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DOCTOR OF PHILOSOPHY

From morphology to microbiome: common garden studies in wild, farmed and hybrid Atlantic salmon (Salmo salar).

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From morphology to microbiome:

common garden studies in wild, farmed and hybrid Atlantic salmon (*Salmo salar*).

A thesis submitted for the degree of Doctor of Philosophy

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Acknowledgements

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Throughout my PhD I have been lucky enough to work with numerous hard working, imaginative and immensely bright individuals, all of which, I know, have had a profound effect on myself and this piece of work.

Bangor University

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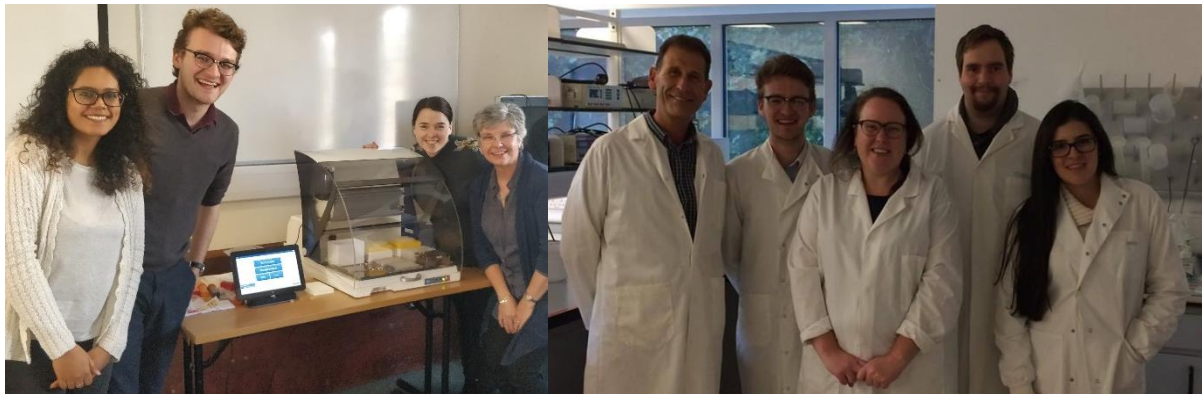
Havforskningsinstituttet

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Abstract

A key consequence to the process of taking organisms into captivity for human use, often with artificial selection of beneficial traits, is domestication. Attention to the domestication of fish has been increasing from the beginning of the 20th century in association with the rapid growth in aquaculture. One such species that typifies the wider growth in aquaculture, as well as domestication, is the Atlantic salmon (*Salmo salar*). Here we assess the impact of domestication on Atlantic salmon morphology and microbiome using a common garden design, whereby fish from different genetic backgrounds including wild, domesticated and reciprocal wild x domesticated hybrids (as well as F2 hybrids and backcrosses) are reared together from the eyed-egg stage. Key phenotypes have been examined, both internal and external, relating to morphology and microbiome. Our pedigree controlled experimental design and use of an array of hybrids has demonstrated genetically additive domestication driven changes, with 1) reduced fork length adjusted kype height in domesticated fish, 2) increased fork length in domesticated fish, 3) increase pectoral fin length in domesticated fish, 4) reduced eye width in domesticated fish and 5) altered body shape in domesticated fish, when compared to wild counterparts - with hybrids showing intermediate phenotypes). In addition to this, the application of both artificial and natural common gardens has highlighted that domestication driven morphological changes are quickly removed from populations through strong stabilising selection to a wild optimum, likely due to reduced fitness. The results shown here not only demonstrate the rapid (~ 12+ generations) total phenotypic changes caused by artificial selection, it also highlights the risks posed to wild populations from aquaculture escapees and introgression. Such findings reinforce the need for continued innovation in preventing fish escapes from aquaculture.

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Publications

Publication 1: "Evolutionary drivers of kype size in Atlantic salmon (Salmo salar): domestication, age and genetics" - *Royal Society of Open Science* https://doi.org/10.1098/rsos.190021

Publication 2: "The role of the gut microbiome in sustainable teleost aquaculture" - *Proceedings of the Royal Society B* https://doi.org/10.1098/rspb.2020.0184

Publication 3 in review: "Disentangling the effects of environment and genetics in Atlantic salmon: growth, heart and liver under common garden conditions" - *Royal Society of Open Science*

Chapter 1: General introduction

1.1 Domestication & aquaculture

1.1.1 Early domestication in fish

The process of domestication has long been part of agriculture, and based on archaeofaunal evidence, it is estimated to have first occurred in animals 10, 000 years ago in goats from the western highlands of Iran (Zeder and Hesse, 2000). Up until the 19th century, the same process of domestication had occurred in only a handful of aquatic organisms. Domestication in fish was first seen in species of Nile tilapia and Asian carp 3000 - 3500 years ago (Teletchea and Fontaine, 2014). Aquaculture, and elements of domestication, were also taking place in Europe in the Middle Ages, with European monks culturing common carp and brown trout (Teletchea and Fontaine, 2014). However, the full domestication of salmonids in Europe did not start until the late 1960s (Harache, 2002). Salmonids, like many fish, have complex life histories which pose an obstacle for cultivation, a problem that is not inherent to many other animals like cattle, sheep or fowl (Stead and Laird, 2002). Looking at domesticated mammals, and their close relatives which were never domesticated, it is possible to identify other reasons why fish were not domesticated for such a long time, including: difficult dietary demands, lack of follow-the-leader dominance hierarchies, and tendency to become stressed in enclosures or when faced with predators (Diamond, 2002).

Intrinsic problems in fish cultivation led to a lag of 8,000 years between the development of aquaculture and the development of agriculture (Liao and Huang, 2000). As domestication is at an early stage in fish such as salmonids, it is an exciting time to document and understand domestication induced change to phenotypes. The focus of this thesis is to explore a range of phenotypic consequences of domestication using the most commercially important aquaculture species, the Atlantic salmon (*Salmo salar* L.), with global production worth $15.4 billion (ISFA, 2018). Phenotypes

examined here include the heart, liver, brain, gut microbiome, external morphology, and secondary sexual characters.

1.1.2 Rise of aquaculture

Despite the later establishment of aquaculture compared to livestock agriculture, numerous aquatic systems are now undergoing rapid change in response to anthropogenic pressures; from the white-legged shrimp (*Litopenaeus vannamei*) in brackish ponds of Southeast Asia, to modern Genetically Improved Farmed Tilapia (GIFT) produced in high density Brazilian freshwater raceways (Kumar and Engle, 2016). The aquaculture industry has grown exponentially throughout the globe since its initial expansion in the 1960s, both in salmonids and more generally, with aquaculture now providing over 45 % of fish-based food products globally (Longo *et al.*, 2019). In addition to this, although the focus of the current study is aquaculture involved in food production, it is important to note that other aquaculture markets have also opened up, such as ornamental fish aquaculture which makes up 40 % of overall production in countries such as Singapore (Ladisa, Bruni and Alessandro, 2017), and globally is worth an estimated $15–30 billion each year (Evers, Pinnegar and Taylor, 2019).

The growth of aquaculture has been particularly pronounced in Asia, which is home to five of the largest aquaculture producing nations, including China (43.5 million tonnes), India (4.5 million tonnes), Indonesia (3.8 million tonnes), Vietnam (3.2 million tonnes), and Bangladesh (1.8 million tonnes), and produced 90 % of global output in 2013 mainly from rural pond based systems (Ottinger, Clauss and Kuenzer, 2016). Products from aquaculture in these rural settings provide a valuable source of animal protein, with China consuming over double the amount of fish protein than those in North America and Europe (Tidwell and Allan, 2001). Other aquaculture intensive nations include Norway, a country of under 5.4 million people, which in 2019 was the sixth largest aquaculture producer in the world (1.4 million tonnes) and focuses almost entirely on aquaculture of Atlantic salmon (1.36 million tonnes of Atlantic salmon and rainbow trout)(Directorate of Fisheries, 2020). Similarly, Chile is among the top

10 global aquaculture producers (0.8 million tonnes), at number nine, due to its production of Atlantic salmon (Ottinger, Clauss and Kuenzer, 2016).

The use of aquaculture to obtain fish-based proteins has been partially driven by the global decline in capture fisheries, driven by overexploitation of wild fish stocks, reduced productivity and ultimately loss in yields (Goldburg and Naylor, 2005). Approximately one third of the world´s fish stocks are currently overexploited (Ding *et al.*, 2017). Indeed, there is some evidence to show that when wild stock management is improved, there is a reduction for the potential in growth of aquaculture (Jensen, Nielsen and Nielsen, 2014). However, even if Atlantic salmon production from capture fisheries were to be improved through better management, and now depleted stocks were able to sustain the peak capture fishery production of 15,387 tonnes seen in 1973, this would not even cover 7 % of current aquaculture production (FAO, 2020a). What this demonstrates is that the global demand for Atlantic salmon massively outstrips the natural supply, which only aquaculture can provide.

The trend of necessary aquaculture production to keep up with demand is not unique to Atlantic salmon, leading to an increase in global aquaculture production, with aquaculture now supplying 47 % of global fish product consumption, soon to surpass that of the stagnated production of capture fisheries (FAO, 2018). Currently, a total of 250 aquatic species are cultivated, with different extents of domestication (Teletchea and Fontaine, 2014). With this increase in aquaculture output, the succession towards more intensive aquaculture is being seen, which mirrors terrestrial livestock domestication, with an aim towards sustainable intensification (Garnett *et al.*, 2013). Human population growth is expected to cause an increase in consumption of fish products by an estimated 1.2 % in the next decade (FAO, 2016a). To keep up with this increased demand for fish products, and to reduce pressures on wild capture fisheries, the need for sustainable intensification of aquaculture is only going to increase.

1.1.3 Variety of aquaculture practices and their environmental impacts

Despite the need for sustainable intensification of aquaculture to provide fish-based protein, the industry is still plagued by many environmental and welfare problems. The nature of the impacts associated with aquaculture have different characteristics depending on the species and region being farmed. Aquaculture can be broken down into the environments that are used for cultivation: freshwater, brackish and marine. Inland freshwater aquaculture is the largest producer of aquaculture products, making up over 63 % of the global production (Ottinger, Clauss and Kuenzer, 2016). Freshwater production can be pond based, especially in countries such as China, with production of finfish such as grass carp, silver and bighead carp, common carp and crucian carp, and crustaceans such as shrimp (Cao *et al.*, 2007). Production in freshwater is highly diverse, and can range from flooded rice fields which also contain shrimp, to concrete raceways, often favoured for the production of freshwater salmonids such as rainbow trout (Bostock *et al.*, 2010). Brackish waters are by far the least utilised aquatic environment, with only 8 % of aquaculture occurring here (Ottinger, Clauss and Kuenzer, 2016), although they provide the perfect environment in tropical climates for the production of penaeid shrimps (Bostock *et al.*, 2010). Finally, marine based aquaculture makes up 29 % of aquaculture production (Ottinger, Clauss and Kuenzer, 2016) and vary from coastal ponds for the production of species such as milkfish, to sea cages, both costal (e.g. farmed salmon), and offshore (e.g. farmed tuna) (Naylor *et al.*, 2000).

The impact on the environment is just as diverse as the methods of farming, but serious environmental issues include habitat modification, dependency on wild seed stock, aquatic biological and chemical waste, salinization of water and soil, parasite transmission, dependency on wild stocks from fishmeal used in feeds, and release of domesticated or non-native fish (see 1.1.4: Aquaculture and its dependence on wild stocks; Primavera, 2006; Cao *et al.*, 2007). Such environmental issues mean that for many forms of aquaculture, sustainability remains an ongoing challenge, exacerbated by problems of fish welfare, particularly in relation to disease and stress (Ashley, 2007).

1.1.4 Aquaculture and its dependence on wild stocks

Aquaculture is often seen as a solution to easing the overexploitation of fish stocks, caused by weak regulatory compliance or inappropriate sustainability targets (Hilborn, Punt and Orensanz, 2004). However, an inherent weakness of such an assertion is that many branches of the aquaculture industry are dependent on capture fisheries and wild fish stocks. Dependence on wild stocks includes the use of wild seed stock for propagation of aquaculture species, and the use of fish in fishmeal for aquaculture feed. These are the two greatest challenges in creating a sustainable aquaculture industry independent of capture fisheries. Many aquaculture species such as milkfish, lumpfish, tuna, shrimp and eel depend on wild seed stock (Naylor *et al.*, 2000). Despite this, the aquaculture industry has managed to close the life cycle in many species, without the need for wild inputs, thus increasing the independence of production from wild stocks.

Even for species in which the entire lifecycle is produced in aquaculture, there may still be links with wild fish stocks. Many aquaculture species are dependent on fish from capture fisheries through fishmeal components in feed. Fishmeal, produced from fish caught in capture fisheries, is a source of high quality protein and energy, and contains highly digestible essential amino and fatty acids (Cho and Kim, 2011). Some aquaculture species require more fishmeal in their feed than others, with carnivorous species such as shrimp, as of 2016, taking up 31 % of the use of fishmeal in aquaculture, and salmonids coming in a close second, using up 23 % (Seafish, 2018). The past decade has, however, seen a global decrease in the production of fishmeal, from an average of 6.0 million tonnes between 2001 - 2005 to 4.9 million tonnes between 2006 - 2010 (Shepherd and Jackson, 2013). The decrease, in part, can be attributed to innovations within the aquaculture industry to provide feeds with less fishmeal. In the 1990s, 90 % of the ingredients in Norwegian salmon feed came from a marine origin, where approximately 2.8 tons wild fish were needed to produce 1 ton of salmon (Naylor *et al.*, 1998). Compare this to 2013, where 30 % of the ingredients in Norwegian salmon feed came from a marine origin, and approximately 0.7 tons of wild fish were needed to produce 1 ton of salmon (Ytrestøyl, Aas

and Åsgård, 2015). Over 20 years of feed development has now made farmed salmon a net producer of marine protein.

1.2 Atlantic salmon in aquaculture

A fish species that has been ranked in the highest tier of domestication is the Atlantic salmon (*Salmo salar*) (Teletchea and Fontaine, 2014). Today, 99 % of salmon consumed is sourced through farmed stocks (Glover *et al.*, 2017), demonstrating the wider trend seen in fisheries across the globe. The growth in Norwegian Atlantic salmon aquaculture occurred initially in the 1970s, when the first 100 tonnes of farmed fish was produced (Harache, 2002). However, Norway is now the leading exporter of farmed salmon, producing over half the world's supply (FAO, 2018) (figure 1.1), as well as contributing to over 80 % of the imports into the European Union (EUMOFA, 2015). The Norwegian Atlantic salmon industry is therefore a well-established system for studying the impacts of domestication on finfish.
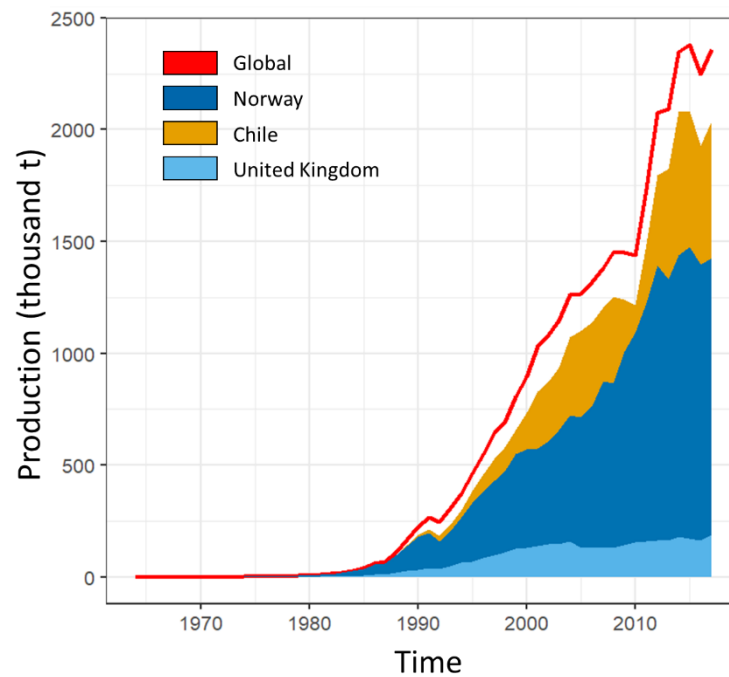
Figure 1.1 Stacked line plot of Atlantic salmon, in thousands of tonnes, between 1964 and 2017 for Norway, Chile and the United Kingdom; also included in this graph is total global production of Atlantic salmon (FAO 2018).

1.3 Artificial selection

The importance of aquaculture in providing fish products in a future world will result in the further domestication of aquaculture species, as has been seen with agricultural livestock over the past 10,000 years, with aquaculture currently in its infancy by comparison. In 2012 it was estimated that less than 10 % of aquaculture products came from genetically improved stock (Gjedrem, 2012), and although this number is likely to have grown since, it demonstrates the embryonic stage of aquaculture, despite its continued expansion globally. One species in which this figure does not apply, however, is the Atlantic salmon, with over 99% of production coming from genetically improved lines of some description (Glover *et al.*, 2017). The widespread use of genetic improved lines and rearing fish in artificial environments has already resulted in considerable shifts in the biology of Atlantic salmon;

not only improving production traits such as growth, but also impacting aspects of behaviour (Huntingford, 2004), disease prevalence (Cabello, 2006) and morphology (Mayer *et al.*, 2011), just to name a few (full impacts reviewed by Glover *et al.*, 2017)). The process of domestication which induces phenotypic change can be split into subcategories: active artificial selection and passive artificial selection. Since the focus of the current study is on exploring various impacts of domestication on Atlantic salmon phenotypes, it is pertinent to consider briefly, the nature of selective forces associated with captive environments, with a brief consideration of key differences to fish living in the wild.

1.3.1 Active artificial selection

Active artificial selection is the process whereby individuals with heritable traits linked to production are bred together in order to control that trait in their offspring. Control of a production trait is mainly achieved by directional selection (figure 1.2a), where individuals at one end of the extreme of an economically important trait are bred together to maximise the trait. Traits that have been under strong active artificial directional selection include growth (Einum and Fleming, 1997; Fleming *et al.*, 2002; Glover, Otterå, *et al.*, 2009; Wolters *et al.*, 2009; Solberg, Skaala, *et al.*, 2013; Harvey, Glover, *et al.*, 2016; Harvey, Solberg, Troianou, *et al.*, 2016; Solberg *et al.*, 2016; Glover *et al.*, 2018a). Stabilising selection is not often used in aquaculture (figure 1.2b), although there are examples, such as when breeding fish for optimum fat content, where extremes in this trait would not be desirable (Gjedrem and Thodesen, 2005). Traditionally, active artificial selection is achieved via an iterative process of measuring a trait in the parent brood stock, extracting gametes from selected brood stock that have the desired trait and conducting artificial fertilisation; or, where trait measurement requires termination of the fish, through family-based selection, whereby siblings from the same family are terminated, and their traits measured. However, modern aquaculture practices have been transformative in how these components of selective breeding are carried out.
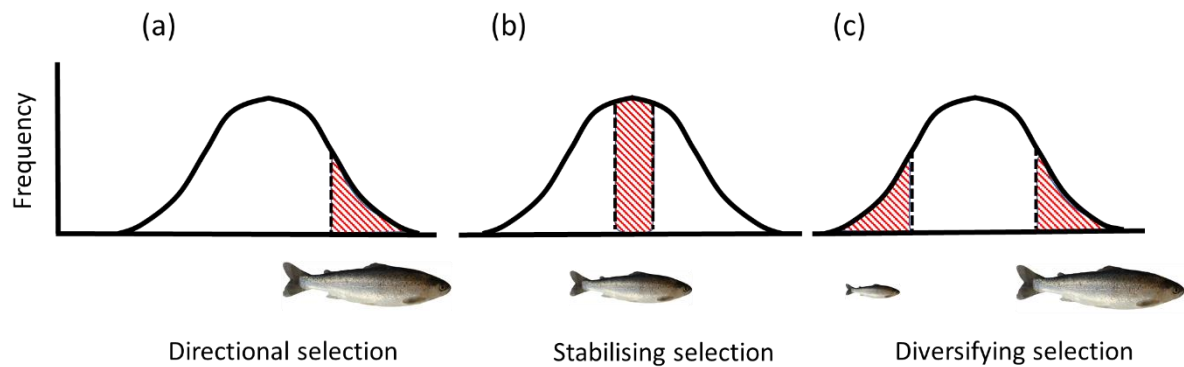
Figure 1.2 Diagrammatic population frequency plots for body size trait in Atlantic salmon, **demonstrating (a) directional, (b) stabilising and (c) diversifying selection on this trait.**

1.3.2 Active artificial selection: selection, crosses and hybridisation

Traditionally, in agriculture, and in more recent years, aquaculture, artificial selection and production of genetically improved lines has been dependant on selection and crosses (Farias, César and Silva, 2017). As discussed above, much of this selection has been directional selection applied to a single production trait, and in addition to this, there are different biological levels of selection pressure, be it individual, family, or a combination of the two. Tandem selection of single traits in succession is a slow way of achieving evolutionary change on multiple traits, and other methods such as independent culling and selection index are often preferred. Independent culling is where criteria are set for multiple traits (body length, weight, condition factor), and all those fish that do not reach a threshold for all these traits are culled (FAO, 2020c). Alternatively, the selection index method (Kang *et al.*, 2013) summarises multiple traits in one index, and considers heritability of traits, genetic correlations between traits, and the importance of the traits; fish with the highest indexes are then used for breeding.

1.3.3 Active artificial selection: Genomics

The ever-decreasing cost and accessibility to next generation sequencing (NGS) is allowing researchers around the globe to apply genomic tools to a broad range of taxa and research questions, where previously it would not have been possible. Therefore, applying genomic tools to Atlantic salmon aquaculture, and aquaculture more broadly, has been of great interest in producing genetically improved stock (Yáñez, Newman and Houston, 2015). One reason that the Atlantic salmon have been a candidate for such tools is that, as discussed previously, they are at a very early stage of domestication, compared to land based livestock that were domesticated 10,000 years ago (Zeder and Hesse, 2000). Recent studies examining domestication induced changes within the Atlantic salmon genome have found F_{ST} values of 0.03 – 0.171 (Mäkinen *et al.*, 2015), which may seem like a low level of genetic differentiation, however, not when put into the context of Norwegian populations separated by approximately 2,000km of coastline that have an F_{ST} value of 0.038 (Karlsson *et al.*, 2011). Considering the natal philopatry of Atlantic salmon preventing these populations from interbreeding, an FST of 0.038 is high. In turn, this shows the large genetic differences that have occurred between domesticated and wild salmon, which have a genetic differentiation of over three times that observed between the most genetically distinct wild Norwegian populations.

As genomics become more widely available to researchers, it has the potential to be applied more widely to the process of selective breeding and artificial selection, also referred to as genomic selection (Heffner, Sorrells and Jannink, 2009). As discussed, growth has been an important trait for selection in many aquaculture breeding programs, but many other traits have also been subject to artificial selection, traits such as time of maturation, resistance to disease and parasites, feed conversion efficiency, environmental tolerance, and fillet quality; although some have been harder to select for using traditional methods of selection due to factors such as low heritability (Zenger *et al.*, 2019). Genomic resources can help increase aquaculture production, and improve traits like those just mentioned through several ways, and these include i) genetic markers for parentage assignment, ii) high-throughput ribonucleic acid (RNA) sequencing and iii) single nucleotide polymorphism arrays to predict genomic breeding values for traits (Yáñez, Newman and Houston, 2015). Parentage

assignment using microsatellite markers have been routinely used for many years, but those techniques involving NGS are far more recent, and are now standard in Atlantic salmon aquaculture.

Firstly, microsatellite markers used for the parentage of wild and farmed fish, as implemented in this thesis, can also be used in sibling selection, also known as family selection, whereby breeding programmes only use between-family genetic variance within a population, rather than within-family genetic variance, thus avoiding inbreeding depression (Norris, Bradley and Cunningham, 2000; Zenger *et al.*, 2019). Beyond this, however, microsatellites have also been used to identify quantitative Trait loci (QTL) for important salmonid aquaculture diseases such as infectious pancreatic necrosis (IPN), identifying linkage between IPN resistant and IPN suspectable strains of rainbow trout (Ozaki *et al.*, 2001).

Secondly, high throughput sequencing of RNA has also advanced our understanding of artificial selection, by going beyond our understanding of what genetic variation exists in an individual, and allowing researchers to understand how this genetic variation is expressed under certain environmental conditions. Examining gene expression during infection of a pathogen or parasite in resistant and susceptible strains, for example, is valuable for understanding the mechanisms of resistance, as has been demonstrated with amoebic gill disease in Atlantic salmon (Robledo *et al.*, 2020). Understanding what genes are being transcribed during such an infection provide breeders with genomic targets for selection, or genome editing (Gratacap *et al.*, 2019). Examples seen in Atlantic salmon include genes involved in apoptosis, or cell death, that were downregulating during an infection of amoebic gill disease (Wynne *et al.*, 2008), and downregulation of energy metabolism and cell proliferation genes during an infection of salmonid alphavirus pancreatic disease (Larsson *et al.*, 2012).

Finally, genomics can also be used in identifying SNPs for important production traits, allowing for the prediction of breeding values for these traits. Techniques such as genome wide association studies (GWAS) between phenotypic extremes allow for the identification of loci contributing to those

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phenotypes, thus allowing targets for genomic selection programs and gene editing (Tsai, Hamilton, Tinch, *et al.*, 2015). Recent examples of the application of GWAS include the discovery of SNPs associated with omega-3 fatty acid composition of Atlantic salmon fillets (Horn *et al.*, 2020).

Genomic resources have not only been useful in categorising host genetic diversity, they have also been instrumental in discovery of host associated bacterial genetic diversity. One of the greatest applications of gut microbial genetic diversity in aquaculture is seen in fish gut microbiomes (Llewellyn *et al.*, 2014). The gut microbiome has been associated with beneficial functions to fish hosts, firstly providing the host with nutrients. For example, the amount of vitamin B12 was seen to be positively correlated with the abundance of anaerobic bacteria in Nile tilapia (*Oreochromis niloticus*) (Sugita, Miyajima and Deguchi, 1990), with anaerobic bacteria also supplying the host with volatile fatty acids (Ramirez and Dixon, 2003). A second function of the gut microbiome is its role in the host's immunity. For example, bacteria belonging to the genera *Bacillus* and *Lactobacillus*, two common probiotic groups 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 goblet cells that are involved in producing the protective intestinal mucus layer (Popovic *et al.*, 2017), and increase phagocytic activity among other innate immune responses (Chen, Liu and Hu, 2019). There have also been causal chains identified in wild three-spined stickleback (*Gasterosteus aculeatus*), whereby diet is able to modulate the immune system, which in turn changes the composition of bacterial communities in the gut of the fish (Friberg, Taylor and Jackson, 2019). Examples such as that demonstrated in the wild three-spined stickleback show the possible power of manipulating the hologenome. The hologenome is the combined genetic material of both host and its gut biota. Genomics and next generation sequencing are giving us greater insight into the relationships between hosts and their gut microbial communities, in such a way that it is feasible that manipulation of the hologenome could take place to improve production in aquaculture. Due to the complex nature of the interacting variables within the hologenome, with the host effecting microbial composition, and the microbial communities modulating host physiology, (through mechanisms such as serotonin signalling (Yano *et al.*, 2015)), a lot of further research is

needed before it can be applied to aquaculture. It does, however, provide grounds for further research.

1.3.4 Active artificial selection: chromosome-set manipulation, hologenome and genetically modified organisms

The process of mixing eggs and milt together in a bucket from desired brood stock has unsurprisingly made way for other more cutting-edge techniques in combining genetic information. Chromosome-set manipulation, for example, is where chromosomes are retained during meiosis of female gametes through the application of hydrostatic pressure or temperature shock, which induces polyploidy, potentially increasing genome flexibility allowing selection for economically desirable traits (Zhou and Gui, 2017). Induced polyploidy has been seen in multiple aquaculture species of carp (Cyprinidae) and salmonids (Salmonidae), with triploid Atlantic salmon showing signs of enhanced growth rates when compared to diploid individuals (Oppedal, Taranger and Hansen, 2003). There is also a dosage effect, whereby traits such as growth are linked to the genetic origin of the second maternal chromosome set, and so selection for commercial traits in the female can have greater effect on resulting offspring (Harvey *et al.*, 2017). Additionally, triploid salmon have the benefit of being functionally sterile, thus eliminating introgression between domesticated escapees and wild populations. However, there is conflicting evidence on the effect of triploidy on enhancing growth rate (Fraser *et al.*, 2013), and it has been shown that triploid Atlantic salmon show increased rates of skeletal abnormalities (Fraser *et al.*, 2013; Peruzzi *et al.*, 2018) and cataracts (Sambraus *et al.*, 2018).

Much like polyploidy, another method of yielding more genetic material for active artificial selection is through the utilisation of the hologenome. As previously mentioned in the genomics section, the hologenome is the combined genome of the host and its microbiome, including the genomes of bacteria, fungi, viruses and other microorganisms. Bacterial cells can equal, or even outnumber, the number of cells in the host (Sender, Fuchs and Milo, 2016), and so provide a huge wealth of genomic

variation which is not available in the host, providing functional capabilities that are not possible in eukaryotes, let alone vertebrates. These functional capabilities can be important in aquaculture traits, such as enzyme production (carbohydrases, cellulases, phosphatases, esterases, lipases and proteases) which contribute to digestion in fish (Ray, Ghosh and Ringø, 2012; Wu et al., 2015) and in disease resistance; *Lactobacillus* in the fish gut, for example, is able to stimulate the production of inflammatory cytokines (He et al., 2017). Therefore, the hologenome is a valuable resource in which active artificial selection can be applied, although like polyploidy, it is just more genetic material for selection in breeding programmes.

More involved genetic engineering than chromosome-set manipulation is now being applied to aquaculture species, producing genetically modified organisms (GMOs). Gene transfer, where transgenic DNA is transferred to the germ cells of a target species is used to produced new, or enhanced, economic traits (Levy, Marins and Sanchez, 2000). Advances in increasing growth rate have been achieved by utilising transgenic Atlantic salmon, as demonstrated in the commercial AquAdvantage strain. This strain is able to reach target weight in half the time of non-transgenic siblings (Ignatz *et al.*, 2020) through an insertion of a growth hormone-regulating gene from the larger Pacific Chinook salmon, in addition to a promoter sequence form ocean pout (*Zoarces americanus*) so that it can grow at low temperatures (all year instead of during growth season). Growth hormone transgenesis has also proven particularly successful in increasing growth rates in coho salmon (*Oncorhynchus kisutch*). Crucially, however, only modest gains in growth have been achieved in already domesticated lines, demonstrating that, at least for growth, GMOs bypass 30-40 years of domestication, but ultimately reach the same end goal (Devlin *et al.*, 2009).

Finally, the CRISPR/Cas9-mediated gene editing capabilities that have emerged in the past decade show great promise in allowing for a more controlled genetic manipulation than all methods before it (artificial selection, chromosome-set manipulation, transgenic lines). Although this technology has not been applied to commercial salmon (Straume *et al.*, 2020) and manipulating has so far only been used

to produce sterile salmon (Wargelius *et al.*, 2016; Kleppe *et al.*, 2017), it remains an exciting area of future research. Ultimately, however, both gene transfers and gene editing aim to increase genetic tractability to enhance production traits, and so are in themselves a form of more controlled active artificial selection, but require an intimate knowledge about the gene that is being targeted.

1.3.4 Passive artificial selection and production trait trade-offs

Passive selection, also known as inadvertent selection, is a by-product of the aquaculture environment, where artificially created environments produce selection pressures different from those in the wild, but unlike active artificial selection, do not target a production trait. It is possible for passive artificial selection to be directional, stabilising or diversifying (figure 1.2). The aquaculture environment varies significantly both between and within cultivated species. In Atlantic salmon aquaculture there are two main environments: the freshwater hatchery and the marine stage.

After fertilisation, eggs can be immersed in iodine to remove pathogenic, but also commensal, bacteria and placed in incubation trays. Following incubation, fry move to the freshwater hatchery stage which typically takes place in tanks where they are fed on a diet of freshwater pellet feeds (starter, grower, smolt transfer) (FAO, 2020b). The freshwater tanks can either be flow-through, where waste from the hatchery is discharged into a river, or, more recently, Recirculating Aquaculture Systems (RAS). Typically, tanks are featureless, with no enrichment for the fry/parr, provide continuous food and have no predation (figure 1.3a). Once the process of smoltification has started, smolt are normally moved into sea cages, although land-based RAS systems for the marine stage also exist. Once in the sea cage, post-smolts are kept in high density and fed on seawater grower pellet feeds containing fish meal (FAO, 2020b), with the possibility of non-natural lighting regimes. Antibiotics and anti-lice treatments may also be applied. The use of antibiotics in Atlantic salmon aquaculture in the northern hemisphere has been greatly reduced. Oxytetracycline is one of the most widely used veterinary antibiotics, belonging to the tetracyline group and is widely used in other salmon producing nations such as Chile, where 1,500 metric tons was applied between 2000 and 2008

(Buschmann *et al.*, 2012). The aquaculture environment is, therefore, very different to that of the wild (figure 1.3b), with a different ecology, requiring evolutionary change to reach the new phenotypic optimal, in addition to the selective pressures of the breeding programmes.
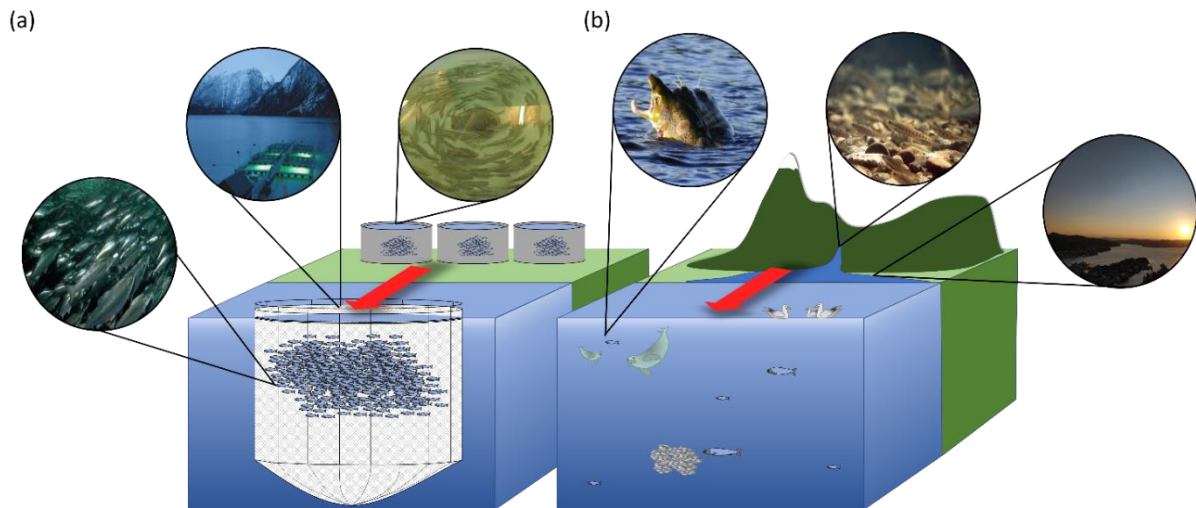
Figure 1.3 Differences in habitat between (a) domesticated Atlantic salmon in aquaculture and (b) wild Atlantic salmon. With emphasis on differences shown in photographs, such as (from left to right) population density (photo credit: WWF), lighting regimes (photo credit: unknown), homogeneous environment (photo credit: William Perry), predation (photo credit: Rob Harris), heterogenous environment (photo credit: U.S. Fish and Wildlife Service) and geographic space (photo credit: William Perry).

Many changes in the biology of Atlantic salmon that have not been directly selected by artificial selection have already been documented, including predator avoidance behaviour (Houde, Fraser and Hutchings, 2010a), sexual morphology (Perry *et al.*, 2019), stress susceptibility (Solberg, Zhang, *et al.*, 2013) and ultimately increased susceptibility to predation (Solberg *et al.*, 2020). It is also possible, however, that not only does the aquaculture come with its own set of passive artificial selection, there may also be trade-offs in order to maximise production traits. One source of these trade-offs could be

hitchhiking-selection, caused by linkage between loci, where strong selection on one locus could reduce gene flow in the region around that locus due to physical linkage (Feder *et al.*, 2012), allowing differentiation from processes such as genetic drift and mutation to accumulate (Via, 2012). Hitchhiking-selection is well studied in relation to sympatric speciation (Malinsky *et al.*, 2015), and the few studies examining domestication induced trait change in vertebrates suggest that a more important contributor to phenotypic change is pleiotropy (Wright *et al.*, 2010). Therefore, strong selection for traits such as growth could also be inducing other phenotypic change due to multiple traits linked with one gene, however, this has not been examined in aquaculture relevant species.

Genetic differentiation caused by domestication has allowed the production of molecular markers to identify wild and domesticated fish. One of the most consistently used molecular markers for parentage are microsatellite markers (Norris, Bradley and Cunningham, 1999; Skaala *et al.*, 2004), although these more traditional markers are now making way for NGS approaches such as SNP-chips, which identify differences at thousands of loci, providing a higher resolution for assessing domesticated introgression into wild populations (Karlsson *et al.*, 2011).

1.4 Aquaculture and escapees

A bourgeoning problem that has been inherent in aquaculture since its creation is the risk of domesticated individuals escaping into the wild, with these individuals referred to as escapees. Escapees from aquaculture have been documented in a variety of species, from aquaculture biocontrol species such as lumpfish (Whittaker, Consuegra and Garcia de Leaniz, 2018) to ornamental species such as topmouth gudgeon (Beyer, 2004). Domesticated fish are released into the wild for a variety of reasons. On one end, hatchery reared fish are used in many systems to supplement local wild stocks, often with negative fitness effects on wild populations (Araki, Cooper and Blouin, 2007; Chilcote, Goodson and Falcy, 2011; Amoroso, Tillotson and Hilborn, 2017). Alternatively, however, there are large scale releases of aquaculture reared fish due to damaged equipment, which can also

have negative fitness effects on wild populations (McGinnity *et al.*, 1997, 2003; Fleming, Hindar, Mjölneröd, *et al.*, 2000; Besnier *et al.*, 2015; Skaala *et al.*, 2019); these effects on wild populations, and the mechanisms behind the reduction in fitness, have been most well studied in Atlantic salmon (Glover *et al.*, 2017). Much of this work is driven by the large number of fish that continue to escape into the waters surrounding Norway and the United Kingdom every year (figure 1.4), along with the chronic under documentation of escapes, which are likely to be 2-4 higher than reported numbers (Skilbrei, Heino and Svåsand, 2015). In addition to the large escapes, which are easier to detect, there are also low-level leakage of domesticated fish from cages; the severity of both can be great (Baskett, Burgess and Waples, 2013), depending on factors such as sexual maturation of the fish escaping.
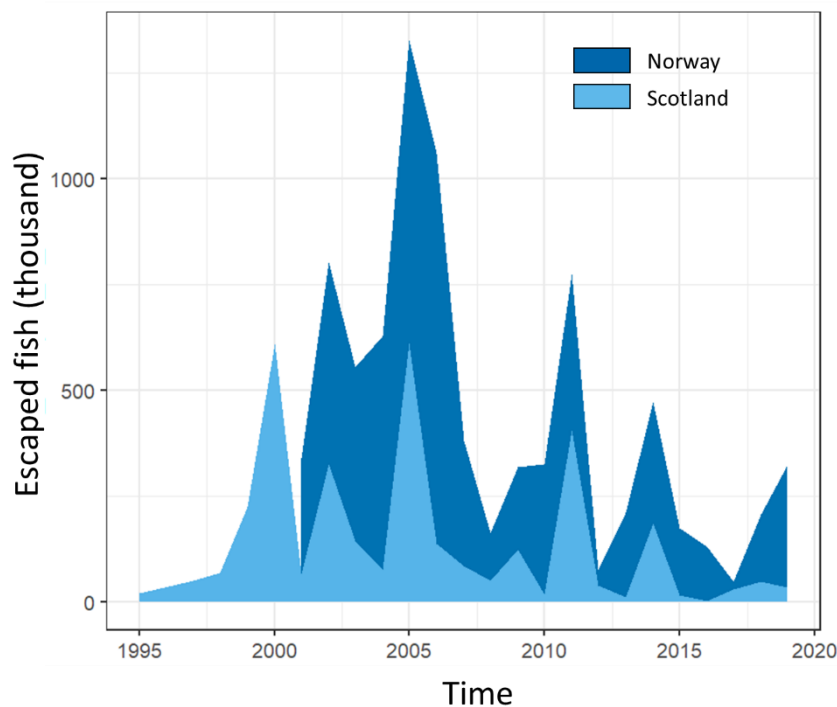
Figure 1.4 Stacked line plot showing the number of reported escapes from farms in Norway *(Directorate of Fisheries, 2020)* and Scotland *(Scotland's aquaculture, 2020)* between 1995 – 2019, for Scotland, and 2001 – *2019* for Norway.

1.5 Hybridisation, fitness and phenotype

1.5.1 Atlantic salmon hybrid fitness

Escaped domesticated Atlantic salmon have an ecological interaction with wild individuals, competing for food, space and mates (Jonsson and Jonsson, 2006a), however, one of the most concerning aspects of domesticated individuals escaping into the wild is the genetic interaction and the process of hybridisation, and genetic introgression. Domesticated and wild individuals can breed and produce fertile offspring, yet there is evidence that these offspring show a reduced fitness when compared to wild counterparts, with no evidence supporting the concept of hybrid vigour populations (McGinnity *et al.*, 1997, 2003; Fleming, Hindar, Mjölneröd, *et al.*, 2000; Besnier *et al.*, 2015; Skaala *et al.*, 2019).

Reduced survival rates in farmed and hybrid individuals in the wild has been documented in all life stages of the Atlantic salmon. Firstly, McGinnity *et al.* (1997), using their experimental system in the Burrishoole, Ireland, showed farmed and first-generation hybrid salmon smolt show reduced survival at the freshwater stage, with the largest reduction in survival seen in second generation hybrids, which has been used as evidence for outbreeding depression. Additionally, farmed parr were also seen to have a reduced fitness compared to wild fish, with hybrids displaying intermediate survival rates. Finally, smolt survival was also seen to be affected, with farmed and farmed x hybrid backcross smolt seen to have lower survival in comparison to wild individuals. In addition to the differences in survival, farmed juveniles grew faster than slow growing wild parr, causing displacement of wild fish, thus potentially reducing wild smolt production (McGinnity *et al.*, 2003). The greatest loss in the survival of farmed and hybrid fish was in the marine environment, where survival was 10 times lower in farmed fish when compared to wild fish; much larger than the 2 times lower survival seen in farmed fish in the river when compared to wild fish (McGinnity *et al.*, 2003; Glover *et al.*, 2017). It should be added, however, that experiments looking at the marine survival were conducted on fish planted in rivers as smolts (after being raised in hatchery conditions), while those experiments conducted on freshwater

survival were conducted on fish planted in rivers as eggs; thus making it difficult to make direct comparisons.

In addition to the Irish experiments, experiments on Norwegian strains of Atlantic salmon have also demonstrated the same patterns of lifetime survival (wild > hybrid > farmed). Additionally, while also demonstrating that farmed fish have a breeding success one third of that of wild fish (Fleming, Hindar, Mjølnerød, *et al.*, 2000), and reduced survival in fish from a farmed background compared to hybrid and wild backgrounds; albeit with variation among families that made up the different backgrounds (Skaala, Glover, *et al.*, 2012a; Skaala *et al.*, 2019). The heritable impact of domestication on the fitness of wild populations is therefore particularly worrying due to its legacy long after an escape has occurred. Introgression may lead to a change in the evolutionary trajectory of wild populations as wild populations are introgressed with fish selected for a different environment and are therefore less fit for a life in the wild. This may change the phenotypic traits in wild populations (Bolstad *et al.*, 2017), and lead to decreased fitness (McGinnity *et al.*, 1997, 2003; Fleming, Hindar, Mjölneröd, *et al.*, 2000; Besnier *et al.*, 2015; Skaala *et al.*, 2019).

1.5.2 Atlantic salmon hybrid phenotypes

The fitness of an individual is based on its phenotype, and how well adapted that phenotype is to the environment it inhabits. Therefore, examining the impact of domestication on phenotype can help us understand the basis of reduced fitness in hybrid individuals. Understanding the variation in phenotypes due to genetic background (domesticated, wild, hybrid etc.) and not due to plasticity and response to environmental cues is vital. Reducing the effect of environmental variation has led to many studies adopting common garden experimental designs, where fish from different genetic backgrounds are reared together in tanks or rivers. A summary of published common garden experiments examining phenotypes between domesticated, wild and hybrid individuals was written by Glover *et al.* (2017a), with an updated graphical summary of these studies outlined in figure 1.4. One important feature highlighted in figure 1.5 is the focus of many studies on body weight, growth,

survival and maturation, due to these traits being most influenced by the artificial selection in aquaculture. This thesis aims to contribute to this body of research, examining aspects such as secondary sexual characters, growth, cardiac performance and external morphology, but also to push this area of research forward, examining novel features with cutting edge techniques; including the application to brain morphology and gut microbiome. By examining these key features, we can better understand the impacts of introgression on lifetime fitness, with secondary sexual characters involved in breeding success, growth, cardiac performance, and the gut microbiome involved in physiology, along with external morphology and brain morphology involved in migration.
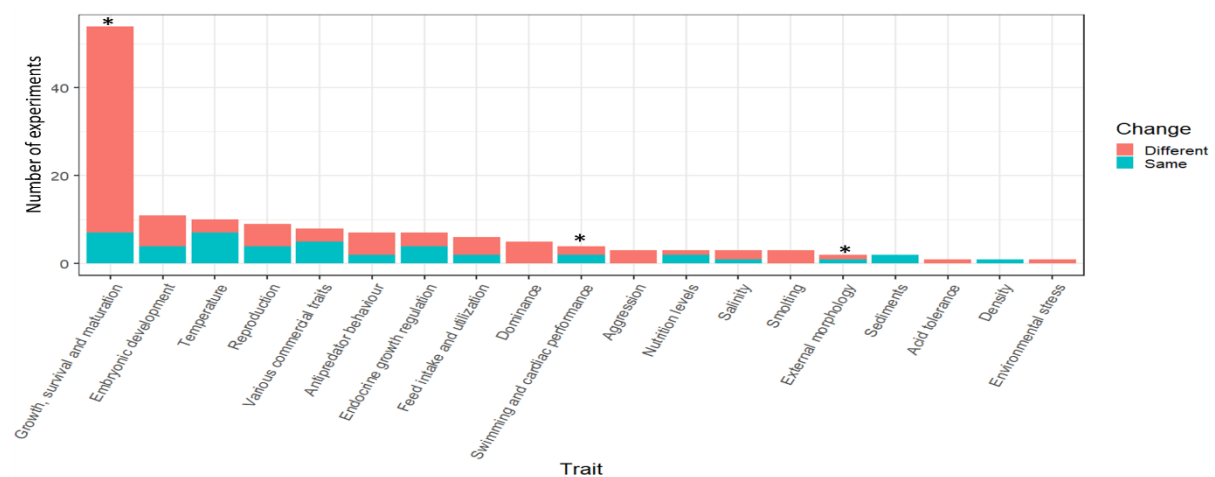
Figure 1.5 Number of experiments examining different traits between domesticated, wild, and in some cased, hybrid, fish in 44 common garden studies. Studies pre-2017 were collated in a previous review (Glover et al., 2017a), but have been updated here. Traits examined in common garden experiments summarised here date from 1997 to 2019, and include phenotypes, behaviour, and the response of traits to experimental manipulation (e.g. environmental stress). Differences or similarities between domesticated and wild fish in different categories of trait are highlighted. Asterisks represent conceptual areas in which this thesis will contribute to, while also adding a new phenotype which has not been examined in a common garden: the gut microbiome and brain morphology. For clarity, trait categories temperature, salinity, sediments, acid tolerance, density and environmental stress refer to plasticity to these abiotic conditions.

1.6 Aims and objectives of this thesis

Here I aim to identify phenotypic changes as a result of domestication in a range of phenotypic traits to better understand the fitness consequences of domesticated individuals in the wild (McGinnity *et al.*, 1997, 2003; Fleming, Hindar, Mjölneröd, *et al.*, 2000; Besnier *et al.*, 2015; Skaala *et al.*, 2019). Phenotypes will be assessed in domesticated, wild, and reciprocal hybrid (domesticated x wild) crosses, as well as in backcross and F2 hybrids in some cases, reared in a common garden, while also considering other factors such as sex and age. The outcomes of this research will provide more evidence on the impact of domestication on evolutionary change in captive reared animals and their progeny, while also providing an understanding of the heritable phenotypic impact of escaped domesticated fish breeding with wild populations. By contrasting phenotypes from salmon of differing genetic backgrounds, this thesis aims to:

1   Assess whether domestication has caused a change in the size of the kype, a secondary sexual trait, in domesticated mature males when compared to wild males, while also examining the influences of age and genetics, in a common garden hatchery (Chapter 2/Paper I: Perry, W.B., Solberg, M.F., Besnier, F., Dyrhovden, L., Matre, I.H., Fjelldal, P.G., Ayllon, F., Creer, S., Llewellyn, M., Taylor, M.I., Carvalho, G. and Glover, K.A, 2019. Evolutionary drivers of kype size in Atlantic salmon (*Salmo salar*): domestication, age and genetics. Royal Society Open Science, 6(4), p.190021. doi.org/10.1098/rsos.190021).

2   Assess whether domestication has caused a change to body shape morphology using geometric morphometrics, along with assessing changes in eye width and pectoral fin length, in both a natural and artificial common garden design (Chapter 3).

3   Assess whether growth is an additive trait, despite recombination, increasing with levels of domestication, and also assessing if domestication has led to detectable changes in heart morphology while also examining the influences of life stage and sex, in a common garden hatchery (Chapter 4/Paper II in review: Perry, W.B., Solberg, M.F., Brodie, C., Medina, A.C., Pillay, K.G., Egerton, A., Harvey, A., Creer, S., Llewellyn, M., Taylor, M.I., Carvalho, G. and Glover, K.A,

2020. Disentangling the effects of environment and genetics in Atlantic salmon: growth, heart and liver under common garden conditions. Scientific Reports).

4   Assess whether domestication has changed the size of brain regions (optic lobe and telencephalon) or changed the morphology of these brain regions (Chapter 5: Getting inside the brain of the Atlantic salmon (*Salmo salar*): examining domestication induced morphological change).

5   Assess whether domestication has changed the diversity of bacterial communities in the gut, in addition to assessing the impact of sex on the diversity of bacterial communities (Chapter 6: Sexual dimorphism in gut bacterial diversity: common garden study in Atlantic salmon (*Salmo salar*)).

Chapter 2: Evolutionary drivers of kype size in Atlantic salmon (*Salmo salar*): domestication, age and genetics

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Abstract

The diversity of reproduction and associated mating patterns in Atlantic salmon (*Salmo salar*) has long captivated evolutionary biologists. *Salmo salar* exhibit strategies involving migration, bold mating behaviours and radical morphological and physiological change. One such radical change is the elongation and curvature of the lower jaw in sexually mature males into a hook-like appendage called

the kype. The kype is a secondary sexual characteristic used in mating hierarchies and a prime candidate for sexual selection. As one of the core global aquaculture fish species, however, mate choice, and thus sexual selection, has been replaced by industrial artificial fertilization seeking to develop more commercially viable strains. Removal of mate choice provides a unique opportunity to examine the kype over successive generations in the absence of sexual selection. Here we use a large-scale common-garden experiment, incorporating six experimental strains (wild, farmed and wild × farmed hybrids), experiencing one to three sea winters, to assess the impact of age and genetic background. After controlling for allometry, fork length-adjusted kype height (AKH) was significantly reduced in the domesticated strain in comparison to two wild strains. Furthermore, genetic variation at a locus on linkage group SSA1 was associated with kype height, and a locus on linkage group SSA23 was associated with fork length-adjusted kype length (AKL). The reduction in fork length-AKH in domesticated salmon suggests that the kype is of importance in mate choice and that it has decreased due to relaxation of sexual selection. Fork length-AKL showed an increase in domesticated individuals, highlighting that it may not be an important cue in mate choice. These results give us insight into the evolutionary significance of the kype, as well as implications of genetic induced phenotypic change caused by domesticated individuals escaping into the natural environment.

2.1 Introduction

Atlantic salmon (*Salmo salar*) are predominantly anadromous salmonid fish that inhabit coldwater streams on both sides of the northern Atlantic during the freshwater stage of their life-cycle. The species is known to display phenotypic variation among individuals and populations, some of which may be adaptive (Garcia de Leaniz *et al.*, 2007). During its life-cycle it undergoes key phenotypic changes manifested in variable morphology (Witten and Hall, 2003), physiology (Prunet *et al.*, 1989), and behaviour (Metcalfe, Huntingford and Thorpe, 1988). Such fundamental biological change is required for survival in both freshwater and marine environments; environments with radically

different abiotic and biotic conditions, ranging from differences in salinity and temperature profiles, to changes in competition with conspecifics. The wealth of life-history variation, both among and within populations (Thorpe *et al.*, 1998), renders Atlantic salmon well suited for investigating the roles of phenotypic plasticity and heritable genetic change in generating variation for maximised reproductive success (Kelly, Panhuis and Stoehr, 2012).

A critical stage in the life-cycle of Atlantic salmon, closely linked with fitness, is spawning. After returning to natal freshwater streams and rivers during the summer and autumn months, females will excavate depressions in the river bed to form nests, where they lay eggs which are simultaneously fertilised by the milt of one or more males (Jones, 1959). Males are able to spawn numerous times in quick succession for up to two months (Jonsson, Jonsson and Hansen, 1991), unlike females who spawn over a more limited time-period, resulting in male-biased operational sex ratios (Fleming, 1996). The disparity between mating males and fertile females generates intense male-male competition, fuelled by the increased fertilization success seen in those males who fertilise eggs first (Mjølnerød *et al.*, 1998).

A phenotypic trait that has been associated with the intense male competition during spawning is the kype, an elongation of the lower jaw forming a hook at the tip (figure 2.1b). Darwin used the male kype in salmon as evidence for the role of sexual traits in natural selection, referencing its defensive use during altercations among spawning males (Darwin, 1859). A century later, Jones (1959) refers again to the kype as a weapon for defence, likening the structure to the antlers of a stag, adding that bodily harm is rarely seen, with most conspecific altercations resolved with agonistic displays. Since these early descriptions, some novel discoveries surrounding the kype have been made (Järvi, 1990; Witten and Hall, 2003), though the fundamental understanding of its purpose has remained mostly true to the definition put forward by Darwin. Behavioural experiments in salmonids have revealed some evidence that the kype is used in both intra and inter-sexual interactions, with correlations seen between kype size and 1) increased rank within local male dominance hierarchies and 2) increased

female mate choice (Järvi, 1990; Fleming, 1996). However, due to issues of correlation with other key characteristics associated with female mate choice, such as body size, these results are not only inconclusive, they are difficult to disentangle in behavioural experiments. Other experimental approaches are required to contribute to our understanding of the significance of the kype in Atlantic salmon breeding systems, as well as its evolution.
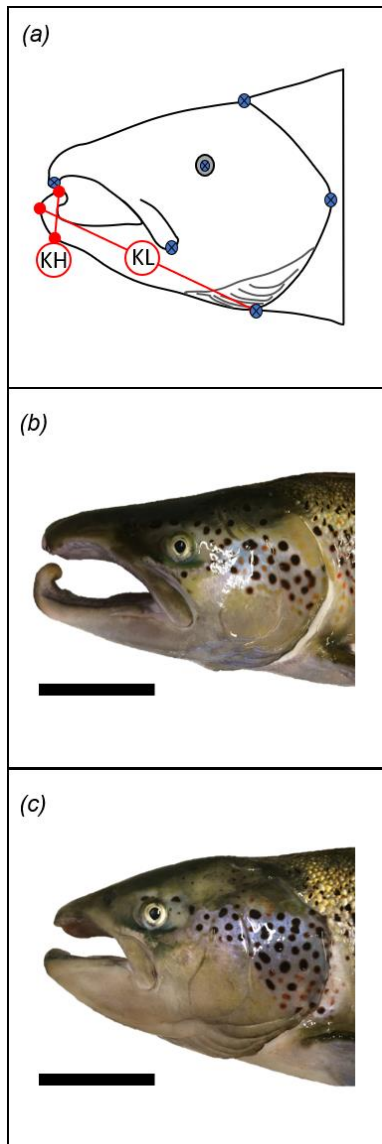
Figure 2.1 (a) Landmarks (blue crosshair) used for the geometric morphometric analysis, kype length (KL) and kype height (KH); in addition to examples demonstrating variation in head morphology, including a mature male showing an elongated kype (b), a mature male with a reduced kype (c) (both mature males are 2SW). Scale bars represent 10cm.

Since the early 1970's, and for more than 12 generations, Atlantic salmon have been subject to domestication and directional selection for economically important traits. As a result, domesticated salmon now display a contrasting array of genetic and phenotypic differences to wild Atlantic salmon (Glover *et al.*, 2017). Salmon breeding programs operate by selection of individuals according to their breeding values, manual stripping of gametes, and thereafter controlled fertilisation for production of families for the next generation of selection. In effect, this practise removes all opportunity for spawning competition and mate choice, and thus renders development of secondary sexual characteristics involved in mate choice or sexual success, potentially redundant (Fleming and Gross, 1989; Petersson and Järvi, 1993; Petersson *et al.*, 1996). Therefore, studying domesticated and wild salmon under controlled conditions may yield insights into the evolutionary significance of head morphology and the kype in Atlantic salmon, as well as wider impacts of domestication.

The few studies examining the kype of male Atlantic salmon and its role in reproduction have focused on behaviour in wild individuals (Jones and King, 1949; Järvi, 1990). While studies in the wild are informative, opportunities that examine the impact of exposure to vastly different selection regimes in captivity, offer an experimental framework for environmental and genetic manipulation under controlled conditions. Such empirical data are of additional interest in the context of domesticated escapees, that typically display a lower spawning success than wild salmon (Fleming *et al.*, 1996; Fleming, Hindar, Mjölneröd, *et al.*, 2000). It remains unclear, however, the degree to which the lower domesticated male spawning success results from phenotypic limitations arising from shifts in head morphology and kype characteristics.

Common-garden experiments, whereby individuals of differing genetic background are reared under identical conditions, can elucidate the degree of genetic influence on traits of interest. Such studies are common in Atlantic salmon, revealing, among other things, differences between domesticated and wild salmon in traits of evolutionary significance such as growth (Solberg, Skaala, *et al.*, 2013; Harvey, Solberg, Troianou, *et al.*, 2016), survival in the wild (McGinnity *et al.*, 1997; Skaala, Kevin A.

Glover, *et al.*, 2012) and precocious male maturation (Debes and Hutchings, 2014). Here, we utilise a pedigree-controlled multi-generation population of domesticated, hybrid and wild Atlantic salmon, alongside a quantitative trait loci analysis, to explore shifts in sexually selected traits influenced by domestication. We implemented both classical and geometric morphometrics on 528 sexually mature adult male Atlantic salmon, of varying age (experiencing 1, 2 and 3 sea winters), to investigate whether domestication, and its associated relaxation of sexual selection, has led to detectable changes in head morphology in ~12 generations.

2.2 Materials and methods

2.2.1 Fish

A range of common-garden experiments on domesticated, hybrid and wild Atlantic salmon have been conducted at the Institute of Marine Research (IMR) for more than a decade (e.g. (Glover, Otterå, *et al.*, 2009; Solberg, Skaala, *et al.*, 2013; Bicskei *et al.*, 2016; Harvey, Glover, *et al.*, 2016)). In this process, a large pedigree-based population of Atlantic salmon consisting of fish originating from multiple wild populations and domesticated strains, including respective hybrids and back-crosses, has been established. Individuals from different backgrounds are reared together under standard farming conditions (i.e., in the same tanks in freshwater, and in the same sea cages for the marine stage), where strains are mixed from the eyed-egg stage onwards. Fish are then allowed to mature naturally under a natural day-length for Bergen at age 1, 2 or 3+ sea winters (SW), all of which smoltified at age 1 (thus total age = SW age +1).

In the present study, we used sexually mature adult males, of which the genetic sex of all individuals was validated by a DNA probe based reverse transcription PCR (RTPCR) presence absence assay (Thermo Fisher Scientific, USA) aimed to detect the presence of the male specific *sdY* gene (Yano *et al.*, 2013; Eisbrenner *et al.*, 2014). The fish originated from the first generation of the main domesticated-wild population established at IMR in 2011, and includes fish from three wild

populations, a domesticated strain and a domesticated-wild F1 hybrid strain (table 2.1). Artificial fertilisation of gametes took place on arrival at the IMR Matre Research station. For further details regarding the parental lines, rivers of origin, and production of these fish see Solberg et al. (2014) (information on the cohort produced in 2012). Fish were reared under the experimental protocol (ID 5296) that was approved by the Norwegian Animal Research Authority (NARA). Procedures included DNA identification and subsequent PIT tagging of all individuals for identification. Upon termination, fish were sedated using Aqui-S, then killed using an anaesthetic overdose of MS-222 and bled by cutting one of the gills. Those working directly with the experimental animals had also undergone Norwegian Food Safety Authority (NFSA) training, as is required with experimentation involving animals that are included in the Norwegian Animal Welfare Act (2010).

Table 2.1 Background of experimental fish, including strains, geographic origin of wild strains, number of families comprising a strain and number of individuals. Numbers inside brackets represent individuals used in kype height analysis, numbers outside the brackets represent individuals used in kype length analysis.

Sex	Type	Origin	One sea winter families (n)	One sea winter individuals (n)	Two sea winter families (n)	Two sea winter individuals (n)	Three sea winter families (n)	Three sea winter individuals (n)
Male	Farmed	Mowi	**5 (4)**	14 (12)	**4 (4)**	16 (12)	**4 (4)**	14 (14)
	Hybrid	Figgjo (♀) x Mowi (♂)	**6 (6)**	95 (91)	**5 (4)**	8 (7)	**5 (4)**	13 (11)
		Mowi (♀) x Figgjo (♂)	**7 (7)**	91 (85)	**6 (4)**	18 (11)	**4 (4)**	4 (4)
	Wild	Arna	60°42´N, 5°46´E	**6 (6)**	63 (62)	**4 (4)**	11 (10)	**3 (3)**	7 (6)
		Figgjo	58°81´N, 5°55´E	**6 (6)**	64 (61)	**6 (5)**	8 (6)	**0**	0
		Vosso	60°64´N, 5°95´E	**7 (7)**	70 (67)	**6 (6)**	20 (12)	**5 (4)**	12 (9)
	Total		**37 (36)**	397 (378)	**31 (27)**	81 (58)	**21 (19)**	50 (44)
Female	Farmed	Mowi			**0**	0		
	Hybrid	Mowi (♀) x Figgjo (♂)			**7**	32		
		Figgjo (♀) x Mowi (♂)			**5**	14		
	Wild	Arna			**3**	13		
		Figgjo			**4**	11		
		Vosso			**2**	4		
		Unknown			**-**	3		
	Total				**21**	77		

Males: established on 16th & 22nd-23rd November 2011. Hatched in Spring 2012. Individuals maturing as one-sea-winter, two-sea-winter and three-sea-winter were terminated on 27th January 2015, 18th January 2016 and 17th January 2017, respectively.
Females: siblings to males. Terminated when ripe (November – January 2016).

2.2.2 Data collection

Photographs of the lateral side of 1, 2 and 3 SW mature male salmon were taken at the end of the spawning season (i.e. January) in 2015, 2016 and 2017 (table 1) using a mounted digital reflex camera and measurement board. Mature female fish, used as an outlier group for the geometric morphometrics analyses, were terminated and photographed when they were ripe through the course of the spawning season in the winter of 2014/2015, thus having completed two sea winters. Milt weight, fork length (most anterior point of the head to the of the middle caudal fin rays) and total wet weight of the mature adult males were also taken during sampling. Milt weight and total wet weight were log10 transformed and used in a linear regression to calculate gonadosomatic residuals (GSR). Internal PIT tags were scanned, which allowed for the pedigree of the fish to be unequivocally identified. Photographs were filtered by technical quality before the application of landmarks and before the individual fish was identified, allowing for the unbiased removal of images. After filtering, the final mature male data set used for both kype length and geometric morphometrics included 397 1SW, 81 2SW and 50 3SW males from three wild populations, a domesticated strain, and a reciprocal F1 hybrid population (a total of 37 families represented in the pedigree) (table 2.1). The mature male photographs were also supplemented by 77 2SW mature females for use in the geometric morphometric analysis, represented by 21 families from the same origins as the males. After the removal of photographs in which the kype was obscured (closed mouths) a subset of individuals were used for kype height comparisons, comprising 378 1SW, 58 2SW and 44 3SW males. All subsequent analysis of photographs was undertaken without prior knowledge of the genetic background of the fish. Additionally, the sequence in which photographs were analysed within sea winters was ordered randomly.

2.2.3 Geometric morphometrics

Positioning of landmarks for the geometric morphometric analysis was based on identifiable external features related to skeletal form of the head (Tchernavin, 1938; Hard *et al.*, 2000), many of which have been outlined in previous literature documenting both salmonid, and other fish morphology. These landmarks (*n* = 6) included: the apex of the upper jaw, the most dorsal (Winans and Nishioka, 1987) and ventral positions of the gill plate, the most posterior point of the gill plate, the maxillary bone (Winans and Nishioka, 1987) and the eye (figure 2.1a). Although of interest, landmarks positioned on the kype were not used in the geometric morphometric analysis due to the influence of mouth opening on head shape. Landmarks were applied by the same observer, using tpsDig version 2.28 (Rohlf, 2016).

Landmark data were analysed using the R package geomorph 2.0 (Adams and Otárola-Castillo, 2013). Generalised Procrustes analysis (GPA) was conducted on landmark data to limit the effect of scale, orientation, and translation between images. Aligned Procrustes coordinates for all landmarks in all individuals were then verified by plotting points around the mean value (figure A2.1). A principle component analysis (PCA) was then conducted on the transformed coordinates for 1SW, 2SW and 3SW individuals, together with 95 % confidence ellipses around respective sea winters, assuming a multivariate t-distribution. All statistical analyses was carried out in R version 3.3.3 (R Core Team, 2017).

2.2.4 Linear measurements

Linear length measurement of the lower jaw (referred to here as kype length), as well as length of the hook forming at the tip of the lower jaw (referred to here as kype height), were taken – both of which comprise the characteristic kype (figure 2.1a). Length of the lower jaw was taken from the most anterior position of the lower jaw to the bottom of the gill plate. Kype height was taken from the most dorsal peak of the hook to the ventral position on the lower jaw where curvature began, as used in previous studies (Järvi, 1990; Petersson and Järvi, 1993; Haugland *et al.*, 2011). If no clear curvature

was found on the lower jaw, the point directly beneath the dorsal peak was used. Lengths were calculated from landmarks placed at these positions.

Single linear regressions were conducted to assess the relationship between fork length and kype length, as well between kype height and fork length, all of which were log10 transformed. The residuals from the regression were used to produce a fork length adjusted kype length (AKL), and a fork length adjusted kype height (AKH).

To assess factors influencing AKL and AKH, two mixed effect models (LME) were constructed using the R package 'lme4' (Bates *et al.*, 2015), one for each response variable. The full models contained the fixed factors: sea winter, strain and GSR, as well as their two-way interactions, with the random intercept factors: family (nested within strain type, to control for the hierarchical structure of the data), sire and dam. The 'step' function within 'lme4' was then used to select the best fitting model through automatic backward elimination, allowing for the removal of fixed terms and random factors which did not contribute to the model.

Analysis of variance type III sum of squares with Satterthwaite approximation for degrees of freedom allowed for the generation of p values between factors in mixed effect models, using the package 'lmerTest' (Kuznetsova, Brockhoff and Christensen, 2017). Sire was added as a random effect to the best fitting model for AKL to calculate with Satterthwaite approximation for degrees of freedom using 'lmerTest', but was removed as a random effect for all other aspects of the analysis. Estimated marginal means and pairwise comparisons between means were calculated using the selected models and the R package 'emmeans' (Lenth, Love and Maintainer, 2018). A Tukey's multiple comparisons test was used to adjust P values, and Kenward-Roger approximations were used to estimate degrees of freedom.

2.2.5 Quantitative Trait Loci analysis (QTL)

All individuals with phenotype measurements, and their parents, were genotyped with 109 SNP markers evenly distributed on the 29 chromosomes of the salmon genome (Besnier *et al.*, 2015). The identity by descent (IBD) relation between offspring was estimated by using both the genotype and pedigree information (Pong-Wong *et al.*, 2001). A Hierarchical Generalized Linear Model (HGLM) (Alam, Rönnegård and Shen, 2015) was then fitted, at each locus, to test for correlation between kype measurement and genotype. $y = XB + Ga + e$ (model 0), $y = XB + Ga + Zq + e$ (model 1), where X is the model matrix for fixed effects (tank, strain and sea winter), B the vector of fixed effect, G the kinship matrix, a the vector of polygenic effects, Z the locus specific IBD matrix, q the vector of QTL effect and e the residuals. To test for a genotype-phenotype correlation at each locus, the likelihood of the model without QTL effect (model 0) and with QTL effect (model 1) were compared in a likelihood ratio test. Each model was fitted in R using the HGLM package (Alam, Rönnegård and Shen, 2015).

2.3 Results

2.3.1 Overview

Kype length, kype height (figure 2.1a) and whole-fish fork length were collected from a total of 528 males originating from six experimental strains that had been reared together from the eyed egg stage until maturity at 1SW-3SW. To control for allometry, kype length and kype height were adjusted for fork length (after being log10 transformed), and the residuals generated from an ordinary least squares linear regression between the two variables were used to produce fork length-adjusted kype length (AKL) and kype height (AKH). Sexing of the fish revealed that 3 individuals used in the study were genetically female, despite these individuals producing a considerable weight of milt (milt weight range: $50.9 - 232.4$ g). The three individuals belonged to three different strains (domesticated, wild and hybrid) and were kept in the analysis after visually assessing them for female phenotypic traits.

2.3.2. Kype length and height

The observed raw kype length and height both showed marked variation within the whole dataset containing all sea winters and all strains, ranging between 5.2 – 28.1 cm (raw kype length) and 0.2 – 6.1 cm (raw kype height). Variation in kype height and kype length was also evident from looking at individual photographs, even within sea winters (figure 2.1b & c), and has been documented in previous studies (Fjelldal $et\ al.$, 2018). From raw data it was also clear that variation existed among strains, both in terms of kype length and height (figure 2.2). Kype length and height were, however, highly correlated with fork length (R^2 = 0.86, $F(1,526)$ = 3284, p <0.01, R^2 = 0.78, $F(1,478)$ = 1680, p <0.01 respectively) (figure 2.3), a correlation that was even stronger when values were log10 transformed (R^2 = 0.87, $F(1,526)$ = 3490, p <0.01, R^2 = 0.81, $F(1,478)$ = 2036, p <0.01 respectively). Residuals from the log-transformed regressions were used as a fork length-adjusted kype length (AKL), and fork length adjusted kype height (AKH).
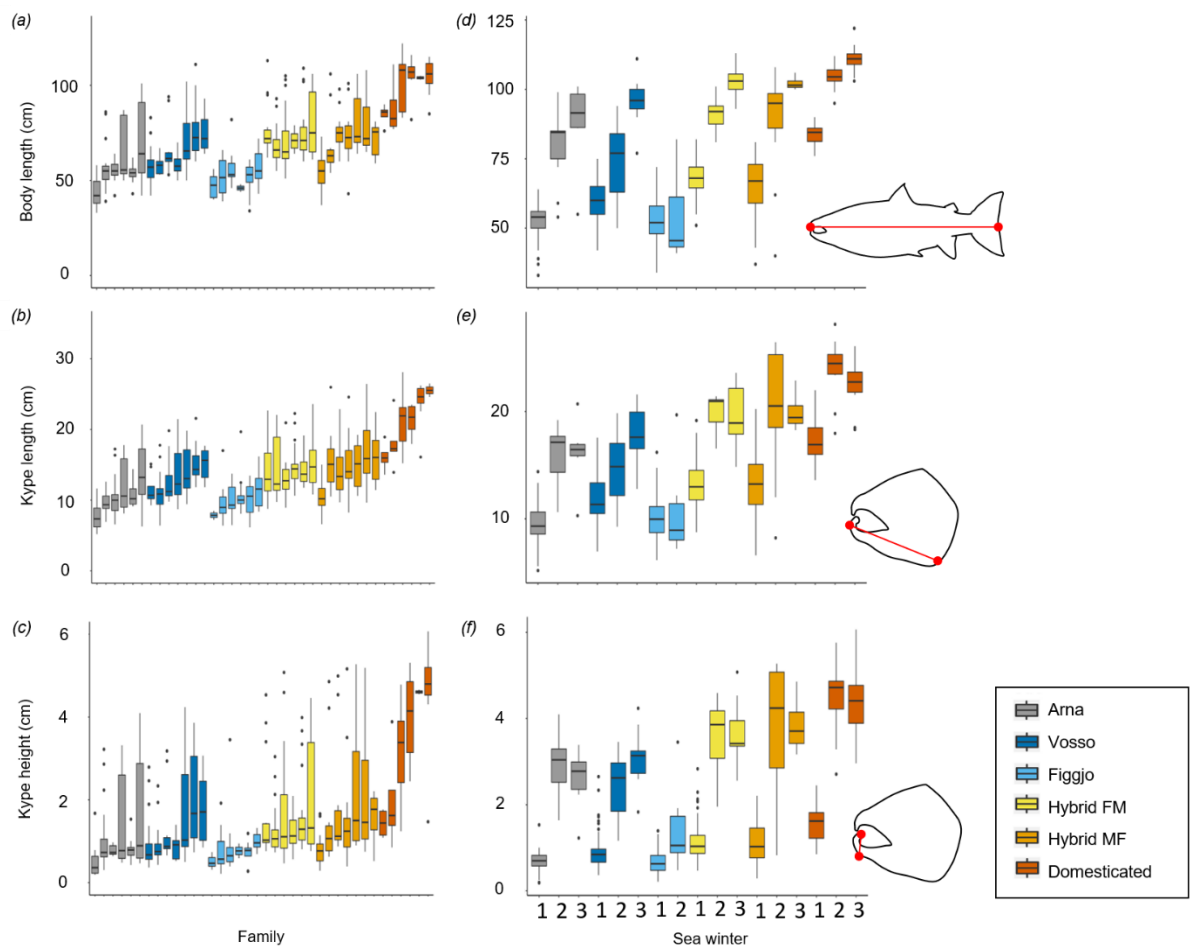
Figure 2.2 Boxplot of observed variation in fork length, kype length and kype height broken down by family (a-c) and sea winter (d-f). Strains consist of wild (Arna, Vosso and Figgjo), hybrid (hybrid Figgjo x Mowi and hybrid Mowi x Figgjo) and domesticated genetic backgrounds.
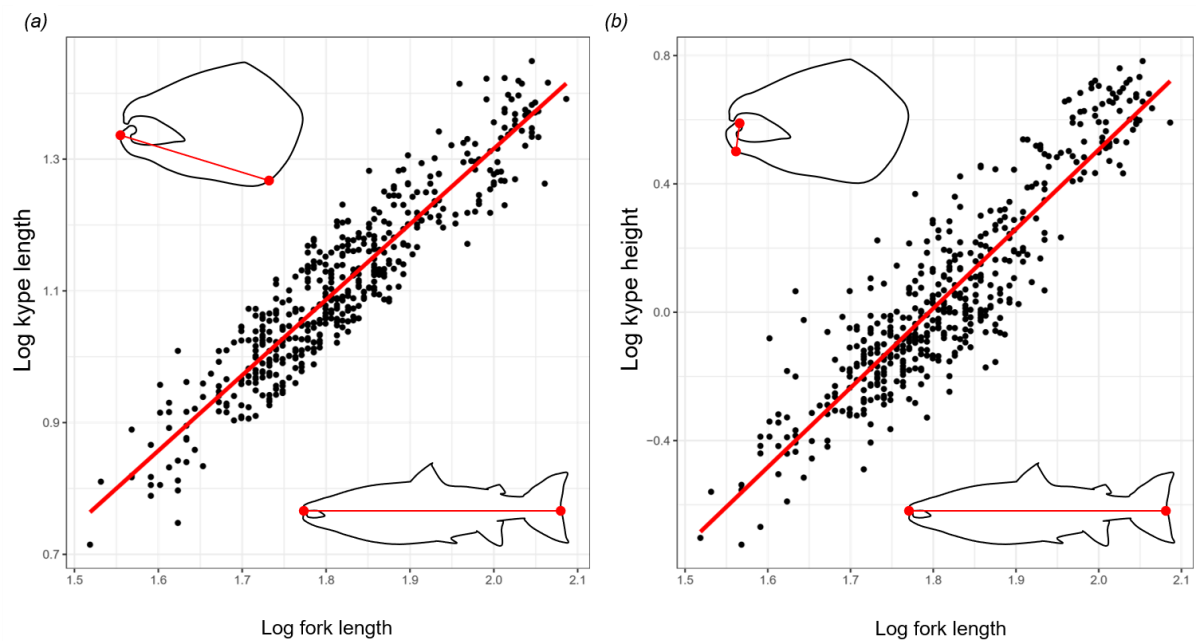
Figure 2.3 Linear regression between log10 fork length and log10 kype length/height, including individuals from all sea winters and strains. Residuals from these regressions were used for fork length adjusted kype length (AKL) and fork length adjusted kype height (AKH).

For adjusted kype length (AKL), the selected model contained terms: strain and SW. AKL showed significant differences among strains (LME Strain: $F_{5,52}$ = 4.37, Sum Sq = 0.050, P < 0.01) (figure 2.4b). The difference in AKL among strains was driven by the significantly larger AKL in the domesticated strain (estimated mean = 0.012) when compared to Arna (estimated mean = - 0.022, t(63) = 3.29, P = 0.02), as well as the significantly larger AKL seen in the hybrid MF strain (Mowi ♀ x Figgjo ♂) (estimated mean = 0.0057, t(30) = 3.30, P = 0.03), when compared to Arna. No other significant effects (P < 0.05) in AKL were found within the pairwise comparisons among strains (table A2.1). AKL also varied among sea winters (LME Sea winter: $F_{2,518}$ = 17.16, Sum Sq = 0.08, P < 0.01) (figure 2.4a), with AKL peaking at 2SW (estimated mean = 0.018), after displaying an increase from 1SW (estimated mean = 0.002), t(520) = 2.66, P = 0.02). A large significant decrease in AKL was also detected between 2SW and 3SW (estimated mean = - 0.032) (t(519) = 5.82, P < 0.01).
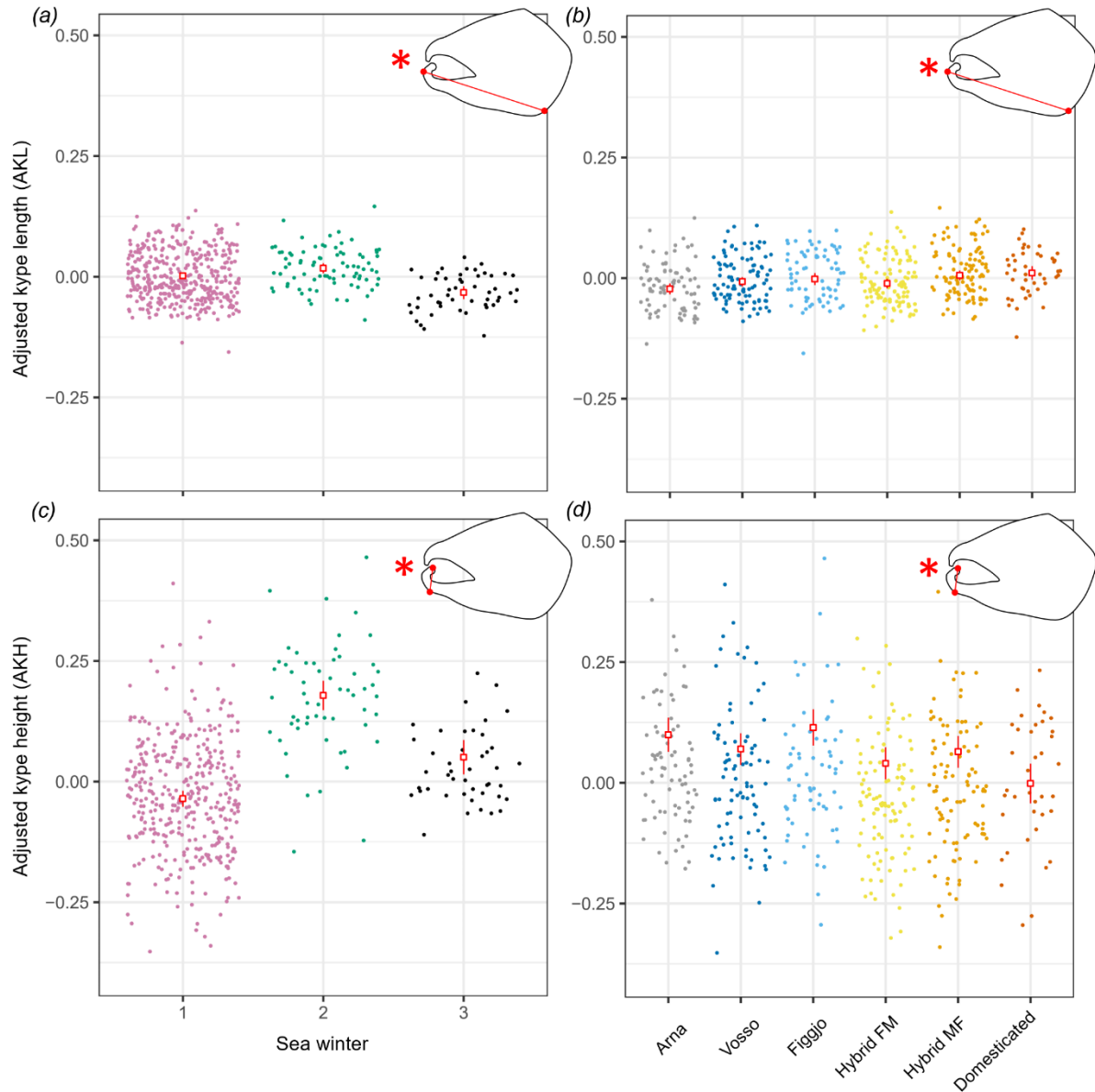
Figure 2.4 Fork length adjusted kype length (AKL) (a-b) and fork length adjusted kype height (AKH) (c-d) broken down by sea winter and strain. Red asterisks represent a significant effect (P ≤ 0.01) of the factor displayed on the x axis for AKL or AKH, as shown from the linear mixed effect model.

For adjusted kype height (AKH), the selected model contained the fixed effect terms strain and SW, as well as sire as a random factor. Strain had a significant effect on AKH (LME Strain: $F_{5,49}$ = 4.78, Sum Sq = 0.282, P < 0.01) with the domesticated strain (estimated mean = - 0.001) showing a decrease in mean AKH when compared to the wild strains Arna (estimated mean = 0.100, t(60) = 3.74, P < 0.01) and

Figgjo (estimated mean = 0.115, t(76) = 4.16, P < 0.01). A reduction in AKH between the domesticated strain and the wild Vosso strain was also detected, although this did not show strong significance (mean = 0.070, t(62) = 2.71, P = 0.09). Finally, a significant reduction in AKH was also seen between Figgjo and the hybrid FM strain (Figgjo ♀ x Mowi ♂) (estimated mean = 0.040, t(32) = 4.16, P < 0.01). No other significant effects (P < 0.05) were found within the pairwise comparisons among strains (table A2.1). A significant effect of sea winter on AKH was observed (LME Strain: $F_{2,469}$ = 91.85, Sum Sq = 2.17, P < 0.01), with an increase seen between 1SW (estimated mean = - 0.036) and 2SW (estimated mean = 0.179) (t(470) = 13.36, P < 0.01), with 2SW having the highest AKH estimated mean, with a decrease in 3SW (estimated mean = 0.051) when compared to 2SW (t(467) = 5.76, P < 0.01).

2.3.3 Geometric morphometrics

There was visible variation in male head shape (figure 2.1b&c) with some individuals showing shorter kype height, a feature shared with female head morphology. The results shown by the geometric morphometrics, however, showed no overlap between female and male head shape (figure 2.5). The lack of overlap in head shape between these groups indicates that while some males exhibit a reduced kype height, this morphological characteristic alone does not constitute female head morphology.
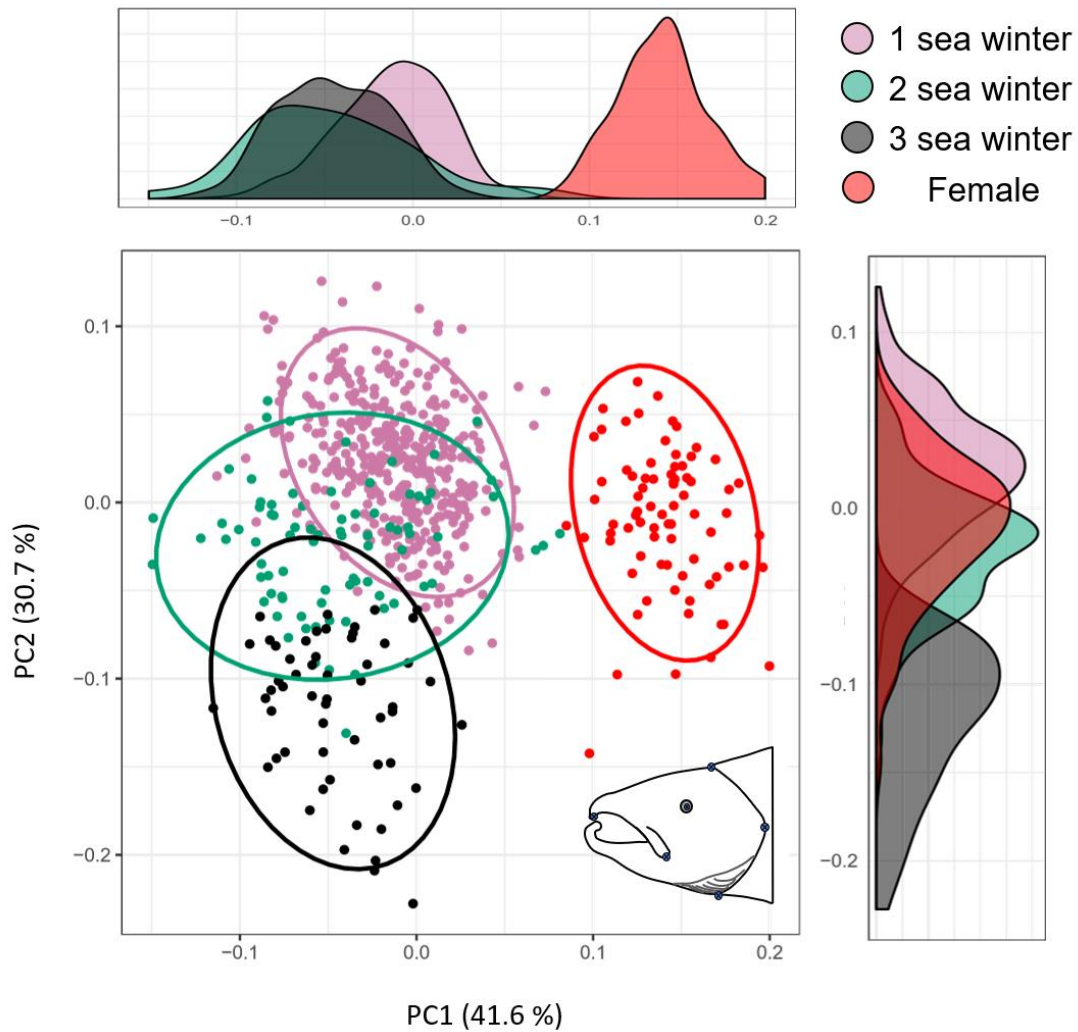
Figure 2.5 Principle component plot summarising the greatest variance in morphospace of salmon head morphology, as represented by the 6 landmarks outlined in figure 2.1a. Groups are split into 1SW-3SW mature males, and 2SW mature females. Groups are eclipsed by 95 % confidence intervals. Principle coordinate density plots have also been included on the x and y axes to better illustrate distribution between groups.

Influence of sea winter on head shape was seen in the landmark based PCA plot, where a predominant difference in clustering can be seen between individuals belonging to 1SW/2SW and 3SW (figure 2.5). Differences in head shape are smaller between individuals from 1SW and 2SW, with distribution

ellipses showing a larger proportion of overlap. Head shape was also assessed using the same methods among strains, however, no clustering was identified (figure A2.2). The differences in male head shape between sea winters was summarised along PC1 (Proportion of variance = 46.5 %) and was characterised by a dorsal shift of features such as the eye, posterior point of the gill plate and the maxillary bone (figure A2.3).

2.3.4 Quantitative Trait Loci analysis (QTL)

Scanning the genome for regions correlated with kype length did not return any significant QTL, though the scan for adjusted kype length (AKL) revealed one QTL on linkage group SSA23 (figure A2.4a). This QTL is for a large part caused by a strong correlation between AKL and the SNP haplotype of each offspring from parent F8. The AKL value of offspring inheriting haplotype 1 and haplotype 2 from F8 was (mean ± sd) -0.03 ± 0.05 and 0.02 ± 0.04, respectively (t = -3-3 df = 37 p= 0.002). Estimated relative proportion of QTL variance attributed to AKL from the Hierarchical Generalized Linear Model (HGLM) was 5.0 %.

The QTL scan for kype height (KH) returned one locus on linkage group SSA1 (figure A2.4b). The correlation between KH and SSA1 was strong among the offspring of parents A9 which displayed significant differences in KH values depending on which parental allele was inherited. The observed HK values were respectively 2.35 ± 1.31 and 0.66 ± 0.16 (t = 3.8, df = 8 p= 0.004) for the offspring that inherited haplotype 1 and haplotype 2 from parent A9. This QTL was, however, non-significant for adjusted kype height (AKH). No genomic regions were significantly associated with AKH in our study. Estimated relative proportion of QTL variance attributed to KH from the HGLM was 6.6 %.

2.4 Discussion

To our knowledge, this is the first study to investigate the potential influence of domestication on head morphology in sexually mature adult male Atlantic salmon where sexual selection has been relaxed compared to the wild. Since the early 1970´s, farmed Atlantic salmon have undergone >12 generations of directional selection for both economically important traits, as well as traits attributed to intrinsic selection pressures of the aquaculture environment, all in addition to the relaxation of natural selection (Gjedrem, 2010a; Glover *et al.*, 2017). We therefore hypothesised that as domesticated salmon have not been exposed to sexual selection since the founding individuals were taken into fish farms more than 12 generations ago, changes in head morphology and in particular the kype, suggested from previous studies to be linked with male reproductive success (Järvi, 1990; Haugland *et al.*, 2011), was likely. After analysing 528 mature male salmon from multiple wild, hybrid and a domesticated strain, all of which had been reared under identical conditions from hatching onwards, we found small yet significant differences in fork length adjusted kype height and length among the strains investigated. The domesticated strain displayed a reduction in fork length adjusted kype height, both compared to the F1 hybrid strains and the two of the wild strains. The reduced kype height trait identified here was not female mimicry, as highlighted by the geometric morphometric analysis, which showed clear head shape separation between female and male fish of all ages and strains. We also found a significant increase in fork length adjusted kype length in the domesticated and hybrid MF strain when compared to the wild Arna strain, suggesting that the length of the lower jaw, or kype length here, is not an important feature in sexual selection. Collectively, our findings suggest that the relaxation of sexual-selection during nearly fifty years selective breeding has driven shifts in kype structure in domesticated Atlantic salmon.

2.4.1 Domestication, sexual selection, and the kype

Earlier studies have strongly indicated that the male kype is important for the reproductive success of adult male Atlantic salmon in the wild (Järvi, 1990), although never independent of other correlative effects (Fleming, 1996). In salmon aquaculture, breeding programs circumvent the potential evolutionary significance of any sexual characteristics as fish are paired after manual stripping of gametes, according to the human-determined breeding value of individual fish. Thus, we predict the loss of female selection and male-male competition, though with continuation of strong trait selection and increased performance under aquaculture conditions will impact the development of sexual characteristics. Resource allocation prioritises traits directly or inadvertently selected for such as fast growth, delayed maturation, high survival, and tempered stress response (Gjedrem, 2010a). Similar trade-offs have been observed in other captively bred salmonids, with reduced kype lengths seen in hatchery reared female Pacific salmon (*Oncorhynchus kisutch*) (Fleming and Gross, 1989) and, to a lesser extent, ranched male sea-trout (*Salmo trutta*) (Petersson and Järvi, 1993), when compared to wild counterparts. The results of the current study support the hypothesis of reduced secondary sexual characters under artificial breeding, in concordance with the previous studies, whereby domesticated individuals exhibit significantly reduced AKH in comparison to two out of three wild strains. It also provides further evidence of the kype's importance in sexual selection, specifically kype height, and the likely energetic cost of producing a larger kype height (Witten and Hall, 2003; Haugland *et al.*, 2011), which has resulted in its reduction within an aquaculture setting.

The domesticated strain used here, Mowi, was founded in part by individuals from the river Vosso. Therefore, the reduction in AKH in the domesticated strain when compared with the Vosso strain was of particular interest, even though not significant ($P > 0.05$). It is noteworthy that other wild populations have also contributed to the Mowi strain, and not exclusively Vosso.

A significant increase in AKL was observed between both the domesticated and hybrid MF strains when compared to the wild Arna strain, suggesting that either AKL is not important in sexual selection,

or it is indirectly selected for in the domestication process. For example, kype length could be more important in male-male competition, rather than in direct female mate choice. Male-male competition may still be present in aquaculture with individuals competing for feed (Kadri *et al.*, 1996) instead of females; therefore, kype length could still be selected for, while kype height would not. Additionally, there could also be possible genetic linkage between traits under selection in aquaculture (e.g. fork length) and kype length. What must also be highlighted, however, is that the prediction of significantly smaller AKL in wild strains was only significant between Arna, and not Vosso or Figgjo when compared to the domesticated strain.

It is also possible that as we detected a very strong relationship between kype length and fork length, with less variance than the relationship between kype height and fork length, this could explain why the domesticated strain has significantly larger AKL than wild strains, even after adjustment for fork length; highlighting the difficulty in removing body size effect in morphological characters. Overall, it suggests that kype length, as described here, is not important in sexual selection.

2.4.2 Genetic basis of the kype

The results indicated the presence of loci that control kype length independently from fork length (a true head morphology QTL), whereas kype height is associated only with a QTL when the measure is not adjusted to fork length. Such an observation indicates that SSA1, which is associated with KH, is likely to be a QTL for body size, rather than for kype production. It is also important to consider that the power to detect QTLs is marginal in this study, and therefore, the fact we do not detect any QTL for AKH does not mean there are no genetic factors controlling kype height. For a QTL to be detected, the following conditions have to be met: 1) the SNP genotype has to be informative within a given family so we can trace back each F1 allele to the parental alleles. 2) the difference in phenotype produced by the two alleles must be significant.

Despite the conflicting results between the QTL for KL and KH, adjusted or otherwise, the QTLs that were identified here demonstrate potential genomic regions for genetic control of kype variability.

Further study into such QTL regions in wild-domesticated experiments would be the next step in understanding the evolutionary history of the kype.

2.4.3 Peak age in sexually mature males

Both the AKL and AKH were significantly larger in 2SW individuals, when compared to 1SW and 3SW individuals, with no significant interaction term between sea winter and strain. Such a trend demonstrates that the increased AKL and AKH seen in 2SW is a general feature of all strains. If a larger kype height is associated with improved reproductive success, as has been suggested in previous studies (Järvi, 1990), this could suggest that mature males reach their maximum physical attractiveness to females in their second sea winter, with this then declining significantly as they enter their third sea winter. It could also be indicative of other life history strategies. As males reach peak body size at the 3SW stage, it is possible that these individuals no longer need to invest as heavily in secondary sexual traits, simply due to their larger size compared to younger males; this would assume that kype size is of secondary importance, after body size, in competing for females, if the males are an order of magnitude larger than their conspecifics. Likewise, 1SW males are going to have the smallest body size, having spent less time at sea, which will also have reduced their risk of mortality at sea, perhaps corresponding to a low-risk low-investment strategy on spawning grounds; depending instead on chance matings rather than competition. Leaving 2SW males as generalists that have to invest in the kype to compete with the larger males, as well as an inability to adopt more chance matings due to their size.

2.4.4 Ecological implications and further research

With the rapid increase in Atlantic salmon in aquaculture, starting in the 1970s, there has been a greater proportion of domesticated individuals escaping into the wild. Research into the impact of these escapees on wild populations first started in the 1990's (Gausen and Moen, 1991) but has continued consistently since (Glover *et al.*, 2017). There is now strong evidence demonstrating that spawning success in domesticated individuals is lower than that of wild individuals, with domesticated

males showing disproportionately lower spawning success than domesticated females (Fleming *et al.*, 1996; Fleming, Hindar, Mjölneröd, *et al.*, 2000; Weir *et al.*, 2004). The reduction in breeding success seen in domesticated males, more so than domesticated females, is due to a reduction in courtship with wild females, which has been attributed to inappropriate mating behaviour (Fleming, Hindar, Mjölneröd, *et al.*, 2000). Results here indicate, however, that domesticated individuals may not only be disadvantaged due to shifts in behaviour as shown in the literature, but also by the size of their kype. Moreover, genetic control over kype height is additive, as shown by the reduction in kype height in the hybrid FM strain (Figgjo ♀ x Mowi ♂), with potential reductions in breeding success in populations with high levels of introgression. Successful spawning between wild males and farmed females during the breeding season is typically a small proportion of spawning individuals, as mentioned previously, and so hybrids such as the hybrid FM strain (Figgjo ♀ x Mowi ♂) here would be in the minority.

To elucidated the determinants and dynamics of observed reductions in fitness in domesticated individuals through disrupted mating strategies, kype measurement should be integrated into behavioural studies examining reproductive behaviour, as in Järvi (1990); while also correcting for body size in a statistically robust manner. Constructing behavioural experiments that utilise the natural variation in AKH, and by selecting individuals with high and low AKH, or even by manipulating kype morphology through prosthetics and 3D printing, are especially potent avenues of investigation. Results here also support the argument that kype length is not important in sexual selection, and that future studies should focus on measurements such as kype height. Fully understanding natural variation in wild mature male head morphology, including kype height, would also be beneficial for our understanding, exploring more Norwegian strains, as well as strains from other locations within the natural range of Atlantic salmon. Examining more wild strains in a wild common garden, to complement the hatchery style common garden shown here, would also be valuable, as intragenerational environmental influences on morphology could also be assessed. Finally, integrating measures of sexually selected traits, such as kype height, into life-history and survival datasets would

provide further insights into how sexual selection operates in Atlantic salmon. What is clear, however, is that there is very little empirical evidence on the kype's role in sexual selection, and there are a multitude of ways in which we can build on the previous literature that has tried to elucidate its role (Järvi, 1990).

In addition to established impacts of hybridisation between wild and domesticated salmon, generally resulting in reduced fitness of offspring, the ongoing high incidence of escapees globally (Glover *et al.*, 2017) and across species (Jensen *et al.*, 2010; Baskett, Burgess and Waples, 2013; Faust *et al.*, 2018; Fukui *et al.*, 2018; Whittaker, Consuegra and Garcia de Leaniz, 2018), is heightening concern that genetic impacts from both pre-zygotic and post-zygotic mechanisms may lower fitness. We show here that the process of domestication is likely to play a disruptive role in sexual selection through morphological change in secondary sexual characteristics; disruption that is also seen in the hybrids between domesticated and wild individuals. Changes in sexually selected traits, as highlighted here, could also be occurring in other finfish species that are also undergoing rapid domestication, in all corners of the globe. Such changes are particularly worrying in systems where there is limited information on life history and ecology, as long-term evolutionary trajectories, as well as wild stock viability, could be undermined through increased domesticated escapees.

2.5 Animal ethics

Those working directly with the experimental animals had undergone Norwegian Food Safety Authority (NFSA) training, as is required with experimentation involving animals that are included in the Norwegian Animal Welfare Act (2010).

2.6 Authors' contributions

William Bernard Perry took photographs and measurements from the fish, applied all landmarks, conducted geometric morphometric analysis, mixed effect modelling analysis, compiled data and wrote the manuscript. Monica Solberg was involved in producing the fish, taking photographs, taking measurements from the fish, constructing the mixed effect models and interpretation of the results, while also being involved in the conception and design of the work. Francois Besnier conducted all QTL analysis. Ivar Helge Matre and Lise Dyrhovden produced the fish used in this study, while also taking measurements from the fish, rearing the fish and taking photographs. Per Gunnar Fjelldal, Simon Creer, Martin Llewellyn and Martin Taylor were involved in the conception and design of the work. Fernando Ayllon conducted genetic sexing of the fish. Kevin Glover and Gary Carvalho. were involved in the conception and design of the work, as well as securing the funding for the project.

Chapter 3: Domestication induced change in body morphology: a study of Atlantic salmon (*Salmo salar*) in an artificial and natural common garden

Abstract

Domestication has contributed to large shifts in production traits of aquaculture species which are under direct artificial selection, but it has also caused unintentional changes to non-production traits. Growth has been an important target of selection since the beginning of Atlantic salmon breeding programmes in the 1970s, yet the impact of domestication on other aspects of body morphology such as the pectoral fin, eye and overall body shape remain relatively underexplored. Here, we compiled one of the largest common garden studies of its kind, comprising of ~4,000 individuals. These were the progeny of 11 populations, of multiple genetic background (wild, domesticated farm, F1 hybrids, F2 hybrids and backcrosses), compared in both freshwater and saltwater environments over various life stages, under reciprocal artificial and natural rearing conditions, in two geographically contrasting European locations (Ireland and Norway). We demonstrate that morphology is influenced by genetic background, environment and ontogenetic drivers. 1) The progeny of artificially reared domesticated fish had significantly larger fork length than those of wild fish, with hybrids and backcrosses showing intermediate phenotypes; but no significant differences in length were found among the progeny of the different groups of naturally reared fish. 2) The progeny of domesticated fish had larger pectoral fin length (APL) adjusted for fork length than wild offspring, but only for those fish living in natural river conditions. 3) The progeny of domesticated fish also had smaller fork length adjusted eye width (AEW) when compared to their wild origin conspecifics, but only when artificially reared; although some indications of this trend were present in naturally reared fish. 4) Significant domestication induced changes in body shape were discovered in all tank reared fish, but not in fish reared in sea cages or in natural river conditions. The results found here in respect of the progeny of the hybrid groups suggests that introgression between domesticated and wild fish is unlikely to result in marked morphological changes in wild populations. However significant morphological differences do appear

to exist among the various hybrid progeny groups and the offspring of pure farm and wild parents when reared artificially, suggesting some level of genetic contribution. Hence, it may not follow, that in the absence of significant differences in body morphology among the farm, wild and hybrid progeny groups in the river that introgression induced changes in morphology have not occurred. It is also plausible that individuals with maladapted body shape traits, in contrast to the hatchery were most individuals are expected to survive, have such reduced fitness in the wild that they are quickly removed from the population through stabilising natural selection and as a consequence were not present at the time of sampling.

3.1 Introduction

For aquatic organisms such as fish, body shape and fin morphology are key to mobility and station holding in water, playing a role in hydrodynamics, as well as both passive (Beal *et al.*, 2006) and active (Thorsen and Westneat, 2005; Li *et al.*, 2012) propulsion. The effective motility of fish is vital for life history traits, their ecology and in many cases fitness, with only a very few examples of sedentary fishes (Johnston, Clarke and Ward, 1991; McCusker and Bentzen, 2010; Caldwell and Vincent, 2012). An exemplar of high vagility is the Atlantic salmon (*Salmo salar*). This species is highly active, first having to have the capacity to hold station against strong river currents in the juvenile life stage, in order to feed (Stradmeyer and Thorpe, 1987) and defend territory (Blanchet, Dodson and Brosse, 2006). Juvenile activity is then followed by passive swimming and long distance migrations, potentially including thousands of kilometres (Mccormick *et al.*, 1998) to and from distant oceanic areas. Besides the physiological capabilities required for such an active life history (Agnisola and Tota, 1994), body morphology is fundamental to biomechanics of movement, through musculature and fins, to drag and passive movement through the water column (Sagnes and Statzner, 2009). One such set of fins are the pectoral fins, whose absolute and relative size are an essential component of a fish's ability to

maintain or correct station, surge and manoeuvre, while feeding or escaping predators in both still and turbulent conditions (Drucker and Lauder, 2002; Drinan *et al.*, 2012).

Recognized drivers of within-species variation in body morphology and pectoral fin length can include ontogeny, genetic background, local adaptation to environmental conditions such as river gradient as well as plastic responses to events experienced during an individual's lifespan (Riddell and Leggett, 1981; Taylor, 1986; Langerhans *et al.*, 2003; Sidlauskas, Chernoff and Machado-Allison, 2006). In salmon, factors such as ontogeny, genetic background and local adaptation have been highlighted in studies on growth rate, demonstrating the strong effect of heritability (Solberg, Zhang, *et al.*, 2013; Reed *et al.*, 2015; Harvey, Solberg, Troianou, *et al.*, 2016), but also plasticity in response to divergent environments (Glover *et al.*, 2018a).

The environments encountered by salmon differ greatly throughout the life cycle, from the beds of rivers and streams used by alevins, fry, parr, smolts and spawning adults, through to the murky brackish waters of estuaries by smolts, to finally, open oceanic habitats encountered by post-smolts and adult salmon. Variations in life history are reflected in body and fin morphology at different life stages, clearly demonstrated by comparison of pre-and post-smolt individuals (Stefansson *et al.*, 2008). While there is a surprising lack of data investigating morphological variation in Atlantic salmon across their life history (von Cramon-Taubadel *et al.*, 2005), studies have been undertaken comparing populations at the same life history stage (e.g. Riddell and Leggett 1981; Hendry and Quinn 1997; Blair, Rogers, and Quinn 2011; Drinan et al. 2012). Findings demonstrate vast morphological diversity within salmonid species, be it the result of plasticity or local adaptation (Obedzinski and Letcher, 2004)).

In addition to variation exhibited in body shape and fin morphology, the eye has long been a prominent feature in comparative morphometric studies of fish (Baumgartner, Bell and Weinberg, 1988; Pakkasmaa, Ranta and Piironen, 1998). Sensory systems such as vision, that allow for an organism to navigate its environment, are also vital for highly mobile species. Not only is vision important in prey detection and predator avoidance, it is thought that visual cues could also play a

role in the migration of salmon back to their spawning grounds; for example, blind sockeye salmon taking longer to return to their natal spawning ground (Ueda *et al.*, 1998). In addition, there is evidence to show that cataract formation developed during smoltification in salmon in sea farms impairs growth performance, due to reduced vision (Sveier, Breck and Sveier, 2001).

A source of large genetic change in recent decades for many aquatic species has been due to the process of domestication. Domestication in this context is a multi-generational process in which humans seek to control an organism through captive rearing and artificial selection, and it has long been part of agriculture. Based on archaeofaunal evidence, it is estimated to have first occurred in animals 10, 000 years ago in the goats from the western highlands of Iran (Zeder and Hesse, 2000). Up until the 19th century, the same process of domestication had occurred in only a handful of aquatic organisms. Domestication in fish was first seen in species of Nile tilapia and Asian carp 3000 - 3500 years ago (Teletchea and Fontaine, 2014). Aquaculture, and elements of domestication, were also taking place in Europe in the Middle Ages, with European monks culturing common carp and brown trout (Teletchea and Fontaine, 2014). Large scale Atlantic salmon aquaculture commenced in the 1970s (Gjedrem, 2000). Despite their relatively recent introduction, the application of modern techniques in Atlantic salmon aquaculture have facilitated the rapid growth of salmon farming into a massive commodity scale industry (Gjøen, 1997). Artificial selection for production traits such as body weight, age of sexual maturation, flesh colour and fat content have successfully improved each of these traits. Such strong selection, along with exposure to artificial environments, have also resulted in many non-production related traits to diverge from those of wild populations (reviewed in Glover et al. 2017). Differentiation between selected farm phenotypes and wild types can be exacerbated by hitchhiking-selection. Hitchhiking-selection is where strong selection on one locus reduces gene flow in the region around a specific locus due to physical linkage (Feder *et al.*, 2012), and can magnify differences through genetic drift and the accumulation of mutations (Via, 2012). Hitchhiking-selection has been previously documented in relation to sympatric speciation (Malinsky *et al.*, 2015), while

evidence of domestication induced trait change in vertebrates suggest that a more important role of pleiotropy (Wright *et al.*, 2010).

There are a plethora of examples of divergent phenotypes between wild salmon and their domesticated counterparts. Some examples include domesticated fish having a reduced maximum rate of oxygen uptake (Zhang *et al.*, 2016), higher levels of aggressive behaviour (Einum and Fleming, 1997) and, as will be discussed here, changes to external morphology (Fleming and Einum, 1997; Wessel, Smoker and Joyce, 2006; Pulcini *et al.*, 2013; Perry *et al.*, 2019). The combined, genetic, morphological and behavioural differences between offspring of wild and domesticated origin are likely to result in the reduced lifetime reproductive success of domesticated progeny in the wild (McGinnity *et al.*, 1997, 2003; Fleming, Hindar, Mjølnerød, *et al.*, 2000; Besnier *et al.*, 2015; Skaala *et al.*, 2019; Solberg *et al.*, 2020).

Despite published studies on the impact of domestication on growth rate, few have examined other forms of external morphological change such as body shape (Einum and Fleming, 1997; Pulcini *et al.*, 2013; Jørgensen *et al.*, 2018; Perry *et al.*, 2019). The common garden design of these studies is crucial as any differences in morphology can be attributed to genetic differences. However, it only examines differences within specific treatments, and so trends seen in a hatchery based common garden studies are not necessarily transferable directly to other environments, such as the wild. To gain a representative ecological perspective of domestication-induced morphological changes, a reciprocal common garden experiment provides a powerful tool to investigate domestication across differing environments. Such a study, involving multiple reciprocated experiments, to assess genetically influenced morphological variation, other than in growth, has not been conducted in Atlantic salmon previously.

Here, we present the most comprehensive Atlantic salmon common garden study to date to examine the effects of domestication on morphological traits in respect of the length of pectoral fins relative to body size, the eye width relative to body size, body size at age and body shape. This included

extensive linear measurements and geometric morphometric analysis of ~4,000 offspring sampled

from contrasting natural and artificial environments, including freshwater and marine environments,

originating from 11 populations (figure 3.1), replicated in both Ireland and Norway. Based on previous

meta analyses examining domestication induced morphological change in fish (Wringe, Purchase and

Fleming, 2016), we predicted that the domesticated progeny of farm parents would have reduced

relative eye size, reduced pectoral fin length and to have altered overall body shape. The results of

this study should show whether domestication selection has had altered the body shape of Atlantic

salmon in both river and hatchery environments and infers whether such changes have fitness

consequences for the progeny of escaping farm salmon, including their hybrid progeny in the wild.

3.2 Materials and methods

Here we combine several common garden experiments, encompassing two life stages (freshwater and saltwater), two experimental countries of origin (Ireland and Norway) (figure 3.1), as well as two environments (artificial and natural).
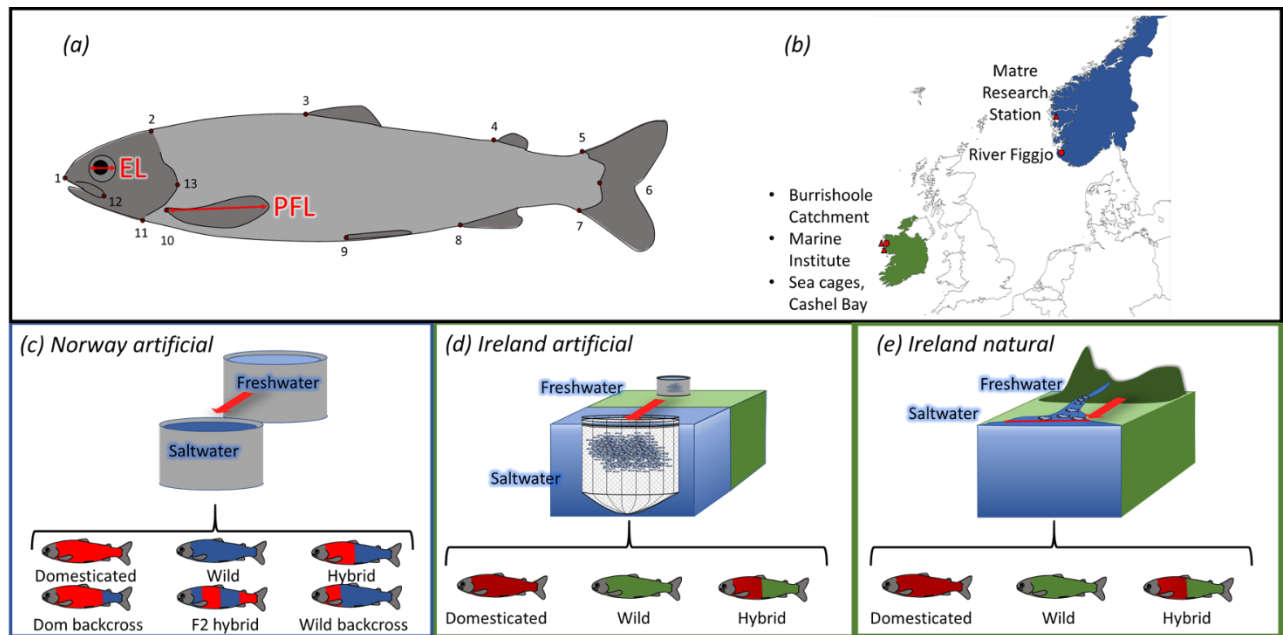
Figure 3.1 Diagram showing the 13 landmarks used for geometric morphometrics and linear measurements on both (a) diagrammatic freshwater Atlantic salmon. Linear measurements include the eye length (EL) and pectoral fin length (PFL). In addition to the breakdown of experimental designs used in this study between the origins of (b) Norway and Ireland, including the sites where the wild genetic backgrounds were acquired. What is also shown is the three different experiment types, including (c) Norwegian artificial (d) Irish artificial and (e) Irish naturally reared, with corresponding genetic backgrounds. It should be highlighted that naturally reared fish in the saltwater life stage are captured before they enter the marine environment.

3.2.1 Experimental design & fish rearing – Norway

Common-garden experiments looking at the performance of the progeny of domesticated farm, hybrid and wild salmon have been undertaken at the Institute of Marine Research (IMR), Hordaland, Norway, for more than a decade (e.g. Glover et al. 2009; Bicskei et al. 2016; Solberg, Skaala, Nilsen, and Glover 2013; Harvey et al. 2016). The process of rearing fish in these common-garden experiments involves a large pedigree-based population of salmon consisting of fish originating from wild and domesticated genetic backgrounds, including respective hybrids and back-crosses. Production of the F1 fish is described in (Solberg *et al.*, 2014)(Cohort 2011 and 2012). The fish are reared under standard farming conditions in tanks for both the freshwater and saltwater life stage, where they are mixed from the eyed-egg stage onwards, with an artificial light regime that simulates natural day-length for Bergen. All fish produced in the Norwegian experiment detailed here were established at the Matre Research Station (60°52'26.4"N, 5°35'09.0"E), with fertilisation of gametes occurring in December 2015. Half of the fish in the freshwater life stage were euthanized in April 2017. Shortly afterwards the other half were moved to saltwater tanks. The fish in the saltwater life stage were terminated a year later in April 2018.

During the freshwater life stage, fish were reared in a flow through system of four replicated octagonal tanks, located in an enclosed outbuilding. Final rearing tanks were 3 m wide and 1.25 m deep with a volume of 6300 L, and a continuous flow rate (60 L/min), supplied with freshwater from several sources surrounding the research station at Matre. Incoming water was passed through 15 m high concrete header tanks and filtered through a 40 μm filtration unit before entering individual tanks. Fish from two of the four freshwater replicates then went on to rearing in flow through saltwater system. Tanks were 5m wide, 1.1m deep, a volume of 15600 L and a flow rate of 170-200 L/min, supplied with water from the surrounding fjord. Both freshwater and saltwater tanks were lit artificially, starting with a 24-hour light regime during first feeding, with the photoperiod simulating that of Bergen post first feeding.

The temperature range of the water during the experiment was 3 °C - 14.2°C. Fish were fed on a diet of pellets produced by Skretting Nutra Olympic (Cheshire, UK). The Norwegian experiment included the genetic backgrounds: domesticated farm (Norwegian Mowi – 12 families), wild (from the river Figgjo, Norway – 6 families), hybrid farmed female and a wild male (HFF – 3 families), hybrid wild female and a farmed male (HWF – 3 families), F2 hybrid (6 families), wild backcross (6 families) and domesticated backcross (6 families) (figure 3.1c).

An anaesthetic overdose of MS-222 was used to euthanize the fish, and all those working directly with the experimental animals had undergone Norwegian Food Safety Authority (NFSA) training, as is required with experimentation involving animals that are included in the Norwegian Animal Welfare Act. As the fish were kept under standard rearing conditions and no procedures were carried out, no specific research permit was required.

3.2.2 Experimental design & fish rearing – Ireland

The Marine Institute and the Srahrevagh river in the Burrishoole catchment in the West of Ireland have been experimental sites for common garden experiments on salmon and brown trout (*Salmo trutta*) since the early 1990s (McGinnity *et al.*, 1997, 2003; de Eyto *et al.*, 2011; O'Toole *et al.*, 2015). As part of this study two types of common garden experiments were conducted in Ireland. The first involved rearing fish under standard farming conditions in hatchery tanks for the duration of the freshwater life stage, and then moved to a sea cage to be reared in the saltwater environment. The second involved rearing fish under natural river conditions in the Srahrevagh river, where ca. 7,250 m^2 of natural juvenile salmonid habitat is contained at its lower end by a high specification trap capable of capturing all life history stages from swimming up, and at its upper end by a series if large waterfalls. Further details on the Srahrevagh river can be found in (McGinnity *et al.*, 1997).

Fish reared artificially were done so under standard farming conditions, in tanks for the freshwater life stage and in sea cages for the saltwater life stage, where they were mixed from the eyed-egg stage onwards. Fish used in the Irish artificial experiment were established at the Marine Institute hatchery

(53°55'22" N 9°34'18" W), with fertilisation of gametes occurring in December 2017. Fish in the freshwater life stage were euthanized in October 2018. Remaining freshwater fish were moved as smolts to sentinel sea cages in the Atlantic Ocean at the Lehanagh Pool site (Cashel Bay, 53°21'11.7"N 9°55'42.7"W) in May 2019. The fish in the sea cage were euthanized between June - August 2019, with the majority being terminated in July.

In the freshwater life stage, fish were reared in a flow through system comprised of four circular outdoor tanks, with natural lighting and nets to exclude avian predators. Final rearing tanks were 2.5 m wide and 0.6 m deep with a volume of 2400 L, and a continuous flow rate (60 L/min), supplied with freshwater from Lough Feeagh, a freshwater lake located upstream from the hatchery. The intake pipes in Lough Feeagh are screened to prevent large debris entering the system but water is otherwise unfiltered. Accordingly, water in the tanks can contain high levels of suspended solids that colour and darken the water, particularly after heavy rain. After rearing in freshwater tanks, smolts were relocated to 4 m diameter x 4 m height sentinel cages placed in a sea pen in the Lehanagh Pool site in the Atlantic Ocean (Cashel Bay, 53°23'43.9"N 9°49'02.7"W).

The temperature range of the water during the study ranged from 3.4 °C – 21.3°C in the freshwater tanks. Freshwater fish were fed ad libitum on a diet of pellets produced by Skretting Nutra Olympic (Cheshire, UK), and saltwater fish in the sea cage were fed daily on a diet of Ewos 75 pellets produced by Cargill (MN, USA). The Irish experiment included the genetic backgrounds: domesticated farm (derived from Norwegian Mowi in the mid 1980's), wild (from the Burrishoole system), farmed female and a wild male hybrids (HFF), and wild female and a farmed male hybrids (HWF) (figure 3.1c). An anaesthetic overdose of MS-222 was used to terminate the fish reared artificially.

Naturally reared fish were reared in the hatchery after first feeding stage, after which they were planted in the Srahrevagh river in Spring 2018 for the freshwater life stage, or in Spring 2017 for saltwater life stage. Fertilization took in December 2017 for the freshwater fish, and in December 2016 for the saltwater fish. The naturally reared freshwater fish were caught in October 2018 using

electrofishing over the entire Srahrevagh river. The naturally reared saltwater life stage were caught during seawards migration at sea entry Wolf traps situated at the Marine Institute in Newport (53°55'13.3"N 9°35'03.3"W) throughout Spring 2019. Therefore, naturally reared fish in the saltwater life stage are captured before they enter the marine environment. The Srahrevagh river is a third-order upland stream with a medium to high gradient (discharge = 25,200 L/min). The relatively high amount of precipitation in this catchment, combined with non-porous blanket peat soil leads to frequent and sudden rainfall-driven high discharge events. This river has variable visibility but usually very high colour (median colour concentration of 130 mg PtCo/L) due to peat soils and high dissolved organic carbon (Doyle *et al.*, 2019). In this river, salmon actively feed on adult flies (Diptera, Plecoptera, Trichoptera) as well as Coleoptera (de Eyto *et al.*, 2020).

The Irish study was carried out under a Health Products Regulatory Authority (HPRA) license number AE19130-P056.

3.2.3 Parentage analysis – Norwegian fish

Genomic DNA was extracted from alcohol-preserved fin-clip samples using the Qiagen DNeasy®96 Blood & Tissue Kit, followed by a multiplex PCR which amplified five microsatellite loci; SsaF43 [GenBank:U37494] (Sánchez *et al.*, 1996), Ssa197 [GenBank:U43694.1] (O'Reilly *et al.*, 1996), SSsp3016 [GenBank:AY372820], MHCI (Grimholt *et al.*, 2002), MHCII (Stet *et al.*, 2002). An ABI Applied Biosystems ABI 3730 Genetic Analyser was used for fragment analysis, the outputs of which were used to call genotypes in GeneMapper (Applied Biosystems, v. 4.0). Further details are outlined by Solberg et al. (2013).

3.2.4 Parentage analysis – Irish fish

Genomic DNA was extracted from alcohol-preserved fin-clip samples using the Promega Wizard® SV 96 Genomic DNA Purification System, followed by a three-panel multiplex PCR which amplified ten

microsatellite loci; (Panel 1, Ssa197 (O'Reilly et al., 1996) and MHC2 (Stet et al., 2002); Panel 2, Ssa202, Ssa171 (both O'Reilly et al., 1996), Sssp2210 (Paterson et al. 2004) and SsaD170 (unpublished; EMBL Accession no. AF525205); Panel 3, Ssp2216, Ssp1605 (both Paterson et al., 2004); SsoSL85 (Slettan et al., 1995) and SsaD157 (King et al., 2005)). An ABI Applied Biosystems ABI 3500xl Genetic Analyser was used for fragment analysis, the outputs of which were used to call genetic background. Further details can be found in appendix 3.

3.2.5 Photograph preparation and analysis

After fork length of the fish had been measured (to the neared 1 mm) using a standard measuring board, photographs were taken on a digital single-lens reflex camera placed above samples on a level surface, with a scale in shot. Fish collected from the river experiment were measured using callipers to the nearest 0.01 mm. Before the addition of landmarks, all photographs were quality checked, with low quality images removed (i.e. when landmarks could not be applied). The process of quality control was conducted without knowledge of genetic background. Landmarks were applied using tpsDig v 2.28 (Rohlf, 2016) by one person, and were based on key external features based on the skeletal form of the fish used previously in the literature (Winans and Nishioka, 1987; Enders, Boisclair and Roy, 2004; Skoglund *et al.*, 2015). The landmarks included the 1) most anterior point of the fish, 2) dorsal point of the head, 3) anterior point of the dorsal fin, 4) anterior point of the adipose fin, 5) dorsal attachment of the caudal fin to the tail trunk, 6) posterior position of the tail trunk, as positioned with the lateral line, 7) ventral attachment of the caudal fin to the tail trunk, 8) anterior point of the anal fin, 9) anterior point of the pelvic fins, posterior position of the pectoral fin (not used in geometric morphometrics), 10) anterior position of the pectoral fin, 11) ventral point of the head, 12) posterior point of the maxillary bone, anterior point of the eye (not used in geometric morphometrics), posterior point of the eye (not used in geometric morphometrics), and the 13) anterior point of the gill plate (figure 3.1a). The landmarks used for geometric morphometrics were also used as endpoints for linear measurements (figure 3.1a). Fork length measured during sampling and body length estimated from the photographs was used to scale the linear measurements obtained from the landmark data. In

addition to landmarks used for geometric morphometrics and linear measurements, 8 landmarks were also placed along the lateral line, from the gill plate to the caudal fin, at regular intervals, these landmarks were used to assess the extend of bending in the fish, and was factored into later morphometrics analysis (figure A3.1). After quality control, a total of 3,970 unique photos were used.

3.2.6 Fork length

Fork length was used as response variable in a single linear mixed effect model, constructed using the R package 'lme4' (Bates *et al.*, 2015). The linear mixed effect model included as fixed factors: genetic background, life stage (freshwater and saltwater), rearing type (artificial and natural) and experimental origin (Norway and Ireland), full interaction terms between factors, as well as the random factor of sampling date. Estimated marginal means and pairwise comparisons between means were calculated using the R package 'emmeans' (Lenth, Love and Maintainer, 2018). Due to the large number of comparisons, z tests were adopted, instead of using the t distribution.

3.2.7 Adjusting linear measurements relative to fork length

Euclidian distances were calculated between the landmarks. To scale the Euclidian distances, the scale between body length measured in the photographs and fork length measured during sampling was used. Once lengths had been scaled, the dataset was split into artificial and natural rearing conditions, due to the contrasting allometry between morphological features and fork length, and the inability for one regression combining all data to remove the effect of fork length on the naturally reared fish. Regression plots between log transformed fork length and log transformed eye width, broken down by life stage and rearing type can be found in figure A3.2. In addition to this, splitting the dataset was necessary for full factorial design within each model, as the Irish artificial was missing the factor of family.

Therefore, to adjust for fork length, three linear regressions were constructed: Norwegian artificially reared, Irish artificially reared and Irish naturally reared. A fork length adjusted measure for eye width/pectoral fin was calculated as the residuals from these two log-log regressions between fork

length and pectoral fin length/eye width, which will now be referred to as adjusted pectoral fin length (APL) and adjusted eye width (AEW). The residuals were then used as response variables in two separate linear mixed effect model in 'lme4' (Bates *et al.*, 2015). An additional linear mixed effect model for the response variable APL in Norwegian artificially reared, without stubby finned individuals removed.

The linear mixed effect model included the fixed factors: genetic background (wild, domesticated, HWF and HFF) and life stage (freshwater and saltwater), full interaction terms between factors, as well as the random factor of sampling date; and family nested in strain in the case of the Norwegian artificially reared fish. Norwegian artificially reared fish also had additional levels for the genetic background factor: wild backcross, domesticated backcross and F2 hybrid. The 'step' function within 'lme4' was used to select the best fitting model through automatic backward elimination, removing fixed terms and random factors which did not contribute to the model. Estimated marginal means and pairwise comparisons between means were calculated using 'emmeans' (Lenth, Love and Maintainer, 2018).

3.2.8 Geometric morphometrics

After landmarks were placed on specimens, the eight landmarks on the lateral line were used in tpsUtility (Rohlf, 2009) to remove the influence of any bending that the specimen might exhibit. Once the specimens had been processed in tpsUtility, these eight landmarks were removed from the TPS file, and were not used in the subsequent geometric morphometric analysis. The R package geomorph 2.0 (Adams and Otárola-Castillo, 2013) was used to analyse the landmark data which firstly involved a Generalized Procrustes analysis (GPA) to remove the effect of scale, absolute size, orientation and translation between individuals. Transformed coordinates from the GPA were then visualised using a principal component analysis (PCA), using the three components that explained the most variation. In addition to the PCA, 95 % confidence ellipses around the different experiments and life stages were generated, assuming a multivariate t-distribution.

The function procD.lm in geomorph was used to identify differences in shape between different factors using a Procrustes ANOVA with permutation procedures on GPA output. A model was applied with the fixed effects: length, life stage, rearing type and genetic background. All interactions between factors were included, apart from rearing, due to the lack of full factorial design between Norwegian and Irish experiments (Norwegian experiment lacking natural rearing element). A series of post-hoc tests focused on identifying differences between genetic backgrounds was then carried out, where the entire dataset was broken down by life stage, rearing type and experimental origin, with independent GPA for each of the six groups. For each group, the function procD.lm was used to construct a model with the factors genetic background and length, along with their interaction term, for the GPA output of the six groups, identifying two-sample z scores between effect sizes of all genetic backgrounds, as well as pairwise comparisons between genetic backgrounds. Coefficient estimation and type III sums of squares calculation were based on 1,000 permutations.

All statistical analyses were carried out in R v. 3.6.2 (R Core Team, 2017). The software PAleontological Statistics (PAST) (Hammer, Harper and Ryan, 2001) was used to produce thin-plate splines to highlight expansion factors in a heat map, imposed on a warped shape grid, using the PCA values.

3.3 Results

3.3.1 Fork length – all experiments and environments

There was a significant effect of genetic background on fork length (LME Genetic background: $F_{6,3653}$ = 346.20, Sum Sq = 14257, p < 0.01) (figure 3.2a&b) in all artificially reared fish. In contrast, no effect of genetic background on fork length was detected between wild and domesticated fish reared naturally, either in the freshwater (z ratio = 2.63, p = 0.92) or saltwater life stage (z ratio = 0.13, p > 0.99) (figure 3.2c). When artificially reared, domesticated fish are always larger than wild fish, and hybrids of all types are intermediate, with a maternal effect. Fish reared artificially were larger than fish reared naturally, while fish from the saltwater stage were larger than fish from the freshwater stage. Means

and standard error for each genetic background in the different experimental groups can be found in table A3.1.
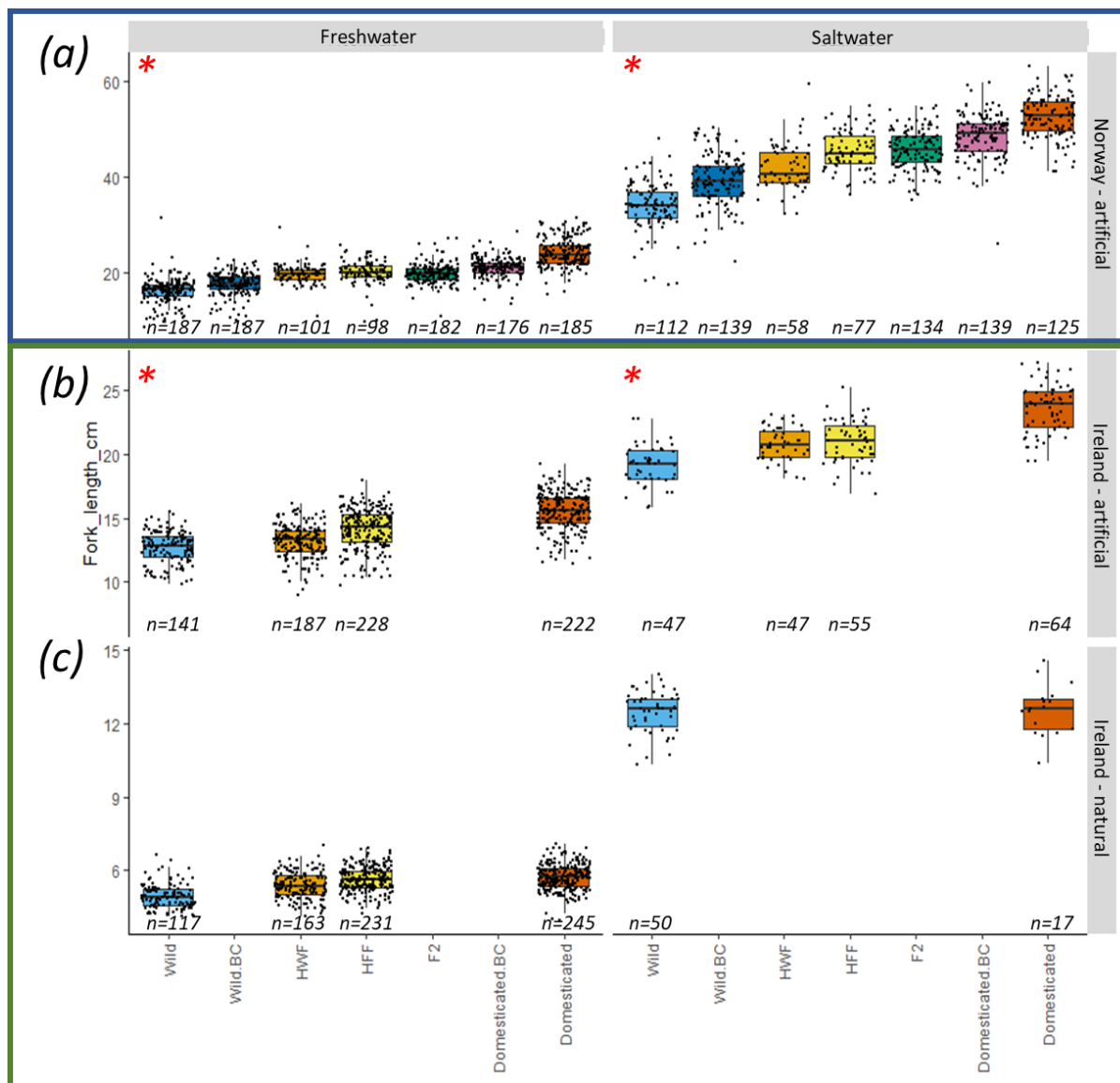
Figure 3.2 Fork length broken down by genetic background and life stage in the (a) Norwegian artificially reared fish, (b) Irish artificially reared fish and (c) Irish naturally reared fish, along with their corresponding samples sizes. It should be highlighted that the y axes show different scales in order to allow for trends within smaller fish. Red asterisk corresponds to a significant effect of genetic background.

3.3.2 Pectoral fin length – Norwegian artificially reared

Genetic background (LME Genetic background: $F_{6,29}$ = 2.68, Sum Sq = 0.02, p = 0.03) had a significant effect on fork length adjusted pectoral fin length (APL) in Norwegian artificially reared fish, as well as the interaction between genetic background and life stage (LME Genetic background *life stage: $F_{6,1851}$ = 2.21, Sum Sq = 0.02, p = 0.04). The wild backcross and domesticated genetic backgrounds in the saltwater life stage were the only significant contrasts (t_{37} = 0.036, p = 0.03) (figure 3.3a), with domesticated fish showing significantly reduced APL when compared to wild fish. When stubby finned individuals with the lowest APL were removed from the analysis (n = 11), all of which were domesticated fish, there was no significant effect of genetic background (LME Genetic background: $F_{6,29}$ = 2.19, Sum Sq = 0.014, p = 0.07).
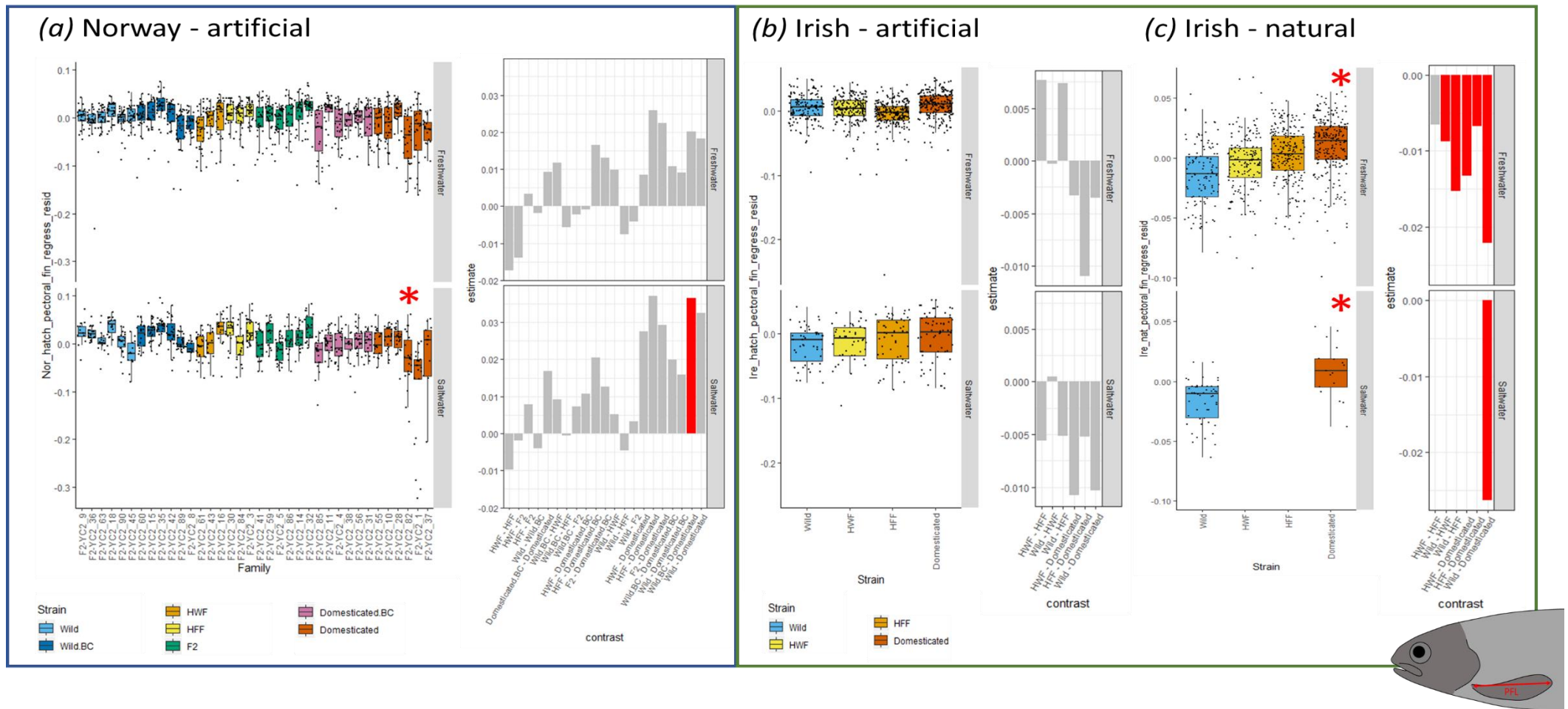
Figure 3.3 Fork length adjusted pectoral fin length (APL) between genetic backgrounds (wild, hybrid farmed female (HFF), hybrid wild female (HWF) and domesticated) split into (a) Norwegian artificially reared (where family information is included) (b) Irish artificially reared and (c) Irish naturally reared. Red asterisk corresponds to significant pairwise differences between genetic backgrounds. Pairwise differences of mean APL between genetic backgrounds are also included, produced from the LME using emmeans, and significant differences in means are coloured red, while grey represent non-significant differences.

3.3.3 Pectoral fin length – Irish artificially reared

Genetic background (LME Genetic background: $F_{3,981}$ = 3.38, Sum Sq = 0.005, p = 0.02) and life stage (LME Life stage: $F_{1,21}$ = 5.62, Sum Sq = 0.003, p = 0.03) both had a significant effect on fork length adjusted pectoral fin length (APL) in Irish artificially reared fish. The trend suggested domesticated fish had greater APL than wild fish in both life stages, with no clear trend seen in the hybrids between life stages. In addition, there was a significant interaction term between genetic background and life stage (LME Genetic background *life stage: $F_{3,981}$ = 2.56, Sum Sq = 0.004, p = 0.05). When looking at pairwise comparisons and correcting for multiple comparisons, however, there were no significant differences between genetic backgrounds in the different life stages (figure 3.3b).

3.3.4 Pectoral fin length – Irish naturally reared

Genetic background (LME Genetic background: $F_{3,763}$ = 17.96, Sum Sq = 0.028, p < 0.01) had a significant effect on fork length adjusted pectoral fin length (APL) in Irish fish sampled from the river. There were many significant pairwise differences between genetic backgrounds (figure 3.3c), which, overall, showed in both the freshwater and saltwater life stages, that APL was larger in domesticated individuals compared to wild individuals, with hybrids showing an intermediate phenotype (hybrids were not available for the saltwater life stage).

3.3.5 Eye width – Norwegian artificially reared

Genetic background (LME Genetic background: $F_{6,30}$ = 23.06, Sum Sq = 0.08, p < 0.01) and the interaction between genetic background and life stage (LME Genetic background *life stage: $F_{6,1851}$ = 5.09, Sum Sq = 0.02, p < 0.01) had significant effects on fork length adjusted eye width (AEW) in Norwegian artificially reared fish. There were many significant pairwise differences between genetic backgrounds (figure 3.4a), however, the overall trend in both the freshwater and saltwater life stages was larger AEW in wild individuals compared to domesticated individuals, with hybrids and backcrosses showing intermediate phenotypes.
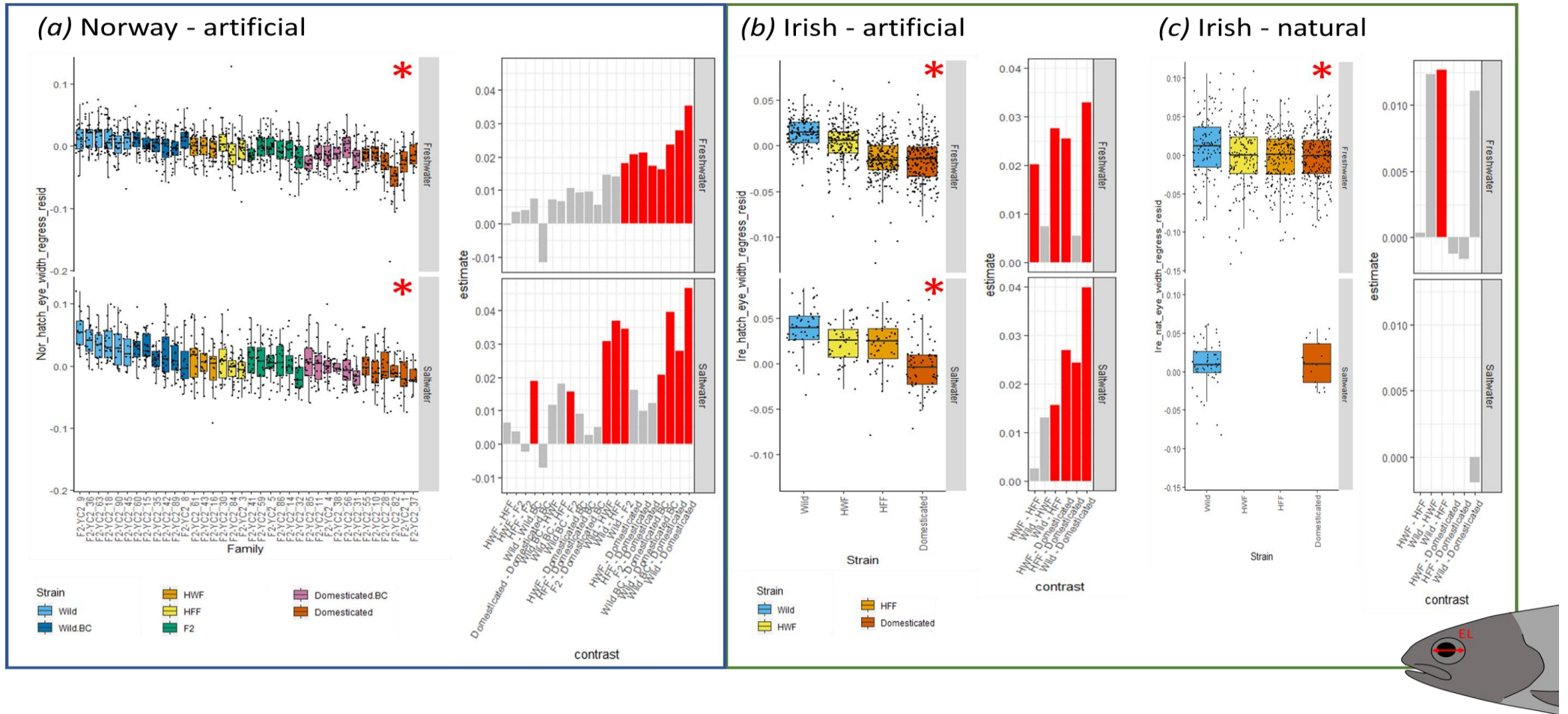
Figure 3.4 Fork length adjusted eye width (AEW) between genetic backgrounds (wild, hybrid farmed female (HFF), hybrid wild female (HWF) and domesticated) split into (a) Norwegian artificially reared (where family information is included) (b) Irish artificially reared and (c) Irish naturally reared. Red asterisk corresponds to significant pairwise differences between genetic backgrounds. Pairwise differences of mean AEW between genetic backgrounds are also included, produced from the LME using emmeans, and significant differences in means are coloured red, while grey represent non-significant differences.

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3.3.6 Eye width – Irish artificially reared

Genetic background (LME Genetic background: $F_{3,976}$ = 75.41, Sum Sq = 0.10, p < 0.01) and life stage (LME Life stage: $F_{1,22}$ =23.6, Sum Sq = 0.01, p < 0.01) had a significant effect on fork length adjusted eye width (AEW) in Irish artificially reared fish, in addition to the interaction between genetic background and life stage (LME Genetic background *life stage: $F_{3,976}$ = 5.72, Sum Sq = 0.01, p < 0.01). There were many significant pairwise differences between genetic backgrounds (figure 3.4b), however, the overall trend in both the freshwater and saltwater life stages was larger AEW in wild individuals compared to domesticated individuals, with hybrids and backcrosses showing intermediate phenotypes (figure 3.4b).

3.3.7 Eye width – Irish naturally reared

We found no significant effect of genetic background on AEW in naturally reared fish (LME Genetic background: $F_{3,796}$ = 2.43, Sum Sq = 0.009, p = 0.06)(figure 4c), life stage (LME Life stage: $F_{1,30}$ = 0.60, Sum Sq = 0.0008, p = 0.44) or the interaction term between genetic background and life stage (LME Genetic background *Life stage: $F_{1,718}$ = 1.47, Sum Sq = 0.002, p = 0.23) (figure 3.4c). However, there was a trend, as well as a significant pairwise comparison between wild and HFF (t_{816} = 3.15, p = 0.04), in the freshwater life stage which mirrored the trend of wild fish having larger AEW than domesticated fish seen in the artificially reared fish.

3.3.8 Geometric morphometrics – all experiments and environments

The principal component analysis summarised 73.9 % of shape variation into PC1 and PC2 (figure 3.5a). Differences in these principal components show shape variation between rearing condition (artificial or natural), life stage (freshwater or saltwater) and experimental origin (Norway or Ireland) (figure 3.5a). There was, however, also a correlation between PC1 and log10 transformed fork length (R^2 = 0.84, p < 0.05) (figure 3.6a). The large differences seen in shape seen between rearing types, and origin of the experiment, differ between the principal components. Thin plate splines show that negative values in PC1 correspond with an expansion of the head, particularly the lower jaw, in conjunction

with a contraction in the midsection of the fish, between the dorsal fin and pelvic fin (figure 3.5a). As PC1 increases to zero and positive values, expansion in the head is reduced, and the contraction is replaced with heavy expansion in a large proportion of the body of the fish. Negative PC2 values are characterised with expansion in both the head and the tail, combined with contractions in the body of the fish, which is reversed when values become positive, with contractions in the dorsal side of the head and dorsal side of the tail, but with large expansions in the ventral region of the head, and body, between the pelvic fin and pectoral fin. Positive values in PC3 show an expansion in the region between the upper head and dorsal fin, a contraction between the pectoral fin base and bottom of the gill plate, and finally, an expansion between the bottom of the gill plate and the end of the maxillary bone, caused by a reduction in the length of the maxillary bone.
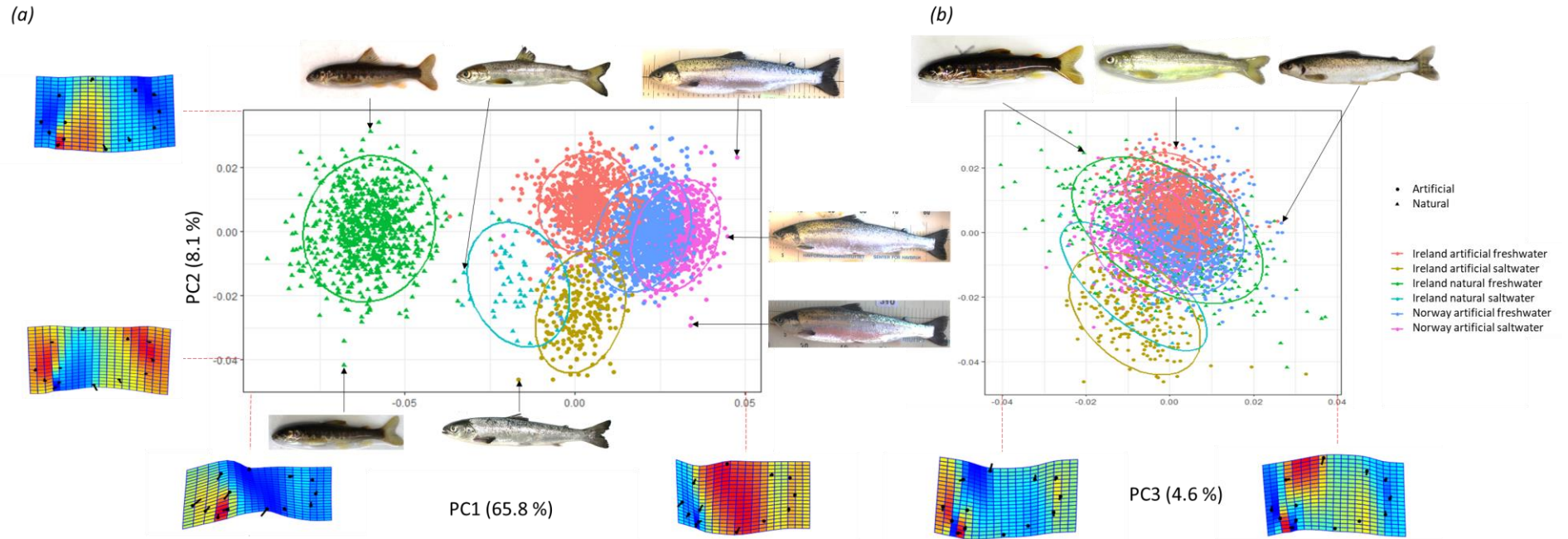
Figure 3.5 Geometric morphometrics output, including the same principle component analysis broken down by experimental key, which consists of experiment origin, rearing type and life stage, for (a) PC1 and PC2, in addition to (b) PC3 and PC2. Also provided are heat map thin-plate splines which, based on the output of the principle component analysis, show areas of expansion (red) and contraction (blue) in shape. In addition to this, examples of individuals on the extremes of their corresponding groups are provided to show real examples of the changes in shape.
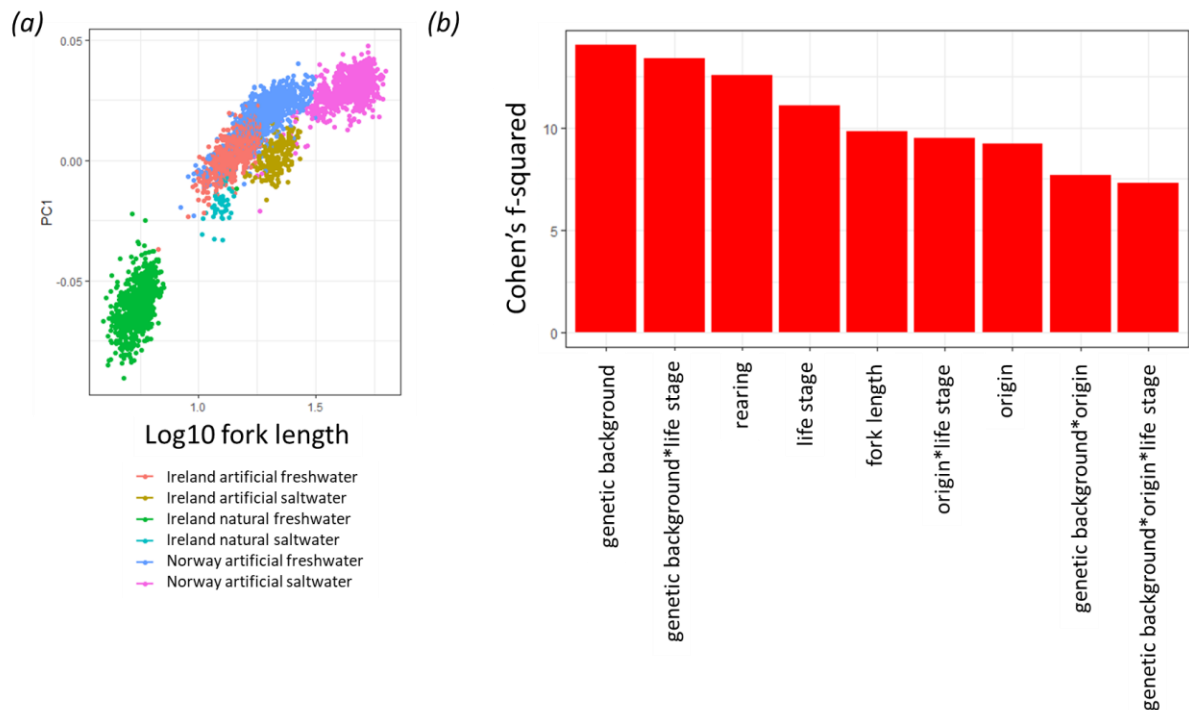
Figure 3.6 Correlation between (a) log10 transformed length and PC1 is also highlighted, with (b) Cohen's f-squared effect size of the different factors, and interactions between factors, on shape.

A Procrustes analysis of variation with permutation procedures showed significant effects of genetic background ($F_{6,3691}$ =14.03, Cohen's f-squared = 14.08, Sum Sq = 0.043, p < 0.01), rearing ($F_{1,3156}$ =993.50, Cohen's f-squared = 12.55, Sum Sq = 0.51, p < 0.01), life stage ($F_{1,3691}$ =143.43, Cohen's f-squared = 11.11, Sum Sq = 0.074, p < 0.01), fork length ($F_{1,3691}$ = 71.44, Cohen's f-squared = 9.81, Sum Sq = 0.037, p < 0.01) and experimental origin ($F_{1,3691}$ =55.67, Cohen's f-squared = 9.21, Sum Sq = 0.029, p < 0.01); in addition to this, all interaction terms between these factors were significant (figure 3.6b). Due to significant interaction terms, to assess the effect of genetic background, the dataset was split up in groups sharing rearing type, life stage and experimental origin. Significant pairwise differences were not observed in the naturally reared Irish experiment (figure 3.7a & b), however, significant pairwise differences in shape between genetic backgrounds were seen in both the Norwegian and Irish artificial experiments (figure 3.7c,d,e &f). Full breakdown of the Cohen's f-squared values

between genetic backgrounds from different experimental origins with, life stages and rearing, with corresponding p values, can be found in table A3.2.
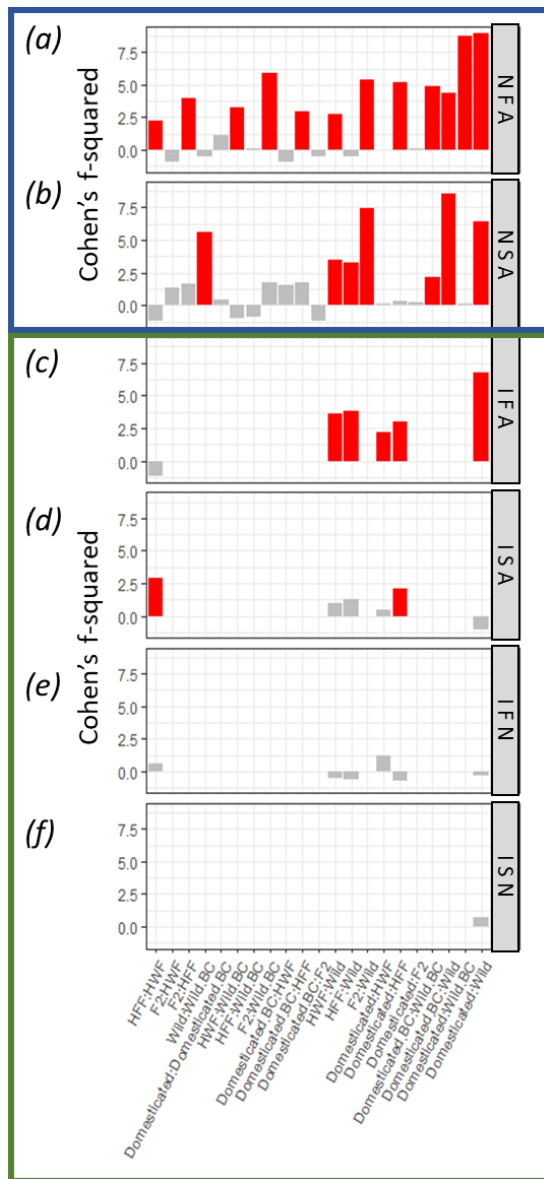
Figure 3.7 Pairwise Cohen's f-squared effect size between genetic backgrounds for Norwegian artificially reared fish, both (a) freshwater (NFA) and (b) saltwater (NSA), Irish artificially reared fish, both (c) freshwater (IFA) and (d) saltwater (ISA), and finally, Irish naturally reared fish, both (e) freshwater (IFN) and (f) saltwater (ISN). Significant differences in means are coloured red, while grey represent non-significant differences.

3.4 Discussion

Using the data from a number of independent common garden experiments carried out under natural river and hatchery tank and sea farm conditions, we demonstrate that Atlantic salmon morphology is influenced by genetic background (wild, domesticated or hybrid), environment and ontogenetic drivers. All morphological features examined here were found to be influenced by genetic background. Firstly, in artificially reared fish, the progeny of farm domesticated fish had significantly longer fork length than the progeny of wild fish, with hybrids and backcrosses showing intermediate length phenotypes; but no significant effect of genetic background was observed in river sampled fish. Secondly, domesticated fish had longer fork length adjusted pectoral fin length (APL) than wild fish in both life stages, smolt and juvenile (disregarding deformities), but only when sampled from the natural environment. Third, domesticated fish had shorter fork length adjusted eye width (AEW) when compared to wild fish in both life stages, but only for those fish reared in the artificial environment; although some indications of this trend were present in fish collected from the wild. Fourth, significant domestication induced changes in body shape were found among all the groups in the artificially tank reared fish. Finally, we also show empirically the changes in body shape associated with smoltification, and that changes are dependent on genetic background, rearing type, and environment; including the contrast in shape between tank and sea cage reared fish.

The results from AEW, body shape and fork length, in respect of the various hybrid groups, indicate that introgression between domesticated and wild fish are unlikely result in marked morphological changes in the wild. Significant domestication induced changes are seen, as shown by AEW, body shape and fork length, when natural selection is not a feature of the experimental environment as was the case in the tank and sea farm experiments. Domestication induced phenotypes shown in artificial environments indicates that the same changes are occurring in the wild, but individuals with these maladapted traits display such reduced fitness that they are quickly removed from the population through stabilising selection to a wild optimum. The exception to this rule is APL, which showed the opposite trend, with domestication induced changes only visible in the naturally reared fish, which

indicates that the increased APL caused by domestication is not detrimental to fitness in the wild. There must also be a source of selection against increased APL in the process of tank rearing, which is discussed below.

3.4.1 Fork length

Under artificial rearing conditions, domesticated fish showed a greater fork length compared to wild fish, with hybrids and backcrosses intermediate (figure 3.2a & 3.c). These results confirm that domestication has a strong effect on growth, that growth is additive, and has high heritability, as has been shown many times in previous artificial rearing common garden studies (Einum and Fleming, 1997; Fleming *et al.*, 2002; Glover, Otterå, *et al.*, 2009; Wolters *et al.*, 2009; Skaala, Glover, *et al.*, 2012; Solberg, Skaala, *et al.*, 2013; Reed *et al.*, 2015; Harvey, Glover, *et al.*, 2016; Harvey, Solberg, Glover, *et al.*, 2016; Solberg *et al.*, 2016). There were no significant differences between genetic backgrounds in fork length discovered in the Irish naturally reared fish, however. The lack of domestication driven growth difference in the natural river environment during the freshwater life stage adds to previous research demonstrating the same trend, thought to be caused by energy-budget plasticity and growth-potential mortality (Skaala, Glover, *et al.*, 2012b; Glover *et al.*, 2018b). However, previously, domesticated Norwegian smolts have been shown to exhibit higher growth rates than wild smolts when reared in a river environment (Skaala, Glover, *et al.*, 2012b). Inconsistencies with the current study and Skaala et al. (2012b) could be due to differences in growth-potential mortality between the Srahrevagh river and the River Guddalselva.

3.4.2 Pectoral fin – deformities

In the data presented here, the only significant effect in APL was due to genetic background was driven entirely by 11 domesticated deformed stub finned individuals in the Norwegian artificially reared saltwater experiment (figure 3.8). The stub fins are likely to be pathology of fin rot disease, usually following abrasion, a common occurrence in hatchery fish. Therefore, the stub fin pathology is not an

example of reduced fin length in an evolutionary context; however, it should be noted that stub fins were not present in wild or hybrid genetic backgrounds.
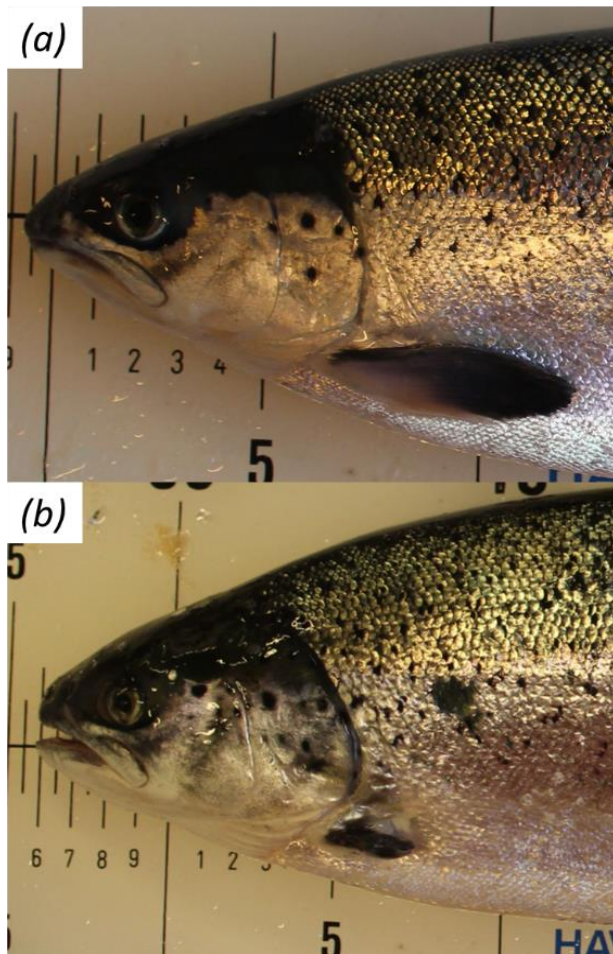
Figure 3.8 Example of a fish with (a) high APL and a fish with (b) extremely low APL, both from the Norwegian artificially reared fish, saltwater life stage. The genetic background of (a) and (b) are hybrid (domesticated mother x wild father) and domesticated, respectively.

3.4.3 Pectoral fin – genetic background

Pectoral fin deformities aside, the results described in this study are not consistent with the previous common garden experiments conducted on fin morphology of Atlantic salmon in the freshwater life stage, where domesticated individuals showed a reduced body size adjusted pectoral fin length (Fleming and Einum, 1997). This older study was, however, based on limited source material, did not have control over DNA pedigree, and the fish were not truly common-garden reared, as they were kept in different tanks. Differences in flow rate during rearing could also have contributed to the inconsistencies with this study and the literature, with I A Fleming and Einum (1997) using a flow rate of just 3 litres per minute, with a flow rate of 60 (Norway and Ireland artificially reared freshwater), and 200 (Norway artificially reared saltwater) litres per minute used here.

In the river, the offspring of domesticated fish showed a significantly larger APL than wild genetic backgrounds, with the hybrid progeny having intermediate lengths. This was true of both life stages, implying a heritable additive effect (although the hybrids were only included in the fresh water stage). Importantly, for fish living under natural conditions, their environment will have heterogenous hydrodynamic properties, enabling individuals to avoid hydrodynamically stressful (Hockley *et al.*, 2014) or abrasive patches of the river. Therefore, results here provide evidence that although genetic background can be important, how it effects APL could be dependent on environmental inputs, such as the hydrodynamic properties, with higher flow rates causing fin deterioration (Roque d'Orbcastel *et al.*, 2009), in addition to abrasion against surfaces (Turnbull *et al.*, 1998). Observations from naturally reared fish indicate that larger APL seen in domesticated individuals are not a suboptimum phenotype in the wild.

3.4.4 Eye width

When artificially reared, domesticated fish had significantly smaller AEW than wild strains, with hybrids showing an intermediate phenotype, suggesting an additive genetic effect on this trait (figure 3.6). The only exception was when the fish were naturally reared. Under natural conditions, no significant differences were seen between wild and domesticated genetic backgrounds, although a suggestive trend was found in the freshwater life stage. For artificially reared fish, where there is no immediate strong selection from aspects such as predation, or lack of food, individuals with reduced AEW can persist.

The trend of significantly smaller AEW in domesticated strains has been documented in other aquaculture species such as Atlantic cod (*Gadus morhua*) (Wringe, Fleming and Purchase, 2015), coho salmon (*Oncorhynchus kisutch*) and rainbow trout (*Oncorhynchus mykiss*), and may be related to regulation of eye size by visual stimulus. Fish reared in aquaculture are not reared in darkness, there will be a reduction selection for acute vision, as they do not need to actively seek food or avoid predators. In the wild, however, vision is an important sensory input for both predation (Leduc *et al.*, 2010) and feeding (Fraser and Metcalfe, 1997), with visual acuity (the ability to resolve spatial detail) correlated with eye size (Caves, Sutton and Johnsen, 2017). It is therefore possible that small AEW is a maladaptive trait removed by natural selection in the wild, thus removing any significant trend, in line with results from fitness models (Castellani *et al.*, 2018) and other natural common garden experiments looking at growth (Skaala, Glover, *et al.*, 2012b; Glover *et al.*, 2018b). In addition to this, for low AEW individuals, the risk of being removed from the population through natural selection is cumulative over time, which could explain why the trend disappears in the saltwater life stage; more so than the freshwater life stage. The trend seen here is therefore evidence of inherited morphological change due to artificial selection and its apparent fitness consequences in the wild.

The proximal causation of reduced AEW could be linked to the pleiotropic effect of the *sonic hedgehog* gene (*SHH*) and growth (Yamamoto *et al.*, 2009). The fundamental reason why domesticated salmon

grow larger than wild under artificial conditions when fed ad lib rations is not yet fully understood. Genetically-increased appetite has been suggested (Harvey, Solberg, Troianou, *et al.*, 2016), but it is also possible that domesticated salmon may have larger oral–pharyngeal phenotypes, in order to consume the amount of feed needed to sustain growth. Evidence of eye degeneration has been well characterised in blind cavefish (*Astyanax mexicanus*), where a pleiotropic effect of *SHH* has been identified, and overexpression induces both taste bud amplification and eye degeneration (Yamamoto *et al.*, 2009). Not only this, but the same study found an inverse relationship between jaw and eye size, further supporting the link between oral–pharyngeal constructive traits and eye degeneration. Strong artificial selection on this trait and expression of the *SHH* could be contributing to the degeneration in eye size that we document here. In addition to this, it has been hypothesized that domestication induced reductions in relative eye size in salmonids could be due to a decoupling of eye growth and somatic growth (Devlin *et al.*, 2012). Devlin et al. (2012) have showed that eyes in growth hormone (GH) transgenic salmon had reduced sensitivity to the GH pathway, and reduced levels of insulin-like growth factor 1 when compared to other tissues. Therefore, artificial selection for somatic growth through the GH pathway, which does not affect eye growth, combined with artificial selection for larger oral–pharyngeal phenotypes and thus an overexpression of the *SHH* gene could be causing the reduction in AEW we see here.

3.4.5 Body shape – genetic background

Domesticated Atlantic salmon in artificially reared conditions have shown consistently higher growth rates than wild individuals, with hybrid strains showing an intermediate growth (Fleming and Einum, 1997; Fleming *et al.*, 2002; McGinnity *et al.*, 2003; Glover, Otterå, *et al.*, 2009; Wolters *et al.*, 2009; Skaala, Glover, *et al.*, 2012b; Solberg, Skaala, *et al.*, 2013; Reed *et al.*, 2015; Harvey, Glover, *et al.*, 2016; Harvey, Solberg, Troianou, *et al.*, 2016). What we demonstrate here is that this trend is also true for shape when fish are artificially reared (figure 3.7), and that it is not only environmental conditions that cause variation in body shape (Drinan *et al.*, 2012; Fenkes, Shiels and Nudds, 2018). Much like our

results for fork length and AEW, however, no significant differences in shape were identified between wild and domesticated fish when naturally reared.

The lack of significant pairwise differences between wild and domesticated fish when naturally reared, and likewise, the artificially reared Irish saltwater (sea cage) fish, could be because these individuals were placed into the natural environment. Much like the results seen for AEW, suboptimum body morphology could have been removed through stabilising selection, again demonstrating the difference between artificial and natural optimums in phenotypes. Previous research has shown that domesticated Atlantic salmon have significantly higher swimming costs than wild fish, which in part is likely to be due to body shape (Enders, Boisclair and Roy, 2004). In the controlled artificial tank-based settings of the Norwegian and Irish (freshwater) artificially reared fish, there is little to no selection against such suboptimum body shapes. The trend seen here is therefore evidence of inherited morphological change due to artificial selection and its apparent fitness consequences in the wild.

3.4.6 Body shape – fork length, life stage and rearing

Absolute length influences shape (figure 3.6a), and as PC1 values increase, large expansions are seen in the body of the fish (figure 3.5a). Despite the interest surrounding the interactions between fork length and shape, it does mask more nuanced differences in shape between the different experimental groups that are not as comprehensively documented as size. PC2 values are not highly correlated with fork length, however, which summarise changes in head and tail shape, but also in the region between head and the pelvic fin. Differences in PC2 were present between Irish artificially reared freshwater fish and Irish artificially reared saltwater fish, with saltwater fish showing expansion in the head and tail, and a contraction in the anterior body region, particularly between the head and pelvic fin, when compared to freshwater fish. Smoltification is known to affect shape of the fish in the wild (Björnsson, Stefansson and Mccormick, 2011) into slender individuals, but shape has never been characterised empirically. In addition to this, the significant interaction terms for body shape between

life stage, genetic background and experimental origin (figure 3.6b) also suggest that the smoltification changes are dependent on genetic background, rearing type, and environment.

The difference between life stages seen in the Irish artificially reared experiments and Norwegian artificially reared experiments are possibly due to the latter experiment housing saltwater individuals in tanks, rather than a sea cage as in the Irish experiment. This adds to the relatively depauperate literature comparing tank and sea cage rearing, which has so far shown that tank rearing can match or even increase growth rates, when compared to sea cage rearing (Espmark *et al.*, 2017). Importantly, however, this demonstrates that different environments influence body shape, with sea cage rearing causing expansion in the head and tail and contraction in the anterior body region of the fish. Changes in these regions are particularly pertinent, as both the head and tail have low commercial value, whereas the anterior body region includes high value cuts such as fillets, therefore making a case for the land-based production. Shape change across PC2 between life stages in the naturally reared Irish fish showed a similar trend to the artificially reared Irish fish, but to a lesser extent, demonstrating that the sea cage reared fish display a more natural shape progression than those reared in tanks, as seen in the Norwegian experiment.

3.5 Summary

We demonstrate here that selection associated with domestication has caused significant changes to morphology in Atlantic salmon, both in Norwegian and Irish fish, however, it is possible that these changes are not being detected in naturally reared populations due to strong natural selection against maladaptive domesticated induced traits. Although because there is no farm domesticated fish based on Irish populations, only Norwegian MOWI, the role of provenance cannot be completely ruled out. Understanding that domestication is influencing morphology, and that it has a fitness consequence in the natural environment, has important ramifications for escapees from aquaculture and the corresponding genetic introgression. In addition to this, it highlights that assessing morphological

features in the wild could be futile in monitoring the effect of introgression, as the true extent of morphological change is masked by strong artificial selection.

3.6 Acknowledgements

3.7 Author contributions

W.B.P organised sampling and conducted DNA parentage analysis on Norwegian specimens, in addition to applying all landmarks, analysing the data and drafting the manuscript. J.K organised sampling for Irish specimens, in addition to statistical advice and drafting the manuscript. K.A.G. and M.F.S produced the fish for the Norwegian experiment. C.B, A.C.M and K.G.P were involved in sampling for the Norwegian experiment. A.E and A.H were involved in the DNA parentage of the fish. All authors, including those mentioned and S.C, M.L and M.T, were involved in the conception and design of the experiment and contributed to data interpretation and editing the final draft of the manuscript. K.A.G, G.C and P.M managed the project.

Chapter 4: Disentangling the effects of environment and genetics in Atlantic salmon: growth, heart and liver under common garden conditions

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Abstract

Animal domestication has long been a part of agriculture, estimated to have first occurred ~10,000 years ago. Despite the plethora of traits studied there is little understanding of the possible impacts domestication has had on internal organs, which are key determinants of survival. Moreover, the genetic basis of observed associated changes in domesticated environments is still puzzling. Here we examine impacts of captivity on two organs in Atlantic salmon (*Salar salar*) that have been domesticated for ~50 years: the heart and liver. The common garden study design enables an examination of genetic effects underlying trait variance in these organs. We studied the growth of multiple families of wild, domesticated, F1 and F2 hybrid, and backcrossed strains of *S. salar* in replicated tanks during the freshwater and marine stages of development. Heart and liver weight were investigated, along with heart morphology metrics examined in just the wild, domesticated and F1 hybrid strains (heart height and width). Growth was positively linked with the proportion of the domesticated line, and recombination in F2 hybrids, and the potential disruption of co-adapted gene complexes, did not influence growth. Despite the influence of domestication on growth, we found no evidence for domestication-driven divergence in heart morphology. However, sexual dimorphism was detected in heart morphology, and after controlling for body size, females exhibited significantly larger heart weight and heart width when compared to males. Wild females also had an increased heart height when compared to wild males, though observed only in the wild strain. Females sampled in saltwater showed significantly larger heart height with rounder hearts, than saltwater males. No effect of genetic background or sex was found on liver weight. Collectively, these results demonstrate an additive basis of growth and despite a strong influence of domestication on growth, no clear evidence of changes in heart or liver morphology associated with domestication was identified.

4.1 Introduction

The process of domestication has long been a part of agriculture, and is estimated to have first occurred ~10, 000 years ago with the domestication of goats in the western highlands of Iran (Zeder and Hesse, 2000). Domestication in fish, however, was first observed in species of tilapia and carp 4,000 years ago (Wohlfarth, 1993), with the aquaculture-driven domestication of salmonids not starting until the 1970s (Gjedrem, 2000, 2010b). Studies examining the impacts of domestication have typically focused on features such as bone shape (Shackelford, Marshall and Peters, 2013), behaviour (Eklund and Jensen, 2011) and genetics (Andersson *et al.*, 1994), and assessment of internal organs in domesticated taxa, including structural heart morphology, is little studied, though with exceptions (Bartyzel, Karbowicz and Bartyzel, 2005; Baranowski *et al.*, 2019). A higher proportion of studies looking at the impact of domestication on heart morphology have done so in fish (Poppe *et al.*, 2003; Mayer *et al.*, 2011; Pombo, Blasco and Climent, 2012). The relatively recent wide-scale domestication of fish, and in particularly salmonids, to domestication offers a valuable insight into the early stages of domestication on organs such as the heart. Moreover, domestication has proven an insightful model for exploring fundamental tenets of the extended evolutionary synthesis (Zeder, 2015, 2017).

Atlantic salmon (*Salmo salar*), an economically major aquaculture species, exhibits an anadromous life history, transitioning between freshwater and marine habitats. Throughout this diverse life history, there is an underlying mosaic of evolutionary selection pressures, acting on all aspects of biology, from morphology to behaviour. While all aspects are important to individual fitness, certain traits can affect survival to a far greater magnitude. Understandably, vital organs provide a key role in survival and success of fish, including the brain, liver, and heart, and this is particularly true of vagile taxa, where energy demands and complex migratory patterns underpin distribution and abundance. Movement in challenging habitats and long-distance migrations characterise the life history of Atlantic salmon where individuals can migrate thousands of kilometres across their life time (Mccormick *et al.*, 1998). Not only are great distances covered, but individuals tackle strong freshwater currents en route to

natal breeding grounds, along with numerous topographic barriers, such as waterfalls and rapids. A component required for such intense and protracted activity, in combination with musculature and body-shape, is strong cardiac function (Agnisola and Tota, 1994).

Although heart morphology has been shaped by natural selection, in recent decades, the evolutionary trajectory of wild fish has changed due to domestication in aquaculture (Glover *et al.*, 2017). Atlantic salmon aquaculture was initiated in the early 1970s, and cultured fish of up to ~13 generations of domestication and directional selection for economically important traits now form the basis of the industry (Gjedrem, 2000). Captive propagation under unnatural conditions has resulted in a wide variety of genetic differences between domesticated and wild salmon (reviewed by (Glover *et al.*, 2017)), the most notable of which, growth, now displays up to several-fold increase in domesticated salmon (Einum and Fleming, 1997; Fleming *et al.*, 2002; Glover, Otterå, *et al.*, 2009; Wolters *et al.*, 2009; Solberg, Skaala, *et al.*, 2013; Harvey, Glover, *et al.*, 2016; Harvey, Solberg, Troianou, *et al.*, 2016; Solberg *et al.*, 2016; Glover *et al.*, 2018a). In addition to growth, directional selection has also impacted traits such as delayed maturation (Glover, Otterå, *et al.*, 2009; Fraser *et al.*, 2010; Morris *et al.*, 2011) and fillet quality (Tsai, Hamilton, Guy, *et al.*, 2015), while also permitting inadvertent or hitchhiking-selection resulting in trait shifts such as predator avoidance behaviour (Houde, Fraser and Hutchings, 2010a), sexual morphology (Perry *et al.*, 2019) and stress susceptibility (Solberg, Zhang, *et al.*, 2013).

Each year, thousands or hundreds of thousands of domesticated salmon escape into the wild, and where it has been studied, extensive introgression of domesticated salmon has been observed in many wild populations (Keyser *et al.*, 2018). Although improvements in infrastructure have reduced the incidence of reported escapees, monitoring programs demonstrate that large numbers of domesticated escapees on spawning grounds of some rivers still remain (Glover *et al.*, 2019). Such wild-farmed interactions are a cause for concern given the shifts in traits of domesticated individuals and wild-domesticated hybrids, as well as domesticated and hybrid fish having reduced fitness in the wild (Fleming, Hindar, Mjölneröd, *et al.*, 2000; McGinnity *et al.*, 2003; Skaala, Kevin A. Glover, *et al.*,

2012; Skaala *et al.*, 2019), thereby compromising the genetic integrity, and long-term fitness of wild populations (Glover *et al.*, 2017). Understanding changes in the biology of domesticated fish is therefore not only relevant to understanding the processes and changes during domestication, they are fundamental to our understanding of how wild-farmed interactions impact on wild populations and communities (Naylor *et al.*, 2005; Jensen *et al.*, 2010).

In addition to the traits discussed above, it has also been suggested that domestication may drive genetic changes in Atlantic salmon heart morphology, going beyond plastic responses to the aquaculture environment (Poppe *et al.*, 2003; Gamperl and Farrell, 2004). These studies have revealed differences in heart morphology between farmed and wild fish. However, as the fish were not reared under common environmental conditions, it remains challenging to disentangle the relative impacts of genetics and environment on observed differences. Other traits, such as spot patterns, also show large differences between wild and farmed Atlantic salmon, and common-garden studies have demonstrated that this is primarily a plastic response (Jørgensen *et al.*, 2018). Here, we investigated whether genetic differences in heart morphology could be detected between domesticated and wild salmon when reared in a common-garden design. We also investigated growth differences among strains, with second-generation crosses, providing a contrast with the influence of domestication on heart morphology.

4.2 Materials and methods

4.2.1 Overall experimental design

In order to investigate potential genetic differences in growth between domesticated, wild, F1 hybrid, F2 hybrid and backcrossed salmon, seven synchronously-produced experimental strains, each with multiple pedigree-controlled families, were reared in a common garden environment from hatching onwards. The same fish were used to assess heart morphology (including adjusted heart height, adjusted heart width and heart width-height residuals) in the domesticated, F1 hybrid and wild strains

(table 4.1). Fish were sampled both as aged 1+ smolts in freshwater after being reared in replicated tanks, and as post-smolts at aged 2+ in replicated saltwater tanks.

Table 4.1 Number of fish used for wet weight, heart weight and heart morphology measures such as adjusted heart height (AHH), adjusted heart width (AHW) and heart width-height residuals (WHR). Counts are broken down by life stage (freshwater or saltwater), experimental strains and sex.

Origin			wet weight		heart weight		heart morphology (AHH, AHW, WHR)	
type	strain	location	freshwater	saltwater	freshwater	saltwater	freshwater	saltwater
domesticated	Mowi		♂ = 98 ♀ = 80	♂ = 72 ♀ = 93	♂ = 43 ♀ = 48	♂ = 34 ♀ = 42	♂ = 8 ♀ = 15	♂ = 12 ♀ = 15
hybrid	Figgjo (♀) × Mowi (♂)		♂ = 59 ♀ = 38	♂ = 52 ♀ = 32	♂ = 34 ♀ = 24	♂ = 24 ♀ = 16	♂ = 14 ♀ = 1	♂ = 13 ♀ = 9
	Mowi (♀) × Figgjo (♂)		♂ = 57 ♀ = 39	♂ = 53 ♀ = 43	♂ = 23 ♀ = 23	♂ = 16 ♀ = 16	♂ = 5 ♀ = 3	♂ = 6 ♀ = 8
F2 hybrid			♂ = 95 ♀ = 76	♂ = 92 ♀ = 98	♂ = 50 ♀ = 43	♂ = 45 ♀ = 43	✕	✕
wild	Figgjo	58°81′N, 5°55′E	♂ = 103 ♀ = 81	♂ = 72 ♀ = 76	♂ = 54 ♀ = 52	♂ = 29 ♀ = 28	♂ = 13 ♀ = 12	♂ = 10 ♀ = 13
wild backcross			♂ = 90 ♀ = 78	♂ = 86 ♀ = 87	♂ = 36 ♀ = 41	♂ = 33 ♀ = 38	✕	✕
domesticated backcross			♂ = 84 ♀ = 78	♂ = 92 ♀ = 79	♂ = 47 ♀ = 44	♂ = 45 ♀ = 30	✕	✕

4.2.2 Experimental Fish

The fish used here were established at the Matre Research Station (60°52'26.4"N, 5°35'09.0"E), with fertilisation of gametes occurring on 1st December 2015 following a wild, domesticated, hybrid, backcross common garden design, implemented over a decade by the Institute of Marine Research (Glover, Otterå, *et al.*, 2009; Solberg, Skaala, *et al.*, 2013; Solberg *et al.*, 2014; Bicskei *et al.*, 2016; Harvey, Glover, *et al.*, 2016). Seven experimental strains were used to identify differences in body weight, heart weight and liver weight: a wild strain, a domesticated strain (developed by Mowi and domesticated for ~13 generations), F1 hybrids, F2 hybrids, wild x hybrid backcrosses, and domesticated x hybrid backcrosses (table 4.1). With a subset of wild, domesticated and reciprocal F1 hybrids used to investigate heart morphology. Individuals from all strains were mixed into four replicate tanks, with the first two tanks representing the freshwater stage of the lifecycle terminated between the dates 25th - 28th April 2017. The second two tanks representing the saltwater stage of the

lifecycle were terminated a year later between the dates 23rd – 27th April 2018, after having been transferred to saltwater in June 2017. Fish were fed on a standard commercial aquaculture diet and experienced natural light regime for Bergen, Norway.

Fish were terminated using an anaesthetic overdose of MS-222. All researchers working directly with the experimental animals had undergone Norwegian Food Safety Authority (NFSA) training, in compliance with experimentation involving live animals, included in the Animal Welfare Act (Norway). As the fish in the experiment were not further manipulated, and exposed only to standard rearing conditions, no specific research permit was required.

4.2.3 Body weight

Wet body weight, hereon referred to as body weight, was measured on 1159 fish in the freshwater life stage, and 1043 fish in the saltwater life stage (table 4.1). A fin-clip for DNA-family identification was taken from all individuals during sampling.

A linear mixed effect model was used to assess factors influencing weight with the R package 'lme4' (Bates *et al.*, 2015). The response variable for the linear mixed effect model was log10 transformed bodyweight. The full models contained the fixed factors: life stage, sex, strain and all two-way interactions, along with random factors: date of dissection, dam, sire, family nested in strain and tank. Random factors in the model included sampling date, tank, dam, sire and family nested in strain. The full model was simplified using the 'step' function within 'lme4' through automatic backward elimination removing fixed terms and random factors which did not contribute to the model. Analysis of variance type III sum of squares with Satterthwaite approximation for degrees of freedom allowed for the generation of p values using the R package 'lmerTest' (Kuznetsova, Brockhoff and Christensen, 2017). Estimated marginal means and pairwise comparisons between means were calculated using the R package 'emmeans' (Lenth, Love and Maintainer, 2018), while generating 95 % confidence intervals, and degrees of freedom using the kenward-roger approximation.

4.2.4 Dissection and heart measurements

Heart ventricles were freed from the thoracic cavity by cutting along the bulbus arteriosus and pulling away the atrium (figure 4.1a). The ventricle was used to assess overall heart morphology due to it being the largest most muscular chamber, pumping blood entering from the atrium, through the bulbus arteriosus (figure 4.1c). The ventricle is also the area of the heart that has been investigated most thoroughly in previous studies (Poppe *et al.*, 2003; Fraser *et al.*, 2015). Livers were freed from the main body cavity by making incisions along the bile duct and hepatic blood vessels. Hearts and livers were immediately weighed and placed in 4 % buffered paraformaldehyde (PFA) for fixation. Heart samples taken from freshwater life stages remained in 4 % PFA for 9 days. Due to the larger size of the heart samples taken from the saltwater life stage, these remained in 4 % PFA for 42 days. Once fixed, tissue samples were then moved to 70 % ethanol for long-term storage.
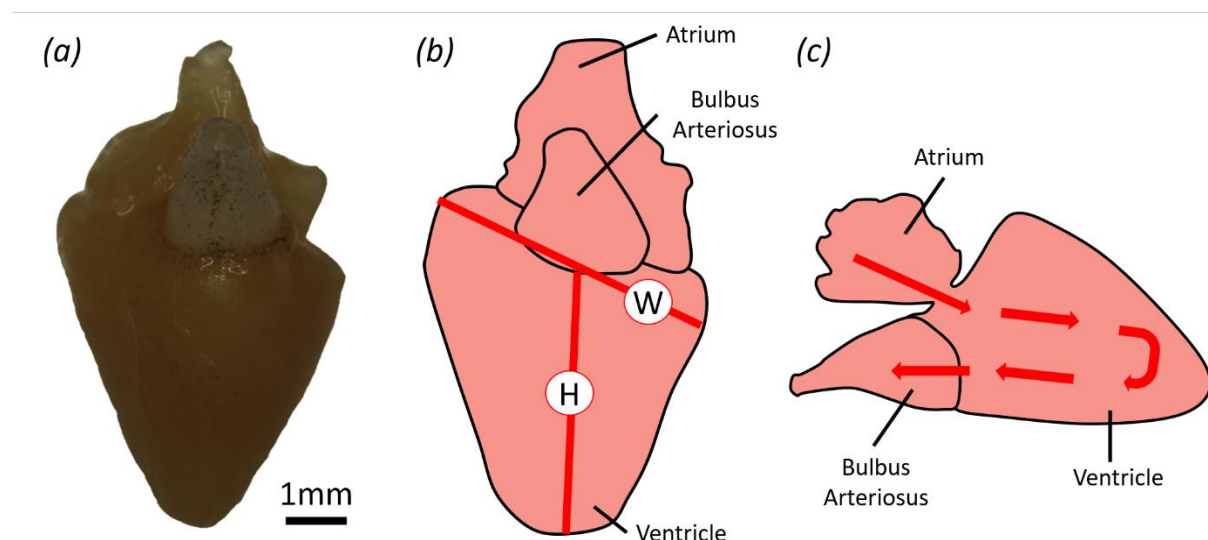
Figure 4.1 Anatomy of the Atlantic salmon (*Salmo salar*) heart, as demonstrated by (a) photograph microscopy of a heart from the freshwater life stage, as well as diagrammatically (b & c). Red lines in (b) labelled with the letters H and W represent the measurements heart height and heart width, respectively. Arrows displayed in (c) show the direction of blood flow within the single circulatory system of the teleost heart.

Height and width of the heart ventricle were measured using callipers, as outlined in figure 4.1b, and were based on previous studies assessing heart morphology in Atlantic salmon (Poppe *et al.*, 2003). The height of the heart is defined here as the line extending from the base of the bulbus arteriosus to the apex of the heart, and the width is defined as the widest length of the ventricle parallel to the base. Heart ventricle height and width are hereon in referred to as heart height and heart width. All heart dissection and measurements were taken by one person without knowledge of the genetic background of the fish.

4.2.5 Secondary morphological measures

A linear regression was constructed between log10 heart height and log10 fork length, as well as log10 heart width and log10 fork length (figure A4.1). Residuals from this linear regression were then used as fork length adjusted heart height (AHH) and fork length adjusted heart width (AH width). A linear regression was also constructed between log10 heart weight and log10 body weight, as well as log10 liver weight and log10 body weight. Residuals from this linear regression were then used as body weight adjusted heart weight (AH weight) and body weight adjusted liver weight (ALW). Finally, a linear regression was constructed between log10 heart width and log10 heart height, residuals from this linear regression, herein described as width-height residuals (WHR). Measurements relating to AHH, AH width and heart WHR were not conducted on backcross or F2 hybrid strains. Backcross and F2 hybrid strains were used for AH weight and ALW, however.

4.2.6 Analysis of heart and liver measurements

When analysing AH weight, AHH, AH width, WHR and ALW each were used as separate response variables in five linear mixed effect models (LME) that were constructed using the R package 'lme4' (Bates *et al.*, 2015). The full models contained the fixed factors: life stage, sex, strain and all two-way interactions, along with random factors: date of dissection, dam, sire, family nested in strain and tank. Models were then simplified using the 'step' function in the package 'lme4'. Estimated marginal means

and pairwise comparisons between means were calculated using the selected models and the R package 'emmeans' using the Tukey's multiple-testing adjustment (Lenth, Love and Maintainer, 2018).

4.3 Results

4.3.1 Body weight

Body weight increased in line with the proportion of the domesticated line within each of the seven experimental groups (LME Strain: $F_{6,28}$ = 38.70, Sum Sq = 5.84, p < 0.01) (figure 4.2). The final model contained the fixed factor strain, sex, life stage, an interaction term between strain and life stage, an interaction term between sex and strain, as well as the random factors family nested in strain, and tank. Significant pairwise differences (P ≤ 0.05) in mean body weight were observed in 15/21 pairwise comparisons (table A4.1). Thus, both F1 and F2 hybrids displayed intermediate body weight to the wild and domesticated strains, while both backcrossed variants displayed body weight intermediate between hybrids and their respective wild (25% domesticated line) or domesticated (75% domesticated line).
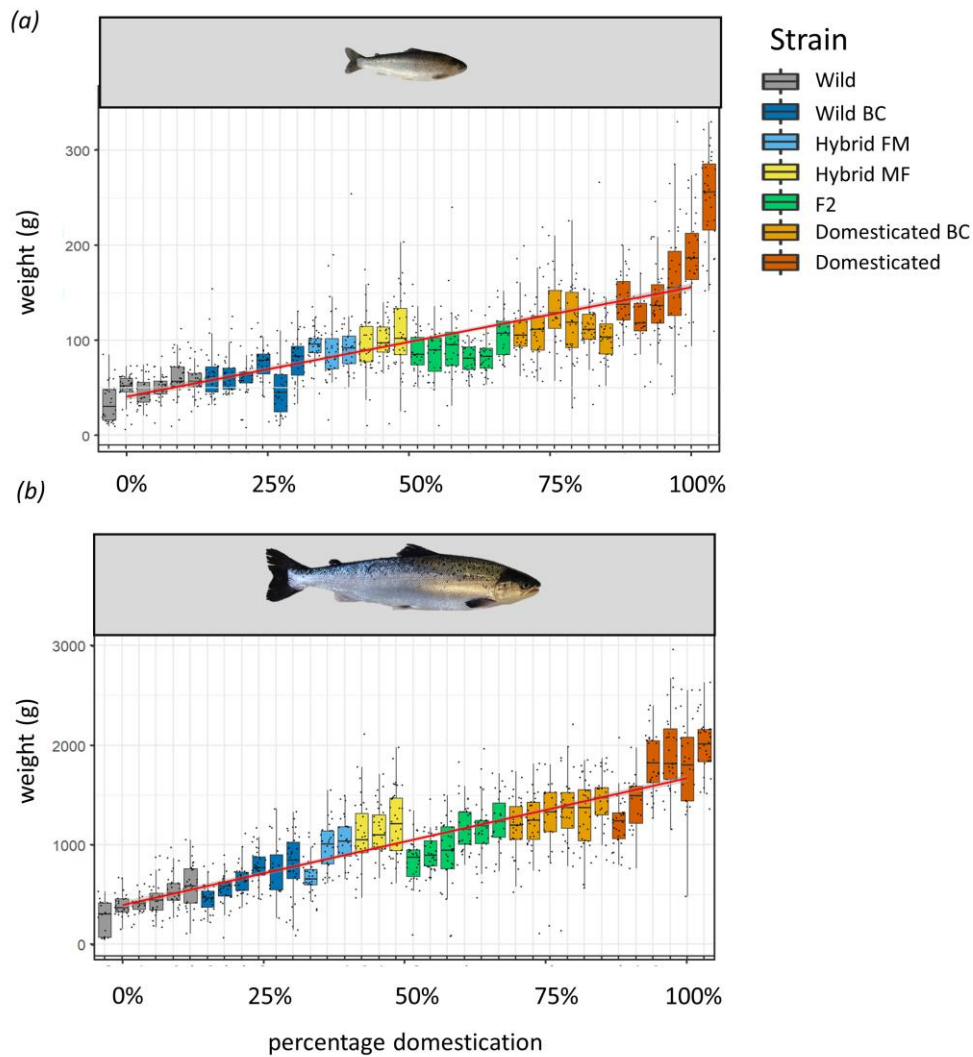
Figure 4.2 Boxplots of wet weight in grams between both (a) freshwater and (b) saltwater individuals, further broken down into the families that make up the seven experimental strains (wild, wild backcross (wild BC), hybrid FM (Figgjo (♀) × Mowi (♂)), hybrid MF (Mowi (♀) × Figgjo (♂)), F2 hybrids, domesticated backcross (domesticated BC) and domesticated (Mowi)), as shown by the different colours. Additionally to the boxplots of wet weight per family, there is also a linear regression, as shown in red, that was run between the percentage levels of domestication, including 0% (wild), 25% (wild BC), 50% (F1 and F2 hybrids), 75% (domesticated BC) and 100% (domesticated).

There was also a significant effect of sex on body weight (LME Sex: $F_{1,2021}$ = 11.28, Sum Sq = 0.283, p < 0.01), in addition to a significant interaction between sex and strain (LME Sex * Strain: $F_{6,2022}$ = 5.50, Sum Sq = 0.83, p < 0.01). A sex difference was only seen in the wild strain (t_{2024} = 4.54, p < 0.01) and the wild backcross strain (t_{2028} = 4.81, p < 0.01), whereby females had larger body weight than males. All other differences between sexes within a strain were non-significant (p > 0.05) (table A4.2).

Table 4.2 p values from the linear mixed effect model for the differences in log10 mean weight between strains, in freshwater and saltwater life stages, along with the trend in log10 mean weight between those strains. All trends between strains were consistent between life stages. Significance of trends, as assessed by p values (P < 0.05), were not consistent between life stages, however. Yellow highlighted trends show that the trend became significant in the saltwater life stage but were not significant in the freshwater life stage. Red highlighted trends show that the trend lost significance in the saltwater life stage but was significant in the freshwater life stage.

contrast	freshwater	saltwater	trend between strains
Wild - Wild.BC	0.2403	0.0193	increase
Wild - Hybrid.FM	0.0004	<.0001	increase
Wild - Hybrid.MF	0.0001	<.0001	increase
Wild - F2	<.0001	<.0001	increase
Wild - Domesticated.BC	<.0001	<.0001	increase
Wild - Domesticated	<.0001	<.0001	increase
Wild.BC - Hybrid.FM	0.158	0.2095	increase
Wild.BC - Hybrid.MF	0.0723	0.001	increase
Wild.BC - F2	0.0795	0.0022	increase
Wild.BC - Domesticated.BC	0.0003	<.0001	increase
Wild.BC - Domesticated	<.0001	<.0001	increase
Hybrid.FM - Hybrid.MF	1	0.8393	increase
Hybrid.FM - F2	1	0.9992	increase
Hybrid.FM - Domesticated.BC	0.9686	0.2008	increase
Hybrid.FM - Domesticated	0.0051	0.0007	increase
Hybrid.MF - F2	1	0.9946	decrease
Hybrid.MF - Domesticated.BC	0.9974	0.9998	increase
Hybrid.MF - Domesticated	0.0134	0.1651	increase
F2 - Domesticated.BC	0.6853	0.4591	increase
F2 - Domesticated	0.0001	0.0008	increase
Domesticated.BC - Domesticated	0.0385	0.3205	increase

As would be expected, life stage showed a strong influence over body weight (LME Life stage: $F_{1,2}$ = 2031.39, Sum Sq = 51.05, p < 0.01). In addition, there was a strong significant interaction between

strain and life stage (LME Strain * Life stage: $F_{6,2018}$ = 7.28, Sum Sq = 1.10, p < 0.01). Once pairwise differences between strains were partitioned into life stages, 11/14 strains showed significant differences in fresh water, compared to 12/14 strains in the saltwater life stage (Table 4.2). Some strains displayed significant differences in body weight in freshwater, but not in saltwater, and vice versa. Differences between the wild backcross strain and the wild strain, the hybrid FM strain and the F2 strain went from being non-significant in the freshwater life stage measurement (P > 0.05) to significantly different in the saltwater life stage measurement (table 4.2). Alternatively, differences between the domesticated strain and the hybrid MF and domesticated backcross strain went from being significant in freshwater to non-significant (P > 0.05) in the saltwater life stage (table 4.2). The difference in significance demonstrates that in the freshwater life stage, the increases in log10 body weight seen with increased domesticated line (up to 50 %) are accentuated to the point of significance, which is not seen in saltwater. The opposite is seen with strains that have over 50 % domesticated line, with the significant increases in log10 body weight minimised in the saltwater life stage, in comparison to the freshwater. Variation in growth due to family background (S.D. = 0.07) and tank (S.D. = 0.02) was detected and controlled for as random factors in the linear mixed effect model.

4.3.2 Liver weight

There were no significant effects of life stage, sex, strain or any of the interaction terms on ALW.

4.3.3 Heart morphology - Adjusted heart weight

Strain and life stage showed no significant effect on adjusted heart (AH) weight. The final model contained sex as a fixed factor, as well as family nested in strain and date of dissection as random factors. Sex was shown to be the only significant factor influencing AH weight (LME Sex: $F_{1,965}$ = 6.28, Sum Sq = 0.04, P = 0.01), with female fish having significantly larger AH weight (estimated mean = 0.011) to their male counterparts (estimated mean = -0.002) (t(964) = 2.50, p = 0.01) (figure 4.3a). Variation in AH weight due to family background (S.D. = 0.03) and dissection date (S.D. = 0.02) were detected and controlled for as random factors in the linear mixed effect model.
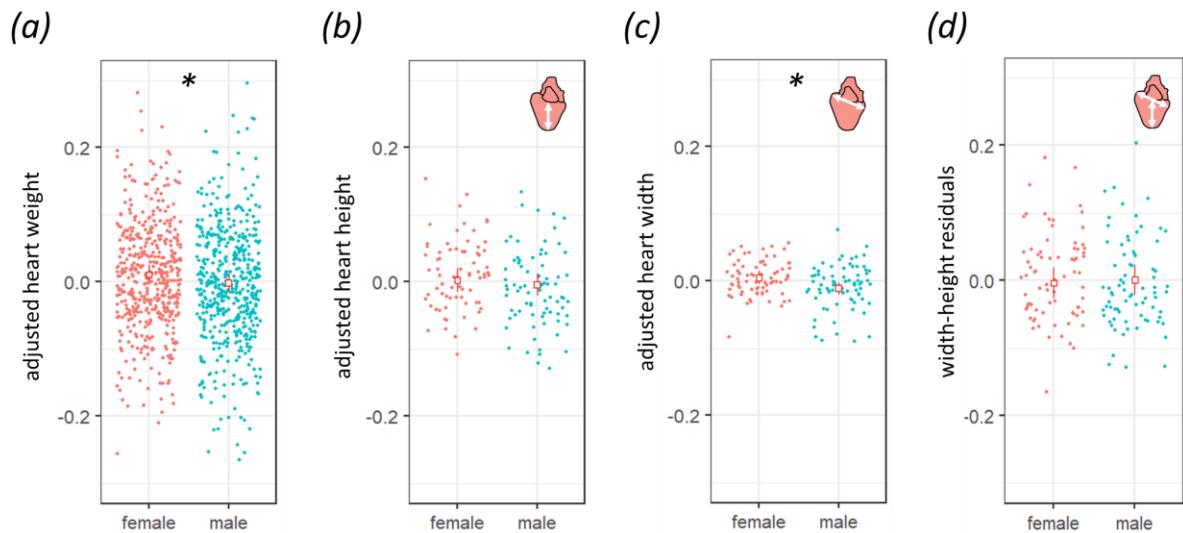
Figure 4.3 Estimated marginal means and confidence intervals from the linear mixed effect models for (a) adjusted heart weight (AH weight), (b) adjusted heart height (AHH), (c) adjusted heart width (AH width) and (d) width-height residuals (WHR). Results are split between sexes. Significant differences between the sexes are indicated with an asterisk. It should also be highlighted that there are significant interaction terms with sex for (b) AHH and (d) WHR.

4.3.4 Heart morphology - Adjusted heart height & adjusted heart width

Strain did not show a significant effect on adjusted heart height (AHH) (LME Strain: $F_{3,13}$ = 1.57, Sum Sq = 0.010, P > 0.05). A LME was run with response variable AHH, and after the step function, the final model contained the fixed factors: life stage, sex, strain, an interaction term between sex and life stage, an interaction term between sex and strain, and the random factor family nested in strain. Life stage showed a significant effect on AHH (LME Life stage: $F_{1,140}$ = 5.80, Sum Sq = 0.013, P = 0.02) (figure 4.4), with a significant difference in estimated means between freshwater (estimated mean = - 0.011) and saltwater (estimated mean = 0.009) (t_{140} = 2.39, p = 0.02); with a further significant interaction between life stage and sex (LME Life stage*sex: $F_{1,137}$ = 7.96, Sum Sq = 0.017, P < 0.01) (figure 4.4). Sex alone was not significant (LME Sex: $F_{1,137}$ = 0.57, Sum Sq = 0.001, P = 0.45). The significant interaction between sex and life stage was driven by a significant difference between freshwater females and

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saltwater females (t_{140} = 3.53, p < 0.01). Saltwater females had a significantly larger AHH (estimated mean = 0.024) than freshwater females (estimated mean = -0.019). The different between freshwater males and saltwater males was not significant (t_{137} = 0.27, p > 0.05). There was also a significant difference between saltwater females and saltwater males (t_{139} = 2.85, p = 0.03), whereby saltwater females had significantly larger AHH (estimated mean = 0.024) than saltwater males (estimated mean = - 0.006). Finally, there was a significant interaction term between sex and strain (LME Sex*strain: $F_{3,135}$ = 3.72, Sum Sq = 0.024, P = 0.01), driven entirely by the difference between male and female wild fish (t_{142} = 3.441, p = 0.02), where female wild fish have a larger AHH (estimated mean = 0.010) than male wild fish (estimated mean = -0.041). Variation in AHH due to family background (S.D. = 0.02) was detected and controlled for as a random factor in the linear mixed effect model.
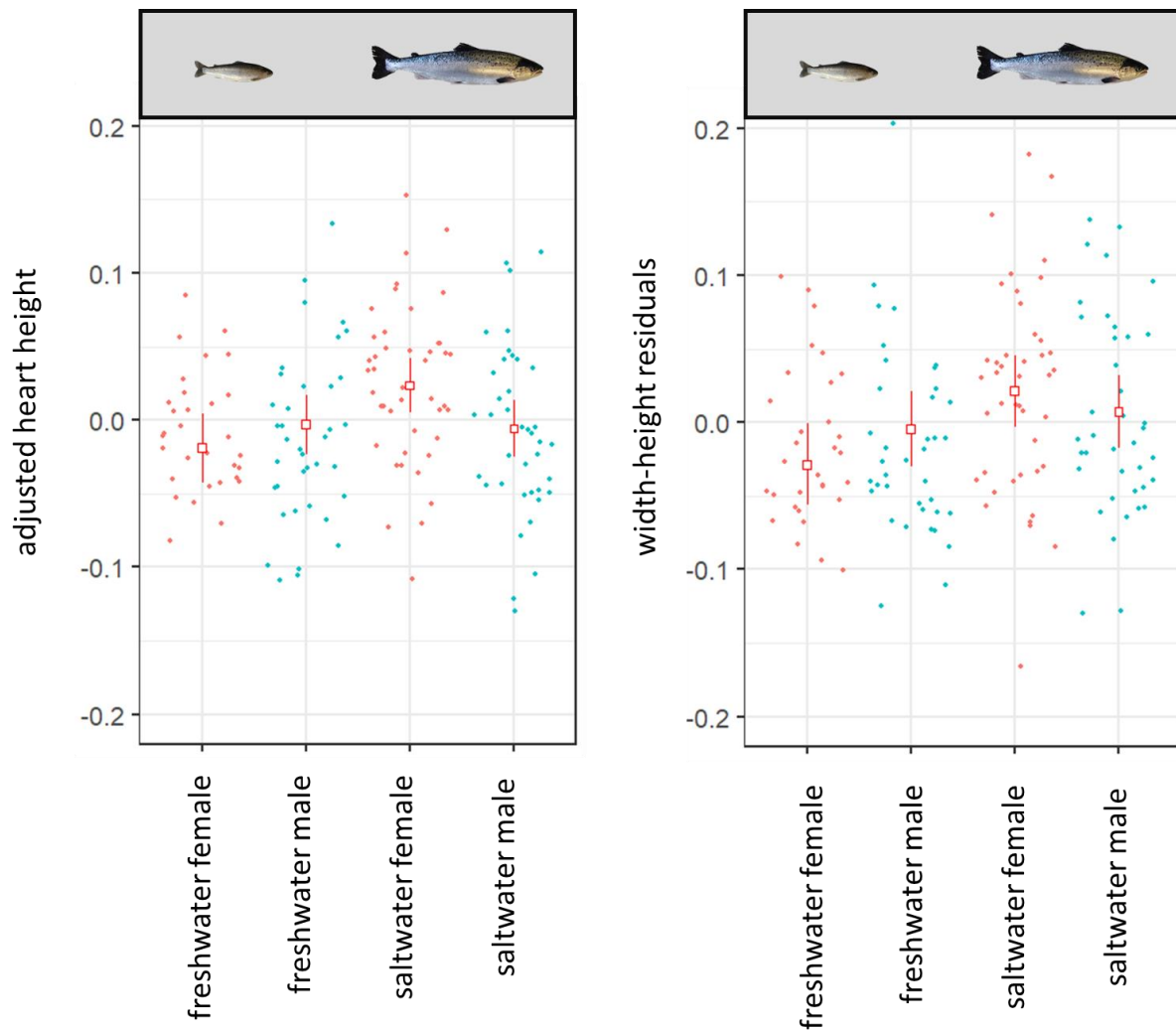
Figure 4.4 Estimated marginal means and confidence intervals from the linear mixed effect models for adjusted heart height (AHH) and width-height residuals (WHR). Results are split into freshwater and saltwater life stages, as well as between sexes.

Strain did not show a significant effect on adjusted heart width (AH width), and neither did life stage. The same full model for AHH was applied to the response variable AH width, and after the step function, the final model contained the fixed factor sex and the random factor family nested in strain. Sex showed a significant effect on AH width (LME Sex: $F_{1,145}$ = 12.74, Sum Sq = 0.009, P < 0.01), with female fish showing significantly larger AH width (estimated mean = 0.0051) when compared to males

(estimated mean = - 0.0111) (figure 4.3c). Variation in AH width due to family background (S.D. = 0.01) was detected and controlled for as a random factor in the linear mixed effect model.

4.3.5 Heart morphology - Heart width-height residuals

Strain did not show a significant effect on heart WHR. The same full model as for AH weight, AHH and AH width was applied to the response variable WHR and after the step function, the final model contained the fixed factor life stage and an interaction term between sex and life stage, as well as the random factor family nested in strain. A significant effect on WHR was seen for life stage (LME Life stage: $F_{1,42}$ = 10.51, Sum Sq = 0.032, P < 0.01), with an increase in WHR seen in saltwater individuals when compared to freshwater; this is equivalent to a lower H:W ratio, and thus a more rounded heart in saltwater individuals, as is described by Poppe *et al.* (2003). We acknowledge here that although individuals with an increased WHR are described as rounded due to overall heart shape, as in Poppe *et al.* (2003), the tip of the ventricle appears less rounded in round hearted individuals (figure 4.5).

	Domesticated	Hybrid	Wild
Rounded			
Not rounded			
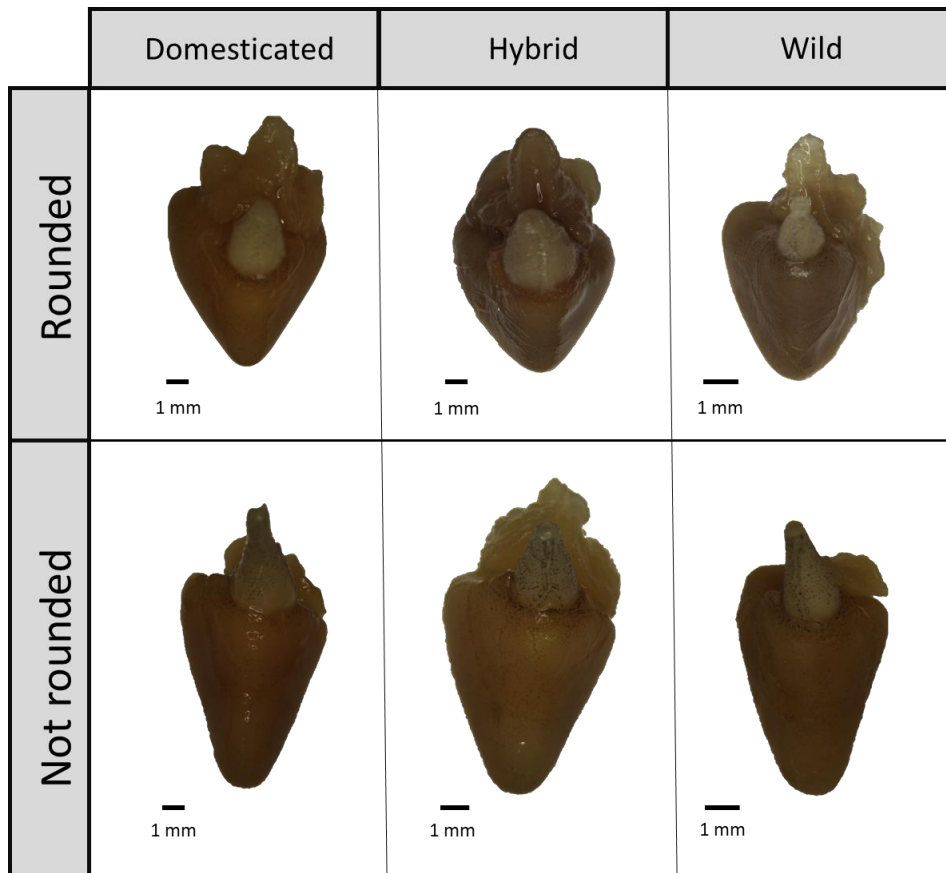
Figure 4.5 Examples of difference in heart shape in domesticated, hybrid and wild fish. As discussed in previous literature, there has been an interest in how round the ventricle is, as defined by the relationship between the height and width. Here, as in Poppe et al. (2003), rounded hearts are those which have more equal height and width measurements, which is characterised by a lower H:W ratio (closer to 1), or a higher width-height residual (WHR). What is demonstrated here is the rounded and not rounded morphology can be found in domesticated, hybrid and wild strains.

There was also a significant interaction term between life stage and sex in the final model (LME Life stage*sex: $F_{1,139}$ = 4.03, Sum Sq = 0.012, P = 0.05) (figure 4.4) which was driven by the significantly larger WHR seen in saltwater females (estimated mean = 0.021) when compared to freshwater females (estimated mean = -0.029) (t_{143} = 3.60, p < 0.01), while males showed no significant difference between life stages. Variation in WHR due to family background (S.D. = 0.03) was detected and controlled for as a random factor in the linear mixed effect model.

4.4 Discussion

To our knowledge, this is the first study to investigate differences in heart-morphology between domesticated and wild Atlantic salmon reared in common garden conditions, and, the first to present extensive growth data on a full matrix of F2-generation crosses and backcrosses. Based upon the described experimental conditions, we observed large differences in body weight between the seven strains investigated, positively linked to the proportion of domesticated line. However, despite domestication playing a major positive role in body weight and thus growth, we found no evidence of domestication-driven divergence in heart morphology. Based on these results, we conclude that while domestication has strongly impacted Atlantic salmon growth capacity, primarily following an additive genetic model (Quaas, 1988), no detectable effects in heart morphology have arisen through ~13 generations of domestication.

4.4.1 Growth

The impact of domestication on growth and body size using common garden experiments has been examined multiple times in Atlantic salmon, under differing experimental manipulations (Einum and Fleming, 1997; Fleming *et al.*, 2002; Glover, Otterå, *et al.*, 2009; Wolters *et al.*, 2009; Solberg, Skaala, *et al.*, 2013; Harvey, Glover, *et al.*, 2016; Harvey, Solberg, Troianou, *et al.*, 2016; Solberg *et al.*, 2016; Glover *et al.*, 2018a). Collectively, these studies demonstrate that under identical hatchery conditions with unlimited access to food, domesticated salmon grow faster than their wild counterparts (typically two to four-fold) due to directional selection for this trait in breeding programmes. However, the present study has the novel addition of both backcrossed variants (i.e., backcrossed to domesticated and wild fish), as well as F2 hybrids. Examining backcross and F2 hybrids allows us to better understand the impact of introgression on phenotypes beyond one generation, involving processes such as recombination; as would be experienced by the progeny of escapees in the wild. The observed relationship between mean strain growth and its proportion of domesticated line, from 0-25-50-75-100%, demonstrate the primarily additive effect of genetic background. I.e., backcrossed strains

showed an intermediate body size between the reciprocal F1 hybrids and their respective generator wild or domesticated strains (figure 4.2). Furthermore, the F2 hybrid strain showed a similar body weight to the F1 hybrid strains (figure 4.2). Therefore, as the genetic contribution of domesticated and wild backgrounds is still 50% in both the F1 and F2 hybrid strains, only being recombined in F2 hybrids, this shows that this recombination, and potential disruption of co-adapted gene complexes, has not influenced growth rates.

Finally, an interaction between sex and strain was shown to be significant across both life stages. The difference in body weight between males and females was being driven by the sex differences within the wild and wild backcross strains, with larger body weight in females. Once the proportion of domesticated line increased >25 %, differences in body weight between the sexes disappeared. Sexual dimorphism in size has been observed in Atlantic salmon previously, showing larger body size in immature smolt males when compared to immature smolt females, the opposite to what described here; however, this previous experiment was conducted on a domesticated strain, rather than on fish from a wild genetic background (Leclercq *et al.*, 2010).

4.4.2 Heart morphology – Life stage

There were significant effects of life stage on two, albeit linked, aspects of heart morphology: adjusted heart height (AHH) and width height residuals (WHR), which were also combined with a significant interaction term with sex in both cases (figure 4.4). Saltwater individuals were seen to have larger AHH which was driven by saltwater females increase over freshwater females, and the increase in saltwater females over freshwater males. Saltwater individuals were also seen to have a higher WHR, with larger WHR values associated with a more rounded heart shape, which again was driven by the increase seen in saltwater females over freshwater females, but also the increase seen between saltwater males over freshwater females. One possible explanation, as the larger AHH is observed in saltwater females, is that this feature could help with the metabolic load associated with gamete production. However,

further study into understand how larger AHH and rounding of the heart relate to stroke volume and overall cardiac performance would be crucial to exploring this hypothesis.

4.4.3 Heart morphology – Sexual dimorphism

Females were seen to have a significantly larger AH weight and AH width when compared to males, as well as saltwater females showing larger AHH when compared to saltwater males and freshwater females (figure 4.3). Sexual dimorphism in heart weight in salmonids has been examined previously, with studies often reporting larger heart sizes in males or no sexual dimorphism at all; however, of these studies, few have done so in a statistically robust manner. For example, studies have either not taken into account variation in body size (Davie and Thorarensen, 1996), or body size is considered through simple divisional indexes such as relative heart mass (RHM) (Graham and Farrell, 1992; Armstrong and West, 1994; Clark *et al.*, 2009; Fraser *et al.*, 2015). On occasions where gonad weight is included in total body weight in mature fish, and is then used to calculate relative heart mass, dimorphism is seen; however, if gonad weight had not been included in total body weight, the significant traces of dimorphism disappear (Clark *et al.*, 2009). The use of ratios and divisional indexes has been widely criticised in the literature, as they are inadequate for removing size correlations from morphological data (Albrecht, Gelvin and Hartman, 1993; García-Berthou, 2001).

The main divisional index used to adjust heart weight for body weight is relative heart mass (RHM). RHM is calculated using the following formula: heart weight (g) / body weight (g) x 100. The regression between AH weight and fish weight for the saltwater individuals was not significant, and had a low R^2 value ($R^2 = 0.006$, $F_{(1,425)} = 2.57$, $P = 0.11$); the regression between RHM and fish weight, however, was significant ($R^2 = 0.060$, $F_{(1,423)} = 26.81$, $P < 0.001$) (figure 4.6 a & b). Randomly simulated data (figure 4.6 c) demonstrates the inverse relationship that RHM has with fish weight, and that when fitted with a linear regression, produces a significant negative correlation under the null model. To further show the problems of using RHM, the same full model for AH weight was fitted sing RHM as the response variable. After the step function, the final model contained the fixed factors sex and life

stage as well as the random factors family nested in strain and dissection date. A significant effect on RHM was seen for life stage (LME Life stage: $F_{1,15}$ = 61.83, Sum Sq = 0.050, P < 0.001), with a significant increase in RHM seen in freshwater individuals when compared to saltwater. Sex was also a significant effect (LME Sex: $F_{1,967}$ = 10.64, Sum Sq = 0.009, P < 0.01), with a significant increase in RHM seen in males when compared to females. This inverse result of sex effect when using RHM rather than AH weight in this dataset, and the inherent significant negative correlation you get under a null model, raises the concern that previous studies reporting larger male heart sizes are doing so due to body size being inadequately controlled for.
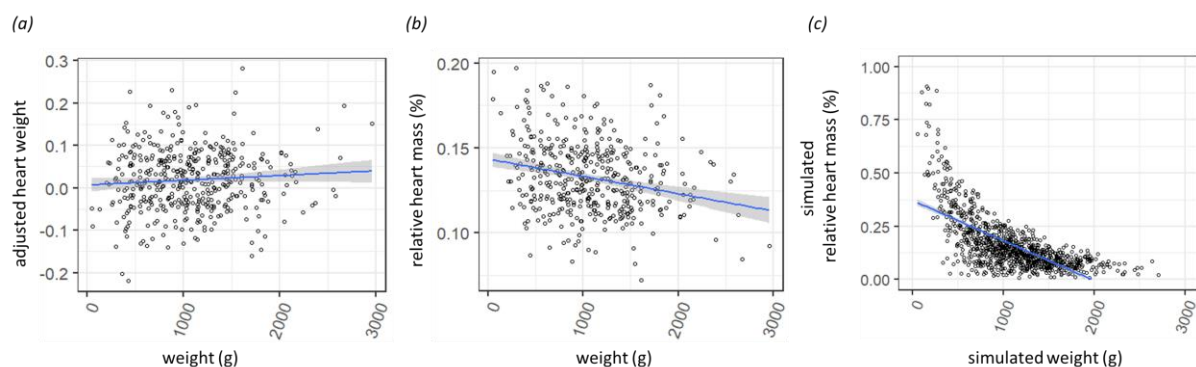
Figure 4.6 Regression plots between saltwater fish weight and two methods of removing the impact of fish weight on heart weight. The first is what is used in this study, (a) adjusted heart weight (AH weight), which are residuals from a regression between fish weight and heart weight. The second is what has been widely used in previous studies, (b) relative heart mass (RHM). Additionally, there is (c) neutrally simulated data, whereby random simulations (using the range, standard deviation and mean of the observed heart and body weight), displays the inverse relationship between RHM and fish weight.

If results from previous studies are generically applicable, that is male salmonids have a larger heart weight than females relative to body weight, then results here indirectly demonstrate that sexual dimorphism in these previous studies is due to environmental factors linked with sex, rather than an

intrinsic feature. Environmentally driven sexual dimorphism with males having a larger heart weight would suggest that males take part in actives that require higher oxygen demands than females. We would therefore not pick up these differences, as our common experimental design controls for environmental conditions. The intrinsically larger AH weight and AH width seen here in females could be linked with metabolically expensive activities that are not dependant on the environment, such as preparation for oogenesis, if indeed a larger AH weight and AH width does relate to increased functional capacity. To test the effect of environment further, reciprocal 'wild' common garden studies must be conducted, in addition to the hatchery common garden study outlined here. Monitoring the levels of activity in the fish and experimental groups of differing aerobic training in a laboratory setting could also elucidate the role of environment on heart sexual dimorphism.

We show here that there was also an interaction term between sex and strain for AHH. Only in the wild strain were there differences in AHH between sexes, with female fish having a significantly larger AHH than males. The sexual dimorphism in AHH disappears in strains that contain domesticated portions of the line, and so it can be assumed that the sexual dimorphism has been selected against, either directly or indirectly in aquaculture. A possible scenario whereby this could be envisaged is through wild females undertaking a metabolically expensive process that is no longer required in aquaculture, and so has been selected against in the trade off with artificial selection for growth. Such an activity could be linked with sexual selection, which is completely removed in the aquaculture setting, and has been reported to have changed other morphological features in Atlantic salmon (Perry *et al.*, 2019). Alternatively, as reproductive success in females is dependent on oogenesis, access to territories and nest quality (Fleming, 1998), and as oogenesis takes place in aquaculture, by deduction, it is possible that a longer AHH could be beneficial in finding access to territories, or for building and maintaining quality nests. To fully understand why there is a sexual dimorphism in wild fish, more wild strains should be examined, as it is possible this dimorphism could differ between populations.

4.4.4 Heart morphology – Strain

We found no effect of genetic background on the heart morphology metrics used here, unlike in a related study by Poppe *et al.* (2003). The lack of differences between domesticated and wild Atlantic salmon seen in this study is not an isolated account, however, with other aspects of cardiac health in Atlantic salmon not differing between the two strains (Dunmall and Schreer, 2003). One difference between this study and the previous study by Poppe *et al.* (2003) is that here we use the width-height residual (WHR) metric instead of the H:W ratio. The problems with divisional indexes and ratios are discussed above, in the case of RHM, with literature outlining how they can contribute to spurious self-correlations (Jackson, Harvey and Somers, 1990). We therefore adopted the use of width-height residuals (WHR) here, to prevent type 1 error.

A second difference between our study and those before, is that here the environment is controlled in a common garden design, whereas Poppe *et al.* (2003) used fish reared in the wild and in an aquaculture setting. Therefore, differences observed by Poppe *et al.* (2003) could be due to environmental plasticity. Finally, different points in the salmon life cycle could play a role. Fish sampled in this previous study were of sizes ranging from 0.5 – 6.4 kg, of which the wild fish could have been multiple sea winter fish and could have spawned multiple times.

4.5 Conclusion

We describe here the largely additive effect of domestication on growth rate, which increases with the percentage of the line that has been domesticated. Additionally, recombination in the F2 hybrids did not disrupt this additive effect, suggesting that co-adapted gene complexes do not play a vital role in growth. Despite the clear changes in growth caused by domestication, we do not see any clear changes in heart morphology between wild, domesticated and hybrid strains. Sex and life stage were seen to influence aspects of heart morphology, however. Sexual dimorphism was seen in adjusted heart weight and adjusted heart width, with females showing larger hearts relative to body size, with future scope to try and link this with function and metabolically expensive processes such as

oogenesis. Similarly, AHH and WHR were also seen to be sexually dimorphic, but this was driven entirely by saltwater females, with again, scope to investigate associations between biomechanics and heart function with processes such as oogenesis. Finally, the observed sexual dimorphism in AHH measurements, with females having larger AHH values, restricted to wild fish only, suggests that domestication may have relaxed selection for sexual dimorphism through direct or indirect artificial selection, as has been seen with other features of Atlantic salmon morphology (Perry *et al.*, 2019).

4.6 Acknowledgements

4.7 Author contributions

W.B.P organised sampling, DNA parentage analysis, sexing, fixation of hearts, measurement of hearts, data analysis and drafting of the original manuscript. K.A.G. and M.F.S produced the fish for the experiment. M.F.S. was involved in constructing the mixed effect models. C.B, A.C.M and K.G.P were involved in sampling, including dissections, weight measurements and tissue samples. A.E and A.H were involved in the DNA parentage and sexing of the fish. All authors, including those mentioned and S.C, M.L and M.T, were involved in the conception and design of the experiment and contributed to data interpretation and editing the final draft of the manuscript. K.A.G and G.C managed the project.

4.8 Animal Ethics

Those working directly with the experimental animals had also undergone Norwegian Food Safety

Authority (NFSA) training, as is required with experimentation involving animals that are included in

the Norwegian Animal Welfare Act (2010).

Chapter 5: Getting inside the brain of the Atlantic salmon (*Salmo salar*): examining domestication induced morphological change

Abstract

The complexity of the vertebrate brain means that its composition and function is only just beginning to be investigated in some species. The most diverse group of vertebrates, teleost fish, are at the centre of a growing research field, trying to understand variation in brain composition and function, and the ecological and evolutionary drivers that underpin it. One element of brain function that has provided insights into the questions of ecological and evolutionary drivers is overall brain morphology; the relative sizes and position of different regions that make up the brain. Traditionally, dissection, histology and photography would have been the methods implemented in collecting data on gross brain anatomy. Here, for the first time, we use digital X-ray microtomography (micro-CT) imaging to build a three-dimensional image of a fish brain, to assess brain anatomy. Measurements of the optic lobe and telencephalon were taken from the brain of Atlantic salmon (*Salmo salar*) parr (juvenile freshwater life stage). In addition to this, measurements were compared between wild, domesticated and partially domesticated ranched fish. No significant differences in optic lobe or telencephalon measurements were discovered between groups, however, this study provides evidence that micro-CT imaging is a valuable technique for uncovering accurate internal imaging of organs without dissection.

5.1 Introduction

Cephalisation of the central nervous system is a key characteristic of vertebrates, a group with complex brains that are roughly composed of three sections: the forebrain, midbrain and hindbrain. Within this basic structure, however, there lies a huge wealth of diversity, and this diversity is non-more apparent than in teleost fish, which make up the largest and most diverse group of vertebrates (Ravi and Venkatesh, 2008). Being a highly diverse group, taxonomic distance is an obvious reason for the diversity in brain structures, however, within highly speciose teleost families, ecology plays a role

(Kotrschal, Van Staaden and Huber, 1998). Cichlids, for example, are one of those highly speciose families that have highlighted the role of ecology in the evolution of the brain, following the adaptive brain component alterations theory, whereby selection can act on different regions of the brain. Throughout their adaptive radiations, effecting form (Huber *et al.*, 1997; Pollen *et al.*, 2007; Gonzalez-Voyer and Kolm, 2010) and also function (Sylvester *et al.*, 2010). Other studies examining intraspecific comparisons, rather than interspecific comparisons discussed above, have also highlighted the importance of predation (Kotrschal *et al.*, 2017) and habitat type (Gonda, Herczeg and Merilä, 2009) on brain structures.

The domesticated environment experienced by fish in aquaculture is often ecologically distinct from that of the wild, with changes in nutrition, predation, stocking density, parasites and disease. Not only are the ecological conditions different, thus contributing to divergent selection pressures, there is also direct artificial selection for favourable traits, such as growth rate, age of maturation and fillet quality. Such drivers typically contrast with natural selection in the wild, where form and function has strong associations with recent evolutionary history. The lack of investigation into the effect of domestication on brain anatomy is typified by the relatively few papers on Atlantic salmon (Fraser *et al.*, 2012; Näslund, Aarestrup, Søren T. Thomassen, *et al.*, 2012), one of the most well characterised domesticated fish (Glover *et al.*, 2017). In addition to relatively few studies, those that have been conducted have relied on more traditional methods of assessing brain anatomy. Primarily, anatomy has been assessed using dissection and photography, rather than advanced imaging techniques which are especially informative in disclosing subtle variations at a microscopic scale (Smith *et al.*, 2016). Exploring the role of domestication on brain morphology elucidates the plasticity of the salmonid brain, informing wider issues such as the impact of escapees into natural systems and the role of environmental enrichment in aquaculture. Both questions are significant in facilitating sustainable salmon aquaculture, the most valuable cultured finfish species globally (Glover *et al.*, 2017).

The studies that have examined the impact of domesticated environments focus on both hatchery reared fish, that have not undergone any direct artificial selection, as well as aquaculture lines that have undergone direct artificial selection. Studies on brain anatomy in hatchery or laboratory reared fish have shown, in examples such as rainbow trout (Marchetti and Nevitt, 2003; Kihslinger and Nevitt, 2006), guppies (Burns and Rodd, 2008), Atlantic cod (Mayer *et al.*, 2011), and Atlantic salmon alevins (Näslund, Aarestrup, Søren T. Thomassen, *et al.*, 2012) reared in non-natural environments, a reduction in brain size when compared to counterparts reared in wild or semi-wild conditions. These trends are not consistent, however. Domesticated rainbow trout have been shown to have larger overall brain size as well as larger olfactory volume (Campbell *et al.*, 2015) than wild strains, hatchery reared chinook salmon showing larger brain mass (Wiper, Britton and Higgs, 2014) than wild counterparts, and hatchery reared Atlantic salmon smolt showing larger brain area (Näslund, Aarestrup, Søren T. Thomassen, *et al.*, 2012) than wild reared fish.

Other studies that have not directly contrasted wild and domesticated fish have shown that conditions associated with domesticated environments can influence brain anatomy. Such studies examining aquaculture associated conditions include: increased stocking density associated with an increase in cerebellum and telencephalon mass in Atlantic salmon (Näslund *et al.*, 2017; Näslund, Rosengren and Johnsson, 2019), female guppies with larger brains showing slower growth (Kotrschal *et al.*, 2015), and larger telencephalon volumes in brook charr that undertake more active foraging behaviours (Wilson and McLaughlin, 2010). As highlighted by Campbell *et al.* (2015), differences in brain morphology between wild and hatchery or domesticated fish could be due to a myriad of factors and mechanisms, which could also be interacting.

Not only are there many ecological conditions within aquaculture that have been observed to change brain anatomy, as highlighted by previous studies, separate regions of the brain are able to show morphological change, rather than the brain as a singular unit. Here we explore the first detailed imaging studies of the impact of environment on fish brains by focusing on the size of different brain

regions to further test the adaptive brain component alterations theory, whereby different regions of the brain can evolve differently, depending on the selection pressures (Campbell *et al.*, 2015). We focus here on the telencephalon and the optic lobe, with multiple metrics for each to investigate multidimensional morphological difference between historically wild, ranched and domesticated Atlantic salmon lines.

5.2 Materials and methods

5.2. 1 Fish

All fish husbandry took place in the Institute of Biodiversity, Animal Health & Comparative Medicine, University of Glasgow. Eggs arrived in February 2017 and first feeding occurred in April 2017. Fish were reared under standard aquaculture conditions, with no environmental enrichment in the tanks. Termination occurred on 14th February 2018 using an overdose of benzocaine and severing of the spinal cord with a scalpel in accordance with Schedule 1 of the Animals (Scientific Procedures) Act 1986. All those terminating the fish were Home Office licenced. The domesticated strain used in this study was provide by Marine Harvest, and is an Irish Mowi strain (Fanad), while the wild and ranched strains were both from the Burrishoole, County Mayo, Ireland. The ranched strain used here was created in the 1960s at the Marine Institute from wild Burrishoole parents, with 10-14 generations of offspring bred in freshwater hatchery, and released as pre-smolts. Before being released into the Burrishoole, the ranched pre smolts were microtagged and their adipose fin clipped for identification on their return journey from the sea. Upon returning to the Burrishoole, some of the ranched fish are used to produce the next generation of ranched fish.

5.2.2 X-ray microtomography

After termination, fork length was measured, and the heads of the fish were dissected by cutting behind the gill covers and placed in 10 % paraformaldehyde (PFA). After fixation, heads were then transferred to 11.25% Lugol's iodine solution. Once stained, heads were scanned using the Nikon

XTH225 X-ray microtomography (micro-CT) at the Advanced Imaging of Materials (AIMs) department, Swansea University. The number of fish scanned included 7 domesticated, 5 ranched and 5 wild.

5.2.3 Measurements

Scans were visualised using the software Dragonfly (Object Research Systems (ORS) Inc, 2016), which was used to estimate distance and area measurements of brain regions. To locate each of the brain regions in a replicable manner, specific landmarks in the brain were used (figure A5.1). In addition to this, once the location had been identified, each measurement was taken in the two adjacent scans to build an average measurement. Average measurements were used to account for error in identifying the exact location in the brain, but also for error in measurement.

A total of six linear length measurements were taken, two in the dorsal ventral (DV) plane, which included the optic lobe width (OLW-DV) and optic lobe depth (OLD-DV), and four in the anterior posterior plane (AP), which included the OLW-AP and OLL-AP, along with the telencephalon width (TW-AP) and telencephalon length (TL-AP) (figure A5.3). To get an idea of shape of the optic lobe, the linear measurement OLL-AP was divided by OLW-AP to get an optic lobe L:W ratio, OLW-DV was divided by OLD-DV to get an optic lobe W:D ratio. For telencephalon shape, TL-AP was divided by TW-AP to get a telencephalon L:W ratio. A ratio was also created by dividing TL-AP by OLL-AP, to understand the size of brain regions relative to each other. Finally, two area measurements were taken in the AP plane, including optic lobe area (OLA-AP) and telencephalon area (TA-AP) (figure 5.1).
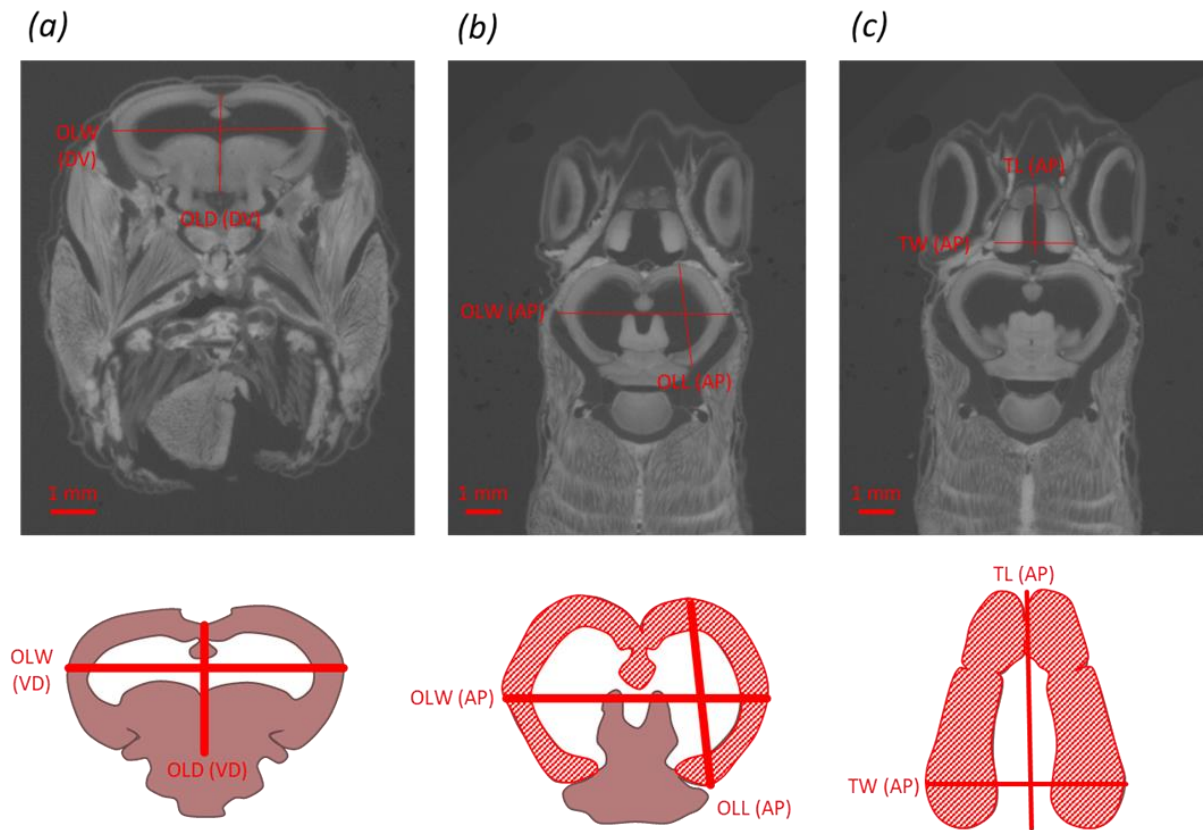
Figure 5.1 Individual X-ray microtomography scans and corresponding diagrammatic sections through the (a) dorsoventral axis and (b & c) anterior-posterior axis. Measurements focus on the (a & b) optic lobe width (OLW-DV & OLW-AP), (a) optic lobe depth (OLD-VD), (b) optic lobe length (OLL-AP), (c) as well as telencephalon width (TW-AP) and telencephalon length (TL-AP). In addition to linear measurements, (b & c) optic lobe area (OLA-AP) and telencephalon area (TA-AP) was also measured, as highlighted by the red crosshatch in the diagrammatic sections.

5.2.4 Statistical models

All statistical analyses was carried out in R version 3.3.3 (R Core Team, 2017). A linear model and an ANOVA was used to assess whether means differed between the three genetic backgrounds (domesticated, wild or hatchery); for both absolute brain measures and fork length adjusted measures. The linear models for all brain length measurements contained the factors genetic background, fork length and an interaction term between fork length and the genetic background. Scheffe's test to identify differences in group means was carried out using the R package 'agricolae'

(De Mendiburu, 2014). Fork length adjusted brain lengths were also created using residuals from regressions between log10 transformed brain measures and log10 transformed fork length.

5.3 Results

Five technical replicates for length and area were measured, each of which were plotted to assess consistency and accuracy of measurements per sample between different regions of the brain (figure A5.2). An average of the five technical replicates were used as the response variable in the final models. There was a significant (P < 0.01) linear relationship identified between all length measurements of brain regions and fork length (figure 5.2), although the linear relationship was stronger in some measures (R^2 range: 0.59 – 0.87). Fork length adjusted brain lengths were created using residuals from these regressions between log10 transformed brain measures and log10 transformed fork length.
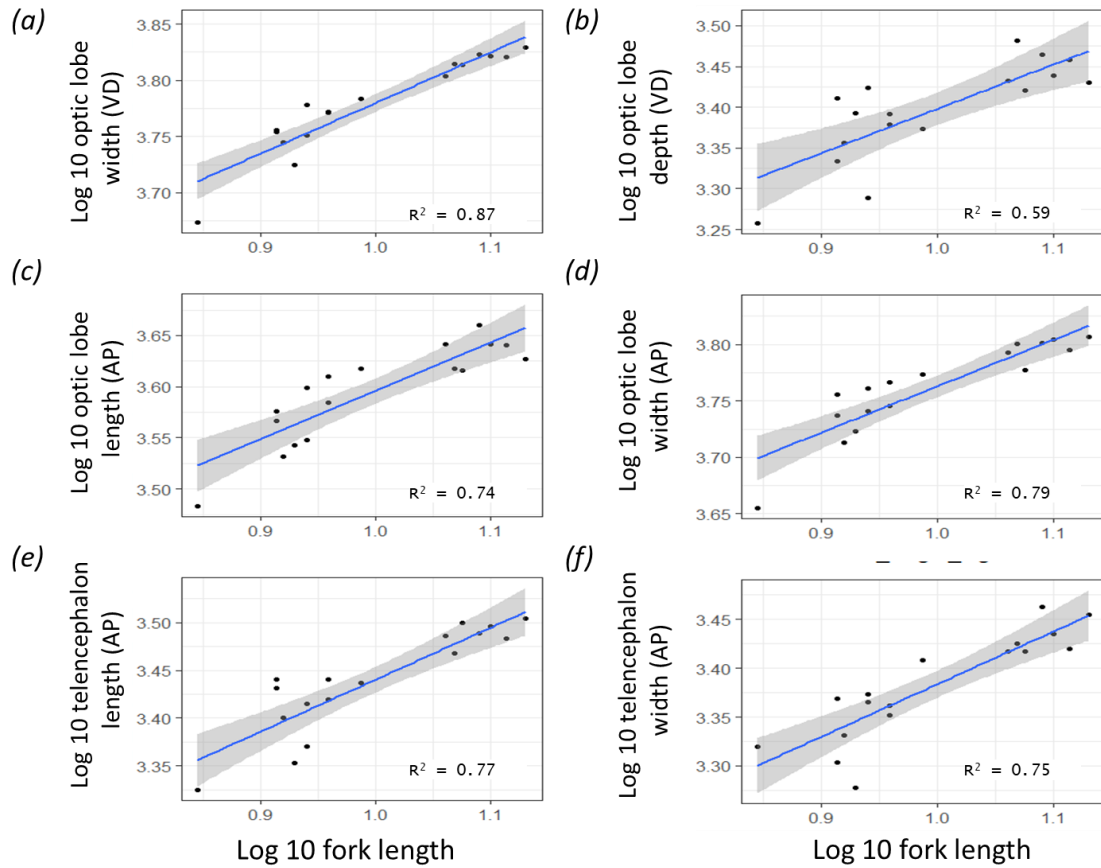
Figure 5.2 Regressions between log10 transformed brain length measurements from ventral-dorsal (VD) and anterior-posterior (AP) planes, and log10 transformed fork length. Length measurements include (a) optic lobe width (VD), (b) optic lobe depth (VD), (c) optic lobe length (AP), (d) optic lobe width (AP), (e) telencephalon length (AP) and (f) telencephalon width (AP). Regression lines are surrounded by 95% confidence intervals, and each plot includes their respective R^2 values.

None of the fork length adjusted measures showed a significant difference ($P < 0.05$) between wild, ranched and domesticated genetic backgrounds. Non-significant differences were found between optic lobe width (VD)($F_{2,14} = 0.17$, Sum Sq = 0.0004, p = 0.84), optic lobe depth (VD)($F_{2,14} = 0.56$, Sum Sq = 0.0035, p = 0.58), optic lobe length (AP)($F_{2,14} = 0.91$, Sum Sq = 0.0034, p = 0.43), optic lobe width (AP)($F_{2,14} = 0.62$, Sum Sq = 0.0020, p = 0.55), telencephalon length (AP)($F_{2,14} = 0.45$, Sum Sq = 0.0016, p = 0.65), and finally, telencephalon width (AP)($F_{2,14} = 0.94$, Sum Sq = 0.0034, p = 0.41). However, there

was a non-significant trend towards wild fish having larger brain regions in comparison to ranched and domesticated groups (figure 5.3).
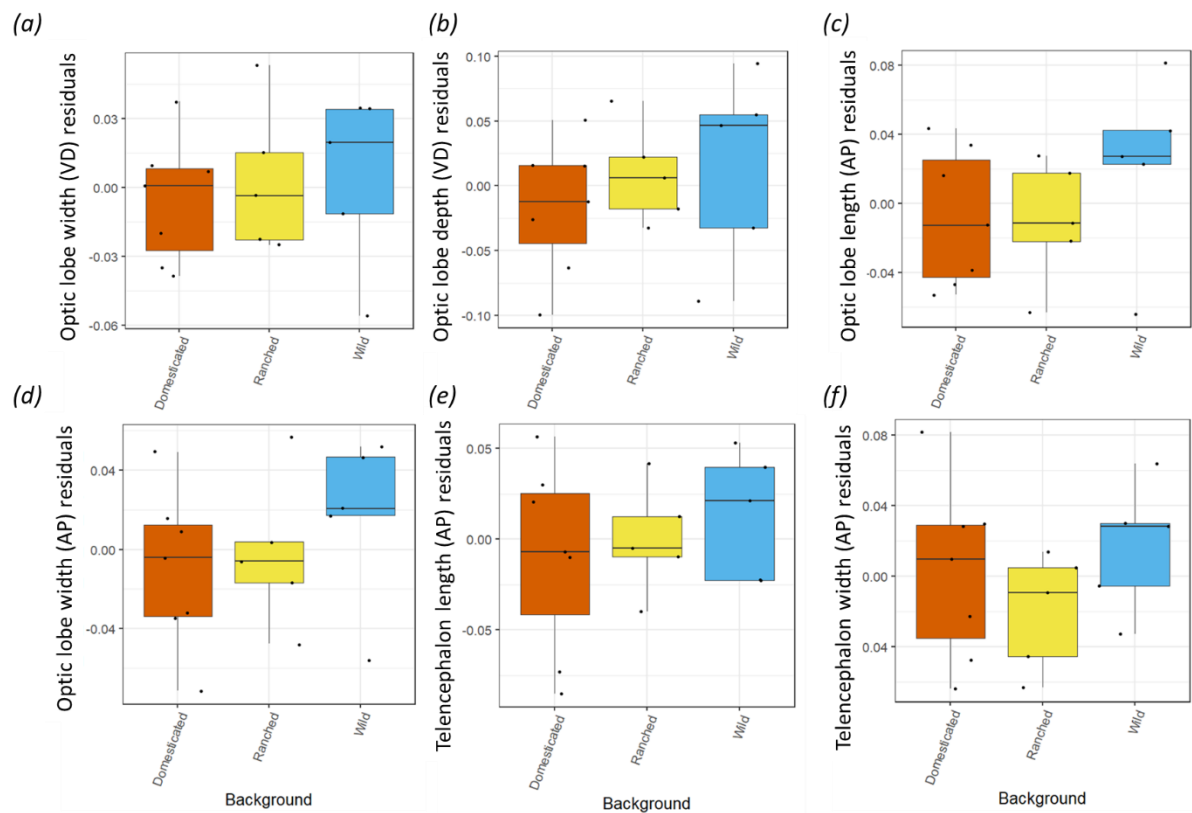
Figure 5.3 Six brain measures examined in this study, after having been adjusted for body size, between domesticated, ranched and wild backgrounds. The six adjusted length measurements are: (a) optic lobe width in the ventral-dorsal plane (VD) (b) optic lobe depth (VD) (c) optic lobe length in the anterior-posterior plane (AP), (d) optic lobe width (AP), (e) telencephalon length (AP) and (f) telencephalon width (AP).

5.4 Discussion

Here, we measured brain regions in fish that had come from: 1) a domesticate background, where strong artificial selection was applied for traits relevant to aquaculture, 2) a ranched background, where fish are reared in hatchery facility during their freshwater life stage, but released into the ocean to grow into a harvestable size, and 3) a wild background from the Burrishoole catchment. All brain regions showed a linear allometric relationship with fork length. The trend seen in the brain

measurements taken here is that fish from wild background have the largest brain structures when compared ranched, and domesticated individuals. The second trend seen here is the similarities seen between individuals from domesticated and ranched backgrounds, when compared to individuals from a wild background. The trend of domesticated fish having smaller brain structures relative to body size, when compared to wild individuals, is consistent with much of the literature.

5.4.1 Ecological interpretation

Two regions of the brain were measured here. First, the telencephalon, where the main cognitive centres are located (Näslund and Johnsson, 2016), involved in spatial learning and memory (Broglio, Rodriguez and Salas, 2003). The second was the optic lobe, and as outlined by Broglio, Rodriguez and Salas (2003), is thought to be the centre for spatial cognition, multisensory integration, sensory–motor transformations and egocentrically referenced actions in space. Therefore, even small anatomical changes to these regions could have dramatic influences on behaviour, and consequently affect how individuals interact with the environment. Such links between telencephalon and behavioural ecology have been demonstrated in fish, many showing that larger telencephalon volume is linked with increased motility in foraging (Wilson and McLaughlin, 2010), occupation of more complex habitats (Bauchot *et al.*, 1977; Huber *et al.*, 1997; Pollen *et al.*, 2007) and a higher incidence of monogamy (Pollen *et al.*, 2007). Examples to the contrary have, however, been identified (Park and Bell, 2010). Links between the optic lobe and behavioural ecology have also been made, but to a far lesser extent, with larger optic lobes associated with enhanced visual stimuli (Brandstätter and Kotrschal, 1990; Espinasa *et al.*, 2001) and piscivorous feeding (Huber *et al.*, 1997).

Based on previous research, the expectation is that wild individuals exhibit the largest telencephalon and optic lobes, with ranched fish as intermediates, and domesticated individuals with smallest. Such a prediction arises from the length of time ancestrally those individuals have spent in artificial, domestic environments which are characterised by a lack of complexity (Marchetti and Nevitt, 2003; Kihslinger and Nevitt, 2006; Burns and Rodd, 2008; Mayer *et al.*, 2011; Näslund, Aarestrup,

Thomassen, *et al.*, 2012). Here we show a trend, which although not significant, shows that the genetic backgrounds which have had a history of active artificial selection (domesticated strain), or being reared in artificial environments (ranched strain), have smaller brain regions when compared to the wild strain.

5.4.2 Further work

We demonstrate here the power of using micro-CT scanning techniques in providing high-quality three-dimensional imaging, at a fine scale, of small soft tissues structures that would be traditionally be too small to dissect and photograph. This study also highlights the important role of sophisticated imaging techniques in quantitative studies on internal organs. Future study, however, must not only focus on a larger sample size, but also take into account other factors which have been shown previously to be important in influencing brain morphology, such as sex (Park and Bell, 2010; Kotrschal *et al.*, 2012), stocking density (Näslund *et al.*, 2017; Näslund, Rosengren and Johnsson, 2019) and behaviour (Campbell *et al.*, 2015). Linking within species brain morphology and behavioural ecology could prove to be a particularly important step forward.

5.5 Animal ethics

Termination of the fish was done in accordance with Schedule 1 of the Animals (Scientific Procedures) Act 1986. All those terminating the fish were Home Office licenced. Fish used in this study were not experimentally manipulated.

5.6 Authors' contributions

Eleanor Lindsay oversaw rearing and termination of the fish. Ria Mitchell and Richard Johnston produced all micro-CT scans. William Bernard Perry stained the samples, processed the micro-CT scan images, analysed the data and drafted the original manuscript. All authors, including Martin Llewellyn, Joshka Kaufmann, Monica Favnebøe Solberg, Martin Taylor, Philip McGinnity, Kevin Alan Glover and

Gary Carvalho were involved in the conception and design of the experiment, and contributed to data interpretation and editing the final draft of the manuscript.

Chapter 6: Sexual dimorphism in gut bacterial diversity: common garden study in Atlantic salmon (*Salmo salar*)

Abstract

Sexual dimorphism has proven to be an important driver in the composition of gut microbial communities in animal hosts, with links to sex biased mucosal immune function and autoimmunity. The impact of sex on the gut microbiome of fish, however, is relatively underexplored, following the trend of overrepresentation of mammalian microbiome studies in the literature. We examine the role of sex on bacterial communities residing within the mid-gastrointestinal tract of male and female Atlantic salmon (*Salmo salar*) post-smolts from wild, hybrid and domesticated backgrounds reared in a common garden. Diversity of the gut microbiome was examined using the 16S ribosomal RNA gene variable region 1-2, indexed, and sequenced on the Illumina MiSeq. Using DADA2 and the SILVA database, operation taxonomic units (ASVs) were identified, as well as relative abundance. Males showed significantly greater bacterial alpha diversity than females, as assessed using the Simpsons ($F_{1,14}$ = 8.27, Sum Sq = 101.49, p = 0.01) and Shannon ($F_{1,12}$ = 7.86, Sum Sq = 193.66, p = 0.02) index. No significant differences in gut microbial alpha or beta diversity were detected between domesticated and wild backgrounds.

6.1 Introduction

Sexual dimorphism is seen in a range of traits in many different species, and particularly in salmon; from the bold displays of sexual dimorphism seen in secondary sexual characteristics such as the kype in male salmon (Perry *et al.*, 2019), to more cryptic sex dependant loci such as VGLL3 that control age of maturity (Barson *et al.*, 2015). Differences between sexes are often driven by sexual selection, either directly or indirectly, and provide insight into the evolutionary forces dictating a phenotypic optimum of a species. A phenotype that has garnered significant interest over recent decades is the gut microbiome, with the number of studies on the fish gut microbiome increasing exponentially in the past 10 years (Perry *et al.*, 2020). The vital role of the gut microbiome in aspects of disease (He *et*

al., 2017), nutrition (Sugita, Miyajima and Deguchi, 1990) and behaviour (Borrelli *et al.*, 2016) is now evident, and is therefore likely an important phenotype that influenced the lifetime success of an individual.

Many factors have been seen to impact the gut microbiome of fish, these include environmental variables such as a diet (Miao *et al.*, 2018a), salinity (Rudi *et al.*, 2018) and dissolved oxygen concentration (Ornelas-García *et al.*, 2018); but they also include intrinsic host properties, such as host gut motility (Wiles *et al.*, 2016), host genetics (Kokou *et al.*, 2018) and host behaviour (Singh *et al.*, 2019). In addition to those listed, another property intrinsic to the host that has been shown to impact the microbiome is sex. Sexual dimorphism in the gut microbiome has been identified in species such as humans and mice, with studies also linking differences in the gut microbiome to aspects of sexually dimorphic immunity; although with human studies, confounding environmental factors make it difficult to identify true sexually dimorphism in the gut microbiome (Elderman, de Vos and Faas, 2018). Sexual dimorphism in the gut microbiome has also been identified in other non-model species of mammal, including northern elephant seals (Morales-Durán and Stoffel, 2019). Despite this, however, very few studies examining sexual dimorphism in gut microbiomes have been performed on fish, reflecting the overall bias in microbiome studies towards mammalian hosts. Some examples do, however, exist, with threespine stickleback (*Gasterosteus aculeatus*) and Eurasian perch (*Perca fluviatilis*), demonstrating sex dependant diet-microbiota associations (Bolnick, Snowberg, Hirsch, *et al.*, 2014).

Globally, the Atlantic salmon is the eighth most cultivated aquaculture fish species, while also being the most valuable (FAO, 2016b). Understanding factors that can influence the gut microbiome in Atlantic salmon is therefore of great value to the aquaculture industry, due primarily to the fact that microbial activity in the gut is linked with two vital processes: immunity (Ellis, 2001) and nutrition (Ray, Ghosh and Ringø, 2012). In addition to sexual dimorphism, identifying possible domestication induced changes to the gut microbiome is also of great interest, including the ongoing significance of assessing

the impact that escaped domesticated fish have on wild populations. Simulations suggest that between 2005 – 2011, approximately 1.5 million salmon escaped from aquaculture facilities per year (Skilbrei, Heino and Svåsand, 2015), a massively underestimated figure than what was reported by facilities. Indeed, the use of microsatellite markers to trace genetic background, has been used in legal cases against aquaculture companies failing to report escapees from their facilities (Glover, Skilbrei and Skaala, 2008). Legacy effects, or the impact early life stage conditions have on the host later on in its life cycle, have been demonstrated with artificial rearing and the fish gut microbiome (Giatsis *et al.*, 2016; Uren Webster *et al.*, 2020), which has important consequences with regard to fitness in aquaculture escapees. In addition to this, however, one of the greatest concerns with large escapes is the impact of introgression, which can lead to changes in the evolutionary trajectory of wild populations beyond the initial escapees, as wild populations are introgressed with fish selected for a different environment. There is a risk that gene flow between wild and farmed individuals may change phenotypic traits, such as the microbiome, in wild populations (Bolstad *et al.*, 2017) with resultant reductions in fitness (McGinnity *et al.*, 1997, 2003; Fleming, Hindar, Mjölneröd, *et al.*, 2000; Besnier *et al.*, 2015; Skaala *et al.*, 2019).

The host genome has been seen to influence the gut microbiome in a variety of different fish species (Smith *et al.*, 2015; Li *et al.*, 2017, 2018), with the influence of domestication also characterised in genetically improved species such as blue tilapia (*Oreochromis aureus*)(Kokou *et al.*, 2018) and rainbow trout (*Oncorhynchus mykiss*)(Brown, Wiens and Salinas, 2019). As Atlantic salmon are in the top tier of domesticated fish species (Teletchea and Fontaine, 2014), it highlights the possibility of strong selection on the host genome also influencing the microbial gut communities. In addition, domesticated fish consistently grow larger than wild fish in common garden hatchery experiments (Einum and Fleming, 1997; Fleming *et al.*, 2002; Glover, Otterå, *et al.*, 2009; Wolters *et al.*, 2009; Skaala, Kevin A. Glover, *et al.*, 2012; Solberg, Skaala, *et al.*, 2013; Reed *et al.*, 2015; Harvey, Glover, *et al.*, 2016; Harvey, Solberg, Glover, *et al.*, 2016; Solberg *et al.*, 2016), with growth rates of domesticated fish up to 3.5 times higher than wild fish (Solberg, Skaala, *et al.*, 2013). The proximal cause for such

disparity in growth rate remains unclear, and therefore, it is possible that it could be, at least in part, microbially mediated.

Here, we add to relatively few studies documenting the impacts of sexual dimorphism and domestication on the gut microbiome of fish, by assessing differences between genetically male and female Atlantic salmon post-smolt in a common garden experiment. The application of next generation sequencing technologies has not only allowed discovery of previously unculturable bacteria, it has also allowed production of high-throughput microbial ecological data, combining widespread species identification with relative abundances. Such ecological data allow us to identify bacterial species richness, and to calculate both alpha and beta diversity metrics. We employ these techniques, in combination with the common garden design to assess how both sex and genetic background (domesticated, wild and reciprocal hybrid) influence microbial diversity of the Atlantic salmon midgut.

6.2 Methods

6.2.1 Fish

Over the past decade, the Institute of Marine Research (IMR) in Norway has undertaken a range of common-garden experiments, as used here, to examine a broad spectrum of biological questions related to domesticated and wild origin Atlantic salmon (e.g. Bicskei, Taggart, Glover, & Bron, 2016; Glover et al., 2009; Harvey, Glover, Taylor, Creer, & Carvalho, 2016; Solberg, Skaala, Nilsen, & Glover, 2013). The setup of these experiments is based on microsatellite parentage of individuals back to their respective genetic origin (wild = 8, domesticated = 7 or hybrid = 11), using DNA extracted from adipose fin clips taken during sampling, allowing for environmental conditions to be kept constant between all backgrounds. Hybrid fish used here include reciprocal crosses between domesticated females and males, with wild males and females. Also included were backcrosses (wild backcross = 3, domesticated backcross = 2), however, these were not used in the analysis looking at strain differences, however,

they were included in the analysis looking at sex differences. Fish used in this study were reared in flow through tanks that were 5m wide, 1.1m deep, and had a volume of 15600 L and a flow rate of 170-200 L/min. Saltwater in these tanks was supplied from the surrounding fjord. The photoperiod within the outbuilding simulates that of Bergen, and the temperature range of the water during the experiment was 3°C - 14.2°C. Fish were fed on a diet of pellets produced by Skretting Nutra Olympic (Cheshire, UK); samples of the bacteria contained on the feed were taken and ultimately sequenced (n = 6).

6.2.2 Sampling

Sampling took place between 23rd – 27th April 2018, at Matre Research Station, Institute of Marine Research (IMR), Hordaland, Norway. An anaesthetic overdose of MS-222 was used to terminate the fish, and all those working directly with the experimental animals had undergone Norwegian Food Safety Authority (NFSA) training, as is required with experimentation involving animals that are included in the Animal Welfare Act. The fish in the experiment were unmanipulated, and were only subject to standard rearing conditions, so no specific research permit was required. Once terminated, incisions were made along the ventral plane of the fish from anterior to posterior, allowing for the removal of the gastrointestinal (GI) tract from the body cavity. The GI tract was removed by cutting at the upper oesophagus and lower hind gut. Each of the three sections were placed on sterilised tin foil, were they were weighed, and microbiome samples taken. A 1 cm long sample was taken from the midpoint of the midgut, as identified by the presence of pyloric caeca, to standardise sample collection between individuals. All dissection equipment was ethanol flamed between use. Microbiome samples comprising of gut tissue and its content were placed into cryotubes and immediately submerged into liquid nitrogen until they were later put into storage in an ultra-low temperature freezer at -80°C.

Sampling of the environmental microbial communities was carried out by passing 1 litre of environmental water through a 0.2 µm Sterivex filter using a peristaltic pump at a speed of 220 rpm.

Prior to filtering, peristaltic pump tubing was sterilised by passing through 1 litre of 10% bleach, followed by 1 litre of the environmental water, at a speed of pump speed of 220 rpm. After filtering, Sterivex filters were capped, placed in a falcon tube, and stored at -80 °C for long term storage.

6.2.3 Parentage of fish

In order to distinguish between wild, domesticated and reciprocal wild x domesticated hybrids in the common garden, there was need to undertake a paternity analysis. Corresponding adipose fin clips to the microbiome samples were also taken and stored in ethanol. DNA was then extracted using the Qiagen DNeasy®96 Blood & Tissue Kit, followed by a multiplex PCR which amplified six microsatellite loci; SsaF43 [GenBank:U37494] (Sánchez et al. 1996), Ssa197 [GenBank:U43694.1] (O'Reilly et al. 1996), SSsp3016[GenBank:AY372820], MHCI (Grimholt et al. 2002), MHCII (Stet et al. 2002). An ABI Applied Biosystems ABI 3730 Genetic Analyser was used for fragment analysis, the outputs of which were used to call genotypes in GeneMapper (Applied Biosystems, v. 4.0). Further details are outlined by (Solberg, Skaala, *et al.*, 2013).

6.2.4 Microbiome DNA extraction

The ordering of microbiome DNA extraction was randomised using a unique sample ID before samples were removed from - 80°C storage. Samples were defrosted on ice, and approximately 180–220 mg of pyloric caeca was used for each extraction. To release and physically lyse bacterial cells from within the lumen of the pyloric caeca, and those bacteria integrated into the pyloric caeca epithelial cells, a bead beating stage was used. MP Biomedicals ¼" ceramic spheres were placed in 2 ml Eppendorf tubes along with MP Biomedicals garnet matrix (enough to cover the ceramic bead). Ceramic spheres, garnet mix and 2 ml Eppendorf tubes were all autoclaved at 134 °C for 10 minutes, and then bathed in UV light for a minimum of 30 minutes before use. The amount of smolt pyloric caeca tissue being used for extraction was weighed, for use in later mixed effect models as a random effect. Manipulation of sub samples from the main pyloric caeca sample taken from the cryotube was conducted with

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disposable, sterile bacterial loops, along with sterile scalpel blades. The individual sterile scalpel blade packaging was used as the cutting surface, being replaced with a new scalpel blade and its packaging per sample. All extraction steps were undertaken in a Thermo Scientific™ Safe 2020 Class II Biological Safety Cabinet.

Once the bead and garnet mix had been sterilised, buffer ASL from the Qiagen AIQamp™ DNA stool minikit was added to the garnet mix and bead within the 1.5ml Eppendorf tube. The samples were then placed in a Retsch MM301 mixer mill, shaking the samples up and down, allowing the bead and garnet mix to interact, thus breaking down the tissue and cells. A frequency of 30Hz was applied for the milling process, for a period of $30 - 60$ seconds, depending on the homogeneity of the tissue. After physical lysing, thermal lysing was undertaken for 10 minutes at $95^{o}C$ on an Eppendorf thermomixer shaking heat block. After the heating step the Qiagen protocol for 'stool pathogen detection' was followed, including a proteinase k chemical lysis phase, without the use of Inhibitex tablets. Extracted DNA was eluted in buffer AE, concentrations were measured using a nanodrop, and all samples were then normalised to a concentration of $\sim$ 20 ng/ul. All cell lysis and subsequent DNA extraction protocols were carried out in the bacteria laboratory at the Institute of Marine Research (IMR), Bergen, Norway, which is not used for any post-PCR laboratory work. DNA from environmental samples was extracted following guidelines outlined by Spens et al. (2017), whereby ATL buffer and proteinase K were added to the filters and incubated at $56\ ^{o}C$ for 24 hours. After lysing, Qiagen QIAamp DNA Mini Kit spin column extraction was conducted on the lysate. Negative controls were taken on every day of DNA extraction, with a selection of negative controls (n = 3) taken through the entire amplification process and subsequently sequenced. Post extraction protocols took place at the Molecular Ecology and Fisheries Genetics Laboratory (MEFGL), Bangor University, Wales.

6.2.5 Amplicon preparation and sequencing

Using DNA extracted from the midgut, tank water, feed, and standards from Zymo Research, the V1-2 region of the 16S rRNA gene (~311bp) was amplified using a two-step polymerase chain reaction (PCR) in triplicate. Although not the most common 16S rRNA gene region (Perry *et al.*, 2020), the V1-2 region showed markedly lower cross amplification with the salmon mitochondrial genome, specifically the 12S rRNA gene region (figure A6.1). Therefore, the template specific primers for the V1-2 region, CS1_27F: 5'- AGAGTTTGATCMTGGCTCAG-3' and CS2_338R: 5' – TCTGCTGCCTCCCGTAGGAGT-3' and universal Illumina tails (Flores *et al.*, 2012; Bista *et al.*, 2017) were amplified in triplicate (figure 6.1a). The PCR mix included 1.3 µl of template DNA, 1 µl CS1_27F, 1 µl CS2_338R, 1 µl Mg^{++}, 10 µl Q5® High-Fidelity 2X master mix and 5.7 µl molecular grade microbial free water. Cycling conditions were 95 °C for 5 minutes, 30 cycles of 30 seconds at 95 °C, 30 cycles of 30 seconds at 55 °C, 30 cycles of 30 seconds at 72 °C and 10 minutes at 72 °C. The samples were then pooled and 5 µl of product from the first PCR was added to a second PCR where a forward or reverse Illumina adaptor and unique i5 or i7 Nextera index was ligated to the corresponding universal tail (figure 6.1b). The PCR mix included 5 µl of template DNA, 0.5 µl i5 index, 0.5 µl i7 index, 12.5 µl Q5® High-Fidelity 2X master mix and 6.5 µl molecular grade microbial free water. Cycling conditions were 98 °C for 3 minutes, 15 cycles of 30 seconds at 98 °C, 15 cycles of 30 seconds at 55 °C, 15 cycles of 30 seconds at 72 °C and 5 minutes at 72 °C. Two 96 well plates of Illumina adaptors were used to uniquely barcode all samples, plate A and plate B; the adaptor plate that was used is included in later analyses to avoid plate effect. Negative controls were included for all PCRs and inspected for a positive band using gel electrophoresis.

After the second PCR, products were purified using Agencourt AMPure magnetic beads, and all samples were pooled into one volume (figure 6.1c). To visualise the amplified product, gel electrophoresis was performed (10 % agarose gel at 100 V) on the final pool (figure 6.1d). Gels were illuminated using a blue light transilluminator, in order to prevent ultraviolet fragmentation, and the desired size band was cut from the gel using sterile blades, and extracted from the agarose using the

Thermo Fisher Scientific PureLink® Quick Gel Extraction Kit. This enabled the removal of both primer dimers and mitochondrial encoded salmonid 12S rRNA gene, which was also cross-amplified using the primers outlined above. Once purified, sequencing of the final library was conducted on an Illumina MiSeq at the University of Glasgow Polyomics facility.
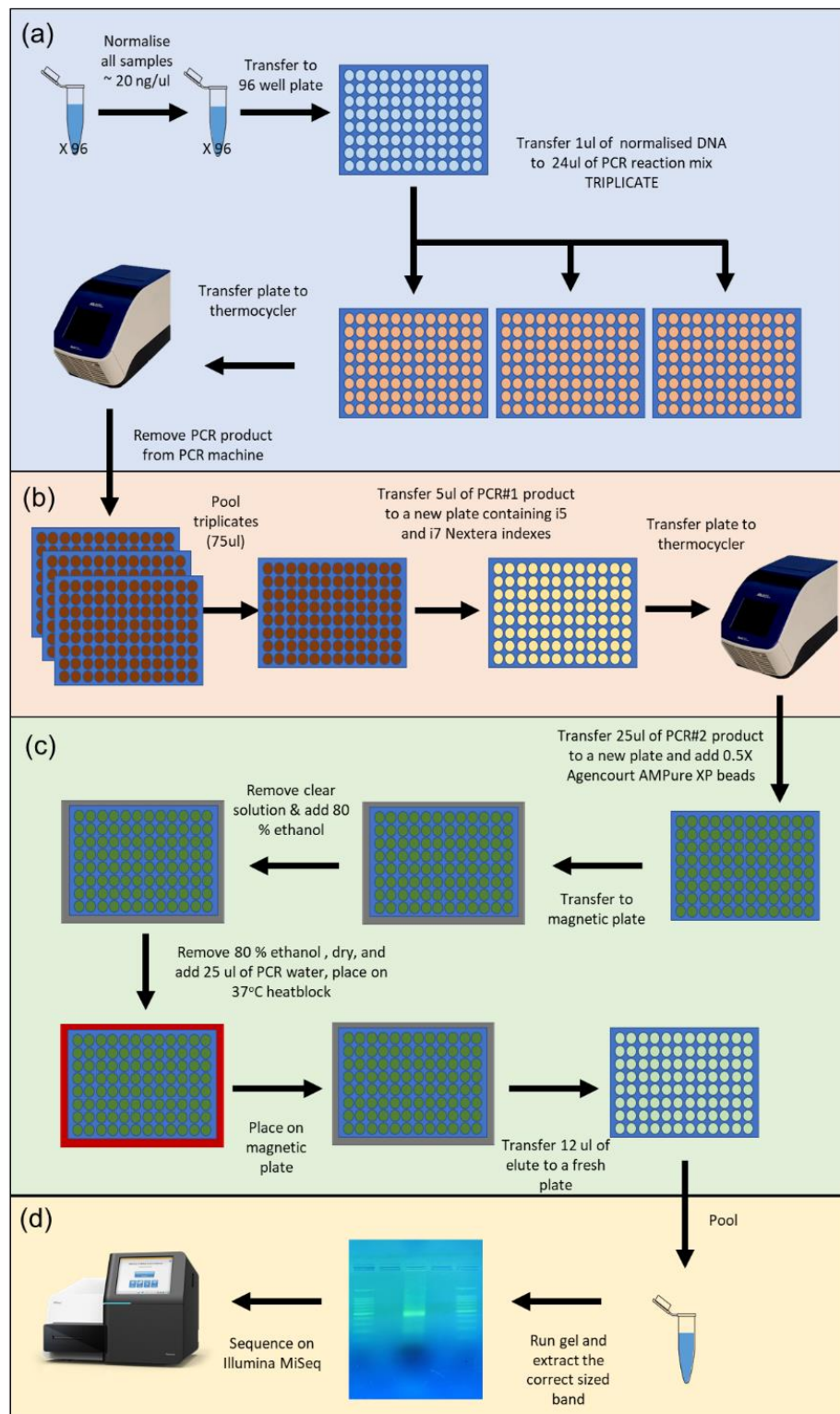
Figure 6.1 Pipeline for polymerase chain reaction (PCR) product preparation, including (a) first PCR amplification of template DNA and universal Illumina tails, (b) second PCR amplification and ligation of i5 and i7 Nextera indexes, (c) Agencourt AMPure XP bead clean up and (d) final pooling, gel extraction and sequencing.

6.2.6 Bioinformatic pipeline

After demultiplexing forward and reverse reads, the R package 'DADA2' (Callahan *et al.*, 2016) was used to filter and dereplicate sequences, merge forward and reverse reads, calculate error rates introduced in variant calling and to remove chimeras, all before taxonomic assignment (in conjunction with the SILVA database (Glöckner *et al.*, 2017)) and creation of an amplicon sequence variant (ASV) table. After creation of the ASV table, sequences that could not have taxonomy assigned to the Kingdom level were removed, as well as any eukaryotic host sequences leaving prokaryotic reads. In addition to this, samples with a read depth less than 4,000 were also removed from the analysis, to remove samples which may have high errors introduced by DNA purification, quantification, PCR amplification, barcoding, or sequencing. No negative controls met filtering criteria, due to low read depth (0 - 5 reads post DADA2 pipeline). The 'Rhea' R pipeline (Lagkouvardos *et al.*, 2017) was used to rarefy the ASV table to the sample with the lowest read depth of 4,136. The rarefied ASV table was then used in a further Rhea pipeline to calculate alpha and beta diversity metrics for each sample. Alpha diversity metrics were then used as the response variable for linear mixed effect models constructed in lme4 (Bates *et al.*, 2015) with the fixed factors sex, midgut weight, fork length and strain, with the random factors index plate, family and DNA extraction date. Estimated marginal means and pairwise comparisons between means were calculated using the selected models and the R package 'emmeans' (Lenth, Love and Maintainer, 2018). A Tukey's multiple comparisons test was used to adjust p-values, and Kenward–Roger approximations were used to estimate degrees of freedom. The 'step' function within 'lme4' was then used to select the best fitting model through automatic backward elimination, allowing for the removal of fixed terms and random factors which did not contribute to the model.

There has been valid criticism on the use of rarefaction in the literature (McMurdie and Holmes, 2014); here we implemented a rarefaction comprising of a simple division to sample size and then multiplication by the size of the smallest sample, which does not introduce random variance (Lagkouvardos *et al.*, 2017). In order to address the other problems associated with rarefaction, such as inflation of false positives, we also removed samples that had a read depth over 4.5 times greater than the lowest read depth to ensure similar read depth among samples. Finally, a baseline effective richness of 0.5% was set, based on the mock communities (figure A6.2), whereby ASVs below this relative abundance per sample were removed.

6.3 Results

6.3.1 Read assignment and rarefaction

Before analysis, sequences that could not have taxonomy assigned to the Kingdom level were removed (6,513 reads), as well as any eukaryotic host sequences (190,686 reads) leaving 847,136 prokaryotic reads (figure 6.2a). The rarefaction curve showed that there was a wide array of variation in species number among samples, however, most samples were seen to reach species saturation at a read depth of 1,500 (figure 6.2b). After rarefaction, three alpha diversity metrics were calculated, including the Shannon diversity index, Simpson diversity index and evenness of species (figure 6.3).
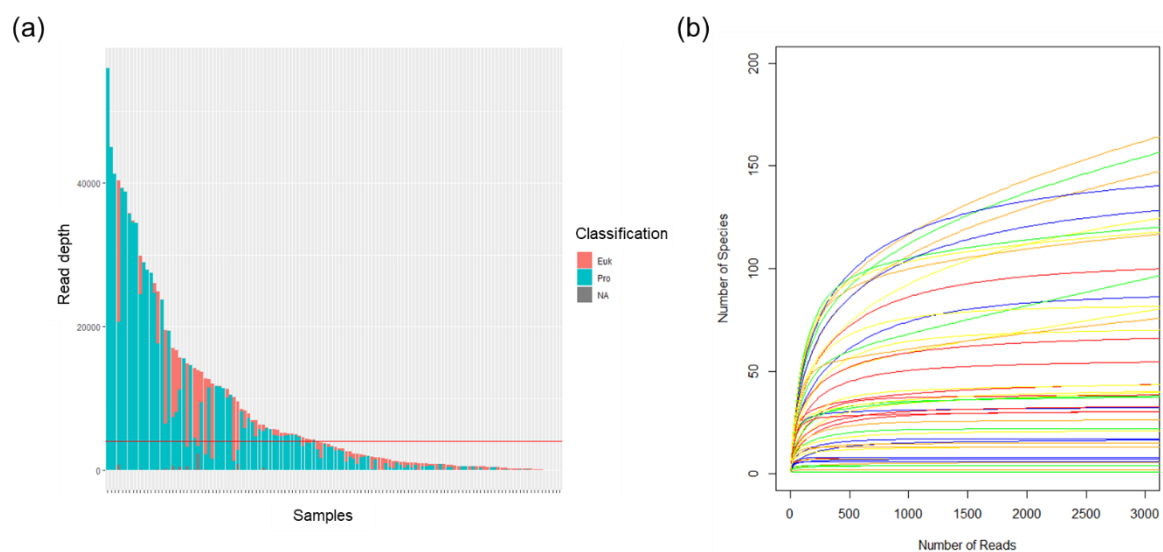
Figure 6.2 (a) read depth between samples, classified into Eukaryotes, Prokaryotes, and those that could not be assigned a taxonomic identity; with a red line transecting the y axis at a read depth of 4,000. In addition to this, (b) rarefaction curves, after the removal of eukaryotic and non-assigned reads, per sample, to show the number of ASVs detected with increasing number of reads.

6.3.2 Alpha diversity metrics – Simpsons index

In the linear mixed effect model assessing Simpsons index, sex, strain (figure A6.3a), midgut weight and fork length were all included as fixed factors. The random effects included family nested in strain, DNA extraction date and index plate. Sex was the only significant effect on the Simpsons index (LME Sex: $F_{1,14}$ = 8.27, Sum Sq = 101.49, p = 0.01), where males (average Simpsons index = 8.39) had a significantly greater average Simpsons index than females (average Simpsons index = 3.14) (t_{19} = 2.52, p = 0.02) (figure 6.3a).
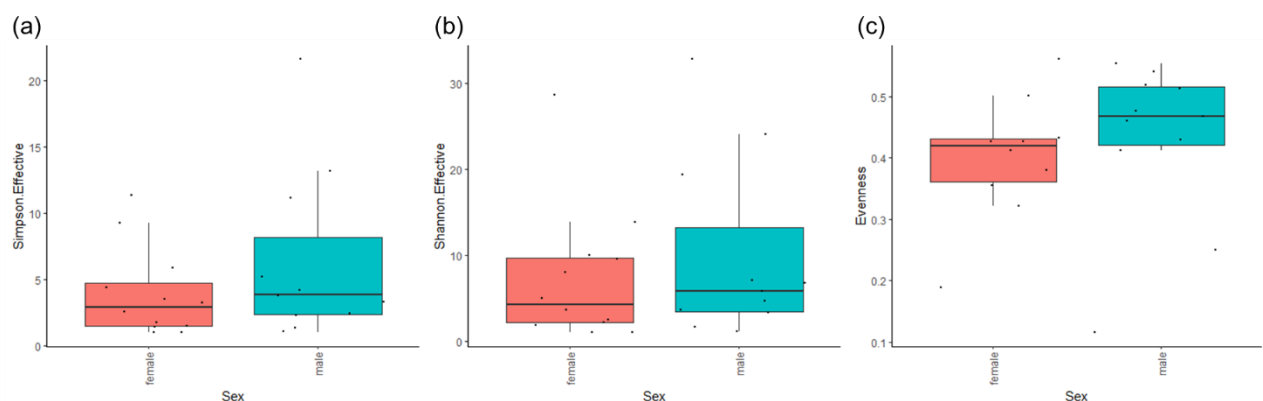
Figure 6.3 Measures of alpha diversity in gut samples, including effective (a) Simpson index, (b) Shannon index and (c) evenness, all split between males and females.

6.3.3 Alpha diversity metrics – Shannon index

In the linear mixed effect model assessing Shannon index, sex, strain (figure A6.3b), midgut weight and fork length were all included as fixed factors. The random effects included family nested in strain, DNA extraction date and index plate. Sex was the only significant effect on the Shannon index (LME Sex: $F_{1,12}$ = 7.86, Sum Sq = 193.66, p = 0.02), where males (average Shannon index = 12.9) had a

significantly greater average Shannon index than females (average Shannon index = 4.7) (t_{11} = 2.46, p = 0.03) (figure 6.3a).

6.3.4 Alpha diversity metrics – Evenness

In the linear mixed effect model assessing evenness, strain (figure A6.3c), midgut weight and fork length were removed from the model by the step function, leaving the fixed effect of sex. The random effects of family nested in strain, as well as DNA extraction date were also removed, leaving the random effect of index plate. There were no significant effects (figure 6.3c).

6.3.5 Beta diversity

The Multidimensional Scaling (MDS) analysis showed no significant differences in beta diversity between samples according to strain (PERMANOVA p = 0.603) (figure A6.4a) or sex (PERMANOVA p = 0.33) (figure A6.4b).

6.4 Discussion

Through our common garden design, and consistent environmental conditions among groups, that there is evidence for sexual dimorphism in the midgut of Atlantic salmon, with male fish showing significantly greater bacterial alpha diversity than females. Despite this, however, there was no evidence to show differences in beta diversity between sexes. In addition to this, we found no significant differences in alpha or beta diversity between wild and domesticated fish. The bacterial genus *Mycoplasma* dominated the relative abundance in the samples making up 57% relative abundance in males and 80% in females.

6.4.1 Sexual dimorphism

We discovered significant differences in alpha diversity metrics between male and female post-smolt Atlantic salmon, adding to sexual dimorphism seen in the mammalian dominated literature (Elderman, de Vos and Faas, 2018; Morales-Durán and Stoffel, 2019), however, unlike the mammalian literature, we find the opposite trend of males having increased bacterial diversity when compared to females.

One of the most obvious, and likely drivers of sexual dimorphism in the gut microbiome, as has been demonstrated in mammals, is the role of sex hormones in modulating the composition of the microbiome (Koren *et al.*, 2012) which impacts mucosal immune function and autoimmunity (Markle *et al.*, 2013). Differences in sex hormones such as steroids have been documented in salmonid species such as the coho salmon (*Oncorhynchus kisutch*) (Patiño and Schreck, 1986), however, little is known about hormone differences in Atlantic salmon. The exact pathways of such hormone-microbiome interactions and potential differences in innate immune factors such as pattern recognition receptors (PRRs) (Chu and Mazmanian, 2013), remain unknown in fish (Bolnick, Snowberg, Hirsch, *et al.*, 2014). In addition to hormone-microbiome interactions, there is evidence to show that allelic diversity for major histocompatibility complex class II (MHCII) receptors of antigen-presenting cells could also be a contributor to sexual dimorphism in the microbiome (Bolnick, Snowberg, Caporaso, *et al.*, 2014). Understanding that sex has the ability to significantly change the alpha diversity in the midgut microbiome of fish means that it should be included as an explanatory variable in more studies assessing factors that can influence the gut microbiome, as it could account for significant variation (Bolnick, Snowberg, Caporaso, *et al.*, 2014). Understanding the mechanisms that contribute to sexual dimorphism in the gut microbiome of fish is an unexplored area for further research.

Getting a better understanding of sexual dimorphism in the gut microbiome of the Atlantic salmon is also important for salmon aquaculture. Today, 99 % of salmon consumed is sourced through farmed stocks (Glover *et al.*, 2017), demonstrating the wider trend seen in fisheries across the globe. Early maturation in male salmonids causes decreased productivity in aquaculture by reducing growth and increasing disease susceptibility and changing, and so females are preferred, with the all-female production seen in other salmonids such as the rainbow trout (*Oncorhynchus mykiss*) (Martínez *et al.*, 2014). Therefore, a combination of female biased sex ratios in aquaculture, and females having reduced bacterial gut diversity, could have implications for production, and would benefit with further research into the functional consequences of having a lower diversity gut microbiome.

6.4.2 Domestication

Although previous studies on genetically improved aquaculture species have shown significant changes in the microbial communities within their gut (Kokou *et al.*, 2018; Brown, Wiens and Salinas, 2019), such a result was not evident between wild and domesticated Atlantic salmon examined here. We did not find any significant differences in either alpha or beta diversity between strains. The lack of significant differences in microbial diversity and composition between domesticated and wild strains is not completely unexpected, however, with previous studies identifying no significant effect of genetic background on the gut microbiome in hatchery reared Atlantic salmon strains (Uren Webster *et al.*, 2020). It is therefore likely that the differences in growth rate seen between domesticated and wild Atlantic salmon in common garden hatchery conditions (Einum and Fleming, 1997; Fleming *et al.*, 2002; Glover, Otterå, *et al.*, 2009; Wolters *et al.*, 2009; Skaala, Kevin A. Glover, *et al.*, 2012; Solberg, Skaala, *et al.*, 2013; Reed *et al.*, 2015; Harvey, Glover, *et al.*, 2016; Harvey, Solberg, Glover, *et al.*, 2016; Solberg *et al.*, 2016) is not microbially induced. It is possible that more nuanced differences in bacterial composition due to domestication may be detectable with greater sequencing power than was applied in this study.

6.4.3 Methodological considerations

Throughout all stages of both the laboratory and bioinformatic pipelines, problems were experienced with confounding cross amplification of host DNA amplification (12S rRNA gene), affecting both V4 and V1-2 primer sets. In addition to this, the short fragment nature of the host amplicons means that they were preferentially sequenced by the Illumina MiSeq flatform, thus reducing read depth for prokaryote amplicons. Therefore, future studies examining the gut microbiome through 16S rRNA gene amplicon sequencing, especially in salmon, should avoid inclusion of excess host tissue in DNA extracts. Such methods as intestinal scraping should be adopted instead of using entire cross sections of the gut, thus retrieving microbes intimately associated with the gut lining, while reducing host contamination.

High levels of the genus Mycoplasma were also detected in our samples, and although not uncommon in fish (Bano *et al.*, 2007), and even Atlantic salmon (Uren Webster *et al.*, 2020), the raised relative abundance of these bacteria are also likely due to the lack of cell wall; thus making them more likely to lyse during DNA extraction. The bias of DNA extraction was also detected in the standards, where gram negative bacteria such as *Salmonella enterica* and *Escherichia coli* that have a thin peptidoglycan layer were overrepresented when compared to the theoretical composition of the standard; demonstrating the importance of standards.

6.4.4 Summary

Using the methodologies outlined here, we did not detect a significant difference in bacterial diversity between domesticated and wild genetic backgrounds reared in a common garden. We did however uncover sexual dimorphism in alpha diversity, with males showing a more diverse microbiome than females. This adds to the few empirical studies on the sexual dimorphism in salmon, while also adding to the even more depauperate literature on sexual dimorphism in gut fish gut microbiomes (Bolnick, Snowberg, Hirsch, *et al.*, 2014); showing a contrasting trend to the sexual dimorphism documented in mammals such as mice (Elderman, de Vos and Faas, 2018). Further research around quantifying key ASVs using quantitative PCR, as well as understanding if there are functional differences between the male and female gut microbiome would be of interest, particularly to salmonid aquaculture where there is often a female sex bias due to the negative production traits associated with males. In addition to the application of a wider variety of molecular techniques, as with many microbiome studies, greater understanding could also be achieved by enhancing the temporal elements of sampling by sampling at different points throughout the salmon's life cycle. It is possible that the impact of genetic background and sex could be different at different stages of the fish's life cycle. Manipulations to the common garden environment could also prove vital in uncovering differences in the gut microbiome between genetic backgrounds and sex, as highlighted by the sex-diet interactions seen in threespine stickleback and Eurasian perch (Bolnick, Snowberg, Hirsch, *et al.*, 2014).

6.5 Acknowledgements

6.6 Author contributions

William Bernard Perry organised the sampling, conducted all laboratory work, bioinformatics, and drafting of the original manuscript. Kevin Alan Glover and Monica Favnebøe Solberg produced the fish for the experiment. Christopher Brodie, Angela Coral Medina and Kirthana G Pillay were involved in sampling, including dissections. Anna Egerton and Alison Harvey were involved in the DNA parentage and sexing of the fish. All authors, including those mentioned and Simon Creer, Martin Llewellyn and Martin Taylor, were involved in the conception and design of the experiment and contributed to data interpretation and editing the final draft of the manuscript. Kevin Glover and Gary Carvalho managed the project.

Chapter 7: General Discussion

7.1 Research highlights

7.1.1 Key findings in morphology

There have been many documented cases on the impact of domestication on the external morphology of fish, with many aquaculture reared fish displaying what has been referred to a 'cultured phenotype' (reviewed in Wringe, Purchase and Fleming, 2016), however, the literature surrounding the morphology of internal features, such as organs, is far more depauperate (Mayer et al., 2011; Joacim Näslund, Aarestrup, Thomassen, & Johnsson, 2012b; Poppe et al., 2003). In addition, many studies examining morphology, be it external or internal, are not conducted in a common garden, making comparisons between fish that are from a wild and domesticated genetic background, that have also been reared in wild and artificial environments, respectively. Without employing a common garden design, it is impossible to elucidate the effect of environment or genetic background and the process of artificial selection. Highlighted here are the contributions to the literature from this thesis, examining both external and internal morphology from common garden experiments.

The first key empirical finding of this work is the influence that domestication and the process of artificial selection has on secondary sexual traits, and thus sexual selection, with the reduction of fork length adjusted kype height (AKH). Until now, although the process of domestication has been known to change secondary sexual characters (Driscoll, Macdonald and O'Brien, 2009), and there have been some empirical studies (Tiemann and Rehkämper, 2009), our multi life stage, common garden experimental design provides compelling evidence to a poorly studied area of artificial selection. Moreover, it is the first study to explore secondary sexual traits in the context of the aquaculture industry, with implications for escaped domesticated fish on the viability of wild populations. Previous behavioural studies have demonstrated that relative kype size is correlated with the dominance rank, and is therefore important in mating success (Järvi, 1990), demonstrating a link between domestication driven morphological change and mating success. Domestication and escapees

influencing mating success in wild populations through morphological change is not only applicable to salmon, however, as there are a plethora of other aquaculture species in which escapees and introgression with wild populations takes place (Jensen *et al.*, 2010; Baskett, Burgess and Waples, 2013; Faust *et al.*, 2018; Fukui *et al.*, 2018; Whittaker, Consuegra and Garcia de Leaniz, 2018).

The second empirical finding, which also relates to the external morphology, is the role of domestication on many traits closely related to fitness in highly vagile species such as salmon, including pectoral fin length, eye width and body shape. Based on a wealth of previous research looking at the impact of domestication on overall body morphology (Wringe, Purchase and Fleming, 2016), this is not unexpected. The truly novel discovery we uncovered, however, is associated with the reciprocal common garden design of both natural and artificially reared fish from both domesticated and wild backgrounds, which showed that differences in these traits is subject to how the fish was reared. Regarding eye width and body shape, our data suggests that maladaptive phenotypes induced by domestication are quickly removed from the population through stabilising natural selection to a wild norm, when fish are reared naturally. Removal of maladaptive phenotypes seen in domesticated individuals demonstrates the fitness consequences of morphological shifts from the wild phenotype, and goes to explain why fish from domesticated backgrounds have been seen to have reduced fitness in the wild (Skaala, Kevin A. Glover, *et al.*, 2012b; Glover *et al.*, 2018b).

The third empirical finding relates to domestication and heart morphology, where we showed that genetic background (wild, domesticated or reciprocal wild x domesticated hybrid) had no significant effect on heart morphology. As comparative studies on domesticated vs wild fish are ever increasing, there is increasing interest in cardiac performance in salmonids; with interest in domesticated induced change to heart morphology (Castro et al., 2011; Claireaux et al., 2005; Jonsson & Jonsson, 2006b). Despite this, however, only two studies have investigated the influence of domestication on heart morphology to date (Poppe *et al.*, 2003; Seierstad *et al.*, 2005). Both studies have been conducted on fish not only from different genetic backgrounds, but also from different rearing environments

(aquaculture or the wild). Therefore, it is not evident if the larger hearts relative to body size seen in these studies is a heritable genetic change, and likely to cause long lasting introgression-linked phenotypic change in wild populations. Our use of a common garden design enabled detection of any signal related to genetic background, which suggests that significant effects in previous studies (Poppe *et al.*, 2003) have likely been linked to environmental rearing and is therefore a plastic response, as has been concluded in other species such as Atlantic cod (*Gadus morhua*) (Mayer *et al.*, 2011). Although another possibility for the conflicting results presented here, is the inadequate statistical approaches for removal of body size.

The work here has demonstrated the importance of using statistically robust methods for accounting for body size and allometry. Previous studies examining the impact of domestication on heart morphology have used simple division metrics to remove the effect of body size, which, as demonstrated here, can lead to type 1 error and false positive results. We discovered that there was no effect of genetic background on different heart metrics. We also discovered sexual dimorphism in body size adjusted heart weight and heart width. The sexual dimorphism discovered here conflicts with the literature, which has documented sexual dimorphism in heart size, but with males having greater body size adjusted heart weights than females (Graham and Farrell, 1992; Armstrong and West, 1994; Clark *et al.*, 2009; Fraser *et al.*, 2015). Here, we document that it is in fact females with the larger relative heat size. However, many experiments in the literature use simple division metrics to remove the effect of body size, introducing false positives. When these same flawed statistical approaches were applied to our dataset, they gave the same trend seen in the literature, with males having larger 'body weight adjusted' hearts.

7.1.2 Key findings in the microbiome

Here, we find no significant signal of domestication induced changes to bacterial diversity in the midgut of Atlantic salmon, which has been highlighted to some extend in other recent studies on Atlantic salmon (Uren Webster *et al.*, 2020). Importantly, it means that the gut microbiome has not

seen extensive changes to bacterial communities due to artificial selection, and so introgression from escapees should not pose any threat to the wild type gut flora in resulting as wild x domesticated hybrids; thus avoiding associated fitness reductions. It also provides evidence that manipulation of the hologenome through artificial selection remains an unexploited resource of genetic and functional diversity, a concept that is far more prominent in plant breeding (Nogales *et al.*, 2016). What we do detect here, however, is sexual dimorphism in alpha diversity of the gut, a phenomenon which has been demonstrated in mammals (Elderman, de Vos and Faas, 2018), albeit an opposite trend to what is observed here; but has only once been previously documented in fish (Bolnick, Snowberg, Hirsch, *et al.*, 2014).

7.1.3 Overview of findings

Here we demonstrate that domestication is having a profound influence on many aspects of the Atlantic salmon's biology, including secondary sexual traits, growth, body shape, eye width and pectoral fin length. Yet we did not detect significant changes in all aspects of biology, for example: heart morphology, liver weight, brain morphology and the gut microbiome. Looking at what elements have been impacted by domestication, one observation is that internal features have not been significantly affected by domestication, or at least to a level where it can be detected using the methods implemented here, yet external features have. One possible explanation for the disparity in trends between internal and external features could be due to the highly conserved nature of genes controlling organ development, with high levels of pleiotropy in genes that are expressed in early development (Cardoso-Moreira *et al.*, 2019). Therefore, the evolutionary drivers associated with domestication, including artificial selection and genetic drift, are constrained, and may be unable to cause morphological shifts in phenotype in the ~13 generations observed in domesticated Atlantic salmon.

7.2 Knowledge gaps

7.2.1 Kype size

There is a considerable lack of literature in relation to secondary sexual traits in Atlantic salmon. We found no relation between fork length adjusted kype height and the body weight adjusted ejaculate weight, though further research could more accurately measure the volume of the ejaculate, alongside quality of the sperm, using important metrics such as relative sperm velocity (Gage *et al.*, 2004). Although domestication has been documented not to impair functional performance of sperm (Yeates *et al.*, 2014), it would be of interest to see how performance would correlate to kype size, with the additional interaction of genetic background. One hypothesis is that a larger kype could signal male fertility and sperm quality to females, as has been observed in red deer (*Cervus elaphus*) (Malo *et al.*, 2005). If this were the case, it would also give an indication of whether the main function of the kype is weaponry for male-male competition, or an ornament conveying fertility (McCullough, Miller and Emlen, 2016). The role of the kype in mating behaviour would also benefit from further research. For example, examining mating success of Atlantic salmon with an artificially increased kype size, before and after it had been increased. One method in which kype size could be artificially increased is through the use of 3D printed prosthetic kypes, using techniques that have been successfully applied to a wide range of animal taxa from parrots to sea turtles (Nickels, 2018). An improved understanding of natural variation in kype size throughout the Atlantic salmon's home range, with the additional interest in rivers with greater introgression, are priority areas for future study.

Finally, additional attention should be paid to other possible secondary sexual traits in Atlantic salmon, which are even well less understood, such as the adipose fin. Much like the kype, there is evidence to show that the adipose fin is involved in female mate choice in Atlantic salmon (Järvi, 1990), and in Arctic charr (*Salvelinus alpinus*) subordinate males show a reduced adipose fin size when compared to dominant males (Haugland *et al.*, 2011). Basic metrics such as adipose fin weight and fin area measured in a similar experimental design as the kype study presented here could delineate whether

removal of mate choice also causes a reduction in its size. Correlations between the kype and adipose fin size would also be of interest, to better understand the mechanisms of sexual selection acting on each. As the adipose fin cannot be used in male-male conflict (unlike the kype), it can only be ornamental, thus, it is possible that if there was a reduction in adipose size between wild and domesticated individuals, the magnitude of the reduction could be used to inform the extent to which the kype is an ornament or a weapon.

7.2.2 Body morphology

Here we comprehensively assessed aspects of body morphology, though without focus on fin measures. Rather than using whole fish images, a specific study on the impact of domestication on fin morphology remains largely unstudied and could build on the insights presented in this thesis. Such a study would require fins to be removed from the fish, pinned in such a manner that opened the entire fin, and photographed independently from the body. Such photographs would allow a great number of fin morphology metrics, rather than just length, including metrics such as fin area, number of rays, and overall fin shape using geometric morphometrics. Methods such as these would allow investigation, not only into the pectoral fin, but also the adipose fin, the dorsal fin, caudal fin, anal fin and pelvic fin.

To be able to assess the hydrodynamic consequences of morphological differences we describe here would add a level of functional understanding and help to further explain the fitness consequences seen in domesticated and hybrid fish in the wild. To achieve this, morphological data would need to be combined with kinematics data in a laboratory setting, using techniques such as scanning particle image velocimetry (PIV) in a flow chamber, allowing for collection of crucial parameters such as thrust and drag (Lauder and Madden, 2007). Finally, understanding habitat choice between domesticated and wild fish in a natural river, or semi-natural environment, and the hydrodynamic properties of these choices, would start to elucidate the behavioural and energetic decisions driven by this change in morphology. Previous studies examining positioning choice in Atlantic salmon have implementing

techniques such as 3D acoustic Doppler velocimeter (ADV), which provides high resolution data on turbulent flow (Wilkes *et al.*, 2017). Combining behavioural data on fish position within a semi-natural experimental arena, along with data on turbulent flow, fin morphology and multiple genetic backgrounds (wild, domesticated and hybrids), it would be possible to start to understand the behavioural and energetic decisions driven by fin morphology. Indeed, similar experimental designs in the literature have shown that 86% of Atlantic salmon chose locations with significantly lower predicted swimming costs than expected at random (Wilkes *et al.*, 2017); comparing data such as these between genetic backgrounds and morphology could identify fitness consequences caused by domestication induced morphological shifts.

7.2.3 Heart morphology

Here, we did no discover any differences between domesticated and wild genetic backgrounds in the heart morphology metrics we measured. Despite this, these metrics were simplistic, and being able to detect domestication induced changes could benefit from more state-of-the-art techniques. Just as we applied micro-CT imaging to brain morphology, the same technique could also be applied to hearts, and would provide a high-resolution 3D model of the heart, as has been demonstrated in the hearts of rodents (Jarvis and Stephenson, 2013). Increasing dimensionality from 2D length metrics used here, could help understand more nuanced differences in heart morphology, and allow the application of geometric morphometrics. It would also allow measurement of internal structures within the heart, such as muscle width across different regions of the heart. These techniques could also give a more detailed understanding of the sexual dimorphism documented here.

7.2.4 Brain morphology

Although no significant differences in brain metrics between domesticated and wild were identified here, an increased sample size could improve our ability to detect more subtle changes in the anatomy of the brain between wild and domesticated individuals. In addition to this, it would also be valuable to combine anatomical data with behavioural data. Finally, increasing the number of samples and

collecting behavioural data should be combined with reciprocal common gardens, whereby these metrics are measured in fish from domesticated and wild genetic backgrounds reared in artificial and natural conditions. There are a number of well documented behavioural differences between wild and domesticated Atlantic salmon that have been assessed in common garden experiments, including domesticated fish showing higher levels of aggression (Fleming and Einum, 1997; Houde, Fraser and Hutchings, 2010b) and dominance (Fleming and Einum, 1997; Metcalfe, Valdimarsson and Morgan, 2003) than wild fish, while also showing reduced levels of antipredator behaviour (Fleming and Einum, 1997; Johnsson, Höjesjö and Fleming, 2001; Houde, Fraser and Hutchings, 2010a). It would then be possible to test if behavioural traits are linked with structural changes within the brain, as has been demonstrated in other fish such as cichlids (Fischer *et al.*, 2015).

Finally, in addition to the gross anatomy of the brain, another important factor that is not assessed using micro-CT scanning is the density of the neurones within brain regions. For example, previous work on rats has shown that multigeneration stress has reduced neural density in areas that regulate the stress response (McCreary *et al.*, 2016), with increased stress being a component of the aquaculture environment (Robinson *et al.*, 2019). Therefore, being able to measure the density of neurones is also an important factor in the structure of the brain. One crude method in achieving this would be to look at the relationship between the mass and length of the brain, giving some index of density, however, a far more precise method would be to apply histological approaches to count the number of neurones within a given area (Kelly and Hawken, 2017).

7.2.5 Gut microbiome

Here we did not detect any changes in alpha or beta diversity between domesticated and wild genetic backgrounds, though the data would benefit from using a more powerful sequencing platform such as the Illumina NextSeq 2000, thus increasing read depth. Greater read depth would give better resolution of data and could allow for the identification of rarer taxa that may have been removed in the bioinformatics filtering pipeline outlined here. Targeting single operation taxonomic units that are

of interest using quantitative polymerase chain reaction (qPCR) could also be of benefit, providing absolute abundances of bacterial taxa, rather than the relative abundances produced from the Illumina sequencing platforms. In addition, the sequencing of gut contents alone could also be considered, so that there is no issue with cross amplification of eukaryotic host DNA. Finally, although the diversity of the gut microbiome does not appear to differ between genetic backgrounds, greater experimental manipulation of factors that are known to impact the microbiome (e.g. diet (Desai *et al.*, 2012), antibiotics (Navarrete *et al.*, 2008), physiochemical properties of the water (Ornelas-García *et al.*, 2018)) may elucidate different microbial responses based on genetic background. Interactions between factors and their effect on the gut microbiome are beginning to be understood in fish, such as the interaction between sex and diet (Bolnick, Snowberg, Hirsch, *et al.*, 2014), but many studies focus on one factor.

7.3 Societal relevance

Not all aspects of morphology and microbiome were affected by domestication, however, features which did show domestication induced change also showed intermediate changes in domesticated x wild hybrids, demonstrating the long-lasting genetic impact on the phenotypes of introgressed wild populations due to escapees from aquaculture. What we also demonstrated in relation to body morphology, with the implementation of a reciprocal wild common garden, is that domestication induced phenotypes are likely to have a considerable fitness consequence. The negative impacts of introgression on fitness demonstrates that if already challenged wild populations of Atlantic salmon are to be conserved, escapees from aquaculture facilities need to be prevented at all costs. In addition, it demonstrates that the consequences of escapees from aquaculture facilities can influence population fitness long after the event.

Appendix 1: The role of the gut microbiome in sustainable teleost aquaculture

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.

A1.1 Introduction

Since its conception in the 1980s describing soil ecology (Whipp, Lewis and Cooke, 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, Morgan and Stewart, 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 and Venkatesh, 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 (a) 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.
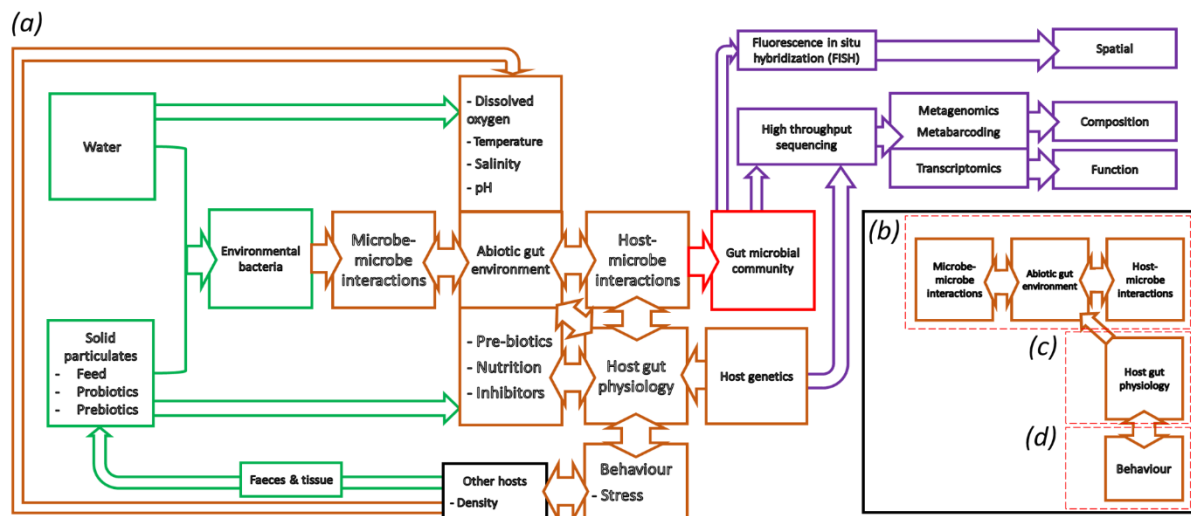
Figure A1.3 (a) Schematic view of the deterministic processes that influence gut microbial communities in fish. Community assemblage of bacteria in the gut starts with inputs from the environment (green), such as the bacteria within the water column, or in solid particulates of biofilm, sediment and 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 boarder interactions is provided, with focus on (b) microbe-environment-host interactions, (c) host gut physiology and (d) behaviour.

A1.2 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, Ghosh and Ringø, 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, Blas, *et al.*, 2006; 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, Miyajima and Deguchi, 1990). Here we discuss this host-microbe relationship in the context of contemporary aquaculture, with a focus on two timely issues: fishmeal and starvation.

A1.2.1 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.*, 2018b). 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.*, 2018b).

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.

A1.2.2 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

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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.

A1.3 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, Decamp, *et al.*, 2006), 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, Liu and Hu, 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, Samuel and Gordon, 2004). Colonising microbes can also modulate host gene expression to create favourable gut environments, thereby constraining invasion by pathogens (Balcázar, Blas, *et al.*, 2006), 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.*, 2010). 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, Taylor and Jackson, 2019). Understanding microbial-host-environmental interactions like this are crucial for aquaculture, where, as previously discussed, diet is often manipulated.

A1.3.1 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.

A1.3.2 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).

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.

A1.4 Artificial selection

Within aquaculture, selection has been applied routinely to increase production by enhancing desirable traits such as growth and disease resilience (Yáñez, Newman and Houston, 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 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, Batra and Srinivasan, 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, Wiens and Salinas, 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.

A1.5 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, Lewis and Volpe, 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).

A1.5.1 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 A1.4). 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.H *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).
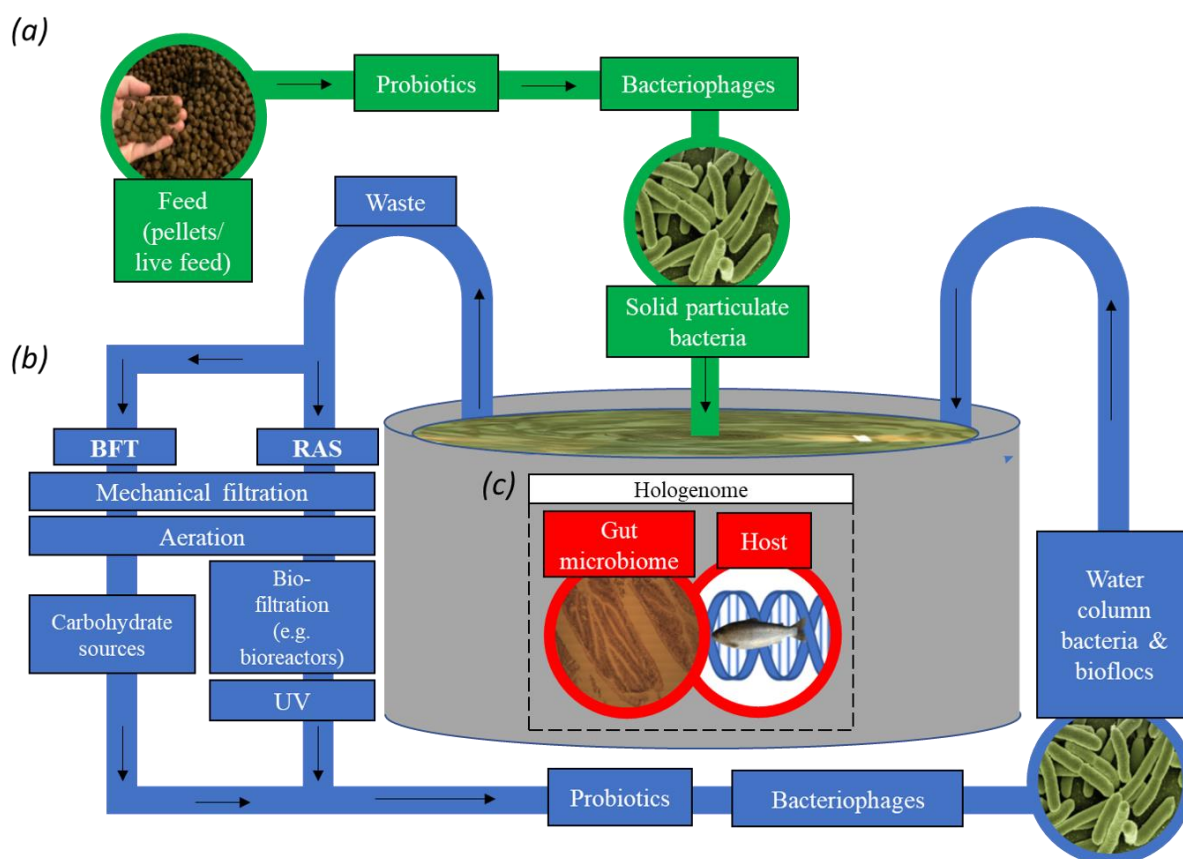
Figure A1.4 Schematic diagram of (a) feed inputs (green), (b) water processing (both recirculating aquaculture systems (RAS) and Biofloc technology (BFT)) (blue) and the (c) species being cultivated, along with its gut microbiome (red).

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 A1.4b); 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.

A1.5.2 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.

A1.5.3 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, Gabriel and Akinrotimi, 2011). More research is needed, however, in assessing the reliability and accuracy of faecal microbiome sampling in identifying stress.

A1.6 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 A1.1b, 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 A1.5). 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.
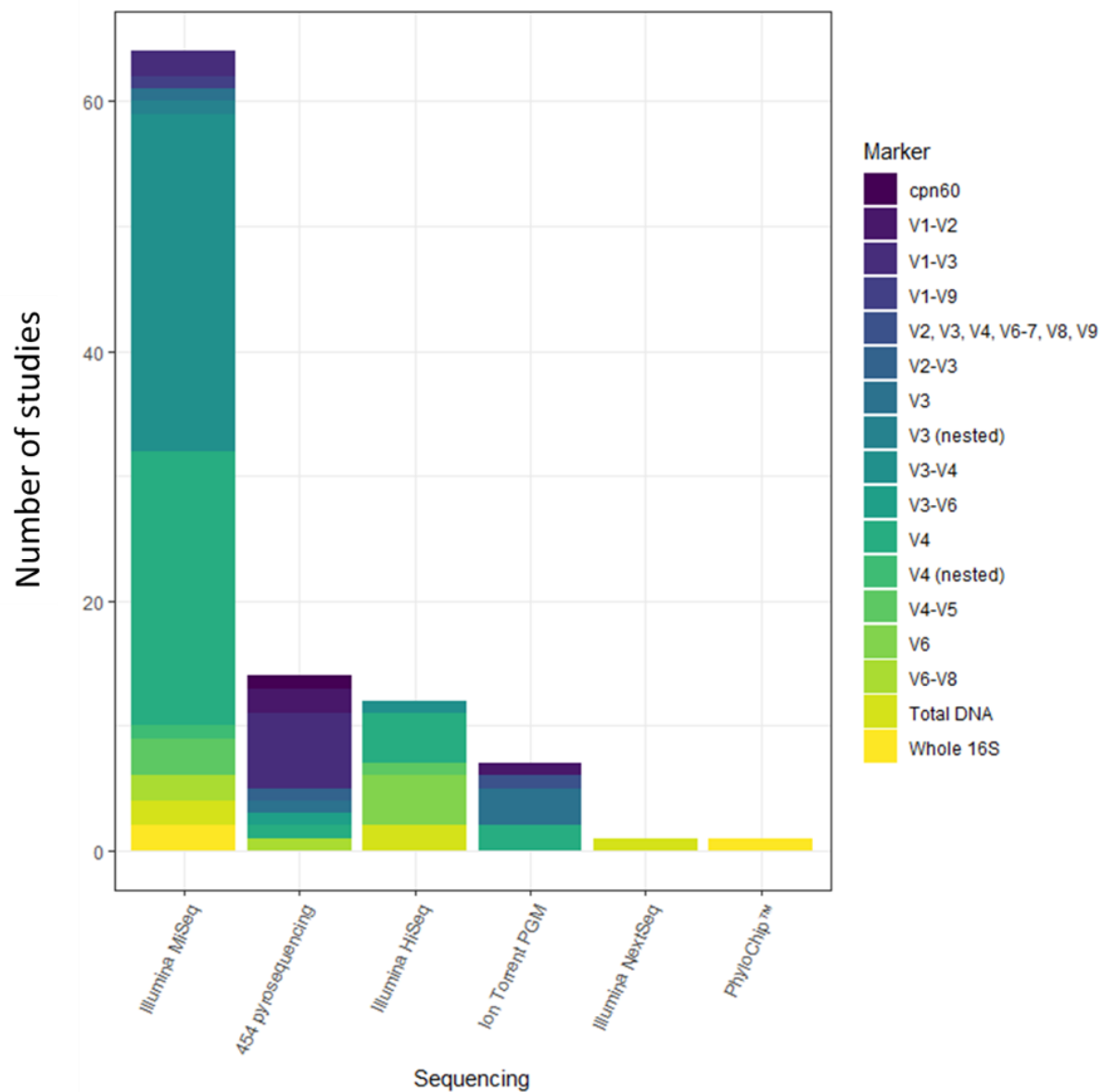
Figure A1.5 Methodological approaches used in high throughput sequencing of fish gut microbiomes, broken down by the type of sequencing platform, and genetic marker. Marker type are predominantly variable regions (V) within the 16S ribosomal RNA gene. Further information on search terms and filtering 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.

A1.7 Authors' contributions

William Bernard Perry organised the creation of the review and facilitated communications between authors. William Bernard Perry, Elle Lindsay, Raminta Kazlauskaite, Christopher James Payne and Christopher Brodie contributed to the concept and writing of the review.

A1.8 Supplementary information

A1.8.1 Systematic review

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 Hybridization (FISH)

Data on the aquaculture status of fish was gathered from FishBase (Froese, 2019).

Appendix 2: Supplementary material for chapter 2 - Evolutionary drivers of kype size in Atlantic salmon (*Salmo salar*): domestication, age and genetics
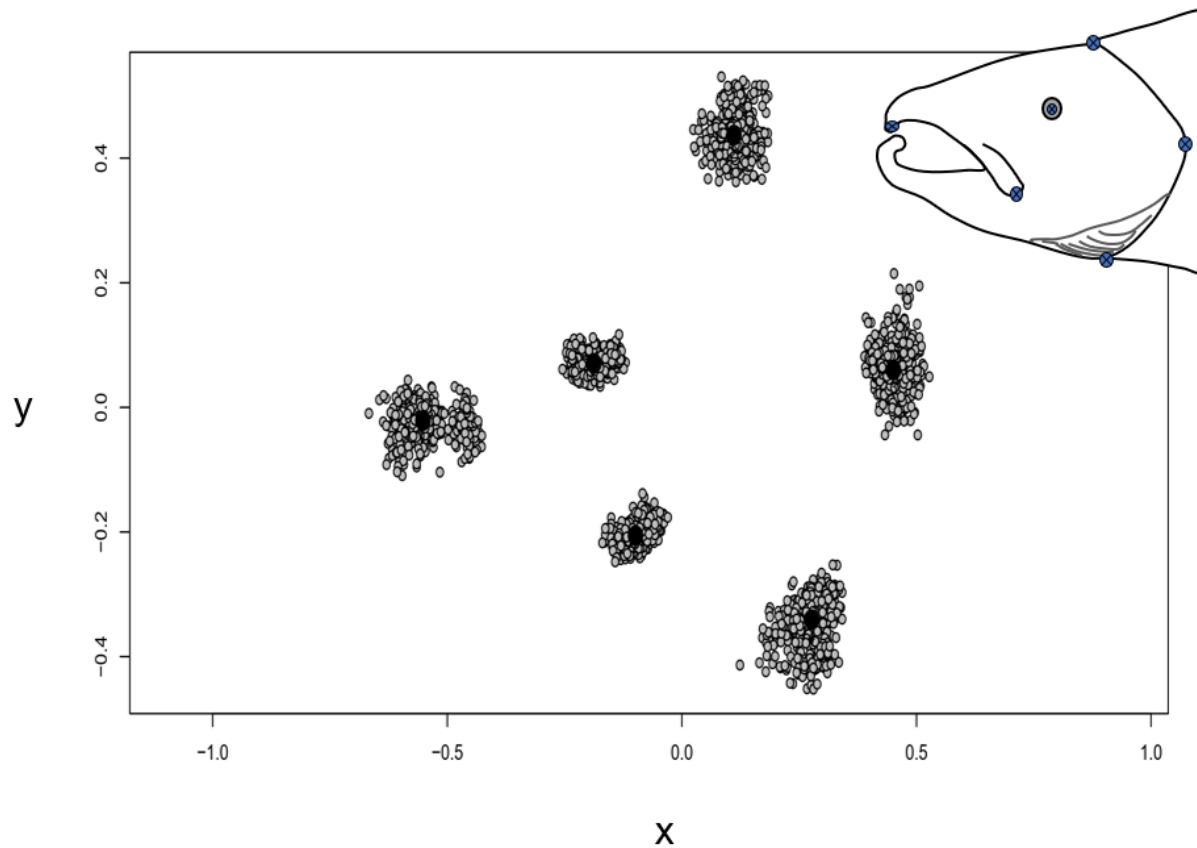
Figure A2.1 Aligned generalised Procrustes analysis points from the 6 landmarks used in the geometric morphometric analysis, produced using the R package 'geomorph' (Adams and Otárola-Castillo, 2013)**. Each grey point represents a landmark on an individual, with the black points representing the mean of those points.**
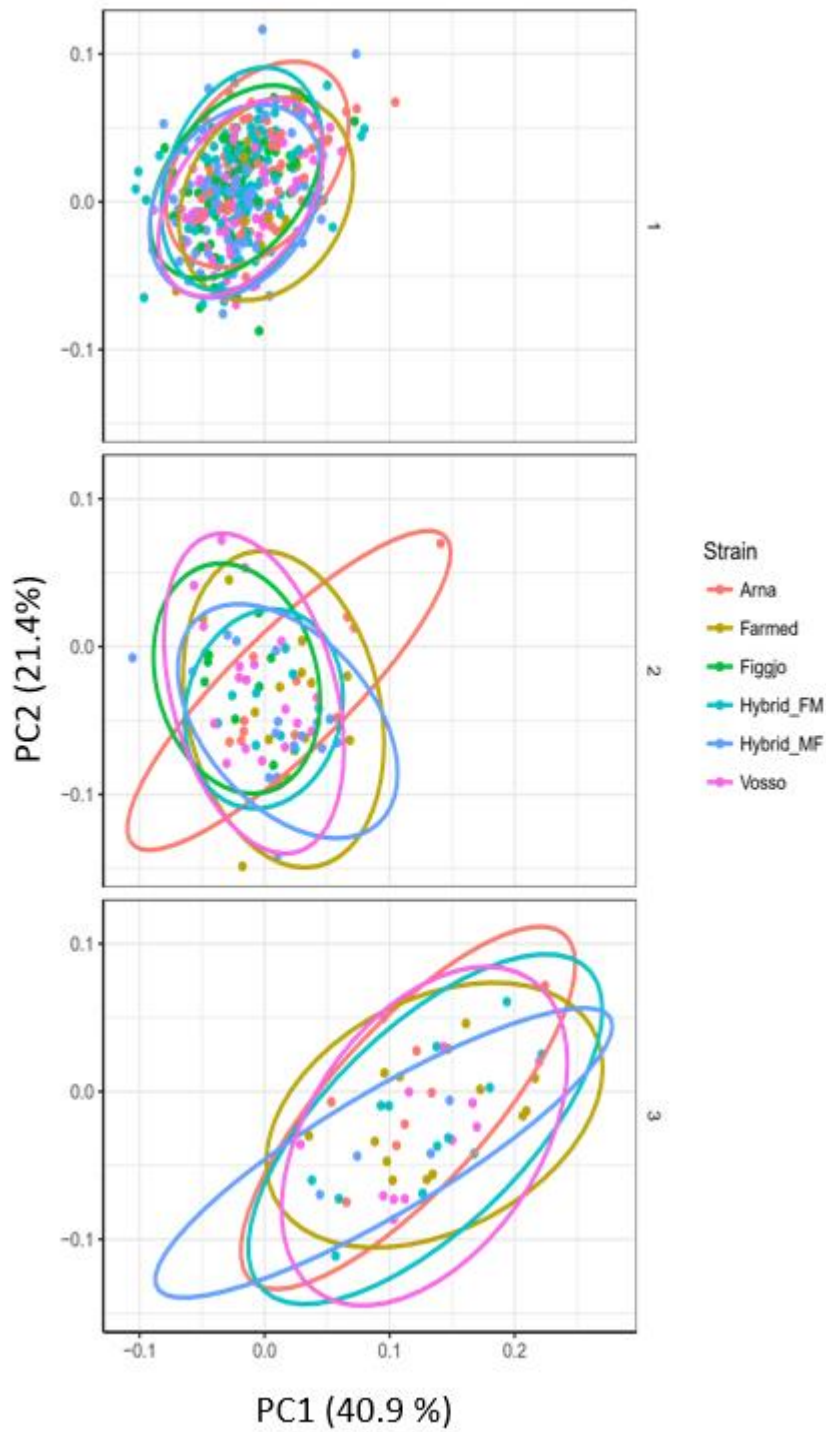
Figure A2.2 Principle component plot summarising the greatest variance in morphospace of salmon head morphology, as represented by the 6 landmarks outlined in figure 1a. Groups are split into 1SW, 2SW and 3SW, with strains broken down by colour. Strains are eclipsed by a 95 % confidence interval.
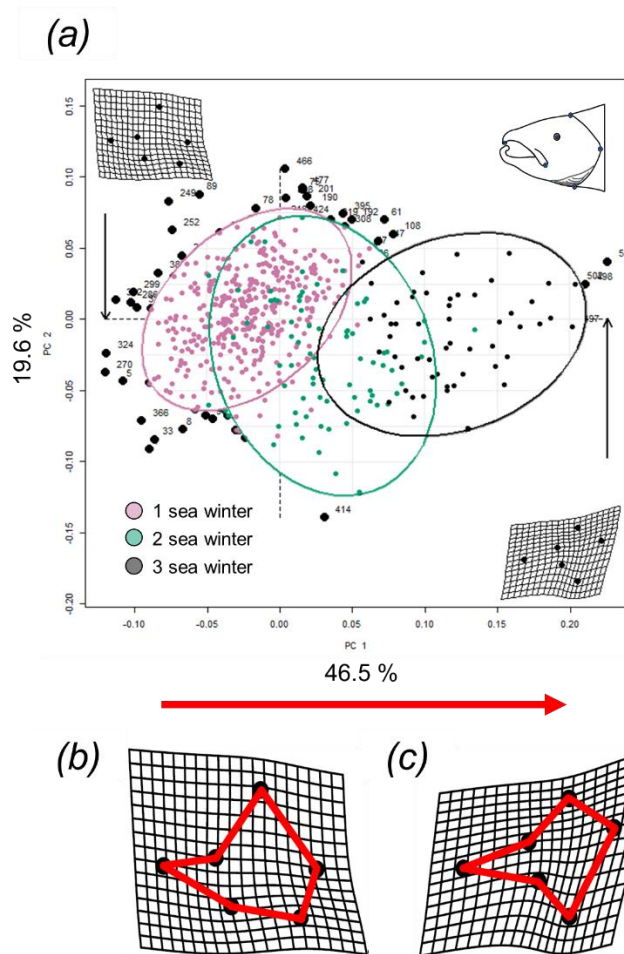
Figure A2.3 (a) Principle component plot summarising the greatest variance in morphospace of male salmon head morphology, as represented by the 6 landmarks outlined in figure 1a. Individuals are grouped by sea winter and are eclipsed by a 95 % confidence interval. Large black dots are outliers outside of the 95 % confidence intervals, and the associated numbers relate to their ID within the landmark TPS file (see data provided). Thin plate splines representing the 6 landmarks at most (b) negative and (c) positive values of PC1 are also displayed, with the landmarks connected to better identify shape change.
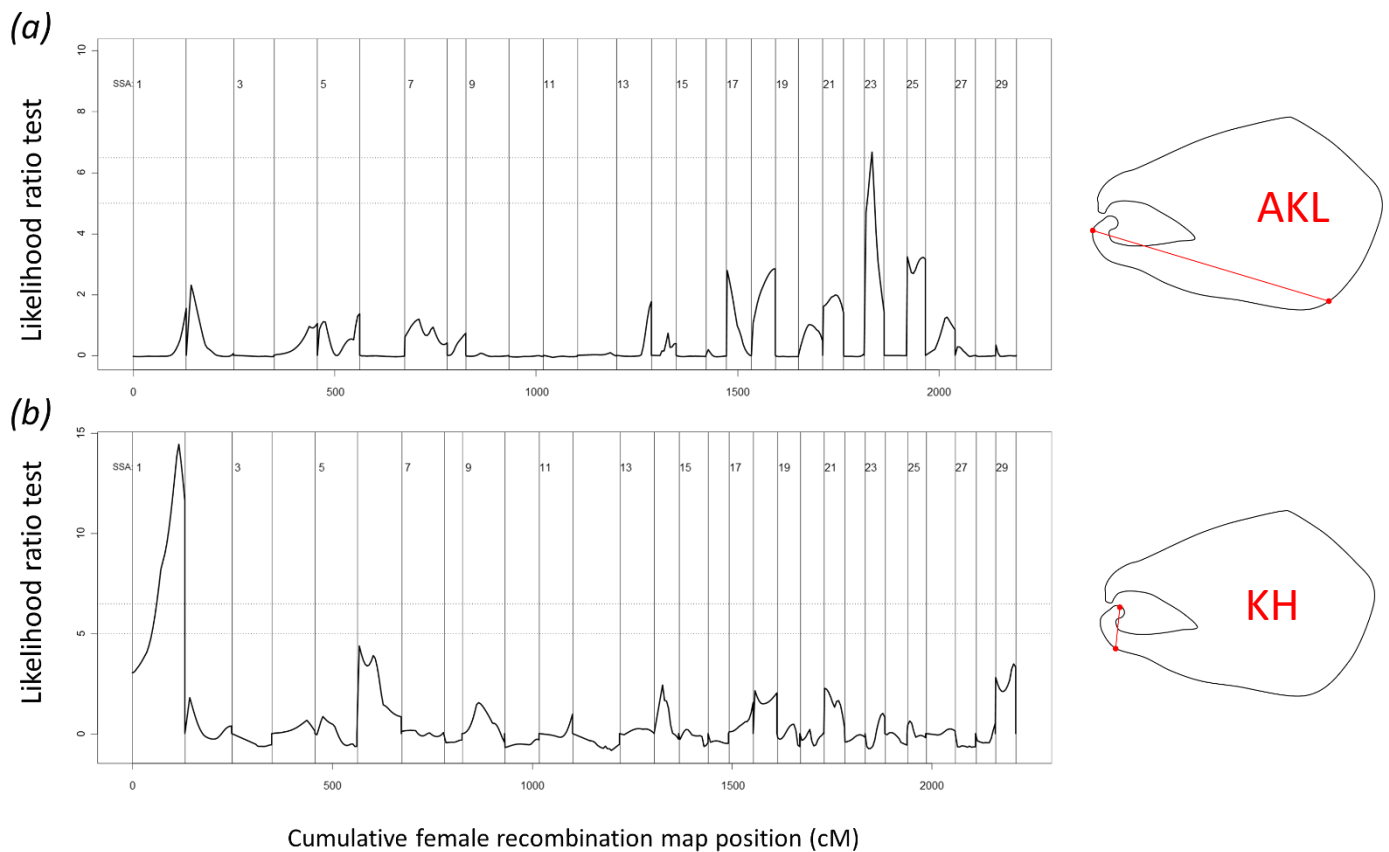
Figure A2.4 Quantitative trait loci scan plots highlighting peaks in likelihood ratio test values for (a) adjusted kype length (AKL) on linkage group SSA23, and (b) kype height (KH) on linkage group SSA1.

Table A2.1 Pairwise comparisons for fork length adjusted kype length (AKL) and fork length adjusted kype height (AKH) between strains, as produced in the R package 'emmeans' (Lenth, Love and Maintainer, 2018)**. Table include estimated mean, standard error (SE), degrees of freedom (DF), t ratio and *P* value. Significant *P* values (P < 0.05) are in bold.**

Measurement	Contrast	Estimate	SE	df	t.ratio	p.value
AKL	**Arna – Farmed**	**-0.03439**	**0.01046 2**	**63.2**	**-3.287**	**0.0197**
AKL	Arna – Figgjo	-0.02214	0.009231	39.38	-2.398	0.1817
AKL	Arna – Hybrid_FM	-0.01071	0.008495	27.94	-1.261	0.8031
AKL	**Arna – Hybrid_MF**	**-0.0279**	**0.00846 8**	**30.05**	**-3.295**	**0.0276**
AKL	Arna – Vosso	-0.01505	0.008574	31.62	-1.755	0.5075
AKL	Farmed – Figgjo	0.012251	0.010811	77.68	1.133	0.866
AKL	Farmed – Hybrid_FM	0.023677	0.008969	485.67	2.64	0.0898

AKL	Farmed – Hybrid_MF	0.006483	0.010044	64.37	0.645	0.987
AKL	Farmed – Vosso	0.019337	0.009987	62.58	1.936	0.3904
AKL	Figgjo – Hybrid_FM	0.011426	0.008756	34.05	1.305	0.7803
AKL	Figgjo – Hybrid_MF	-0.00577	0.007575	375.32	-0.761	0.9737
AKL	Figgjo – Vosso	0.007086	0.00887	38.85	0.799	0.966
AKL	Hybrid_FM – Hybrid_MF	-0.01719	0.007962	24.98	-2.16	0.2911
AKL	Hybrid_FM – Vosso	-0.00434	0.008073	26.52	-0.538	0.994
AKL	Hybrid_MF – Vosso	0.012854	0.008034	28.73	1.6	0.6052
AKH	**Arna – Farmed**	**0.101016**	**0.027007**	**59.67**	**3.74**	**0.0053**
AKH	Arna – Figgjo	-0.01513	0.023605	36.15	-0.641	0.987
AKH	Arna – Hybrid_FM	0.059163	0.021985	25.76	2.691	0.1116
AKH	Arna – Hybrid_MF	0.035335	0.022015	28.38	1.605	0.602
AKH	Arna – Vosso	0.029822	0.022322	29.57	1.336	0.7631
AKH	**Farmed – Figgjo**	**-0.11615**	**0.027898**	**76.14**	**-4.163**	**0.0011**
AKH	Farmed – Hybrid_FM	-0.04185	0.022363	463.42	-1.872	0.4211
AKH	Farmed – Hybrid_MF	-0.06568	0.026279	64.01	-2.499	0.1397
AKH	Farmed – Vosso	-0.07119	0.026248	62.3	-2.712	0.0871
AKH	**Figgjo – Hybrid_FM**	**0.074292**	**0.022594**	**32.35**	**3.288**	**0.0269**
AKH	Figgjo – Hybrid_MF	0.050464	0.018605	392.65	2.712	0.0751
AKH	Figgjo – Vosso	0.04495	0.022988	37.39	1.955	0.3862
AKH	Hybrid_FM – Hybrid_MF	-0.02383	0.020944	24.7	-1.138	0.8609
AKH	Hybrid_FM – Vosso	-0.02934	0.021267	25.91	-1.38	0.7383
AKH	Hybrid_MF – Vosso	-0.00551	0.021311	28.91	-0.259	0.9998

Appendix 3: Supplementary material for chapter 3 - Domestication induced change in body morphology: a study of Atlantic salmon (*Salmo salar*) in an artificial and natural common garden

Parentage analysis – Irish fish (extended)

Genomic DNA was extracted from alcohol-preserved fin-clip samples using the Promega Wizard® SV 96 Genomic DNA Purification System. DNA quality was assessed on agarose gels by comparison with a Quick-Load® Purple 100 bp DNA Ladder (New England Biolabs) and concentration was estimated using a Nanodrop microvolume spectrophotometer (Thermo Fisher Scientific). Aliquots of DNA were diluted to approximately 2-10ng/μL for microsatellite locus amplification.

Ten microsatellite DNA loci were amplified in three multiplex panels (Panel 1, Ssa197 (O'Reilly et al., 1996) and MHC2 (Stet et al., 2002); Panel 2, Ssa202, Ssa171 (both O'Reilly et al., 1996), Sssp2210 (Paterson et al. 2004) and SsaD170 (unpublished; EMBL Accession no. AF525205); Panel 3, Ssp2216, Ssp1605 (both Paterson et al., 2004); SsoSL85 (Slettan et al., 1995) and SsaD157 (King et al., 2005)). All PCRs were performed in a total volume of 3.5μL, including 1μL of genomic DNA and 1.75μL Plain Combi PP Master Mix (TopBio). Primer concentrations (same for forward and reverse primers for each locus) and fluorescent label employed in each panel were as follows: Ssa197(VIC) 0.02μM, MHC2(NED) 0.04μM, Ssa202(FAM) 0.06μM, Sssp2210(VIC) 0.03μM, SsaD170(NED) 0.06μM, Ssa171(PET) 0.06μM, Ssp2216(VIC) 0.02μM, SsoSL85(NED) 0.04μM, SsaD157(NED) 0.12μM and Sssp1605(PET) 0.06μM. Forward primers included fluorescent labels from the Applied Biosystems (ABI) standard dye sets to enable visualisation on ABI genetic analysers and reverse primers included a GTTT 'pig-tail' to minimise stuttering. Primers to amplify a locus for sex determination was included in Panel 1, SalmoYF (forward primer labelled with VIC (0.015μM forward and reverse primer concentration)) (Paulo Prodohl, pers.comm.). Cycling conditions included an initial denaturing period of 15 minutes at 95°C followed

by: five cycles of 30 seconds at 94°C, 90 seconds at 55°C, 1 minute at 72°C; then 22 cycles of 30 seconds at 94°C, 90 seconds at 57°C and 1 minute at 72°C; and a final incubation at60°C for 30 minutes.

Each sample was diluted in Hi-Di™ Formamide with GeneScan™ 600 LIZ™ Dye Size Standard (ThermoFisher Scientific) as an internal size ladder, comparison with which enabled allele size estimation. Samples were denatured by incubation at 95C for 3 minutes and snap-chilled prior to Electrophoresis performed on an ABI3500xl DNA analyser using POP-7™ Polymer. Alleles and genotype calling for each microsatellite locus in each individual were executed using GeneMarker (SoftGenetics).

A holistic approach to determine a consensus genetic provenance was developed using (1) a maximum likelihood method to assign sibship with COLONY (Jones et al 2010), (2) a systematic Bayesian clustering approach with STRUCTURE (Pritchard, Stephens & Donnelly 2000), (3) a discriminant analysis of principal components trained on parental genotypes with adegenet (Jombart T 2008), and (4) group-individual relatedness (SUPP MAT KAAAARL to be provided by Karlito Master).
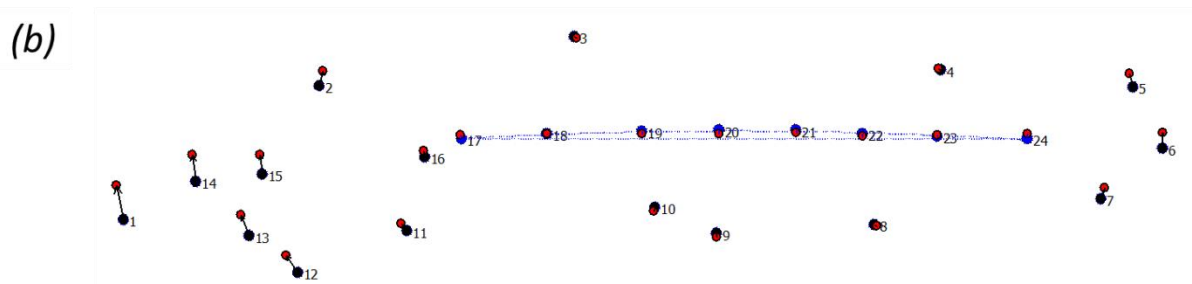
Figure A3.1 (a) Landmarks (yellow) applied along the lateral line to remove the effect of fish bending on shape. Landmarks were not based on any morphological structure on the landmarks and were simply added as equidistantly as possible. Due to the position of the landmarks in the y plane not being based on any morphological feature, they were not included in the geometric morphometrics. In addition, (b)an example of alterations made by tspUtility to remove the effect of bending, based on the 8 landmarks placed along the lateral line.
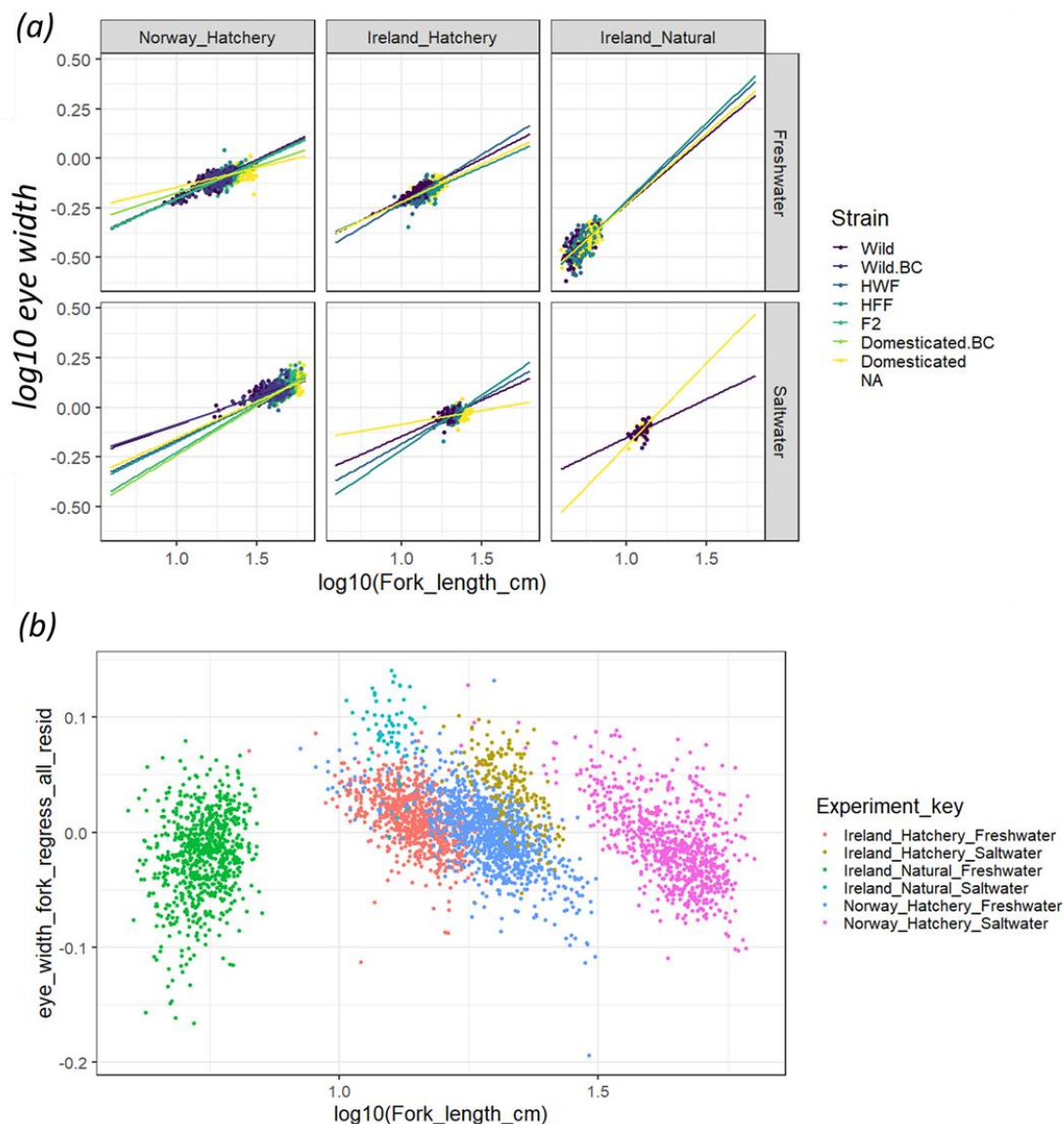
Figure A3.2 (a) Regressions between log10 transformed fork length and log10 transformed eye width, broken down by genetic background and categorised by life stage, experiment origin and rearing type. The difference in allometry between artificially reared and naturally reared fish meant

that (b) the residuals from a regression including all datapoints (not partitioned) did not remove the effect of fork length on characters. Eye width is shown here as an example, but similar results were seen for the pectoral fin length. Based on this, data was partitioned into three groups: Norway artificially reared, Ireland artificially reared and Ireland naturally reared.

Table A3.1 Estimated marginal means and corresponding standard errors for the linear mixed effect model examining fork length in cm between genetic backgrounds within each of the experimental groups.

Strain	lifestage	Experiment	Rearing	emmean	SE	asymp.LCL	asymp.UCL
Wild	Freshwater	Ireland	Hatchery	12.57345433	0.258557	12.06669254	13.08021611
HWF	Freshwater	Ireland	Hatchery	13.1799698	0.232806	12.72367931	13.6362603
HFF	Freshwater	Ireland	Hatchery	14.15591225	0.225364	13.71420757	14.59761693
Domesticated	Freshwater	Ireland	Hatchery	15.53377267	0.227059	15.08874478	15.97880056
Wild	Saltwater	Ireland	Hatchery	19.10173483	0.386514	18.34418114	19.85928852
HWF	Saltwater	Ireland	Hatchery	20.75762721	0.388007	19.99714735	21.51810707
HFF	Saltwater	Ireland	Hatchery	20.95067914	0.359551	20.24597117	21.6553871
Domesticated	Saltwater	Ireland	Hatchery	23.55688352	0.333131	22.9039587	24.20980834
Wild	Freshwater	Norway	Hatchery	15.85638106	0.217738	15.4296216	16.28314052
Wild.BC	Freshwater	Norway	Hatchery	17.20656322	0.221953	16.77154253	17.64158392
HWF	Freshwater	Norway	Hatchery	19.35660505	0.2809	18.80605093	19.90715918
HFF	Freshwater	Norway	Hatchery	19.69636488	0.287717	19.1324492	20.26028056
F2	Freshwater	Norway	Hatchery	19.38069805	0.223516	18.94261467	19.81878144
Domesticated.BC	Freshwater	Norway	Hatchery	20.72378644	0.226306	20.28023451	21.16733837
Domesticated	Freshwater	Norway	Hatchery	23.78398574	0.221152	23.35053599	24.2174355
Wild	Saltwater	Norway	Hatchery	33.85565976	0.297725	33.27212901	34.43919052
Wild.BC	Saltwater	Norway	Hatchery	39.22222546	0.275036	38.68316482	39.76128609
HWF	Saltwater	Norway	Hatchery	41.95673567	0.380829	41.21032518	42.70314616
HFF	Saltwater	Norway	Hatchery	45.42656916	0.342125	44.75601623	46.09712209
F2	Saltwater	Norway	Hatchery	45.73946339	0.279002	45.19263003	46.28629675
Domesticated.BC	Saltwater	Norway	Hatchery	48.54982818	0.276755	48.00739808	49.09225828
Domesticated	Saltwater	Norway	Hatchery	52.55245373	0.284304	51.99522849	53.10967896
Wild	Freshwater	Ireland	Natural	4.929426445	0.28218	4.376363605	5.482489285
HWF	Freshwater	Ireland	Natural	5.366467866	0.251063	4.874394009	5.858541722
HFF	Freshwater	Ireland	Natural	5.611454287	0.225296	5.1698828	6.053025773
Domesticated	Freshwater	Ireland	Natural	5.713724401	0.224242	5.274218414	6.153230388
Wild	Saltwater	Ireland	Natural	12.4641014	0.378862	11.72154648	13.20665632
Domesticated	Saltwater	Ireland	Natural	12.56061538	0.640861	11.30455101	13.81667975

Table A3.2 Pairwise comparisons between shape produced from generalised Procrustes analysis, including Cohen's f-squared and p values. Comparisons are between genetic backgrounds.

Origin	Lifestage	Rearing	Compare	z	p
Ireland	Freshwater	Hatchery	Domesticated:HFF	3.03762	0.012
Ireland	Freshwater	Hatchery	Domesticated:HWF	2.137004	0.038
Ireland	Freshwater	Hatchery	Domesticated:Wild	6.705047	0.001
Ireland	Freshwater	Hatchery	HFF:HWF	-1.161836	0.906
Ireland	Freshwater	Hatchery	HFF:Wild	3.82139	0.003
Ireland	Freshwater	Hatchery	HWF:Wild	3.609361	0.002
Ireland	Freshwater	Natural	Domesticated:HFF	-0.7351355	0.707
Ireland	Freshwater	Natural	Domesticated:HWF	1.2074857	0.132
Ireland	Freshwater	Natural	Domesticated:Wild	-0.2801987	0.524
Ireland	Freshwater	Natural	HFF:HWF	0.6492396	0.24
Ireland	Freshwater	Natural	HFF:Wild	-0.6665089	0.688
Ireland	Freshwater	Natural	HWF:Wild	-0.48437	0.594
Ireland	Saltwater	Natural	Domesticated:Wild	0.6779787	0.236
Norway	Freshwater	Hatchery	Domesticated:Domesticated.BC	1.08251421	0.136
Norway	Freshwater	Hatchery	Domesticated:F2	0.13029815	0.384
Norway	Freshwater	Hatchery	Domesticated:HFF	5.16268864	0.001
Norway	Freshwater	Hatchery	Domesticated:HWF	-0.02090374	0.414
Norway	Freshwater	Hatchery	Domesticated:Wild	8.96798485	0.001
Norway	Freshwater	Hatchery	Domesticated:Wild.BC	8.76015868	0.001
Norway	Freshwater	Hatchery	Domesticated.BC:F2	-0.49646542	0.615
Norway	Freshwater	Hatchery	Domesticated.BC:HFF	2.99807573	0.009
Norway	Freshwater	Hatchery	Domesticated.BC:HWF	-0.9066297	0.839
Norway	Freshwater	Hatchery	Domesticated.BC:Wild	4.36859116	0.001
Norway	Freshwater	Hatchery	Domesticated.BC:Wild.BC	4.8704854	0.001
Norway	Freshwater	Hatchery	F2:HFF	3.95489116	0.004
Norway	Freshwater	Hatchery	F2:HWF	-0.92835525	0.825
Norway	Freshwater	Hatchery	F2:Wild	5.35849876	0.001
Norway	Freshwater	Hatchery	F2:Wild.BC	5.88969748	0.002
Norway	Freshwater	Hatchery	HFF:HWF	2.24542913	0.031
Norway	Freshwater	Hatchery	HFF:Wild	-0.49681392	0.618
Norway	Freshwater	Hatchery	HFF:Wild.BC	0.12022301	0.383
Norway	Freshwater	Hatchery	HWF:Wild	2.79315841	0.016
Norway	Freshwater	Hatchery	HWF:Wild.BC	3.21152203	0.011
Norway	Freshwater	Hatchery	Wild:Wild.BC	-0.51720718	0.623
Norway	Saltwater	Hatchery	Domesticated:Domesticated.BC	0.462546	0.281
Norway	Saltwater	Hatchery	Domesticated:F2	0.2361522	0.348
Norway	Saltwater	Hatchery	Domesticated:HFF	0.3182119	0.311
Norway	Saltwater	Hatchery	Domesticated:HWF	0.1252737	0.391
Norway	Saltwater	Hatchery	Domesticated:Wild	6.3803865	0.001
Norway	Saltwater	Hatchery	Domesticated:Wild.BC	0.1149749	0.378
Norway	Saltwater	Hatchery	Domesticated.BC:F2	-1.1504168	0.926
Norway	Saltwater	Hatchery	Domesticated.BC:HFF	1.7268783	0.065
Norway	Saltwater	Hatchery	Domesticated.BC:HWF	1.589333	0.084
Norway	Saltwater	Hatchery	Domesticated.BC:Wild	8.5986246	0.001
Norway	Saltwater	Hatchery	Domesticated.BC:Wild.BC	2.162311	0.032
Norway	Saltwater	Hatchery	F2:HFF	1.6185068	0.07
Norway	Saltwater	Hatchery	F2:HWF	1.3902456	0.104
Norway	Saltwater	Hatchery	F2:Wild	7.4883125	0.001
Norway	Saltwater	Hatchery	F2:Wild.BC	1.7090568	0.061
Norway	Saltwater	Hatchery	HFF:HWF	-1.176006	0.928
Norway	Saltwater	Hatchery	HFF:Wild	3.2312005	0.006
Norway	Saltwater	Hatchery	HFF:Wild.BC	-0.8456592	0.761
Norway	Saltwater	Hatchery	HWF:Wild	3.4548234	0.005
Norway	Saltwater	Hatchery	HWF:Wild.BC	-1.0089083	0.845
Norway	Saltwater	Hatchery	Wild:Wild.BC	5.6020979	0.001
Ireland	Saltwater	Hatchery	Domesticated:HFF	2.1060237	0.04
Ireland	Saltwater	Hatchery	Domesticated:HWF	0.4408259	0.29
Ireland	Saltwater	Hatchery	Domesticated:Wild	-1.0187598	0.86
Ireland	Saltwater	Hatchery	HFF:HWF	2.9357294	0.017
Ireland	Saltwater	Hatchery	HFF:Wild	1.2985972	0.12
Ireland	Saltwater	Hatchery	HWF:Wild	0.9959475	0.16

Appendix 4: Supplementary Material for chapter 4 - Disentangling the effects of environment and genetics in Atlantic salmon: growth, heart and liver under common garden conditions
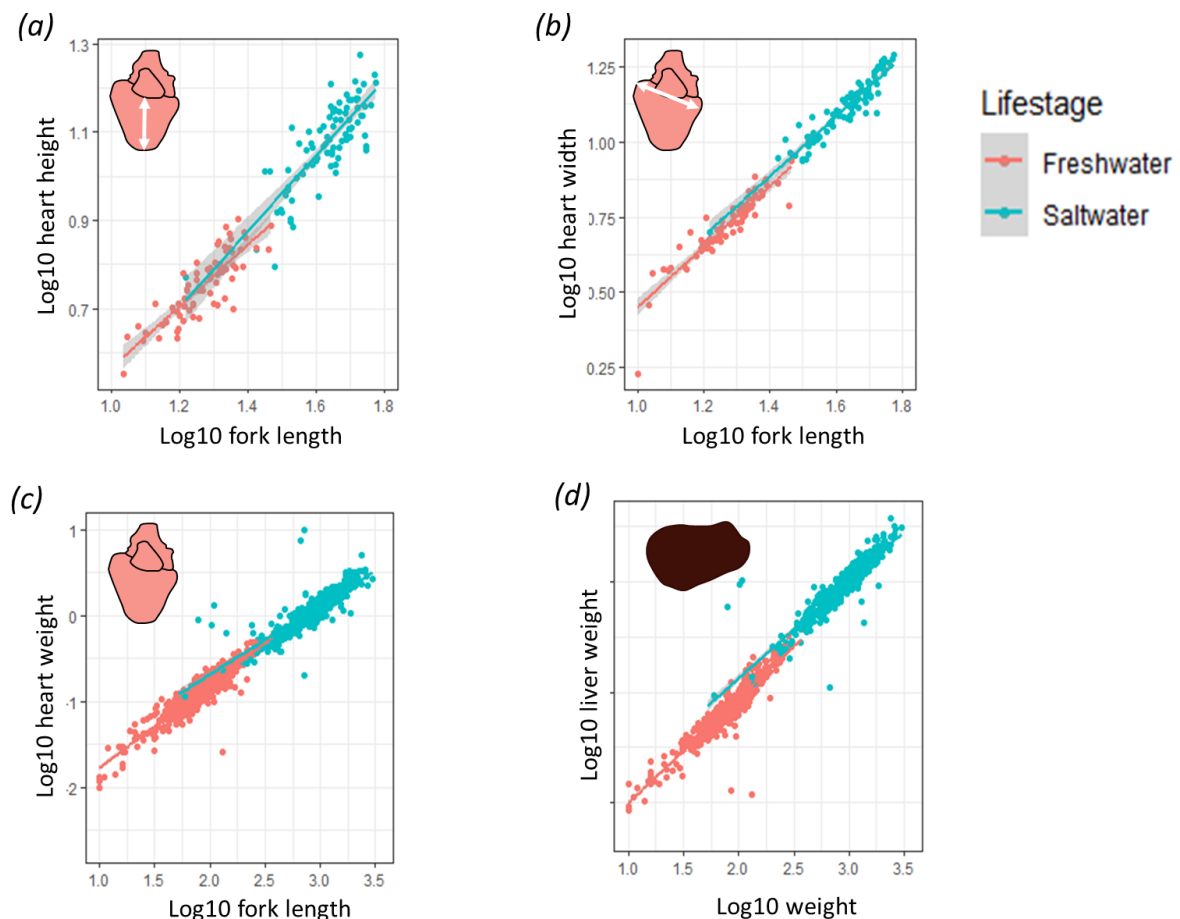
Figure A4.1 Linear regressions between log10 transformed (a) heart height, (b) heart width, (c) heart weight, (d) liver weight and log10 transformed fork length/log10 transformed body weight. Life stages are separated here, with independent regression lines, however, for the creation of adjusted measurements used in the study, one regression was used between both freshwater and saltwater life stages.

Table A4.1 Pairwise differences in mean wet body weight (kg) between the seven experimental strains, with significant (p < 0.03) contrasts between means highlighted in bold. Results are based on freshwater and saltwater body weight combined.

contrast	estimate	SE	df	t.ratio	p.value
Wild - Wild.BC	-0.1533	0.042991	29.14	-3.566	**0.0193**
Wild - Hybrid.FM	-0.31793	0.052619	29.07	-6.042	**<.0001**
Wild - Hybrid.MF	-0.38588	0.052478	28.76	-7.353	**<.0001**
Wild - F2	-0.3363	0.042933	28.99	-7.833	**<.0001**
Wild - Domesticated.BC	-0.43844	0.043013	29.2	-10.193	**<.0001**
Wild - Domesticated	-0.58184	0.043003	29.17	-13.53	**<.0001**
Wild.BC - Hybrid.FM	-0.16463	0.05259	29.01	-3.13	**0.0539**
Wild.BC - Hybrid.MF	-0.23258	0.052451	28.7	-4.434	**0.0021**
Wild.BC - F2	-0.183	0.042901	28.9	-4.266	**0.0033**
Wild.BC - Domesticated.BC	-0.28514	0.042982	29.11	-6.634	**<.0001**
Wild.BC - Domesticated	-0.42854	0.04297	29.08	-9.973	**<.0001**
Hybrid.FM - Hybrid.MF	-0.06795	0.060595	28.76	-1.121	0.9163
Hybrid.FM - F2	-0.01836	0.052546	28.91	-0.349	0.9998
Hybrid.FM - Domesticated.BC	-0.12051	0.052613	29.05	-2.29	0.2822
Hybrid.FM - Domesticated	-0.26391	0.052602	29.03	-5.017	**0.0004**
Hybrid.MF - F2	0.049582	0.052403	28.6	0.946	0.9613
Hybrid.MF - Domesticated.BC	-0.05256	0.052467	28.74	-1.002	0.9495
Hybrid.MF - Domesticated	-0.19596	0.05246	28.72	-3.736	**0.0129**
F2 - Domesticated.BC	-0.10215	0.042921	28.95	-2.38	0.243
F2 - Domesticated	-0.24555	0.042912	28.93	-5.722	**0.0001**
Domesticated.BC - Domesticated	-0.1434	0.042991	29.14	-3.336	**0.0336**

Table A4.2 Pairwise differences in mean wet body weight (kg) between sexes in the seven experimental strains, with significant (p < 0.001) contrasts between means highlighted in bold. Results are based on freshwater and saltwater body weight combined.

contrast	estimate	SE	df	t.ratio	p.value
female,Wild - male,Wild	0.080344	0.017711	2024.87	4.536	**0.0005**
female,Wild.BC - male,Wild.BC	0.084307	0.017542	2027.73	4.806	**0.0001**
female,Hybrid.FM - male,Hybrid.FM	0.010119	0.02436	2019.06	0.415	1
female,Hybrid.MF - male,Hybrid.MF	0.006145	0.023227	2019.06	0.265	1
female,F2 - male,F2	0.022058	0.016844	2019.8	1.31	0.9901
female,Domesticated.BC - male,Domesticated.BC	-0.00781	0.017617	2024.36	-0.443	1
female,Domesticated - male,Domesticated	-0.02227	0.017472	2022.86	-1.275	0.9923

Appendix 5: Supplementary material for chapter 5 - Getting inside the brain of the Atlantic salmon (*Salmo salar*): examining domestication induced morphological change
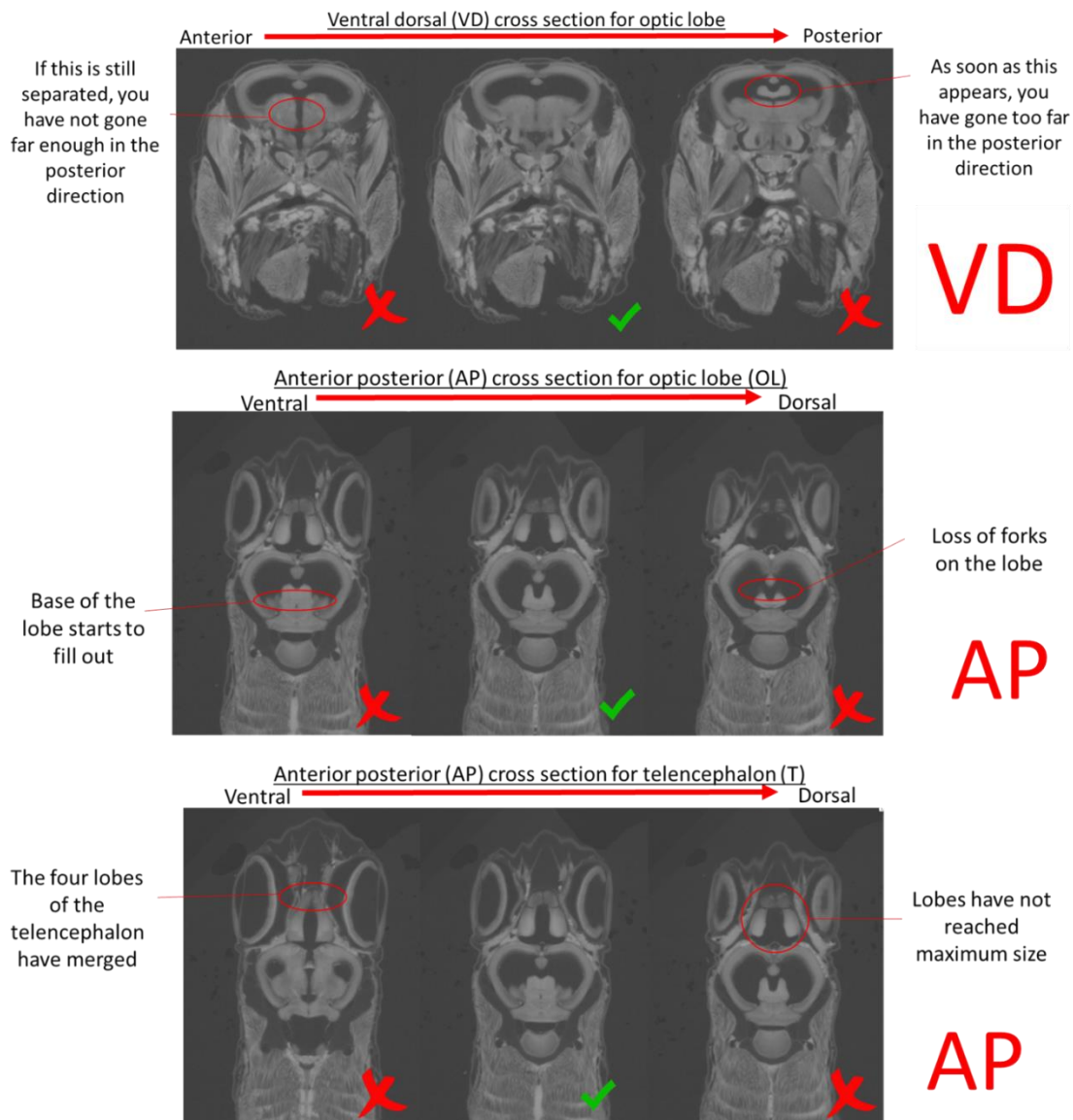
Figure A5.1 Landmarks in the brain used to identify replicable linear measurements, both in the dorsal-ventral (DV) plane and the anterior posterior (AP) plane.
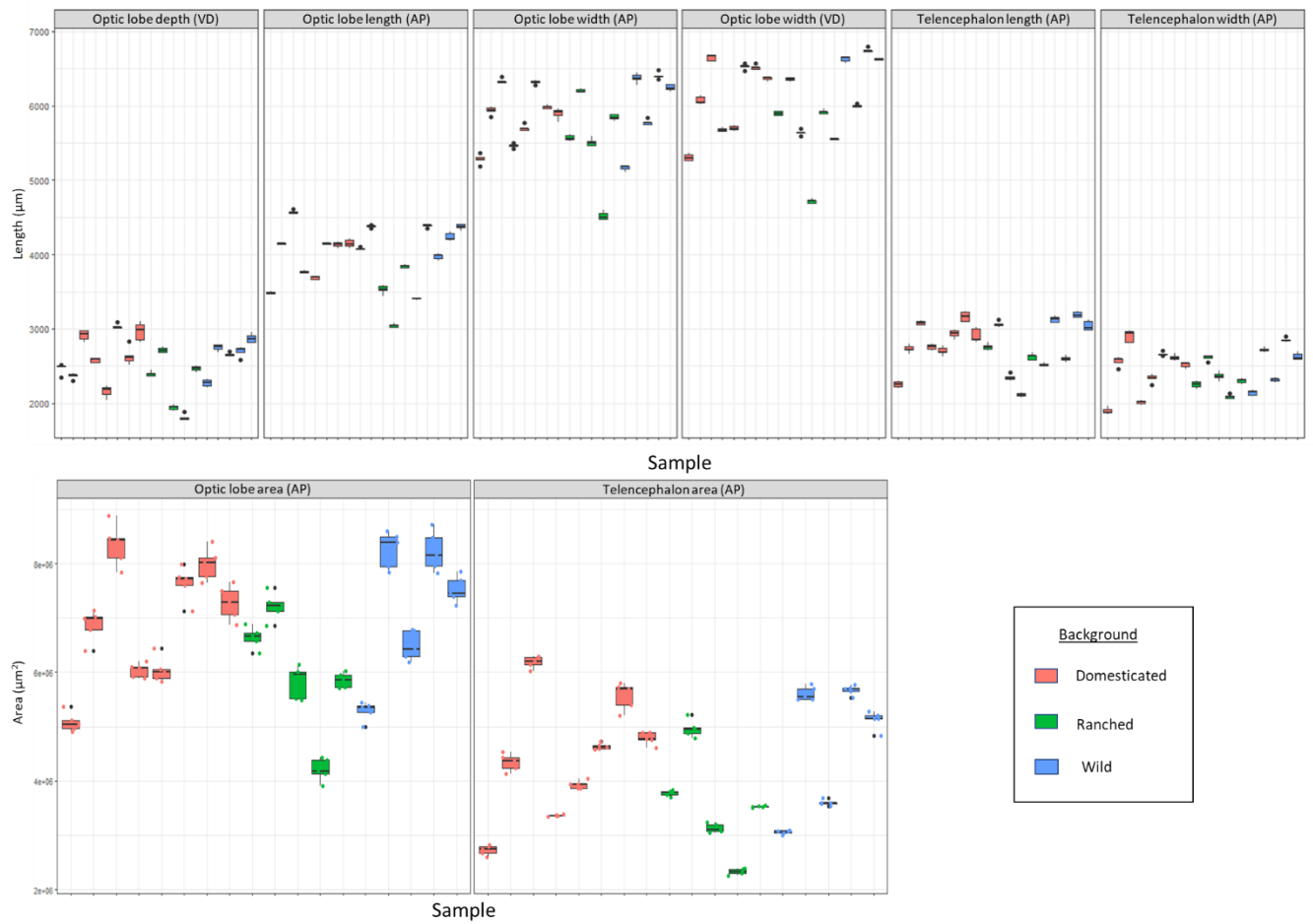
Figure A5.2 Boxplots of technical replicates for each of the eight brain measurements, broken down by sample and coloured by genetic background.
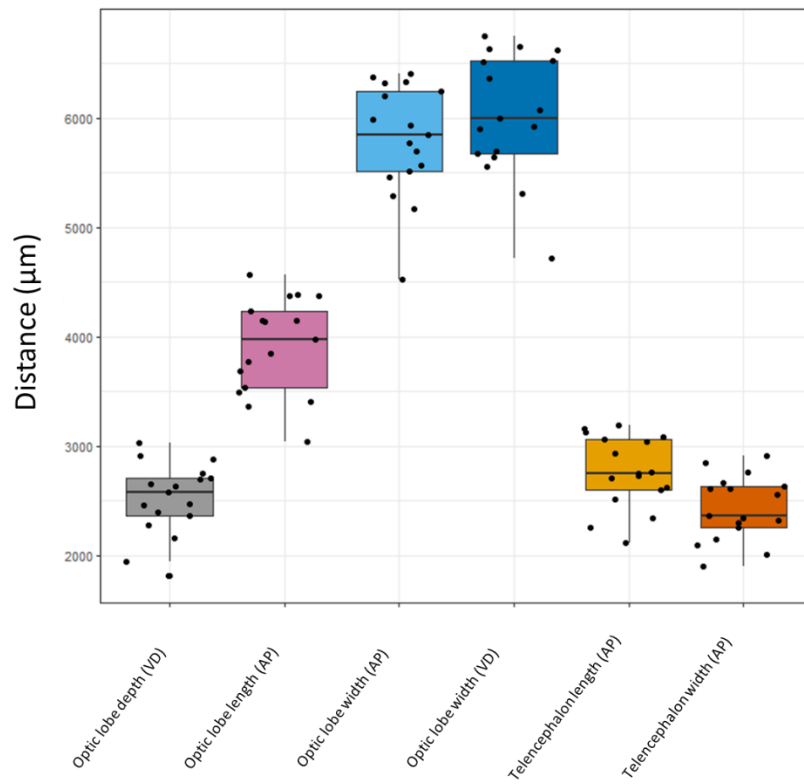
Figure A5.3 Measures of distance in µm between the different brain regions measured in this study.

Appendix 6: Supplementary material for chapter 6 - Sexual dimorphism in gut bacterial diversity: common garden study in Atlantic salmon (*Salmo salar*)
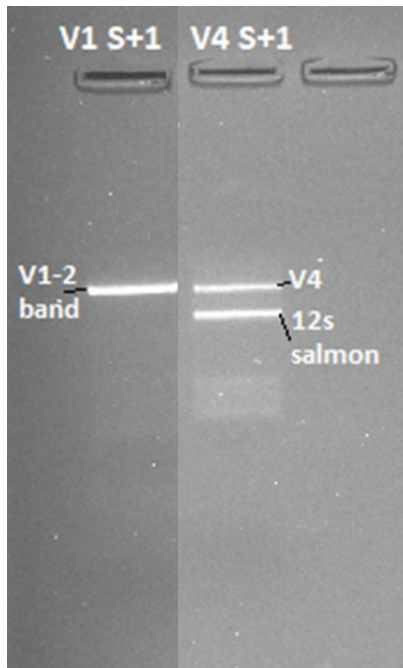
Figure A6.1 Gel electrophoresis output showing the amplification of the 16S V1-2 region in the first round PCR (V1 S+1) in addition to the amplification of the V4 region and 12S mitochondrial salmon amplicon.
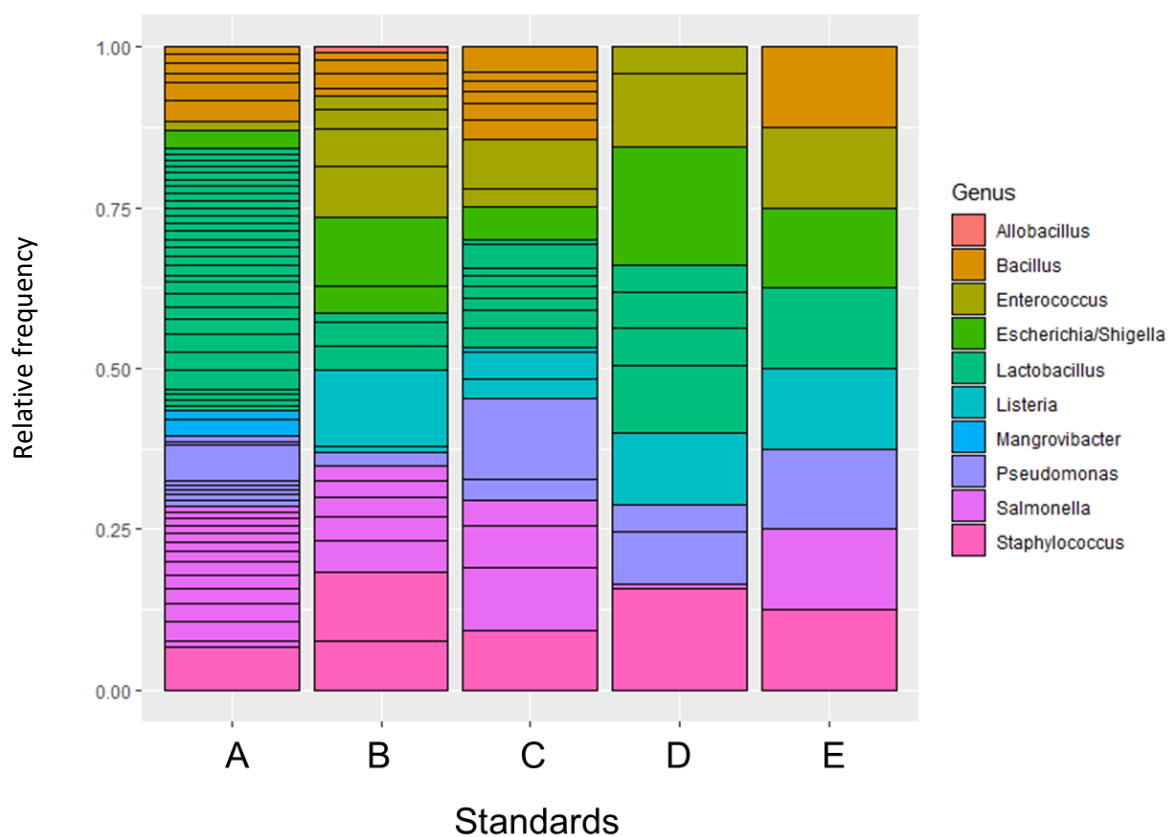
Figure A6.2 stacked bar plot showing genus level composition in five standards process and sequences with samples, with each segment representing an ASV. Sample A went through the entire laboratory pipeline, including extraction, amplification and sequencing. Samples B-D were already extracted, and so were amplified using our pipeline and sequenced. Sample E is the theoretical composition of the standard from Zymo Research. A baseline effective richness of 0.5% removed spurious ASV classification from the standards, and so this level was chosen to be applied to samples.
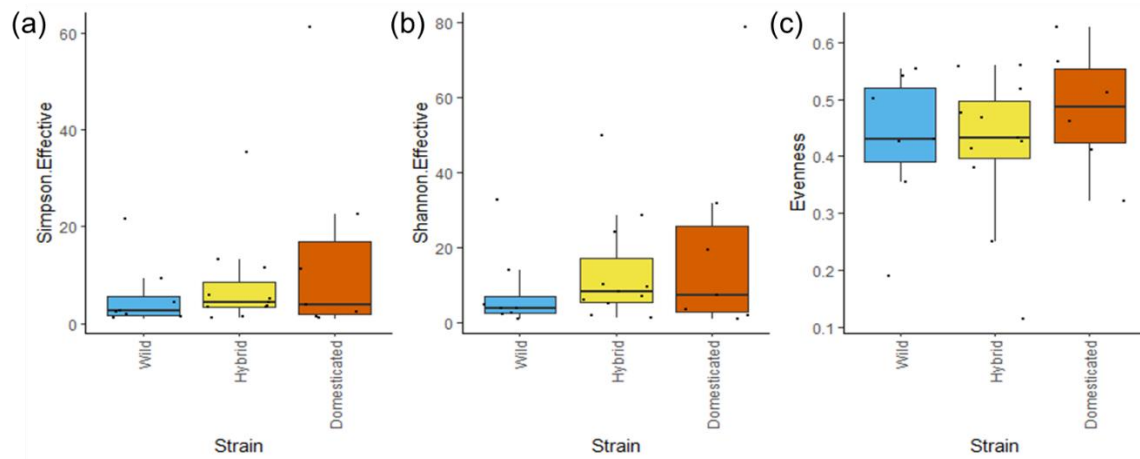
Figure A6.3 Measures of alpha diversity in gut samples, including effective (a) Simpson index, (b) Shannon index and (c) evenness, all split between three experimental strains.
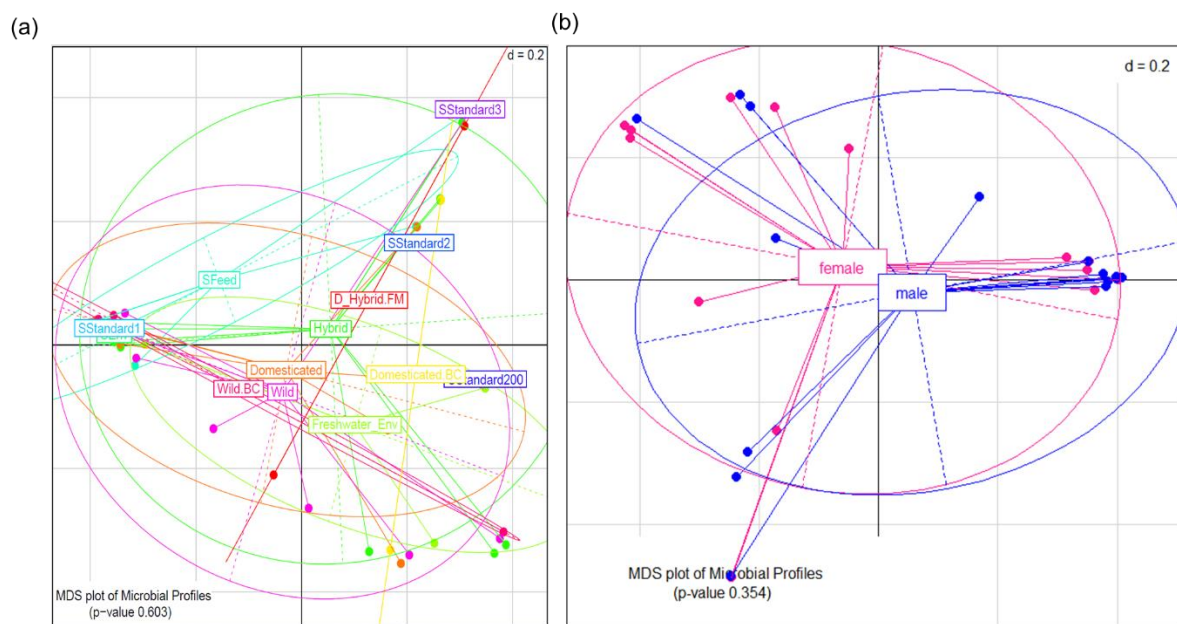
Figure A6.4 Multidimensional Scaling (MDS) plot showing beta diversity metrics for samples, including different (a) experimental strains, environmental samples, and standards, as well as (b) between sexes. Dissimilarity between two grid lines represents 20 % dissimilarity between samples.

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