UNIVERSITY *of* GLASGOW

Ecophysiological Exploration:

The Microbiota, Metabolic Rate and Behaviour of Juvenile Atlantic Salmon (*Salmo salar*).

A thesis submitted for the degree of Doctor of Philosophy

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Author's Declaration

I declare that, with the exception of the aid listed below, this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

All Illumina sequencing was carried out by Glasgow Polyomics, University of Glasgow. Bomb Calorimetry was carried out by Graeme McWhinnie at the University of Stirling. The behavioural experiment in Newport was conducted by Dr Joshka Kaufmann and Amandine Tauzin. Philipp Schwabbl, Luis Enrique Hernandez Castro, Alessandro Bussetti and Raminta Kazlauskaite all helped gather data measuring the opercular beat of Atlantic salmon for use in Chapter 3. However, I curated all of the resulting data, and all statistical analyses in the thesis were performed by me. Finally, the review paper in Chapter 2 was overseen by Dr Martin Llewellyn and Professor Neil Metcalfe and the review paper in Appendix 1 was co-authored by William Bernard Perry, Christopher Payne, Christopher Brodie and Raminta Kazlauskaite.

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1.1. The Gut Microbiota

1.1.1. Characterisation of the Gut Microbiota

In recent years, much work has been carried out in an effort to characterize the endogenous microbiota of various metazoan species, elucidate the function of microbial communities and better understand host-microbiota interactions (Rawls et al., 2004b; Consortium, 2012). The microbiome incorporates the collective genomic content of the microbiota; the microbiota includes all of the microorganisms, incorporating bacteria, fungi, archaea, viruses and protozoans, inhabiting an environment (Tremaroli and Backhed, 2012; Jandhyala et al., 2015). There are huge disparities between microbial communities at different locations on a single host; the diversity observed is largely unexplained, though host genetics, diet, environment and early exposure to microbes are thought to each have a role in determining an organism's microbiota assemblage (Navarrete et al., 2009; The Human Microbiome Project Consortium, 2012; Tremaroli and Backhed, 2012; Alberdi et al., 2016; Zarkasi et al., 2016). Whilst the host impacts microbial composition, microbial symbionts in turn are thought to affect many aspects of the host's metabolism and physiology and they also have a role in nutritional provisioning (e.g. synthesizing amino acids), regulating fat storage and interacting with the immune system (Gomez and Balcazar, 2008; Navarrete et al., 2009; Nicholson et al., 2012; Semova et al., 2012; Tremaroli and Backhed, 2012; Abid et al., 2013; Asakura et al., 2014; Dehler et al., 2016). Gut microbiota have attracted a lot of attention as they are one of the largest and most diverse compared to microbial communities at other locations within a host (Tremaroli and Backhed, 2012). This high alpha diversity is coupled with variable beta diversity, as many species are conserved between individuals (Consortium, 2012; Tremaroli and Backhed, 2012). Differences in pH and tissue types within the GI tract create a variety of environments within the gut, so when examining the microbiota from the oesophagus to the colon, large diversity will be seen in a single host (Jandhyala et al., 2015). The gut microbiome is thought to interact with the host in a variety of ways (Gómez and Balcázar, 2008; Gajardo, et

al., 2016); consequently, many links between the gut microbiota and an organism's physiology and immunology are now being explored.

Technological advances have increased our ability to explore microbial communities and their interactions with the host organism. Previously, knowledge of microbial community composition relied on culturing techniques; however, difficulties associated with identifying the specific growth environments necessary for each species meant these methods were not sensitive enough to truly represent communities present (Hovda et al., 2007; Navarrete et al., 2009). In a direct comparison, Hovda et al. (2007) revealed that direct DNA extraction from samples compared to DNA obtained from cultivation methods produced different summaries of bacterial community composition, as cultivation underestimated diversity. In recent years, high-throughput DNA sequencing methodologies have allowed rapid characterization of microbial communities (Gajardo et al., 2016) and techniques such as RT-Q-PCR are able to quantify the mRNA or tRNA within samples (Olsvik et al., 2013), whilst next-generation sequencing can identify transcriptional responses in host species (Smith and Osborn, 2009; Olsvik et al., 2013). These technologies allow researchers to detect gene expression. In most studies, a fragment of the 16S rRNA gene is used to 'barcode' the bacteria present within microbial communities, using universal primers that are capable of amplifying hypervariable regions of the gene (Hovda et al., 2007). These amplified products can then be sequenced using next generation technologies in order to characterise the bacteria present (Hovda et al., 2007; Navarrete et al., 2009; Gajardo et al., 2016).

Characterisation of the gut microbiota has been attempted in many species, from red bellied lemurs (*Eulemur rubriventer*) (Raulo et al., 2018) to the common fruit fly (*Drosophila melanogaster*) (Morimoto et al., 2017). Whilst large intraspecific variation in the abundances of several microbial taxa is common (Burns et al., 2016), interindividual and even interspecific commonalities are also observed: for example, phylogenetic analyses cluster fish gut communities with those of mammals and insects (Sullam et al., 2012). Interspecific similarities can in part be explained by the fact that host-microbial interactions have been refined on an evolutionary timescale (Rawls et al., 2004b). Furthermore, the microbial community will in part be dictated

by interactions between microbes, which will create analogous compositions within and between species, as it is likely that some microbial species are reliant upon one another's metabolic activities and therefore always concur, whilst other species must compete for the same niche and might be mutually exclusive (Schroeder and Bäckhed, 2016). Nonetheless, many processes are thought to impact microbial assembly within a host, with evidence for both stochastic neutral processes (Sieber et al., 2018) and non-neutral processes, such as host-selection, microbial interactions and active dispersal (Burns et al., 2016). The presence of certain microbial species are necessary for host survival, so it would be unreasonable to suggest that microbial assembly always occurs purely stochastically.

Identification of gut microbial community composition may be informative in certain respects; however, information such as functional profiles of the microbiota can only be gathered via non-targeted shotgun meta-omics studies. In these cases, metagenomics can be combined with processes such as metatranscriptomics, metaproteomics and metabolomics, as information regarding gene abundance alone does not necessarily indicate microbial gene-expression, nor the ways in which a certain microbial composition impacts the host (Tremaroli and Backhed, 2012; Alberdi et al., 2016). Of the vast amount of information available about the gut microbiota, 16S data reveal very little regarding the complex host-microbiota axes. Current literature focuses on characterisation of the microbiome, but meta-omic approaches are needed to reveal functional variation of the gut microbiota (Alberdi et al., 2016). A complementary approach is to use gnotobiotic techniques, in which a germ-free host is examined in the absence of all microbes or selectively colonized with specific microbial communities. These have revealed specific host genes regulated by the gut microbiota (Rawls et al., 2004b; Marques et al., 2006; Turnbaugh et al., 2009; Consuegra et al., 2020). One such study found 59 responses conserved between the mouse and zebrafish (*Danio rerio*) gut microbiota, showing the role of the gut microbiota in epithelial proliferation, nutrient metabolism and innate immune responses across species (Rawls et al., 2004b). Research has also shown certain metabolic pathways between the host and gut microbiota are highly conserved between species, since the transplantation of the gut microbiota from human donors with improved glucose metabolism into a gnotobiotic mouse gut resulted in the same metabolic gene expression (Kovatcheva-Datchary et al., 2015).

Studies using these approaches have therefore given further insight into the functional relationship between the host and its gut microbiota.

1.1.2. Interplay Between the Gut Microbiota and the Host

The production of metabolites by the gut microbiota is well established – gut bacteria produce bioactive compounds, such as short chain fatty acids (SCFA), as a result of fermentation of soluble fibres (Nicholson et al., 2012; Karasov and Douglas, 2013; Brüssow and Parkinson, 2014; Schroeder and Bäckhed, 2016). Acetate, propionate and butyrate are abundant SCFAs, each with their own particular effect on host physiology, but all have the common features that they diffuse or are transported across the enterocytes, improve glucose regulation and are used as substrates for lipogenesis and aerobic respiration (Karasov and Douglas, 2013; Brüssow and Parkinson, 2014). Butyrate is of extreme importance within the mammalian colon as it regulates cell proliferation and acts as a primary carbon source for colonocytes (Karasov and Douglas, 2013). Examining the full range of microbial metabolites and their physiological consequences on the host is beyond the scope of any one study, but research has thus far revealed many complex interactions between the host and microbiota. The gut microbiota acts not only on the gut itself, but also influences functioning of the host's liver, muscle, adipose tissue and brain (Nicholson et al., 2012). It also has implications for host behaviour, since SCFAs have the capacity to stimulate the sympathetic nervous system and have been reported to have an effect on social behaviour and cognition (MacFabe et al., 2011).

Unsurprisingly, the gut microbiota is intricately linked with host diet and digestion: in zebrafish, the gut microbiota play a substantial role in dietary fatty acid metabolism and absorption (Semova et al., 2012), whilst studies in humans have revealed that a change in diet impacts gut microbiota composition within days, resulting in changes in microbial gene expression (Martínez et al., 2010; Tremaroli and Backhed, 2012; David et al., 2013).

The microbial organisms also produce digestive enzymes or carry out fermentative processes (Krogdahl et al., 2005; Sugita and Ito, 2006; Askarian et al., 2012; Ray et al., 2012; Hang et al., 2013; Sanders et al., 2015). Some species must rely on the

presence of symbiotic gut microbes to aid in digestion more than others – animals which feed primarily on indigestible compounds such as chitin, cellulose and certain fibres benefit from morphological adaptations and a gut microbiota adapted to their specialised diet (Depauw et al., 2015; Xue et al., 2015). It has been suggested that the gut microbiota community composition can alter in response to a changing environment (Alberdi et al., 2016) and examples of this resonate within studies that have altered the gut microbiota by changing the host diet (Martínez et al., 2010; Tremaroli and Backhed, 2012; Abid et al., 2013; David et al., 2013). Alberdi et al. (2016) suggested that the capacity of a host to acclimate and adapt to various changing conditions is partially associated with whether the gut microbiota can change its composition or metagenome in response to these changes.

The plastic nature of the gut microbiota is widely acknowledged: the microbial community composition within the host gut has been shown to alter in response to diet (Abid et al., 2013; Bergmann et al., 2015), environment (Dehler et al., 2016) and ontogeny (Burns et al., 2016), so producing longitudinal intraindividual variation. The multitude of both biotic and abiotic factors that can impact the gut microbiota suggests that different microbial communities and their resulting functional profile can have varying effects on the host. Cross-talk between gut microbiota and the host can occur through signalling to peripheral host organs via the previously discussed microbial metabolites, structural components of the microbes themselves or via nervous/hormonal signalling (Nicholson et al., 2012; Schroeder and Bäckhed, 2016). SCFAs, produced as a result of microbial fermentation, can stimulate gut motility and affect many host processes such as cellular signalling, control of gut pH and can even alter the host metabolic phenotype (Nicholson et al., 2012). In fact, a major influence of the gut microbiota is over host metabolism. Microbial metabolites influence the host metabolic phenotype, since certain microbial signals are capable of regulating host transcription (Nicholson et al., 2012; Tremaroli and Backhed, 2012; Alberdi et al., 2016). The gut microbiota has an influence over processes such as thermogenesis, lipogenesis, energy expenditure, insulin secretion, gluconeogenesis and whole body growth (Schroeder and Bäckhed, 2016), as well as nutritional energy harvest (Turnbaugh et al., 2006). For example, Chevalier et al. (2015) found that exposure to the cold produced marked shifts in the composition of the gut microbiota in mice and that transplantation of this cold-exposed microbiota into germ

free mice increased host tolerance to low temperatures. This study evidenced a role of the gut microbiota in tissue remodelling and altering intestinal gene expression (Chevalier et al., 2015). Given the importance of metabolic rate on host functioning, microbiota-level effects are now beginning to be explored. The links between the gut microbiota and host metabolic phenotype are discussed further within Chapter 2 of this thesis; however, the relevance of considering the gut microbiota in studies examining host metabolic rate cannot be understated. The host-microbiota interactions in fish, the focal taxon of this thesis, will now be discussed.

1.1.3. The Teleost Gut Microbiota

Based on the interest in its effects on human health, it is unsurprising that most research on the gut microbiota revolves around mammalian microbiomes. At present, there are comparatively fewer studies revealing information on teleost gut microbiota. This may be partially due to the increased complications arising from their pokilothermic nature, since external environmental factors such as water temperature can have a greater effect on the gut microbiota (Gajardo et al., 2016). Bacterial growth is hugely influenced by temperature, so microbial assemblages within a teleost gut will be significantly influenced by the ambient environmental temperature, in comparison to endothermic mammals (Zarkasi et al., 2016). In spite of this, fish represent an interesting microbiome model as their aquatic environment allows characterization of both internal and external microbial species pools, which is a substantially more complex undertaking in a terrestrial environment; by sampling the water in which the fish reside, it is possible to examine the processes of microbiome community assembly (Schmidt et al., 2015). Interestingly, Navarrete et al. (2009) found that the dominant bacteria from the external aquatic environment and pelleted food of juvenile Atlantic salmon (*Salmo salar*) were not detected in the salmon GI tract; however, the dominant bacteria within the salmon gut microbiota were derived from minor bacteria present within their holding water. Similarly, in Atlantic salmon early life stages, *Pseudomonas* sp. are dominant within the gastrointestinal tract and are found on the eggs; however, they are not identified in the water or the food, indicating colonization must occur by other means (reviewed in Llewellyn et al. (2014)). Fish larvae are colonized by bacteria from ova debris and the environment itself upon hatching and the gut becomes colonized with

microorganisms as soon as ingestion of their medium initiates, but the initial microbial community is unstable until first-feeding occurs (Llewellyn et al., 2014; Dehler et al., 2016).

Fish are ancestral to other extant vertebrates, with the earliest originating over 600 million years ago, and so research regarding their microbiotas is the first step in understanding the co-evolution of vertebrate-microbial symbiotic relationships. Previous studies have shown similarities between the bacterial communities found in the fish gut and those found in the mammalian GI system (Asakura et al., 2014; Gajardo et al., 2016) giving yet further incitement of the use of fish in microbiome research. A common method of gut microbiota characterisation discussed in the literature is the study of faecal microbiota (Tremaroli and Backhed, 2012; Asakura et al., 2014; Zarkasi et al., 2016); however, much microbial activity occurs in the small intestine, so studies benefit from focal targeting of microbial assemblages from different gut compartments, since this allows identification of an increased volume of microbial biomarkers, such as SCFAs (Hovda et al., 2007; Tremaroli and Backhed, 2012; Schroeder and Bäckhed, 2016). As within other animals, studies on teleosts have revealed that the gut microbiota can alter in response to diet (Askarian et al., 2012; Abid et al., 2013) and abiotic factors such as salinity (Sullam et al., 2012). Other studies have highlighted the role of the gut microbiota in nutrition (Nayak, 2010) and the immune system (Gomez and Balcazar, 2008). Across the host species examined, common colonisers of the gut in freshwater and marine fish species include *Vibrio*, *Aeromonas*, *Flavobacterium*, *Plesiomonas*, *Pseudomonas*, *Microbacterium*, *Enterobacteriaceae*, *Micrococcus*, *Acinetobacter*, *Clostridium*, *Fusarium* and *Bacteroides* (as reviewed in Gomez and Balcazar (2008) and Ray et al. (2012)).

Since the gut microbiota has implications for host functioning, studies examining the effect of gut microbial communities on their fish host should be of particular interest to the aquaculture industry (as discussed in Appendix 1). In 2018, aquaculture accounted for 52% of the 156 million tonnes of fish consumed by humans, with finfish dominating aquaculture production (FAO, 2020). Globally, fish production and fish consumption continue to increase, so the aquaculture industry is of huge economic importance globally, with exports being essential to the economies of

many regions (FAO, 2020). Therefore, it is imperative that losses are minimised and production remains efficient. Indeed, the future of fish farming at its current scale is called into question, because of negative consequences on the environment, fish stock diseases and the unsustainability of intensive fed aquaculture (Chopin et al., 2001; Marques et al., 2006; Mente et al., 2006; Harvey et al., 2016a). Moving forward, an approach that considers fish welfare and sustainability, whilst prioritizing efficient growth, will be necessary. The links between the gut microbiota and host physiology and growth outlined above are thus highly relevant to the aquaculture industry. Furthermore, the microbiome has also been observed to shift in response to stress, which can impact the physiological, hormonal and cellular functions within the host (Nicholson et al., 2012; Llewellyn et al., 2014; Gajardo et al., 2016). Therefore, changes in the microbial community have the potential to negatively impact the host in ways which might be immunocompromising or result in less efficient nutrient assimilation. Consequently, studies examining the host-microbiota relationship might highlight functional links and subsequently inform fish husbandry.

1.2. Atlantic Salmon

1.2.1. The Metabolic Rate and Gut Microbiota of Atlantic Salmon

Gut microbiota research using teleosts will benefit from focusing on species in which a large amount about their biology is known; if the general physiology of the host is understood, it will be easier to interpret any effects caused by the gut microbiota in both manipulative and observational studies. The biology of Atlantic salmon is well-established due to the commercial and recreational importance of salmonids and their consequent wide use as experimental species. The life-history of salmonids is interesting as it is shows both inter- and intraspecific variation. Salmonids spawn in fresh water in the autumn/winter, burying their eggs in gravel substrates (usually a riverbed), with the eggs hatching and young fish emerging in the spring. Many species of salmonid are anadromous, with juvenile fish migrating from their freshwater habitat (usually streams or rivers, occasionally lakes) to the sea, where they remain until they return to fresh water to spawn. In species such as the Atlantic salmon the amount of time spent as juveniles (called parr) in fresh water before migrating to the ocean is variable (Thorpe, 1989). Departure from the river is dependent upon smolt transformation, which allows the fish to move from a

freshwater to saltwater environment. The migration of smolts occurs in the late spring, but determination of whether smoltification will occur in a given year is largely dependent upon resource levels, such as lipids, many months earlier (Thorpe et al., 1998).

During the time spent as parr, salmonids are usually inhabiting mid- to high latitude oligotrophic streams and rivers, so must cope with seasonal changes in environmental conditions. There is intrapopulation variation in their response to this seasonality. For example, those fish that will remain as parr for at least a further year show a reduction in appetite and become semi-dormant in the winter, whereas those that will transform into smolts the coming spring continue to forage actively throughout the winter months (Thorpe et al., 1998). Smolt transformation allows migration to the ocean, where fish undergo the majority of their somatic growth, and they remain in this new environment for a variable number of years (usually 1-2 but occasionally 3). Following this period of growth and sexual maturation, fish then return to their natal streams to reproduce (Thorpe, 1989). It must be noted that although usually anadromous, some salmonids can complete their full life cycle within the freshwater environment, since sexual maturation can occur at the parr stage (usually in males, but very occasionally also in females) (Klemetsen et al., 2003). This flexibility extends to their reproductive strategy, since Atlantic salmon are iteroparous, meaning they can spawn repeatedly (Klemetsen et al., 2003). The heterogeneity in Atlantic salmon life cycles can be due to ecological considerations or genetics, but fish with discrete life history strategies can co-exist within the same environment (Thorpe, 1989). Seasonal variations, environmental stochasticity and their anadromous nature means that Atlantic salmon in the wild experience very different environmental conditions and energetic demands over the course of their lives. To manage such environmental variation, Atlantic salmon undergo phenotypic changes impacting their physiology (Fleming et al., 2019), morphology (Kacem et al., 1998) and even behaviour (Metcalfe et al., 1995).

The energetic status of salmonids has been of particular interest to researchers, since metabolic rate has been found to impact upon their growth and survival (Álvarez and Nicieza, 2005), and to vary with their environment and life history (Seppanen et al., 2010). Metabolic rate in fish is important due to their poikilothermic nature; their standard metabolic rate (SMR) represents the minimum level of energy

required to sustain the most basic requirements of life, below which an organism faces physiological impairment (Chabot et al., 2016). Indeed, survival of juvenile Atlantic salmon when environmental conditions deteriorate in the winter has been linked to energy levels (Finstad et al., 2011). Due to their complex life histories, salmonids in the wild can experience large variations in their energetic demands on both temporal and spatial scales. Energetically expensive tasks such as competitive interactions and predator avoidance are also commonplace. The standard metabolic rate within salmonids has been shown to be flexible (Cutts et al., 1998; Auer et al., 2016b), which means that subsequent impacts on growth and survival can vary temporally.

The resulting inter- and intraindividual variation in the metabolic rate of Atlantic salmon not only has implications for overall functioning of the fish, but also gives another reason for gut microbiota research to be carried out within this species. Given the suggested links between an organism's metabolic rate and its gut microbiota, Atlantic salmon provide an excellent opportunity to study the gut microbiota in the context of different metabolic phenotypes. The benefit of a given metabolic phenotype in Atlantic salmon is thought to be context-dependent, where a high metabolic rate can result in increased growth when conditions are favourable, but is not a predictor of growth when resources become patchier (Reid et al., 2012). This interplay between metabolic phenotype and the environment could have implications for the gut microbiota, particularly given that biotic and abiotic factors affect both an individual's metabolic rate and the gut microbiota (Ghanbari et al., 2015).

Diet composition has been shown to affect the gut microbial communities and physiology of salmon (Abid et al., 2013; Gajardo et al., 2016; Zarkasi et al., 2016). For example, probiotic and prebiotic use have led to upregulation of host immunological responses via modification of the intestinal microbial community (Abid et al., 2013), whilst Zarkasi et al. (2016) established that diets with different lipid, protein and fishmeal proportions impact the structure of microbial communities present within Atlantic salmon. The combined influence of the environmental temperature along with the composition and energy levels of the diet affected the growth performance of the salmon (Zarkasi et al., 2016). Nonetheless, despite the importance of metabolic rate for overall host functioning, there have been few

studies that have explored the relationship between metabolism and the gut microbiota in teleosts. Since inter- and intraindividual variation in metabolic phenotype is seen within Atlantic salmon, this species offers an intriguing opportunity to study the gut microbiota the context of differing metabolic rates.

High throughput sequencing studies have revealed consistency regarding the major microbial genera present within the gut of Atlantic salmon (Dehler et al., 2016; Gajardo et al., 2016; Zarkasi et al., 2016). Species from the Firmicutes phylum are characteristic within the allochthonous communities and the autochthonous communities are often predominated by Proteobacteria (Abid et al., 2013; Dehler et al., 2016; Gajardo et al., 2016). Navarrete et al. (2009) found that the microbial composition across all sections of the gut was similar in juvenile Atlantic salmon and dominated primarily by several *Pseudomonas* strains, which belong to phylum Proteobacteria. Similarly, Hovda et al. (2007) found that microbiota diversity varied little across the gut of Atlantic salmon, but found that the Proteobacterium *Photobacterium phosphoreum* dominated in the hindgut and common species in the foregut include *Janthinobacterium* species, as well as species from the *Pseudomonas*, *Acinetobacter* and *Vibrio* genera. Heterogeneity between individuals is of course observed, both between salmon reared in different locations as well as between salmon within the same treatment groups (Dehler et al., 2016; Gajardo et al., 2016; Zarkasi et al., 2016). Potential factors influencing microbial community composition include individual differences in feeding (Zarkasi et al., 2016), water temperature and salinity (Dehler et al., 2017; Rudi et al., 2018), stochastic colonization processes, effects of farm/wild conditions, seasonality, developmental stage (Llewellyn et al., 2015), stress and geographical location (Gajardo et al., 2016; Zarkasi et al., 2016)**.** Despite intraspecific differences in gut microbiota, the significant homogeneity observed indicates that whilst microbial community composition is influenced by a range of host-mediated and environmental factors, certain bacterial species are maintained regardless of the external environment (Dehler et al., 2016). As discussed within the context of other species, this may indicate the presence of certain species that are necessary for healthy functioning of the host, which would mean that host physiological selection has a role in determining community composition (Llewellyn et al., 2015; Alberdi et al., 2016; Dehler et al., 2016).

Thus far, researchers have been unable to establish mechanistic links between the gut microbiota community composition in Atlantic salmon and physiological/morphological consequences which may influence performance outcomes; however, it is possible that studies are able to find a relationship between the gut microbiota and metabolic rate in these fish. Given the link between diet and the gut microbiota in fish species (Ghanbari et al., 2015), the interplay between diet and growth, and the impact of the host's metabolic rate on growth (Reid et al., 2011), it seems logical that host gut microbiota and metabolic rate will interact, which will have repercussions for host physiology. The ability of Atlantic salmon to perform well under a variety of environmental conditions could in part be due to the relationship between their metabolic rate and gut microbiota.

1.2.2. Atlantic Salmon in Aquaculture

As previously mentioned, gut microbiota studies on teleosts have often focused on species of economic importance due to their use within the aquaculture industry. Within aquaculture, Atlantic salmon are indisputably one of the world's most socio-economically important farmed fish, in terms of both volume and value (Mente et al., 2006; Harvey et al., 2016a): Atlantic salmon aquaculture is now one of the most profitable fish production industries worldwide (FAO, 2020). Aquaculture itself is young in comparison to land-based agriculture, but for the most economically important species it has existed long enough to allow domestication, in which selective breeding genetically separates animals from members of their species that exist in the wild (Teletchea and Fontaine, 2014). Domestication of salmonids in Europe began in the late 1960s, with the Norwegian Atlantic salmon aquaculture industry dominating salmon production since the 1970s (Liu, 2011). Norway now produces over half of the global supply of farmed salmon, making the country the leading exporter (FAO, 2020). Farmed Atlantic salmon from Norway are therefore good experimental strains on which the effects of domestication can be examined, since they have undergone artificial selection for desirable traits (Gjedrem and Thodesen, 2005) and the main selection lines have been reared for >12 generations (Glover et al., 2017).

As aquaculture is of economic importance globally and has a role in reducing the pressure on wild fish stocks (Teletchea and Fontaine, 2014), the industry will seek to continuously improve performance. This means expanding the industry, whilst minimising food loss and waste, ensuring economical use of resources and targeting any inefficiencies that might result in loss of wealth (FAO, 2020). In this vein, directional selection in aquaculture has prioritised delayed maturation, desirable flesh characteristics and increased growth rate (Glover et al., 2018). Consequently, any effect of the gut microbiota on growth of the host will be important within the industry that aims to maximise growth efficiency. Furthermore, as metabolism can directly impact growth, any functional interactions that exist between the gut microbiota and host metabolism will be of interest, since this information might lead to increased efficiency of feed utilisation and fish growth, thereby enhancing profitability.

1.2.3. Diverse Genetic Backgrounds in Atlantic Salmon

Atlantic salmon provide the opportunity to examine both metabolic rate and the gut microbiota in the context of different strains of the same species. Populations of Atlantic salmon in the wild are often genetically distinct from one another due to local adaptation (Normandeau et al., 2009; Glover et al., 2017), but most notably, directional selection in aquaculture has resulted in domesticated fish that are now distinct from their wild ancestors. Unusually, there is also a recognised intermediate phenotype between wild and farmed (domesticated) fish, in the form of ‘ranched’ fish: these are reared throughout the freshwater phase in a hatchery, but at the time of smolting are then released into the wild, to complete the marine stage of their lifecycle. These fish can be recaptured if they return to freshwater to spawn (McGinnity et al., 2003). Furthermore, interbreeding between farmed and wild Atlantic salmon following accidental escapes of farmed salmon into the wild environment can result in hybrid fish. The existence of these different strains provides the chance to examine whether Atlantic salmon from different genetic origins differ not only in morphology, but also in their physiology, behaviour and even their gut microbiota. Previous studies have shown differences in growth (Solberg et al., 2013; Harvey et al., 2016b) and behaviour (Metcalfe et al., 2003; Huntingford and Adams, 2005) between Atlantic salmon of farmed and wild origins, so it is not

unreasonable to suggest that a difference in the gut microbial community composition might also exist between these two groups. Although Roeselers et al. (2011) found the gut microbiota community composition of domesticated zebrafish and those of wild origin to be extremely similar, interindividual variation in many other traits are widespread in Atlantic salmon. Although the process of domestication focuses on directional selection for economically important traits, inadvertent selection can also occur (McGinnity et al., 2003) and there are likely to be other effects on the physiology and behaviour of domesticated Atlantic salmon due to general adaptation to the aquaculture environment (Glover et al., 2017). Individual differences in behaviour can have implications for survival (Moiron et al., 2019), whilst differences in growth can impact host fitness. If differences in the gut microbiota do exist between Atlantic salmon of farmed and wild genetic origin, these could also have implications for host fitness.

Examining the physiological and behavioural traits of Atlantic salmon in the context of genetic origin is important for two main reasons. Firstly, to see if there are genetic drivers to behavioural traits, metabolic rate, microbial community composition and growth. Secondly, to better understand the potential impacts of aquaculture escapees in the context of introgression and the resulting effect on the aforementioned traits. Every year it is estimated that approximately two million farmed salmon escape into the wild in the North Atlantic alone (McGinnity et al., 2003). The influx of domesticated salmon into the wild environment can have ecological repercussions for the wild populations as the presence of the additional fish increases competition for resources within the environment. Studies have shown that Atlantic salmon of farmed and wild origin perform differently. For example, farmed Atlantic salmon show reduced anti-predator responses (Houde et al., 2010), enhanced appetite (Harvey et al., 2016b) and behavioural differences compared to their wild conspecifics in traits such as aggression and reproductive behaviour (reviewed in Huntingford (2004)). Understanding the disparities in the behaviour of fish from different origins will better inform the potential outcomes when these fish occupy the same environment following escapee invasion.

Alongside ecological consequences of aquaculture escapees, there are also genetic repercussions for the wild populations. Interbreeding between farmed and wild fish can result in genotypic changes, including loss of genetic variation in wild

populations (Roberge et al., 2008). As domestication has resulted in genetic divergence between farmed and wild stock, introgression can therefore have fitness consequences on the resulting generations, especially in populations in which local adaptation by the wild population has occurred (Garcia de Leaniz et al., 2007). Although studies have shown that Atlantic salmon of farmed origin show reduced overall survival in the wild (McGinnity et al., 2003), spawning by mature male parr means that introgression can occur without the farmed escapees having to survive until adulthood (Glover et al., 2017). Predicting the impact of introgression is challenging as it will vary between populations (Normandeau et al., 2009); however, given that important characteristics, such as the metabolic phenotype, are heritable (White and Kearney, 2013), it is unsurprising that interbreeding between farmed and wild fish can negatively impact overall population fitness. Additionally, if a relationship exists between the gut microbiota and metabolic rate, interbreeding between farmed and wild Atlantic salmon might have implications for both. It is clear that introgression from domesticated salmon can impact on the life history of salmon in wild populations (Bolstad et al., 2017); it has also been shown that artificial selection can lead to heritable changes in gene transcription profiles in as few as 5 – 7 generations (Roberge et al., 2006), highlighting the rate at which gene flow from domesticated fish might affect wild populations. Any genetically-based behavioural changes induced by domestication are likely to result in hybrids exhibiting maladaptive behaviours (Houde et al., 2010). Furthermore, whilst hybrids often show reduced fitness and survival compared to their wild conspecifics, farmed and hybrid parr grow faster and can therefore displace wild parr (McGinnity et al., 2003). This indicates that it will not only necessarily be the direct impacts of introgression that negatively affect wild Atlantic salmon populations. Studies assessing behavioural and physiological variation between salmon from different genetic backgrounds can therefore be informative when trying to understand the effects of interactions between these fish.

Finally, it must also be recognised that in addition to genetic differences between Atlantic salmon of farmed and wild origin, the rearing environment experienced by the fish might also impact the traits of interest: behaviour, metabolic rate, gut microbiota and growth. The phenotype of fish can develop differently in response to their rearing environment due to interactions between their genes and the

environment (Johnsson et al., 2014). Consequently, although genetic differences between Atlantic salmon of farmed and wild origin are inescapable, the effect of the rearing environment could also explain some of the outcomes of wild-farmed ecological interactions. The rearing environment has been shown to affect the behaviour of salmon, as shown by different experimental approaches. For instance, Atlantic salmon progeny from a common genetic stock reared in either the hatchery or wild environment showed different proportions of shelter use at high population densities (Griffiths and Armstrong, 2002). An alternative way of disentangling genetic from environmental effects is to use the common-garden approach, in which individuals from different origins are reared in the same environment, thereby potentially revealing genetic effects on the phenotype independent from environmental effects. In one such study, Metcalfe et al. (2003) showed that domesticated Atlantic salmon were dominant over fish of wild origin when reared in a common-garden hatchery environment, but that wild-origin fish that had been reared in the wild were generally dominant over both domesticated and wild-origin fish reared in the hatchery. Common-garden studies can therefore reveal the influences of both environment and genetics. Additionally, Solberg et al. (2020) found that the susceptibility of fish of farmed, hybrid and wild origins to predation varied with their rearing environment, but also between fish from different origins within the same rearing environment. These studies were able to separate genetic from environmental effects on the fish by drawing comparisons between different strains reared across multiple environments.

The complex relationship between physiology, behaviour and the gut microbiota in Atlantic salmon can therefore be examined in the context of different genetic origins and different rearing environments. The relative importance of both on overall fitness of the fish will be largely context-dependent; however, enhanced understanding of genetic and environmental impacts will be of interest to the aquaculture industry, due to the economic importance of the species. The incorporation of physiological, microbiological and behavioural studies will provide insight into how the physiology of Atlantic salmon is impacted by their gut microbiota and whether inherent differences in the traits of interest have resulted from domestication of the salmon or their rearing environment. This might also inform husbandry practices if genetic or environmental variation exists.

1.3. Aims and Objectives of This Thesis

Across many taxa, there is wide intra- and interspecific variation in metabolic rate (White and Seymour, 2005; White and Kearney, 2013; Killen et al., 2016; Salin et al., 2016) and in the microbial community composition within the gut (Burns et al., 2016). In separate studies, both the metabolic rate and the gut microbiota of an organism have been shown to be related to diet (O'Connor et al., 2000; Abid et al., 2013; Bergmann et al., 2015), environment (Dehler et al., 2016; Norin and Clark, 2016) and ontogeny or life history (Burns et al., 2016; Pettersen et al., 2018), all of which can have repercussions on overall fitness and survival. Nonetheless, few studies have examined a host's gut microbiota and metabolic rate concurrently. The experiments within this thesis therefore aim to explore whether a relationship exists between the gut microbiota and metabolic rate in juvenile Atlantic salmon. In addition, this thesis examines whether the genetic origin of the fish can account for any variation in the gut microbial community composition, host physiology, or in host behaviour. The outcomes of this research will not only highlight relationships between the gut microbiota and host physiology in juvenile Atlantic salmon, but also examine the impacts of different host genetic backgrounds and rearing environments. Following a detailed review of the relationship between the gut microbiota and host energetics across multiple taxa (Chapter 2: Lindsay et al. (2020)) to put this work into a wider context, this thesis aims to:

1. Assess whether the gut microbiota differs between juvenile Atlantic salmon with different metabolic phenotypes, as characterised by either a 'low' or 'high' metabolic rate. The fundamentals of the way in which microbiota interacts with metabolism and subsequent impacts the host will also be examined in the context of host physiological characteristics, such as body composition and growth efficiency (Chapter 3).
2. Examine whether genetic origin (farmed, ranched and wild) has an impact on the metabolic rate and the gut microbiota of juvenile Atlantic salmon, including the assessment of standard metabolic rate (SMR), maximum metabolic rate (MMR) and specific dynamic action (the metabolic cost of processing food; SDA) (Chapter 4).
3. Explore whether domestication has resulted in a change to behavioural characteristics by assessing the exploratory behaviour in farmed, wild and farmed x wild hybrid juvenile Atlantic salmon. In addition, the impact of rearing environment

(common-garden, hatchery or river) will also be examined to unpick the effects of genetics and environment (Chapter 5).

Chapter 2: The Potential Role of the Gut Microbiota in Shaping Host Energetics and Metabolic Rate.

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2.1. Abstract

It is increasingly recognised that symbiotic microbiota (especially those present in the gut) have important influences on the functioning of their host. Here the interplay between this microbial community and the growth, metabolic rate and nutritional energy harvest of the host is reviewed. The review shows how recent developments in experimental and analytical methods have allowed much easier characterisation of the nature, and increasingly the functioning, of the gut microbiota. Manipulation studies that remove or augment gut microorganisms or transfer them between hosts have allowed unprecedented insights into their impact. While much of the information to date has come from studies of laboratory model organisms, recent studies have used a more diverse range of host species, including those living in natural conditions, revealing their ecological relevance. The gut microbiota can provide the host with dietary nutrients that would be otherwise unobtainable, as well as allow the host flexibility in its capacity to cope with changing environments. The composition of the gut microbial community of a species can vary seasonally or when the host moves between environments (e.g. fresh and sea water in the case of migratory fish). It can also change with host diet choice, metabolic rate (or demands) and life stage. These changes in gut microbial community composition enable the host to live within different environments, adapt to seasonal changes in diet and maintain performance throughout its entire life history, highlighting the ecological relevance of the gut microbiota. While it is evident that gut microbes can underpin host metabolic plasticity, the causal nature of associations between particular microorganisms and host performance is not always clear unless a manipulative approach has been used. Many studies have focussed on a correlative approach by characterising

microbial community composition, but there is now a need for more experimental studies in both wild and laboratory-based environments to reveal the true role of gut microbiota in influencing the functioning of their hosts, including its capacity to tolerate environmental change. Areas where these would be particularly fruitful in the context of ecological energetics are highlighted.

2.2. Introduction

While ecologists have appreciated the key role of energy flow in structuring ecological communities, and hence the importance of inter- and intraspecific variation in metabolic rate, there has been growing recognition of the fact that an animal's metabolism can be significantly influenced by the microbial communities in its gut. These communities, henceforth termed the gut 'microbiota' (see Glossary box 2-1 for definitions), are fundamentally ecological in nature in that they interact with each other (competitively and symbiotically) and with the host upon which they reside (mutualistically and commensally), and are dependent on the biophysical environment that the host creates (Tremaroli and Backhed, 2012; Jandhyala et al., 2015). There are frequently differences in species composition and abundance between the microbial communities found at different locations on a single host, underpinned by variation in the micro-environments that the microbes encounter. For example, within the vertebrate gut the dynamics of cell turnover, secretions and peristalsis all drive micro-variation in microbial community structure (Rolig et al., 2017). The complexity of the microbial community can also differ markedly between hosts, for example, the gut microbiota is simpler in *Drosophila* than within mammals (Erkosar et al., 2013). Significant microbial diversity also exists between the same intra-host niche among different individuals (Burns et al., 2016). Most observed interindividual diversity is as yet generally unexplained, though host genetics, diet, environment and early exposure to microbes are each thought to have a role (Navarrete et al., 2009; Consortium, 2012; Tremaroli and Backhed, 2012; Alberdi et al., 2016; Zarkasi et al., 2016).

The gut microbiota is the most diverse and populous microbial assemblage on the host (Senghor et al., 2018) and is thought to interact with the host in a myriad of ways (Gomez and Balcazar, 2008; Gajardo et al., 2016). Microbial symbionts are thought to affect many aspects of the host's metabolism and physiology, and hence have direct relevance for ecological studies, since effects of the microbiota can have marked impacts on the host and the way in which it interacts with its environment. Microbes interact with the immune system (Mackos et al., 2017) and aid in the regulation of fat storage (Cani and Delzenne, 2009), but their most direct role is in supplying nutrients to the host via the digestion of components of the host's diet or the synthesis of amino acids (Carey et al., 2013; den Besten et al., 2013), so

influencing its ability to compete for scarce resources. Therefore, the gut microbiota can influence the host's food assimilation efficiency, energy consumption and metabolic rate (collectively comprising its energetic phenotype). Through discussing the links between the gut microbiota and host lifestyle, genotype and environment, the impacts of the microbiota on host ecology are explored. In so doing, this review highlights the need for future ecological research to focus not only on the host, but on the 'holobiont' (Bordenstein and Theis, 2015), which comprises the host and its associated microbiota.

Research on host-microbiota interactions has to date largely been focused on laboratory-based studies and model organisms, but clearly has broader relevance; for example, studies of hosts such as gorillas (Hicks et al., 2018) and house sparrows (Teyssier et al., 2018) have provided ecological insight into host-microbiota interactions specifically relevant to natural systems by assessing spatio-temporal effects on the host microbiota. This review aims to highlight the multiple advantages of conducting such studies on wild animals, specifically in relation to the ecological understanding this might provide, including generating insights into how gut microbes can underpin host energetic plasticity in changing environments. This burgeoning research area is not without its complications however, and this review aims to identify many of the limitations involved with the exploration of ecological questions in the context of wild and laboratory-reared host associated microbiota.

Glossary	
Axenic	An environment devoid of contaminating microorganisms.
Gnotobiotic	An environment in which all microorganisms are absent, used to establish gnotobiotic (germ-free) animals.
Holobiont	The combination of different species that form an ecological unit. In the context of the microbial ecology, the holobiont incorporates the host and its microbiota.
Hologenome	The collective genomes of the holobiont. In the context of microbial ecology, the hologenome considers the genomic content of the host and its symbiotic microbes.
Microbiota	Microorganisms found both within and on every multicellular species.

Box 2-1: Glossary of relevant terms.

2.3. Old and New Tools for Determining the Impact of Microbiota on Host Energetics

Table 2-1: an overview of current approaches to research the ecology and function of gut microbiota.

Approach	Description	Use	Limitations	Reference
Next Generation Sequencing (NGS)	DNA sequencing using the concept of massively parallel sequencing, which describes the high-throughput and high speed of the technology.	Identifies the diversity of microorganisms present via targeted (e.g. 16S rDNA) or non-targeted (e.g. shotgun metagenomics) approaches.	Issues with reliability of library preparation (i.e. selectivity of primers). Provides information only on functional capacity, not function.	(Hovda et al., 2007; Kimura et al., 2020).
Meta-omics	Metagenomics, genome; metatranscriptomics, transcriptome; metaproteomics, proteome and; metabolomics, metabolome. Following these -omics approaches, mass spectrometry (MS) and nuclear magnetic resonance (NMR) allow characterisation of compounds/metabolites that microorganisms are producing.	Used in combination to analyse the complex ecology of microbiota – characterising communities but also providing detail on alpha and beta diversity and metabolic functions. Metatranscriptomics are typically targeted at microbial mRNA to reveal community level gene expression. MS and NMR allow metabolic profiles of a species or population of species to be assessed. Characterisation of these metabolites (such as short-chain fatty acids and volatile fatty acids) can indicate function of the microbial species or population.	Expensive. Difficult to scale-up to population samples. Transcriptomics are subject to contamination by ribosomal RNA. Often highly sensitive to sample preparation.	(Ni and Tokuda, 2013; Xie et al., 2013; Rambold et al., 2019).

Reverse Transcription Quantitative PCR (RT-qPCR)	RNA is first reverse transcribed into complementary DNA, before this is then used as a template for qPCR. qPCR quantifies presence and abundance of this DNA.	Quick and targeted quantitative measurement of microbial gene transcription (e.g. CAZenzymes, see text), allowing identification of which microbial genes are being expressed.	Difficulties associated with RNA work. Same PCR issues as found within library preparation for NGS.	(Smith and Osborn, 2009; Olsvik et al., 2013; Gajardo et al., 2016; Bredon et al., 2018).
Gnotobiotic manipulations	Complete removal of microbiota – the host is reared in an axenic environment.	Can be used to examine physiology of the host in the absence of all specific microbial symbionts, thereby allowing identification of the role of the microbiota. Facilitates testing of role of individual microbes or microbial communities in defining host phenotype. Creating axenic hosts and environments can be challenging. Eggs of oviparous species can be sterilised by antiseptics and antibiotics. Germ-free viviparous species can currently only be achieved via aseptic caesarean or hysterectomy.	Limited to sterile lab environments. Costly to establish and maintain gnotobiotic lines. Findings not always transferable to natural conditions.	(Rawls et al., 2004b; Marques et al., 2006; De Swaef et al., 2016; Martin et al., 2016; Zhang et al., 2020a).
Mono-associations	The host is inoculated with a single microbial taxon.	Enables researchers to view the impact of a single microbial taxon on a (gnotobiotic) host.	Limited to sterile lab environments. Biologically unrealistic.	(Morimoto et al., 2017; Lee et al., 2020).

Microbiota-transplants	A gnotobiotic host is inoculated with the microbiota of another individual.	Enables researchers to view the impact of a full microbial community on the host. Can be deployed xenobiotically (e.g. bear to mouse, human to mouse) in order to use sterile lab conditions. Can be used to determine the extent to which the microbiota can influence the host phenome.	Limited to laboratory environments. Findings not always transferable to natural conditions.	(Rawls et al., 2006; Crawford et al., 2009; Chevalier et al., 2015; Sommer et al., 2016).
Antibiotics	Single strains or multiple varieties of antibiotics are administered to a host.	Reveals how reduction of the microbiota can impact the host. Can be deployed to specifically target sensitive classes of microbes. Can be used to sterilise eggs of oviparous species. Can be used in species in which gnotobiotic methods would not be feasible.	Difficulties in repeatability as different antibiotics and dosages can have varying effects. Some bacterial taxa are difficult to eliminate; higher antibiotic doses may have toxic effects on the host. Problem of antibiotic resistance, especially in the field.	(Hu et al., 2013; Lin et al., 2015; Morgun et al., 2015; De Swaef et al., 2016; Raymann et al., 2018).

To make sense of so many recent advances, it is important to first describe the 'toolkit' of approaches that are now available to researchers in this field. Characterisation of the composition and function of the microbiota has classically relied on DNA and RNA sequencing techniques. Most commonly, high throughput sequencing of the 16S rRNA gene is used to identify the bacteria present within microbial communities (Table 2-1). However, research is gradually moving from simply measuring the diversity of bacteria present to determining microbial expression profiles, to delineate the functional basis of the host-microbiota relationship. Techniques such as reverse transcription quantitative PCR (RT-qPCR) can identify transcriptional responses in host species (Table 2-1), whilst various meta-omics approaches used in tandem with 16S rRNA sequencing can describe not only what bacteria are present, but the impact that their presence has on the host (Tremaroli and Backhed, 2012; Alberdi et al., 2016). Metabolomic approaches, for example, can identify metabolites produced by gut microbiota, especially volatile fatty acids (VFAs), short chain fatty acids (SCFAs), lactic acid and aromatic amino acids, so revealing how the microbiota can make specific nutrients available to the host or other members of the bacterial community (Mashego et al., 2007; Le Gall et al., 2011; Zheng et al., 2011; Sridharan et al., 2014).

A complication consistently encountered when studying host-microbiota relationships is disentangling cause from effect, since studies are often correlational. Progress has been made to overcome this limitation via the use of germ-free technologies, in which animals are reared in axenic environments, allowing the host to remain entirely devoid of microbes. Gnotobiotic, or germ-free, models have been successfully established in order to both determine how hosts perform in the absence of all microbes and to measure how this changes when the 'clean' animal is then seeded with selected microbial taxa (Table 2-1). Such studies have revealed various effects of gut microbiota on the host, including modulation of bone-mass density, fat storage and the immune system in mice (Tremaroli and Backhed, 2012) and regulation of fatty acid metabolism in zebrafish (Semova et al., 2012). In one such landmark study, transplantation of the gut microbiota from an obese host into a gnotobiotic recipient mouse led to an improved capacity for energy harvest and higher levels of fat deposition in comparison to when a host was colonised with a 'lean microbiota' (Turnbaugh et al., 2006). This early study highlighted the link

between gut microbiota and metabolism and showed that traits can be transmissible via microbiota transplants; another example is shown in Figure 2-1. Now, mono associations (in which a gnotobiotic host is the recipient of a single microbial taxon) and the transplantation of microbial communities between hosts (Table 2-1) have the potential to reveal the effect that the microbiota have on host phenomes.

It is difficult to render a host germ-free once it has already been colonised with microbes (i.e. once it is free-living), but antibiotics can be used to examine the impacts of a disrupted gut microbiota (Table 2-1), whereby antimicrobial compounds are used in ecological research as a tool to knock out groups of microbes in order to explore their function (Lin et al., 2015; Morgun et al., 2015; Raymann et al., 2018). Antimicrobial knock-out approaches have revealed, for example, the effects of the microbiota on host metabolism: the standard metabolic rate (SMR) of *P. americana* cockroaches was altered when the gut microbiota was disrupted by antibiotics (Ayayee et al., 2018). Unsurprisingly, antibiotic administration resulted in a reduction of bacterial load within the cockroach gut, but interestingly, this led to a decrease in host metabolic rates. Fine-scale effects on bacterial taxa remained unquantified, so these physiological effects could not be ascribed to specific microbes. However, other studies have shown antibiotics to cause changes in gut microbial community composition in mice (Yoon and Yoon, 2018) and honeybees (Raymann et al., 2018); the latter study showed that two key bacterial species of the bee gut responded differently to antibiotics, with *Gillamella apicola* experiencing a large reduction in genetic diversity, whilst *Snodgrassella alvi* remained largely unaffected. However, the use of antibiotics in ecological and microbiota research is not without its limitations (Table 2-1).

While several of these promising new experimental approaches, such as gnotobiotic treatments followed by seeding with selected microbial communities, are now available, to date their use has been restricted to a small number of laboratory model organisms. These allow determination of the causal role of host associated microbial communities, but field-based studies remain the best way of truly understanding host-microbiota relationships since they place host-microbe interactions in an ecological context (Figure 2-1). An example of this is the demonstration that the social environment of red-bellied lemurs – which can only be realised fully in groups of wild animals - plays a role in modifying their microbial community (Raulo et al.,

2018). However, there remain significant logistical challenges to implementing some of these lab-derived approaches in the field (Table 2-1). Therefore, this review draws attention to the range of approaches being used to explore the host-microbiota relationship, which are allowing direct links to be found between host-associated microbial communities and host energetics.

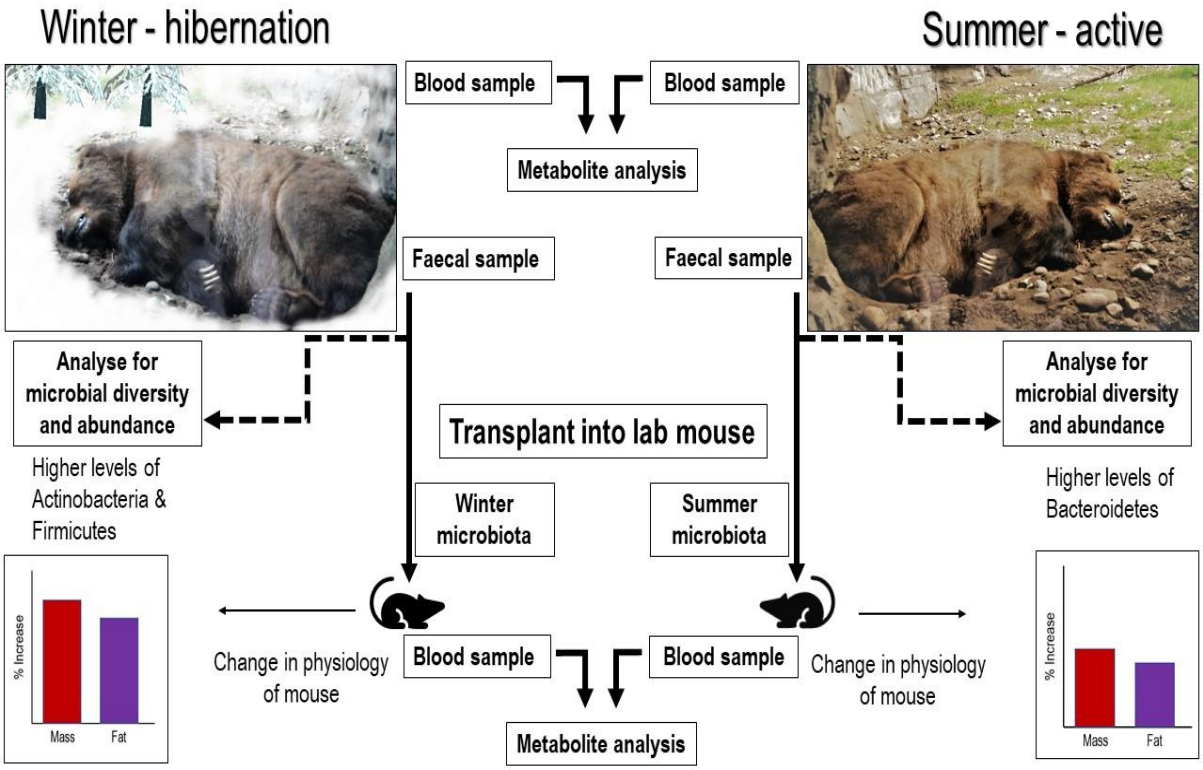

Figure 2-1: An illustration of how an experimental approach can be used to determine microbiome-host relationships in an ecological context. *This single study utilised many of the methods discussed within this review. Researchers used 16S rRNA profiling of brown bear (Ursus arctos) faeces and the ceca of colonised mice to assess diversity and abundance of the microbes present within the gut. Blood metabolites were also analysed and compared between hosts to examine metabolites relevant to metabolism. Transplantation of gut microbiota from a wild bear to gnotobiotic mice demonstrated that the seasonally-dependent energetic phenotype was transmissible, as shown by the gut microbiota, blood metabolite profiles and the resulting physiological state of the new host being dependent on the season of transfer. This study highlights the range of ways a single study can examine the gut microbiota in relation to the host metabolic phenotype and combines lab and field-based approaches. Information taken from Sommer et al. (2016).*

2.4. Gut Microbiota Nutritional Niches and Host Nutritional Energy Harvest

Many host species consume diets for which they lack the endogenous enzymatic repertoire to fully exploit, and so depend on their gut microbiota to produce the key digestive enzymes. Obvious examples of this are termites and ruminants, which rely on microbial hydrolase enzymes to break down the cellulose in their plant-based diet into monosaccharides and oligosaccharides (Varel and Dehority, 1989; Ni and Tokuda, 2013). These are then fermented by microbes such as saccharolytic bacteria to produce short chain fatty acids (den Besten et al., 2013). The cell walls of woody plants contain lignocellulose, a complex composed of lignin, cellulose and hemicellulose. Digestion of lignocellulose requires multiple carbohydrate-active enzymes (CAZymes), only some of which may be produced by the host (Bredon et al., 2018). For example, while mealworms (*Tenebrio molitor*) and woodlice have been found to produce degradation enzymes such as cellulases, they rely on their gut microbiota to produce the enzymes that break down compounds like lignin and phenols (Genta et al., 2006; Bredon et al., 2018). Similarly, termites symbiotically combine with their microbiota to produce the range of CAZymes needed to break down lignocellulose, producing metabolites which drive the termite's energy metabolism (Ni and Tokuda, 2013). The hindgut microbiota of higher termites also has a role in fixing, recycling and upgrading nitrogen, without which termite growth would be constrained (Brune and Dietrich, 2015). These examples highlight how the gut microbiota allows the host to exploit otherwise inaccessible niches.

An analogous phenomenon is also observed in some cetaceans (Sanders et al., 2015). Baleen whales (Mysticeti) require CAZymes to break down the large quantities of the polysaccharide chitin that they consume in the form of krill and other zooplankton. Sanders *et al*. (2015) found that the microbiome of baleen whales shares characteristics with those of both terrestrial carnivores and herbivores, with an amino acid metabolism gene profile resembling that of a carnivore, but a gene profile associated with energy metabolism and lipid metabolism reflecting those of herbivores. Similarities of the cetacean gut microbiota to that of a fermentative herbivore is thought to aid release of chitin nutrients to the whales (Sanders et al., 2015).

The waste-products of microbial metabolism, such as the acetate and butyrate produced by fermentative bacteria, can have significant effects on host metabolism since they are involved in the regulation of fatty acid, glucose and cholesterol metabolism, as well as being used by the host as an energy source (den Besten et al., 2013). For example, the symbiotic class Mollicutes was found to convert dietary citrate into acetate to fuel host metabolism in Panamanian fungus-growing leaf-cutter ants (Sapountzis et al., 2018). Short chain fatty acids such as acetate and butyrate provide much of the energy needed to sustain the high turnover rate of colonocytes and enterocytes (epithelial cells of the colon and cells of the intestinal lining, respectively) within the host gut, with oxidation of butyrate alone able to provide up to 70% of energy needed by colonocytes in rats (Roediger, 1982).

As well as impacting the nutritional niche of organisms by allowing them to digest complex and otherwise inaccessible biological polymers, the gut microbiota is also thought to play a role in the detoxification of dietary components, allowing the host to exploit a niche intolerable to most other species (Genta et al., 2006; Wienemann et al., 2011; Heys et al., 2019). One such example is that of the coffee berry borer (*Hypothenemus hampei*), an insect pest of coffee. Caffeine is a known toxicant that has negative effects on insects, including impacting DNA repair and phosphodiesterase activity (Ceja-Navarro et al., 2015); nonetheless, the coffee berry borer completes its entire life cycle on the coffee plant. This is made possible due to caffeine degradation carried out by its gut microbiota. When the gut microbiota is incapacitated with antibiotics, the host loses its ability to degrade caffeine, but this is restored by reinfection with *Pseudomonas fulva*, known to produce an enzyme that causes demethylation of caffeine (Ceja-Navarro et al., 2015). A similar study in mealworms compared germ-free larvae with conventionally reared individuals and found the gut microbiota had a role in detoxifying allelochemicals within *T. molitor's* plant-based diet. Detoxification in this context was thought to be due to the microorganisms' ability to catabolise toxic plant glycosides and aglycones, with some bacterial species using aglycones as a carbon source (Genta et al., 2006). Similar relationships are seen in diverse host species: the caecal microbiota of the Western capercaillie (*Tetrao urogallus*) allows its host to survive on its potentially toxic resin-rich winter diet (Wienemann et al., 2011), whilst the unusual tolerance of sheep and goats to toxins in the ragwort (*Jacobaea vulgaris*) is thought to be due to the

detoxification role of the rumen microbiota (Rattray and Craig, 2007). In allowing hosts to exploit otherwise indigestible or toxic dietary compounds, gut microbiota thus permit hosts to expand their niche and reduce interspecific competition by feeding on underutilised sources.

2.5. Studying Gut Microbial Energetics in their Ecological Context

As researchers strive to understand specific functional benefits of the microbiota, an advantage to studying holobiont dynamics in wild animals is the existence of pronounced variation in environmental conditions experienced by the hosts. This allows researchers to examine whether there is selection for microbial taxa that are more effective under different environmental conditions, which in turn allows the host to function across a broader range of environments. Of particular relevance is the influence of dietary composition, which can show pronounced seasonal changes that have a powerful influence on gut microbial communities (Hang et al., 2013). By way of example, seasonal changes in gut microbial community composition have been found in the giant panda (*Ailuropoda melanoleuca*) as its diet transitions from protein-rich bamboo shoots to bamboo leaves that are less rich with a higher cellulose content (Wu et al., 2017). The shift to the poorer quality diet is associated with the gut microbiome becoming less diverse, but more specialized on breaking down cellulose. The presence of cellulose-digesting bacteria in the gut of the panda also help to explain how a carnivore can feed on plants (Xue et al., 2015; Wu et al., 2017).

Similar effects of diet on microbial community composition have been seen across a variety of species; often the consequences for the host are unknown, but there are some suggestive examples. In American bison (*Bison bison*) an increased abundance of the Phylum Tenericutes, which metabolise simple sugars, is found when the diet is biased more towards plants lower in secondary metabolites (Bergmann et al., 2015). Human studies are informative: microbial community structure within the human gut differs between subjects consuming an animal-based diet compared to a plant-based diet, which has consequences for microbial gene expression and activity: animal-based diets result in greater activity of amino acid catabolism pathways whereas plant-based diets lead to an emphasis on

biosynthesis pathways (David et al., 2013). Energy requirements in the Western capercaillie are met primarily by foraging on resinous coniferous needles during winter, reducing the diversity of the caecal bacterial community in comparison to when the birds have a more diverse diet (Wienemann et al., 2011). Interestingly though, greater differences in community composition exist between wild and captive individuals. Within captive capercaillie, there is an absence of certain fermentative bacterial species, such as those from the *Synegistes* phylum. These species ferment carbohydrates to produce acetate, propionate and succinate, so contributing to succinate turnover and supplying energy to the host. Reduced fermentative capacity compromises detoxification activities within the gut, which is necessary to tolerate the birds' resin- and phenol-rich winter diet (Wienemann et al., 2011). The gut microbiota differences between wild and captive individuals could in part explain why reintroductions using captive-bred birds have thus far largely been unsuccessful (Wienemann et al., 2011).

Though the complex interplay between host diet and the gut microbiota has been examined in many contexts, reproducibility can remain poor, so attempts to define the diet-host-microbiota relationship remain a challenge. Understanding can be further complicated due to variation in the ecology of bacterial species: Holmes et al. (2017) found that responses to dietary nitrogen levels were divergent between bacterial taxa, which had repercussions for host health. Specifically, the taxa positively responding to limited protein availability (endogenous nitrogen users), such as members of the Phylum Bacteroidetes, included species known to provide maintenance to intestinal barrier functions and immunoregulation within the murine host and promote good overall 'cardiometabolic health' (avoidance of cardiovascular disease). This was in contrast to the poorer cardiometabolic health phenotype seen in mice administered with a higher protein diet that favoured microbes that rely upon dietary nitrogen (Holmes et al., 2017). Other human and laboratory animal studies also indicate complex interactions between diet, gut microbiota and host metabolism and health (Cani and Delzenne, 2009; Musso et al., 2011; Ayayee et al., 2018), emphasizing the importance of taking into account the dietary factors impacting microbial community dynamics and assembly.

In addition to coping with changes in dietary composition, wild animals often have to withstand significant fluctuations in the quantity of food available, both directly due to

seasonal changes and indirectly as a result of their life history: e.g. when they migrate, hibernate or otherwise become dormant, or show ontogenetic niche shifts. These periods in which the energetic phenotype of the host changes can reveal potential functional links between the energetics of the gut microbiota and that of the host. Short-term fasts can induce responses from the gut microbiota that benefit the host, e.g. by increasing the supply of SCFAs through fermentation of glycans (Crawford et al., 2009). A more extreme fast is experienced by species that hibernate – although it is important to note that hibernation and fasting are not equivalent physiological states for endotherms. In contrast to fasting, hibernation is often characterised by the lowering of the core body temperature to <10^{o}C, producing a much reduced metabolism of around <4% of the level seen in the active mammal (Carey et al., 2013). As a consequence, the gut microbiota may respond differently to the two situations: Syrian hamsters (*Mesocricetus auratus*) showed no reduction in total bacterial numbers or SCFA concentrations when entering hibernation, but showed significant decreases in both measures of microbial activity when involuntarily starved (Sonoyama et al., 2009), suggesting that the microbiota are more resilient to a predictable seasonal change in host energetic status than to an unexpected (and potentially more stressful) crash in food intake.

The diversity of the gut microbiota can nonetheless decrease during a period of hibernation, with an increase in the preponderance of bacteria that can live directly off the host (e.g. feeding off host mucins) and a loss of species that are reliant on host dietary compounds (Carey et al., 2013). Studies of the metabolomics of hibernating species have identified compounds produced by microbes that will affect host energetics during the period of hibernation. The shift in the composition of the gut microbiota in ground squirrels (*Ictidomys tridecemlineatus*) as they prepare for and enter torpor may contribute to the build-up of fat stores and leads to an increase in the relative production of acetate, which can be used as an alternative to glucose for energy in certain organs (Carey et al., 2013). Hibernating and active ground squirrels differ in the levels of SCFAs known to play key roles in host energy metabolism (Carey et al., 2013). A direct effect of the microbiota on the physiology of a hibernating host, allowing it to conserve energy, was demonstrated by showing that the transfer of the ‘winter microbiota’ (i.e. that present within the gut during winter hibernation) of wild brown bears (*Ursus arctos*) into gnotobiotic mice had

different effects on the mice than did the transfer of the ‘summer microbiota’ (Sommer et al., 2016) (Figure 2-1). Moreover, metabolites in the blood of these mice correlated with those observed in the wild bears in the appropriate season, further demonstrating that the modulation of host energy metabolism was a direct result of the microbiota (Sommer et al., 2016). That a seasonal metabolic phenotype was in part transferable even between host species (Figure 2-1) provides dramatic empirical evidence that the microbiota can provide the means by which a host shows metabolic acclimation under different environmental conditions.

Some host species are adapted to prolonged period of fasting regardless of environmental conditions: Burmese pythons (*Python molurus*) experience extended periods of time without food before consuming an exceedingly large meal (sometimes exceeding 50% of their body weight) (Costello et al., 2010). This host therefore offers a different insight into host-microbiota-metabolism interactions, due to the altered circumstances in which nutrient-deprivation occurs. The snake undergoes large physiological and morphological changes when it feeds, including enteric hypertrophy, and experiences dramatic but short-term changes in its metabolic demands and energy flux (Costello et al., 2010). There are parallel changes in the python’s gut microbiota: Costello *et al*. (2010) discovered that the gut microbiota of a fed python was characterised by a higher proportion of taxa associated with proteolytic activity, including an increase in Firmicutes, known to increase energy harvest in other animals.

2.6. Adaptability, Plasticity and Host Energetics

Flexibility in the microbial community composition or activity can potentially be beneficial to a host, since it can allow the host to respond to changing food availability or metabolic demands (Sommer et al., 2016; Foster et al., 2017); conversely, the benefit of a particular functional profile of microbes can vary in time and space (Sommer et al., 2016; Risely et al., 2017). The gut microbiota-host relationship can vary temporally in response to changes in environmental factors other than simply diet (Burns et al., 2016; Uren Webster et al., 2020). This should be most evident in animals that experience large environmental shifts over their lifetime. Thus the transition from fresh to salt water in Atlantic salmon has been found to

influence the number of microbial species present in different regions of the gut and the overall bacterial load (Llewellyn et al., 2015; Rudi et al., 2018). The microbiota might be especially relevant at key developmental stages, as found for Wood frogs where disruption of the microbiota in early larval life was found to have legacy effects on development that lasted until after metamorphosis, long after the microbiome had recovered from the perturbation (Warne et al., 2019). This highlights the value of considering a host's lifecycle and changing energetic demands when elucidating the impact of the gut microbiota. As a further example, Gould *et al*. (2018) found the diversity of the gut microbiota of *Drosophila melanogaster* influenced the life history of the host, with interactions between the 5 major bacterial species commonly found in the fruit fly affecting the scheduling of reproduction. Germ-free flies had an increased lifespan, but a lower reproductive rate (Gould et al., 2018). The complexity of the host-microbial relationship is thus increased when considered in the host's ecological context, highlighting the dynamic nature of the association.

Longitudinal studies examining changes in energy demand benefit from being able to compare the gut microbiota within the same host under different conditions, but always have the confounding factor of time (or host age). This can be circumvented where host species exhibit intraspecific variation in energy demand at the same time point. Risely *et al*. (2017) simultaneously compared the gut microbiota of migratory *Calidris spp.* shorebirds to that of their non-migratory conspecific counterparts. Long-distance migration can represent physiological and morphological challenges for the host (such as the need to reduce body mass in order to reduce the costs of locomotion), often in association with high energy demands. Migrant individuals of two species were found to have a 30-fold higher abundance of the *Corynebacterium* genus in their guts in comparison to conspecific residents, though the remaining community structure remained broadly similar (Risely et al., 2017). The reason for this dramatic increase in the prevalence of *Corynebacterium* species in migrants is as yet unknown.

The dynamic nature of the microbiota-host relationship means that it can be difficult to determine the relative importance of the microbiota in determining the phenotype of the host. Recent work has begun to revolve around the holobiont and to incorporate the 'hologenome' concept, in which the evolutionary capacity of both the host and its associated microorganisms are considered together (Bordenstein and

Theis, 2015; Alberdi et al., 2016). Within the field of ecology, consideration of the hologenome/holobiont allows researchers to, for instance, more properly evaluate the potential for phenotypic plasticity or adaptation in the face of changing environments.

If gut microbial plasticity is to enhance the host's utilization of its niche, the composition and activity of the microbiota must be capable of altering with changing environmental conditions, resulting in the provision of different services to the host (Alberdi et al., 2016). Studies that simply identify shifts in microbial community composition in response to environmental changes cannot identify the functional mechanism that underpins any such effect, but have nonetheless proved useful, for instance in showing how the microbiota changes over time within an individual as a result of ontogenetic (Burns et al., 2016), dietary (Abid et al., 2013; Carmody et al., 2015) or other environmental changes (Candela et al., 2012). This longitudinal intraindividual variation in microbiota diversity can exceed interindividual variation, particularly when hosts have been exposed to similar environmental conditions (Schmidt et al., 2015; Rudi et al., 2018).

2.7. Future Research Directions

As the focus moves to wild and non-model organisms and more importance is placed on the function rather than simply the characterisation of the microbiota, the important questions in an ecological setting include: how stable is the gut microbial community across different life stages, environments or seasons? Does it truly offer phenotypic plasticity to the host? How much does it impact on host metabolism? And given this impact, how might modulating the microbiota affect the energy balance of the host? Answering these complex questions will require integration of knowledge from a variety of biological fields.

Many different techniques are being used to characterise the gut microbiota and untangle the complicated host-microbiota-physiology axis, with the ultimate aim of detecting causal rather than just correlational relationships, but not all can be combined with an ecological approach. Whilst gnotobiotic studies have allowed researchers to examine the physiological impact of mono-associations and specific community compositions of microbes (Rawls et al., 2004b; Marques et al., 2006; Lee

et al., 2020), they are restricted to sterile laboratory environments and usually involve a limited range of model organisms (Table 2-1). Antibiotics can be used to examine the effect of disrupting the gut microbiota (Lin et al., 2015; Gao et al., 2018; Raymann et al., 2018; Yoon and Yoon, 2018; Zhou et al., 2018), but studies to date have focussed on the impacts on either the host, or on microbial community composition. Future studies would benefit from combining these two in order to deepen our understanding of the functional profile of specific taxa, but there may be too many ethical issues with the use of antibiotics to make this a commonly adopted approach in ecological studies.

A more promising technique to disrupt microbiome identity and/or function is to administer probiotic bacteria to the host. Probiotics are live bacteria chosen specifically for their potential beneficial effects on host health, including acting as antagonists against pathogenic bacteria as well as aiding the host immune system development and homeostasis (Ringø et al., 2007; Abid et al., 2013). Depending on the treatment chosen, probiotics have the capacity to alter microbial load and change community composition, and as a result impact the host's intestinal immunity (Abid et al., 2013) as well as its growth rate and survival (Bagheri et al., 2008). The complication lies in understanding which bacterial taxa should be targeted in such interventions. One option is to adopt the approach of Holmes et al. (2017) who recommend describing the composition of microbial communities by their requirements rather than by their function. By looking at responses to dietary interventions at the community level, bacterial communities could be broadly targeted, as opposed to trying to predict the response of individual bacterial taxa. If this information is combined with the resulting impact on host energetic phenotype, the targets of probiotic intervention might then be identified. Consideration must also be given to the fact that the most beneficial functional profile in terms of host-fitness will likely vary spatially, temporally and ontogenetically, reflecting the changing environment faced by the host.

Understanding the complexities of host-microbiota interactions remains at the forefront of gut microbiota research and to take this research further, a wide variety of studies will be necessary: mono-associations with just one microbial species can elucidate functions of specific bacterial taxa, wild-based studies can characterise how the prevalence of certain bacteria changes within the natural environment,

whilst studies in a laboratory environment may inform the best dietary interventions. From an ecological perspective, increasing knowledge of spatial and temporal changes in the gut microbiota as a result of environmental change remains a priority. The seasonal, life-history and genetic diversity seen in nature necessitates a breadth of approaches in order to understand the impact of the gut microbiota on host metabolism under these different conditions. As yet, these approaches are in their infancy, but some studies are beginning to adopt a more integrative approach: a study of three species of small mammal that compared the effects of genetics versus environment on gut microbiota composition found environment to be of secondary importance in comparison to host genetic similarity. Specifically, the gut microbiota of mice, voles and shrews were more similar within species at different locations than between different species living in sympatry (Knowles et al., 2019). Since many gut microbiota-host associations are highly conserved (Rawls et al., 2004b; Erkosar et al., 2013) and there is increasing emphasis on the concept of co-evolution (Chevalier et al., 2015), this idea could be integrated with such studies incorporating both inter- and intraspecific comparisons, in order to provide greater resolution.

It is clear that longitudinal studies in the wild would be most insightful, but sample size and repeatability often suffer, and studies to date have tended to be correlational and so cannot explicitly separate cause from effect. The growing assumption that the microbiota of the gut is both beneficial and essential needs to be continually challenged, since there is now evidence of species that have no such reliance on gut microbes and their associated services (Hammer et al., 2017), and colonisation models suggest that many microbes do not appear to adapt to the host environment, simply passing through alongside food items (Heys et al 2019). These colonisation models, such as those proposed by Sloan and others (Sloan et al., 2006; Burns et al., 2016), can be useful in clearly identifying those microbial taxa that are responding to the host environment, and so narrowing the focus onto a subset of organisms that may have some functional role (positive or negative) on host fitness.

To further understand the relationship between host energetics and gut microbiota, characterisation of the enteric bacteria must occur alongside robust phenotyping of the metabolic status of the host. This can be achieved via metabolite profile analysis of host blood, urine and faeces (Xie et al., 2013), in combination with techniques providing a greater overview of host metabolic rate, such as respirometry. These top-

down techniques will allow information on host energetics to complement quantification of bacterial community composition and their functional profiles, allowing greater understanding of the interface between microbial complement and host dynamics. Non-invasive metabolomic techniques will allow for longitudinal data collection, enabling researchers to examine how microbial community profile and host metabolic profile covary under a range of conditions.

2.8. Summary

This review has highlighted the increasing number of studies now finding direct links between gut microbiota and host energetics. Given the plastic nature of both the host and microbe phenotypes, it clear that the gut microbiota should be a key consideration of host adaptability in changing environmental conditions. Research should now move from broad characterisation of community composition to elucidation of impacts on the host, in both laboratory- and field-based studies, to allow a broader understanding of the ecological perspectives of these dynamic relationships. This will require us to define the function of specific microbial taxa in an effort to reliably inform the ways in which gut microbiota impact host metabolism.

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2.10. Authors' Contributions

All authors contributed substantially to the concepts and revising of the manuscript; E.L. led the writing. All authors approved the final version for publication.

Chapter 3: Does Gut Microbial Composition Vary with Host Metabolic Phenotype in Juvenile Atlantic Salmon?

3.1. Abstract

Standard metabolic rate (SMR) describes the minimum energetic cost of living, below which an organism's physiological functions will be compromised. An individual's metabolic rate has consequences for its growth, behaviour and overall fitness, and in turn is influenced by a variety of factors, including diet and the environment (in particular temperature in the case of ectothermic/poikilothermic animals). The physiological factors associated with metabolic rate have also been shown to have a functional relationship with the gut microbiota. In parallel to an organism's metabolic rate, the gut microbiota is also impacted by diet and environmental effects. Although both metabolic rate and the gut microbiota have consequences for host physiology and therefore host fitness, only a limited number of studies have examined both concurrently. This study explored the potential interplay between the microbial composition in the gut and host metabolic rate in juvenile Atlantic salmon by examining the gut microbiota in fish of contrasting metabolic phenotypes: 'low' SMR and 'high' SMR. Additionally, physiological measures such as growth efficiency and body composition were assessed, in order to investigate whether relationships exist between these factors, the metabolic rate and the gut microbiota of Atlantic salmon. It was anticipated that growth efficiency and fat content would be higher in fish with a high SMR due to the necessity that they retain more energy to maintain their higher baseline energetic cost of living. Additionally, it was hypothesised that the gut microbial community composition would differ between fish from the 'low' and 'high' SMR groups. Salmon with a higher SMR were indeed found to grow more efficiently and have a higher fat content than their conspecifics with a lower metabolic rate. In addition, fish from the 'high' metabolic rate group had reduced microbial alpha diversity within the foregut. Beta diversity and differential abundance analyses highlighted that gut microbial community composition was divergent between fish from the two metabolic rate groups. Intriguingly, distance-based redundancy analysis (dbRDA) suggested an influence of fish body mass and rSMR on the gut microbiota, whilst correlation analysis identified

an OTU from the family Rhodobacteraceae as being negatively correlated with metabolic rate. The differential abundance analysis also highlighted that genera from the Rhodobacteraceae family were overabundant within the guts of fish with a low metabolic rate. Although it is challenging to elucidate functional links, the gut microbial community composition differences found within this study are discussed in the context of the discrepancy in metabolic phenotype and other physiological parameters between the two groups of fish. Seemingly, this is the first study to examine the gut microbiota in Atlantic salmon in the context of metabolic rate. To build upon this work, possible future research directions are discussed, including the importance of studies that will elucidate the role function of microbial taxa, such as the Rhodobacteraceae member identified here in influencing host metabolic phenotype.

3.2. Introduction

Metabolic rate offers insight into many key traits in living organisms, such as growth, reproductive capacity and survival (Biro and Stamps, 2010; Burton et al., 2011; Pettersen et al., 2018). An organism's metabolic phenotype has fitness consequences; however, there is seemingly no single metabolic phenotype that is more beneficial than another. Whole-animal metabolic rate can vary within a single individual due to factors such as activity level, temperature and body size (Chabot et al., 2016), so comparisons within and between species are often based on the standardised measurement of the minimum cost of living, termed basal metabolic rate (BMR) in endotherms and standard metabolic rate (SMR) in ectotherms (White and Kearney, 2013). Substantial interspecific and intraspecific variation is still seen in this minimal metabolic rate (Pettersen et al., 2018). Whilst differences in mass can account for much interspecific variation, BMR has been shown to vary several-fold between species of the same size (White and Seymour, 2004). To understand intraspecific variation, many studies have also assessed the repeatability of metabolic rate in an effort to establish whether metabolism can respond to selection (Artacho and Nespolo, 2009; Auer et al., 2016a), because natural selection shapes heritable differences among species (Pettersen et al., 2018). Though metabolic rate can also vary throughout ontogeny (Pettersen et al., 2018) and the degree of

repeatability can depend upon the time between measurements and the organism's environment (Auer et al., 2016a), it is now widely accepted that there is a heritable component to metabolism (White and Kearney, 2013). In spite of this heritability, variation in metabolic phenotypes persists.

Within teleosts, metabolic rate impacts many aspects of an individual's life, including growth rate (Auer et al., 2015c) and the processing of food (Millidine et al., 2009). The size of an individual's aerobic scope (AS, which describes an individual's capacity for aerobic activity) has been shown to determine the ability of Atlantic cod (*Gadus morhua*) to forage in a hypoxic environment, as individuals with a higher AS could forage for longer in the hypoxic demersal zone (Behrens et al., 2018). Many studies have highlighted the link between individual variation in metabolic phenotype and behavioural traits: metabolic rate has been linked to angling vulnerability in largemouth bass (*Micropterus salmoides*) (Redpath et al., 2010), dominance and aggression in Atlantic salmon (Metcalfe et al., 1995; Cutts et al., 1998), as well as risk taking in common carp (*Cyprinus carpio*) (Huntingford et al., 2010). While a high SMR might result in an increased capacity to win competitive interactions (Metcalfe et al., 1995), the benefit of this higher metabolic rate will be attenuated in periods of food scarcity, since this might result in a fish being unable to meet its higher energetic demands. Indeed, it may be that increased dominance is necessitated by a higher SMR in order to obtain the food and territory required by its metabolism (Cutts et al., 2002). Maintenance of a higher metabolic rate will put more pressure on a fish to outperform conspecifics, but also result in an increased need to gain as much as possible from its diet. Juvenile Atlantic salmon with a higher SMR have been shown to be able to process their meals faster, allowing them to make use of ingested food more rapidly; however, they also incur greater metabolic costs when processing their meals, which might be related to their assimilation efficiency and could also reflect a greater growth potential in fish with a higher metabolic rate (Millidine et al., 2009).

Metabolic studies such as these highlight that benefits associated with certain metabolic phenotypes are often highly context dependent, further explaining the maintenance of variation (Burton et al., 2011). Such context-dependent benefits have been widely reported in the literature: it has been shown that juvenile Atlantic salmon with a high RMR thrived in less complex habitats with a predictable food

supply, but that this benefit diminished in worse environmental conditions (Reid et al., 2012). Further, although laboratory based studies have reported a positive correlation between SMR and growth rate in salmonids, a study on brown trout (*Salmo trutta*) in the wild found no trend or a negative relationship between the two (Álvarez and Nicieza, 2005). In Atlantic salmon, the relationship between metabolic rate and survival ranged from negative to positive amongst different environments, highlighting that environmental heterogeneity can act to maintain variation in metabolic phenotype (Robertson et al., 2014).

Across all species, it is accepted that intraspecific variation in metabolic rate is partly due to environmental conditions: metabolic rate has been shown to respond to food availability (Auer et al., 2015b), temperature (Khaliq and Hof, 2018) and hypoxia (Jordan and Steffensen, 2007). This has recently been extended to include examination of the influence of the gut microbiota on metabolic rate (Cani and Delzenne, 2009; Ayayee et al., 2020), due to research uncovering interplay between the gut microbiota and host physiology. The ways in which the gut microbiota might influence an organism's metabolic phenotype, discussed in detail in Chapter 2, has been examined across a wide variety of taxa. Results have shown that the gut microbiota can adapt to changes in host diet (Bergmann et al., 2015), which has implications for the production of microbial metabolites which in turn can affect host metabolic rate (Kovatcheva-Datchary et al., 2015). Microbiota metabolites have also been shown to signal to other host organs, which can also regulate host metabolism (Schroeder and Bäckhed, 2016). Like metabolic rate, the composition of the gut microbiota is context-dependent: the microbial community has been shown to shift with ontogeny (Burns et al., 2016), environment (Candela et al., 2012; Rudi et al., 2018) and changes in metabolic state, such as during hibernation (Carey et al., 2013). Feedback between the gut microbiota and host metabolism appears to be bidirectional, but the complex interplay between the two means that when studying metabolic rate, the gut microbiota is an important consideration.

Though studies on the metabolic rate of teleosts and those on the teleost gut microbiota are common, few studies have examined the links between the two. As SMR reflects the energy required to sustain the most basic requirements of life, an organism faces physiological impairment if its metabolic rate drops below this level

(Chabot et al., 2016). This makes it imperative that a fish can always meet its basic energy requirements, regardless of the environmental conditions it faces. Within salmonids, there are large variations in their energetic demands on both temporal and spatial scales, with daily and seasonal fluctuations in response to food abundance, temperature and environmental conditions. Fish are also faced with energetically expensive tasks such as competitive interactions and predator avoidance. Such variation in biotic and abiotic factors also has repercussions for the community composition of the gut microbiota (Ghanbari et al., 2015). Due to their links with host physiology, plasticity in an individual's metabolic rate and the gut microbiota would function to allow a fish to cope with a broad range of environmental conditions.

Consideration of both the metabolic rate and the gut microbiota simultaneously could uncover relationships between the metabolism, the gut microbiota and the performance of teleosts, thereby enabling researchers to investigate the capacity of fish to thrive in different environments. For example, fish can reduce metabolic costs during periods of low food availability, but their capacity to do so varies between individuals (O'Connor et al., 2000). Meanwhile, it has been shown separately that changes in food input (amount and composition) has repercussions for both the host's metabolic rate (Auer et al., 2015b) and its gut microbiota (Heikkinen et al., 2006; Green et al., 2013), both of which will have repercussions for host fitness. In addition, given that the gut microbiota can function to aid in host digestion (Nayak, 2010), which could have implications for fish growth, it is therefore possible that there will be a relationship between the metabolic phenotype, the gut microbiota and the growth efficiency of a teleost host.

As detailed in Chapter 2, a link between host metabolism, the gut microbial community and host physiological state is suggested by previous research in other taxa: for instance, in humans, obesity has been shown to have a microbial component (Ley et al., 2006; Turnbaugh et al., 2006), which is thought to interact with host metabolism due to an improved energy yield from food (Tremaroli and Backhed, 2012). This might mean that there is a relationship between an individual's fat content and metabolic rate. Fish such as Atlantic salmon experience large seasonal variation in food consumption due to changing environmental conditions (Fraser et al., 1995). This can impact their physiology, because during the winter

months, it is harder for their food intake to meet their energy demands (Finstad et al., 2011). The body composition of Atlantic salmon can vary dramatically throughout the lifespan of a fish, most markedly when fish build up both lean mass and fat reserves prior to their upstream migration to spawn (Kadri et al., 1995). This would be similar to physiological responses found in migratory birds that deposit greater volumes of fat prior to migratory flight (Rutkowska et al., 2016; Watts et al., 2017). Salmonid migration has high associated metabolic costs, further suggesting there might be a link between fat content and SMR. The relationship between the two could be positive or negative, where fish with a high SMR have high living costs therefore burn off more fat, or where a high SMR results in high daily food intake and so a greater retention of fat. It is most likely that as with other aspects of metabolic rate, the relationship will be context-dependent (i.e. the relationship could swing from positive to negative as food supply drops). Taken together, any relationship between the metabolic phenotype, the gut microbiota and the growth efficiency of a teleost host might also have repercussions for body composition and individual fitness.

Consequently, this study seeks to understand the physiological results of different metabolic phenotypes in Atlantic salmon of wild origin. As costs and benefits of given metabolic phenotypes are dependent on the environment, the study will assess the growth efficiency and fat content of individual fish with contrasting metabolic rates in a common environment in order to assess whether metabolic rate directly impacts fish physiology. Due to the hypothesised links between digestion, metabolism and the gut microbiota in Atlantic salmon, the microbial load of both low and high metabolic rate fish will also be examined. In addition, this study aims to characterise the gut microbiota of Atlantic salmon with both metabolic phenotypes, in order to highlight any significant differences in microbial diversity or community composition between the two.

This study will therefore test the following hypotheses:

- Growth efficiency will be higher in fish with a high SMR due to their increased need to retain more energy to compensate for a higher baseline energetic cost of living.

- Fat content will be related to SMR and growth efficiency, with individuals that grow more efficiently being able to lay down an increased amount of fat.
- The microbial community in fish from 'low' and 'high' metabolic rate groups will differ, which could be linked to the difference in SMR.

3.3. Methods

3.3.1. Fish Husbandry and Acclimation

The Atlantic salmon used in this experiment were of wild origin, derived from parents caught in the River Conon, Northern Scotland during their spawning migration as part of mitigation measures for hydropower installation. The fish, part of a larger experiment described in Auer et al. (2018), were reared under hatchery conditions from the egg stage onwards in the aquarium facilities at the Institute of Biodiversity, Animal Health and Comparative Medicine, University of Glasgow. All fish were immature juveniles in their second summer of life at the start of this experiment (July 2017). 60 fish were selected at random from a 400L circular stock tank containing several hundred offspring from 30 full sibling families (see Auer et al. (2018) for details of family selection and early rearing). These 60 fish were transferred into individual compartments (190 x 130 x 200mm) within a recirculating stream system in the same aquarium room. All 60 fish shared a common water source, sterilised by a UV filter. The room was kept on a 12L:12D photoperiod and the water temperature within the stream system was maintained at 11.9˚C ±1.0˚C, similar to the temperature of the stock tank from which they were taken. The fish were left to acclimate within these compartments for a period of 2 weeks, during which time each individual was hand fed a ration of EWOS MICRO 5P LR pellets (EWOS Ltd, Bathgate, UK) daily. This ration was determined by first selecting 5 fish at random from the initial stock tank and weighing them to gain an approximate average mass of the experimental fish (mean: 15.8g, ±4.5g S.D.). This average mass was used to determine the daily ration to feed all experimental fish during the acclimation period based on the following equation from Auer et al. (2015c), itself derived from Elliott (1976b), that originally described the energetics and growth of the closely related

and ecologically similar brown trout; the ration was calculated to be midway between a maintenance and maximum daily food intake:

$$\text{Ration (MJ.day}^{-1}) = (2.91M^{0.737} e^{(0.154T)}) * 0.000004184 \quad (1)$$

Where M = body mass (g) and T = water temperature (˚C). Using knowledge regarding the energetic content of the feed (19.53 MJ.kg^{-1}), it was possible to determine the daily ration (mg pellets) for the 60 experimental fish, as a function of the average body mass of the 5 stock fish (M, g) and water temperature (T, ˚C) for the acclimation period. Two individuals died during the acclimation period of unknown causes, leaving a sample size of 58 fish.

3.3.2. Experimental Group Formation

To accurately measure SMR in fish, it is necessary for individuals to be thermally acclimated, in a post-absorptive state and as inactive as possible (except for minimal movement to remain stationary within the water if necessary) (Chabot et al., 2016). Though respirometry is often used, ventilation rate has previously been shown to correlate closely with metabolic rate (oxygen consumption) in juvenile salmon (Millidine et al., 2008); this method minimises disturbance and was therefore used to distinguish between fish with a ‘low’ or ‘high’ SMR. Following 2 weeks of acclimation, 2 experimental groups (‘high’ SMR and ‘low’ SMR) were formed as follows. First, the SMR of each individual was estimated by recording its opercular ventilation rate (VR). This approach allowed metabolic rates to be estimated at a time when the fish, having been starved for 48 hours, were *in situ* within their individual tanks and so were undisturbed. VR was recorded for 20s at a time but expressed as beats.min^{-1} in analyses. This procedure was repeated 3 times for each fish, with each measurement being taken an hour apart, and the mean of the 3 VR measurements then calculated. All measurements were conducted during the light period on the same day, and except for the observers, the aquarium remained undisturbed throughout the day. In order for VR measurements to give an accurate indication of SMR, it was important for fish to remain undisturbed and inactive. During observation, fish were resting on the substrate as the water flow throughout the stream compartments was sufficient to ensure water turnover, but slow enough so that individual fish did not need to swim actively to maintain their position. Following

the acclimation period and implementation of the feeding regime, the fish were habituated to observation and did not react to the presence of the observer. Individual VR measurements of <30 beats.min^{-1} were not included when calculating a fish's mean VR, as such a low rate (analogous to bradycardia) indicates a brief stress response and therefore does not represent the true standard metabolic rate of an individual; the range of the accepted mean VR measurements was from 34 – 101 beats.min^{-1}.

In order to correct SMR for body mass, it was necessary to measure fish mass. Therefore, immediately following their final VR measurement, each individual was anaesthetised using benzocaine solution, weighed to the nearest 0.1g and measured (fork length) to the nearest 0.1mm. During the measurement process, 3 individuals were discovered to have a fungal infection, so were removed from the study. The mean mass of the remaining 55 individuals was 13.9 ± 3.9g (range 7.1 – 27.8g) and their mean length was 107.1mm±10.5mm (range 85.7 – 136.7mm).

Regression equations from Millidine, Metcalfe & Armstrong (2008) were then used to relate VR to SMR for the remaining 55 individuals, with knowledge of fish weight (M, in g) and water temperature (T, in ˚C):

$$SMR = m(VR) + c \quad (2)$$

where

$$m = 0.2773 - [0.2350 \times \log_{10}(M)] - [0.01838\ T] + [0.05813\ T \times \log_{10}(M)]] / 9 \quad (3)$$

and

$$c = -3.4078 + 0.2958\ T + [2.1956 \times \log_{10}(M)] - [0.82057\ T \times \log_{10}(M)] + 0.5335\ M \quad (4)$$

and VR is expressed as beats.min^{-1}. Note that the value for *m* includes a correction for an error in the published equation.

If an individual had not been feeding or producing faeces over the acclimation period, they were not considered for further analysis, regardless of their SMR measurement. The estimated SMR values (mg O_2.hr^{-1}) of the remaining fish (n=55) needed to be corrected for their body mass, since body mass can influence metabolic rate and growth rate (Auer et al., 2015c). SMR (mg O_2.hr^{-1}) was therefore plotted against mass (M, g) and the resulting regression (SMR = 0.3544M – 2.4856,

R^2 = 0.70) was used to calculate the expected SMR for each individual, given its mass. This 'expected SMR' value was subtracted from the actual SMR (as calculated from VR), to give the relative SMR (rSMR). A positive residual indicates the fish had a higher SMR than expected from its mass, whilst negative residuals indicate a lower SMR than expected from mass. The fish were then ranked based on their rSMR and the 15 individuals with the highest and lowest rSMR values were chosen for further study. This resulted in two groups (n=15 per group) consisting of individuals with distinct metabolic phenotypes; one with high SMR and one with low SMR.

3.3.3. Feeding Regime and Growth Measurements

In order for growth efficiency to be calculated, it was necessary to know the exact amount of food pellets (EWOS Ltd, Bathgate, UK) each fish consumed over the 2-week experimental period. Each fish was fed an 'intermediate' ration, which represented an amount smaller than if they were fed *ad libitum*, but that would be expected to be eaten in full. Unique rations were calculated for each fish using Equation 1 above, except that the value for an individual fish's wet mass used in Equation 1 was in this instance estimated from the equation linking fish fork length L (mm) to mass M (g) for the experimental population of fish: $M = 2.956x10^{-5}(L)^{2.789}$. This gave a predicted mass for each fish, based on its length.

The reason for using the predicted mass rather than its measured mass was that an aim of Equation 1 is to correct ration size for fish size, and using length gave a more accurate representation of the fish's size, uninfluenced by its current body condition. The energy value of the daily ration ($MJ.day^{-1}$) derived from Equation 1 was used together with the energy content of the feed (19.53 $MJ.kg^{-1}$) to determine the mg of feed to be fed per day to each fish. Each individual was fed its daily ration in one meal. Prior to feeding, it was noted whether the previous day's full ration had been consumed and if not, any remaining food was removed by siphoning. Knowledge of food consumption, fish body mass, body length and fish energy content was necessary for subsequent fish growth efficiency calculations.

To calculate the changes in energy content of the fish during the experiment, the initial energy density (kJ) of each individual was estimated using equations derived from Elliott (1976a):

$$E_i = (4608L^{-0.962}M_i^{0.391}) \times 0.004184 \quad (5)$$

Where E_i is the initial energy density ($kJ.g^{-1}$, wet mass), L is fork length (cm) and M_i is the initial wet mass (g); the final term (0.004184) converts the calories in the original equation to kJ. The value given by Equation 5 was multiplied by the wet mass of the fish to give an estimate of its total energy content (G_i kJ) at the beginning of the experiment.

At the end of the experimental period, the final energy density (E_f, $kJ.g^{-1}$) of each fish was estimated using a further equation from Elliott (1976a):

$$E_f = (7303 - 77.9W) \times 0.004184 \quad (6)$$

Where W is the percentage water content.

The % water content of each individual was determined as follows. Firstly, upon termination of the experiment, fish were culled via benzocaine overdose followed by severing the spinal cord. The wet mass (to nearest 0.01g) and fork length (to nearest 0.1mm) of each individual was recorded; mean mass and length were 15.43 ± 4.82g and 113.9 ± 11.3mm respectively. The entire length of the gut, from the stomach to the anus, was removed. Following dissection, the pyloric caecae and the hind gut were separated into labelled cryotubes, and the stomach was disposed of. A total of 60 samples, 2 from each individual, were collected and stored in liquid nitrogen for subsequent analysis. To account for the missing gut in water content calculations, final wet mass M_f was recorded to 0.01g (mean 14.38±4.34g; range 7.93 – 24.47g) following removal of tissue samples. Each individual carcass was then partitioned into 3, before being placed in a drying oven at 60˚C. After approximately 70 hours, the fish carcasses were removed, and an individual's dry mass was recorded to 0.01g (mean 3.73 ± 1.20g; range 1.93 – 6.19g). This information was used to determine their % water content (mean 73.65 ± 2.26%; range 67.09 – 75.61%). Following calculation of percentage water content, one individual (from the high metabolic rate group) was dropped from water content analyses due to clearly incorrect original inputting of data. In addition to being used to calculate the growth

efficiency of each fish, the % water content was also used to indicate 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 (r = -0.98; since fat has a decreased volume of water than muscle, as protein binds water whereas fat does not) (Elliott, 1976a).

The wet weight energy density ($kJ.g^{-1}$) could then be calculated using Equation 6 above. This figure was multiplied by an individual's mass in order to calculate the total final energy content of each fish (G_f kJ). Energy gained by the fish during the experiment (E_{gain} kJ) was calculated by subtracting G_i from G_f. This figure was scaled using equations by Elliott and Hurley (2000) to give the energy gained by a fish of standardised size of 10g (E_{corr}, kJ) in order to make the data comparable for fish of different size,

$$E_{corr} = E_{gain} \times 10^{0.766} / ((M_i + M_f)/2)^{0.766} \quad (7)$$

Finally, this figure was divided by energy consumed by each individual (also standardised to that of a 10g fish using a variant of equation 7 with energy consumed replacing E_{gain}) in order to give growth efficiency (which hypothetically ranged from 0 to 1.0, where 1.0 would correspond to a fish that converted all of its ingested energy into new energy content). For growth efficiency analyses, only data collected from fish that consumed their full ration each day for over 90% of the experimental period were considered, since these offered the most accurate values for total energy consumed; therefore, growth efficiency was collected for 18 fish in total (10 from the high SMR and 8 from the low SMR group).

3.3.4. Environmental and Atlantic Salmon Parr Samples: Collection and Processing

3.3.4.1. *Atlantic salmon faeces for bomb calorimetry*

Faecal samples for bomb calorimetry were collected so that the energy content of faeces of each fish could be determined, and hence (by subtraction) the energy that it had gained from the feed. To accumulate enough faecal material for analysis, samples were collected over 10 days and pooled for each individual. On each of the 10 days, all faecal material that had been produced over the preceding 24 hours was

removed from an individual's tank using a siphon, provided that the fish had eaten its full ration on the previous day, since only then could its energy intake be quantified. Samples were stored in 1L containers at -20˚C and added to daily.

At the end of this period, the faecal samples and residual water were defrosted, transferred into 50ml centrifuge tubes and centrifuged at 1000rpm for 5 minutes. Excess water was drained, and the process repeated until the entire sample had been centrifuged and a single pellet per individual (representing 1-10 days of sample collection) had been produced. These samples were stored at -20˚C for subsequent analysis.

The energy content of an individual's faeces was determined by bomb calorimetry, which gave the energy content ($kJ.g^{-1}$) of faecal material collected. The energy content of the food pellets was $19.53kJ.g^{-1}$ (value supplied by manufacturer). By taking into account the mass of food consumed and quantifying the remaining energy in faeces, it was possible to determine the nutritional energy each individual was gaining from their feed. These data were collected for 23 out of the 30 fish within the study (the masses of faecal material from the remaining 7 individuals being too low for successful bomb calorimeter analyses).

The daily relative energy retained (DRER) (kJ) of each individual was defined as:

DRER = daily energy in (kJ) – daily energy out (kJ) (8)

where daily energy in = caloric content of ration ($kJ.g^{-1}$) x mass of ration (g)

and daily energy out = (energy in faeces ($kj.g^{-1}$) x faecal pellet mass (g)) / number of sampling days

The term 'relative' is used because the absolute energy value an individual retained from its food each day cannot be calculated as the 'daily energy out' value was derived from the bomb calorimetry result ($kJ.g^{-1}$) multiplied by the mass of the faecal pellet (g), but the pellet would have contained some water. The dry mass of the faeces was unknown, so 'daily relative energy retained' was chosen on the assumption that all faecal samples would have had an equivalent water content.

3.3.4.2. *Atlantic salmon faeces for microbial load analysis*

To determine microbial load, further faecal samples were collected in order to perform DNA extraction and qPCR analysis. These samples were collected on days other than when faeces were collected for DRER. All faeces produced were collected from each tank via a pipette on 2 separate days providing duplicate samples for each individual. The samples were stored in 15ml centrifuge tubes and centrifuged at 4500rpm for 5 minutes, after which excess water was drained. The resulting pellets were stored at -80˚C for subsequent analysis.

For DNA extraction from faecal samples, the QIAamp DNA Stool Mini Kit (Qiagen) was used according to the manufacturer's protocol, with the following modifications: buffer ASL was added directly to the frozen faecal samples within their 15ml centrifuge tubes. The volume of buffer ASL added to each sample was relative to the weight of the faecal sample and tubes were vortexed thoroughly prior to the incubation step. Lysis temperature was raised to 90˚C for an extended period of 30 minutes to increase the break-down of bacteria that are difficult to lyse. Following the remainder of the manufacturer's protocol, DNA was quantified by NanoDrop spectrometry.

Bacterial load in faeces was assessed using qPCR, which measures fluorescence in relation to presence of target DNA. The output from qPCR analysis was the C_T value, the cycle at which amplification of the target DNA causes the fluorescence to surpass a threshold; it is proportional to the log of the number of bacteria in the sample, so indicates the sample's microbial load (Nadkarni et al., 2002). In order to create a standard curve for qPCR, competent *E. coli* taken from a StrataClone PCR Cloning Kit (Agilent Technologies) were grown overnight in Luria-Burtani (LB) broth at 37˚C in an incubator. 2 cultures were prepared from this inoculum: LBB1 (30ml LBB + 1ml inoculum) and LBB5 (30ml LBB + 5ml inoculum). Optical density measurements (absorbance, nm) of the cultures were taken at various timepoints over the following 5 hours in order to gauge growth rate of *E. coli* from different starting concentrations. Viable cell counts of the cultures were determined by plating a series of dilutions (10^0 – 10^{-11}; made with PBS) of each culture on LB agar plates. Each dilution was plated in duplicate, with each plate containing between 6-8 20ul drops of culture. All plates were then incubated at 37˚C for 12h, following which it

was possible to count the colonies (CFU: colony forming units) from the 10^{-5} dilution plate.

DNA was isolated from the *E. coli* culture using the QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's protocol, with the alteration of disregarding the inhibit EX tablet step. The extracted DNA was quantified with a Qubit fluorometer. The CFU information was combined with the DNA concentration to ascertain that the inoculum formed 117, 897.7 CFUs.ng^{-1} of DNA.

Serial dilutions of the extracted *E. coli* DNA (1, ½, ¼, 1/8 and 1/16 in triplicate) were quantified using real-time PCR in order to create a standard curve for determining bacterial number (standard curves shown in Appendix 3-1). This curve allowed quantification of the faecal samples collected from the experimental fish. 30 faecal samples were quantified from the first sampling session and 27 samples were quantified from the second sampling session, as 3 individuals did not produce faeces on the 2nd occasion (amplification curves shown in Appendix 3-2). CFU data were log transformed prior to analyses to normalise the distributions and an average value was taken from the duplicate data for each individual.

The primers used in the qPCR reactions were U16SRT-F, 5' ACTCCTACGGGAGGCAGCAGT 3' and U16SRT-R, 5' TATTACCGCGGCTGCTGGC 3'. This primer set, taken from Clifford et al. (2012), were designed to amplify products from bacterial 16S rRNA genes without the need for a probe, by aligning >960,000 bacterial *16S* rRNA sequences. Amplification and detection of DNA by real-time PCR were performed with the MX3000P qPCR System (Agilent Technologies). All samples were run in duplicate for the determination of DNA by real-time PCR. The reaction was performed using a total volume of 20µl: 10µl SensiMix™ SYBR No-ROX Master Mix (Bioline), 1µl of each the forward and reverse primers, 3µl water and 5µl of template DNA per well. The reaction conditions were as follows: 95˚C for 10:00; 40 cycles of 95˚C for 0:15, 58˚C for 0:15 and 72˚C for 0:15; followed by a single cycle of 95˚C for 1:00, 58˚C for 0:30 and 95˚C for 0:30. For subsequent data analysis, the MxPro qPCR software was used.

3.3.4.3. *Environmental samples*

Throughout the experimental period, environmental samples were collected every 4 days, in order to assess and control for the background microbial diversity in the tank environment in which the fish were living. Biofilm samples were taken by swabbing the inside of 2 randomly selected stream tank compartments and water samples were taken by passing 1L of water through a filter (Minisart single use filter, 16534-K, CE 0120) using a peristaltic pump. Each filter paper was manually removed from the filter and immediately placed into a cryotube (Cryo-Vial Int Thd FS, Ref:LW3534) before being stored in liquid nitrogen for subsequent analysis. These environmental samples were taken in triplicate and processed (DNA extraction, PCR and sequencing) alongside tissue samples.

3.3.4.4.*Environmental and Atlantic salmon gastrointestinal tissue samples: DNA extraction and PCR*

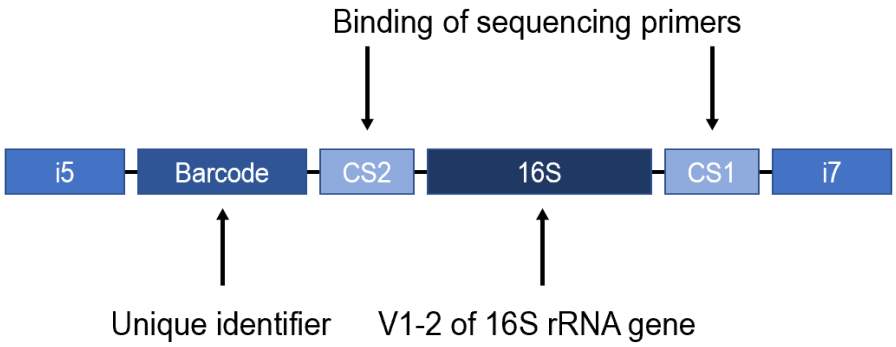

Figure 3-1: The construct of the product generated after primary and second round PCR. *Amplification of variable region (V) 1-2 of the 16S rRNA gene was achieved, alongside addition of CS1 and CS2 tags in primary PCR. A DNA barcode for identification and Illumina index sequences i5 and i7 were added during second round PCR. This product was then sequenced.*

For DNA extraction from tissue and environmental samples, the QIAamp DNA Stool Mini Kit (Qiagen) was used according to the protocol described above, with the following differences: buffer ASL was added directly to the frozen samples and this mixture was then transferred to a 2.0ml microcentrifuge tube (Thermo-scientific #3469-11) containing a ¼" ceramic bead and lysing matrix A garnet (MP Biomedicals). Tubes were homogenised using a fast prep machine: speed 4 for 4

rounds of 25 seconds prior to the lysis step. Following the remainder of the manufacturer's protocol, DNA concentration was quantified by NanoDrop spectrometry.

For primary PCR reactions, variable region 1-2 of the 16S rRNA gene was targeted with the primer pair CS1_27F and CS2_338R, adapted from those used to previously examine the gut microbiota of Atlantic salmon (Gajardo et al., 2016). The forward primer had the sequence 5' **ACA CTG ACG ACA TGG TTC TAC A**AG AGT TTG ATC MTG GCT CAG 3', and the reverse primer had the sequence 5' **TAC GGT AGC AGA GAC TTG GTC T**GC TGC CTC CCG TAG GAG T 3'. These primers were tagged CS1 for the forward sequence and CS2 for reverse (as shown in bold within the sequences) (Figure 3-1). In order to avoid amplification biases, primary PCR reactions were performed in triplicate and pooled after amplification. 30µl reactions were used, consisting of 1.5µl of each forward and reverse primer (10µM), 15µl of Q5 Hot Start High-Fidelity 2X Master Mix (New England BioLabs Inc.) and 2µl of DNA template. PCR conditions were as follows: initial denaturation of 95˚C for 10 minutes; 30 cycles of 0:30 at 95˚C, 0:30 at 55˚C and 0:30 at 72˚C; followed by a final extension step for 10 minutes at 72˚C. The PCR product was verified on a 1.7% agarose gel using TBE buffer.

These PCR products were then used as templates for 2nd round PCR, in which DNA barcodes were attached in order for subsequent sequencing to be performed and latterly demultiplexed for each sample. In this instance, a universal forward primer PE1_CS1_Fwr with the sequence 5' AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT GAC GAC ATG GTT CTA 3' was used alongside a barcoded reverse primer 5' CAA GCA GAA GAC GGC ATA CGA GAT XXX XXX XXT ACG GTA GCA GAG ACT TGG TCT 3', which was specific to each sample. A map of the final sequencing construct is shown in Figure 3-1. Reaction volumes were 25µl and contained 12.5µl Q5 Hot Start High-Fidelity 2X Master Mix (New England BioLabs Inc.), 1µl forward primer, 1µl barcode and 8µl of DNA template. PCR conditions were as follows: initial denaturation of 95˚C for 10 minutes; 8 cycles of 0:10 at 95˚C, 0:30 at 60˚C and 1:00 at 72˚C; followed by a final extension step for 3 minutes at 72˚C. The barcoded PCR products were verified on a 1.7% agarose gel using TBE buffer.

Upon visualisation, if a product produced >1 band, it was manually excised from the gel using a scalpel and purified using PureLink Quick Gel Extraction Kit (Invitrogen). If only the single target band was produced, the remaining product was purified using the Agencourt paramagnetic bead clean-up (Agencourt AMPure XP, Beckman Coulter) with a modified 0.8:1 volume of AMPure XP beads to PCR product. Following these protocols, the concentration (ug/ml) of each product was determined with a Qubit fluorometer. The amplicons were then pooled in equal concentration and the final library was sequenced using the Illumina Mi Seq® NGS system at Glasgow Polyomics, University of Glasgow.

3.3.5. Data Analyses

3.3.5.1. *Statistical analyses*

All statistical analyses were performed in R.3.5.1 (R Development Core Team) using moments (Komsta and Novomestky, 2015), rms (Harrell Jr, 2019) and e1071 (Meyer et al., 2019) packages for model diagnostics. Prior to analysis via models, all continuous variables were mean-centred and scaled to reduce the risk of multicollinearity, tested for via the rms package in R (Harrell Jr, 2019). Generalised Linear Models (GLMs) and Linear Models (LMs) were used to explore potential relationships between microbial load, metabolism, growth efficiency and nutritional energy harvest.

Due to the lower number of Atlantic salmon for which growth efficiency was calculated (n=18 out of the full sample size of 30 fish), parallel models were run that included/excluded growth efficiency as an explanatory variable, to check for the effects of growth efficiency whilst retaining statistical power when growth efficiency was found not to be important. Non-significant terms were removed, and final models were chosen based on AICc and visual inspection of residual plots (scale-location, Cook’s distance, q-q plots). Significance testing was used to indicate the strength of observed relationships.

When creating graphs to represent the significant relationships found via the linear models with more than one explanatory variable, the residual value of the response variable was plotted instead of the raw data. This allowed the focal relationship to be represented accurately, by controlling for other covariates within the original model.

To achieve this, residual values were calculated by removing the explanatory variable of interest from the original model in order to gain expected measures of the response variable based on the remaining covariates. These expected values were then subtracted from the actual values to give the residual values, which could be plotted against the response variable of interest. Further details are given throughout the results section wherever this method was deployed.

3.3.5.2. *Bioinformatic analyses*

Quality curves of the sequencing data showed that reverse reads were of higher quality than forward in Miseq Illumina paired-end sequencing. Therefore, reverse reads were trimmed and filtered using sickle (Joshi and Fass, 2011) with an average quality threshold above a Phred score of 30. Filtered sequences were decontaminated against the *S. salar* genome using DeconSeq (Schmieder and Edwards, 2011). Then sequences were treated to remove chimeras and afterwards clustered at a similarity threshold of 97% using VSEARCH (Rognes et al., 2016). Firstly de-novo sequence clustering was performed with VSEARCH and then operational taxonomic units (OTUs) were taxonomically classified against the SILVA database (Quast et al., 2013) and annotated using the QIIME2 classifiers (Bolyen et al., 2019). OTUs were processed for multiple sequence alignment using MAFFT (Katoh and Standley, 2013) and an OTU tree was built using the software FASTTREE (Price et al., 2009) to assist with the calculation of beta diversity metrics (e.g. generalised UNIFRAC).

For downstream analysis of sequencing data, samples were separated by section of the gut from which they were taken (foregut or hindgut) and the two gut sections were analysed separately. In total, there were 27 foregut samples (13 from low SMR fish and 14 from high SMR) and 27 hindgut (14 from low SMR fish and 13 from high SMR) that yielded sufficient sequencing depth for downstream analysis; not all samples could be analysed due to low quality of the sequencing data.

To assess the diversity of OTUs within each sample, alpha diversity was evaluated in terms of both species richness and the Shannon effective number of species counts. The Shannon effective counts represent the Shannon diversity index as true alpha diversity of OTUs, as first proposed by Lu Jost (2006). Briefly, the effective

number of species is the number of equally-common species that will give a particular value of the Shannon-Wiener diversity index. Use of this “effective” number allows easier comparison and interpretation since, unlike the raw Shannon-Wiener index, the converted numbers ensure homogeneous properties (Jost, 2006). Linear Models (LMs) were used to explore relationships between microbial alpha diversity traits and metabolic rate (rSMR), percentage water content, fish mass, fish length, log average microbial load and experimental group. In these models, microbial species richness or Shannon effective was used as the response variable, and experimental group (categorical variable with two levels: low or high rSMR), fish mass, percentage water content, DRER, rSMR and average microbial load as explanatory variables. LMs were also used to examine the effect of each alpha diversity metric on growth efficiency, where growth efficiency was included as the response variable and microbial richness, Shannon effective, experimental group, fish mass, percentage water content, DRER, rSMR and average microbial load were included as explanatory variables. For all LMs, non-significant terms were removed, and final models were chosen based on AICc (using the MuMin package in R (Barton, 2019)) and visual inspection of residual plots (scale-location, Cook’s distance, q-q plots). Significance testing was used to indicate the strength of observed relationships.

As when forming the graphs showing the other significant statistical relationships, when visualising the alpha diversity data, the residual value of the response variable was plotted instead of the raw data. This again allowed accurate presentation of the data when the original model included multiple explanatory variables.

To assess similarity between different microbial profiles, beta diversity was assessed using generalised UniFrac. This method offers a balance between weighted and unweighted UniFrac, which assign too much weight to rare or abundant lineages when used in isolation (Chen et al., 2012). Visualisation of beta-diversity was performed via unconstrained non-metric Multi-Dimensional Scaling (NMDS), in which separation of the microbial communities was assessed via PERMANOVA. Beta diversity analyses including environmental samples can be seen in Appendix 3-3. Both alpha and beta diversity analyses were performed in R using the Rhea package (Lagkouvardos et al., 2017).

To gain an overview of the genera present within each sample, taxonomic binning was performed using the SILVA database (Quast et al., 2013) as a reference. Stacked bar plots were formed to show the taxonomic composition and relative abundance across the foregut and hindgut samples, with the threshold abundance of OTUs set to 0.25 or 0.5 to allow for better visualisation within the plots (Appendix 3-4 and 3-5). To find microbial genera that were significantly different in their relative abundance between fish from each metabolic rate group, the DESeqDataSetFromMatrix function from DESeq2 package was used (Love et al., 2014), with the adjusted p-value cut-off of 0.005 and log2fold chance cut-off of 2. This function uses negative binomial GLM to obtain maximum likelihood estimates for OTUs log fold change between the two experimental groups. Then Bayesian shrinkage was applied to obtain shrunken log fold changes before the Wald test was used for obtaining significance in each pairwise comparison. Log2fold was chosen in order to better visualise the data (Ijaz et al., 2018) and the Cox-Reid adjusted profile likelihood correction was used (Cox and Reid, 1987).

To illustrate how different explanatory variables contributed to any variation in microbial communities, distance-based redundancy analysis (dbRDA) was used. dbRDA is a constrained (canonical) ordination analysis, which only analyses the variation in the microbial communities that can be explained by the environmental variables. By using the generalised UniFrac distance-based matrix, dbRDA could take into account the phylogenetic makeup of the microbial communities (Shankar et al., 2017). Redundancy analysis with forward selection was performed to specifically select the environmental variables that explained variation within the microbial communities (Vass et al., 2020). Once the forward selection was carried out with the ordistep function in the vegan package of R (Oksanen et al., 2019), dbRDA was applied on the significant variables using the capscale command (also in the vegan package of R). These processes were carried out separately for the foregut and hindgut bioinformatic data. Statistical analyses of the dbRDA data were then performed using the adonis2 function within the vegan package, as this uses PERMANOVA which can test for similarity among samples based on the chosen distance measure of generalised UniFrac. To complement the dbRDA analysis, Pearson correlation coefficients were calculated across the metavariables (fish mass, fish length, percentage water content, rSMR and average microbial load) to

assess any correlations between these and the OTUs. The Pearson correlation for all pairs was calculated and a false discovery rate (FDR) correction was applied to correct for type I errors. Correlation analyses were performed in R using the Rhea package (Lagkouvardos et al., 2017), using a p-value significance level of 0.05. To avoid underpowered analysis, OTUs that were present in <30% of the samples were removed and the minimum number of pairs necessary to support a correlation was set to 4. The resulting correlation analyses were visualised in a graphical display to showcase whether metabolic rate correlated with the presence of any OTUs.

3.4. Results

3.4.1. Experimental Groups: Metabolic Rate and Morphological Data

In order to understand whether metabolic rate is influenced by the gut microbiota in Atlantic salmon and whether metabolic rate or microbial community composition in the gut impacted growth efficiency, morphological properties such as mass and percentage water content were analysed in combination with the metabolic rate data and the sequence data. Following measurement of the ventilation rate (VR) and calculation of rSMR for all individuals (n = 55), two experimental groups with divergent metabolic phenotypes were formed from the 15 fish with the highest and the lowest SMR relative to their body mass (rSMR; Table 3-1). rSMR was used in order to account for any effect of mass; however, an unpaired two-sample t-test confirmed there was no significant difference in mass between the fish in the 'low' and 'high' metabolic rate groups ($t_{28} = 1.05$, $p = 0.30$).

Table 3-1: The metabolic and weight data of the 'high' and 'low' SMR groups as quantified by ventilation rate of each individual. SMR (mg O2.hr^{-1}) was predicted from ventilation rate (VR) of each fish, before being converted to residual SMR (rSMR) which corrects for fish mass (see text for details). rSMR values were used to form the two experimental groups.

	High SMR Group	Low SMR Group
Average SMR (mg O_2.hr^{-1})	3.43	1.57
Average rSMR	0.54	-0.70
rSMR Range	-0.15 to 2.65	- 1.35 to -0.38
Average Mass (g)	15.16	13.43
Mass Range (g)	8.4 to 27.8	8.2 to 19.2
Mass SD	4.97	3.66

3.4.2. Microbial Load

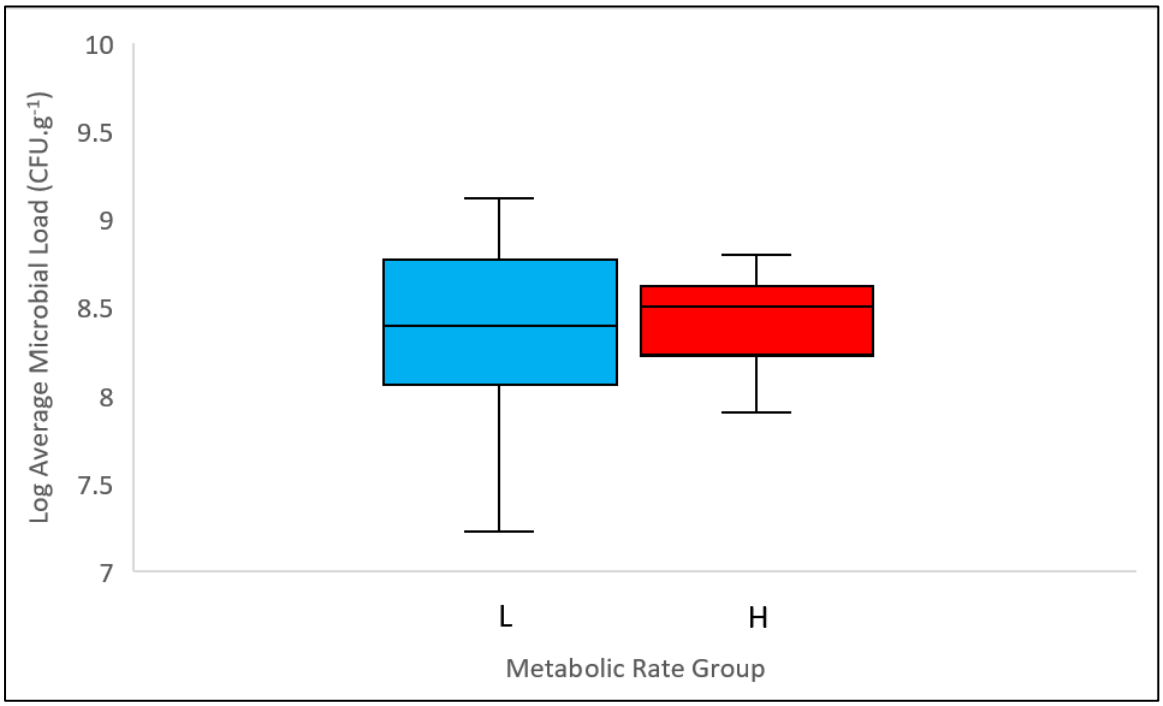

Figure 3-2: The log-transformed average microbial load (cfu.g^{-1}) in the faeces of the two metabolic rate groups of juvenile Atlantic salmon *('L': low; 'H': high relative SMR).*

There was no significant difference in log-adjusted average microbial load between the low and high rSMR groups according to a Welch's t-test ($t_{23.6}$ = 0.18, P = 0.86; Figure 3-2). A linear model was used to examine the effect of individual physiology on microbial load, with log-transformed average microbial load as the response variable, and DRER, percentage water content, rSMR and fish mass as explanatory variables. None of the considered variables were found to explain variation in the log-adjusted average microbial load (LM, all P > 0.23).

3.4.3. Nutritional Energy Harvest

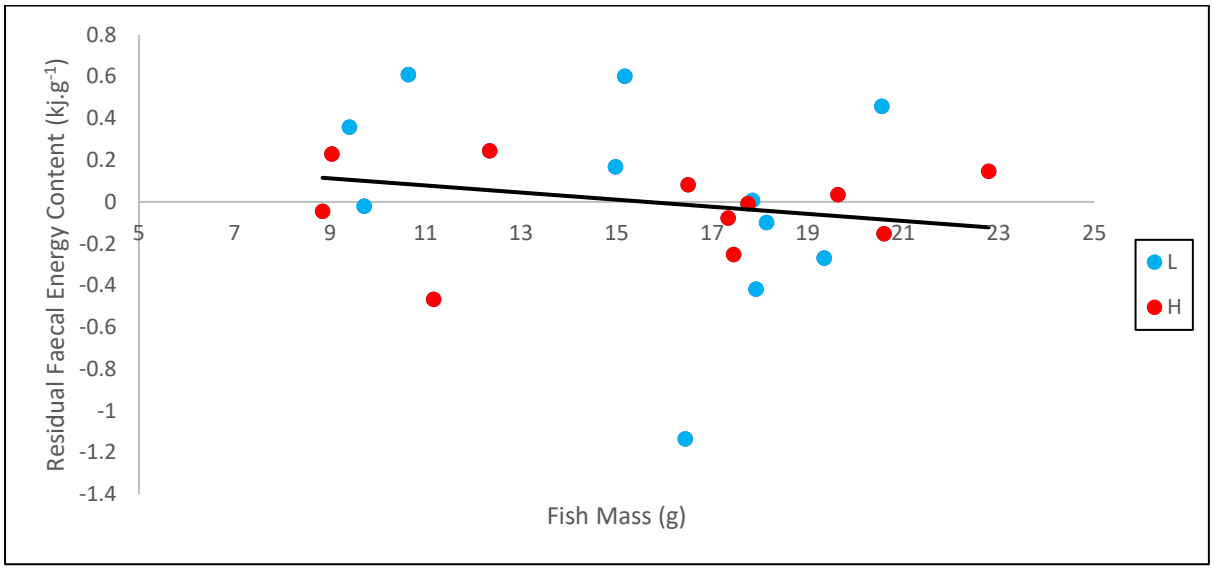

Figure 3-3: The relationship between residual energy content in a juvenile Atlantic salmon's faeces (kJ.g^{-1}) as quantified by bomb calorimetry and its body mass (g) (n = 22 in total). *Blue and red points represent individuals from the low and high relative standard metabolic rate groups, respectively. Note that the statistical analyses were based on the (absolute) energy content of the faeces, but here the residual values after controlling for DRER, log-transformed average microbial load, percentage water content and rSMR are plotted, in order to illustrate the relationship with fish mass (g). See text for analysis.*

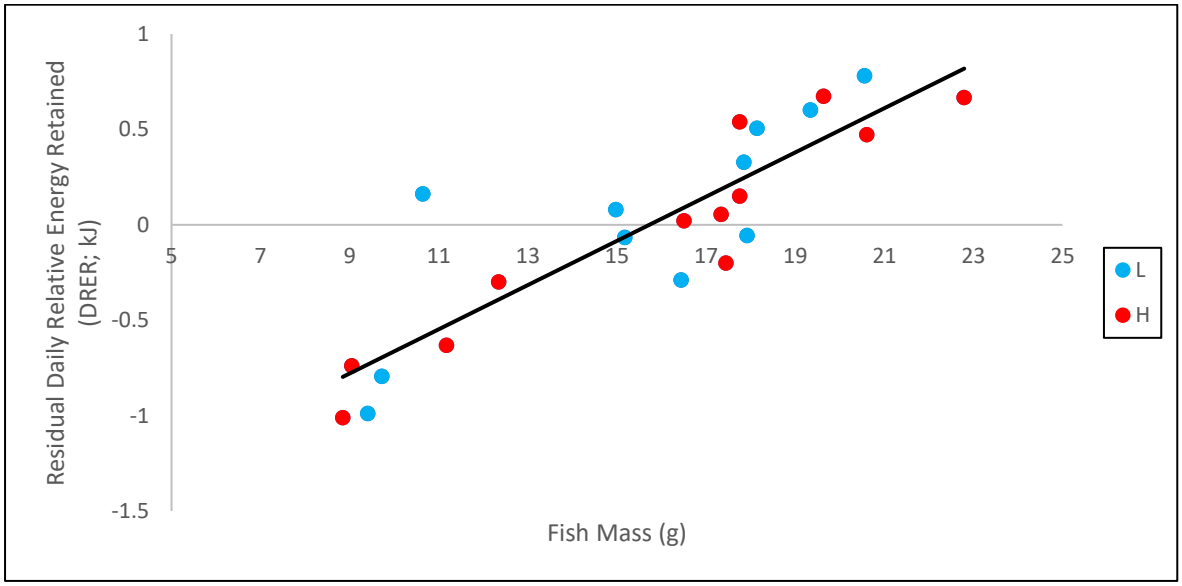

Figure 3-4: The relationship between residual daily relative energy retained (DRER; kJ) and mass (g) of juvenile Atlantic salmon (n = 23 in total). *Blue and red points represent individuals from the low and high relative standard metabolic rate groups respectively. Note that the statistical analyses were based on the (absolute) DRER, but here the residual values after controlling for energy*

consumed by the fish (kJ) are plotted, in order to illustrate the relationship with the fish's body mass (g) See text for analysis.

The energy in the faeces was assessed by including mass, DRER, percentage water content, log-transformed average microbial load and rSMR as explanatory variables. Faecal energy content ($kJ.g^{-1}$) was only found to be impacted by the fish's mass (Figure 3-3; LM, $F_{5, 16} = 3.66$, $P = 0.0105$), where the faeces of larger fish contained a decreased amount of energy per unit mass. To present the relationship between faecal energy content ($kJ.g^{-1}$) and fish mass (g) accurately, the other covariates within the original model had to be controlled for. To achieve this, residual energy content was calculated by first removing mass from the original model to gain a measure of expected faecal energy content (when accounting for DRER, log-transformed average microbial load, percentage water content and rSMR), where expected faecal energy content = ((-0.34309 * DRER) + (0.11825 * microbial load) + (-0.06416 * water) + (-0.18841 * rSMR) + 5.95731). This expected faecal energy content was then subtracted from the actual faecal energy content to give the residual value, which could be plotted against fish mass (Figure 3-3).

The variation in daily relative energy retained (DRER; kJ) was assessed in relation to fish mass and energy consumed (kJ; scaled to 10g fish). DRER was found to increase with fish mass (Figure 3-4; LM, $F_{2, 20} = 364.7$, $p<0.001$), as well as to the energy each fish consumed (kJ, scaled to 10g mass of fish) ($p<0.001$). To present the relationship between DRER and fish mass, the same approach as above was used to calculate residual DRER; first expected DRER was calculated as ((-0.0247 * scaled kJ consumed) + 4.1439). This expected DRER was subtracted from actual DRER to give the residual values, which could then be plotted against fish mass (Figure 3-4). Taken together, Figures 3-3 and 3-4 show that larger fish retained more energy in absolute terms, and also had faeces that were lower in energy.

3.4.4. Growth Efficiency

For growth efficiency analyses, only data collected from fish that consumed their full ration each day for over 90% of the experimental period were considered since these offered the most accurate values for total energy consumed. Fortunately, rSMR was found not to impact the proportion of days on which fish consumed their full ration (LM, $F_{1, 28} = 2.37$, $P = 0.14$), resulting in accurate growth efficiency calculations for roughly equal numbers of low and high relative standard metabolic rate fish (8 from the low rSMR group and 10 from the high rSMR group). Growth efficiency for the 18 individuals averaged 0.40 (± 0.20) but showed wide variation (range 0.06 – 0.87). Growth efficiency is defined here by the body energy gained by an individual during the experiment in relation to the energy it consumed; therefore, individuals that converted more of the energy that they consumed throughout the experimental period into growth had higher 'growth efficiency' (a similar concept to the 'feed conversion efficiency' used in aquaculture).

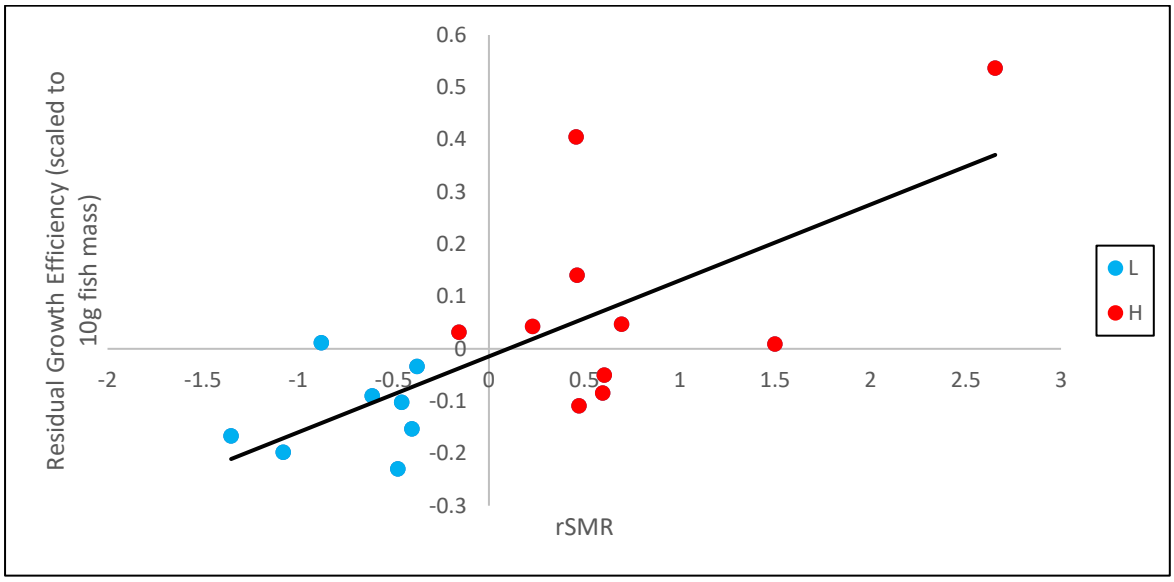

Figure 3-5: The relationship between residual growth efficiency and rSMR in juvenile Atlantic salmon, where residual growth efficiency is scaled to that of a 10g fish (n = 18 in total). *Individuals within the low and high relative standard metabolic rate groups are represented by blue and red points, respectively. Note that the statistical analyses were based on the (absolute) growth efficiency, but here the residual values after controlling for fish mass (g) are plotted, in order to illustrate the relationship with rSMR. See text for analysis.*

Growth efficiency (standardised to that of a 10g fish) was found to increase with rSMR (Figure 3-5; LM, $F_{2, 15} = 10.26$, P = 0.0011) and fish mass (p = 0.017). This meant that salmon with a higher rSMR or a higher mass showed a more efficient conversion of food energy into growth throughout the experimental period. The relationship between the total energy consumed over the experimental period (kJ, standardised to that of a 10g fish) and growth efficiency was not significant (LM, $F_{1, 16} = 0.57$, P = 0.46), nor was the relationships between rSMR and total energy consumed (LM, $F_{1, 28} = 0.63$, P = 0.44), so individuals with a higher rSMR were not simply consuming more food, but were more efficient at harvesting the energy within the food that they did consume. To present the relationship between growth efficiency and rSMR, the same approach as above was used, with expected scaled growth efficiency calculated as ((0.018 * fish mass) + 0.1138). This expected growth efficiency was subtracted from actual growth efficiency to give the residual values, which could then be plotted against the rSMR (Figure 3-5).

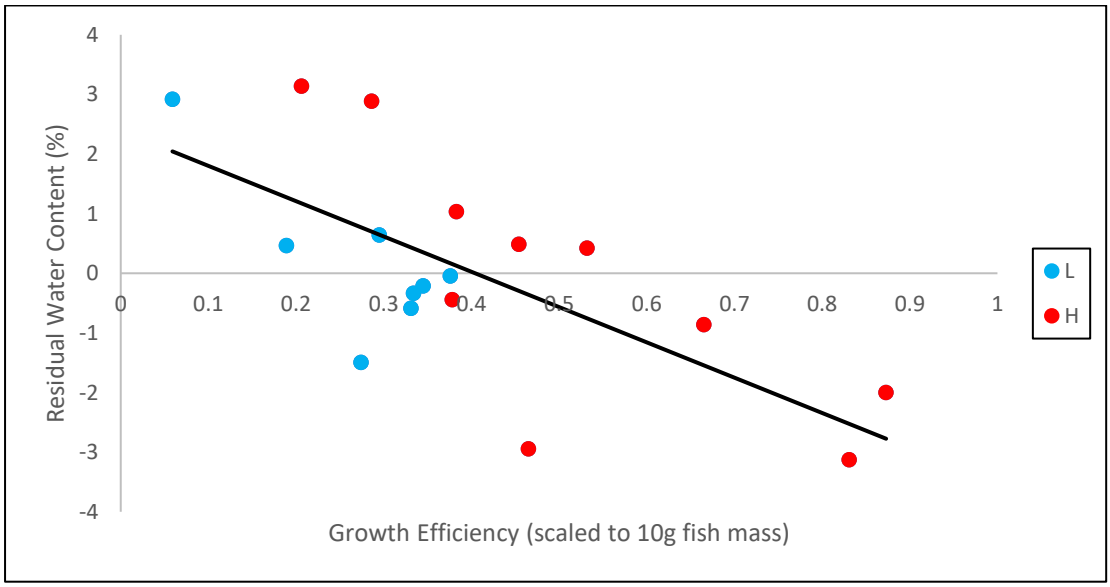

Figure 3-6: The relationship between residual percentage water content and growth efficiency (standardised to that of a 10g fish) in juvenile Atlantic salmon (n = 18 in total). *Fish with low and high relative standard metabolic rates are represented by blue and red points, respectively. Note that the statistical analyses were based on the (absolute) water content (%), but here the residual values after controlling for rSMR are plotted, in order to illustrate the relationship with the growth efficiency. See text for analysis.*

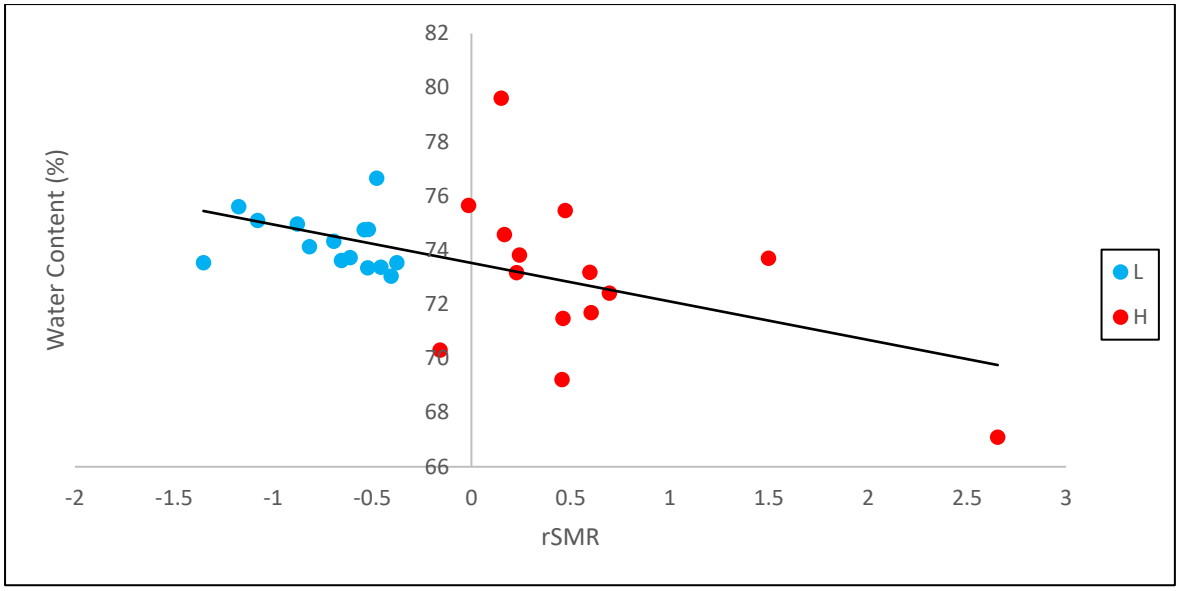

Figure 3-7: The relationship between water content (%) and rSMR in juvenile Atlantic salmon (n = 29 in total). *Fish with low and high relative standard metabolic rates are represented by blue and red points, respectively.*

The drivers of percentage water content of the juvenile Atlantic salmon were assessed by including growth efficiency (scaled to that of a 10g fish) and rSMR as explanatory variables. Water content (%) was negatively related to growth efficiency (Figure 3-6; LM, $F_{2, 15} = 44.94$, $P < 0.001$), indicating that individuals that converted more of their ingested energy into growth had a lower % water content. To present the relationship between water content (%) and growth efficiency (standardised to 10g fish mass) accurately, the expected percentage water content was first calculated as expected water content (%) = ((-1.48 * rSMR) + 73.021). This expected percentage water content was subtracted from actual percentage water content to give the residual values, which could then be plotted against the growth efficiency (standardised to 10g fish mass) (Figure 3-6).

For the subset of individuals for which growth efficiency data were collected (n = 18), there was no relationship between percentage water content and rSMR ($P = 0.859$); however, the relationship was significant when the data for all individuals were included (n = 29, Figure 3-7; LM, $F_{1, 27} = 9.94$, $P = 0.0039$), with fish with a higher relative standard metabolic rate having a lower final % water content. As a lower water content is indicative of higher fat content, this suggests that within this experiment, fish with a higher rSMR had higher fat levels.

3.4.5. 16S Sequence Data: Exploring the Drivers Behind Variation in Metabolic Rate of Atlantic Salmon Parr

Gut microbiota samples were isolated from homogenates derived from two separate gut compartments. The foregut and hindgut of 29 fish were sampled, producing a total of 58 samples. Following 16S rRNA extraction and amplification, 54 of these samples were successfully sequenced (27 foregut and 27 hindgut). These samples contained a total of 6678 operational taxonomic units (OTUs), of which 4928 could be assigned to at least Phylum level. Downstream analysis of sequencing data separating the foregut and the hindgut increased the accuracy of the overall analyses. The data were analysed to assess whether the gut microbiota of the Atlantic salmon differed between individuals of the low and high metabolic rate groups. Specifically, alpha diversity and beta diversity were assessed to examine the microbial diversity within the samples and the similarity of different microbial community compositions; differential abundance analysis was used to find any OTUs within the gastrointestinal tract of the fish that differed in abundance between the low and high metabolic rate groups; distance-based redundancy analysis assessed overall drivers of variation in microbial community composition; and correlation analysis was used to assess possible relationships between physiological metavariables and specific microbial OTUs whilst treating SMR as a linear variable.

3.4.5.1. *Microbial alpha diversity within the gut of Atlantic salmon parr*

3.4.5.1.1. Alpha diversity metrics within the Atlantic salmon foregut

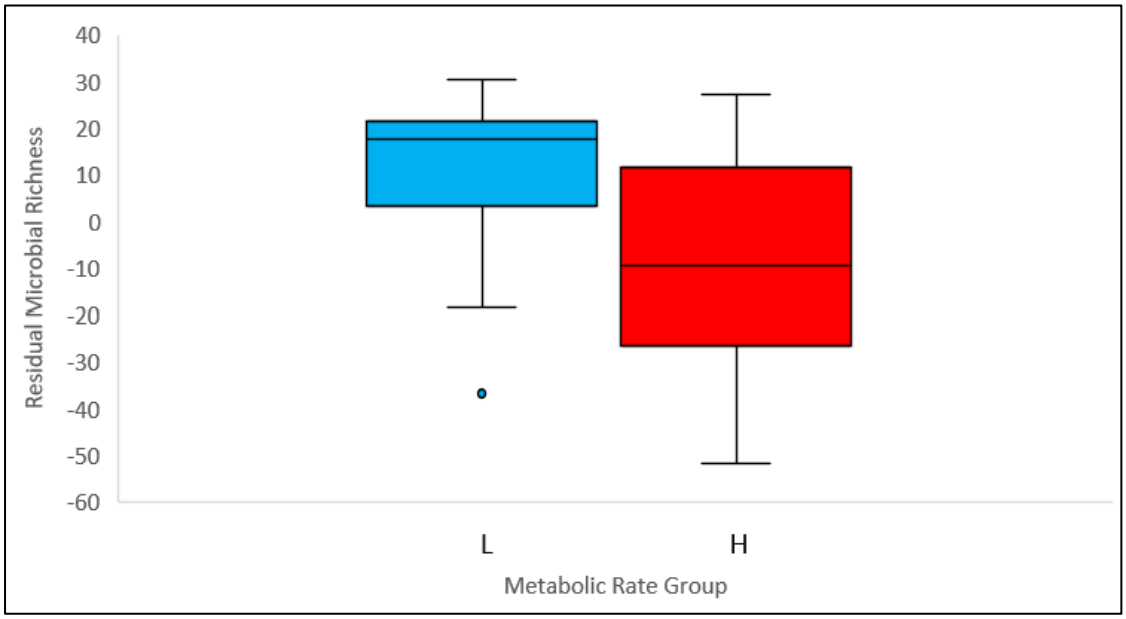

Figure 3-8: The difference in foregut microbial richness between Atlantic salmon with low and high metabolic rates (n = 27 in total). *Fish with low and high relative standard metabolic rates are represented by blue and red, respectively. Note that the statistical analyses were based on the (absolute) microbial richness, but here the residual values after controlling for microbial load are plotted, in order to illustrate the difference between the two experimental groups. See text for analysis.*

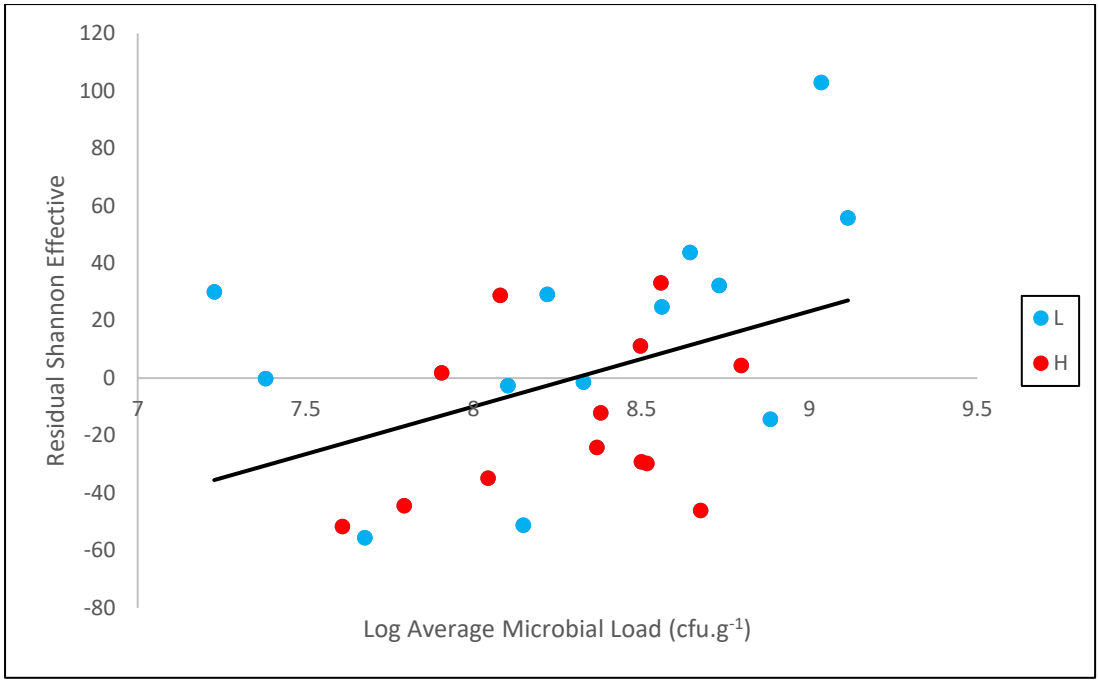

Figure 3-9: The relationship between the residual Shannon effective (a measure of alpha diversity) and log-transformed average microbial load (cfu.g^{-1}) in the foregut of juvenile Atlantic salmon with 'low' or 'high' metabolic rates (n = 26 in total). *Fish with low and high relative standard metabolic rates are represented by blue and red points, respectively. Note that the statistical analyses were based on the (absolute) Shannon effective, but here the residual values after controlling for water content (%) are plotted, in order to illustrate the relationship with the log-transformed microbial load (cfu.g^{-1}). See text for analysis.*

The alpha diversity metrics, microbial richness and Shannon effective, were used to assess the diversity of OTUs within the foregut samples of the juvenile Atlantic salmon. These metrics were chosen because microbial richness describes the number of OTUs present within a sample, whilst the Shannon effective also accounts for the differential abundance of those OTUs. The microbial richness within the foregut of the fish was assessed by including experimental group and the log-transformed average microbial load (cfu.g^{-1}) as explanatory variables. Microbial richness differed significantly between the low and high rSMR groups (Figure 3-8; LM, $F_{2, 24} = 5.05$, $P = 0.023$), with samples from Atlantic salmon with a lower rSMR having a higher microbial richness. There was a non-significant trend for microbial richness to be positively related to microbial load ($P = 0.056$), as shown in Table 3-2. To present the difference in microbial richness between the two experimental groups, the residual microbial richness was calculated by removing experimental group from the original model in order to gain a measure of expected microbial

richness (when accounting for the log-transformed average microbial load), where expected microbial richness = (7.689 * average microbial load) – 87.491). This expected microbial richness was subtracted from actual microbial richness to give the residual values, which could then be plotted for each metabolic rate group (Figure 3-8).

The Shannon effective diversity within the foregut of juvenile Atlantic salmon was assessed by including experimental group, log-transformed average microbial load (cfu.g^{-1}) and water content (%) as explanatory variables. The Shannon effective was significantly different between fish from the low and high rSMR groups (LM, $F_{3, 22}$ = 7.17, P = 0.019), where foregut samples from Atlantic salmon with a lower rSMR had an increased Shannon effective. The Shannon effective was also found to increase with the log-transformed average microbial load (Figure 3-9; P = 0.024), but there was no relationship between the Shannon effective and percentage water content (P = 0.16), as summarised in Table 3-2. The relationship between the Shannon effective and the log-transformed average microbial load for each experimental group was presented using residual values for the Shannon effective; first the expected Shannon effective (when accounting for percentage water content) was calculated as ((9.493 * percentage water content) – 636.03). This expected Shannon effective was then subtracted from actual Shannon effective to give the residual values, which were then plotted against the log-transformed average microbial load (Figure 3-9).

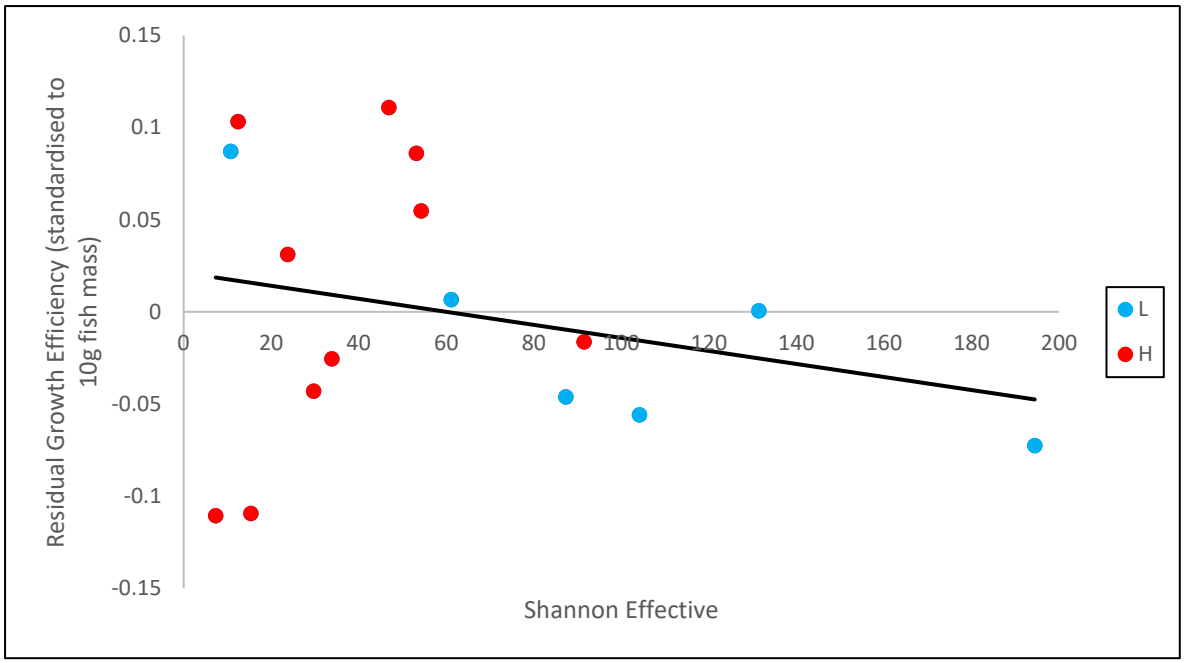

Figure 3-10: The relationship between growth efficiency (standardised to 10g fish mass) and the Shannon effective (an alpha diversity metric) in the foregut of juvenile Atlantic salmon (n = 16 in total), p = 0.043. *Fish with low and high relative standard metabolic rates are represented by blue and red points, respectively. Note that the statistical analyses were based on the (absolute) growth efficiency, but here the residual values after controlling for microbial richness, log-transformed average microbial load (cfu.g^{-1}) and water content (%) are plotted, in order to illustrate the relationship with the Shannon effective. See text for analysis.*

The impact of both alpha diversity metrics of the foregut microbiota on the growth efficiency (standardised to 10g fish mass) of juvenile Atlantic salmon was also assessed. The final linear model examining the effect of the foregut alpha diversity on fish growth efficiency contained microbial richness, Shannon effective, log-transformed average microbial load (cfu.g^{-1}) and fish water content (%) as explanatory variables. Growth efficiency increased with microbial richness (LM, $F_{4,11}$ = 32.22, P = 0.014), but decreased with the Shannon effective (Figure 3-10; P = 0.043) and percentage water content (P<0.001), as summarised in Table 3-2. Here, microbial richness and the Shannon effective seemed to be working in different directions (albeit the Shannon effective p-value is borderline), as they measure slightly different aspects of alpha diversity: richness simply describes the number of OTUs present within the sample, whilst the Shannon effective also accounts for abundance. Therefore, a high Shannon effective reflects fairly even abundance

across the OTUs present, whilst a low Shannon effective indicates that the number of equally common species is low (i.e. a few OTUs dominate the sample as a whole). In this context, this indicates that an increased number of OTUs (microbial richness) within the foregut could contribute to an increased growth efficiency, but that if these OTUs were broadly even in abundance, growth efficiency decreased.

As before, the relationship between growth efficiency (standardised to 10g fish mass) and the Shannon effective was presented using residual values. This time, Shannon effective was removed from the original model in order to gain a measure of expected growth efficiency (when accounting for microbial richness, log-transformed average microbial load and percentage water content), where expected growth efficiency = ((0.0014180 * microbial richness) + (0.0139520 * log-transformed average microbial load) + (-0.0915599 * percentage water content) + 6.7316). This expected growth efficiency was then subtracted from actual growth efficiency to give the residual values, which were then plotted against the Shannon effective (Figure 3-10).

Table 3-2: A summary of the results from the linear models testing the relationships between microbial alpha diversity metrics within the Atlantic salmon foregut and fish physiological measures.

Response	**Explanatory**	**t-value**	**P-value**
Richness	Experimental group – low rSMR	2.43	0.023
	Log-transformed average microbial load (cfu.g^{-1})	2.01	0.056
Shannon effective	Experimental group – low rSMR	2.54	0.019
	Log-transformed average microbial load (cfu.g^{-1})	2.42	0.024
	Fish water content (%)	1.46	0.16
Growth efficiency	Richness	2.93	0.014
	Shannon effective	-2.29	0.043
	Log-transformed average microbial load (cfu.g^{-1})	1.39	0.19
	Fish water content (%)	-8.22	<0.001

3.4.5.1.2. Alpha diversity metrics within the Atlantic salmon hindgut

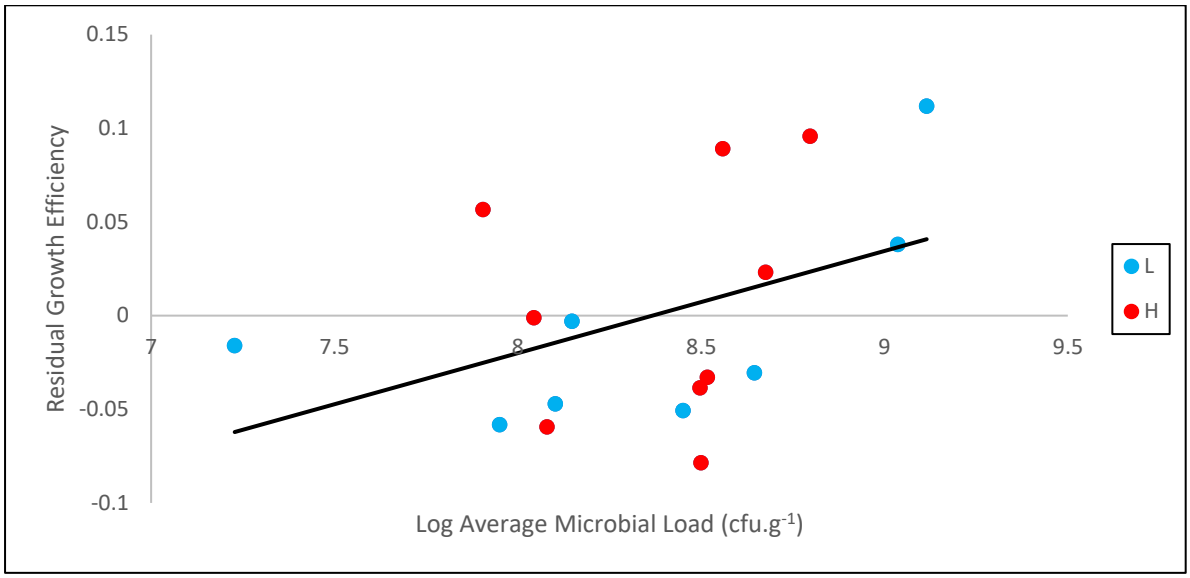

Figure 3-11: The relationship between growth efficiency (scaled to 10g fish mass) and the log-transformed microbial load (cfu.g^{-1}) in the hindgut of Atlantic salmon with a 'low' or 'high' rSMR (n = 17 in total). *Fish with low and high relative standard metabolic rates are represented by blue and red points, respectively. Note that the statistical analyses were based on the (absolute) growth efficiency, but here the residual values after controlling for the Shannon effective, fish mass (g), daily relative energy retained (DRER; kJ) and water content (%) are plotted, in order to illustrate the relationship with the log-transformed microbial load (cfu.g^{-1}). See text for analysis.*

Within the hindgut of juvenile Atlantic salmon, no explanatory variable was found to impact either the microbial richness or the Shannon effective. As with the foregut samples, the impact of both alpha diversity metrics (this time pertaining to the hindgut) and the other metavariables on the growth efficiency (standardised to 10g fish mass) was also assessed. The final model had growth efficiency (standardised to 10g fish mass) as the response variable and the Shannon effective, fish mass, daily relative energy retained (DRER), log-transformed average microbial load and percentage water content of the fish as explanatory variables (as summarised in Table 3-3). Growth efficiency was found to increase with the log-transformed average microbial load (Figure 3-11; LM, $F_{5, 11} = 42.72$, $P = 0.034$) and, as with the foregut, decrease with percentage water content ($P < 0.001$). To present the relationship between growth efficiency (standardised to 10g fish mass) and the log-transformed average microbial load, the expected growth efficiency (when accounting for the Shannon effective, fish mass, DRER and the fish percentage water content) was calculated as ((0.0011870 * Shannon effective) + (-0.0105962 *

fish mass) + (0.0571968 * DRER) + (-0.0921459 * percentage water content) + 7.0513818). This expected growth efficiency was then subtracted from actual growth efficiency to give the residual values, which were then plotted against the log-transformed average microbial load (Figure 3-11).

Table 3-3: A summary of the results from the linear model testing the relationships between microbial alpha diversity metrics within the Atlantic salmon hindgut and fish growth efficiency.

Response	**Explanatory**	**t-value**	**P-value**
Growth efficiency	Shannon effective	2.03	0.068
	Mass	-1.47	0.17
	Daily relative energy retained (DRER; kJ)	1.71	0.12
	Log-transformed average microbial load ($cfu.g^{-1}$)	2.42	0.034
	Fish water content (%)	-13.60	<0.001

3.4.5.2. *Microbial community-composition differences within the gut of Atlantic salmon parr*

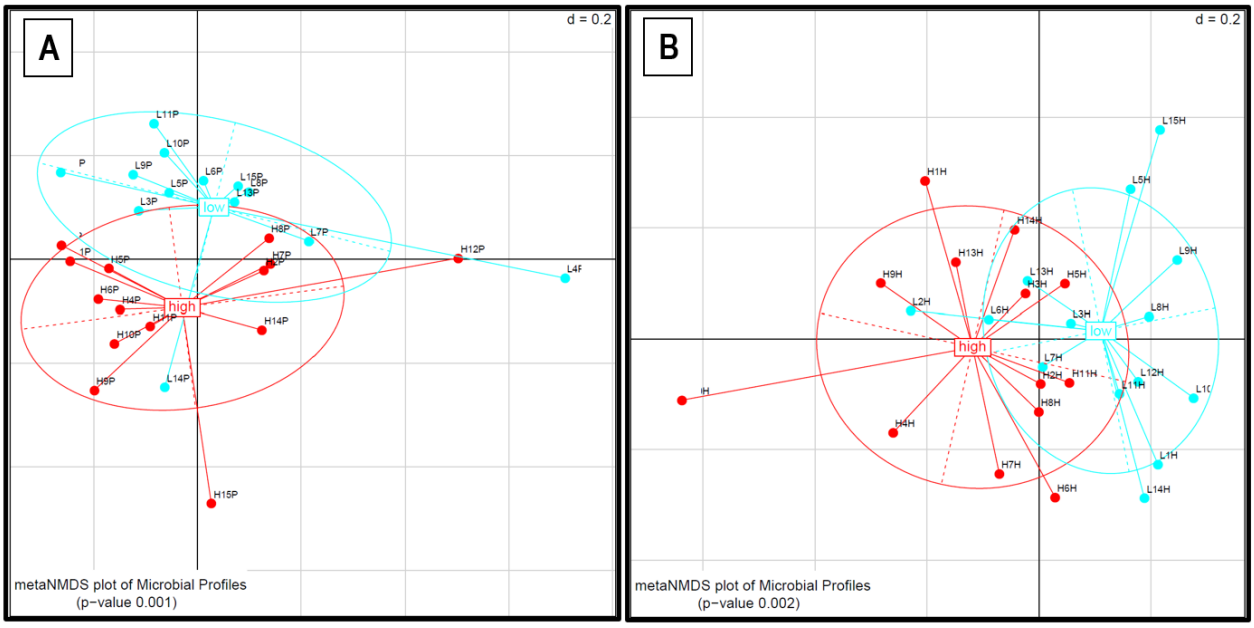

Figure 3-12: The difference in [A] foregut and [B] hindgut beta diversity of juvenile Atlantic salmon with 'low' or 'high' metabolic rates (rSMR), illustrated by non-metric multidimensional scaling (NMDS) based upon generalised UniFrac. *The dissimilarity scale of the grid, d = 0.2, indicates the distance between two grid lines represent approximately 20% dissimilarity between the samples. The p-values were calculated by permutational multivariate analysis of variance, which used the distance matrix to assess whether the separation of groups (samples from fish with either a high or low metabolic rate) was significant. Blue and red points represent fish with low and high metabolic rates, respectively.*

To examine the difference in microbial beta diversity between the juvenile Atlantic salmon from the ‘low’ and ‘high’ rSMR groups, multivariate analysis was used, which utilises generalised UniFrac metrics to account for the phylogenetic distance between OTUs. There was a significant difference in the microbial profiles between the two experimental groups in both the foregut (Figure 3-12 [A], P = 0.001) and the hindgut (Figure 3-12 [B], P = 0.002) of the fish.

Upon finding a difference in beta diversity between juvenile Atlantic salmon with ‘low’ and ‘high’ metabolic rates, this divergence was further explored to understand whether the difference between the two groups was due to specific taxa being differentially abundant. Differential abundance analysis was conducted separately for the foregut and hindgut samples. Microbial taxa that were significantly different in abundance between the low and high metabolic rate groups were identified in a

pairwise manner using the Wald test: a significant p-value was obtained when the abundance of a genus in one experimental group was log2 fold different from its abundance in the other, following Cox-Reid adjustment. There were 63 differentially abundant microbial genera between the foreguts of 'low' and 'high' rSMR groups (Figure 3-13) and 55 microbial genera that were differentially abundant between the hindguts (Figure 3-14).

Within the foregut of Atlantic salmon, 47 of the 63 differentially abundant genera were significantly more abundant within fish from the 'low' rSMR group than within the 'high' rSMR group. The most common genera to be differentially abundant between the two groups belonged to the phylum Proteobacteria (e.g. *Methylotenera* and *Stenotrophomonas*), accounting for 54% of all differentially abundant microbes. Indeed, genera from Proteobacteria represented 55% (n = 26 genera) and 50% (n = 8 genera) of the overabundant microbes in the foregut of fish with low and high metabolic rates, respectively. The other most commonly overabundant taxa in the foreguts of fish from both the 'low' and 'high' rSMR groups were microbes from Actinobacteria (e.g. *Microbacterium* and *Friedmanniella*), Bacteroidetes (e.g. *Polaribacter* and *Chryseobacterium*) and Firmicutes (e.g. *Trichococcus* and *Bacillus*). Actinobacteria and Bacteroidetes each accounted for 14% (n = 9 genera) of the total of differentially abundant genera between the two metabolic rate groups, though microbes from both of these phyla were more abundant in the foreguts of fish with a low rSMR (6 of the 9 Actinobacteria genera and 7 of the 9 Bacteroidetes genera were overabundant in fish with a low rSMR). The phylum Firmicutes represented 8% (n = 5 genera) of the total amount of differentially abundant genera, where these genera once more had an increased presence within fish from the low rSMR group (4 of the 5 Firmicutes genera were overabundant in fish with a low rSMR).

Within the hindgut of the juvenile Atlantic salmon, 32 of the 55 differentially abundant genera were overabundant within fish from the 'low' rSMR group in comparison to those from the 'high' rSMR group (Figure 3-13). Once more, the most common genera to be differentially abundant between the two groups belonged to the phyla Proteobacteria (e.g. *Loktanella* and *Brevundimonas*). Genera from Proteobacteria accounted for 56% of the total of differentially abundant microbes between the two metabolic rate groups, representing 59% (n = 19 genera) and 52% (n = 12 genera) of the overabundant genera in the hindgut of fish with low and high metabolic rates,

respectively. As found within the foregut, in addition to Proteobacteria, the overabundant genera within the hindguts of fish most commonly belonged to Actinobacteria (e.g. *HGCL clade* and *Streptomyces*), Bacteroidetes (e.g. *Ulvibacter*) and Firmicutes (e.g. *Lactococcus* and *Weisella*). Actinobacteria accounted for 16% (n = 9 genera) of the total of differentially abundant genera between the two metabolic rate groups, but unlike in the foregut, genera belonging to this phylum were more likely to be overabundant within fish with a higher rSMR (8 of the 9 Actinobacteria genera were overabundant within the hindgut of fish from the 'high' rSMR group). Meanwhile, the phyla Bacteroidetes and Firmicutes represented 13% (n = 7 genera) and 7% (n = 4 genera) of the total amount of differentially abundant genera between the two origins, respectively. As in the foregut, genera from these phyla were more commonly overabundant within the hindgut of fish with a lower rSMR (all 7 of the differentially abundant Bacteroidetes genera and 3 of the 4 differentially abundant Firmicutes genera were overabundant in fish from the 'low' rSMR group).

Figure 3-13: A heatmap showing the subset of microbial OTUs within the foregut of Atlantic salmon classified as significantly differing in abundance between 'low' and 'high' metabolic rate groups (rSMR). *Microbial genera were classified as significantly different based upon a log2 fold threshold. Each column represents a different sample and the bottom row colour-codes the sample based upon metabolic rate group, with blue and red representing foregut samples from fish with low and high metabolic rates (rSMR), respectively. Within the heatmap, pink-red represents increased abundance and grey-black represents decreased abundance.*

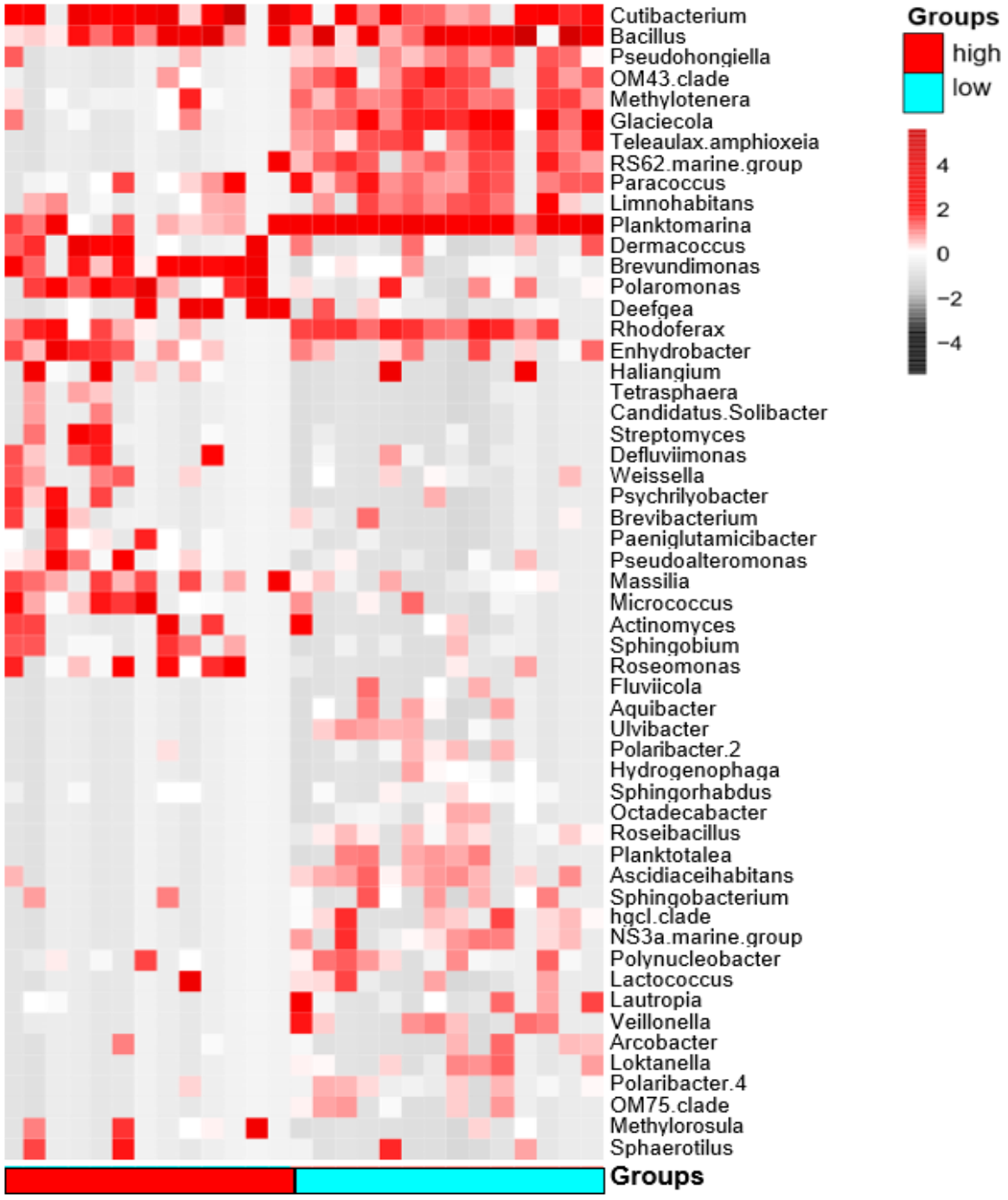

Figure 3-14: A heatmap showing the subset of microbial OTUs within the hindgut of Atlantic salmon classified as significantly differing un abundance between 'low' and 'high' metabolic rate groups (rSMR). *Microbial genera were classified as significantly different based upon a log2 fold threshold. Each column represents a different sample and the bottom row colour-codes the sample based upon metabolic rate group, with blue and red representing hindgut samples from fish with low and high metabolic rates (rSMR), respectively. Within the heatmap, pink-red represents increased abundance and grey-black represents decreased abundance.*

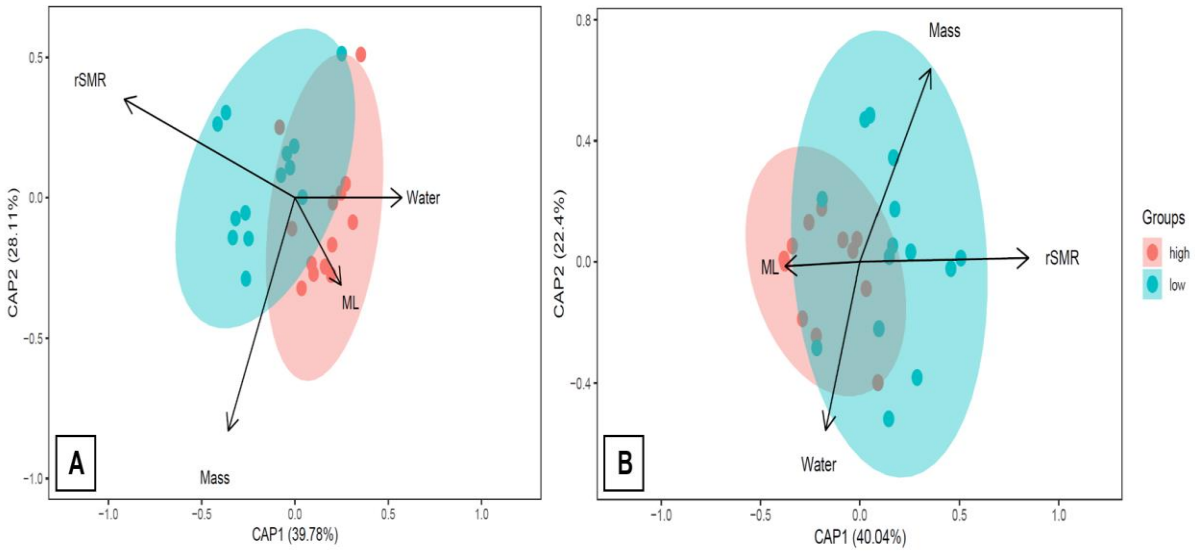

Figure 3-15: Distance-based redundancy analysis (dbRDA) illustrating the drivers of differences in [A] foregut and [B] hindgut beta diversity between juvenile Atlantic salmon with 'low' and 'high' metabolic rates (rSMR). *Blue and red points represent fish from 'low' and 'high' rSMR groups, respectively. Arrows in the plot denote the magnitudes and directions of the effects of explanatory variables. The total variance (in percent) explained by each axis is indicated. In the hindgut, mass (p = 0.017) and rSMR (p = 0.0073) were found to be significant drivers within the foregut.*

The potential drivers of microbial community composition (as shown in Figure 3-12) were also assessed using distance-based redundancy analysis (dbRDA) to explore whether the variation seen within microbial communities between the 'low' and 'high' rSMR groups was attributable to environmental variables. Initially, fish mass, daily relative energy retained (DRER), growth efficiency, log-transformed average microbial load, fish water content and rSMR were considered, and the foregut and hindgut data were analysed separately. Forward selection (as discussed within the methods section, 3.3.5.2.) then identified the variables to be retained within the models.

Within the foregut of the juvenile Atlantic salmon, fish mass, log-transformed average microbial load, fish water content and rSMR were included within the final model. Permutational ANOVA for dbRDA confirmed that the overall model was significant (P = 0.0012), showing that the explanatory variables accounted for 22.27% of the observed variance. Specifically, mass (P = 0.017) and rSMR (P = 0.0073) accounted for 6.26% and 6.90% of the variation in microbial community composition between the two metabolic rate groups, respectively (Figure 3-15, A). Within the hindgut, fish

mass, log-transformed average microbial load, fish water content and rSMR were once more included in the final model. Permutational ANOVA for dbRDA revealed that the overall model was not significant, showing that none of the considered variables explained variation in microbial community structure within the hindgut of the Atlantic salmon (Figure 3-15, B).

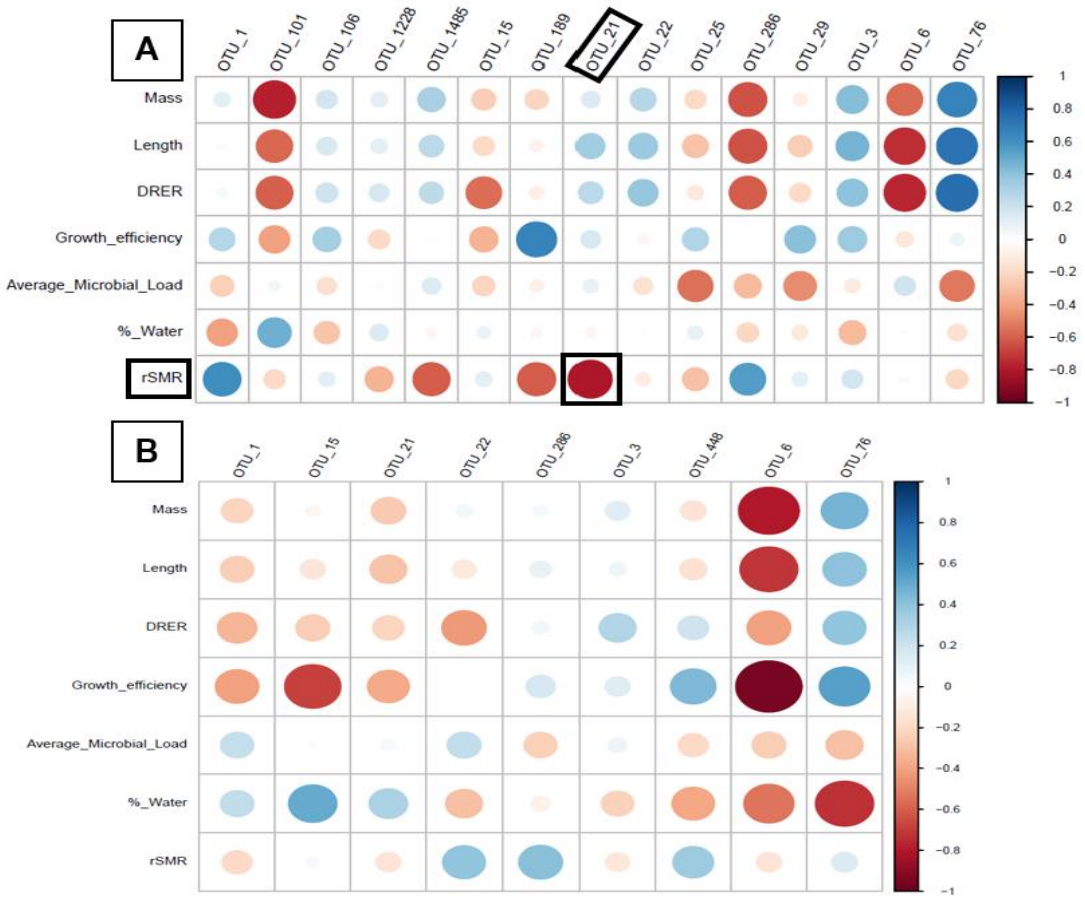

Figure 3-16: A correlations plot showing the correlations between metavariables and OTUs present within the foregut [A] and hindgut [B] of juvenile Atlantic salmon. *Each correlation is shown as a circle that is coloured to indicate direction of the correlation coefficient, where red is negative, and blue is positive. The size of each circle relates to the uncorrected p-value of the corresponding relationship, with larger circles indicating lower uncorrected p-values. Any statistically significant correlation remaining following an FDR correction is indicated in a bold black box.*

Finally, Pearson correlation coefficients were calculated to assess whether any metavariable (fish mass, fish length, daily relative energy retained (DRER), growth efficiency, log-transformed average microbial load, fish water content and rSMR) correlated with any of the OTUs identified within the gastrointestinal samples. Samples from the foregut and hindgut of juvenile Atlantic salmon were assessed

separately, but all fish from both the ‘low’ and ‘high’ rSMR groups were analysed together, treating rSMR as a continuous variable to understand the relationships between the metavariables and OTUs. In both analyses, an FDR (false discovery rate) correction was applied before assessing significance. Within the foregut of the juvenile Atlantic salmon, there was a significant negative correlation between rSMR and OTU 21 (r_{14} = -0.81, p = 0.017), which is a member of the Rhodobacteraceae family, belonging to the Proteobacteria phylum (Figure 3-16, A). Within the foregut, an increased abundance of this Rhodobacteraceae was found in fish with a lower metabolic rate. Within the hindgut of all juvenile Atlantic salmon, there was no significant correlation between any pair of OTUs and metavariable after application of an FDR correction (Figure 3-16, B). Stacked bar plots showing taxonomic composition at the family level also indicate that microbial taxa belonging to the Rhodobacteraceae family are more common within the foreguts of fish with a low metabolic rate (Figure 3-17).

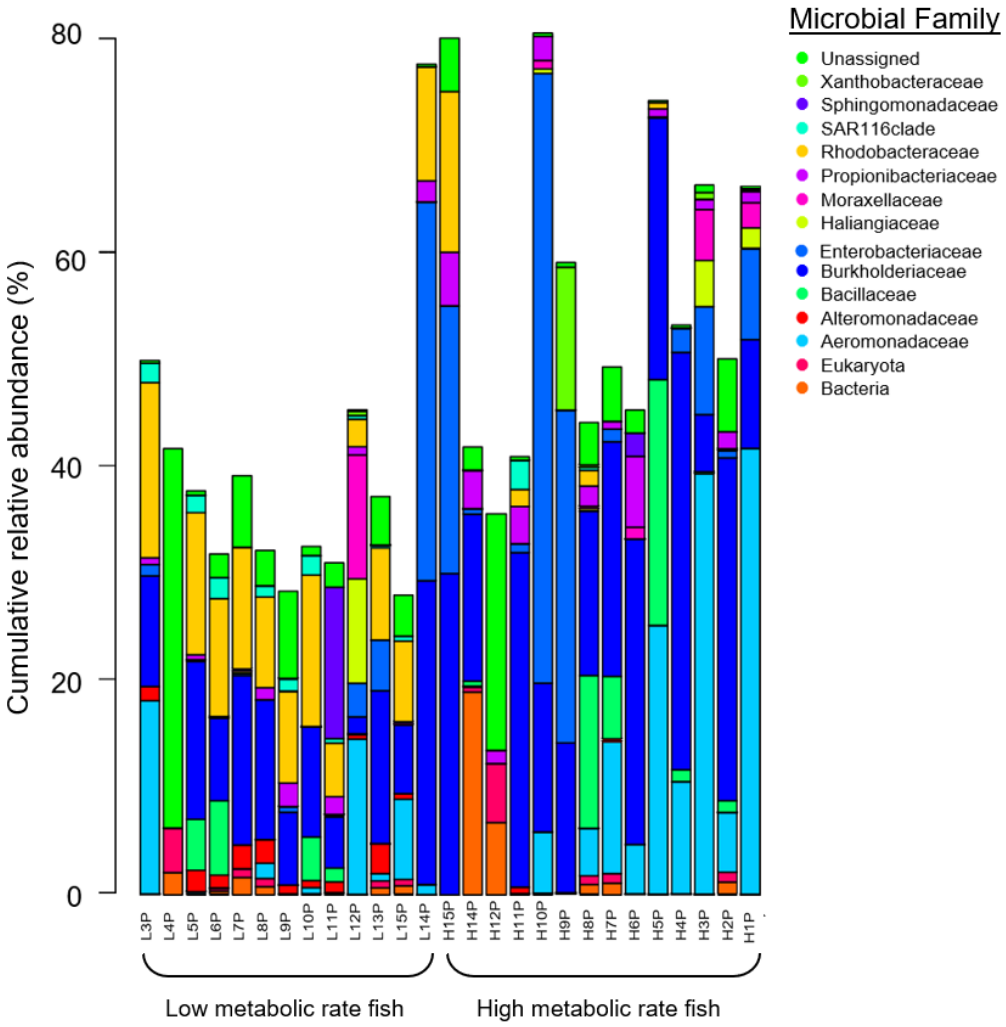

Figure 3-17: Stacked bar plots showing the taxonomic composition and cumulative abundance (%) of microbes within the foregut of Atlantic salmon with high and low metabolic rates. *Microbes are shown at the family level, with proportions of the bar coloured according to relative abundance of that family. Threshold abundance was set to a cut-off of 0.5 to allow for better visualisation of the most abundant microbial families.*

3.5. Discussion

Within this study, relationships were found between metabolic rate, growth efficiency and percentage water content in Atlantic salmon. As initially predicated, fish with a higher SMR grew more efficiently and had a lower percentage water content than their conspecifics with a lower metabolic rate. This study also sought to examine if differences in the microbial community existed between fish with different metabolic phenotypes. Differences were found in the microbial alpha diversity within the foregut, where fish from the 'high' metabolic rate group had reduced diversity within the foregut. In addition, in line with the original prediction, gut microbial community composition was divergent between fish from the two metabolic rate groups, with beta diversity and differential abundance analyses highlighting variation in the microbial taxa present within the gut. Finally, distance-based redundancy analysis suggested fish body mass and rSMR influence gut microbial community composition, whilst correlation analysis identified an OTU from the family Rhodobacteraceae as being negatively correlated with metabolic rate. Other OTUs from this family were identified in analyses treating rSMR as a categorical variable, where differential abundance analysis highlighted presence of genera from the Rhodobacteraceae family as being overabundant within the guts of fish with a low metabolic rate.

3.5.1. Metabolic Rate and Growth Efficiency in Atlantic Salmon with Different Metabolic Phenotypes

A variety of studies have tried to establish significant links between metabolic rate and the body mass of individuals, but by controlling for the size of an individual, it is possible to examine the potential impact of their size-corrected metabolic rate on a range of parameters such as growth efficiency, nutritional energy harvest and their gut microbial community. It was hypothesised that Atlantic salmon with a higher SMR would grow more efficiently, since they would have an increased need to retain more energy to compensate for their higher baseline costs of living. Within this study, fish with a higher SMR relative to their size did grow more efficiently (i.e. they incorporated a higher proportion of their ingested energy into body growth), but interestingly, there was no relationship between metabolic rate and the absolute amount of energy an individual retained from its food (defined as DRER). DRER was

only related to its mass and the energy it consumed, where larger fish and fish that ate more retained more energy from their diet. The lack of relationship between DRER, growth efficiency and metabolic rate was surprising: although fish with a higher SMR did grow more efficiently (which was shown to be independent of the amount of food consumed), they didn't seem to retain more energy from their food. Interestingly, the relationship between metabolic rate and growth rate was previously shown to be either non-significant or a negative correlation in juvenile brown trout living in natural streams (Álvarez and Nicieza, 2005). Of course, whilst growth efficiency was measured in the current study, the actual growth rate of the fish was not. Nonetheless, the Álvarez and Nicieza (2005) study highlights that results derived in a laboratory might not always be apparent in the more complex natural environment.

Individuals with a larger mass had a higher growth efficiency. Within this study, there was an overlap in mass between the 'low' and 'high' metabolic rate groups, and this relationship between mass and growth efficiency remained even once metabolic rate was taken into account. Growth efficiency was also negatively related to an individual's percentage water content – fish with a lower water content have more fat and less muscle (Elliott, 1976a; Berg and Bremset, 1998), so within this study, as predicted, those able to assimilate more of their ingested energy were able to lay down more fat. In addition, when the entire sample of Atlantic salmon were considered, there was a negative relationship between percentage water content and metabolic rate, showing that fish with more fat had a higher SMR. As SMR reflects the cost of maintaining metabolic machinery (SMR represents the baseline of maintaining total energy expenditure), then it is usually assumed that this cost will be higher in individuals that must invest more in maintaining high volumes of energetically expensive material, such as muscle (Auer et al., 2017). In contrast, within the current study, it was individuals with higher fat levels that had a higher SMR, which possibly reflects that they were able to invest more into growth, so more of their mass was the result of fat deposits. The discrepancy here could be that under stable conditions, fish with a higher SMR in this study were able not only to maintain their somatic tissues, but also store excess energy, as energy reserves have been shown to be stored as fat within salmonids (Swift, 1955). Indeed, changes in body composition caused by seasonal variability in food availability are more

associated with altered proportions of fat and water as opposed to muscle (Berg and Bremset, 1998; Finstad et al., 2009), suggesting that if excess energy is being assimilated it is going to be stored as fat, not muscle. Also, within this study, fish with a higher mass had a higher metabolic rate and a higher proportion of fat, which reflects the fact that larger fish have previously been shown to deplete their fat stores more readily than smaller juveniles; larger fish have been shown to increase their fat content above that of small fry throughout summer, as smaller fish prioritise protein to increase their size, as doing so reduces predation risk and cost of competitive interactions with other fish (Berg and Bremset, 1998).

The link between fat content, growth efficiency and metabolic rate is particularly interesting given that across their lifespan, salmonids in the wild can show large variation in body composition (Swift, 1955; Elliott, 1976a; Berg and Bremset, 1998). This variation is primarily due to seasonal changes in both biotic and abiotic environmental factors, such as water temperature, photoperiod and prey availability. As well as changes in an individual's metabolic rate (O'Connor et al., 2000), the result is often a reduction in somatic energy content over the winter, followed by a subsequent increase over the spring/summer months (Berg and Bremset, 1998). Body composition is therefore important within salmonids, as energy deficiency is thought to be a major cause of winter mortality in juvenile Atlantic salmon (Finstad et al., 2011). Consequently, individuals with larger energy stores have increased insurance under more challenging environmental conditions, which could explain why within this study, fish with a higher SMR had an increased proportion of fat – these individuals might need increased fat deposits as insurance to allow them to maintain their higher metabolic rate regardless of environmental conditions. Nonetheless, the link between growth efficiency, body composition and metabolic rate within this study must be put within the wider context that the nature of this relationship is likely to vary with environmental conditions.

It is well established that the performance of Atlantic salmon with different metabolic rates in the wild is largely dependent on the environment. For example, fish with an increased metabolic rate have been shown to grow faster and obtain better territories in simple habitats with a consistent food supply, but this performance advantage diminishes as environmental conditions worsen (Reid et al., 2012). Further, juvenile brown trout are able to lower their metabolic rate during periods of food deprivation

and increase it when resources are more plentiful (Auer et al., 2015b). Indeed, flexibility in metabolic rate shows intraspecific variation (O'Connor et al., 2000), which results in individuals responding differently to challenging environmental conditions. One example is the way in which fish in the wild cope with winter conditions – some display an anorexic response to minimise aerobic activity (Bull et al., 1996), whilst others continue to feed during the winter months (Grade and Letcher, 2006). Regardless of behaviour, an individual's intake in winter is usually insufficient to maintain energy reserves (Bull et al., 1996), further highlighting the importance of energy reserves in salmonids. The relevance of body composition in salmonids is underscored by the fact that fat levels in these fish are also thought to influence life-history decisions relating to sexual maturation, spawning and migration (Jonsson and Jonsson, 2005), which are processes that will all have repercussions for the growth and metabolic rate of the fish. Therefore, any links between fat levels, growth efficiency and metabolic rate can have wide repercussions for the ecology of Atlantic salmon.

The hypothesis that there would be a relationship between metabolic rate, growth efficiency and fat levels was based upon previous research highlighting the variation of growth (Grade and Letcher, 2006), metabolic rate (O'Connor et al., 2000) and body composition (Kadri et al., 1995) in Atlantic salmon across seasons and their entire lifespan. The results of this study suggest that fat content might be related to an increased SMR and greater growth efficiency, which is thought to be associated with fish with a higher SMR depositing an increased proportion of fat as physiological insurance for if environmental conditions were to deteriorate. Indeed, seasonal matching of foraging requirements has been studied in juvenile Atlantic salmon, showing that fish are capable of matching their energy stores to their anticipated needs (Bull et al., 1996). This study also provides evidence that even when environmental conditions are stable (such as within aquaculture where efforts are made to provide standardised conditions and equitable food intakes for all fish), inherent differences in metabolic rate might be a cause of differential growth amongst fish, which is often seen even within the same production environment (Huntingford and Adams, 2005).

3.5.2. Microbial Load and Gut Microbiota Diversity in Atlantic Salmon with Different Metabolic Phenotypes

In addition to the relationship between the metabolic rate and growth in Atlantic salmon within this experiment, it was also thought that there could be a link between a fish's metabolic phenotype and its gut microbiota. Therefore, microbial load, microbial diversity and microbial community composition were examined in fish from both the 'low' and 'high' metabolic rate groups. It was hypothesised that fish from the 'low' and 'high' metabolic rate groups would have a different microbial load and microbial diversity within the gut, which might reflect a role of the gut microbiota in enabling the host to meet its energetic requirements. However, contrary to this prediction, the microbial load present within the faeces did not differ between fish with distinct metabolic phenotypes. In addition, bomb calorimetry revealed that energy within the faeces was not impacted by microbial load or the energy an individual retained from its diet. Previously, a higher microbial load has been found in Atlantic salmon gut during their marine phase in comparison to their juvenile freshwater phase, which could be due to continued maturation and proliferation of the gut microbiota across the lifespan of the host (Rudi et al., 2018). Given that all of the fish within the current experiment were at the same life history stage, were fed the same diet and had lived their lives in the same environment, a stable microbial load found across individuals may not be unexpected. Regardless of metabolic phenotype, it seemed logical that an increased abundance of microbes within the gut would influence host nutritional harvest, either via competition for resources, or via facilitation of digestion, or a combination of both. However, as no such relationship was found, potentially the diversity and community composition of gut bacteria is of greater importance than simple abundance.

Given the literature showing differences in microbial community composition in association with variation in metabolic rate in various hosts (as discussed extensively within Chapter 2), it was hypothesised that the gut microbial community would differ between fish from the 'low' and 'high' metabolic groups. This was first assessed by comparing the alpha diversity (microbial richness and Shannon effective) of the gut microbial communities in Atlantic salmon from both 'low' and 'high' metabolic rate groups. Within the foregut, both microbial richness and the Shannon effective were higher in fish with a low SMR (although the p-values were borderline, being 0.023 for

richness and 0.019 for the Shannon effective). No relationship between metabolic rate and either alpha diversity metric was found within the hindgut. Within the foregut, the Shannon effective was also related to the average microbial load, where fish with an increased volume of bacteria also had an increased Shannon effective measure, perhaps suggesting that the more abundant the microbes in the gut, the more taxa that can be recovered.

The role of the gut microbiota in nutrition is well established in many animals, including within teleosts, where studies have shown that gut bacteria produce enzymes (e.g. carbohydrases, cellulase, lipase, etc) that contribute to digestion in fish, whilst anaerobic bacteria can provide volatile fatty acids, amino acids and different vitamins to aid the host digestion (Nayak, 2010; Ray et al., 2012). Therefore, the alpha diversity metrics in both the foregut and the hindgut were also examined in relation to growth efficiency. Across all Atlantic salmon tested, growth efficiency increased with an increased foregut microbial richness, a decreased foregut Shannon effective and, as previously established, a decreased water content. Microbial richness and the Shannon effective therefore seemed to be working in different directions (although once more the p-values were borderline, being 0.014 and 0.043 respectively), suggesting that growth efficiency was highest in fish in which many OTUs were present (high microbial richness), but a small number of these OTUs dominated the community (low Shannon effective). Foregut microbial richness (when the larger sample size of n = 27 was considered instead of being constrained by the smaller sample size from which growth efficiency data was collected) was higher in fish with a lower SMR overall. However, when considered in relation to growth efficiency, fish that grew most efficiently had a higher SMR, a higher proportion of fat and a rich but uneven foregut microbial community. Potentially, as fish with a rich foregut microbial community dominated by a few bacterial taxa show increased growth efficiency, the bacterial species present in higher proportions have a larger role in digestion within the host. It is not possible to mechanistically define the links found between the alpha diversity of the gut microbiota, metabolic rate and growth. In particular, alpha diversity associations with salmon phenotypes are only borderline significant, and their directionality not clear cut. Nonetheless, the results of this study provide evidence that there might be an

interplay between these factors within Atlantic salmon. This relationship is explored further in Chapter 4.

3.5.3. The Community Composition of the Gut Microbiota in Atlantic Salmon with Different Metabolic Phenotypes

In addition to examining diversity metrics, the gut microbial community composition was also assessed. Within this study, there was a significant difference in microbial beta diversity within both the foregut and the hindgut of the Atlantic salmon between the 'low' and 'high rSMR groups. It was hypothesised that Atlantic salmon from the 'low' and 'high' metabolic rate groups would differ in their gut microbial communities. Differential abundance analysis revealed that overall, genera from the Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes phyla were most likely to be present in significantly different proportions between fish with a 'low' or a 'high' metabolic rate. Previous research has shown that Tenericutes, Firmicutes, Bacteroidetes and Proteobacteria dominate the gastrointestinal tract of Atlantic salmon (Fogarty et al., 2019), though this can vary with geography of the gut, with Proteobacteria dominating in the mucosa and Proteobacteria and Firmicutes showing highest abundance in the digesta (Gajardo et al., 2016). Meanwhile, presence or absence of certain bacterial taxa can also alter in response to environment – Rudi et al. (2018) found that the transition from freshwater to saltwater led to an increase in Firmicutes and a decrease in Actinobacteria and Proteobacteria within Atlantic salmon. Further, Xia et al. (2014) found that Bacteroidetes increased as Betaproteobacteria decreased in the gut of Asian seabass (*Lates calcarifer*) in response to starvation. Within the current study, most of the genera that were overabundant in either metabolic rate group belonged to Proteobacteria, but in the foregut, Actinobacteria, Bacteroidetes and Firmicutes were more likely to be overabundant in fish from the 'low' rSMR group. This relationship remained in the hindgut, with the exception that genera from Actinobacteria were more likely to be overabundant in the hindgut of fish from the 'high' rSMR group.

It is well-established that different microbial communities have different functional profiles that will impact the host in a variety of ways. For example, short-chain fatty acids are produced by anaerobic members of the intestinal microbiota, and these

can have effects on the host, for example by impacting lipid, glucose and cholesterol metabolism (den Besten et al., 2013). Many studies have found *Pseudomonas* (a Proteobacteria) to be a recurrent members of the gut microbiota (Nayak, 2010) – these bacteria regulate a selection of genes associated with host nutrient metabolism (Navarrete et al., 2009). The links between gut microbiota and energetics in the literature (see Chapter 2) formed the hypothesis for the current study: that the metabolic rate of an individual would vary with microbial composition. Certainly, the empirical evidence linking specific bacterial taxa to host metabolic phenotype is beyond the scope of this study; however, the distance-based redundancy analysis did suggest that within the foregut, microbial community composition was in part driven by both mass and metabolic rate. Further, the correlation analyses found a negative correlation between SMR and a member of the Rhodobacteraceae family (a Proteobacteria). Therefore, within the foregut, an increased abundance of this Rhodobacteraceae was found in fish with a lower metabolic rate. Interestingly, though this specific OTU cannot be identified at the genus level, other members of the Rhodobacteraceae family (e.g. *Ascidiaceihabitans* in the foregut and *Octadecabacter* in the hindgut) were identified as being overabundant in fish with a low SMR when 'low' and 'high' rSMR groups of fish were compared (Figures 3-13 and 3-14). Additionally, stacked bar plots showing the taxonomic composition of the gut microbiota indicated that OTUs belonging to Rhodobacteraceae were more common in the foregut of fish from the 'low' metabolic rate group (Figure 3-17).

The striking relationship between the Rhodobacteraceae bacterial family and the metabolic rate of Atlantic salmon would benefit from further investigation. Though determining causal links conclusively can be challenging, there is the potential for different experimental approaches (discussed within Chapter 2) to provide insight into the role of specific microbial taxa. Such approaches include the assessment of microbial metabolites and targeted knock-out studies. Following on from this experiment, these methods might improve our understanding of the function of members of Rhodobacteraceae: whether these bacteria produce secondary metabolites that have an impact on host metabolic rate could be assessed, whilst examining host performance in the absence of species from Rhodobacteraceae might reveal a subsequent effect on host physiology. Additionally, the family could

also be targeted via supplementation, which could indicate whether an increased abundance of bacterial members belonging to Rhodobacteraceae has an impact on the host's metabolic rate.

Research exploring the relationship between the gut microbiota and host growth is extensive within teleosts, as shown by the volume of studies examining the impact of host diet on the gut microbiota (see Chapter 2). Such studies have revealed that the community composition of the gut microbiota can alter in response to a change in diet composition (Green et al., 2013). Similar research has also sought to explore the use of probiotics and prebiotics to target specific bacterial taxa in an effort to benefit host health and growth (Nayak, 2010; Llewellyn et al., 2014). For example, one such study showed that within Atlantic salmon, dietary synbiotic supplementation led to increased microbial community diversity and richness in comparison to control fish (Abid et al., 2013). Meanwhile, the gut microbiota impacts energy harvest from the diet, resulting in host physiological changes (Xia et al., 2014), which will likely impact the host metabolic rate. The context of these studies highlights the overlap between the gut microbiota and teleost physiology. In addition, research in other taxa has focused on temporal differences in the gut microbiota, with seasonal variation in gut microbial community composition revealed in many animals, including giant pandas (Wu et al., 2017), American bison (Bergmann et al., 2015) and capercaillie (Wienemann et al., 2011). The drivers of these changes are often environmental, especially due to seasonal variation in diet. As previously discussed, wild Atlantic salmon experience large seasonal differences in environmental conditions and prey availability, which impact body composition and metabolic rate. Given the extensive evidence of such factors impacting the gut microbiota of the host, it is possible that there is feedback between the gut microbiota and host physiology (metabolic rate and body composition), which could differ spatially and temporally. As this experiment has highlighted these relationships between the gut microbiota and physiology of Atlantic salmon within a stable laboratory environment, future work would benefit from examining these links across different environments, seasons, and life stages, thereby incorporating different diets, body compositions and physiological states of the host. Additionally, as previously discussed within the context of Rhodobacteraceae, the function of specifically identified microbial taxa could then be elucidated. Upon understanding their function, studies could be

designed to target these bacterial species via probiotics/prebiotics with the aim of having a direct benefit for the host.

3.6. Conclusion

This study sought to examine whether there were any links between metabolic rate, growth efficiency and the gut microbiota in Atlantic salmon. Growth efficiency was higher in fish with a high SMR, which could reflect their increased need to retain more energy to compensate for a higher baseline energetic cost of living. Interestingly, body composition was also related to growth efficiency and SMR, where individuals with an increased proportion of fat had a higher metabolic rate and showed increased growth efficiency. This finding highlights the interplay between an organism's metabolic phenotype and body composition, both of which have implications for an animal's fitness. The Atlantic salmon within this study also had divergent microbial community compositions within the gut depending upon which metabolic rate group they were in – both microbial alpha diversity and beta diversity differed between the two groups of fish. It is challenging to suggest a functional explanation for the findings, so future work would benefit from assessing the production of microbial metabolites to establish whether production of secondary metabolites differs between fish with distinct metabolic phenotypes. In addition, diet has repeatedly been associated with variation in the community composition of the gut microbiota and host metabolic rate. Though the composition of the diet within this study was consistent for all fish, intake levels would have varied and within the natural environment, diet will show large variation between individuals. Consequently, intraspecific disparities in the composition of the gut microbiota and host metabolic rate due to differences in nutrient input cannot be overlooked.

The combination of the adaptive nature of both host metabolism and gut microbiota could be a powerful means by which a host organism can cope with a vast variety of environmental and physiological pressures. This makes understanding links between the two in relation to growth and body composition important, especially given that such impacts on host physiology has implications for survival in Atlantic salmon who experience vast seasonal variation in metabolic rate and fat levels.

Chapter 4: The Impact of Origin on the Metabolic Rate and Gut Microbiota of Atlantic Salmon: A Comparison of Wild, Ranched and Farmed Fish.

4.1. Abstract

Differences between wild and captive individuals have been studied across a variety of taxa, with variation being reported in behaviour, host physiology and even the host's microbiota. Such intraspecific variation is of particular interest within Atlantic salmon, as the species' economic importance has led to generations of fish being bred to thrive within an aquaculture setting, in stark contrast to the environment faced by their wild conspecifics. The result of historical selective breeding has implications for when farmed and wild-origin Atlantic salmon interact, as occurs when farmed fish escape from fish farms. In the freshwater environment, competition between the two origins of fish is often to the detriment (at least in the short term) of those from the wild origin. The factors driving the superior performance of farmed fish are not fully understood but must be due to more than simply environmental effects. It is widely accepted that the metabolic rate, the gut microbiota and the interplay between the two can have large repercussions for the performance of teleosts. This study therefore examined the metabolic rate, the Specific Dynamic Action (SDA), and the gut microbiota across three origins of Atlantic salmon: farmed, ranched and wild. The largest differences were expected between farmed and wild individuals, as it was anticipated that the ranched fish would reflect an intermediate position between the other two origins. Links between host metabolism and community composition of the gut microbiota were investigated to identify any correlations between these factors and highlight any differences between all three origins. Wild-origin Atlantic salmon were found to have a higher aerobic scope (AS) than their conspecifics and also a higher peak SDA, which could reflect the increased demands on the metabolic rate of wild fish due to their more stochastic environment. Bioinformatic analyses revealed differences between the three origins of fish in the abundance of microbial taxa, with many genera belonging to the Proteobacteria phylum being differentially abundant across the guts of all three origins. These analyses also highlighted some interesting relationships between the gut microbiota and host physiology: independent of origin, Atlantic salmon with an increased rSMR had decreased percentage water content, increased mass and

decreased gut microbial alpha diversity. Although further work will be required to elucidate causation, this study highlights the variety of factors that could drive intraspecific differences in Atlantic salmon alongside the already well-established environmental influences.

4.2. Introduction

An animal's metabolism can impact its physiology, ecology and behaviour (Careau et al., 2008; Burton et al., 2011; Killen et al., 2013; Mathot and Dingemanse, 2015). There have therefore been many studies examining the causes and consequences of variation in metabolic rate (MR), covering a wide range of taxa, from invertebrates (Artacho and Nespolo, 2009), to teleosts (Killen et al., 2016) and endotherms (Khaliq and Hof, 2018). These studies have highlighted that MR shows both large interspecific and intraspecific variation. Many factors are thought to impact an organism's MR independent of its body size, with previous studies revealing the impact of environmental conditions (Hopkins et al., 1999), morphology (Killen et al., 2016) and even mitochondrial respiratory capacity (Salin et al., 2016). In spite of the range of these influences, studies have shown that MR is a reasonably repeatable trait (Nespolo and Franco, 2007; Auer et al., 2016a), and an organism's metabolic phenotype is believed to have a degree of heritability (White and Kearney, 2013), with research showing that selection can act upon metabolic traits, which might therefore evolve over generations (Artacho and Nespolo, 2009; Boratyński and Koteja, 2010).

Due to the relative ease with which they can be standardised, the majority of studies of metabolism have focussed on minimum metabolic rate (MR_{min}) and maximum metabolic rate (MMR). MR_{min} describes the minimum cost of living (Hulbert and Else, 2004) and is referred to as standard metabolic rate (SMR) in ectotherms and basal metabolic rate (BMR) in endotherms. Though MR_{min} is correlated with the body mass of an animal, once mass is corrected for, it has become a powerful tool when comparing metabolic rates within and between species. This "baseline" metabolic rate reflects the essential homeostasis of cells and tissues, but does not cover the metabolic cost of activity, digestion, growth or reproduction Therefore, measures of MR_{min} allow researchers to assess the bare necessities of an organism's oxygen uptake requirements, if there were no other requirements other than to simply

subsist. On the other hand, MMR describes the upper limits to an organism's ability to take up oxygen, so sets a limit on an animal's capacity to move, grow, digest, reproduce and carry out any other behaviours (Auer et al., 2017). Both measures can provide ecological insight, since they describe the cost of living; moreover, the difference between MR_{min} and MMR defines the scope for aerobic metabolism within which an animal has to live, termed its "aerobic scope" (AS) (Chabot et al., 2016).

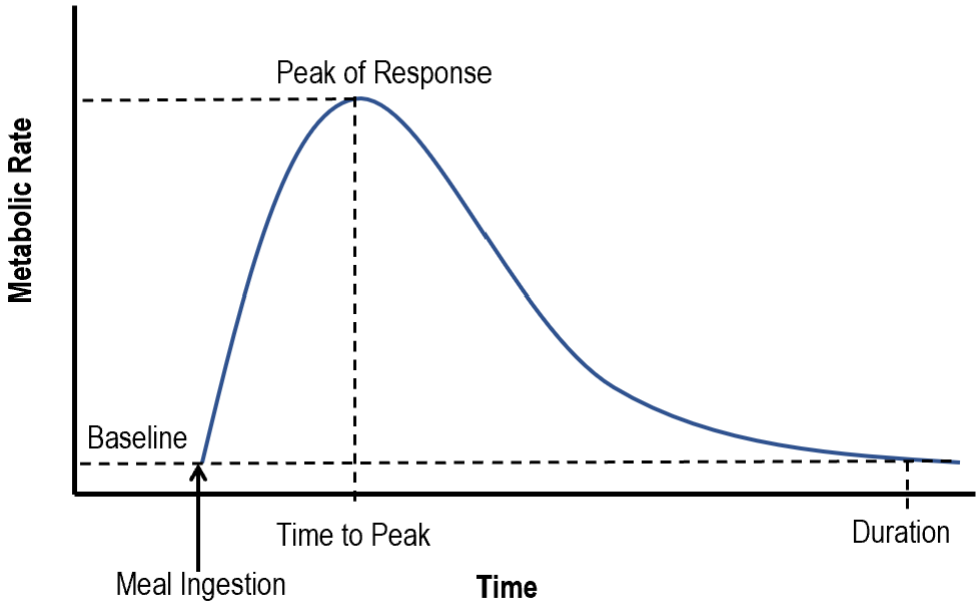

Figure 4-1: The typical metabolic profile of an SDA response. *The postprandial response shows the peak, time to the peak and the duration. After Secor (2009).*

The SMR of an ectotherm such as a fish is measured when an individual is exhibiting no activity, so its oxygen usage reflects purely that which is necessary to maintain its tissues. It is therefore important that an individual is not digesting any food during SMR measurement, due to the metabolic cost associated with digestion. This postprandial metabolic response is termed the Specific Dynamic Action (SDA), and describes the impact of processing a meal on an individual's MR (Secor, 2009; Tirsgaard et al., 2014). The SDA response is therefore ecologically relevant, since it gives insight into the energetic cost of digestion and assimilation of nutrients – energy that might otherwise be put toward movement, reproduction or interactions with conspecifics. As with all other aspects of metabolism, in fish there is intra- and interspecific variation in the SDA response, and it is also influenced by environmental factors such as hypoxia (Jordan and Steffensen, 2007) and temperature (Tirsgaard et al., 2014). Nonetheless, the SDA profile remains

predictable: following ingestion of a meal, MR raises from its baseline (e.g. its SMR, if the animal is inactive) to a “peak”, before declining more slowly back to the baseline (Figure 4-1). If the baseline is known and the MR is then measured throughout the postprandial response, then data regarding the duration of the response, the peak oxygen consumption and time to reach this peak can be collected. Individuals with a more efficient SDA response (i.e. one that involves less of an increase in metabolic rate over the baseline) might have a fitness advantage over others, as they will have to apportion less of their energy budget to the necessary processes associated with digestion (Secor, 2009). Similarly, individuals that have a shorter duration of SDA response can potentially resume feeding sooner, taking greater advantage of abundant but transient food supplies (Millidine et al., 2009). Further, an understanding of the energetic costs of digestion gives insight into the overall energy necessary for growth (Peck et al., 2005).

The literature regarding performance outcomes of an organism’s metabolic phenotype can be contradictory, with conflicting results on the relationship between MR and growth (McCarthy, 2000; Norin and Malte, 2011), reproduction (Blackmer et al., 2005; Sadowska et al., 2013) and survival (Artacho and Nespolo, 2009; Niitepõld and Hanski, 2013). Research has highlighted therefore, that the costs or benefits associated with a given metabolic phenotype are often context-dependent, whereby the performance outcomes associated with a given MR depend upon environmental conditions (Auer et al., 2015c; Auer et al., 2015b). In the wild, the stochasticity of the environment will result in a variation in food supply and its quality. In Atlantic salmon, individuals have been shown to decrease their SMR during periods of food deprivation, only to increase it again once food became more widely accessible (O'Connor et al., 2000).

This is in contrast however, to members of economically important species that spend their entire lives within fish farms, which offer a comparatively stable environment: the food supply is steady and environmental conditions usually less variable than those found in the wild. The marked environmental differences experienced by farmed and wild fish might select for different metabolic phenotypes, as the guaranteed food supply provided by aquaculture will mitigate the “cost” of having a higher MR, which could be detrimental to wild fish in times of food scarcity (Reid et al., 2012). The metabolism of farmed fish is clearly of economic importance since the aquaculture industry is aiming to increase the efficiency of feed utilisation

and fish growth in order to enhance profitability (Oliva-Teles and Peres, 2015). Given the heritable nature of the metabolic phenotype, it is likely that generations of selection in farmed animals to increase their growth efficiency will have indirectly impacted the MR of these individuals; however, it might also have created differences in the SDA response between farmed and wild individuals. A higher SMR has been shown to result in a shorter duration of SDA and hence faster processing of meals (Millidine et al., 2009); whilst farmed individuals might have a higher MR due to their stable food supply, there could be increased pressure on wild individuals to digest their meals faster in order to effectively cope with the increased energetic demands associated with a more dynamic environment (Secor, 2009; Norin and Clark, 2017).

Of equal importance to the aquaculture industry should be the relationship between an individual's gut microbiota and its MR. Since the gut microbial community has been shown to impact host energetics across a variety of taxa (Bagheri et al., 2008; Chevalier et al., 2015; Ayayee et al., 2018; for detailed discussion see Chapter 2), the intricate relationship between gut microbes and their host has repercussions for host fitness. The gut microbiota has also been implicated in the immunocompetence of teleost hosts, including Atlantic salmon (Navarrete et al., 2009). Since Atlantic salmon are of global commercial importance within the aquaculture industry (Fogarty et al., 2019; Uren Webster et al., 2020), examining the relationships between metabolic rate, the gut microbiota and their consequences for host performance within this species is particularly relevant. Just as divergent husbandry between farmed and wild Atlantic salmon might have resulted in metabolic differences between the two origins, it is possible that their gut microbiota have also been impacted: the gut microbiota has been shown to alter in response to environment (Rudi et al., 2018), host physiology (Dehler et al., 2016) and diet (Zarkasi et al., 2016), all of which will differ between the aquaculture and natural environments.

Any differences in metabolic rate or the gut microbiota between farmed and wild-origin Atlantic salmon are likely due to historic selective breeding of farmed Atlantic salmon for favourable traits and the environmental differences experienced by the different origins of fish. Though understandable, if such variances between the two origins result in differential fitness, this has implications for populations of wild Atlantic salmon that encounter farmed salmon due to aquaculture escapees. The risk of farmed aquaculture species escaping into the wild is a concern as it has

implications for habitats, biodiversity and wild stocks (FAO, 2020). Hundreds of thousands of farmed salmon escape into the wild annually and interact with wild populations, resulting in direct and indirect competition, transfer of pathogens (such as sea lice) and in some cases, genetic introgression (Glover et al., 2017). It is thought that farmed individuals show reduced overall survival in comparison to wild across both marine and freshwater natural environments, though the largest differential in survival exists in the marine environment (McGinnity et al., 2003); however, farmed juveniles have more rapid growth, whilst post-smolt and adult individuals are able to displace wild salmon of the same life stage (Glover et al., 2017). Farmed Atlantic salmon will tend to outgrow their wild and hybrid conspecifics in both natural and hatchery environments (Harvey et al., 2016b). Meanwhile, if interbreeding between farmed and wild individuals occurs, the resulting hybrids have also been shown to have reduced survival in the wild (McGinnity et al., 2003; Solberg et al., 2020), whilst any backcrossing will result in a loss of genetic heterogeneity, which might therefore reduce the fitness of the wild population (McGinnity et al., 2003). Indeed, in Atlantic salmon, hybrid vigour has not been reported, with the hybrid offspring of farmed and wild fish often being intermediate in terms of both performance and fitness (McGinnity et al., 2003; Harvey et al., 2016b).

Whilst the implications for the encounters between wild fish and farm escapees are broad, the extent to which physiological, behavioural and genetic differences between farmed and wild Atlantic salmon impact the outcomes of such interactions remains unknown. As environmental cues, the gut microbiota and metabolic rate all influence the life history of Atlantic salmon, it remains possible that these factors have differential impacts upon different origins of Atlantic salmon. Although some studies have examined the links between differential metabolic rate (Robertson et al., 2019) and gut microbiota community composition (Dehler et al., 2016) in wild and farmed Atlantic salmon, to the author's knowledge, none have examined both simultaneously. Increasing the understanding regarding any differences that exist between distinct origins of Atlantic salmon might help to explain why fish from different origins thrive in different environments.

Consequently, this study seeks to understand whether the metabolic rate, the community composition of the gut microbiota and the relationship between the two differs between Atlantic salmon of three different origins: farmed (i.e. semi-domesticated fish bred for multiple generations in the farm environment), wild, and

ranched (which are of intermediate genetic status between farmed and wild, being reared in farms when juvenile and then released to the wild). In addition, it is hypothesised that any differences found in metabolic phenotype between the three origins might be in part linked to differences within their gut microbiota. Therefore, links between the metabolic and microbial differences will be explored, both among individual fish and among the three origins of fish.

This study will therefore test the following hypotheses:

1) The SMR of farmed Atlantic salmon will be higher than that of wild or ranched fish due to the stability of food supply experienced by generations of farmed individuals.
2) The AS of wild Atlantic salmon will be higher than that of farmed or ranched fish due to the additional energetic demands and variability in energetic demand associated with their wild environment. Consequently, the MMR of wild fish will be higher than that of their conspecifics.
3) Wild Atlantic salmon will have a shorter duration of SDA response than their conspecifics due to the necessity of balancing the energetic costs of feeding with predator avoidance and social interactions.
4) The microbial diversity within the gut of wild Atlantic salmon will be more variable than that of farmed, due to a more varied genetic background than that of farmed fish that have been bred selectively for generations.
5) There will be a difference in gut microbial community composition between farmed, ranched and wild Atlantic salmon.
6) Ranched fish will largely reflect an intermediate position between farmed and wild with respect to both metabolic phenotype and gut microbiota parameters.

4.3. Methods

4.3.1. Fish Husbandry and Acclimation

The Atlantic salmon used in this experiment came from one of three origins: domesticated, ranched and wild. Marine Harvest provided the domesticated fish, which were a Norwegian Mowi strain. Both the wild and ranched fish came from the Burrishoole catchment, County Mayo, Ireland. The ranched strain was gradually isolated from the wild population from 1960 to 1964, and since then 10-14

generations have been maintained as a ranched population (i.e. using parents that had been raised as juveniles in the hatchery until the presmolt stage, then released into the wild to be recaptured and used as broodstock when returning as adults to the river to spawn). Offspring were first produced from wild fish and then only by pure crosses between recaptured ranched parents. Ranched fish were microtagged and their adipose fin clipped before being released as presmolts in Lough Furnace, to assist with their identification as ranched stock on their return to freshwater to breed. The ranched fish eggs provided for this experiment were produced from ranched parents that had returned to the Burrishoole and were caught upon return at the sea entry/exit traps between Lough Feeagh and Lough Furnace. The parents of the wild fish were caught in these same traps (Figure 4-2).

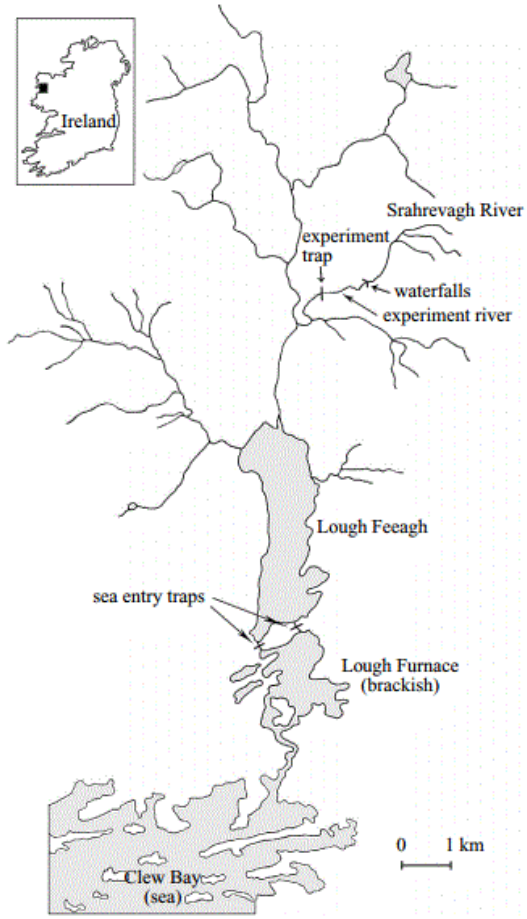

***Figure 4-2: A map of the Burrishoole catchment**, showing the sea entry/exit traps between Lough Feeagh and Lough Furnace in which the parents for the ranched and wild Atlantic salmon used within this experiment were caught.*

All three categories of egg were generated by stripping sexually mature fish of eggs and sperm in winter 2016. All crosses were between fish of the same origin. To

produce the eggs used in this experiment, each ranched female was fertilised by a single male to produce 10 full sibling families, whilst 10 families of wild fish were derived from 5 females and 10 males (the eggs from each female being divided into two batches, fertilised by separate males). For the farmed fish, eggs of mixed parentage were sourced from the Mowi ASA, Norway, stock population (unknown number of families). Eggs from all three origins were initially raised under identical conditions (with families within an origin being pooled) at the Marine Institute hatchery at the Burrishoole. They were then transferred at the eyed stage to the aquarium facilities at the Institute of Biodiversity, Animal Health and Comparative Medicine, University of Glasgow. Once in Glasgow, the eggs of each origin were kept within separate buckets within the same larger tank, allowing water exchange across all eggs. The room was kept on a 12L:12D photoperiod. Feeding of each group commenced once the eggs had all hatched and the yolk sacs had been depleted. Fry were hand fed on commercial salmon fry food pellets (EWOS MICRO 5P LR; EWOS Ltd, Bathgate, UK) twice daily; all had begun first feeding by 24/04/17. Fry were then transferred into identical 400L stock tanks; one for each origin. Flow rate was continuous to minimise aggression and individuals were fed a combination of bloodworms (Chironomid midge larvae) and EWOS pellets daily. Throughout the rearing period, all fish shared water from a common recirculation system and were kept at a constant temperature of approximately 12˚C.

All fish were reared under these conditions, with the size of feed pellet being increased to match fish size, until the experiment commenced in April 2018. At this point a total of 90 fish were selected (30 per origin); fish were chosen at random, but to include a range of sizes within each origin, ensuring a size overlap between origins. As respirometry could only be performed on 15 fish at any one time, the fish were acclimated in batches of 15 in order to ensure equal treatment of all experimental individuals. In order to acclimate individuals, 5 randomly selected fish from each origin group (farmed, ranched and wild) were transferred from their respective 400L stock tanks into individual compartments (190 x 130 x 200mm) within a recirculating stream system. The individual compartments, each with mesh upstream and downstream walls and one opaque and one glass side wall (to allow observations), allowed each individual fish to be fed its own ration. All fish shared a common water source, sterilised by a UV filter. The water temperature was maintained at 11.8˚C ± 1.0˚C, similar to the temperature of the stock tanks from

which they were taken. The fish were left to acclimate for a period of 12 days, during which each individual was fed bloodworms to satiation daily. The fish were then deprived of food for two days to ensure that they had an empty gut prior to mass measurements and respirometry; this period has proven to be sufficient time for Atlantic salmon to evacuate their guts on a variety of diets at 9.0˚C (Storebakken et al., 1999), so would be more than adequate at the temperature used in the present experiment. A fresh batch of 15 fish was moved into the stream tank system to acclimate every 4 days, resulting in 6 batches of 15 fish in total; this staggered start to the acclimation was designed to match the rate at which the fish could be processed in the respirometers. One wild fish died during the acclimation period.

4.3.2. Mass Measurements of Atlantic Salmon Parr

The mass of each fish was measured (± 0.1g) immediately prior to transfer to a respirometry chamber, so that metabolic rates could be corrected for body mass. Mass measurements were also used when calculating the mass of food to give each fish during the measurements of Specific Dynamic Action (SDA) (see below).

4.3.3. Respirometry Measurements in Atlantic Salmon Parr

4.3.3.1. *General respirometry methods*

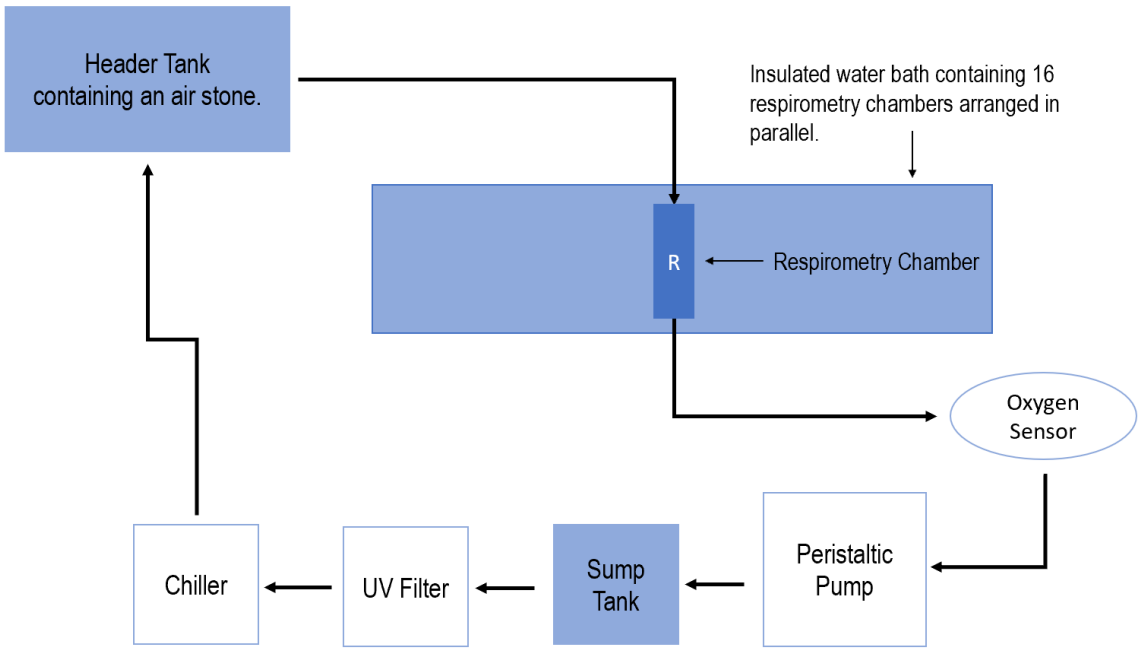

Figure 4-3: An overview of the respirometer set up. *The system had capacity for 16 respirometer chambers (R), allowing 15 fish to be measured at any one time, with one chamber used as a control to record background microbial respiration. Bold black arrows indicate the direction of water flow.*

Sixteen chambers were arranged in parallel within a water bath, allowing data for 15 fish to be collected simultaneously, whilst one empty chamber acted as a control measure of background (microbial) oxygen consumption. Downstream of each respirometry chamber, an oxygen sensor (PyroScience GmbH, Aachen, Germany) contained within a glass case measured the oxygen concentration of each channel after it had been depleted by the metabolism of the fish. Water in a header tank was oxygenated by use of an air stone and was pulled through the respirometry chambers (400ml) using a peristaltic pump (Cole Palmer, London, UK), which also allowed for the control of the flow rate of the water. From a sump tank it was then pumped through a UV steriliser (v2 Vecton 600, Tropical Marine Centre, Bristol, UK) back up to the header tank (Figure 4-3). The water bath was insulated to maintain water temperature and the bath was covered with a sheet to minimise fish activity. Water temperature in the respirometer system was maintained at 12.5˚C by a chiller and noted in order to estimate the capacitance of oxygen in the water (βWO_2, ml $O_2.L^{-1}$).

During respirometry measurements, the oxygen concentration of the water flowing out of the respirometry chamber was measured using FireSting software (PyroScience). Four multichannel oxygen meters (FireStungO2, PyroScience) each simultaneously captured the oxygen levels of four respirometry chambers; this system was replicated four times to allow monitoring of the sixteen chambers. The oxygen meters also included a temperature sensor to capture continuous temperature data for oxygen concentration calculations. During measurements, data were recorded every two seconds. Prior to each batch of measurements, each oxygen electrode was calibrated against both fully aerated water (100% saturation) and a solution with 0% oxygen saturation (desaturation achieved via sodium sulphite in 0.01M sodium tetraborate), both at the same temperature as the water within the respirometry system. Further details of the system can be found in Appendix S2 of Auer et al. (2015).

All respirometry was carried out April – May 2018. Following acclimation and a 48-hour starvation period, maximum metabolic rate (MMR) data was gathered first, followed by collection of standard metabolic rate (SMR) data and finally the SDA of the fish was measured. The timeline of the experiment is detailed in Figure 4-4.

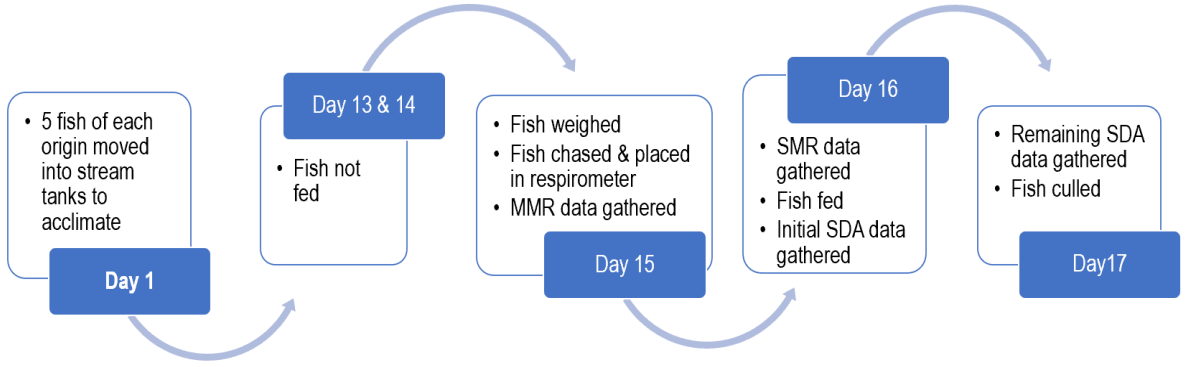

Figure 4-4: An overview of the timeline of the experiment. *5 Atlantic salmon from each origin (farmed, ranched and wild) went through this experimental process at a time. There were six replicates of this 17-day protocol (with staggered start dates), so that 90 experimental fish were processed in total.*

4.3.3.2. *Maximum metabolic rate measurements*

The maximum metabolic rate (MMR) of each individual was recorded using continuous flow respirometry, measuring oxygen consumption immediately after the fish had undergone exhaustive exercise (a protocol recommended for measuring

MMR (Norin and Clark, 2016; Zhang et al., 2020b)). This burst swimming performance was elicited by chasing each fish within a circular bucket, until it could no longer swim and ceased to evade capture in a net. The circular bucket was independent of the respirometry system but was filled with water from the water bath of the respirometry system in order to keep the water temperature the fish was exposed to consistent. At this point, the fish was immediately placed in its respirometry chamber and post-exercise oxygen consumption was measured, during which the flow rate of the water was 1.98 $L.h^{-1}$. The water temperature within both the chase-protocol bucket and the respirometry system was maintained at ~12.5˚C by control of air temperature and by using a water bath respectively.

MMR (mg $O_2.h^{-1}$) was calculated for each fish from the data collected during the first 3 minutes from when the fish was placed in the chamber after the exhaustive exercise, after allowing for a 30-second lag between the time the fish was placed in the chamber and the initial detection of the decrease in oxygen concentration by the sensor. The mean of the highest 10% of measurements of O_2 consumption was used to calculate MMR using the following equation:

$$M_{O2} = V_W\,(C_{WO2}\ control - C_{WO2}\ fish)$$

Where V_W is the flow rate through the chamber ($L.h^{-1}$), $C_{WO2}\ control$ is the concentration of oxygen ($mg.L^{-1}$) in the outflow of the empty control chamber and $C_{WO2}\ fish$ is the concentration of oxygen ($mg.L^{-1}$) in the outflow of the experimental chambers containing a fish.

Residual MMR (rMMR) was calculated by plotting MMR (mg $O_2.h^{-1}$) against body mass (g) and using the resulting regression ($y = 0.2312x + 3.2116$, $R^2 = 0.70$, $n = 88$) to determine an individual's expected MMR given its mass. The rMMR value was given by subtracting the expected MMR value from the actual MMR value. A positive residual indicated the fish had a higher MMR than expected from its mass, whilst a negative residual indicated an MMR that was lower than expected from the mass of the fish. rMMR was not calculated for one fish due to a fault with an oxygen electrode during measurement. These mass-independent rMMR values were used in any subsequent analyses that used MMR as a predictor variable, whereas the raw MMR values were used when MMR was being used as the response variable (but with body mass included as an explanatory variable). This ensured that mass was

always being controlled for when forming statistical models, but also that it was only being controlled for once.

4.3.3.3. *Standard metabolic rate measurements*

Following MMR measurement, fish were left to settle within the respirometry system, and their oxygen consumption was measured continuously for the following 20 hours in order to calculate their Standard Metabolic Rate (SMR) (Figure 4-4). Flow rate was maintained at 1.98 $L.h^{-1}$ for the duration of the SMR data collection period. This flow rate was chosen in order to maintain oxygen concentration at >80% within the chambers. Oxygen consumption (mg $O_2.h^{-1}$) during this period were measured using the same equation as for MMR.

SMR was then calculated for each fish by taking the mean of the lowest 10% of O_2 consumption measurements over the 20h period following MMR measurements and excluding any outliers, which were classified as any values greater than 2 standard deviations from the mean (Clark et al., 2013). All measurements were adjusted for both temperature and barometric pressure. Calculating the mean of the lowest 10^{th} percentile of M_{O2} facilitated consistency and accounted for any spontaneous activity performed by the fish throughout the measurement period.

rSMR was calculated in the same way as rMMR: the regression relating SMR ($mg.O_2.h^{-1}$) to fish mass (g) ($y = 0.1274x - 0.128$, $R^2 = 0.70$, $n = 77$) was used to determine each individual's expected SMR given its mass. It was only possible to collect SMR data for 77 out of 89 individuals due to oxygen sensor failures. The rSMR value was given by subtracting the expected SMR value from the actual SMR value. A positive residual indicated the fish had a higher SMR than expected from its mass, whilst a negative residual indicated an SMR that was lower than expected from the mass of the fish. As for rMMR, the choice between using SMR and rSMR values depended on the nature of the analysis, to ensure that mass was always controlled for.

4.3.3.4. *Specific dynamic action measurements*

In order to quantify their specific dynamic action (SDA), the fish were fed bloodworms at the end of the SMR measurement period but whilst they were still inside the respirometry chambers. At this point, they had been deprived of food for

72 hours (the 48 hours prior to MMR measurement and during the 24hr SMR measurement), as outlined in Figure 4-4. All fish were fed 0.15% of their body mass (measured prior to MMR measurements) in order to obtain comparable digestive response data; the amount was chosen to represent a small-medium sized meal that all fish would be capable of consuming. Once all the bloodworms had been consumed, respirometry was carried out with the same methods used to gain SMR data, but with a reduced flow rate (1.59L.h^{-1}). This reduced flow rate was chosen as SDA metabolic rates during digestion of a comparatively small meal are far lower than those of MMR, so the flow rate did not need to be as high as earlier in order maintain water oxygen saturation at a high level. Oxygen consumption (mg O_2.h^{-1}) was recorded over the following 24 hours, which has previously proven to be sufficient time to allow the metabolic rate of an individual to return to its pre-prandial level (Millidine et al., 2009).

SDA was analysed in relation to 3 parameters: peak SDA (mg O_2.hr^{-1}), measured by subtracting the SMR from the maximum postprandial MR; time taken after food ingestion to reach this peak SDA (minutes); and duration of the SDA response (minutes), quantified as the time elapsed between completion of the meal and the point when the rate of oxygen consumption returned to the pre-prandial level. The 'pre-prandial' level was determined by taking the average MR for the 4 hours preceding feeding. Oxygen consumption was classified as having returned to this level as the point at which the individual maintained this MR (or lower) for > 5 minutes subsequent to feeding. These parameters were quantified for all 26 individuals that consumed the full meal within an hour of being fed (the remainder only ate a partial meal and so their SDA response would not be comparable).

4.3.3.5. *Aerobic scope measurements*

Absolute aerobic scope (AS) for each fish was calculated by subtracting its standard metabolic rate from its maximum metabolic rate (mg O_2.h^{-1}). Plotting AS (mg$O2.h^{-1}$) against mass (g) resulted in the regression ($y = 0.1375x + 2.7576$, $R^2 = 0.43$, $n = 77$), which was used to determine residual aerobic scope (rAS = actual AS – expected AS) in the same manner as rMMR and rSMR.

At the mid-point of the experiment, once respirometry measurements for 3 of the 6 batches of fish had been completed, the respirometry equipment was cleaned with

bleach, drained and refilled before being used for the final batches of fish. These respirometry protocols were repeated across 6 batches of fish, allowing data to be collected for 88 individuals (30 farmed, 30 ranched and 28 wild) for rMMR and 77 individuals (29 farmed, 25 ranched and 23 wild) for rSMR and rAS, across a mass range of 7.32 – 43.19g (mean mass = 17.21 ± 7.89g S.D.).

4.3.4. Environmental and Atlantic Salmon Parr Gut Samples: Collection and Processing

Following collection of all respirometry measurements, fish were killed by anaesthesia overdose (benzocaine), followed by severing of the spinal cord. The wet weight (to nearest 0.01g) and fork length (to nearest 0.1mm) of each individual were measured. To collect the gastrointestinal samples, an incision was made along the ventral side of the fish. The gut, from the stomach to the anus, was removed and the pyloric caecae and hindgut were separated into labelled cryotubes. These were then immediately stored in liquid nitrogen for subsequent analysis.

Each individual was re-weighed (to nearest 0.01g) to gain the individual's wet mass without inclusion of the gastrointestinal tract. Each fish was then cut into three pieces and placed into a drying oven at 60˚C. After approximately 70 hours, the fish carcass was removed from the drying oven and the total dry mass of the three parts recorded (to nearest 0.01g). The wet mass (g, excluding the gut) and dry mass (g) were then used to determine the % water content where

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

Throughout the experimental period, environmental samples were collected every 4 days as follows: on each occasion, 3 biofilm and 3 water samples were taken from randomly selected stream tank compartments (each from a separate compartment, so 6 compartments in total). Biofilm samples were taken with cotton swabs by swabbing the inside of the stream tank compartment. Water samples were taken by passing 1L of water through a filter (Minisart single use filter, 16534-K, CE 0120) using a peristaltic pump. Each filter paper and swab was immediately placed into its own cryotube (Cryo-Vial Int Thd FS, Ref:LW3534) and stored in liquid nitrogen for subsequent analysis. These were subsequently processed (DNA extraction, PCR and sequencing) alongside tissue samples.

4.3.4.1. *Environmental and gastrointestinal samples: DNA extraction*

In order to extract bacterial DNA from the gastrointestinal and environmental samples, the QIAamp DNA Stool Mini Kit (Qiagen) was used according to the manufacturer's protocol with the modifications described in the previous chapter. Following extraction, DNA concentration was quantified by NanoDrop spectrometry.

4.3.4.2. *Environmental and gastrointestinal samples: PCR amplification and sequencing*

For primary PCR reactions, variable region 1-2 of the 16S rRNA gene was targeted with the primer pair CS1_27F and CS2_338R (full details of which are given in the previous chapter). These primers were again tagged CS1 for the forward sequence and CS2 for reverse (Figure 3-1). Primary PCR reactions were performed in triplicate and pooled after amplification, using the same reaction volumes and PCR conditions as described in the previous chapter. The PCR product was verified on a 1.7% agarose gel using TBE buffer.

As described in the previous chapter, the first round PCR products were then used as templates for 2nd round PCR, in which DNA barcodes were attached in order for subsequent sequencing to be performed and latterly demultiplexed for each sample. The same universal forward primer PE1_CS1_Fwr was used (the sequence of which is given in the previous chapter), alongside a barcoded reverse primer 5' CAA GCA GAA GAC GGC ATA CGA GAT XXX XXX XXT ACG GTA GCA GAG ACT TGG TCT 3', specific to each sample. A map of the final sequencing construct can be seen in Figure 4-1. The reaction volumes and PCR conditions used in the previous experiment were used again, the barcoded PCR products were verified on a 1.7% agarose gel using TBE buffer.

Upon visualisation, if a product produced >1 band, it was manually excised from the gel using a scalpel blade and purified using PureLink Quick Gel Extraction Kit (Invitrogen). If only the single, target band was produced, the remaining product was purified using the Agencourt paramagnetic bead clean-up (Agencourt AMPure XP,

Beckman Coulter): a 0.8:1 volume of AMPure XP beads to PCR product was used to remove primer dimers. The final concentration (µg/ml) of each product was then determined with a Qubit fluorometer. The amplicons were then pooled in equal concentration and the final library was sequenced using an Illumina Mi Seq® NGS system at Glasgow Polyomics, University of Glasgow.

4.3.5. Data Analyses

4.3.5.1. *Statistical analyses of metabolic rate data*

All statistical analyses of metabolic rate data were performed in R.3.5.1 (R Development Core Team) using moments (Komsta and Novomestky, 2015), rms (Harrell Jr, 2019), MuMin (Barton, 2019) and e1071 (Meyer et al., 2019) packages for model diagnostics.

Following calculation of residuals, two individuals (one of wild origin and one of ranched) were dropped from all subsequent analyses due to clearly incorrect original inputting of data. Prior to analysis, all continuous data within each data frame were centred in order to reduce the risk of multicollinearity, tested for via the rms package in R (Harrell Jr, 2019).

Linear Models (LMs) were used to explore relationships between metabolic rate, water content, mass and origin. The effect of origin and mass on metabolic traits was investigated using linear models (LMs) with metabolic rate (SMR, MMR or AS, $mgO_2.h^{-1}$) as the response variable, and origin (categorical variable with three levels: farmed, ranched or wild) and mass, including their interaction, as explanatory variables. If not significant, interactions were removed sequentially, starting with those with the smallest F-values. Similarly, models with SDA parameter (time to peak SDA (min), SDA duration (min) and peak SDA ($mgO_2.h^{-1}$)) as the response variable were run with metabolic traits (rSMR, rMMR and rAS) and mass, each interacting with fish origin, as explanatory variables. Final models were chosen based on AICc and visual inspection of residual plots (scale-location, Cook's distance, q-q plots). Continuous variables were log-transformed if doing so improved the fit of the model. Significance testing was used to indicate the strength of observed relationships. When the factor 'origin' was found to be significant for a

level, F-tests were used to determine whether the overall effect of origin was significant.

As fish underwent respirometry in batches of 15, the effect of batch was tested for using linear models and found to be significant within the SMR ($mgO_2.h^{-1}$), MMR ($mgO_2.h^{-1}$), time to peak SDA (min) and mass data. Consequently, linear mixed effects models were used when exploring these response variables, using the lmerTest package in R (Kuznetsova, 2017) (which automatically calculates t-tests using Satterthwaite approximations to degrees of freedom), with the inclusion of (1|batch) as a random effect.

4.3.5.2. *Bioinformatic analyses*

Sequencing data from all experiments pertaining to this thesis were processed together, so the same processes as described in the previous chapter were used for the bioinformatic data from this experiment. As before, the reverse reads were of higher quality than forward so only the reverse reads were trimmed, filtered and decontaminated as previously described. De-novo sequence clustering was once more performed with VSEARCH before operational taxonomic units (OTUs) were taxonomically classified against the SILVA database (Quast et al., 2013) and annotated using the QIIME2 classifiers (Bolyen et al., 2019), before the software FASTTREE (Price et al., 2009) was used to build an OTU tree specific to the bioinformatic data resulting from this experiment.

As in the previous chapter, for downstream analysis of sequencing data, samples were separated by section of the gut from which they were taken (i.e. foregut or hindgut) and data from the two gut sections were analysed separately. In total, there were 61 foregut samples (23 farmed, 22 ranched and 16 wild) and 71 hindgut samples (26 farmed, 26 ranched and 19 wild) that yielded sufficient sequencing depth for downstream analysis; not all samples could be analysed due to low quality of the sequencing data.

To assess the diversity of OTUs within each sample, alpha-diversity was evaluated in terms of both species richness and the Shannon effective number of species counts. The Shannon effective counts represent the Shannon diversity index as true alpha diversity of OTUs, as first proposed by Lu Jost (Jost, 2006); the use of this

"effective" number allows easier comparison and interpretation between populations. Linear Models (LMs) were used to explore relationships between microbial alpha diversity traits and metabolic rate (rSMR, rMMR, rAS), percentage water content, fish mass and fish origin. In these models, microbial species richness or Shannon effective was used as the response variable, and origin (categorical variable with three levels: farmed, ranched or wild), mass and metabolic traits (rSMR, rMMR and rAS) as explanatory variables. Initially, interactions between fish origin and each other explanatory variable were included. Due to the lower sample size of the SDA data, models including SDA parameters (time to peak SDA (min), SDA duration (min) and peak SDA ($mgO_2.h^{-1}$)) as explanatory variables were run separately. As before, these models were initially formed by including fish origin both as a main effect and via its interactions with each explanatory variable. Within all models, if interactions were not significant, they were removed sequentially, starting with those with the smallest F-values. Final models were chosen based on AICc and visual inspection of residual plots (scale-location, Cook's distance, q-q plots). Significance testing was used to indicate the strength of observed relationships. When the factor 'origin' was found to be significant for a level, F-tests were used to determine whether the overall effect of origin was significant.

To assess similarity between different microbial profiles, beta diversity was assessed using generalised UniFrac, which offers a balance between weighted and unweighted UniFrac, as these assign too much weight to rare or abundant lineages when used in isolation (Chen et al., 2012). Unconstrained non-metric Multi-Dimensional Scaling (NMDS) was used to visualise the beta-diversity of the gut microbiota, in which separation of the microbial communities was assessed via PERMANOVA. Beta diversity analyses including environmental samples can be seen in Appendix 4-1. Both alpha and beta diversity analyses were performed in R using the Rhea package (Lagkouvardos et al., 2017).

Taxonomic binning was performed using the SILVA database (Quast et al., 2013) as a reference. Stacked bar plots were formed to show the taxonomic composition and relative abundance across the foregut and hindgut samples, with the threshold abundance of OTUs set to 0.25 to allow for better visualisation within the plots (Appendix 4-2 and 4-3). As in Chapter 3, differential abundance analyses were performed, but this time to find microbial genera that were significantly different in their relative abundance between origins. The DESeqDataSetFromMatrix function

from DESeq2 package (Love et al., 2014) was once again used, with the same adjusted p-value cut-off of 0.005 and log2fold chance cut-off of 2. This function uses negative binomial GLM to obtain maximum likelihood estimates for OTUs log fold change between two origins at a time. Then Bayesian shrinkage was applied to obtain shrunken log fold changes before the Wald test was used for obtaining significance in each pairwise comparison. Log2fold was chosen in order to better visualise the data (Ijaz et al., 2018).

To illustrate how different explanatory variables contributed to any variation in microbial communities, distance-based redundancy analysis (dbRDA) was used. dbRDA is a constrained (canonical) ordination analysis, the details of which are given in the previous chapter. Redundancy analysis with forward selection was performed to specifically select the environmental variables that explained variation within the microbial communities (Vass et al., 2020). After forward selection was carried out with the ordistep function in the vegan package of R (Oksanen et al., 2019), dbRDA was applied on the significant variables using the capscale command (also in the vegan package of R). These processes were carried out separately for the foregut and hindgut bioinformatic data. The adonis2 function within the vegan package was again used to carry out statistical analyses of the dbRDA data, which uses PERMANOVA to test for similarity among samples based upon generalised UniFrac. Pearson correlation coefficients were once more calculated to complement the dbRDA analysis: this time, any correlations between the metavariables (fish mass, fish length, percentage water content, rSMR, rMMR and rAS) and the OTUs were assessed. The Pearson correlation for all pairs was calculated in R using the Rhea package (Lagkouvardos et al., 2017), with a p-value significance level of 0.05. To avoid underpowered analysis, the same cut-offs as applied in Chapter 3 were used, whereby OTUs that were present in <30% of the samples were removed and the number of pairs necessary to support a correlation was set to 4. An FDR (false discovery rate) correction was applied, and the resulting correlation analyses were visualised in a graphical display to showcase whether metabolic rate or any fish physiological measure correlated with the presence of any OTUs.

4.4. Results

4.4.1. Morphological Data: Establishing the Initial Differences Between Farmed, Ranched and Wild Origins of Atlantic Salmon Parr

In order to understand whether origin (i.e. whether the fish was from farmed, ranched, or wild parents) might explain variation in host metabolic phenotype and gut microbial communities, other phenotypic differences between the origins had to be accounted for and the variance in data collected needed to be understood. Therefore, morphological properties such as mass and water content were assessed alongside metabolic rate data. An overview of the metabolic rate and weight data is shown in Table 4-1.

Table 4-1: The metabolic and weight data of experimental fish from the three origins (farmed, ranched and wild). Metabolic rate data was quantified by continuous flow respirometry. Each metabolic rate (mg O_2.hr^{-1}) was converted into its respective residual value (rSMR, rMMR and rAS) which corrects for fish mass (see text for details).

	Farmed	**Ranched**	**Wild**
Mean Mass (g)	14.76 (n=30)	13.36 (n=29)	23.82 (n=28)
Mass Range (g)	7.52 – 30.52	7.32 – 21.73	10.22 – 43.19
Mean SMR (mg O_2.hr^{-1})	1.70 (n=29)	1.64 (n=25)	2.68 (n=23)
Mean rSMR (mg.O_2.h^{-1})	-0.01	0.09	-0.09
Mean MMR (mg O_2.hr^{-1})	6.31 (n=30)	6.27 (n=29)	9.15 (n=28)
Mean rMMR (mg.O_2.h^{-1})	-0.31	-0.04	0.43
Mean AS (mg O_2.hr^{-1})	4.43 (n=29)	4.56 (n=25)	6.29 (n=23)
Mean rAS (mg.O_2.hr^{-1})	-0.31	-0.02	0.41

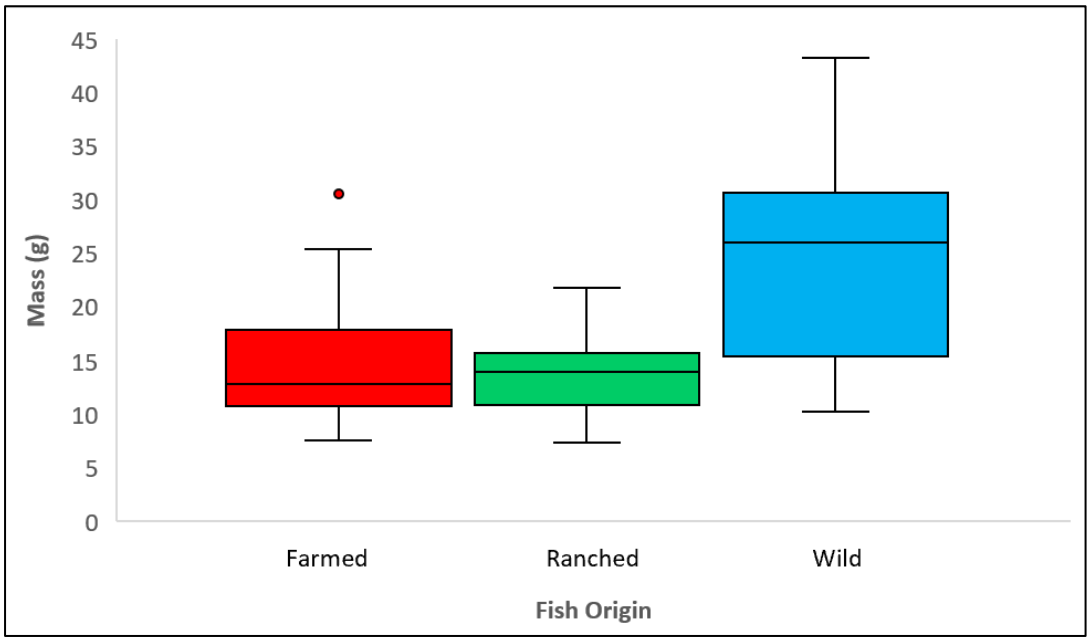

Figure 4-5: A boxplot showing the body mass (g) of farmed, ranched and wild Atlantic salmon parr *used in the experiment, with median, minimum and maximum values shown.*

Table 4-2: Results of linear mixed effects model examining the impact of origin on mass of the fish, with batch included as a random effect.

Fixed effects	Estimate	Standard Error	df	t-value	P
(Intercept)	-2.451	1.857	7.592	-1.319	0.225
Origin – Ranched	-1.403	1.432	79.006	-0.980	0.330
Origin – Wild	9.337	1.445	79.020	6.461	<0.001

While the mass range of experimental fish showed extensive overlaps between the three origins, there was a difference in mean size (Figure 4-5; Table 4-2), confirmed by an F-test ($F_{2, 79.027} = 32.00$, $P < 0.001$), with wild fish being significantly heavier for their age than ranched or farmed. Mass was therefore controlled for throughout all statistical analyses by including mass as a covariate when raw metabolic rate (SMR, MMR or AS, $mgO_2.h^{-1}$) was the response variable, or by using residual metabolic rate (rSMR, rMMR and rAS) when metabolic rate was included as an explanatory variable, as these residual values control for mass.

4.4.2. Metabolic Rate Data: Understanding the Drivers Behind Variation in Metabolic Rate of Atlantic Salmon Parr

4.4.2.1. *Standard metabolic rate of Atlantic salmon parr*

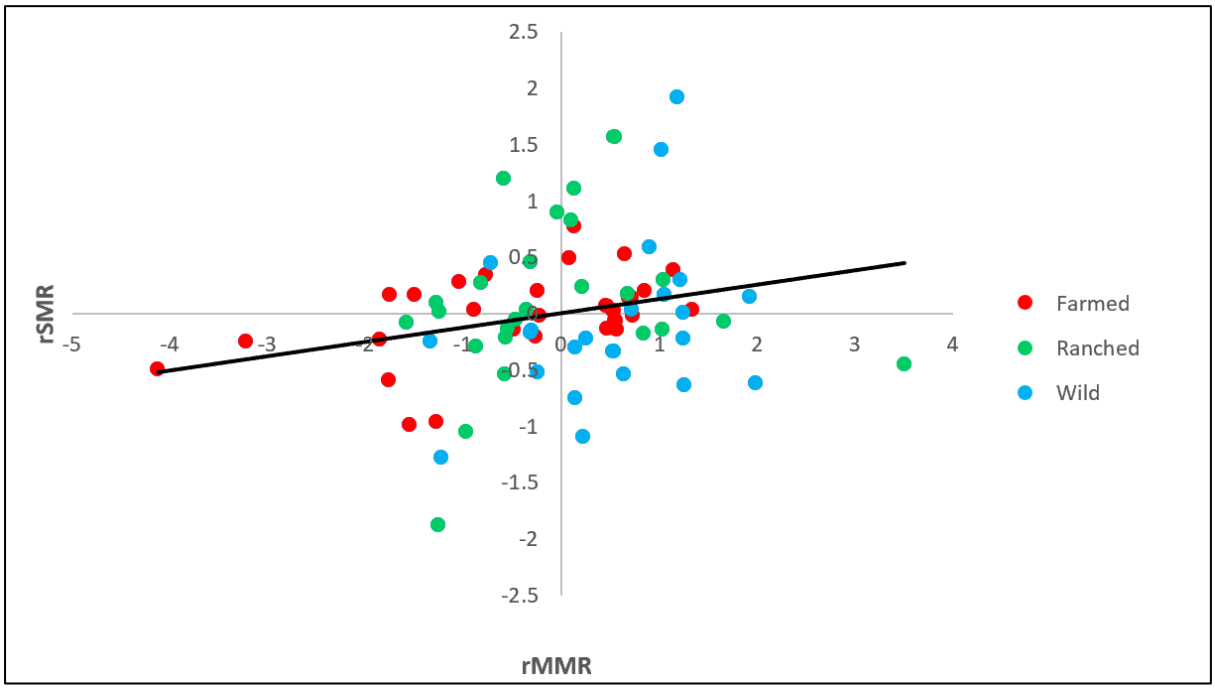

Figure 4-6: The relationship between rSMR (mg $O_2.hr^{-1}$) and rMMR (mg $O_2.hr^{-1}$) in Atlantic salmon parr. *Red, green and blue points represent farmed, ranched and wild individuals respectively (n = 77 in total). Note that the statistical analysis presented in Table 4-3 is based on SMR with mass included as a covariate, but here rSMR is used in order to show values corrected for body mass. See text and Table 4-3 for analysis.*

Table 4-3: *Results of linear mixed effects model examining the impact of origin, mass (g) and rMMR (mg $O_2.hr^{-1}$) on the SMR (mg $O_2.h^{-1}$) of the fish, with batch as a random effect.*

Fixed effects	Estimate	Standard Error	df	t-value	Pr (>|t|)
(Intercept)	0.099	0.195	6.597	0.505	0.630
rMMR (mg.$O_2.h^{-1}$)	0.145	0.052	67.037	2.813	0.006
Mass (g)	0.104	0.011	71.454	9.500	<0.001
Origin – Ranched	0.027	0.138	66.638	0.197	0.844
Origin – Wild	0.044	0.175	69.205	0.251	0.803

Standard metabolic rate measurements were available for 77 fish. The relationship between SMR and MMR was investigated: rSMR was found to increase with an individual's rMMR (Figure 4-6; LM, $F_{1, 75} = 4.78$, P = 0.032). A linear mixed effect model showed that there was a positive effect of both mass and rMMR on SMR (mg $O_2.h^{-1}$), whilst fish origin had no effect (Table 4-3). This shows that overall, as an individual's MMR increased, so too did its SMR, but there was no difference in this relationship between the three fish origins (farmed, ranched and wild).

4.4.2.2. *Maximum metabolic rate of Atlantic salmon parr*

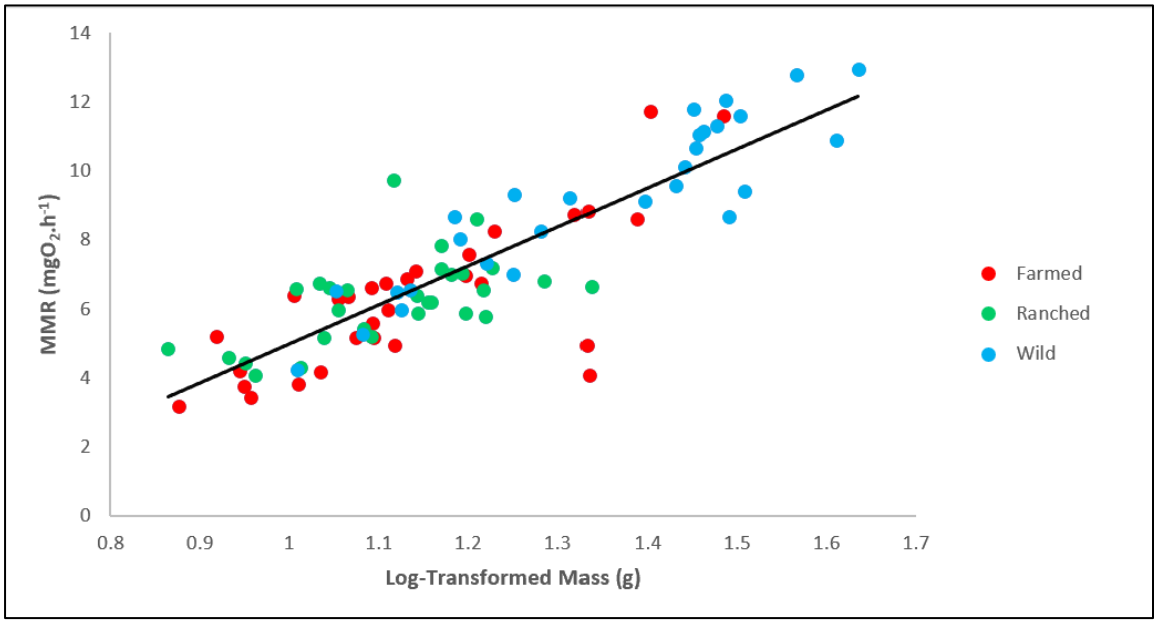

Figure 4-7: The relationship between MMR (mg $O_2.hr^{-1}$) and log-transformed mass (g) in Atlantic salmon parr. *Red, green and blue points represent farmed, ranched and wild individuals respectively (n = 87 in total). See text and Table 4-4 for analysis.*

Table 4-4: results of linear mixed effects model examining the impact of origin and log-mass (g) on the MMR (mg O2.hr-1) of the fish, with batch as a random effect.

Fixed effects	Estimate	Standard Error	df	t-value	P
(Intercept)	-0.317	0.221	83.0	-1.433	0.156
Log-Transformed Mass (g)	4.537	0.369	83.0	12.288	<0.001
Origin – Ranched	0.246	0.309	83.0	0.797	0.428
Origin – Wild	0.730	0.355	83.0	2.054	0.043

Maximum metabolic rate measurements were available for 87 fish. A linear mixed effect model showed a relationship between both log-transformed mass (Figure 4-7) and origin with MMR (mg $O_2.hr^{-1}$) (Table 4-4), revealing that MMR increased with body mass, but an F-test confirmed that the overall effect of origin was not significant (P = 0.13). Overall therefore, neither SMR nor MMR different significantly between farmed, ranched and wild fish, whilst both metabolic traits were found to increase with mass across all individuals.

4.4.2.3. *Aerobic scope of Atlantic salmon parr*

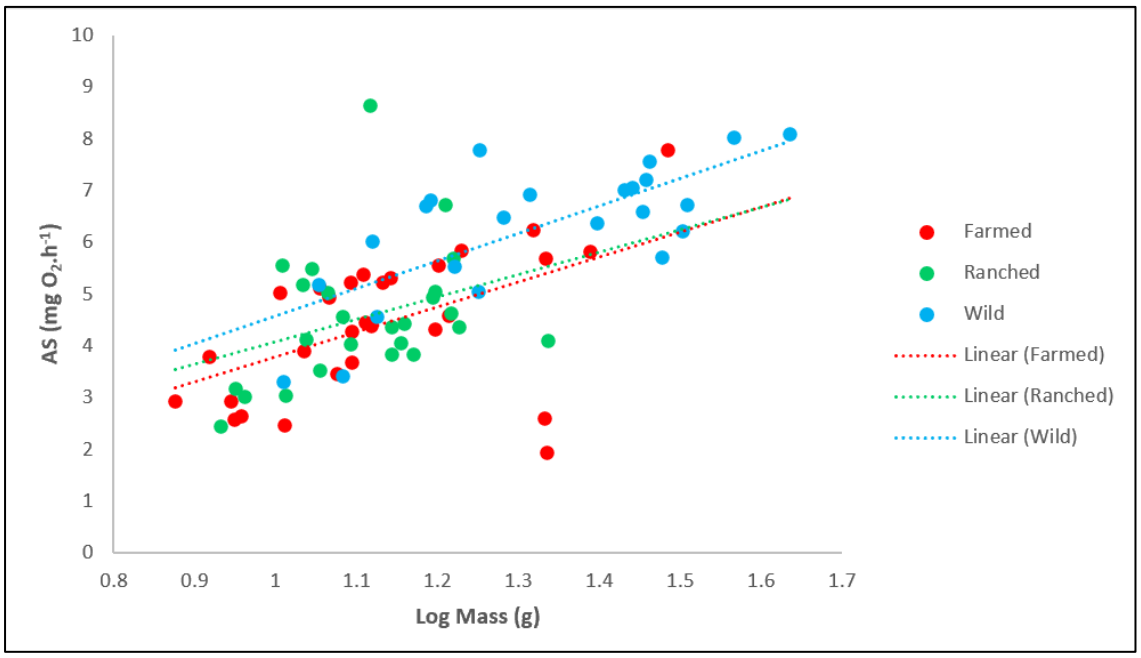

Figure 4-8: The relationship between AS (mg $O_2.hr^{-1}$) and log mass (g) for each origin of Atlantic salmon parr. *Red, green and blue represent farmed, ranched and wild individuals respectively (n = 77 in total). See text for analysis.*

Aerobic scope was calculated for 77 fish from the SMR and MMR data collected. There was a relationship between AS (mg $O_2.hr^{-1}$) and both log-transformed mass (LM, $F_{3, 73}$ = 24.76, P < 0.001) and origin, which an F-test confirmed was significant overall (Figure 4-8; $F_{2, 73}$ = 3.36, P = 0.04). A post hoc Tukey test showed that this was driven by the difference in aerobic scope between wild and farmed fish (P= 0.031), where that of wild fish was significantly greater. Fish of ranched origin were intermediate and not significantly different from those of wild or farmed.

In summary, these analyses of variation in metabolic traits indicate that fish origin only impacted aerobic scope, and the effect of body mass on host metabolic phenotype was more consistently prominent than the effect of origin.

4.4.2.4. *Water content of Atlantic salmon parr*

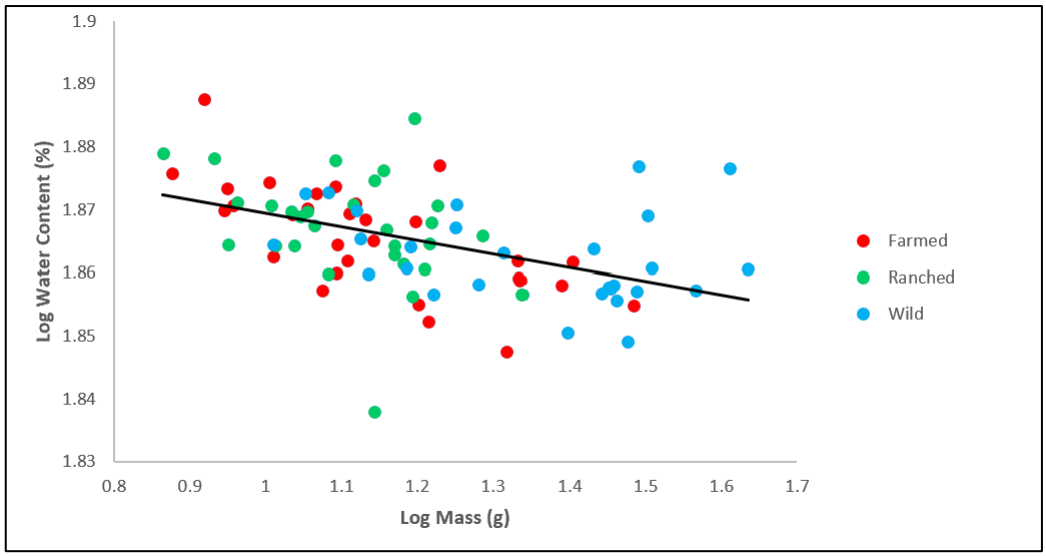

Figure 4-9: The relationship between log-transformed water content (%) and log-transformed mass (g) in Atlantic salmon parr. *Red, green and blue points represent farmed, ranched and wild individuals respectively (n = 87 in total). See text for analysis.*

Log-transformed water content (%) showed a decline with both an increase in log-transformed mass (Figure 4-9; LM, $F_{2, 74} = 16.61$, $P < 0.001$) and an increase in rSMR, but the trend with rSMR was not significant ($P = 0.232$). The increase in mass with a decrease in percentage water content is expected due to a lower percentage water content being indicative of a greater proportion of fat.

4.4.2.5. *Specific dynamic action in Atlantic salmon parr*

The cost of digestion (specific dynamic action, SDA) was analysed for the 26 fish that consumed their full ration within an hour of being fed. Following the meal, all fish exhibited a postprandial rise in MR, which is the prominent feature of an SDA response (Figure 4-1). The size of the peak of the SDA response (maximum MR during meal processing, measured in mg $O_2.hr^{-1}$) was used as a measure of the highest cost of digestion at any one time. The time taken to reach this peak (minutes) and total duration of the response (time taken in minutes between

consumption of the meal to MR returning to the pre-prandial level) were also analysed, to examine how long an individual's MR was impacted by meal consumption. Metabolic responses to feeding were examined within the 26 fish in relation to their origin and MR (rSMR, rMMR and rAS), their interactions were also tested, but none were found. As rSMR increased, all three SDA parameters (peak SDA (mg O_2.hr^{-1}), time to peak SDA (minutes) and SDA duration (minutes)) tended to decrease; however, the relationships between metabolic rate and SDA parameters were not found to be significant.

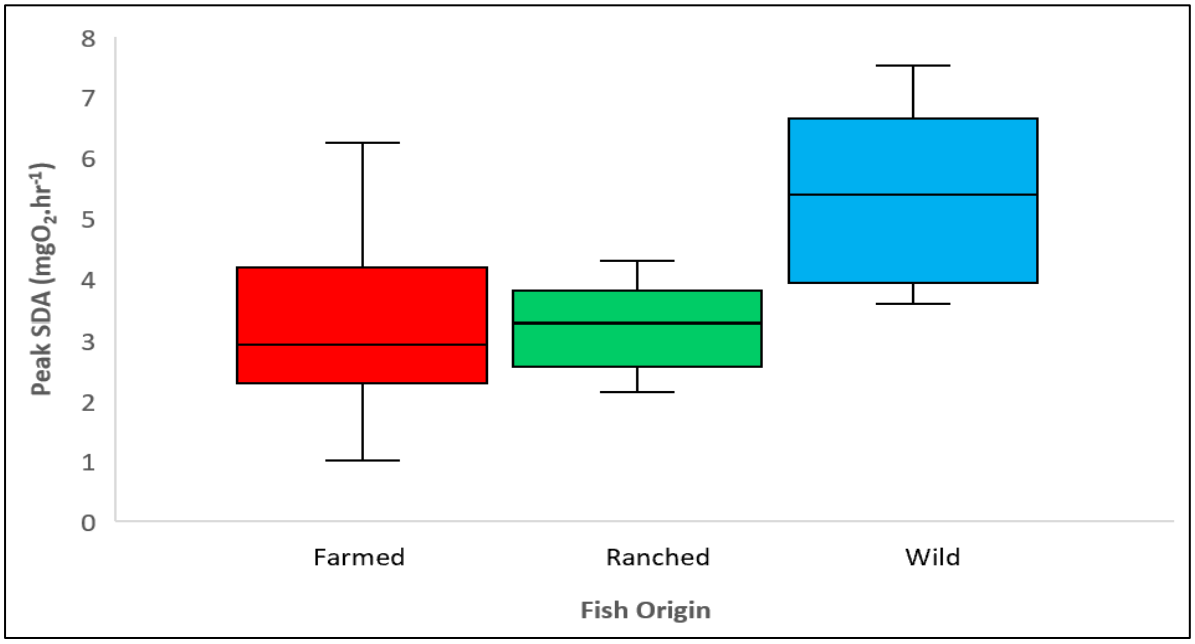

Figure 4-10: A boxplot showing the peak of the SDA response (mg O_2.hr^{-1}) for Atlantic salmon parr*, with median, minimum and maximum values shown. Red, green and blue represent farmed, ranched and wild individuals respectively (n = 26 in total). See text for analysis.*

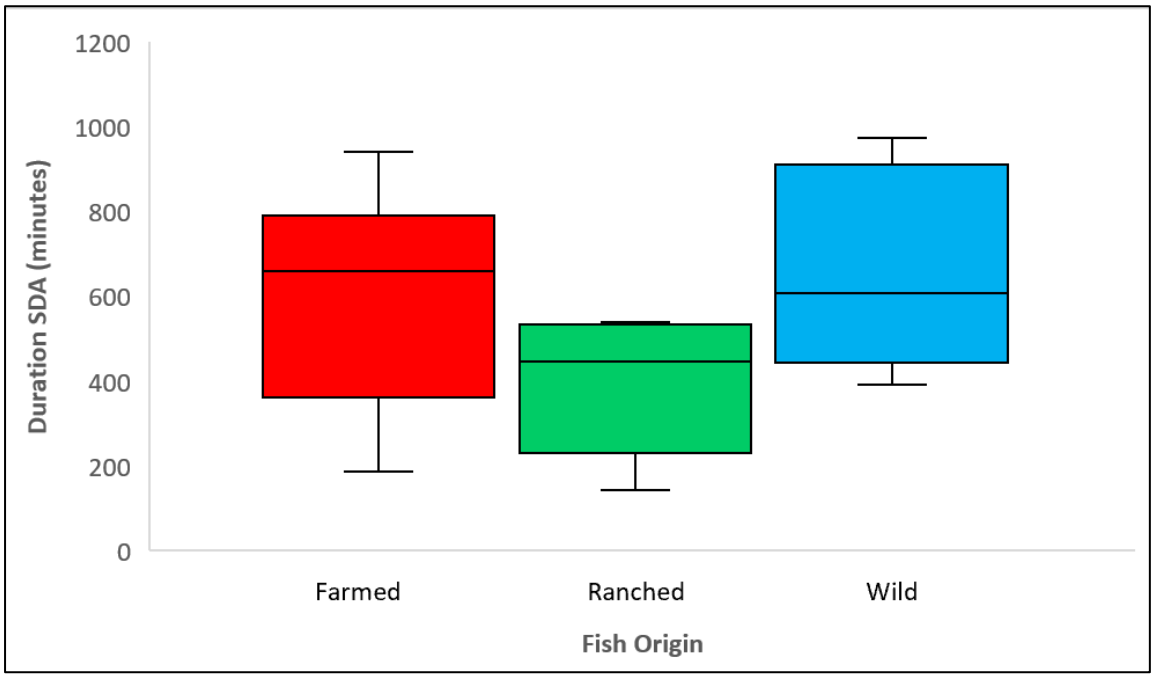

Figure 4-11: A boxplot showing the duration of the SDA response (minutes) for Atlantic salmon parr*, with median, minimum and maximum values shown. Red, green and blue represent farmed, ranched and wild individuals respectively (n = 26 in total). See text for analysis.*

The peak of the SDA response (mg O_2.hr^{-1}) was examined in relation to origin and mass. While mass was found not to be significant, there was a significant effect of origin (F-test, $F_{2, 22} = 5.26$, $P = 0.014$), with wild fish having a greater peak SDA response for a given size of meal relative to their body mass (Figure 4-10). No relationship was found between time taken for a fish to reach its peak SDA (minutes) and body mass or any metabolic variable (rSMR, rMMR and rAS), nor did this parameter differ between origins. However, the total duration of the SDA response (minutes) differed between the three origins of fish (F-test, $F_{2, 22} = 4.63$, $P = 0.021$), with the metabolic rates of ranched fish returning to the pre-prandial level more quickly than those of farmed or wild origin (Figure 4-11).

4.4.3. Bioinformatic Data: Assessing the Microbial Community within the Gut of Atlantic Salmon Parr

Gut microbiota samples were isolated from homogenates derived from two separate gut compartments. Since the foregut and hindgut of 89 fish were sampled, this produced a total of 178 samples. Following 16S rRNA extraction and amplification, 132 of these samples were successfully sequenced at the V1-2 region of the 16S rRNA gene. The total number of operational taxonomic units (OTUs) produced was 15,508, of which 13,857 could be assigned to a Phylum. By analysing the foregut (n = 61 samples; 23 farmed, 22 ranched, 16 wild) and hindgut (n = 71 samples; 26 farmed, 26 ranched, 19 wild) microbial profiles separately, it was possible to highlight any inherent differences between the two locations of the alimentary canal, whilst increasing the accuracy of the overall analyses. The data were examined in relation to alpha diversity to assess the diversity of OTUs within a sample; beta diversity to assess the similarity between different microbial profiles; differential abundance of OTUs to identify any taxa that had a different abundance between the three fish origins (farmed, ranched and wild); distance-based redundancy analysis to assess the main drivers of any variation in microbial community composition between the fish; and finally correlation analysis to identify any relationships between physiological variables and specific OTUs.

4.4.3.1. *Microbial alpha diversity within the gut of farmed, ranched and wild Atlantic salmon*

4.4.3.1.1. Alpha diversity metrics within the Atlantic salmon foregut

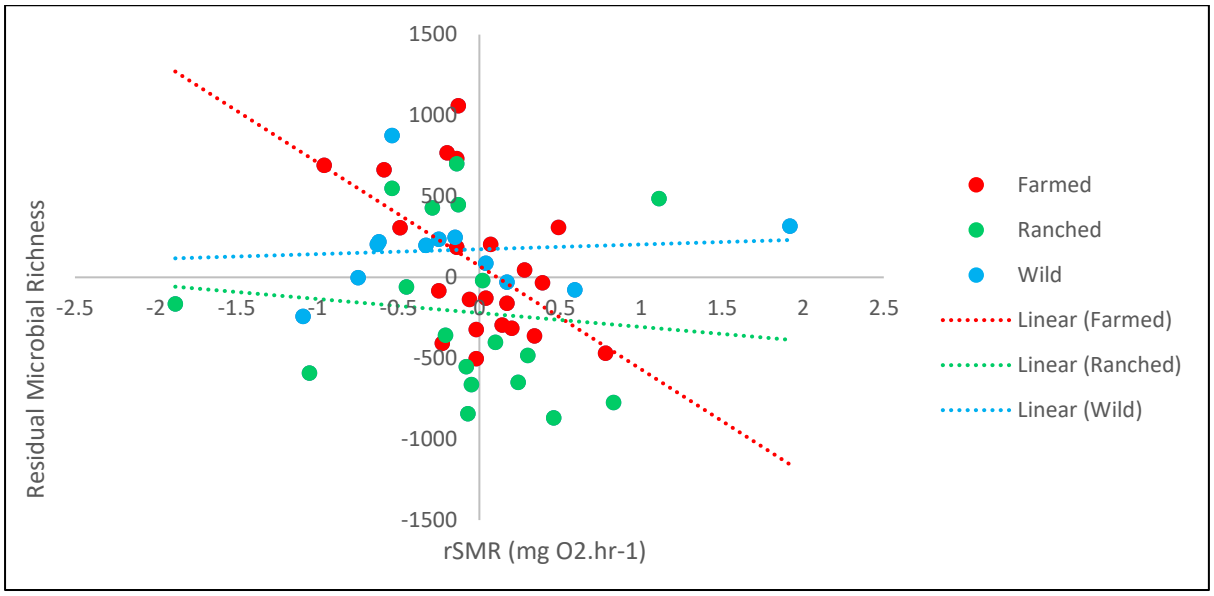

Figure 4-12: The relationship between residual microbial richness and rSMR (*mg O_2.hr^{-1}*) in the foregut of Atlantic salmon parr*, including the interaction between origin and rSMR. Red, green and blue represent farmed, ranched and wild individuals respectively (n = 52 in total). Note that though the statistical analysis was based on the (absolute) microbial richness, here the microbial richness is plotted as residual values after controlling for water content and rMMR, in order to illustrate the relationship with rSMR. See text for analysis.*

The alpha diversity metrics, microbial species richness and Shannon effective, were analysed both with and without the inclusion of SDA data. This allowed any relationships between alpha diversity and SDA data to be found, but due to the smaller SDA sample size, models without the inclusion of SDA data had greater power. Though SDA data was collected for 26 Atlantic salmon, sequencing data with adequate depth was not produced for every individual, resulting in 19 foregut samples with reciprocal SDA measures that could be used within linear models. Similarly, though sequencing data was produced from 61 foregut samples, 52 of these samples had associated metabolic rate data to use within linear models. The two separate alpha diversity measures were both assessed because microbial richness simply describes the number of OTUs within a sample, whilst the Shannon effective also considers the structure of the microbial community, by accounting for differential abundance of OTUs. The results of all linear models exploring alpha

diversity in the foregut of Atlantic salmon are summarised in Table 4-5. The microbial richness within the foregut of fish was assessed by including origin, water content, rSMR, rMMR and the interaction between rSMR and origin as explanatory variables. Within the foregut, richness was found to decrease with rSMR (LM; $F_{7, 44} = 2.616$, P = 0.008) and increase with percentage water content (P = 0.043), but there was also a significant interaction between origin and rSMR (Figure 4-12; F-test, $F_{2, 22} = 3.257$, P = 0.048), since the relationship between rSMR and richness was marginally positive for wild fish, marginally negative for ranched and strongly negative for farmed fish. To present the relationship between rSMR and richness accurately, the other covariates within the original model had to be controlled for by calculating residual microbial richness. To do so, rSMR and origin were removed from the original model in order to gain a measure of expected richness (when accounting for water content and rMMR), where expected richness = ((56.78 * water content) + (62.69 * rMMR) - 3111.07). This expected richness was subtracted from actual richness to give residual microbial richness, which could then be plotted against rSMR (Figure 4-12).

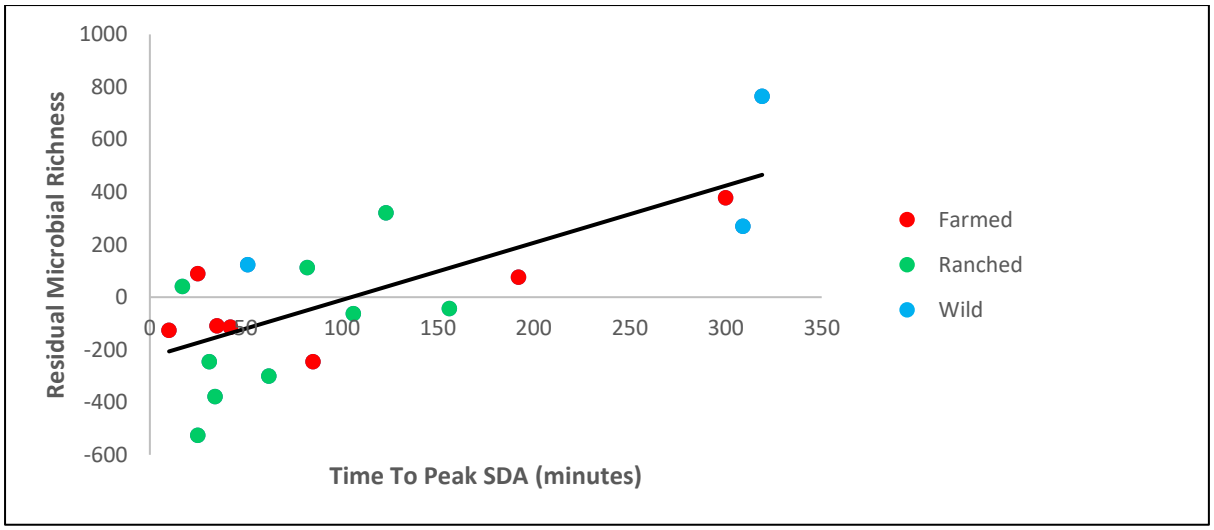

Figure 4-13: The relationship between residual microbial richness and time to peak SDA (minutes) in the foregut of Atlantic salmon parr. *Red, green and blue represent farmed, ranched and wild individuals respectively (n = 19 in total). Note that while the statistical analysis was based on the (absolute) microbial richness, here the microbial richness is plotted as residual values after controlling for water content and rSMR, in order to illustrate the relationship with time to peak SDA. See text for analysis.*

In spite of the smaller sample size for which SDA data was gathered, microbial community richness was analysed in relation to all SDA parameters (the peak of the

SDA response (mg $O_2.hr^{-1}$), the time taken to reach this peak (mins) and total duration of the response (mins)) to see whether an individual's metabolic response to feeding had an impact on microbial richness. Once the SDA data were included, the final model included water content, rSMR and time to the peak of the SDA response as explanatory variables. As found when using the full dataset, a positive relationship between microbial richness and percentage water content remained (LM; $F_{3, 15} = 26.49$, $P < 0.001$), as did the negative relationship between richness and rSMR ($P < 0.001$). There was no effect of origin, but time to reach the peak SDA increased with microbial richness (Figure 4-13; $P < 0.001$). To present the relationship between time to the peak of the SDA response and microbial richness accurately, the same approach as above was used to calculate residual microbial richness, this time removing time to peak SDA from the original model in order to gain a measure of expected richness (when accounting for water content and rSMR), where expected richness = ((217.6 * water content) + (-997.6 * rSMR) - 15101.6). This expected richness was subtracted from actual richness to give residual richness, which could then be plotted against time to peak SDA (Figure 4-13).

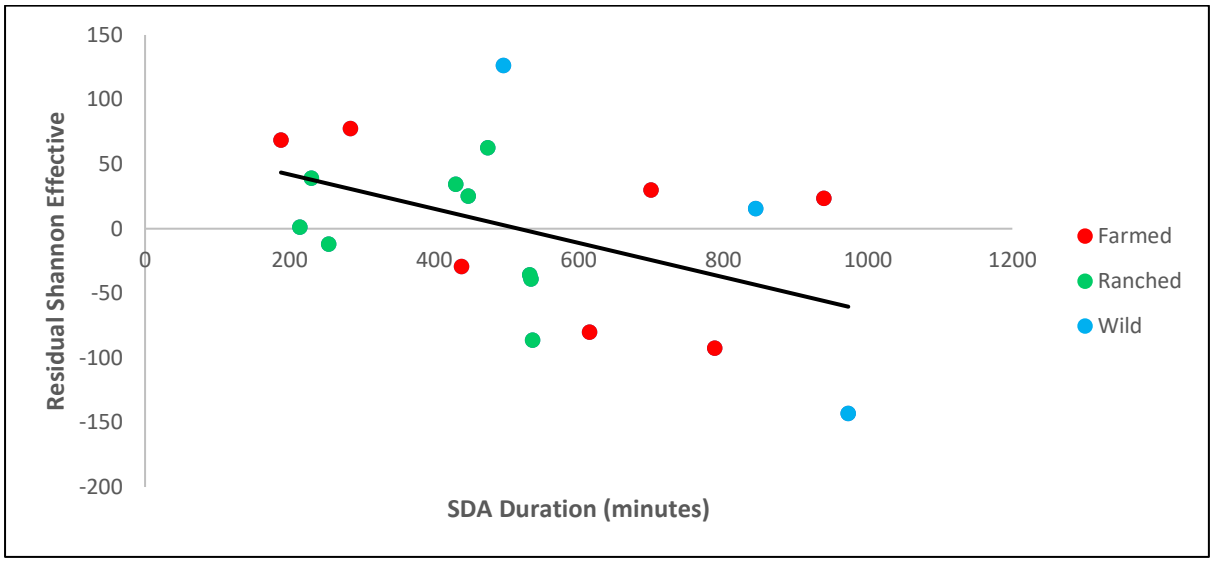

Figure 4-14: The relationship between residual Shannon effective (a measure of microbial diversity that controls for the effect of percentage water content and origin) and SDA duration (minutes) in the foregut of Atlantic salmon parr. *Red, green and blue represent farmed, ranched and wild individuals respectively (n = 19 in total). Note that while the statistical analysis was based on the (absolute) Shannon effective, here the Shannon effective is plotted as residual values after controlling for water content and origin, in order to illustrate the relationship SDA duration. See text for analysis.*

Initially, there was no relationship between the Shannon effective number of species and any explanatory variable tested, including no difference between the origins of fish. Once SDA parameters were included as explanatory variables, the final model included water content, origin and duration of the SDA response as explanatory variables. The Shannon effective was found increase with water content (LM, $F_{4, 14}$ = 12.1, P < 0.001) and decrease with SDA duration (Figure 4-14; P = 0.0038), and there was an overall effect of origin (F-test, $F_{2, 14}$ = 17.74, P < 0.001), driven by the higher Shannon effective in the foregut of wild fish in comparison to farmed (P = 0.0019) and ranched (P < 0.001). To present the relationship between SDA duration and Shannon effective accurately, the same approach as above was used, this time to calculate the residual Shannon effective by removing SDA duration from the original model, where expected Shannon effective = ((38.29 * water content) – 2677.61) for farmed, ((38.29 * water content) – 2677.61 – 60.37) for ranched and ((38.29 * water content) – 2677.61 + 120.56) for wild. This expected Shannon effective was subtracted from actual Shannon effective to give the residual Shannon effective, which could then be plotted against the SDA duration (Figure 4-14).

Table 4-5: A summary of the relationships between alpha diversity metrics and physiological measures within the Atlantic salmon foregut. For microbial richness, the results of two analyses are presented – that based on the larger sample size (n = 52 foregut samples) that excluded SDA as an explanatory variable, and on the smaller sample (n = 19 foregut samples) in which SDA was measured and included in the model. For the Shannon effective, the presented model also used the smaller sample (n = 19).

Response	**Explanatory**	**F value**	**P-value**
Richness (no SDA data)	Origin*rSMR	3.25	0.048
	Water content (%)	3.26	0.043
	rMMR (mg O_2.hr^{-1})	3.74	0.060
Richness (SDA data)	Water content (%)	24.66	<0.001
	rSMR (mg O_2.hr^{-1})	43.88	<0.001
	Time to peak SDA (minutes)	19.40	<0.001
Shannon Effective (SDA data)	Water content (%)	23.96	<0.001
	Origin	17.74	<0.001
	Duration SDA (minutes)	11.99	0.0038

4.4.3.1.2. Alpha diversity metrics within the Atlantic salmon hindgut

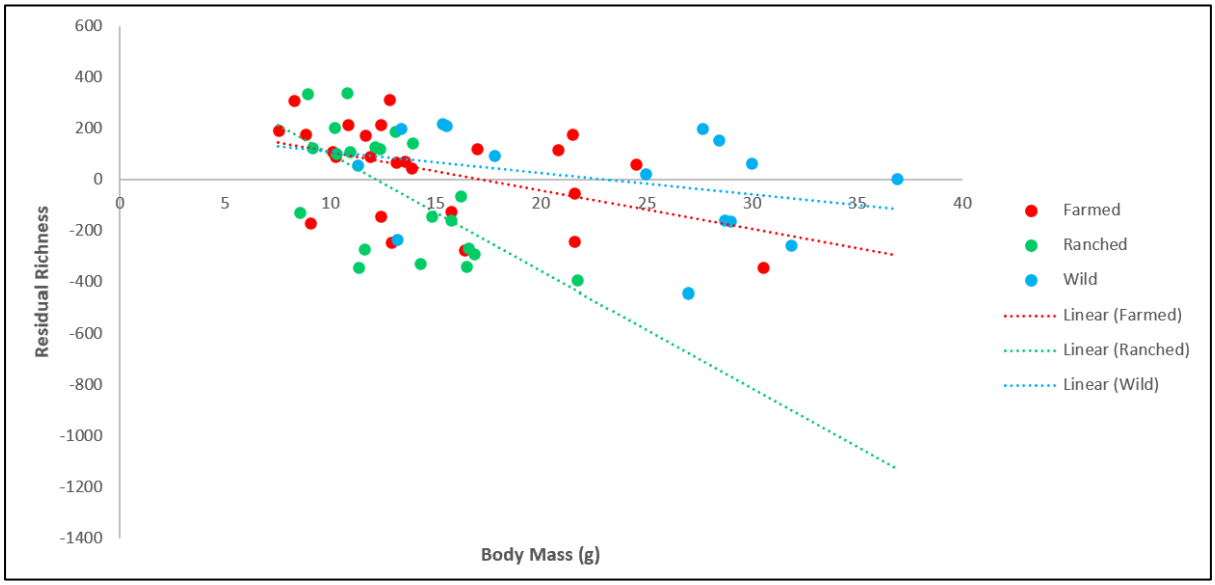

Figure 4-15: The relationship between residual microbial richness and body mass (g) in the hindgut of fish. *Red, green and blue represent farmed, ranched and wild individuals respectively (n = 61 in total). Note that while the statistical analysis was based on the (absolute) microbial richness, here microbial richness is plotted as residual values after controlling for rSMR in order to illustrate the relationship with body mass. See text for analysis.*

As with the alpha diversity models for the foregut data, models examining hindgut microbial richness and Shannon effective had reduced sample sizes: although SDA data was collected for 26 Atlantic salmon, sequencing data with adequate depth was not produced from the hindgut of every fish, resulting in 19 hindgut samples with reciprocal SDA measures that could be used within linear models. Similarly, though sequencing data was produced from a total of 71 hindgut samples, 61 of these samples had associated metabolic rate data to use within linear models. To assess microbial richness within the hindgut of fish, mass, rSMR and the interaction between mass and origin were included as covariates. Within hindgut samples, microbial richness decreased with an increase in rSMR (LM, $F_{6, 54} = 5.048$, $P = 0.013$). There was a significant interaction between mass and origin (F-test, $F_{2, 54} = 3.36$, $P = 0.042$), showing that the negative relationship between richness and mass was different in fish from the different origins (Figure 4-15). To present the relationship between body mass and microbial richness accurately, residual microbial richness by removing mass and origin from the original model in order to gain a measure of expected richness (when accounting for rSMR), where expected richness = ((-104.54 * rSMR) + 501.84). This expected richness was subtracted from actual richness to give residual microbial richness, which could then be plotted

against body mass (Figure 4-15). The results of all linear models exploring alpha diversity in the hindgut of Atlantic salmon are summarised in Table 4-6.

Once SDA parameters were included as explanatory variables, the final model included mass, water content, origin, rSMR and time to the peak of the SDA response as explanatory variables. Microbial richness was still found to decrease with an increase in mass (LM, $F_{6, 12} = 4.57$, P = 0.0042) and rSMR (P = 0.035), but the overall effect of origin was not significant (P = 0.12).

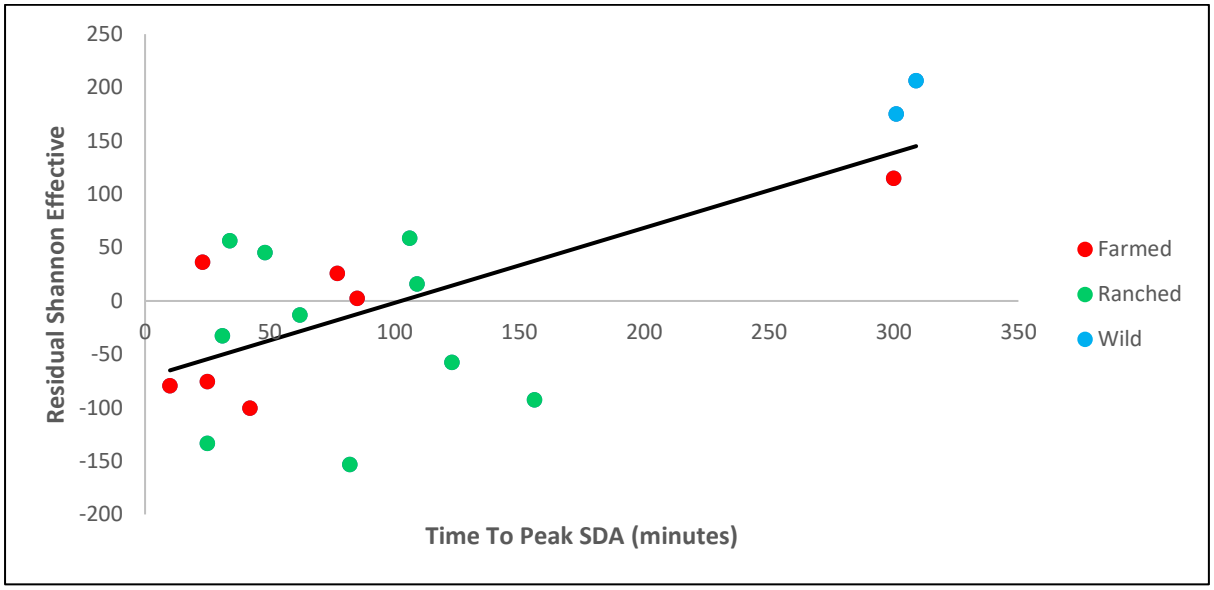

Figure 4-16: The relationship between the residual Shannon effective and time to peak SDA (minutes) in the hindgut of Atlantic salmon parr. *Red, green and blue represent farmed, ranched and wild individuals respectively (n = 19 in total). Note that while the statistical analysis was based on the (absolute) Shannon effective, here the Shannon effective is plotted as residual values after controlling for mass and length in order to illustrate the relationship with time to peak SDA. See text for analysis.*

When analysing the full data set in relation to the Shannon effective, the original model included mass, length, water content, origin, rSMR and rMMR. The Shannon effective was negatively related to mass (LM, $F_{6, 54} = 1.84$, P = 0.0097). Once SDA parameters were included as explanatory variables, the final model included mass, length and time to the peak of the SDA response as explanatory variables. As with the full data set, the Shannon effective was negatively related to mass (LM, $F_{3, 15} = 8.56$, P = 0.031), but was also found to be positively related to the time taken to reach the peak of the SDA response (Figure 4-16; P = 0.0014). To present the relationship between the time to the peak SDA and the Shannon effective accurately, the residual Shannon effective was calculated by removing the time to the peak SDA

from the original model in order to gain a measure of expected Shannon effective (when accounting for body mass and length), where expected Shannon effective = ((-29.259 * body mass) + (8.037 * length) – 316.289). This expected Shannon effective was subtracted from actual Shannon effective to give residual Shannon effective, which could then be plotted against the time to the peak SDA (Figure 4-16).

Table 4-6: A summary of the relationships between alpha diversity metrics and physiological measures within the Atlantic salmon hindgut. For microbial richness and the Shannon effective, the results of two analyses are presented – that based on the larger sample size (n = 61 hindgut samples) that excluded SDA as an explanatory variable, and on the smaller sample (n = 19 hindgut samples) in which SDA was measured and included in the model.

Response	**Explanatory**	**F value**	**P-value**
Richness (no SDA data)	rSMR (mg O_2.hr^{-1})	6.55	0.013
	Mass*Origin	3.36	0.042
Richness (SDA data)	Mass (g)	12.43	0.0042
	Water content (%)	3.69	0.079
	Origin	2.58	0.12
	rSMR (mg O_2.hr^{-1})	5.65	0.035
	Time to peak SDA (minutes)	3.13	0.102
Shannon Effective (no SDA data)	Mass (g)	7.18	0.0097
	Water content (%)	0.62	0.43
	Origin	2.79	0.070
	rSMR (mg O_2.hr^{-1})	0.33	0.57
	rMMR (mg O_2.hr^{-1})	1.29	0.26
Shannon Effective (SDA data)	Mass (g)	5.67	0.031
	Length (mm)	3.33	0.088
	Time to peak SDA (minutes)	15.24	0.0014

4.4.3.2. *Microbial community-composition differences within the gut of farmed, ranched and wild Atlantic salmon*

By using generalised UniFrac metrics to account for the phylogenetic distance between OTUs, multivariate analysis showed that there was a significant difference in microbial beta diversity between farmed, ranched and wild fish within both the foregut (Figure 4-17 [A], P = 0.003) and hindgut (Figure 4-17 [B], P = 0.006). The differences in composition were further explored by assessing which microbial genera were differentially abundant between the three origins of fish.

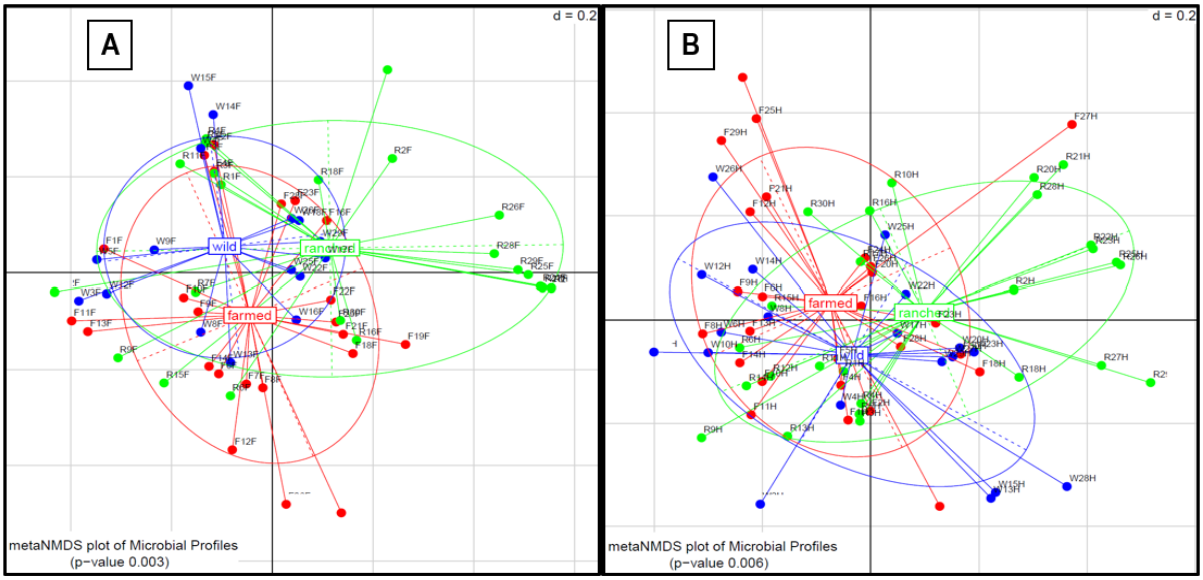

Figure 4-17: The difference in [A] foregut and [B] hindgut beta diversity of each origin illustrated by non-metric multidimensional scaling (NMDS) based upon generalised UniFrac. The dissimilarity scale of the grid, d = 0.2, indicates the distance between two grid lines represent approximately 20% dissimilarity between the samples. The p-values were calculated by permutational multivariate analysis of variance, which used the distance matrix to assess whether the separation of groups (samples from fish of either farmed, ranched or wild origin) was significant. *Green, red and blue points represent farmed, ranched and wild individuals respectively. Distinct clustering is observed between the origins.*

To assess which OTUs were differentially abundant between origins, the abundance of microbial genera within the guts of farmed, ranched and wild Atlantic salmon were compared in a pairwise manner. A significant p-value indicated that the abundance of a genus in one origin was significantly different from its abundance in another based upon the Wald test. Within both the foregut and hindgut, there were microbial genera that were log2 fold different in abundance between fish origins, shown in Figures 4-18 and 4-19 respectively. Within the foregut of Atlantic salmon, there were 30 microbial genera differentially abundant between farmed and ranched fish, 25 of

which were significantly more abundant within the foregut of farmed fish than within that of ranched. The genera differentially abundant between the foreguts of farmed and ranched Atlantic salmon were predominantly of the phyla Proteobacteria (e.g. *Legionella* and *Plesiomonas*), Actinobacteria (e.g. *Rothia* and *Friedmanniella*) and Firmicutes (e.g. *Thermicanus* and *Lactobacillus*) (Figure 4-18 [A]). Proteobacteria represented 10 of the 30 genera that were differentially abundant between the foreguts of farmed and ranched fish, but were most commonly over abundant within the foreguts of farmed fish when compared to those of the ranched, representing 36% (9 of the 25 genera) of the over abundant genera found within the foreguts of farmed individuals. Within ranched fish, genera from Actinobacteria and Firmicutes were most commonly over abundant, with each phylum accounting for 2 of the 5 over abundant genera within the foregut of ranched fish. There were 48 microbial genera differentially abundant between the foreguts of ranched and wild Atlantic salmon, 40 of which were significantly more abundant within wild fish than within ranched. The phylum Proteobacteria (e.g. *Ezakiella* and *Thermomonas*) characterised most of those microbial genera that were differentially abundant between ranched and wild fish, representing 50% (n = 4 genera) and 40% (n = 16 genera) of the microbial genera over abundant in ranched and wild fish foreguts respectively (Figure 4-18 [B]). Finally, within the foregut, 36 microbial genera were differentially abundant between farmed and wild Atlantic salmon, with 23 of these being over abundant within wild fish. Within both farmed and wild fish, Proteobacteria (e.g. *Aquabacterium* and *Reyranella*) once more accounted for the highest proportion of microbial genera that were differentially abundant between the two origins (17 of 36 differentially abundant genera), representing 46% (n = 6 genera) and 48% (n = 11 genera) of the over abundant genera in the foreguts of farmed and wild fish respectively. The other differentially abundant microbes between the foreguts of farmed and wild fish most commonly belonged to phyla Firmicutes (n = 7 genera: 2 in farmed, 5 in wild), Actinobacteria (n = 5 genera: 4 in farmed, 1 in wild) or Bacteroidetes (n = 5 genera: 1 in farmed, 4 in wild) (Figure 4-18 [C]).

Comparing the hindgut of Atlantic salmon, there were 15 microbial genera differentially abundant between farmed and ranched fish, 13 of which were significantly more abundant within the hindgut of farmed fish than within those of ranched. The phylum Proteobacteria (e.g. *Aliivibrio* and *Aeromonas*) accounted for 6 of these 15 genera, all of which were overabundant within the hindgut of farmed fish,

reflecting 46% of the over abundant microbial genera within the hindgut of farmed fish. Within the hindgut of ranched fish, the only 2 over abundant microbial genera belonged to phyla Actinobacteria (e.g. *Marmoricola*) and Deinococcus-Thermus (e.g. *Deinococcus*) (Figure 4-19 [A]). When comparing the hindguts of ranched and wild fish, 34 microbial genera were differentially abundant between the two origins, 25 of which were over abundant within wild fish. Within the hindguts of ranched fish, the over abundant microbial genera were primarily of Actinobacteria (e.g. *Brooklawnia* and *Sanguibacter*) and Firmicutes (e.g. *Acetivibrio* and *Carnobacterium*), with each phylum representing 3 of the 9 over abundant genera. Of the over abundant microbial genera within the wild fish hindgut, 60% (15 of the 25 genera) belonged to Proteobacteria (e.g. *Roseomonas* and *Massilia*) (Figure 4-19 [B]). Finally, within the hindgut, a total of 30 microbial genera were differentially abundant between farmed and wild fish, with 17 being over abundant within fish of wild origin. 50% (n = 15 genera; 3 in farmed, 12 in wild) of the overabundant genera belonged to Proteobacteria (e.g. *Lautropia* and *Sphingobium*). Firmicutes (e.g. *Turicibacter* and *Paraclostridium*) accounted for the next highest proportion of microbial genera that were differentially abundant between the hindguts of farmed and wild fish (n = 8 genera: 6 in farmed, 2 in wild). The other differentially abundant microbes between the hindguts of farmed and wild fish most commonly belonged to Actinobacteria (n = 3 genera: 2 in farmed, 1 in wild) or Bacteroidetes (n = 3 genera: 1 in farmed, 2 in wild) (Figure 4-19 [C]).

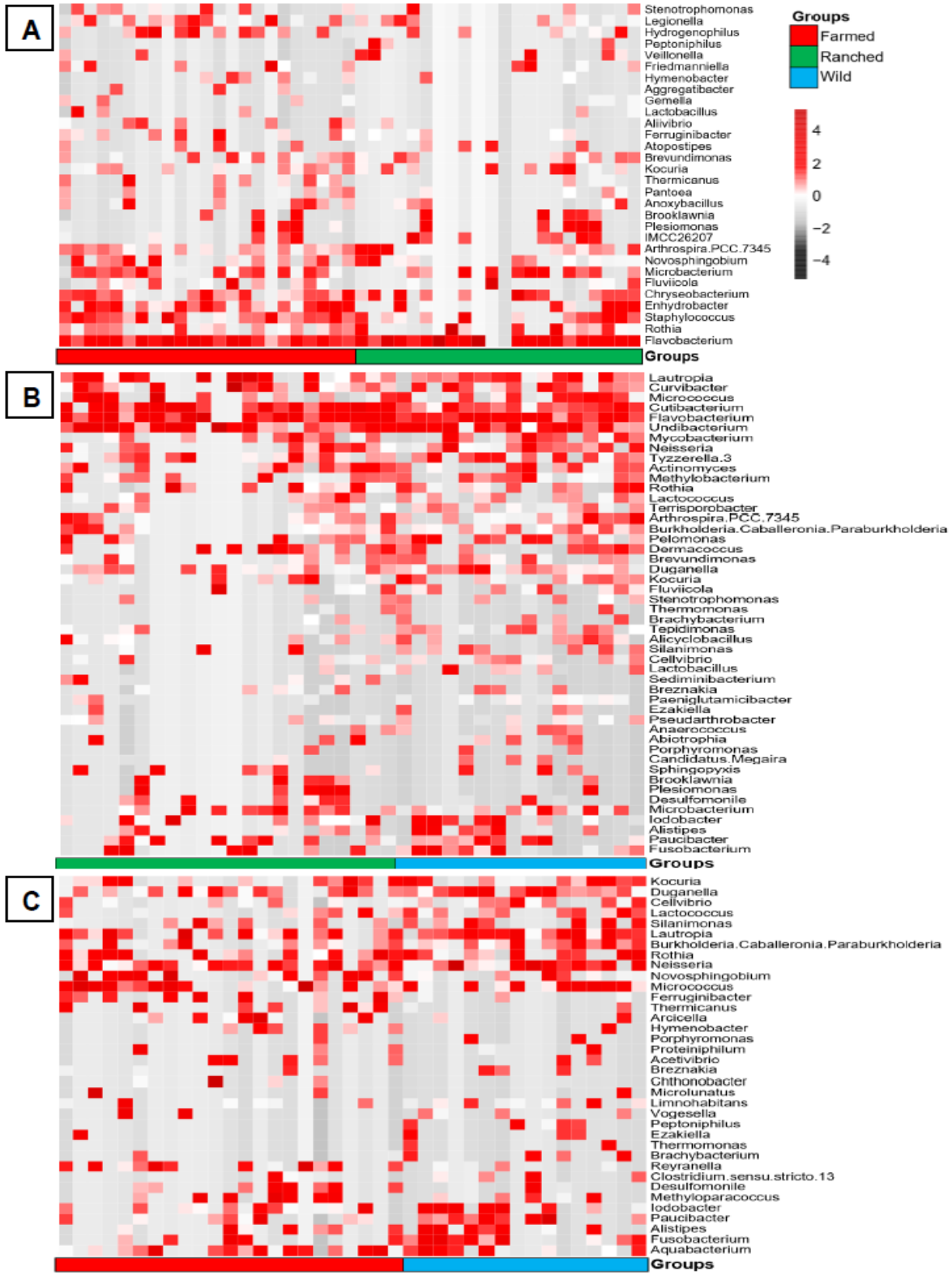

Figure 4-18: Heatmaps showing the subset of microbial OTUs within the foregut of Atlantic salmon classified as significant on pairwise differential analysis, where [A] farmed vs ranched, [B] ranched vs wild and [C] farmed vs wild. *Each column represents a different sample (individual fish) and the bottom row colour-codes the sample based upon origin, with red, green and blue indicating foregut samples from farmed, ranched and wild fish respectively. The heatmap is shaded whereby pink-red indicates increased abundance and grey-black represents decreased abundance.*

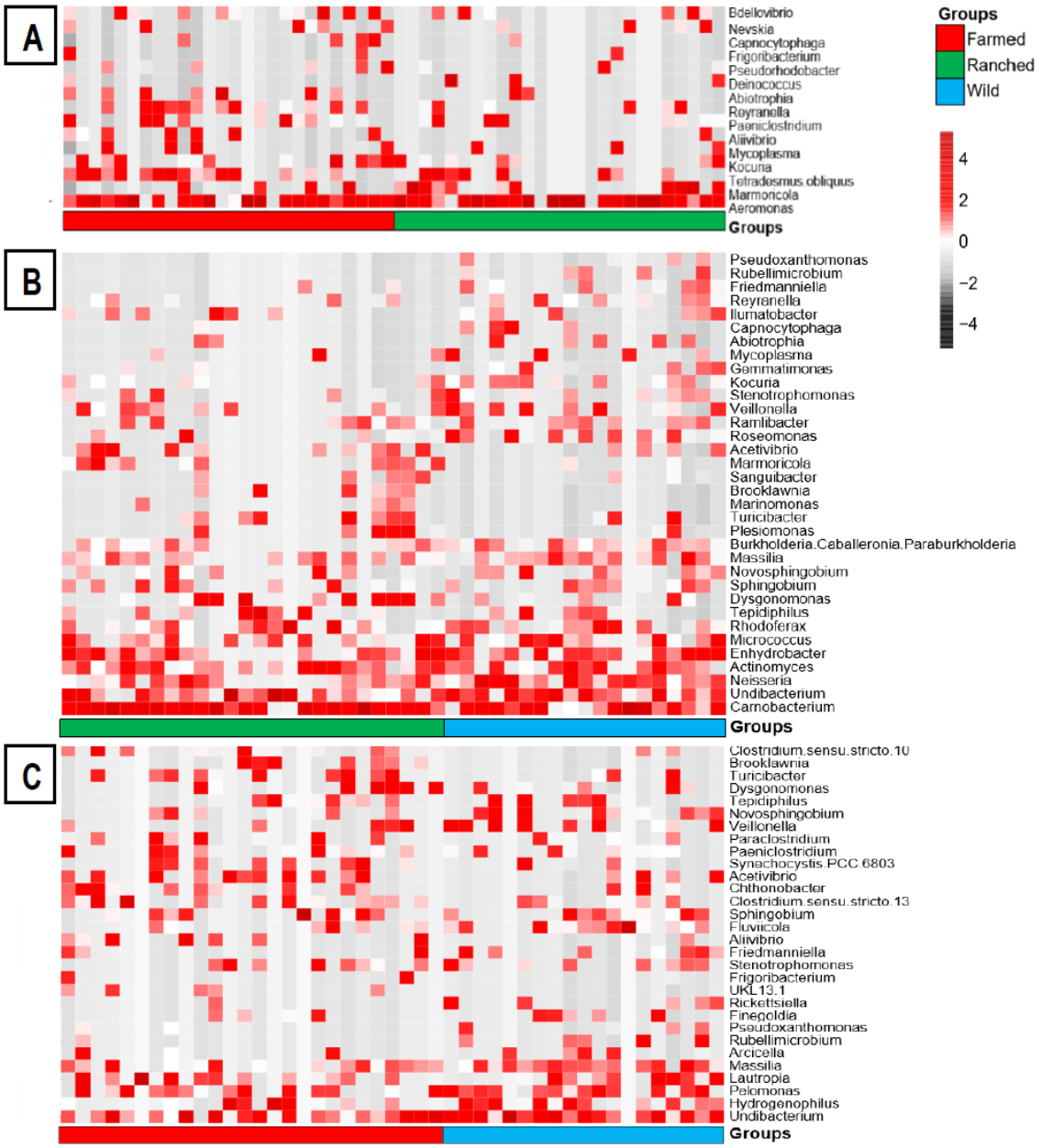

Figure 4-19: Heatmaps showing the subset of microbial OTUs within the hindgut of Atlantic salmon classified as significant on pairwise differential analysis, where [A] farmed vs ranched, [B] ranched vs wild and [C] farmed vs wild. *Each column represents a different sample (individual fish) and the bottom row colour-codes the sample based upon origin, with red, green and blue indicating foregut samples from farmed, ranched and wild fish respectively. The heatmap is shaded whereby pink-red indicates increased abundance and grey-black represents decreased abundance.*

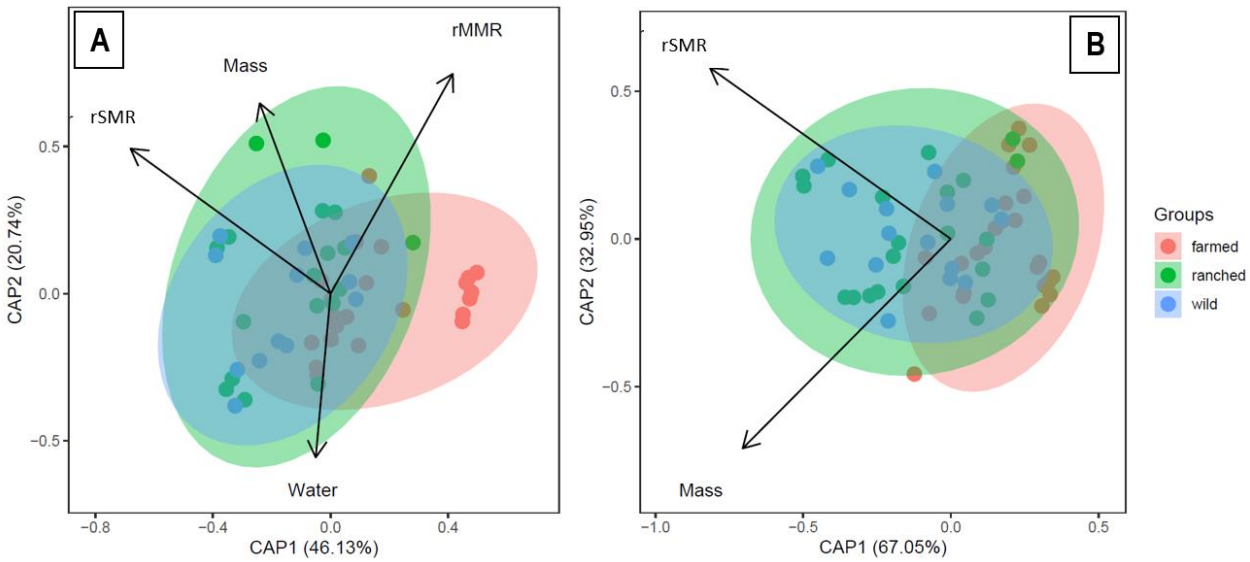

Figure 4-20: ***Distance-based redundancy analysis (dbRDA) illustrating the drivers of differences in [A] foregut and [B] hindgut beta diversity between the three origins of Atlantic salmon****; green, red and blue points represent farmed, ranched and wild individuals respectively. Arrows in the plot denote the magnitudes and directions of the effects of explanatory variables. 'Water' is an abbreviation of '% water content'. The total variance (in percent) explained by each axis is indicated.*

Drivers of microbial community composition were also assessed using distance-based redundancy analysis (dbRDA), which illustrates how different explanatory variables contribute to the variation seen within microbial communities. dbRDA analyses the variation in the microbial communities that can be explained by the environmental variables. The environmental variables considered were the metabolic variables (rSMR and rMMR), fish morphological data (body mass and % water content), as well as the SDA data (peak SDA, time to peak SDA and duration of the SDA). rAS and length were not included as they show collinearity with rMMR and mass, respectively, which can decrease the reliability of dbRDA analyses. As with the alpha diversity analyses, dbRDA analysis was also performed without the SDA data. This allowed analysis of the potential drivers of microbial community variation without the restriction of the smaller SDA sample size, which would reduce the statistical power. With and without the inclusion of the SDA data, dbRDA was performed for both the foregut and the hindgut bioinformatic data.

When SDA data were included, no explanatory variable was found to drive variation in microbial structure within either the foregut or the hindgut of the Atlantic salmon. Without the inclusion of SDA data, forward selection revealed that no explanatory variable (rSMR, rMMR, fish body mass and % water content) was more important

than any other within the foregut of the fish. dbRDA was therefore run with all environmental variables. The overall model was not significant, showing no explanatory variable was responsible for variation in the structure of microbial communities within the foregut (Figure 4-20, A). Forward selection on bioinformatic data from the hindgut of Atlantic salmon selected fish body mass (g) and rSMR (mg $O_2.hr^{-1}$) as significant, so subsequent dbRDA was performed on these two variables. Permutational ANOVA for dbRDA confirmed that the overall model was significant ($p = 0.02$), suggesting that mass and rSMR together accounted for 5.04% of the variation seen in microbial community composition. However, the marginal significance meant that impacts of mass and rSMR on microbial community structure within the Atlantic salmon hindgut were borderline ($p = 0.059$ and $p = 0.053$, respectively), so the dbRDA results are inconclusive overall (Figure 4-20, B).

To assess whether there were any correlations between the metavariables (fish body mass, fish body length, % water content, rSMR, rMMR and rAS) and individual OTUs, Pearson correlation coefficients were calculated within both the foregut and the hindgut samples taken from all 3 origins of the Atlantic salmon. Within the foregut of all Atlantic salmon, there was no significant correlation between any pair of OTU and metavariable once an FDR (false discovery rate) correction was applied (Figure 4-21, A). Within the hindgut, there was a significant negative correlation between rSMR and OTU 5 ($r_{26} = -0.66$, $p = 0.023$), which belongs to the bacterial Phylum of Actinobacteria (Figure 4-21, B). This indicates abundance of this Actinobacteria was lower within the hindgut of Atlantic salmon with a higher metabolic rate.

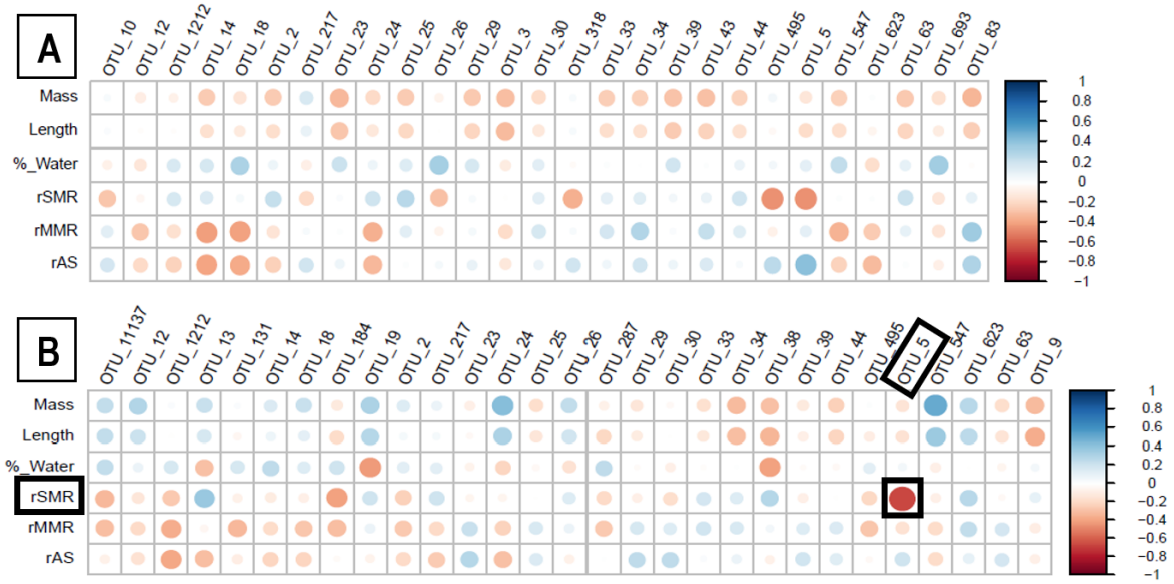

Figure 4-21: A correlations plot to show the correlations between metavariables and OTUs present within the foregut [A] and hindgut [B] of Atlantic salmon. *Each correlation is shown as a circle, coloured according to the strength and direction of correlation coefficients, where red is negative, and blue is positive. The size of each circle relates to the uncorrected p-value of the corresponding relationship, with larger circles indicating lower uncorrected p-values.. Any statistically significant correlation that remained following an FDR correction is indicated by a bold black box.*

4.5. Discussion

4.5.1. The Metabolic Rate of Atlantic Salmon Parr

It was hypothesised that the SMR of farmed Atlantic salmon would be higher than that of wild or ranched fish, due to the stable food supply experienced by generations of farmed individuals mitigating the costs associated with a high SMR. However, no difference was found in the rSMR or rMMR between fish of different origins, whilst the AS of wild fish was higher than that of farmed fish. In this case, the AS of ranched fish represented an intermediate between farmed and wild fish, which could have arisen after several generations of domestic selection in the freshwater environment. AS can vary considerably between individuals (Auer et al., 2015c), and because it represents the ability of an individual to move, digest, grow and reproduce, a higher AS could be beneficial in many circumstances. For example, in Atlantic cod, a larger AS allowed fish to forage for longer in a hypoxic environment when compared to fish with a lower AS (Behrens et al., 2018). Also, in juvenile mullet (*Liza aurata*), AS has been linked to the position an individual takes within a school of fish, where a larger AS allows fish to take a leading position in faster-swimming fish schools (where they can maximise food intake), as their additional aerobic capacity allows them to maintain swimming speed and other physiological functions in spite of the increased drag associated with this frontal position. Meanwhile fish with a smaller AS benefit from reduced swimming costs associated with posterior positions (Killen et al., 2012). These context-dependent benefits could explain why this study found that wild fish had an increased AS. Throughout their lifetime, wild fish experience much more complex environments than their farmed conspecifics. Within aquaculture, Atlantic salmon expend energy on interactions with conspecifics and feeding, but their conditions remain fairly stable and their locomotion is often only moderate. Even within sea cages, Atlantic salmon will not truly experience the wild environment, because the surface of the water is protected from wind and water currents and so water mixing is reduced (Johansson et al., 2006). Also in sea cages, husbandry methods that control factors including predation, food input and light levels are commonplace (Oppedal et al., 2011). In contrast, wild fish have both inter- and intraspecific interactions, including with predators and prey. They must actively forage to meet their energy requirements and must do so in a more stochastic environment than that offered within an aquaculture setting, experiencing greater variation in water currents, which will increase the cost of locomotion.

It may also be relevant that wild Atlantic salmon must be able to respond to seasonal cues, which is not the case (and may even be detrimental) within farmed fish. For example, it has been established that upon maturation, wild Atlantic salmon will often enter a period of anorexia before migrating upstream to spawn (Kadri et al., 1995). Prior to spawning, which generally takes place in the autumn, salmon cease feeding, which is thought to occur when a fish has adequate reserves for the subsequent energetic costly migration. Consequently, the timing of the onset of anorexia varies between individuals, but is recognised as an adaptive response (Kadri et al., 1995). This seasonal diminishment in appetite has been reported within fish farms, though the onset of anorexia within aquaculture is maladaptive due to the sudden growth termination. Unlike in the wild, sexual maturation of farmed Atlantic salmon is avoided, as the resulting reduction in product quality leads to an economic loss (McClure et al., 2007). Within both farmed and wild Atlantic salmon, growth rate is prioritised, but there is a disparate benefit of a high growth rate between the two origins; in wild fish faster growth reduces vulnerability and allows an individual to attain sexual maturity (Grade and Letcher, 2006), whilst in farmed fish, the advantages are purely economic. The aquaculture industry therefore aims to reduce the risk of early sexual maturation in Atlantic salmon by disrupting physiological processes whilst still promoting growth (McClure et al., 2007).

Along with endogenously controlled sexual maturation, wild Atlantic salmon are also exposed to large seasonal variation in temperature and food availability, whilst farmed fish have steady access to food and are comparatively shielded from large variations in biotic factors. In the winter, wild Atlantic salmon must cope with lower temperatures and reduced food levels, which can lead to a period of energy deficiency (Finstad et al., 2011). It has been shown that juvenile Atlantic salmon living in their natural freshwater environment are likely to reduce their levels of activity in the day during such periods, which is thought to be an adaptive strategy due to a reduction in prey and the presence of warm-blooded predators making daytime foraging riskier (Fraser et al., 1995; Finstad et al., 2009). Though wild fish must continue to forage at night to survive the winter, the depletion of lipid in wild Atlantic salmon during winter is well established. The concept of ‘catch-up’ growth, in which an individual might show increased appetite following a period of deprivation, is not fully understood in Atlantic salmon, with long-term costs having been reported in fish that initially showed compensatory growth (Morgan and Metcalfe, 2001).

Nonetheless, a higher AS would be beneficial if an individual were to go through a period of hyperphagia. Such seasonal disparities experienced by wild Atlantic salmon highlight that they are faced with much more complex, energetically demanding circumstances than their farmed conspecifics. Thus, even though arguably an increased AS is important for all Atlantic salmon as this will enable more energy to be directed towards growth, it is unsurprising that wild Atlantic salmon might naturally have a higher AS, because this will allow individuals to undertake energetically costly activities such as migration, spawning, surviving winter and even catch-up growth. As the timing of such activities is in part driven by seasonality and photoperiod cues (Finstad et al., 2009), an increased AS overall will enable wild Atlantic salmon to cope with seasonal requirements. Meanwhile, the aquaculture industry's use of tools such as underwater lights reduces the necessity for this increased AS within farmed fish.

Though the difference in AS between farmed and wild Atlantic salmon could have a genetic element and therefore partly be due to the differential energetic requirements imposed by the environments experienced by previous generations of fish, the expectation was that this would have also therefore impacted the SMR of these fish. Previous research has shown that a higher SMR can be beneficial when resources are plentiful, but detrimental if environmental conditions deteriorate (Metcalfe et al., 2016). Consequently, as there is more likely to be a cost associated with a high SMR within a natural environment, the expectation was that the wild Atlantic salmon would have a lower SMR than the farmed. Potentially, the lack of variation in SMR and MMR between the three origins reflects the strong impact the environment has on an individual's metabolic phenotype. Within this common-garden experiment, Atlantic salmon from all three origins were reared under uniform conditions, seeking to remove the influence of environmental factors in order to highlight any genetic influences on metabolism. It is possible that the context-dependent benefits associated with specific metabolic phenotypes have a stronger influence than that of genetics. It might have been expected for there to have been a difference in all three metabolic measures (SMR, MMR and AS) between origins, or none at all, especially as MMR and AS are often correlated. Nonetheless, the difference seen in AS between farmed and wild Atlantic salmon could reflect the higher importance of AS in allowing flexibility of behaviour in wild fish, in comparison to SMR. In other words, the benefits of a higher SMR or MMR might be context-dependent, whilst a higher AS

might simply be necessary within wild Atlantic salmon, for the aforementioned reasons.

4.5.2. The Cost of Digestion in Atlantic Salmon Parr

This experiment sought to understand the metabolic cost of digestion in Atlantic salmon parr from different origins as well as its links to metabolic rate. The feeding behaviours of farmed and wild fish will be exceptionally different, with farmed Atlantic salmon having a dependable source of food throughout the year and wild salmon experiencing significant daily and seasonal fluctuations in food levels. Whilst the feeding regime within aquaculture can vary, it is chosen based upon expected growth performance (Johansen and Jobling, 1998) and farmed fish are therefore usually provided with a constant supply of food throughout the day, the level of which can remain consistent throughout the year. In contrast, wild fish rely on either drifting invertebrate food ('drift') or on the benthos in the winter (Grade and Letcher, 2006). The density of this 'drift' is variable, since prey abundance (Fraser et al., 1995) and composition (Grade and Letcher, 2006) show seasonal variation. Meanwhile, as previously established, wild fish can exhibit marked seasonal variation in appetite and feeding behaviour, often linked to life history stage and photoperiod, respectively. Gut fullness in Atlantic salmon parr within their natural environment has been shown to vary markedly within a single 24-hour period (Amundsen et al., 1999; Grade and Letcher, 2006), as the drift will often peak at dawn and dusk. Although some studies have found peak fullness in the Atlantic salmon gut to occur at night (Amundsen et al., 1999) and others in the morning (Grade and Letcher, 2006), this variation highlights the lack of consistency in the feeding behaviour of wild Atlantic salmon when compared to farmed.

These inherent differences in feeding behaviour between farmed and wild Atlantic salmon could therefore influence the way in which feeding impacts the metabolism of each origin. The SDA (as measured by peak of the response, time to reach the peak of the response and overall duration of the response) is of ecological relevance because when a fish is digesting and processing a meal, this energy is directed away from other metabolic activities (Jordan and Steffensen, 2007). Any energy required in the SDA comes from an individual's AS; consequently, a trade-off has been described, between larger meals that might increase growth efficiency and smaller meals that might retain aerobic capacity (Norin and Clark, 2017). Whilst

processing a meal, this redirection of available energy is of no concern within aquaculture but could be deadly within natural conditions, where there will be a trade-off between increased vigilance and metabolic demand (Killen et al., 2015). It is therefore less critical for farmed fish to have a shorter SDA duration as they only need to swim and digest (Norin and Clark, 2017). For this reason, it was hypothesised that when fed comparable rations, wild Atlantic salmon would have a shorter SDA duration than their conspecifics. It was hypothesised that a shorter SDA duration would have evolved in wild fish due to the necessity of balancing the energetic costs of feeding with predator avoidance and other costly behaviours required by their more complex environment. Since retaining aerobic capacity is arguably more important in wild fish than in farmed, there is a clear benefit for wild fish to be able to process their food faster. Within this study, ranched fish had the shortest SDA duration, with the variation among fish origins primarily driven by the difference between ranched and wild fish. This was surprising, as the inclusion of ranched in the study was to establish whether they truly reflected a physiological intermediate between farmed and wild Atlantic salmon. In this case, they did not, and it is unclear why ranched fish might have a reduced SDA duration overall.

In contrast, wild Atlantic salmon had a higher peak SDA than either of the other two origins (where ranched did represent an intermediate as expected). This discrepancy in peak SDA between the origins might reflect the timing of meals experienced by farmed and wild fish over previous generations, where digestion in farmed fish might have less acute costs than within wild fish, as they eat more continuously across the day. Potentially, because wild Atlantic salmon rely on pulses of food instead of a constant supply, their SDA has a higher peak which would not be sustainable in farmed fish which must more consistently process food. The higher peak within wild Atlantic salmon could be made possible in part due to their higher AS, which allows a greater peak demand in aerobic activities.

Previous literature has suggested that fish with a higher SMR have a higher SDA (Reid, 2012) and decreased SDA duration (Millidine et al., 2009). These findings suggest that the metabolic cost of digestion is more generously met by fish with a higher SMR, which in turn shortens the duration. Within this study, there was a negative nonsignificant trend between SMR and all three SDA parameters (duration, peak and time to the peak). This supports the theory that individuals with a higher SMR process meals faster (Millidine et al., 2009), but it has not been established that

fish with a higher metabolic rate might have a reduced peak in their SDA response. SDA analyses within this study were hindered by a small sample size resulting from only a small proportion of fish consuming their full ration. A more robust sample size would have allowed a more thorough analysis of the relationships between metabolic rate and the cost of digestion. If a higher SMR allows individuals to process meals faster, thereby allowing energy to be directed into other activities sooner, this would be beneficial for wild Atlantic salmon; however, as previously established, there are greater costs associated with a high SMR in wild salmon than their farmed conspecifics (Auer et al., 2015a; Metcalfe et al., 2016). As there was no difference in SMR between the three origins, nor was it the case that wild fish had a shorter SDA duration overall, it is possible that other factors overshadowed these biological mechanisms, such as composition of the food. Perhaps, as discussed with SMR and MMR, the SDA response is the result of the interplay between both genetic and environmental factors.

4.5.3. Microbial Diversity within the Atlantic Salmon Parr Gut

An aim of the present study was to establish whether there are any differences within the gut microbial community between farmed, ranched and wild Atlantic salmon. If so, a further aim was to understand whether such differences had any genetic basis. When assessing foregut alpha diversity, a strongly negative relationship was found between rSMR and microbial richness within farmed fish, whilst slight negative and positive trends were found within ranched and wild fish respectively (Figure 4-12). Here, ranched fish did reflect an intermediate position between farmed and wild Atlantic salmon. This negative relationship was found across all three origins within the hindgut samples. Also across all origins, microbial richness within both the foregut and the hindgut, and the Shannon effective diversity within the hindgut, all increased with an increase in water content. Fish with an increased water content have lower energy content, % protein and % fat (Elliott, 1976b), suggesting that fish with greater alpha diversity had lower levels of body fat. Within the present study, fish with a higher mass had a lower water content (and therefore a higher proportion of fat). Further, an increase in body mass was associated with a decrease in richness and in the Shannon effective within the hindgut of fish from all three origins. The relationship between body mass and microbial richness was most negative

within ranched fish, which did not reflect an intermediate between farmed and wild as expected. These results indicate that across all origins, heavier fish have an increased proportion of body fat and a decreased microbial diversity within their foregut and hindgut. In relation to the metabolic rate data, it was also the heavier fish that had an increased rSMR. Though the main difference in alpha diversity between the three origins was in the positive relationship between rSMR and microbial richness within the wild Atlantic salmon foregut, the overall pattern was for individuals with a high rSMR to have decreased microbial richness (as seen within the previous chapter). As with Chapter 3, the drivers of this relationship are far from clear. Microbial diversity is mediated by multiple ecological factors including competition and adaptation, but fundamentally by 'niche opportunity', including access to different resources provided by the host diet (Scanlan, 2019). Therefore, if there is a link between higher body fat, body mass, rSMR and gut microbial alpha diversity, this could be associated with levels of food intake, that were not measured within this experiment.

Interestingly, time to reach the peak SDA increased with microbial richness in the foregut, and with Shannon effective in the hindgut. This indicates that fish with an increased microbial alpha diversity took longer to reach the peak meal processing period. A large proportion of the increase in metabolism after a meal is thought to be associated with amino acid flux and protein turnover (Carter et al., 2001), processes essential to growth. Reaching the peak of the SDA rapidly can therefore indicate a faster digestion of food and accumulation of somatic tissues (Millidine et al., 2009). Nonetheless, overall SDA duration gives a better view of the time burden associated with digestion, because it captures the full time taken for the metabolic rate to return to its pre-prandial level. Indeed, although there was a positive correlation between microbial alpha diversity and the time taken to reach the peak SDA, the overall SDA duration increased as the foregut Shannon effective decreased. This suggests that a less even abundance of different microbial taxa within the foregut was associated with a slower return to the baseline metabolic rate. Though the function of the gut microbiota will vary with microbial community composition and alter with life history stage and environment, the impact of diet on the gut microbiota has been established across a wide array of species (Karasov and Douglas, 2013) and feeding habit is thought to be a key driver of interspecific differences in the gut microbiota of

teleosts (Asakura et al., 2014). Consequently, it would not be surprising to find that the gut microbiota impacts the SDA within Atlantic salmon.

How long an individual takes to reach the peak of its SDA response has less consequence than the overall duration of the response, which is more important in relation to returning an individual to its full capacity to perform aerobic activity. Similarly, the time to the peak of the response is arguably less biologically important than the size of the peak itself, which reduces the metabolic scope available for other aerobic activities (Norin and Clark, 2017; Jutfelt et al., 2020). Taken together, the time to the peak SDA and SDA duration signify the total time investment associated with processing a meal. The alpha diversity analyses here indicate that the time to the peak of the SDA and the full SDA duration were shorter in fish with decreased foregut richness, increased foregut Shannon effective and decreased hindgut Shannon effective. This means that in fish that showed quicker digestion, the foregut microbial community was less rich but had a more even abundance of different microbes, whilst the hindgut community had a less even abundance. Fully understanding the variation in alpha diversity in relation to SDA would require greater insight into microbial function. Nonetheless, studies have shown that an increase in microbial alpha diversity will result when diet variety is increased (Abid et al., 2013; Uren Webster et al., 2020). If this has a functional basis, reflecting that more complex diets require more complex gut microbial communities, then within this study, the commercial diet might have rendered some microbial taxa functionally redundant. In this case, quick digestion would still be achieved within a less rich microbial community, as a greater proportion of microbes were contributing in equivalent ways. In this case, a more even microbial abundance would provide good functionality, as has been seen within other biological communities (Wittebolle et al., 2009).

The specific gut microbial community structure described here (a foregut in which richness was lower and evenness was higher, and a hindgut in which evenness was lower) is reflected in the literature; a reduced Shannon diversity in the distal intestine compared to the proximal intestine has previously been reported in Atlantic salmon (Fogarty et al., 2019). The reasons for variation in microbial Shannon diversity within teleosts remain unconfirmed. The variation could have a functional basis, as studies have shown that a higher Shannon diversity is seen in herbivorous than omnivorous fish (Yan et al., 2016) and differs between fish in fresh water and salt water (Rudi et

al., 2018). However, the variation within the gut itself could simply be due to physiochemical differences along the gastrointestinal tract of Atlantic salmon, since the pH is around 7.0 in the foregut but closer to 9.0 in the hindgut (Navarrete et al., 2009). If variation in Shannon diversity has a functional basis, then fish with the gut microbial community structure associated with a faster SDA were potentially able to process their meal quickly as the foregut microbial community effectively carried out the initial digestive processes (as previously discussed), whilst within the hindgut, a lower evenness might result in the presence of dominant taxa particularly adapted to carry out the remaining digestive processes more efficiently.

Within this study, wild-origin fish had a higher foregut Shannon effective than farmed fish. In other species, differences in the gut microbial communities have been found between wild and captive individuals (Wienemann et al., 2011). In Atlantic salmon however, it has previously been shown that fish origin has no effect on diversity or richness of the microbiome: a translocation experiment between wild and hatchery-reared Atlantic salmon highlighted that richness and diversity were entirely determined by the environment (Uren Webster et al., 2020). Though the current study found microbiota-level differences between the origins, it must be recognised that the farmed, ranched and wild Atlantic salmon were kept in separate stock tanks from when they were fry until the acclimation period of the current experiment. It is possible that microbe-level differences between the origins were due to the resulting tank effects, as the gut microbiota has been shown to respond to environmental changes: intraspecific variation has been reported due to geographic location (Zhao et al., 2018) and within teleosts, between freshwater and saltwater environments (Rudi et al., 2018). There was also no way to standardise the amount of food consumed between individuals, which might have varied based upon social hierarchies which are known to form in Atlantic salmon (Ashley, 2007). Nonetheless, for the fish used within this study, the recirculatory nature of the aquarium meant that water between the stock tanks was continuously mixed. Further, fish were acclimated within the stream tank system for 2 weeks before the beginning of the experiment. Adaptation of the gut microbiota can be rapid, with a change in diet resulting in compositional microbiota changes in as little as 1 – 3 days (Candela et al., 2012). It was therefore assumed that the 2-week acclimation period would act to standardise the environmental impacts on the gut microbiota between different origins.

The hypothesis that wild Atlantic salmon would have a greater gut microbial diversity than farmed was based on the assumption that the gut microbiota of wild salmon would have been shaped by the exposure over previous generations to a more varied diet than that of farmed fish. Although assembly of the gut microbiota has neutral stochastic elements (Heys et al., 2020), deterministic processes can also occur (Schmidt et al., 2015). Whilst the environment is inarguably important, co-evolution between the host and microbes can occur, and host phylogeny is speculated to have a role in shaping the microbial community composition in the teleost gut (Sullam et al., 2012). Due to the substantial difference in diet between farmed and wild Atlantic salmon, diet could lead to selection for particular microbial communities, producing a genetic basis for alpha diversity in the Atlantic salmon gut microbiota. However, although the differences in SDA response and microbial diversity between origins presented here have been linked to the differential ability of farmed and wild fish to benefit from their diet of commercial food, previous work has failed to find evidence that farmed Atlantic salmon parr are more adapted to exploit commercial food than their wild counterparts (Harvey et al., 2016a). This led the authors to suggest that farmed fish had evolved an enhanced appetite. Nonetheless, other factors could impact the microbial alpha diversity, as the current study also found that fish with a higher rSMR had a decreased microbial richness. With countless examples of links between the gut microbiota and an organism's metabolism (as summarised in Chapter 2), these results may support the theory that microbial diversity is impacted by far more than simply the diet and environment.

Interestingly, the gut microbiota is thought to become more specialised as the host develops (Stephens et al., 2016), which is often characterised by a reduction in alpha diversity (Yan et al., 2016). Though all experimental fish were at the same life history stage, the results presented here suggest that the microbiota of the farmed fish might be more specialised than that of the wild, as farmed fish had a lower alpha diversity. Within the context of this experiment, this could reflect the fact that the farmed Atlantic salmon were able to develop a more specialised gut microbiota due to their increased familiarity of the commercial diet in comparison to the wild fish. Nonetheless, greater insight into microbial function would be necessary to draw firm conclusions, especially given that whilst some taxa of the gut microbiota are adapted to their environment and are present due to deterministic processes, there are also

many (even the majority) that are simply there by chance (Yan et al., 2016; Heys et al., 2020).

Beta diversity measures in this study paint a complex picture. Uren Webster et al. (2020) found effects of fish origin on beta but not alpha diversity in a translocation experiment: *Mycoplasma sp.* and *Lactobacillus sp*. were dominant in the gut microbiota of salmon of hatchery origin, regardless of their new environment, which was not the case within wild-origin fish. Also, microbes from Family Brevinemataceae were rare before translocation but became more abundant in hatchery-origin fish across all experimental groups, regardless of their final environment. Within the current study, there was a significant difference in microbial beta diversity between the three origins of fish (farmed, ranched and wild). Though Uren Webster et al. (2020) posit that their results are due to colonisation history, this study does not support that possibility, since fish were exposed to the same conditions from the egg stage (albeit in different tanks of the same recirculation system as previously discussed). Consequently, the differential abundance of microbial taxa between the three host origins could be related to genetically determined phenotypic differences between them, perhaps in gut physiochemical architecture or immune system.

Previous literature has shown that Firmicutes dominate wild and farmed parr and postsmolt stages, but that wild salmon are dominated by Proteobacteria in their later life stages (Rudi et al., 2018). Within this study, Proteobacteria were more likely to be overabundant in farmed and wild than in ranched parr, but though Proteobacteria characterised most of the overabundant genera in the foregut of farmed fish and both the foregut and hindgut of wild fish, in the hindgut of farmed fish, most overabundant genera belonged to phylum Firmicutes. Interestingly, in mice an increased ratio of Firmicutes to Proteobacteria has been linked to a high fat diet (Kim et al., 2012). Studies in humans have conversely found that a diet higher in fat leads to an increase in Actinobacteria and Bacteroidetes, with a decrease in Firmicutes and Proteobacteria as a consequence (Senghor et al., 2018). The contradictory nature of literature on this topic highlights that the links between the gut microbiota and host physiology will be context dependent; nonetheless, within the present study, farmed fish had an increased ratio of Firmicutes to Proteobacteria in comparison to fish of wild origin. Actinobacteria and Bacteroidetes genera were found to be overabundant in farmed, ranched and wild fish across the whole gut, but

genera belonging to Actinobacteria were more likely to be overabundant in farmed and ranched fish and those belonging Bacteroides were more likely to be overabundant in fish of wild origin. As the SDA and alpha diversity analyses have indicated, wild Atlantic salmon within this experiment had an increased peak SDA and Shannon effective. As discussed, one possible reason for this is that wild Atlantic salmon were gaining less from the commercial food than the farmed fish. The overabundance of Firmicutes within farmed, but not wild, Atlantic salmon hindguts might reflect this; if an increased ratio of Firmicutes to Proteobacteria is associated with a high fat diet, though all fish were fed the same food, potentially Firmicutes were more abundant in farmed fish as they were able to make the most of their diet. Firmicutes are known to metabolise dietary polysaccharides (Carey et al., 2013; David et al., 2013), which would give the farmed Atlantic salmon additional help in digesting their food. Overall, there were fewer genera that were differentially abundant between farmed and ranched than there were between either farmed and wild or ranched and wild Atlantic salmon. Whilst the initial hypothesis was that the ranched fish will always represent an intermediate between the fish of farmed and wild origins, this has not been the case with every physiological measure, such as duration of the SDA response. Nonetheless, the differential abundance analyses indicate that there is more separation in gut microbial communities between ranched and wild than between ranched and farmed.

Alongside the impact of diet and fish origin previously discussed, there are likely to be many drivers of variation in microbial community composition, but the dbRDA results were not robust enough to draw strong conclusions. In relation to the other metavariables, the dbRDA loosely suggests that mass and rSMR might have an impact on microbial community composition**.** The biggest differences in microbial communities might therefore be due to origin as opposed to metabolic rate (as there were few differences between metabolic rate across the origins anyway). The previous chapter revealed metabolic rate to be a driver of microbial diversity in the foregut of Atlantic salmon, in which a member of the *Rhodobacteraceae* family was also found to be negatively correlated with metabolic rate. Possibly, when fish from different origins are examined, differences in genetic provenance overshadow those of metabolic rate. Meanwhile, further understanding of the drivers of physiological differences between the origins was gained by the correlation analyses, which showed that a higher abundance of Actinobacteria in the hindgut led to a decrease in

rSMR. Though rSMR was not significantly different between the three origins, Actinobacteria was more likely to be overabundant within the guts of ranched and farmed fish than their wild counterparts. As Actinobacteria has been linked to diets high in fat within humans (Senghor et al., 2018), this phylum could be implicated in a lower metabolism and obesity across different taxa. In contrast, within the experiment examining whether gut microbial composition varied with host metabolic phenotype (Chapter 3), genera from Actinobacteria were found to be overabundant within fish with both 'low' and 'high' metabolic rates, with 67% of the overabundant Actinobacteria found in the foregut genera (6 of the 9 of the overabundant Actinobacteria) being within fish with a 'low' rSMR and 89% of the overabundant Actinobacteria genera found within the hindgut (8 of the 9 overabundant Actinobacteria) being within fish with a 'high' rSMR. These conflicting results highlight the need for a greater level of detail to be applied when studying the microbiota, as species-level differences might provide increased clarity.

4.6. Conclusion

Taken together, we can see that there were significant differences in mass, AS, peak SDA and SDA duration between the farmed, ranched and wild Atlantic salmon. There were also differences in the foregut microbial Shannon effective and the hindgut microbial richness between origins. Though this study has revealed inherent differences between the three origins of Atlantic salmon, they were not always what would have been expected. Ranched fish did not always represent an intermediate between farmed and wild fish, and wild fish did not have a higher SMR or a shorter SDA duration. This could in part be due to the common garden approach that was adopted, indicating that environment is a large driver of differences. The uniformity of the aquarium environment experienced by all three origins of fish may account for the lack of variation seen in metabolic rate, but it therefore might also suggest a host genetic component to microbial community composition, given the differences seen that arguably should have been reduced due to the recirculating nature of the aquarium and the acclimation period (such as differentially abundant microbial taxa across the different origins of fish). Genetic drivers of the gut microbiota might result from contrasting selection pressures over many generations between fish in the wild and those reared on a uniform diet within the aquaculture setting.

By exploring the metabolic rate, the gut microbiota and physiological measures of the fish together, it has been possible to identify some consistent features associated with these variables: overall, fish with a lower metabolic rate (SMR, MMR and AS) also had a lower mass, a higher water content (and therefore less fat) and an increase in microbial alpha diversity. The contradictory nature of some of these results (such as wild fish having a higher mass overall, but also increased Shannon effective in the hindgut) highlights the complex nature of the interactions between the gut microbiota and host physiology. As researchers seek to understand these complexities, this study highlights the intricacies of the relationship and the broad range of factors that have the potential to drive differences between different origins of Atlantic salmon. As both metabolic rate and the gut microbiota community composition are context dependent, this will necessitate the exploration of differences between farmed and wild Atlantic salmon across a variety of contexts. This will be particularly important when addressing the potential impacts of interactions between wild and escaped farm-origin Atlantic salmon.

Chapter 5: The Impact of Provenance on the Exploratory and Food Seeking Behaviour of Juvenile Atlantic Salmon: A Comparison of Fish of Farmed and Wild Origins and their Hybrids Reared in Aquarium, Hatchery and Wild Environments.

5.1. Abstract

Intraspecific variation in behaviour is widely reported across a broad range of taxa; however, it is of particular interest when examining the impact of domestication on a species. Globally, Atlantic salmon are of huge importance to the aquaculture industry, but directional selection for economically important traits can have unintended consequences, including upon the behaviour of the farmed fish. Additionally, the environment an individual experiences during ontogeny can also impact its behavioural phenotype. Disentangling genetic effects from those imposed by the environment can be challenging. Using a common garden approach, this study examined the impact of genetic origin on both the exploratory behaviour and the food-seeking behaviour of juvenile Atlantic salmon from farmed and wild origins, along with their hybrids. Two separate experiments were carried out: fish were reared in either an aquarium (the Glasgow experiment), or in a hatchery or river (the Newport experiment). Both exploratory and food-reaching behaviour of the fish was found to be impacted by their provenance, with farmed x wild hybrid individuals having an intermediate behavioural phenotype between the two parental strains. Interestingly, fish reared in the Glasgow aquarium showed inverse behavioural patterns to those reared in Newport (whether these were reared in the hatchery or river). Whilst the common garden method allowed genetic drivers of behaviour to be assessed independently of environmental ones, by rearing fish in both a hatchery and a river in Newport, the impact of rearing environment was assessed independent of genetic origin. Rearing environment was not found to impact exploratory behaviour, but Atlantic salmon reared in the river were less likely to have reached the food at any given point than their conspecifics that had been reared in the hatchery. Taken together, these experiments indicate the importance of both genetic

origin and environmental factors on the behaviour of Atlantic salmon. No interaction between genetic origin and rearing environment was found, but the inversion of behaviours observed between the Glasgow and Newport experiments indicates that the environment has a role in determining fish behaviour. The implications of behavioural differences are discussed within the context of aquaculture escapees.

5.2. Introduction

Atlantic salmon are amongst the most well-researched teleosts, primarily due to their economic importance: in 2018 alone, Atlantic salmon made up 4.5% of total finfish production in aquaculture, representing the 9th largest 2018 share (FAO, 2020). Aquaculture production for Atlantic salmon has been expanding since 1990 and high demand globally has resulted in Atlantic salmon fish farms across the entire world, from Norway to Chile (FAO, 2020). Although the aquaculture industry provides food, wealth and seeks to minimise the depletion of wild fish stocks, the reality is that fish farms result in issues such as habitat destruction, unsustainable consumption of marine-sourced fishmeal and fish oil, the use of harmful chemicals/drugs and the impact of farmed escapees on wild populations (FAO, 2020). Farmed escapees have repercussions on the environment and on wild populations, as the escapees often colonise the same habitats as their conspecifics. For example, farmed salmon in Norwegian rivers are thought to account for an average of 14 – 36% of spawning populations, but could be as high as 80% in some rivers (Liu, 2011).

Populations of wild Atlantic salmon are often genetically distinct from one another, in part due to local adaptation (Glover et al., 2017). As anadromous fish, Atlantic salmon in the wild show large variation in life history strategy, reflected in the varying lengths of time spent in freshwater as parr, as well as discrepancies in the number of winters spent at sea, even within a single population (Klemetsen et al., 2003). Such variation is minimised among farmed fish, as environmental heterogeneity is limited and sexual maturation is avoided (McClure et al., 2007). Meanwhile, domestication of Atlantic salmon results in differentiation between farmed and wild populations due to different geographical origins of farmed source populations, inadvertent selection and directional selection (McGinnity et al., 2003), as farmed salmon have been deliberately bred for desirable traits, including fast growth and delayed maturation

(Huntingford and Adams, 2005; Solberg et al., 2020). Genetic differences between salmon of farmed and wild populations are coupled with behavioural differences, as each origin of fish experience very different environments. As a result, phenotypic differences between strains of Atlantic salmon can occur not only due to genetic factors, but also due to their rearing environment. Within aquaculture, conditions are those that will promote growth of the fish in order to increase production. Food availability is stable in comparison to the conditions experienced by wild populations, as this has an important role in minimising aggression between fish (Ashley, 2007). Social hierarchies are formed within Atlantic salmon, which can lead to aggression within densely stocked fish farms; therefore, ensuring regular access to food reduces aggressive interactions (Ashley, 2007), and also minimises growth rate variation that can occur due to competitive interactions for food (Cutts et al., 1998). In comparison, wild freshwater populations must rely upon drifting invertebrates for food or on the benthos in the winter (Grade and Letcher, 2006), resulting in marked differences in the gut fullness of wild Atlantic salmon over a single day (Amundsen et al., 1999; Grade and Letcher, 2006). Moreover, wild populations must also cope with seasonal variation, which impacts prey composition and availability (Fraser et al., 1995).

In addition to differences in food availability and interactions with conspecifics, the life histories of farmed and wild Atlantic salmon differ in a myriad of ways. Just as annual changes influence the feeding pattern in wild Atlantic salmon, the resulting change to environmental conditions also leads to seasonal variation in their energetic requirements. For example, during the winter months, when temperatures and daylight hours are both reduced, wild Atlantic salmon can show varying coping mechanisms – some juvenile salmon become relatively inactive in the winter months (Fraser et al., 1995) and some individuals show an anorexic response even when food is available (Finstad et al., 2009). Both of these behaviours would be economically unviable in an aquaculture setting, so seasonal variation in environmental conditions is often mitigated within aquaculture, with farmed fish being relatively shielded from changes in the abiotic and biotic enviroment. Even within sea cages, where factors such as water temperature are harder to control, many husbandry methods, such as the control of light levels, can alleviate seasonal variation (Oppedal et al., 2011).

Just as there are differences in energetic requirements between Atlantic salmon of farmed and wild origin (as discussed in Chapter 4), there may also be differences in their behaviour. Aside from the behavioural variation resulting from seasonality in the wild environment, Atlantic salmon in the wild must navigate much more complex scenarios in their daily lives. From the moment juvenile salmonids leave their redds, they are in intense competition with their conspecifics for feeding territories, as these increase survival probability and reproductive success (Johnsson et al., 2004). Owners of a territory often defeat intruders that seek to displace them, which has been linked to both duration of residency (Metcalfe et al., 2003) and also the perceived value of a territory to the owner (e.g. the presence of a shelter increases value as it reduces the risk of predation) (Johnsson et al., 2004). These territories are where the fish will hold station to be able to intercept passing food items, whilst shelter will allow them to avoid predation from avian or other teleost predators. Although the benefits associated with certain behaviours can be context-dependent, one study found that regardless of feeding regime, growth in juvenile salmonids increased when they actively foraged and used shelters, whilst territorial aggressive behaviours only increased growth when food supply was predictable (Hoogenboom et al., 2013). Indeed, across many species that show behavioural variation, different behavioural phenotypes perform best under different circumstances (Huntingford and Adams, 2005).

The spatial heterogeneity experienced by salmonids in the wild is in stark contrast to the uniform conditions met by farmed fish. Territoriality, foraging behaviour, predator avoidance and the ability to rapidly adapt to heterogeneous environments remains of the utmost importance amongst wild fish, whilst farmed fish experience reduced competition and a less stochastic environment. For domesticated fish, retaining such behaviours may be maladaptive if they are not providing a competitive advantage – domesticated animals are better adapted to their captive lives (Mignon-Grasteau, 2005). In addition, behaviour that is beneficial in captivity may be maladaptive in the wild (Alioravainen et al., 2020).

The differing behavioural requirements for Atlantic salmon of wild and farmed origin could have repercussions in instances where these fish come into contact. As previously mentioned, farmed salmon have been known to escape from aquaculture

settings – tens of millions of farmed salmon are thought to have escaped into the wild since the 1970s (Glover et al., 2017). Escapees can be directly and indirectly problematic for native wild populations by introducing parasites and disease (Liu, 2011), competing for limited resources and inter-breeding with them (Garant et al., 2003), resulting in maladaptive phenotypes in their offspring in relation to survival and reproduction (McGinnity et al., 2003; Houde et al., 2010). The aforementioned extreme competition experienced by Atlantic salmon in the wild is further intensified if farmed fish invade. Juvenile Atlantic salmon of farmed origin have been shown to be more aggressive than wild fish (Metcalfe et al., 2003). As a result, farmed fish often displace wild counterparts from their territories; however, although farmed fish have much higher growth rates than their wild counterparts in aquaculture settings, this difference is significantly reduced in the wild (Harvey et al., 2016b). In fact, whilst farmed Atlantic salmon parr initially outcompete those of wild origin, they show reduced survival in comparison to wild juveniles in the natural freshwater environment (McGinnity et al., 2003). Interestingly, domesticated Atlantic salmon have also been found to be more susceptible to predation in comparison to wild fish (Houde et al., 2010; Solberg et al., 2020), thought to be partially due to a trade-off between increased growth and predation susceptibility (Solberg et al., 2020), as well as an increase in risk-taking (Houde et al., 2010).

A further complication resulting from interactions between Atlantic salmon of different genetic origins is that of introgression. Such cases, in which genetic material from the domesticated fish enters the wild population due to hybridisation and backcrossing, can negatively affect wild Atlantic salmon populations (Normandeau et al., 2009; Glover et al., 2017; Robertson et al., 2019; Solberg et al., 2020). McGinnity et al. (2003) have shown that hybrid offspring of farmed and wild Atlantic salmon have reduced survival in comparison to wild fish, potentially because genetically encoded traits that promote survival and growth in aquaculture are maladaptive in the wild (Glover et al., 2017). When part of juvenile wild recruitment involves hybrids (and then backcrosses in subsequent generations), this will therefore reduce the overall fitness of the wild population (McGinnity et al., 2003). Though hybrid vigour, in which cross-bred individuals have enhanced fitness in comparison to either parent, is a widely accepted phenomena, it has not been seen in studies examining the impacts of introgression in Atlantic salmon – hybrids have been shown to

represent an intermediate between Atlantic salmon of farmed and wild origins in terms of growth and survival (McGinnity et al., 2003), and also to be no less susceptible to predation than fish of wild origin (Solberg et al., 2020).

The ecological and genetic interactions between Atlantic salmon of farmed and wild origin are of importance if we are to understand the relative risk farmed escapees pose on wild populations. Many common-garden experiments have compared the behaviours of Atlantic salmon of farmed and wild origins under controlled hatchery conditions across all life stages; such experiments have assessed traits including growth, reproduction, dominance and reactions to changes in environmental conditions (Glover et al., 2017). Whilst it is widely acknowledged that domesticated Atlantic salmon show reduced survival in the wild due to directional and inadvertent selection (McGinnity et al., 2003; Houde et al., 2010; Solberg et al., 2020), much of the focus within this field has been on those traits that are relevant to direct competition between fish of different genetic origins. Less work has been carried out regarding general behavioural traits, such as exploratory behaviour, that might have fitness consequences independent of direct competition between fish of different origins. Though research on these traits have been carried out in other salmonids, such as brown trout (Alioravainen et al., 2020). Interestingly, it has been found that farmed Atlantic salmon show reduced environmental sensitivity than their wild conspecifics, with genetic transcripts associated with environmental information processing being down-regulated in domesticated fish when reared under controlled conditions (Bicskei et al., 2014). In addition, farmed Atlantic salmon have shown reduced response to stress (Solberg et al., 2013). This shows that regardless of whether or not fish of different genetic origin directly interact with one another, fish might still have different performance outcomes. If heritable, these traits would have repercussions for future generations if introgression does occur. Insight into the effect of genetic differences versus the effect of rearing environment is also of interest, as performance of fish of farmed, hybrid and wild origins have been shown to vary according to rearing environment (Solberg et al., 2020). There are also phenotypic differences that might occur due to the effect of the different environments the fish have been exposed to (Metcalfe et al., 2003). Experience-dependent differences between domesticated and wild fish resulting from differences in rearing environment often lead to behavioural variation (Huntingford, 2004). Such

developmental effects can be independent of any genetic differences between the fish. Consequently, this study seeks to examine differences in behaviours between Atlantic salmon of different origins, specifically pertaining to traits that are of less relevance to competitive interactions. By assessing the willingness of fish to emerge from a shelter and their ability to find food at the end of a simple maze, this study aims to address whether there are inherent differences in the ways fish from farmed and wild origins approach the same context. By carrying out the experiment across two distinct locations (Glasgow and Newport), it will also be possible to assess whether reciprocal rearing environments (aquarium in Glasgow; hatchery versus wild river in Newport) influence behavioural comparisons, so helping disentangle genetic effects from environmental ones. In addition, in Newport, testing farmed x wild hybrid fish along with those of farmed and wild origin will give further insight into the potential effects of introgression.

This study therefore has the following aims:

1) To assess whether there is a difference between fish of farmed and wild origin in emergence behaviour (likelihood to emerge from a shelter and the time taken to do so).
2) To assess whether there is a difference between fish of farmed and wild origin in the total time they spend emerged from the shelter and the total time they spend moving.
3) To assess whether there is a difference between fish of farmed and wild origin in food-reaching behaviour (likelihood to reach the food and the time taken to do so).
4) To assess whether hybrid fish represent an intermediate between farmed and wild fish in their behaviour.
5) To assess whether there is a difference in behaviour between fish that were reared in contrasting environments (hatchery versus river).
6) To examine whether fish of farmed and wild origin show the same behavioural patterns when reared in two separate locations (Glasgow and Newport).

5.3. Methods

5.3.1. Common-Garden Environment (Glasgow) Experiment: Fish Husbandry and Acclimation

This experiment was performed in the aquarium facilities of the University of Glasgow and so is referred to as the Glasgow experiment. The Atlantic salmon used in this experiment were from two distinct origins: farmed and wild. These fish were the same stock as used in the experiment described in Chapter 4. Briefly, the domesticated fish were a Norwegian Mowi strain provided by Marine Harvest, whilst the wild fish were produced from wild anadromous parents caught in the Burrishoole catchment, county Mayo, Ireland. The eggs used in these experiments were produced in winter 2016, where 10 families of wild fish were derived from 5 females and 10 males, whilst the farmed fish developed from eggs of mixed parentage from an unknown number of families. Upon transfer at the eyed stage to the aquarium facilities at the Institute of Biodiversity, Animal Health and Comparative Medicine, University of Glasgow, the eggs of each origin were kept within separate buckets within the same larger tank, allowing water exchange across all eggs. The room was kept on a 12L:12D photoperiod. Fry were hand fed on commercial salmon fry food pellets (EWOS MICRO 5P LR; EWOS Ltd, Bathgate, UK) twice daily; all had begun first feeding by 24/04/17. Fry were then transferred into identical 400L stock tanks (one for farmed origin and one for wild) and individuals were fed a combination of bloodworms (Chironomid midge larvae) and EWOS pellets daily. Throughout the rearing period, all fish shared water from a common recirculation system and were kept at a constant temperature of approximately 12˚C. All fish were reared under these conditions, with the size of feed pellet being increased to match fish size, until the experiment commenced in November 2018 (i.e. when the fish were approximately 19 months old).

A total of 40 fish were used within this experiment: 20 of farmed origin and 20 of wild origin. Fish were chosen at random from their respective stock tanks but to include a range of sizes, ensuring a size overlap between the two origins: farmed fish had a mass range of 58.7 – 137.5g (mean mass = 86.6 ± 16.8g S.D.) and wild fish had a mass range of 70.6 – 157.2g (mean mass = 107.1 ± 21.1g S.D.) (as shown in Table 5-3). As the behavioural assays lasted 135 minutes in total (three 45 minute trials per fish) and could be conducted on 2 fish at a time, only 4 fish could be tested each day

(2 farmed and 2 wild), so the fish were acclimated in batches of 4 to ensure equal treatment of all experimental individuals. Atlantic salmon were transferred from their stock tanks into two identical 160L circular holding tanks (one for farmed and one for wild) 48 hours prior to behavioural trials, during which time food was withheld. The temperature of the water in these tanks was consistent with that of the original stock tanks. A batch of 4 fish was moved into the holding tanks every day, resulting in a total of 10 batches of 4 fish.

5.3.2. Experimental Arena Design

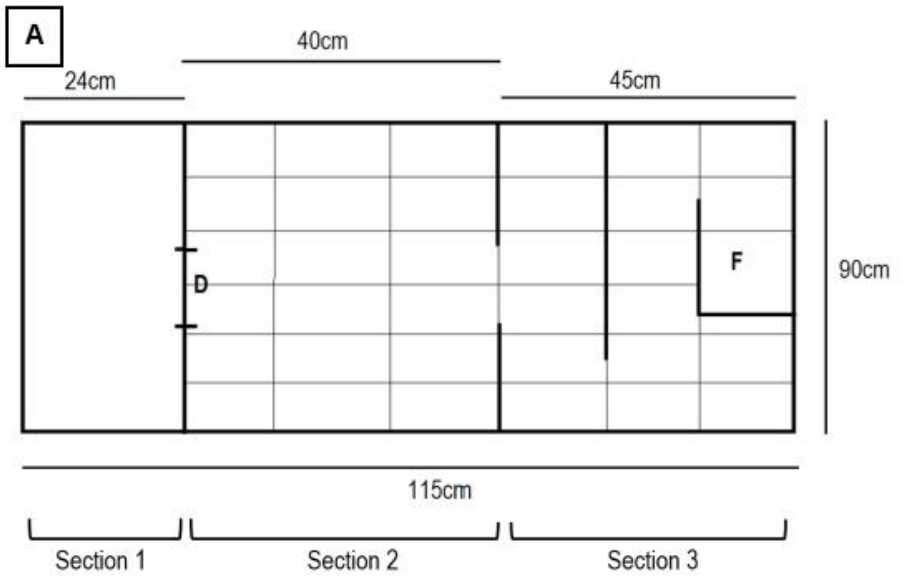

Figure 5-1: The design of the arena used for the behavioural experiments. *[A] is a graphic of in which dimensions are indicated in cm; arena side walls had a height of 42cm and water depth was 18 – 20cm. Bold black lines indicate opaque plastic separators and thin black lines represent the grid system marked on the bottom of the arena in sections 2 and 3. 'D' and 'F' identify the locations of the door and the food, respectively. Section 1 was covered to provide a shelter and sections 2 and 3 were left uncovered as shown in the photo in [B].*

The set up for the behavioural experiments consisted of two identical custom-made rectangular tanks, to allow 2 fish to be tested at once. These two identical arenas (arena A and arena B) were made out of grey PVC (l x w x h: 115 x 90 x 42cm) (Figure 5-1) and were positioned in an undisturbed room lit by fluorescent roof lights.

Each tank was partitioned into 3 with thin sheets of opaque plastic: section 1 was roofed with black plastic sheeting to provide a darkened shelter in which the fish would acclimate, whilst sections 2 and 3 remained uncovered so behaviour could be recorded. Section 2 was an open arena, while the third section contained a simple maze made from further thin opaque sheets of plastic. Food (bloodworm) was suspended at the end of this maze in a latex tube perforated with holes in order to allow scent to diffuse through the water. Sections 1 and 2 were separated by a door operated remotely by a pulley system once the acclimation period had elapsed, whilst access between sections 2 and 3 remained unconstrained throughout each trial. Sections 2 and 3 had gridlines on the bottom of the tank in order to more easily assess when an individual was moving. Water was kept at a depth of between 18 – 20cm and the ambient temperature in the room was maintained at 12˚C so that it was not necessary to chill the water within the tanks during the trials.

In order to capture the behaviour of each subject, a camera (Logitech HD Pro Webcam C920) was positioned above each arena. The live streams from the cameras were fed to a tablet (Samsung Galaxy Tab), each of which remained connected to an external hard drive so that the saved trial footage could be analysed at a later time.

5.3.3. Experimental Trial Protocol

Table 5-1: The organisation of trials each day in the Glasgow experiment using the two identical arenas (A and B) and four Atlantic salmon (two of farmed origin and two of wild origin). Each fish received three trials, and trials of the two fish origins (farmed and wild) were equally distributed across the two arenas.

		Experimental Trial Repeat		
		A	**B**	**C**
Fish ID	**Wild 1**	Arena 1	Arena 2	Arena 1
	Farmed 1	Arena 2	Arena 1	Arena 2
	Wild 2	Arena 2	Arena 1	Arena 2
	Farmed 2	Arena 1	Arena 2	Arena 1

For each trial, an Atlantic salmon from each origin (farmed and wild) was moved into section 1 of an experimental arena. The fish was retained within this covered section with the door closed for a 15-minute acclimation period, after which the door to section 2 was remotely opened. The remainder of the trial then lasted a further 30 minutes, throughout which the motion of the fish was captured by the overhead camera. The door separating sections 1 and 2 was left open, allowing the fish to retreat into section 1 once it had emerged. Once 30 minutes had elapsed, video recording ceased, and each fish was returned to its separate holding tank where it was left to recover. Water in the arena was mixed to disrupt scent trails and an air stone was placed in it to maintain oxygen saturation of the water. The bloodworm food was also replaced within the latex tube. During the recovery period of the two fish that had most recently been trialled, the remaining 2 fish that had been acclimated were trialled in the same way. Each experimental individual was put through 3 trials in one day, with each trial taking place in an alternate tank (Table 5-1). Though conducting 3 trials meant that each individual was not tested equally often in the two arenas, the overall pattern was that both origins of fish were tested an equal number of times (n = 60) in each arena.

At the end of each day, each arena was drained entirely of water and then refilled. The 4 fish that had been trialled were killed by anaesthesia overdose (benzocaine), followed by severing of the spinal cord. The wet weight (to nearest 0.1g) and fork length (to nearest 0.1mm) were measured. These measures were then used to calculate an individual's condition factor,

$k = mass/length^{x}$

where x is the coefficient for length given by the linear model log(mass) ~ log(length).

5.3.4. Footage Analysis of Behavioural Assays

Table 5-2: The ethogram used when reviewing the trial footage in BORIS (Friard, 2016). State events captured continuous actions that stopped and started throughout each trial (in seconds), whilst point evens captured discrete events that happened a discrete number of times (frequency count).

Ethogram Letter	Type of Event	Activity
E	State (continuous)	The fish was emerged from shelter (section 1)
M		The fish was actively moving in sections 2 and 3
D	Point (discrete)	The door opened (start of trial)
T		The fish emerged from shelter (section 1)
F		The fish reached the food

To ensure consistent analysis of the video footage, BORIS (v.7.2) was used (Friard, 2016), which allowed an ethogram to be created for each video. Creation of an ethogram allowed both state events (continuous actions) and point events (singular, discrete action) to be noted (Table 5-2). For each trial, two separate state events were recorded: whenever the fish was in the open (E; classified as whenever the fish was fully out of the covered section 1 of the arena) and whenever it was moving (M; classified as actively swimming). Recording as state events meant that throughout the trial, the timer was started when the activity began and stopped when the activity ended, allowing the recording of activities that frequently stopped and started. This resulted in a total time for 'time spent emerged' and 'time spent moving' for each fish in each trial. Along with these two state events, three point events were also recorded: when the door opened (D; which indicated the official start of the trial), when the fish emerged from the shelter (T; capturing the number of times the fish emerged from section 1) and when the fish reached the food (F; classified as when the fish entered the grid marked on the bottom of the arena in which the food was located). Whilst 'D' occurred only once in each trial, the 'T' and 'F' events could occur multiple times throughout the trial (e.g. a fish might commonly retreat and re-emerge from the shelter). Note that the trial did not end once the fish reached the food – behaviour was recorded for the full 30-minute duration. It was important to note the time of the door opening (D) as this marked the official start of the trial from which subsequent behaviours would be measured and also ensured analysis of each trial only covered the first 30 minutes after the door opened, even if the video footage

extended beyond this period. By recording the 5 events described, the following measures could be calculated for each trial: time until emergence (time taken for the fish to first emerge from section 1), number of emergences (a count of the number of times the fish emerged from section 1, as individuals often returned to it after having emerged), total time emerged (the full time an individual spent in sections 2 and 3 throughout the 30 minute trial), total movement time (the full time an individual spent actively moving in sections 2 and 3 throughout the trial), and food first reached (the time at which the fish first entered the grid section containing the food).

5.3.5. Hatchery vs Wild (Newport) Experiment: Fish Husbandry, Acclimation and Experimental Trial Protocol

Once the behavioural experiment had been completed at the University of Glasgow, comparing wild and farm origin fish reared in a common-garden aquarium environment, the same experiment was carried out at the Marine Institute in Newport, County Mayo, Ireland, to compare the behaviour of juvenile Atlantic salmon from different genetic backgrounds and rearing environments. In this second experiment (also termed the Newport experiment), the fish within both rearing environments represented four different genetic backgrounds: farmed (the same as the farmed stock used in the Glasgow experiment; wild (again, the same as the wild stock used in Glasgow; hybrid farmed female (HFF), descended from crosses between a female from the farmed stock (issued from the Mowi strain) and a male from the wild stock from the Burrishoole catchment; and hybrid wild female (HWF), produced by crossing a male from the farmed stock (issued from the Mowi strain) and a female from the wild stock from the Burrrishoole catchment. *In vitro* fertilisation took place in December 2017.

The two different rearing environments were hatchery and river. The hatchery fish were reared at the Marine Institute's hatchery facility, consisting of a flow through system that included four circular outdoor tanks. The tanks (2.5m wide, 0.6m deep, 2.4m^2 volume) had natural lighting and nets to deter avian predators. The tanks were supplied with unfiltered freshwater from Lough Feeagh (located upstream of the hatchery) and had a continuous flow rate of 60 $l.min^{-1}$. As the tanks were situated outdoors, water temperature ranged from 3.4˚C – 21.3˚C. Within the hatchery facility,

fish were grown at high density and fed *ad libitum* with pellets produced by Skretting Nutra Olympic (Cheshire, UK). Fish of different genetic origins were initially kept separate before then being PIT-tagged in October 2018 (when aged around 6 months), allowing the mixing of fish of different origins into 3 separate tanks, each containing the same number of individuals from each origin.

The river environment fish were created by releasing first-feeding fry of the same four genetic backgrounds into the Srahrevagh River, a natural river which experiences no artificial nutrient input. These fish were allowed to grow up under natural conditions until they were captured by electrofishing and brought into the hatchery just prior to the behavioural experiments (see below for details). These fish were retrospectively assigned to their respective genetic origin upon termination of the experiment using microsatellite markers (performed at University College Cork). This genetic analysis revealed that of the 68 Atlantic salmon that came from the river environment, 21 were farmed, 10 were wild, 23 were hybrid farmed female and 14 were hybrid wild female.

The Newport experiment took place in Spring-Summer 2019 (so all of the fish were 1+ parr at the time of testing). Fish from the hatchery rearing environment were tested first. Between March 27 and April 18 2019, 78 fish were captured by net from their rearing tanks: 6 fish were captured each day, as only 6 individuals could be tested in a single day. Fish were sampled from a single tank each day, with the PIT tags ensuring that one or two (but not zero) fish from each genetic origin were sampled each day (i.e. if a third fish of one origin was caught on any one day, it was put back). Of the 78 fish tested within the hatchery rearing environment, 19 were farmed, 19 were wild, 20 were HFF and 20 were HWF. Then, between May 9 and May 24 2019, 68 fish were captured by electrofishing from the Srahrevagh River. For both rearing environments, after capture, fish were weighed and put in individual numbered buckets and starved for 48 hours. The 6 fish selected each day were introduced in random order to an experimental arena. The number of trials per day (n = 2) was chosen to overcome any arena effect. Fish from the hatchery rearing environment had a mass range of 29.3 – 154.5g (mean mass = 66.9 ± 23.5g S.D.) and those from the wild river rearing environment had a mass range of 0.9 – 10.8g (mean mass = 4.8 ± 1.9g S.D.). Full details of the body mass of all tested salmon from each genetic origin and rearing environment are shown in Table 5-3.

To ensure consistency, the original arenas used within Glasgow's experiment were sent to Ireland. The trial structure was repeated; thus there was an initial 15 minute acclimation period in the sheltered section 1 of the arena in order to reduce stress, followed by the trial itself lasting 30 minutes once the door between sections 1 and 2 had been opened. As in the Glasgow experiment, fish were acclimated in batches and food was withheld from experimental individuals for the 48 hours preceding their trials. Whilst only bloodworm was used in the perforated latex tube in the Glasgow experiment, a combination of bloodworm and commercial pellets was used in the Newport experiment. Whilst each day in the Glasgow experiment four Atlantic salmon were trialled three times, in the Newport experiment six Atlantic salmon were trialled two times, due to the larger number of fish to be tested. To account for arena effects, each fish was trialled in both arenas, with the order of use randomised. As before, the footage of each trial was captured using overhead cameras (Logitech HD Pro Webcam C920) and water within the arenas was changed at the end of each day of testing. 292 trials were carried out in total.

Video analysis was conducted in the Newport experiment using the software BORIS 7.5.1. (Friard, 2016). Only data on time taken to emerge from section 1 (seconds) and time taken to first reach the food (in seconds) was collected for each trial.

Table 5-3: The mass measurements (range and mean (g)) of Atlantic salmon parr used within behavioural experiments in both Glasgow and Newport. Fish of farmed and wild origin were used in Glasgow and Newport, whilst hybrid fish with either a farmed female (HFF) or wild female (HWF) parent were also included in the Newport experiment. In addition, while all Glasgow fish were reared in the same environment (the University aquarium), fish in the Newport experiment were reared in two different rearing environments: hatchery and river.

Experiment:	**Glasgow**	**Newport**	
Rearing Environment:	**Aquarium**	**Hatchery**	**River**
Farmed mass range (g)	58.7 – 137.5g	68. 0 – 154.5g	3.5 – 10.8.5g
Farmed mean mass (g)	86.6 ± 16.8g S.D.	96.9 ± 22.4g S.D.	5.8 ± 22.4g S.D.
Wild mass range (g)	70.6 – 157.2g	36.5 – 70.5g	2.0 – 5.6g
Wild mean mass (g)	107.1 ± 21.1g S.D.	52.9 ± 10.8g S.D.	3.4 ± 1.1g S.D.
HFF mass range (g)	N/A	29.3 – 86.0g	2.5 – 8.5g
HFF mean mass (g)	N/A	59.8 ± 14.2g S.D.	5.0 ± 1.6g S.D.
HWF mass range (g)	N/A	42.5 – 88.8g	0.9 – 7.4g
HWF mean mass (g)	N/A	58.9 ± 14.3g S.D.	4.1 ± 1.8g S.D.

5.3.6. Statistical Analyses

Due to the difference in the number of replicate trials per fish between the experiments carried out in Glasgow (n=3) and Newport (n=2), the data from the two locations were not directly comparable so therefore had to be analysed separately. All statistical analyses were performed in R.3.5.1. (R Development Core Team) using lme4 (Bates, 2015), survminer (Kassambara, 2020), dplyr (Wickham, 2020), survival (Therneau, 2020b) and coxme (Therneau, 2020a) packages.

For the data generated by the experiment in Glasgow, generalised linear mixed models (binomial or poisson based upon response variable data type) or linear mixed models were used to examine the relationship between the continuous behavioural measures (time spent emerged (s) and time spent moving (s)) or discrete behavioural measures (time of emergence, time taken to reach food and number of emergences) and the explanatory variables (origin, trial number, fish mass and arena ID). Firstly, data from all trials (n=120) were analysed to assess both the likelihood of a fish ever emerging during the 30-minute trial and the number of emergences, across trials, arenas, and origins. Data were separated for the two origins and Cochran's Q tests were used to determine whether farmed and wild fish showed differences in whether or not they emerged at all across their three trials. This class of test determines whether there are differences on a dichotomous dependent variable (emergence or no emergence, in this instance).

The data were then subdivided based upon whether the fish emerged. For trials in which the fish did emerge, analyses examining the amount of time fish spent emerged (s) and the amount of time fish spent moving (s) were carried out. The transformtukey() function of the RCompanion package in R (Mangiafico, 2020) was used to assess whether the fit of the model would be improved with transformation of the response variable: the function conducts Tukey's ladder of powers on a vector to produce a more-normally distributed vector of values. This function returned a value of lambda, which was then used to transform the data where necessary. Within all models, arena ID (categorical variable with two levels: 1 and 2) was initially included as a random effect to account for any effect of using two separate arenas; however, the different arenas accounted for such little variance that models incorporating this random effect returned singularity warnings. Consequently, arena was always included as an explanatory variable, to check for any effect. Trial (categorical

variable with 3 levels: A, B and C) was included as an explanatory variable to assess whether behavioural differences existed between trials. To account for each fish being used on three occasions, fish ID was incorporated as a random effect within these models. Final models were chosen based on AIC, Log-Likelihood and visual inspection of residual plots using the DHARMa package (Hartig, 2020) in R.

In addition to generalised linear model analysis, ‘emergence behaviour’ (whether or not a fish emerged and how long it took to first do so) and ‘food-reaching behaviour’ (whether or not a fish reached the food and how long it took to first do so) were analysed via survival analysis using the R packages survival (Therneau, 2020b) and survminer (Kassambara, 2020). The time to first emerge was defined as the time, in seconds, from the door separating sections 1 and 2 opening to the experimental individual first entering section 2, whilst the time to reach the food was defined as the time, in seconds, from an individual’s initial emergence to when it first reached the food in section 3. This class of analysis gave an overview of ‘emergence behaviour’ and ‘food-reaching behaviour’ by accounting for both the time taken in each scenario, whilst also accounting for the fact that not every individual actually performed the action (i.e. not all fish emerged or reached the food). Survival analysis was therefore the best way to account for these censored observations. The survminer package was used to form Kaplan-Meier survival curves, before statistical significance was assessed using Cox proportional-hazards mixed effects models, using the coxme package in R (Therneau, 2020a). These Cox models relate the time that passes before an event (here, time to emerge/food) to explanatory variables (fish mass, origin, trial number and arena). This class of model also allowed for fish ID to be incorporated as a random effect. Cox model diagnostics were run using the survival and survminer packages in R: the proportional hazards assumption was tested using the cox.zph() function, influential observations were assessed using the ggcoxdiagnostics() function and for models containing continuous covariates, non-linearity was tested for using the function ggcoxfunctional(). Final models were chosen based on AIC and Log-Likelihood.

For the data generated by the experiment in Newport, only survival analysis was performed, as fewer explanatory variables were collected in this experiment than in Glasgow’s. As with the data produced in Glasgow, survival analysis was carried out for both emergence behaviour (whether or not the fish emerged and how long it took

to first do so) and food-reaching behaviour (whether or not the fish reached food and how long it took to first do so). Initially, data from the fish taken from both of the rearing environments (hatchery and river) were pooled, to examine any differences in behaviour across origins (farmed, wild, hybrid farmed female and hybrid wild female). Data were then further stratified by rearing environment to see whether there were any differences in behaviour amongst the origins that had come from different environments. The survminer package (Kassambara, 2020) was used to form Kaplan-Meier survival curves. The coxme package in R (Therneau, 2020a) was then again used to form Cox proportional-hazards mixed effects models to determine whether any significant differences in behaviour existed between fish from different origins or environments. Unlike within the experiment in Glasgow, only two trials were carried out per individual (one in each of the two arenas), so it was unnecessary to include both trial and arena as explanatory variables within the mixed effects Cox proportional-hazards models. Fish ID was still included as a random effect. Models initially included origin, rearing environment, fish mass, and arena as explanatory variables. Any interaction between origin and rearing environment was investigated, but none were found to be significant. Once more, the R packages survival and survminer were used to run Cox model diagnostics to test the proportional hazards assumption, for influential observations and for non-linearity, before final models were chosen based upon AIC and Log-likelihood. Due to the increased number of levels in the origin explanatory variable within the Irish dataset (due to the addition of two groups of hybrid fish), if any level of origin was significant within any mixed effects Cox model, *post hoc* pairwise comparisons were performed using partial likelihood-ratio tests to determine whether there was an overall effect of origin.

5.4. Results

5.4.1. Common-Garden Rearing Environment (Glasgow) Experiment: Behaviour of Farmed and Wild Atlantic Salmon

5.4.1.1. Initial emergence behaviour

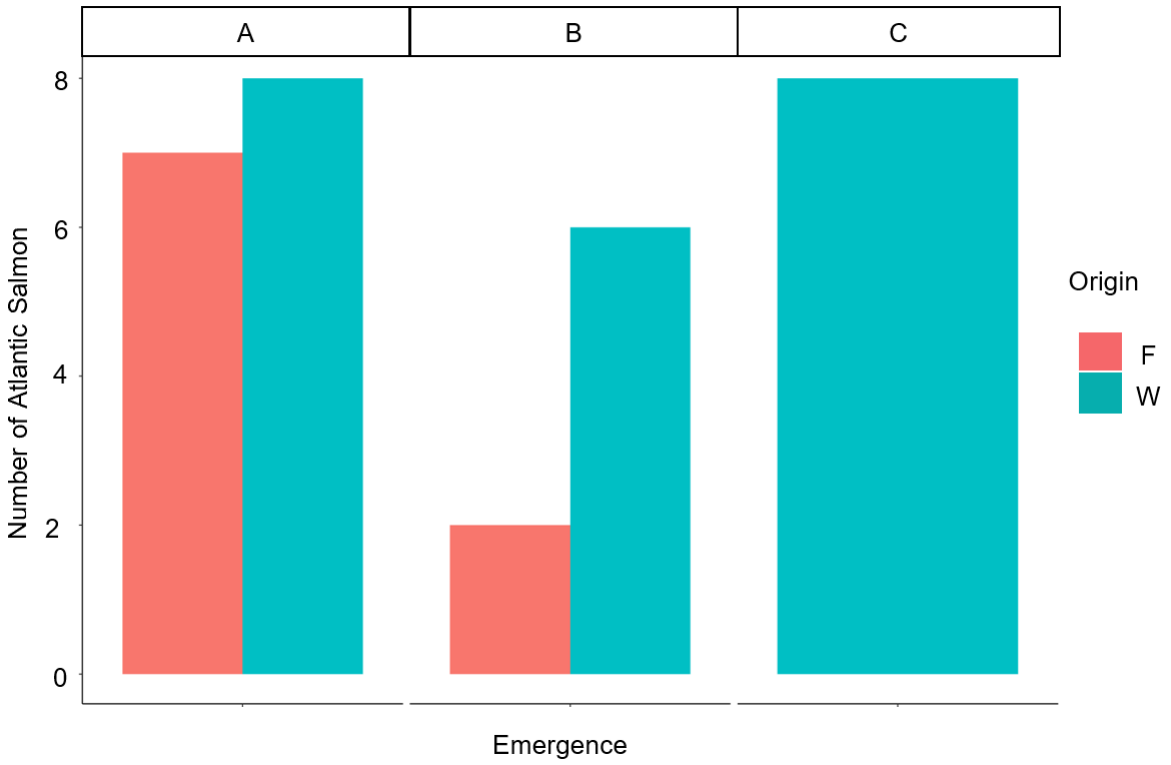

Figure 5-2: The number of farmed and wild Atlantic salmon (out of a maximum of 20 per origin) that emerged from the sheltered section of the arena across 3 trials (A, B and C) in the common-garden (Glasgow) experiment*. Atlantic salmon of farmed and wild origin are shown in orange and blue, respectively.*

Table 5-4: The overall number of farmed and wild Atlantic salmon to emerge and reach food across all three trials (A, B and C) in the common-garden (Glasgow) experiment (n = 40 fish in total, 20 of each origin).

	Farmed	Wild
Total number of fish that emerged in any of their 3 trials	9/20	15/20
Total number of trials in which fish emerged	9/60	22/60
Total number of fish that reached the food in any 3 of their trials	4/20	7/20
Total number of trials in which fish reached the food	4/60	10/60
Total number of fish that emerged in trial A	7/20	8/20
Total number of fish that emerged in trial B	2/20	6/20
Total number of fish that emerged in trial C	0/20	8/20
Total number of fish that emerged in 0 trials	11/20	5/20
Total number of fish that emerged in 1 trial	9/20	9/20
Total number of fish that emerged in 2 trials	0/20	5/20
Total number of fish that emerged in 3 trials	0/20	1/20

Of the 40 fish tested, 16 did not emerge at all in any of their three trials, and only one emerged in all three (Figure 5-2; Table 5-4). To assess whether the behaviour of each origin was maintained across all three trials, data were initially separated by origin; Cochran's Q test on the farmed origin salmon indicated that there was a significant difference in their likelihood to emerge across trials ($X^2_{(2)} = 8.67$, P = 0.013), with *post hoc* pairwise McNemar analysis showing a significant difference between trials A and C (adjusted P = 0.024, FDR adjusted). The same analysis on wild origin salmon showed no significant difference in their likelihood to emerge across the three trials (P = 0.75). A binomial generalised linear mixed model was then used to examine the likelihood of emergence of all fish from both origins. Emergence was the binomial response variable (yes or no) and origin, mass, length, arena and trial were included as explanatory variables. To account for each fish being tested multiple times, fish ID was included as a random effect. Of all explanatory variables, origin was found to be significant (P = 0.029), showing that wild Atlantic were more likely to emerge than their farmed conspecifics. This analysis also confirmed that there was no effect of arena (P = 0.76) or overall effect of trial (P = 0.077) (Table 5-5).

Table 5-5: The results from the binomial generalised linear mixed effects model testing which variables explained whether or not Atlantic salmon of wild and farmed origin would emerge from their shelter (section 1 of the tank; binomial response variable) in the common-garden (Glasgow) experiment. To account for each fish being tested multiple times, fish ID was included as a random effect in the original model, but is not shown as it did not account for any variance.

Fixed effects	Estimate	Standard Error	z value	P
Intercept	2.75	4.15	0.66	0.51
Origin - Wild	-1.22	0.56	-2.19	0.029
Fish Mass (g)	0.0045	0.032	0.14	0.89
Fish Length (mm)	-0.092	0.32	-0.29	0.78
Arena - 2	-0.14	0.44	-0.31	0.76
Trial - B	0.94	0.53	1.77	0.077
Trial - C	0.94	0.53	1.77	0.078

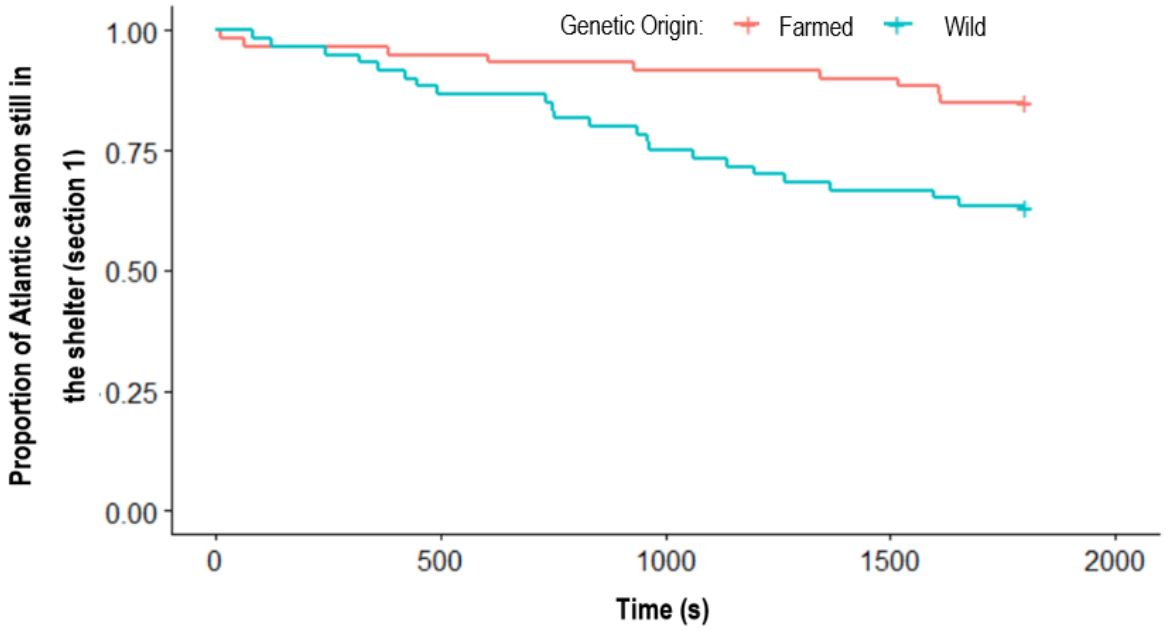

Figure 5-3: Kaplan-Meier "survival" curves showing the emergence behaviour in farmed and wild Atlantic salmon in the common-garden (Glasgow) experiment. *The curves indicate the proportion of farmed and wild fish that remained in their shelter throughout the 30-minute trial period (1800 seconds). Data are combined across the three trials of fish, but trial number, arena and fish ID were controlled for in the original analysis. Atlantic salmon of farmed and wild origin are represented by orange and blue lines, respectively.*

Table 5-6: The results of a Cox proportional-hazards mixed effects model exploring the impact of explanatory variables on the emergence behaviour of Atlantic salmon in the common-garden (Glasgow) experiment. The three explanatory variables used within the model are shown in the 'Fixed Effects' column. Fish ID was also included as a random effect The coefficient illustrates the likelihood of emergence, where a positive value indicates that the likelihood of emergence is higher for subjects with higher values of the corresponding variable; the hazard ratio is the exponentiated coefficient (exp(coef)), which gives the effect size for the coefficient; the 'z' value gives the Wald statistic; and the final column shows the statistical significance.

Fixed effects	**Coefficient**	**Standard Error**	**Hazard Ratio**	**z value**	**P**
Origin – Wild	1.031	0.40	2.81	2.60	0.0095
Trial – B	-0.69	0.44	0.50	-1.58	0.11
Trial – C	-0.69	0.44	0.50	-1.57	0.12
Arena - 2	0.19	0.36	1.21	0.54	0.59
Random effects	**Std Dev**	**Variance**			
Fish ID	0.020	0.00040			

Survival analysis was used to analyse the overall emergence behaviour of the Atlantic salmon. Time to emerge was regarded as the time elapsed between the start of the trial (the point at which the door between sections 1 and 2 opened) and when the fish first emerged into section 2 of the arena. By using survival analysis, time to emerge was treated as a time-to-event variable, instead of a simple quantitative continuous variable. This approach allowed the analysis to account for the fact that some outcomes had censored observations, meaning that not all fish had emerged within the 30-minute trial period (Figure 5-3). This approach, by retaining all of the observations in the analysis, therefore gave a better understanding of overall emergence behaviour and also allowed a Cox proportional-hazards mixed effects model to be used to test statistical significance of the explanatory variables. The Cox model found to fit best included origin, trial and arena as explanatory variables, with fish ID included as a random effect. Origin was significant (Table 5-6), indicating that the emergence behaviour was significantly different between farmed and wild fish. Hazard ratios were calculated for each explanatory variable, to show the effect of each variable on the likelihood of the event (emergence) occurring. For wild fish, the

hazard ratio = 2.81, which indicates that wild fish were more likely to have emerged at any given time point than their farmed conspecifics.

5.4.1.2. Multiple emergence behaviour

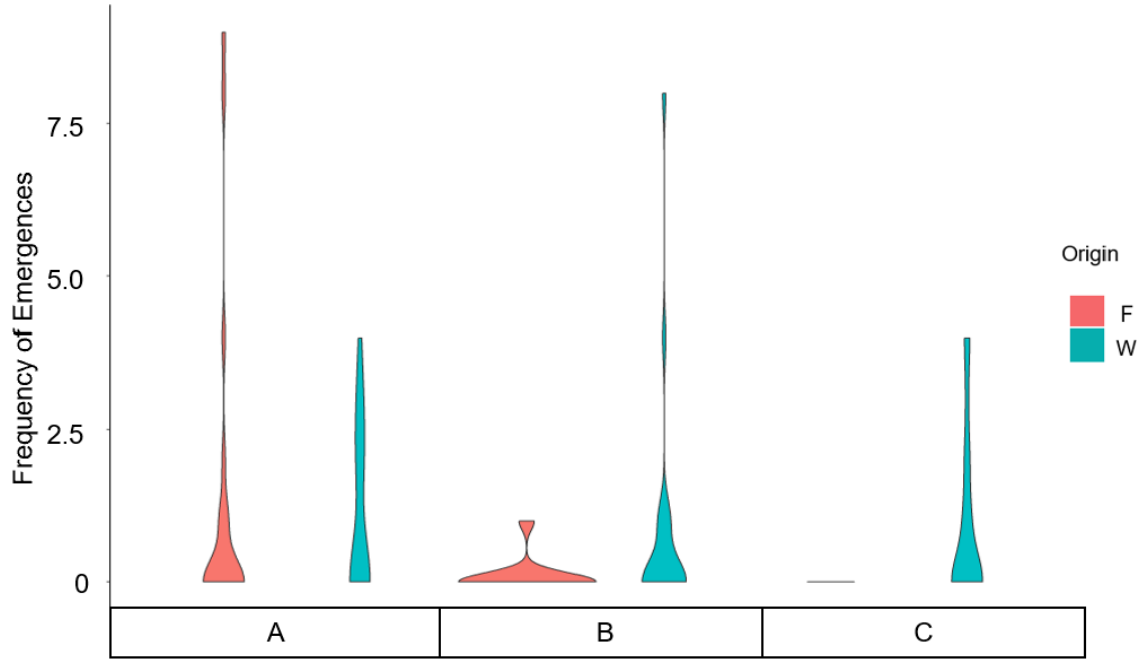

Figure 5-4: The number of times that Atlantic salmon of farmed and wild origin emerged from shelter, shown for the 3 separate trials (A, B and C) of each fish in the common-garden (Glasgow) experiment (n = 40 fish in total, 20 of each origin). *The total number of trials = 120 and fish emerged in 31 of these. In a single trial, fish emerged between 0 and 8 times. Atlantic salmon of farmed and wild origin are shown in orange and blue, respectively.*

As previously stated, in total, Atlantic salmon emerged in only 31 out of the 120 trials run. However, some individuals emerged multiple times within a trial, since they could retreat back into section 1 of the arena during the trial. Consequently, the frequency of emergence events varied between 0 and 8 across all trials (Figure 5-4). The number of times an individual emerged was explored with a generalised linear mixed effect model of the Poisson (log) family, as the response variable could be treated as count data. When forming the model, all trials were considered (n = 120), thereby incorporating data for all individuals, regardless of whether they emerged or not. Origin, trial and arena were included as explanatory variables, with fish ID included as a random effect (Table 5-7). Origin was significant (P = 0.0088), showing that wild individuals emerged more frequently in any one trial than their farmed

conspecifics, likely reflecting that wild fish were more likely to emerge overall (Figure 5-3; Table 5-6). Interestingly, the effect of trial was significant, with fish emerging less frequently in later trials (Figure 5-4).

Table 5-7: The results from the Poisson generalised linear mixed effects model exploring the impact of explanatory variables on the number of emergences of an Atlantic salmon from its shelter in a single trial in the common-garden (Glasgow) experiment. Fish ID was included as a random effect but is not shown as it did not account for any variance.

Fixed effects	Estimate	Standard Error	z value	P
Intercept	-1.88	0.65	-2.91	0.0036
Origin - Wild	1.42	0.54	2.62	0.0088
Trial – B	-1.40	0.63	-2.24	0.025
Trial – C	-1.29	0.62	-2.08	0.038
Arena - 2	-0.057	0.51	-0.11	0.91

5.4.1.3. Time spent emerged

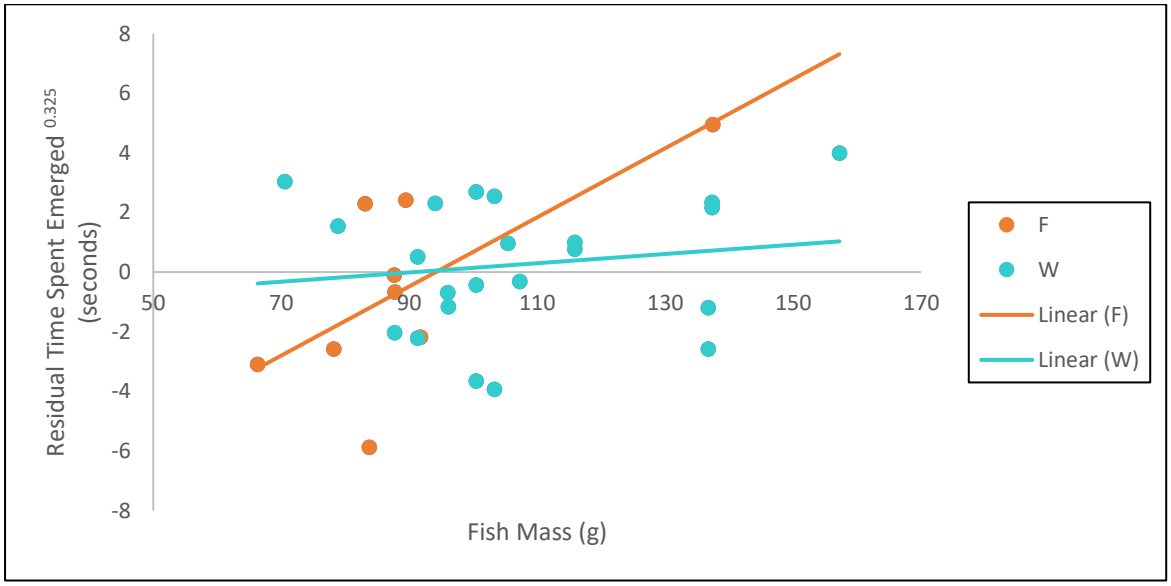

Figure 5-5: The relationship between the residual time spent emerged (transformed to the power of 0.325 to normalise the data) (s) in behavioural trials and fish mass (g) in farmed and wild Atlantic salmon in the common-garden (Glasgow) experiment (n=40 in total). *Orange and blue points and lines represent farmed and wild fish, respectively. Note that though the statistical analysis was based on the (absolute) time spent emerged, here the time spent emerged is plotted as residual values after controlling for arena (1 or 2) and trial (A, B, or C), in order to illustrate the relationship with fish mass.*

Table 5-8: The results of a linear mixed effects model examining the impact of trial, arena, origin, fish mass and the interaction between origin and fish mass on the time farmed and wild Atlantic salmon spent emerged from a shelter during a behavioural trial in the common-garden (Glasgow) experiment. Time spent emerged was transformed based upon Tukey's ladder of powers (λ = 0.325) to normalise the data and fish ID was included as a random effect.

Fixed effects	**Estimate**	**Standard Error**	**t value**	**P**
Intercept	-6.86	4.25	-1.61	0.12
Origin - Wild	12.09	5.13	2.36	0.029
Fish Mass (g)	0.15	0.05	3.15	0.0045
Trial – B	-2.57	1.10	-2.33	0.040
Trial – C	-2.06	1.07	-1.94	0.10
Arena – 2	1.27	0.89	1.43	0.17
Origin – Wild * Mass	-0.12	0.05	-2.31	0.033
Random effects	**Std Dev**	**Variance**		
Fish ID	1.44	2.09		

Of the 9 farmed and 15 wild Atlantic salmon that emerged, individuals spent varying amounts of time outside the shelter of section 1. Data from trials in which the fish emerged showed that farmed fish spent between 2.8 – 1736.7s emerged (mean time = 520.7 ± 607.42s S.D., n = 9), whilst wild fish spent between 21.3 – 1715.9s emerged (mean time = 565.8 ± 475.9s S.D., n = 22). A linear mixed effect model was used to explore the relationship between the time the Atlantic salmon spent emerged and trial, arena and the interaction between origin and fish mass (Table 5-8). Only data from trials in which the fish had emerged were included (n=31). The time emerged was transformed based upon Tukey's ladder of powers (λ = 0.325) to normalise the data and fish ID was included as a random effect. Origin, mass and the comparison of trials A and B were each found to be significant, but an F-test showed that the effect of trial was not significant overall (P = 0.091). Whilst the results of the model indicated that both wild fish and larger fish spent more time emerged overall, the interaction between origin and mass showed that the effect of mass was much stronger in the farmed fish. An unpaired two-sample t-test confirmed there was a significant difference in mass between the farmed and wild salmon (t_{38} = -3.32, P = 0.0028), where wild fish were significantly larger than their farmed conspecifics. To present the relationship between time spent emerged (s), fish mass

(g) and fish origin, the other covariates within the original model had to be controlled for by calculating residual time spent emerged. To do so, fish mass and origin were removed from the original model in order to gain a measure of expected time spent emerged (when accounting for arena and trial number). Expected time spent emerged (as shown in Table 5-9) was then subtracted from the transformed actual time emerged to give residual time spent emerged, which could then be plotted against fish mass (Figure 5-5).

Table 5-9: The values calculated for the expected time spent emerged in the common-garden (Glasgow) experiment, which were then used when calculating the residual values to plot against fish mass (g).

	Arena 1	**Arena 2**
Trial A	6.6783	7.5927
Trial B	6.3478	7.2622
Trial C	6.0805	6.9949

5.4.1.4. Time spent moving

As with the varying amount of time spent emerged by each individual, in each of the trials in which the fish emerged (farmed n = 9; wild n = 22), there was also variation in the amount of time spent actively moving. Even once emerged from the shelter of section 1, some individuals would remain stationary for large periods of time. Within trials in which fish emerged, farmed fish spent between 2.8 – 326.8s actively moving (mean time = 98.4 ± 104.94s S.D., n = 9), whilst wild fish spent between 15.2 – 961.1s actively moving (mean time = 175.6 ± 230.4s S.D., n = 22). The time spent moving was log-transformed (based upon Tukey's ladder of powers) and a linear mixed effect model explored the impact of origin, mass, trial and arena on movement time, with fish ID included as a random effect. Only data from trials in which fish had emerged was included (n = 31). No explanatory variable was found to significantly impact the time an individual spent moving in any of the trials overall.

5.4.1.5. Food-reaching behaviour

Food was available to all Atlantic salmon at the end of section 3 within each behavioural arena. This food was not directly visible upon emergence; to reach the bloodworm, the fish had to navigate through section 2 and plastic partitions forming a simple maze in section 3. Of the 9 farmed and 15 wild Atlantic salmon that emerged, 4 farmed and 7 wild fish reached the food in one or more of their trials. In total, out of the 31 trials in which fish emerged, fish reached food in 14 trials (4 farmed and 10 wild trials; summarised in Table 5-4). Data from all trials were used to explore whether or not the fish reached food: a binomial generalised linear mixed model, with origin, trial, arena as explanatory variables and fish ID as a random effect showed that no explanatory variable impacted an individual's likelihood of reaching the food.

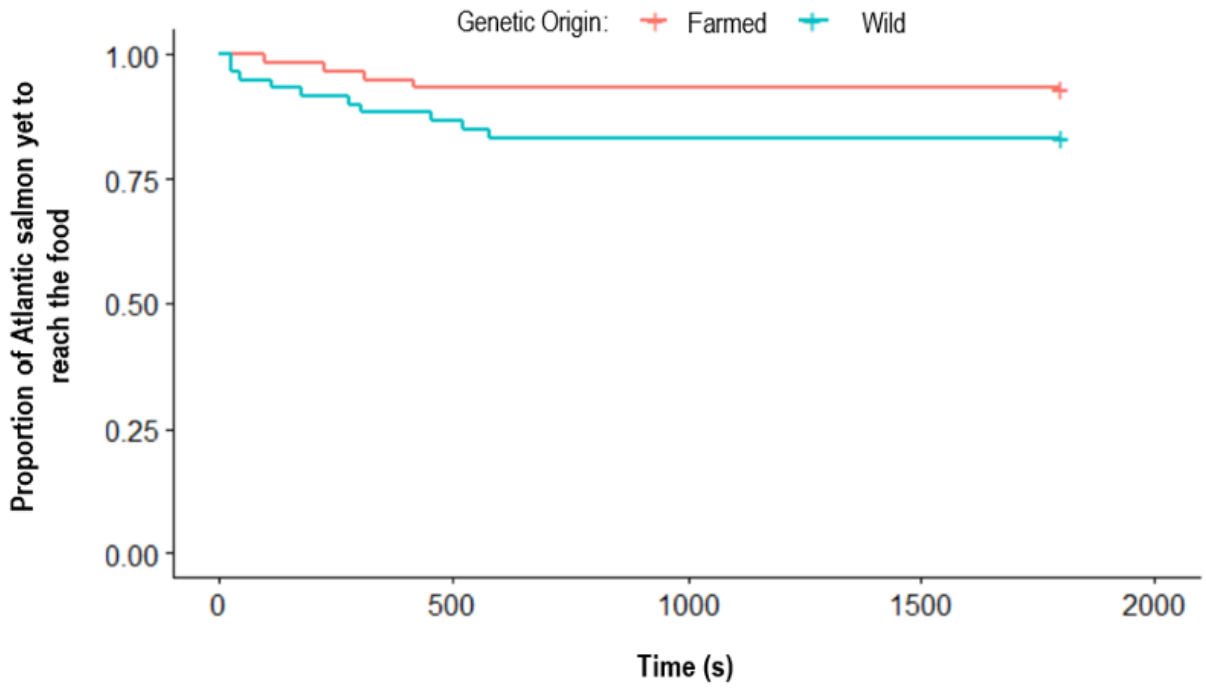

Figure 5-6: Kaplan-Meier "survival" curves showing the food-reaching behaviour in farmed and wild Atlantic salmon in the common-garden (Glasgow) experiment. *The curves indicate the proportion of farmed and wild fish that had yet to reach the food throughout the 30-minute trial period (1800 seconds). Atlantic salmon of farmed and wild origin are represented by orange and blue lines, respectively.*

Survival analysis was used to analyse the behaviour of the Atlantic salmon with regards to reaching the food. As with the emergence behaviour, survival analysis was chosen because it allowed time to reach the food as a time-to-event variable, which once more accounted for the censored observations (in this instance, the fact

that not all individuals reached the food). Again, this avoided biased estimates that would have occurred by treating the time to reach the food as a quantitative variable. All 120 trials were included within the analysis. Here, time to reach the food was defined as the time from the fish' initial emergence until the time at which the fish reached the bloodworm food in section 3 of the arena (in seconds). The food-reaching behaviour of Atlantic salmon was illustrated by Kaplan-Meier curves (Figure 5-6), whilst a Cox proportional hazards mixed effects model was used to explore the relationship between the behaviour and explanatory variables. The final Cox model included origin, trial and arena as explanatory variables, with fish ID included as a random effect. No explanatory variable was found to be significant ($P > 0.05$), indicating that there was no difference between farmed and wild Atlantic salmon in their food-reaching behaviour (incorporating their likelihood of reaching food and the time taken to do so).

5.4.2. Rearing Environment (Newport) Experiment: Behaviour of Farmed and Wild Origin Atlantic Salmon and their Hybrids

5.4.2.1. Emergence behaviour

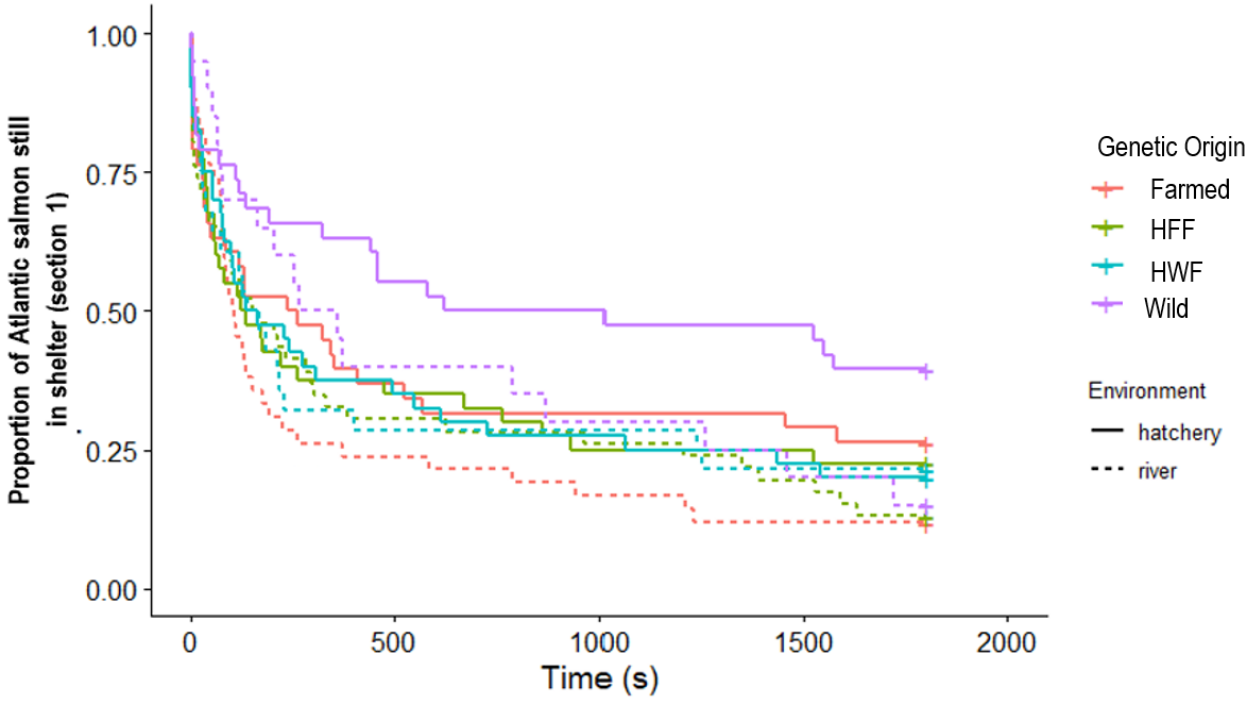

Figure 5-7: Kaplan-Meier "survival" curves showing the emergence behaviour in farmed, wild and hybrid Atlantic salmon in the hatchery vs wild (Newport) experiment. *The curves indicate the proportion of farmed, wild and hybrid (HFF and HWF) fish that had yet to emerge from the shelter throughout the 30-minute trial period (1800 seconds). Eight curves are shown, coloured by genetic origin of the fish and styled (solid or dashed) according to the environment in which they were reared (hatchery or river).*

Table 5-10: The results of a Cox proportional-hazards mixed effects model exploring the impact of explanatory variables on the emergence behaviour of farmed, wild and hybrid (HFF and HWF) Atlantic salmon in the hatchery vs wild (Newport) experiment. The four explanatory variables used within the model are shown in the 'Fixed effects' column. Fish ID was also included as a random effect. The coefficient illustrates the likelihood of emergence, where a positive value indicates that the likelihood of emergence is higher for subjects with higher values of the corresponding variable; the hazard ratio is the exponentiated coefficient (exp(coef)), which gives the effect size for the coefficient; the 'z' value gives the Wald statistic; and the final column shows the statistical significance.

Fixed effects	**Coefficient**	**Standard Error**	**Hazard Ratio**	**z value**	**P**
Environment – River	-0.33	0.42	0.72	-0.78	0.43
Origin – HFF	-0.14	0.24	0.87	-0.57	0.57
Origin – HWF	-0.18	0.26	0.83	-0.70	0.48
Origin – Wild	-0.75	0.29	0.47	-2.55	0.011
Fish Mass (g)	-0.0093	0.00603	0.99	-1.54	0.12
Trial - B	-0.89	0.14	0.41	-6.16	< 0.001
Random effects	**Std Dev**	**Variance**			
Fish ID	0.61	0.37			

Survival analysis was used to analyse the overall emergence behaviour of the four origins of Atlantic salmon (farmed, wild, hybrid farmed female (HFF) and hybrid wild female (HWF)) from the two different rearing environments (hatchery and river). Time to emerge was again defined as the time between the start of the trial (the point at which the door between sections 1 and 2 opened) and when the fish first emerged into section 2 of the arena. As with the data generated in Glasgow, using survival analysis allowed time to emerge to be treated as a time-to-event variable, instead of a simple quantitative continuous variable, and allowed inclusion of censored observations (i.e. where the fish did not emerge during the 30-minute trial). Kaplan-Meier curves were initially formed to show the emergence behaviour of all origins of fish from both rearing environments (Figure 5-7). A Cox proportional-hazards mixed effects model was used to test the statistical significance of the explanatory variables. The final Cox model included origin (W, F, HFF or HWF), rearing environment (hatchery or river), fish mass (g) and trial (A or B) as explanatory variables. Fish ID was included as a random effect to account for each individual

being tested twice. Neither rearing environment (hatchery or river) or fish mass (g) were significant, but there was a significant effect of trial and a significant difference between farmed and wild origin fish (Table 5-10). For wild fish, the hazard ratio = 0.47, which indicates that compared to their farmed conspecifics, wild fish were less likely to have emerged at any given time than their farmed conspecifics, whilst for trial, fish were less likely to emerge in the second trial. *Post hoc* analysis using a partial likelihood-ratio test showed that the overall effect of origin was significant (X^2 (3) = 8.11, P = 0.044), whilst a *post hoc* Tukey test showed that differences between origins were largest between wild and farmed fish and smallest between HWF and HFF (Table 5-11).

Table 5-11: The pairwise comparisons resulting from a Cox proportional-hazards mixed effects model, which used survival analysis to explore the emergence behaviour juvenile Atlantic salmon from four different origins: farmed (F), wild (W), hybrid farmed female (HFF) and hybrid wild female (HWF) in the hatchery vs wild (Newport) experiment. The coefficient and hazard ratio for each comparison is given.

Origins	Coefficient	Hazard Ratio
HFF – F	- 0.14	0.87
HWF – F	-0.18	0.83
W – F	-0.75	0.47
HWF – HFF	-0.05	0.95
W – HFF	-0.61	0.54
W – HWF	-0.56	0.57

5.4.2.2. Food-reaching behaviour

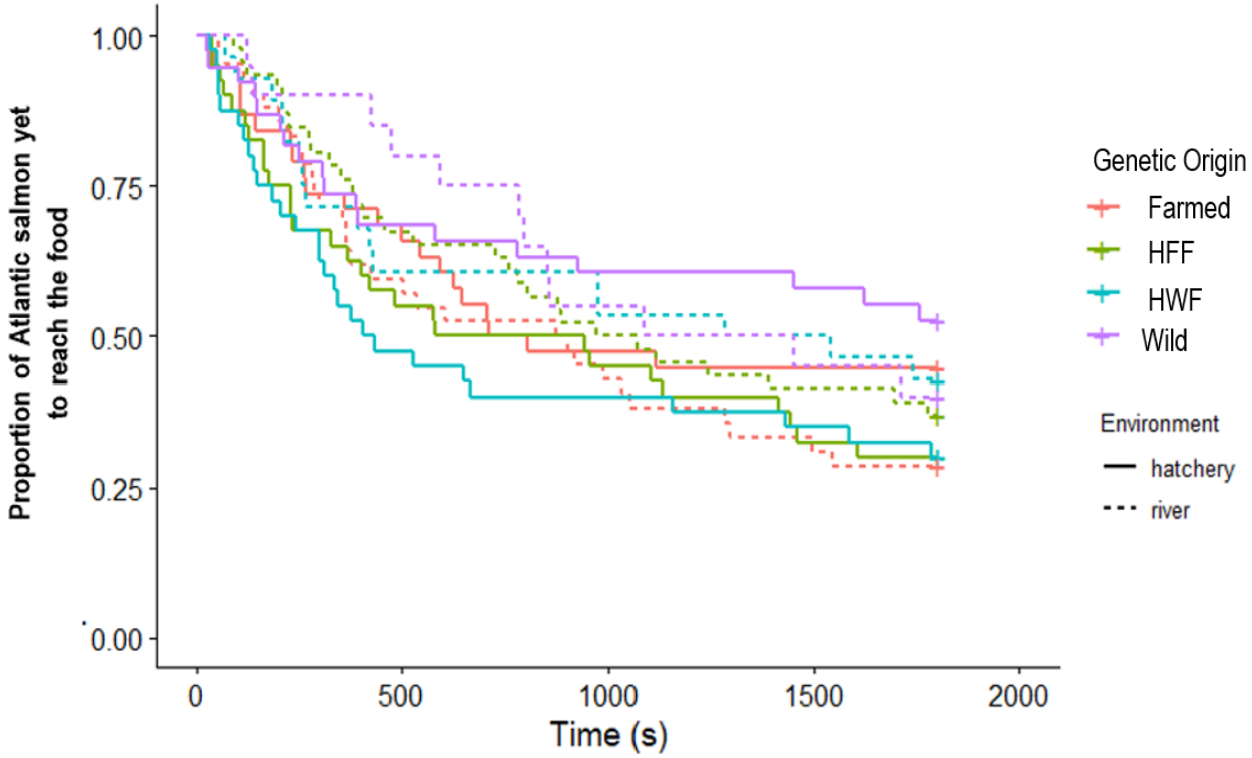

Figure 5-8: Kaplan-Meier "survival" curves showing the food-reaching behaviour in farmed, wild and hybrid Atlantic salmon in the hatchery vs wild (Newport) experiment. *The curves indicate the proportion of farmed, wild and hybrid (HFF and HWF) fish that had yet to reach the food throughout the 30-minute trial period (1800 seconds). Eight curves are shown, coloured genetic origin of the fish and styled (solid or dashed) according to the environment in which they were reared (hatchery or river).*

Table 5-12: The results of a Cox proportional-hazards mixed effects model exploring the impact of explanatory variables on the food-reaching behaviour of farmed, wild and hybrid (HFF and HWF) Atlantic salmon in the hatchery vs wild (Newport) experiment. The explanatory variables used are shown in the 'Fixed Effects' column. Fish ID was also included as a random effect. The coefficient illustrates the likelihood of reaching the food, where a positive value indicates that the likelihood of reaching the food is higher for subjects with higher values of the corresponding variable; the hazard ratio is the exponentiated coefficient (exp(coef)), which gives the effect size for the coefficient; the 'z' value gives the Wald statistic; and the final column shows the statistical significance.

Fixed effects	**Coefficient**	**Standard Error**	**Hazard Ratio**	**z value**	**P**
Environment – River	-1.67	0.52	0.19	-3.22	0.0013
Origin – HFF	-0.47	0.30	0.63	-1.57	0.12
Origin – HWF	-0.29	0.32	0.75	-0.92	0.36
Origin – Wild	-1.03	0.36	0.36	-2.88	0.004
Fish Mass (g)	-0.02	0.0075	0.98	-3.14	0.0017
Trial - B	-1.22	0.17	0.29	-7.40	< 0.001
Random effects	**Std Dev**	**Variance**			
Fish ID	0.83	0.69			

Survival analysis was also used to analyse the behaviour of the Atlantic salmon with regards to time taken to reach the food (defined as the time from the fish's initial emergence until the time at which the fish reached the food in section 3 of the arena (in seconds)). All 292 trials were included within the analysis, so therefore included trials in which fish did not emerge at all. The food-reaching behaviour of Atlantic salmon was illustrated by Kaplan-Meier curves (Figure 5-8). The final Cox model included origin, rearing environment, fish mass (g) and trial, with fish ID included as a random effect. There was a significant effect of rearing environment, fish mass and trial, and also a significant difference between farmed and wild origin fish (Table 5-12). This indicated that fish reared in the river, larger fish and fish in their second trial were less likely to have reached the food at any given time, whilst fish of wild origin were less likely than farmed to have reached the food. Specifically, the exponentiated coefficients showed that compared to their farmed conspecifics, the chance that wild fish would reach the food at any given time was 0.36; compared to fish reared in the hatchery, the chance that fish from the river would reach the food at any given time was 0.19; and compared to fish in their first trial, the chance that

fish in their second trial would reach the food at any given time was 0.29. *Post hoc* analysis using a partial likelihood-ratio test indicated that the overall effect of origin was significant (X^2 (3) = 9.30, P = 0.026), whilst a *post hoc* Tukey test showed that differences between origins were again largest between wild and farmed fish and smallest between HWF and HFF (Table 5-13).

Table 5-13: The pairwise comparisons resulting from a Cox proportional-hazards mixed effects model, which used survival analysis to explore the food-reaching behaviour juvenile Atlantic salmon from four different origins: farmed (F), wild (W), hybrid farmed female (HFF) and hybrid wild female (HWF) in the hatchery vs wild (Newport) experiment. The coefficient and hazard ratio for each comparison is given.

Origins	Coefficient	Hazard Ratio
HFF – F	-0.47	0.63
HWF – F	-0.29	0.75
W – F	-1.03	0.36
HWF – HFF	0.17	1.19
W – HFF	-0.56	0.57
W – HWF	-0.74	0.48

5.5. Discussion

This study sought to examine whether there are inherent differences in exploratory behaviour between juvenile Atlantic salmon of different genetic origins: farmed, wild and hybrid (HFF and HWF), and whether any differences were affected by the rearing environment. Behavioural experiments were conducted on fish that had been reared in three different 'common garden' environments: aquarium, hatchery and river. All experiments found a difference in the emergence behaviour of fish of farmed and wild origin; however, behavioural differences between wild and farm origin fish were dependent upon the rearing environment. If reared in the aquarium, wild origin fish were more likely to emerge from shelter than those of farmed origin. However, if reared in either the hatchery or wild, the pattern was reversed, with farmed origin individuals being more likely to emerge than those of wild origin.

Data collected from aquarium-reared fish also indicated that there was an effect of fish origin on the number of times a fish emerged in any one trial and the total time spent emerged, though origin did not dictate the time an individual spent moving. Unlike emergence behaviour, an impact of origin on the likelihood of locating the food was not found in aquarium-reared fish and was only found among hatchery- and wild-reared fish, in which fish of wild origin were less likely to have reached the food at any given time than were those of farm origin. This study also examined the behaviour of farmed x wild hybrid strains and found that these fish displayed intermediate behaviour compared to the two parental lines. Finally, this study allowed a direct comparison of whether two different rearing environments (hatchery or river) had an effect on behaviour of the fish. Whilst rearing environment had no impact on emergence behaviour, Atlantic salmon reared in the river showed reduced food-reaching behaviour than their conspecifics that had been reared in the hatchery, independent of their genetic origin.

5.5.1. The Impact of Genetic Origin on the Behaviour of Juvenile Atlantic Salmon

5.5.1.1. Exploratory behaviour

Within the experiment carried out on fish reared in the aquarium common garden (i.e. in Glasgow), survival analysis revealed that Atlantic salmon of wild origin were

more likely to have emerged from shelter at any given time than their farmed conspecifics. Subsequent analyses also showed that within a single experimental trial, fish of wild origin were more likely to emerge from the shelter multiple times and also spend more time emerged overall. The time spent emerged was also related to fish mass, with larger fish spending a greater proportion of the trial emerged from the shelter, the effect of which was particularly strong amongst individuals of farmed origin. Interestingly, amongst fish reared in the aquarium, wild fish seemed equally likely to emerge in all replicate trials, whilst fish of farmed origin became increasingly less likely to emerge with each subsequent trial, culminating in no farmed fish emerging in their final trial. This reflects what has been seen in brown trout, where parr of wild origin that had been reared in the wild were found to be more consistent in their exploratory behaviour than hatchery-reared parr (Adriaenssens and Johnsson, 2011).

The discrepancy between fish of farmed and wild origin in the time spent in the open is striking, especially since the pattern was reversed in the two experiments: the experiment carried out in Newport found that, regardless of whether they had been reared in the hatchery or the river, Atlantic salmon of farmed origin were more likely to emerge from shelter and did so more quickly, than those of wild origin. Concordant with the results produced from the farmed fish reared in the aquarium environment, the Atlantic salmon tested in Newport also showed reduced emergence behaviour in their later trial. The reduced emergence/increased time to emerge in the latter trials could indicate a fatigue effect (as all trials for each fish took place within the same day). In fish of farmed origin that had been reared in the hatchery or the river, the increased willingness to emerge and their reduced latency to do so was not expected, since the same behavioural patterns would have been expected across all three common garden environments. Although genetic origin of the Atlantic salmon was a significant predictor of behaviour, this indicates that genetics may determine a behavioural response to a given environment, but not necessarily the behaviour itself.

A difference in behaviour between fish of different origins was anticipated, since contrasting behaviours between teleosts of wild and farmed origin are widely reported within the literature. For example, higher risk-taking behaviour has been

noted in species of farmed fish (Berejikian et al., 1996; Reinhardt, 2001). Risky behaviours are thought to be more common in farmed teleost species as an unintended result of selection for fast growth, where these individuals are more active and aggressive, but also more prone to predation (Biro and Post, 2008). Such inherent genetic differences between fish of different origins are likely to be the result of directional selection during domestication and relaxed natural selection in the captive environment (Christie et al., 2012). When considering genetic drivers of exploratory behaviour in Atlantic salmon, the divergent behavioural requirements throughout the lives of fish of different origins must be considered. In the wild, Atlantic salmon often show strong site fidelity (Cunjak, 1992), but the environment experienced by salmonids is dynamic and even those that defend territories often make foraging excursions (Elliott, 1990). Additionally, the more complex life histories of wild salmon mean that they range much farther, especially during migration (Klemetsen et al., 2003). In comparison, juvenile farmed salmon stay within their tanks, and whilst their movement is continuous (Ashley, 2007), their environment is less dynamic. Indeed, for the fish that did emerge, there was no significant difference in the time the fish spent moving between the two origins of fish, which could reflect that regardless of genetic origin, continuous swimming is often a necessity. The discrepancies in behaviour of salmon of farmed and wild origin across their whole life cycles might result in salmon of wild origin having a genetic predisposition to be more exploratory. In contrast, due to domestication, fish of farmed origin might be less willing to emerge from a shelter and move around an unfamiliar arena, since generations of selective breeding have reduced the potential benefits of exploration, since this is not possible within an aquaculture setting. Nonetheless, behaviours have been shown to be strain-specific, with Alioravainen et al. (2020) finding that hatchery-reared brown trout were more likely than wild fish to disperse downstream, as the wild strain was mainly resident.

The contrasting results indicate an interaction between genetic factors and the environment. It is possible that fish of farmed origin that had been reared in the hatchery and river environments showed increased emergence behaviour as a result of their rearing environment, resulting in the farmed fish having a riskier behavioural phenotype than their wild conspecifics. Meanwhile, the aquarium rearing environment did not have the same impact on fish phenotype, resulting in the inverse

behaviours being observed between fish of different origins. The preference of a fish to remain within the shelter in the present experiment might reflect that the individual has placed greater importance on the value of shelter: territorial salmonids have shown a preference for territories with protective cover, which is related to perceived levels of predation risk (Johnsson et al., 2004). If that is the case, then the value of shelter to Atlantic salmon of different origins could in part be dependent the environment that they have experienced up until then

5.5.1.2. Food-reaching behaviour

Interestingly, though contrasting emergence behaviour was noted between the fish of farmed and wild origins, within fish reared in the aquarium (the Glasgow experiment) there was no effect of origin nor any other explanatory variable on the likelihood of an individual reaching food, nor how quickly they might do so. Arguably, the ability of a fish to reach the food at the end of the short maze could also be considered exploratory behaviour, so it was surprising that the differences in emergence behaviour between the two origins were not reflected in the food-reaching behaviour. Nonetheless, the food-reaching behaviour in fish reared in the hatchery and the river (tested in Newport) showed differences to that of the fish reared in the aquarium (tested in Glasgow). In Newport, there was a significant effect of origin, where fish of wild origin were less likely to have reached the food at any given time than their farmed conspecifics.

Within aquarium-reared fish at least, a genetic component to food reaching behaviour in juvenile Atlantic salmon was not observed. Nonetheless, amongst the fish reared in the hatchery and the river environments, as with exploratory behaviour, a genetic component to food-reaching behaviour was observed. As with exploratory behaviour, it is likely that the process of domestication has led to differences in the food-reaching behaviour of Atlantic salmon from different genetic backgrounds. In general, domesticated individuals show reduced motivation for foraging due to generations in which they have not had to actively seek food or assess differences in food quality (Mignon-Grasteau, 2005). There are wide differences in the foraging activity necessitated by the aquaculture environment in comparison to the wild environment, since individuals in fish farms are provided with food, whilst those in

the wild must actively forage and resources are often patchier (Cunjak, 1992). Generations of adaptation to feeding within either the aquaculture or wild environment would have therefore been expected to influence the foraging activity of the fish.

Another possible explanation for the observed behavioural patterns could be linked to photoperiod and the circadian rhythms of fish from different genetic backgrounds. The activity patterns of wild salmonids is largely dictated by photoperiod and temperature, which leads to daily fluctuations in foraging activity (Fraser et al., 1995). Whilst timing of foraging activity varies seasonally, primarily in response to temperature changes, salmonids in the wild largely also show diel rhythms in feeding rate (Fraser et al., 1995; Amundsen et al., 1999; Grade and Letcher, 2006). In contrast, fish in an aquaculture setting often have largely continuous access to food. As a result, it is possible that within the Newport experiment, fish of farmed origin sought food that was available, unconstrained by any inherent circadian rhythms in appetite. This effect has previously been noted in salmonids, where circadian rhythmicity in the behaviour of fish is often lost in domesticated individuals as they have not experienced the same environmental selection pressures that drive circadian patterns in behaviour (Alioravainen et al., 2020). Indeed, salmonids classified as active or inactive in a laboratory setting can show different patterns of behaviour in the wild environment, which is thought to be due to differences in light levels (Závorka et al., 2015). In the context of the experiment conducted in Newport, it could be that fish of wild origin were less likely to display food-reaching behaviour within the experimental setting due to the lack of circadian cues, since all behavioural trials were carried out indoors, under constant lighting. Although all fish reared in the hatchery and the river remained outside until the commencement of the experiment, potentially fish of farmed origin were unhindered by the lack of circadian cues, as the importance of these cues has been negated as multiple previous generations have been fed consistently, unconstrained by the rhythmicity experienced by wild fish. This could also be a driver for the lack of difference in food-reaching behaviour seen between fish of farmed and wild origin reared in the aquarium, as all fish in Glasgow spent their entire lives inside on a 12L:12D photoperiod. Therefore, when reared in the aquarium, the fish would have been acclimated to the constant artificial light used throughout the experiment, whereas

upon being reared in the outdoor hatchery or river, fish would be unfamiliar with the artificial light used. This environmental change for the fish in Newport could have triggered the contrasting behavioural phenotypes observed between fish of different origins.

When assessing the genetic drivers of behaviour in Atlantic salmon of different origins, it is important to consider that there are interactions between different behaviours and that it is challenging to consider any one behavioural characteristic in isolation. For example, emergence latency can be linked to foraging activity, but also to stress coping styles (Näslund and Sandquist, 2017). Meanwhile, when quantifying the foraging activity in brook charr, aggressive individuals made more foraging attempts than their non-aggressive conspecifics (McLaughlin et al., 1999) and aggression is more commonly associated with fish of farmed origin than those of wild origin (Berejikian et al., 1996; Metcalfe et al., 2003). Additionally, feeding behaviour is thought to be influenced by traits such as aggression and exploratory behaviour (Závorka et al., 2016), which are often positively correlated with activity (Adriaenssens and Johnsson, 2013), so it is unsurprising that within fish that were reared in the hatchery and the river (Newport), the fish that displayed greater emergence behaviour (i.e. the farmed individuals) also showed increased food-reaching behaviour.

5.5.1.3. Behaviour of hybrid Atlantic salmon

Due to the prevalence of aquaculture escapees and risks of domesticated salmon interbreeding with wild conspecifics, it is important for studies to assess hybrid physiology and performance. The hybrid Atlantic salmon (HFF and HWF) always represented an intermediate between the behaviours exhibited by fish of farmed and wild origin. Pairwise comparisons revealed that in the case of both emergence and food-reaching behaviours, fish of farmed and wild origins showed the most divergence, followed by the differences between wild and hybrid fish, then between farmed and hybrid fish, with the hybrid strains (HFF and HWF) displaying the most similar behaviours. This could imply that these behavioural traits are polygenic and additive, as hybrid animals, with a mix of parental alleles, show behaviours that are intermediate relative to the two pure lines. The intermediate position of hybrid

Atlantic salmon relative to pure domesticated and wild strains has previously been reported in the context of survival (McGinnity et al., 2003), breeding success (Garant et al., 2003), growth (Solberg et al., 2013; Harvey et al., 2016a; Harvey et al., 2016b) and anti-predatory performance (Houde et al., 2010).

In captivity, the reduced strength of natural selection can lead to more variation in traits unimportant in the captive environment (Mignon-Grasteau, 2005); however, domesticated strains often show reduced genetic variability for many important traits (Alioravainen et al., 2020). The result of this is the risk that introgression between fish of farmed and wild origins will reduce genetic variability in wild populations, which could lead to inbreeding depression (McGinnity et al., 2003), particularly if the population shows local adaptation. The lack of evidence of hybrid vigour within this study and others examining hybrid performance indicates that introgression is not likely to benefit local populations of Atlantic salmon. Nonetheless, the complex interplay between genetics and environment can have varying effects on an individual's phenotype, which makes it a challenge to understand exactly how hybridisation will impact populations at the transcription level (Glover et al., 2017). Moreover, the impact of introgression has been shown to be population-specific due to genetic differences between wild populations of Atlantic salmon (Normandeau et al., 2009).

5.5.1.4. Understanding the genetic differences in behaviour between different rearing environments

Although genetic origin seemingly had a role in the behaviours displayed by all Atlantic salmon reared in the aquarium (Glasgow) and in the hatchery and the river (Newport), this of course does not explain why fish of farmed and wild genetic origins showed inverse behaviours if they had been reared in the aquarium compared to having been reared in the hatchery or the river. The explanation can only be posited but it must be acknowledged that there will be context-dependent differences in the outcomes of certain behaviours, in that some behaviours will only provide advantages for the individual under specific circumstances. Moreover, the exhibition of certain behaviours is often related to other factors, such as environmental conditions or even an individual's physiology. For example, a previous study found

that whilst active foraging behaviour and use of shelter always led to increased growth of juvenile brown trout, territorial aggressive behaviour only benefited juvenile brown trout when the food supply was predictable and only within the fish that had high metabolic rates (Hoogenboom et al., 2013). Just as certain behaviours, such as aggression, territoriality and exploratory seem to be correlated and therefore form "behavioural syndromes" (Adriaenssens and Johnsson, 2013), behaviour has also been linked to metabolic rate (Metcalfe et al., 1995; Biro and Stamps, 2010) and other life history traits (Biro and Stamps, 2008). Within this experiment, physiological measures like metabolic rate were not assessed, but could have explained some of the variation in behaviour. Truthfully, behavioural research often finds contradictions between studies. For example, although the reduced survival of aquaculture escapees in the wild is often attributed to increased predation susceptibility and risk-taking (Houde et al., 2010; Solberg et al., 2020), a meta-analysis found that individuals with a "risky" behavioural type lived longer in the wild than individuals that were classified as "shy" (Moiron et al., 2019). Meanwhile, a review into fish behavioural types found that behavioural consequences on the fitness and survival of fish were often variable (Mittelbach et al., 2014). It is therefore probable that drivers of behaviour are multifaceted and include a mixture of environmental, physiological and genetic factors – whilst some genetic variation will be additive (McGinnity et al., 2003), this will not always be the case.

Whilst all fish were tested using the same tanks and protocol, and fish were starved for 48 hours prior to the commencement of the experiment since foraging behaviour can be state-dependent (Werner and Anholt, 1993), it was not possible to ensure uniform conditions between Glasgow and Newport. Differences in light levels, ambient temperature, noise and water quality were unavoidable. Furthermore, it must be noted that whilst fish of different origins were mixed in the hatchery and river rearing environments, Atlantic salmon of wild and farmed origin were housed separately within the aquarium. When being reared, fish across all three rearing environments would have also been at different densities, with the groups of fish in Newport being much larger. Additionally, it is difficult to compare directly between the experiments carried out in Glasgow and Newport due to the discrepancy in the number of trial repeats. Whilst there is no clear evidence that these factors would have had an effect on behaviour, this possibility cannot be ruled out. In spite of these

confounding factors and the contrasting results, origin was shown to be a significant driver of the behavioural traits examined, indicating that genetic origin has behavioural implications.

Certain behavioural traits, including responsiveness to stressors, are believed to be heritable (Koolhaas et al., 2007), such as farmed salmon in general showing reduced responsiveness to stress (Solberg et al., 2013). As a result, there are now strains of animals that have been bred to display a specific behavioural phenotype, such as proactive versus reactive stress coping styles (Schjolden and Winberg, 2007). In such strains, individuals with low responsiveness have lower plasma cortisol levels, are more aggressive, feed more rapidly after experiencing stress and are socially dominant in comparison to individuals with high responsiveness (Schjolden and Winberg, 2007; Ruiz-Gomez et al., 2008). Interestingly, in two studies using the same cohort of rainbow trout (*Oncorhynchus mykiss*) selectively bred for either low or high responsiveness, a switch in behavioural profiles was reported when the fish were reared and tested in a different environment: the first study found that in comparison to the high responsive strain, the low responsive strain were socially dominant and began feeding more quickly when transferred to an unfamiliar tank, in line with predictions (Øverli et al., 2002). However, when a batch of these trout were transported to Norway from their original rearing site in the UK, the strains switched behavioural profiles – the high responsive strain became dominant and resumed feeding more rapidly (Ruiz-Gomez et al., 2008). The change in behavioural profiles was not reflected in plasma cortisol levels, which remained the same across transported and non-transported fish from the same generation (Ruiz-Gomez et al., 2008). Prior to these experiments, the characteristics of low responsiveness and high responsiveness within the rainbow trout had been conserved for at least 3 generations, but a reversal in behavioural phenotype was observed following the transportation, which included a 7-day period of starvation (Ruiz-Gomez et al., 2008). Although Ruiz-Gomez et al. (2008) suggested that the inversion might be a result of the high responsive strain having lost more mass during transportation, therefore becoming bolder due to experiencing a higher degree of hunger, this would have to have had long-term effects since the switch in behavioural profiles was still evident one year after transportation. Interestingly, within the next generation, no differences in risk-taking during feeding were observed, but the low responsive strain

were once more dominant over the high responsive strain (Ruiz-Gomez et al., 2008). Within the present study, the Atlantic salmon were transported from Newport to Glasgow at the egg stage, which is likely to have had a negligible impact on their phenotype; however, inverse behaviours were observed between the fish of farmed and wild genetic origins depending on their rearing environment. Behavioural plasticity might vary according to the relationship between the behaviour and an individual's physiological trait, where behaviours strongly associated with specific genes are less plastic (Ruiz-Gomez et al., 2008). Nonetheless, that behaviour became uncoupled from physiology in rainbow trout suggests that the interplay between genetics, physiology and behaviour can also be impacted by the experiences of an individual (Ruiz-Gomez et al., 2008), which naturally would have been divergent between the Glasgow and Newport experiments.

5.5.2. The Impact of Rearing Environment on the Behaviour of Juvenile Atlantic Salmon

In addition to examining behavioural differences in Atlantic salmon due to genetic origin, the impact of different rearing environments was also assessed. Common-garden experiments remain important within research examining differences between animals from different genetic backgrounds, as they allow quantitative examination of genetic differences in phenotype (Glover et al., 2017). Meanwhile, studies comparing rearing environments allow researchers to assess whether experience removes or reduces genetic effects. Examining the effect of the rearing environment is important because it allows researchers to attempt to disentangle genetic effects from environmental ones. Just as there are genetic components to behaviour (Koolhaas et al., 2007), behavioural plasticity can occur due to individual experience (Ruiz-Gomez et al., 2008). In juvenile Atlantic salmon, domesticated fish were dominant over fish of wild-origin when reared in a common-garden hatchery environment, but wild-origin fish that had been reared in the wild were generally dominant over both domesticated and wild-origin fish reared in the hatchery (Metcalfe et al., 2003). Both experiments within the present study used a common garden approach, as all experimental fish had been exposed to the same conditions since hatching. Whilst the experiment in Glasgow only assessed genetic drivers of

behaviour, Newport's experiment examined fish that had come from one of two rearing environments, the hatchery or the river. This made it possible to directly examine the impact of rearing environment independent from that of genetic origin in the Newport experiment.

Rearing environment (hatchery versus river) had no effect on the exploratory behaviour of fish; however, Atlantic salmon reared in the river were significantly less likely to have reached the food at any given time than fish reared in the hatchery. A hatchery environment is less dynamic and complex than a river, as within the river, fish will experience the additional pressures of more variable environmental conditions and the risk of predation. Whilst both the hatchery and the river rearing environments were outside and so exposed to ambient light and temperature levels and precipitation, the hatchery tanks were covered with a net to deter avian predators, which would have been a threat within the river environment. Within the Srahevagh river, salmon actively feed on a wide range of invertebrates including larval and adult Diptera, Plecoptera and Trichoptera as well as Coleoptera (de Eyto et al., 2020), whilst fish in the hatchery would have been reared solely on commercial pellets. The hatchery-reared fish would have therefore been more accustomed to the food (bloodworms and commercial pellets) used within this experiment. Familiarity with the food could explain the divergence in food-reaching behaviour between Atlantic salmon reared in different environments. Additionally, the aforementioned circadian rhythmicity considerations that could explain why fish of farmed origin were more likely to reach the food than fish of wild origin might also be relevant here. In this case, fish reared in the hatchery might have less notion of the rhythmicity of food availability than their conspecifics reared in the river.

The small effect of rearing environment was surprising, given the large differences between the hatchery and river environments. Fish reared in the river would have been through stronger selection than their conspecifics reared in the hatchery, as mortality in the wild environment will be substantially higher than that within the hatchery, due to factors such as competition, energy depletion (Finstad et al., 2011), density dependent mortality (Heggberget, 1993) and predation. This might have

been expected to lead to a reduction in the apparent effects of origin, since fish of farmed origin that displayed the most maladaptive phenotype for the wild environment might have already died before the sample of survivors was collected for behavioural tests. In addition, fish are considered to be quite plastic in their development and behavioural traits are thought to vary based upon the environment experienced during ontogeny (Johnsson et al., 2014). In spite of selective breeding, genetic variability in behaviour is often still seen within domesticated populations – behavioural traits do not disappear as a result of domestication, but levels of expression can change (Mignon-Grasteau, 2005). The impact of rearing environment on food-reaching behaviour independent of the genetic effects of origin implies that although behavioural traits are heritable, an individual's experience can also shape their behavioural phenotype.

5.6. Conclusion

Within these common garden experiments, the genetic origin of the Atlantic salmon proved to be a significant driver of their exploratory and food-reaching behaviour. Domestication of Atlantic salmon has purposefully selected for fish with economically important traits; however, it is likely that directional selection also leads to inadvertent selection for other traits, which can impact behaviour. Therefore, different behavioural phenotypes between fish with distinct genetic backgrounds was expected and as predicted, farmed x wild hybrids displayed behaviours that were intermediate between the two parental strains. Nonetheless, the behavioural patterns were not consistent across rearing environments, with fish reared in the hatchery and the river (Newport's experiment) displaying the inverse behaviours to those reared in the aquarium (Glasgow's experiment). These results indicate that whilst domestication has led to distinct behavioural phenotypes separating fish of different origins, the presentation of behaviours can be influenced by the environment experienced during ontogeny.

Using a common garden approach allowed for the genetic drivers of behaviour to be assessed, independent of environment; however, by examining Atlantic salmon from two separate common garden environments in Newport, it was also possible to

quantify the impact of the hatchery and the river rearing environments on behaviour, independent of genetic effects. That analysis showed that the behavioural differences associated with genetic origin were not overridden by the environmental conditions experienced by a fish in its early life. Although rearing environment impacted food-reaching behaviour, within this study there was no evidence that being reared in a natural environment acted to reduce the genetic effects of domestication. This has implications for interactions between Atlantic salmon of farmed and wild origins, as it suggests that being reared in the wild does not remove the behavioural effects of domestication. Indeed, even the hybrid fish from the river rearing environment did not show closer alignment to fish of wild origin (there was no interaction between genetic origin and rearing environment). Therefore, if farmed fish escape into the wild and breed with fish of wild origin, although their offspring will develop within the wild environment, this won't necessarily overcome maladaptive traits that might be associated with the introgression of farmed genes.

Chapter 6: General Discussion.

6.1. Research Highlights

As characterisation of the gut microbiota continues across many taxa, the evidence for the wide-reaching impacts of this diverse microbial community on the host increases. However, although an organism's metabolic rate is intricately linked to its fitness and survival, studies examining links between the host metabolic rate and the gut microbiota remain scarce. An aim of this thesis was to address both factors simultaneously in relation to the physiology of juvenile Atlantic salmon.

The first notable finding of this thesis was the link between metabolic rate and body state. Fish with a higher metabolic rate had a higher mass and a lower percentage water content, which indicated that they had more fat than their conspecifics with a lower metabolic rate. This was probably due to the fact that within the stable aquarium environment, fish with a higher metabolic rate were able to invest more energy into growth. Indeed, it is these fish that showed greater growth efficiency. As food supply and abiotic conditions were consistent, fish with a higher metabolic rate were potentially able to accumulate excess energy, which in salmonids is stored as fat (Swift, 1955). In the wild environment, a body composition with a higher proportion of fat could act as physiological insurance if environmental conditions were to decline (Bull et al., 1996); however, accumulating these stores would be more challenging due to additional energetic requirements, such as foraging and predator avoidance. This offers further insight into why the benefits of a given metabolic phenotype are context-dependent, such as Atlantic salmon losing the benefits associated with a higher metabolic rate in worse environmental conditions (Reid et al., 2012).

Interestingly, there were differences in growth efficiency between fish with the two distinct metabolic rates, where fish with a higher metabolic rate gained more body energy throughout the experiment in relation to the energy they consumed. Variation in growth rate is common in aquaculture (Huntingford and Adams, 2005), but in an industry that actively applies directional selection for economically important traits, such as growth (Glover et al., 2018), this is inefficient. This thesis has highlighted that aside from any effects due to husbandry practices, differences in growth rate can be associated with an individual's metabolic rate and independent of the volume

of food consumed. This has implications for the way in which artificial selection might be approached within Atlantic salmon aquaculture production, as to improve growth efficiency, directional selection could also be applied to the metabolic phenotype of the fish.

A surprising outcome of this research was the finding that SMR did not differ between Atlantic salmon from three distinct genetic backgrounds: farmed, ranched and wild. It was hypothesised that due to the heritable nature of metabolic rate (White and Kearney, 2013), there would be disparity between the three groups of fish. However, the result is in agreement with Robertson et al. (2019), who were unable to conclude that domestication induced an increased SMR in Atlantic salmon. Nonetheless, some metabolic differences were seen: aerobic scope was significantly higher in wild fish than their farmed conspecifics, as was the peak of their SDA response (mg O_2.hr^{-1}). Additionally, the SDA response of ranched fish was shorter in duration than that of fish with farmed or wild genetic backgrounds. Any genetic differences between the three origins are likely to be driven by the artificial selection experienced by previous generations of farmed fish in the aquaculture setting that contrasts with the selection pressures experienced by fish of wild origin in the wild environment. Ranched fish represented an interesting intermediate, as they are reared as juveniles in a hatchery environment and then released into the wild at the presmolt stage. Consequently, ranched fish experience both a stable artificial environment and the more stochastic wild environment. This could explain why the discrepancy in aerobic scope was higher between the fish of wild and farmed origin than between those of wild and ranched.

Although it was initially hypothesised that SMR would differ between the Atlantic salmon of different origins due to the divergent selection imposed by the environments experienced by previous generations, a potential explanation for the lack of contrast could be due to the common garden nature of this research. Standard metabolic rate is largely dependent upon the current environmental conditions, as evidenced by reduction of metabolic rate over winter in salmonids (Auer et al., 2016b). Therefore, given that the fish used within Chapter 4 were all reared within a common garden setting and with a constant food supply, differences in SMR might have been lessened due to the lack of environmental drivers.

Although this study suggests that domestication may have had little effect on metabolic rates in Atlantic salmon, it is notable that it may have influenced their gut microbiota. This was evidenced by the significant differences in microbial beta diversity between fish of farmed, ranched and wild salmon outlined in Chapter 4. Such differences could be due to subtle phenotypic differences between fish from the three origins, as factors such as gut morphology can impact the gut microbiota (Yan et al., 2016). The differential abundance analyses indicated that genera from Proteobacteria were more common in the guts of fish from farmed and wild origin, than in those of ranched; however, Firmicutes were more likely to be overabundant in the hindgut of farmed fish. The microbial community composition and alpha diversity analysis of each origin described in Chapter 4 led to the hypothesis that the gut microbiota of farmed Atlantic salmon might be more specialised than that of the wild fish. As discussed, it is possible that the farmed fish are better adapted to the commercial diet used within the experiments, as evidenced by an increased abundance of Firmicutes within farmed fish, which are known to metabolise dietary polysaccharides (Carey et al., 2013; David et al., 2013).

A major finding in this thesis were associations that revealed – in two independent experiments - a relationship between metabolic rate and the gut microbiota. In the experiment described in Chapter 4, a higher abundance of Actinobacteria in the hindgut was associated with a lower rSMR. Actinobacteria were more likely to be overabundant in the guts of fish from farmed and ranched origin, with genera from Bacteroidetes more likely to be overabundant in fish of wild origin; however, the experiment described in Chapter 3 found that genera from Actinobacteria were common within fish from both ‘low’ and ‘high’ metabolic rate groups. In that experiment, at the family level, a higher abundance of Rhodobacteraceae in the foregut of fish was found to correlate with a decrease in rSMR. Although this suggests that members of the Actinobacteria phylum and Rhodobacteraceae family have implications for host metabolic rate (or vice versa), these findings highlight the need for greater granularity when characterising the gut microbiota, as identifying the OTUs at genus or species level might reveal functional relationships and even divergence between the Actinobacteria taxa present in each metabolic rate group. Interestingly, the increased Firmicutes:Proteobacteria ratio found within fish of farmed origin in Chapter 4 was also found within fish from the ‘low’ metabolic rate

group in Chapter 3. This ratio has previously been linked to a high fat diet in mice (Kim et al., 2012; Senghor et al., 2018), but as all fish within this experiment were fed the same diet, this suggests that an increased ratio is not only caused by a fatty diet, but also relates to the metabolic rate of the host. That this ratio was also higher in the guts of fish of farmed origin within Chapter 4 could reflect an adaptation to the commercial diet or differences in the metabolic phenotype between fish from different genetic backgrounds.

Alpha diversity analyses were less clear cut, however, across both experiments examining the gut microbiota there was a relationship between microbial alpha diversity and fish metabolic rate. Overall, there was a negative relationship between rSMR and microbial richness within the fish foregut. This relationship was also found within the fish hindgut in the experiment described in Chapter 4. In the experiment described in Chapter 3, microbial alpha diversity metrics were found to be associated with salmon growth efficiency, but worked in divergent directions, where the fish that grew most efficiently had a higher SMR, higher fat levels and increased microbial richness and decreased Shannon effective within the foregut. In Chapter 4, the microbial alpha diversity metrics were also associated with the cost of digestion in the fish, being related to both the time to the peak of the SDA and the SDA duration. Once more, microbial richness and Shannon effective were observed to be working in different directions: fish that showed faster digestion had decreased foregut microbial richness, increased Shannon effective and decreased hindgut Shannon effective. These results imply that both the abundance and the evenness of microbes within the gut microbiota have implications for the physiology of Atlantic salmon; however, as discussed within Chapter 4, the drivers of this relationship are far from clear. As microbial diversity is mediated by multiple ecological factors, elucidating causation would require an experiment designed to control for any confounding factors, such as food intake.

Differences in microbiota often studied in the context of dietary changes (Abid et al., 2013; Zarkasi et al., 2016) or environment (Rudi et al., 2018; Uren Webster et al., 2020), but given that fish within these experiments had the same dietary regime and experienced the same husbandry conditions, this work explicitly indicates that divergence in gut microbial community composition has both genetic (Chapter 4) and physiological (Chapter 3) drivers. The common OTUs identified within the

experiments characterising the gut microbiota concur with studies in other fish, from Atlantic salmon (Rudi et al., 2018) to grass carp (*Ctenopharyngodon idellus*) (Wu et al., 2015). These and other such studies have suggested Proteobacteria, Firmicutes, Fusobacteria and Bacteriodetes are common members of the gut microbial community in fish (Wu et al., 2015; Gajardo et al., 2016; Rudi et al., 2018; Fogarty et al., 2019), and this is largely reflected by the genera present in the taxonomic bar plots found in Appendices 3 and 4. Nonetheless, beta diversity and differential abundance analyses highlighted differences in microbial community composition between fish from different genetic backgrounds and those with different metabolic phenotypes. Although the function of many microbial taxa within the intestines of fish remain unexplored, it has been established that the gut microbiota aids in host digestion, such as that of the grass carp functioning in carbohydrate turnover and fermentation (Wu et al., 2015). Moreover, in Atlantic salmon, bacteria that produce enzymes including lipase, chitinase, cellulase and amylase have been identified (Askarian et al., 2012). Additionally, Chapter 4 discussed the relationship between SDA parameters and the gut microbiota, where microbial alpha diversity was related to both the time to reach the peak SDA and SDA duration. These studies, along with those discussed in Chapter 2, highlight the links between the diet, the gut microbiota and host metabolism. It is therefore unsurprising that this thesis found relationships between the metabolic rate and the gut microbiota in Atlantic salmon.

Finally, it must be acknowledged that in addition to the genetic and physiological drivers of gut microbial community composition, genetic and environmental factors were also found to drive Atlantic salmon behaviour. The behaviour of Atlantic salmon has repercussions for their fitness, physiology and survival both within aquaculture and wild environments. Within aquaculture, the behaviour of fish is driven by social interactions and abiotic factors such as temperature and light (Føre et al., 2009), whilst in the wild, behaviour is additionally impacted by predator avoidance (Johnsson et al., 2004) and the need to actively forage for food (Grade and Letcher, 2006; Syrjänen et al., 2011). Populations of Atlantic salmon in the wild can show local adaptation to their environment (Glover et al., 2017) as a result of adaptive variation resulting in an interaction between the environment and an individual's genotype (Garcia de Leaniz et al., 2007). Populations might therefore be genetically distinct from one another (Normandeau et al., 2009), so there could also be

geographic effects on behaviour. Asides from these environmental effects, there are genetic drivers of behaviour (Koolhaas et al., 2007). Although directional selection within aquaculture focuses on economically important traits, inadvertent selection might occur as a consequence (McGinnity et al., 2003), which could have repercussions on the behaviour of domesticated fish. Behavioural studies examining Atlantic salmon of different origins often investigate the potential impacts of aquaculture escapees; competitive interactions between Atlantic salmon of farmed and wild origins have been investigated (Metcalfe et al., 2003), as has the survival differential between farmed fish, wild fish, and their hybrid offspring (McGinnity et al., 2003). By assessing the behaviour of farmed, wild and hybrid Atlantic salmon in the absence of conspecifics, it was possible to assess whether a behavioural phenotype had resulted due to genetic or environmental effects. Once more, a common garden approach allowed genetic impacts to be isolated from those of an environmental nature; however, rearing environment was also investigated by carrying out the experiment in two separate locations. The effect of rearing environment on the foraging behaviour of the Atlantic salmon suggests that early life exposure can influence the fitness of the fish regardless of genetic origin. Meanwhile, whilst the divergent exploratory behaviour between fish of farmed and wild origins was expected, the inversion in the behaviour each origin showed between the Glasgow and Newport experiments was surprising. When assessing the behaviour of hybrid offspring of farmed and wild parents, the results concurred with that of other studies examining hybrid fitness which have found that hybrid vigour does not occur, with hybrids often representing an intermediate position between that of the parental strains (McGinnity et al., 2003; Solberg et al., 2013). When put into the context of aquaculture escapees, these results clearly demonstrate that there are both genetic and environmental drivers of Atlantic salmon behaviour. Consequently, phenotypic divergence in behaviour between Atlantic salmon of farmed and wild origin might be enhanced by the process of domestication. Not only is behavioural research therefore important to increase understanding of general salmonid biology, but the field is also relevant to the aquaculture industry due to the ecological and genetic interactions between fish of different origins. Furthermore, an individual's behaviour can be assessed alongside physiological measures to assess fish welfare in an aquaculture setting (Ashley, 2007). Nonetheless, the combined impact of both genetics and environment also means that careful consideration must be made when

extrapolating results from behavioural studies in any environment, as behavioural consequences on fish survival can be variable and highly dependent on external factors.

6.2. Knowledge Gaps and Future Research

6.2.1. The Gut Microbiota

Characterisation of the gut microbiota is insightful and allows comparisons to be made, such as here between fish with different metabolic phenotypes or fish from different genetic origins. Nonetheless, whilst an OTU from the Actinobacteria phylum and an OTU from the Rhodobacteraceae were correlated with a reduced SMR in the Atlantic salmon gut, short sequencing reads meant that greater taxonomic granularity could not be gained. The resolution of microbiome analysis is often limited to genus or species (Scanlan, 2019), but being unable to reach those taxonomic levels for every OTU represents an impediment to significant conclusions being drawn. As previously acknowledged, more insight is to be gained from studies that are able to assess the functional significance of the taxa present. Future work should therefore aim to identify the OTUs implicated in Atlantic salmon metabolic rate at species level. If successful, studies making use of gnotobiotic hosts could examine the impact of a mono-association of a specific microbial taxon on host physiology.

6.2.2. Metabolic Rate and Behaviour

The major theme from research examining the metabolic rate and the behaviour of Atlantic salmon is that any implications for host fitness are highly context-dependent – this is seen within a broad range of studies, from those examining aggressive behaviour (Hoogenboom et al., 2013) to those examining the impact of metabolic rate on growth (Auer et al., 2015c). The wide life history variations seen within salmonids echo the importance of studying their biology in the context of their environment, as different populations can show different adaptive strategies based upon geography (Finstad et al., 2009). The work within this thesis highlights the need to consider the environment when extrapolating any results. Both biotic and abiotic

factors specific to the environment have the propensity to impact both metabolic rate and behaviour. This also has implications for research examining the outcome of interactions between Atlantic salmon of different origins, as different strains are likely to prosper dependent upon the environment.

6.2.3. A Multidisciplinary Approach

Atlantic salmon are the focus of considerable research effort due to their economic importance within the aquaculture industry, and studies examining the effect of chemicals/prebiotics/probiotics on fish health and performance are becoming more common (Jaramillo-Torres et al., 2019; Klakegg et al., 2020). Many such dietary impacts are thought to be mediated in part by alterations to the gut microbiota (Gupta et al., 2019; Jaramillo-Torres et al., 2019; Hoseinifar et al., 2020). Although literature regarding interindividual variation in the Atlantic salmon gut microbiota can be conflicting, this thesis has provided evidence that the gut microbiota shows divergence between fish from different genetic backgrounds, even when a common garden approach was used, suggesting a heritable component of gut microbial community composition. If farmed fish have diets supplemented with probiotics or prebiotics, this could increase such divergence. Moreover, since the gut microbiota also showed links to the metabolic rate of Atlantic salmon, which has implications for their fitness, the gut microbiota should be taken into account when contemplating the impact of interbreeding between aquaculture escapees and fish from wild populations.

Although challenging, future work should (as here) incorporate research into Atlantic salmon physiology, behaviour and the gut microbiota within a single study. Metabolic rate, behaviour and gut microbiota all have a relationship with fish growth rate or body size. Studies examining genetic differences need to take this into account, because fish of different genetic backgrounds might have quite substantial discrepancies in size-at-age (as in Chapters 4 and 5). Previous studies have found relationships between metabolic rate and behaviour (Metcalfe et al., 1995; Biro and Stamps, 2010). Disregarding behaviour when considering an animal's metabolic rate might risk oversimplification when interpreting results, since behaviours such as aggression and foraging activity can be intimately linked to metabolic rate (Careau et

al., 2008). Moreover, behaviour and metabolic rate are intimately linked with Atlantic salmon physiology; for example, proactive fish have shown faster growth than reactive conspecifics (Damsgard et al., 2019). Although gut microbiota research has focused on microbial impacts on host physiology, there is some evidence of a relationship between the gut's microbial community and behaviour (MacFabe et al., 2011). The work here suggests the interplay between host metabolic rate, physiology, gut microbiota and behaviour. Building upon this thesis, future studies could determine whether the gut microbiota drives behavioural phenotypes in Atlantic salmon. Moving forward, it will also be important to focus on elucidating the function of key microbial taxa implicated in host physiology and behaviour. This thesis has made important discoveries in relation to associations between host physiology and microbial taxa. Complex relationships between metabolic rate, host physiology, behaviour and the gut microbiota clearly exist. Future research can now address clear hypotheses, developing from these findings to provide further, definitive insight into the role microbes in the gut play in shaping host physiology and behaviour.

Appendix 1: The Role of the Gut Microbiome in Sustainable Teleost Aquaculture.

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A1.1. Abstract

As the most diverse vertebrate group and a major component of a growing global aquaculture industry, teleosts continue to attract significant scientific attention. The growth in global aquaculture, driven by declines in wild stocks, has provided additional empirical demand, and thus opportunities, to explore teleost diversity. Among key developments is the recent growth in microbiome exploration, facilitated by advances in high throughput sequencing technologies. Here we consider studies on teleost gut microbiomes in the context of sustainable aquaculture, which we have discussed in four themes: diet, immunity, artificial selection, closed-loop systems. We demonstrate the influence aquaculture has had on gut microbiome research, while also providing a road map for the main deterministic forces that influence the gut microbiome, with topical applications to aquaculture. Functional significance is considered within an

aquaculture context with reference to impacts on nutrition and immunity. Finally, we identify key knowledge gaps, both methodological and conceptual, and propose promising applications of gut microbiome manipulation to aquaculture, and future priorities in microbiome research. These include insect-based feeds, vaccination, mechanism of pro- and pre-biotics, artificial selection on the hologenome, in-water bacteriophages in recirculating aquaculture systems (RAS), physiochemical properties of water, and dysbiosis as a biomarker.

Key words: Fish, teleost, gut, microbiome, aquaculture, review

A1.2. Introduction

Since its conception in the 1980s describing soil ecology (Whipp et al., 1987), the term microbiome has evolved into an intensely studied area of research. In recent decades, this area has begun expanding from an anthropocentric and medically dominated field, into a taxonomically broad field, examining research questions in non-model species, from trees (Denman et al., 2018) to frogs (Kohl et al., 2015), and increasingly, fish. The diversification in microbiome studies has been driven by increased access to next generation sequencing (NGS), a tool that is not reliant upon culture-based techniques, which often require previous knowledge of target microbes.

Currently, gut bacterial communities have been assessed in over 145 species of teleosts from 111 genera, representing a diverse range of physiology and ecology (Figure A1-1a), often with similarities in bacterial phyla composition between fish species, dominated by Bacteroidetes and Firmicutes (Sullam et al., 2012; Givens et al., 2015). Non-model taxa from an array of aquatic ecosystems have had their gut microbiomes sequenced using NGS, with studies extending beyond species identification, into hypothesis testing which was once only feasible in model systems. Examples of studies on non-model teleost gut microbiomes range from those demonstrating rapid gut microbiome restructuring after feeding in clownfish (*Premnas biaculeatus*) (Parris et al., 2019) to the effect of differing environmental conditions, such as dissolved oxygen content, on the gut microbial diversity of blind cave fish (*Astyanax mexicanus*) (Ornelas-García et al., 2018). Interest in the gut microbiome of fish has accelerated for many reasons, as not only do teleosts represent the most

diverse vertebrate group (Ravi, 2008), they are also of significant economic importance, including in aquaculture (Wu et al., 2015). Aquaculture now provides over 45% of fish-based food products globally (Longo et al., 2019), and influence of the aquaculture industry on teleost gut microbiome research is demonstrated by the research questions tackled, with a clear bias towards salmonids (genera: *Oncorhynchus* and *Salmo*), carp (genera: *Hypophthalmichthys, Carassius, Cyprinus* and *Ctenopharyngodon*) and tilapia (genus: *Oreochromis*) (Figure A1-2).

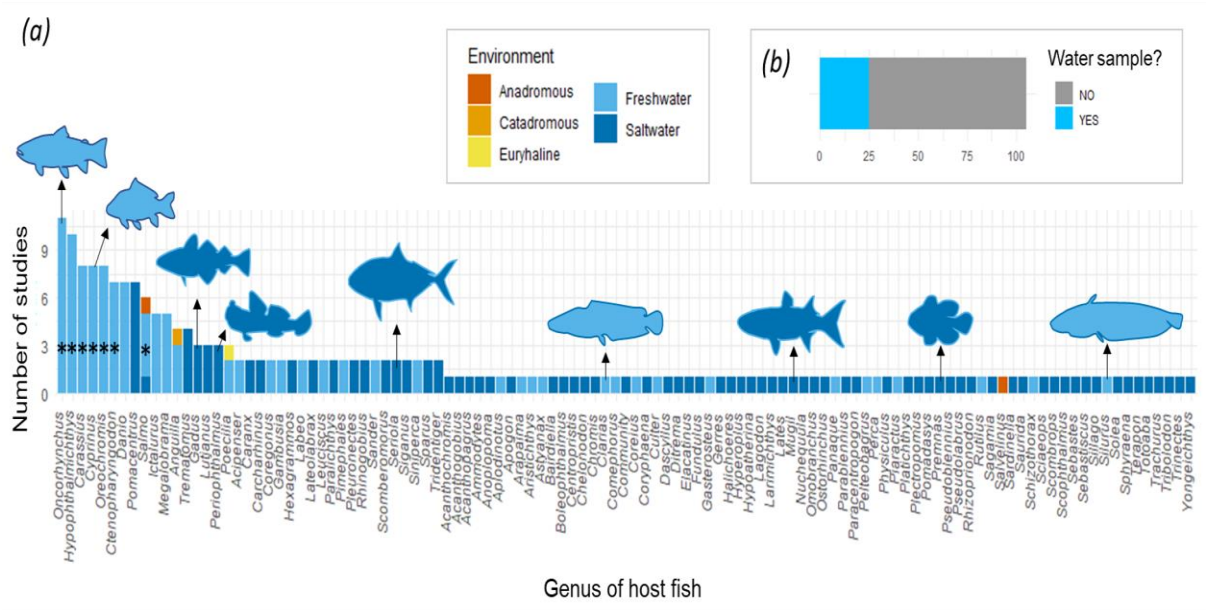

Figure A1-1: An overview of the assessment of the gut microbiota in teleosts. *(a) The number of studies on the gut microbiome using next generation sequencing (NGS) broken down by the genus of fish that the study was conducted on, as well as the environment those fish same from. Asterisk represent salmonid, carp and tilapia. Additionally, (b) shows the number of studies that assessed the water microbial communities. Gut microbiome studies were compiled using Web of Science (Reuters, 2012), and only include studies that implemented NGS. It is acknowledged that total microbiome research extends further than this. Further information on search terms and filtering can be found in the supplementary information.*

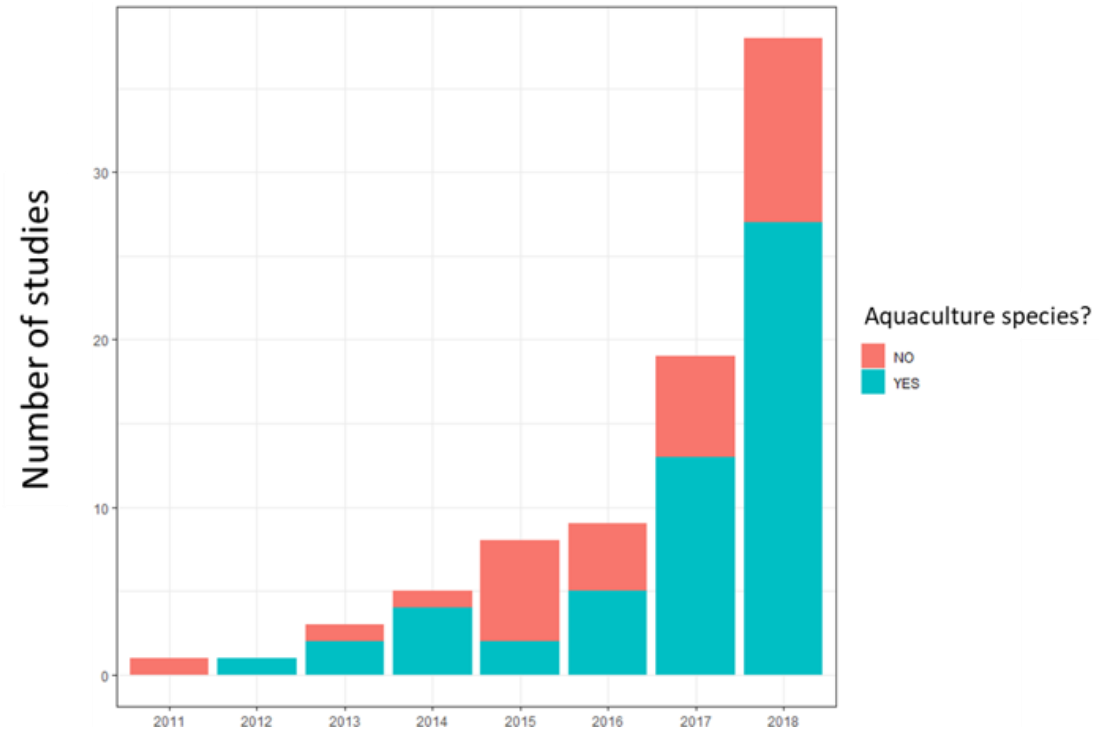

***Figure A1-2: Growth in the studies using next generation sequencing on fish gut microbiomes**, including food aquaculture species (aquaculture status taken from FishBase (Froese, 2019)). Further information on search terms and filtering can be found in the supplementary information.*

Rapid growth of the aquaculture industry has led to mounting pressure to make it more sustainable (Naylor et al., 2000), and here we discuss four key components relevant to its sustainability in the context of the teleost gut microbiome: diet, immunity, artificial selection, and closed-loop systems. We highlight some key deterministic factors important to aquaculture, although as shown in Figure A1-3, there are numerous interacting ecological processes. More in-depth reviews focusing on these specific interactions are available, for example, interactions between the gut microbiome and the immune system (Kelly and Salinas, 2017), energy homeostasis (Butt and Volkoff, 2019) and physiology (Yukgehnaish et al., 2020). Understanding and manipulating microbial-host-environmental interactions (Figure A1-3a) and associated functional capacity in these areas could contribute substantially towards achieving a more sustainable aquaculture industry. We identify potential for future research, both methodological and conceptual. Other microbiomes are known to impact host function, in particular, the skin microbiome and its relationship to immunity (Azimirad et al., 2016), however, due to their differing ecology (Sylvain et al., 2016) and aquaculture applications (Llewellyn et al., 2017), the gut microbiome will remain our focus here.

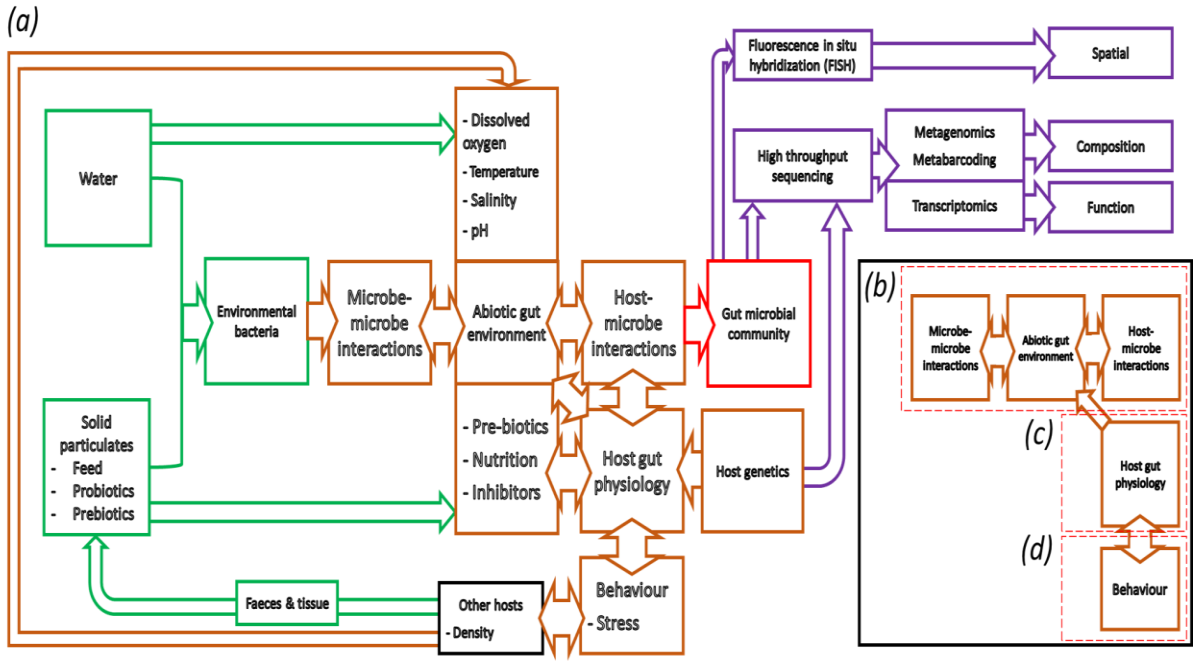

Figure A1-3: Schematic *view of the deterministic processes that influence gut microbial communities in fish.* *(a) Community assemblage of bacteria in the gut starts with inputs from the environment (green), such as the bacteria within the water column, or in solid particulates of biofilm, sediment and feed. Once ingested, these bacteria are influenced by interacting deterministic processes (brown) such as the host's abiotic gut environment, interaction with the hosts' physiology through the gut lining and its secretions, as well as interactions between other microbiomes. The outcome (red) is final community assembly, which can be characterised using an array of cutting-edge molecular techniques (purple). A subset of the broader interactions is provided, with focus on (b) microbe-environment-host interactions, (c) host gut physiology and (d) behaviour.*

A1.3. Diet

The gut microbiome has long been linked with diet, yielding insights into the commensal relationship between certain microbes and host. It has been shown that the teleost gut microbiome produces a range of enzymes (carbohydrases, cellulases, phosphatases, esterases, lipases and proteases) which contribute to digestion (Ray et al., 2012; Wu et al., 2015). More intimate relationships also exist, for example, anaerobic bacteria in the teleost gut have a role in supplying the host with volatile fatty acids (Ramirez and Dixon, 2003), an end-product of anaerobic fermentation that provides energy for intestinal epithelial cells (Clements, 1997). Gut microbes also synthesise vitamins and amino acids in the gut of aquatic vertebrates (Balcázar et al.,

2006a; Nayak, 2010). For example, the amount of vitamin B_{12} positively correlated with the abundance of anaerobic bacteria belonging to the genera *Bacteroides* and *Clostridium*, in Nile tilapia (*Oreochromis niloticus*) (Sugita et al., 1990). Here we discuss this host-microbe relationship in the context of contemporary aquaculture, with a focus on two timely issues: fishmeal and starvation.

A1.3.1. Fishmeal

Fishmeal is an efficient energy source containing high-quality protein, as well as highly digestible essential amino and fatty acids (Cho and Kim, 2011), which is included in feed for a range of teleost species. Fish used in fishmeal production is, however, predominantly sourced from capture fisheries, putting pressure on already overfished stocks (Naylor et al., 2000). Despite a global decrease in fishmeal production, from an average of 6.0 million tonnes between 2001-2005 to 4.9 million tonnes between 2006-2010 (Shepherd and Jackson, 2013), and growth in plant-based substitutes (e.g. wheat gluten, soybean protein, and pea protein), some aquaculture species still require a proportion of fish-sourced amino acids and proteins (Pratoomyot et al., 2010).

As dietary changes can alter the fish gut microbiome (Ingerslev et al., 2014), there has been a considerable rise in the number of studies investigating the influence of alternative plant-protein sources on host-microbe interactions. Plant-protein sources have been shown to disturb the gut microbiota of some fish, with the production of antinutritional factors (factors that reduce the availability of nutrients) and antigens, impeding host resilience to stress (Batista et al., 2016), metabolism (Gatesoupe et al., 2018) and immune functioning (Miao et al., 2018). Fish fed plant-protein based diets can exhibit alterations in their intestinal morphology including disruption to the lamina propria and mucosal folds (Wang et al., 2017), which may modify attachment sites for commensal bacteria (Ringø and Gatesoupe, 1998), and can therefore impact microbial composition (Desai et al., 2012; Miao et al., 2018).

Insect meal is increasingly used in aquafeed as a protein source with a high nutritional value (Magalhães et al., 2017), and several studies have demonstrated its potential use in manipulating the gut microbiome in fish (Bruni et al., 2018; Huyben et al., 2019). As insects are chitin rich, these diets have been associated with prebiotic effects,

through increased representation of beneficial commensal bacteria such as *Pseudomonas* sp. and *Lactobacillus* sp., which in turn improves performance and health in some fish (Bruni et al., 2018). Despite this, however, the beneficial effects of chitin are species specific, with Atlantic cod (*Gadus morhua*) and several cyprinid species demonstrating increased growth rates on diets with varying levels of chitin, whereas tilapia hybrids (*Oreochromis niloticus* × *O. aureus*) and rainbow trout (*Oncorhynchus mykiss*) both display decreased growth rates (Ringø et al., 2012). Chitin can therefore not be described as a probiotic for all species. The influence of insect meal on microbial-mediated functions also remains underexplored, with little known about the extent to which species-specific responses to a chitin rich diet are microbially mediated (Fines and Holt, 2010), offering scope for future research.

A1.3.2. Starvation

Starvation is common in the production of valuable species such as salmon (Waagbø et al., 2017), sea bream (Ginés et al., 2003), halibut (Foss et al., 2009) and cod (Bjørnevik et al., 2017), prior to handling, transportation and harvest, but is also used as a method to improve fillet quality. However, starvation is likely to have a substantial impact on host-microbe interactions (Figure A1-3b). Gut microbial communities of the Asian seabass (*Lates calcarifer*), for example, shifted markedly in response to an 8-day starvation period, causing enrichment of the phylum Bacteroidetes, but a reduction of Betaproteobacteria, resulting in transcriptional changes in both host and microbial genes (Xia et al., 2014). Perturbation to the gut microbiome could lead to the opening of niches for other commensal or even pathogenic bacteria (Wiles et al., 2016), especially if this is combined with the compromised immune system of a stressed host (Ellison et al., 2018) (Figure A1-3d). Even if all fish are terminated shortly after starvation, gut microbial community changes before termination could cause long term impacts to the microbial composition of water and biofilters in closed recirculating aquaculture systems (RAS). RAS systems will be discussed in greater detail later in this review.

A1.4. Immunity

Gut microbial communities have strong links to immunity (Raulo et al., 2018), which is pertinent in fish as they are in constant contact with water, a source of pathogenic and opportunistic commensal microbes (Ellis, 2001). In addition to this, fish cultured intensively are often stocked at high densities, allowing for easier transmission of microbes. Therefore, a microbially diverse gut microbiome in aquaculture is important to prevent unfavourable microbial colonisation (Balcázar et al., 2006b), and although the mechanisms are not fully understood, some key processes have been identified. For example, *Bacillus* and *Lactobacillus*, two common probiotic genera of bacteria used in aquaculture, are able to stimulate expression of inflammatory cytokines in the fish gut (He et al., 2017), increase the number of mucus layer producing goblet cells (Popovic et al., 2017), and increase phagocytic activity (Chen et al., 2019). Furthermore, comparison in gene expression between gnotobiotic zebrafish and conventionally reared zebrafish have shown bacteria induced expression of myeloperoxidase, an enzyme that allows neutrophil granulocytes to carry out antimicrobial activity (Rawls et al., 2004a). Colonising microbes can also modulate host gene expression to create favourable gut environments, thereby constraining invasion by pathogens (Balcázar et al., 2006a), whilst also promoting expression of proinflammatory and antiviral mediators genes, leading to higher viral resistance (Galindo-Villegas et al., 2012). Reducing viral and bacterial pathogens, such as *Vibrio* sp. and *Aeromonas* sp., is important for fish health in aquaculture, and will be discussed further in the context of closed-loop systems later in the review.

The interaction between the gut microbiome and the immune system is bilateral, for example, secretory immunoglobulins in fish recognise and coat intestinal bacteria to prevent them from invading the gut epithelium (Zhang et al., 2020a). Similarly, in wild three-spined stickleback (*Gasterosteus aculeatus*), a causal chain (diet→immunity→microbiome) was discovered, demonstrating the impact of diet on fish immunity and thus the microbial composition of the gut (Friberg et al., 2019). Understanding microbial-host-environmental interactions like this are crucial for aquaculture, where, as previously discussed, diet is often manipulated.

A1.4.1. Antibiotics

As most antibiotics used in aquaculture display broad-spectrum activity, they can affect both pathogens and non-target commensal microbes (Ubeda and Pamer, 2012). Oxytetracycline is one of the most widely used veterinary antibiotics, with 1,500 metric tons applied between 2000-2008 to salmon aquaculture in Chile (Buschmann et al., 2012). However, oxytetracycline was seen to reduce gut microbial diversity in Atlantic salmon (*Salmo salar*), while enriching possible opportunistic pathogens belonging to the genus *Aeromonas*, and leading to a high prevalence of multiple tetracycline resistance-encoding bacterial genes (Navarrete et al., 2008). Long-term exposure to oxytetracycline has also been reported to negatively affect growth, immunity and nutrient digestion/metabolism in Nile tilapia (*Oreochromis niloticus*) through antibiotic-induced disruption to the microbiota (Limbu et al., 2018), causing considerable changes in the representation of Bacteroidetes and Firmicutes.

Vaccination has become a widespread prophylactic measure applied in aquaculture to improve immune functioning and disease resilience in farmed fish (Sudheesh and Cain, 2017). One study attempted to identify potential alterations in the microbiota structure and localised immune responses caused by a novel recombinant vaccine against *Aeromonas hydrophila* in grass carp (*Ctenopharyngodon idella*) (Liu et al., 2015). Results from their study suggest that oral vaccines can target *Aeromonas* sp. through activation of innate and adaptive immune defences within the intestine without causing large disturbances in non-target microbiota populations. Given the importance of the immune response in regulating the gut microbiome (Llewellyn et al., 2014), only a small number of studies have investigated the influence of vaccines on the resident microbiota composition and function in fish, providing grounds for future study.

A1.4.2. Pro- and Prebiotic Supplementation

In view of the challenges associated with antibiotics, studies have examined the impact of alternative, prophylactic measures such as pro- and prebiotics (Figure A1-4a). As literature on the types of pro- and prebiotics used in aquaculture have been reviewed elsewhere (Hai, 2015; Dawood and Koshio, 2016), as well as their effectiveness (Zorriehzahra et al., 2016; Hoseinifar et al., 2018), we focus here on the ability of these compounds to induce changes in host physiology and function through

shifts in the gut microbiome. As has already been discussed, *Bacillus* sp. and *Lactobacillus* sp. have a beneficial effect on immunity and are suggested to provide an alternative approach to controlling disease in aquaculture. Targeted microbiota manipulation using these same bacteria have also been reported to exert beneficial effects on fish growth through i) alterations in gut morphology (Elsabagh et al., 2018), leading to improved digestion and metabolism (Falcinelli et al., 2015), and ii) microbial-mediated regulation of the genetic components involved in growth and appetite control (Falcinelli et al., 2016; Gioacchini et al., 2018). Recently, the establishment of *Lactobacillus* probiotic bacteria within the gut microbiota was also associated with improved learning/memory capacity and changes in shoaling of zebrafish (Borrelli et al., 2016; Zang et al., 2019), indicating a potential gut-brain interaction pathway similar to what is described in higher vertebrates (Mayer et al., 2015).

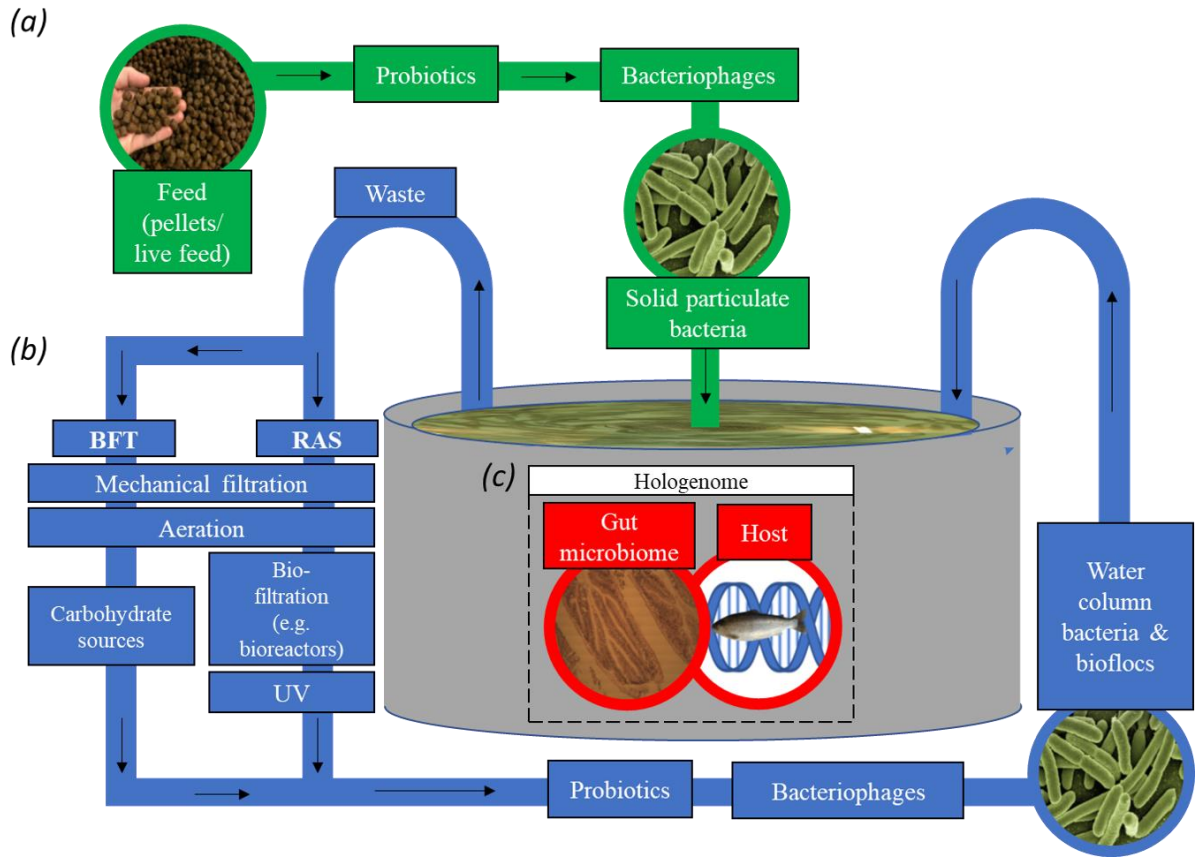

Figure A1-4: A *Schematic diagram representing recirculating aquaculture systems (RAS) and Biofloc technology (BFT) in aquaculture.* *(a) feed inputs (green), (b) water processing (both RAS and BFT) (blue) and the (c) species being cultivated, along with its gut microbiome (red).*

Research into the modulation of gut microbial communities using prebiotic compounds has expanded also. Certain dietary components have been reported to induce changes in gut morphology within the fish host, including vacuolation of enterocytes (Cerezuela et al., 2013) and enhancing mucosal barrier integrity (Yang et al., 2018). Improved mucosal protection and disease resilience are thought to be driven by microbes and associated microbial metabolites. Several prebiotics have been reported to manipulate the resident microbiota community of a host in favour of Firmicutes and short-chain fatty acid producing communities (Piazzon et al., 2017). Mechanistic pathways remain elusive, however, with additional research required.

A1.5. Artificial Selection

Within aquaculture, selection has been applied routinely to increase production by enhancing desirable traits such as growth and disease resilience (Yáñez et al., 2015; Zenger et al., 2019). Recent evidence suggests, however, that host genetics plays a fundamental role in determining the gut microbiota in fish (Li et al., 2018). The "hologenome" concept proposes that the host organism, along with their commensal microbial community, form one unit of selection (Zilber-Rosenberg and Rosenberg, 2008). Host physiology, for example, is determined in part by the host's genome, and has the ability to shift gut microbiome composition, as demonstrated in zebrafish, whereby host neural activity and subsequent gut motility is able to destabilise microbial communities (Wiles et al., 2016) (Figure A1-3c). Although not described in teleosts, the reverse has also been seen, whereby microbial communities are able to regulate the host's gut through: i) serotonin signaling (Yano et al., 2015; De Vadder et al., 2018), ii) macrophages and enteric neurons interactions (Muller et al., 2014), iii) metabolism of bile salts (Dey et al., 2015), and possibly, iv) metabolism of short-chain fatty acids such as butyrate (Raja et al., 2018). The host-microbe relationship means that traits selected during breeding programs may be traits from the hologenome. Pyrosequencing studies have also shown significant changes in the microbial community composition of genetically improved fish compared with domesticated individuals (Kokou et al., 2018; Brown et al., 2019). Artificial selection has also been demonstrated on single species of bacteria, with *Aeromonas veronii* selected to exhibit greater colonisation success in gnotobiotic zebrafish (Robinson et al., 2018). Environmental filtering of the reservoir of bacteria surrounding the fish generates the potential for improving colonisation success of commensal bacteria. Currently,

bacterial communities selected by breeding programs could be neutral, sympathetic or antagonistic to the goals of artificial selection, and understanding this relationship will be vital in manipulating the hologenome.

A1.6. Closed Aquaculture Systems

Many environmental problems plague current aquaculture practices. In addition to those already discussed, there are also issues with parasite transmission to wild fish (Krkošek et al., 2005), interactions between wild and escaped farmed fish (Glover et al., 2017), and release of faeces and excess feed into the environment (Primavera, 2006). One way to better control these problems is to remove aquaculture from ecosystems and bring it into a land-based setting (Tal et al., 2009).

A1.6.1. Manipulating Environmental Microbiota

RAS and Biofloc technology (BFT) are forms of aquaculture which utilise microbial communities to minimize excess nutrients and pathogens in rearing water (Figure 4). In these systems, microbial reconditioning of the rearing water is vital as fish are stocked at high densities, resulting in elevated levels of organic material, which can promote microbial growth (Aruety et al., 2016). Selection of competitive, slow-growing K-strategist bacteria shift the community from autotrophy to heterotrophy activity. Such shifts allow for a microbial community which maintains both water quality, through nutrient recycling, and inhibits the growth of fast-growing, opportunistic r-strategists, which include many bacterial pathogens such as *Aeromonas* sp. (Skjermo et al., 1997; Ahmad et al., 2016). RAS and BFT could therefore be combined with vaccination against bacterial pathogens such as *Aeromonas* sp., as previously discussed, to reduce infections. The selection of K-strategist microbial communities differ between RAS and BFT. In RAS; K-selection is achieved by passing rearing water through heterotrophic biofilters (Vadstein et al., 2018), whereas in BFT, a high carbon to nitrogen ratio within rearing water is conditioned by the addition of carbohydrate sources, favouring heterotrophic K-strategist bacteria (Liu et al., 2019). High carbon conditions in BFT systems also promote nitrogen uptake into microbial biomass, which forms protein-rich bacterial “flocs” that supplement feed (Pérez-Fuentes et al., 2016).

Manipulation of microbes associated with live feed cultures is critical to the production of fish larvae as live feeds often contain opportunistic pathogens (Figure 4a), resulting

in stochastic mortality (Llewellyn et al., 2014). While traditional approaches involve non-selective, temporary methods, i.e. physical/chemical disinfection (Skjermo and Vadstein, 1999), more recent efforts have shifted towards targeted manipulation through probiotics, for example, the successful use of *Phenylobacterium* sp., *Gluconobacter* sp. and *Paracoccus denitrificans* in rotifer (*Brachionus plicatilis*) production (Qi et al., 2009). Lytic bacteriophages have also proven somewhat successful in reducing the prevalence of opportunistic pathogens, such as *Vibrio* sp. (Karunasagar et al., 2007; Higuera et al., 2013; Kalatzis et al., 2016). Live feed also appears to play a critical role in the delivery and establishment of colonising gut microbiota in fish larvae upon first feeding (Reid et al., 2009). Supplementation of live feed cultures with beneficial microbes, such as the previously mentioned *Lactobacillus* spp., and *Pediococcus* sp., has become common practice in hatcheries, with beneficial effects on growth, mucosal immunity and stress tolerance of larvae (Carnevali et al., 2004; Rollo et al., 2006; Azimirad et al., 2016). Bacteriophages and probiotics have also been applied directly to tank water (Figure 4b); probiotics such as *Bacillus* spp. preventing fish mortality from *Vibrio* spp. infections (Moriarty, 1998) and *Flavobacterium columnare* -infecting phages have been shown to persist in RAS for up to 21 days (Almeida et al., 2019). Far less is known about the application of probiotics directly to tank water when compared to feed application (Jahangiri and Esteban, 2018), however, and the use of bacteriophages is still in its infancy, providing potential for future research.

A1.6.2. Controlling Environmental Variables

Changes in abiotic conditions in the water column propagate into the gut, as seen with dissolved oxygen concentration (Ornelas-García et al., 2018). Such parameters are hard to control within the natural environment, but closed-loop systems provide consistent abiotic conditions, and allow for other variables, such as hologenome (Figure 4c), to be manipulated with greater ease. The effect of many important physiochemical water properties (e.g. nitrate, ammonia and phosphate) on the teleost gut microbiome have not been studied, however, let alone how these properties interact (Ruiz et al., 2019). Salinity is another important physiochemical property for the gut microbiome in many aquaculture species. When Atlantic salmon transition from freshwater to saltwater, individuals can experience a 100-fold increase in gut bacteria, combined with a shift in dominant microbial taxa (Rudi et al., 2018). Increasing salinity

in RAS systems can, however, negatively impact nitrate removal in bioreactors (von Ahnen et al., 2019), highlighting the importance of understanding interacting physiochemical properties.

A1.6.3. Dysbiosis as a Stress Biomarker

The use of closed-loop systems is a progression to a more intensive method of aquaculture, mirroring the progression seen in animal agriculture, and a crucial element to sustainable intensification is welfare. It is possible to measure fish welfare through physiological and behavioural indicators, with a current focus on identifying stress. The microbiome has been identified as another potential biomarker (Llewellyn et al., 2014) due to its interaction with the host immune system, and its responsive nature to stressors (Boutin et al., 2013; Webster et al., 2019). Therefore, identifying imbalances in the gut microbiome, or dysbiosis, could be a useful predictor of stress-related syndromes, which could ultimately lead to mortality. Using non-invasive faecal samples could complement other non-invasive stress biomarkers, such as water cortisol (Fanouraki et al., 2008), allowing for the optimisation of husbandry, alerting operators to chemical (e.g. poor water quality, diet composition imbalance, accumulation of wastes), biological (e.g. overcrowding, social dominance, pathogens), physical (e.g. temperature, light, sounds, dissolved gases) or procedural (e.g. handling, transportation, grading, disease treatment) stressors (Gabriel et al., 2011). More research is needed, however, in assessing the reliability and accuracy of faecal microbiome sampling in identifying stress.

A1.7. Conclusions and Future Applications

The teleost gut microbiome has a clear role in the future of aquaculture, and although research has come a long way in recent decades, there are still many areas of gut microbiome research that require further development. As highlighted in Figure 1b, there are still key elements lacking from many studies, particularly those assessing metacommunity composition, with the lack of water samples being particularly glaring. The ability to sample the environmental metacommunity with ease is one of the strengths of using a teleost model. Another methodological problem that will hinder comparability, reproducibility and metanalysis of fish gut microbiome datasets is the varying degree of sequencing platforms and markers (Figure 5). A solution to this

problem would be to focus on one marker, and one sequencing platform, with many metabarcoding microbiome studies adopting the V3 and V4 regions, sequenced on Illumina platforms. It is noted, however, that different markers and sequencing platforms work better in some systems with no simple fit-all approach. Therefore, tools that incorporate differences in taxonomic identification that arise through using different methodological approaches will be vital in comparing datasets.

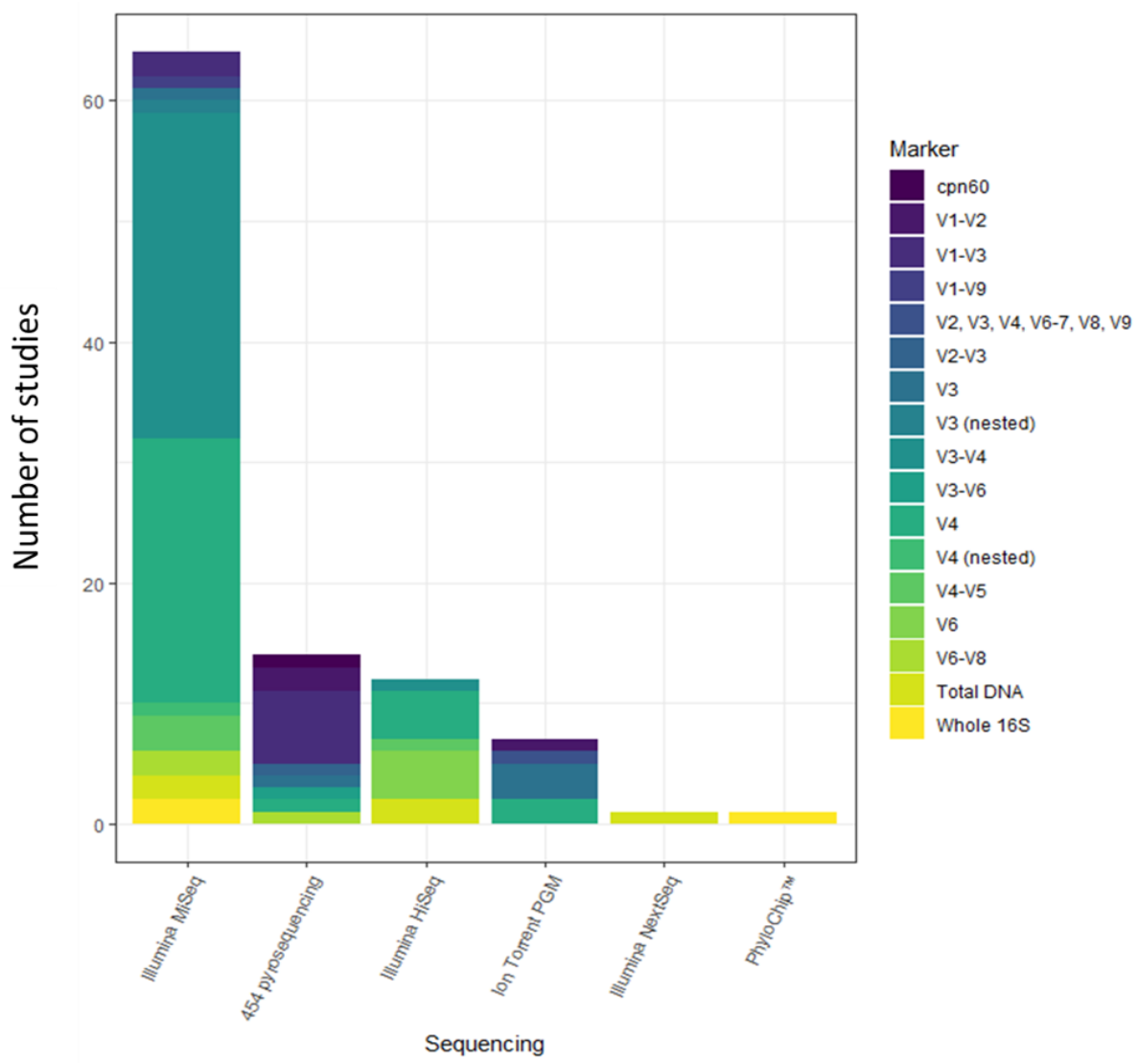

Figure A1 5: Methodological *approaches used in high throughput sequencing of fish gut microbiomes, broken down by the type of sequencing platform, and genetic marker.* *Marker type are predominantly variable regions (V) within the 16S ribosomal RNA gene. Further information on search terms and filtering can be found in the supplementary information.*

Current findings, as summarised here, show that the teleost gut microbiome plays an important role in aquaculture, however, the literature is dominated with studies performed on mammals, leading to limited data on functional capacity of fish gut microbiomes (Llewellyn et al., 2014). Furthermore, a knowledge gap exists between ascertaining the composition of the microbiome and understanding its function, partly due to the complexity and variability in the ecology of teleost gastrointestinal tracts (Egerton et al., 2018) and unknown bacterial taxa. More specifically, however, it has been caused by the lack of synthesis between multiple cutting-edge molecular techniques. Progression in teleost gut microbiome research will depend on combining function (RNA sequencing), composition (metabarcoding and metagenomics) and spatial distribution (fluorescence in situ hybridization). Understanding host genetic diversity (population genomics) and expression (RNA sequencing) of that diversity, all while incorporating environmental variation, will also be vital.

Finally, there are many areas in which synergies between gut microbiomes and aquaculture can be made. These have been highlighted through the review, but in summary, include a better understanding of the gut microbiome and: insect-based feeds, vaccination, mechanism of pro- and pre-biotics, artificial selection on the hologenome, in-water bacteriophages in RAS/BFT, physiochemical properties of water, and dysbiosis as a biomarker.

A1.8. Acknowledgements

Thanks to Professor Gary Carvalho and Dr Martin Llewellyn for providing feedback on the review. This work was supported by the Natural Environment Research Council.

A1.9. Authors' Contributions

W.B.P organised the creation of the review and facilitated communications between authors. W.B.P, E.L, R.K, C.P and C.B contributed to the concept and writing of the review.

A1.10. Supplementary Information

Data collected in the systematic review used for Figure 1 and Figure 2 in the main document were collected from Web of Science (Reuters, 2012) using the search

terms ‘fish’, ‘gut’ and ‘microbiome’. Studies were not included in the database if they contained:

- Non-community-based studies
- No high throughput sequencing
- Methods paper with no novel data
- A focus on fungi or other microorganisms that are not bacteria
- Skin or gill microbiomes
- Fluorescence In Situ Hybridisation (FISH)
- Data on the aquaculture status of fish was gathered from FishBase (Froese, 2019)

Appendix 3: Supplementary Material for Chapter 3 - Does Gut Microbial Composition Vary with Host Metabolic Phenotype in Juvenile Atlantic Salmon?

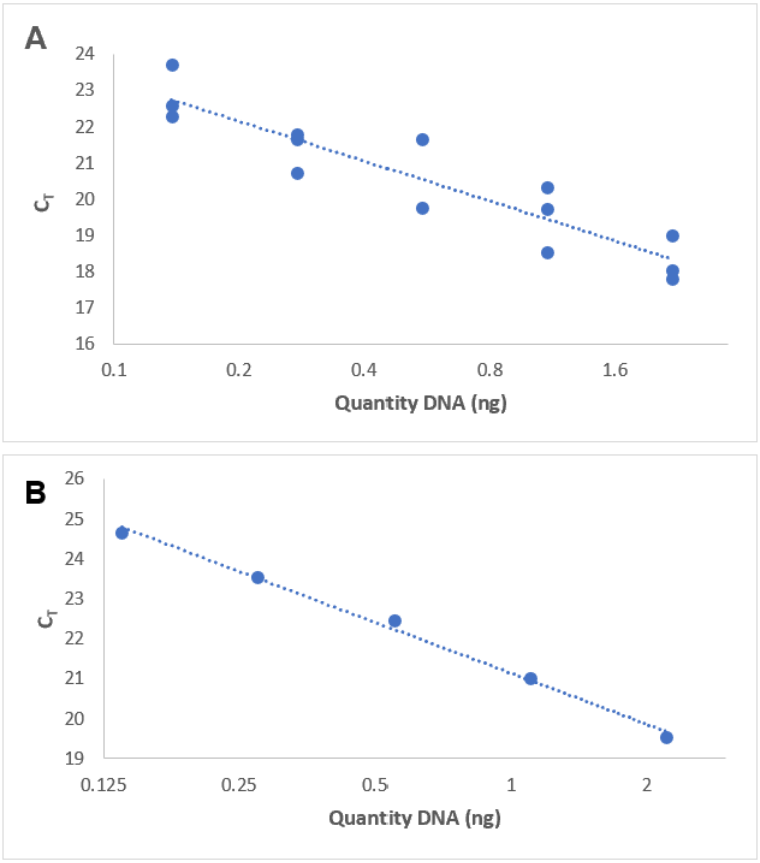

Figure A3- 1: The standard curves from qPCR analysis, for which E. coli was serially diluted (1, 0.5, 0.25, 0.125 and 0.0625) and run in duplicate. *Curve [A] was formed using 3 samples of E. coli and [B] was formed using 1 sample.*

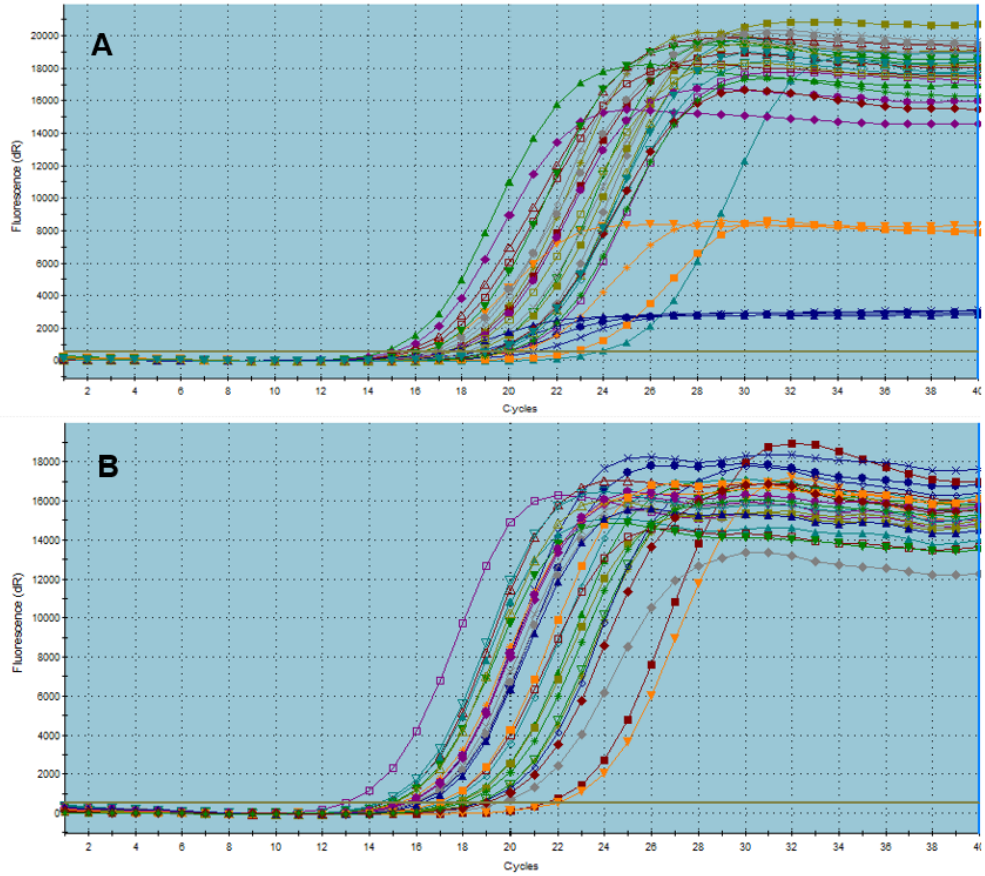

Figure A3- 2: qPCR amplification curves from Atlantic salmon faecal samples. *The curves in [A] were created on 11/01/18 and show amplification of 30 faecal samples, no-template controls (NTCs) and 3 samples of E. coli for standard creation; each run in duplicate. The curves in [B] were created on 15/01/18 and show amplification of 27 faecal samples, no-template controls (NTCs) and E. coli for standard creation; each run in duplicate.*

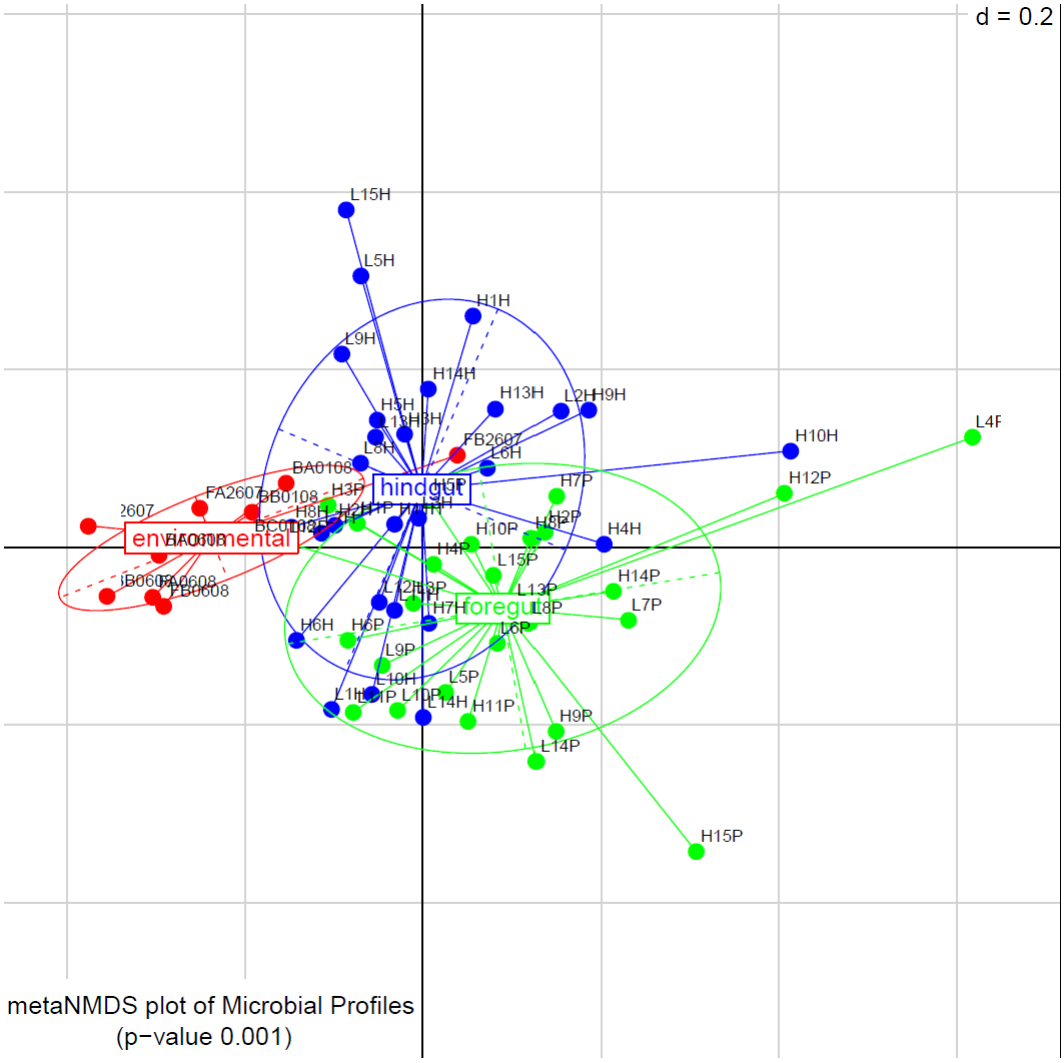

Figure A3- 3: A non-metric multidimensional scaling (NMDS) plot showing the beta diversity of different sample types: foregut and hindgut Atlantic salmon samples and environmental samples (biofilm and water) taken from the tanks in which the fish were kept. *The NMDS plot was calculated from the generalised UniFrac dissimilarity matrix. The dissimilarity scale of the grid, d = 0.2, indicates the distance between two grid lines represent approximately 20% dissimilarity between the samples. A permutational multivariate analysis of variance used the distance matrix to assess whether the separation of groups (sample types) was significant (p = 0.001).*

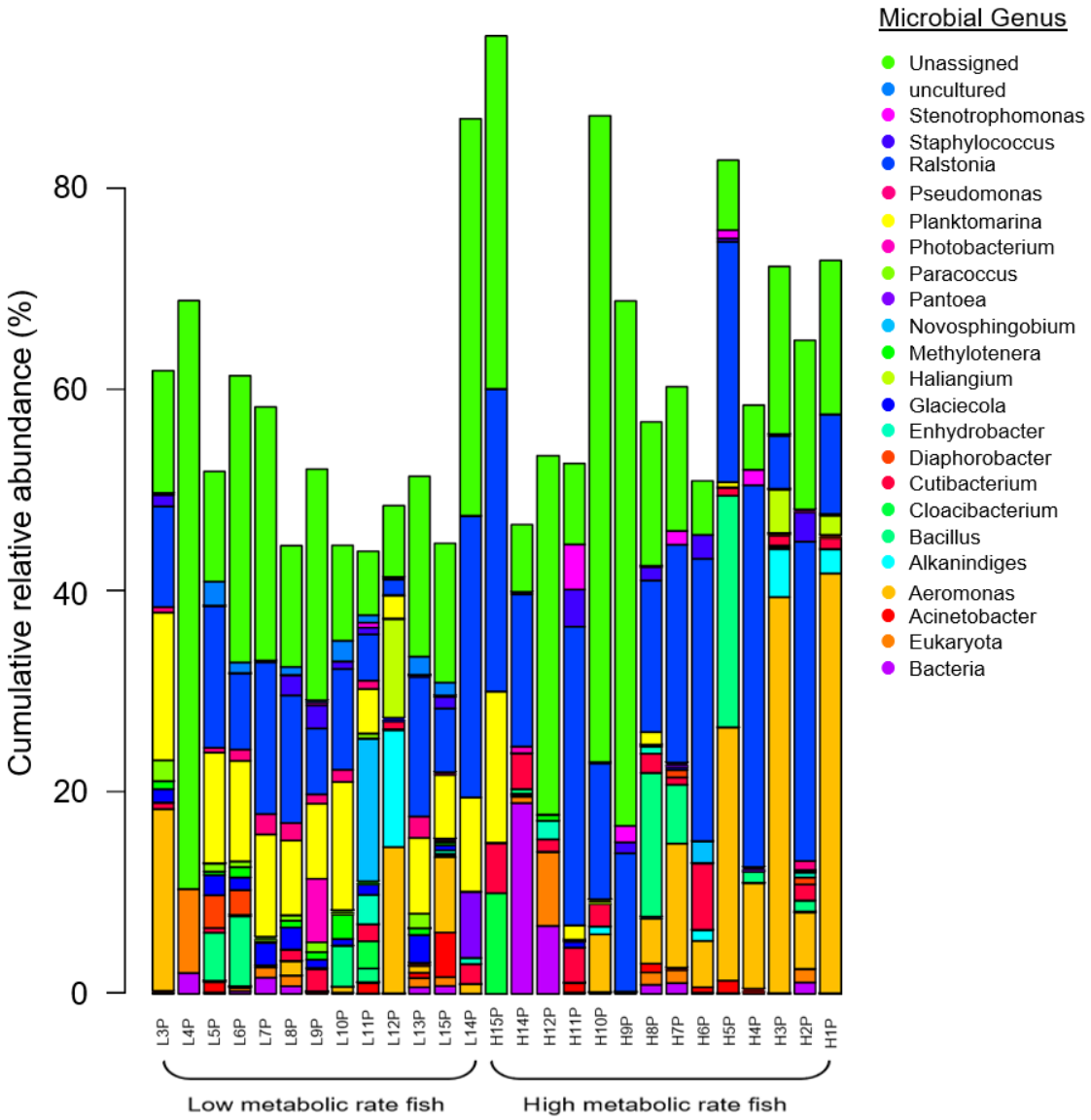

Figure A3- 4: ***Stacked bar plots showing the taxonomic composition and cumulative abundance (%) of microbes within the foregut of Atlantic salmon with high and low metabolic rates.*** *Microbes are shown at the genus level, with proportions of the bar coloured according to relative abundance of that genus. Threshold abundance was set to a cut-off of 0.25 to allow for better visualisation of the most abundant microbial genera.*

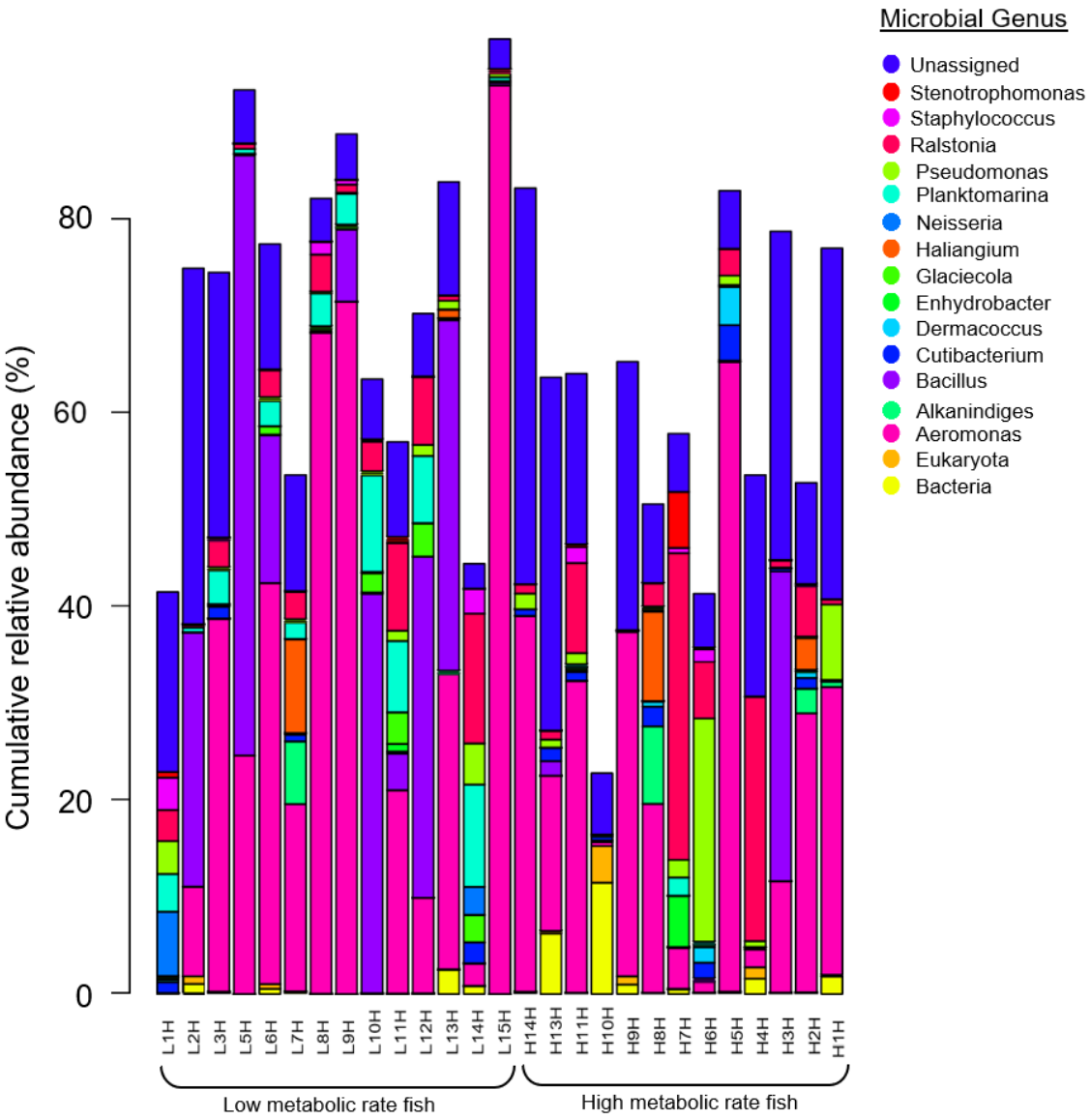

Figure A3- 5: Stacked bar plots showing the taxonomic composition and cumulative abundance (%) of microbes within the hindgut of Atlantic salmon with high and low metabolic rates. Microbes are shown *at the genus level, with proportions of the bar coloured according to relative abundance of that genus. Threshold abundance was set to a cut-off of 0.25 to allow for better visualisation of the most abundant microbial genera.*

Appendix 4: Supplementary Material for Chapter 4 – The Impact of Origin on the Metabolic Rate and Gut Microbiota of Atlantic Salmon: A Comparison of Wild, Ranched and Farmed Fish.

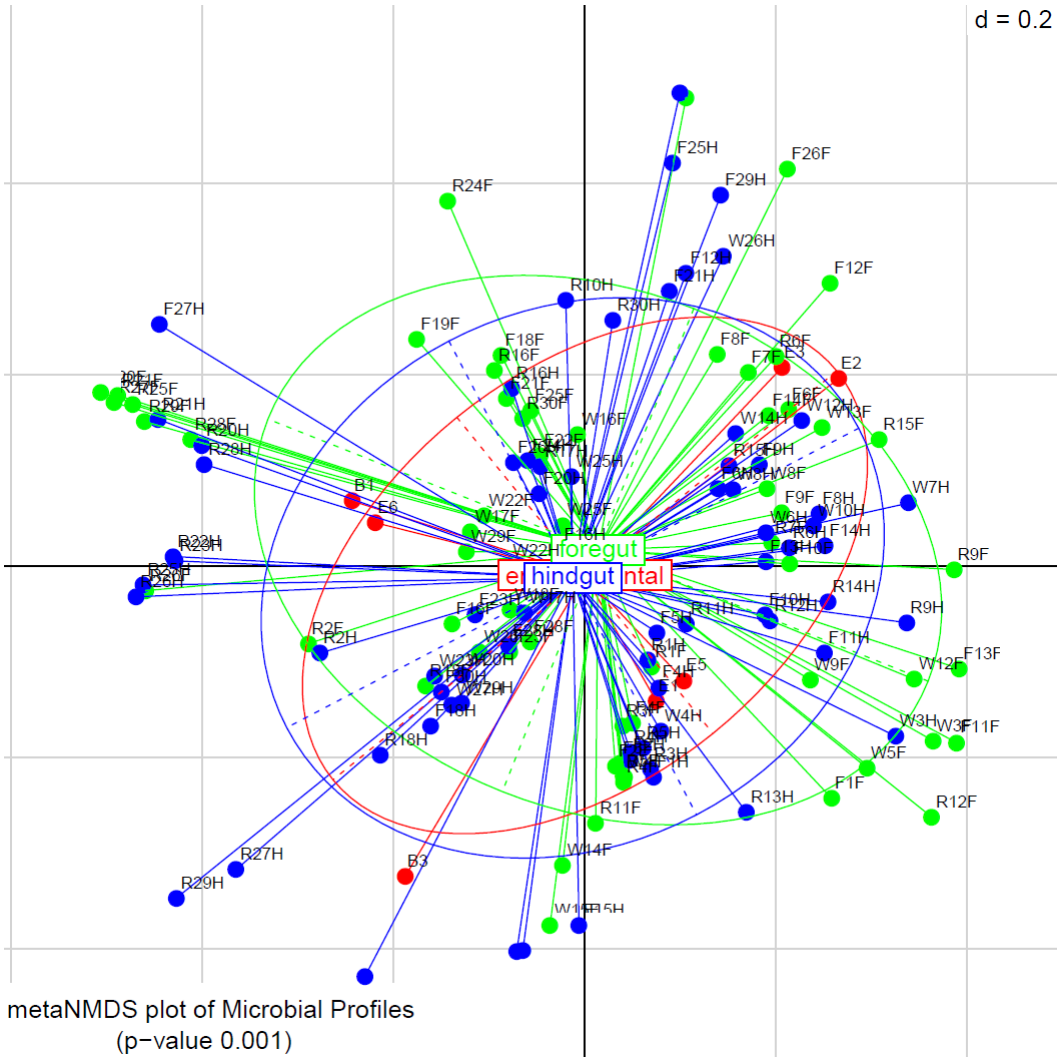

Figure A4- 1: A non-metric multidimensional scaling (NMDS) plot showing the difference in beta diversity between different sample types: foregut and hindgut Atlantic salmon samples and environmental samples (biofilm and water) taken from the tanks in which the fish were kept. *The NMDS plot was calculated from the generalised UniFrac dissimilarity matrix. The dissimilarity scale of the grid, d = 0.2, indicates the distance between two grid lines represent approximately 20% dissimilarity between the samples. A permutational multivariate analysis of variance used the distance matrix to assess whether the separation of groups (sample types) was significant (p = 0.001). Green, blue and red points represent fish with foregut, hindgut and environmental samples, respectively.*

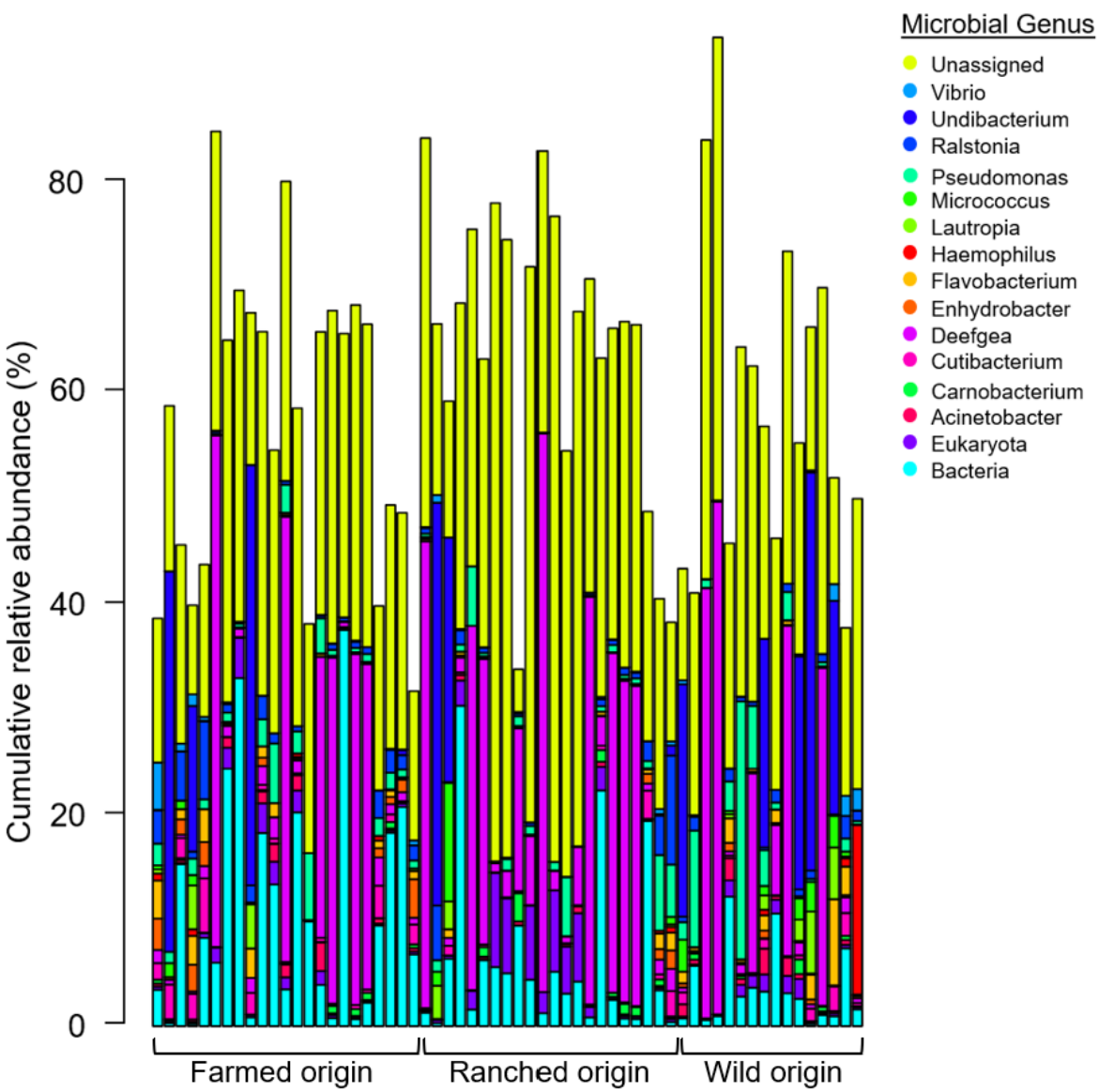

Figure A4-2: Stacked bar plots showing the taxonomic composition and cumulative abundance (%) of microbes within the foregut of Atlantic salmon from farmed, ranched and wild genetic backgrounds. *Microbes are shown at the genus level, with proportions of the bar coloured according to relative abundance of that genus. Threshold abundance was set to a cut-off of 0.25 to allow for better visualisation of the most abundant microbial genera.*

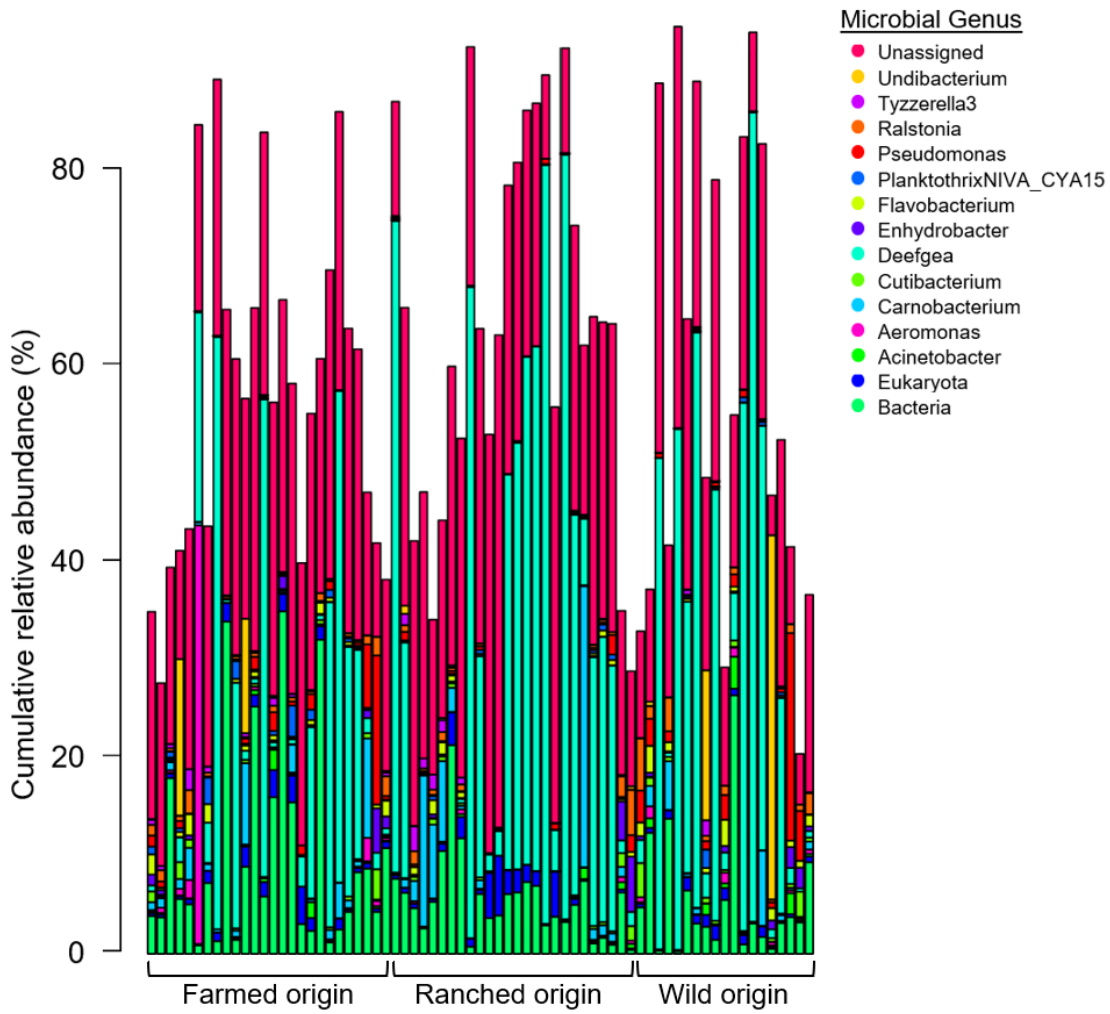

Figure A4- 3: Stacked bar plots showing the taxonomic composition and cumulative abundance (%) of microbes within the hindgut of Atlantic salmon from farmed, ranched and wild genetic backgrounds. *Microbes are shown at the genus level, with proportions of the bar coloured according to relative abundance of that genus. Threshold abundance was set to a cut-off of 0.25 to allow for better visualisation of the most abundant microbial genera.*

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