0 standard error) of pairwise Bray-Curtis distance between eyed egg microbiomes for each dam versus all other dams was calculated and used to create a summary histogram in PAST (v3.25; Hammer *et al.,* 2001).

Passive vertical transmission analyses

Microbiome sequence data from the ovarian fluid and eyed egg samples for 11 dams were used to assess the role of the maternal ovarian fluid as a source of the egg-surface microbiome. Only 11 of the 39 maternal families were used due to rejection of ovarian fluid microbiome sequencing data (i.e. failed QC). To test whether possible dam effects resulted from maternal vertical transmission, we identified the OTUs shared between eyed egg surface and ovarian fluid microbiomes within families. The OTUs that were shared between eyed egg and ovarian fluid samples from the same family was determined across all families; however, very few shared OTUs were found in more than 2 maternal families (most were shared in only one family, while some were shared in two families, out of 11). We then calculated the ovarian fluid and eyed egg OTU overlap for all "common" shared OTUs, that is, those found in at least 3 (out of 11) ovarian-fluid and eyed egg sample pairs. The shared OTUs represent evidence for possible vertical transmission. The OTU table for 11 pairs of dam and offspring was normalized using CSS, and a pairwise Bray-Curtis distance matrix was used to estimate PC1 and PC2 scores of ovarian fluid microbiome and mean eyed egg surface microbiome OTU composition. Pearson correlation coefficients were calculated between ovarian fluid microbiome PCs and eyed egg surface microbiomes using the PCoA scores from PC1 and PC2. Finally, the Chao1 index was estimated for each microbiome from the 11 family pairs (i.e. ovarian fluid and eyed egg surface) in QIIME v1.9.1 (Caporaso *et al.,*

2010) using OTU tables rarefied to minimum library size (3268 reads). We then tested for a correlation between ovarian fluid and eyed egg surface microbiome Chao1 index, PC1 and PC2 values using Pearson's correlation in PAST (v3.25; Hammer *et al.,* 2011).

Ovarian fluid and dam gut content analyses

To explore the potential for the dam gut microbiome as a source for the ovarian fluid microbiome, we compared the microbiomes for 10 dams (these are dams which had both ovarian fluid and gut microbiome data). To test for an association between ovarian fluid and gut microbiomes, we first normalized the OTU table for the 10 families of interest using CSS normalization. We then calculated the pairwise distance matrix using the Bray-Curtis dissimilarity index and tested for correlations between ovarian fluid and gut microbiome PC1 and PC2 scores using Pearson's correlation in PAST v3.25 (Hammer *et al.,* 2011). To test for correlations in alpha diversity between the two sample types, the Chao1 index was estimated for each microbiome from 10 pairs of maternal gut content and ovarian fluid sample pairs in QIIME v1.9.1 (Caporaso *et al.,* 2010) using OTU tables rarefied to minimum library size (3317 reads). Using the Chao1 index, we tested for correlations (using Pearson's correlation) between ovarian fluid and dam gut diversity in PAST v3.25 (Hammer *et al.,* 2011).

Results

Ordination analysis and community composition across all samples

To ensure adequate sequencing depth, samples with less than 3,000 sequence reads were removed from the study; the minimum number of reads was 3,024. After removing low-read samples, a total of 1,094,241 sequences were recovered across all ovarian fluid (n =

11), eyed egg surface (n = 116), and dam gut (n = 25) samples. The highest number of unique OTUs were found for eyed eggs (8,697), followed by dam gut (1,782) and ovarian fluid (912). In total, 7.15% of the OTUs found in the eyed eggs were also found in the ovarian fluid, and 40.7% of the OTUs found in the ovarian fluid microbiome were also found in the gut microbiome.

The first two PCs generated from the all microbiome data Bray-Curtis distance matrix accounted for 42.1% and 5.0% of the variance, respectively (Figure 3.1). Substantial separation was observed between the eyed egg microbiome and the ovarian fluid/ gut content microbiome PCs, while the gut and ovarian microbiome composition showed substantial overlap (Figure 1). Using permutational multivariate analysis of variance (PERMANOVA) of the Bray-Curtis matrix, a significant overall effect was found (F-model: 54.82; $P = 0.0001$) among three microbiomes (ovarian fluid, maternal gut and eyed egg surface). Pairwise PERMANOVA tests showed significant differences between eyed egg surface versus ovarian fluid (F-model: 38.79; $P = 0.0001$) and gut content (F-model: 88.74; $P = 0.0001$) OTU composition, but not between ovarian fluid and gut content OTU composition (F-model: 1.62; $P = 0.076$).

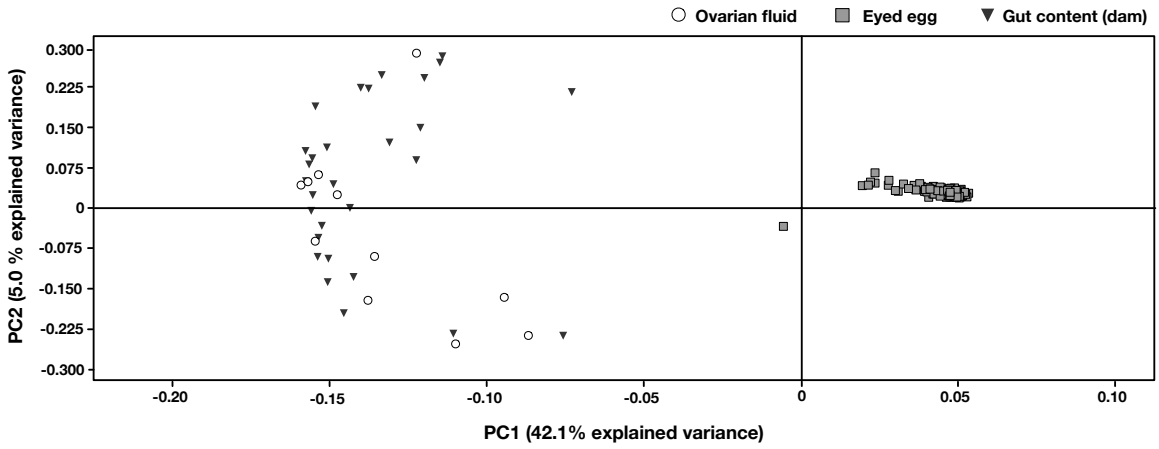

Figure 3.1. Scatterplot of the PC1 and PC2 from the principal coordinates analysis of pairwise Bray-Curtis dissimilarity distances. Samples include ovarian fluid (n = 11), dam gut content (n = 25) and eyed egg surface (n = 116) microbiomes from Chinook salmon. Bray-Curtis distances were calculated at the OTU-level for bacterial OTUs.

Testing maternal effects on eyed egg surface microbiome

After sequence filtration and using our minimum read depth cut-off, 116 eyed egg surface microbiome samples remained for the microbiome analyses. Of those, 16 families had four replicates, 10 had either two or three replicates, and 3 had a single replicate (Sample distribution in Appendix C1). After singleton, doubleton and tripleton removal, 764,838 reads were used for the analysis with 8,697 OTUs. Using nested PERMANOVA, significant dam (Df = 38, F-model = 1.53, $R^2 = 0.41$, $P < 0.001$) and replicate effects (Df = 32, F-model = 1.20, $R^2 = 0.27$, $P < 0.001$) were found for the microbial community composition of the eyed egg surface microbiome. The mean Bray-Curtis dissimilarity distances for eyed egg samples from each dam against all other unrelated dams ranged from 0.50 to 0.59 (Figure 3.2).

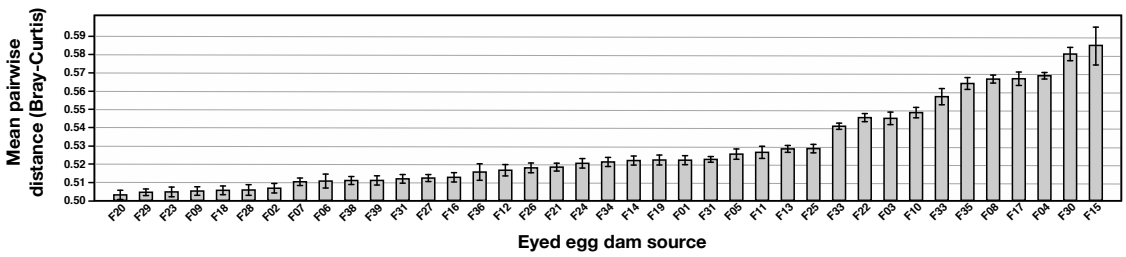

Figure 3.2. Histogram showing mean pairwise Bray-Curtis dissimilarities of egg eye surface microbiome composition among dams (X-axis). Dams are arranged from lowest (0.50) to highest (0.59) mean pairwise distance. Error bars represent one standard error.

Passive vertical transmission analyses

Our correlation analyses used 11 dam-offspring pairs as it was limited by low sequencing depth achieved for ovarian fluid samples. These analyses were performed to test for vertical transmission of specific microbial taxa from mother (ovarian fluid) to offspring (eyed egg surface). A total of 367 OTUs were found to be shared between eyed egg surface and ovarian fluid samples from at least a single same family (out of 11). This drops to 41 shared OTUs with a two-family threshold, and 7 OTUs when at least three-family overlap is set as the threshold. We tested for presence/absence patterns indicative of passive vertical transmission between ovarian fluid and eyed egg surface relative abundance for the 7 overlapping OTUs (three-pair threshold criterion) to allow for a conservative analysis (Figure 3.3). Considering the same family, 3 OTUs (OTU 818052, genus: *Lactococcus;* OTU 396697, *Clostridium perfingens;* OTU 398350, *family: Bacillaceae*) were shared in three families, 2 OTUs (OTU 465079, genus: *Bacillus*; OTU 31013, genus: *Carnobacterium*) were shared in four families, and 2 OTUs (OTU 112, genus: *Bacillus*; OTU 820837, genus: *Bacillus*) were shared in five families. Using pairs of ovarian fluid and eyed eggs corresponding to 11 families, we found no correlation between ovarian fluid and eyed egg surface composition based on the first two principal

coordinate scores of Bray-Curtis distances (PC1, R = -0.44, $P = 0.18$; PC2, R = 0.38, $P = 0.25$). Further, no significant correlation was found between the Chao1 index for ovarian fluid and eyed egg samples from the same family (Pearson's R = -0.46, P = 0.18). These correlation analyses indicate that simple maternal vertical transmission of the ovarian fluid microbial community to the surface of the eyed eggs is not likely responsible for the observed maternal effects on eyed egg surface microbiome composition.

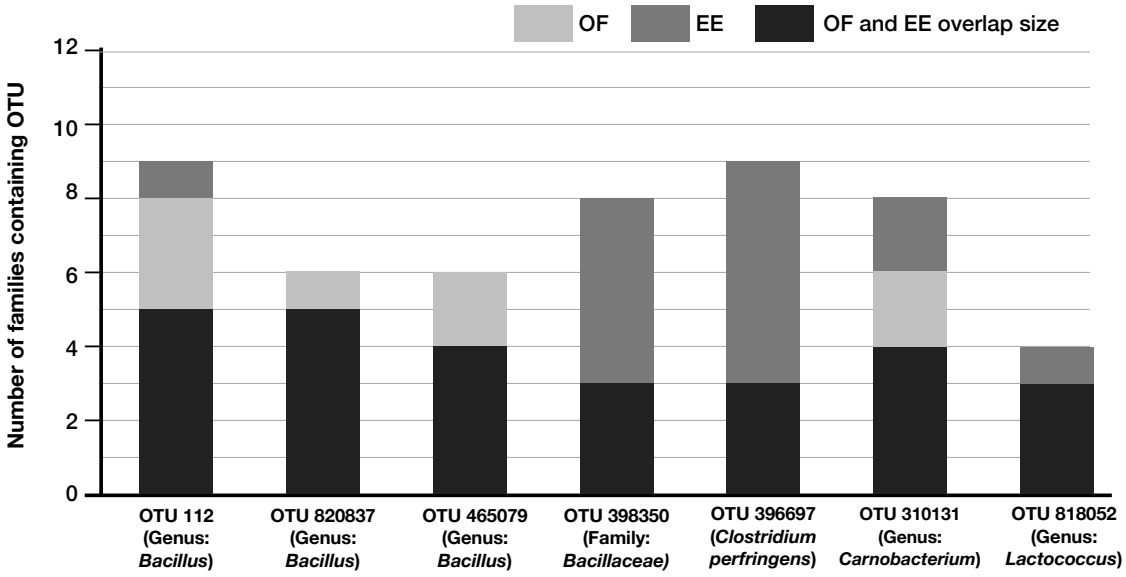

Figure 3.3. Stacked histogram showing OTU presence/ absence patterns across ovarian fluid (OF) and eyed eggs surface (EE) from 11 tested Chinook salmon families. Stacked bars represent the number of families in which the candidate OTU was detected (OF, or EE or both).

Ovarian fluid and dam gut analyses

40.7% of the OTUs found in the ovarian fluid microbiome were also found in the gut microbiome. Using pairs of ovarian fluid and dam gut samples corresponding to 10 different dams (due to read depth limitations for the gut and ovarian fluid samples), we found no evidence for a correlation between ovarian fluid and dam gut microbiomes based on the first two principal coordinates scores of Bray-Curtis distances (PC1, R = -

0.22, $P = 0.54$; PC2, R = 0.63, $P = 0.051$). However, a significant correlation was found for the Chao1 alpha diversity index between the ovarian fluid and dam gut microbial communities (R = 0.73, $P = 0.017$).

Discussion

We detected substantial dam effects on the surface bacterial community composition of eyed eggs in Chinook salmon. The dam effects reported here reflect a combination of maternal and additive genetic effects based on our breeding design (Aykanat *et al.,* 2012a; Wilkins *et al.,* 2016). Although a previous study showed differences among-dams on the eyed egg surface microbial community in brown trout (Wilkins *et al.,* 2016), our study presents exciting avenues to explore the mechanisms that underpin microbial community composition differences among dams. This includes an analysis of passive vertical transmission between ovarian fluid and eyed egg samples, and potential gut-level inoculation of the ovarian fluid microbial composition. Determining the role of maternal contribution in the developing microbiome community composition of eyed eggs will be critical to characterize microbial colonization and functional patterns at this life-history stage (Wilkins *et al.,* 2016).

Although we expected the environment to dominate in determining the composition of the eyed egg surface microbiome, we found unexpected but substantial dam effects. There are two possible mechanisms that can explain the observed dam-related variation among eyed egg surface microbiomes: 1) Additive effects mediated through embryo gene expression and 2) maternal effects, which may be adaptive or non-adaptive. First, the dam effects found in our study may result from host additive genetics-driven selection of bacterial assemblages, guided by known egg surface receptors (Hansen & Olfasen, 1999;

Olfasen 2001; de Brujin *et al.,* 2018) and dictated by host (embryo) genotype (Llewellyn *et al.,* 2014). Second, our observed dam effects may reflect non-genetic maternal effects, specifically, passive vertical transmission of bacteria (Brown, 1995). The final, and most likely explanation of the observed differences in the surface microbiome among eyed eggs due to dam of origin is a non-genetic, potentially adaptive, true maternal effect (Heath & Blouw, 1998; Heath *et al.,* 1999; Allen *et al.,* 2008).

Eyed egg embryos are known to express genes that code for proteins critical for their development and immune regulatory function in rainbow trout (*Oncorhynchus mykiss*; Wang *et al.,* 2010) and for immune system and various cell communication functions in Atlantic salmon (Bicskei *et al.,* 2016). For example, the immune regulatory FoxP3 protein expressed in rainbow trout (Wang *et al.,* 2010) is known to promote microbiome diversification (Kawamoto *et al.,* 2014). The chorion of Chinook salmon eggs is known to be permeable to large proteins, such as steroids (Warriner *et al.,* 2020), providing a mechanism for embryo-expressed proteins to be displayed on the eyed egg surface and hence modify egg surface microbiome composition. Finally, large maternal additive and dominance components drive expression patterns for the genes expressed in embryos in Atlantic salmon embryos (Bicskei *et al.,* 2016). Therefore, a maternally driven additive genetic component of egg surface microbial colonization by embryonic control is possible.

In salmonids, dams may directly transfer specific (potentially pathogenic) bacteria to eggs, (e.g. Evelyn *et al.,* 1986b; reviewed in Funkhouser & Bordenstein, 2014) which prompted us to use the ovarian fluid bacterial microbiome data to study passive transmission of bacteria to eyed eggs as a form of non-adaptive maternal effect. Although

over 900 and 8,000 OTUs were detected for ovarian fluid and eyed eggs, respectively, only 7 were found to overlap the two sample types in three or more families out of 11 tested. Although we found a high degree of overlap of OTUs classified as *Bacillus* and *Lactococcus* between the ovarian fluid and the eyed eggs, 5 (of 7) OTUs that showed three or more families with sharing between ovarian fluid and eyed egg surface microbiomes were also found in in more eyed egg surface microbiomes but without corresponding maternal ovarian fluid presence. This suggests that the majority of our identified "shared" OTUs are likely actually acquired from the environment. Furthermore, there was a lack of microbial community composition and alpha diversity correlation between ovarian fluid and egg surface abundance for these 7 overlapping OTUs – making simple vertical transmission unlikely to be a factor in our observed dam effects. Despite the lack of bacterial vertical transmission observed, it is critical to note that the power of our analysis was limited by a small number of ovarian fluid samples (n = 11) achieving sufficient sequencing depth. Nevertheless, given the very strong dam effect we detected, the potentially subtle role of bacterial vertical transmission in driving microbiome community structure on eyed egg surfaces is unlikely to be a major contributor.

Various molecules are acquired through maternal provisioning of the egg, such as yolk proteins in various fishes (Arukwe & Goksøyr, 2003), immune factors in Atlantic salmon and rainbow trout (Lillehaug, 1996; Løvoll *et al.*, 2006; Løvoll *et al.,* 2007) and mRNAs in sea bream (*Sparus aurata* L.; Picchietti *et al.,* 2006). Such provisioning plays a significant role in the survival and performance of the offspring in early-life stages by supporting its growth and protecting it against pathogens (reviewed in Zhang *et al.,*

2013). Perhaps the strongest indication of the adaptive significance of actively transmitted maternal components are immune factors (Seppola *et al.,* 2009; Zhang *et al.,* 2013). For example, this was shown for antibacterial properties of maternally transferred complement proteins in zebrafish (Wang *et al.,* 2008). Furthermore, variation in the inheritance of various maternally-derived immune factors exists (Swain & Nayak, 2009), and this may contribute to variation in egg-associated bacterial communities. In salmonids, maternal effects in general are known to be greater during the early development stages, decreasing in later life stages (Kinghorn, 1983; Heath & Blouw, 1998; Heath *et al.*, 1999; Aykanat *et al.,* 2012b). Finally, the mechanisms of active maternal transfer of such parental signals are currently unknown, although the ovarian fluid may be one mode of delivery. This study presents the first report of the ovarian fluid microbiome. Surprisingly, the composition of the ovarian fluid microbiome is correlated with that of the maternal gut microbiome at the alpha and beta-diversity level, with considerable overlap in OTU composition. This is counterintuitive: Fish are known to host diverse microbiomes that vary considerably by body site (Ye *et al.*, 2014; Gajardo *et al.*, 2016; Zhang *et al.*, 2017; Zhang *et al.,* 2019). However, as the urogenital papilla and the anus are proximal in salmonids (see Peaks *et al.,* 1997), the inoculation of ovarian fluid with bacteria from the gut is possible. If the ovarian fluid microbiome originates from the gut microbiome via inoculation effects, then the ovarian fluid would be expected to have a lower alpha diversity. Indeed, our alpha diversity analysis shows that ovarian fluid has a significantly lower Chao1 index. Previous studies have shown the presence of bacteria in the ovarian fluid in salmon using culture-techniques (Barnes *et al.,* 2010). Despite this, ovarian fluid is commonly studied exclusively for its role in reproduction

(e.g. see review in Johnson *et al.,* 2014), with disregard to its role in microbiome ontogenesis. Although this significance has not been investigated in fish, a recent study in humans showed that beneficial intestinal bacteria such as *Bifidobacterium* are transmitted from mother to infant during childbirth (Makino *et al.,* 2011; Milani *et al.,* 2015). Given the weak evidence found in this study for passive transmission of bacteria to the eggs, a gut-level inoculation of the eyed egg surface is unlikely, but further research employing a larger sample size or with larger sequencing depth may uncover evidence of passive (or active) maternal transmission. Finally, it is also possible that following ovarian fluid inoculation from the environment, host immune function may equally apply to the ovarian fluid microbiome and the gut microbiome, resulting in a similar composition. This would be possible since the ovarian fluid is known to be partially derived from filtered maternal blood plasma (Lahnsteiner *et al.,* 1995), and that previous studies detected bacterial response proteins (e.g. matrix metalloproteinase-9 precursor) in its composition (Johnson *et al.,* 2014). Therefore, ovarian fluid microbiome research provides exciting opportunities to study the role of maternal transfer in offspring microbiome composition and, ultimately, fitness.

In conclusion, we determined the maternal component of variation in the eyed egg surface microbiome community in Chinook salmon. Surprisingly, we found a strong dam component on the surface microbiota on eyed eggs, indicative of previously unreported maternal effects in Chinook salmon. However, what the mechanism driving these maternal effects might be, and how the processes underlying it are regulated by the developing embryo or by the mother remain unclear. Characterizing the mechanisms

driving these differences will be critical to determining whether they are truly adaptive or merely physiological by-products.

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CHAPTER FOUR
General Discussion

One of the fundamental pre-requisites for selection in any population is phenotypic variation, as determined by the host genetic architecture, the environment, and their interaction. As populations experience various evolutionary pressures, the genetic architecture of their fitness traits changes, leading populations to change in their responses to selective pressures (Kawecki and Ebert 2004). Quantifying the relative contributions of these components is critical to predicting host fitness and response to stressors. A critical emerging factor in the fitness of individuals is their microbiome, defined as the consortium of microorganisms found in and on the body of an individual (Llewellyn *et al.*, 2014; Ghanbari *et al.*, 2015). The microbiome is shown to be affected by various environmental factors across many animal taxa, from invertebrates such as squids and fruit flies, to vertebrates such as fish, mice, and humans (reviewed in Kostic *et al.*, 2013). However, the degree to which sources of genetic variation affecting microbiome composition have been characterized varies considerably among animal models. For example, the genetic architecture underlying microbiome composition is arguably best characterized in humans, with studies establishing many heritable taxa in the gut (Goodrich *et al.*, 2016) and determining single nucleotide polymorphisms (SNPs) in the host associated with specific microbiome taxa (Bleckman *et al.*, 2015; Wang *et al.*, 2016). These studies generally show that the environment dominates host-genetics in determining the microbiome composition and diversity (Rothschild *et al.*, 2018). This is contrasted in fish, where a few key knowledge gaps still exist and impact our understanding of the role of host genome versus environmental effects. As shown and reviewed in this thesis, numerous studies show that the microbiome is controlled by a

combination of environmental and host-related factors. The environmental factors range from application of dietary treatments (Ingerslev *et al.*, 2014; Ye at al. 2014; Sullam *et al.*, 2015) and probiotic applications in aquaculture (Ringø *et al.*, 2016) to those effects of rearing conditions and temporal effects (Sullam *et al.*, 2015). However, studies determining host-related drivers of the microbiome in fish have largely disregarded the overall effects of the underlying genetic architecture, while instead focusing on other host factors such as feeding behavior (Ingerslev *et al.*, 2014), intestinal structure (Ye *et al.*, 2014), or metabolic characteristics (Li *et al.*, 2013). Overall, these knowledge gaps can be summarized into three main categories: 1) the lack of fish-based experimental studies partitioning the effects of host-genetics and the environment; 2) the relative scarcity of studies employing multiple populations to examine among- and within- population genetic architecture components, especially under controlled settings, such as a common environment; and 3) the role of parental non-genetic effects, especially maternal effects, in driving microbial community composition and function. These knowledge gaps result in an overall weak characterization of genetic architecture underlying variation in the microbiome with respect to drivers occurring at the within-population and among-population levels in fish-microbiome research.

The overall goal of my thesis was to answer the question: What is the role of the host genetic architecture in driving the microbiome composition and diversity in Chinook salmon? To address that goal, my thesis made several contributions to the characterization of the microbiome in Chinook salmon, showing patterns of microbiome inheritance at two important life stages (eyed egg and salt-water juveniles) of Chinook salmon. As described in chapter 2, I characterized among-population and within-

population genetic variance components as factors in the composition of the gut microbiome in salt-water juvenile Chinook salmon. Despite small environmental effects, I showed strong evidence for 1) among-cross effects, indicative of population effects, and 2) within-cross, among families (sires) additive genetics effects driving the microbiome at the community (beta-diversity) and individual OTU levels. These effects were found for fish that were reared in the same environment — a necessary form of control for addressing possible confounding environmental effects that is rarely used to study host-genetics drivers of microbiome diversity and composition in fish. In chapter 3, I determined that the eyed-egg surface microbiome community composition is highly impacted by dam effects. Moreover, I provided the first report of Chinook salmon ovarian fluid microbiome but showed that there is no correlation or overlap with the eyed egg surface microbiomes. Interestingly, I found a surprising and previously unreported similarity between ovarian fluid and dam gut microbiome composition in terms of beta-diversity, and a significant positive correlation in alpha-diversity (Chao1 index). Finally, while my work on maternal effects on egg surface microbiome indicated a strong dam effect (41% of the variance explained by dam), there was a significant incubation cell effect (~27% of the variance explained) likely indicating significant environmental effects, and possibly (but unlikely) dam-by-environment interaction effects. Taken together, my findings show that in a controlled setting, microbiome acquisition from the environment undergoes selection by host factors. The outcomes of this thesis add to our knowledge of the effects of the underlying host genetic architecture on the microbiome at the among- and within-population levels.

Based on findings from both of my data chapters, I propose that the host and its microbiome have co-evolved for mutual benefit, reflective of local adaptation. A critical pre-requisite for these co-evolutionary dynamics is bi-directional, genetic-based effects of host-microbiome interactions (O'Brien *et al.*, 2019). Here, I postulate that selective pressures directly affecting the host also indirectly shape its microbiome, leading to host-microbiome co-evolution. To support this, I first showed that among-cross effects (population effects, Chapter 2), reflective of the native environment of sires, drive microbiome compositional differences. Although not conclusive evidence, I maintain that these results reflect additive among-population effects. Inter-population diversification of the microbiome phenotype may be random or adaptive. Despite short generation time and high rates of mutation in most bacteria (Linz *et al.*, 2014), evidence for neutral processes influencing microbiome evolution is weak, and deterministic processes (e.g. mutualism and parasitism and their associated selection pressures) appear to be the main driver (Kostella *et al.*, 2018). Therefore, the among-cross effects on I observed on the microbiome phenotype likely reflect non-random effects that are possibly an adaptive product of local adaptation (Taylor, 1991). Second, I found significant, cross (population)-specific, additive genetics effects on the composition of the gut microbiome (sire effects, Chapter 2). Additive effects are required for evolution by natural selection (Fisher, 1958; Aykanat *et al.*, 2012) and have been shown to drive the eyed egg surface microbiome in brown trout (*Salmo trutta*; e.g. Wilkins *et al.*, 2016) and in skin microbiomes of brook charr (*Salvelinus fontinalis*; Boutin *et al.*, 2014). Partitioning additive and non-additive genetic variance components for microbiomes associated with host fitness (e.g. gut microbiome, Vasemägi *et al.*, 2017; or eyed eggs, Wilkins *et al.*,

2016) will be critical to determining if local adaptation patterns are mediated by additive or non-additive gene action models. Third, I showed that the surface-microbiome of eyed eggs is controlled by maternal effects (Chapter 3). Fitness related early-life trait variation among populations in salmonids have been shown to be primarily driven by maternal effects (Aykanat *et al.*, 2012). Thus, the strong maternal component in eyed egg surface microbiome composition I reported may well have a population-specific component, a potentially fascinating line of research. In summary, although my data did not allow for an explicit test of local adaptation, evidence of host genetic architecture effects across my two data chapters supports patterns of local adaptation, in the forms of 1) additive among-population genetic variance, 2) within-populations, among-families additive genetics effects and 3) maternal effects on early-life history stages. Since there is an abundance of evidence for local adaptation effects on a wide range of phenotypic traits in salmonids (Taylor, 1991; Garcia de Leaniz *et al.*, 2007; Fraser *et al.*, 2011), it is critical to conduct further research on the adaptive potential of microbiome diversity in salmon. Determining the extent of adaption for traits related to host-fitness in populations of salmon is important for maintaining biodiversity (Fraser & Bernatchez, 2001).

By utilizing a common garden experiment (Chapter 2), I have shown that even when placed within the same environment, individuals from various crosses or families differ dramatically in the composition of the microbiome they host, challenging the notion that "the environment selects (Sanghera, 2015)." This adds to a growing body of literature showing host-mediated microbiome acquisition and colonization effects comprising host factors are driven by a genetic component, such as those observed in studies utilizing classical breeding designs for salmon (Wilkins *et al.*, 2016). By testing

microbiome differences associated with genetic architecture effects, I show that host-factors with a genetic basis allow individuals to select for microbial symbionts from the environment, establishing microbiomes that vary considerably in their composition. This begs the question: What mechanism underlies microbiome acquisition differences among related individuals in a common habitat? One common explanation for this observation is feeding behavior (Bolnick *et al.*, 2014b); however, as feeding behaviors are often linked to species-level differences (Ghanbari *et al.*, 2015), a more likely explanation of these host-mediated differences, at least partly, are variations in immune defenses (Bolnick *et al.*, 2014a). Variations in immune defenses impact both the hosts ability to evade or counter (i.e. select) microbial symbionts (Van Opstal & Bordenstein, 2015), indirectly contributing to microbiome variation. Overall, I have shown that microbiome selection from the environment could indirectly be influenced by the host genetic architecture.

A central purpose of this thesis was to fill the knowledge gap concerning the underlying genetic architecture associated with salmon microbiome composition, allowing us to better predict future microbiome evolution under changing environments and management strategies (Waples *et al.*, 2019). Despite increasing census population sizes, declining effective population sizes of Chinook salmon have been reported (Shrimpton & Heath, 2002), necessitating management efforts to maintain their potential adaptive genetic variation and to minimize inbreeding effects (Rieman & Allendorf, 2011). Microsatellite analyses reveal a highly structured underlying genetic architecture among populations of Chinook salmon (Bartley & Gall, 1990; Shirmpton & Heath, 2003), and in Pacific salmon in general (Waples *et al.*, 2019). Evidence presented in this thesis supports this, as indications of local adaptation are supported by demonstrating

additive among-population effects and maternal effects on early-life traits. Further, salmonids maintain a high capacity for evolution (Fraser *et al.*, 2011), and despite being anadromous and hence experiencing diverse aquatic environments, they evolve rapidly (Garcia de Leaniz *et al.*, 2007). As highlighted in chapter 2, perhaps this explains the presence of an additive genetic component to gut microbiome variation in Chinook salmon, defining the scope for traditional evolutionary response to selection (Visscher *et al.*, 2008). Nonetheless, because of the small effective population sizes reported for Chinook salmon (Heath *et al.*, 2002), theory predicts that responses to selection under an additive gene action model should decline over selection cycles, with epistatic gene action providing more long-term responses to environmental perturbations (Jannink *et al.*, 2003). Therefore, it is necessary to determine the potential for non-additive genetic components of host to contribute to the microbiome composition if the goal is to achieve a complete holistic of the evolutionary responses in rapidly changing environments (Jannink 2013). Finally, although genetic improvement programs have been initiated for Chinook salmon for traits relating to higher performance such as increased growth and feeding efficiency (Devlin *et al.*, 1995; Fjalestad *et al.*, 2003), no work has been attempted to utilize microbiome manipulation in these programs. This is perhaps due to the lack of knowledge of the microbiome's underlying genetic architecture in salmon. To artificially select for microbiomes that may be useful for hatcheries, two conditions should be met: 1) it must first demonstrated that the microbiome's diversity or composition (e.g. at the OTU or community level) has an additive genetics effect basis and 2) the microbiome should be either correlated directly to higher fitness in stocks or indirectly correlated with traits that confer such advantage. The reported additive genetics

effects (Chapter 2) contribute to the potential of selecting for microbiomes associated with higher fitness (Reed *et al.*, 2015). Thus, determining the role of host genetic architecture in driving microbiome composition and function might allow us to select for microbiomes (Zilber-Rosenberg & Rosenberg, 2008; Llewellyn *et al.*, 2014) that are possibly more diversified and stabilized (Llewellyn *et al.*, 2014). Once we master some level of control of microbiome function, we may be able to achieve the ultimate goal of fish farmers and conservation hatcheries: to select for microbial communities associated with lower mortality and better performance in fish.

Future directions

Although the work presented in this thesis contributes to our understanding of transgenerational control of the microbiome, the fields of quantitative genetics and population genetics have yet to answer many questions to better define this process. The host-associated microbiome is characterized by many factors that highlight its high capacity to evolve, including: fast generational turn-over, potential horizontal gene transfer, variable capacity of vertical transmission, capability to evolve community-level functions, and exposure to a wide-range of biotic selective pressures (Koskella *et al.*, 2018). With the advent of high throughput 'omics' approaches, we are on the cusp of new era in biology characterized by the rapid generation of large host-microbiome datasets (Misra *et al.*, 2019). These approaches capitalize on various levels of biological organization, with genomics focusing on DNA, transcriptomics on RNA, and proteomics on proteins to name a few. The integration of these tools into microbiome research will be key in advancing future research efforts, permitting studies to transition from focusing on taxonomic profiling to taxonomic and functional profiling (Ghanbari *et al.*, 2015).

Here, I propose some avenues for future microbiome research efforts as it relates to genetics and microbial ecology and propose areas where meta- 'omics' tools could be usefully integrated.

Many questions regarding the role of the host genetic architecture remain unanswered, making it a major line of future microbiome research. Opportunities to further explore the role of the host genetic architecture in controlling the microbiome range from interactions within and among loci (non-additive genetics), to whole-genome studies, and epigenetic effects. Utilizing tools in genomics will allow us to study the interaction of the host genome and its microbiome to determine, for example, if the additive genetic effect detected in my thesis (sire effects; chapter 2) are driven by a few major loci or many loci with small effects. Since the microbiome is a host phenotype with a complex genetic architecture (i.e. one that is polygenic; Benson *et al.*, 2016), genome-wide association studies (GWAS) may be the best approach to quantify the effects of various loci contributing to microbiome variation (e.g. Blekhman *et al.*, 2015). Further, by utilizing whole-genome comparative sequencing tools ("metagenomics"), more insight will be gained on the dynamics behind host genetic architecture variation and the microbiome response to it. For example, since mutations have a larger impact on microbial symbionts (due to their small genomes; Koskella *et al.*, 2018), the true the genetic potential for microbiome evolution is of critical interest (Ghanbari *et al.*, 2015). Finally, what is the role of host epigenetics on microbiome dynamics, and how does the microbiome itself influence host epigenetics? Surprisingly, even some the most comprehensive reviews on the microbiome do not address this problem (Nayak, 2010; Ghanbari *et al.*, 2015; Koskella *et al.*, 2018). Excitingly, evidence in human studies show

that microbial symbionts can indirectly guide epigenetic mechanisms by changing the repertoire of available metabolites utilized in epigenetic pathways (Hullar & Fu, 2015).

The function of the microbiome will likely continue to be another growing line of future microbiome research as 'omics' tools become more affordable (Misra *et al.*, 2019). Studies utilizing such tools can advance our knowledge of the function of the microbiome. For example, metatranscriptomics relies on cloning and sequencing messenger RNA (mRNA) molecules to determine active bacterial taxa in a microbiome, and which genes are being expressed in a specific environment (Franzosa *et al.*, 2015). These will include well-established functions such as nutrition (e.g. Nayak, 2010), or less understood ones such as the role of the microbiome in host ontology (Bledstoe *et al.*, 2016) or social behaviour (Soares *et al.*, 2019). Examples of studies using these tools are becoming more frequent (Misra *et al.*, 2019) and their applications will allow us to better characterize the role of microbiome in host health and disease.

The functions studied for the microbiome give evidence of its significance, but the adaptive potential of the microbiome is still to be quantified. The findings in this thesis suggest that population-level patterns of host-microbiome co-diversification exist (Chapter 2); however, further research is required to determine if the patterns observed among populations and among families within populations are truly a result of local adaptation. Ideally, future research should focus on utilizing natural populations (Hird, 2017) and utilize reciprocal transplants to determine if microbiome variation observed among populations (Chapter 2) is truly adaptive (Garcia de Leaniz *et al.*, 2007). Moreover, the role of microbiome variation in early-life stages remains unclear, and so does its adaptive potential. The maternal effects I found on the surface microbiome add to

growing knowledge that non-genetic maternal effects contribute significantly to population-level effects for fitness traits in salmon, sometimes more strongly than additive genetics (Aykanat *et al.*, 2012). Therefore, quantifying maternal effects will be critical to determining the adaptive potential of the microbiome. Finally, quantifying the adaptive potential of the microbiome will require a better characterization of host-responses to the microbiome. To measure host-mediated selection of the microbiome, the mechanisms underlying host immunity and protection against pathogens (Van Opstal & Bordenstein, 2015), competition for nutrients (in the gut; Coyte *et al.*, 2015), and development-based factors (Llewellyn *et al.*, 2014) will be the major areas of focus.

Conclusions

In addition to providing the first microbiome community characterization for Chinook salmon eyed eggs, my work contributes important findings to the genetic architecture effects on the microbiome composition and diversity in Chinook salmon. Specifically, I demonstrate, for the first time, genetic effects at the among-population and among-family (within-population) levels in a controlled semi-natural environment. Furthermore, I demonstrate significant maternal effects on the surface microbiome of eyed eggs, and the first description of the ovarian fluid microbiome in fish, and the similarity to that of the gut microbiome in dams. Overall, this thesis will lead the way to conducting more research into the adaptive potential of the microbiome in salmon and will have critical implications for conservation biology and commercial aquaculture.

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APPENDICES

Appendix A1. Distribution of the samples used in Chapter 2. Crosses are divided into two columns representing the pens, and the sires used in each replicate pen are listed beside it. The original number of samples surviving to the sea net pen are shown, and the numbers in brackets indicate the samples realized in the final study analysis with overall depth of 3000 sequences or higher.

Population hybrid cross	Pen 1	Sires in Pen 1	No. of samples (Individuals with ≥3000 reads)	Pen 2	Sires in Pen 2	No. of samples (Individuals with ≥3000 reads)
Big Qualicum (BQ) N = 69 (40)	2BN N = 40 (22)	BQ1	6 (3)	2BS N = 29 (18)	BQ1	3 (2)
		BQ2	4 (3)		BQ2	2 (1)
		BQ3	6 (5)		BQ3	1 (1)
		BQ4	5 (4)		BQ4	11 (7)
		BQ5	4 (1)		BQ5	5 (3)
		BQ6	6 (5)		BQ6	2 (1)
		BQ7	2 (1)		BQ7	2 (2)
		BQ8	1 (0)		BQ8	1 (1)
		BQ9	5 (0)		BQ9	1 (0)
		Unrecorded	1 (0)		Unrecorded	1 (0)
Capillano (CAP) N = 50 (33)	7A N = 20 (15)	C1	4 (3)	21BS N = 30 (18)	C1	4 (2)
		C2	3 (3)		C2	5 (3)
		C3	3 (1)		C3	1 (1)
		C4	4 (4)		C4	6 (5)
		C5	2 (2)		C5	3 (1)
		C6	1 (0)		C6	4 (3)
		C7	0 (0)		C7	0 (0)
		C8	2 (2)		C8	2 (2)
		C9	1 (0)		C9	1 (1)
		C10	0 (0)		C10	3 (0)
Chilliwack (CHILL) N = 53 (27)	19BN N = 24 (10)	Ch1	1 (0)	22BS N = 29 (17)	Ch1	3 (2)
		Ch2	4 (2)		Ch2	3 (2)
		Ch3	1 (0)		Ch3	1 (1)
		Ch4	3 (1)		Ch4	5 (4)
		Ch5	5 (1)		Ch5	3 (2)
		Ch6	3 (2)		Ch6	4 (2)
		Ch7	4 (3)		Ch7	3 (3)
		Ch8	1 (1)		Ch8	3 (1)

		Ch9	0 (0)		Ch9	1 (0)
		Ch10	2 (0)		Ch10	0 (0)
		Unrecorded	0 (0)		Unrecorded	3 (0)
Puntledge (PUNT) N = 59 (35)	4BS N = 26 (15)	P1	1 (1)	10BS N = 33 (20)	P1	4 (2)
		P2	4 (2)		P2	2 (1)
		P3	4 (3)		P3	7 (5)
		P4	2 (1)		P4	4 (2)
		P5	4 (3)		P5	5 (4)
		P6	3 (1)		P6	2 (2)
		P7	1 (1)		P7	0 (0)
		P8	5 (3)		P8	2 (2)
		P9	1 (0)		P9	5 (2)
		P10	1 (0)		P10	2 (0)
Nitinat (NIT) N = 55 (37)	10BN N = 26 (20)	N1	6 (6)	7BS N = 29 (17)	N1	2 (2)
		N2	1 (1)		N2	5 (3)
		N3	3 (1)		N3	2 (2)
		N4	3 (2)		N4	4 (3)
		N5	0 (0)		N5	3 (2)
		N6	5 (5)		N6	3 (2)
		N7	2 (2)		N7	0 (0)
		N8	2 (2)		N8	7 (3)
		N9	2 (0)		N9	0 (0)
		N10	1 (1)		N10	1 (0)
		Unrecorded	1 (0)		Unrecorded	2 (0)
Quinsam (QUIN) N = 49 (33)	21BN N = 25 (19)	Q1	1 (1)	19A N = 24 (14)	Q1	4 (2)
		Q2	2 (2)		Q2	0 (0)
		Q3	2 (2)		Q3	1 (1)
		Q4	4 (4)		Q4	3 (3)
		Q5	3 (3)		Q5	4 (3)
		Q6	3 (2)		Q6	4 (2)
		Q7	3 (2)		Q7	1 (0)
		Q8	2 (2)		Q8	3 (2)
		Q9	4 (0)		Q9	1 (0)
		Q10	1 (1)		Q10	3 (1)
Robertson Creek (RC) N = 51 (32)	19BS N = 29 (17)	RC1	3 (2)	22BS N = 22 (15)	RC1	4 (4)
		RC2	2 (2)		RC2	1 (0)
		RC3	3 (3)		RC3	2 (1)
		RC4	2 (2)		RC4	4 (3)
		RC5	4 (3)		RC5	2 (2)

		RC6	2 (2)		RC6	2 (1)
		RC7	3 (3)		RC7	2 (2)
		RC8	0 (0)		RC8	2 (2)
		RC9	4 (0)		RC9	1 (0)
		RC10	6 (0)		RC10	2 (0)
Yellow Island (YIAL) N = 80 (41)	4BN N = 30 (16)	Y1	3 (2)	7BN N = 50 (25)	Y1	6 (5)
		Y2	3 (2)		Y2	6 (4)
		Y3	5 (3)		Y3	4 (3)
		Y4	2 (1)		Y4	5 (4)
		Y5	4 (3)		Y5	5 (2)
		Y6	2 (1)		Y6	2 (1)
		Y7	3 (3)		Y7	4 (3)
		Y8	2 (1)		Y8	7 (3)
		Y9	1 (0)		Y9	6 (0)
		Y10	4 (0)		Y10	5 (0)
		Unrecorded	1 (0)		Unrecorded	0 (0)

Appendix B1. OTU-level differential abundance analysis results corresponding to LRT on fitted LMMs. The Benjamini-Hochberg method was used to adjust for multiple comparisons. Abbreviations: NR = "New Reference". Significance codes: '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1)

OTU	Cross LRT P-value	Pen LRT P-value	Sire LRT P-value	OTU taxonomic classification (Lowest identified rank)
OTU.511679	0.25	1.00	1.00	Genus: Mycoplasma
OTU.145914	0.021*	1.00	1.00	Family: Mycoplasmataceae
OTU.147311	0.77	1.00	1.00	Family: Mycoplasmataceae
OTU.816470	0.11	1.00	1.00	Genus: Bacillus
OTU.349839	0.27	0.68	1.00	Genus: Photobacterium
OTU.1823053	0.11	1.00	1.00	Genus: Lactococcus
OTU.1061429	0.044*	0.68	1.00	Family: Comamonadaceae
NR.OTU.145	0.18	1.00	1.00	Class: ZB2
NR.OTU.6	0.84	1.00	1.00	Genus: Mycoplasma
OTU.785565	0.43	1.00	1.00	Class: Gammaproteobacteria
NR.OTU.188	0.11	1.00	1.00	Family: Micrococcaceae
OTU.106476	0.75	1.00	1.00	Order: Vibrionales
NR.OTU.125	0.37	1.00	1.00	Family: Mycoplasmataceae
NR.OTU.163	0.49	1.00	1.00	Genus: Mycoplasma
OTU.974121	0.05	1.00	1.00	Genus: Pseudomonas
OTU.84937	0.20	1.00	1.00	Family: Vibrionaceae
OTU.594370	0.36	1.00	1.00	Family: Bacillaceae
OTU.995978	0.07	1.00	1.00	Family: Comamonadaceae
NR.OTU.282	0.66	1.00	1.00	Genus: Mycoplasma
OTU.538602	0.16	1.00	1.00	Family: Vibrionaceae
OTU.854050	0.11	1.00	1.00	Genus: Bacillus
OTU.592425	0.34	1.00	1.00	Family: Desulfovibrionaceae
OTU.939811	0.31	1.00	1.00	Genus: Vibrio
OTU.396697	0.58	1.00	1.00	*Clostridium perfringens*
OTU.516115	0.31	1.00	1.00	Genus: Lactococcus

OTU.783719	0.25	1.00	1.00	Genus: Ralstonia
OTU.586387	0.07	1.00	1.00	Genus: Lactococcus
OTU.1097359	0.63	1.00	1.00	Genus: Acinetobacter
OTU.567840	0.044*	0.68	1.00	Family: Bradyrhizobiaceae
OTU.815406	0.13	1.00	1.00	Genus: Acidovorax
OTU.739614	0.17	1.00	1.00	Genus: Vibrio
OTU.818603	0.42	1.00	1.00	Genus: Bacillus
NR.OTU.43	0.49	1.00	1.00	Genus: Photobacterium
OTU.1076969	0.16	1.00	1.00	Genus: Streptococcus
NR.OTU.76	0.64	1.00	1.00	Class: Gammaproteobacteria
OTU.120952	0.13	1.00	1.00	Genus: Burkholderia
OTU.805055	0.09	1.00	1.00	Genus: Lactococcus
OTU.540940	0.27	1.00	1.00	Genus: Leuconostoc
OTU.556100	0.044*	1.00	1.00	Genus: Bdellovibrio
OTU.1716185	0.43	1.00	1.00	Genus: Enterovibrio
OTU.584580	0.16	1.00	1.00	Genus: Hydrogenophaga
OTU.326324	0.36	1.00	1.00	Order: Vibrionales
OTU.580625	0.07	0.90	1.00	*Bosea genosp.*
OTU.712047	0.48	1.00	1.00	Family: Clostridiaceae
OTU.877752	0.25	1.00	1.00	Genus: Pseudomonas
NR.OTU.26	0.42	1.00	1.00	Family: Mycoplasmataceae
NR.OTU.184	0.43	1.00	1.00	Genus: Mycoplasma
NR.OTU.75	0.45	1.00	1.00	Class: Gammaproteobacteria
NR.OTU.130	0.84	1.00	1.00	Family: Bacillaceae
NR.OTU.106	0.95	1.00	1.00	Family: Mycoplasmataceae
OTU.818052	0.67	1.00	1.00	Genus: Lactococcus
OTU.244657	0.40	1.00	1.00	*Bosea genosp.*
NR.OTU.231	0.62	1.00	1.00	Family: Mycoplasmataceae
OTU.1110763	0.25	1.00	1.00	Family: Enterobacteriaceae
OTU.18223	0.58	1.00	1.00	Family: Vibrionaceae
OTU.1085832	0.043*	0.90	1.00	Genus: Streptococcus

OTU.331652	0.43	1.00	1.00	Family: Vibrionaceae
OTU.144640	0.09	1.00	1.00	Family: Comamonadaceae
OTU.106340	0.044*	0.68	1.00	Family: Vibrionaceae
NR.OTU.14	0.45	1.00	1.00	Genus: Mycoplasma
OTU.930834	0.18	1.00	1.00	*Pseudomonas veronii*
OTU.1074801	0.18	1.00	1.00	Genus: Sphingomonas
NR.OTU.242	0.84	1.00	1.00	Genus: Bacillus
NR.OTU.56	0.85	1.00	1.00	Genus: Mycoplasma
OTU.310131	0.0066**	1.00	1.00	Genus: Carnobacterium
OTU.553472	0.42	1.00	1.00	Genus: Vibrio
OTU.200890	0.18	1.00	1.00	Class: Betaproteobacteria
NR.OTU.197	0.40	1.00	1.00	Genus: Mycoplasma
NR.OTU.110	0.45	1.00	1.00	Class: Gammaproteobacteria
NR.OTU.118	0.90	1.00	1.00	Family: Mycoplasmataceae
OTU.590960	0.23	1.00	1.00	Family: Comamonadaceae
OTU.668105	0.15	1.00	1.00	Family: Sphingomonadaceae
NR.OTU.108	0.11	1.00	1.00	Genus: Lactococcus
OTU.366419	0.60	1.00	1.00	Genus: Geobacillus
OTU.750840	0.29	1.00	1.00	Family: Comamonadaceae
NR.OTU.92	0.044*	1.00	1.00	Family: Sphingomonadaceae
NR.OTU.225	0.77	1.00	1.00	Family: Mycoplasmataceae
OTU.2874742	0.77	1.00	1.00	Genus: Bacillus
OTU.319533	0.45	1.00	1.00	Class: Gammaproteobacteria
NR.OTU.170	0.18	1.00	1.00	Genus: Vibrio
NR.OTU.208	0.37	1.00	1.00	Family: Vibrionaceae
OTU.554346	0.36	1.00	1.00	Genus: Photobacterium
NR.OTU.166	0.42	1.00	1.00	Family: Mycoplasmataceae
NR.OTU.21	0.15	1.00	1.00	*Mycoplasma microti*
NR.OTU.16	0.25	1.00	1.00	*Shewanella benthica*
NR.OTU.10	0.30	1.00	1.00	Genus: Bacillus
OTU.731707	0.0097**	0.22	1.00	Family: Comamonadaceae

OTU.668303	0.40	1.00	1.00	Genus: Burkholderia
NR.OTU.216	0.20	1.00	1.00	Family: Comamonadaceae
OTU.323791	0.17	1.00	1.00	Family: Rhizobiaceae
OTU.820837	0.91	1.00	1.00	Genus: Bacillus
OTU.315506	$8.13E^{-7***}$	$2.32E^{-5***}$	0.017^{***}	Order: Lactobacillales
NR.OTU.273	0.18	1.00	1.00	Family: Vibrionaceae
NR.OTU.79	0.36	1.00	1.00	Genus: Hyphomicrobium
OTU.928776	0.27	1.00	1.00	Genus: Acinetobacter
OTU.780555	0.044^{*}	0.68	1.00	Family: Comamonadaceae
OTU.928829	0.38	1.00	1.00	Genus: Pseudomonas
NR.OTU.230	0.45	1.00	1.00	Family: Mycoplasmataceae
NR.OTU.278	0.30	1.00	1.00	*Clostridium perfringens*
OTU.1101451	0.021^{*}	0.52	1.00	*Micrococcus luteus*
OTU.306996	0.42	1.00	1.00	Genus: Burkholderia
OTU.1108275	0.45	1.00	1.00	Family: Comamonadaceae
NR.OTU.192	0.12	1.00	1.00	Family: Vibrionaceae
NR.OTU.281	0.71	1.00	1.00	Order: Bacillales
OTU.874999	0.36	1.00	1.00	*Enhydrobacter aerosaccus*
OTU.415661	0.033^{*}	1.00	1.00	Genus: Ralstonia
NR.OTU.12	0.25	1.00	1.00	Family: Mycoplasmataceae
OTU.938794	0.20	1.00	1.00	Genus: Acinetobacter
NR.OTU.191	0.33	1.00	1.00	Family: Bradyrhizobiaceae
NR.OTU.207	0.07	1.00	1.00	Family: Sphingomonadaceae

Appendix B2. Taxonomic family-level differential abundance analysis results corresponding to LRT on fitted LMMs. The Benjamini-Hochberg method was used to adjust for multiple comparisons. Significance codes: '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1).

Bacterial Family	Cross LRT P-value	Pen LRT P-value	Sire LRT P-value
Mycoplasmataceae	0.17	1.00	0.68
Vibrionaceae	0.55	0.60	1
Bacillaceae	4.92E-05*	0.87	1.73E-03*
Streptococcaceae	5.35E-04*	0.74	0.052
Comamonadaceae	0.23	0.38	0.016
Micrococcaceae	0.022	1.00	0.17
Pseudomonadaceae	0.16	0.090.	0.0063
Clostridiaceae	0.045	0.68	1
Bradyrhizobiaceae	0.31	0.36	9.63E-04*
Moraxellaceae	0.13	0.58	2.80E-03*
Oxalobacteraceae	0.15	0.23	0.68
Desulfovibrionaceae	0.16	1.00	0.046
Sphingomonadaceae	0.92	0.47	0.15
Burkholderiaceae	0.7	0.40	0.036
Enterobacteriaceae	0.011	0.10	0.18
Leuconostocaceae	7.48E-06*	2.99E-04***	0.64
Bdellovibrionaceae	0.015	0.11	0.011
Carnobacteriaceae	5.31E-06*	2.66E-04***	0.80
Rhodobacteraceae	0.29	0.98	0.98
Hyphomicrobiaceae	0.43	0.53	0.51
Enterococcaceae	0.1	1.00	0.38
Shewanellaceae	0.16	0.79	1
Rhizobiaceae	0.068	0.14	0.47
Flavobacteriaceae	0.29	0.82	0.20
[Weeksellaceae]	0.033	0.68	0.80

Rhodocyclaceae	0.017	0.12	0.11
Rhodospirillaceae	0.011	0.350	0.081
Propionibacteriaceae	0.19	0.63	0.79
Chitinophagaceae	7.73E-03*	0.663	0.066
Methylobacteriaceae	0.057	0.35	0.077
Xanthomonadaceae	0.2	0.90	0.90
Corynebacteriaceae	0.063	0.39	0.15
Methylophilaceae	0.89	1.00	1
Lactobacillaceae	5.32E-05*	1.20E-03**	0.082
Staphylococcaceae	0.18	0.80	0.81
Phyllobacteriaceae	0.038	0.20	0.45
Nitrospiraceae	0.73	1.00	0.011
[Borreliaceae]	0.99	1.00	1
Pirellulaceae	0.7	1.00	0.84
Listeriaceae	0.25	0.76	0.63
Alcaligenaceae	0.062	0.34	0.34
Mycobacteriaceae	2.54E-03*	0.025	0.025
Endozoicimonaceae	0.63	0.89	0.95
Pseudoalteromonadaceae	0.26	1.00	1

Appendix C1. Sample distribution of eyed egg samples used in Chapter 3. Eyed eggs were divided between replicate cells across all dams. Replicate cells were divided either between replicate trays (F1-F16 and F17-F32) or within the same tray (F33-F39).

No. of eyed egg samples		***No. of dams***
Replicate cell 1	***Replicate cell 2***	
2	*2*	*16*
2	*1*	*10*
1	*1*	*7*
2	*0*	*3*
1	*0*	*3*

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