**Ollscoil na hÉireann, Corcaigh**

**National University of Ireland, Cork**


# Exploring the microbial ecology and energetics of wild and domesticated Atlantic salmon (*Salmo salar*).

Thesis presented by

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for the degree of

**Doctor of Philosophy**

**University College Cork**

**School of Biological, Earth and Environmental Sciences**

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October 2023

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## DECLARATION

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism and intellectual property.

Patrick Schaal

## ABSTRACT

Since the inception of Atlantic salmon (*Salmo salar*) aquaculture in the 1970s, millions of domesticated fish have escaped from aquaculture facilities into the wild. This phenomenon raises concerns about the ecological and genetic consequences of farmed fish interbreeding with their wild counterparts. The fitness of hybrid offspring from such interactions has long been recognised as diminished compared to pure wild salmon populations, thereby posing a substantial threat to the overall health and robustness of Atlantic salmon populations.

This study takes a comprehensive approach to investigate the impact of domestication on two vital aspects of Atlantic salmon biology: gut microbial communities and metabolism. Both traits have been identified as critical determinants of fish health and well-being. To disentangle the genetic effects from confounding environmental factors, this study employed common garden experiments. These experiments involved rearing fish with diverse genetic backgrounds, including wild, domesticated and reciprocal hybrids, together from the eyed-egg stage through both freshwater and marine phases. This design allowed us to simulate farmed escape and hybridisation events that naturally occur in the wild.

The first data chapter examines the drivers and sources that shape gut microbial assembly over time in juvenile Atlantic salmon in a natural river system. The study shows that the major contributors to the salmon intestine's microbial taxa come from macroinvertebrates, a potential food source, rather than the water column. Moreover, results suggest a possible role of host genetics in driving inter-individual differences in gut microbial community composition, leading to distinct microbiota

assemblages between farmed, wild and hybrid fish. Neutral modelling further revealed that the majority of gut taxa are transient, underscoring the dynamic nature of these microbial communities and emphasizing the need to distinguish between transient and resident taxa within the gut environment.

The second data chapter examines the seasonal dynamics of gut microbial communities and energetics in juvenile Atlantic salmon, considering potential variations among farmed, wild and hybrid fish. The study unveils genetic factors as significant influencers of metabolic flexibility in Atlantic salmon. Wild fish exhibit lower metabolic rates in winter and higher rates in summer compared to farmed salmon, indicating their adaptability to seasonal environmental changes. This metabolic flexibility potentially enhances their chances of survival in variable wild environments compared to their farmed counterparts, which might exhibit less adaptability due to artificial selection for commercially favoured traits. Furthermore, our research unveils shifts in gut microbial communities during the winter months, particularly among the offspring of wild fish, possibly attributable to reduced feeding activity. This reduced activity, in turn, might be associated with their generally lower metabolic demands in winter.

The third data chapter assesses whether survivability, gut microbial structure and metabolic rate of Atlantic salmon reared in marine sea pens are affected by amoebic gill disease (AGD), a parasitic infection that poses a significant challenge to Atlantic salmon reared in aquaculture facilities, and if those effects vary between fish from farmed, wild and hybrid origins. Wild fish exhibited substantially higher mortality rates compared to their farmed counterparts, while hybrids fell in between. All fish,

regardless of genetic origin, showed significantly lower metabolic rates with increased AGD infection rates. In addition, gut microbial diversity significantly declined in AGD-infected fish.

In summary, our study significantly contributes to our understanding of the complex interactions between host genetics, environmental factors and gut microbiota in Atlantic salmon. It offers indications that the domestication process in Atlantic salmon has influenced both host-associated microbiota and metabolism. As aquaculture continues to expand, these findings underscore the need for comprehensive conservation strategies to safeguard the ecological integrity of wild Atlantic salmon populations in the face of evolving aquaculture practices and the potential consequences of farmed fish escape events.

## ACKNOWLEDGMENTS

What an incredible journey this PhD has been. During this time, I had the privilege of crossing paths with numerous remarkable people who have left a lasting impact on both my personal growth and the development of this thesis. I had the opportunity to experience the breathtaking landscapes of Western Ireland and the vibrant city of Glasgow. Both places hold a special place in my heart, and I will always look forward to return.

I want to thank my supervisors Phil McGinnity, Thomas Reed and Martin Llewellyn for granting me the opportunity to pursue this PhD. This experience not only allowed me to follow my scientific interests but also led me to forge lifelong friendships and connect with wonderful people beyond the realm of academia. Your unwavering guidance and support, particularly during challenging moments, have been invaluable. It is not to be taken for granted that supervisors are as warm, welcoming and supportive as you have been. Thank you for providing such a brilliant working environment.

### Ireland

I want to thank the Marine Institute Ireland and its research members for providing me with a welcoming environment at their facilities. It was a fantastic experience working in Newport. Thanks to Russell, Elvira, Alan and the “lads” for your support. A special thanks to Pat, who generously assisted me twice a week in obtaining saltwater from the bay for my respirometry experiment. I hold dear the memories of our drives, shared laughter and your amusement each time I returned from our trips, inevitably soaked wet once again.

Thanks to the members of Phil's working group, Cat and Ronan, for their efforts in organising and conducting lab work. Thanks also to Joshka and Karl, whose expertise and guidance in statistics were invaluable. I learned a great deal from both of you. Special thanks to Karl for the (mostly) enjoyable experience of tinkering with the respirometry setup, in hindsight it was actually good fun and I even picked up some handy DIY skills along the way. I also want to thank Flo and Sarah, my fellow PhD candidates, for the shared laughs and chitchat. Finally, a heartfelt thanks to Liz, who I consider the unsung hero of our lab. Your dedication, especially in making the trip to the sea pens twice a week, was truly remarkable. You always found the right words when I was worried about a project or my PhD in general. I'm proud to have had the opportunity to learn from your extensive field experience.

**Scotland**

I also want to thank the University of Glasgow and the Institute of Biodiversity, Animal Health & Comparative Medicine for treating me as one of their own. I met so many wonderful people at the institute and the city of Glasgow. I made so many fantastic memories, the institute and Glasgow became a true home.

I want to thank the members of Martins working group, Michele, Raminta, Elle, Luis, Phillipp, Tony, Pei, Chloe and Bach for the great working environment and good laughs in and outside of work. Special thanks to Bach: You are brilliant! Thanks for introducing me to the world of bioinformatics and thanks for your valuable input and discussions about microbiomes, statistics and modelling.

### Viva Voce Examination

I’d like to express my sincere gratitude to the examiners of this thesis, Prof. Sofia Consuegra del Olmo and Prof. Catherine Stanton. Their thorough review and insightful feedback have been instrumental in refining and improving the quality of my work. I greatly appreciate the time and effort they dedicated to examining my thesis and their valuable suggestions which have significantly contributed to the final version of this document.

### Personal

I'd like to give a shoutout to my friends—no matter the distance, you always make me feel like I'm right there with you. I am genuinely fortunate to have such incredible people supporting me.

Thanks to my family for consistently showing interest in my life and research. Special thanks to my grandparents, who gave me a wonderful childhood. Although some of you may not be here to witness the completion of this work, I believe you'll be smiling down on me from above.

And last but certainly not least, my parents. You are the best parents a child could wish for. You raised me to be curious, independent and to always pursue my dreams. No matter what decisions I have made in my life, you have had my back. When I have faced challenges, you have been there to lift me up and set me back on my feet. This thesis is dedicated to you! Thanks for everything you have done for me.

## IMPACT OF COVID-19

Covid-19 had a widespread impact and I consider myself fortunate that my family and friends did not suffer severe consequences from it. Unfortunately, I personally contracted Covid twice, and the first bout affected me for several months. Aside from the physical challenges, the prolonged lockdown periods took a toll on my mental well-being. It is very important for me to emphasize the significance of mental health within the context of this thesis. Pursuing a PhD is demanding, often deviating from planned paths and the self-imposed pressure can become overwhelming at times.

Apart from my personal struggles, Covid-19 also caused delays in some aspects of my thesis. Although most of the data had been collected in the field prior to January 2020, the crucial laboratory work required for analysing the gathered material faced setbacks during the initial lockdown when laboratories were closed. Following the reopening of laboratory facilities, the analysis of samples was notably hindered due to restrictions and the limited number of individuals allowed to work in the laboratory simultaneously. This change in the sample processing timeline subsequently resulted in delays in data analysis. Additionally, the transition from face-to-face meetings to online interactions influenced the quality and timelines of feedback and support from colleagues and external sources, further extending the timeline required to achieve consistent results.

I want to thank the University College Cork for granting me the Covid extension award "Higher Education Authority Covid-19 Cost extension (Call Three).

## THESIS STRUCTURE

Data chapters in this thesis are either already published in a peer-reviewed journal, under review or about to be submitted. Each data chapter is accompanied by supplemental materials at the end of the chapter and includes a reference list. While chapters are cross-referenced when relevant, they can also be read as standalone documents.

I acknowledge the contributions to the data chapters in my thesis here. However, specific author's contribution statements and acknowledgments for each chapter can be found at the end of that respective chapter.

I conducted all fieldwork, laboratory work, data analysis and writing for this thesis, with the following exceptions:

- Parentage and fish sex was determined by Dr. Jamie Coughlan at UCC, the statistical parental assignments were made by Dr. Karl Phillips and Dr. Joshka Kaufmann
- Dr. Elizabeth Ryder and Dr. Catherine Waters conducted macroinvertebrate sampling and sorting.
- Dr. Chloe Heys performed ventilation rate counts in Chapter 3.
- Amplicon sequencing was carried out by Novogene (UK) Company Limited.

# CHAPTER 1: GENERAL INTRODUCTION

## 1.1 Gut microbiome

### 1.1.1 Community development and host-microbe interactions

The assemblage of bacteria, archaea, viruses, fungi and their genetic material residing in a specific environment is termed the microbiome (Savage, 1977). However, this thesis will exclusively focus on bacteria. The relationship between a host and its associated microorganisms is exceedingly complex. Intestinal microbiomes display significant variations among individuals (e.g., Turnbaugh et al., 2007). A key objective is to discern the circumstances under which bacterial strains are advantageous or harmful. Investigating the interplay among the environment, the development of the intestinal community, and destabilizing factors is a central focus in microbiome research. The following section provides a summary of gut microbial community acquisition, development, interactions and host benefits, with a primary emphasis on human microbiomes.

Humans are traditionally believed to acquire their initial microbiota at birth. However, recent research has suggested the possible presence of bacteria in placental material (Aagaard et al., 2014) and even *in utero* (Collado et al., 2016), indicating potential transmission of bacteria from the mother's womb to the infant during birth (Baker et al., 2018; Rackaityte et al., 2021). The subsequent colonisation process seems to be primarily driven by stochastic processes. Notably, the microbial community in the infant does not closely resemble that of any specific maternal body site (Ferretti et al., 2018). During the first three years of an infant's life, the intestinal

microbiome undergoes rapid diversification, followed by stabilization throughout childhood and early adulthood, before gradually shifting again in middle age (Bäckhed et al., 2015; Enav et al., 2022; Roswall et al., 2021; Wilmanski et al., 2021). Environmental factors, particularly diet composition and volume, play a significant role in perturbing microbiomes (David et al., 2014; Turnbaugh et al., 2009; Wilson et al., 2020). For example, diets with a high contribution of fat and low plant-based fibre content have been shown to reduce microbial diversity and alter community composition, thus affecting overall microbial function (David et al., 2014). Microbial community composition can also change due to factors such as antimicrobial treatment (Schwartz et al., 2020; Yassour et al., 2016), age (Bosco & Noti, 2021; de la Cuesta-Zuluaga et al., 2019), lifestyle changes (Keohane et al., 2020; Yatsunenko et al., 2012) and health status (Lynch & Pedersen, 2016). While inter-individual variation is primarily influenced by environmental factors (Rothschild et al., 2018), there is evidence for heritable variation in microbiome composition (Sanna et al., 2022). Studies on twins have identified many microbial taxa influenced by host genetics (Goodrich et al., 2014). In addition, microbiome-wide association studies (mbGWAS) conducted in various populations have revealed associations between specific genetic loci and the abundance of different bacteria and microbial traits, encompassing aspects such as β-diversity, microbial pathways and bacterial presence traits (Bonder et al., 2016; Sanna et al., 2022; Turpin et al., 2016; J. Wang et al., 2016).

In death our microbiome inevitably overcomes us. To maintain stability in the relationship between the living host and its microbiome, microbial growth must be constrained to some extent. In the intestinal tract the host accomplishes this by

combining a strong immune response with special anatomical features. The surface epithelium in the gut produces mucus in form of glycoproteins which certain microbial species attach and feed on (Arike & Hansson, 2016; Johansson et al., 2011; Tailford et al., 2015). This mucus lining, although partially permeable to bacteria, serves as a barrier to protect host cells from systemic bacterial invasion (Schroeder, 2019). In addition, the quantity of microorganisms is mediated by a cocktail of antimicrobial peptides and antibodies (Hooper et al., 2012; Thaiss et al., 2016; Zong et al., 2020). For instance, mucosal tissues in the intestine produce Immunoglobulin A (IgA), which not only offers protection against pathogens and toxins but also directly interacts with resident bacteria (Donaldson et al., 2018; McLoughlin et al., 2016). Consequently, IgA plays a significant role in shaping the composition of the gut microbial community and maintaining host-microbiota homeostasis Furthermore, the release of oxygen and acids also influences the composition of the gut microbial community by altering ecological niches within the gut "ecosystem" (Ridlon et al., 2014; Staley et al., 2017). Whether a bacterial species is able to thrive in the gut environment depends not only on its capability to survive in the intestinal ecosystem provided by the host but also on its interaction with other microorganisms (Hibbing et al., 2010). A key determinant of this is competition for resources (Ghoul & Mitri, 2016). The availability of nutrients in the gut, whether from the host's diet or through mucin secretion, plays a pivotal role in driving competition among microbial species (Coyte et al., 2015; Koropatkin et al., 2012; La Rosa et al., 2022). In fact, this nutrient-driven competition can potentially drive adaptive radiations, causing strains to diverge into distinct ecological niches in order to reduce

competition (Foster et al., 2017; Rainey & Travisano, 1998). For example, the presence of organic (derived from host mucins or amino acids) or inorganic (sulphates or sulphites) sulphated compounds facilitates the growth of sulphate-reducing bacteria, such as bacterial strains belonging to the *Desulfobacter* or *Streptococcus* genus (Carbonero et al., 2012). Microbes also attempt to outcompete their competitors through biological warfare, including the production of antibiotics, bacteriocins or specific proteins (Abrudan et al., 2015; Cornforth & Foster, 2015; Kommineni et al., 2015). However, competition is not the sole factor shaping microbial communities. These interactions contribute to the nutritional niche available to other microbes in the gut. Stochastic processes, such as founder effects, microbial dispersal and random events involving death, reproduction and replacement, play a crucial role in determining whether a bacterial species can establish itself in the gut (Jones et al., 2022; Verster & Borenstein, 2018). For instance, when multiple bacterial species compete for the same ecological niche, the first coloniser may gain a growth advantage over later arrivals (Fukami, 2015; Sprockett et al., 2018). Additionally, interactions can occur between resident gut bacteria and transient bacteria introduced through the process of feeding (David et al., 2014). Food-borne bacteria can temporarily integrate into the resident gut microbiome, influencing both the composition and overall function of the microbial community (Derrien & Vlieg, 2015; Zhang et al., 2016). Furthermore, mutations and lateral gene transfer can drive diversification within microbial populations (Brito, 2021; Gordo, 2019). The introduction of new bacterial functions can create positive

feedback loops, promoting niche variation and further diversification (Emerson & Kolm, 2005; Svanbäck & Bolnick, 2006; Thursby & Juge, 2017).

Variations in environmental factors that impact gut microbial community composition can lead to changes in microbial community structure and function, resulting in a range of potential outcomes for the host. Bacteria contribute significantly to host well-being by fulfilling various roles, including defending against pathogens (Bäumler & Sperandio, 2016; Cameron & Sperandio, 2015; Sassone-Corsi & Raffatellu, 2015), influencing epithelial homeostasis (Lee et al., 2022; Natividad & Verdu, 2013) mediating biochemical signalling between gut and central nervous system (Fung et al., 2017; Jones & Neish, 2017), the biosynthesis of vitamins (Karlsen et al., 2022; Koh et al., 2016; LeBlanc et al., 2017; Patterson et al., 2014), essential amino acids (Lin et al., 2017) and the breakdown of otherwise undigestible food items, like fibre (David et al., 2014; Tremaroli & Bäckhed, 2012).

To accomplish these functions, microbes deploy their enzymatic arsenal to ferment carbohydrates, yielding short-chain fatty acids (SCFAs) in the process (Koh 2016; Rios-Covian 2016, Louis 2016). The three most abundant SCFAs, acetate, propionate and butyrate, are usually found in a proportion of 3:1:1 in the gut (Cummings et al., 1987; Louis et al., 2014). These SCFAs are efficiently absorbed by cells in the gut epithelium, with each metabolite exerting specific effects on host physiology (Besten et al., 2013). For instance, butyrate serves as the primary energy source for epithelial cells (Hague et al., 1996) and strengthens the mucus barrier's function against microorganisms (Gao et al., 2020; Ghosh et al., 2021). Additionally, butyrate provides anti-inflammatory effects and modulates host immune functions (Salvi & Cowles,

2021; Siddiqui & Cresci, 2021). In general, all SCFAs play crucial roles as energy sources, gene expression regulators and signalling molecules involved in cellular processes such as proliferation, differentiation, or apoptosis (Corrêa-Oliveira et al., 2016; Martin-Gallausiaux et al., 2021; Sanna et al., 2019).

### 1.1.2 Fish microbiomes

Compared to humans, research on fish microbiomes is still in its infancy (Legrand et al., 2020). There are profound differences to be considered in the study of host-associated microbiomes in fish compared to land-based animals. The direct link between teleost species and their aquatic living environment highlights the importance of research of the source pool from which the fish acquires its intestinal microbiota. Teleost intestines differ from the ones of mammals in their complexity and compartmentalisation (Harder & Sokoloff, 1976). Furthermore, teleosts are the most speciate group of living vertebrates (Ravi & Venkatesh, 2018). As such, the structure and functional characteristics of the gastrointestinal tract exhibit significant variations among fish species, mostly reflecting the adaptability to the diverse feeding habits and environmental conditions exploited by fish (Ray & Ringø, 2014). The intestine of salmonids can be roughly divided into five sections (Bjørgen et al., 2020; Løkka et al., 2013). The stomach, which is acidic and responsible for the initial unspecific digestion of food. The pyloric caecum, which consists of multiple finger-like excrescences arranged in a longitudinal pattern. These surface area-increasing structures are thought to be the key area of nutrient absorption within the salmon intestine (Buddington & Diamond, 1986; Denstadli et al., 2004), accounting for around 70% of total nutrient absorption capacity within the intestine (Bakke-

McKellep et al., 2000; Denstadli et al., 2004). First (referred to as midgut) and second segment of the mid intestine and the posterior segment follow on the pyloric caeca and further aid in digestion and the absorption of nutrients (Løkka et al., 2013).

Most teleost species are oviparous. Initial colonisation of microorganisms usually occurs after birth when environmental bacteria colonise the chorion of the eggs (Hansen & Olafsen, 1999; Llewellyn et al., 2014; Olafsen, 2001). Bacteria can be found not only on the surface but also within the internal tissue of eggs from both freshwater and marine fish (Hansen & Olafsen, 1999). While vertical transmission from the mother has been demonstrated in a few cases of fish egg microbiota, such as the pathogenic bacteria *Renibacterium salmoninarum* (Bruno & Munro, 1985) and *Flavobacterium psychrophilum* (Long et al., 2014), it remains to be determined whether maternal transfer plays a significant role in shaping fish gut microbial communities. Recent findings by (Rasmussen et al., 2023) suggest co-evolution of Mycoplasma species, indicating a potential for maternal transfer. After hatching, larvae quickly become colonised by surrounding environmental bacteria (Hansen & Olafsen, 1999). The initial colonisation of the intestinal tracts occurs through the ingestion of the surrounding medium, a process that has often been assumed to be purely stochastic (Hansen & Olafsen, 1999; Ringo & Birkbeck, 1999). As the host matures, better-adapted microorganisms eventually outcompete the initial colonisers, leading to increased stability in the intestinal microbial communities (Burns et al., 2016). In contrast to homeothermic animals, where internal factors dominate, external environmental factors such as water temperature are expected to play a more prominent role in poikilotherms like fish due to their temperature-

dependent metabolism. Bacterial growth is temperature-dependent, and some flexibility to respond to temperature variability might provide fitness advantages for certain bacterial species over their competitors (Corkrey et al., 2012). The gut microbiome in fish, akin to that in humans, is significantly influenced by the host's diet. The composition of their food has a profound impact on the available resources for gut microorganisms and exposes the hosts to the microbiomes of their prey (Ringø et al., 2016; Sullam et al., 2012). Moreover, fish exhibit specific anatomical adaptations in their digestive systems, which vary depending on their dietary preferences. For instance, species that consume sediment may possess a gizzard, while herbivorous fish may feature a specialised hindgut. These structural modifications create distinct conditions in the gut that favour specific types of microorganisms (Clements et al., 2009; Egerton et al., 2018; Escalas et al., 2021; Mountfort et al., 2002). The host's living environment also plays a pivotal role in shaping the microbial community within the intestine. Variations in microbial composition among different host populations are often linked to disparities in their food sources, which fluctuate across habitats (Uren Webster et al., 2018). However, noteworthy alterations in environmental conditions, such as fluctuations in salinity, have been observed to particularly impact the gut microbial community compositions of certain species, including anadromous fish like Atlantic salmon (Dehler et al., 2017). Similar to what has been discussed in the context of humans, there is evidence of interactions between the gut microbiome and aspects such as the immune system (Kelly & Salinas, 2017), energy homeostasis (Butt & Volkoff, 2019) and physiology (Yukgehnaish et al., 2020) in fish.

### 1.1.3 Knowledge gap

Despite the progress made in studying gut microbial communities in fish, several questions remain unanswered. Host genetics has been shown to play a role in shaping gut microbial communities, as evidenced among divergent populations of sticklebacks (Smith et al., 2015), guppies (Sullam et al., 2015) and very recently in Chinook salmon, *Oncorhynchus tshawytscha* (Ziab et al., 2023). Genetics also influences host growth and behaviour, directly impacting the amount and types of food fish consume, which, in turn, might influence the composition of the gut microbial community (Li et al., 2018). In aquaculture species, such as Atlantic salmon, selective breeding aims to enhance productivity (Glover et al., 2017; Janssen et al., 2017). However, when escaped fish interbreed with native wild populations, hybrid offspring with distinct genetic and behavioural traits are formed (Glover et al., 2017), an aspect which will be reviewed in detail later in this introduction. These traits might also contribute to differences in the composition of the gut microbial community, potentially affecting host metabolism and health. Another closely related question concerns how changes in food availability affect the fish gut microbiome. Understanding the variation of microbial communities during periods of food scarcity, changing environmental conditions or host health is crucial for understanding the overall gut microbial dynamics. Additionally, research indicates that stochastic processes might be important contributors to inter-individual differences in gut microbial community compositions between hosts (Burns et al., 2016; Heys et al., 2020). Understanding the interplay of environmental drivers and stochastic forces is essential when designing interventions to preserve or restore

microbiome compositions in captive-bred and reared animals, such as farmed salmon, including the use of pro and prebiotics or managing animal nutrition. To achieve this, it is also crucial to determine which bacterial taxa are genuinely resident in the intestine, as the significant inter-individual variation observed among hosts may be attributed to differences in the environmental bacteria present at any given time.

## 1.2 Atlantic salmon

### 1.2.1 Life cycle

Most Atlantic salmon populations are anadromous and undergo seven major life cycle stages: Egg, alevin, fry, parr, smolt, post-smolt and adult. In anadromous populations, the juvenile phase in freshwater is followed by a marine phase for feeding and growth and a return migration phase to freshwater for reproduction (Aas et al., 2010). Atlantic salmon inhabit temperate and subarctic regions of the North Atlantic Ocean and freshwater habitats bordering the Baltic Sea and both sides of the North Atlantic Ocean (Klemetsen et al., 2003). In anadromous salmon, spawning occurs between September and February, with timing influenced by water temperature, resulting in earlier spawning for northern populations. Eggs typically hatch in early spring and the newly hatched fish, known as alevins, remain concealed in gravel, relying on their yolk sacs for nutrition. Initial feeding can begin when the yolk sac is depleted by 30% (Skoglund & Barlaup, 2006), but most juveniles start feeding after three to eight weeks when their yolk sacs are fully absorbed (Jones et al., 2003). Alevins acquire many of their initial gut bacteria from the surrounding environment. However, as mentioned previously, there is the potential of specific

bacterial species being vertically transmitted from the mother (e.g., *Mycoplasma*; Rasmussen et al., 2023). After emerging from their gravel hiding spots, the fry search for a suitable habitat and feed on microscopic life in the stream. Once they reach a length of five to eight centimetres, the fry mature into parr, which can be identified by the dark vertical markings on their sides known as parr marks. Salmon are specialized carnivores and as parr, they primarily feed on macroinvertebrates. However, their diet may opportunistically change depending on the availability of food sources and differences in prey and fish size (Gibson, 1993). During spring, Atlantic salmon exhibit increased feeding activity, which gradually declines throughout the summer. In winter, juvenile salmonids reduce their activity levels and seek shelter in streambed refuges during the daytime (Cunjak et al., 1998; Metcalfe et al., 1999). In these refuges, their feeding activity is minimal, and they rely heavily on their lipid stores for sustenance (O. K. Berg & Bremset, 1998; Finstad et al., 2004). Anadromous salmon parr remain in freshwater for one to eight years before undergoing a process called smoltification. Smolting involves a range of morphological, biochemical, behavioural and physiological changes that enable them to adapt to a marine life (Björnsson et al., 2011; Hoar, 1988; McCormick, 2012). Smolts begin to leave the river in late spring and are called post-smolts upon entering the marine environment. Post-smolts remain in the ocean for one to four years before returning to their native spawning grounds to reproduce. It is noteworthy that some populations of Atlantic salmon or certain individuals can complete their entire life cycle within freshwater (Kazakov, 1992).

### 1.2.2 Atlantic salmon energetics

Throughout their lives, Atlantic salmon encounter significant environmental challenges, resulting in substantial variations in their energy demands depending on the season and life cycle stage. To survive and reproduce successfully, individuals must allocate their energy effectively to processes such as growth, predator avoidance, foraging and long-term energy storage (N. Jonsson & Jonsson, 2003; Metcalfe et al., 2002; Post & Parkinson, 2001). A crucial trait related to this energy allocation is the standard metabolic rate (SMR), which represents the minimum energy level necessary for basic life functions (Chabot et al., 2016). Variations in metabolic rates between individuals can be explained by differences in body mass, feeding status and a range of environmental factors (Burton et al., 2011; Clarke & Johnston, 1999; McKechnie, 2008). However, even after adjusting for these factors, metabolic rates exhibit significant and consistent interindividual variation, which correlate with variations in behaviour and critical life-history traits (Stearns, 1992). For instance, individual variations in metabolic rate can influence intraspecific aggression, social dominance and subsequently impact resource acquisition, growth and reproduction (Metcalfe et al., 2016). However, it is important to note that the relationship between metabolic rate, behaviour and performance is context-dependent (Burton et al., 2011; Reid et al., 2012). For instance, higher metabolic rates may confer advantages in situations characterised by intense interference competition, while lower metabolic rates may be beneficial in resource-limited environments or under other stressful conditions, such as food deprivation or hypoxia (Armstrong et al., 2011; Auer et al., 2015; Bochdansky et al., 2005; Killen et

al., 2011). The underlying cause of inter-individual differences in metabolic rates is not entirely clear (Metcalfe et al., 2016). The size of metabolically expensive organs, such as the heart, liver and brain, may significantly influence the metabolic rate of organisms (Konarzewski & Książek, 2013). Moreover, studies in fish suggest a potential link between enzyme activity and metabolic rates (Norin & Malte, 2012). There is also evidence suggesting that interindividual differences in metabolic rate may, to some extent, have a genetic basis and be subject to selection (Nilsson et al., 2009; Pakkasmaa et al., 2006). Given the increasing rate of environmental change, there has been a rising interest in investigating whether individuals can adapt their metabolic rates in response to these dynamic conditions (Norin & Metcalfe, 2019). Metabolic alterations could be programmed to enable animals to cope with predictable changes in their energetic state or demands, such as the reduction of SMR observed during hibernation (Staples, 2016). In Atlantic salmon, it is particularly intriguing to explore how the heritage of an individual (e.g., farmed, wild or hybrid background) may influence metabolic rates. These rates may differ due to substantial directional selection for higher growth rates and the predominance of Norwegian strains in farming practices in Great Britain and Ireland. In addition, investigating whether the ability to adapt to different environmental conditions varies according to the individual's provenance would provide valuable insights into the species' adaptive capacity. Moreover, given the significant impact of metabolism on growth, there is a rising interest in studying the functional interactions between the gut microbiota and host metabolism (Lindsay et al., 2020). This understanding holds the

potential to optimize feed utilization efficiency, enhance fish growth and ultimately improve profitability within the aquaculture industry (Lindsay, 2021).

### 1.2.3 Aquaculture

Atlantic salmon stand out as one of the economically most important farmed fish globally, both in terms of volume and value (Bostock et al., 2010; FAO, 2020). The domestication of Atlantic salmon, which involves selectively breeding animals was initiated in Norway during the late 1960s, with the aim of improving economically significant traits (Gjedrem, 2010; Teletchea & Fontaine, 2014). These traits primarily include growth rate, but also encompass characteristics such as age at maturity, filet colour, disease resistance and various other desirable attributes (Gjøen & Bentsen, 1997). Norway has emerged as the largest player in the salmonid aquaculture industry (Harache, 2002; Liu et al., 2011). Currently, Norway accounts for over half of the world's farmed salmon supply and holds a leading position as an exporter (FAO, 2020). The initial achievements of the Scandinavian country served as a catalyst for the expansion of salmon farming in other regions, including Scotland, Ireland, the Faroe Islands, Canada, USA, Chile and Australia (FAO, 2020). However, as the industry expanded, it encountered an increasing number of challenges related to sustainability, disease management and the conservation of wild fish populations (Boerlage et al., 2020; Forseth et al., 2017; Glover et al., 2020; Semple & Dixon, 2020; Teletchea & Fontaine, 2014).

As with many other cultured marine organisms, farmed Atlantic salmon rely on fish meal as energy and protein source (Naylor et al., 2009). Fish meal and fish oil, which is produced primarily from wild-caught fish, is known for its high protein and energy

content and serves as valuable source of essential fatty and amino acids (Tacon & Metian, 2008). To reduce the pressure on wild fish stocks and to secure the long-term viability of the aquacultural sector effort is underway to find suitable substitutes for this finite and potentially unsustainable resource (Naylor et al., 2000). In recent years, alternative feed ingredients, such as plant-based oils, insect meals, algae and microbial-derived products, have been tested in order to reduce the reliance on marine resources while maintaining or improving fish growth and health (Albrektsen et al., 2022; Alfiko et al., 2022; Bandara, 2018; Gatlin III et al., 2007; Hua et al., 2019). As dietary changes can alter the fish gut microbiome, which is in turn connected to various host physiological traits (Ingerslev et al., 2014), there has been an increased focus on studying the impact of alternative feed sources on host microbiomes (Consuegra et al., 2023; Sagaram et al., 2021). However, an investigation of the impact of natural food sources on gut microbial community composition is lacking.

### 1.2.4 Disease

High fish densities in aquaculture facilities lead to an easier transmission of diseases among the fish population (Crosbie et al., 2010; Perry et al., 2020). In addition, the often-low genetic diversity of aquaculture stocks can increase the susceptibility of hosts and increase the virulence of pathogens (Kennedy et al., 2016). The economic impact of disease outbreaks in aquaculture is immense, encompassing substantial costs for disease control measures. A notable example is the prevalence of sea lice infections in Norwegian salmon farming, which account for estimated economic losses amounting to 13% of farm revenues (Abolofia et al., 2017). In 2011 alone, these infections resulted in estimated damages surpassing 400 million US$ (Abolofia

et al., 2017). The most severe and persistent problems include the formerly mentioned proliferation of sea lice, various forms of gill diseases, especially amoebic gill disease (AGD) as well as bacterial infections. All having potentially detrimental consequences not only for caged fish but also for wild fish in and transitioning through surrounding habitats (Boerlage et al., 2020; Costello, 2009; Frazer, 2009; Kristoffersen et al., 2018; Krkošek, 2017; Krkošek et al., 2005; Semple & Dixon, 2020; Shephard & Gargan, 2021).

Sea lice are small parasitic copepods that attach themselves to skin, fins and gills of the fish thereby feeding on blood and tissue (Pike, 1989). They can cause skin and tissue damage, leading to open wounds that may become susceptible to secondary infections. Severe infestations can result in decreased growth, increased stress levels and even mortality among the affected fish (Pike, 1989). Treatment includes chemical interventions like bath treatments and medicated feed, as well as the usage of cleaner fish (Aaen et al., 2015; Bravo & Treasurer, 2023; Overton et al., 2020).

AGD is caused by the marine amphizoic amoeba *Neoparamoeba perurans* (Crosbie et al., 2012). The amoebae attach themselves to the gill tissue, leading to inflammation and damage. An infection with *N. perurans* can cause alterations in the gills, impacting gas exchange and ion regulation (Hvas et al., 2017). The initial clinical signs of AGD often include decreased appetite, lethargy and changes in swimming behaviour, such as fish swimming near the water surface (Boerlage et al., 2020). As the disease progresses additional clinical signs can emerge including respiratory distress, which can lead to high mortality rates (Steinum et al., 2008). Gross examination of the gills reveals pale lesions in multiple areas on the gill surface or the

presence of raised white mucoid spots and plaques (M. B. Adams et al., 2004). AGD has several environmental risk factors, including high salinity (Clark & Nowak, 1999) proximity to infected sites (Boerlage et al., 2020) and elevated temperatures (Douglas-Helders et al., 2001). Furthermore, studies showed that the genetic characteristics of fish stocks can influence their susceptibility to AGD (Kube et al., 2012; Lillehammer et al., 2019; Robledo et al., 2020; Taylor et al., 2009). Hybrid fish, such as Atlantic salmon x brown trout (*Salmo trutta*) hybrids, have shown increased resistance to AGD compared to pure species (M. B. Adams et al., 2023). However, whether this observation is also valid for intraspecies hybrids between farmed and wild Atlantic salmon has yet to be determined.

### 1.2.5 Microbes and disease mitigation

Atlantic salmon raised in aquaculture are vulnerable to various bacterial pathogens (Semple & Dixon, 2020). Stress factors like overcrowding, temperature fluctuations or parasitic infections can weaken the fish's immune system, allowing normally harmless microorganisms to become opportunistic pathogens (Meyer, 1991). Some examples for bacterial pathogens include *Yersinia ruckeri* causing enteric redmouth disease (Tobback et al., 2007), *Piscirickettsia salmonis* causing Piscirickettsiosis (Rozas & Enríquez, 2014), *Aeromonas salmonicida* causing Furunculosis (Menanteau-Ledouble et al., 2016) or certain *Vibrio* species causing Vibriosis (Ina-Salwany et al., 2019; Larsen et al., 1994). Chemicals and antibiotics have traditionally been used in aquaculture to combat disease outbreaks, alongside vaccines and other biocontrol agents (A. Adams, 2019; Assefa & Abunna, 2018; Dadar et al., 2017; Gudding & Van Muiswinkel, 2013; Pérez-Sánchez et al., 2018; Romero et al., 2012; Soliman et al.,

2019). However, the use of these treatments has shown limited efficiency in case of vaccines (Berg et al., 2007; Figueroa et al., 2020) or has been restricted or banned in several countries due to concerns about their impact on consumers and the environment (Alderman & Hastings, 1998; Domingo, 2016; Watts et al., 2017). For example, the prolonged exposure to antibiotics at sub-therapeutic levels can lead to the evolution of antibiotic-resistant genes or plasmids in bacteria (Cabello et al., 2016; Santos & Ramos, 2018). Once a bacterial strain is resistant, this resistance can be transferred to other bacterial species and strains via horizontal gene transfer (von Wintersdorff et al., 2016). Therefore, efforts are underway to find safe and environmentally friendly alternatives to antibiotics to prevent the adverse effects associated with their use (Abdel-Latif et al., 2020; Bondad-Reantaso et al., 2023; Dawood et al., 2021; Lieke et al., 2020; Santos & Ramos, 2018). Hence, the administration of pro and prebiotics have gained significant attention as potential methods to enhance the immune defences of fish in a safe and natural way (Llewellyn et al., 2014; Pereira et al., 2022; Ringø, 2020; Yousuf et al., 2022). Probiotics are defined by the FAO/WHO as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Hill et al., 2014). Probiotics are typically bacteria belonging to the Firmicutes phylum (e.g., lactic acid producing bacteria (LAB) and *Bacillus* species) that are similar to the beneficial microorganisms naturally found in the gut (El-Saadony et al., 2021; Sumon et al., 2022). So far, several theories have emerged on how probiotics exert their beneficial effects. Those theories include competitive exclusion of pathogenic bacteria, stimulation of immune cells, production of antimicrobial compounds (e.g., bacteriocins and organic

acids), promoting the production of mucus, enhancing the production of beneficial metabolites or increasing microbial diversity (Balcázar et al., 2006; Cunningham et al., 2021; Dawood et al., 2020; Pereira et al., 2022; Zorriehzahra et al., 2016). *Carnobacterium divergens* for example has been shown to inhibit the growth of pathogen *Yersinia ruckeri* (Kristiansen & Ringø, 2011). Furthermore, the utilization of probiotics has demonstrated positive effects on fish growth, digestive enzyme activities and feed utilization (Akbari Nargesi et al., 2020; Naderi Farsani et al., 2021). This has generated significant interest in exploring their potential to aid in the digestion of alternative food sources as mentioned previously (Wang et al., 2020). Prebiotics on the other hand are non-digestible food ingredients that stimulate the growth and activity of beneficial microorganisms, such as probiotics in the gut (Davani-Davari et al., 2019). Prebiotics are typically carbohydrates, such as fibres or oligosaccharides, that resist digestion in the stomach and reach the pyloric caecum intact, where they selectively stimulate the growth and metabolic activity of beneficial bacteria (Ringø et al., 2014; Song et al., 2014). Pre and probiotics are often administered simultaneously. Those combined treatments are termed “synbiotic” (Huynh et al., 2017; Yilmaz et al., 2022). Despite the growing utilization of pro and prebiotics in the aquaculture sector, there remain significant gaps in our understanding that impede the realization of their full potential. Specifically, we lack comprehensive knowledge regarding the specific beneficial bacteria and their contextual relevance. Thus, it is crucial to comprehend the intricate interplay among the environment, host and its microbiome within a natural setting to derive conclusions applicable to a broader scale.

### 1.2.6 Hybridisation and Introgression

Atlantic salmon aquaculture has been dealing with an ongoing problem of domesticated fish escaping into the wild, commonly referred to as escapees. It is estimated that millions of farmed salmon escape from aquaculture facilities into the wild yearly (Glover et al., 2017; Skilbrei et al., 2015; Figure 1.1), most likely because of a combination of natural events like storms, floods or high tides and damaged nets or cages. Whilst most of these escapees die due to predation or starvation, some manage to enter rivers and reach native salmon spawning grounds. Thereby, escapees might engage in both ecological (Jonsson & Jonsson, 2006; Thorstad et al., 2008) and genetic interactions with wild populations (Crozier, 1993; Ferguson et al., 2007). For example, farmed escapees can compete with wild salmon for resources such as food and spawning grounds (B. Jonsson & Jonsson, 2006). In addition, as mentioned previously escaped fish might carry parasites or diseases that can spread to wild populations (Naylor et al., 2005). However, most concerning is the hybridisation of farmed and wild salmon, leading to genetic introgression and potential loss of genetic diversity in wild populations (Forseth et al., 2017; Glover et al., 2020). These hybrids can reproduce and produce fertile offspring that often exhibits different phenotypic traits and reduced fitness compared to their wild counterparts, with no evidence supporting the concept of hybrid vigour populations (Bolstad et al., 2021; McGinnity et al., 1997, 2003; Skaala et al., 2019; Wacker et al., 2021). Research has provided evidence of reduced egg size (Hagen et al., 2019), as well as decreased survival of juveniles following introgression (Sylvester et al., 2019; Wacker et al., 2021). Due to the deliberate selection for faster growth in farmed

salmon, their developmental speed has significantly increased (Thodesen et al., 1999). As a result, the timing of various life-history decisions might be influenced by the introgression of farmed genetic traits (Besnier et al., 2022; Bolstad et al., 2017). Studies have suggested that if interbreeding between farmed and wild salmon continues to occur frequently and accumulates over time, the detrimental impacts on the fitness and genetic integrity of wild populations may become increasingly pronounced (Castellani et al., 2018; Glover et al., 2017; McGinnity et al., 2003; Sylvester et al., 2019). Understanding the phenotypic variations resulting from different genetic backgrounds is crucial for comprehending the reduced fitness observed in hybrids. Common garden experimental designs have been employed to study the effects on fish phenotype, with a particular focus on traits influenced by artificial selection in aquaculture, such as body weight, growth, survival, morphology and maturation (Perry, 2020). However, information about disease susceptibility, behaviour, metabolism and the gut microbiome are still limited.

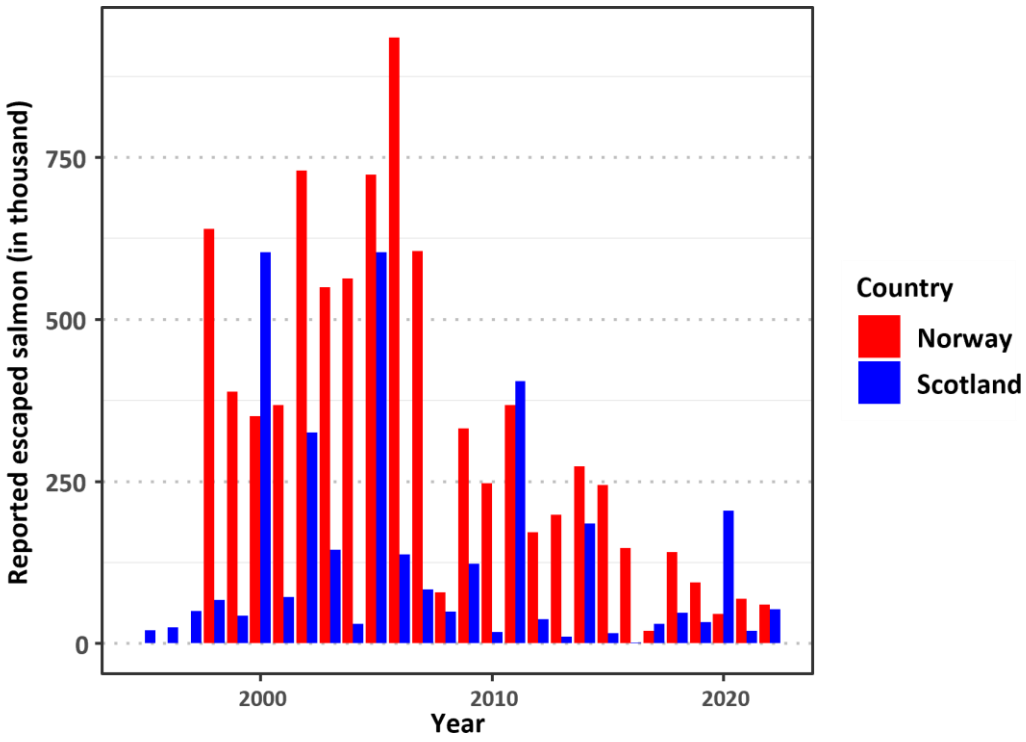


**Figure 1.1** Barplot showing the reported numbers of farmed escaped Atlantic salmon in Norway between 1998 and 2022 (www.fiskeridir.no) and in Scotland between 1995 and 2022 (www.aquaculture.scotland.gov.uk).

## 1.3 Aims

In this study, I aim to understand if a fish's genetic origin influences its gut microbiome, energetics and if yes in what context. Additionally, I aim to explore potential links between fish origin, energetics and susceptibility to disease and subsequently evaluate whether these factors are linked to variations in the fish's gut microbiome. The outcome of this research might help to gain a better understanding of the broader impacts of farmed escape events on wild populations, whilst also enhancing our general understanding of gut microbial communities in Atlantic Salmon. This knowledge may contribute to conservation efforts and be valuable for the aquaculture industry. Thereby, this thesis aims to:

## Chapter 1

1. Examine the drivers and sources that shape gut microbial assembly over time in juvenile Atlantic salmon in a natural river system (Chapter 2; Schaal P, Cheaib B, Heys C, et al. Gut microbial assembly among freshwater Atlantic salmon reared in a natural stream system during a simulated farm escape and introgression event. Authorea Preprints; 2023. DOI: 10.22541/au.168873131.14015465/v1.)
2. Examine the seasonal dynamics of gut microbial communities and energetics in juvenile Atlantic salmon, considering the potential variations between farmed, wild and hybrid fish (Chapter 3)
3. Assess whether survivability, gut microbial structure and metabolic rate of Atlantic salmon reared in marine sea pens is affected by AGD and if those effects vary between fish from farmed, wild and hybrid origin (Chapter 4; Schaal, P., Cheaib, B., Kaufmann, J. et al. Links between host genetics, metabolism, gut microbiome and amoebic gill disease (AGD) in Atlantic salmon. Anim microbiome 4, 53 (2022). https://doi.org/10.1186/s42523-022-00203-x).

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# Chapter 2: : GUT MICROBIAL ASSEMBLY AMONG FRESHWATER ATLANTIC SALMON REARED IN A NATURAL STREAM SYSTEM DURING A SIMULATED FARM ESCAPE AND HYBRIDISATION EVENT

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## 2.1 Abstract

Intestinal microbial communities are influenced by a confluence of ecological forces. Understanding the dynamics between environment, microbiota and host is essential to gain insights into microbial community assembly processes. However, few studies systematically assess the contribution of different environmental sources to gut microbial community composition. We used a common garden experiment to determine the roles of biotic, abiotic and stochastic processes shaping gut microbial communities in Atlantic salmon (*Salmo salar*) in a natural river during a simulated 10-month farm escape scenario. Most of the taxa found in the salmon intestine originated from macroinvertebrates (the potential food source) rather than the water column, indicating that diet might be an important factor in community assembly. The contribution of macroinvertebrates to the fish gut community was lowest in winter and increased over March and May, reflecting seasonality in fish appetite. Our results suggest a possible host genetic effect explaining inter-individual differences in gut microbial community composition, whereby distinct assemblages were noted between farmed, wild and hybrid fish. Neutral modelling estimated that the majority (86%) of taxa present in the gut are transient. Overall, our data highlight the significance of both deterministic and stochastic drivers influencing the seasonal fluctuations of gut microbial communities in young Atlantic Salmon and hint at potential genetic or maternal effects on fish microbiota. These findings greatly enhance our understanding of the complex interactions between hosts, their living environment and associated microbiota.

## 2.2 Introduction

Host-associated microbiota play a vital role for host health (Hooper et al., 2012; Ray et al., 2012) and development (J. M. Bates et al., 2006; Llewellyn et al., 2014; Sommer & Bäckhed, 2013). Intestinal bacteria, for example, are known to facilitate digestion of otherwise inaccessible feeds (Ray et al., 2012; Tremaroli & Bäckhed, 2012), stimulate the immune system (Stagaman et al., 2017), protect the host from pathogens due to competitive exclusion (Lawley & Walker, 2013) and may even influence host-behaviour (Cusick et al., 2021; Davis et al., 2016).

The expansion of the aquaculture industry has led to an increased interest in manipulating gut microbiota to improve fish welfare and nutritional absorption capacity (Egerton et al., 2018; Perry et al., 2020). However, to induce desired microbial traits one must understand the dynamics of the gut ecosystem, including the underlying processes of microbial community assembly, its temporal development and gut transit rates (Clements et al., 2014; Dittmann et al., 2017). In theory, fish acquire their intestinal microbiota from the surrounding environment, e.g., by swallowing water or due to bacteria attached to food items (Hansen & Olafsen, 1999). As the individual matures, its gut microbial community composition is shaped by a confluence of ecological forces, which interact but can be grouped into two main factors: deterministic/selective and stochastic/neutral (Chase & Leibold, 2009; Hubbell, 2005; Stegen et al., 2012). Deterministic factors create specific conditions and selective pressures that favour the growth and colonisation of certain microbial taxa, leading to the establishment of a unique gut microbial community in each individual. Deterministic factors include host-specific factors such as genetics (Smith et al., 2015), immune response (Kelly & Salinas, 2017) and physiology (Dehler

et al., 2017) as well as environmental factors such as food source and food availability (Gajardo et al., 2017; Li et al., 2022; Ringø et al., 2016), parasite presence (Llewellyn et al., 2017; Schaal et al., 2022), temperature (Ghosh et al., 2022; Kokou et al., 2018), pH (Sylvain et al., 2016) and other microbes (Coyte et al., 2015; Kokou et al., 2019). Stochastic processes, on the other hand, are not guided by specific host or environmental factors but are rather influenced by random events, such as dispersal, ecological drift and priority effects (Costello et al., 2012; Hanson et al., 2012; Vellend, 2010). Dispersal is a process where microorganisms are introduced to the gut from external sources, such as the environment or other individuals. Ecological drift refers to random events of microbial birth, death and replacement and can lead to variability in the gut microbial community even in the absence of strong selective pressures. Priority effects, or historical contingency, play a significant role in shaping gut microbial communities. During the process of colonization, the first species to establish themselves may gain a competitive advantage over later arrivals if both are competing for the same ecological niche within the gut (Fukami, 2015; Sprockett et al., 2018). Furthermore, these initial colonisers can influence the gut environment, potentially creating conditions that favour specific microbial species over others as the community develops over time. In fish, neutral community assembly can explain a substantial amount of observable differences in gut microbial community structure (Burns et al., 2016; Heys et al., 2020). Despite advances in microbiome research, there are still substantial knowledge gaps regarding the dynamics of gut microbial communities over time and the origin of microbial taxa within these communities. One key unanswered question is whether the microbial taxa that are detectable in

the gut are established, long-term residents, or if they are transient, externally sourced and passing through the intestine without establishing a lasting presence.

Most studies investigate microbial assembly processes using laboratory models or artificial systems. Yet, insights must also be acquired from natural environments (Cusick et al., 2021) because these provide valuable insights into the complex dynamics, ecological relationships and adaptations that have evolved over time (Friberg et al., 2019). Atlantic salmon is one of the ecologically and economically most important fish species worldwide and is extensively researched to examine various aspects of fish biology, aquaculture practices and ecosystem dynamics (Aas et al., 2010; Houston & Macqueen, 2019). One significant research focus revolves around the ramifications of domesticated fish escaping from aquaculture facilities, which pose a serious threat to wild populations (Forseth et al., 2017; Thorstad et al., 2008). These escapes can have detrimental effects on wild fish due to competition for limited habitat and food resources, as well as the potential for genetic interactions through interbreeding with wild individuals (Jonsson & Jonsson, 2006; McGinnity et al., 2003; Reed et al., 2015). Most Atlantic salmon populations have an anadromous life cycle. After hatching in spring, the majority of Atlantic salmon remain in their freshwater habitat for two years, before migrating to the ocean where they undergo most of their somatic growth (Hoar, 1988). During their juvenile phase, Atlantic Salmon face seasonal variations in food supply (e.g., macroinvertebrate type and quantity; de Eyto et al., 2020) and environmental conditions (e.g., temperature, oxygen concentration and water pH) that might directly or indirectly affect the structure of gut bacteria. Understanding the role of environmental factors determining gut microbial community composition in the wild will enable better

predictions of the impact of future environmental changes on fish health in both natural and aquaculture populations. For example, changes in food availability or rising water temperatures due to global warming will likely perturb microbial communities, with direct consequences for fish survival and welfare (Harvell et al., 2002).

In the present study, we take advantage of a large-scale common garden experimental setup in the wild to investigate gut microbial community assembly and development in Atlantic salmon. In a natural river environment, we examined the gut microbiota of juvenile Atlantic salmon sampled over a 10-month period. We evaluated the role of different drivers of gut microbial assembly including abiotic variables, host genetics and water- and feed-associated microbiota. In addition, we utilized Sloan's neutral model to estimate the importance of neutral community assembly processes and to determine potential, resident community members. We used source tracking analysis to understand how the composition and abundance of environmental bacteria influences the gut microbiota throughout different seasons in both transient and resident microbiota. By exploring the intricate interplay between stochastic and deterministic factors driving community assembly, our study offers a comprehensive perspective on the ecological succession of the wild gut microbial community of juvenile Atlantic salmon.

## 2.3 Materials and Methods

### 2.3.1 Study area and sampling

Atlantic salmon were bred at the Marine Institute in Furnace, Newport, Co. Mayo, Ireland (53°55'22"N 9°34'18"W) located at the Burrishoole river system and

consisted of four genetic groups: domesticated farmed fish (F) from the "Fanad MOWI" strain, native wild fish (W) from the Burrishoole river system and their reciprocal hybrids (denoted hybrid farmed female (HFF) and hybrid wild female (HWF, Figure 2.1a,b). In April 2018 at the swim-up stage, prior to the commencement of exogenous feeding, fry were introduced into a section of the Srahrevagh river in the Burrishoole catchment. The experimental river consists of approximately 7520$m^2$ of high-quality Atlantic salmon habitat. It is contained at its upper end by a series of large waterfalls and at its lower end by a fish trap capable of capturing all life cycle stages from egg to adult. A detailed description of the system is reported in (McGinnity et al., 1997, 2003; Perry et al., 2021).

A total of 80 fish were captured in the Srahrevagh River across a 10-month period in 2019: January (n=16); March (n=14); May (n=8); June (n=13); July (n=11); November (n=18). Fish were caught via electrofishing and transported in buckets filled with oxygenated river water to the Marine Institute Newport Research Station for processing. The feeding status of all fish was unknown. All fish were euthanized by an anaesthetic overdose of methane tricaine sulphonate (MS-222, 80ml/l, FVG, Ireland) and their fork length (mm) and wet weight (g) measured. The intestines of sampled fish were dissected aseptically via an incision along the fish's ventral side. The pyloric caecum was removed, cut into pieces, put into sterile cryotubes and immediately placed on dry ice. An overview of samples taken is shown in Table S 2.1. This study only investigated the gut microbial community composition in the pyloric caecum.

In order to identify bacteria in the water column that might serve as a dispersal source for fish gut communities, three water samples were collected at each sampling timepoint at locations at the bottom (bot), middle (mid) and top of the study section of the Srahrevagh river (Figure 2.1c). The water was collected in sterile water bottles (1.5 L). Within one hour of collection the water was filtered in a sterile environment using 0.2µm filters (Whatman, Chicago, IL, USA) at the Marine Institute Newport Research Station. After filtration the filter papers were placed into cryotubes, immediately placed on dry ice and stored at -80°C.

To evaluate the potential contribution of prey organisms to gut microbial community composition, a sample of the macroinvertebrate community was collected at each fish sampling timepoint. Macroinvertebrate samples were retrieved from the Srahrevagh river using a surber sampler (Surber, 1937). The surber sampler was placed in three different sections on the riverbed allowing three macroinvertebrate sample replicates to be obtained at each of the three sampling sites. Larger stones were overturned and wiped to collect attached invertebrates and the riverbed was agitated for three minutes allowing macroinvertebrates to flow into the collection net. Samples were collected in an upstream direction (bottom, middle and top). All macroinvertebrates present, were sorted into common taxa on the riverbank. Macroinvertebrates of the same order (two of each replicate) were pooled into sterile cryotubes on each sampling occasion. The cryotubes were immediately stored on dry ice in the field until samples were taken back to the Marine Institute Newport Research Station and stored in the -80 freezer. We limited the analysis to the five most abundant taxonomic orders of macroinvertebrates in the experimental river:

Mayfly (Ephemeroptera), Stonefly (Plecoptera), Fly (Diptera), Caddis fly (Trichoptera) and Beetles (Coleoptera).

All gut, water and macroinvertebrate samples were transported on dry ice to the University of Glasgow for subsequent microbial profiling.

A suite of environmental parameters in the Burrishoole catchment and the Srahrevagh river are measured continuously as part of an ongoing LTER (long-term ecological research) program of monitoring. Parameters that were used in this study include water temperature, water level and water discharge, dissolved oxygen (DO) and conductivity.

To determine the sex and genetic origin (farmed, wild or hybrid provenance) of each fish in the common garden river experiment, fin clips were taken and preserved in absolute ethanol for subsequent genetic profiling and parentage assignment. Parentage analysis was conducted at the University College Cork using a three-panel multiplex PCR which amplified 10 microsatellites loci (for details see Perry et al., 2021).

The study was carried out under the Health Products Regulatory Authority (HPRA) licence number AE19130-P056 in Ireland.

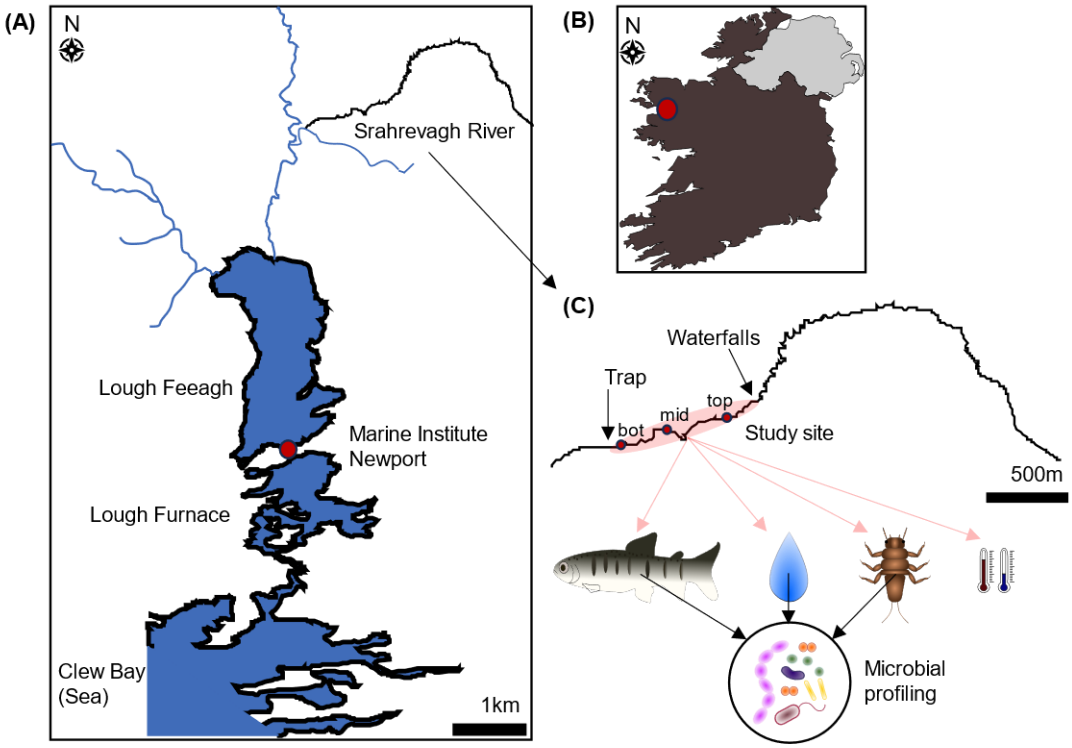


**Figure 2.1:** Map highlighting the location of the Marine Institute Research Station in Newport and the experiment river (Shrarevagh river) located within the Burrishoole river system (A) in Western Ireland (B). (C) shows the study site within a section of the experiment river. The study site is contained on its lower end by a trap and its upper end by waterfalls. Atlantic Salmon of four genetic origins (farmed, wild and their reciprocal hybrids) were introduced into the experimental river to simulate a farm escape and hybridisation event. Environmental parameters, including water temperature, dissolved oxygen, conductivity and river discharge were continuously measured over the course of the 10-month experiment. At each sampling timepoint replicates of water and macroinvertebrate samples were taken at three sections of the river, marked as bot (bottom), mid (middle) and top. Atlantic Salmon were caught via electrofishing across the whole section of the study site. Microbial profiling of Atlantic salmon intestines, water column and macroinvertebrates were conducted at the University of Glasgow.

### 2.3.2 Microbial DNA extraction and NGS library preparation

DNA extraction and NGS library preparation protocols used were based on methods established and summarized in Kazlauskaite et al., (2021) and Schaal et al., (2022). The frozen gut tissue (100mg) and filter papers were cut up into pieces using sterilized equipment and DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol (Claassen et al., 2013). The pooled macroinvertebrates were crushed with a sterile pestle before DNA

extraction. Extracted DNA was amplified using primers targeting the V1 hypervariable 16S rDNA region (Gajardo et al., 2016). V1 was chosen over V4 because the primers are less liable to cross-hybridisation with salmon DNA (Heys et al., 2020; Werner et al., 2012). The reaction mix contained a total volume of 15 µl and consisted of 0.7 µl of each internal forward and reverse primer, 7µl of Q5 Hot Start High-Fidelity 2X Master Mix (New England Biolabs Ltd, UK) and 1µl of DNA template. Amplification of the target region was achieved using tagged barcodes 27F and 338R at a concentration of 1pM for each primer. PCR included an initial denaturation step at 95°C for 10min; 30 cycles at 95°C for 30s, 55°C for 30s and 72°C for 30s; and a final elongation step of 72°C for 10min. First-round PCR products were then used for a subsequent second round of PCR, in which external multiplex identifiers (barcodes) were added. The second round PCR reaction mix contained a total volume of 25 µl and consisted of 1.25 µl of external revers primer (10µM), 1.25 µl external forward primer (10µM), 12.5µl of Q5 Hot Start High-Fidelity 2X Master Mix (New England BioLabs Ltd, UK) and 1.3 µl of first round PCR template. Cycle number was reduced to eight and reaction conditions were identical as to mentioned before. All primer sequences are detailed in (Schaal et al., 2022). Second round amplicons were gel-purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) and quantified using a Qubit fluorometer (Thermo Fisher Scientific, USA). Final amplicons were pooled equimolarly at a concentration of 10nM, and paired-end sequencing was carried out using a NovaSeq 6000 system provided by NovoGene.

### 2.3.3 Bioinformatic pipeline

Sequence analysis was performed with our bioinformatic pipeline as described previously by Kazlauskaite et al. (2021) and Schaal et al. (2022).

Quality filtering and trimming (>Q33 Phred score) was performed on all sequence reads of the target region using the Sickle (v.1.2) software (Joshi & Fass, 2011). Read error correction was carried out using the BayesHammer module within the SPAdes (v.2.5.0) software to obtain high-quality assemblies (Nikolenko et al., 2013). Paired-end reads were merged (overlap length 50bp) using PANDAseq (v.2.11) with the simple Bayesian read merging algorithm (Masella et al., 2012; Schirmer et al., 2016). Thereafter, merged reads were dereplicated, sorted, and chimaeras and singletons were removed by using VSEARCH (v.2.3.4; Rognes et al., 2016). Sequences were decontaminated against the last assembled version of *Salmo salar* genome using DeconSeq (v.0.4.3; Schmieder and Edwards, 2011) and overlapped reads were clustered into operational taxonomic units (OTUs) using VSEARCH at 97% sequence identity. Naïve Bayesian classifiers, implemented in QIIME2 (Bolyen et al., 2019; Pedregosa et al., 2011) were used to classify OTUs against the Silva 138 database (Quast et al., 2012). Phylogenetic trees were generated using FastTree (Price et al., 2010).

### 2.3.4 Statistical analysis

All data were analysed in R version 3.41 (R Core Team, 2022) using the packages PhyloSeq (McMurdie & Holmes, 2013), microeco (Liu et al., 2021), metacoder (Foster et al., 2017) and vegan (Oksanen, 2007). Packages lme4 (D. Bates et al., 2015) and MuMIn (Barton, 2009) were utilized for data model fitting. The R code is illustrated in chapter 4.89. OTUs that were not assigned to the kingdoms of Bacteria and Archaea were removed, as were sequences assigned as chloroplast or mitochondria. To limit sample depth effects on diversity measurements, samples were rarefied to 10,000 reads. Alpha diversity was assessed via Chao1 richness and the Shannon's

index of entropy. Generalized Linear Models (GLMs) were employed to explore the relationship between alpha diversity measures and predictor variables, considering potential nonlinear relationships and distributional assumptions. Predictor variables encompassed temperature, fish sex, host genetic origin, fish length, fish weight and sampling month. The package "performance" was used to assess the distribution of the data (Lüdecke et al., 2021). Model performance was evaluated using Akaike Information Criterion corrected for small sample sizes (AICc), Bayesian Information Criterion (BIC), deviance, pseudo-R-squared and cross-validation. The MuMIn package (Barton, 2009) facilitated systematic evaluation of predictor variables, selecting the best-fitting model based on AIC scores. The selected model for both Chao1 richness and Shannon diversity assumed a gamma distribution for the response variable with a logarithmic link function, using Month as the predictor variable. Beta-diversity measures were visualised by principal coordinates analysis (PCoA) plots using Bray-Curtis and weighted UniFrac distance measures. Permutational multivariate analysis of variance (PERMANOVA) was used to test the effects of sampling date (month), sex and genetic origin on the intestinal microbial communities among individual fish.

### 2.3.5 Distance-based redundancy analysis (dbRDA)

We used distance-based redundancy analysis (dbRDA) to determine how much of the variation in intestinal or environmental microbial communities could be explained by external environmental factors. Environmental variables were log10 transformed to improve comparability of canonical coefficients (Buttigieg & Ramette, 2014). Candidate predictors tested were host-specific factors such as fish length or weight and environmental factors such as water temperature, water level and river

discharge, dissolved oxygen (DO) and conductivity. To identify multicollinearity among the predictor variables, we calculated Variance Inflation Factors (VIF) for each predictor. Additionally, we computed a correlation matrix to visualize the correlations among predictors (Figure S 2.1). Environmental factors were used to predict seasonal differences in the bacterial communities collected from the water column. To estimate the perturbation of aquatic bacteria due to flooding events, the average river discharge was calculated as the seven-day mean prior to sampling timepoints.

### 2.3.6 Source tracking analysis

Fast expectation-maximization for microbial source tracking (FEAST, Shenhav et al., 2019) was used to analyse the contribution and the relative importance of fish feed (macroinvertebrates) and planktonic water bacteria to intestinal microbial community composition of the individual fish. The tool estimates the contribution of different source environments to a microbial community, referred to as the sink. It also identifies the fraction of the sink attributed to other unidentified origins, known as the unknown source. Mixing proportions were calculated for each individual fish by using five macroinvertebrate samples (each from one order) and three water samples (reflecting top, mid and bottom locations within the study site). These eight "sources" were sampled at the same timepoint as the respective fish.

### 2.3.7 Abundance-occupancy analysis and neutral model fitting

We employed a Shade-Stopnisek abundancy-occupancy analysis to identify potential 'core' OTUs in the intestinal microbiota of Atlantic salmon (Shade & Stopnisek, 2019), which uses abundance-occupancy distributions fitted to Sloan's neutral model (Sloan

et al., 2006). To determine the core OTUs, we ranked the OTUs based on their occupancy (the frequency of their occurrence in the samples) and weighted them by their abundance. Only OTUs present in all sampling timepoints were considered core. In addition, a potential core OTU had to pass a core inclusion threshold. Therefore, we quantified the contribution of the core subset of taxa to beta diversity using the Bray-Curtis resemblance. To determine the core inclusion threshold, we used a minimum percentage increase in beta diversity of 4% to identify the point at which further increases would offer marginal returns in explanatory value. Important to note is that the inclusion threshold percentage depends on the study design and must be chosen accordingly. To estimate the importance of neutral processes in the assembly of the intestinal microbiota, we applied the Sloan neutral model. The model assumes that community composition dynamics are primarily driven by random processes such as ecological drift and dispersal rather than by species-specific interactions or adaptations. OTUs that occur more frequently than expected are interpreted as potentially having additional factors influencing their abundance, such as microbe-microbe interactions, niche differentiation or host filtering. In this study we refer to OTUs that occur more frequently as expected as being positively selected. Conversely, if certain OTUs occur less frequently than expected, it may suggest that they are subject to competitive exclusion, environmental filtering or other processes that limit their abundance. To fit the occupancy of OTUs and their mean relative abundances across the metacommunity to the model, we used the R code of Burns et al., 2016.

### 2.3.8 Differential abundance testing

We used analysis of compositions of microbiomes with bias correction (ANCOM-BC; Lin & Peddada, 2020) to identify differentially abundant taxa between environments. ANCOM-BC was recently recommended as a very robust method for accurately determining differentially abundant taxa (Nearing et al., 2022). The method is based on a log-linear model that accounts for sampling fractions across samples and deals with the sparse compositionality of microbiome data. In addition, ANCOM-BC can deal with different scenarios for zero-counts. Here, we did not correct for structural zeros since we assumed that the presence of taxa was not unique to a certain sampling timepoint. The iteration convergence tolerance for the expectation-maximization algorithm was kept at its default value of $1e^{-5}$. Significance was determined by using Benjamini-Hochberg corrected p-values (Benjamini & Hochberg, 1995).

In addition, we used random forest analysis implemented in the microeco package (Liu et al., 2021), to highlight the relative abundance of core OTUs grouped on genera with respect to the sampling month. The aim was to determine if seasonality in gut microbial community composition persists in core OTUs and to identify which positively selected core OTUs exhibit seasonal patterns (Beck & Foster, 2014; White et al., 2009; Yatsunenko et al., 2012). MeanDecreaseGini was used to determine the importance of differentially expressed taxa per sampling month. P-values were adjusted for multiple comparisons using the Benjamini–Hochberg method (Benjamini & Hochberg, 1995).

## 2.4 Results

### 2.4.1 Distinct compositions of gut, water and macroinvertebrate microbial communities

After quality filtering, database alignment and removal of potential chimeras, a total of 12,842,387 reads and 44,946 OTUs were obtained. For diversity analysis, samples were rarefied to 10,000 reads, resulting in the identification of 7,377 OTUs in macroinvertebrate samples, 20,287 OTUs in water samples and 8,214 OTUs in gut (pyloric caecum) samples, which were retained for further analysis.

PERMANOVA and PCoA analysis revealed that the microbial community compositions of gut, water and macroinvertebrate samples differed significantly ($F=20.67$; $R^2=0.21$; $p=0.001$; Figure 2.2a). Water communities showed significantly more diversity than gut and macroinvertebrate communities (Figure 2.2b, c). ANCOM-BC detected 513 taxa at genus level that were differentially abundant between at least two bacterial environments (gut, macroinvertebrate, water). *Schlegellela*, *Corynebacterium*, *Staphylococcus* and *Acidovorax* were most abundant in fish guts, whereas *Flavobacterium*, *Sphingorhabdus*, *Novosphingobium* and *Methylotenera* were dominant in macroinvertebrates (Figure 2.2d). 108 genera showed negative log abundances in water samples but were positively enriched in gut communities. Biggest log fold changes between the gut and water environment were observed for *Schlegella* (W= -16.48, p<0.0001), *Corynebacterium* (W= -15.01, p<0.0001), *Staphylococcus* (W= -14.08, p<0.0001), *Delftia* (W= -16.85, p<0.0001) and *Acidovorax* (W= -13.26, p<0.0001). Many genera belonging to the phylum of Firmicutes (new: *Bacillota*) e.g., *Mycoplasma*, *Clostridium sensu stricto, Bacillus* and

several taxa belonging to the order of *Lactobacillales* were also positively enriched in salmon guts.

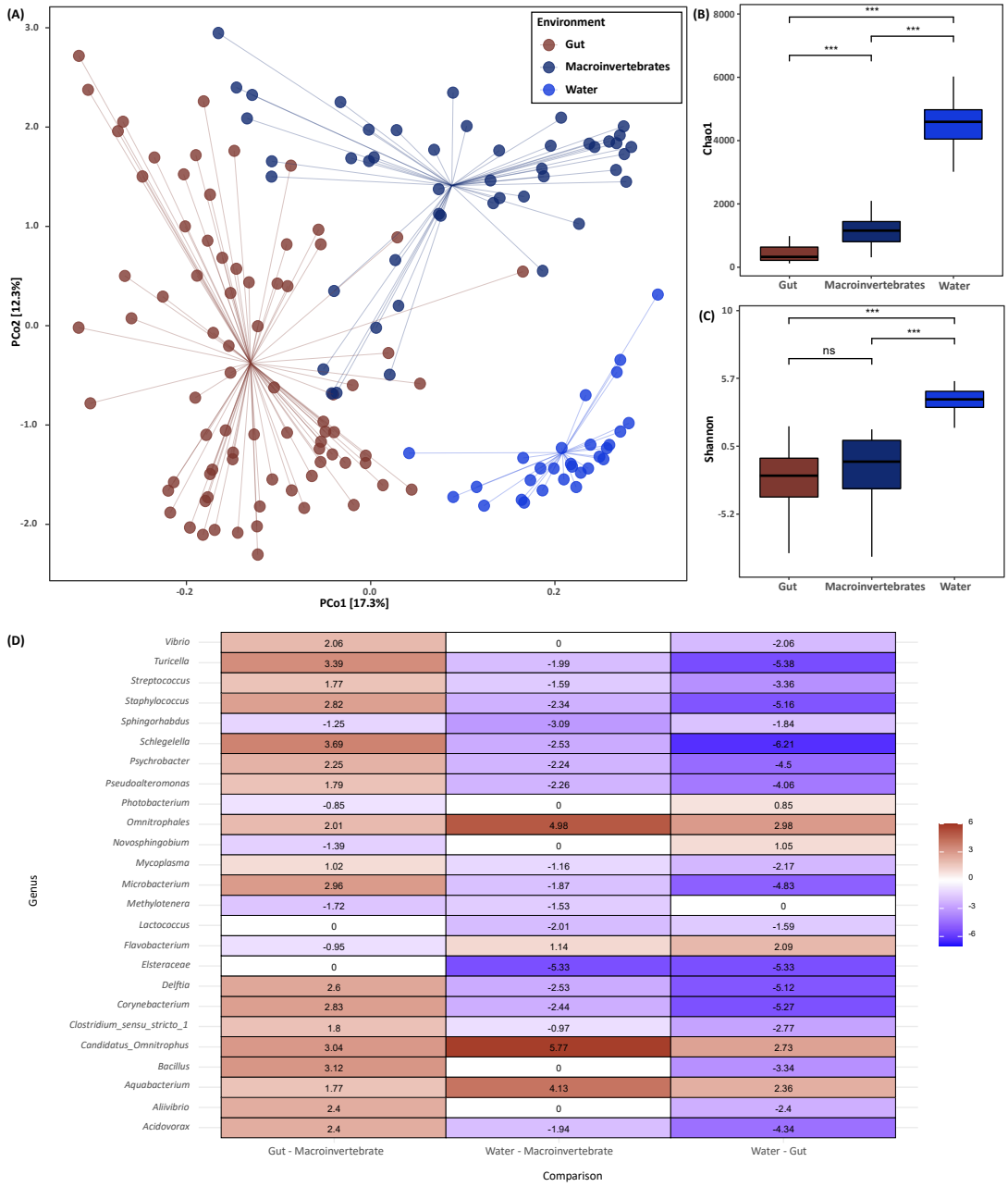


**Figure 2.2:** Principal coordinate analysis (PCoA) of bacterial communities from gut, water and macroinvertebrate samples. (B) Chao1 richness of bacterial communities collected from gut, macroinvertebrate and water environments. (C) Shannon diversity index of bacterial communities collected from different environments. Significance was determined by pairwise t-testing. Significance codes: ***<0.001; ns=not significant. (D) Heatmap of ANCOM-BC2 pairwise analysis for the effect of the three environments on microbial taxa. The analysis included multiple pairwise comparisons among the three groups: Gut (pyloric caecum), macroinvertebrate and

water while controlling the overall modified false discovery rate (FDR) at 0.05. The X-axis represents the specific comparisons made between gut vs. macroinvertebrate, water vs. macroinvertebrate and water vs. gut. The Y-axis displays a selection of significant genera identified by ANCOM-BC2. Each cell in the heatmap is color-coded with blue representing reduced abundance and red representing increased abundance and the numbers on each cell indicate the log fold-change. The Holm-Bonferroni method was utilised to correct for multiple testing.

### 2.4.2 Influences of seasonality and host genetic origin on gut microbial communities

Gut microbial community composition of Atlantic salmon juveniles varied considerably among sampling months (Figure 2.3a). However, monthly clusters did not align sequentially from January to November and instead appeared to be randomly separated by PCoA ordination. Interestingly, PCoA ordination clustered fish samples from the wild and hybrid wild female origin in March, May and November. However, it is important to note that our sampling design lacks statistical power in terms of the number of fish per sampling time point, specifically in relation to their genetic origin (see Table S 2.1). Therefore, results indicating a genetic effect associated with the origin of the fish (farm, wild or hybrid) must be treated with care. PERMANOVA indicated statistical support for the differences observed in PCoA ordination. Sampling month (16.4%), genetic origin (5.4%) and their interaction term (20.5%) explained almost half of the observable variation. Fish sex had no significant effect on gut microbial community composition, and 56.5% of the variation remained unexplained (Table S 2.2). Pairwise testing revealed that the microbial community composition significantly differed between all sampling months (Table S 2.3). For genetic origin, we found that the microbial communities in wild fish differed significantly from those of farmed fish ($F=2.121$, $p=0.01$) and hybrid farmed female ($F=1.858$, $p= 0.01$) fish but not to hybrid wild female fish ($F=0.897$, $p=0.66$, Table S 2.4). However, these significant differences might only be present in certain sampling

months. Unfortunately, we could not elaborate on this further due to the formerly mentioned flaws in our sampling design.

Fish gut microbiota showed their highest average diversity in January (Figure 2.3b, c). Alpha diversity measures were lowest in March and May and increased again over the summer months. The results from evaluating several predictors in generalised linear models showed that the sampling month was the best predictor for alpha diversity measures. No significant effects on alpha diversity measures were found for genetic origin, sex or fish length and weight (Table S 2.5, Table S 2.6, Table S 2.7).

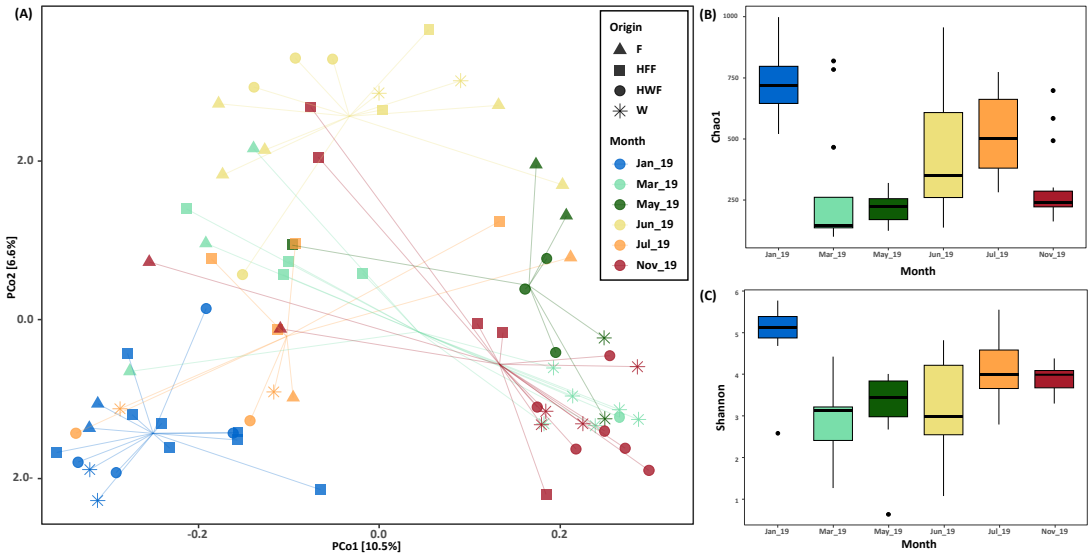


**Figure 2.3:** Alpha and beta diversity of Atlantic salmon gut microbiota obtained from samples collected in the river environment. (A) Principal coordinate analysis (PCoA) of gut samples grouped by sampling month. Each data point represents an individual sample. Shapes represent the genetic background of fish (F=Farmed, HFF=Hybrid Farmed Female, HWF=Hybrid Wild Female, W=Wild). Percentages in parenthesis indicate the amount of variation shown on each axis. Lines mark the centroids of each group. Distance matrix was calculated based on Bray-Curtis dissimilarities. (B) Chao1 richness for gut samples grouped by sampling month. (C) Shannon diversity index for gut samples grouped by sampling month.

### 2.4.3 Environmental and host-specific factors correlate with gut microbial community composition

We used distance-based redundancy analysis (dbRDA) to attempt to explain monthly differences in gut microbial community composition in terms of environmental (e.g., temporal) and host-specific (e.g., host developmental stage) factors. Distance-based linear models revealed that water temperature ($F=2.56$; $R^2=0.03$) and fish age ($F=3.58$; $R^2=0.04$) were significant predictors of gut microbial community patterns (Table S 2.8).

However, inspection of Figure 2.4a, combined with the statistical evaluation of the model, suggests that the combination of temperature and fish age accounts for only about 7.3% of the total variation. This finding implies that in our study, the direct effects of water temperature and host age on gut bacteria may be minor.

Temperature was lowest for the January sampling event at around 6°C (Figure 2.4b). Intermediate temperatures were detected in March, May, June and November (app. 9°C, 10°C, 12°C and 8°C, respectively) and highest in July (app. 16°C). We observed increased frequencies of high river flow rates during spring and autumn (Figure S 2.2). March had the highest mean discharge rate (747(l/s)) followed by November (729(l/s)) and January (570(l/s)). Lower discharge rates were recorded in May (78(l/s)), June (88(l/s)) and July (128(l/s)). Observed conductivity was highest in May (0.14(mS/cm) and June (0.13(mS/cm)). Other sampling timepoints showed lower conductivity (0.09(mS/cm), Figure S 2.2). Dissolved oxygen in the Srahrevagh river was negatively correlated with temperature and showed corresponding patterns over time (Figure 2.4c).

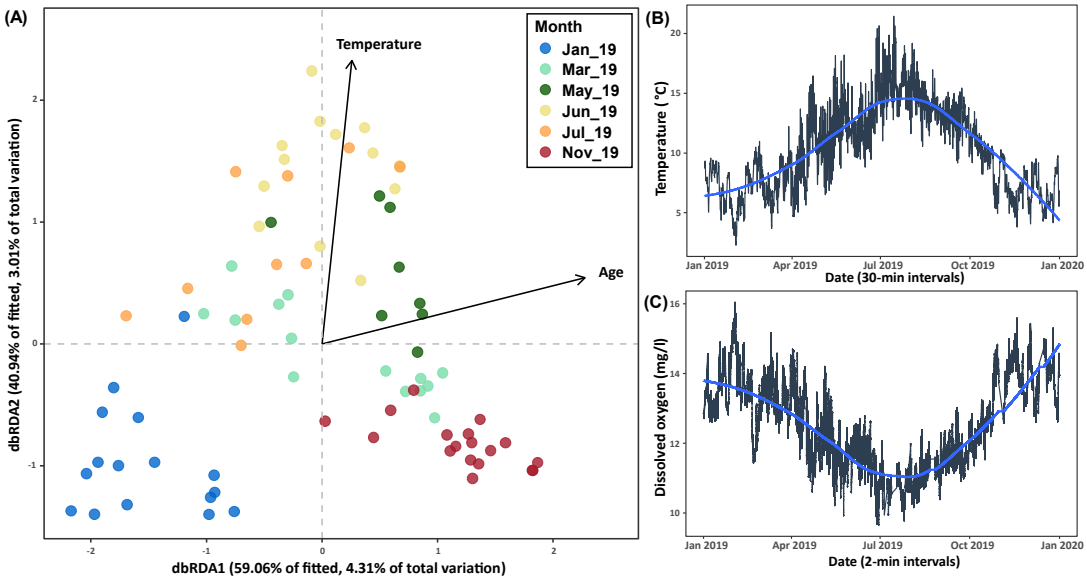


**Figure 2.4:** Distance-based Redundancy Analysis (dbRDA) shows correlations between gut microbial community composition and temperature, fish age and dissolved oxygen (A). Relative position of gut samples in the bi-plot is based on Bray-Curtis dissimilarities. Vectors indicate the weight and direction of those environmental and host-specific factors that were best predictors of gut bacterial composition as suggested by the results of the distance-based linear model. The dbRDA axes describe the percentage of the fitted or total variation explained by each axis while being constrained to account for group differences. (B) Water temperature [°C] of the Srahrevagh river in 2019 measured in 30-minute intervals. (C) Dissolved oxygen [mg/l] of the Srahrevagh river in 2019 measured in 2-minute intervals. Blue lines depict mean values fitted with loess regression.

### 2.4.4 The role of environmental bacteria in shaping gut microbial communities

Microbial communities in the water column showed a pronounced seasonal pattern ($F=10.34$; $R^2=0.81$; $p=0.001$). DbRDA analysis revealed that water temperature ($F=13.09$; $R^2=0.29$; $p=0.001$), conductivity ($F=11.34$; $R^2=0.25$; $p=0.001$), dissolved oxygen ($F=4.52$; $R^2=0.10$; $p=0.009$) and river discharge ($F=5.60$; $R^2=0.08$; $p=0.024$) were all significant predictors of the seasonal changes in bacterial community composition in the water column (Figure 2.5a). Microbial communities derived from macroinvertebrate samples were clustered by their taxonomic order, but also showed temporal trends within groups (Figure 2.5b). PERMANOVA supports this observation. Macroinvertebrate origin (taxonomic order) explained 30.6% of the

observable variation in macroinvertebrate community composition and sampling month explained 14.8%, with 54.5% of variation remaining unexplained. Pairwise testing revealed that all macroinvertebrate orders showed significant differences in microbial community composition except Ephemeroptera and Plecoptera ($F=1.05$; $R^2=0.09$; $p=0.37$), which both showed temporal differences (Table S 2.9).

Source tracking analysis revealed monthly variations in mixing proportions in gut microbial communities (Figure 2.5c). In January, bacteria from the water body contributed approximately 36% of intestinal genera, whereas taxa from the potential food sources (macroinvertebrates) shared21% community identity overlap. The macroinvertebrate overlap steadily increased over the following sampling months, to around 50% in June, July and November. The contribution of taxa which could not be associated with either water or food sources declined after May (53%) reaching its lowest percentage in November (23%). Water source contributions were lowest in June at about 8% and highest in January (36%) and November (23%). Within food sources, macroinvertebrates from the order Diptera were the most dominant source of bacteria for gut communities. Microbial taxa derived from Diptera were almost completely absent in November, when Ephemeroptera became the largest source contributor. Similar observations were made for March. Source contributions for individual fish are shown in Figure S 2.3.

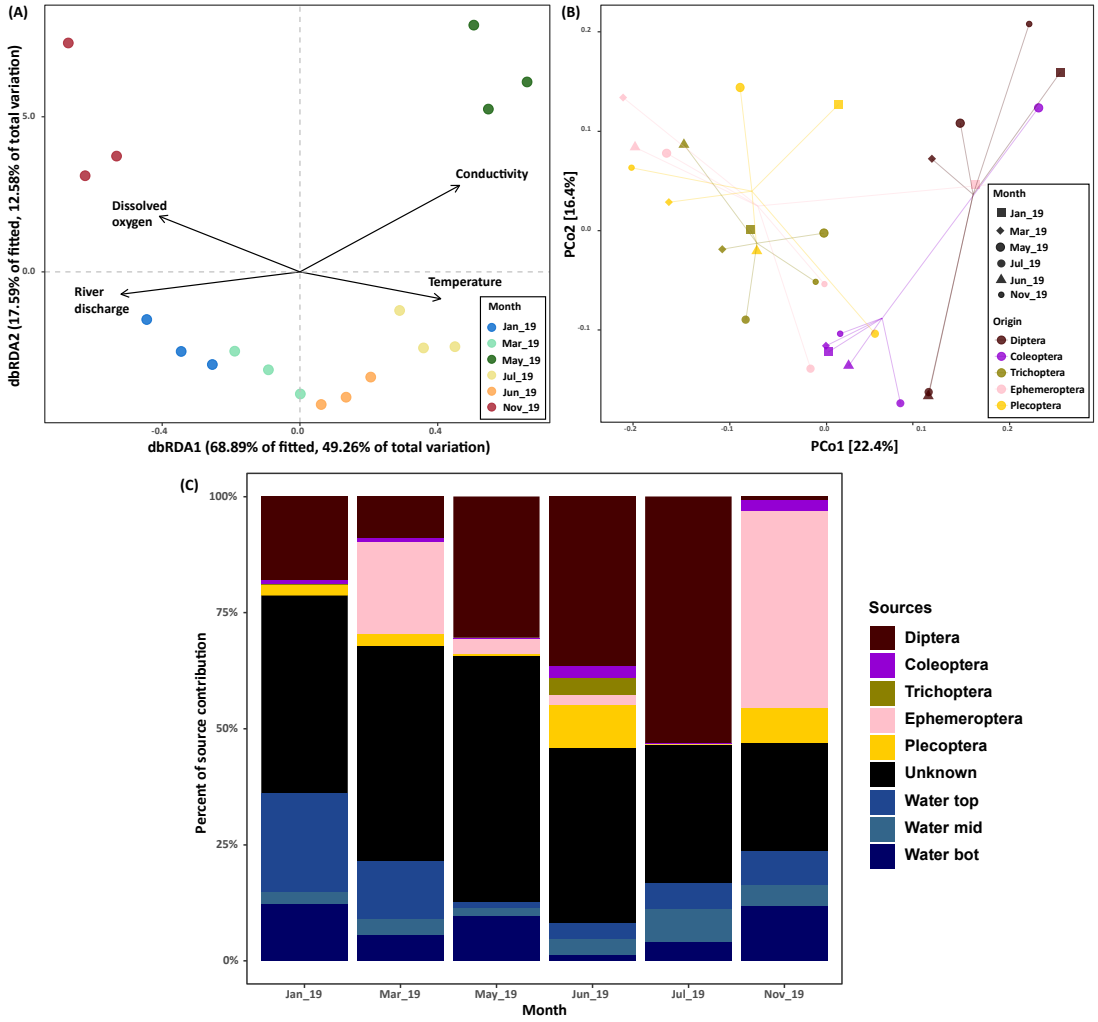


**Figure 2.5:** (A) Distance based Redundancy Analysis (dbRDA) shows correlations between microbial community composition of the water column and temperature, dissolved oxygen, conductivity and river flow rate (discharge rate). (B) Principal coordinate analysis (PCoA) of microbial communities from macroinvertebrate samples grouped by origin. (C) Fast expectation-maximization for microbial source tracking (FEAST) estimations of average microbial source contributions for Atlantic salmon gut communities per sampling month. Mixing proportions were calculated by using taxa counts on genus level. Sources contain five different macroinvertebrate orders (potential food source) and three different water samples, collected from the top, the middle (mid) and the bottom (bot) section of the Srahrevagh river (see Figure 2.1). Source samples were collected at the same sampling day as the fish gut samples.

### 2.4.5 Potential core taxa of juvenile Atlantic Salmon intestinal microbiota

Our neutral model estimated that 6821/7911 (86.22%) of OTUs detected in our study were randomly present in fish intestines, whereas 987 (12.47%) occurred more frequently than expected by the neutral model (Figure 2.6a, b). The model assigned 117 OTUs as 'core' OTUs, of which 78 occurred significantly more frequently than

expected by the neutral model, 17 were considered neutral, and 22 OTUs occurred less frequently than expected.

After grouping the 117 core OTUs by genus, we discovered that certain genera appeared in multiple sections of the neutral model (Figure S 2.4): these genera were observed more frequently than expected, less frequently than expected, or with the same frequency as expected. *Corynebacterium* and *Pseudoalteromonas* were the only genera that appeared in all three sections of the neutral model. Additionally, OTUs associated with *Pseudomonas*, *Acidovorax*, *Turicella*, *Schlegellea*, and *Xanthobacteriaceae* were present in two sections of the neutral model.

We used a taxonomic heat tree to illustrate the distribution of the 78 core OTUs that did occur more frequently than expected in the salmon gut (Figure S 2.5). Those OTUs were dominated by Proteobacteria (85.7%), followed by Firmicutes (10%) and Actinobacteriota (3.5%). At genus level we identified 25 different taxa. Here, *Variovorax* (18.4%), *Pseudomonas* (11.2), *Staphylococcus* (6.7%), *Delftia* (5.8%), *Pseudoalteromonas* (4.4%) and *Schlegella* (4.4%) were the most dominant contributors to the deterministically selected core taxa. Other notable genera included *Acinetobacter* (2.5%), *Streptococcus* (1.8%), *Carnobacterium* (1.3%) and *Fibrobacter* (0.9%). A further 16 OTUs could not be assigned to a specific genus by the reference database, most of those OTUs belonged to the *Comamonadaceae* family.

When restricted to just core taxa we found that 17 out of 25 genera were differentially abundant in at least one sampling month. Interestingly, genera *Pseudomonas*, *Streptococcus*, *Carnobacterium*, *Acientobacter*, *Bosea*,

*Methylothenera*, *Gallionella* and *Sphingomonas* showed no significant seasonal differences in their relative abundance (Figure 2.6c).

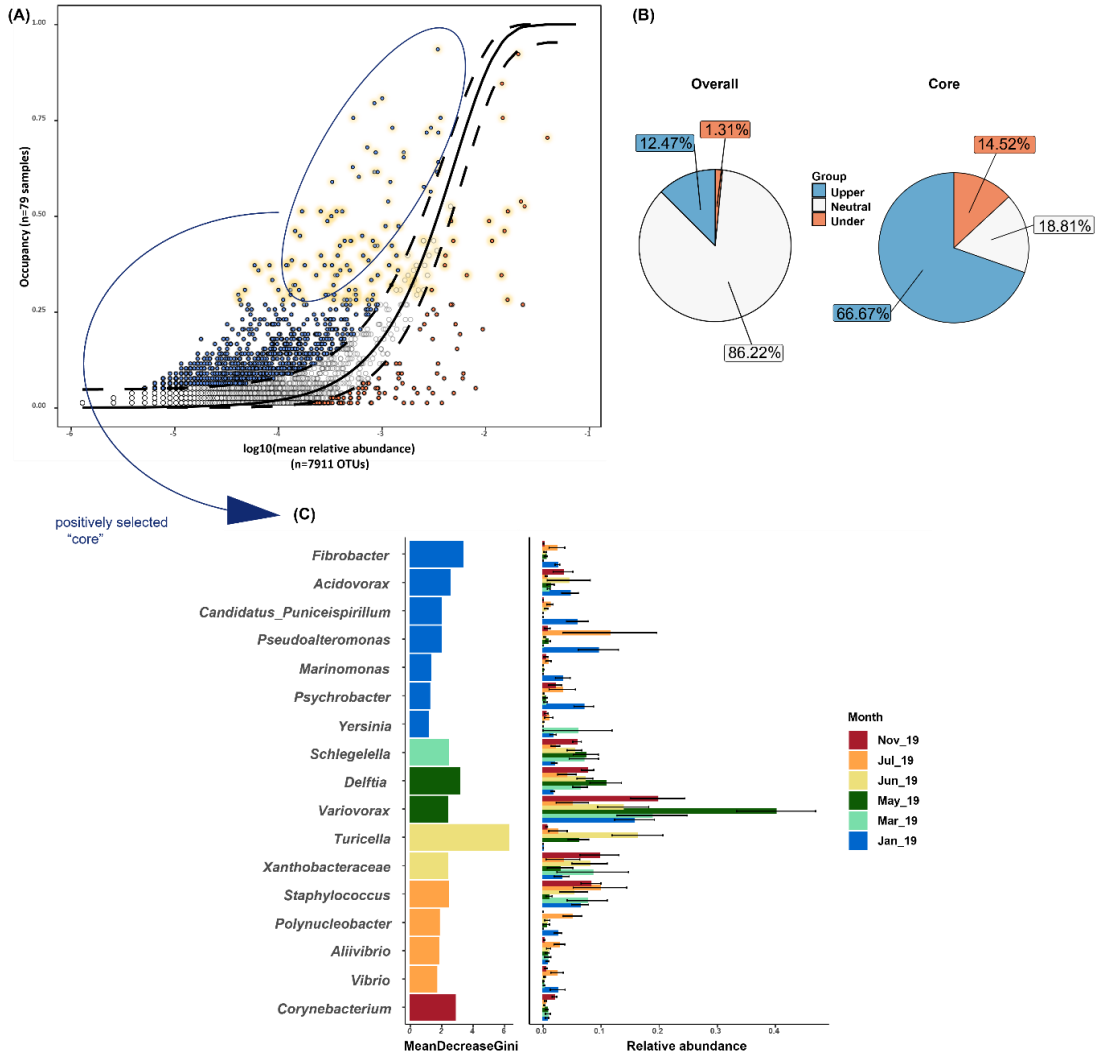


**Figure 2.6:** (A) Abundance-occupancy distribution of OTUs from Atlantic salmon intestines sampled at six sampling timepoints over a period of 10 months. Each point represents an OTU. The black line represents the fit of the neutral model, and the dashed lines represents 95% confidence intervals around the model prediction. OTUs that occur more frequently than predicted by the model are shown above (upper) the interval and are marked in blue. OTUs that occur less frequently than predicted are shown below (under) the interval and are marked in orange. OTUs that fit the neutral model are marked in white. OTUs that are classified as upper and under are likely to be deterministically selected by the intestinal environment. Yellow glowing OTUs represent core OTUs and were estimated by abundance–occurrence relationships and their contribution to Bray-Curtis similarity according to (Shade & Stopnisek, 2019). Core OTUs that appear more frequently than expected by the neutral model were used for further analysis (roughly highlighted by the blue ellipsis). (B) Pie plots of percentages of OTUs that fit the neutral model (white), occur more frequently than predicted (blue) or less frequently than predicted (orange). Second

pie plot depicts percentages for the core OTUs highlighted by the yellow glow in the abundance-occupancy plot. (C) Differential abundance analysis of deterministically selected core OTUs grouped on genus level. 17 out of 25 genera were differently abundant between at least two sampling months. Mean Decrease Gini indicator represents the importance of each genus in distinguishing between sampling months.

## 2.5 Discussion

We assessed the role of host specific and environmental factors, as well as stochastic processes, in shaping the gut microbial development of Atlantic salmon living in a natural river. We found that bacterial community compositions collected from water and macroinvertebrate samples were significantly different from each other and the salmon intestine. This aligns with findings that gut microbial communities in fish are shaped by the environmental filter conditioned by the gut ecosystem (Cheaib et al., 2020; Kim et al., 2021). In theory, most microbes found in the gut originate from the surrounding environment. However, low-abundance environmental bacteria can further evolve to be more prolific colonisers of a fish's intestine, whereas highly abundant environmental bacteria might lack the capability of surviving in the gut environment (López Nadal et al., 2020; Robinson et al., 2018). We found that certain taxa of Firmicutes, such as *Mycoplasma*, *Clostridium sensu stricto*, *Bacillus*, as well as several taxa belonging to the order *Lactobacillus*, were present in very low abundance in environmental samples. However, the same taxa were significantly enriched in fish intestines, indicating their capability to thrive and multiply in the fish gut environment.

Gut microbial community composition varied significantly between all sampling months. Fish caught in January had on average, a significantly more diverse gut microbiota than fish caught in the other months. Microbial richness was lowest in

spring and autumn, with intermediate levels in summer. The magnitude of the observed difference in diversity between January and the other months was a rather surprising find. In theory, the reduction of feeding in winter (Elliott & Elliott, 2010; Volkoff & Rønnestad, 2020) should limit the amount of different ecological niches in the gut, leading to less diversity rather than more. However, based on the findings of the FEAST analysis we noticed that more bacterial taxa from the water column were present in the gut in January compared to other sampling months. Given the high microbial diversity of the water column, this carryover might explain the high gut alpha diversity in January. Bray-Curtis distances between sampling months did not follow a sequential pattern, and Bray-Curtis distances within sampling months did not increase gradually over time. The results suggest that Atlantic salmon juveniles' gut microbial community composition is highly dynamic and undergoes considerable changes over time. This indicates that the factors driving the changes in the gut microbiota may vary from one month to another and that multiple factors may be involved.

Potential deterministic processes could be seasonal temperature, genetic differences between hosts and diet volume or composition. Whilst temperature is a probable cause of differences in gut microbial community composition between sampling months, genetic background is a potential cause of inter-individual differences within a sampling timepoint. Dietary differences might drive monthly differences as well as inter-individual differences. In our study, we observed that water temperature accounted for approximately 3% of the total variation in gut microbial community composition. Although it was a statistically significant predictor, we determined that the overall impact of temperature on the gut microbiota was relatively minor. Fish

are poikilothermic, and each bacterial species has an optimum growing temperature determined by their thermodynamic limitations (Corkrey et al., 2012). Hence, environmental temperature variations might lead to microbial abundance changes (Steiner et al., 2022). Indeed some studies have suggested that elevated temperature can drive overabundance of pathogenic *Vibrionaceae* in some freshwater systems (Suzzi et al., 2023). PERMANOVA results also suggest that a fish's genetic origin impacts gut microbial communities. However, given that we had an uneven and non-constant distribution of the genetic origins across our sampling months, we do not believe we can confidently say that there are effects of both month and genetic origin.

If gut microbial community composition is indeed driven by host genetics, it would not be the first such example in fish. Genetic impacts on fish gut microbiota have been observed among genetically divergent populations of sticklebacks (Smith et al., 2015), guppies (Sullam et al., 2015) and very recently in Chinook salmon (*Oncorhynchus tshawytscha*, Ziab et al., 2023). In our system, genetic background might impact gut microbial communities in several ways. First, due to differences in feeding habits between farmed and wild fish. Farmed fish are from lineages that have been fed *ad libitum* in a sheltered environment for multiple generations. The metabolic conditioning of farmed fish may lead to earlier feeding in the year and prolonged activity in autumn compared to their wild counterparts. This hypothesis is grounded in research that has observed several alterations in farmed fish compared to wild fish, particularly regarding behaviour and life history traits (Besnier et al., 2022; Skaala et al., 2019; Solberg et al., 2020; Sylvester et al., 2019). . A second possibility could be that genetic differences between farmed and wild fish create

different selective pressures in the gut environment, possibly in respect to variation among fish of different provenances in immune response genes (de Eyto et al., 2007, 2011). Consequently, this genetic variation may lead to the proliferation of different bacterial species. However, we also cannot discount the possibility of maternal effects, whereby microbes may be transferred during oviposition (e.g., Long et al., 2014). Close co-diversification between adult salmon lineages and *Mycoplasma* strains observed recently would be consistent with this hypothesis (Rasmussen et al., 2023). To assess the implications of genetic effects on gut microbial communities it is important to understand if differences in microbial communities between farmed, wild and hybrid fish persist over time and if those changes correlate with variations in host metabolism or disease susceptibility. This knowledge is not only relevant for assessing the impact of introgression events to the fitness of salmonid populations but also for developing targeted strategies to promote fish health and mitigate potential negative effects.

Diet is one of the most important drivers of gut microbial community composition (Gajardo et al., 2017; Zarkasi et al., 2016). Intestinal microorganisms feed on food items that the host cannot digest, producing many host-beneficial metabolites in the process (Koh et al., 2016; Ríos-Covián et al., 2016). A change of diet composition or volume might therefore have major implications for gut bacteria growth and consequently for fish physiology and health (Ni et al., 2014; Weththasinghe et al., 2022). It is known that fish have reduced appetite in the winter months (Volkoff & Rønnestad, 2020). Hence, it is unsurprising that we found a distinct cluster of winter samples in our PCoA. FEAST analysis showed that the contribution percentages of food-derived bacteria were found to be the lowest in January, which is in agreement

with a decrease in appetite of juvenile salmon during the winter season (Volkoff & Rønnestad, 2020).We found traces of bacterial taxa from all five macroinvertebrate groups in our fish, with Diptera (Fly) and Ephemeroptera (Mayfly) potentially being favoured food sources for salmon based on microbiota sharing. However, it should be noted that these observations do not confirm a direct dietary link, as taxa were pooled by order and the specific diets of individual fish were not examined. Our data suggest that a significant portion of the taxa found in fish guts originated from the macroinvertebrate order Diptera. Notably, in March and particularly in November, the contribution percentages shifted towards Ephemeroptera. This could imply a change in diet or simply reflect a seasonal variation in macroinvertebrate abundance. Our results align well with a prior stomach count analysis, which also identified Diptera and Ephemeroptera as significant food sources for juvenile Atlantic salmon in our experimental river (de Eyto et al., 2020). While not the primary focus of this manuscript, it is noteworthy that the investigated macroinvertebrates themselves appear to host order-specific microbiota. As with most host associated intestinal microbiota it is likely that diet is of major influence for the microbial structure in macroinvertebrates (Kroetsch et al., 2020). Depending on their life stage aquatic insects feed on algae or leaf litter but may also prey on other macroinvertebrates when older. As adults, certain Diptera species, commonly known as midges, also interact with terrestrial livestock by feeding on their blood (Walker, 2001). It would be interesting to investigate a potential carryover from bacteria associated with terrestrial animals (skin or faeces) through the macroinvertebrate as intermediate host into salmon.

An important aspect in determining the impact of dietary shifts on host health is whether the bacteria introduced through food consumption persist in the gut or if they are merely transient. Our abundance-occupancy analyses allowed further exploration of this question. The model implied that the majority of OTUs (86%) were neutrally assembled, suggesting that the presence of those OTUs in fish guts is determined by ecological drift or random dispersal from environmental sources. This high percentage is consistent with previous studies, which have also shown that the majority of taxa in salmon microbial communities are neutrally assembled (Burns et al., 2016; Heys et al., 2020). However, it is worth noting that the neutral model approach used in our study has been subject to criticism for potentially overestimating the significance of stochastic processes in shaping community outcomes (Ning et al., 2019, 2020). To identify potentially important taxa, we employed an abundance-occupancy threshold. All taxa that passed the threshold were considered “core”, but we remind readers that “core” taxa should be treated as study specific. We identified 78 OTUs as being positively selected core microbiota. These core microorganisms can be considered versatile species that can adapt to the gut environment and persist across individual fish and over time. However, no OTU was present in all our samples, revealing inter-individual variations among hosts. Most positively selected core genera displayed significant differences in their relative abundance across sampling timepoints, suggesting a pronounced temporal variation in the composition of core microbial communities in the intestines of juvenile Atlantic salmon and strong links to seasonally influenced diet. As mentioned in the beginning of this discussion, some taxa were positively enriched in the gut compared to the environment. Out of these taxa our model identified positively selected core OTUs

associated with *Turicella*, *Staphylocccus*, *Schlegella*, *Pseudoalteromonas*, *Delftia*, *Aliivibrio*, *Axicodovorax* and *Psychrobacter*. *Mycoplasma*, a genus that was found to be an important member in other studies of Atlantic salmon intestines, especially in the marine phase (Cheaib et al., 2021; Heys et al., 2020; Llewellyn et al., 2016; Rasmussen et al., 2021, 2023), was only observed in small numbers of fish in our study. However, all OTUs associated with *Mycoplasma* were predicted to be more frequently abundant than estimated by our neutral model, indicating deterministic selection. It is possible that this genus establishes predominance over the course of Atlantic salmon development (Llewellyn et al., 2016). Whilst characterising core microbiota, we found several other similarities to previous studies that have characterised core members of gut microbial communities in juvenile Atlantic salmon sampled in freshwater. For instance, *Corynebacterium*, several Firmicutes and Vibrionales are often reported as core contributors of Atlantic salmon gut microbial communities (Gajardo et al., 2016; Uren Webster et al., 2018). The model used in this study also classified OTUs associated to bacteria of marine origin (*Marinomonas* and *Pseudoalteromonas*) as core microbiota. These bacteria are known to be highly abundant in marine fish (Schaal et al., 2022) and as mentioned previously, their presence in juvenile freshwater fish could possibly be due to maternal transmission during birth. However, knowledge about maternal effects on microbiota in oviparous fish is lacking. It is crucial to recognise that even though certain OTUs may be classified as neutral, indicating a higher probability of having a short presence in the gut, they can still play a significant role in shaping the overall community structure. This is because these OTUs may interact with the resident microbiota, influencing their composition and function. The question remains how those taxa influence

residence bacteria and under what (environmental) circumstances those taxa can incorporate themselves into the resident community. Understanding this process would be especially beneficial to improve the applicability of pre and probiotic treatments.

## 2.6 Conclusion

This study provides a comprehensive overview about the factors governing gut microbial succession in Atlantic salmon utilising a large-scale common garden experiment undertaken in the wild. The results suggest that gut microbial community composition is highly dynamic and undergoes considerable changes over time, driven by both deterministic and stochastic processes. We found distinct microbial profiles of water, macroinvertebrate and intestine communities, which indicates that microbial communities in fish are shaped by the environmental filter conditioned by the gut ecosystem. Microbial taxa associated with macroinvertebrates were more abundant in the gut than bacteria associated with the water column, indicating that macroinvertebrates might be an important factor in gut community assembly. We estimated that most detectable OTUs in our study were assembled stochastically. Deterministic factors impacting gut microbial community composition, like temperature and fish age had a significant influence on the fish's microbiota but their overall importance was estimated to be minor. Monthly differences in gut microbial community composition also persisted in deterministically selected core taxa. Our results also suggest that there might be an additive host genetic effect determining differences observed between farmed and wild fish, thereby promoting inter-individual differences in gut microbial community composition within sampling months. Previous work in salmon has hinted at a role for maternal effects in driving

inter-generational sharing of microbial taxa and our study, although lacking in statistical power, points in that direction. Future work could explore such a genetic and/or maternally determined relationship.

## 2.7 Author Contributions

P.S organised sampling, conducted laboratory work, analysed the data and drafted the manuscript. B.C programmed the bioinformatic pipelines and gave statistical advice. J.K, K.P, L.R and C.H organised sampling, conducted laboratory work and gave statistical advice. All authors were involved in the conception and design of the experiment and contributed to data interpretation and editing of the final draft of the manuscript. P.McG and M.L managed the project.

## 2.8 Acknowledgements

This research has been carried out with the support of the Marine Institute's Newport Research Section and Aquaculture Section, contributing to all field, hatchery, laboratory and logistic support.
Special thanks also to Fintan Egan, Elvira de Eyto, Mary Dillane, Alan Drumm, Jamie Coughlan, Floriane Leseur, Sarah Ryan, Ronan Grealis and Catherine Waters.

## 2.9 Funding Information

PS, JK, KP, ER and PMcG were supported by the Science Foundation Ireland grant award (grant no. 16/BBSRC/3316). PS was also granted the Covid extension award "Higher Education Authority Covid-19 Cost extension (Call Three). This research was further supported in part by research grants from the Biotechnology and Biological Science Research Council (grant no. BB/T016280/1, BB/P001203/1 & BB/N024028/1) and by the Science Foundation Ireland, the Marine Institute Ireland and the Department for the Economy, Northern Ireland, under the Investigators Program (grant no. SFI/15/IA/3028). JK is currently supported by Science Foundation Ireland / Ireland Research council grant, under the Pathway programme (grant no. SFI-IRC/21/PATH-S/9671). The funding bodies did not influence data interpretation or in writing of the manuscript.

## 2.10 Competing Interests

The authors declare that they have no competing interests.

## 2.11 Data Availability Statement

The raw 16S rRNA gene sequence files and metadata are deposited at the NCBI SRA database under the BioProject PRJNA977248. The scripts and codes generated during the current study are available from the corresponding author on reasonable request.

## 2.12 Benefit Sharing

Benefits from this research accrue from the sharing of our data and results on public databases, as described in the data availability section. The present work strongly benefitted from a collaboration between the University of Glasgow, Scotland, the Marine Institute Newport, Ireland and the University College Cork, Ireland.

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## 2.14 Additional Files

### 2.14.1 Supplementary Figures

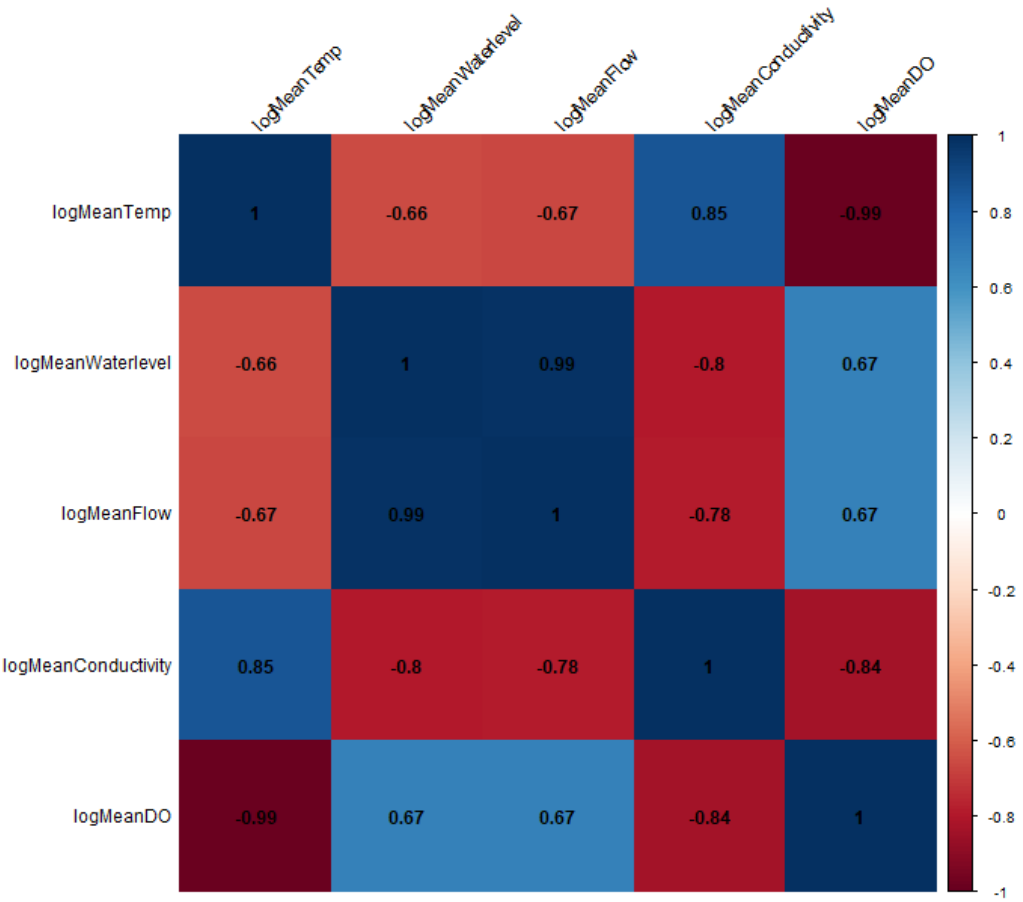


**Figure S 2.1**: The correlation plot illustrates the relationships between environmental variables in the dataset. Each cell displays the Pearson correlation coefficient between pairs of variables, with color intensity and direction indicating the strength and direction of the correlation. Variables include: logMeanTemp (logarithm of mean temperature), logMeanWaterlevel (logarithm of mean water level), logMeanFlow (logarithm of mean flow), logMeanConductivity (logarithm of mean conductivity), logMeanDO (logarithm of mean dissolved oxygen). Strong correlations are

represented by darker shades of blue (positive correlation) or red (negative correlation), while weaker correlations are depicted in lighter colors.

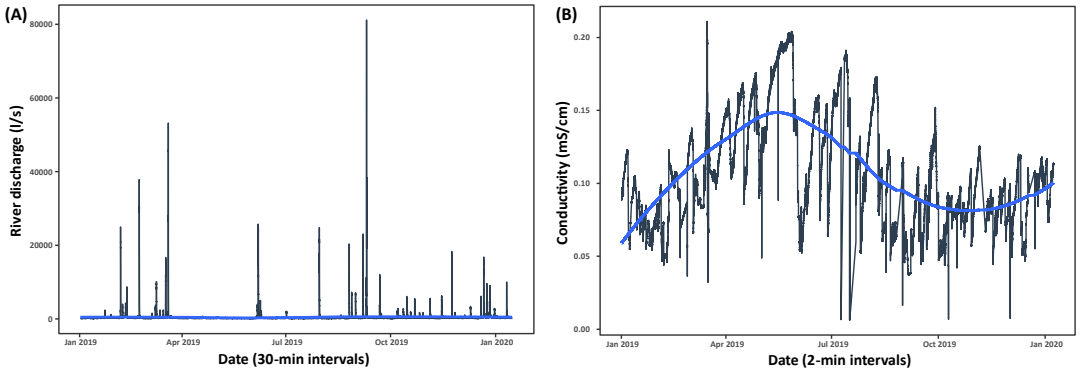


**Figure S 2.2**: (A) Mean river discharge (l/s) of the Srahrevagh river in 2019 measured in 30-minute intervals. (B) Mean conductivity (mS/cm) of the Srahrevagh river in 2019 measured in 2-minute intervals. Blue lines depict mean values fitted with loess regression.

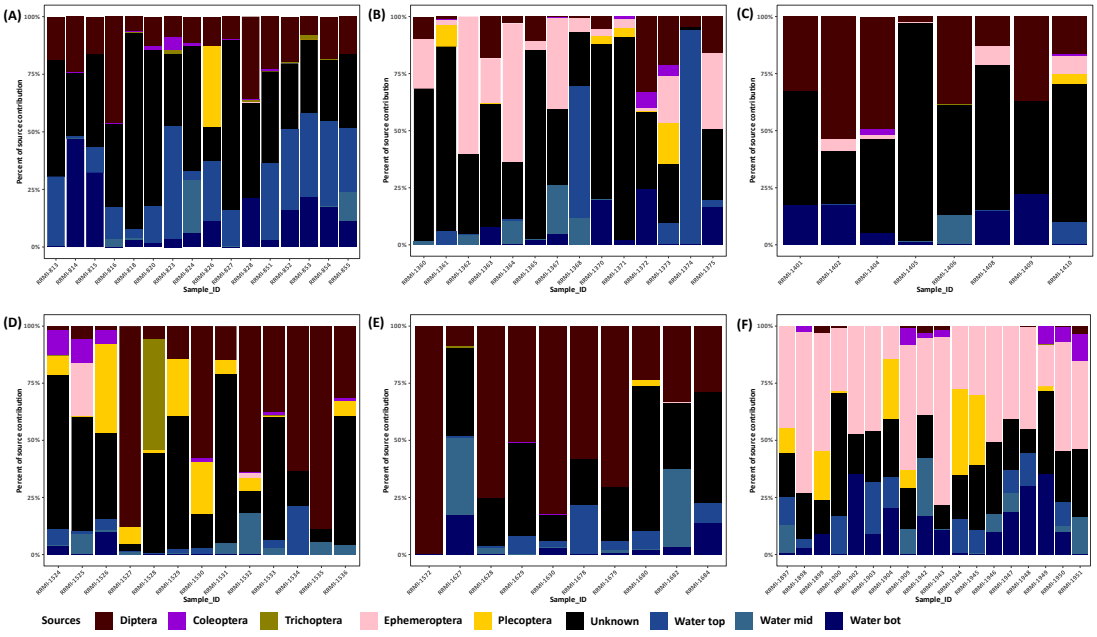


**Figure S 2.3:** Fast expectation-maximization for microbial source tracking (FEAST) estimations of microbial source contributions for Atlantic salmon gut communities. Each bar represents one individual sample. Each plot represents one sampling month. (A) January, (B) March, (C) May, (D) June, (E) July, (F) November. Mixing proportions were calculated by using taxa counts on genus level. Sources contain five different macroinvertebrates (feed samples) and three different water samples, collected from the top, the middle (mid) and the bottom (bot) of the experimental study area in the Srahrevagh river. Source samples were collected at the same sampling day as fish guts.

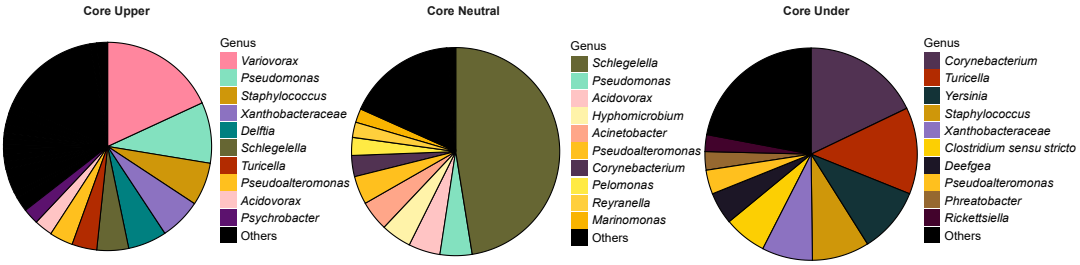


**Figure S 2.4**: Pie plots of ten most abundant core genera as determined by the abundance-occupancy model. The model was used to determine core OTUs, which had to surpass a specific core inclusion threshold to be included in the analysis. The core OTUs were further divided into three categories: Core upper, which were positively selected by the intestinal environment and appeared more frequently than expected by a neutral model; Core neutral, which occurred as frequently as predicted by the neutral model; and Core under, which appeared less frequently than expected by the neutral model. For the plots OTUs were grouped on genus level.

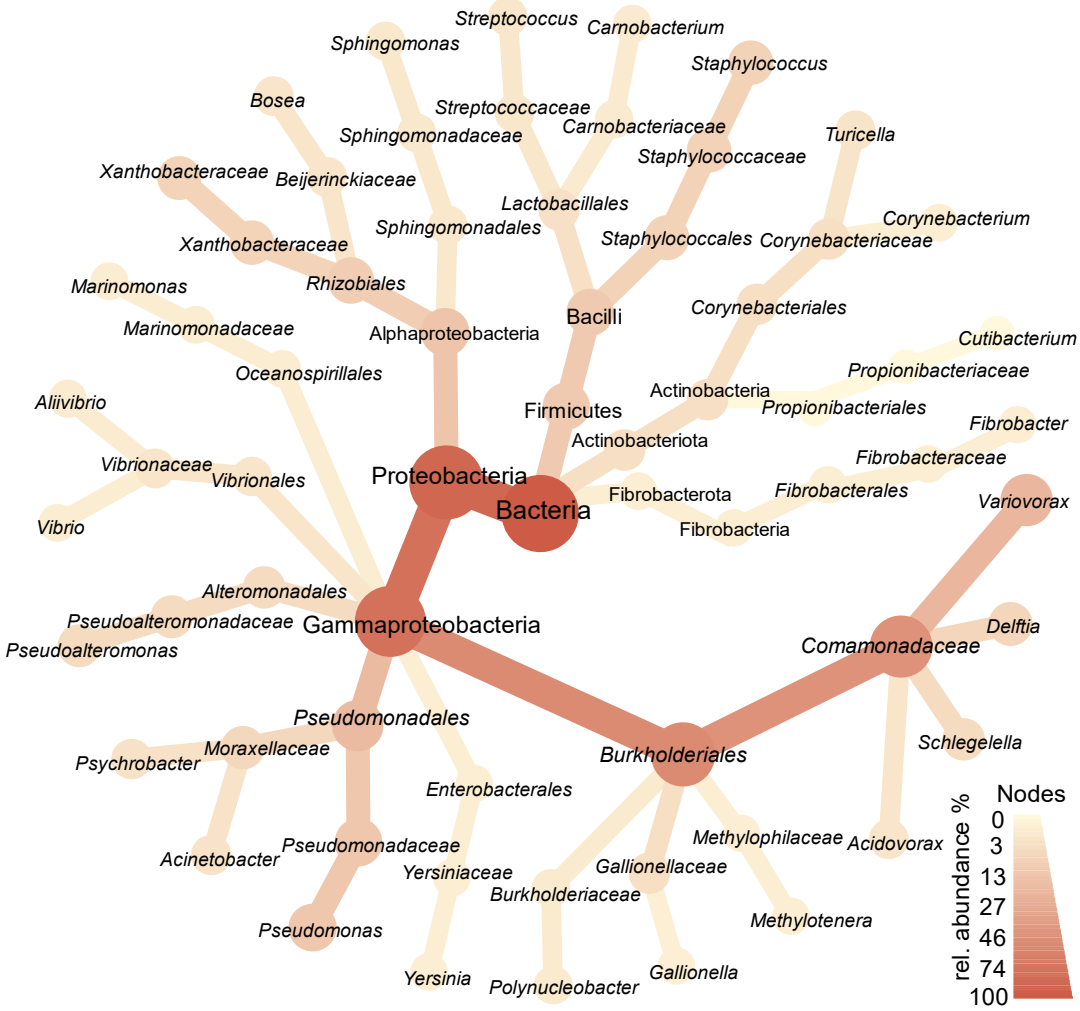


**Figure S 2.5**: Taxonomic heat map of deterministically selected core OTUs as determined by the abundance-occupancy model from Figure 2.6. Node size and colour reflect the mean relative abundance of taxa per taxonomic rank.

## 2.14.2 Supplementary Tables

**Table S 2.1:** Overview of sampled fish during the 10-month experiment. 80 fish were sampled across six sampling timepoints. Fish age is depicted in months. Number of samples taken is shown for each sex per origin per month. Respective mean length (mm) and weight (g) measurements are included. Brackets show standard deviations. F=Farmed, HFF=Hybrid Farmed Female, HWF=Hybrid Wild Female, W=Wild.

| Month | Age (Months) | Origin | Male (N) | Female (N) | Length (mm) | Weight (g) |
|---|---|---|---|---|---|---|
| **Jan_19 (N=16)** | 9 | F | 1 | 1 | 61.91 (±1.85) | 2.39 (±0.33) |
| | | HFF | 4 | 4 | 62.76 (±4.13) | 2.36 (±0.49) |
| | | HWF | 3 | 1 | 55.35 (±7.52) | 1.67 (±0.79) |
| | | W | 0 | 2 | 56.62 (±14.07) | 1.78 (±1.27) |
| **Mar_19 (N=14)** | 11 | F | 1 | 2 | 66.45 (±6.32) | 2.82 (±0.82) |
| | | HFF | 2 | 2 | 63.88 (±7.23) | 2.50 (±0.88) |
| | | HWF | 1 | 0 | 53.38 | 1.28 |
| | | W | 3 | 3 | 53.29 (±4.77) | 1.37 (±0.44) |
| **May_19 (N=8)** | 13 | F | 2 | 0 | 80.00 (±1.41) | 4.9 (±0.13) |
| | | HFF | 1 | 0 | 78.00 | 4.87 |
| | | HWF | 1 | 2 | 73.67 (±1.53) | 3.76 (±0.22) |
| | | W | 2 | 0 | 69.50 (±3.54) | 2.92 (±0.28) |
| **Jun_19 (N=13)** | 14 | F | 1 | 4 | 90.80 (±7.36) | 7.91 (±2.50) |
| | | HFF | 0 | 2 | 85.67 (±10.07) | 5.91 (±2.23) |
| | | HWF | 4 | 0 | 89.50 (±5.45) | 6.57 (±0.60) |
| | | W | 1 | 1 | 73.00 (±4.24) | 4.24 (±0.01) |
| **Jul_19 (N=11)** | 15 | F | 1 | 1 | 107.50 (±2.12) | 13.55 (±1.51) |
| | | HFF | 1 | 3 | 94.33 (±11.5) | 8.36 (±2.93) |
| | | HWF | 1 | 1 | 87.50 (±3.54) | 6.34 (±0.66) |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | W | 2 | 1 | 79.00 (±1.41) | 5.21 (±0.04) |
| **Nov_19 (N=18)** | 19 | F | 1 | 1 | 112.50 (±6.36) | 14.59 (±2.41) |
| | | HFF | 5 | 0 | 111.00 (±14.71) | 16.25 (±6.65) |
| | | HWF | 3 | 4 | 103.43 (±8.73) | 11.85 (±2.14) |
| | | W | 2 | 2 | 102.25 (±6.34) | 10.72 (±2.35) |

**Table S 2.2:** PERMANOVA results to assess the effects of genetic origin, sampling month and sex on the composition of the Atlantic salmon gut microbiome in the river habitat. Significance code: ***<0.001

| Group | Df | SumOfSqs | R2 | F | Pr(>F) | Signif. |
|---|---|---|---|---|---|---|
| **Origin** | 3 | 1.690 | 0.054 | 1.707 | 0.001 | *** |
| **Month** | 5 | 5.098 | 0.164 | 3.089 | 0.001 | *** |
| **Sex** | 1 | 0.278 | 0.009 | 0.843 | 0.791 | |
| **Origin:Month** | 15 | 6.358 | 0.205 | 1.284 | 0.001 | *** |
| **Residual** | 53 | 17.495 | 0.565 | | | |
| **Total** | 77 | 30.922 | 1 | | | |

**Table S 2.3:** PERMANOVA testing pairwise comparisons of gut microbial samples grouped by their sampling timepoint (month) in the river habitat. Significance codes: **<0.01; *<0.05.

| Groups | measure | F | R2 | p.value | p.adjusted | Signif. |
|---|---|---|---|---|---|---|
| **Jan_19 vs Mar_19** | bray | 3.627 | 0.115 | 0.001 | 0.002 | ** |
| **Jan_19 vs May_19** | bray | 4.299 | 0.163 | 0.001 | 0.002 | ** |
| **Jan_19 vs Jun_19** | bray | 4.856 | 0.152 | 0.001 | 0.002 | ** |
| **Jan_19 vs Jul_19** | bray | 1.885 | 0.073 | 0.003 | 0.004 | ** |
| **Jan_19 vs Nov_19** | bray | 5.469 | 0.150 | 0.001 | 0.002 | ** |
| **Mar_19 vs May_19** | bray | 1.808 | 0.083 | 0.008 | 0.009 | ** |
| **Mar_19 vs Jun_19** | bray | 2.706 | 0.098 | 0.001 | 0.002 | ** |
| **Mar_19 vs Jul_19** | bray | 1.772 | 0.075 | 0.004 | 0.005 | ** |
| **Mar_19 vs Nov_19** | bray | 1.661 | 0.054 | 0.018 | 0.018 | * |
| **May_19 vs Jun_19** | bray | 2.360 | 0.110 | 0.001 | 0.002 | ** |
| **May_19 vs Jul_19** | bray | 2.044 | 0.113 | 0.004 | 0.005 | ** |
| **May_19 vs Nov_19** | bray | 2.385 | 0.094 | 0.001 | 0.002 | ** |
| **Jun_19 vs Jul_19** | bray | 2.183 | 0.094 | 0.001 | 0.002 | ** |
| **Jun_19 vs Nov_19** | bray | 4.201 | 0.130 | 0.001 | 0.002 | ** |
| **Jul_19 vs Nov_19** | bray | 2.930 | 0.105 | 0.001 | 0.002 | ** |

**Table S 2.4:** PERMANOVA testing pairwise comparisons of gut microbial samples grouped by their associated genetic origin in the river habitat. F=Farmed, HFF=Hybrid Farmed Female, HWF=Hybrid Wild Female, W=Wild. Significance codes: *<0.05.

| Groups | measure | F | R2 | p.value | p.adjusted | Signif. |
|---|---|---|---|---|---|---|
| **HWF vs HFF** | bray | 1.247 | 0.028 | 0.076 | 0.114 | |
| **HWF vs W** | bray | 0.897 | 0.024 | 0.662 | 0.662 | |
| **HWF vs F** | bray | 1.545 | 0.043 | 0.036 | 0.072 | |
| **HFF vs W** | bray | 1.858 | 0.044 | 0.004 | 0.012 | * |
| **HFF vs F** | bray | 1.002 | 0.025 | 0.446 | 0.535 | |
| **W vs F** | bray | 2.121 | 0.062 | 0.002 | 0.012 | * |

**Table S 2.5:** Generalised linear model results to assess the effects of sampling time (Month) on Chao1 richness. The best model fit was determined using the MuMIn package, with the Akaike Information Criterion (AIC) used as the selection criterion. The chosen model was: glm(formula = Chao1 ~ Month, family = Gamma(link = "log"), data = data), with an AIC of 1046.2. The coefficients are interpreted on the log scale, with the exponentiated coefficients representing multiplicative effects on Chao1 richness. For example, a coefficient of -0.9753 for Mar_19 indicates that the expected Chao1 richness in March is approximately 37.7% of the expected richness in the reference month (January). Significance codes: *** < 0.001; ** < 0.01.

| | Estimate | Std. Error | t value | Pr(>\|t\|) | Signif. |
|---|---|---|---|---|---|
| **(Intercept)** | 6.605 | 0.1365 | 48.383 | < 2e-16 | *** |
| **Mar_19** | -0.975 | 0.1999 | -4.88 | 6.05E-06 | *** |
| **May_19** | -1.209 | 0.2365 | -5.113 | 2.46E-06 | *** |
| **Jun_19** | -0.432 | 0.2039 | -2.121 | 0.0373 | * |
| **Jul_19** | -0.472 | 0.2201 | -2.143 | 0.0354 | * |
| **Nov_19** | -0.956 | 0.1876 | -5.098 | 2.61E-06 | *** |

**Table S 2.6:** Generalised linear model results to assess the effects of sampling time (Month) on Shannon diversity. The best model fit was determined using the MuMIn package, with the Akaike Information Criterion (AIC) used as the selection criterion. The chosen model was: glm(formula = Shannon ~ Month, family = Gamma(link = "log"), data = data), with an AIC of 1046.2. The coefficients are interpreted on the log scale, with the exponentiated coefficients representing multiplicative effects on Chao1 richness. Significance codes: ***<0.001; **<0.01

| | Estimate | Std. Error | t value | Pr(>\|t\|) | Signif. |
|---|---|---|---|---|---|
| **(Intercept)** | 1.609 | 0.064 | 25.21 | < 2e-16 | *** |
| **Mar_19** | -0.585 | 0.093 | -6.26 | 2.38E-08 | *** |
| **May_19** | -0.431 | 0.111 | -3.9 | 0.00022 | *** |
| **Jun_19** | -0.413 | 0.095 | -4.33 | 4.62E-05 | *** |
| **Jul_19** | -0.223 | 0.103 | -2.17 | 0.03344 | * |
| **Nov_19** | -0.251 | 0.088 | -2.86 | 0.00545 | ** |

**Table S 2.7:** Summary of the top 6 generalised linear models for predicting Chao1 richness, using the MuMIn package to rank models based on the corrected Akaike Information Criterion (AICc). The global model included the predictor variables: Fish length, sampling month, sex, fish genetic origin (Orig), temperature (Temp) and utilised a Gamma distribution with a log link function. The table displays the models with the lowest AICc values, indicating the best fit among the models considered.

| No. | Intrcpt | Length | Month | Orig | Sex | Temp | df | logLik | AICc | delta | w. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **3** | 6.605 | | + | | | | 7 | -516.09 | 1047.8 | 0 | 0.117 |
| **4** | 6.024 | 0.009 | + | | | | 8 | -514.88 | 1047.8 | 0.05 | 0.113 |
| **7** | 6.75 | | + | + | | | 10 | -512.37 | 1048 | 0.2 | 0.105 |
| **12** | 5.958 | 0.01 | + | | + | | 9 | -514.38 | 1049.4 | 1.6 | 0.052 |
| **11** | 6.559 | | + | | + | | 8 | -515.66 | 1049.4 | 1.61 | 0.052 |
| **20** | 5.481 | 0.01 | + | | | 0.081 | 9 | -514.45 | 1049.5 | 1.73 | 0.049 |

**Table S 2.8:** Distanced-based linear model showing the best environmental and host specific predictors of gut microbial community composition in Atlantic Salmon parr in a river habitat. Model assessed the marginal effects of the predictors ("by margin"). Significance code: ***<0.001

| Predictor | Df | SumOfSqs | R2 | F | Pr(>F) | Signif. |
|---|---|---|---|---|---|---|
| **Temperature** | 1 | 0.972 | 0.031 | 2.556 | 1.00E-04 | *** |
| **Age** | 1 | 1.364 | 0.044 | 3.587 | 4.00E-04 | *** |
| **Residual** | 75 | 28.903 | 0.885 | | | |
| **Total** | 78 | 31.221 | 0.926 | | | |

**Table S 2.9:** PERMANOVA testing pairwise comparisons of macroinvertebrate samples grouped by their origin. Significance codes: **<0.01; *<0.05.

| Groups | measure | F | R2 | p.value | p.adjusted | Signif. |
|---|---|---|---|---|---|---|
| **Diptera vs Coleoptera** | wei_unifr | 2.695 | 0.212 | 0.019 | 0.024 | * |
| **Diptera vs Trichoptera** | wei_unifr | 4.427 | 0.307 | 0.001 | 0.007 | ** |
| **Diptera vs Ephemeroptera** | wei_unifr | 2.992 | 0.230 | 0.004 | 0.007 | ** |
| **Diptera vs Plecoptera** | wei_unifr | 3.365 | 0.252 | 0.005 | 0.007 | ** |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Coleoptera vs Trichoptera** | wei_unifr | 2.675 | 0.211 | 0.004 | 0.007 | ** |
| **Coleoptera vs Ephemeroptera** | wei_unifr | 2.458 | 0.197 | 0.004 | 0.007 | ** |
| **Coleoptera vs Plecoptera** | wei_unifr | 3.551 | 0.262 | 0.002 | 0.007 | ** |
| **Trichoptera vs Ephemeroptera** | wei_unifr | 2.010 | 0.167 | 0.032 | 0.036 | * |
| **Trichoptera vs Plecoptera** | wei_unifr | 2.596 | 0.206 | 0.003 | 0.007 | ** |
| **Ephemeroptera vs Plecoptera** | wei_unif | 1.055 | 0.095 | 0.369 | 0.369 | |

# Chapter 3: METABOLIC TRAITS AND GUT MICROBIOTA: IMPLICATIONS FOR THE PERFORMANCE IN THE WILD OF FARM INTROGRESSED ATLANTIC SALMON POPULATIONS

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## 3.1 Abstract

The escape of farmed Atlantic salmon into the wild has raised concerns about their ecological and genetic impact on recipient wild populations. Differences in the fitness of the progeny from farmed and wild parents, including their hybrid offspring, have been known for some time. However, less well-known are the mechanisms underlying these differences. Here, we measured the gut microbial community composition and standard metabolic rate (SMR), traits likely associated with fitness, in juvenile salmon of known farmed and wild parentage collected in the wild seasonally over a period of 11 months. In winter, the SMR, as determined by ventilation rates, in the wild progeny was significantly lower than in their farmed and hybrid conspecifics. In contrast, in the summer, SMR was higher in the wild fish relative to those with either one or two farmed parents. These results suggest greater metabolic flexibility in the progeny of wild fish, which makes an important contribution to fitness in variable wild environments. The data illustrates the role of genetics in shaping a fish's capacity to regulate its metabolic response to seasonal changes in the environment, particularly during low-energy winter conditions. Locally adapted wild populations appear better equipped to cope, while farm strains may be less adaptable due to artificial selection for commercially favoured traits. Additionally, we observed a significant disparity in the gut microbial community composition of wild offspring during winter compared to both farmed and hybrid offspring. However, we did not find a direct link between gut microbial composition and standard metabolic rate. Our results provide evidence of some of the biological differences between the progeny of farm and wild parents, contributing to an understanding of the poorer performance of farm fish progeny in the wild.

## 3.2 Introduction

The commercial farming of Atlantic salmon (*Salmo salar*; henceforth "salmon") dates to the 1970s (Gjedrem, 2010). Since then, salmon have been selectively bred for greater feeding efficiency, faster growth rates, disease resistance, improved flesh quality and other desirable attributes to meet the increasing global demand for salmon among consumers (Gjerde et al., 2007). The expansion of aquaculture production has presented numerous challenges, one of the most significant being the escape of farmed fish into the wild and their ecological and genetic interactions with recipient wild populations (Gilbey et al., 2021; Glover et al., 2017; Karlsson et al., 2011; Palm et al., 2021; Thorstad et al., 2008). Estimates suggest that the annual worldwide escape rate range from several thousands to millions of fish (Glover et al., 2017, 2019; Skilbrei et al., 2015). As a consequence, many rivers across the range are substantially introgressed, resulting in significant changes in the biology of recipient wild populations (Bolstad et al., 2021; Taranger et al., 2015; Wacker et al., 2021, 2023).

While domestication selection has conferred advantages for fish in aquaculture settings characterized by *ad libitum* feeding and the absence of predators, these domesticated traits are likely to render the progeny of farmed fish poorly suited for life in the wild (McGinnity et al 2003; Fleming et al; Reed et al. Skaala et al., 2012). Furthermore, there is a phylogeographic aspect, as the fish farmed in many parts of the North Atlantic are sourced and developed from Norwegian wild populations (Gjedrem, 2010; Skaala et al., 2005). Finally, the hybrid offspring resulting from mixed spawning often inherit phenotypic traits from their farmed parents, including high

growth rates, behaviour and life-history characteristics (Bolstad et al., 2017; Reed et al., 2015; Wacker et al., 2021).

While it is known that the progeny of farm escapes and their subsequent F1 and F2 generations have reduced Darwinian fitness in the wild (McGinnity et al. 2003), our understanding of the mechanisms, both proximate and ultimate, underlying the differences between farmed and wild salmon remains limited. For instance, in experiments conducted in controlled environments resembling farming conditions or semi-natural settings, domesticated Atlantic salmon grew faster but also had diminished awareness and hence heightened vulnerability to predators (Solberg et al., 2020), earlier maturation and consequent altered life history traits (Besnier et al., 2022; Skaala et al., 2019; Sylvester et al., 2019). Research suggests that if interbreeding between farmed and wild salmon continues to occur frequently and accumulates over time, it could increasingly undermine the fitness and genetic integrity of wild populations (Bolstad et al., 2021; Castellani et al., 2018; McGinnity et al., 2003; Skaala et al., 2019). Therefore, gaining an understanding of the phenotypic variations arising from different genetic backgrounds is essential for comprehending the reduced fitness observed in hybrids. So far only few studies have investigated how hybridisation affects fish metabolic rates and gut microbiomes (Lindsay et al., 2020; Robertsen et al., 2019). Both traits are known (reviewed below) to be important for determining host fitness and alterations between farmed, wild and hybrid fish might contribute to the reduced fitness observed in hybrid populations.

Standard Metabolic Rate (SMR) quantifies the minimum energy an organism requires to maintain essential physiological functions while at rest (Chabot et al., 2016). It acts as a proxy for the underlying process of cellular respiration, during which ATP is produced from nutrient molecules (Norin & Metcalfe, 2019). Hence, SMR is an important measure for understanding an organism's energy requirements and can be estimated by calculating opercular movements within a specific time frame (Millidine et al., 2008). Studies have demonstrated significant variation in SMR between individuals, even after accounting for confounding factors such as fish weight (Burton et al., 2011). This variation is noteworthy because it has been shown previously that fish with high SMRs often display increased aggression and dominance, potentially conferring a competitive edge in occupying areas with greater food availability (Metcalfe et al., 2016). While a high SMR can be advantageous in resource-rich environments, it may be a disadvantage during periods of food scarcity due to the increased metabolic demands exceeding energy intake (Auer et al., 2015; Killen et al., 2011; Reid et al., 2012). However, it is not just the absolute level of SMR at a given time that is of significant interest; as important or maybe more important, is the capacity of individual fish to adapt their metabolic rates to fluctuating environmental conditions (Norin & Metcalfe, 2019; Staples, 2016) and sustained and rapid directional changes in seasonal aquatic thermal states associated with global warming (Alfonso et al., 2021). Flexibility in metabolism would bolster resilience-an idea that has been examined previously in the context of inter species comparisons (Magozzi & Calosi, 2015), but currently lacks empirical support at the intra-species level (Norin & Metcalfe, 2019). Many animals, including fish, exhibit metabolic (energy management) adaptations such as reducing their activity

levels and metabolism to conserve energy during winter (Bull et al., 1997; Finstad et al., 2007, 2010; Geiser, 2013; Volkoff & Rønnestad, 2020). Given that SMR is a heritable trait (Pettersen et al., 2018) an important research question arises: As a result of the process of domestication selection on metabolic flexibility, are the progeny of farmed salmon diminished in their ability to effectively adapt to different environmental conditions, which in turn reduces their chances of surviving in the wild relative to the chances of locally adapted wild fish?

Gut microbiomes have been shown to be closely linked to host metabolism (reviewed by Lindsay et al., 2021). A direct demonstration of this relationship in hibernating hosts, such as wild brown bears (*Ursos arctos*), was shown in an experiment conducted by (Sommer et al., 2016). The study observed distinct effects of the "winter microbiota" and "summer microbiota" from the bear's gut during hibernation and active periods, respectively, when transferred into gnotobiotic mice. The winter microbiota not only exhibited a distinct microbial community composition compared to the summer microbiota but also induced significant shifts in the metabolic profiles of the recipient mice. Moreover, analysis of metabolites in the blood of these mice revealed correlations with metabolite profiles observed in wild bears during the corresponding seasons.

The gut microbiome is a complex and diverse community of microorganisms, including bacteria, archaea, viruses and fungi, residing within the gastrointestinal tract. Beyond microbial diversity, it encompasses their genetic material and metabolic by-products, playing a pivotal role in regulating host health across all animal species (Egerton et al., 2018; Lynch & Pedersen, 2016; Vos et al., 2022). This

intricate microbial ecosystem is involved in a multitude of functions, including but not limited to aiding in the digestion and absorption of nutrients (David et al., 2014), supporting the host's immune system (Shi et al., 2017), synthesizing essential vitamins (LeBlanc et al., 2013) as well as metabolites (Krautkramer et al., 2021). Recent research has also highlighted its role in influencing disease susceptibility, metabolism (Tremaroli & Bäckhed, 2012) and even host behaviour (Burokas et al., 2015). Atlantic salmon predominantly acquire their gut microbiota from their surrounding environment, though the possibility of direct or indirect maternal microbial transfer is debated (Rasmussen et al., 2023; Chapter 2). Seasonal variations in gut microbial community composition in Atlantic salmon are strongly influenced by diet composition and volume (Leeper et al., 2023). Individual differences in gut microbiota likely arise from variations within the gut ecosystem, potentially influenced by genetic predispositions (Small et al., 2023; Ziab et al., 2023). While it is increasingly evident that host genetics can shape gut microbiomes, uncertainties remain regarding whether the domestication process in Atlantic salmon has led to distinct gut microbial profiles and their potential implications for fish fitness.

In this study, we conducted sampling of juvenile salmon within an ongoing common garden setup to simulate a farmed escape and hybridisation event in a natural environment. Over the course of a year, we measured ventilation rates (VR) and assessed the composition of gut microbial communities in juvenile Atlantic salmon at four different sampling timepoints. Our primary objective was to investigate whether disparities in metabolic rates and gut microbial community composition exist among farmed, wild and hybrid fish and whether these distinctions persist over time. This

research aims to determine whether these variations contribute to observed declines in fitness within hybrid salmon populations, providing valuable insights into factors influencing the health and sustainability of salmonid populations.

## 3.3 Methods

### 3.3.1 Sampling

An experimental population of Atlantic salmon of farmed, wild and hybrid origin was established in December 2017 at the Marine Institute's hatchery facility on the Burrishoole river system in the west of Ireland (coordinates: 53°55'22"N 9°34'18"W). The wild broodstock consisted of 13 females and 16 male adults captured on their upstream migration in the Burrishoole river system. The farmed broodstock, sourced from the "Fanad MOWI" strain, comprised 15 females and 15 males. In total 55 families were produced, 15 pure farmed (F), 13 pure wild (W) and 24 reciprocal hybrid families, designated as hybrid farmed female (HFF, 12 families) and hybrid wild female (HWF, 12 families; Figure 3.1). Eggs were held under ambient hatchery conditions until May 2018. After hatching and prior to exogenous feeding juvenile swim-up fry were introduced into an experimental section of the Srahrevagh river, located within the Burrishoole catchment (n(F)= 11253; n(W)=9683; n(HFF)= 11815; n(HWF) 8294). The experimental section of the river consists of an area of approximately 7,250 $m^2$ of habitat enclosed by a series of large waterfalls at its top and a high specification trap capable of capturing of life history stages from swim up fry to adult at its lower end. Further comprehensive descriptions of the river are provided in McGinnity et al., 1997, 2003, 2009; Perry et al., 2021 and chapter 2).

Samples of individual parr were collected by electrofishing on four occasions: 9th-12th October 2018 (referred to as Autumn 2018: n(F)=13, n(W)=19, n(HFF)=16, n(HWF)=16), 5th-7th February 2019 (referred to as Winter 2019: n(F)=19, n(W)=22, n(HFF)=20, n(HWF)=19), 9th-11th July 2019 (referred to as Summer 2019: n(F)=13, n(W)=15, n(HFF)=17, n(HWF)=14) and 17th-19th September 2019 (referred to as Autumn 2019: n(F)=13, n(W)=12, n(HFF)=16, n(HWF)=8; Figure 3.1b). The fish were transported in buckets filled with oxygenated river water to the Marine Institute Newport Research Station. They were then acclimated in dark, transparent containers containing oxygenated river water for 24 hours before ventilation rate (VR) measurements were conducted. Subsequently fish were euthanized by an anaesthetic overdose of methane tricaine sulphonate (MS-222, 80ml/l, FVG, Ireland) and their fork length (mm) and wet weight (g) measured. Fin clips were taken and preserved in ethanol to enable genetic analysis and identification of parentage. The intestines of sampled fish were dissected aseptically via an incision along the fish's ventral side. The pyloric caecum was removed, cut into pieces, put into sterile cryotubes and immediately placed on dry ice. Genetic profiling of fish sex and parentage analysis was conducted at the University College Cork population genetics laboratory using a three-panel multiplex PCR technique, which amplified 10 microsatellite loci to achieve accurate parental assignments (for details see Perry et al., 2021). Water samples were collected to identify bacteria in the water column that might serve as a dispersal source for fish gut communities (see chapter 2). The water was collected in sterile water bottles (1.5 L). Within one hour of collection the water was filtered in a sterile environment using 0.2µm filters (Whatman, Chicago, IL, USA) at the Marine Institute Newport Research Station. After filtration the filter papers

were placed into cryotubes, immediately placed on dry ice and stored at -80°C. Intestinal and water samples were transferred on dry ice to the University of Glasgow for microbial profiling.

The study was carried out under the Health Products Regulatory Authority (HPRA) licence number AE19130-P056 in Ireland.

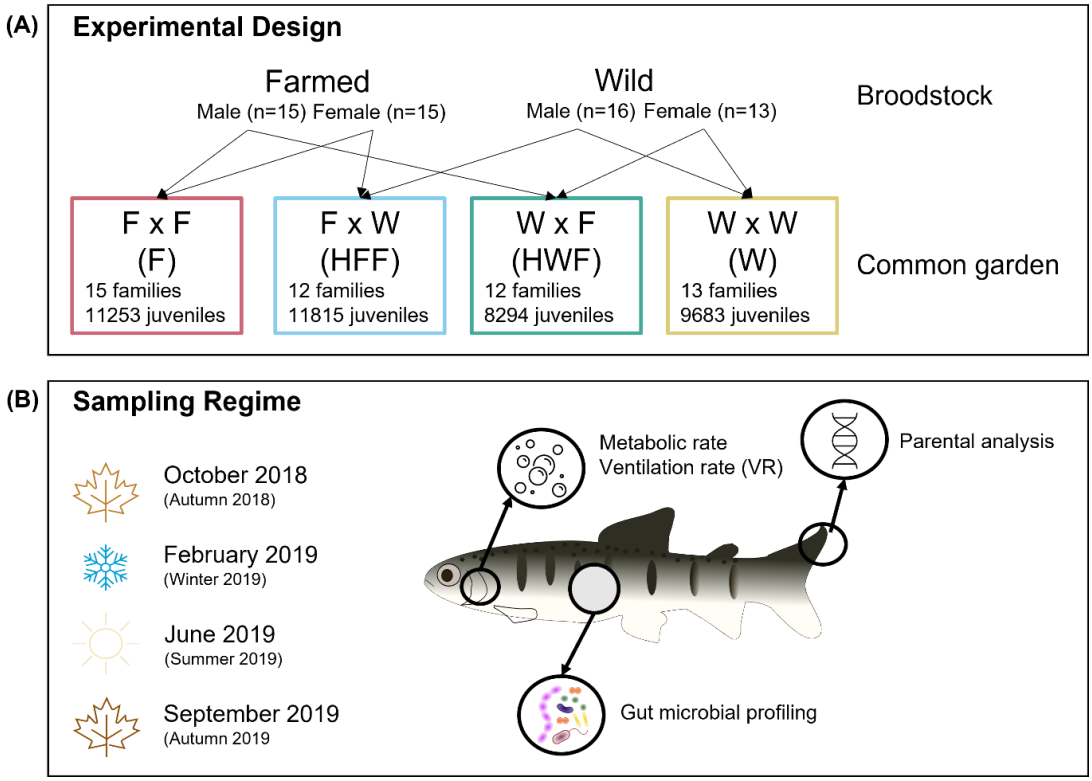


**Figure 3.1:** (A) Illustrates the composition of the experimental population. Farmed and wild adults were bred to produce pure farmed offspring (F), pure wild offspring (W) and hybrid offspring with a farmed mother (hybrid farmed female=HFF) and a wild mother (hybrid farmed wild=HWF). Eggs were held in ambient conditions at a facility in County Galway until the eyed egg stage of development and transferred to Marine Institute facility in County Mayo. In May 2018 after hatching swim-up fry (fry which have part of their yolk sack still attached) were equally distributed in the experimental part of the Srahrevagh river, located within the Burrishoole catchment. (B) The first out of four sampling events took place in October 2018 (n(F)=13, n(W)=19, n(HFF)=16, n(HWF)=16), followed by a Winter sampling: n(F)=19, n(W)=22, n(HFF)=20, n(HWF)=19), Summer 2019: n(F)=13, n(W)=15, n(HFF)=17, n(HWF)=14) Autumn 2019: n(F)=13, n(W)=12, n(HFF)=16, n(HWF)=8. In addition to the described sampling regime, water samples were collected at each sampling timepoint for water bacterial profiling.

### 3.3.2 **Ventilation rate analysis**

To estimate the rate of fish metabolism and associated energy expenditure we calculated, as a surrogate of metabolic rate, the opercular ventilatory beat rate (Millidine et al., 2008). Ensuring a consistent baseline, we subjected the fish to a 24-hour period of food withdrawal to achieve a post-absorptive state. To measure the ventilation rate (VR), we observed and recorded the number of opercular beats per minute for each fish, using a 30-second counting interval. The counts were repeated twice, with an hour-long interval between each repetition. Statistical analysis were conducted in R (R Core Team, 2022). We used ANCOVA (Analysis of Covariance) models to assess whether there are significant differences in VR between the four origins among and between seasons while accounting for the potential confounding effect of fish size (using fish weight as covariate). To validate our model, we examined the significance of the interaction terms between weight, origin and season by applying mean centering to VR and weight and running individual ANCOVAS. After determining that the interactions of weight with the other predictors were not significant, we conducted further testing by removing all non-significant predictors one at a time in the main ANCOVA model. Tukey's Honest Significant Difference (HSD) test was used to assess the significance of differences between pairs of group means. Data was visualised by plotting the partial residuals calculated by applying the “partialize” function of the jtools package (Long, 2022). The coefficient of variation (CV) was utilized to assess the relative variability of VR measurements within different fish origins across seasonal sampling points. CV was calculated as the ratio of the standard deviation to the mean VR across all individuals within each group and expressed as a percentage: CV = (SD / mean VR) * 100.

### 3.3.3 Microbial DNA extraction and NGS library preparation

The DNA extraction of the gut samples and NGS library preparation protocols employed in this study were based on established methods outlined in Heys et al., 2020, Kazlauskaite et al., 2021; Llewellyn et al., 2016. Frozen gut tissue (100mg) and filter papers were prepared using sterilized equipment, and DNA extraction was performed using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol (Claassen et al., 2013). The V1 hypervariable region of the 16S rDNA was targeted for amplification using primers described in chapter 4. V1 was chosen over V4 due to the primers' reduced susceptibility to cross-hybridization with salmon DNA (Heys et al., 2020; Werner et al., 2012). Amplification was carried out using tagged barcodes (27F and 338R), with PCR conditions consisting of an initial denaturation step at 95°C for 10 minutes, followed by 30 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, and a final elongation step at 72°C for 10 minutes. The first-round PCR products were then subjected to a second round of PCR to incorporate external multiplex identifiers (barcodes), with the same PCR conditions but only eight cycles. The second-round amplicons were gel-purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA), quantified using a Qubit fluorometer (Thermo Fisher Scientific, USA), and pooled equimolarly at a concentration of 10nM. Paired-end sequencing was performed using a NovaSeq 6000 system provided by NovaGene.

### 3.3.4 Bioinformatic pipeline

Quality filtering and trimming (>Q33 Phred quality score) was performed on all the reads of the 16s rRNA V1 hypervariable region using the Sickle (v.1.2) software (Joshi

& Fass, 2011). Read error correction was carried out by using the BayesHammer module within the SPAdes (v.2.5.0) software to obtain high-quality assemblies (Nikolenko et al., 2013). The paired-end reads were merged using PANDAseq (v.2.11) with a simple Bayesian read merging algorithm, considering an overlap length of 50bp (Masella et al., 2012; Schirmer et al., 2016). Subsequently, the merged reads underwent dereplication, sorting, and removal of chimeras and singletons using VSEARCH (v.2.3.4, Rognes et al., 2016). Decontamination against the *Salmo salar* genome was performed using DeconSeq (v.0.4.3, (Schmieder & Edwards, 2011) and the overlapped reads were clustered into operational taxonomic units (OTUs) at a 97% sequence identity using VSEARCH. For taxonomic classification of the OTUs, we employed naïve Bayes classifiers implemented in QIIME2 (Bolyen et al., 2019; Pedregosa et al., 2011), utilizing the Silva 132 database (Quast et al., 2012). Finally, phylogenetic trees were generated using FastTree (Price et al., 2010).

### 3.3.5 **Microbiota analysis**

All data were analysed in R (R Core Team, 2022) using the packages phyloseq (McMurdie & Holmes, 2013), microeco (Liu et al., 2021), metacoder (Foster et al., 2017) and vegan (Oksanen, 2007). The R code is illustrated in chapter 4.89. OTUs which were not assigned to the kingdoms of bacteria and archaea were removed. In addition, taxa classified as chloroplast or mitochondria were considered as contamination and discarded. To limit the number of effects on diversity measurements samples were rarefied to 10000 reads. After removing the environmental bacteria from further analysis (see section “Differential abundance testing” below), the remaining samples were rarefied to 1000 reads for alpha and

beta diversity measurements. Alpha diversity was assessed via Chao1 richness and the Shannon's index of entropy. Generalised Linear Models (GLMs) were employed to investigate the relationships between alpha diversity measures and a suite of predictor variables, accounting for potential nonlinearities and distributional assumptions. The predictor variables included temperature, fish sex, host genetic origin, fish length, fish weight, and sampling season. Prior to model fitting, data distributions were assessed using the "performance" package (Lüdecke et al., 2021). Model performance was evaluated using several metrics, including the Akaike Information Criterion corrected for small sample sizes (AICc), Bayesian Information Criterion (BIC), deviance, pseudo-R-squared and cross-validation. The MuMIn package (Barton, 2009) facilitated systematic evaluation of predictor variables, aiding in the selection of the best-fitting model based on AIC scores. For both Chao1 richness and Shannon diversity, the selected GLM assumed a gamma distribution for the response variable with a logarithmic link function. Month was utilized as the primary predictor variable to examine seasonal effects on alpha diversity measures. Beta-diversity measures were visualised by principal coordinates analysis (PCoA) plots using Bray-Curtis and weighted UniFrac distance measures. Permutational multivariate analysis of variance (PERMANOVA) was used to test the effects of sampling timepoint (season), host genetic origin and sex on the intestinal microbial communities among individual fish. Significance was determined by using Benjamini-Hochberg corrected p-values (Benjamini & Hochberg, 1995). To explore the relationship between ventilation rate and beta diversity, we categorized VR values into four distinct groups: low, medium, high and very high. Quantiles were used to ensure that each group contained an approximately equal number of observations.

Each season's VR values were grouped separately to account for seasonal variations, and an additional grouping was performed on the combined data from all seasons.

### 3.3.6 **Differential abundance testing**

In order to discern whether fish from different genetic origins harbour distinct microbial profiles, we focussed on bacteria with a high likelihood of being associated with the host. This emphasis was driven by the need to remove potentially confounding environmental bacteria, as these microbes might obscure the impact of genetics on host-associated microbial communities. There are several approaches to detect and filter out environmental bacteria from the analysis, with advantages and disadvantages associated with each. Here, we used analysis of compositions of microbiomes with bias correction (ANCOM-BC) in order to distinguish between environmental and gut enriched taxa (Lin & Peddada, 2020). This analysis enabled us to selectively include only those taxa that exhibited a positive enrichment within the gut compared to the water column. While we recognize the inherent limitations of this method, such as the possibility of transient water column bacteria temporarily colonizing the gut environment, our approach enhances the likelihood of identifying bacteria closely associated with the host. This, in turn, allows us to exclude taxa that could potentially obscure the influence of host genetic origin on microbial community composition.

ANCOM-BC addresses the challenges of compositional data by applying a log-ratio transformation to mitigate spurious associations. The method performs pairwise, or multi-group comparisons of the log-ratio transformed abundances and adjusts p-values to control false discoveries.

To distinguish between environmental and gut associated bacteria, we opted to group the operational taxonomic units (OTUs) at the family level. This decision was made to obtain a more reliable and robust estimate, specifically suited for the sample processing in our study.

To analyse differences in taxa abundance between sampling seasons and host genetic origin, we utilized raw counts of genera as input for the "ANCOM-BC" function. To account for multiple testing and reduce the likelihood of false discoveries, the resulting p-values were adjusted using the Benjamini-Hochberg method (Benjamini & Hochberg, 1995). Taxon prevalence threshold was set to 0.05 and the library threshold was set to 500, in order to remove samples with very low read counts. The rest of the parameters were left as default.

## 3.4 Results

### 3.4.1 Impact of fish genetic origin on ventilation rates

We sampled juvenile Atlantic salmon at four different seasonal time points over the course of one year. At the first sampling time point in October 2018 (late autumn 2018) all fish were approximately seven months old. Here, the dataset included 13 farmed fish, 16 hybrid farmed female fish, 16 hybrid wild female fish and 19 wild fish. The mean lengths ranged from 50.89 mm to 55.94 mm and the mean weights ranged from 1.28 g to 1.64 g. Size and weight differences among the farmed, wild and their reciprocal hybrid groups increased over the course of the study (Table 3.1). During Winter 2019, 19 farmed fish, 20 hybrid farmed female fish, 19 hybrid wild female fish and 22 wild fish were studied. Mean lengths varied from 52.51 mm to 60.32 mm and weights ranged from 1.31 g to 2.05 g. In Summer 2019, 13 farmed fish, 17 fish hybrid

farmed female fish, 14 hybrid wild female fish and 15 wild fish were sampled. Mean lengths ranged from 89.06 mm to 94.23 mm and weights from 6.84 g to 8.87 g. Finally, Autumn 2019 included 13 farmed fish, 16 hybrid farmed female fish, 8 hybrid wild female fish and 12 wild fish. The mean lengths ranged from 98.55 mm to 109.40 mm, and weights ranged from 10.59 g to 15.53 g. At the last sampling time point in September 2019 (early autumn 2019), farmed fish were significantly bigger than hybrid farmed female ($t(19.7)=2.86$, $p=0.04$), hybrid wild female ($t(21.4)=3.56$, $p=0.01$) and wild ($t(19.4)=4.02$, $p=0.006$) fish. There was no significant difference in fish condition factor among fish from different genetic origins within the same sampling season. However, Fulton's k was lowest in winter ($k_{winter19}=0.88\pm0.05$) and increased for all fish thereafter ($k_{summer19}=1.01\pm0.07$, $k_{autumn19}=1.1\pm0.13$). It is important to note that despite sharing the same names, the timepoints in autumn 2018 and autumn 2019 exhibited highly different mean temperatures (9.1°C vs. 12.8°C).

Our ANCOVA model revealed that fish ventilation rate was significantly predicted by fish weight ($F(1,250)=78.39$, $p<0.001$)), sampling season ($F(3,250)=49.95$, $p<0.001$) and the interaction term of origin and season ($F(9,250)=2.0$, $p=0.04$; Table S 3.1). All fish regardless of their origin showed significantly lower VR in winter (73.47 (±9.50) bpm) compared to the other sampling seasons (Figure S 3.1), however, post hoc testing also revealed that wild fish had a significantly lower VR in winter than farmed and hybrid farmed female fish.

Highest mean VR measurements were observed in summer (100.12 (±11.07) bpm). It is noteworthy, however, that the overall spread of mean VR differed among fish

origins. For instance, farmed fish exhibited the highest mean ventilation rate in winter (79.14 ± 9.76 bpm) and the lowest in summer (96.82 ± 10.98 bpm), showcasing a narrower range of variation. In contrast, wild fish displayed the lowest VR in winter (67.93 ± 6.02 bpm) and the highest VR in summer (103.01 ± 7.76 bpm), demonstrating a wider range of variation across seasons. This observation is illustrated further by differences in the coefficient of variation. In winter, the coefficient of variation (CV) in ventilation rates (VR) across farmed, hybrid farmed female, hybrid wild female and wild fish ranged from 8.93% to 14.4%. Wild fish displayed the lowest CV, indicating relatively stable VR compared to other origins. In summer, CV values ranged from 7.53% to 13.9%, with wild fish again exhibiting the lowest variability in VR. To further understand the physiological responses of different fish origin to water temperature, we performed linear regression analyses of weight-adjusted ventilation rate (VR) values against temperature. Here, weight-adjusted VR measurements of fish from hybrid farmed female ($F(1,77)=30.09$, $p<0.001$, $R2=0.3$) and farmed ($F(1,52)=27.75$, $p<0.001$, $R2=0.37$) origin showed lower correlation with temperature then hybrid wild female ($F(1, 61)=60.39$, $p<0.001$, $R2=0.51$) and wild fish ($F(1,69)=137.4$, $p<0.001$, $R2=0.68$; Figure S 3.2).

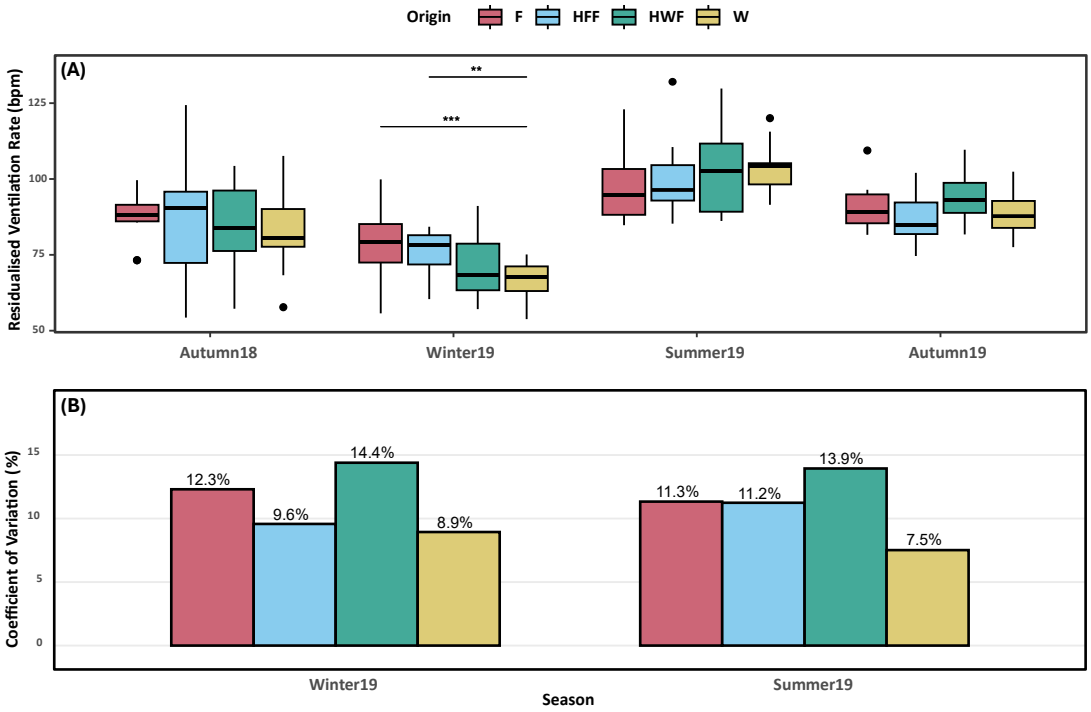


**Figure 3.2:** (A) Boxplot displaying the seasonal variations in weight-adjusted ventilation rates of juvenile Atlantic salmon among individuals from different genetic origins. (B) illustrates the coefficient of variation (%) of ventilation rates across different origins of juvenile Atlantic salmon sampled during summer and winter seasons. F=Farmed, HFF=Hybrid Farmed Female, HWF=Hybrid Wild Female, W=Wild. Bpm= Opercular beats per minute. Significance codes: ***<0.001; **<0.01.

**Table 3.1** Mean values of fish characteristics by season, origin and measurement. Measurements include fish length (mm), fish weight (g), raw values of fish ventilation rate (VR; bpm) and fish condition factor (Fulton's k). F=Farmed, HFF=Hybrid Farmed Female, HWF=Hybrid Wild Female, W=Wild. Four sampling efforts were undertaken over the course of four sampling seasons. Average daily temperature of each sampling event is displayed underneath each sampling season. Values depict mean values for the respective season, origin and measurement. Brackets after mean values show standard deviations. Brackets after the fish origin shows the respective number (N) of samples taken.

| Season Temperature | Origin (N) | Length (mm) | Weight (g) | VR (bpm) | Fulton's k |
|---|---|---|---|---|---|
| **Autumn 18 9.1 (±2.0)°C** | F (13) | 55.94 (±6.15) | 1.58 (±0.70) | 87.79 (±8.99) | 0.85 (±0.13) |
| | HFF (16) | 55.93 (±4.94) | 1.64 (±0.44) | 85.47 (±17.07) | 0.91 (±0.04) |
| | HWF (16) | 53.28 (±5.43) | 1.43 (±0.55) | 85.02 (±14.42) | 0.91 (±0.11) |
| | W (19) | 50.89 (±7.06) | 1.28 (±0.51) | 83.19 (±11.75) | 0.94 (±0.14) |

| | | | | | |
|---|---|---|---|---|---|
| **Winter 19 5.6 (±1.3)°C** | F (19) | 60.32 (±7.23) | 2.05 (±0.73) | 79.14 (±9.76) | 0.90 (±0.05) |
| | HFF (20) | 59.21 (±5.77) | 1.92 (±0.59) | 76.03 (±7.27) | 0.90 (±0.05) |
| | HWF (19) | 58.13 (±6.88) | 1.76 (±0.64) | 71.35 (±10.27) | 0.86 (±0.06) |
| | W (22) | 52.51 (±5.92) | 1.31 (±0.43) | 67.93 (±6.02) | 0.88 (±0.04) |
| **Summer 19 15.2 (±1.3)°C** | F (13) | 94.23 (±9.99) | 8.87 (±2.81) | 96.82 (±10.98) | 1.03 (±0.04) |
| | HFF (17) | 91.50 (±9.85) | 8.03 (±2.47) | 98.63 (±11.08) | 1.01 (±0.05) |
| | HWF (14) | 89.16 (±8.17) | 7.48 (±2.05) | 102.33 (±14.26) | 1.03 (±0.10) |
| | W (15) | 89.06 (±6.29) | 6.84 (±1.14) | 103.01 (±7.76) | 0.96 (±0.07) |
| **Autumn 19 12.8 (±0.6)°C** | F (13) | 109.40 (±6.65) | 15.53 (±2.32) | 90.13 (±8.36) | 1.19 (±0.14) |
| | HFF (16) | 101.78 (±7.30) | 11.30 (±2.65) | 86.57 (±8.37) | 1.05 (±0.06) |
| | HWF (8) | 98.59 (±8.19) | 10.59 (±3.02) | 93.62 (±7.12) | 1.09 (±0.18) |
| | W (12) | 98.55 (±6.73) | 10.67 (±2.42) | 87.92 (±7.10) | 1.10 (±0.11) |

### 3.4.2 **Identification of host- associated microbiota in Atlantic salmon**

After quality filtering, database alignment and removal of potential chimeras, a total of 11,173,771 reads and 42,127 OTUs were obtained. For diversity analysis, samples were rarefied to 10,000 reads, resulting in the identification of 31559 OTUs in water and gut samples combined.

PCoA ordination clearly separated microbial community compositions of Atlantic salmon intestines and the water column (Figure 3.3a). PCoA1 captured 22.2% of the variation whilst PCoA2 captured 7.6%. PERMANOVA verified the visible distinctions between the two environments to be significant ($F=69.05$, $R^2=0.19$, $p=0.001$). Water samples showed a significantly higher alpha diversity than samples derived from

salmon intestines (Chao1: t=52.15, p=6.39e$^{-50}$, Shannon: t=36.08, p=1.34e$^{-49}$; Figure 3.3b, c).

All OTUs, from intestine and water combined, were grouped on family level. Thereby, we determined 391 microbial families. Analysis of Compositions of Microbiomes with Bias Correction determined 144 families to be positively enriched in the gut environment compared to the water column. The biggest differences were observed for families *Propionibacteriaceae*, *Vibrionaceae*, *Streptococcaceae*, *Clostridiaceae* and *Staphylococcaceae* (Figure 3.3d). Since we were only interested in OTUs that potentially proliferate in the intestinal environment we kept all OTUs (n=3215), which were associated to the positively enriched families. Due to the grouping on family level some OTUs were only present in water samples. After discarding these OTUs we obtained 1575 OTUs, which were retained for further analysis.

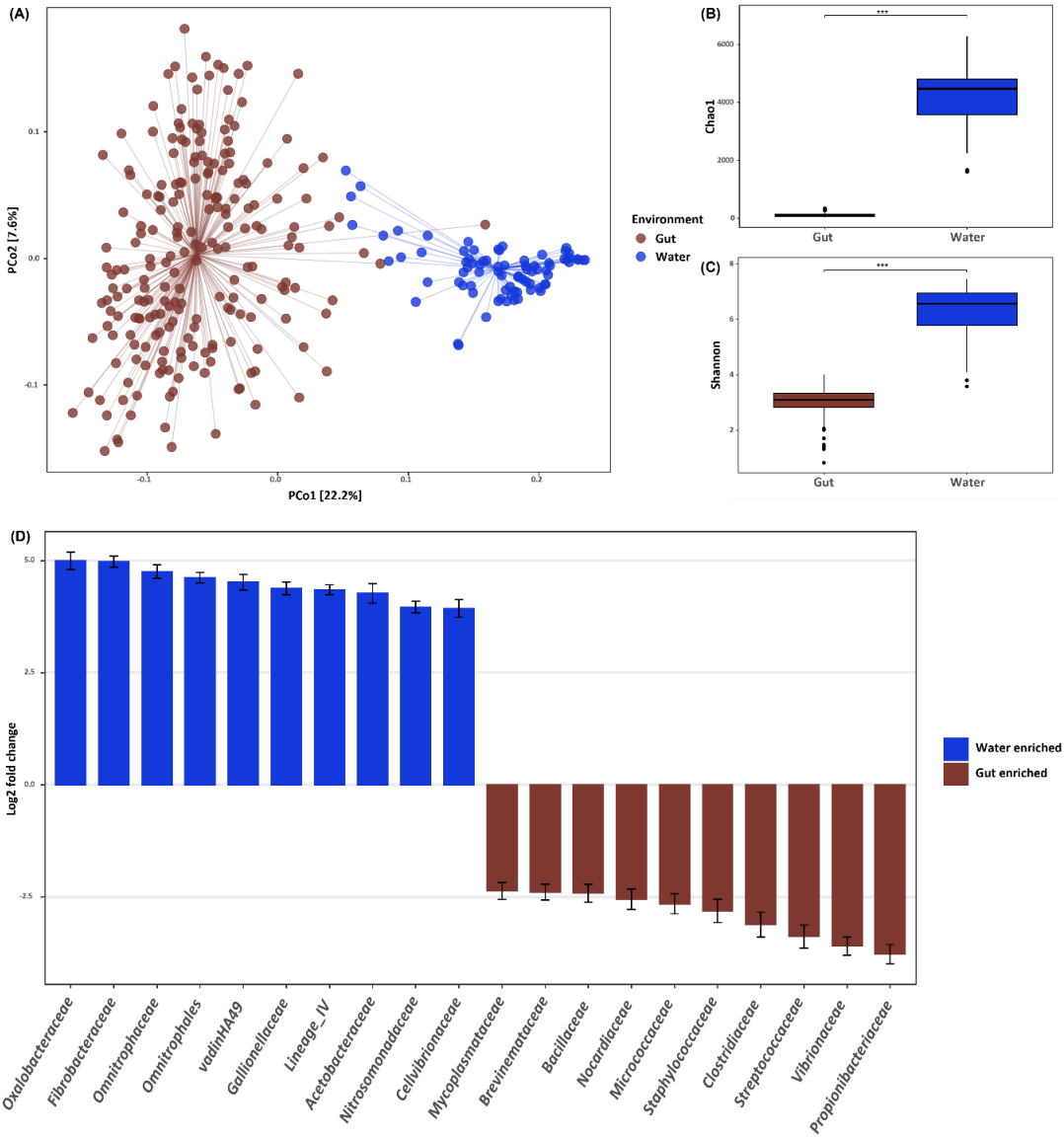


**Figure 3.3:** (A) Principal coordinates analysis (PCoA) derived from weighted UniFrac distances between samples from Atlantic salmon guts and the water column. Gut samples were collected at four sampling time points (Autumn 2018, Winter 2019, Summer 2019 and Autumn 2019). Each data point represents an individual sample. (B) Chao1 richness of bacterial communities collected from salmon gut and the water column of the experimental river. (C) Shannon diversity index of bacterial communities collected from gut and water environments. Significance was determined by pairwise t-testing. Significance codes: ***<0.001; ns=not significant. (D) Waterfall plot of the 20 most differently abundant microbial taxa on family level between the gut and water environment determined by Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC). Taxa with a positive log fold change were significantly enriched in the water column when compared to the gut. Taxa with a negative log fold change were significantly enriched in the salmon intestine compared to the water column. 144 out of 391 taxonomic families were positively

enriched in the salmon intestine compared to the water column. OTUs associated with those families were kept for further analysis.

### 3.4.3 **Characterisation of host-associated microbiota**

OTUs that were positively enriched in the gut environment mostly belonged to the Phyla of *Proteobacteria* (30% of relative abundance), *Actinobacteria* (30%), *Firmicutes* (26%) and *Patescibacteria* (10%; Figure 3.4). In total OTUs were associated with 17 different Phyla and 205 different genera. In *Proteobacteria*, genera *Psychrobacter* (10.5% of overall relative abundance), *Acinetobacter* (8.6%), *Arsenophonus* (3%) and *Photobacterium* (1%) showed the highest relative abundance. *Actinobacteria* were mostly represented by the genus *Corynebacterium* (6.8%) and *Microbacterium* (4.4%). In Firmicutes the most abundant OTUs belonged to the genus *Clostridium sensu stricto* (9% of overall relative abundance). However, we also discovered a wide range of taxa belonging to the taxonomic class of *Bacilli*. Some examples include *Staphylococcus* (4.9 %), *Streptococcus* (2.7%), *Carnobacterium* (1.7), *Turicibacter* (1.3%) and *Mycoplasma* (1%). *Patescibacteria* were mostly represented by *Saccharimonadales* (8.7% of overall relative abundance). Other interesting taxa included *Fusobacterium* (0.4%) belonging to the Phylum of *Fusobacteriota* and *Marinomonas* (0.2%), as well as *Pseudoalteromonas* (0.3%) belonging to the Phylum *Proteobacteria*.

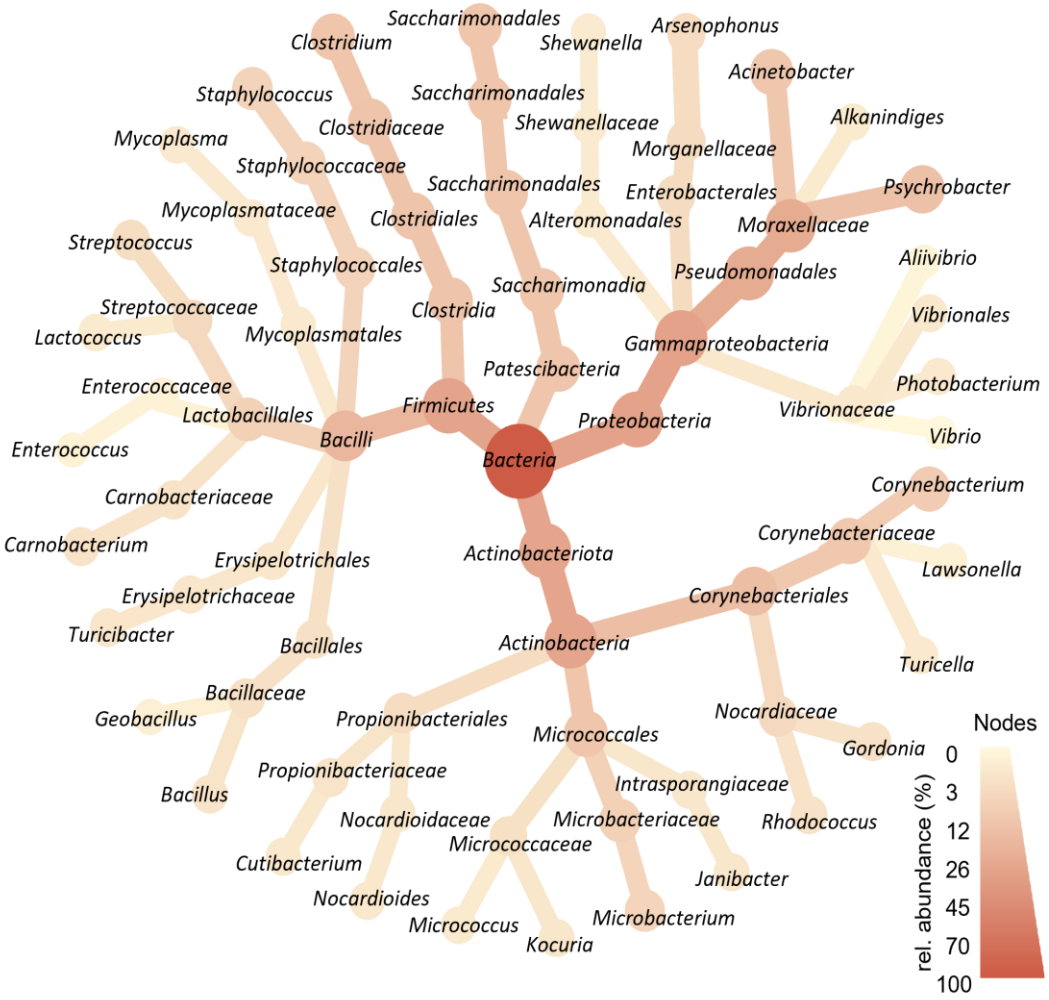


**Figure 3.4:** Taxonomic heat tree of the abundance of bacterial taxa at different ranks present in the Atlantic salmon intestine after filtering out taxa that were associated with the water column. Node size and colour reflect the mean relative abundance of taxa per taxonomic rank. For visualisation purposes only the most abundant taxa of the four most abundant phyla are illustrated.

### 3.4.4 **Seasonal dynamics of gut microbial communities in Atlantic salmon**

PCoA ordination of gut microbial community composition showed distinct seasonal clusters among three out of four sampling time points (Figure 3.5a). No clear clusters were observed between sampling time points in summer 2019 and autumn 2019. PCo1 explained 21.1% of variation in gut microbial community composition. This component was able to differentiate samples taken at the same sampling time points, indicating inter-individual variations in the gut microbial community

composition among hosts within seasons. Most of the variation between sampling seasons was captured by PCo2, which explained 13.9% of the overall variation. PERMANOVA confirmed the observed variations. In addition to the sampling season, which explained 10.9% of total variation, host genetic origin ($F=2.28$, $R^2=0.028$, $p=0.001$) and the interaction term of season and origin ($F=1.92$, $R^2=0.07$, $p=0.001$) were also significant contributors in explaining differences in gut microbial community composition (Table S 3.2). Host sex had no significant impact on gut microbial community composition. 78.5% of variation remained unexplained. Pairwise testing also confirmed non-significant differences between gut microbial community compositions between the sampling time points in summer 2019 and autumn 2019 ($F=1.54$, $R^2=0.01$, $p=0.09$; Table S 3.7). PERMANOVA was conducted to examine the effect of ventilation rate (VR) on beta diversity. In Autumn 2019, VR groups did not significantly affect beta diversity ($F = 0.9642$, $p = 0.498$), with VR explaining 6% of the variance. Similarly, in Winter 2019, VR groups had no significant impact ($F = 0.7757$, $p = 0.828$), accounting for only 3% of the variance. When data from all seasons were combined, the VR groups still showed no significant effect ($F = 0.8475$, $p = 0.716$), explaining just 1% of the variance. Overall, these results indicate that there is no significant relationship between VR and beta diversity.

Microbial richness was highest in autumn 2018 and lowest in winter 2019 (Figure 3.5b). Shannon diversity showed a similar trend (Figure 3.5c). In winter alpha diversity was significantly lower compared to autumn 2018 ($p<0.001$) and autumn 2019 ($p=0.001$). The results from generalised linear models showed that only the sampling season had a significant effect on alpha diversity measures (Table S 3.3,

Table S 3.4). Model selection analysis indicated that predictors such as host genetic origin, weight, sex, and ventilation rate did not demonstrate significant associations with alpha diversity measures and were therefore not retained in the final models (Figure S 3.3, Table S 3.5, Table S 3.6).

We were interested in understanding the underlying community compositions of the observed seasonal differences in beta diversity, particularly for the winter sampling time point. Here, *Corynebacterium* (9.5%), *Acinetobacter* (7.9%) and *Saccharimonadales* (7.6%) showed the highest mean relative abundance. The top ten relative abundant taxa for each sampling season are displayed in Figure S 3.4. ANCOM-BC revealed that 15 genera were differently abundant in winter compared to the three other sampling seasons (Figure 3.5d). *Clostridium sensu stricto*, *Mycoplasma*, *Saccharimonadales* and *Stenotrophomonas* all showed their lowest abundance in winter and were positively enriched in the other sampling timepoints. *Cutibacterium*, on the other hand, was most abundant in winter when compared to the other seasons.

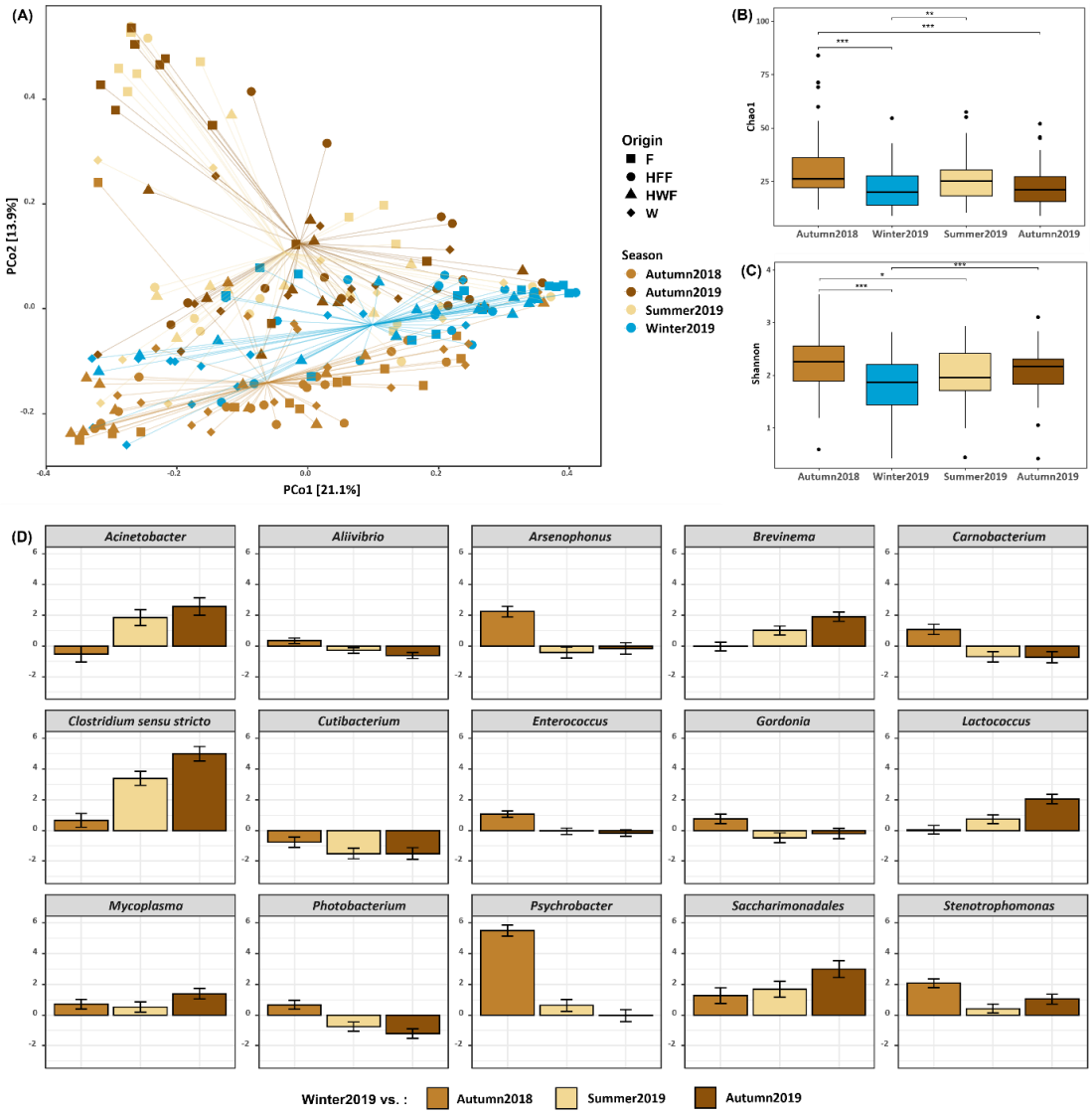


**Figure 3.5:** Alpha and beta diversity of Atlantic salmon gut microbiomes obtained from samples collected in the river environment. (A) Principal coordinate analysis (PCoA) of gut samples grouped by sampling season. Each data point represents an individual sample. Shapes represent the genetic background of fish (F=Farmed, HFF=Hybrid Farmed Female, HWF=Hybrid Wild Female, W=Wild). Percentages in parenthesis indicate the amount of variation shown on each axis. Lines mark the centroids of each group. Distance matrix was calculated based on the weighted UniFrac distance metric (B) Chao1 richness for gut samples grouped by sampling season. (C) Shannon diversity index for gut samples grouped by sampling season. Lines connecting sampling season show significant relationships (pairwise t-test). Significance codes: ***<0.001; **<0.01; *<0.05. (D) Boxplots showing 15 differently abundant genera between winter and three other sampling seasons autumn 2018, summer 2019 and autumn 2019. Data are represented by log fold change (shown as a column) ±SE (shown as error bars) derived from the ANCOM-BC model.

### 3.4.5 Gut microbial community composition varies with fish origin

As previously stated, the PERMANOVA results of Table S 3.2 revealed not only a pronounced impact of seasonal factors on gut microbial communities but also a noteworthy association between host genetic origin and these microbial communities. Pairwise testing revealed that the progeny of farmed parents had a significantly different gut microbial community composition compared to that of wild offspring ($F=3.75$, $R^2=0.04$, $P=0.006$) and hybrid offspring with a wild female parent ($F=2.7$, $R^2=0.28$, $p=0.02$) when considering the samples of all four sampling seasons combined (Table S 3.8). ANCOM-BC revealed that a single genus called *Candidatus Levybacterium* ($W=28.58$, $p=0.0001$) was significantly more abundant in farmed fish compared to fish from the other cohorts.

In autumn 2018 ($F=1.4$, $R^2=0.07$, $p=0.20$) and summer 2019 ($F=1.4$, $R^2=0.07$, $p= 0.10$) no significant impact of host genetic origin was detected. However, in winter 2019, from the progeny of wild origin parents had a significantly different gut microbial community composition to that of the offspring of farmed ($F=5.15$, $R^2=0.16$, $p= 0.01$), hybrid farmed female ($F=2.78$, $R^2=0.01$, $p= 0.03$) and hybrid wild female ($F=2.78$, $R^2=0.02$, $p= 0.03$) origin (Figure 3.6a, Table S 3.9). In addition, microbial communities in the gut of fish of farmed parentage differed significantly from hybrid offspring with a wild mother ($F=2.39$, $R^2=0.08$, $p= 0.03$). In autumn 2019, we found that fish where both parents were of farmed origin had a significant different gut microbial community composition compared to fish with two wild parents ($F=3.45$, $R^2=0.15$, $p= 0.02$), hybrids with a farmed female parent ($F=3.12$, $R^2=0.12$, $p= 0.03$) and hybrid offspring with a wild female parent ($F=3.02$, $R^2=0.16$, $p= 0.03$) (Figure 3.6b, Table S

3.10). In addition, microbial communities of pure wild offspring differed significantly from hybrid progeny with a farmed female parent (F=1.8, $R^2$=0.06, p= 0.04). Generalised linear models showed that farmed offspring had a significantly higher Shannon diversity and Chao1 richness than fish with other parental combinations (Table S 3.11, Table S 3.12). We found that for the sampling period of autumn 2019, five OTUs were differentially abundant between the four origins (Figure S 3.5). All of them belonged to the genus *Clostridium sensu stricto*. In winter 2019 we did not find any differently abundant OTUs.

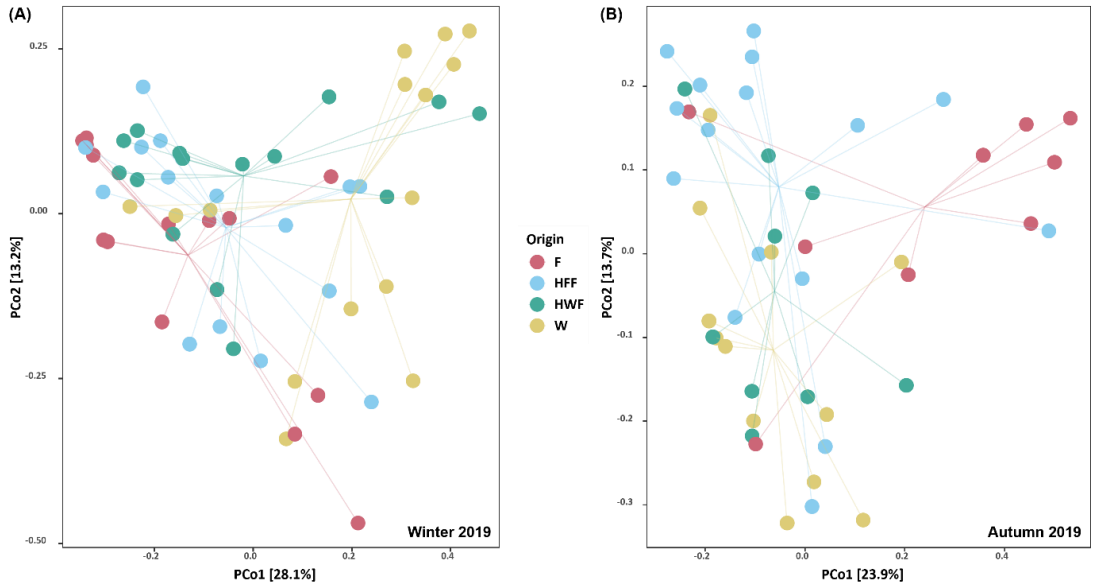


**Figure 3.6:** Principal coordinate analysis (PCoA) of gut samples from juvenile Atlantic salmon collected in winter19 (A) and autumn19 (B) grouped by genetic origin. Each data point represents an individual sample. Colours represent genetic origin of fish (F=Farmed, HFF=Hybrid Farmed Female, HWF=Hybrid Wild Female, W=Wild). Percentages in parenthesis indicate the amount of variation shown on each axis. Lines mark the centroids of each group. Distance matrix was calculated based on the weighted UniFrac distance metric.

## 3.5 Discussion

Juvenile Atlantic salmon, regardless of their genetic origin, exhibited significantly reduced ventilation rates during winter compared to summer. This observation is

consistent with the poikilothermic nature of most fish species, where biochemical processes, metabolic respiration, oxygen consumption and consequently metabolic rates, correlate with temperature variations (Y. Liu et al., 2019; Logan & Buckley, 2015). This phenomenon reflects a well-documented trend among both endothermic and ectothermic organisms, where metabolic demands are decreased in response to energy limited environments (Guppy & Withers, 1999; Staples, 2016). For instance during winter, many animals adopt strategies to conserve energy, such as reducing activity levels through hibernation (Geiser, 2013; Nedergaard et al., 1997) or decreased foraging (Cotton & Parker, 2000; Volkoff & Rønnestad, 2020). Ectotherms in particular, frequently enter dormancy as temperatures decline, relying heavily on stored energy reserves to endure the period of reduced metabolic activity and maintain their physiological functions (Shuter et al., 2012).

In this study we observed that the progeny wild fish in their local environment exhibited significantly lower ventilation rates in winter compared to farmed and hybrid farmed female fish, which is in contrast to other studies carried out under controlled conditions (Lindsay, 2021) and under semi-natural conditions (Robertsen et al., 2019). In addition, wild fish displayed the widest range of ventilation rates between seasons, as evidenced by their highest mean ventilation rates during summer and lowest rates during winter. Similarly, the offspring of wild fish show a consistent pattern of a lower coefficient of variation in both summer and winter, suggesting a potential adaptation to environmental conditions. In contrast, farmed fish exhibit more variability in VR within a given season, potentially indicating less adaptation to environmental conditions. The reason for this observation might be

rooted in the evolutionary histories of wild and domesticated fish, as well as their phylogenetic and phylogeographic origin (Gjedrem, 2010). Wild fish inhabit natural ecosystems characterised by significant variability in temperature, food availability, water flow and other environmental factors. This variability possibly drove the development of diverse physiological and behavioural adaptations in wild fish, enabling them to thrive in diverse and variable conditions. This phenomenon is well-documented in various species and reflects the capacity of organisms to adapt to their specific ecological niches (Norin & Metcalfe, 2019). In contrast, domesticated fish are typically provided with consistent food sources, potentially reducing the necessity for the same level of adaptability seen in wild fish (Glover et al., 2017).

We hypothesize that fish with high standard metabolic rates will utilise energy in foraging during the winter months to meet their heightened metabolic demands, thereby depleting their energy reserves faster. The depletion of energy stores can have severe consequences, as individuals become more susceptible to mortality due to starvation (Finstad et al., 2004; Krams et al., 2013). Additionally, it is possible that in feeding to defend energy levels (Bull et al., 1997) farmed and hybrid fish increase their exposure to predation ( Solberg et al., 2020), while wild fish can stay hidden and survive with minimal energy expenditure. Hence, the lower adaptability to seasonal conditions in pure farm and farm hybrid fish could indeed contribute to the observed lower fitness in hybridised populations (Bolstad et al., 2021; McGinnity et al., 1997, 2003; Wacker et al., 2021). With future climate change causing shifts in food webs and the onset of more extreme temperatures, the capability to adapt is paramount (Canale & Henry, 2010; Seebacher et al., 2015). In such an environment, the reduced

adaptiveness observed in hybrid populations could spell trouble for the entire salmonid population, especially if escape events persist and genetic introgression continues to dilute the wild genetic traits (Bolstad et al., 2021; Castellani et al., 2018; McGinnity et al., 2009; Skaala et al., 2019).

We also observed differences in gut microbial community compositions among the progeny of farmed and wild fish, including their hybrid progeny. Intriguingly, these were found exclusively during autumn 2019 and in winter of 2019 and, with no discernible disparities observed during the autumn 2018 and the summer of 2019 sampling period. However, we did not find a statistically significant correlation between the ventilation rate in fish and certain community compositions or over or underabundance of specific taxa. This absence of a clear individual-level relationship between gut microbiome and ventilation rates is indeed understandable, considering the high variability and lack of precision in microbial profiling methodologies. Microbial communities exhibit substantial inter-individual variability and the impact of even a few outlier measurements can diminish the strength of correlations. In addition, our sample size might not have been sufficiently large to overcome these challenges. Nevertheless, the absence of a clear correlation does not necessarily negate the potential interactions between gut microbiomes and host metabolism. It is well-known that gut microbiota can impact the metabolism of their host by producing metabolites as a result of their own metabolic processes (Besten et al., 2013). Butyrate especially, plays a crucial role as a key regulator in mediating microbiota metabolic control (Zhang et al., 2021). Therefore, future research should delve into the metabolic processes of microbiomes, aiming to achieve a finer

understanding of microbiome-metabolism interactions in fish. Despite our inability to identify correlations, there could potentially be a common link between the low ventilation rates observed in wild fish and the observed differences in beta diversity between the fish cohorts. This link might stem from the distinct feeding behaviours exhibited by wild and farmed fish during winter. Diet is known to be an important driver of gut microbial community composition (Escalas et al., 2021; Leeper et al., 2023). If indeed, domestication leads to heightened feeding activity during winter, gut microbial communities would likely diverge between actively feeding farmed and hybrid fish and more sedentary wild conspecifics due to the distinct nutritional niches created in the gut environment.

As previously highlighted, our study revealed significant differences in gut microbial community composition between farmed fish and other provenances during autumn 2019, while no such differences were apparent in the preceding summer or the previous year's autumn. Presently, our understanding of the potential causes for this phenomenon remains speculative. While differences in feeding behaviour stand as a conceivable factor in winter, robust empirical evidence to substantiate this hypothesis for the autumn2019 sampling period is lacking, since we did not find significant differences in VR among the cohorts for this sampling timepoint. It seems reasonable to infer that the observed dissimilarities in microbial communities are influenced by factors intrinsic to the gut ecosystem, potentially due to differences in immune response genes (de Eyto et al., 2007, 2011) or other yet unknown factors, for instance ontogenetic changes in gut physiology. There is also evidence pointing towards the possibility of maternal transfer of certain bacterial strains during

oviposition (Rasmussen et al., 2023). However, such differences should, in theory, be consistently evident across all sampling periods, but our findings indicate that they were only present in two out of four sampling time points.

In addition to the variations in gut microbial communities attributed to different fish origins, our study also revealed distinct seasonal differences that were independent of fish provenance. While we had already observed these seasonal dynamics previously in chapter 2, our current research offers confirmation that these changes are not solely driven by presence of environmental bacteria in the gut, but also affect the structure of host-associated microbiota. Our findings further support previous results, as we observed the highest alpha diversity during the winter sampling period in chapter 2. We hypothesised that reduced feeding activity in winter should lead to a lower microbial diversity rather than the highest. At the time we attributed this phenomenon to the influx of taxa from the water column into the gut ecosystem. By deliberately excluding water-associated bacteria in this study from our analysis we indeed confirmed that gut microbial diversity was at its lowest during the winter season. Overall, we suggest that seasonal changes in gut microbial communities are likely a natural process that may not have adverse effects on the health of fish. However, even though fish display high inter-individual variations within one sampling time point, clustering of samples in this context could mean that effects are at play which might have the potential to be maladaptive. Both theories require testing through the examination of microbial activity and metabolic profiles to gain a comprehensive understanding of the impact on host health.

## 3.6 Conclusion

This study had access to a large-scale common garden experiment which sought to mimic a farmed escape and hybridisation event of Atlantic salmon in the wild. During the winter season, we observed significant lower ventilation rates in wild fish compared to hybrid farmed females and pure farmed fish. This suggests a strong genetic basis for the observed seasonal variation in basal levels of metabolism. Furthermore, wild fish demonstrated the highest level of metabolic flexibility between seasons and the lowest coefficient of variation within summer and winter seasons, indicating a higher adaptability to present environmental conditions compared to farmed and hybrid offspring. Additionally, we noted significant differences in gut microbial community composition between the progeny of wild and farmed individuals during winter, possibly attributable to reduced feeding activity, which in turn might be associated to the generally lower metabolic demands in winter. Our findings provide some new insights into the mechanisms underlying previously reported differences in the performance of the offspring of wild and farmed parents under natural conditions. Continued farm escape events could further compromise the adaptability and fitness of salmon, which in the short-term may impact on their productivity and ultimately heighten their risk of extirpation. As we face an era of climate change marked by unpredictable environmental shifts and temperature extremes throughout the seasons, particularly winter, preserving genetic diversity and adaptive potential of wild traits becomes crucial. Conservation efforts should prioritize the protection of wild salmonid populations and the prevention of genetic dilution through farm escape events.

## 3.7 References

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## 3.8 Appendix

### 3.8.1 **Supplementary Figures**

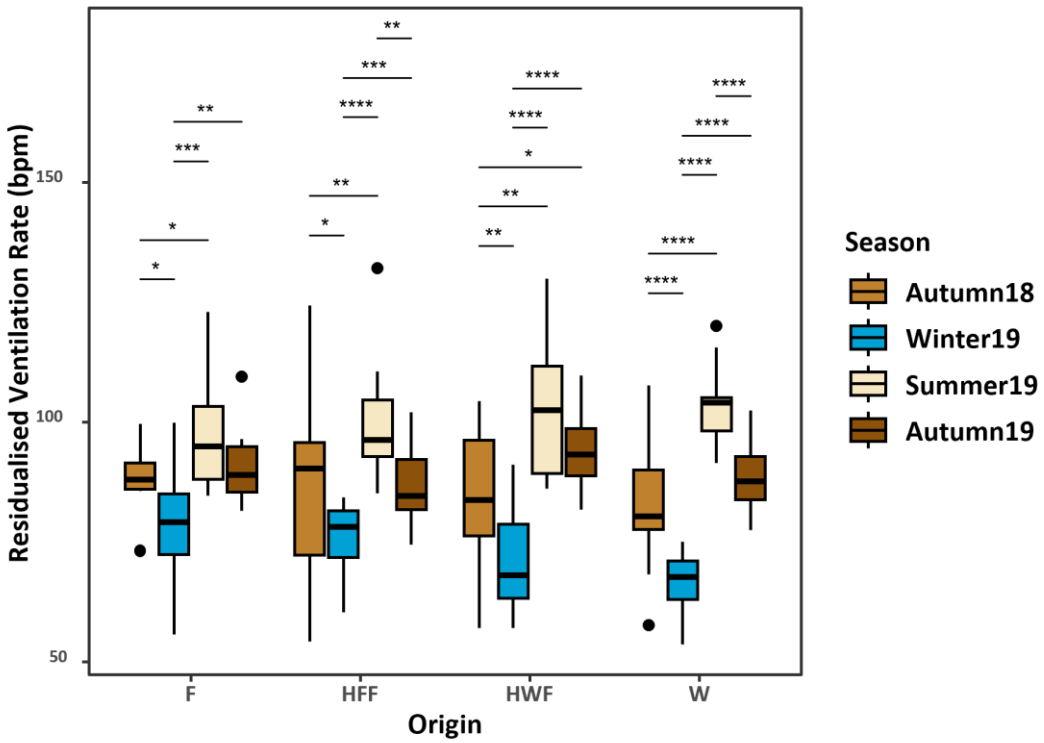


**Figure S 3.1:** Boxplot showing differences in the residualised ventilation rates of juvenile Atlantic salmon per origin between different seasons after adjusting for fish weight. F=Farmed, HFF=Hybrid Farmed Female, HWF=Hybrid Wild Female, W=Wild. Bpm= Opercular beats per minute. Significance codes: ****< 0.0001, ***<0.001; **<0.01; *<0.05.

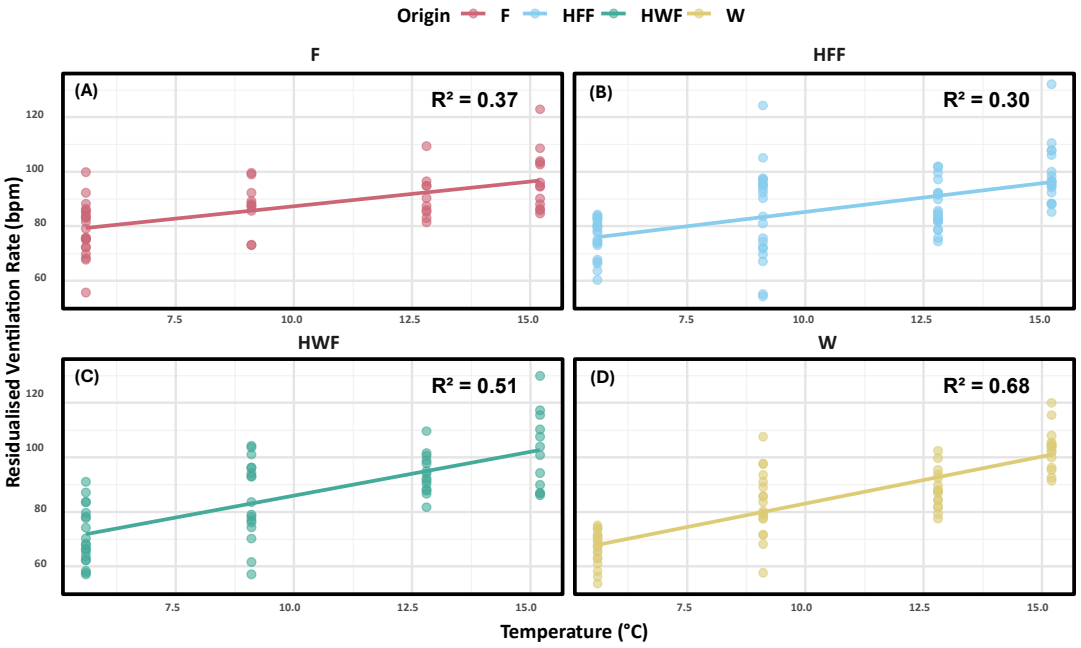


**Figure S 3.2**: Linear regression analyses of weight-adjusted ventilation rate (VR) values against temperature (°C) for distinct genetic origins. (A) F=Farmed, (B) HFF=Hybrid Farmed Female, (C) HWF=Hybrid Wild Female, (D) W=Wild. Bpm= Opercular beats per minute.

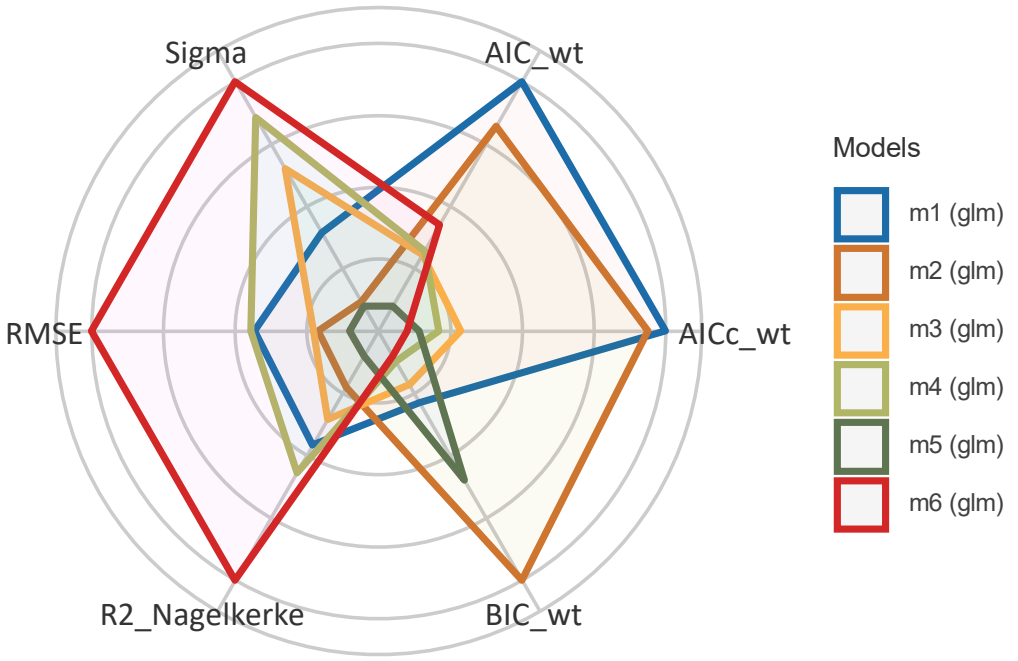


**Figure S 3.3:** Visualisation of performance metrics for 6 generalized linear models (GLMs) for predicting Chao1 richness using the performance package. Each row corresponds to a different GLM (m1-m6) fitted to predict Chao1 richness. Models

were fitted using a Gamma family with a log link function. Model formulas are as follows: m1: Chao1 ~ Season + VR + Weight; m2: Chao1 ~ Season + Weight; m3: Chao1 ~ Season + Sex + Weight; m4: Chao1 ~ Season + VR + Sex + Weight; m5: Chao1 ~ Season + VR; m6: Chao1 ~ Season + Origin + VR + Weight. Models are compared based on Akaike Information Criterion (AIC), corrected AIC (AICc), Bayesian Information Criterion (BIC) and associated weights, Nagelkerke'sR$^2$ indicating model fit, root mean square error (RMSE) indicating prediction error, and standard deviation (Sigma) indicating model dispersion. Lower AIC, AICc, and BIC values suggest better model fit, while higher Nagelkerke's $R^2$ values indicate better explanatory power.

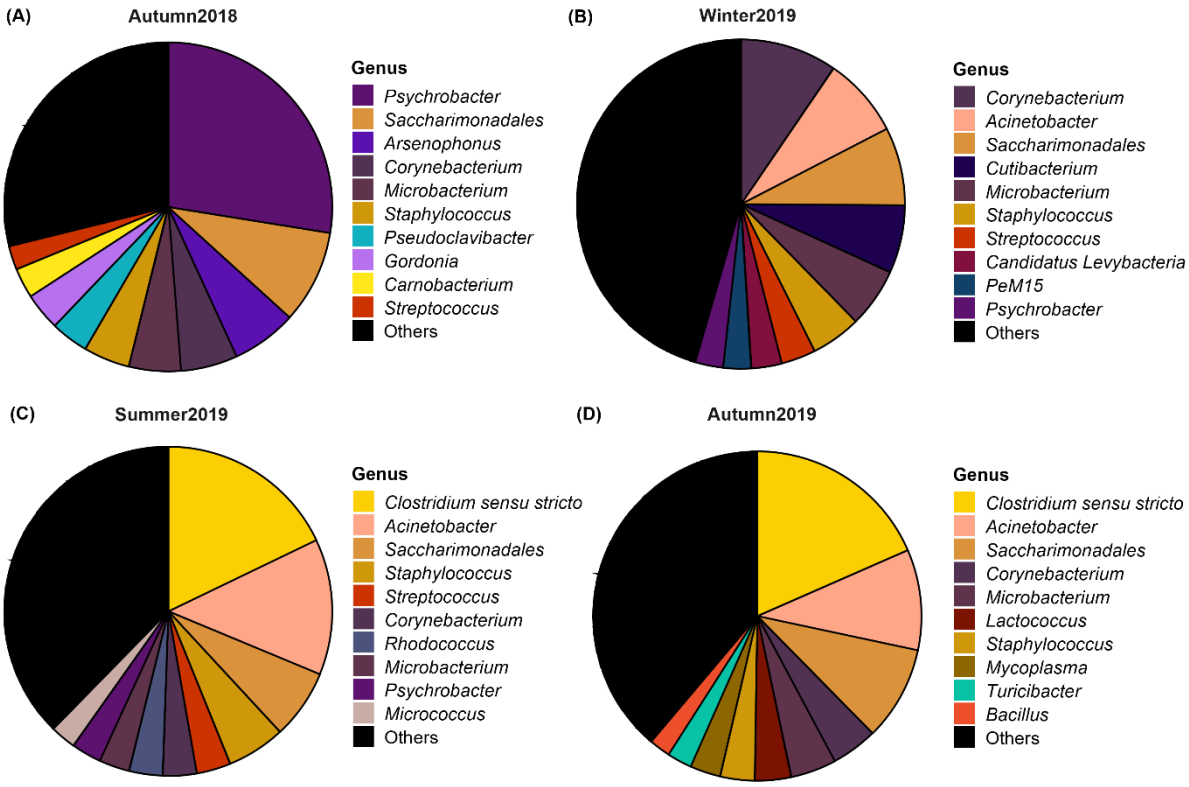


**Figure S 3.4**: Pie charts showing the mean relative abundance of the 10 most abundant taxa on genus level for the four sampling seasons. (A) Autumn 2018, (B) Winter 2019, (C) Summer 2019, (D) Autumn 2019.

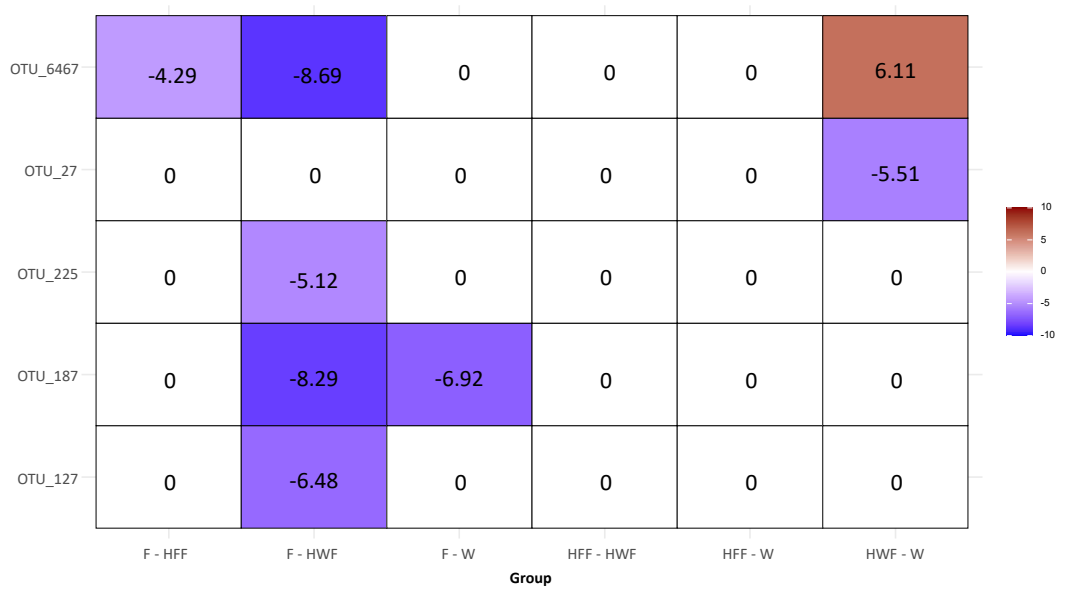


**Figure S 3.5:** Heatmap showing the log-fold changes of differentially abundant OTUs according to fish origin for autumn 2019, as determined by ANCOM-BC2. The groups compared are Farmed (F), Hybrid Farmed Female (HFF), Hybrid Wild Female (HWF) and Wild (W).

### 3.8.2 **Supplementary Tables**

**Table S 3.1**: ANCOVA results of the effect of fish weight, origin, sampling season and the interaction term of origin and season on ventilation rates of juvenile Atlantic salmon. Significance codes: ***< 0.001; *< 0.05.

| | Df | Sum Sq | Mean Sq | F value | Pr(>F) | Signif. |
|---|---|---|---|---|---|---|
| **Weight** | 1 | 8777 | 8777 | 78.39 | <2e-16 | *** |
| **Origin** | 3 | 138 | 46 | 0.41 | 0.7456 | |
| **Season** | 3 | 16779 | 5593 | 49.95 | <2e-16 | *** |
| **Origin:Season** | 9 | 2015 | 224 | 2 | 0.0398 | * |
| **Residuals** | 250 | 27990 | 112 | | | |

**Table S 3.2:** PERMANOVA results to assess the effects of host genetic origin, sampling season, their interaction effect and host sex on the composition of the Atlantic salmon gut microbiome in the river habitat. Significance code: ***<0.001

| | Df | SumOfSqs | R2 | F | Pr(>F) | Signif. |
|---|---|---|---|---|---|---|
| **Season** | 3 | 4.872 | 0.109 | 8.756 | 0.001 | *** |
| **Origin** | 3 | 1.27 | 0.028 | 2.282 | 0.001 | *** |
| **Sex** | 2 | 0.225 | 0.005 | 0.608 | 0.96 | |
| **Season:Origin** | 9 | 3.213 | 0.072 | 1.925 | 0.001 | *** |
| **Residual** | 189 | 35.053 | 0.785 | | | |
| **Total** | 206 | 44.633 | 1 | | | |

**Table S 3.3**: Generalised linear model showing the effects of sampling season on the Shannon diversity index. Autumn18 served as reference level. AIC: 319.48. AIC served as indicator to determine the best model fit. Significance codes: ***<0.001; **<0.01

| | Estimate | Std. Error | t value | Pr(>\|t\|) | Signif. |
|---|---|---|---|---|---|
| **(Intercept)** | 2.231 | 0.066 | 33.501 | < 2e-16 | *** |
| **Winter2019** | -0.454 | 0.094 | -4.805 | 3.01E-06 | *** |
| **Summer2019** | -0.244 | 0.103 | -2.36 | 0.0192 | * |
| **Autumn2019** | -0.159 | 0.101 | -1.576 | 0.1165 | |

**Table S 3.4:** Generalised linear model showing the effects of sampling season on Chao1 richness. Fish weight was not significant. Autumn18 served as reference level. AIC: 1543. AIC served as indicator to determine the best model fit. Significance codes: ***<0.001; **<0.01

| | Estimate | Std. Error | t value | Pr(>\|t\|) | Signif. |
|---|---|---|---|---|---|
| **(Intercept)** | 3.01 | 0.25 | 12.10 | < 2e-16 | *** |
| **Winter19** | -0.24 | 0.09 | -2.82 | 0.01 | ** |
| **Summer19** | -0.37 | 0.15 | -2.55 | 0.01 | * |
| **Autumn19** | -0.57 | 0.19 | -2.92 | 0.00 | ** |
| **Weight** | 0.03 | 0.02 | 1.66 | 0.10 | |
| **VR** | 0.00 | 0.00 | 1.40 | 0.16 | |

**Table S 3.5**: Summary of the top 6 generalised linear models for predicting Chao1 richness, using the MuMIn package to rank models based on the corrected Akaike Information Criterion (AICc). The global model included the predictor variables: Fish genetic origin, sampling season, sex, ventilation rate (VR) and fish weight. The models utilised a Gamma distribution with a log link function. The table displays the models with the lowest AICc values, indicating the best fit among the models considered.

| No. | (Intrc) | Origin | Season | Sex | VR | Weight | df | logLik | AICc | delta | weight |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **101** | 3.012 | | + | | 0.004 | 0.029 | 7 | -764.2 | 1543 | 0 | 0.039 |
| **69** | 3.352 | | + | | | 0.030 | 6 | -765.3 | 1543 | 0.07 | 0.038 |
| **77** | 3.38 | | + | + | | 0.027 | 7 | -764.7 | 1544 | 0.97 | 0.024 |
| **109** | 3.052 | | + | + | 0.004 | 0.027 | 8 | -763.6 | 1544 | 1.11 | 0.022 |
| **125** | 3.052 | | + | + | 0.004 | 0.027 | 8 | -763.6 | 1544 | 1.11 | 0.022 |
| **39** | 3.129 | + | + | | 0.004 | | 9 | -762.6 | 1544 | 1.21 | 0.021 |

**Table S 3.6**: Performance metrics for 6 generalized linear models (GLMs) for predicting Chao1 richness using the performance package. Each row corresponds to a different GLM (m1-m6) fitted to predict Chao1 richness. Models were fitted using a

Gamma family with a log link function. Model formulas are as follows: m1: Chao1 ~ Season + VR + Weight; m2: Chao1 ~ Season + Weight; m3: Chao1 ~ Season + Sex + Weight; m4: Chao1 ~ Season + VR + Sex + Weight; m5: Chao1 ~ Season + VR; m6: Chao1 ~ Season + Origin + VR + Weight. Models are compared based on Akaike Information Criterion (AIC), corrected AIC (AICc), Bayesian Information Criterion (BIC) and associated weights, Nagelkerke's$R^2$ indicating model fit, root mean square error (RMSE) indicating prediction error, and standard deviation (Sigma) indicating model dispersion. Lower AIC, AICc, and BIC values suggest better model fit, while higher Nagelkerke's $R^2$ values indicate better explanatory power.

| Name | Model | AIC (weights) | AICc (weights) | BIC (weights) | Nagelkerke's R2 | RMSE | Sigma |
|---|---|---|---|---|---|---|---|
| **m1** | glm | 1542.3 (0.231) | 1542.9 (0.238) | 1565.7 (0.109) | 0.109 | 10.871 | 0.438 |
| **m2** | glm | 1542.5 (0.208) | 1543.0 (0.230) | 1562.5 (0.521) | 0.099 | 10.934 | 0.439 |
| **m3** | glm | 1543.3 (0.142) | 1543.9 (0.146) | 1566.6 (0.067) | 0.105 | 10.929 | 0.436 |
| **m4** | glm | 1543.3 (0.144) | 1544.0 (0.136) | 1569.9 (0.013) | 0.114 | 10.867 | 0.435 |
| **m5** | glm | 1543.7 (0.116) | 1544.1 (0.128) | 1563.7 (0.290) | 0.094 | 10.966 | 0.44 |
| **m6** | glm | 1543.1 (0.158) | 1544.2 (0.122) | 1576.4 (<.001) | 0.133 | 10.707 | 0.434 |

**Table S 3.7**: PERMANOVA testing pairwise comparisons of gut microbial samples grouped by their sampling timepoint (season) in the river habitat. Wei_uni=weighted UniFrac. Significance codes: **<0.01

| Groups | measure | F | R2 | p.value | p.adjusted | Signif. |
|---|---|---|---|---|---|---|
| **Winter2019 vs Summer2019** | wei_uni. | 7.318 | 0.051 | 0.001 | 0.001 | ** |
| **Winter2019 vs Autumn2019** | wei_uni. | 8.271 | 0.061 | 0.001 | 0.001 | ** |
| **Winter2019 vs Autumn2018** | wei_uni. | 13.799 | 0.089 | 0.001 | 0.001 | ** |
| **Summer2019 vs Autumn2019** | wei_uni. | 1.542 | 0.014 | 0.093 | 0.093 | |
| **Summer2019 vs Autumn2018** | wei_uni. | 11.812 | 0.089 | 0.001 | 0.001 | ** |
| **Autumn2019 vs Autumn2018** | wei_uni. | 13.907 | 0.111 | 0.001 | 0.001 | ** |

**Table S 3.8:** PERMANOVA of pairwise comparisons of samples grouped by host genetic origin. F=Farmed, HFF=Hybrid Farmed Female, HWF=Hybrid Wild Female,

W=Wild. Distance matrix calculated by weighted UniFrac measure. Permutations used: 9999. Significance codes: ***< 0.001; **< 0.01; *< 0.05.

| Groups | measure | F | R2 | p.value | p.adjusted | Signif. |
|---|---|---|---|---|---|---|
| **W vs HFF** | wei_unifrac | 1.705 | 0.015 | 0.065 | 0.098 | |
| **W vs HWF** | wei_unifrac | 0.754 | 0.007 | 0.697 | 0.697 | |
| **W vs F** | wei_unifrac | 3.747 | 0.035 | 0.001 | 0.006 | ** |
| **HFF vs HWF** | wei_unifrac | 1.325 | 0.013 | 0.167 | 0.200 | |
| **HFF vs F** | wei_unifrac | 1.943 | 0.019 | 0.053 | 0.098 | |
| **HWF vs F** | wei_unifrac | 2.690 | 0.028 | 0.005 | 0.015 | * |

**Table S 3.9**: PERMANOVA of pairwise comparisons of samples grouped by host genetic origin for winter 2019. F=Farmed (Reference category), HFF=Hybrid Farmed Female, HWF=Hybrid Wild Female, W=Wild. Distance matrix calculated by weighted UniFrac measure. Permutations used: 9999. Significance codes: ***< 0.001; **< 0.01; *< 0.05.

| Groups | measure | F | R2 | p.value | p.adjusted | Signif. |
|---|---|---|---|---|---|---|
| **F vs HWF** | wei_unifrac | 2.392 | 0.081 | 0.016 | 0.028 | * |
| **F vs W** | wei_unifrac | 5.154 | 0.160 | 0.002 | 0.012 | * |
| **F vs HFF** | wei_unifrac | 1.116 | 0.039 | 0.331 | 0.331 | |
| **HWF vs W** | wei_unifrac | 2.781 | 0.090 | 0.019 | 0.028 | * |
| **HWF vs HFF** | wei_unifrac | 1.185 | 0.040 | 0.254 | 0.304 | |
| **W vs HFF** | wei_unifrac | 2.777 | 0.090 | 0.012 | 0.028 | * |

**Table S 3.10**: PERMANOVA of pairwise comparisons of samples grouped by host genetic origin for autumn 2019. F=Farmed, HFF=Hybrid Farmed Female, HWF=Hybrid Wild Female, W=Wild. Distance matrix calculated by weighted UniFrac measure. Permutations used: 9999. Significance codes: ***< 0.001; **< 0.01; *< 0.05.

| Groups | measure | F | R2 | p.value | p.adjusted | Signif. |
|---|---|---|---|---|---|---|
| **F vs W** | wei_unifrac | 3.446 | 0.154 | 0.004 | 0.024 | * |
| **F vs HWF** | wei_unifrac | 3.016 | 0.159 | 0.014 | 0.034 | * |
| **F vs HFF** | wei_unifrac | 3.119 | 0.119 | 0.017 | 0.034 | * |
| **W vs HWF** | wei_unifrac | 1.085 | 0.054 | 0.346 | 0.346 | |
| **W vs HFF** | wei_unifrac | 1.801 | 0.065 | 0.03 | 0.045 | * |
| **HWF vs HFF** | wei_unifrac | 1.149 | 0.048 | 0.298 | 0.346 | |

**Table S 3.11**: Generalised linear model showing the effects of fish origin on Shannon diversity index for samples collected in autumn 2019. AIC: 38.77. AIC

served as indicator to determine the goodness of fit. F=Farmed (Reference category), HFF=Hybrid Farmed Female, HWF=Hybrid Wild Female, W=Wild. Significance codes: ***<0.001; **<0.01, *< 0.05.

| | Estimate | Std. Error | t value | Pr(>\|t\|) | Signif. |
|---|---|---|---|---|---|
| **(Intercept)** | 2.3778 | 0.1183 | 20.092 | < 2e-16 | *** |
| **HFF** | -0.3647 | 0.1479 | -2.466 | 0.01818 | * |
| **HWF** | -0.3887 | 0.1725 | -2.253 | 0.02994 | * |
| **W** | -0.4561 | 0.1631 | -2.796 | 0.00799 | ** |

**Table S 3.12**: Generalised linear model showing the effects of fish origin on Chao1 richness for samples collected in autumn 2019. AIC: 421.35. AIC served as indicator to determine the goodness of fit. F=Farmed, HFF=Hybrid Farmed Female, HWF=Hybrid Wild Female, W=Wild. Significance codes: ***<0.001; **<0.01, *< 0.05.

| | Estimate | Std. Error | t value | Pr(>\|t\|) | Signif. |
|---|---|---|---|---|---|
| **(Intercept)** | 30.717 | 2.637 | 11.647 | 2.88E-14 | *** |
| **HFF** | -8.621 | 3.297 | -2.615 | 0.01262 | * |
| **HWF** | -13.154 | 3.844 | -3.422 | 0.00147 | ** |
| **W** | -13.367 | 3.635 | -3.677 | 0.00071 | *** |

# Chapter 4: LINKS BETWEEN HOST GENETICS, METABOLISM, GUT MICROBIOME AND AMOEBIC GILL DISEASE (AGD) IN ATLANTIC SALMON

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A version of this manuscript has been published in Animal Microbiome (https://doi.org/10.1186/s42523-022-00203-x)

## 4.1 Abstract

Rapidly spreading parasitic infections like amoebic gill disease (AGD) are increasingly problematic for Atlantic salmon reared in aquaculture facilities and potentially pose a risk to wild fish species in surrounding waters. Currently, it is not known whether susceptibility to AGD differs between wild and farmed salmon. Wild Atlantic salmon populations are declining, and this emerging disease could represent an additional threat to their long-term viability. A better understanding of how AGD affects fish health is therefore relevant for the accurate assessment of the associated risk, both to farming and to the well-being of wild populations. In this study, we assessed the impact of natural exposure to AGD on wild, hybrid and farmed post-smolt Atlantic salmon reared in a sea farm together under common garden conditions.

Wild fish showed substantially higher mortality levels (64%) than farmed fish (25%), with intermediate levels for hybrid fish (39%) suggesting that AGD susceptibility has an additive genetic basis. Metabolic rate measures representing physiological performance were similar among the genetic groups but were significantly lower in AGD-symptomatic fish than healthy fish. Gut microbial diversity was significantly lower in infected fish. We observed major shifts in gut microbial community composition in response to AGD infections. In symptomatic fish the relative abundance of key taxa *Aliivibrio*, *Marinomonas* and *Pseudoalteromonas* declined, whereas the abundance of *Photobacterium* and *Vibrio* increased compared to healthy fish.

Our results highlight the stress AGD imposes on fish physiology and suggest that low metabolic-rate fish phenotypes may be associated with better infection outcomes.

We consider the role increased AGD outbreak events and a warmer future may have in driving secondary bacterial infections and in reducing performance in farmed and wild fish.

## 4.2 Introduction

Atlantic salmon aquaculture is one of the largest and most profitable fish production industries worldwide (FAO, 2020). Large scale production accompanied by high fish densities has led to ecological and economical challenges and increased the pressure to make aquaculture more sustainable (Naylor et al., 2000, 2021). Whilst the salmonid aquaculture industry is undergoing rapid expansion, fish are increasingly prone to numerous stressors associated with pen-rearing in the marine environment. Anthropogenic stressors include behavioural and physiological stress induced by high stocking densities (Baldwin, 2011), the use of terrestrial protein and lipid sources that impact gut health (Ciji & Akhtar, 2021; Egerton et al., 2020; Hardy, 2010) and enhanced handling stress associated with the treatment of fish for several important parasitic diseases (Murray & Peeler, 2005; Overton et al., 2019). Biological stressors include a variety of plankton-borne threats, especially sea lice (e.g., parasitic copepods; Costello, 2009; Torrissen et al., 2013), micro-jellyfish and harmful algal blooms (Díaz et al., 2019) and amoebic gill disease (Oldham et al., 2016). The presence of large volumes of farmed fish along North Atlantic coastlines also impacts wild salmon populations via epizootics and genetic introgression from farm stocks (Glover et al., 2017; McGinnity et al., 2003).

Amoebic gill disease (AGD) is increasingly problematic for the marine phase of salmonid aquaculture globally (Oldham et al., 2016). In recent years, this disease has

caused substantial economic losses on Scottish and Irish salmon farms amounting to millions of British Pound Sterling (Hjeltnes et al., 2017; Shinn et al., 2015). AGD is caused by the ectoparasitic protozoan *Neoparamoeba perurans*, which colonises fish's gill epithelium inducing lamellar fusion resulting in anorexia, increased ventilation rates and eventually death if not treated (Munday et al., 1990; Young et al., 2007). Whilst AGD infections are well documented in farmed salmonids little is known about their effect on wild fish (Hellebø et al., 2017). In the wild, predators may rapidly eliminate affected wild fish making an assessment difficult (Taranger et al., 2015). There is growing evidence of significant transmission of infectious diseases from farmed fish to wild fish, which occurs either due to farmed escapes or by wild fish being in close proximity to aquaculture pens while feeding or in process of migrating (Lafferty et al., 2015; Mordecai et al., 2021). Climate change accompanied by rising water temperatures make AGD outbreaks more likely and therefore a pressing subject for future research (Oldham et al., 2016; Steinum et al., 2008). It is especially important to assess the sublethal effects of AGD infections, their impact on fish physiology and especially the potential impact of AGD on wild salmonids.

Two important physiological measures of interest are standard metabolic rate (SMR) and maximum metabolic rate (MMR). SMR is the minimal maintenance metabolic rate of an ectotherm in a post-absorptive and inactive state (Chabot et al., 2016). MMR describes the upper limit to an organism's ability to take up oxygen (Norin & Clark, 2016). The difference between MMR and SMR is the aerobic scope (AS), which represents the amount of energy available for activities like locomotion, feeding, digestion, growth, and reproduction (Claireaux & Lefrançois, 2007; Priede, 1985). Host metabolic rate has strong ecological relevance (Burton et al., 2011) by impacting

fish growth and survival (Álvarez & Nicieza, 2005). Variation in metabolic rate measures between individuals are likely to have fitness consequences (Steyermark et al., 2005). For example, fish with high metabolic rates might be able to grow faster in favourable living conditions but struggle during times of food scarcity or stress (e.g., parasitic infections) due to their high maintenance costs (Metcalfe et al., 2016; Reid et al., 2011; Rosenfeld et al., 2015). High SMR individuals have also been shown to digest meals faster, potentially resulting in higher food intake and consequentially a greater growth potential (Millidine et al., 2009). Since farmed fish show an upregulation of genes associated with energy metabolism (Debes et al., 2012), have bigger food intake and also tend to be bigger than their wild counterparts (Glover et al., 2017) one might assume underlying differences in metabolic phenotype between the groups. It is known that AGD infections compromise gill functions, resulting in lower MMR values (Hvas et al., 2017). However, whether there is a differential impact of AGD on individuals with distinct genetic backgrounds and metabolic rates has yet to be determined.

Microbiomes can be defined as microbial communities, their genomes and surrounding environmental conditions in well-defined habitats (Berg et al., 2020; Whiteside et al., 2015). Gut microbial communities are known to impact host metabolism (Visconti et al., 2019), growth (Li et al., 2013), behaviour (Mayer, 2011), immune response (Thaiss et al., 2016) and pathogen defence (Kamada et al., 2013). Microbial communities are shaped by selective (Dehler et al., 2017; Tarnecki et al., 2017; Uren Webster et al., 2018) and stochastic processes (Burns et al., 2016; Heys et al., 2020). Different gut compartments e.g., the pyloric caeca (PC) or midgut (MG) have distinct microbial profiles (Kazlauskaite et al., 2021; Llewellyn et al., 2016) which

might be connected to their different functionalities (Egerton et al., 2018; Krogdahl et al., 2015; Sahlmann et al., 2015). Correlations between host phenotype and microbiome are commonly reported (Lynch & Hsiao, 2019) with stress being an important driver of microbiome change in aquaculture systems (Boutin et al., 2013). In addition, host-associated microbial communities can be a source of opportunistic pathogens associated with parasite infection (Llewellyn et al., 2017). Another poorly studied aspect of fish microbiome ecology is the effect of fasting (Kohl et al., 2014; Xia et al., 2014). It is not uncommon for fish in the wild to spend prolonged periods of time without food, e.g. due to seasonal changes in food availability (Green & Farwell, 1971). It is a widespread practice to starve fish prior to major farming operations like crowding, pumping, delousing, and transportation (Hvas et al., 2022). Also, fish will often lose appetite on transfer from freshwater to the sea and may take some time acclimatising and resuming feeding in the new environment (Usher et al., 1991). We hypothesize that deteriorating host health, induced by external stressors like parasitic infections also affects gut microbial community composition. Hence, tracking host-associated microbiota can provide clues to changing host physiological status and performance as a result of primary parasitic and possible secondary infections.

In the current study, we aim to understand the impact of natural exposure of common garden-reared wild, hybrid and farmed fish to amoebic gill disease. Alongside classical measures of the fish condition and AGD-status, we also monitored the impact of AGD on host respiratory physiology across the different fish cohorts. Finally, we also explored the impact of AGD infection on the host's gut microbiology.

## 4.3 Methods

### 4.3.1 Experimental setup

Atlantic salmon of four different genetic origins were raised in hatchery ponds at the Marine Institute research facility at Newport, County Mayo, Ireland. The four groups consisted of fish from the progeny of a commonly reared farmed strain (F), wild fish sourced from the Burrishoole river in the west of Ireland (W) and their reciprocal hybrid progeny of farm and wild parents (HFF and HWF, respectively). The fish were fed *ad libitum* on a diet of pellets produced by Skretting Nutra Olympic (Cheshire, UK) during freshwater rearing. Individuals were tagged with passive transponder tags (PIT tags) for later identification. On smoltification, May 2019, the four groups of smolts were transferred to a sea pen at the Marine Institutes Lehanagh Pool research site, Ireland (Cashel Bay, 53.401116, -9.819287). Within the pen were four sentinel pens (4x4 m). A subset of farmed, wild and hybrid fish groups was introduced into each of three of the sentinel pens, one for each of farmed (app. 380 fish), wild (app. 360 fish) and combined hybrid groups (app. 700 fish). The fish in these pens were used for the tracking of length and weight trajectories of the groups throughout the study. Twenty fish of each cohort (40 in the case of hybrids) were measured weekly. The fourth sentinel pen contained a mixture of PIT tagged fish representing all four experimental groups (F: n=207; HFF: n= 204; HWF: n=194; W: n=198)). Post smolts in the sea pen were fed a maintenance diet of Ewos 75 pellets produced by Cargill (MN, USA) on a five-day cycle. Mortality per genetic group in the mixed sentinel pen was assessed by comparing the fish counts from the start and end of the experiment. After a settlement period of six weeks at sea 16 fish (four fish of each genetic background) were transferred from the mixed ($4^{th}$) sentinel pen twice a week to the Newport

research facility for metabolic rate measurements and subsequent dissections of organs and guts (Figure 4.1). In total, we had 11 sampling days over the course of 49 days. During this time some fish showed signs of AGD, especially at the later stages of the experiment. Gut microbial profiling was carried out at the University of Glasgow. An overview of sample identification number, sampling days (the date when fish were caught), water temperatures and processing days (the day fish were dissected) is shown in Table S 4.1.

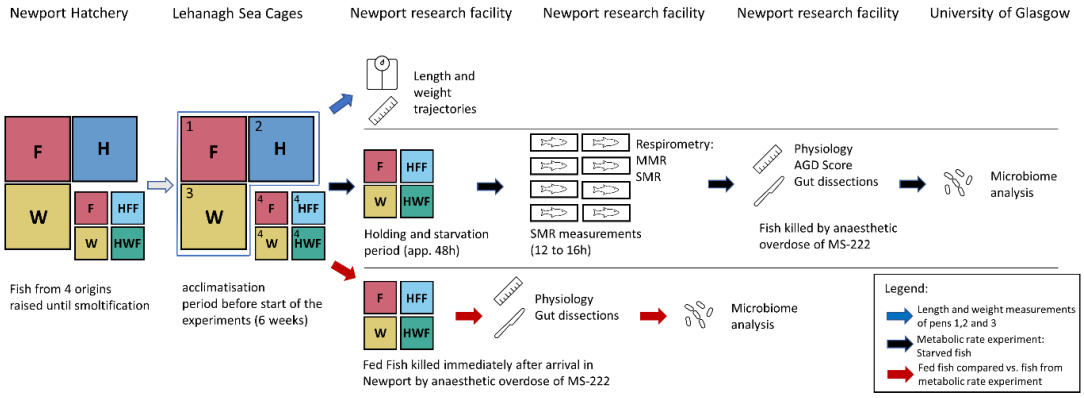


**Figure 4.1:** Flow chart of the experimental setup. Fish from four genetic backgrounds were used: Farmed (F), wild (W) and their reciprocal hybrids named after the origin of the mother (hybrid farmed female (HFF) and hybrid wild female (HWF)). Fish from pens 1-3 were used for length and weight trajectories (blue arrow). PIT tagged fish from pen 4 were used for respirometry experiment and microbial profiling (black arrow). In addition, we compared starved fish from the metabolic rate experiment with recently fed fish at two different sampling timepoints T0 (day0 of respirometry experiment) and T1 (day14 of the respirometry experiment) (red arrow).

### 4.3.2 Metabolic rate measurements

The oxygen consumption ($\dot{M}O_2$) of individual fish was determined using automated intermittent respirometry as described previously (Clark et al., 2011; Norin & Malte, 2011) and using best practices outlined in (Steffensen, 1989), (Clark et al., 2013) and (Chabot et al., 2016).

After transfer to the Marine Institute respirometry laboratory, fish were placed in holding tanks for 24 h. Natural hiding spots were provided to reduce fish stress. Fish were not fed for a period of 48 h to ensure that they were in a post-absorptive state required for accurate metabolic rate measurements. To determine the fish's maximum metabolic rate (MMR) fish were chased in a bucket for 60 s and immediately transferred to the respirometry setup (Chabot et al., 2016). The setup consisted of respirometry chambers (rectangular sealable plastic boxes with a total volume of 2 litres), which were contained within an oxygenated and temperature-controlled reservoir filled with seawater. Oxygen concentration in the respirometry chambers was measured constantly by using fibre-optic oxygen meter probes (FireStingO2; Pyro Science GmbH, Aachen, Germany) and accompanying software (Pyro Oxygen Logger; Pyro Science GmbH, Germany). Automated flush pumps refreshed the water in the respirometers for 6 min in every 10 min period and $\dot{M}O2$ was calculated from the decline in oxygen concentration in the respirometers between flush cycles (Norin et al., 2014).

### 4.3.3 Metabolic rate data analysis

Data obtained from the respirometry measurements was uploaded into R (R Core Team, 2022). Analysis was carried out using the R packages respirometry (Birk, 2021) and custom functions. For MMR analysis we first plotted the slope of the decline in $MO_2$ over time. Then, we assessed the start and end times of the measurement cycle visually for each chamber (fish) individually. To provide clean slopes, we chose to cut the observed slope 5 s after the maximum $MO_2$ value and 5 s before the lowest $MO_2$ value of the measurement cycle.

SMR was calculated for each fish by calculating the quantiles that assign 20% of the data below mean SMR ($q_{0.2}$). Mean SMR was assessed from slopes that had an $R_2$ of at least 0.8 ($r_{2min}$=0.8). This method accounts for periods of high activity by the fish throughout the measurement period and was determined as per (Chabot et al., 2016) as best estimate for SMR, when fish activity levels cannot be observed visually.

Metabolic rates were size corrected using general linear models with body mass (weight) as covariate (ANCOVA) (Schurmann & Steffensen, 1997). This allows a direct comparison of individuals of different masses and consequently accounts for any differences in metabolism arising from size differences found among fish from the different groups. To linearise the data, metabolic rate and body mass measures were log10-transformed. To maintain comparability within the study 19 out of 145 metabolic rate measurements were excluded from the analysis due to inconsistencies in fish handling time or equipment set-up errors.

### 4.3.4 Gut microbiota collection and AGD scoring

After SMR measurements fish were euthanized by an anaesthetic overdose of methane tricaine sulphonate (MS-222, 300mg/l, FVG, Ireland). Histological AGD lesion assessment is known to be good qualitative tool for AGD scoring (Chang et al., 2019). Therefore, fish gills were visually examined for a potential AGD infection and AGD severity was determined by using the scoring system described by (Taylor et al., 2016; Table 4.1).

Wet weight (g) and fork length (mm) were measured. Fulton's condition factor (K) was calculated as follows (Ricker, 1975):

$K = 100 \times \text{weight (g)} / \text{length (cm)}^3$

Fish were dissected aseptically via an incision along the ventral side. Organ weights and gut length were measured, and sections of the gut compartments pyloric caeca and mid-gut were separated, put into cryotubes and immediately placed on dry ice. Samples were subsequently stored at -80°C and later shipped on dry ice to the University of Glasgow for microbial profiling.

Each dissected fish was reweighed without its gut and placed into a drying oven at 60°C for 72h before measuring its dry weight. The wet mass (g, excluding the gut) and dry mass (g) was then used to determine the % water content as follows:

% water content = 100 ((wet mass – dry mass) / wet mass)

% water content was also used to estimate the fat content of the fish, since it has previously been shown that there is a strong negative correlation between % water content and % fat content (Elliott, 1976).

**Table 4.1:** AGD scoring system (Taylor et al., 2016). Fish were classified depending on visual inspection of the gills. White spots and mucus patches on the gills are typical indicators of an AGD infection.

| AGD Score | AGD severity | Description |
|---|---|---|
| **0** | Clear | No sign of infection and healthy red colour (non-symptomatic) |
| **1** | Very Light | 1 white spot, light scarring or undefined necrotic streaking |
| **2** | Light | 2-3 white spots/ small mucus patch |
| **3** | Moderate | Established thickened mucus patch/ white spot groupings up to 20% of gill area |
| **4** | Advanced | Established lesions covering up to 50% of gill area |
| **5** | Heavy | Extensive lesions covering most of the gill surface (over 50%) |

### 4.3.5 Environmental controls and determination of feeding status

At each sampling date environmental samples were taken to identify bacteria in the water column. Sterile water bottles (1.5 litres) were used to collect seawater at a depth of app. one meter inside sentinel cage four. The seawater was filtered in a sterile environment using 0.2 µm filters (Whatman, Chicago, IL, USA). After filtration the filters were placed into cryotubes, immediately placed on dry ice and stored at -80°C prior to transportation to the University of Glasgow and microbiome profiling.

To account for the influence of food withdrawal and holding times on fish gut microbial community composition, recently fed control fish were sampled in addition to the regular sampling procedure. Eight fish (two fish of each genetic background) were sampled right after the 6 weeks acclimatisation period, which equals the start of the metabolic rate experiments. Those fish were labelled “T0Fed”. Fourteen days later another 8 recently fed fish (again two fish of each genetic background) were sampled and labelled “T1Fed”. At each of the two timepoints the gut microbial community composition of the fed fish was compared with 8 starved fish. Those fish were sampled (caught) at the same day but were starved for 48 hours during metabolic rate measurements. These samples are labelled T0Starved and T1Starved, respectively. All fish in this control study were asymptomatic for AGD.

### 4.3.6 Microbial DNA extraction and NGS library preparation

The DNA extraction and NGS library preparation protocols used were based on methods established and summarized in (Heys et al., 2020; Kazlauskaite et al., 2021).

To extract bacterial DNA from gut samples, the frozen gut tissue (200 mg) and filter papers were cut up into pieces using sterilized equipment and DNA was extracted

using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol (Claassen et al., 2013).

Extracted DNA was amplified using primers targeting the V1 hypervariable 16S rDNA region (Gajardo et al., 2016). V1 was chosen over V4 because it showed less cross-contamination with salmon DNA (Heys et al., 2020; Werner et al., 2012). Amplification of the target region was achieved by using tagged barcodes 27F and 338R at a concentration of 1pM for each primer. Primer sequences are shown in Table S 4.2: First round PCR primers used for NGS library preparation. The reaction mix contained a total volume of 15 µl and consisted of 0.7 µl of each internal forward and reverse primer, 7µl of Q5 Hot Start High-Fidelity 2X Master Mix (New England Biolabs Ltd, UK) and 1µl of DNA template. PCR conditions were initial denaturation at 95°C for 10min; 30 cycles at 95°C for 30s, 55°C for 30s and 72°C for 30s; and a final elongation step of 72°C for 10min.First round PCR products were then used for a subsequent second round of PCR in which external multiplex identifiers (barcodes) were added. The second round PCR reaction mix contained a total volume of 25 µl and consisted of 1.25 µl of external revers primer (10µM), 1.25 µl external forward primer (10µM), 12.5µl of Q5 Hot Start High-Fidelity 2X Master Mix (New England BioLabs Ltd, UK) and 1.3 µl of first round PCR template. PCR conditions were the same as before but only eight cycles were used. Barcode sequences are shown in Table S 4.3.

The cleaned DNA was then gel-purified by using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) and quantified by using Qubit® (Thermo Fisher Scientific,

USA). PCR products were pooled together at a concentration of 10nM and paired-end sequencing was carried out using a NovaSeq 6000 system.

### 4.3.7 Bioinformatic pipeline

Sequence analysis was performed with our bioinformatic pipeline as described previously (Heys et al., 2020; Kazlauskaite et al., 2021; Llewellyn et al., 2016).Firstly, quality filtering and trimming (>Q33 Phred quality score) was performed on all the reads of the 16s rRNA V1 hypervariable region using the Sickle (v.1.2) software (Joshi & Fass, 2011). Read error correction was carried out by using the BayesHammer module within the SPAdes (v.2.5.0) software to obtain high-quality assemblies (Nikolenko et al., 2013). Paired-end reads were merged (overlap length 50bp) by using PANDAseq (v.2.11) with the simple Bayesian read merging algorithm (Masella et al., 2012; Schirmer et al., 2016). Thereafter, merged reads were dereplicated, sorted, and chimaeras and singletons were removed by using VSEARCH (v.2.3.4; (Rognes et al., 2016). Sequences were decontaminated against the *Salmo salar* genome using DeconSeq (v.0.4.3;(Schmieder & Edwards, 2011) and overlapped reads were clustered into operational taxonomic units (OTUs) using VSEARCH at 97% sequence identity. OTUs were taxonomically classified against the SILVA 132 database (Quast et al., 2012) and annotated using the Scikit-learn algorithm implemented in QIIME2 (Bolyen et al., 2019; Pedregosa et al., 2011).

### 4.3.8 Post OTU statistics

All data were analysed in R (R Core Team, 2022) using the microeco package (An et al., 2019; Liu et al., 2021). After uploading the required files OTUs which were not assigned to the kingdoms of bacteria and archaea were removed. In addition, taxa

classified as chloroplast or mitochondria were considered as contamination and discarded. Samples were rarefied to 10000 reads to limit the number of effects on diversity measurements. Samples of the gut compartments PC and MG were analysed separately. The significance of alpha diversity indices was assessed using Kruskal-Wallis (KW) rank sum test in combination with pairwise Wilcoxon for multi-level factors. In addition, we used a linear model to display significant variables. Beta diversity was measured using weighted UniFrac distances (Lozupone et al., 2011). To identify significant differences among grouping variables (e.g., genetic origin, AGD severity), permutational multivariate analysis of variance (PERMANOVA) was performed based on the weighted unifrac dissimilarity matrix. Fast expectation-maximization for microbial source tracking (FEAST) (Shenhav et al., 2019) was used to assess the contribution and the relative importance of fish feed and water bacteria to intestinal microbial communities of starved and fed fish. Thereby, the microbial communities of individual fish served as sink and feed and water samples (both collected at the same sampling timepoint) served as source. Differential abundance was carried out to obtain important indicator taxa by using random forest analysis (Beck & Foster, 2014; White et al., 2009; Yatsunenko et al., 2012). MeanDecreaseGini was used to determine the importance of differentially expressed taxa. P values were adjusted for multiple comparisons using the Benjamini-Hochberg method (Benjamini & Hochberg, 1995).

The experiments and measurements carried out in this study were conducted under licence (AE19130-P056) of the Health Products Regulatory Authority (HPRA) of Ireland.

## 4.4 Results

### 4.4.1 Health trajectories – Wild fish show the highest mortality.

From the end of March to August 2019 length and weight measurements for 1,949 fish were obtained. For this analysis the two hybrid groups were combined. The farmed fish were bigger than the other two groups (Figure 4.2; Table S 4.4) . Fish grew most during *ad libitum* feeding in the hatchery. After sea site transfer farmed fish gained on average 11cm in length, whilst maintaining the same weight. Hybrid fish increased by 23cm in length and 7g in weight, whilst wild fish increased in length by 31cm and 14g in weight. During the *ad libitum* feeding in the hatchery phase all fish had a Fulton's condition factor (k) of above 1, regardless of their origin. After the sea site transfer the condition factor declined for all groups, with farmed fish (K(F) =0.9) having on average a higher condition than hybrid or wild fish (K(H)=0.85; K(W)=0.82; Figure 4.2c). Farmed fish had on average significantly lower the lowest % water content (74.5 ± 2.7%), followed by the two hybrid groups (HFF: 76.1 ± 1.8%, HWF: 76.8 ± 2.0%) and the progeny of wild fish (77.3 ± 2.32%; Figure S 2.2).

Mortality was calculated as the difference in fish counts between the start and the end of the experiment for the mixed ($4^{th}$) sentinel pen. Here, wild fish showed by far the highest mortality rate (64.1%), followed by the hybrid group (39.2%) and farmed fish (24.6%; Figure 4.2d).

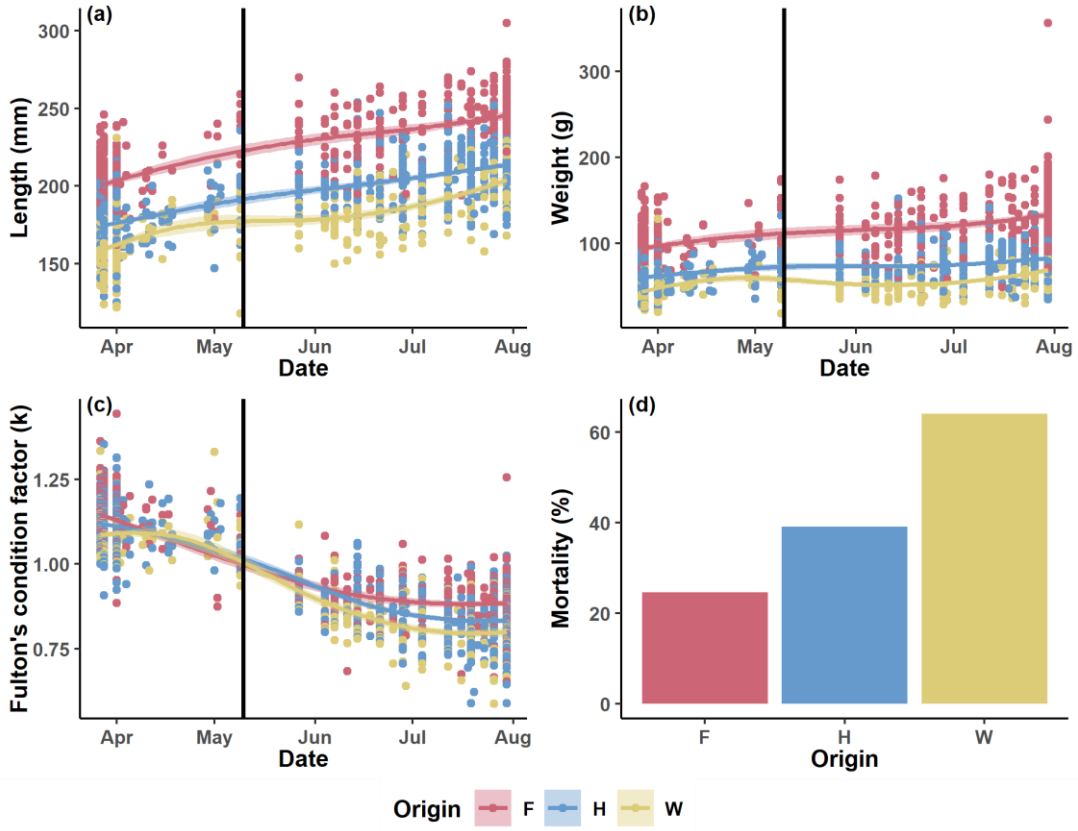


**Figure 4.2**: Loess regression of growth trajectories (length (a), weight (b) and Fulton's condition factor (c)) of fish from different genetic backgrounds throughout the experiment. The black line indicates the point in time of the transfer from the freshwater stage to the sea pens. Mortality (d) was calculated as the difference in fish counts between the start and end of the experiment. Origin: F=Farmed, H= Hybrid, W=Wild

### 4.4.2 Metabolic rate – AGD severity leads to a decline in oxygen consumption.

The metabolic rates of post-smolt Atlantic salmon were determined over 6 weeks. 55 fish showed signs of AGD with their AGD severity scores ranging from very light (score 1: 17 fish) to moderate (score 3: 23 fish). Signs of AGD were mostly observed towards the end of the experiment. Overall, the fish's origin had no significant impact on neither SMR (p=0.25), MMR (p=0.51) or AS (p=0.43). Simple pairwise testing of weight-adjusted metabolic rate measures revealed a significant decline of such with increasing AGD severity (SMR: AGD0 vs. AGD 2: p=0.01; AGD0 vs. AGD3: p=0.004; MMR: AGD0 vs. AGD 2: p=0.02; AGD0 vs. AGD3: p=0.005; AS: AGD0 vs. AGD3: p=0.03;

Figure 4.3). We chose to confirm pairwise testing results by an ANCOVA (Anova Type III), using weight as a covariate. The model revealed significant differences in SMR and MMR means between fish with different AGD severity scores (SMR: F=7.38, p=0.0001; MMR: F=3.79, p=0.01). However, in contrast to the formerly mentioned pairwise t-testing no significant differences in AS were observed (AS: F=2.15, p=0.09; Table S 4.5). Multiple comparisons of means (Tukey post-hoc) revealed significantly lower metabolic rate measures between AGD 0 vs. AGD 2 and 3, respectively (SMR: p(0/2)=0.002, p(0/3)=0.002; MMR: p(0/3)=0.03; Table S 4.6). Important to note that the observable dispersion of metabolic rate measurements for fish with an AGD score of 0 (SD(SMR=21.22; MMR=168.07)) is larger than for fish with AGD scores of 3 (SD(SMR=18.52; MMR=83.49); Figure 4.3).

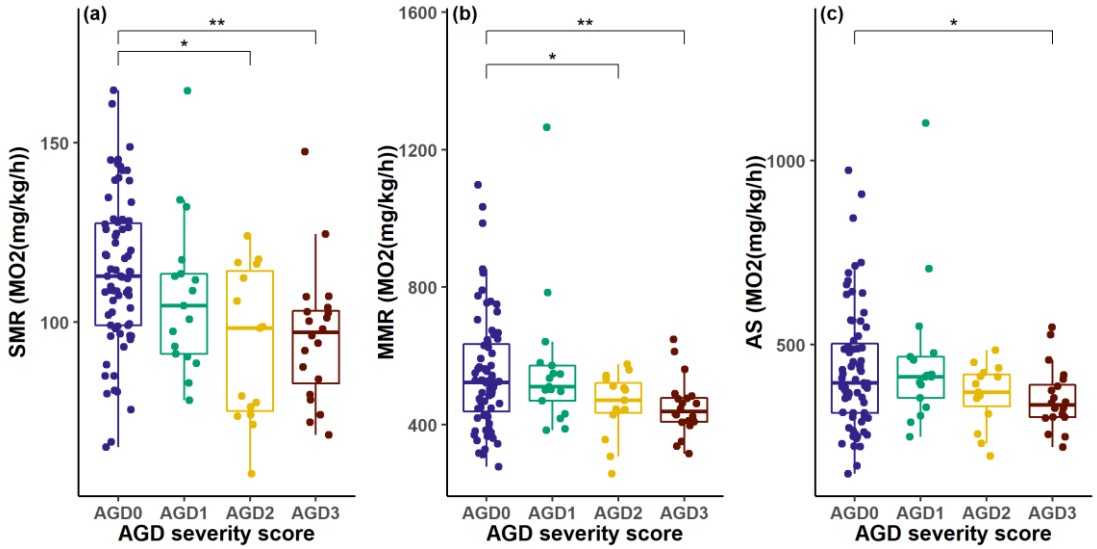


**Figure 4.3:** Calculated oxygen consumption ($MO_2$(mg/kg/h)) for fish affected by AGD at different severity levels. No differentiation between genetic background was made. Standard metabolic rate (a), maximum metabolic rate (b) and aerobic scope (c). Significance was determined by pairwise t-testing against weight adjusted metabolic rate measures of fish with an AGD score of 0. P-values adjusted by

Bonferroni correction. Significance codes are only shown for significant results: ** p< 0.01; *p<0.05.

### 4.4.3 The gut microbiota of starved and fed fish – Feeding status matters

In an auxiliary experiment, we compared recently fed and starved fish at two different timepoints (14 days apart). The results in this paragraph are based on samples from this side experiment. At a phylum level, gut microbial communities were mostly dominated by *Proteobacteria*. However, there was an observable difference in relative abundance between fed and starved fish. In fed fish the mean relative abundance of *Actinobacteria* (Fed: PC 14.6%, MG 13.8%; Starved: PC 1.3%, MG 4.3%), *Firmicutes* (Fed: PC 8.7%, MG 8.4%; Starved: PC 0.7%, MG 2.0%) and *Fusobacteria* (Fed: PC 6.7%, MG 8.4%; Starved: PC 0.09%, MG 0.01%) were higher than in starved fish, whereas *Proteobacteria* (Fed: PC 57.5%, MG 54.3%; Starved: PC 83.4%, MG 86.5%) were less abundant (Figure S 4.2). At genus level, starved fish guts were dominated by *Marinomonas*, *Vibrio*, *Photobacterium*, *Aliivibrio*, *Pseudoalteromonas* and *Tenacibaculum* (Figure 4.4a, b). The top 10 most relative abundant genera accounted for app. 75% of total relative abundance in both PC and MG samples. In recently fed fish, the top 10 genera accounted for app. 55% of total relative abundance. Here, most abundant taxa were *Paracoccus*, *Turicella*, *Mycoplasma*, *Fusobacterium* and *Aliivribio* (Figure 4.4d, e). Feed samples were mostly dominated by *Paracoccus* and *Pseudomonas* and water samples (MW) were dominated by genera *Clade Ia*, *Planktomarina* and *Pseudohongiella* (Figure 4.4c, f). Differential abundance analysis revealed that in PC samples 69 taxa were significantly different between fed and starved fish and 31 taxa in MG samples. The most striking differences between starved and fed fish were observed for *Marinomonas* (Fed: PC

1.2%, MG 0.3%; Starved: PC 18.6%, MG 22.3%), *Pseudoalteromonas* (Fed: PC 2.4%, MG 2.8%; Starved: PC 13.5%, MG 8.4%), *Vibrio* (Fed: PC 0.2%, MG 0.2%; Starved: PC 7.2%, MG 9.6%), *Acinetobacter* (Fed: PC 2.6%, MG 3.4%; Starved: PC 0.03%, MG 0.9%), *Fusobacterium* (Fed: PC 5.6%, MG 7.0%; Starved: PC 0.04%, MG 0.01%) and *Paracoccus* (Fed: PC 16.8%, MG 10.9%; Starved: PC 0.1%, MG 0.01%; Figure 4.5a, b). We used source tracking analysis to determine the overall contribution of feed and water bacteria to fed and starved fish guts. In fed fish around 50 % of observed bacteria originated from feed samples, whereas water bacteria were only a minor source for the gut microbial community composition (Figure 4.5c). PCoA analysis separated starved, fed and environmental samples, confirming differences in community composition between the groups (Figure 4.5d). Within groups, recently fed fish showed significant differences between timepoints T0 and T1 in MG samples ($F=2.2$, $R^2=0.14$, $p=0.03$) but not in PC samples ($F=1.8$, $R^2=0.14$, $p=0.09$). There was also no significant difference within the two timepoints of starved fish, regardless of the sampled gut compartment (PC: $F=0.93$, $R^2=0.07$, $p=0.48$; MG: $F=1.3$, $R^2=0.09$, $p=0.27$; Table S 4.7 and Table S 4.8).

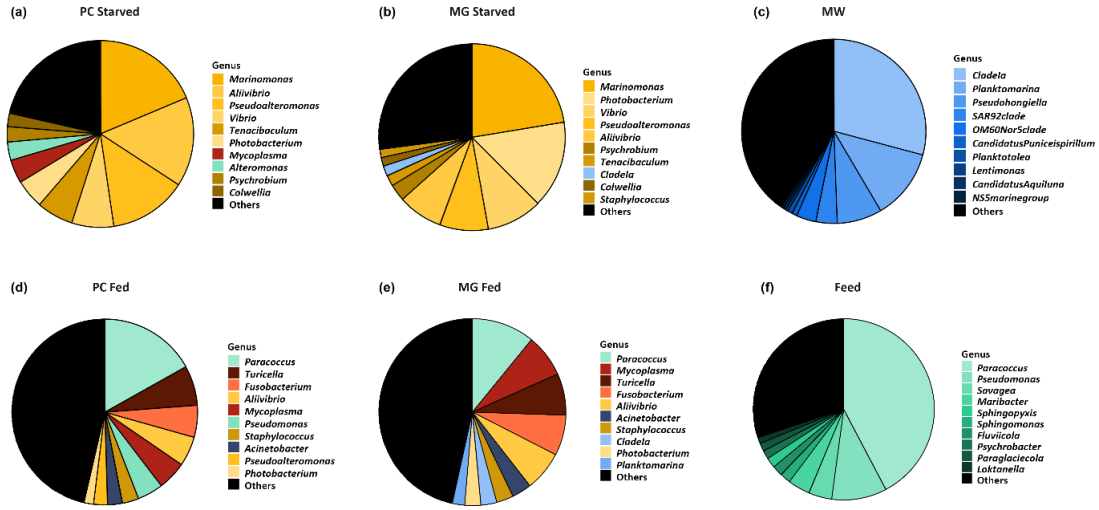


**Figure 4.4:** Pie charts showing the mean relative abundance of the 10 most abundant taxa on genus level for pyloric caeca of starved fish (a), midgut of starved fish (b), marine water (MW; (c)), pyloric caeca of recently fed fish (d), midgut of recently fed fish (e) and fish feed (f). Starved fish were without food for at least 48 hours. For illustration purposes samples of T0 and T1 were pooled together.

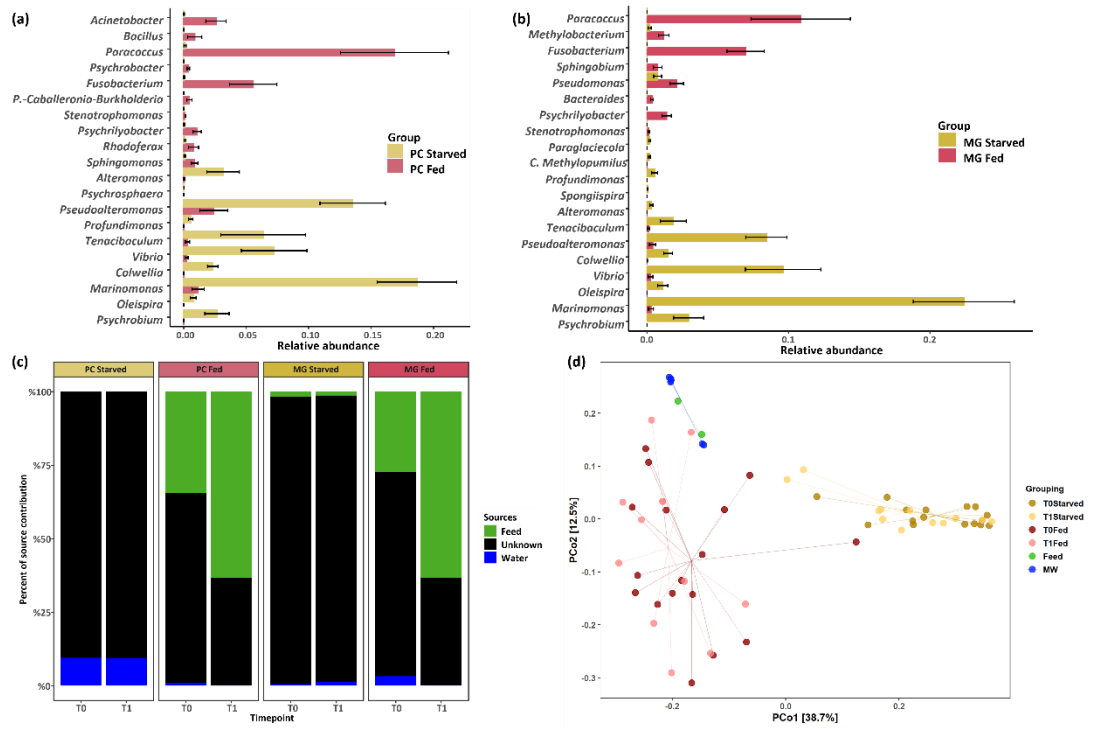


**Figure 4.5:** Differential abundance analysis between starved and fed fish in PC samples (a) and MG samples (b). Important genera were determined with random forest classification. Mean decrease gini served as indicator value. (c) shows the source tracking analysis determined by the FEAST framework. Bars show the average contribution of each source (either feed, water or unknown) to the overall microbial community for different gut compartments, feeding status and timepoints. (d) illustrates a principal coordinates analysis (PCoA) derived from weighted UniFrac

distances between environmental samples (MW= Marine water and feed), starved fish ((48 hours feed withdrawal) and recently fed fish at two different points in time (T0 and T1). T1 was sampled 14 days after T0. Lines mark the position of the centroids of each group. For illustration purposes no differentiation between PC and MG samples was made. However, differentiation was included in PERMANOVA calculations (Additional File 1 Table S 7 and Table S 8).

### 4.4.4 **Alpha Diversity – Lower microbial richness in AGD infected fish**

All following analysis refer to samples from the metabolic rate experiment. PC samples showed a significantly higher alpha diversity than MG samples (Chao1: KW, p=0.001; Shannon: KW, p=0.001; Figure 4.6a). In PC samples pairwise testing revealed no significant impact of genetic origin on diversity measures. However, Shannon and InvSimpson indices showed a significantly lower diversity for the samples with the highest AGD score 3 compared to AGD score 0 (InvSimpson: KW, Z=3.44, p=0.003) and AGD score 1 (Shannon: KW, Z=2.91, p=0.02; InvSimpson: KW, Z=2.92, p=0.01). In MG samples several diversity indices showed significantly lower values for samples with AGD score 3 than AGD score of 0 (Chao1: KW, Z=3.20, p=0.008; Shannon: KW, Z=3.00, p=0.01; InvSimpson: KW, Z=3.05, p=0.01; Figure 4.6c). In addition, Chao1 richness estimates were lower in fish of wild origin than in farmed (Chao1: KW, Z=2.69, p=0.04; Figure 4.6b). To investigate this relationship further we tested the combination of influencing factors by fitting linear models. The model results for the interaction term of AGD severity score and genetic origin on Chao1 richness estimates are displayed in Table S 4.9. Fish with an AGD score of 3 showed a significantly lower richness than fish with a score of 0 (t=-3.1, p=0.002). Fish from HFF and wild origin had significantly lower richness compared to farmed fish (HFF: t=-2.39, p=0.01; W: t=-2.29, p=0.02). When affected by AGD (AGD score=3) our model detected a significant lower microbial richness in farmed fish compared to wild (t=2.08, p=0.04) and hybrid farmed fish (t=2.70, p=0.007). However, due to the

low number of observations (n=3) per origin for an AGD score of 3 these interaction results must be treated with care.

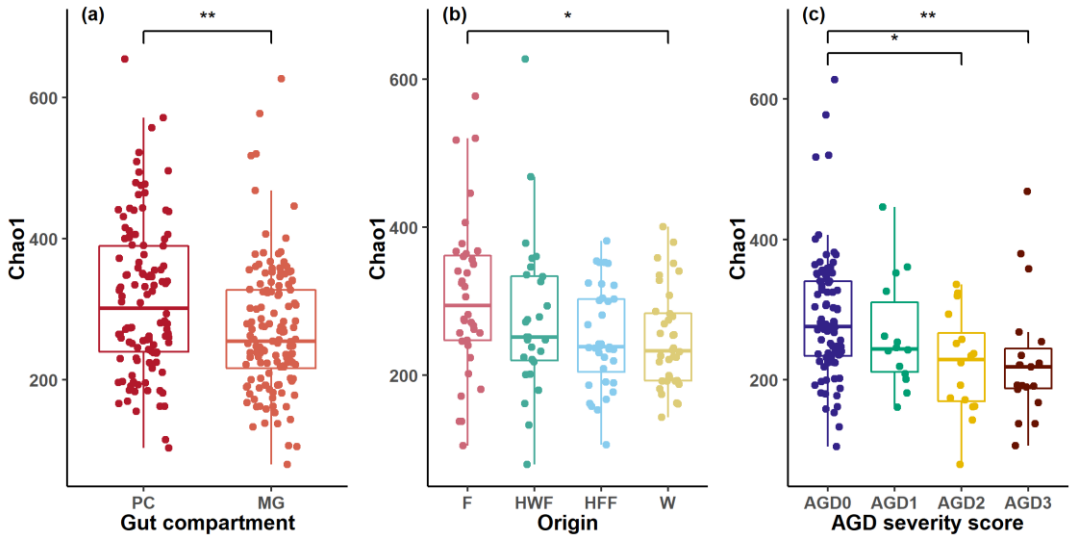


**Figure 4.6:** Chao1 richness estimates for multiple comparisons for starved fish between gut compartments (a), genetic background in MG samples (b) and AGD severity in MG samples (c). Lower microbial richness in MG samples compared to PC samples. In MG samples wild fish show a lower microbial richness than farmed fish. Fish with an AGD severity score of 2 and 3 showed a lower microbial richness than non-symptomatic fish (AGD 0). PC=Pyloric caeca, MG=Midgut, F=Farmed, HWF=Hybrid Wild Female, HFF= Hybrid Farmed Female, W=Wild. Significance was determined by Dunn's test for multiple comparisons of groups of significant Kruskal-Wallis results. Significance codes shown for significant differences: **p< 0.01; *p< 0.05.

### 4.4.5 **Beta Diversity – Community shift in AGD infected fish**

PCoA ordination separated microbial communities of PC samples by processing days and AGD severity score along PCoA1, which captured 39.2% of the variation, whilst most fish associated with an AGD score of 3 were separated by PCoA2 (Figure 4.7). Whilst samples at the earlier stages of the experiment seem to cluster together randomly, samples taken at the later stages of the experiment, especially from Day 46 onwards seem to have a higher likelihood to have a different community structure. Most of those samples had a higher AGD severity score, which implies an effect of symptomatic AGD infections on gut microbial communities. To confirm our

observations, we tested our variables of interest by applying PERMANOVA sequentially. Processing day (dissection day of the fish) explained 25.2% of variation ($F=2.2$, $R^2=0.25$, $p=0.0001$), AGD severity score was also significant and explained 9% of variation ($F=4.6$, $R^2=0.09$, $p=0.0001$), but there were no significant differences regarding genetic origin ($F=1.32$, $R^2=0.02$, $p=0.16$). 62.6 % of variation remained unexplained (Table S 4.10). To test for differences within the two significant factors of interest we conducted groupwise comparisons. We observed significant differences in community composition between samples with an associated AGD score of 0 and other AGD severity groups. In addition, samples with an AGD score of 1 differed significantly from samples with an AGD score of 3, whilst there were no significant differences between groupings of AGD score 1 and 2, nor between AGD score 2 and 3 (Table 4.2). Permutation test for homogeneity of multivariate dispersion revealed that the dispersion between AGD groups did not differ ($F=0.97$, $p=0.411$). As for day-by-day comparisons, samples taken at the beginning of the study do show similarities with each other and differ significantly from samples of later stages (Table S 4.11), which matches the difference in AGD scores and a gradual rise in temperature ($R^2= 0.99$). However, there are several “outlier” days where community compositions do not differ significantly e.g., the community composition from processing days 36 and 39 are not significantly different from the community composition of samples on day 0. Similar patterns were observed for samples derived from fish midguts. Results are displayed in Table S 4.12 and Table S 4.13.

**Table 4.2:** Permutational analysis of variance (PERMANOVA) testing pairwise comparisons for pyloric caeca (PC) samples from different AGD severity groups.

Distance matrix calculated by weighted UniFrac measure. Permutations used: 9999. Significance codes: **p< 0.01; *p< 0.05

| Groups | F | $R^2$ | p.value | p.adjusted | Significance |
|---|---|---|---|---|---|
| **AGD0 vs AGD1** | 2.97640 | 0.03167 | 0.017 | 0.025 | * |
| **AGD0 vs AGD2** | 3.91483 | 0.04306 | 0.002 | 0.006 | ** |
| **AGD0 vs AGD3** | 6.17319 | 0.06288 | 0.001 | 0.006 | ** |
| **AGD1 vs AGD2** | 1.50253 | 0.06987 | 0.161 | 0.161 | |
| **AGD1 vs AGD3** | 3.43869 | 0.12091 | 0.012 | 0.024 | * |
| **AGD2 vs AGD3** | 1.96371 | 0.08551 | 0.078 | 0.093 | |

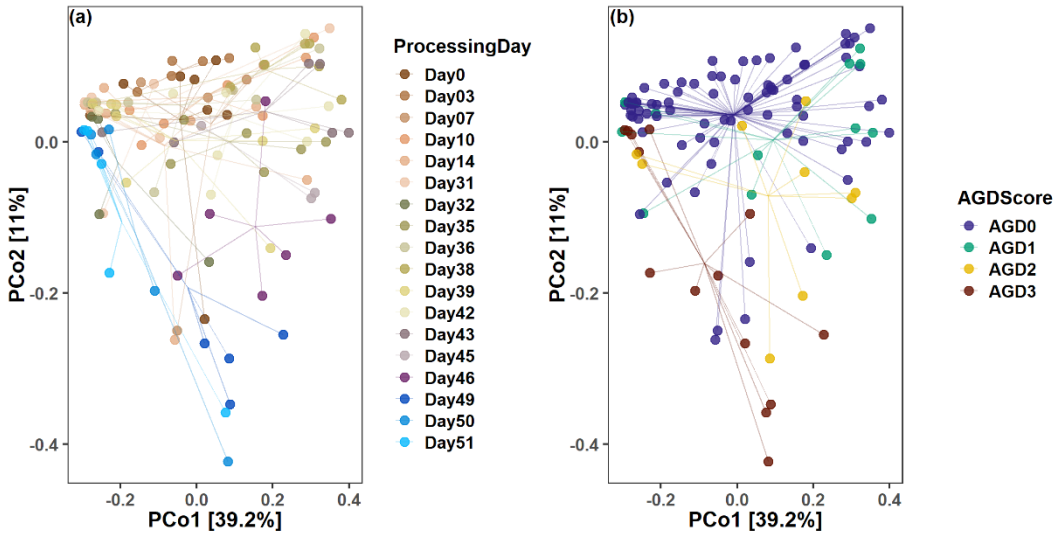


**Figure 4.7:** Principal coordinates analysis (PCoA) derived from weighted UniFrac distances among PC samples from starved fish. Samples are coloured according to the day when fish were dissected (a). Thereby, Day0 marks the start of the respirometry experiment. Figure (b) shows the same distribution, but here samples are coloured according to the associated AGD severity level. Connected lines mark the position of the different group centroids.

### 4.4.6 Differential abundance

We found for both PC and MG samples 45 taxa which differed significantly between the four AGD severity stages. *Psychrobium* showed the highest measure of importance in differential abundance for AGD severity score (Figure 4.8a, b). However, overall *Psychrobium* only shows a low relative abundance. In PC samples, *Aliivibrio*, *Marinomonas* and *Pseudoalteromonas* show a lower abundance in fish

with an AGD score of 3 compared to AGD 0, whereas *Polaribacter* and *Vibrio* show a higher abundance in symptomatic fish (Figure 4.8).

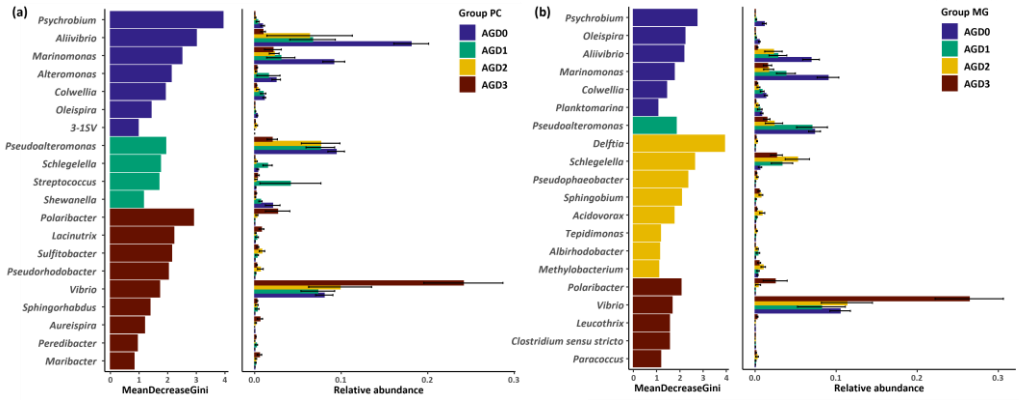


**Figure 4.8**: Top 20 differentially abundant genera in PC samples (a) and MG samples (b) in starved fish determined by random forest analysis. Mean Decrease Gini indicator represents the importance of each genus in distinguishing between AGD severity scores. Right side of each graph depicts the relative abundance of genera per AGD severity group. Error bars indicate standard deviation.

## 4.5 Discussion

All fish, regardless of genetic origin, showed a decline in Fulton's condition factor after transfer to the sea pens. A common observation since fish will often lose appetite on transfer from fresh to seawater (Usher et al., 1991). In addition, changes in food availability (ad libitum vs. maintenance feeding) and rising water temperatures accompanied by emerging AGD infections might have contributed to this reduction in condition. Surprisingly, wild fish gained on average more weight and length than hybrid and farmed fish after the sea pen transfer. However, wild fish also showed higher mortality. Size-selective mortality may explain the relative increase in size. Body size appears to have impacted the survival of the progeny of the farmed group to a lesser degree. There are several possible explanations for this observation. Firstly, farmed fish are genetically selected to maximize growth and are therefore bigger and heavier than their wild and hybrid counterparts (Glover et al., 2017). In

our study farmed fish showed on average the lowest percentage of water content. Since a low water content is negatively correlated with fat content (Elliott, 1976), one might hypothesise that farmed fish had more fat reserves and therefore potentially a higher chance of survival under our experimental conditions. In addition, farmed fish might have developed different coping mechanisms for stressors compared to wild and hybrid fish (reviewed by Castanheira et al., 2017), eventually leading to the observed differences in cohort mortalities.

Towards the end of the experiment fish had clear signs of AGD regardless of their genetic background. Fish with more severe AGD infections had on average a significantly lower standard metabolic rate and maximum metabolic rate compared to the non-symptomatic fish at the beginning of the experiment. ANCOVA revealed no significant differences in AS. At first glance this might be a surprising find. However, AS is calculated as the difference of MMR and SMR. Hence, if SMR and MMR "decline" at a similar rate, the AS value will still stay roughly consistent between groups. Respirometry uses oxygen consumption as a proxy for host metabolic rate (Lighton & Halsey, 2011). AGD infections likely lead to a decrease in oxygen absorption capacity of fish gills, resulting in lower metabolic rate measurements. Hvas et al., 2017 showed that AGD infected Atlantic salmon had significantly lower MMR and AS compared to healthy fish. They concluded that changes occurring in the gill because of an infection with *N. perurans* can lead to compromised gas exchange and ion regulation across the gills, potentially affecting appetite, growth and overall survival (Hvas et al., 2017). This finding corresponds with our findings of lower MMR values in AGD infected fish. However, we also noted a reduction in SMR. Previous studies have not detected any differences in SMR either

between healthy and sick fish with advanced signs of AGD (AGD Score of 4 and above; Hvas et al., 2017) or between different fish species (Dupont-Prinet et al., 2013; Petersen & Gamperl, 2010). In addition to the significant decline in SMR, we also observed a greater dispersion of metabolic rate measures in fish with no signs of infection. For symptomatic fish this dispersion declines. Individuals with high metabolic rates were rarely observed in symptomatic fish. AGD infections might have led to higher mortalities in fish with high SMR and/or MMR during the experiment, resulting in selection for fish with comparatively lower metabolic rates. Previous research has shown that a higher SMR can be beneficial when resources are plentiful, but detrimental should environmental conditions deteriorate (Metcalfe et al., 2016). This phenomenon is also consistent with the "allocation hypothesis" described in other animals. The allocation hypothesis suggests a fitness advantage for lower standard metabolic rates because of lower maintenance costs, which allows for the reallocation of energy towards growth and increased immune function (Downs et al., 2013; Larivée et al., 2010; Pettersen et al., 2016; Steyermark, 2002). Consequently, low oxygen intakes induced by AGD might lead to an earlier state of hypoxia and eventually death in fish with high metabolic rates compared to fish with a lower maintenance cost. To establish the true metabolic status of AGD-infected fish and the validity of the allocation hypothesis, it may be valuable to establish more direct measures of metabolic rate, for example via measures of muscular mitochondrial efficiency and ATP production (Salin et al., 2016, 2019). Such direct measures would establish whether AGD truly selects for fish with lower metabolic demands, rather than simply inducing a state of chronic hypoxia.

Metabolic rate differences among fish from different genetic backgrounds were not observed. Fish with a higher SMR have been shown to harvest energy from ingested food more rapidly, which could be reflected in a greater growth potential (Millidine et al., 2009). By this logic, we hypothesized that farmed fish should have a higher SMR than wild fish. In addition, in a common garden experiment (Lindsay, 2021) found higher AS among the progeny of wild fish from the Burrishoole catchment than the offspring of farmed fish, potentially due to differences in life history traits (Lindsay, 2021). It is possible that within the context of our common garden experiment, uniform environmental conditions counterbalanced potential genetic differences in metabolic phenotypes. Therefore, we assume a strong impact of the environment on a fish's metabolic profile. However, genetic effects might still be very important in the wild, especially in the context of evaluating fitness differences between wild and hybrid populations.

To accurately estimate metabolic rates fish were starved before going into respirometry. Whilst it is commonly accepted that diet can affect microbial community composition (H. Liu et al., 2016; Yukgehnaish et al., 2020) the prior feeding status of a fish is hardly considered when investigating gut microbial communities (Parris et al., 2019). However, due to the process of feeding and the consequent introduction of associated bacteria, an individual's observable microbiota at the time of sampling might be a carryover from the individual's last meal (Hehemann et al., 2010). The consequential variation makes a precise discrimination between drivers of community composition and noise extremely difficult. We found significant differences in gut microbial community composition between fed and starved fish. In fed fish three of the top ten most abundant taxa

likely originated from the environment. *Paracoccus* and *Pseudomonas* from feed samples and *Clade Ia* from the surrounding water. Since those taxa were not abundant in starved fish, we can assume they are allochthonous in origin. By comparing gut microbial community composition at two points in time 14 days apart we found in MG samples significant differences between fed fish but not between starved fish. It so seems that the presence of food increases the relative abundance of certain taxa ( e.g., *Fusobacteria*, *Psychryliobacter* and *Acinetobacter*), whereas the relative abundance of taxa, which are dominant in starved fish decreases (e.g., *Marinomonas*, *Vibrio* and *Pseudoalteromonas*). It is possible that the presence of food changes the nutritional niche within the gut, thereby favouring taxa which metabolise the introduced nutrients. In times of food sparsity those taxa are outcompeted by bacteria found in starved fish.. We conclude that in our experiment starved fish harbour mostly autochthonous bacteria and an investigation of changes in community composition will not be biased by fish feeding status. In addition, since there was no difference between gut microbial community composition in starved fish between T0 and T1 for neither gut compartment, short periods of food withdrawal do not seem to impose changes on overall gut microbial community composition. However, an observation over longer time periods is warranted to draw definite conclusions.

We used a common garden approach undertaken under farm conditions to evaluate the impact of AGD and co-occurring environmental factors on gut microbial community composition. By doing so we kept diet and environmental conditions constant for all fish throughout their life, thereby reducing the number of factors impacting host microbiota, opening the possibility to estimate the potential influence

of host genetics. In our experimental setup a fish's genetic background played only a minor role in explaining inter-individual differences. In midgut samples, we did find a significantly higher diversity in farmed fish compared to wild and hybrid-farmed-female fish. Interestingly, this finding was reversed when fish were affected by AGD. However, given the high variability between fish, the latter observation must be treated with care as we just had three samples per origin for that category. A big part of the observed variation in beta diversity could not be explained by environmental drivers. An expected result, since each individual has its own gut microbial ecosystem, which is impacted by its very own intrinsic dynamics (Vandeputte et al., 2021). Thereby, an individual's "ecosystem" is not only shaped by environmentally induced selective pressures but also by stochastic processes, like dispersal (e.g., the exchange of microbes with the environment) or drift (the natural occurrence of death, reproduction and replacement (Burns et al., 2016; Chase & Myers, 2011; Heys et al., 2020). Nevertheless, PERMANOVA testing also revealed that community composition differed by time (processing/sampling day) and a fish's AGD severity score, indicating a role of environmental factors to influence gut bacteria profiles. Rising water temperatures throughout the experiment might be a possible explanation for the time component. In ectotherms temperature is known to play a major role in shaping gut microbial communities (Givens, 2012; Hylander & Repasky, 2019; Kokou et al., 2018). All bacteria have optimal growing temperatures determined by thermodynamic limitations (Corkrey et al., 2012). It is therefore plausible that some taxa were affected by the temperature increase, consequently leading to a change in abundance due to changes in their relative ecological fitness. AGD severity score was also highly significant in explaining inter-individual

differences in community composition. In fish with an AGD severity score of 3 we observed significantly less microbial richness and evenness compared to non-symptomatic fish. This implies a decline in the overall number of taxa as well as a dominating effect resulting in fewer highly abundant taxa. In addition, beta diversity analysis showed a shift in taxa abundance between symptomatic and non-symptomatic fish. Differential abundance analysis in combination with correlation analysis showed abundance changes for several taxa among disease states. Thereby we observed a gradual decline in highly abundant taxa like *Aliivibrio*, *Marinomonas* and *Pseudoalteromonas*, whereas *Vibrio* increased in symptomatic fish. Perturbation of microbial communities due to fish disease has been described in several studies (Bozzi et al., 2021; Llewellyn et al., 2014, 2017; Vasemägi et al., 2017). In homeostatic conditions commensal bacteria can resist colonization or growth of potentially pathogenic taxa by occupying all available ecological niches (Lawley & Walker, 2013). However, if conditions change e.g., due to host stress or infection, the microbial barrier functions might be disturbed (Bozzi et al., 2021; Llewellyn et al., 2017). The degree of community perturbation might be linked to the host's stress tolerance e.g., maintaining appetite and immune function. It Is known that communities showing reduced diversity are less likely of having a species with an opposing trait towards an invader or pathogen (Fargione & Tilman, 2005; Levine & D'Antonio, 1999). Hence, we predict that a decline in diversity might have a negative impact on the ability of bacterial communities to prevent secondary infections. In addition, the replacement of taxa by few and highly abundant bacteria might result in a reduced capacity to digest a diverse diet, which could negatively impact fish growth and wellbeing (Makki et al., 2018). A question very relevant to aquaculture research would be to test if a

perturbation of microbial communities is reversible when host health improves (e.g., due to freshwater treatment against AGD infections) and to determine the long-term effects on fish growth. Finally, in the context of a warming future AGD infections might become an even more severe threat for captive and wild populations alike. Due to the inverse relationship between water temperature and water oxygen levels, fish hypoxia and stress are likely to increase. Coupled with an infectious disease like AGD implications for farmed and wild fish might be devastating.

## 4.6 Conclusion

This study provides a novel and multi-approach exploration of AGD infection on Atlantic salmon physiology. Wild fish showed a higher mortality relative to the progeny of hybrid or farmed fish. However, we could not determine categorically if vulnerability to AGD infection was a direct function of a genetically determined immunological response or some other factor associated with genetic background. It is just as likely that a fish's initial condition or size, also traits governed by genetic background, determines its chances of survival. We thereby assume that a combination of stressors, that include the initial saltwater transfer, the sea pen environment and AGD infections, contributed the higher mortality in the progeny of wild fish. We also show that infection with AGD limits the respiration capacity of fish, likely leading to hypoxia and health deterioration. Due to our findings, we hypothesize that high standard metabolic rates and associated high maintenance costs, negatively impact a fish's chances of survival when affected by AGD. Future research might address this question by using more direct measures of metabolic rates e.g., via measures of muscular mitochondrial efficiency and ATP production.

Future studies of gut microbial communities in fish should consider a fish's feeding status. We show that feeding promotes inter-individual differences, which might limit the explanatory power of a study depending on its context. Our study also shows an effect of AGD infections on gut microbial balance such that AGD infected fish had fewer but highly abundant taxa. Microbial communities that show a low diversity are less likely to have species with an opposing trait towards pathogens and might have a diminished capability to digest a diverse diet. Hence, a perturbation of gut microbial community composition might have severe implications for fish growth and general wellbeing, threatening farmed fish and wild populations alike. A question very relevant to aquacultural research would be to test if such a dysbiosis is reversible when host health improves (e.g., due to freshwater treatment against AGD infections) and to determine the long-term effects on fish growth.

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## 4.8 Appendix

### 4.8.1 Supplementary Figures

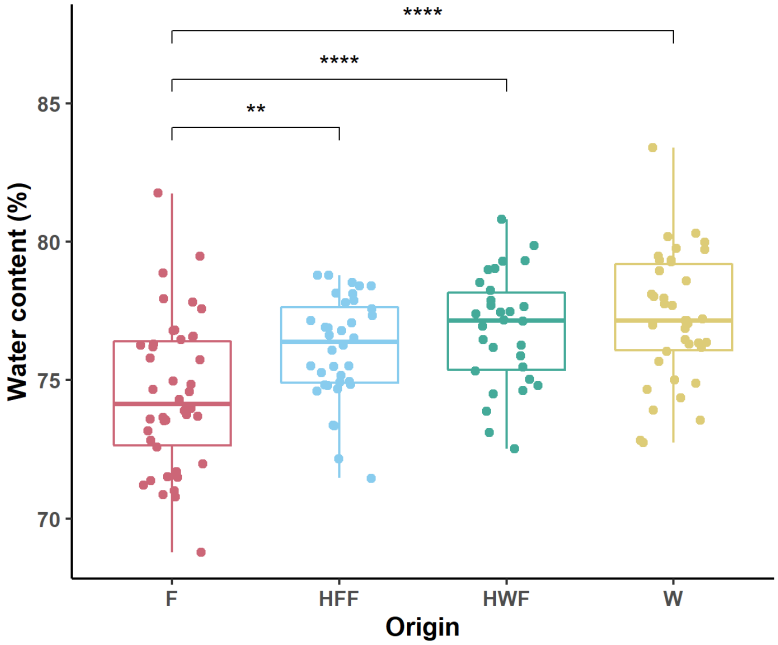


**Figure S 4.1:** Percent water content of fish from different origins during the sea pen experiments. Data derived from fish of mixed (4th) sentinel pen over the course of the experiment. Significance was determined by pairwise t-testing against fish from farmed origin. F= Farmed, HFF= Hybrid Farmed Female, HWF= Hybrid Wild Female, W=Wild, Significance codes: ***p< 0.001; **p< 0.01.

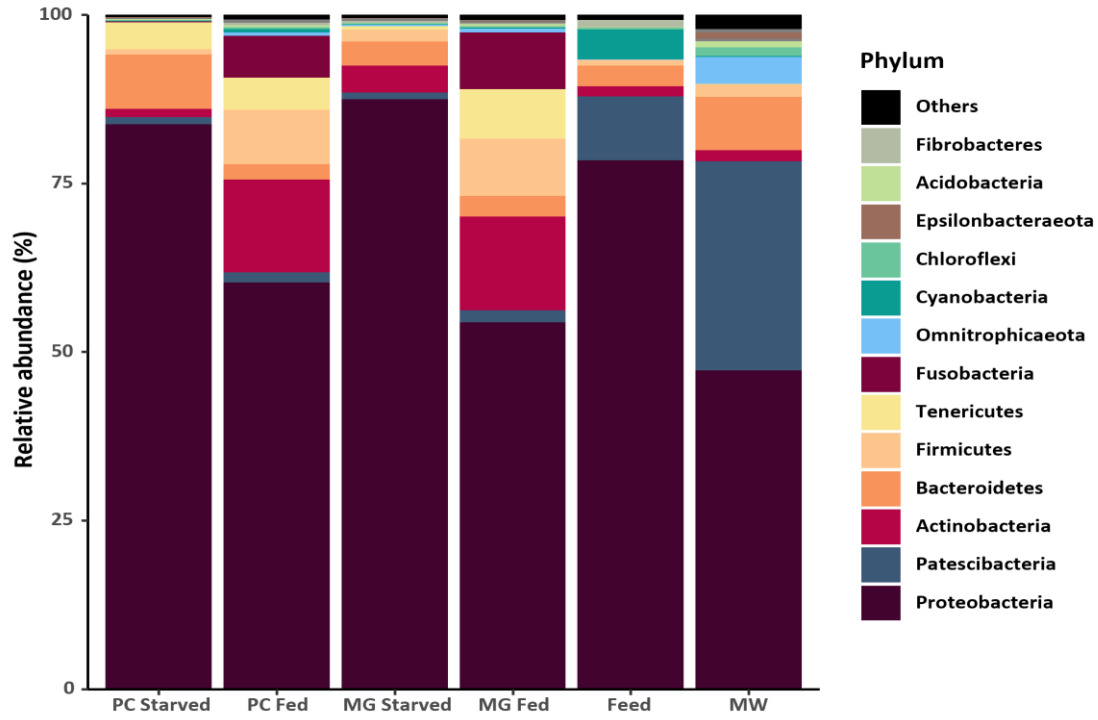


**Figure S 4.2:** Stacked bar plot showing the mean relative abundance of gut microbiota on phylum level for recently fed fish, starved fish (48 h feed withdrawal) and environmental control samples (feed and marine water (MW)).

### 4.8.2 Supplementary Tables

**Table S 4.1:** Overview of the metabolic rate experiment. Sample_Id refers to individual fish. Sampling day refers to the day when fish were caught. Processing day refers to the day fish were dissected. Experiment day equals processing days, only in another format. Temperature refers to the water temperature at the sea pens on each sampling day.

| Sample_ID | Sampling Day | Processing Day | Experiment Day | Temperature [°C] |
|---|---|---|---|---|
| **H18D_1000** | 11/06/2019 | 14/06/2019 | Day0 | 14.17 |
| **H18D_1001** | 11/06/2019 | 14/06/2019 | Day0 | 14.17 |
| **H18D_1002** | 11/06/2019 | 14/06/2019 | Day0 | 14.17 |
| **H18D_1003** | 11/06/2019 | 14/06/2019 | Day0 | 14.17 |
| **H18D_1004** | 11/06/2019 | 14/06/2019 | Day0 | 14.17 |
| **H18D_1005** | 11/06/2019 | 14/06/2019 | Day0 | 14.17 |
| **H18D_1006** | 11/06/2019 | 14/06/2019 | Day0 | 14.17 |
| **H18D_1007** | 11/06/2019 | 14/06/2019 | Day0 | 14.17 |
| **H18D_1008** | 14/06/2019 | 17/06/2019 | Day03 | 13.62 |
| **H18D_1009** | 14/06/2019 | 17/06/2019 | Day03 | 13.62 |
| **H18D_1010** | 14/06/2019 | 17/06/2019 | Day03 | 13.62 |
| **H18D_1011** | 14/06/2019 | 17/06/2019 | Day03 | 13.62 |

| | | | | |
|---|---|---|---|---|
| **H18D_1012** | 14/06/2019 | 17/06/2019 | Day03 | 13.62 |
| **H18D_1013** | 14/06/2019 | 17/06/2019 | Day03 | 13.62 |
| **H18D_1014** | 14/06/2019 | 17/06/2019 | Day03 | 13.62 |
| **H18D_1015** | 14/06/2019 | 17/06/2019 | Day03 | 13.62 |
| **H18D_1016** | 18/06/2019 | 21/06/2019 | Day07 | 14.22 |
| **H18D_1017** | 18/06/2019 | 21/06/2019 | Day07 | 14.22 |
| **H18D_1018** | 18/06/2019 | 21/06/2019 | Day07 | 14.22 |
| **H18D_1019** | 18/06/2019 | 21/06/2019 | Day07 | 14.22 |
| **H18D_1020** | 18/06/2019 | 21/06/2019 | Day07 | 14.22 |
| **H18D_1021** | 18/06/2019 | 21/06/2019 | Day07 | 14.22 |
| **H18D_1022** | 18/06/2019 | 21/06/2019 | Day07 | 14.22 |
| **H18D_1023** | 21/06/2019 | 24/06/2019 | Day10 | 14.83 |
| **H18D_1024** | 21/06/2019 | 24/06/2019 | Day10 | 14.83 |
| **H18D_1025** | 21/06/2019 | 24/06/2019 | Day10 | 14.83 |
| **H18D_1026** | 21/06/2019 | 24/06/2019 | Day10 | 14.83 |
| **H18D_1027** | 21/06/2019 | 24/06/2019 | Day10 | 14.83 |
| **H18D_1028** | 21/06/2019 | 24/06/2019 | Day10 | 14.83 |
| **H18D_1029** | 21/06/2019 | 24/06/2019 | Day10 | 14.83 |
| **H18D_1030** | 21/06/2019 | 24/06/2019 | Day10 | 14.83 |
| **H18D_1031** | 25/06/2019 | 28/06/2019 | Day14 | 14.95 |
| **H18D_1032** | 25/06/2019 | 28/06/2019 | Day14 | 14.95 |
| **H18D_1033** | 25/06/2019 | 28/06/2019 | Day14 | 14.95 |
| **H18D_1034** | 25/06/2019 | 28/06/2019 | Day14 | 14.95 |
| **H18D_1035** | 25/06/2019 | 28/06/2019 | Day14 | 14.95 |
| **H18D_1036** | 25/06/2019 | 28/06/2019 | Day14 | 14.95 |
| **H18D_1037** | 25/06/2019 | 28/06/2019 | Day14 | 14.95 |
| **H18D_1038** | 25/06/2019 | 28/06/2019 | Day14 | 14.95 |
| **H18D_1039** | 12/07/2019 | 14/07/2019 | Day31 | 16.53 |
| **H18D_1040** | 12/07/2019 | 14/07/2019 | Day31 | 16.53 |
| **H18D_1041** | 12/07/2019 | 14/07/2019 | Day31 | 16.53 |
| **H18D_1042** | 12/07/2019 | 14/07/2019 | Day31 | 16.53 |
| **H18D_1043** | 12/07/2019 | 14/07/2019 | Day31 | 16.53 |
| **H18D_1044** | 12/07/2019 | 14/07/2019 | Day31 | 16.53 |
| **H18D_1045** | 12/07/2019 | 14/07/2019 | Day31 | 16.53 |
| **H18D_1046** | 12/07/2019 | 15/07/2019 | Day32 | 16.53 |
| **H18D_1047** | 12/07/2019 | 15/07/2019 | Day32 | 16.53 |
| **H18D_1048** | 12/07/2019 | 15/07/2019 | Day32 | 16.53 |
| **H18D_1049** | 12/07/2019 | 15/07/2019 | Day32 | 16.53 |
| **H18D_1050** | 12/07/2019 | 15/07/2019 | Day32 | 16.53 |
| **H18D_1051** | 12/07/2019 | 15/07/2019 | Day32 | 16.53 |
| **H18D_1052** | 16/07/2019 | 18/07/2019 | Day35 | 17.13 |
| **H18D_1053** | 16/07/2019 | 18/07/2019 | Day35 | 17.13 |
| **H18D_1054** | 16/07/2019 | 18/07/2019 | Day35 | 17.13 |
| **H18D_1055** | 16/07/2019 | 18/07/2019 | Day35 | 17.13 |
| **H18D_1056** | 16/07/2019 | 18/07/2019 | Day35 | 17.13 |
| **H18D_1057** | 16/07/2019 | 18/07/2019 | Day35 | 17.13 |

| | | | | |
|---|---|---|---|---|
| **H18D_1058** | 16/07/2019 | 18/07/2019 | Day35 | 17.13 |
| **H18D_1059** | 16/07/2019 | 18/07/2019 | Day35 | 17.13 |
| **H18D_1060** | 16/07/2019 | 18/07/2019 | Day35 | 17.13 |
| **H18D_1061** | 16/07/2019 | 19/07/2019 | Day36 | 17.13 |
| **H18D_1062** | 16/07/2019 | 19/07/2019 | Day36 | 17.13 |
| **H18D_1063** | 16/07/2019 | 19/07/2019 | Day36 | 17.13 |
| **H18D_1064** | 16/07/2019 | 19/07/2019 | Day36 | 17.13 |
| **H18D_1065** | 16/07/2019 | 19/07/2019 | Day36 | 17.13 |
| **H18D_1066** | 16/07/2019 | 19/07/2019 | Day36 | 17.13 |
| **H18D_1067** | 16/07/2019 | 19/07/2019 | Day36 | 17.13 |
| **H18D_1068** | 16/07/2019 | 19/07/2019 | Day36 | 17.13 |
| **H18D_1069** | 19/07/2019 | 21/07/2019 | Day38 | 17.31 |
| **H18D_1070** | 19/07/2019 | 21/07/2019 | Day38 | 17.31 |
| **H18D_1071** | 19/07/2019 | 21/07/2019 | Day38 | 17.31 |
| **H18D_1072** | 19/07/2019 | 21/07/2019 | Day38 | 17.31 |
| **H18D_1073** | 19/07/2019 | 21/07/2019 | Day38 | 17.31 |
| **H18D_1074** | 19/07/2019 | 21/07/2019 | Day38 | 17.31 |
| **H18D_1075** | 19/07/2019 | 21/07/2019 | Day38 | 17.31 |
| **H18D_1076** | 19/07/2019 | 21/07/2019 | Day38 | 17.31 |
| **H18D_1077** | 19/07/2019 | 22/07/2019 | Day39 | 17.31 |
| **H18D_1078** | 19/07/2019 | 22/07/2019 | Day39 | 17.31 |
| **H18D_1079** | 19/07/2019 | 22/07/2019 | Day39 | 17.31 |
| **H18D_1080** | 19/07/2019 | 22/07/2019 | Day39 | 17.31 |
| **H18D_1081** | 19/07/2019 | 22/07/2019 | Day39 | 17.31 |
| **H18D_1082** | 19/07/2019 | 22/07/2019 | Day39 | 17.31 |
| **H18D_1083** | 19/07/2019 | 22/07/2019 | Day39 | 17.31 |
| **H18D_1084** | 19/07/2019 | 22/07/2019 | Day39 | 17.31 |
| **H18D_1085** | 23/07/2019 | 25/07/2019 | Day42 | 17.73 |
| **H18D_1086** | 23/07/2019 | 25/07/2019 | Day42 | 17.73 |
| **H18D_1087** | 23/07/2019 | 25/07/2019 | Day42 | 17.73 |
| **H18D_1088** | 23/07/2019 | 25/07/2019 | Day42 | 17.73 |
| **H18D_1089** | 23/07/2019 | 25/07/2019 | Day42 | 17.73 |
| **H18D_1090** | 23/07/2019 | 25/07/2019 | Day42 | 17.73 |
| **H18D_1091** | 23/07/2019 | 25/07/2019 | Day42 | 17.73 |
| **H18D_1092** | 23/07/2019 | 25/07/2019 | Day42 | 17.73 |
| **H18D_1093** | 23/07/2019 | 26/07/2019 | Day43 | 17.73 |
| **H18D_1094** | 23/07/2019 | 26/07/2019 | Day43 | 17.73 |
| **H18D_1095** | 23/07/2019 | 26/07/2019 | Day43 | 17.73 |
| **H18D_1096** | 23/07/2019 | 26/07/2019 | Day43 | 17.73 |
| **H18D_1097** | 23/07/2019 | 26/07/2019 | Day43 | 17.73 |
| **H18D_1098** | 23/07/2019 | 26/07/2019 | Day43 | 17.73 |
| **H18D_1099** | 23/07/2019 | 26/07/2019 | Day43 | 17.73 |
| **H18D_1100** | 23/07/2019 | 26/07/2019 | Day43 | 17.73 |
| **H18D_1101** | 26/07/2019 | 28/07/2019 | Day45 | 18.25 |
| **H18D_1102** | 26/07/2019 | 28/07/2019 | Day45 | 18.25 |
| **H18D_1103** | 26/07/2019 | 28/07/2019 | Day45 | 18.25 |

| | | | | |
|---|---|---|---|---|
| **H18D_1104** | 26/07/2019 | 28/07/2019 | Day45 | 18.25 |
| **H18D_1105** | 26/07/2019 | 28/07/2019 | Day45 | 18.25 |
| **H18D_1106** | 26/07/2019 | 28/07/2019 | Day45 | 18.25 |
| **H18D_1107** | 26/07/2019 | 28/07/2019 | Day45 | 18.25 |
| **H18D_1108** | 26/07/2019 | 28/07/2019 | Day45 | 18.25 |
| **H18D_1109** | 26/07/2019 | 29/07/2019 | Day46 | 18.25 |
| **H18D_1110** | 26/07/2019 | 29/07/2019 | Day46 | 18.25 |
| **H18D_1111** | 26/07/2019 | 29/07/2019 | Day46 | 18.25 |
| **H18D_1112** | 26/07/2019 | 29/07/2019 | Day46 | 18.25 |
| **H18D_1113** | 26/07/2019 | 29/07/2019 | Day46 | 18.25 |
| **H18D_1114** | 26/07/2019 | 29/07/2019 | Day46 | 18.25 |
| **H18D_1115** | 26/07/2019 | 29/07/2019 | Day46 | 18.25 |
| **H18D_1116** | 26/07/2019 | 29/07/2019 | Day46 | 18.25 |
| **H18D_1117** | 26/07/2019 | 30/07/2019 | Day47 | 18.25 |
| **H18D_1118** | 26/07/2019 | 30/07/2019 | Day47 | 18.25 |
| **H18D_1119** | 26/07/2019 | 30/07/2019 | Day47 | 18.25 |
| **H18D_1120** | 26/07/2019 | 30/07/2019 | Day47 | 18.25 |
| **H18D_1121** | 26/07/2019 | 30/07/2019 | Day47 | 18.25 |
| **H18D_1122** | 30/07/2019 | 01/08/2019 | Day49 | 18.26 |
| **H18D_1123** | 30/07/2019 | 01/08/2019 | Day49 | 18.26 |
| **H18D_1124** | 30/07/2019 | 01/08/2019 | Day49 | 18.26 |
| **H18D_1125** | 30/07/2019 | 01/08/2019 | Day49 | 18.26 |
| **H18D_1126** | 30/07/2019 | 01/08/2019 | Day49 | 18.26 |
| **H18D_1127** | 30/07/2019 | 01/08/2019 | Day49 | 18.26 |
| **H18D_1128** | 30/07/2019 | 01/08/2019 | Day49 | 18.26 |
| **H18D_1129** | 30/07/2019 | 01/08/2019 | Day49 | 18.26 |
| **H18D_1130** | 30/07/2019 | 02/08/2019 | Day50 | 18.26 |
| **H18D_1131** | 30/07/2019 | 02/08/2019 | Day50 | 18.26 |
| **H18D_1132** | 30/07/2019 | 02/08/2019 | Day50 | 18.26 |
| **H18D_1133** | 30/07/2019 | 02/08/2019 | Day50 | 18.26 |
| **H18D_1134** | 30/07/2019 | 02/08/2019 | Day50 | 18.26 |
| **H18D_1135** | 30/07/2019 | 02/08/2019 | Day50 | 18.26 |
| **H18D_1136** | 30/07/2019 | 02/08/2019 | Day50 | 18.26 |
| **H18D_1137** | 30/07/2019 | 02/08/2019 | Day50 | 18.26 |
| **H18D_1138** | 30/07/2019 | 03/08/2019 | Day50 | 18.26 |
| **H18D_1139** | 30/07/2019 | 03/08/2019 | Day50 | 18.26 |
| **H18D_1140** | 30/07/2019 | 03/08/2019 | Day50 | 18.26 |
| **H18D_1141** | 30/07/2019 | 03/08/2019 | Day50 | 18.26 |
| **H18D_1142** | 30/07/2019 | 03/08/2019 | Day50 | 18.26 |
| **H18D_1143** | 30/07/2019 | 03/08/2019 | Day50 | 18.26 |
| **H18D_1144** | 30/07/2019 | 03/08/2019 | Day50 | 18.26 |
| **H18D_1145** | 30/07/2019 | 03/08/2019 | Day50 | 18.26 |

**Table S 4.2:** First round PCR primers used for NGS library preparation.

| Primer | Label | Illumina 5' sequencing primer (CS1/CS2) | Internal index length | Heterogeneity spacer | spacer 16S rRNA gene v1 primer |
|---|---|---|---|---|---|
| **Fwd** | 27F | ACACTCTTTCCCTACACGACGCTCTTCCGATCT | 8bp | NNNN or NNN (5/3bp) | AGAGTTTGATCMTGGCTCAG |
| **Rev** | 338R | GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT | 8bp | none | GCTGCCTCCCGTAGGAGT |

**Table S 4.3:** Second round PCR primers used for NGS library preparation.

| Primer | Illumina 3' flow cell linker (i5/i7) | External index length | Illumina 5' sequencing primer (CS1/CS2) |
|---|---|---|---|
| **Fwd** | AATGATACGGCGACCACCGAGATC TACAC | 8bp | CACTCTTTCCCTACACGACGCT |
| **Rev** | CAAGCAGAAGACGGCATACGAGAT | 8bp | GTGACTGGAGTTCAGACGTGTGCTC |

**Table S 4.4:** Mean length, weight and Fulton's condition factor per genetic origin for 3 points in time (pre sea pen hatchery, date of sea pen transfer and at the termination point of the experiment)

| Points in time | Origin | Length (mm) +/- SD | Weight (g) +/-SD | Fulton's K |
|---|---|---|---|---|
| **Hatchery (27.03.2019)** | Farmed | 200.10 +/- 19.07 | 94.45 +/- 25.12 | 1.17 |
| | Hybrid | 175.64 +/- 13.12 | 61.84 +/- 14.37 | 1.14 |
| | Wild | 160.90 +/- 11.50 | 46.18 +/- 10.23 | 1.10 |
| **Sea Pen Transfer (09.05.2019)** | Farmed | 235.70 +/- 18.07 | 137.60 +/- 28.67 | 1.05 |
| | Hybrid | 191.85 +/- 23.78 | 78.04 +/- 21.08 | 1.10 |
| | Wild | 173.00 +/- 25.51 | 56.28 +/- 21.38 | 1.08 |
| **End of experiment (30.07.2019)** | Farmed | 246.34 +/- 18.75 | 135.44 +/- 37.50 | 0.90 |
| | Hybrid | 214.46 +/- 16.13 | 84.76 +/- 21.69 | 0.85 |
| | Wild | 204.55 +/- 12.81 | 70.35 +/- 14.36 | 0.82 |

**Table S 4.5:** ANCOVA results of the effect of AGD severity Score on weight adjusted SMR (left), MMR (middle) and AS (right) measures. Significance codes: ***< 0.001; **< 0.01; *< 0.05.

| | | **SMR** | | | **MMR** | | | **AS** | |
|---|---|---|---|---|---|---|---|---|---|
| | Sum Sq | F value | Sig. | Sum Sq | F value | Sign. | Sum Sq | F value | Sign. |
| **(Intercept)** | 7.4 | 1060.75 | *** | 14.34 | 1125.61 | *** | 12.48 | 602.1 | *** |
| **log.weight** | 1.99 | 283.79 | *** | 1.877 | 147.355 | *** | 1.71 | 82.86 | *** |
| **AGDScore** | 0.15 | 7.38 | *** | 0.147 | 3.8611 | * | 0.14 | 2.26 | |
| **Residuals** | 0.82 | | | 1.554 | | | 2.50 | | |

**Table S 4.6:** Multiple comparisons of means of metabolic rate measures between different AGD severity scores (Tukey post-hoc for weight-adjusted model). Significance codes: **< 0.01; *< 0.05.

| | | **SMR** | | | **MMR** | | | **AS** | |
|---|---|---|---|---|---|---|---|---|---|
| **Coeff.** | Estimate | Pr(>\|t\|) | Sign. | Estimate | Pr(>\|t\|) | Sign. | Estimate | Pr(>\|t\|) | Sig. |
| **AGD 0: AGD 1** | -0.0252 | 0.688 | | 0.0087 | 0.991 | | 0.0240 | 0.924 | |
| **AGD 0: AGD 2** | -0.0872 | 0.002 | ** | -0.0695 | 0.132 | | -0.0553 | 0.523 | |
| **AGD 0: AGD 3** | -0.0765 | 0.002 | ** | -0.0745 | 0.033 | * | -0.0722 | 0.159 | |
| **AGD 1: AGD 2** | -0.0622 | 0.170 | | -0.0783 | 0.203 | | -0.0794 | 0.399 | |
| **AGD 1: AGD 3** | -0.0322 | 0.636 | | -0.0832 | 0.099 | | -0.0963 | 0.158 | |
| **AGD 2: AGD 3** | 0.0299 | 0.713 | | -0.0049 | 0.999 | | -0.0169 | 0.984 | |

**Table S 4.7:** PERMANOVA results showing differences between gut microbial community compositions of starved and fed fish for two different timepoints T0 and T1 in PC samples. Significance codes: **< 0.01; *< 0.05.

| **Groups** | **F** | **R2** | **p.value** | **p.adjusted** | **Significance** |
|---|---|---|---|---|---|
| **T0Fed vs T1Fed** | 1.817 | 0.132 | 0.078 | 0.094 | |
| **T0Fed vs T0Starved** | 12.588 | 0.473 | 0.001 | 0.003 | ** |
| **T0Fed vs T1Starved** | 7.932 | 0.398 | 0.001 | 0.003 | ** |

| | | | | | |
|---|---|---|---|---|---|
| **T1Fed vs T0Starved** | 10.960 | 0.477 | 0.002 | 0.004 | ** |
| **T1Fed vs T1Starved** | 6.944 | 0.410 | 0.003 | 0.005 | ** |
| **T0Starved vs T1Starved** | 0.973 | 0.075 | 0.489 | 0.489 | |

**Table S 4.8:** PERMANOVA results showing differences between gut microbial community compositions of starved and fed fish for two different timepoints T0 and T1 in MG samples. Significance codes: **< 0.01; *< 0.05.

| Groups | F | R2 | p.value | p.adjusted | Significance |
|---|---|---|---|---|---|
| **T0Fed vs T1Fed** | 2.208 | 0.145 | 0.024 | 0.029 | * |
| **T0Fed vs T0Starved** | 11.593 | 0.436 | 0.001 | 0.002 | ** |
| **T0Fed vs T1Starved** | 7.975 | 0.380 | 0.001 | 0.002 | ** |
| **T1Fed vs T0Starved** | 13.785 | 0.535 | 0.001 | 0.002 | ** |
| **T1Fed vs T1Starved** | 10.265 | 0.507 | 0.006 | 0.009 | ** |
| **T0Starved vs T1Starved** | 1.303 | 0.098 | 0.276 | 0.276 | |

**Table S 4.9:** Linear model for Chao1 richness estimates in MG samples calculated by the interaction term of AGD severity score and genetic origin. AGD severity of 0 and farmed origin served as baseline for comparisons. Significance codes: ***< 0.001; **< 0.01; *< 0.05.

| Coefficients | Estimate | Std. Error | t value | Pr(>\|t\|) | Sign. |
|---|---|---|---|---|---|
| **(Intercept)** | 324.71 | 16.396 | 19.804 | < 2e-16 | *** |
| **AGDScore 1** | 3.968 | 44.903 | 0.088 | 0.92974 | |
| **AGDScore 2** | -75.249 | 50.978 | -1.476 | 0.14253 | |
| **AGDScore 3** | -158.505 | 50.978 | -3.109 | 0.00234 | ** |
| **Origin HFF** | -57.854 | 24.219 | -2.389 | 0.01846 | * |
| **Origin HWF** | -37.119 | 26.565 | -1.397 | 0.1649 | |
| **Origin W** | -54.733 | 23.932 | -2.287 | 0.02395 | * |
| **AGDScore 1: Origin HFF** | -18.122 | 61.089 | -0.297 | 0.76725 | |
| **AGDScore 2: Origin HFF** | 88.655 | 80.071 | 1.107 | 0.27042 | |
| **AGDScore 3: Origin HFF** | 83.501 | 62.57 | 1.335 | 0.18456 | |
| **AGDScore 1: Origin HWF** | -27.902 | 77.123 | -0.362 | 0.71815 | |
| **AGDScore 2: Origin HWF** | 11.971 | 64.812 | 0.185 | 0.85377 | |
| **AGDScore 3: Origin HWF** | 187.101 | 69.159 | 2.705 | 0.00782 | ** |
| **AGDScore 1: Origin W** | -75.08 | 68.191 | -1.101 | 0.27309 | |
| **AGDScore 2: Origin W** | -13.624 | 65.579 | -0.208 | 0.83577 | |
| **AGDScore 3: Origin W** | 136.461 | 65.579 | 2.081 | 0.03957 | * |

**Table S 4.10:** Permutational analysis of variance (PERMANOVA) results testing the effect of AGD severity score, genetic origin and processing day (day of gut dissection)

on pyloric caeca (PC) microbial community composition based on weighted unifrac distance matrices.

| | Df | SumsOfSqs | MeanSqs | F.Model | $R^2$ | Pr(>F) | Significance |
|---|---|---|---|---|---|---|---|
| **Origin** | 3 | 0.3681 | 0.12268 | 1.3178 | 0.02693 | 0.1597 | |
| **AGDScore** | 3 | 1.2882 | 0.42941 | 4.6123 | 0.09426 | 0.0001 | *** |
| **ProcessingDay** | 17 | 3.4451 | 0.20265 | 2.1767 | 0.25208 | 0.0001 | *** |
| **Residuals** | 92 | 8.5652 | 0.0931 | | 0.62672 | | |
| **Total** | 115 | 13.6666 | | | 1 | | |

**Table S 4.11:** PERMANOVA of pairwise comparisons of PC samples grouped by day of their processing. Distance matrix calculated by weighted UniFrac measure. Permutations used: 9999. Significance codes: ***< 0.001; **< 0.01; *< 0.05.

| Groups | F | R2 | p.value | p.adjusted | Significance |
|---|---|---|---|---|---|
| | | | | | |
| **Day0 vs Day03** | 1.5352 | 0.1225 | 0.143 | 0.179 | |
| **Day0 vs Day07** | 0.9736 | 0.0750 | 0.465 | 0.474 | |
| **Day0 vs Day10** | 2.4742 | 0.1502 | 0.049 | 0.084 | |
| **Day0 vs Day14** | 1.1710 | 0.0772 | 0.324 | 0.344 | |
| **Day0 vs Day31** | 2.3879 | 0.1552 | 0.033 | 0.070 | |
| **Day0 vs Day32** | 2.7732 | 0.2013 | 0.047 | 0.082 | |
| **Day0 vs Day35** | 3.0856 | 0.1918 | 0.012 | 0.047 | * |
| **Day0 vs Day36** | 1.0803 | 0.0767 | 0.336 | 0.352 | |
| **Day0 vs Day38** | 3.9314 | 0.2192 | 0.002 | 0.045 | * |
| **Day0 vs Day39** | 1.9212 | 0.1207 | 0.112 | 0.149 | |
| **Day0 vs Day42** | 3.0127 | 0.1771 | 0.015 | 0.048 | * |
| **Day0 vs Day43** | 4.9092 | 0.2903 | 0.008 | 0.045 | * |
| **Day0 vs Day45** | 4.3044 | 0.3235 | 0.016 | 0.050 | * |
| **Day0 vs Day46** | 5.7595 | 0.3243 | 0.001 | 0.045 | * |
| **Day0 vs Day49** | 4.5736 | 0.2760 | 0.003 | 0.045 | * |
| **Day0 vs Day50** | 2.9574 | 0.2119 | 0.013 | 0.047 | * |
| **Day0 vs Day51** | 4.1226 | 0.2726 | 0.011 | 0.045 | * |
| **Day03 vs Day07** | 1.5755 | 0.1490 | 0.073 | 0.115 | |
| **Day03 vs Day10** | 2.5267 | 0.1868 | 0.041 | 0.077 | |
| **Day03 vs Day14** | 2.2803 | 0.1717 | 0.051 | 0.087 | |
| **Day03 vs Day31** | 2.5915 | 0.2058 | 0.037 | 0.074 | |
| **Day03 vs Day32** | 4.9486 | 0.3822 | 0.014 | 0.048 | * |
| **Day03 vs Day35** | 4.5657 | 0.3135 | 0.005 | 0.045 | * |
| **Day03 vs Day36** | 1.4466 | 0.1264 | 0.220 | 0.250 | |
| **Day03 vs Day38** | 4.3128 | 0.2816 | 0.015 | 0.048 | * |
| **Day03 vs Day39** | 2.0583 | 0.1576 | 0.097 | 0.135 | |
| **Day03 vs Day42** | 3.8761 | 0.2606 | 0.010 | 0.045 | * |
| **Day03 vs Day43** | 4.0509 | 0.3104 | 0.037 | 0.074 | |
| **Day03 vs Day45** | 5.1336 | 0.4611 | 0.008 | 0.045 | * |

| | | | | | |
|---|---|---|---|---|---|
| **Day03 vs Day46** | 5.2137 | 0.3668 | 0.002 | 0.045 | * |
| **Day03 vs Day49** | 5.7744 | 0.3908 | 0.013 | 0.047 | * |
| **Day03 vs Day50** | 3.8175 | 0.3230 | 0.012 | 0.047 | * |
| **Day03 vs Day51** | 5.8675 | 0.4231 | 0.009 | 0.045 | * |
| **Day07 vs Day10** | 1.6207 | 0.1190 | 0.149 | 0.181 | |
| **Day07 vs Day14** | 1.5978 | 0.1175 | 0.131 | 0.168 | |
| **Day07 vs Day31** | 2.8324 | 0.2048 | 0.031 | 0.070 | |
| **Day07 vs Day32** | 3.7058 | 0.2917 | 0.028 | 0.070 | |
| **Day07 vs Day35** | 2.2914 | 0.1724 | 0.033 | 0.070 | |
| **Day07 vs Day36** | 1.0918 | 0.0903 | 0.348 | 0.362 | |
| **Day07 vs Day38** | 2.5783 | 0.1769 | 0.039 | 0.076 | |
| **Day07 vs Day39** | 1.7671 | 0.1284 | 0.118 | 0.154 | |
| **Day07 vs Day42** | 2.2628 | 0.1587 | 0.033 | 0.070 | |
| **Day07 vs Day43** | 2.9900 | 0.2302 | 0.030 | 0.070 | |
| **Day07 vs Day45** | 2.8454 | 0.2890 | 0.018 | 0.053 | |
| **Day07 vs Day46** | 3.7716 | 0.2739 | 0.003 | 0.045 | * |
| **Day07 vs Day49** | 3.8465 | 0.2778 | 0.009 | 0.045 | * |
| **Day07 vs Day50** | 3.1367 | 0.2584 | 0.008 | 0.045 | * |
| **Day07 vs Day51** | 4.3731 | 0.3270 | 0.008 | 0.045 | * |
| **Day10 vs Day14** | 1.4252 | 0.0924 | 0.200 | 0.230 | |
| **Day10 vs Day31** | 1.9501 | 0.1304 | 0.144 | 0.179 | |
| **Day10 vs Day32** | 3.0422 | 0.2166 | 0.046 | 0.081 | |
| **Day10 vs Day35** | 2.9741 | 0.1862 | 0.033 | 0.070 | |
| **Day10 vs Day36** | 1.6349 | 0.1117 | 0.167 | 0.197 | |
| **Day10 vs Day38** | 3.0795 | 0.1803 | 0.025 | 0.068 | |
| **Day10 vs Day39** | 1.7370 | 0.1104 | 0.149 | 0.181 | |
| **Day10 vs Day42** | 2.5770 | 0.1555 | 0.040 | 0.077 | |
| **Day10 vs Day43** | 2.1912 | 0.1544 | 0.092 | 0.129 | |
| **Day10 vs Day45** | 2.4604 | 0.2147 | 0.077 | 0.118 | |
| **Day10 vs Day46** | 3.3144 | 0.2164 | 0.011 | 0.045 | * |
| **Day10 vs Day49** | 4.0095 | 0.2504 | 0.007 | 0.045 | * |
| **Day10 vs Day50** | 2.8555 | 0.2061 | 0.031 | 0.070 | |
| **Day10 vs Day51** | 3.5398 | 0.2435 | 0.027 | 0.069 | |
| **Day14 vs Day31** | 1.0922 | 0.0775 | 0.298 | 0.326 | |
| **Day14 vs Day32** | 1.2409 | 0.1014 | 0.221 | 0.250 | |
| **Day14 vs Day35** | 2.6096 | 0.1672 | 0.045 | 0.080 | |
| **Day14 vs Day36** | 1.1270 | 0.0798 | 0.268 | 0.297 | |
| **Day14 vs Day38** | 3.9922 | 0.2219 | 0.013 | 0.047 | * |
| **Day14 vs Day39** | 0.8313 | 0.0561 | 0.416 | 0.427 | |
| **Day14 vs Day42** | 2.2595 | 0.1390 | 0.078 | 0.118 | |
| **Day14 vs Day43** | 3.6594 | 0.2337 | 0.027 | 0.069 | |
| **Day14 vs Day45** | 3.3503 | 0.2713 | 0.019 | 0.054 | |
| **Day14 vs Day46** | 3.9976 | 0.2499 | 0.006 | 0.045 | * |
| **Day14 vs Day49** | 2.6480 | 0.1808 | 0.053 | 0.088 | |

| | | | | | |
|---|---|---|---|---|---|
| **Day14 vs Day50** | 1.5705 | 0.1249 | 0.146 | 0.180 | |
| **Day14 vs Day51** | 1.7792 | 0.1392 | 0.121 | 0.157 | |
| **Day31 vs Day32** | 0.7709 | 0.0716 | 0.613 | 0.617 | |
| **Day31 vs Day35** | 3.8317 | 0.2420 | 0.032 | 0.070 | |
| **Day31 vs Day36** | 1.2211 | 0.0924 | 0.280 | 0.308 | |
| **Day31 vs Day38** | 5.3988 | 0.2934 | 0.010 | 0.045 | * |
| **Day31 vs Day39** | 1.1236 | 0.0796 | 0.313 | 0.337 | |
| **Day31 vs Day42** | 3.5564 | 0.2148 | 0.032 | 0.070 | |
| **Day31 vs Day43** | 3.7913 | 0.2563 | 0.039 | 0.076 | |
| **Day31 vs Day45** | 4.1483 | 0.3415 | 0.009 | 0.045 | * |
| **Day31 vs Day46** | 5.0366 | 0.3141 | 0.006 | 0.045 | * |
| **Day31 vs Day49** | 3.9833 | 0.2658 | 0.024 | 0.067 | |
| **Day31 vs Day50** | 1.5790 | 0.1364 | 0.116 | 0.153 | |
| **Day31 vs Day51** | 1.8778 | 0.1581 | 0.080 | 0.119 | |
| **Day32 vs Day35** | 5.4287 | 0.3519 | 0.017 | 0.052 | |
| **Day32 vs Day36** | 2.0729 | 0.1717 | 0.101 | 0.139 | |
| **Day32 vs Day38** | 7.0285 | 0.3899 | 0.007 | 0.045 | * |
| **Day32 vs Day39** | 1.6173 | 0.1282 | 0.183 | 0.214 | |
| **Day32 vs Day42** | 4.7344 | 0.3009 | 0.007 | 0.045 | * |
| **Day32 vs Day43** | 5.8827 | 0.3953 | 0.026 | 0.069 | |
| **Day32 vs Day45** | 6.6813 | 0.5269 | 0.015 | 0.048 | * |
| **Day32 vs Day46** | 6.5139 | 0.4199 | 0.004 | 0.045 | * |
| **Day32 vs Day49** | 4.1401 | 0.3151 | 0.041 | 0.077 | |
| **Day32 vs Day50** | 1.0875 | 0.1197 | 0.265 | 0.296 | |
| **Day32 vs Day51** | 1.5661 | 0.1637 | 0.265 | 0.296 | |
| **Day35 vs Day36** | 2.3011 | 0.1609 | 0.080 | 0.119 | |
| **Day35 vs Day38** | 2.2100 | 0.1453 | 0.087 | 0.123 | |
| **Day35 vs Day39** | 2.5061 | 0.1616 | 0.075 | 0.116 | |
| **Day35 vs Day42** | 1.0879 | 0.0772 | 0.324 | 0.344 | |
| **Day35 vs Day43** | 2.8545 | 0.2060 | 0.035 | 0.073 | |
| **Day35 vs Day45** | 1.9966 | 0.1997 | 0.112 | 0.149 | |
| **Day35 vs Day46** | 3.4179 | 0.2371 | 0.006 | 0.045 | * |
| **Day35 vs Day49** | 3.9837 | 0.2659 | 0.010 | 0.045 | * |
| **Day35 vs Day50** | 4.1991 | 0.2957 | 0.011 | 0.045 | * |
| **Day35 vs Day51** | 5.8217 | 0.3680 | 0.008 | 0.045 | * |
| **Day36 vs Day38** | 3.3525 | 0.2050 | 0.033 | 0.070 | |
| **Day36 vs Day39** | 0.6851 | 0.0501 | 0.553 | 0.560 | |
| **Day36 vs Day42** | 2.2257 | 0.1462 | 0.084 | 0.122 | |
| **Day36 vs Day43** | 2.6332 | 0.1931 | 0.081 | 0.119 | |
| **Day36 vs Day45** | 3.1445 | 0.2822 | 0.044 | 0.080 | |
| **Day36 vs Day46** | 3.8792 | 0.2607 | 0.005 | 0.045 | * |
| **Day36 vs Day49** | 3.5907 | 0.2461 | 0.027 | 0.069 | |
| **Day36 vs Day50** | 2.2658 | 0.1847 | 0.036 | 0.074 | |
| **Day36 vs Day51** | 2.9654 | 0.2287 | 0.059 | 0.096 | |

| | | | | | |
|---|---|---|---|---|---|
| **Day38 vs Day39** | 3.9359 | 0.2194 | 0.019 | 0.054 | |
| **Day38 vs Day42** | 1.0716 | 0.0711 | 0.313 | 0.337 | |
| **Day38 vs Day43** | 2.2068 | 0.1553 | 0.085 | 0.123 | |
| **Day38 vs Day45** | 1.3900 | 0.1338 | 0.186 | 0.216 | |
| **Day38 vs Day46** | 3.7774 | 0.2394 | 0.004 | 0.045 | * |
| **Day38 vs Day49** | 6.0716 | 0.3360 | 0.002 | 0.045 | * |
| **Day38 vs Day50** | 5.5075 | 0.3336 | 0.007 | 0.045 | * |
| **Day38 vs Day51** | 7.4306 | 0.4032 | 0.005 | 0.045 | * |
| **Day39 vs Day42** | 2.2601 | 0.1390 | 0.062 | 0.099 | |
| **Day39 vs Day43** | 2.7851 | 0.1884 | 0.087 | 0.123 | |
| **Day39 vs Day45** | 2.9966 | 0.2498 | 0.054 | 0.089 | |
| **Day39 vs Day46** | 3.6074 | 0.2311 | 0.014 | 0.048 | * |
| **Day39 vs Day49** | 2.9182 | 0.1956 | 0.061 | 0.098 | |
| **Day39 vs Day50** | 1.9330 | 0.1495 | 0.111 | 0.149 | |
| **Day39 vs Day51** | 2.3200 | 0.1742 | 0.111 | 0.149 | |
| **Day42 vs Day43** | 2.1847 | 0.1540 | 0.075 | 0.116 | |
| **Day42 vs Day45** | 1.6229 | 0.1528 | 0.152 | 0.182 | |
| **Day42 vs Day46** | 3.0323 | 0.2017 | 0.006 | 0.045 | * |
| **Day42 vs Day49** | 3.8052 | 0.2408 | 0.011 | 0.045 | * |
| **Day42 vs Day50** | 4.0408 | 0.2687 | 0.011 | 0.045 | * |
| **Day42 vs Day51** | 5.1583 | 0.3192 | 0.005 | 0.045 | * |
| **Day43 vs Day45** | 1.1901 | 0.1453 | 0.394 | 0.407 | |
| **Day43 vs Day46** | 1.5201 | 0.1320 | 0.165 | 0.196 | |
| **Day43 vs Day49** | 4.1499 | 0.2933 | 0.018 | 0.053 | |
| **Day43 vs Day50** | 3.7812 | 0.2958 | 0.032 | 0.070 | |
| **Day43 vs Day51** | 5.3108 | 0.3711 | 0.043 | 0.079 | |
| **Day45 vs Day46** | 1.1855 | 0.1448 | 0.328 | 0.346 | |
| **Day45 vs Day49** | 2.8385 | 0.2885 | 0.052 | 0.087 | |
| **Day45 vs Day50** | 3.0760 | 0.3389 | 0.045 | 0.080 | |
| **Day45 vs Day51** | 5.2185 | 0.4652 | 0.039 | 0.076 | |
| **Day46 vs Day49** | 1.7118 | 0.1462 | 0.142 | 0.179 | |
| **Day46 vs Day50** | 3.5315 | 0.2818 | 0.010 | 0.045 | * |
| **Day46 vs Day51** | 4.6658 | 0.3414 | 0.014 | 0.048 | * |
| **Day49 vs Day50** | 1.9563 | 0.1786 | 0.136 | 0.173 | |
| **Day49 vs Day51** | 2.2097 | 0.1971 | 0.150 | 0.181 | |
| **Day50 vs Day51** | 0.3347 | 0.0402 | 0.844 | 0.844 | |

**Table S 4.12:** Permutational analysis of variance (PERMANOVA) results testing the effect of AGD severity score, genetic origin and processing day (day of gut dissection) on midgut (MG) microbial community composition based on weighted unifrac distance matrices.

| Groups | Df | SumOfSqs | R2 | F | Pr(>F) | Significance |
|---|---|---|---|---|---|---|

| | | | | | | |
|---|---|---|---|---|---|---|
| **Origin** | 3 | 0.4172 | 0.0279 | 1.5823 | 0.093 | |
| **AGDScore** | 3 | 1.0738 | 0.0718 | 4.0724 | 0.001 | *** |
| **ProcessingDay** | 17 | 3.6207 | 0.2421 | 2.4233 | 0.001 | *** |
| **Residual** | 112 | 9.8436 | 0.6582 | | | |
| **Total** | 135 | 14.9553 | 1 | | | |

**Table S 4.13:** Permutational analysis of variance (PERMANOVA) testing pairwise comparisons for midgut (MG) samples from different AGD severity groups. Distance matrix calculated by weighted UniFrac measure. Permutations used: 9999. Significance codes: **p< 0.01; *p< 0.05

| Groups | F | R2 | p.value | p.adjusted | Significance |
|---|---|---|---|---|---|
| **AGD0 vs AGD1** | 1.804173 | 0.0178978 | 0.1 | 0.12 | |
| **AGD0 vs AGD2** | 3.953787 | 0.0376717 | 0.008 | 0.024 | * |
| **AGD0 vs AGD3** | 5.421323 | 0.0495454 | 0.001 | 0.006 | ** |
| **AGD1 vs AGD2** | 1.060662 | 0.03649819 | 0.301 | 0.301 | |
| **AGD1 vs AGD3** | 3.204815 | 0.09369486 | 0.014 | 0.028 | * |
| **AGD2 vs AGD3** | 2.742479 | 0.07672884 | 0.06 | 0.09 | |

### 4.8.3 Acknowledgements

This research has been carried out with the support of the Marine Institute's Newport Research Section and Aquaculture Section, contributing to all field, hatchery, laboratory and logistic support.

Special thanks also to Clear Seas Aqua for support at Lehanagh Pool and Fintan Egan, Alan Drumm, Pat Hughes, Ronan Grealish and Catherine Waters.

### 4.8.4 Author contributions statement

P.D.S organised sampling, conducted laboratory work, analysed the data and drafted the manuscript. B.C programmed the bioinformatic pipelines and gave statistical advice. J.K organised sampling and gave statistical advice. K.P wrote the R-code for metabolic rate analysis and gave statistical advice. L.R organised sampling and logistics. All authors were involved in the conception and design of the experiment and contributed to data interpretation and editing of the final draft of the manuscript. P.McG and M.L managed the project.

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### 4.8.6 Competing interests

The authors declare that they have no competing interests.

### 4.8.7 Data availability statement

The raw 16S rRNA gene sequence files and metadata are deposited at the NCBI SRA database under the BioProject PRJNA866155. The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

### 4.8.8 Funding

PS, JK, KP, ER and PMcG were supported by the Science Foundation Ireland grant award (grant no. 16/BBSRC/3316). PS was also granted the Covid extension award "Higher Education Authority Covid-19 Cost extension (Call Three). This research was further supported in part by research grants from the Biotechnology and Biological Science Research Council (grant no. BB/T016280/1, BB/P001203/1 & BB/N024028/1) and by the Science Foundation Ireland, the Marine Institute Ireland and the Department for the Economy, Northern Ireland, under the Investigators Program (grant no. SFI/15/IA/3028). JK is currently supported by Science Foundation Ireland / Ireland Research council grant, under the Pathway programme (grant no. SFI-IRC/21/PATH-S/9671). The funding bodies did not influence data interpretation or in writing of the manuscript.

### 4.8.9 R.Script

The following R Script contains basic code, which was used in an adapted form for all research chapters.

```
rm(list=ls())

library(ggplot2)
library(microeco)
library(mecodev)
library(ape)
library(tidyverse)
library(magrittr)
library(picante)
library(dplyr)
library(FSA)
library(rcompanion)
library(ggsignif)
library(aplot)
library(heatmaply)
library(reshape2)
library(phyloseq)
library(vegan)
```

```
library (performance)
library(ggpubr)
library(extrafont)

#if(!require("V8")) install.packages("V8")
#if(!require("devtools")) install.packages("devtools")
#devtools::install_github("bhaskarvk/colormap")
library(colormap)
#if (!require("BiocManager", quietly = TRUE))
# install.packages("BiocManager")
#library(BiocManager)
#BiocManager::install("microbiome")
#BiocManager::install("ANCOMBC2")
#BiocManager::install("ALDEx2")

library(ANCOMBC)
library(microbiome)
library(DT)
library(file2meco)
library(MicrobiomeStat)
library(ALDEx2)
# Load the phylogenetic tree calculated from the OTU sequences
phylo_tree <- read.tree("tree.nwk")
# check tip labels and fix if there is anything odd
phylo_tree$tip.label
phylo_tree$tip.label <- gsub("'", "", phylo_tree$tip.label)
phylo_tree$tip.label
# check whether the tree is rooted, if unrooted, transform to rooted
if(!is.rooted(phylo_tree)){
  phylo_tree <- multi2di(phylo_tree)
}


# Load sample info
sample_info <- read.csv("meta.csv",row.names = 1,header=T, stringsAsFactors = FALSE)
sample_info <- read.csv("meta_VR.csv",row.names = 1, header=T, stringsAsFactors = FALSE)

#----------subset if necessary (examples)----------
#Macro
sample_info <- subset(sample_info, SampleType %in% c("Macroinvertebrate"))
#Water
sample_info <- subset(sample_info, SampleType %in% c("Water"))
#PC
sample_info <- subset(sample_info, SampleType %in% c("Gut"))

#Calculate Quantiles for VR
row_names <- rownames(sample_info)
quantiles <- c(0, 0.25, 0.5, 0.75, 1)
labels <- c('low', 'medium', 'high', 'very high')

# Grouping by Season and creating VR groups
```

```
sample_info <- sample_info %>%
  group_by(Season) %>%
  mutate(VR_group_season = cut(VR, breaks = quantile(VR, probs = quantiles), labels = labels,
include.lowest = TRUE))

# Creating VR groups for all seasons combined
sample_info <- sample_info %>%
  mutate(VR_group_all = cut(VR, breaks = quantile(VR, probs = quantiles), labels = labels,
include.lowest = TRUE))

write.csv(sample_info,"meta_VR.csv", row.names = TRUE)
#-------------
#load otu_table
file.path="otu_table.csv"
file.path="VIP_otu_byfam.csv"
otu_table_1<-read.table (file.path, check.names = FALSE, header = TRUE, dec = ".", sep = ",",
row.names = 1, comment.char = "")
otu_table_1[,"taxonomy"]<-NULL
otu_table_1[,"lineage"]<-NULL

# load taxonomy file
taxonomy_table_1 <- read.table ("taxonomy.txt", check.names = FALSE, header = TRUE, dec
= ".", sep = "\t", row.names = 1, comment.char = "")
#taxonomy_table_1 <- read.table ("tax_for_genus_reomval.txt", check.names = FALSE,
header = #TRUE, dec = ".", sep = "\t", row.names = 1, comment.char = "")
taxonomy_table_1 %<>% tidy_taxonomy

# create a microtable object
dataset <- microtable$new(sample_table = sample_info, otu_table = otu_table_1, tax_table
= taxonomy_table_1, phylo_tree = phylo_tree)


#-----------------------------------------------------------------remove contaminants-----------------------
----###
print(dataset)
dataset$sample_table
# To make the species and sample information consistent across different files in the dataset
object, #we can use function tidy_dataset() to trim the dataset.
dataset$tidy_dataset()
print(dataset)

#we remove OTUs which are not assigned in the Kingdom "k__Archaea" or "k__Bacteria".
dataset$tax_table %<>% base::subset(Domain == "d__Archaea" | Domain == "d__Bacteria")
print(dataset)

# This will remove the lines containing the taxa word regardless of taxonomic ranks and
ignoring #word case in the tax_table.
# So if you want to filter some taxa not considerd pollutions, please use subset like the
previous #operation.
dataset$filter_pollution(taxa = c("mitochondria", "chloroplast"))
```

```
# to make the OTUs same in otu_table, tax_table and phylo_tree, we use tidy_dataset() again.
dataset$tidy_dataset()
print(dataset)

#-----------------------------------------------create phyloseq-----------------------------------------
# from microtable to phyloseq object
library(file2meco)
data("dataset")
pseq <- meco2phyloseq(dataset)
pseq

# SourceTracking
write.csv(dataset$otu_table,"otu_rar.csv", row.names = TRUE)
write.csv(dataset$sample_table,"meta_rar.csv", row.names = TRUE)
write.csv(dataset$tax_table,"tax_for_stats_tree.csv", row.names = TRUE)

write.csv(Water$otu_table,"otu_table_Water.csv", row.names = TRUE)

#---------------------------------------standardize the data for diversity measurements-----------------
set.seed(123)

#use sample_sums() to check the sequence numbers in each sample.
dataset$sample_sums() %>% range
dataset$sample_sums()

#write.csv(dataset$sample_names(), file = "sample_names.csv", row.names = FALSE)
#write.csv(dataset$sample_sums(), file = "sample_sums.csv", row.names = FALSE)
# check rarefaction curves

t1 <- trans_rarefy$new(dataset, alphadiv = "Shannon", depth = c(0, 10, 50, 100, 200, 400, 800, 2000))
t1$plot_rarefy(color_values = rep("black", 252), show_point = TRUE, add_fitting = FALSE, show_legend = FALSE)

#rarefy samples accordingly

dataset$rarefy_samples(sample.size = 500)
dataset$sample_sums() %>% range
print(dataset)

#-------------------------------------------calculate rel. abundance----------------------------

dataset$cal_abund()
# return dataset$taxa_abund
class(dataset$taxa_abund)
# save the taxa abundance file to a local place
dataset$taxa_abund
```

```
dir.create("taxa_abund")

#-----------------------------------------Caluculate Diversity ---------------------------------
#Alpha
dataset$cal_alphadiv(PD = TRUE)

#safe (optional)
dir.create("alpha_diversity")
dataset$save_alphadiv(dirpath="alpha_diversity")

#Beta
dataset$cal_betadiv(unifrac = TRUE)
dataset$beta_diversity

#safe beta (optional)
dir.create("beta_diversity")
dataset$save_alphadiv(dirpath="beta_diversity")

#---------------------------------alpha diversity modelling-----------------------------------------------------
rm(list=ls())
library(lme4)
library(MASS)
library(ggfortify)
library(boot)
library(car)
library(pscl)
library(ggpmisc)
library(MuMIn)
library (performance)
library(corrplot)
library(ggcorrplot)
library(gridExtra)
library(see)

setwd("C:/Users/Paddy/Documents/PhD/Chapter Torpor/River/NEW/alpha_diversity")
data <- read.csv("merged_alpha.csv",row.names = 1, header=T)

# Examine summary statistics
summary(data)

#----Chao1
summary(data$Chao1)
data$Chao1 <- round(data$Chao1)

#distribution examples:
hist(rnorm(1000), main = "Histogram of Normal Distribution", xlab = "Value")
hist(rpois(1000, lambda = 2), main = "Histogram of Poisson Distribution", xlab = "Value")
hist(rnbinom(1000, size = 2, mu = 5), main = "Histogram of Negative Binomial Distribution",
xlab = "Value")
hist(rexp(1000, rate = 1), main = "Histogram of Exponential Distribution", xlab = "Value")
```

```
hist(rgamma(1000, shape = 2, scale = 2), main = "Histogram of Gamma Distribution", xlab =
"Value")
hist(rbinom(1000, size = 10, prob = 0.5), main = "Histogram of Binomial Distribution", xlab =
"Value")

# Check the distribution of Chao1 alpha diversity
hist(data$Chao1, breaks = 50, main = "Histogram of Chao1 Alpha Diversity", xlab = "Chao1")

#------gamma distribution
fit_gamma <- fitdistr(data$Chao1, "gamma")
shape <- fit_gamma$estimate["shape"]
rate <- fit_gamma$estimate["rate"]

# Overlay the fitted Gamma distribution on the histogram
hist(data$Chao1, breaks = 30, probability = TRUE, main = "Histogram of Chao1 with Fitted
Gamma", xlab = "Chao1")
curve(dgamma(x, shape = shape, rate = rate), col = "blue", lwd = 2, add = TRUE)

# Q-Q plot to compare the quantiles of the observed data with the quantiles of the Gamma
#distribution

qqPlot(data$Chao1, dist = "gamma", shape = shape, rate = rate, main = "Q-Q Plot for Gamma
Distribution")

#------fit gamma model
model_gamma_glm <- glm(Chao1 ~ Season + Temperature + Sex + Origin + Length + Weight
+ VR, family = Gamma(link = "log"), data = data)
summary(model_gamma_glm)
check_model(model_gamma_glm)

par(mfrow = c(2, 2))
plot(model_gamma_glm)

#-------Fit normal distribution
fit_normal <- fitdistr(data$Chao1, "normal")

print(fit_normal)

#visualize fit
# Create histogram
hist(data$Chao1, breaks = 20, probability = TRUE,
    main = "Histogram of Chao1 with Fitted Normal Distribution",
    xlab = "Chao1", col = "lightblue")

# Overlay the fitted normal distribution
curve(dnorm(x, mean = fit_normal$estimate["mean"], sd = fit_normal$estimate["sd"]),
     col = "darkblue", lwd = 2, add = TRUE)

# Generate Q-Q plot
qqnorm(data$Chao1, main = "Q-Q Plot of Chao1")
qqline(data$Chao1, col = "red")
```

```
#---- Fit a linear regression model
model_lm <- lm(Chao1 ~ Temperature + Sex + Origin + Length + Weight + Season + VR, data = data)

#check Variance Inflation Factor (VIF) for colinearity
vif(model_lm)

# Check normality of residuals
residuals <- resid(model_lm)
hist(residuals, breaks = 20, main = "Histogram of Residuals", xlab = "Residuals")

# Shapiro-Wilk test for normality
shapiro.test(residuals)

#Performance
check_model(model_lm)

#-------------Example for Poisson
hist(data$Chao1, probability = TRUE, main = "Histogram of Chao1 with Poisson Fit", xlab = "Chao1")
curve(dpois(x, lambda = mean(data$Chao1)), col = "red", add = TRUE)

#-------Example for Negative Binomial
hist(data$Chao1, probability = TRUE, main = "Histogram of Chao1 with Negative Binomial Fit", xlab = "Chao1")
fit_nb <- fitdistr(data$Chao1, "Negative Binomial")
curve(dnbinom(x, size = fit_nb$estimate["size"], mu = fit_nb$estimate["mu"]), col = "blue", add = TRUE)

# ----fit other model types

model_poisson <- glm(Chao1 ~ Temperature + Sex + Origin + Length + Weight +Month, data = data, family = poisson)
model_glmnb <- glm.nb(Chao1 ~ Temperature + Sex + Origin + Length + Weight +Month, data = data, maxit = 1000)

autoplot(model_lm)
autoplot(model_poisson)
autoplot(model_glmnb)
autoplot(model_gamma_glm)

# Compare models using AIC
AIC(model_lm, model_glmnb, model_poisson, model_gamma_glm)
BIC(model_lm, model_glmnb, model_poisson, model_gamma_glm)

#Deviance (Lower deviance values indicate a better fit to the data)
deviance(model_glmnb)
deviance(model_gamma_glm)
```

```
#pseud r squared (Pseudo R-squared values, indicating a similar proportion of variance explained)
pR2(model_glmnb)
pR2(model_gamma_glm)

#cross validation (Lower delta values in cross-validation indicate better predictive performance)
cv_glm_nb <- cv.glm(data, model_glmnb, K = 10)
cv_glm_gamma <- cv.glm(data, model_gamma_glm, K = 10)
print(cv_glm_nb$delta)
print(cv_glm_gamma$delta)

# check collinearity of number predictors
predictors <- c("logMeanTemp", "logMeanWaterlevel", "logMeanFlow", "logMeanConductivity", "logMeanDO")

# Create a formula for the linear model
formula <- as.formula(paste("Chao1 ~", paste(predictors, collapse = " + ")))

# Fit a linear model using the predictors
model <- lm(formula, data = data)

check_model(model)
vif_values <- vif(model)
print(vif_values)

# Select the predictor columns from the dataset
predictor_data <- data[predictors]
cor_matrix <- cor(predictor_data, use = "complete.obs")

# Save the correlation plot to a PNG file
png("correlation_plot.png", width = 800, height = 600)
corrplot(cor_matrix, method = "color", addCoef.col = "black", tl.col = "black", tl.srt = 45)
dev.off()

# use MuMIn for best model

options(na.action= "na.fail")

model <- glm(Chao1 ~ Season + Temperature + Sex + Origin + Length + Weight + VR , family = Gamma(link = "log"), data = data)
summary(model)

# Create all possible combinations of predictors

MuMIn::dredge(global.model = model)


model.best <- glm(Chao1 ~ Season + Weight + VR, family = Gamma(link = "log"), data = data)
summary(model.best)
check_model(model.best)
```

```
check_predictions(model.best)

ggsave(filename = "Chao1_checkmodel.svg", plot = check, width = 14, height = 10, dpi = 300,
units = "cm")

deviance(model.best)
pR2(model.best)
cv_model.best <- cv.glm(data, model.best, K = 10)
print(cv_model.best$delta)

m1 <- glm(Chao1 ~ Season + VR + Weight,family = Gamma(link = "log"), data=data)
m2 <- glm(Chao1 ~ Season + Weight ,family = Gamma(link = "log"), data=data)
m3 <- glm(Chao1 ~ Season + Sex + Weight,family = Gamma(link = "log"), data=data)
m4 <- glm(Chao1 ~ Season + VR + Sex + Weight,family = Gamma(link = "log"), data=data)
m5 <- glm(Chao1 ~ Season + VR,family = Gamma(link = "log"), data=data)
m6 <- glm(Chao1 ~ Season + Origin + VR + Weight,family = Gamma(link = "log"), data=data)

#check 6 best models
check_model (m1)

library(see)
compare_performance(m1, m2, m3, m4, m5, m6)
p1 <- plot(compare_performance(m1, m2, m3, m4, m5, m6))
test_performance(m1, m2, m3, m4, m5, m6)
p1


ggsave(filename = "Chao1_indices.svg", plot = p1, width = 14, height = 10, dpi = 300, units =
"cm")

#----------------------------Shannon-------------------------

#----Shannon
summary(data$Shannon)
data$Shannon <- round(data$Shannon)

#distribution examples:
hist(rnorm(1000), main = "Histogram of Normal Distribution", xlab = "Value")
hist(rpois(1000, lambda = 2), main = "Histogram of Poisson Distribution", xlab = "Value")
hist(rnbinom(1000, size = 2, mu = 5), main = "Histogram of Negative Binomial Distribution",
xlab = "Value")
hist(rexp(1000, rate = 1), main = "Histogram of Exponential Distribution", xlab = "Value")
hist(rgamma(1000, shape = 2, scale = 2), main = "Histogram of Gamma Distribution", xlab =
"Value")
hist(rbinom(1000, size = 10, prob = 0.5), main = "Histogram of Binomial Distribution", xlab =
"Value")

# Check the distribution of Shannon alpha diversity
hist(data$Shannon, breaks = 20, main = "Histogram of Shannon Alpha Diversity", xlab =
"Shannon")
```

```
#Example for Poisson
hist(data$Shannon, probability = TRUE, main = "Histogram of Shannon with Poisson Fit", xlab = "Shannon")
curve(dpois(x, lambda = mean(data$Shannon)), col = "red", add = TRUE)

# Example for Negative Binomial
hist(data$Shannon, probability = TRUE, main = "Histogram of Shannon with Negative Binomial Fit", xlab = "Shannon")
fit_nb <- fitdistr(data$Shannon, "Negative Binomial")
curve(dnbinom(x, size = fit_nb$estimate["size"], mu = fit_nb$estimate["mu"]), col = "blue", add = TRUE)

#--------gamma distribution
fit_gamma <- fitdistr(data$Shannon, "gamma")
shape <- fit_gamma$estimate["shape"]
rate <- fit_gamma$estimate["rate"]

# Overlay the fitted Gamma distribution on the histogram
hist(data$Shannon, breaks = 30, probability = TRUE, main = "Histogram of Shannon with Fitted Gamma", xlab = "Shannon")
curve(dgamma(x, shape = shape, rate = rate), col = "blue", lwd = 2, add = TRUE)

# Q-Q plot to compare the quantiles of the observed data with the quantiles of the Gamma
#distribution

qqPlot(data$Shannon, dist = "gamma", shape = shape, rate = rate, main = "Q-Q Plot for Gamma Distribution")

#------fit gamma model
model_gamma_glm <- glm(Shannon ~ Temperature + Sex + Origin + Length + Weight + Season, family = Gamma(link = "log"), data = data)
summary(model_gamma_glm)

par(mfrow = c(2, 2))
plot(model_gamma_glm)


#----normal distribution

# Fit normal distribution
fit_normal_shannon <- fitdistr(data$Shannon, "normal")

print(fit_normal_shannon)

# Create histogram
hist(data$Shannon, breaks = 20, probability = TRUE,
    main = "Histogram of Shannon Diversity with Fitted Normal Distribution",
    xlab = "Shannon Diversity", col = "lightblue")

# Overlay the fitted normal distribution
```

```
curve(dnorm(x, mean = fit_normal_shannon$estimate["mean"], sd = fit_normal_shannon$estimate["sd"]),
    col = "darkblue", lwd = 2, add = TRUE)

# Generate Q-Q plot
qqnorm(data$Shannon, main = "Q-Q Plot of Shannon Diversity")
qqline(data$Shannon, col = "red")

#---- Fit a linear regression model

model_lm <- lm(Shannon ~ Temperature + Sex + Origin + Length + Weight + Season, data = data)

# Check normality of residuals
residuals <- resid(model_lm)
hist(residuals, breaks = 20, main = "Histogram of Residuals", xlab = "Residuals")

# Shapiro-Wilk test for normality
shapiro.test(residuals)

# ----fit other model types

model_poisson <- glm(Shannon ~ Temperature + Sex + Origin + Length + Weight +Month, data = data, family = poisson)
model_glmnb <- glm.nb(Shannon ~ Temperature + Sex + Origin + Length + Weight +Month, data = data)

autoplot(model_lm)
autoplot(model_poisson)
autoplot(model_glmnb)
autoplot(model_gamma_glm)

# Compare models using AIC
AIC(model_lm, model_poisson,model_glmnb, model_gamma_glm)
BIC(model_lm, model_poisson,model_glmnb, model_gamma_glm)

#Deviance (Lower deviance values indicate a better fit to the data)
deviance(model_lm)
deviance(model_gamma_glm)

#pseud r squared (Pseudo R-squared values, indicating a similar proportion of variance explained)
pR2(model_lm)
pR2(model_gamma_glm)

#cross validation (Lower delta values in cross-validation indicate better predictive performance)
cv_lm_nb <- cv.glm(data, model_lm, K = 10)
cv_glm_gamma <- cv.glm(data, model_gamma_glm, K = 10)
print(cv_lm_nb$delta)
print(cv_glm_gamma$delta)
```

```
# use MuMIn for best model

# Define all possible predictors
predictors <- c("Temperature", "Sex", "Origin", "Length", "Weight", "Month")

options(na.action= "na.fail")

model <- lm(Shannon ~ Season + Temperature + Sex + Origin + VR + Weight, data=data)
summary(model)
# Create all possible combinations of predictors

MuMIn::dredge(global.model = model)

m1 <- lm(Shannon ~ Season + VR, data=data)
m2 <- lm(Shannon ~ Season , data=data)
m3 <- lm(Shannon ~ Season + VR + Weight, data=data)
m4 <- lm(Shannon ~ Season + VR + Sex, data=data)
m5 <- lm(Shannon ~ Season + Weight, data=data)
m6 <- lm(Shannon ~ Season + Origin + VR, data=data)
summary(model.best)

#check 6 best models
check_model (m1)

library(see)
plot(compare_performance(m1, m2, m3, m4, m5, m6))

#--plot
myCol2 <- c( AJan_19="#0066CC",  CMar_19="#79deaa" ,EMay_19="#0e5b05",
FJun_19="#ede07b", GJul_19="#ffa346", KNov_19="#a71b2b")

bxp <- ggboxplot(data, x = "Month", y = "Chao1", fill = "Month",
palette = myCol2 )
bxp

bxp <- bxp + theme_bw() + theme(panel.grid.major = element_blank(),
                        panel.grid.minor = element_blank()) +
  theme(text=element_text(family="Calibri", face="bold", size=11))+
  theme(legend.position = "none")
#+
#   scale_x_discrete(labels=c("Jan_19" = "Jan19", "Mar_19" = "Mar19",
#  "May_19" = "May19","Jun_19" = "Jun19", "Jul_19" = "Jul19",
#   "Nov_19" = "Nov19", "May_19_H" = "May19",
#   "Jun_19_S" = "Jun19"))

bxp

ggsave(filename = "Shannon_alphadiversity_Month_nostat.svg", plot = bxp, width = 14,
height = 10, dpi = 300, units = "cm")
```

```
#-------------------------------------------------Beta diversity--------------------------
#calculate beta diversity
dataset$cal_betadiv(unifrac = T)
dataset$beta_diversity

# clone dataset
Autumn19 <- clone(dataset)
Autumn19$sample_table <- subset(Autumn19$sample_table, Season == "DAutumn19")
Autumn19$tidy_dataset()

Winter19 <- clone(dataset)
Winter19$sample_table <- subset(Winter19$sample_table, Season == "BWinter19")
Winter19$tidy_dataset()

# create, use dataset depending on the question
t1 <- trans_beta$new(dataset = dataset, group = "VR_group_all", measure = "wei_unifrac")
# t1$res_ordination is the ordination result list
t1$cal_ordination(ordination = "PCoA")
class(t1$res_ordination)

myCol2 <- c(F = "#CC6677", HFF = "#88CCEE", HWF ="#44AA99", W = "#DDCC77")
myCol2 <- c(low = "#66c2a5", medium= "#fc8d62", high= "#8da0cb", veryhigh = "#e78ac3")

b1 <- t1$plot_ordination(plot_color = "VR_group_all", plot_shape= , plot_type = c("point",
"centroid"),color_values = myCol2,
point_size = 4, point_alpha = 1, centroid_segment_alpha = 0.3, centroid_segment_size = 0.3,
centroid_segment_linetype = 1,
shape_values =c(15,16,17,18,19,20,21),
add_sample_label =  )

b1 <- b1 + theme_bw() + theme(panel.grid.major = element_blank(),
panel.grid.minor = element_blank()) +
 theme(text=element_text(family="Calibri", face="bold", size=11))+
 theme(legend.key.size = unit(0.5, 'cm'))+
 theme(legend.title = element_text(size=9))

b1

ggsave(filename = "Winter19_Origin.svg", plot = b1, width = 14, height = 14, dpi = 300, units
= "cm")

# manova for all groups when manova_all = TRUE
t1$cal_manova(manova_all = TRUE)
t1$res_manova

# manova for each paired groups
t1$cal_manova(manova_all = FALSE)
t1$res_manova

# manova for specified group set:
t1$cal_manova(manova_set = "Origin + VR")
```

```
t1$res_manova

# PERMDISP for the whole comparison and for each paired groups
t1$cal_betadisper()
t1$res_betadisper

#-------------------------------------------------Diff abundance-------------------------
pseq <- meco2phyloseq(dataset)
pseq

#AncomBC2
#create microeco dataset and transform to physeq...

#add otu column
taxa_table <- as.data.frame(tax_table(pseq))
head(taxa_table)
taxa_table$OTU <- rownames(taxa_table)
taxa_table_matrix <- as.matrix(taxa_table)
tax_table(pseq) <- taxa_table_matrix
head(tax_table(pseq))

#change levels
sample_data(pseq)$SampleType <- factor(sample_data(pseq)$SampleType, levels =
c("Gut","Macroinvertebrate","Water"))

# Check the levels to ensure the referenc
levels(sample_data(pseq)$SampleType)

# Check the structure of the SampleType variable
str(sample_data(pseq)$SampleType)

# Print each level individually to see if there are hidden characters
for (level in levels(sample_data(pseq)$SampleType)) {
  cat(paste0("'", level, "'"), "\n")
}

#Run ANCOM
output = ancombc2(data = pseq, assay_name = "Environmental", tax_level = "OTU",
                  fix_formula = "SampleType", rand_formula = NULL,
                  p_adj_method = "holm", pseudo_sens = TRUE,
                  prv_cut = 0.05, lib_cut = 1500, s0_perc = 0.05,
                  group = "SampleType", struc_zero = FALSE, neg_lb = TRUE,
                  alpha = 0.05, n_cl = 6, verbose = TRUE,
                  global = FALSE, pairwise = FALSE, dunnet = TRUE, trend = FALSE,
                  iter_control = list(tol = 1e-2, max_iter = 20,
                                      verbose = TRUE),
                  em_control = list(tol = 1e-5, max_iter = 100),
                  lme_control = lme4::lmerControl(),
                  mdfdr_control = list(fwer_ctrl_method = "holm", B = 100),
                  trend_control = list(contrast = list(matrix(c(1, 0, -1, 1),
                  nrow = 2,
```

```
byrow = TRUE),
matrix(c(-1, 0, 1, -1),
nrow = 2,
byrow = TRUE),
matrix(c(1, 0, 1, -1),
nrow = 2,
byrow = TRUE)),
node = list(2, 2, 1),
solver = "ECOS",
B = 8))

dunn <- output$res_dunn
write.csv(dunn,"AncomBC_dunn_OTU_0.05.csv", row.names = TRUE)

#structural zeros
tab_zero = output$zero_ind
tab_zero %>%
datatable(caption = "The detection of structural zeros")

#bias correcte dlog abundances
bias_correct_log_table = output$bias_correct_log_table

#Multiple pairwise comparisons

ancombc_table = output$res_pair

df_fig_pair1 <- ancombc_table %>%
 filter(diff_SampleTypePC == TRUE | diff_SampleTypeWater == TRUE |
diff_SampleTypeWater_SampleTypePC == TRUE) %>%
 mutate(lfc1 = ifelse(diff_SampleTypePC == TRUE, round(lfc_SampleTypePC, 2), 0),
 lfc2 = ifelse(diff_SampleTypeWater == TRUE, round(lfc_SampleTypeWater, 2), 0),
 lfc3 = ifelse(diff_SampleTypeWater_SampleTypePC == TRUE,
round(lfc_SampleTypeWater_SampleTypePC, 2), 0)) %>%
 pivot_longer(cols = lfc1:lfc3, names_to = "group", values_to = "value") %>%
 arrange(taxon)

df_fig_pair2 <- ancombc_table %>%
  filter(diff_SampleTypePC == TRUE | diff_SampleTypeWater == TRUE |
diff_SampleTypeWater_SampleTypePC == TRUE) %>%
  mutate(lfc1 = ifelse(passed_ss_SampleTypePC == TRUE & diff_SampleTypePC == TRUE,
"aquamarine3", "black"),
 lfc2 = ifelse(passed_ss_SampleTypeWater == TRUE & diff_SampleTypeWater == TRUE,
"aquamarine3", "black"),
 lfc3 = ifelse(passed_ss_SampleTypeWater_SampleTypePC == TRUE &
diff_SampleTypeWater_SampleTypePC == TRUE, "aquamarine3", "black")) %>%
  pivot_longer(cols = lfc1:lfc3, names_to = "group", values_to = "color") %>%
  arrange(taxon)

df_fig_pair <- df_fig_pair1 %>%
  left_join(df_fig_pair2, by = c("taxon", "group"))
```

```
df_fig_pair <- df_fig_pair %>%
  mutate(group = case_when(
    group == "lfc1" ~ "PC - Makroinvertebrate",
    group == "lfc2" ~ "Water - Makroinvertebrate",
    group == "lfc3" ~ "Water - PC",
    TRUE ~ as.character(group)
  ))

df_fig_pair$group = factor(df_fig_pair$group,
levels = c("PC - Makroinvertebrate",
"Water - Makroinvertebrate",
"Water - PC"))
# Remove "g__" from the taxon column
df_fig_pair <- df_fig_pair %>%
  mutate(taxon = sub("^g__", "", taxon))

#choose taxa
# Create a ranking metric (not always required
ranking_metric <- df_fig_pair %>%
  mutate(
    rank_metric = -log10(p_SampleTypePC.x + 1e-10) * abs(lfc_SampleTypePC.x) +
      -log10(p_SampleTypeWater.x + 1e-10) * abs(lfc_SampleTypeWater.x) +
      -log10(p_SampleTypeWater_SampleTypePC.x + 1e-10) *
abs(lfc_SampleTypeWater_SampleTypePC.x)
  )

# Select top 20 taxa based on ranking metric
top_taxa <- ranking_metric %>%
  arrange(desc(rank_metric)) %>%
  slice(1:60)

#manually choose taxa (not always required)
df_fig_pair <- df_fig_pair[df_fig_pair$taxon %in%
c("Flavobacterium","Methylotenera","Sphingorhabdus", "Novosphingobium","Elsteraceae",
"Candidatus_Omnitrophus", "Omnitrophales", "Aquabacterium", "Schlegelella",
"Corynebacterium",

"Staphylococcus","Acidovorax","Turicella","Streptococcus","Clostridium_sensu_stricto_1",
 "Vibrio", "Psychrobacter", "Aliivibrio", "Delftia", "Microbacterium", "Bacillus",
"Photobacterium" ,"Mycoplasma", "Lactococcus", "Streptococcus", "Pseudoalteromonas"),
]

#heatmap

fig_pair <- df_fig_pair %>%
  ggplot(aes(x = group, y = taxon, fill = value)) +
  geom_tile(color = "black") +
  scale_fill_gradient2(low = "blue", high = "darkred", mid = "white",
 na.value = "white", midpoint = 0,
= c(lo, up), name = NULL) +
  geom_text(aes(label = value), color = "black", size = 3) +
```

```
 scale_color_identity(guide = FALSE) +
 theme_minimal() +
 theme(
  plot.title = element_text(hjust = 0.5),
  axis.text.y = element_text(face = "italic")  # Italicize the y-axis text
 ) +
 scale_y_discrete(labels = function(x) parse(text = paste0("italic('", x, "')")))

fig_pair


ggsave(filename = "Ancom2_Heatmap_Environment.svg", plot = fig_pair, width = 26, height = 14, dpi = 300, units = "cm")

##----------Core taxa and Neutral model----------

library(tidyverse)
library(reshape2)
library(vegan)
library(ggsci)
library(dplyr)


setwd("C:/Users/Paddy/Documents/PhD/Chapter River(new)/Assembly/Month/Viva")

#Input rarefied data !

nReads=9300
file.path="otu_rar.csv"
otu<-read.table (file.path, check.names = FALSE, header = TRUE, dec = ".", sep = ",", row.names = 1, comment.char = "")
map <- read.csv("meta_rar.csv",row.names = 1, header=T, stringsAsFactors = FALSE)

otu_PA <- 1*((otu>0)==1)                                    # presence-absence data
otu_occ <- rowSums(otu_PA)/ncol(otu_PA)                     # occupancy calculation
otu_rel <- apply(decostand(otu, method="total", MARGIN=2),1, mean)        # relative abundance   occ_abun <- data.frame(otu_occ=otu_occ, otu_rel=otu_rel) %>%              # combining df
 rownames_to_column('otu')

ggplot(data=occ_abun, aes(x=log10(otu_rel), y=otu_occ)) +
 geom_point(pch=21, fill='white') +
 labs(x="log10(mean relative abundance)", y="Occupancy")


PresenceSum <- data.frame(otu = as.factor(row.names(otu)), otu) %>%
 gather(sample_ID, abun, -otu) %>%
 left_join(map, by = 'sample_ID') %>%
 group_by(otu, Month) %>%
 summarise(plot_freq=sum(abun>0)/length(abun),
```

```
        coreSite=ifelse(plot_freq == 1, 1, 0),
        detect=ifelse(plot_freq > 0, 1, 0)) %>%
 group_by(otu) %>%
 summarise(sumF=sum(plot_freq),
        sumG=sum(coreSite),
        nS=length(Month)*2,
        Index=(sumF+sumG)/nS)

otu_ranked <- occ_abun %>%
 left_join(PresenceSum, by='otu') %>%
 transmute(otu=otu,
        rank=Index) %>%
 arrange(desc(rank))

BCaddition <- NULL


otu_start=otu_ranked$otu[1]
start_matrix <- as.matrix(otu[otu_start,])
#start_matrix <- t(start_matrix)
x <- apply(combn(ncol(start_matrix), 2), 2, function(x) sum(abs(start_matrix[,x[1]]-
start_matrix[,x[2]]))/(2*nReads))
x_names <- apply(combn(ncol(start_matrix), 2), 2, function(x)
paste(colnames(start_matrix)[x], collapse=' - '))
df_s <- data.frame(x_names,x)
names(df_s)[2] <- 1
BCaddition <- rbind(BCaddition,df_s)

for(i in 2:500){
 otu_add=otu_ranked$otu[i]
 add_matrix <- as.matrix(otu[otu_add,])
# add_matrix <- t(add_matrix)
 start_matrix <- rbind(start_matrix, add_matrix)
 x <- apply(combn(ncol(start_matrix), 2), 2, function(x) sum(abs(start_matrix[,x[1]]-
start_matrix[,x[2]]))/(2*nReads))
 x_names <- apply(combn(ncol(start_matrix), 2), 2, function(x)
paste(colnames(start_matrix)[x], collapse=' - '))
 df_a <- data.frame(x_names,x)
 names(df_a)[2] <- i
 BCaddition <- left_join(BCaddition, df_a, by=c('x_names'))
}

x <-  apply(combn(ncol(otu), 2), 2, function(x) sum(abs(otu[,x[1]]-otu[,x[2]]))/(2*nReads))
x_names <- apply(combn(ncol(otu), 2), 2, function(x) paste(colnames(otu)[x], collapse=' - '))
df_full <- data.frame(x_names,x)
names(df_full)[2] <- length(rownames(otu))
BCfull <- left_join(BCaddition,df_full, by='x_names')

rownames(BCfull) <- BCfull$x_names
temp_BC <- BCfull
temp_BC$x_names <- NULL
```

```
temp_BC_matrix <- as.matrix(temp_BC)

BC_ranked <- data.frame(rank = as.factor(row.names(t(temp_BC_matrix))),t(temp_BC_matrix)) %>%
 gather(comparison, BC, -rank) %>%
 group_by(rank) %>%
 summarise(MeanBC=mean(BC)) %>%
 arrange(-desc(MeanBC)) %>%
 mutate(proportionBC=MeanBC/max(MeanBC))
Increase=BC_ranked$MeanBC[-1]/BC_ranked$MeanBC[-length(BC_ranked$MeanBC)]
increaseDF <- data.frame(IncreaseBC=c(0,(Increase)), rank=factor(c(1:(length(Increase)+1))))
BC_ranked <- left_join(BC_ranked, increaseDF)
BC_ranked <- BC_ranked[-nrow(BC_ranked),]

#Creating thresholds for core inclusion

#Method:
#A) Elbow method (first order difference) (script modified from https://pommevilla.github.io/random/elbows.html)
fo_difference <- function(pos){
 left <- (BC_ranked[pos, 2] - BC_ranked[1, 2]) / pos
 right <- (BC_ranked[nrow(BC_ranked), 2] - BC_ranked[pos, 2]) / (nrow(BC_ranked) - pos)
 return(left - right)
}
BC_ranked$fo_diffs <- sapply(1:nrow(BC_ranked), fo_difference)
elbow <- which.max(BC_ranked$fo_diffs)

#B) Final increase in BC similarity of equal or greater then x%; choose x accordingly
lastCall <- last(as.numeric(as.character(BC_ranked$rank[(BC_ranked$IncreaseBC>=1.04)])))
ggplot(BC_ranked[1:400,], aes(x=factor(BC_ranked$rank[1:400], levels=BC_ranked$rank[1:400]))) +
 geom_point(aes(y=proportionBC)) +
 theme_classic() + theme(strip.background = element_blank(),axis.text.x = element_text(size=7, angle=45)) +
 geom_vline(xintercept=elbow, lty=3, col='red', cex=.5) +
 geom_vline(xintercept=lastCall, lty=3, col='blue', cex=.5) +
 labs(x='ranked OTUs',y='Bray-Curtis similarity') +
 annotate(geom="text", x=elbow+10, y=.15, label=paste("Elbow method"," (",elbow,")", sep=''), color="red")+
 annotate(geom="text", x=lastCall-4, y=.08, label=paste("Last 2% increase (",lastCall,')',sep=''), color="blue")

#Create a column defining "core" OTUs

occ_abun$fill <- 'no'
occ_abun$fill[occ_abun$otu %in% otu_ranked$otu[1:lastCall]] <- 'core'

spp=t(otu)
taxon=as.vector(rownames(otu))
pool=t(otu)
```

```
#log transform spp test
#spp <- log(spp + 1)
#pool<- log(pool + 1)
#define env as pool
#file.path2="otu_table_Jan_19E.csv"
#pool<-read.table (file.path2, check.names = FALSE, header = TRUE, dec = ".", sep = ",",
row.names = 1, comment.char = "")

#pool=t(pool)
#file.path2="tax_table_rar.csv"
#taxon<-read.table(file.path2, check.names = FALSE, header = TRUE, dec = ".", sep = ",",
row.names = 1, comment.char = "")


#Models for the whole community

#------------------------- Burns Model scnm.fit

sncm.fit <- function(spp, pool=NULL, stats=TRUE, taxon=NULL){
 require(minpack.lm)
 require(Hmisc)
 require(stats4)

 options(warn=-1)

 #Calculate the number of individuals per community
 N <- mean(apply(spp, 1, sum))

 #Calculate the average relative abundance of each taxa across communities
 if(is.null(pool)){
  p.m <- apply(spp, 2, mean)
  p.m <- p.m[p.m != 0]
  p <- p.m/N
 } else {
  p.m <- apply(pool, 2, mean)
  p.m <- p.m[p.m != 0]
  p <- p.m/N
 }

 #Calculate the occurrence frequency of each taxa across communities
 spp.bi <- 1*(spp>0)
 freq <- apply(spp.bi, 2, mean)
 freq <- freq[freq != 0]

 #Combine
 C <- merge(p, freq, by=0)
 C <- C[order(C[,2]),]
 C <- as.data.frame(C)
 C.0 <- C[!(apply(C, 1, function(y) any(y == 0))),] #Removes rows with any zero (absent in
either source pool or local communities)
```

```
p <- C.0[,2]
freq <- C.0[,3]
names(p) <- C.0[,1]
names(freq) <- C.0[,1]

#Calculate the limit of detection
d = 1/N

##Fit model parameter m (or Nm) using Non-linear least squares (NLS)
m.fit <- nlsLM(freq ~ pbeta(d, N*m*p, N*m*(1-p), lower.tail=FALSE), start=list(m=0.1))
m.ci <- confint(m.fit, 'm', level=0.95)

##Fit neutral model parameter m (or Nm) using Maximum likelihood estimation (MLE)
sncm.LL <- function(m, sigma){
  R = freq - pbeta(d, N*m*p, N*m*(1-p), lower.tail=FALSE)
  R = dnorm(R, 0, sigma)
  -sum(log(R))
}
m.mle <- mle(sncm.LL, start=list(m=0.1, sigma=0.1), nobs=length(p))

##Calculate Akaike's Information Criterion (AIC)
aic.fit <- AIC(m.mle, k=2)
bic.fit <- BIC(m.mle)

##Calculate goodness-of-fit (R-squared and Root Mean Squared Error)
freq.pred <- pbeta(d, N*coef(m.fit)*p, N*coef(m.fit)*(1-p), lower.tail=FALSE)
Rsqr <- 1 - (sum((freq - freq.pred)^2))/(sum((freq - mean(freq))^2))
RMSE <- sqrt(sum((freq-freq.pred)^2)/(length(freq)-1))

pred.ci <- binconf(freq.pred*nrow(spp), nrow(spp), alpha=0.05, method="wilson",
return.df=TRUE)

##Calculate AIC for binomial model
bino.LL <- function(mu, sigma){
  R = freq - pbinom(d, N, p, lower.tail=FALSE)
  R = dnorm(R, mu, sigma)
  -sum(log(R))
}
bino.mle <- mle(bino.LL, start=list(mu=0, sigma=0.1), nobs=length(p))

aic.bino <- AIC(bino.mle, k=2)
bic.bino <- BIC(bino.mle)

##Goodness of fit for binomial model
bino.pred <- pbinom(d, N, p, lower.tail=FALSE)
Rsqr.bino <- 1 - (sum((freq - bino.pred)^2))/(sum((freq - mean(freq))^2))
RMSE.bino <- sqrt(sum((freq - bino.pred)^2)/(length(freq) - 1))

bino.pred.ci <- binconf(bino.pred*nrow(spp), nrow(spp), alpha=0.05, method="wilson",
return.df=TRUE)
```

```
 ##Calculate AIC for Poisson model
 pois.LL <- function(mu, sigma){
  R = freq - ppois(d, N*p, lower.tail=FALSE)
  R = dnorm(R, mu, sigma)
  -sum(log(R))
 }
 pois.mle <- mle(pois.LL, start=list(mu=0, sigma=0.1), nobs=length(p))

 aic.pois <- AIC(pois.mle, k=2)
 bic.pois <- BIC(pois.mle)

 ##Goodness of fit for Poisson model
 pois.pred <- ppois(d, N*p, lower.tail=FALSE)
 Rsqr.pois <- 1 - (sum((freq - pois.pred)^2))/(sum((freq - mean(freq))^2))
 RMSE.pois <- sqrt(sum((freq - pois.pred)^2)/(length(freq) - 1))

 pois.pred.ci <- binconf(pois.pred*nrow(spp), nrow(spp), alpha=0.05, method="wilson",
return.df=TRUE)

 ##Results
 if(stats==TRUE){
  fitstats <- data.frame(m=numeric(), m.ci=numeric(), m.mle=numeric(), maxLL=numeric(),
binoLL=numeric(), poisLL=numeric(), Rsqr=numeric(), Rsqr.bino=numeric(),
Rsqr.pois=numeric(), RMSE=numeric(), RMSE.bino=numeric(), RMSE.pois=numeric(),
AIC=numeric(), BIC=numeric(), AIC.bino=numeric(), BIC.bino=numeric(), AIC.pois=numeric(),
BIC.pois=numeric(), N=numeric(), Samples=numeric(), Richness=numeric(),
Detect=numeric())
  fitstats[1,] <- c(coef(m.fit), coef(m.fit)-m.ci[1], m.mle@coef['m'], m.mle@details$value,
bino.mle@details$value, pois.mle@details$value, Rsqr, Rsqr.bino, Rsqr.pois, RMSE,
RMSE.bino, RMSE.pois, aic.fit, bic.fit, aic.bino, bic.bino, aic.pois, bic.pois, N, nrow(spp),
length(p), d)
  return(fitstats)
 } else {
  A <- cbind(p, freq, freq.pred, pred.ci[,2:3], bino.pred, bino.pred.ci[,2:3])
  A <- as.data.frame(A)
  colnames(A) <- c('p', 'freq', 'freq.pred', 'pred.lwr', 'pred.upr', 'bino.pred', 'bino.lwr',
'bino.upr')
  if(is.null(taxon)){
   B <- A[order(A[,1]),]
  } else {
   B <- merge(A, taxon, by=0, all=TRUE)
   row.names(B) <- B[,1]
   B <- B[,-1]
   B <- B[order(B[,1]),]
  }
  return(B)
 }
}

# run model
#file.path3="otu_table_env.csv"
```

```
#pool<-read.table (file.path3, check.names = FALSE, header = TRUE, dec = ".", sep = ",", row.names = 1, comment.char = "")

obs.np=sncm.fit(spp, taxon, stats=FALSE, pool=NULL)
obs.np <- subset(obs.np, select = -c(y))
obs.np <- na.omit(obs.np)
sta.np=sncm.fit(spp, taxon, stats=TRUE, pool=NULL)
sta.np.16S <- sta.np

above.pred=sum(obs.np$freq > (obs.np$pred.upr), na.rm=TRUE)/sta.np$Richness
below.pred=sum(obs.np$freq < (obs.np$pred.lwr), na.rm=TRUE)/sta.np$Richness

ap = obs.np$freq > (obs.np$pred.upr)
bp = obs.np$freq < (obs.np$pred.lwr)

b1 <- ggplot() +
 geom_point(data=occ_abun[occ_abun$fill=='no',], aes(x=log10(otu_rel), y=otu_occ), pch=21, fill='cornsilk', alpha=.3)+
 geom_point(data=occ_abun[occ_abun$fill!='no',], aes(x=log10(otu_rel), y=otu_occ), pch=21, fill='darkblue',size=2)+
 geom_line(color='black', data=obs.np, size=1, aes(y=obs.np$freq.pred, x=log10(obs.np$p)), alpha=1) +
 geom_line(color='black', lty='dashed', size=1, data=obs.np, aes(y=obs.np$pred.upr, x=log10(obs.np$p)), alpha=1)+
 geom_line(color='black', lty='dashed', size=1, data=obs.np, aes(y=obs.np$pred.lwr, x=log10(obs.np$p)), alpha=1)+
 labs(x="log10(mean relative abundance)", y="Occupancy")

b1 <- b1 + theme_bw() + theme(panel.grid.major = element_blank(),
                              panel.grid.minor = element_blank()) +
 theme(text=element_text(family="Calibri", face="bold", size=11))+
 theme(legend.key.size = unit(0.5, 'cm'))+
 theme(legend.title = element_text(size=9))

b1

ggsave(filename = "Core_Occupancy.svg", plot = b1, width = 20, height = 14, dpi = 300, units = "cm")


# merge dataframes to obtain core taxa

library(tibble)
df <- tibble::rownames_to_column(obs.np, "otu")

df_merge <- merge(df,occ_abun,by="otu",all.x = TRUE, all.y=TRUE)


#find upper and lower OTUs + middle

otu.upper<-df_merge[df_merge$freq>df_merge$pred.upr,]
```

```
otu.under<-df_merge[df_merge$freq<df_merge$pred.lwr,]
otu.middle <- df_merge[df_merge$freq >= df_merge$pred.lwr & df_merge$freq <= df_merge$pred.upr, ]

# add upper,neutral and lower tag to the df_merge dataframe

# Create a new column called 'model' in df
df_merge$model <- NA

# Loop through each row in df
for (i in 1:nrow(df_merge)) {

  # Check if the otu_ID is in otu.upper
  if (df_merge[i, "otu"] %in% otu.upper$otu) {
    df_merge[i, "model"] <- "upper"
  }

  # Check if the otu_ID is in otu.middle
  else if (df_merge[i, "otu"] %in% otu.middle$otu) {
    df_merge[i, "model"] <- "neutral"
  }

  # Check if the otu_ID is in otu.under
  else if (df_merge[i, "otu"] %in% otu.under$otu) {
    df_merge[i, "model"] <- "under"
  }
}
write.csv(df_merge, "model_stats_complete.csv", row.names = FALSE)

#plot with upper/under
b1 <- ggplot() +
 geom_point(data=occ_abun[occ_abun$fill=='no',], aes(x=log10(otu_rel), y=otu_occ), pch=21, fill='white', alpha=.3)+
  geom_point(data=otu.middle, aes(x=log10(otu_rel), y=otu_occ), pch=21, fill='white',size=1.5)+
  geom_point(data=otu.upper, aes(x=log10(otu_rel), y=otu_occ), pch=21, fill='cornflowerblue',size=1.5)+
  geom_point(data=otu.under, aes(x=log10(otu_rel), y=otu_occ), pch=21, fill='coral',size=1.5)+
 geom_point(data=occ_abun[occ_abun$fill!='no',], aes(x=log10(otu_rel), y=otu_occ), pch=21,shape = 21,size=2)+
  geom_line(color='black', data=obs.np, size=1, aes(y=obs.np$freq.pred, x=log10(obs.np$p)), alpha=1) +
  geom_line(color='black', lty='dashed', size=1, data=obs.np, aes(y=obs.np$pred.upr, x=log10(obs.np$p)), alpha=1)+
  geom_line(color='black', lty='dashed', size=1, data=obs.np, aes(y=obs.np$pred.lwr, x=log10(obs.np$p)), alpha=1)+
  labs(x="log10(mean relative abundance)", y="Occurance frequency")

b1 <- b1 + theme_bw() + theme(panel.grid.major = element_blank(),
                              panel.grid.minor = element_blank()) +
```

```
 theme(text=element_text(family="Calibri", face="bold", size=11))+
 theme(legend.key.size = unit(0.5, 'cm'))+
 theme(legend.title = element_text(size=9))

b1

ggsave(filename = "Neutral_model_New.svg", plot = b1, width = 18, height = 14, dpi = 300,
units = "cm")

otu.upper %>% count(fill)
otu.under %>% count(fill)
otu.middle %>% count(fill)
occ_abun %>% count(fill)

# viva
otu.under
otu.upper
otu_new <- tibble::rownames_to_column(otu, "otu")

otu_model <- otu_new %>%
 filter(otu %in% otu.upper$otu | otu %in% otu.under$otu)
write.csv(otu_model,"otu_deterministic.csv", row.names = FALSE)

# subset otu.s upper dataframe to have only the core OTUs, in order to get only core otus
that are selected by the host
CoreDF <- otu.upper %>% filter(fill=="core")

otu_new <- tibble::rownames_to_column(otu, "otu")

# subset to genereta upper core otu table
otu_core<- otu_new %>%
 filter(otu %in% CoreDF$otu)

write.csv(otu_core,"otu_core_upper_new.csv", row.names = FALSE)

# subset otu.s under dataframe to have only the core OTUs that are under
CoreDF2 <- otu.under %>% filter(fill=="core")

otu_new2 <- tibble::rownames_to_column(otu, "otu")

# subset to generet under core otu table
otu_core2<- otu_new2 %>%
 filter(otu %in% CoreDF2$otu)

write.csv(otu_core2,"otu_core_under_new.csv", row.names = FALSE)

# subset otu.s under dataframe to have only the core OTUs that are neutral
CoreDF3 <- otu.middle %>% filter(fill=="core")

otu_new3 <- tibble::rownames_to_column(otu, "otu")
```

```
# subset to generet neutral core otu table
otu_core3<- otu_new3 %>%
  filter(otu %in% CoreDF3$otu)

write.csv(otu_core3,"otu_core_neutral_new.csv", row.names = FALSE)


#subset only upper OTUs
otu_model<- otu_new %>%
  filter(otu %in% otus.upper$otu)

write.csv(otu_model,"otu_upper.csv", row.names = FALSE)

#save model stats
write.csv(sta.np,"model_Stats_new.csv", row.names = T)
write.csv(df_merge,"otu_stats_new.csv", row.names = T)

#otu all; to include stats in all models by running river_eco and combine it with tax table
write.csv(otu,"otu_all.csv", row.names = T)

### CORE pie

pie <- read.csv("core_barchart.csv",row.names = 1, header=T, stringsAsFactors = FALSE)

library(ggplot2)
library(ggrepel)
library(tidyverse)

# Get the positions
df2 <- pie %>%
  mutate(csum = rev(cumsum(rev(value))),
         pos = value/2 + lead(csum, 1),
         pos = if_else(is.na(pos), value/2, pos))

b1 <- ggplot(pie, aes(x = "" , y = value, fill = fct_inorder(group))) +
  geom_col(width = 1, color = 1) +
  coord_polar(theta = "y") +
  scale_fill_brewer(palette = "RdBu", direction=-1) +
  geom_label_repel(data = df2,
                   aes(y = pos, label = paste0(value, "%")),
                   size = 6.5, nudge_x = 1, show.legend = FALSE) +
  guides(fill = guide_legend(title = "Group")) +
  theme_void()

b1 <- b1 +
  theme(text=element_text(family="Calibri", face="bold", size=11))

b1

ggsave(filename = "NoCore_OTU_pie_reverse.svg", plot = b1, width = 14, height = 14, dpi =
300, units = "cm")
```

```
#-------------------------------------------------CHAPTER 3-------------------

#Ventilation Rate

rm(list=ls())

library(ggplot2)
library(dplyr)
library(broom)
library(tidyr)
library(ez)
library(car)
library(rstatix)
library(ggpubr)
library(jtools)
library(lme4)
library(lmerTest)
library(nlme)
library(emmeans)
library(patchwork)
library (reshape2)
library(gridExtra)


VR_meta<- read.csv("VR_meta.csv",header=T)


# Prepare data for heatmap
heatmap_data <- dcast(VR_meta, Origin ~ Season, value.var = "VR", mean)
heatmap_matrix <- as.matrix(heatmap_data[, -1])
rownames(heatmap_matrix) <- heatmap_data$Origin

# Create heatmap
heatmap_plot <- ggplot(melt(heatmap_matrix), aes(Var2, Var1, fill = value)) +
 geom_tile(color = "white") +
 scale_fill_gradient(low = "white", high = "steelblue") +
 labs(title = "Heatmap of Mean Ventilation Rates by Season and Origin",
      x = "Season",
      y = "Origin",
      fill = "Mean VR") +
 theme_minimal()

heatmap_plot

#calculate sd
mean_sd_data <- VR_meta %>%
 group_by(Season) %>%
 summarize(mean_VR = sprintf("%.2f", mean(VR, na.rm = TRUE)),
           sd_VR = sprintf("%.2f", sd(VR, na.rm = TRUE)))

# View the resulting data
```

```
print(mean_sd_data)

VR_meta$Or_Se <- paste0(VR_meta$Origin, "__", VR_meta$Season)
#Ancova to explain VR by season, origin and accounting for the influence of weight.
#effect of Season on VR is different across the different Origin groups?.

# Perform the ANCOVA with interactions to adjust for weight
adjusted_ancova_result <- aov(VR ~ Weight + Origin + Season + Origin:Season, data = VR_meta)

# View the summary of the adjusted ANCOVA model
summary(adjusted_ancova_result)

# Perform Tukey HSD test for the interaction term
tukey_results <- TukeyHSD(adjusted_ancova_result, which = "Origin:Season")
print(tukey_results)

df <- partialize(adjusted_ancova_result, vars = c("Season", "Origin"))
df
VR_meta$VR_adjust <- df$VR

df2 <- partialize(adjusted_ancova_result, vars= "Origin")

VR_meta$VR_Origin <- df2$VR

# define colors
myCol2 <- c(F = "#CC6677", HFF = "#88CCEE", HWF ="#44AA99", W = "#DDCC77")

# t-test
stat.test <- VR_meta %>%
  group_by(Season) %>%
  t_test(VR_adjust ~ Origin) %>%
  adjust_pvalue(method = "fdr") %>%
  add_significance("p.adj")
stat.test

#plot
bxp <- ggboxplot(VR_meta, x = "Season", y = "VR_adjust", fill = "Origin",
                 palette = myCol2 )+
  labs(y = "Residualised Ventilation Rate (bpm)")
bxp

#include stats
stat.test <- stat.test %>%
  add_xy_position(x = "Season", dodge = 0.8)

bxp <- bxp + stat_pvalue_manual(
  stat.test,  label = "p.adj.signif", tip.length = 0, hide.ns = TRUE
)

bxp
```

```
b1 <- bxp  + theme_bw() + theme(panel.grid.major = element_blank(),
                        panel.grid.minor = element_blank()) +
 theme(legend.position = "top")+
 theme(text=element_text(family="Calibri", face="bold", size=11))
b1

ggsave(filename = "resVR_Season.svg", plot = b1, width = 14, height = 14, dpi = 300, units =
"cm")

# Temperature - adjusted VR correlation

# Plotting
custom_colors <- c(F = "#CC6677", HFF = "#88CCEE", HWF ="#44AA99", W = "#DDCC77")

# Calculate R-squared values for each origin
r_squared_values <- VR_meta %>%
 group_by(Origin) %>%
 summarize(R_squared = summary(lm(VR_adjust ~ Temperature))$r.squared)

# Create the plot with jittering
vr_temp_plot <- ggplot(VR_meta, aes(x = Temperature, y = VR_adjust, color = Origin)) +
 geom_jitter(width = 0.2, height = 0, alpha = 0.6, size = 2) +  # Jittered scatter plot points
 geom_smooth(method = "lm", se = FALSE, size = 1) +  # Linear regression lines
 scale_color_manual(values = custom_colors) +  # Custom colors
 labs(
  title = "Relationship between Weight-Adjusted Ventilation Rate and Temperature",
  x = "Temperature (°C)",
  y = "Weight-Adjusted Ventilation Rate (VR_adjust)",
  color = "Origin"
 ) +
 theme_minimal(base_size = 14) +
 theme(
  panel.grid.major = element_line(color = "gray90"),
  panel.grid.minor = element_line(color = "gray95"),
  plot.title = element_text(hjust = 0.5, face = "bold"),
  legend.position = "top"
 ) +
 geom_text(
  data = r_squared_values,
  aes(
   x = Inf, y = Inf,
   label = sprintf("R² = %.2f", R_squared),
   color = Origin
  ),
  hjust = 1.1, vjust = 1.1, size = 4,
  show.legend = FALSE
 )

# Print the plot
print(vr_temp_plot)
```

```
#other plot
vr_temp_plot_facet <- ggplot(VR_meta, aes(x = Temperature, y = VR_adjust, color = Origin))
+
 geom_point(alpha = 0.6, size = 2) +  # Scatter plot points
 geom_smooth(method = "lm", se = FALSE, size = 1) +  # Linear regression lines
 scale_color_manual(values = custom_colors) +  # Custom colors
 labs(
  x = "Temperature (°C)",
  y = "Residualised Ventilation Rate (bpm)",
  color = "Origin"
 ) +
 theme_minimal(base_size = 14) +
 theme(
  panel.grid.major = element_line(color = "gray90"),
  panel.grid.minor = element_line(color = "gray95"),
  plot.title = element_text(hjust = 0.5, face = "bold"),
  legend.position = "top",
  text = element_text(family = "Calibri", face = "bold", size = 11),
  panel.border = element_rect(color = "black", fill = NA, size = 1)  # Frame around each plot
 ) +
 facet_wrap(~ Origin) +  # Facet by Origin
 geom_text(
  data = r_squared_values,
  aes(
   x = Inf, y = Inf,
   label = sprintf("R² = %.2f", R_squared),
   color = Origin
  ),
  hjust = 1.1, vjust = 1.1, size = 4,
  show.legend = FALSE
 )

# Print the plot
print(vr_temp_plot_facet)

ggsave(filename = "VR_Temp_plot.svg", plot = vr_temp_plot_facet, width = 22, height = 14,
dpi = 300, units = "cm")

# Summarize the data to get mean VR and coefficient of variation (CV)
cv_data <- VR_meta %>%
 group_by(Season, Origin) %>%
 summarise(mean_VR = mean(VR, na.rm = TRUE),
        sd_VR = sd(VR, na.rm = TRUE),
        cv_VR = sd_VR / mean_VR * 100)  # CV = (SD / Mean) * 100

autumn18_data <- subset(cv_data, Season == "Autumn18")
winter19_data <- subset(cv_data, Season == "Winter19")
summer19_data <- subset(cv_data, Season == "Summer19")
autumn19_data <- subset(cv_data, Season == "Autumn19")
```

```
cv_data

cv_data_filtered <- cv_data %>%
  filter(Season %in% c("BWinter19", "CSummer19"))


anova_result <- aov(cv_VR ~ Origin, data = cv_data_filtered)
summary(anova_result)

# Post-hoc tests (Tukey's HSD) to compare means between groups
posthoc <- TukeyHSD(anova_result)
print(posthoc)

cv_plot <- ggplot(cv_data_filtered, aes(x = Season, y = cv_VR, fill = Origin)) +
  geom_bar(stat = "identity", position = position_dodge(width = 0.7), width = 0.7,
           color = "black", size = 1) +  # Add black frame around bars
  labs(x = "Season",
       y = "Coefficient of Variation (CV)",
       fill = "Origin") +
  scale_fill_manual(values = c(F = "#CC6677", HFF = "#88CCEE", HWF = "#44AA99", W =
"#DDCC77")) +
  theme_minimal() +
  theme(panel.grid.major.x = element_blank(),  # Remove vertical grid lines
        panel.grid.minor = element_blank(),
        text = element_text(family = "Calibri", face = "bold", size = 11),
        panel.border = element_rect(color = "black", fill = NA, size = 1)) +  # Adjust line properties
  # Add CV values above bars
  geom_text(aes(label = paste0(round(cv_VR, 1), "%"), y = cv_VR + 1),
            position = position_dodge(width = 0.7),
            vjust = 1, size = 3.5) +
  # Remove legend
  guides(fill = FALSE)

# Display the plot
print(cv_plot)

ggsave(filename = "cv_plot.svg", plot = cv_plot, width = 22, height = 14, dpi = 300, units =
"cm")

combined_plot <- grid.arrange(b1, cv_plot, ncol = 1, heights = c(0.6, 0.4))
ggsave(filename = "combined_plot2.svg", plot = combined_plot, width = 22, height = 14, dpi
= 300, units = "cm")


#--------------------------------------------------Diff abundance--------------------------
Autumn19 <- clone(dataset)
Autumn19$sample_table <- subset(Autumn19$sample_table, Season == "DAutumn19")
Autumn19$tidy_dataset()

Winter19 <- clone(dataset)
Winter19$sample_table <- subset(Winter19$sample_table, Season == "BWinter19")
```

```
Winter19$tidy_dataset()

pseq <- meco2phyloseq(Winter19)
pseq

#AncomBC2
#create microeco dataset and transform to physeq...

#add otu column
taxa_table <- as.data.frame(tax_table(pseq))
head(taxa_table)
taxa_table$OTU <- rownames(taxa_table)
taxa_table_matrix <- as.matrix(taxa_table)
tax_table(pseq) <- taxa_table_matrix
head(tax_table(pseq))


#Run ANCOM
output = ancombc2(data = pseq, assay_name = "Environmental", tax_level = "OTU",
            fix_formula = "Origin", rand_formula = NULL,
            p_adj_method = "holm", pseudo_sens = TRUE,
            prv_cut = 0.05, lib_cut = 1500, s0_perc = 0.05,
            group = "Origin", struc_zero = FALSE, neg_lb = TRUE,
            alpha = 0.05, n_cl = 6, verbose = TRUE,
            global = TRUE, pairwise = TRUE, dunnet = TRUE, trend = FALSE,
            iter_control = list(tol = 1e-2, max_iter = 20,
                        verbose = TRUE),
            em_control = list(tol = 1e-5, max_iter = 100),
            lme_control = lme4::lmerControl(),
            mdfdr_control = list(fwer_ctrl_method = "holm", B = 100),
            trend_control = list(contrast = list(matrix(c(1, 0, -1, 1),
                                        nrow = 2,
                                        byrow = TRUE),
                                    matrix(c(-1, 0, 1, -1),
                                        nrow = 2,
                                        byrow = TRUE),
                                    matrix(c(1, 0, 1, -1),
                                        nrow = 2,
                                        byrow = TRUE)),
                                node = list(2, 2, 1),
                                solver = "ECOS",
                                B = 8))

res = output$res
res_global = output$res_global
dunn <- output$res_dunn
write.csv(dunn,"AncomBC_dunn_OTU_0.05.csv", row.names = TRUE)
#structural zeros
tab_zero = output$zero_ind
tab_zero %>%
  datatable(caption = "The detection of structural zeros")
```

```
#bias correcte dlog abundances
bias_correct_log_table = output$bias_correct_log_table

#Multiple pairwise comparisons

ancombc_table = output$res_pair

# Filter for differentially abundant OTUs and create lfc columns for each pairwise comparison
df_fig_pair1 <- ancombc_table %>%
 filter(diff_OriginHFF == TRUE | diff_OriginHWF == TRUE | diff_OriginW == TRUE |
        diff_OriginHWF_OriginHFF == TRUE | diff_OriginW_OriginHFF == TRUE |
diff_OriginW_OriginHWF == TRUE) %>%
 mutate(lfc1 = ifelse(diff_OriginHFF == TRUE, round(lfc_OriginHFF, 2), 0),
       lfc2 = ifelse(diff_OriginHWF == TRUE, round(lfc_OriginHWF, 2), 0),
       lfc3 = ifelse(diff_OriginW == TRUE, round(lfc_OriginW, 2), 0),
       lfc4 = ifelse(diff_OriginHWF_OriginHFF == TRUE, round(lfc_OriginHWF_OriginHFF, 2),
0),
       lfc5 = ifelse(diff_OriginW_OriginHFF == TRUE, round(lfc_OriginW_OriginHFF, 2), 0),
       lfc6 = ifelse(diff_OriginW_OriginHWF == TRUE, round(lfc_OriginW_OriginHWF, 2), 0))
%>%
 pivot_longer(cols = lfc1:lfc6, names_to = "group", values_to = "value") %>%
 arrange(taxon)

# Create color coding based on significance
df_fig_pair2 <- ancombc_table %>%
 filter(diff_OriginHFF == TRUE | diff_OriginHWF == TRUE | diff_OriginW == TRUE |
        diff_OriginHWF_OriginHFF == TRUE | diff_OriginW_OriginHFF == TRUE |
diff_OriginW_OriginHWF == TRUE) %>%
 mutate(lfc1 = ifelse(passed_ss_OriginHFF == TRUE & diff_OriginHFF == TRUE,
"aquamarine3", "black"),
       lfc2 = ifelse(passed_ss_OriginHWF == TRUE & diff_OriginHWF == TRUE, "aquamarine3",
"black"),
       lfc3 = ifelse(passed_ss_OriginW == TRUE & diff_OriginW == TRUE, "aquamarine3",
"black"),
       lfc4 = ifelse(passed_ss_OriginHWF_OriginHFF == TRUE & diff_OriginHWF_OriginHFF ==
TRUE, "aquamarine3", "black"),
       lfc5 = ifelse(passed_ss_OriginW_OriginHFF == TRUE & diff_OriginW_OriginHFF == TRUE,
"aquamarine3", "black"),
       lfc6 = ifelse(passed_ss_OriginW_OriginHWF == TRUE & diff_OriginW_OriginHWF ==
TRUE, "aquamarine3", "black")) %>%
 pivot_longer(cols = lfc1:lfc6, names_to = "group", values_to = "color") %>%
 arrange(taxon)

# Merge the two data frames
df_fig_pair <- df_fig_pair1 %>%
 left_join(df_fig_pair2, by = c("taxon", "group"))

# Rename the groups for clarity
df_fig_pair <- df_fig_pair %>%
```

```
  mutate(group = case_when(
    group == "lfc1" ~ "F - HFF",
    group == "lfc2" ~ "F - HWF",
    group == "lfc3" ~ "F - W",
    group == "lfc4" ~ "HFF - HWF",
    group == "lfc5" ~ "HFF - W",
    group == "lfc6" ~ "HWF - W",
    TRUE ~ as.character(group)
  ))

df_fig_pair$group = factor(df_fig_pair$group,
                           levels = c("F - HFF", "F - HWF", "F - W",
                                      "HFF - HWF", "HFF - W", "HWF - W"))

# Heatmap
fig_pair <- df_fig_pair %>%
  ggplot(aes(x = group, y = taxon, fill = value)) +
  geom_tile(color = "black") +
  scale_fill_gradient2(low = "blue", high = "darkred", mid = "white",
                       na.value = "white", midpoint = 0,
                       limit = c(-10, 10), name = NULL) +
  geom_text(aes(label = value), color = "black", size = 4) +
  scale_color_identity(guide = FALSE) +
  theme_minimal() +
  theme(
    plot.title = element_text(hjust = 0.5),
    axis.text.y = element_text(face = "italic")  # Italicize the y-axis text
  ) +
  scale_y_discrete(labels = function(x) parse(text = paste0("italic('", x, "')")))

fig_pair

write.csv(df_fig_pair,"OTU_Diff_Abund_Winter19.csv", row.names = TRUE)
ggsave(filename = "Ancom2_Heatmap_Winter19.svg", plot = fig_pair, width = 26, height =
14, dpi = 300, units = "cm")


#----------------------------------------------Ventilation Rate----------------------------------------------
rm(list=ls())

library(ggplot2)
library(dplyr)
library(viridis)
library(Interpol.T)
library(lubridate)
library(ggExtra)
library(tidyr)
library(tidyverse)
library(timetk)
```

```
df<- read.csv("Temperature.csv",header=T)

df<- df %>% mutate(year = year(Date),
                   month = month(Date, label=TRUE),
                   day = day(Date))

p <-ggplot(df,aes(day,hour,fill=Temperature))+
 geom_tile(color= "white",size=0.1) +
 scale_fill_viridis(name="Hrly Temps ?C",option ="C")
p <-p + facet_grid(year~month)
p <-p + scale_y_continuous(trans = "reverse", breaks = unique(df$hour))
p <-p + scale_x_continuous(breaks =c(1,10,20,31))
p <-p + theme_minimal(base_size = 8)
p <-p + theme(legend.position = "bottom")+
 theme(plot.title=element_text(size = 14))+
 theme(axis.text.y=element_text(size=6)) +
 theme(strip.background = element_rect(colour="white"))+
 theme(plot.title=element_text(hjust=0))+
 theme(axis.ticks=element_blank())+
 theme(axis.text=element_text(size=7))+
 theme(legend.title=element_text(size=8))+
 theme(legend.text=element_text(size=6))+
 removeGrid()#ggExtra
p

#-----------------------------------------------------------------------------------------

data<- read.csv("WaterLevel19.csv",header=T)

data$Day <- as.POSIXct((paste(data$Date, data$Time)), format="%Y-%m-%d %H:%M:%S")

data <- na.omit(data)

# Setup for the plotly charts (# FALSE returns ggplots)
interactive <- FALSE

p1 <-data %>%
 plot_time_series(Day,WaterLevel,
                  .color_var = year(Day),      # Color applied to Week transformation
                  # Facet formatting
                  .interactive = interactive,
                  .title = NULL,
                  .legend_show = FALSE,
                  .x_lab = "Date (30-min intervals)",
                  .y_lab = "Water level (m) ",)
b1 <- p1 + theme_bw() + theme(panel.grid.major = element_blank(),
                              panel.grid.minor = element_blank()) +
 theme(legend.position="none")+
 theme(text=element_text(family="Calibri", face="bold", size=11))

b1
```

```
ggsave(filename = "WaterLevel.svg", plot = b1, width = 14, height = 10, dpi = 300, units =
"cm")

p1<-data %>%
 plot_time_series(Day,Flow,
             .color_var = year(Day),     # Color applied to Week transformation
             # Facet formatting
             .facet_scales = "free",
             .interactive = interactive,
             .title = NULL,
             .legend_show = FALSE,
             .x_lab = "Date (30-min intervals)",
             .y_lab = "Flow rate (l/s) ",)
b1 <- p1 + theme_bw() + theme(panel.grid.major = element_blank(),
                      panel.grid.minor = element_blank()) +
 theme(legend.position="none")+
 theme(text=element_text(family="Calibri", face="bold", size=11))

b1

ggsave(filename = "Flow.png", plot = b1, width = 14, height = 10, dpi = 300, units = "cm")

#------------------------ conductivity/DO------------------------

data<- read.csv("Conductivity19.csv",header=T)

data$Day <- as.POSIXct((paste(data$Date, data$Time)), format="%Y-%m-%d %H:%M:%S")

data <- na.omit(data)

interactive <- FALSE

b1<-data %>%
 plot_time_series(Day,Conductivity,
             .color_var = year(Day),     # Color applied to Week transformation
             # Facet formatting
             .facet_ncol = 2,
             .facet_scales = "free",
             .interactive = interactive,
             .title = NULL,
             .legend_show = FALSE,
             .x_lab = "Date (2-min intervals)",
             .y_lab = "Conductivity (mS/cm) ",)

b1 <- b1 + theme_bw() + theme(panel.grid.major = element_blank(),
                      panel.grid.minor = element_blank()) +
 theme(legend.position="none")+
 theme(text=element_text(family="Calibri", face="bold", size=11))

b1
```

```
ggsave(filename = "Conductivity.png", plot = b1, width = 14, height = 10, dpi = 300, units = "cm")

p1 <- data %>%
  plot_time_series(Day,(DO_mgl),
                   .color_var = year(Day),     # Color applied to Week transformation
                   # Facet formatting
                   .facet_scales = "free",
                   .interactive = interactive,
                   .title = NULL,
                   .legend_show = FALSE,
                   .x_lab = "Date (2-min intervals)",
                   .y_lab = "Dissolved oxygen (mg/l) ",)
b1 <- p1 + theme_bw() + theme(panel.grid.major = element_blank(),
                              panel.grid.minor = element_blank()) +
  theme(legend.position="none")+
  theme(text=element_text(family="Calibri", face="bold", size=11))

b1

ggsave(filename = "DO.png", plot = b1, width = 14, height = 10, dpi = 300, units = "cm")
#------------------------------Temperature-------------------
data<- read.csv("Temperature19.csv",header=T)
myCol <- c(Jan19="#0066CC", Feb19="#003366", Mar19="#33CC99", Apr19="#33CC66",May19="#00FF00",
           Jun19="#990000", Jul19="#993300", Aug19="#660000", Sep19="#FF9900", Oct19="#CC6600", Nov19="#CC9966", Dec19="#99CCFF", Jan20="#0099FF")

myCol <- c("#0066CC","#003366","#33CC99","#33CC66","#00FF00","#990000","#993300","#660000","#FF9900","#CC6600","#CC9966","#99CCFF")

data$Day <- as.POSIXct((paste(data$Date, data$Time)), format="%Y-%m-%d %H:%M:%S")

data <- na.omit(data)

interactive <- FALSE

p1 <- data %>%
  plot_time_series(Day,Temperature,
                   .color_var = year(Day),     # Color applied to Week transformation
                   # Facet formatting
                   .facet_ncol = 2,
                   .facet_scales = "free",
                   .interactive = interactive,
                   .title = NULL,
                   .legend_show = FALSE,
                   .x_lab = "Date (30-min intervals)",
                   .y_lab = "Temperature (°C) ",)
b1 <- p1 + theme_bw() + theme(panel.grid.major = element_blank(),
```

```
                    panel.grid.minor = element_blank()) +
 theme(legend.position="none")+
 theme(text=element_text(family="Calibri", face="bold", size=11))

b1

ggsave(filename = "Temp.svg", plot = b1, width = 14, height = 10, dpi = 300, units = "cm")

#-----------------------Dayly median, average---------------------------
options(max.print=10000)

data<- read.csv("WaterLevel.csv",header=T)
data<- read.csv("Conductivity.csv",header=T)
data<- read.csv("Temperature.csv",header=T)

data$Day <- as.POSIXct((paste(data$Date, data$Time)), format="%Y-%m-%d %H:%M:%S")

data <- na.omit(data)

aggregate(list(Level = data$Temperature),
      list(Date = cut(data$Day, "day")),
      min)

aggregate(list(Level = data$DO_mgl),
      list(Date = cut(data$Day, "day")),
      min)

#-----------------------------------VR GLMS---------------------------
rm(list=ls())

library(ggplot2)
library(dplyr)
library(broom)
library(tidyr)
library(ez)
library(car)
library(rstatix)
library(ggpubr)
library(jtools)

setwd("C:/Users/Paddy/Documents/PhD/Chapter Torpor/River/Metabolism")
setwd("C:/Users/Paddy/Documents/PhD/Chapter Torpor/River/NEW")
fish_data<- read.csv("meta.csv",header=T)

# Convert 'Origin' to a factor variable
fish_data$Origin <- factor(fish_data$Origin)

# look at data: Calculate mean and standard deviation for each combination of Origin and
Season
mean_sd_data <- fish_data %>%
 group_by(Season) %>%
```

```
  summarize(mean_VR = sprintf("%.2f", mean(VR, na.rm = TRUE)),
            sd_VR = sprintf("%.2f", sd(VR, na.rm = TRUE)))

# View the resulting data
print(mean_sd_data)

fish_data$Or_Se <- paste0(fish_data$Origin, "__", fish_data$Season)
#Ancova to explain VR by season, origin and accounting for the influence of weight.
#effect of Season on VR is different across the different Origin groups?.

# Perform the ANCOVA with interactions to adjust for weight
adjusted_ancova_result <- aov(VR ~ Weight + Origin + Season + Origin:Season, data =
fish_data)

# View the summary of the adjusted ANCOVA model
summary(adjusted_ancova_result)

# Perform Tukey HSD test for the interaction term
tukey_results <- TukeyHSD(adjusted_ancova_result, which = "Origin:Season")
print(tukey_results)

df <- partialize(adjusted_ancova_result, vars = c("Season", "Origin"))
df
fish_data$VR_adjust <- df$VR

df2 <- partialize(adjusted_ancova_result, vars= "Origin")

fish_data$VR_Origin <- df2$VR

# define colors
myCol2 <- c(F = "#CC6677", HFF = "#88CCEE", HWF ="#44AA99", W = "#DDCC77")

# t-test
stat.test <- fish_data %>%
  group_by(Season) %>%
  t_test(VR_adjust ~ Origin) %>%
  adjust_pvalue(method = "fdr") %>%
  add_significance("p.adj")
stat.test


#plot
bxp <- ggboxplot(fish_data, x = "Season", y = "VR_adjust", fill = "Origin",
                 palette = myCol2 )+
  labs(y = "Residualised Ventilation Rate (bpm)")
bxp

#include stats
stat.test <- stat.test %>%
  add_xy_position(x = "Season", dodge = 0.8)
```

```
bxp <- bxp + stat_pvalue_manual(
  stat.test,  label = "p.adj.signif", tip.length = 0, hide.ns = TRUE
)

bxp

b1 <- bxp  + theme_bw() + theme(panel.grid.major = element_blank(),
                          panel.grid.minor = element_blank()) +
  theme(legend.position = "top")+
  theme(text=element_text(family="Calibri", face="bold", size=11))
b1

ggsave(filename = "resVR_Season.svg", plot = b1, width = 14, height = 14, dpi = 300, units =
"cm")

myCol3 <- c(Autumn18 = "#BF812D", BWinter19 = "#00A1D5FF", CSummer19="#F6E8C3",
DAutumn19="#8C510A")

stat.test2 <- fish_data %>%
  group_by(Origin) %>%
  t_test(VR_adjust ~ Season) %>%
  adjust_pvalue(method = "fdr") %>%
  add_significance("p.adj")
stat.test2


bxp2 <- ggboxplot(fish_data, x = "Origin", y = "VR_adjust", fill = "Season",
               palette = myCol3 )+
  labs(y = "Residualised Ventilation Rate (bpm)")
bxp2

stat.test2 <- stat.test2 %>%
  add_xy_position(x = "Origin", dodge = 0.8)

bxp2 <- bxp2 + stat_pvalue_manual(
  stat.test2,  label = "p.adj.signif", tip.length = 0, hide.ns = TRUE
)

bxp2

b2 <- bxp2  + theme_bw() + theme(panel.grid.major = element_blank(),
                           panel.grid.minor = element_blank()) +
  theme(legend.position = "top")+
  theme(text=element_text(family="Calibri", face="bold", size=11))
b2

ggsave(filename = "resVR_Origin.svg", plot = b2, width = 14, height = 14, dpi = 300, units =
"cm")


# --> here farmed winter and summer VR is significantly different.
```

```
# So far so good. Now I would like to plot whether VR changes between seasons
# and if this change "adaptation" differs between origins.
# I also want to plot origins per season...
# use adjusted values to account for the variability that the model couldn't explain

#calculate residuals
residuals <- residuals(ancova_result)

#predicted VR values from the ANCOVA model
predicted_VR <- fitted.values(ancova_result)

# Calculate the adjusted VR
adjusted_VR <- predicted_VR

#add adjsuted VR
fish_data$Adjusted_VR <- adjusted_VR


#plot and examine residuals
par(mfrow=c(2,2)) # init 4 charts in 1 panel
plot(ancova_result)

par(mfrow=c(1,1))
plot(x = fitted(ancova_result), y = residuals,
    xlab = "Fitted Values", ylab = "Residuals",
    main = "Residuals vs. Fitted Values")

hist(residuals, main = "Histogram of Residuals", xlab = "Residuals")

qqnorm(residuals)
qqline(residuals)


shapiro.test(residuals)

# -> residuals non normally distributed, but homoscedasticity seems fine

# check if t-test is ok
# Function to check assumptions for each group
check_t_test_assumptions <- function(group_data) {
 # Check normality assumption
 norm_test <- shapiro.test(group_data$Adjusted_VR)

 # Check homoscedasticity assumption
 homoscedasticity_test <- bartlett.test(Adjusted_VR ~ Season, data = group_data)

 # Create a data frame to store the results
 assumption_results <- data.frame(
  Is_Normal = ifelse(norm_test$p.value >= 0.05, "Yes", "No"),
  Homoscedasticity_Test = homoscedasticity_test$p.value >= 0.05
```

```
 )
 return(assumption_results)
}

# Check the assumptions for the t-test within each 'Origin' group
assumption_check <- fish_data %>%
 group_by(Origin) %>%
 group_modify(~check_t_test_assumptions(.))

# Add 'Origin' as a separate column in the results
assumption_check$Group <- levels(fish_data$Origin)

# View the results
print(assumption_check)

#-------------------------------------- adaptation

adjusted_ancova_result <- aov(VR ~ Weight + Origin + Season + Origin:Season + Weight:Origin + Weight:Season + Weight:Origin:Season, data = fish_data)

## Make a new variable that combines Origin and Season
fish_data$Or_Se <- paste0(fish_data$Origin, "__", fish_data$Season)

## Make three new versions of VR and Weight that are:
##   - A) centred within origins
##   - B) centred within seasons
##   - C) centred within Or_Se

## To do this, get group means of each variable for each predictor
grpMeans_VR_Or <- unclass(tapply(fish_data$VR, fish_data$Origin, mean))
grpMeans_VR_Se <- unclass(tapply(fish_data$VR, fish_data$Season  , mean))
grpMeans_VR_OrSe <- unclass(tapply(fish_data$VR, fish_data$Or_Se , mean))

grpMeans_wght_Or <- unclass(tapply(fish_data$Weight, fish_data$Origin, mean))
grpMeans_wght_Se <- unclass(tapply(fish_data$Weight, fish_data$Season  , mean))
grpMeans_wght_OrSe <- unclass(tapply(fish_data$Weight, fish_data$Or_Se , mean))

## Use these to do the centering
fish_data$VR_C_Or <- fish_data$VR - grpMeans_VR_Or[fish_data$Origin]
fish_data$Weight_C_Or <- fish_data$Weight - grpMeans_wght_Or[fish_data$Origin]

fish_data$VR_C_Se <- fish_data$VR - grpMeans_VR_Se[fish_data$Season]
fish_data$Weight_C_Se <- fish_data$Weight - grpMeans_wght_Se[fish_data$Season]

fish_data$VR_C_OrSe <- fish_data$VR - grpMeans_VR_OrSe[fish_data$Or_Se]
fish_data$Weight_C_OrSe <- fish_data$Weight - grpMeans_wght_OrSe[fish_data$Or_Se]

## Then, do the following three ANCOVAs, and look specifically at whether the interaction term in each is significant
##   - OrSe first because if it's sig, the next two aren't worth bothering with
##   - in an ideal world, the interaction in none of these will be significant
```

```
adjusted_ancova_result <- aov(VR ~ Weight + Origin + Season + Origin:Season + Weight:Origin + Weight:Season + Weight:Origin:Season, data = fish_data)
ancova_C_OrSe <- aov(VR_C_OrSe ~ Weight_C_OrSe*Or_Se, data = fish_data)
ancova_C_Or <- aov(VR_C_Or ~ Weight_C_Or*Origin, data = fish_data)
ancova_C_Se <- aov(VR_C_Se ~ Weight_C_Se*Season, data = fish_data)

summary(adjusted_ancova_result)
summary(ancova_C_OrSe)
summary(ancova_C_Or)
summary(ancova_C_Se)


#----------Heat tree----------

#devtools::install_github("ropensci/taxa")

rm(list=ls())
set.seed(123)
library(metacoder)
library(gridExtra)
library(taxa)

sample_data <- read.csv("meta_heat_core.csv",row.names = 1, header=T, stringsAsFactors = FALSE)
otu <- read.csv("otu_heat_core.csv",row.names = 1, header=T, stringsAsFactors = FALSE)

print(sample_data)

# regex "From the start of the string, (^) match any character between"a" and "z" ([a-z]) zero or one times ({0,1}) f
# ollowed by an underscore (_) occurring between zero and 2 times ({0,2}), followed by any character (.) occurring zero or more times (*),
# followed by the end of the text ($)."

obj <- parse_tax_data(otu,
                      class_cols = "lineage",
                      class_sep = ";",
                      class_regex = "^([a-z]{0,1})_{0,2}(.*)$",
                      class_key = c("tax_rank" = "taxon_rank", "name" = "taxon_name"))


obj$data$tax_data <- zero_low_counts(obj, dataset = "tax_data", min_count = 5)

no_reads <- rowSums(obj$data$tax_data[, sample_data$sample_id]) == 0
sum(no_reads)

#drop taxa
obj <- filter_obs(obj, target = "tax_data", ! no_reads, drop_taxa = TRUE)
print(obj)
```

```
# uneven sampling
# getting per taxon info
obj$data$tax_abund <- calc_taxon_abund(obj, "tax_data",
                          cols = sample_data$sample_id)
print(obj$data$tax_abund)
# calculate the number of samples that have reads for each taxon:

obj$data$tax_occ <- calc_n_samples(obj, "tax_abund", groups = sample_data$Season, cols
= sample_data$sample_id)


# or: calculategroup mean
obj$data$type_abund <- calc_group_mean(obj, "tax_abund",
                          cols = sample_data$sample_id,
                          groups = sample_data$Season)

print(obj$data$type_abund)

# Make plots
dont_print <- c("unknown", "unidentified")
my_plots <- lapply(c("Autumn2018", 'Winter2019', 'Summer2019',
"Autumn2019"),function(type) {

# Make each plot layout look the same if using another layout
 heat_tree(obj, node_label = taxon_names,
        node_size = n_obs,
        node_color = obj$data$tax_occ[[type]],
        node_color_range = c("beige", "tan", "blue"),
        node_size_range = c(0.013, 0.04),# makes scale range the same in both
        layout = "davidson-harel",
        initial_layout = "reingold-tilford",
        title = type)
})
combined <- grid.arrange(grobs = my_plots, nrow = 2)

# single plot for overall

obj <- parse_tax_data(otu,
                class_cols = "lineage",
                class_sep = ";",
                class_regex = "^([a-z]{0,1})_{0,2}(.*)$",
                class_key = c(tax_rank = "taxon_rank", name = "taxon_name"))

print(obj$data$n_obs)

p1 <- obj %>%
 filter_taxa(grepl(pattern = "^[a-zA-Z]+$", taxon_names)) %>% # remove "odd" taxa
 filter_taxa(taxon_ranks == "g", supertaxa = TRUE) %>% # subset to the order rank
 heat_tree(node_label = gsub(pattern = "\\[|\\]", replacement = "", taxon_names),
        node_size = n_obs,
        node_color = n_obs,
```

```
        node_color_axis_label = "Core otu count",
        node_color_range = c("cornsilk", "blue4"),
        node_size_range = c(0.018, 0.04),
        overlap_avoidance = 0.2,
        layout = "reingold-tilford", initial_layout = "reingold-tilford")
p1 <- p1 +
 theme(text=element_text(family="Calibri"))

p1
ggsave(filename = "HeatTree_core.svg", plot = p1, width = 18, height = 18, dpi = 300, units =
"cm")
```

# Chapter 5: DISCUSSION

## 5.1 Research highlights

### 5.1.1 Study design and its effect on microbial community composition

Intestinal microbial communities vary significantly between hosts. In order to disentangle the impact of host genetic origin from other drivers one has to establish a baseline of evaluation. Many studies evaluate the impact of host genetics on gut microbiota between different species (e.g, Kim et al., 2021) or when considering one species between different habitats, e.g., different rivers (Uren Webster et al., 2018). However, the observed differences in microbial communities might be primarily due to variations in diet or environmental conditions specific to each habitat, rather than genetic differences between the host populations. To overcome this challenge, we conducted common garden experiments in our study. These experiments provided a controlled environment where we could eliminate the confounding factors of diet and environmental conditions, allowing us to directly examine the impact of host genetic background on intestinal microbial differences, at least in theory.

When investigating whether gut microbiomes differ between individuals with different genetic backgrounds, it becomes crucial to consider the precise nature of what we are measuring. When we sample a fish at a specific timepoint, the microbial community we observe is a complex combination of both environmental bacteria, which are brought in from the surroundings through food or the water column and bacteria already residing within the gut (Sullam et al., 2012). This interplay creates a dynamic situation where the observed variation between individual samples might be primarily influenced by the presence of environmental bacteria at that specific moment. To illustrate, one fish may have recently fed, leading to an abundance of bacteria associated with the ingested food items (Karlsen et al.,

2022). Conversely, another fish might not have consumed food recently, but instead it could have ingested a larger volume of water. We made use of this variation in chapter 2 where we investigated potential microbial source communities. The problem is that the huge and potentially random variation between gut microbial communities of different fish might overshadow underlaying host genetic effects. To explore the immediate impacts of host genetics on gut microbiomes it is necessary to focus on resident bacteria. These bacteria are adapted to their specific hosts and their community composition can provide insights into the influence of host genetics. Scientists developed several strategies to disentangle resident from transient bacteria, all having their pros and cons. For instance, one might distinguish between viable and nonviable microbial cells in the intestine to identify prolific colonizers. Several methods have been developed over the years. One approach involves using RNA instead of genomic DNA (gDNA) to create sequencing libraries, which can help to better identify the active members of the host gut microbiota (De Vrieze et al., 2018; Legrand et al., 2020, 2021). Other methods that assess microbial viability in samples include molecular viability testing (MVT) and viability PCR (vPCR; Cangelosi & Meschke, 2014). To do this, samples are treated with a membrane-impermeative reagent like propidium monoazide (PMA; Cangelosi & Meschke, 2014; Nocker et al., 2009). In salmonids it is common practice to collect intestinal *digesta* to capture the transient community and then wash the intestine wall with saline buffer to obtain mucosal swabs for the resident microbial community (Gajardo et al., 2016; Nyholm et al., 2022). Another possible solution is to collect samples after a period of food deprivation. We chose this method in chapters 3 and 4 because it also provided the opportunity to link microbial community compositions to host physiological markers like metabolic rate measurements. To accurately measure metabolic rates, it was essential to starve the fish for a 24 to 48-hour period, ensuring a post-absorptive state. This strategy

helped establish a standardised condition among the fish samples, reducing the potential confounding effects of recent food consumption and enabling a more accurate assessment of the relationship between gut microbiomes and host physiological markers.

### 5.1.2 **Microbiota**

In this study, we observed distinct gut microbial profiles between farmed and wild fish when investigating both transient and resident communities combined, as well as when focusing only on what we determined as host associated communities. However, the differences were circumstantial and occurred for example in chapter 3 in winter and autumn19 but not in the other two sampling events. Nevertheless, our findings suggest that host genetics plays a role in shaping gut microbial community composition, which aligns with recent discoveries by Rasmussen et al., 2023 and Ziab et al., 2023. A possible explanation for the observed differences in gut microbial communities between fish from different provenances might be the influence of distinct selective pressures within their gut environments, for instance due to differences in immune response genes as demonstrated by de Eyto et al., 2007, 2011. It is possible that farmed and wild fish have reacted differently to an unknown environmental pressure that occurred in autumn and winter, thereby developing different immune responses, leading to differences in gut microbial beta diversity. Another theory explaining the differences in gut microbial community composition between the cohorts evolves around differences in feeding behaviour, which might translate into different gut microbial profiles. For example, during the winter sampling event farmed fish exhibited significantly higher metabolic rates (chapter 3), suggesting increased energy demands compared to wild fish. As a result, farmed fish might have been compelled to feed more frequently during winter to meet their metabolic needs. Diet is known to play a vital role in shaping gut microbial communities, influencing their composition either by introducing new taxa attached to food

particles or by changing the nutritional niches in the gut (Escalas et al., 2021). Another theory that could explain the observed distinct microbial profiles is the potential maternal transfer of bacteria from parents to offspring. In this scenario, one would assume that a maternal transmission would result in distinct profiles throughout every part of the salmon's life, which we did not observe in our study. Nevertheless, there is a possibility that those transferred bacteria only proliferate in certain environmental conditions, such as stress or lack of food. Interestingly, a similar ongoing debate exists in humans concerning whether or not foetuses develop in a germ-free environment (Kuperman et al., 2020; Mishra et al., 2021; Walker et al., 2017). Even if the pre-birth exposure to microbes in humans may be relatively minimal, it is widely acknowledged that during the process of birth and subsequent nursery care, human infants share a significant number of microbial taxa with their mothers (Ferretti et al., 2018). However, this starkly contrasts with the microbial transmission patterns observed in most fish species. In Atlantic salmon, eggs are released from the mother and are immediately fertilized with milt from the males. After the fertilised eggs are buried in redds, they are no longer in direct contact with their parents. This means that the duration during which maternal transmission of microbes from the parents to the eggs might occur is relatively brief. In addition, the presence of microbes on the outer surface of the egg doesn't ensure their survival or successful colonization of the intestine. However, a maternal transfer of microbes could still serve as a protective layer against pathogenic microorganisms. For instance, in lizards, it has been demonstrated that a vertical transfer of microbes from the maternal cloaca during oviposition resulted in reduced fungal colonisation and infection of the eggs (Bunker et al., 2021). Despite the observed host genetic effects on gut microbiota, we did not identify any differentially abundant taxa at the genus level. At first glance, this may appear to contradict the hypothesis proposed previously. However, several factors may have

contributed to conceal the detection of significant patterns. For instance, the use of genus-level classification might not capture variations that manifest at lower taxonomic levels (such as species or strain), which will be visible in beta diversity analysis. In addition, the sample sizes employed might have been insufficient to discern statistically significant trends, particularly if the observed effects were subtle.

In Chapter 2, we observed significant seasonal variations in gut microbial communities. FEAST analysis suggested that fish might have altered their diet throughout the year, consuming different macroinvertebrates. Notably, these macroinvertebrates carried distinct microbial profiles, leading us to conclude that different bacteria were introduced into the gut, influencing the transient community. Interestingly, in Chapter 3, we also demonstrated seasonal changes in resident microbial communities. However, unlike the more chaotic changes observed in Chapter 2, PCoA analysis revealed that resident communities seemed to develop gradually over time, suggesting a more stable pattern of change. Apart from seasonal changes in dietary intake other factors like host age (Bosco & Noti, 2021; Xiao et al., 2021; Yan et al., 2016), water temperature (Steiner et al., 2022), microbial succession (Lokesh et al., 2019) or microbe-microbe interactions (Faust & Raes, 2012; Kommineni et al., 2015; Woyke et al., 2006) might play a role in the observed changes. These alterations can lead to a scenario where distinct microbial groups successively replace each other over time, thereby capitalising on their competitive advantages within varying environmental conditions of the gut habitat (Faust & Raes, 2012). The complexity amplifies further as these changes can set off a cascading effect of further alterations, given that numerous bacterial taxa coexist in states of symbiosis or dysbiosis with one another (Faust & Raes, 2012).

Perturbations within microbial communities are frequently documented when the host becomes ill (Bozzi et al., 2021; Rosado et al., 2019; Turnbaugh et al., 2007; Vasemägi et al., 2017; Wynne et al., 2020). In Chapter 4, we observed a significant decline in microbial diversity in fish that exhibited visible signs of AGD. Several factors may contribute to this observation. First, the parasitic infection could trigger a stress response in the fish's body, leading to the release of stress hormones like cortisol (Allan et al., 2020; Hvas et al., 2017). Elevated cortisol levels have previously been shown to lead to reduce gut microbial diversity (Uren Webster et al., 2020). Second, AGD might reduce the appetite of infected fish (Boerlage et al., 2020), thereby altering the nutritional niches within the gut. Furthermore, the immune system combats the parasitic infection and this immune activation can have both direct and indirect effects on the gut microbiota (Gómez & Balcázar, 2008). Inflammation can reshape the gut environment and potentially impact microbial populations. Some microbes may thrive in the altered environment, while others may struggle to survive or reproduce. In a balanced microbial ecosystem, community members compete for nutrients and space (Ghoul & Mitri, 2016). If all microbial niches within the ecosystem are occupied, this creates a barrier towards invading and potentially pathogenic new members (Lawley & Walker, 2013). However, when the equilibrium is disrupted, for example, by the aforementioned factors, the community becomes susceptible to invasion by outsiders because there is a reduced likelihood of the community having a species with traits opposing the invader or pathogen(Fargione & Tilman, 2005; Levine & D'Antonio, 1999). Furthermore, the decline of specific community members that regulate others can lead to the proliferation of previously benign microbes, potentially rendering them harmful due to their newfound abundance. For instance, in this study, we found that the genus Vibrio became highly abundant in AGD-infected fish, and some Vibrio

species, such as Vibrio vulnificus, are known to be opportunistic pathogens (Baker-Austin & Oliver, 2018).

A fundamental question posed to researchers investigating host-associated microbiomes in any animal is to define the characteristics of a “healthy” or “optimal” microbiome (Ikeda-Ohtsubo et al., 2018; Lloyd-Price et al., 2016; Shanahan et al., 2021). Unfortunately, this question remains impossible to answer definitively due to the significant inter-individual variability among hosts. Additionally, what constitutes a healthy microbial community for one host could potentially be an unhealthy state for another. Nevertheless, in this study, we identified key players that appear to hold significance as integral members of the Atlantic salmon gut microbiome. In freshwater juveniles most of these taxa belonged to Proteobacteria, Actinobacteria and Firmicutes. On genus level *Corynebacterium*, *Staphylococcus*, *Streptococcus*, *Acinetobacter* and *Clostridium sensu stricto* seem to be key players. Whilst we did not statistically analyse the differences between fresh and saltwater microbial profiles in this study, it has been shown that the transition from fresh to saltwater alters gut microbial community compositions (Dehler et al., 2017; Lorgen-Ritchie et al., 2021). In chapter 4, we found that communities in marine post-smolts were mainly dominated by Proteobacteria, in particular by the family of *Vibrionaceae*. *Mycoplasma* which was identified as a key member of fish gut communities in several studies (Cheaib et al., 2020; Heys et al., 2020; Rasmussen et al., 2023), were found in both freshwater and marine fish but seemed to be more abundant in the marine phase. The exact role of these taxa, how they interact with each other and what benefits they have for their salmonid host has yet to be determined. While some other studies have identified similar key taxa in their investigations, it's important to note that our primary emphasis in this study was on sampling the pyloric caeca, whereas many other studies tend to prioritize the sampling of hindgut content (Bugten et al., 2022;

Lorgen-Ritchie et al., 2023; Wang et al., 2018; Zarkasi et al., 2016 and more). Consequently, making a direct comparison of community compositions between studies may not be warranted. An intriguing observation in our study was the relatively higher gut microbial diversity found in the pyloric caecum as opposed to the midgut in chapter 4. This discovery stands in contrast to human gut microbiota, where the gut compartment immediately following the stomach, specifically the duodenum in the small intestine, is typically colonised by only a limited number of bacterial species (Leite et al., 2020; Takakura & Pimentel, 2020). An excess of bacteria in the small intestine is even associated with a condition known as small intestinal bacterial overgrowth (SIBO), which is characterised by a variety of clinical symptoms (Erdogan et al., 2015; Pimentel et al., 2020).

### 5.1.3 **Energetics**

This study showed that juvenile Atlantic salmon exhibited distinct seasonal changes in their metabolic rates, indicating a capacity to adjust their energy needs in response to prevailing environmental conditions. In Chapter 3 we observed lowest metabolic rates during winter, which aligns with a common trend among various animal species, where metabolic demands are downregulated in response to limited food availability (Staples, 2016). Many animals reduce their activity levels during winter months, either by entering hibernation or by decreasing their foraging rates (Geiser, 2013; Volkoff & Rønnestad, 2020). Interestingly, we found that wild fish showed significantly lower metabolic rates than farmed and hybrid-farmed-female fish in winter. This is in contrast to studies conducted by Lindsay, 2021 and Robertsen et al., 2019 who were unable to conclude that domestication induced significant alteration in SMR in Atlantic salmon. The reason for the discrepancy between the study results remains speculative; however, it is possible that variations in fish age and the experimental settings (natural stream system in our study vs. semi-natural experimental stream channels)

may have contributed to the differing outcomes. In our study, wild fish showed a consistent pattern of a lower coefficient of variation (CV) in both summer and winter, suggesting a potential adaptation to seasonal changes in environmental conditions. In contrast, farmed fish and hybrid fish showed a higher variation of metabolic rates within a given season. These findings indicate a substantial genetic influence on metabolic traits. It is possible that the differences observed between fish from different provenances may be attributed to the distinct selective pressures experienced in their respective environments over evolutionary time. This phenomenon is well-documented in various species and reflects the capacity of organisms to adapt to their specific ecological niches (Norin & Metcalfe, 2019). For example, studies on the high-latitude subspecies of the Atlantic killifish (*Fundulus heteroclitus*) have shown that in response to cold acclimation, these fish increase their mitochondrial volume density and surface area to a greater extent than their low-latitude counterparts (Dhillon & Schulte, 2011). These adaptations are associated with higher whole-animal metabolic rates in high-latitude fish (Fangue et al., 2009). The farmed fish from the Norwegian “MOWI” strain, that we used in this study have geographical habitat differences and might be used to different temperature regimes than wild Atlantic salmon, native in Ireland. In addition, the artificial selection of farmed salmon over generations may lead to specific genetic changes in these fish, tailored to the controlled conditions of captivity (Glover et al., 2017). On the other hand, wild-origin fish experience natural selection, adapting to the challenges of their native environment. We hypothesize that the combination of a lower coefficient of variation in wild fish with the fact the wild fish showed the lowest VR in winter and highest in summer indicates a higher degree of adaptability to the changing environmental conditions during different seasons. The ability to adapt could provide wild fish with fitness advantages, especially in challenging environmental conditions, such as periods of food scarcity or droughts. Since,

hybrid offspring seem to have inherited a loss of adaptability from their farmed parent, this could become problematic for the fitness of salmonid populations in a climate changing future.

In Chapter 4, it became evident that fish exhibiting advanced signs of AGD also displayed notably lower metabolic rates compared to their healthy counterparts. This finding aligns with our expectations, given that AGD disrupts the gills' capacity to efficiently uptake oxygen, primarily due to the formation of mucus patches on the gill surfaces (Boerlage et al., 2020; Hvas et al., 2017). Consequently, the observed reduction in maximum metabolic rates among AGD-affected fish is consistent with these physiological constraints. We highlighted previously the ability of fish to adapt their SMR in response to varying temperature regimes. Furthermore, research has demonstrated that fish can adjust their metabolic rates within days or weeks when confronted with changing feeding conditions (Auer et al., 2015). Although feeding conditions remained constant in our study, it is plausible that the AGD-infected fish, facing the adverse effects of the disease, ceased feeding, resulting in a reduction in their standard metabolic rates. However, in chapter 4 we also propose an alternative explanation for our observations. It is conceivable that fish with high SMR might have succumbed to the later stages of the infection, leading to an overrepresentation of fish with low SMR in our dataset. This theory suggests that fish with lower SMR may have possessed a fitness advantage over their high-SMR counterparts in the context of AGD infection. Prior research has demonstrated that a higher SMR could prove beneficial when resources are abundant but could become detrimental when environmental conditions worsen (Armstrong et al., 2011; Auer et al., 2015; Bochdansky et al., 2005; Killen et al., 2011; Metcalfe et al., 2016). This phenomenon echoes the “allocation hypothesis” seen in other animals. According to this hypothesis, lower SMR confer a fitness advantage due to reduced maintenance costs,

facilitating energy reallocation towards growth and bolstered immune function (Downs et al., 2013; Larivée et al., 2010; Pettersen et al., 2016; Steyermark, 2002). Consequently, the AGD-induced reduction in oxygen intake might accelerate the onset of hypoxia and eventual death in fish with high metabolic rates compared to their counterparts with lower maintenance costs. Our study did not yield any evidence indicating that the origin of the fish influenced their susceptibility to AGD. Nevertheless, it is worth noting that wild fish exhibited the lowest survivability percentage in the sea pen environment. This discrepancy might be attributed to the better stress-coping mechanisms developed by farmed fish in this environment (Solberg et al., 2013), as well as their higher fat content which potentially rendered them more resilient under these specific environmental conditions.

### 5.1.4 **Conclusion**

The objective of this study was to explore potential disparities in gut microbiota and metabolic profiles between domesticated, wild and hybrid Atlantic salmon. By simulating farm escape and hybridisation events, we aimed to ascertain whether such differences exist and, if they do, to identify the specific conditions under which they manifest and determine potential consequences for salmon population fitness.

Our investigation revealed circumstantial variations in metabolic rates and gut microbial communities between the fish provenances. For instance, during winter, farmed fish exhibited higher metabolic rates and distinct gut microbial community compositions compared to their wild counterparts. Although we did not establish a direct correlation among metabolic rates and gut microbial profiles, it might be possible that an underlying connection exists. One hypothesis is that the variation in feeding behaviour exhibited by farmed fish during winter could induce dissimilar selective pressures on gut microbiota for

farmed and wild fish, thus resulting in different microbial profiles. These distinct microbial compositions could potentially harbour diverse genetic elements and might process nutrients in varying ways, leading to the creation of distinct metabolites or differences in the quantity of produced metabolites. For instance, compounds like butyrate, a main product of gut microbial fermentation, are recognised for their ability to modify host metabolic pathways (Zhang et al., 2021). Hence, alterations in the signalling pathways might foster a feedback loop where an altered microbiome again influences host behaviour and host metabolism.

In summary, our study offers indications that the domestication process in Atlantic salmon has exerted an influence on both host-associated microbiota and metabolism. Some of these changes were also observed in hybrid offspring, especially when the mother was a farmed fish. The observed differences only occurred circumstantially and are likely triggered by environmental conditions. It appears that there is an inherent interplay between host genetics, variations in behaviour, metabolism, gut bacteria and the surrounding environment. However, the challenge lies in establishing causality - identifying which factor influences the others. Within the scope of this thesis, providing an answer to this question remains elusive. Nonetheless, the potential consequences are significant. If escape events persist and wild populations are compromised by hybridisation, there is a risk that the observed gut microbial and metabolic changes might be maladaptive for the survival and ecological integrity of wild Atlantic salmon populations. Further research and monitoring are crucial to better understand these dynamics and guide conservation efforts to protect the genetic diversity and health of Atlantic salmon populations.

## 5.2 Future Research

### 5.2.1 Gut microbiota

While this work has contributed to advance our understanding of microbial assembly processes throughout a salmon's life, it did not specifically investigate the initial assembly processes of gut microbiota. Based on the findings that hosts with different genetic origins exhibit circumstantially distinct gut microbial profiles, it would be intriguing to investigate whether bacteria can be transferred from parents to offspring and if those bacteria can establish themselves in the intestine. This exploration could shed light on the heritability of microbial traits and provide a deeper understanding of the intricate interplay between genetics, environment and the gut microbiome in shaping an individual's overall well-being. It is recognised that the initial colonisers play a pivotal role in shaping their environment and influencing subsequent colonisation events (Fukami, 2015; Sprockett et al., 2018), presenting a compelling avenue for microbiome manipulation. By comprehending the early assembly of microbial communities, we could potentially unlock the key to cultivate a beneficial gut microbiome during later stages of a fish's life. Alongside this pursuit, it becomes essential to evaluate the stability of these microbial communities and explore the feasibility of introducing novel bacteria, such as probiotics (complemented by prebiotics for enhanced growth). Critical questions encompass whether those introduced bacteria can implement themselves into the resident community and under which circumstances, how these bacteria interact and whether these introduced bacteria persist after treatment discontinuation or if the gut reverts to its original state. This understanding seamlessly aligns with the exploration of the functional roles carried out by the gut microbiota, in order to test if those introduced bacteria actually benefit the host. In this study we did not determine the functional activities of the microbes themselves—namely, the specific roles they are fulfilling within this intestinal

ecosystem. While methods like PICRUSt (Langille et al., 2013) or tax4fun2 (Wemheuer et al., 2020) could potentially infer metabolic information from 16S sequencing data, we chose not to use it in this study since the reliability of this approach in fish remains questionable due to the lack of a robust database for non-human animals (Sun et al., 2020). To really understand host microbiome interactions, we emphasize the comprehensive adoption of a diverse array of metagenomics techniques. While human microbiome research has already embraced a wide spectrum of those techniques, it is important to highlight that the field of fish microbiome research has yet to fully harness the potential of these methodologies. Despite the differences, the underlying questions remain fundamentally the same. Shotgun metagenome sequencing would be the gold standard to view all genetic material in a sample, including bacteria, archaea, viruses and fungi, thereby achieving a deeper taxonomic resolution than amplicon sequencing. It is worth noting, however, that while shotgun metagenomics offers remarkable insights, its substantial cost, challenges in handling low biomass samples and susceptibility to host contamination pose notable limitations. Given these considerations, alternative strategies remain relevant for addressing the intricate complexities of microbial community analysis. While this study exclusively utilised 16S rRNA amplicon sequencing to answer the question “Who is there?”, techniques such as rRNA metatranscriptomics and mRNA analysis hold the potential to uncover the question of “What are they doing?” by delving into the active gene expression and functional dynamics within the microbial community. Different microbial species can perform similar functions, which means even if the taxonomic composition differs between hosts, the presence of functionally equivalent microorganisms might still maintain the same metabolic functions (Blakeley-Ruiz et al., 2019; Eng & Borenstein, 2018). In the context of our research question, it becomes particularly intriguing to investigate whether the observed circumstantial differences in

microbial profiles among farmed, wild and hybrid fish also translate into distinct microbial gene activities and metabolite patterns. Comparing these metabolic activities to host physiological markers-such as blood markers-can yield a direct measure of the impact of specific microbes on the host, surpassing the limitations of correlating community compositions to long-term host phenotype markers like body size and mass or fat content. However, it is important to note that in order to truly understand biological functions, deeper taxonomic resolution up to strain level might be required. To establish the causality of findings, a promising avenue involves isolating and cultivating microbial strains from community samples to investigate their effects in artificial gut systems (Kazlauskaite et al., 2021) or gnotobiotic models (Llewellyn et al., 2014; Luna et al., 2022). In this context, zebrafish emerge as ideal candidates, given their adaptability for a wide range of research inquiries (Stagaman et al., 2020). For the aquaculture industry, there is a potential opportunity not only to explore the impacts of pro and prebiotic treatments on fish, as currently practiced, but also to consider an alternative approach by understanding and enhancing specific metabolic pathways, which could then guide the development of targeted treatments.

### 5.2.2 **Energetics**

One of the most interesting finds of this study was that wild fish seemed to be able to adapt their metabolic rates more to varying environmental conditions than farmed fish. It would be intriguing to investigate the underlying cause for the observation. For instance, the variations in metabolic rates could arise from differing life history traits between farmed and wild fish (either geographically or genetically), potentially leading to the development of distinct epigenetic marks. Epigenetic factors, such as DNA methylation or histone modifications can influence how genes are turned on or off, affecting an organism's characteristics and

responses to the environment. While the underlying DNA sequence remains unchanged, epigenetic marks acquired during an individual's lifetime might be inherited by its offspring. We hypothesized that fish with lower metabolic rates might possess a survival advantage during periods of food scarcity, such as winter. Their reduced metabolic demands could negate the urgency to seek food. It would be intriguing to investigate whether this hypothesis holds true and to examine the dynamics of fat storage depletion during winter. Overall, we need to determine if our research findings are generalisable beyond our common garden experiments.

Our study primarily focused on differences observed within entire populations, with each individual being sampled only once. However, gaining a deeper understanding of adaptation dynamics would greatly benefit from monitoring the adaptation rate of a single individual over an extended period. This approach has the potential to offer invaluable insights into the mechanisms that underlie individual-level responses to changing environmental conditions, particularly in the context of climate change. Furthermore, an intriguing avenue for exploration lies in investigating the interplay between metabolic rates, diverse feeding behaviours and habitat preferences. Research has indicated that heightened metabolic rates are associated with more assertive behaviours, which in turn could influence the selection of habitats rich food resources. This behaviour-driven habitat selection could potentially confer growth and overall fitness advantages to the individuals displaying such tendencies. This becomes particularly intriguing considering interactions between farmed, wild, and hybrid populations in their natural environment. This perspective offers the opportunity to assess potential trends in salmonid population fitness for the future.

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**Chapter 5**

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