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Exploring epigenetic resistance to infectious salmon anaemia in Atlantic salmon by genome-wide bisulfite sequencing in heart tissue

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Preface

This master thesis is submitted in fulfilment of the requirements for the Erasmus Mundus Joint European Master's degree in animal breeding and genetics, University of Göttingen, Göttingen, Germany and the Norwegian University of Life Sciences, Ås, Norway. The thesis represents original research that was carried out in the period from 16.11.2020 to 31.08.2021.

This thesis was under the supervision of the following:

Jorge Fernandes, Professor, FBA, Nord University: primary supervisor

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Abstract

The viral disease infectious salmon anaemia (ISA) is an ever-present threat to salmon farmers globally. Since its appearance it has been responsible for crises in the industry and has changed the way in which salmon are farmed. Disease resistance is an important phenotype that contributes to sustainable aquaculture production. The increasing understanding of epigenetics suggest a role for DNA methylation in the plasticity of an immune response and thereby an involvement in resistance to disease. Differences in methylation profiles between fish that are resistant or susceptible to the virus could reveal DNA methylation's role in ISA resistance. Immune responses in Viral target tissues, particularly tissues affected early in disease progression, have the potential to slow and hinder disease before systemic effects take hold. In ISA this is well represented by heart tissue. To this aim, the most thorough method for whole genome DNA methylation profiling was used to revealing 163 differentially methylated genes between survivors and early mortalities in an ISA cohabitation challenge test. Amongst these genes are genes involved in viral replication, antiviral innate immune response, and viral immune evasion that suggests a role played by epigenetics in resistance to ISA.

Contents

Introduction:

Aquaculture and aquatic animal diseases

Global capture fisheries production has remained constant since the 1990s. By contrast, aquaculture, the fastest growing food production sector, has seen a growth rate of more than 8% per year, currently producing nearly half of the seafood consumed (FAO, 2020). An Increase in sustainable aquaculture production will remain critical in meeting the growing global demand for animal protein (Gephart *et al.*, 2021). The aquaculture sector also employs more than 20 million people globally and is an important source of export income for several countries (FAO, 2020). Salmon is the largest single fish commodity by value accounting for 19% of the total value of internationally traded fish products and provides rural employment in coastal communities, particularly in the main salmon producing countries, Norway, Chile, Canada and Scotland (FAO, 2020).

Disease risk is central in the farming of aquatic animals and disease outbreaks cost the global aquaculture industry some US$ 6 billion per year and impact food security and livelihoods. It represents one of the main hurdles to sustainable aquaculture production (World Bank, 2014; Rodger, 2016; FAO, 2020). The Atlantic salmon farming industry combines intensive farming and exposure to the marine environment in open net pens, making it prone to disease outbreak. It remains vulnerable to existing and emerging pathogens (Pettersen *et al.*, 2015). An increased understanding of salmonid diseases and scientific innovation in fish vaccinology has been and continues to be instrumental to the sustainability of the salmon industry (Gudding and Muiswinkel, 2013). There has been much success of this approach to bacterial diseases. However, viral outbreaks have seen less success in terms of treatment, and vaccines currently used offer only partial efficacy in preventing and controlling disease. (Gudding and Muiswinkel, 2013; Rodger, 2016)

Advances in genomics, the increased understanding of the genetics of disease resistance and the use of disease challenge test have supported breeding programs contribution to reducing the impact of disease in aquaculture species particularly in Atlantic salmon (Gjedrem, 2015). A focused selective breeding approach to disease resistance in salmon started early in 1993, applying family selection followed by marker-assisted selection (MAS) and genomic selection to breed for resistance (Houston, 2017) MAS has seen success in the cases of less polygenic trait genetic architectures, including viral disease. Resistance to infectious pancreatic necrosis stands out as a major success with this approach and to a lesser degree with pancreas disease but is undergoing current developments (Gonen *et al.*, 2015; Moen *et al.*, 2015). However, MAS based on few quantitative trait loci (QTL) has not been routinely successful in animal breeding for disease resistance or selective breeding in general

due to the polygenic nature of most production relevant traits (Meuwissen, Hayes and Goddard, 2016; Houston, 2017). Another outcome of the growing understanding of genomics is genomic selection (GS), which is more effective than MAS for polygenic traits (Meuwissen, Hayes and Goddard, 2016). GS has been validated and is being applied in salmonid breeding programs for resistance to several diseases and parasites, amongst which, *Piscericketsia salmonis* (Bangera *et al.*, 2017), amoebic gill disease (Robledo *et al.*, 2017; Boison *et al.*, 2019), and bacterial cold water disease (Vallejo *et al.*, 2017). The importance and contribution of applying advances in genomics are many, and filling key knowledge gaps in understanding resistance to disease and host-infectious agent dynamics will continue to contribute to controlling aquaculture diseases in the future (Lafferty *et al.*, 2015)

Infectious salmon anaemia

Infectious salmon anaemia (ISA), first identified in Norway 1984, is a vertically and horizontally transmissible disease caused by the ISA virus (ISAV) that generally affects salmonids but has been chiefly described in Atlantic salmon (Thorud and Djupvik, 1988; Marshall *et al.*, 2014). ISA outbreaks without intervention result in high accumulated fish mortality have had severe production, economic and welfare implications for salmon farming. ISA outbreaks in Norway reached a peak in 1990, with 80 outbreaks diagnosed, instigating the introduction of several hygiene and management measures to control the disease. These included regulations on fish transport, smolt production slaughter procedures, year class separation in production and control zones around infected sites affecting most aspects of production. These biosecurity measures have been effective at reducing the number of outbreaks but have also incurred economic loss associated with management-related costs associated with regulations aimed at controlling the disease (Rimstad, Dale and Falk, 2011). The 2007 outbreak of ISA in Chile caused more than a 60% drop in production by 2009, with broad consequences for farmers, livelihoods and fish welfare (Asche, Hansen and Tveteras, 2010; Kibenge *et al.*, 2012). The severe consequences following outbreaks of ISA make it arguably the most important viral disease of farmed Atlantic salmon, pivotal to introducing biosecurity standards. It is a notifiable disease in Norway and the EU and a listed disease internationally (Kibenge *et al.*, 2012; Jansen and Oliveira, 2021). It remains a threat in all major salmon producing countries, where it has been identified, first in Norway then Canada followed by Scotland, the United States and Chile (Cottet *et al.*, 2011). Biosecurity management regulation has been credited with the decrease in ISA outbreaks over other strategies to a multifaceted approach to managing ISAV.

The pathology of ISA is characterized by varying macroscopic lesions, gill paleness, liver congestion, spleen inflammation and intestinal congestion. Externally infected fish exhibit

exophthalmos, abdominal petechiae and oedema, all of which are associated with severe anaemia, the main clinical characteristic present in all cases of ISA (Cottet *et al.*, 2011; Figueroa *et al.*, 2019). 2019). *Figure 1* shows Images of some of the more consistent lesions associated with ISA mortalities. The causative agent ISAV was identified in 1997 and is an 8 segment, single-, negative-stranded RNA virus of the Orthomyxoviridae family (Aamelfot *et al.*, 2012). The ISAV genome is capable of coding for 10 – 11 different proteins depending on isolate with segments identified having been predicted to encode polymerase complex proteins, nucleoprotein, hemagglutinin esterase (HE), matrix proteins and viral surface proteins and fusion proteins (Cottet *et al.*, 2011; Valenzuela-miranda *et al.*, 2015). ISAV has two distinct pathotypes associated with sequence differences at high polymorphic region (HPR) which is also the largest genetic variability marker in ISAV separating virus isolates in from different regions (Cottet *et al.*, 2011). The highly pathogenic HPR-deleted strains and the non-pathogenic, gill infecting ISAV-HPR0, which is widespread and proposed to act as progenitor and reservoir for all virulent ISAVs strains (Aamelfot, Dale and Falk, 2014; Christiansen *et al.*, 2017)

Figure 1. Common lesions observed at necropsy: a) exophalmia and gill paleness, b) abdominal petechiae and odeama, c) dark liver and heamorrhaging in the visceral adipose tissue. Images from (Godoy *et al.*, 2008).

ISAV has been shown to enter the host by crossing the mucosal barrier in the gills. However ISAV has been shown to also enter through the skin and fins and to replicate early in epithelial cells before shifting to endothelial infection (Weli *et al.*, 2013; Aamelfot, Dale and Falk, 2014). The ISAV viral attachment protein was shown to have a greater affinity for host membrane proteins with a specific sialic acid modification Neu4,4Ac. This sialic acid modified membrane protein has been located on gill and hindgut epithelial cells but is concentrated on the luminal surface of endothelial cells and likely drives the final observed tissue tropism for vascular endothelial cells including cells that line heart lumen and red blood cells (RBC). The heart lumen has the largest surface concentration of endothelial cells and has been shown early on to be the most infected tissue and is used as the main tissue for routine detection of viral load (Gregory, 2002). It is the vascular and heart tropism along with viral *in situ* hemagglutination and attachment to RBCs that is thought to possibly underly the increased haemophagocytosis, severe anaemia and circulatory disturbance that characterizes ISA

(Aamelfot *et al.*, 2012; Aamelfot, Dale and Falk, 2014). Cell infection begins upon the binding to host cell membrane sialic acid receptor by the virus haemagglutinin esterase initiating the infection process and viral replication (*figure 10*). The attached virus is then internalized by receptor-mediated endocytosis through a process that is not well described but may involve interaction with more cell receptors and may in Orthomyxoviridae viruses involve viral non-structural proteins, fusion protein and/or the receptor destroying enzyme (Aamelfot, Dale and Falk, 2014). A lowering of pH in the endosome has been shown to trigger the fusion of the virus and endosomal membranes (Eliassen *et al.*, 2000). After unknown processes, some viral proteins, nucleoprotein and matrix proteins accumulation can be detected in the nucleus confirming that like other Orthomyxoviridae viruses that part of replication occurs in the nucleus, unique amongst RNA viruses other than retroviruses. The haemagglutinin esterase has been traced to the endoplasmic reticulum and the Golgi apparatus prior to incorporation in the host cell membrane (Falk *et al.*, 2004). The virus has then been shown with electron microscopy to exit the host cell by budding from the cell membrane facing the lumen and into the bloodstream and is the possible driver of viremia (Aamelfot *et al.*, 2012). It is clear that ISAV haemagglutinates RBCs and RBCs are shown to be virus coated but conflicting evidence on weather ISAV replicates within erythrocytes (Workenhe *et al.*, 2008; Aamelfot, Dale and Falk, 2014). The coating and or the infection of erythrocytes and resultant heamophagocytosis has been suggested as a potential major cause of severe anaemia that in turn results in haemorrhagic necrosis in the liver gut and kidney in the later stages of the disease but the details of disease progression past endothelial infection have not been elucidated (Aamelfot *et al.*, 2015).

ISA resistance has been identified like most disease resistant traits to be polygenic and estimated to have a moderate heritability and has been included in some genetic improvement programs since the mid-1990s (Moen *et al.*, 2007; Kjöglum *et al.*, 2008). The ISA vaccine is amongst the viral vaccines showing low efficacy and does not give satisfactory protection to salmon in the field (Evensen and Leong, 2013; Robertsen, Chang and Bratland, 2016). Disease resistance could be functionally determined by several factors that influence the successful viral replication within the host from, viral entry, viral spread, replication efficiency, and immune evasion. In a recent exploration of the genetic basis of ISA resistance, a GWAS confirmed the polygenic nature of the trait and detected one significant QTL on chromosome 13 explaining 3% of the variance in ISAV resistance (Gervais *et al.*, 2021). Heart tissue transcriptomics comparing high and low resistance breeding value individuals revealed a small number of differentially expressed genes in heart that included several innate immune response genes (Gervais *et al.*, 2021). The same study identified 1511 differentially expressed genes (DEG) in heart that persisted throughout infection when compared to uninfected controls but

4344 DEGs in total over two timepoints indicating the complex nature of host viral interactions at the gene regulatory level that could form the basis of resistance to ISA.

Epigenetics in Teleosts

Waddington (1942) first coined the term epigenetics to describe the study of causal interactions between genes and their products that bring about varying phenotypes, primarily in the context of the establishment of different cell lineages during development. The contemporary definition is more general, as it refers to the inheritance of chromatin states and modifications in the absence of changes in DNA sequence (Bird, 2007). Inheritance can refer to the maintenance of epigenetic modifications that are retained in subsequent mitosis and can also refer to intergenerational inheritance in immediate offspring and further transgenerational inheritance in subsequent generations (Lacal and Ventura, 2018; Cavalli and Heard, 2019). The modifications that act as carriers of epigenetic information and modulators of gene expression are histone variants and modifications, DNA methylation, and non-coding RNAs (Gavery and Roberts, 2017; Cavalli and Heard, 2019). Chromatin proteins and DNA methylation have been shown to, for example change the accessibility to DNA binding proteins to underlying genetic sequence and thereby, directly, and indirectly, influencing gene transcription. Whereas a growing list of characterized non-coding RNAs are generally recognized to act as post-transcriptional regulators of mRNA abundance and translation in addition to a well-established role in orchestrating the stable epigenetic inactivation of the X chromosome in mammals (Gendrel and Heard, 2014; Wei *et al.*, 2017; Cavalli and Heard, 2019).

The study of epigenetics in animals has focused more on mammalian model systems than teleost model systems, and differences between these models exist. The evolution of teleosts and salmonids' molecular epigenetic machinery has been affected by the teleost genome duplication and further in the salmoniform specific genome duplication (Ss4R). Teleosts possess more paralogues for many genes involved in histone modification and novel histone variants, more miRNA loci and, particularly in the case of salmoniforms, more paralogues for miRNA biogenesis pathway genes (Best *et al.*, 2018). The duplicated de novo methyltransferases have been retained in paralogues in zebrafish, and their different expression profiles indicating their potential subfunctionalization (Campos et al., 2012). These multiple paralogues have also been retained to different degrees in the salmoniform lineage (Best, Ikert, D. J. Kostyniuk, *et al.*, 2018). The DNMT1 duplicate, on the other hand, has not been retained as a paralogue in zebrafish but has in salmonids (Best *et al.*, 2018; Liu *et al.*, 2020).

Evidence of generational transmission of epigenetic modifications shows it is a common mechanism in plants. In metazoans, however, the indications of transgenerational inheritance differ between phyla (Skvortsova, Iovino and Bogdanović, 2018). In teleosts, the growing body of epigenetics research reveals differences to mammalian patterns of parental inheritance of DNA methylation (Best *et al.*, 2018). While many studies confirm the critical role of epigenetics in regulating gene expression in several contexts, few studies have explored transgenerational epigenetic inheritance (Best *et al.*, 2018). The studies that address transgenerational inheritance have found hypoxia tolerance in zebrafish (Ho and Burggren, 2012), hypoxia-induced reproductive impairment in marine medaka (Wang *et al.*, 2016) along with several studies on toxicological epigenetics (Carvan III *et al.*, 2017; Kamstra *et al.*, 2017; Olsvik *et al.*, 2020) to show indications or evidence of transgenerational epigenetic inheritance. In addition, micronutrient deficiency in parental zebrafish show locus-specific methylation marks that persist in descendants (Skjærven *et al.*, 2018). In salmonids for example studies show distinct sperm DNA methylation profiles between hatchery-reared and wild Atlantic salmon, some of which persisted in the sperm of the hatchery-reared offspring (Rodriguez Barreto *et al.*, 2019). The increasing number of examples of transgenerational inheritance in teleost has direct translational implications for selective breeding in aquaculture (Feeney, Nilsson and Skinner, 2014; Moghadam, Mørkøre and Robinson, 2015; Triantaphyllopoulos, Ikonomopoulos and Bannister, 2016). Biological processes, like disease resistance, where the adaptive embedding and transmission of environmental memory would confer the most selective advantage to offspring, are amongst the most likely to have evolved transgenerational epigenetic inheritance.

DNA methylation

DNA methylation is found preferentially but not exclusively at the cytosine guanine dinucleotide (CpG) in animals. The degree of methylation in animals displays a broad variability between phyla with *Caenorhabditis elegans* on one end lacking detectable 5 methyl cytosine (5mC) to vertebrates on the other end with the highest proportion of methylated cytocines (C) (Bird, 2002) 2002). The identification of the major known functions of 5mC has grown to include X chromosome inactivation, genomic imprinting, the repression of transposable elements and the regulation of transcription, particularly during development and cellular differentiation (Bird, 2002; Goll and Halpern, 2011; Hervouet *et al.*, 2018). The methylation of cytosine bases is an enzymatically driven process catalysed by a family of DNA binding proteins, DNA methyltransferases (DNMT), that covalently bind a methyl group from S-adenosylmethionine to the 5^{th} carbon position of cytosine (Denis, Ndlovu and Fuks, 2011). The DNMTs in mammals that were first identified and best

characterized are the maintenance methyltransferase DNMT1 followed by the de novo methyltransferases DNMT3a DNMT3b in 1999 (Okano *et al.*, 1999). DNMT1 functions by identifying hemimethylated DNA, such as would occur on the daughter strand following replication, and proceeds to methylate the complementary C on CpG dinucleotide (Okano *et al.*, 1999; Hervouet *et al.*, 2018). This function is central to having some degree of mitotic heritability that would be a vital characteristic of a heritable epigenetic marker and the maintenance process has been shown to occur with a high degree of fidelity (Bird, 2002; Breiling and Lyko, 2015). The de novo methyltransferases DNMT3a and DNMT3b that methylate cytosines outside of the hemimethylated maintenance context are responsible for new methylation marks. Although many reports reveal primarily overlapping targets for these two de novo methyl transferases, differing post-translational modifications and protein complex interactions could impact the selectivity of methylation targets and preference for some genomic features, developmental states, and tissues (Okano *et al.*, 1999; Hervouet *et al.*, 2018; Lyko, 2018). Both the addition of methyl groups by the DNMTs but also demethylation is required to produce the observed methylation profiles of a genome. Demethylation can occur passively during replication in the absence of maintenance methylation but also actively when catalysed by the ten-eleven translocation family of enzymes TET1, TET2, TET3, which oxidize the 5mC to 5hmC (Tahiliani *et al.*, 2009; Branco, Ficz and Reik, 2011). The TET mediated demethylation pathways are proposed to then either have the further oxidized 5hmC site be passively demethylated in subsequent replication or undergo base excision repair mediated by thymine DNA glycosylase, resulting in demethylation independent of replication (Wu and Zhang, 2017).

A functional analysis of cytosine methylation has proven to be complex and difficult even in well characterized mammalian organisms, and DNA methylation has been shown to be associated with varying regulatory outcomes depending on the genomic feature and specific gene context (Breiling and Lyko, 2015). CpG islands, mostly unmethylated C and G rich regions with higher relative densities of CpG found at the 5’ end of many vertebrate genes, form a distinct feature in genomic methylation patterns. These CpG-rich regions, for the most part, remain unmethylated whether associated genes are silenced or being expressed in a tissue-specific manner. However, in the instances where CpG islands are methylated there is a consistent stable silencing of promoters within CpG islands, particularly during development (Bird, 2002). In general, when present near regulatory regions, 5mC is traditionally linked with the downregulation of associated genes. An example of one of the exceptions is transcription factors that show preferential binding to methylated cytosines, resulting in an upregulation of the associated gene (Breiling and Lyko, 2015; Lyko, 2018). There are also indications that gene body methylation, particularly on exons is associated with actively expressed genes, most evident in the context of housekeeping genes (Denis, Ndlovu and Fuks, 2011;

Suzuki *et al.*, 2013; Lyko, 2018). First intron methylation has also been shown to be associated with increased expression across multiple tissues in vertebrates (Anastasiadi, Esteve-Codina and Piferrer, 2018) In addition, gene body methylation differences between introns and exons have also been associated with alternative splicing and a change in gene product isoforms (Gelfman *et al.*, 2013). Epigenome mapping studies have identified tissue-specific differentially methylated sites that are associated with gene transcription. These are CpG island shores, the less CpG dense regions flanking CpG islands, as well as distal promoter sites that exhibit enhancer activity (Milosavljevic, 2011). Despite the exceptions, 5mC is primarily considered a repressor of gene expression and is thought to bring about this effect on associated genes by three main proposed basic mechanisms. These include 5mC blocking methylation-sensitive transcription activators from binding or allowing the binding of methylated CpG binding proteins like MeCP2 that in turn block transcription and finally 5mC promoting the formation of inactive chromatin structure all of which promote the downregulation of transcription (Branco, Ficz and Reik, 2011).

Epigenetics of Disease Response in Fish

One of the frontiers in the study of disease resistance in fish is the role epigenetics plays in host disease response to pathogens. Recent research has started to reveal to which extent epigenetics is involved in regulating and balancing the transcriptional response to infection in teleosts. Similarly, to environmental factors, exposure to pathogens has resulted in histone modifications, changes in miRNA abundance and DNA methylation, with several examples from immune tissues in teleosts (Best *et al.*, 2018). The most thoroughly studied epigenetic mechanism is in relation to pathogen response in fish is the role of miRNAs, with investigations into several aquaculture species, including salmonids (Andreassen and Høyheim, 2017). Several conserved miRNAs associated with bacterial and virus response have been identified, including some teleost-specific miRNAs. The immune response genes and gene network targets for some of the miRNAs have been validated, and the understanding of their role in the intricate balancing of immune response and homeostasis is growing. (Andreassen and Høyheim, 2017). The epigenetic role of histone modifications has been explored to a lesser degree in teleosts. Studies in zebrafish models indicate that covalent histone modifications orchestrate immune genes prior to the development of the adaptive immune system (Galindo-Villegas *et al.*, 2012) and regulate key immune system process genes that control the response to viral infections (Medina-Gali *et al.*, 2018). Recently, chromatin immunoprecipitation sequencing of pooled kidney, liver, spleen and heart revealed gene enrichment of histone 3 methylation was associated with higher transcription

levels of key immune genes in zebrafish infected with spring viremia of carp virus (Medina-Gali *et al.*, 2018).

DNA methylation's role in the coordination of the disease response has been reported in several fish species, both animal models and aquaculture species. A genome-wide methylome study of Trinidadian guppy (*Poecilia reticulata*) challenged with the ectoparasite monogenean (*Gyrodactylus turnbulli*) revealed changing patterns of methylation in skin tissue immune response genes that coincided with changing phases of infection (Hu *et al.*, 2018). In a parasite-host interaction between three-spined stickleback (*Gasterosteus aculeatus)* and the nematode (*Camallanus lacustris)* was shown to exhibit transgenerational resistance effects (Kaufmann *et al.*, 2014). A separate experiment exploring the underlying mechanisms showed this infection to be associated with DNA hypermethylation that in part, affect immune pathways genes in liver cells (Sagonas *et al.*, 2020). DNA methylation's role in pathogen response has also been studied in diverse aquaculture species, generally focusing on immune relevant tissues. In grass carp (*Ctenopharyngodon idellus*), grass carp reovirus infection is associated with increased expression of DNMTs and TET genes, followed by hypermethylation in spleen tissue (Xiong *et al.*, 2018). Changes in the expression of complement and coagulation cascade genes following infection were also associated with upstream changes in DNA methylation (Xiong *et al.*, 2018). Also, grass carp reovirus resistant fish show an increased expression and decreased methylation of the melanoma differentiation-associated gene 5 in the spleen that recognizes intracellular viral infection and induces an antiviral response (Shang *et al.*, 2015). Differences in Nile tilapia (*Oreochromis niloticus*) resistance to *Streptococcus agalactie* were found in genetically similar fish, associated with differences in gene expression and DNA methylation in spleen tissue (Hu *et al.*, 2020). In the aquaculture species, Chinese tongue sole (*Cynoglossus semilaevis*) artificial selection for resistance to *Vibrio harveyi* infection was accompanied by broad differences in methylation profiles in pooled immune-related tissues between resistant and susceptible fish challenged with infection (Xiu *et al.*, 2019). Pacific salmon (*Oncorhynchus kisutch*) challenged with *Piscirickettsia salmonis* show a time-dependant dynamic change in methylation profiles in spleen tissue. Infected groups had more differences in methylated regions, yet together distinct differences compared to uninfected controls with a subset of persistent differentially methylated regions (DMR), highlighting processes pathways involved in cellular responses to infection (Leiva *et al.*, 2020). In Atlantic salmon, chronic and acute stress on the gill epigenome suggests that early life stress can have long-term effects on immunocompetence in later life stages, indicating a mechanistic role for DNA methylation in hormesis in fish (Webster *et al.*, 2018). Several examples across the diversity of teleost species and pathogen types indicate a mechanistic role for DNA methylation in the plasticity of cellular

reactions to disease. However, there has been an almost exclusive focus on tissues involved in the immune response, particularly the spleen, less so on pathogen tissue targets.

Genome wide profiling of DNA methylation methodology

There are three main experimental approaches to genome wide DNA methylation profiling, enzyme digestion, affinity enrichment and bisulfite conversion (Yong, Hsu and Chen, 2016; Pajares *et al.*, 2021). Restriction enzyme-based methods make use of the difference in isoschizomers sensitivity to DNA methylation state and is followed by using microarrays or sequencing to reveal the location and methylation of restriction sites. Although single base resolution is attained the main limitation to this method is that only CpG containing recognition sites are screened for methylation status leaving the majority of CpGs, those outside of restriction sites, uninvestigated (Yong, Hsu and Chen, 2016). Affinity enrichment-based methods utilize either methyl CpG binding domain proteins or antibodies with specificity for methylated cytosines to enrich methylated DNA regions which is followed by profiling by microarray or sequencing. Methylated DNA immunoprecipitation, for example, is considered a cost-effective method requiring low DNA input, is effective with low coverage utilizing standard mapping tools but shares some of the limitations common to affinity enrichment methods. The main limitations are that the method while covering most of the genomes CpG sites does not yield a single base resolution or methylation context but rather a resolution in the range of 100 – 300 bp representing relative enrichment of DNA methylation, biased depending on region CpG density (Yong, Hsu and Chen, 2016; Beck, Maamar and Skinner, 2021).

The bisulfite conversion-based methods treat DNA with sodium bisulfite, which deaminates unmethylated C residues to uracil (U) leaving methylated C unconverted. Subsequent Polymerase chain reactions (PCR) converts the U to thymine (T). The bisulfite conversion thereby reveals 5mC as the only cytosines remaining in a converted DNA fragment, after which genome wide methylation can be profiled using methylation arrays or sequencing (Yong, Hsu and Chen, 2016; Beck, Maamar and Skinner, 2021). The two main bisulfite sequencing based approaches are reduced representation bisulfite sequencing (RRBS) and whole genome bisulfite sequencing (WGBS) both providing single base resolution. These two approaches share some limitations in that they depend on the efficiency of bisulfite conversion, and both generate converted fragments with reduced sequence complexity, requiring specialized mapping approaches that result in lower alignment efficiency and neither approach can differentiate between 5hmC and 5mC (Beck, Maamar and Skinner, 2021). RRBS uses an enzymatic DNA digestion MspI and size selection to target a reduced portion of the genome with high CpG densities (Meissner *et al.*, 2005; Beck, Maamar and Skinner, 2021). The advantage of this targeted approach ensures concentrated coverage particularly around CpG islands. This approach lowers

sequencing costs and increases power to detect differentially methylated cytosines or regions (DMC/DMR) but is limited to representing a reduced component of the genome with many regions outside CpG islands not investigated (Yong, Hsu and Chen, 2016; Beck, Maamar and Skinner, 2021) WGBS main advantage is that it has the potential to assesses the methylation state of nearly all CpGs and unlike RRBS covers low CpG-density regions that are generally less studied, such as intergenic gene deserts and distal regulatory elements. Untargeted WGBS has high sequencing depth requirements but in contrast to RRBS also provides more accurate assessments of absolute methylation levels and is the most thorough method for detection of genome wide differential methylation with the least CpG density bias (Yong, Hsu and Chen, 2016; Beck, Maamar and Skinner, 2021; Pajares *et al.*, 2021).

Methods

ISA challenge test and sample selection

The challenge test was performed by VESO Vikan to document the susceptibility to ISA in different salmon families, the results of which are for use in breeding and selection as described in the study report (Ramstad, 2020) according to the regulations and compliance of the study protocol V-4461.pro. The cohabitation challenge test was carried out after a week of acclimatization between 16.07.2020 and 08.09.2020 when more than 50% mortality was obtained. A total of 4992 PIT tagged parr from both the Salmobreed and Stofnfiskur strains weighed an average of 25.7 were used as test fish. The 400 shedder fish (representing 8% of all the fish) were parr from the Stofiskur strain weighing an average of 19.6 grams that were intraperitoneally injected with 0.1 ml ISAV 10log4.5 using the ISAV isolate Glaesvær 080411. The test was carried out in a 4 m tank with water temperatures at 12°C ± 1°C, a photoperiod regime of L:D = 24:0 and the fish were fed according to standard procedures at VESO Vikan. PIT tag numbers where recorded and heart samples collected from dead or moribund fish and all surviving fish at termination and stored in RNAlater. In addition, 4 test fish were randomly sampled five days a week, and a large random sampling of 1575 test fish was carried out 3.5 weeks post-challenge.

To represent the 6 ISA susceptible individuals, heart samples from 6 individuals from early mortalities in the first week of the challenge test were selected. The susceptible fish were chosen randomly from the two families with the most mortalities in the first week. Six ISA-resistant fish were

chosen from fish that survived until the termination of the challenge, from each of the two families with the most remaining survivors at termination. Heart samples from fish selected for the study were received from VESO Vikan stored in RNAlater™.

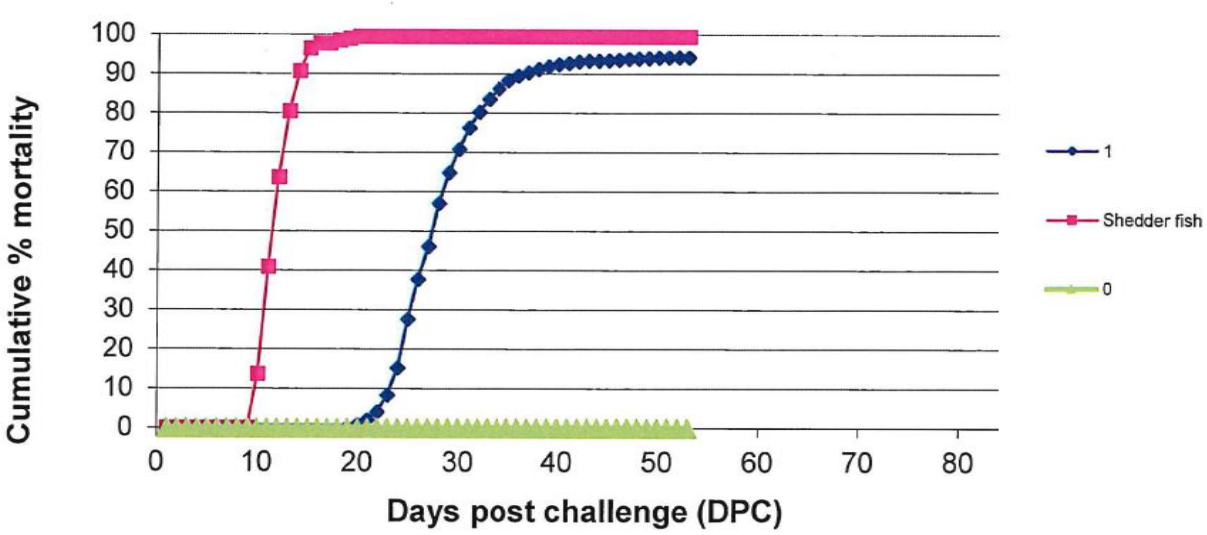


Figure 2. Cumulative mortality curve for the cohabitation challenge test. Pink squared line showing cumulative % mortality over time of shedder fish intraperitoneally injected with ISAV. Blue diamond line showing cumulative % mortality of test fish infected during cohabitation with shedder fish (Ramstad, 2020).

Whole-genome bisulfite sequencing library preparation

DNA isolation

Genomic DNA from heart samples was extracted using the Qiagen DNeasy Blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. 20 µl of proteinase K was added to the samples and incubated at 56°C after which heart tissue was completely lysed. Subsequent steps followed the manufacturers Spin Column Protocol for animal tissues using the provided reagents resulting in elution of purified DNA in 20 µl of Buffer AE to be used downstream for preparing the WGBS libraries. The DNA concentration was determined using Qubit® 3.0 fluorometer with the dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, United States) and purity of the DNA extractions was assessed using the nanodrop. Using the DNA concentration as measured by Qubit, the DNA was diluted with Milli q water to 100ng of DNA in 20 µl of solution for the DNA input

in library preparation**.** DNA concentration and absorption ratios at 260/280nm and 260/230nm are shown in *Table 1.*

Library preparation

Whole-genome bisulfite libraries were prepared using the Pico Methyl-Seq library prep kit (Zymo Research Corp, Irvine, CA, United States) for Illumina-based sequencing following the manufacturer's protocol. An overview of the workflow for the WGBS protocol can be found in *figure 3*. This approach performs bisulfite conversion of the natural DNA as the first step in the protocol, which also results in the random fragmentation of the DNA. Bisulfite treatment results in the conversion of unmethylated cytosines into uracil, leaving methylated cytocines unchanged. This is followed by 2 cycles of PCR whereby the uracil and the guanine on the opposing strand of the base pair are respectively repaired as thymine and adenine during elongation. The creation and sequencing of these libraries where the cytosines present originate only from native methylated cytosines allow for the genome-wide analysis of methylation. WGBS library preparation was performed using reagents supplied with the kit unless otherwise stated. The maximum recommended amount of input DNA of 100 ng per sample was used to prepare the libraries.

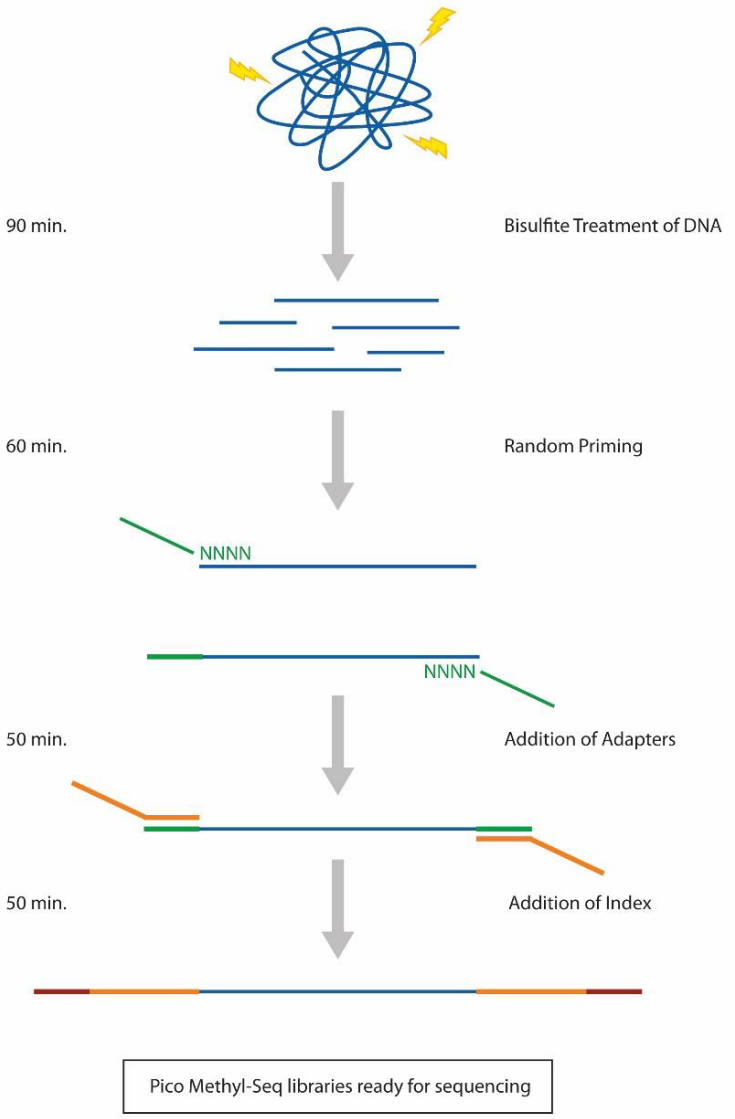


Figure 3. Overview of Zymo Pico Methyl-Seq Library preparation workflow adapted from the protocol (Zymo Research Corp, Irvine, CA, United States)

Bisulfite conversion of genomic DNA

Input DNA in 20µl of purified water was added to 130 µl of Lightning conversion reagent in PCR tubes and incubated in a thermocycler at 98°C for 8 min followed by 54°C for 1 hour and finally a storage step at 4°C. The bisulfite-treated DNA was then mixed with 600 µl of M-binding buffer in a Zymo-spin Column and spun down at 10,000 x g for 30 seconds discarding the flow-through. 100 µl of M-Wash buffer was added to the column and spun down for 10,000 x g for 30 seconds. The converted DNA solution was then desulfonated. 200 µl of L-desulfonation buffer was then added to the column and incubated at room temperature for exactly 20 min and spun down at 10,000 x g for 30 seconds

discarding the flow-through. 200µl of M-Wash buffer was added to the column and spun down for 10,000 x g for 30 seconds, and repeated. The bisulfite converted DNA solution now desulfonated was eluted by adding 8 µl of elution buffer to the spin column and after 1 min was spun down at 10,000 x g for 30 seconds.

PCR amplification of bisulfite converted DNA

A priming reaction mix made with 7 µl of converted DNA was added to PCR tubes on ice with 2 µl of PrepAmp Buffer (5X), 1 µl of PrepAmp primer (40 µM). In addition, a PrepAmp Mix was prepared with 1µl PrepAmp Buffer (5X), 3.75µl PrepAmp Pre-Mix and 0.3 µl of prep amp polymerase (13 U/µl). The 2 cycles of PCR were carried out as follows. The priming reaction mix was placed in a thermocycler held at 98°C for 2 min and held at 8°C to add 3 µl the PrepAmp mix to each reaction which was pulse spun and continued in the following thermocycling program: 16°C for 1min, 22°C for 1 min, 28°C for 1 min, 36°C for one min 36.5°C for 1 min, 37°C for 8 min. Ramp rates of 0.1°C s^{-1} were used for each temperature change and repeated for the second cycle. After denaturing step at 98°, 0.3 µl of polymerase was added to the reaction during the second cycle. The PCR product was then purified using the DNA Cleanup and Concentrator™. The PCR product was mixed with DNA binding buffer at a ratio of 7:1, added to a spin column, and spun down for 10,000 x g for 30 seconds. 200 µl of DNA Wash Buffer was then added to the column and spun down for 10,000 x g for 30 seconds, and repeated. The cleaned and concentrated converted DNA was then eluted with 12 µl of elution buffer and spun down for 10,000 x g for 30 seconds.

Adapter ligation, dual indexing, library validation and quantification

For the ligation of the adaptors, a 25 µl PCR reaction was prepared using 11.5 µl of eluted DNA from the previous PCR product and adding 12.5 µl LibraryAmp Master Mix (2X) and 1µl of LibraryAmp primers (10 µM) in a 0.2 ml PCR tube. The reaction was incubated in a thermocycler for 6 amplification cycles specified for 100 ng or initial DNA input with the following program 94°C for 30sec, 45°C for 30sec, 55°C for 30 sec, 68°C 1 min. The final cycle ended with an additional 5 min at 67°C and held at 4°C. The PCR product was then purified using the DNA Cleanup and Concentrator™ as described previously but eluted with 12.5 µl of DNA elution buffer. For indexing, a dual indexing approach was used instead of the indexes supplied with the Pico methyl-Seq kit to help resolve potential index hopping during sequencing. The indices used were Illumina TruSEQ CD P5-P7 index pairs. Index pairs

used for each sample are shown in table 2. The 25 µl PCR amplification reaction included 12 µl of purified adapters DNA, 25.5 µl of LibraryAmp Master Mix (2X) and 0.5µl of each pair of index primers and cycled with the following program for 10 cycles. 94 °C for 30 sec, 58 °C for 30 sec, 68 for 1 min. The final cycle was kept at 68°C for 5 min then held at 4°C. The PCR product was then purified using the DNA Cleanup and Concentrator™ as described previously but eluted with 12 µl of DNA elution buffer finalizing the libraries preparation. The libraries were validated and quantified using the 2200 Tapestation (Agilent Technologies Santa Clara, CA, USA). Libraries were then pooled at equal concentration before being sent for sequencing.

Sequencing and bioinformatics analyses

The libraries were sent to the Norwegian Sequencing Center (University of Oslo, Oslo, Norway) for sequencing. Libraries were sequenced for 150bp pair end in one lane of an S4 flowcell on the Hiseq4000 platform (Illumina, CA, USA) with 10% PhiX spike in to balance the nucleotide distribution bias of bisulfited converted fragments. Prior to any downstream analysis, the quality of the raw reads was assessed using FastQC v0.11.5 (https://www.bioinformatics.babraham.ac.uk) to check for low quality scores and the level of duplications. The reads were then trimmed and filtered to remove low quality sequences retaining sequences with Phred33 scores higher than 30 and to identify and remove adaptor sequences. This was followed by a second trimming of the first 4 base pairs from the 5' end of every read. All trimming was performed in paired-end mode with TrimGalore v0.4.4 (http://www.bioinformatics.brabraham.ac.uk). Bismark v0.23.0 (Krueger and Andrews, 2011) with Bowtie2 v2.3.1 (Langmead and Salzberg, 2012) was used to align the reads to the salmon reference genome ICSASG_v2 as single end reads using the parameter --score_min L,0,-0.4. Duplicate aligned reads are likely to be PCR duplicates and were removed using the deduplicate Bismark module in Bismark and only unique alignments with no duplicates were used in further downstream analysis. Methylation status of CpG dinucleotides in aligned reads was identified using the Bismark methylation extractor excluding methylation calls in the first 5 bases on the 5' end of reads and the last base on the 3' end of reads. Bismark methylation calls were used as input in the R package methylKit (Akalin *et al.*, 2012) for the analysis of differential methylation, applying a logistic regression test with p-values corrected to q-values using a sliding linear model. Only CpGs with at least 10x coverage in all samples were tested and q-values lower than 0.01 with methylation differences greater than 25% were considered as differentially methylated. Annotation of all differential methylation was performed using the software HOMER, annotatePeaks.pl (Heinz *et al.*, 2010) and the NCBI *Salmo salar* annotation release 100 (Lien *et al.*, 2016) . Gene ontology and KEGG pathway enrichment analysis were performed

for genes associated with differential methylation using enrichr (Chen *et al.*, 2013) for genes with identifiable human (*Homo sapiens*) orthologues.

Results

DNA Extraction, library validation, and alignment

Concentration of extracted DNA varied from 13.4 to 50.2 ng/µl. DNA concentrations and Nanodrop absorbance ratio measurements are shown in *Table 1*. The library regions containing fragment sizes of interest between 150 bp and 950 bp had an average fragment size of 363 bp (SD 21.3 bp) and average molarity of 21.2 nmol/l (SD 4.21). *Table 2* shows the individual library concentrations, average fragment size and molarity for each library region of interest based on fragment size. *Figure 4* is a representative tape station electropherogram for the susceptible fish 6 library. A total of 4,844,022,594 paired end reads where obtained from sequencing with the lowest read count per sample, being 156,360,780 the highest 764,952,460 average read count per sample 403,668,549 (SD 153,155,133) giving an average raw read coverage of 20.2 x (SD 7.66). The variation in raw read coverage is shown in the boxplot in figure 5b. All reads obtained from sequencing had mean phred33 quality scores of 30 or higher mean scores along the read length are shown in figure 5a. The average proportion of duplicate reads was 20.69% (SD 0.02%). On average 59.02% of reads aligned as single end reads to the genome 13.13% of these where multiple alignments with 45.89% of the alignments being unique and used for further analysis. An overview of alignment efficiency per sample R1 and R2 reads is shown in figure 6a.

Table 1. Heart sample DNA absorbance ratios measured with Nano Drop, concentrations measured with Qubit 3.0 fluorometer and final library preparation input volume.

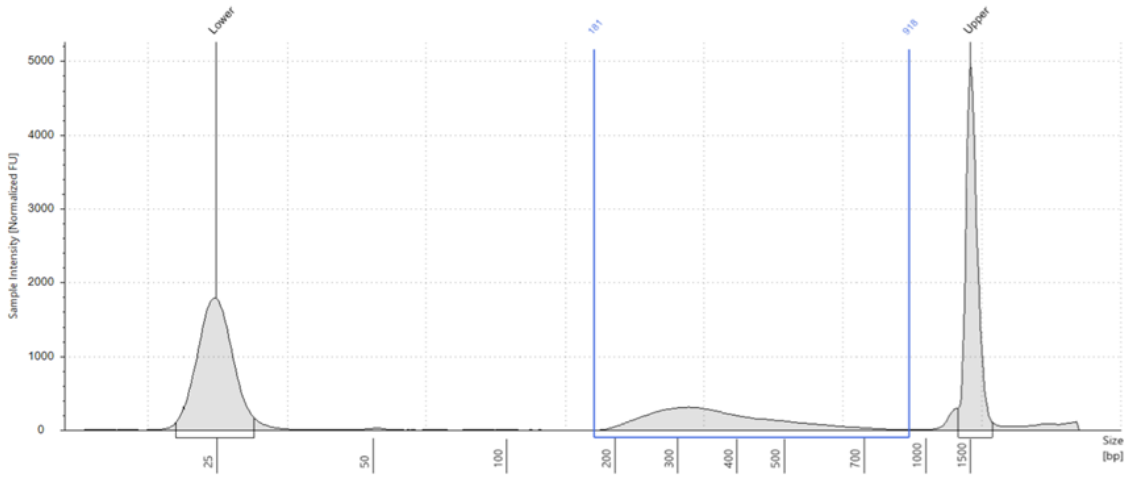
Concentration and purity of extracted DNA

Sample	DNA ng/µl	260/280 nm	260/230 nm	Library input (µl)
Susceptible 1a	50.2	1.93	1.76	2
Susceptible 2a	24.2	1.9	1.51	4.1
Susceptible 3a	13.4	1.87	1.28	7.5
Susceptible 4b	36.5	1.92	1.58	2.7
Susceptible 5b	24.5	1.87	1.06	4.1
Susceptible 6b	35.7	1.88	1.07	2.8
Resistant 1c	40.3	1.92	2.00	2.5
Resistant 2c	20.3	1.9	1.03	4.9
Resistant 3c	31.7	1.93	1.23	3.2
Resistant 4d	29	1.96	1.12	3.4
Resistant 5d	30.2	1.97	0.96	3.3
Resistant 6d	28	2.04	0.89	3.6

Table 2. Index pairs and Tapestation library validation measurements showing library concentrations. Mean fragment size and molarity within library region between 150 bp and 950 bp are also shown.

Library Index Pairs and Quantification

Sample	Unique Index P5/P7 Pair	Library Concentration (ng/ml)	Library Region Mean Fragment Size [bp]	Library Region Molarity (nmol/l)
Susceptible 1a	D505/D705	33.6	359	15.6
Susceptible 2a	D501/D705	26.4	350	10.4
Susceptible 3a	D503/D702	22.2	303	17.5
Susceptible 4b	D507/D701	21.2	349	17.8
Susceptible 5b	D501/D706	20.4	385	10.6
Susceptible 6b	D506/D701	19.7	360	15.8
Resistant 1c	D507/D705	22.8	357	13.4
Resistant 2c	D503/D703	12.2	375	19.7
Resistant 3c	D501/D702	19.7	379	12.2
Resistant 4d	D506/D705	14.7	362	23.8
Resistant 5d	D502/D702	19.2	352	21.4
Resistant 6d	D506/D702	15.4	376	18.4



Region Table

From [bp]	To [bp]	Average Size [bp]	Conc. [ng/µl]	Region Molarity [nmol/l]	%of Total	Region Comment	Color
181	918	376	4.12	18.4	76.52		

Figure 4. Representative Tapestation electropherogram of a WGBS library (ID suscepltable 6b), showing the selected library region in the blue section.

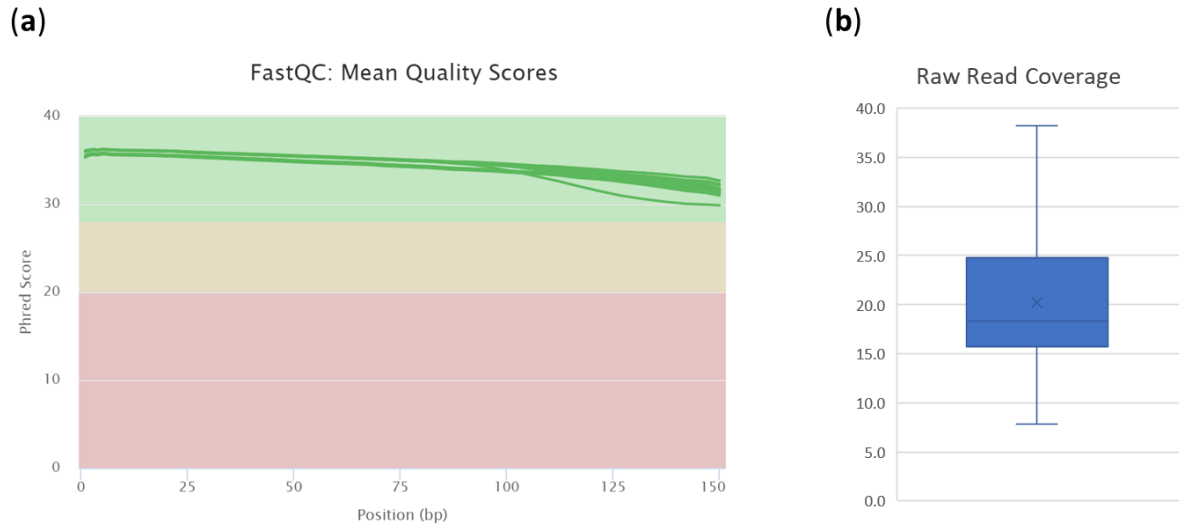


Figure 5 ***a)*** *Mean fastQC sequence quality scores for all libraries generated with MultiQC* ***b)*** *Box plot of sample raw read coverage.*

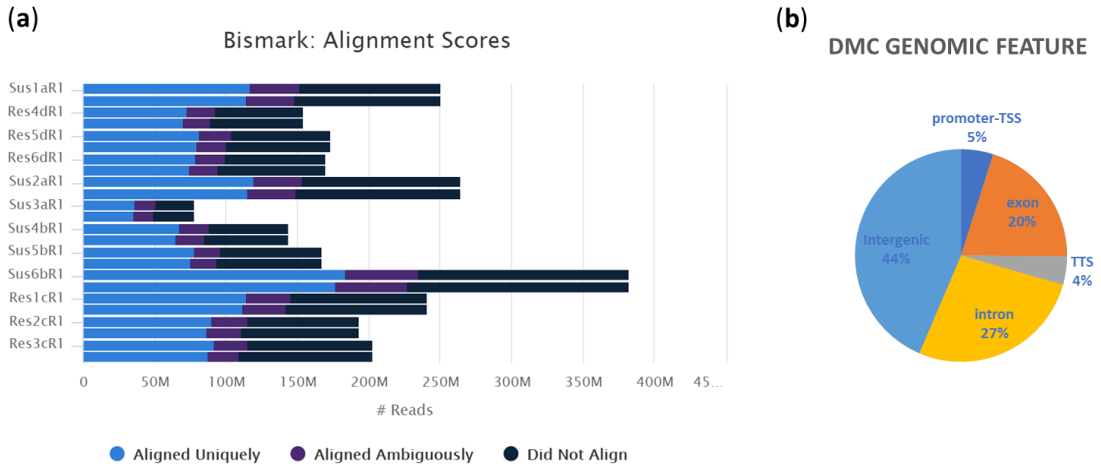


Figure 6. a) Mapping efficiency overview for R1 followed by R2 reads for each sample generated with MultiQC. b) percentage distribution of genomic feature location of differentially methylated cytosines.

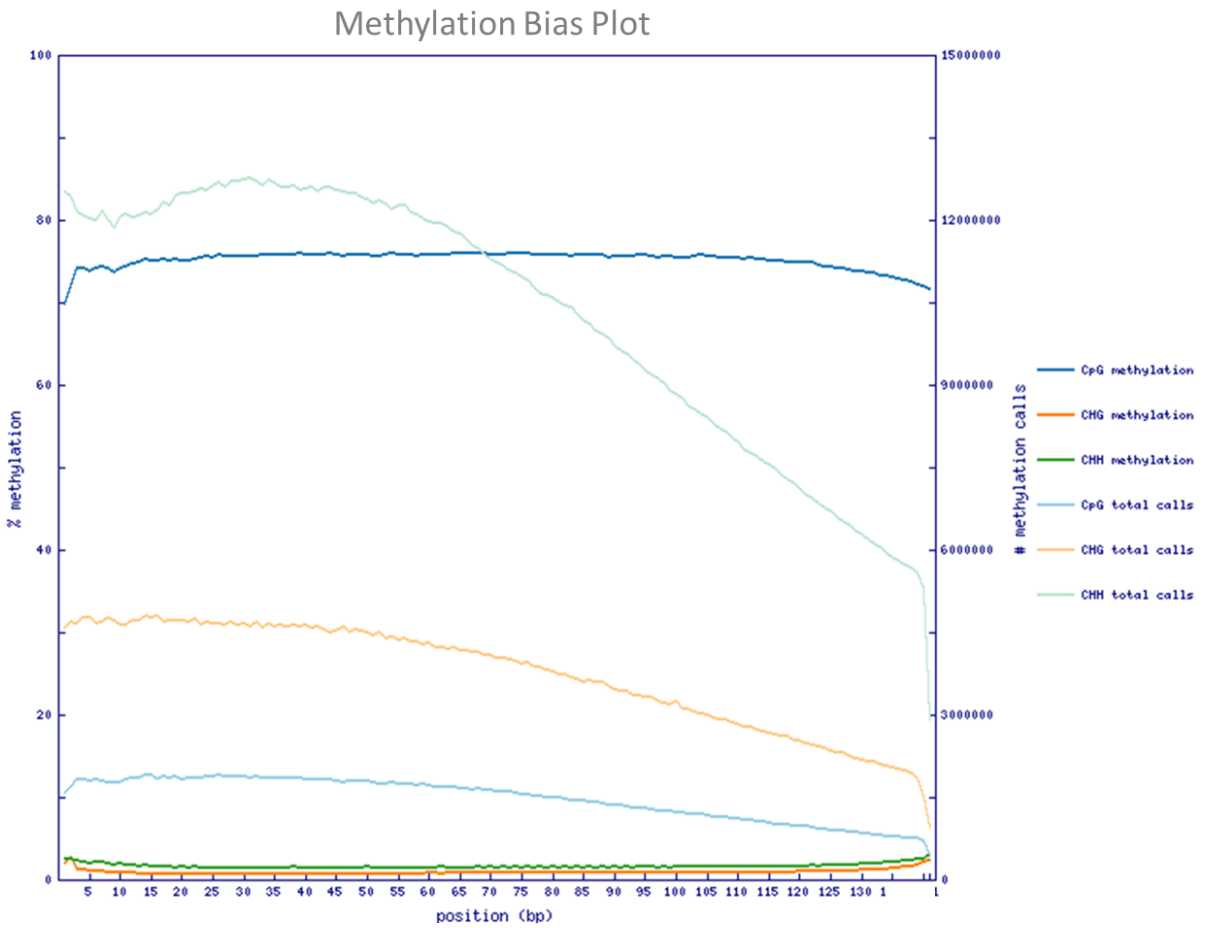


Figure 7. Representative Methylation Bias plot (R1 reads sus1a) showing the distribution of methylation calls in bold darker lines. In non-bold lighter lines are total calls distribution along reads that have 9 bp trimmed on the 5' end and 1 bp trimmed on the 3' end.

Methylation Calling, Exploratory Analysis and Differential Methylation

Of all the CpGs covered in the data, approximately 75% were identified to be in a methylated state 1.61% of cytosines in CHH context were identified as methylated. *Figure 7.* shows the methylation bias along the reads for susceptible sample 1a similar methylation bias plots were seen for all samples. Methylation calls are even across the samples, but the total calls decrease towards the end of both R1 and R2 reads for all samples. A total of 250,000 CpGs had 10x coverage in all samples of these 163 were found to be differentially methylated between the susceptible and resistant groups. A total of 75 significantly hypomethylated and 88 significantly hypermethylated cytosines detected in the susceptible group (qvalue < 0.01). The chromosomal distribution of differential methylation is shown in Figure 8a with chromosome 3 showing the most DMCs composed of mostly hypermethylation in susceptible fish. No DMC were detected on chromosomes 2, 8, 17, 18, 21, 26, 28 and 29. The cytosines with at least 10x coverage in all samples were most often found as 100% methylated; the frequency of different methylation percentages is shown in figure 8b for susceptible

sample 6 with other samples following a similar pattern of % methylation difference. The principal component analysis plot of all the CpGs passing the filters can be seen in figure 9a, showing no clear clustering of samples in PC1 and PC2. The scree plot for the PCA figure 9b also shows little increase in variance explained by the first 10 principal components.

The genomic feature distribution of DMC figure 6b was mostly intergenic with 44% followed by introns at 27% and exons at 22% of DMCs, and finally putative promoter regions 1 kb upstream of TSS containing 5% of identified DMC and transcription termination sites 4%. All genes closest to DMCs can be seen in *table 4* in the appendix. Table 3 shows 11 genes identified as particularly relevant to the experimental context and their comparative methylation status in susceptible fish. These genes where chosen based on their more direct involvement in viral processes and viral immunity as well as their expression in cell types present in the sampled tissue.

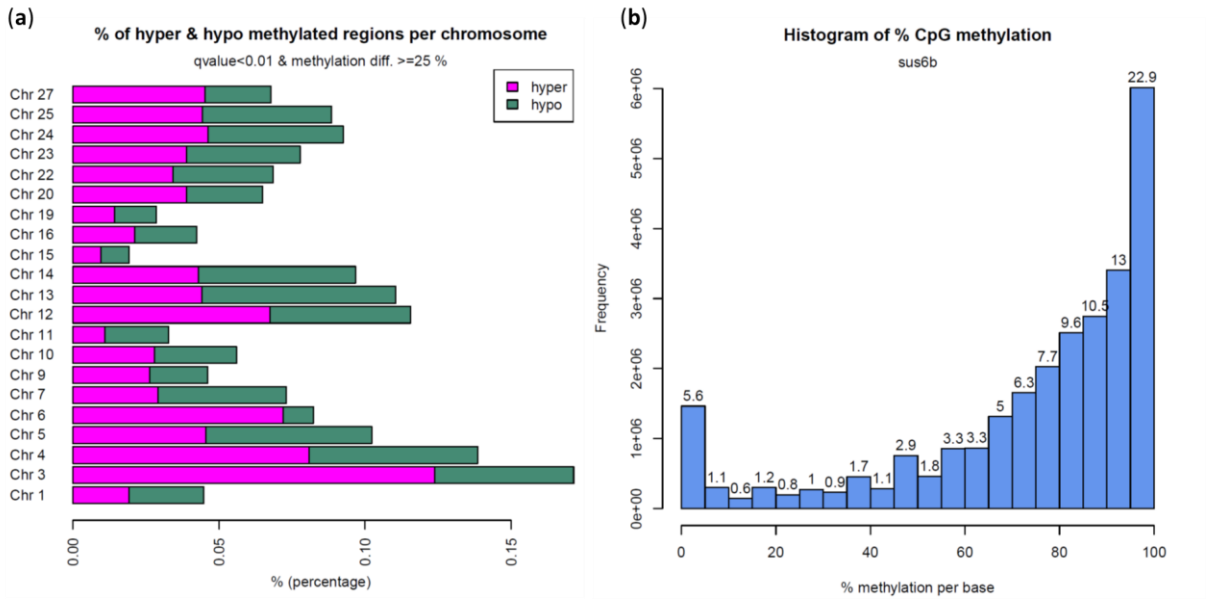


Figure 8. ***a)*** *Chromosomal distribution of differential methylation showing hypo and hyper differential methylation relative to resistant fish.* ***b)*** *Representative histogram of % methylation in CpG context for susceptible fish 6.*

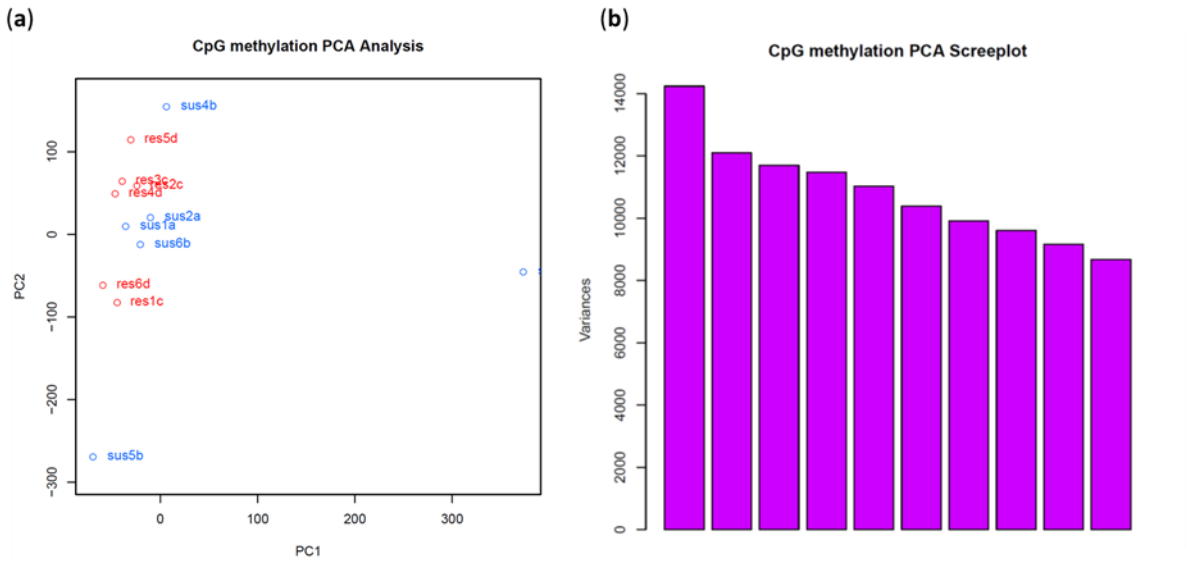


Figure 9. ***a)*** *Principal component analysis of methylated CpG with 10x coverage blue labels representing susceptible fish and red labels resistant fish.* ***b)*** *Scree plot of PCA analysis showing components 1 – 10 from left to right.*

Table 3. Selected ISA-relevant genes associated with differentially methylated cytosine. Showing human ortholog ID, Salmon chromosome, Genomic feature, and percent methylation difference in susceptible compared to resistant fish along with relevant biological function in the context of viral infection.

Entrez ID	Chr	Annotation	Distance to TSS	%Meth.diff	Relevant Biological Function
ptprs	chr 16	Intergenic	-229623	-38.1	negative regulation of TLR, interferon-alpha in pDCs
tnfrsf10b	chr 20	intron	-13056	-36.8	virus infeciton induced apoptosis
npm1	chr 2	exon	5420	-35.8	viral rRNA export from nucleus
usp14	chr 3	promoter	-701	-32.6	deubiquitinase and inactivator of RIG1, proteosome homeostasis
pias1	chr 11	exon	3802	-32.2	inhibitor of IFN1 induciton
lrrc59	chr 23	Intron	-3022	-31.5	negative regulation of pattern recognition receptors, IFN1 signalling
c1r	chr 5	Intergenic	-3359	-30.7	component of antibody binding C1q in classical complement pathway
thoc2	chr 13	intron	69060	-25.2	viral mRNA transport from nucleus and through cytoplasm
gbp1	chr 21	exon	4253	26.8	IFN induced antiviral effector
hnrnpa1	chr 12	promoter	-884	30.9	viral process, RNA transport from nucleus
cacna1c	chr 17	Intergenic	-15949	55.7	membrane ion channel target for influenza binding and entry

Discussion

Assay Quality

This research project has analysed the differential genome-wide distribution of CpG methylation marks in heart in the context of ISA resistance using whole-genome bisulfite sequencing. The project represents one of the few times the thorough WGBS method has been applied to salmonids in any experimental context and the first in relation ISA. The sequencing quality suggests an overall robust assay starting with a high proportion of good quality reads (*figure 5a*). DNA purity is an important requirement for bisulfite sequencing (Olova *et al.*, 2018) and the 260/280 absorption ratios (*table 1*) indicate low contaminating protein content and, therefore, unlikely to influence downstream processes. The 260/230 absorption ratios (*table 1*) show some variation across samples. However, there is little relationship between the 260/230 ratio and sequencing quality or final methylation bias of the individual samples. Even sample coverage is important to gain the full benefit of the sequencing effort, particularly in how the data has been analysed in this instance by strictly filtering all CpGs with less than 10x coverage in any single sample. There is variation in raw read coverage (*figure 5b*). The lowest sequence coverage is from the library from the sample susceptible fish 3a. This sample's raw mean sequence quality is over Phred score 28 but overall lower than other libraries and is visible as the lowest curve in *figure 5a*. The only pre sequencing indicators that separate this sample from the rest are the low DNA extraction concentrations (*table 1*), and the lowest library mean fragment size (*table 2*). The smaller mean fragment size is potentially an indicator of higher fragmentation in the sample and library preparation of susceptible 3a. However, the library electropherogram and the M bias plot do not show clear indications of any difference in this sample. Susceptible fish 3a does not show any major signs of divergence from the other samples but represents a limitation to achieving 10x coverage across all samples. Future analysis performed for this study might need to consider the impact on achieved coverage due to this sample and perform an analysis that better utilizes the sequencing effort by better balancing coverage stringencies or the more common approach, reducing the analysis resolution to detect DMRs as opposed to DMCs.

In this study, no spike-in control was used to estimate bisulfite conversion efficiency. The methylation bias plots and the proportion of non-CpG methylation calls in the aligned data are similar to the low levels reported in zebrafish and other teleosts (Goll and Halpern, 2011; Wan *et al.*, 2016). The low non-GpG methylation indicates a high bisulfite conversion efficiency for this study. However, without a spike in methylation control, it is difficult to estimate non-appropriate methylated cytosine to thymine bisulfite conversions. However, these methylated C to T conversions remain a small

fraction in other studies with similar BS conversion efficiencies and are likely a small influence on final methylation calling (Moghadam *et al.*, 2017; Webster *et al.*, 2018) The overall mapped reads (56% - 66%) are lower than reported in other Atlantic salmon experiments aligning bisulfite-converted reads 80% and 90.6% respectively (Moghadam *et al.*, 2017; Webster *et al.*, 2018) The higher overall alignment efficiency might be due to the experimental population's closer similarity to the reference genome used in either of these cases, alignment parameters or possible differences between RRBS and WGBS methodologies. The alignment was performed, treating paired-end reads as single-end reads. Using this approach can leas to a miscounting of methylation calls in shorter fragments (<300bp) with overlapping sequence pairs. These short read overlapping sequences about 10% of the sequence data should be randomly distributed amongst samples and loci, considering that the average library fragment length was similar between samples. The few overlapping sequence where this would occur would be perceived as random noise in the analysis. The follow up analysis should however be carried out on reads that have been aligned as pair ends to reduce random noise, particularly in data with few and subtle differences. A realignment of the data in pair end mode could help reduce some of the random noise that could be present in the data. This along with a reduction in resolution by the detection of DMRs could resolve DMC acting as peaks in DMRs as well as could act to better utilise the available coverage. However, the analysis presented in this thesis used strict stringencies in terms of coverage per sample, statistical significance and resolution giving a high level of confidence in the results.

Differential methylation

Resistant and susceptible fish showed overall similar methylation profiles in heart tissue with few differences distributed along the genome (*figure 8a*). This would indicate a largely stable methylation profile in heart tissue with few specific differences separating resistant and susceptible fish. Tissue sample cell type heterogeneity is a factor that could be contributing to the observed differences in methylation profiles that must be considered in the interpretation of the results. The frequency of methylation between both extremes more than 0% and less than 100% (*figure 8b*) could be both an indicator of differential methylation due to cell heterogeneity observed as intermediate percent methylation or varying methylation at the same locus within the same cell type. *Figure 8b,* however, is general and does not show the distribution of methylation % within a specific genomic feature. Where this has been done in other experiments; the methylation differences are more distinct in gene bodies and putative promotors, showing clearer hyper and hypomethylation (Webster *et al.*, 2018). The cell type heterogeneity could also be contributing to a levelling of the observed

differential methylation which is 55.8% at its highest (*table 4*). In addition, the significant methylation differences between groups are few in relation to the number of CpGs that pass the filters. This subtlety is reflected in the PCA results figure 9a, 9b. The analysis parameters used are restrictive, while there is confidence in the significance of the results the approach might not be making the best use of the available coverage. The small proportion of CpGs passing filters prior to testing would be randomly dispersed along the genome. It will likely capture a small subset of already subtle differences that separate the groups. This would further prevent the segregation of groups using methods such as PCA. The available data and coverage achieved could again benefit from an alternative analysis based on either detecting DMR or relaxing coverage stringencies allowing larger parts of the genome to pass coverage filters. However, the differential methylation that has been detected with the current analysis parameters used in this thesis represents high stringencies in terms of resolution, coverage in all samples and statistical significance. Therefore, the DMCs represented in *table 4* and *table 3* are likely to be present following the use of an alternative analysis method and would represent the DMC with the highest confidence level.

The DMC distribution between intragenic and intergenic regions is similar to DMCs in other recent studies in Atlantic salmon using the RRBS method to profile gills and whole fry respectively (Moghadam *et al.*, 2017; Webster *et al.*, 2018). The proportion of DMC detected in putative promoter regions is also similar between 5-6%. However, within intragenic regions, the DMC detected in exons, 20%, differs from DMC from one RRBS study 12% in exons (5% exons, 5' UTR 4%, 3' UTR 3%) (Moghadam *et al.*, 2017). The differences could result from method choice and inherent genomic region bias but are likely driven by differences in experimental epigenators and the cell type heterogeneity of the tissues profiled.

The gene enrichment analysis performed did not reveal significant enrichment of functional gene ontology terms or pathway enrichment. The recognized regulatory effect on transcription of differential methylation places DNA methylation amongst a range of other layered regulatory mechanisms that lead to the final differences in phenotypic response. As such, differential methylation may produce less clear enrichment results, being one aspect of a complex regulatory system than, for example, transcriptomics that is already the result of some regulatory influence and is further upstream towards proteome and is thereby closer to generating the final phenotypic differences. Further confounding to enrichment results would be the reduced characterization of teleost genes where the conserved functions could vary compared to human homologues on which the enrichment analysis is performed. Out of 163 DMC-associated genes, 11 have been highlighted as more clearly relevant to potential ISA resistance and viral host interactions. The proximity of the DMC in intergenic regions of these key genes varies, with some DMC several Kb distant to their associated genes.

Methylated CpGs are associated with neighbouring CpGs also being methylated (Peters *et al.*, 2015). The coverage stringencies used in the analysis is much more likely to filter out most neighbouring CpGs rather than to test and detecting them as differentially methylated. Therefore, DMCs could represent coverage peaks in larger DMRs bringing differential methylation closer to the TSS and genomic regions in which their association with transcriptional regulation is better understood. An analysis approach that identifies DMR could aid in resolving the overall differential methylation status of nearby CpGs.

Virus host interaction genes associated with differential methylation

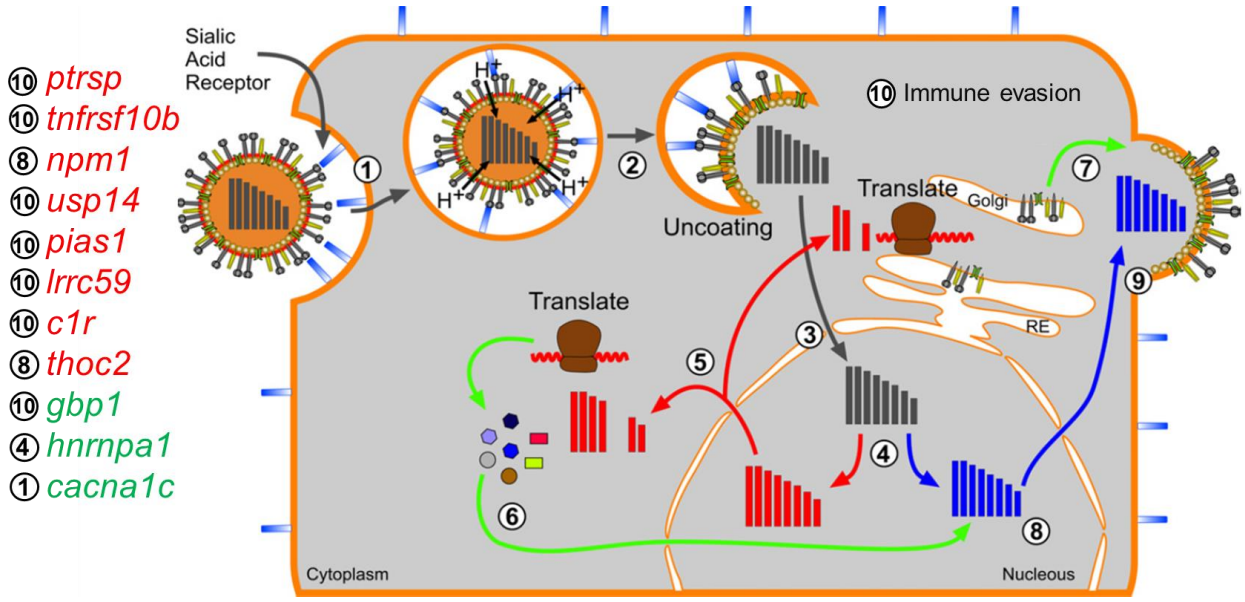


Figure 10. Aspects of the ISAV replication process adapted from (Cottet *et al.*, 2011) *and key DMC associated genes putative influence on viral process numbered. 1) hemagglutinin binding and endocytosis. 2) PH change in the endosome releasing the viral genome. 3) vRNP transport into the nucleus. 4) transcription and replication of the viral genome. 5) Viral transcript export and transcription. 6) Import of some viral proteins into the nucleoplasm. 7) Surface glycoproteins follow secretory pathway to the plasma membrane. 8) vRNP formation and export through the cytoplasm. 9) Formation and budding of new virion. 10) Host immune response and viral evasion. The key genes in red indicate an association with hypomethylated DMC in susceptible fish and green indicates hypermethylation.*

Hypomethylated genes related to viral processes in susceptible fish

Fish that succumbed to ISA early had hypomethylation associated with a range of genes involved in viral host interactions, including immune response regulating genes and genes with protein products known to be utilized in the viral replication process. Most of these genes are expressed in the major cell type constituents of the heart. One is particular to immune cells are also likely present

to a lesser extent in infected heart tissue in ISA mortalities. Receptor protein tyrosine phosphatase sigma (PTPRS) is expressed specifically on plasmacytoid dendritic cells (pDC), the primary producers of type I interferon (IFN) in response to viruses. Initially defined by their ability to respond to purified influenza virus in vitro, pDCs express high levels of nucleic acid sensing toll like receptors (TLRs) and play a central role in antiviral responses with massive and rapid production of IFN1 (Reizis, 2019). Only PTPRS downregulated pDC are known to produce IFN and thereby marks activated pDC cells (Bunin *et al.*, 2015). If hypomethylation of PTPRS, in this case, is associated with upregulation of PTPRS and inactivation of the primary IFN producing immune cell this could indicate a reduced viral innate immune response in susceptible fish. However, the detected intergenic DMC for PTPRS is the most distant to the TSS of the key genes representing a less likely association with transcription due to lack of proximity to the TSS. Alternatively, the detection of DMC near a pDC specific gene could indicate the differential presence of pDC in the samples that could also be associated with the effectiveness of a host's response to the ISA virus.

The infected cell's production of IFN1 plays an essential central role as the first line of defence in the antiviral immune response. IFN1 contributes to the inflammatory response and induces an antiviral state in uninfected neighbouring cells (Ivashkiv and Donlin, 2014). IFN1 starts with the host recognition of pathogen-associated molecular patterns (PAMPs), of which the retinoic acid inducible gene 1 (RIG-1) is a key cytoplasmic pathogen recognition receptor (Kell and Gale, 2015). RIG-1 was primarily associated with dsRNA PAMPs but was first shown to trigger IFN1 production when detecting negative sense ssRNA virus ligands from Orthomyxoviridae viruses (Pichlmair *et al.*, 2006). Toll like receptors that are also expressed in endothelial cells and are effective when in lysosomes, like TLR3, TLR7 and TLR8 all of which detect negative sense ssRNA (Lee and Barton, 2014). One important regulator of both these pattern recognition receptor types is the leucine rich repeat containing protein 59 (LRRC59) that prevents autophagosome degradation of RIG-1 and is necessary for the trafficking and endosomal localization of a range of nucleic acid sensing TLRs (Xian *et al.*, 2020; Tatematsu *et al.*, 2021). In susceptible fish, the second intron of the LRRC59 homologue is hypomethylated. First, intron methylation is correlated with reduced expression. However, the hypomethylated LRRC59 intron is early in the second of 28 introns in a context where its association with transcription is less understood. If it affects LRRC59 activity, this differential methylation could alter the host cell's ability to detect ISAV and influence the effectiveness of IFN1 antiviral immune response.

Efficient viral replication involves immune evasion, and viruses have evolved several mechanisms to interfere with immune pathways in infected cells. Two ISAV viral proteins have been shown to exert interferon antagonistic activity (García-Rosado *et al.*, 2008). One of the target mechanisms evolved in different virus types is the manipulation of SUMO E3 ligases, of which the

protein inhibitor of activated STAT (PIAS) family of proteins is the most investigated (Lowrey, Cramblet and Bentz, 2017). PIAS1 inhibition of IFN1 is a recognized immune evading tactic in several viruses and increases in global sumoylation is a characteristic of influenza infected cells (Lowrey, Cramblet and Bentz, 2017). Susceptible fish show hypomethylation in an exon for PIAS1 which if associated with more effective inhibition of IFN1 would allow for greater viral immune evasion in susceptible fish.

The extrinsic apoptotic pathway plays a role in eliminating dangerous cells infected with pathogens and thereby contributes to control the spreading of viral infections (Mehrbod *et al.*, 2019). Tumour necrosis factor receptor superfamily member 10b (TNFRSF10B), when ligand activated, transduces extrinsic apoptosis signalling and have been shown, at the proteomic level to be associated with influenza induced apoptosis in humans (Kroeker *et al.*, 2012). TNFRSF10B has been shown to be upregulated in gill tissue in later stages of ISAV infection an indication that this role is conserved in ISAV infection in salmon (Valenzuela-Miranda *et al.*, 2015). Susceptible fish show hypomethylation in a late intron on the salmon homologue for TNFRSF10B. Intragenic methylation has been associated with increased expression in early introns, however patterns of hyper and hypo methylation/hydroxymethylation at intron exon boundaries has been associated with alternative splicing. This is thought more likely to be a regulating factor in cases where exon sequence is methylated, and intron sequence is unmethylated at the splice site (Lev Maor, Yearim and Ast, 2015). In human, there are 2 known largely uncharacterized alterative isoforms of TNFRSF10B. The non-canonical isoforms contain premature stop codons which may make them susceptible to nonsense-mediated mRNA decay (Uniprot, 2020). Intronic differential methylation at TNFRSF10B could affect the efficiency of apoptotic clearing of infected cells contributing to viral susceptibility, however the details of DNA methylation regulation of alternative splicing remain partly understood.

The complement system is an important part of the innate immune defence, and one well characterized function of the classical complement pathway is to interact with the adaptive immune system by recognizing antibody opsonized targets and contributing to their destruction via the forming the membrane attack complex or recruiting phagocytes to the opsonized target (Lubbers *et al.*, 2017). Complement component 1 subcomponent R (C1r), unlike some other soluble complement components, is extrahepatic and expressed widely in many tissues and forms part of the C1q molecule the antibody binding component of the complement system (Lubbers *et al.*, 2017). The activated complement system has antiviral effects but is normally highly regulated to prevent overstimulation and damage to uninfected cells (Merle *et al.*, 2015). ISA is characterized by severe anaemia and RBCs have been shown to be coated in virions but with conflicting evidence of viral replication in nucleated RBCs. Haemophagocytosis has also been regularly observed in ISAV infected fish and the resulting anaemia has been suggested to be a main driver of the systemic outcomes of ISAV infection (Aamelfot

et al., 2012). Upregulation of classical complement components could be a contributing factor to the haemophagocytocisis of opsonized virion coated RBCs. Susceptible fish genomes are hypomethylated in the intergenic region upstream of the putative promotor for the C1r homologue. Although not in the putative promoter itself, the DMC could be part of a DMR that effects the putative promotor. Hypomethylation in this case would be associated with the upregulation of C1r with potentially both antiviral outcomes as well as detrimentally increasing the haemophagocytosis and anaemia observed in ISA infected fish. That strong upregulation of complement components is a feature of ISAV that is also supported by a transcriptomics study that shows complement component 3 to be the most upregulated of a subset of immune genes between early and late stages of infection (Valenzuela-Miranda *et al.*, 2015)

Viruses require the use of several components of the host cell for replication. A total of 1292 proteins have been shown to coprecipitate with the proteins of one member of the Orthomyxoviridae family in humans (Watanabe *et al*., 2014). The cellular availability of some of these proteins have been shown to impact the replication efficiency of Orthomyxoviridae viruses (Watanabe *et al*., 2014). Orthomyxoviridae viruses are one of the few RNA viruses that perform part of their replication process in the nucleus and ISAV virus nucleoprotein localize to the nucleus early in infection (Falk *et al.*, 2004). Influenza viral protein and host nuclear protein-protein interactions have highlighted the RNA chaperone protein nucleophosmin 1 (NPM1) to play a role in viral ribonucleoprotein complex (vRNP) export from the nucleus. Furthermore, in vitro NPM1 knockdown has been shown to inhibit Orthomyxoviridae replication (Bortz *et al.*, 2011). NPM1 is upregulated in later stages of ISAV infection in the head kidney which could indicate its conserved role in ISAV and Salmon (LeBlanc, Laflamme and Gagné, 2010). Susceptible fish show hypomethylation in an exon for the homologue of NPM1. The regulatory effect of exon methylation can regulate both alternative splicing and if representing part of a larger DMR covering promoter regions could be influencing the transcription of NPM1. If the differential hypomethylation in susceptible fish is indicative of increased translation of NPM1, it would imply the increased availability of a viral replication enhancing host protein that could play a role in reduced resilience and resistance to ISA. In addition to nuclear export, vRNP transport within the cytoplasm is an important part of viral replication and necessary to complete the creation of new virions. Influenza utilizes several host proteins for the intracellular transport of progeny vRNP including THO complex subunit 2 (THOC2) primarily involved in host mRNA nuclear transport THOC2 downregulation has been shown to impeded early stages of the viral lifecycle (Watanabe *et al.*, 2014). A late intron in THOC2 is significantly hypomethylated in susceptible fish. If this hypomethylation is associated with an increase in THOC2 transcription and translation it would in turn increase a viral

enabling component of the host proteome likely utilized by ISAV for replication essential, vRNP transport.

The Ubiquitin (Ub) conjugating system is a conserved part of the host cell machinery that viruses have evolved mechanisms to bypass, in the case of processes that challenge them. As well as to co-opt ubiquitin driven processes to maximize their chance to replicate. The varied viral exploitation of the most studied Ub process, the ubiquitin proteosome system, is so common that proteosome inhibitors have been shown to interfere with the replication of ten major pathogenic virus families including orthomyxoviruses (Calistri *et al.*, 2014). Ubiquitin specific protease 14 (USP14) is a deubiquitinating enzyme that has been shown to be critical for proteosome homeostasis and as a regulator of the innate immune response (Liu *et al.*, 2018). In addition, inhibition of USP14 has been shown to interfere with the replication of some virus families (Nag and Finley, 2012). USP14 has also been shown to act in the deubiquitinating and thereby deactivation of RIG1, acting as a negative regulator of the IFN1 antiviral response. Its importance in viral replication makes it a potential therapeutic target for a range of viruses (Li *et al.*, 2019). The general virus enabling activity of USP14 is also evident in teleosts where USP14 acts as a significant pro-viral factor for grouper iridovirus (Huang *et al.*, 2020) The USP14 homologue is hypomethylated in the putative promoter region in susceptible fish. Promoter methylation and its association with transcription is the most well described of the methylation contexts and most often results in repression of gene transcription. If susceptible fish with unmethylated promoters have more transcriptionally active USP14 than resistant fish, the increase in this virus enabling protein could play a role in the establishment of their divergent phenotypes.

Hypermethylated genes associated with viral processes in susceptible fish

Virus surface proteins usually have highly specific host membrane targets that they bind to prior to gaining entry to and infecting cells. The specific sialic acid modification on the host target has been identified for ISAV hemagglutinin as Neu4,5Ac (Aamelfot *et al.*, 2012). The likely membrane protein that carries the specific sialic acid modification that is required for influenza A infection in humans has been relatively recently identified to be the voltage dependent Ca^{2+} Channel (CACNA1C) (Fujioka *et al.*, 2018). It has also been shown that the viral interaction with CACNA1C triggers intracellular Ca^{2+} oscillations that are required for viral entry and subsequent replication (Fujioka *et al.*, 2018). ISAV however binds to a slightly different sialic acid modification to influenza A but neither its specific sialic acid containing target membrane protein has been identified nor the details of its

means of entry to host cells (Aamelfot *et al.*, 2012). Interestingly, the salmon homologue for CACNA1C is associated with the second biggest % difference in hypermethylation in susceptible fish. The DMC is however several Kb upstream of the TSS and would only cover the putative promoter region if it represented part of a very large DMR. A plausible reason for this DMC to be influencing the resistance to ISAV would require the salmon homologue for CACNA1C to be a main membrane protein target for ISAV. Many viruses contain a receptor destroying enzyme gene, consequently a rather large portion of the small viral genome is dedicated to the destruction of the membrane protein that they bind to. In ISAV this enzyme is sialate-4-O esterase (Hellebø *et al.*, 2004) and like other viruses removes the target membrane protein. This is done to prevent superinfection and reinfection and importantly the agglutination of newly budded virions. Where virions that display both hemagglutinate and its target protein bind to each other. All these factors are so detrimental to infective efficiency that the receptor destroying enzyme is the most used licenced viral drug target for influenza, as Relenza™, Tamiflu™, Inavir™, and Rapivab™ all inhibit the receptor destroying enzyme. However unlikely this phenotypic outcome of upregulation of CACNA1C is, it remains an intriguing DMC associated gene and a reminder that the main sialic acid modified protein targeted and entry method for ISAV remains undiscovered.

Orthomyxoviridae genomes contain a specialized virus polymerase that interacts with co-opted host proteins to perform the essential negative sense ssRNA replication required for the creation of new virions (Choi, 2012). Proteomics experiments have identified several interaction partners for influenza polymerase some of which are shown, following RNAi knockdown, to be required for transcription broadly across strains. RNA binding host proteins whose viral polymerase interaction is hypothesized to be more direct have been shown to be particularly relevant to reducing viral replication (Bortz *et al.*, 2011). A hijacking of similar host proteins for transcription seems to occur during ISAV infections where various ribonucleoproteins show upregulation coinciding with the peak in viral replication (LeBlanc, Laflamme and Gagné, 2010). Heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1) is one of the RNA binding influenza polymerase interactome proteins that reduces viral replication when knocked down. The HNRNPA1 homologue's putative promoter region is significantly methylated in susceptible fish. This methylation context is potentially associated with downregulation of this gene, which is required for the effective replication of viral RNA essential to the creation new virions. If this interaction is conserved in ISAV and salmon, it would suggest that susceptible fish are counterintuitively less enabling of viral RNA replication.

In head kidney and liver tissue ISAV has been shown to induce a strong response in select genes of the IFN1 system but ISAV also show signs of adept immune evasion as the triggered main IFN system genes like MX1 and ISG15 do not impede viral replication (Kileng, Brundtland and Robertsen, 2007). IFN however induces a broad range of proteins some of which might be less susceptible to viral

evasion or have different outcomes in other tissues. Guanylate binding protein 1 (GBP1) is an interferon inducible antiviral effector that exerts direct antiviral functions effecting multiple steps in viral reproduction in a range of pathogenic viral families (Zhang *et al.*, 2021). In orthomyxoviruses, GBP1 and two splice variants of GBP3 have been shown to suppress viral replication of several strains in vitro and the influenza A virus non-structural protein has evolved to interact with and inhibit GBP1's antiviral activity (Zhu *et al.*, 2013). GBP1 has been identified as upregulated in the early stages of ISAV infection in the head kidney and liver indicating that its role in early antiviral response is conserved in salmonids (Valenzuela-Miranda *et al.*, 2015). Heart tissue of susceptible fish show hypermethylation in a late exon for the GBP1 homologue. This is a context that could be associated with alternative splicing and if indicative of a large DMR potentially gene silencing of a well characterized antiviral protein in susceptible fish.

Conclusion

The most thorough method for methylation profiling has been applied to ascertain differences in methylation that could underly the observed resistance to ISAV in Atlantic salmon. The heart tissue was chosen for profiling as it represents one of the main target tissues with the highest viral loads early in infection. The findings indicate that overall methylation profiles in salmon heart tissue of susceptible and resistant fish are similar. The differences however highlight genes that have been shown to be involved in viral replication, and innate immune response and evasion. These findings point towards a role for methylation in the regulation of the antiviral immune response in infected tissue that could confer part of the observed resistance to ISAV. However, the regulatory effect of these methylation marks remains elusive. As such, would benefit from the quantification of the expression of the differentially methylated genes to ascertain if these methylation marks are indeed candidates indicating resistance to ISAV.

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Appendix

Table 4. Differentially methylated DMC (q value <0.01). Listing chromosome, position and differential methylation in the ISAV susceptible group. The genomic feature for the DMC and the distance to the transcription start site to the closest associated gene. The closest associated gene symbol and the human homologue gene ID is given where available along with the homologue gene description. A subset of genes selected for their relevance to ISAV resistance are highlighted in green (hypomethylated in susceptible fish) and red (hypermethylated in susceptible fish)

Chr	Position	Meth %	q.val	Annotation	Distance to TSS	Gene symbol	Homologue ID	Description
chr 13	17776956	-43.4	0.00000	Intergenic	-18596	*trh*	TRH	Thyrotropin-Releasing Hormone
chr 12	22475933	-43.3	0.00000	exon (12 of 12)	8531	*LOC106564748*	MRPL45	Mitochondrial Ribosomal Protein L45
chr 12	58280292	-42.9	0.00000	intron (1 of 28)	-2052	*LOC106565537*	ERBB3	Erb-B2 Receptor Tyrosine Kinase 3
chr 26	33025600	-41.7	0.00001	Intergenic	-1604	*rbpms_1*	RBPMS	RNA Binding Protein, MRNA Processing Factor
chr 9	1.05E+08	-39.7	0.00001	Intergenic	6254	*trnae-uuc_9*	trnae-uuc_9	tRNA
chr 3	60022676	-38.5	0.00024	intron (1 of 10)	4639	*LOC106601018*	HSPA1L	Heat Shock Protein Family A (Hsp70) Member 1 Like
chr 16	51648378	-38.1	0.00000	Intergenic	-229623	*LOC106574153*	PTPRS	Protein Tyrosine Phosphatase Receptor Type S
chr 19	6613475	-37.4	0.00110	promoter-TSS	-348	*ccdc186*	CCDC186	Coiled-Coil Domain Containing 186
chr 20	35477155	-36.8	0.00076	intron (13 of 15)	-13056	*LOC106580524*	TNFRSF10B	Tumor Necrosis Factor Receptor Superfamily Member 10b
chr 13	14757732	-36.8	0.00010	Intergenic	-4096	*LOC106566581*	unchar	unchar
chr 3	30830592	-36.5	0.00000	intron (2 of 18)	-6186	*sntg1*	SNTG1	Syntrophin Gamma 1
chr 2	42561848	-35.8	0.00045	exon (3 of 5)	5420	*LOC106585752*	NPM1	Nucleophosmin 1
chr 2	29779322	-35.0	0.00000	Intergenic	-160943	*LOC106584618*	unchar	ncRNA
chr 10	43729837	-34.5	0.00261	intron (1 of 3)	15131	*LOC106560509*	LRP8	LDL Receptor Related Protein 8
chr 1	24317923	-34.1	0.00023	TTS	9123	*LOC106589786*	VASH1	Vasohibin 1
chr 4	410485	-33.9	0.00232	TTS	2190	*LOC106602095*	SMIM18	Small Integral Membrane Protein 18
chr 26	9992670	-33.6	0.00020	Intergenic	-4195	*LOC106586984*	HE1.2	hatching enzyme 1
chr 11	41799177	-33.5	0.00023	TTS	69871	*LOC106562738*	HIPK3	Homeodomain Interacting Protein Kinase 3
chr 14	19942770	-33.5	0.00005	intron (14 of 24)	-66489	*lactb2*	LACTB2	Lactamase Beta 2
chr 5	54618063	-33.4	0.00155	exon (2 of 2)	1162	*LOC106605204*	unchar	unchar
chr 13	86485153	-33.3	0.00209	intron (12 of 20)	14431	*LOC106568226*	KDM2B	Lysine Demethylase 2B

chr 6	22338004	-33.2	0.00001	exon (15 of 15)	-2800	*LOC106606819*	ATXN7	Ataxin 7
chr 4	37521133	-33.0	0.00545	intron (1 of 2)	2980	*LOC106603148*	GNG3	G Protein Subunit Gamma 3
chr 1	13001880	-32.7	0.00000	Intergenic	-160475	*LOC106565829*	unchar	ncRNA
chr 3	33163379	-32.6	0.00007	promoter-TSS	-701	*LOC106600434*	USP14	Ubiquitin Specific Peptidase 14
chr 2	22514395	-32.6	0.00003	intron (10 of 14)	9787	*LOC106581241*	EAF1	ELL Associated Factor 1
chr 26	29123328	-32.4	0.00256	intron (9 of 17)	4027	*LOC106587569*	GPI	Glucose-6-Phosphate Isomerase
chr 14	78935452	-32.3	0.00046	intron (2 of 10)	20540	*LOC106570498*	TINAGL1	Tubulointerstitial Nephritis Antigen Like 1
chr 11	38648895	-32.2	0.00130	exon (4 of 8)	3802	*LOC106562666*	PIAS1	Protein Inhibitor Of Activated STAT 1
chr 13	65864330	-32.2	0.00165	Intergenic	28476	*LOC106567745*	unchar	ncRNA
chr 23	27642993	-31.5	0.00562	intron (2 of 28)	-3022	*LOC106584340*	LRRC59	Leucine Rich Repeat Containing 59
chr 12	88331790	-31.5	0.00244	Intergenic	-52179	*LOC106566224*	ACAP1	ArfGAP With Coiled-Coil, Ankyrin Repeat And PH Domains 1
chr 10	11134211	-31.5	0.00040	exon (3 of 4)	1136	*dio1*	DIO1	Iodothyronine Deiodinase 1
chr 25	21293216	-31.5	0.00190	Intergenic	13243	*LOC106586366*	CRYGM2D3	crystallin, gamma M2d3 (Danio rerio)
chr 20	68884726	-31.3	0.00003	intron (6 of 12)	7560	*LOC106581318*	GACK	RhoGAP domain-containing protein
chr 22	4957566	-31.2	0.00743	intron (2 of 5)	15277	*LOC106582599*	unchar	protein coding
chr 24	33908682	-31.0	0.00369	Intergenic	-89661	*LOC106585766*	COX7C	Cytochrome C Oxidase Subunit 7C
chr 26	27415447	-30.9	0.00002	Intergenic	-6800	*calc1*	CALCA	Calcitonin Related Polypeptide Alpha
chr 26	38541501	-30.8	0.00517	exon (3 of 3)	8675	*LOC106587841*	CHSY1	Chondroitin Sulfate Synthase 1
chr 5	68604059	-30.7	0.00042	Intergenic	-3359	*LOC106605721*	C1R	Complement C1r
chr 7	56302274	-30.7	0.00889	Intergenic	12128	*LOC106609794*	GXYLT1	Glucoside Xylosyltransferase 1
chr 5	15792532	-30.7	0.00910	exon (2 of 2)	1296	*LOC106604380*	INTS5	Integrator Complex Subunit 5
chr 2	1927871	-30.1	0.00234	intron (2 of 5)	10115	*LOC106573409*	CREB5	CAMP Responsive Element Binding Protein 5
chr 9	20763878	-30.0	0.00020	Intergenic	-53597	*fsip1*	FSIP1	Fibrous Sheath Interacting Protein 1
chr 14	49466491	-29.7	0.00035	exon (3 of 16)	2353	*eif2b5*	EIF2B5	Eukaryotic Translation Initiation Factor 2B Subunit Epsilon
chr 24	16869328	-29.6	0.00099	Intergenic	-4326	*LOC106585258*	EIF4EBP1	Eukaryotic Translation Initiation factor 4E Binding Protein 1
chr 16	28011681	-28.9	0.00517	exon (6 of 9)	8505	*plin1*	PLIN1	Perilipin 1
chr 29	37064219	-28.9	0.00301	intron (1 of 1)	5153	*LOC106590659*	unchar	protein coding
chr 3	75155813	-28.7	0.00129	exon (27 of 27)	4871	*LOC106601548*	unchar	protein coding
chr 7	36028266	-28.7	0.00000	Intergenic	-242335	*LOC106609201*	GPR37	G Protein-Coupled Receptor 37
chr 29	17967168	-28.6	0.00628	intron (2 of 30)	8760	*LOC106590290*	COL21A1	Collagen Type XXI Alpha 1 Chain

chr 13	40601152	-28.3	0.00866	Intergenic	-3512	*LOC106567208*	XK	X-Linked Kx Blood Group
chr 4	62837115	-28.2	0.00256	Intergenic	47927	*LOC106603720*	YWHAE	Tyrosine 3-Monooxygenase Activation Protein
chr 2	29786448	-28.2	0.00611	Intergenic	-168069	*LOC106584618*	unchar	ncRNA
chr 14	53173655	-28.0	0.00112	exon (27 of 30)	23957	*LOC106570082*	ADGRB1	Adhesion G Protein-Coupled Receptor B1
chr 12	33265496	-27.9	0.00894	Intergenic	-18260	*LOC106565045*	unchar	ncRNA
chr 3	65401622	-27.9	0.00057	TTS	3431	*LOC106601177*	CDC42SE1	CDC42 Small Effector 1
chr 15	70559616	-27.8	0.00085	Intergenic	-5980	*kiaa2013*	KIAA2013	Uncharacterized Protein KIAA2013
chr 23	35851634	-27.5	0.00005	Intergenic	4615	*LOC106584486*	unchar	protein coding
chr 5	78374774	-27.5	0.00725	Intergenic	6744	*LOC106606140*	EPR1	Proline-Rich Extensin-Like Protein EPR1
chr 25	547335	-27.5	0.00136	Intergenic	124938	*LOC106586021*	SHOX	Short Stature Homeobox
chr 4	50275398	-27.4	0.00937	Intergenic	-132223	*LOC106603524*	AUTS2	Activator Of Transcription And Developmental Regulator
chr 28	22772990	-27.1	0.00123	Intergenic	-3201	*LOC106589742*	unchar	ncRNA
chr 1	1.14E+08	-26.8	0.00063	intron (14 of 21)	32463	*tmem232*	TMEM232	Transmembrane Protein 232
chr 22	3566717	-26.8	0.00984	intron (6 of 10)	40823	*LOC106582652*	MATN4	Matrilin 4
chr 10	80858430	-26.6	0.00001	Intergenic	10196	*LOC106560814*	SYT1	Synaptotagmin 1
chr 5	73054831	-26.2	0.00155	Intergenic	-6758	*LOC106605933*	EFNA4	Ephrin A4
chr 7	54885860	-26.2	0.00005	intron (4 of 9)	5400	*LOC106609767*	KRR1	KRR1 Small Subunit Processome Component Homolog
chr 12	21233982	-26.2	0.00629	promoter-TSS	-175	*LOC106564643*	GFI1B	Growth Factor Independent 1B Transcriptional Repressor
chr 14	68755943	-26.1	0.00026	exon (27 of 35)	40943	*LOC106570304*	TOP2B	DNA Topoisomerase II Beta
chr 1	21684513	-25.9	0.00613	Intergenic	-3126	*LOC106581189*	unchar	ncRNA
chr 4	19157888	-25.9	0.00366	Intergenic	6907	*LOC106602669*	MUC5AC	Mucin 5AC, Oligomeric Mucus/Gel-Forming
chr 27	35750312	-25.5	0.00465	intron (1 of 2)	5719	*LOC106589030*	TMEM170B	Transmembrane Protein 170B
chr 13	78941611	-25.2	0.00649	intron (36 of 38)	69060	*LOC106567846*	THOC2	invoved in viral mrna trasport from nuc
chr 9	66290555	-25.1	0.00721	intron (3 of 11)	-153408	*LOC106611818*	ETF1	Eukaryotic Translation Termination Factor 1
chr 18	55051110	25.1	0.00289	Intergenic	-3271	*LOC106577690*	UGT1A5	UDP Glucuronosyltransferase Family 1 Member A5
chr 22	54581662	25.2	0.00801	exon (4 of 9)	718	*LOC106583725*	CSL3	L-rhamnose-binding lectin CSL3-like
chr 3	57371246	25.3	0.00747	exon (1 of 2)	398	*hoxb3aa*	HOXB3	Homeobox B3
chr 12	75311851	25.3	0.00198	intron (1 of 6)	-16245	*LOC106565963*	unchar	protein coding
chr 7	4753879	25.6	0.00822	Intergenic	-3267	*pcolce*	PCOLCE	Procollagen C-Endopeptidase Enhancer
chr 4	39435001	25.7	0.00045	exon (1 of 6)	1828	*LOC106603249*	PCDHGC5	Protocadherin Gamma Subfamily C, 5

chr 27	16263607	25.7	0.00064	exon (4 of 4)	37125	*LOC106588589*	KCNA2	Potassium Voltage-Gated Channel Subfamily A Member 2
chr 27	10852462	25.8	0.00827	intron (5 of 5)	15089	*coea1*	COL14A1	Collagen Type XIV Alpha 1 Chain
chr 5	78927672	25.9	0.00138	intron (5 of 7)	5826	*cep76*	CEP76	Centrosomal Protein 76
chr 13	67955634	25.9	0.00076	Intergenic	-4567	*LOC106567708*	unchar	ncRNA
chr 20	22370986	25.9	0.00304	Intergenic	-13333	*vgll4*	VGLL4	Vestigial Like Family Member 4
chr 23	38589607	26.0	0.00822	Intergenic	-101444	*LOC106584577*	GNPDA2	Glucosamine-6-Phosphate Deaminase 2
chr 1	45654313	26.1	0.00042	TTS	428	*LOC106603840*	unchar	ncRNA
chr 13	22453549	26.2	0.00637	intron (8 of 20)	17015	*LOC106566915*	unchar	ncRNA
chr 14	37399454	26.2	0.00003	Intergenic	-16407	*erich2*	ERICH2	Glutamate Rich 2
chr 12	75311649	26.2	0.00000	intron (1 of 6)	-16447	*LOC106565963*	unchar	protein coding
chr 9	10609543	26.3	0.00129	Intergenic	-2873	*cmpk2*	CMPK2	Cytidine/Uridine Monophosphate Kinase 2
chr 17	53018323	26.4	0.00351	exon (1 of 8)	269	*LOC106576554*	TMTC2	Transmembrane O-Mannosyltransferase Targeting Cadherins
chr 14	33662191	26.4	0.00391	promoter-TSS	-47	*LOC106569404*	unchar	ncRNA
chr 3	57371025	26.5	0.00374	exon (1 of 2)	619	*hoxb3aa*	HOXB3	Homeobox B3
chr 21	34784057	26.8	0.00135	exon (7 of 10)	4253	*LOC106582237*	GBP1	Guanylate Binding Protein 1
chr 5	34639904	27.0	0.00450	exon (13 of 16)	3116	*prp19*	PRPF19	Pre-MRNA Processing Factor 19
chr 4	77962158	27.1	0.00484	promoter-TSS	46	*LOC106603979*	CORO6	Coronin 6
chr 3	57371054	27.2	0.00059	exon (1 of 2)	590	*hoxb3aa*	HOXB3	Homeobox B3
chr 24	41027782	27.2	0.00637	Intergenic	-6988	*LOC106585883*	MYH7	Myosin Heavy Chain 7
chr 17	10682755	27.3	0.00801	Intergenic	9538	*LOC106575277*	SLITRK1	SLIT And NTRK Like Family Member 1
chr 21	57543223	27.7	0.00015	intron (7 of 15)	-3630	*LOC106582585*	unchar	ncRNA
chr 1	50710411	28.1	0.00463	intron (1 of 1)	-28832	*LOC106605336*	unchar	ncRNA
chr 17	39726570	28.1	0.00517	Intergenic	-9452	*LOC106576147*	ATP2B1	ATPase Plasma Membrane Ca2+ Transporting 1
chr 3	57371182	28.1	0.00830	exon (1 of 2)	462	*hoxb3aa*	HOXB3	Homeobox B3
chr 18	9841155	28.3	0.00076	intron (17 of 17)	17820	*cssa18h2orf61*	STPG4	Sperm-Tail PG-Rich Repeat Containing 4
chr 21	14520316	28.3	0.00070	intron (4 of 25)	89275	*tanc1*	TANC1	TPR Domain, Ankyrin-Repeat And Coiled-Coil-Containing
chr 10	10262966	28.3	0.00221	Intergenic	17361	*LOC106613513*	HES1	Hes Family BHLH Transcription Factor 1
chr 5	65673261	28.4	0.00821	Intergenic	-5533	*fbrl*	FBL	Fibrillarin
chr 12	58023004	28.4	0.00052	intron (1 of 17)	6369	*LOC106565545*	PRKCD	apoptosis and inflamatory proccess

chr 3	84967070	28.4	0.00184	Intergenic	-36224	*LOC106601888*	SRL	Sarcalumenin
chr 3	57371096	28.5	0.00014	exon (1 of 2)	548	*hoxb3aa*	HOXB3	hoxb3aa
chr 20	35565839	28.7	0.00426	promoter-TSS	-320	*LOC106580525*	UNC5D	Unc-5 Netrin Receptor D
chr 12	42085136	28.7	0.00155	intron (1 of 2)	-6372	*LOC106565215*	LPIN1	lipin 1
chr 10	96008258	29.1	0.00059	intron (5 of 13)	58014	*LOC106561393*	TBXAS1	Thromboxane A Synthase 1
chr 19	52565631	29.2	0.00662	Intergenic	24581	*LOC106579252*	KNCJ12A	fish specific potasium channel
chr 6	75805250	29.3	0.00154	Intergenic	37806	*LOC106608164*	ZFP36L1	ZFP36 Ring Finger Protein Like 1
chr 22	52890826	29.5	0.00004	Intergenic	-4877	*asic1*	ASIC1	Acid Sensing Ion Channel Subunit 1
chr 4	77962201	29.5	0.00023	promoter-TSS	3	*LOC106603979*	CORO6	Coronin 6
chr 9	10561272	29.6	0.00000	Intergenic	45398	*cmpk2*	CMPK2	Cytidine/Uridine Monophosphate Kinase 2
chr 20	43829770	29.7	0.00002	Intergenic	-9591	*LOC106580685*	C19ORF47	Chromosome 19 Open Reading Frame 47
chr 25	9713748	29.8	0.00439	Intergenic	-13333	*itb2*	ITGB2	Integrin Subunit Beta 2
chr 14	63222803	30.0	0.00002	Intergenic	-17828	*LOC106570181*	unchar	protein coding
chr 4	28585072	30.3	0.00328	intron (2 of 11)	1168	*pdzd3*	PDZD3	PDZ Domain Containing 3
chr 7	57387036	30.3	0.00000	Intergenic	12510	*LOC106609820*	TMEM60	Transmembrane Protein 60
chr 6	76582912	30.5	0.00000	exon (1 of 3)	304	*rdh14*	RDH14	Retinol Dehydrogenase 14
chr 12	54014328	30.9	0.00783	promoter-TSS	-884	*LOC106565459*	HNRNPA1	Heterogeneous Nuclear Ribonucleoprotein A1
chr 9	4098912	30.9	0.00001	Intergenic	39099	*LOC106610637*	unchar	ncRNA
chr 3	57371034	31.0	0.00008	exon (1 of 2)	610	*hoxb3aa*	HOXB3	Homeobox B3
chr 6	73537376	31.4	0.00062	Intergenic	-86785	*LOC106608111*	FLRT2	Fibronectin Leucine Rich Transmembrane Protein 2
chr 4	58679291	31.5	0.00015	intron (4 of 20)	93870	*LOC106603676*	IGSF9B	Immunoglobulin Superfamily Member 9B
chr 4	19738629	31.6	0.00070	Intergenic	-1596	*LOC106602692*	LONRF1	LON Peptidase N-Terminal Domain And Ring Finger 1
chr 3	57371234	32.3	0.00006	exon (1 of 2)	410	*hoxb3aa*	HOXB3	Homeobox B3
chr 16	78554422	32.6	0.00012	Intergenic	-18730	*LOC106574803*	TRAK2	Trafficking Kinesin Protein 2
chr 24	12007375	32.8	0.00052	intron (1 of 6)	21374	*LOC106585085*	unchar	protein coding
chr 13	73842954	32.8	0.00249	TTS	11277	*LOC106567964*	RPS6KB1	Ribosomal Protein S6 Kinase B1
chr 18	13167276	33.1	0.00170	Intergenic	-5255	*LOC106576686*	HELLS	lymphocite specific non heart
chr 14	5591440	33.1	0.00270	Intergenic	122256	*LOC106568678*	TMX3	Thioredoxin Related Transmembrane Protein 3
chr 3	57371075	33.2	0.00000	exon (1 of 2)	569	*hoxb3aa*	HOXB3	Homeobox B3
chr 23	20904938	33.4	0.00050	Intergenic	-5921	*LOC106584107*	DCUN1D1	Defective In Cullin Neddylation 1 Domain Containing 1

chr 3	57371045	33.7	0.00000	exon (1 of 2)	599	*hoxb3aa*	HOXB3	Homeobox B3
chr 5	61623828	33.7	0.00020	Intergenic	19436	*LOC106605486*	MROH1	Maestro Heat Like Repeat Family Member 1
chr 4	79367426	33.7	0.00000	Intergenic	-5329	*LOC106604013*	OLR1361	Olfactory receptor 1361
chr 3	57371073	34.3	0.00000	exon (1 of 2)	571	*hoxb3aa*	HOXB3	Homeobox B3
chr 6	2001209	34.4	0.00018	Intergenic	4720	*LOC106606255*	PIGQ	Phosphatidylinositol Glycan Anchor Biosynthesis Class Q
chr 11	3091094	34.7	0.00126	exon (6 of 26)	45210	*daam2*	DAAM2	Dishevelled Associated Activator Of Morphogenesis 2
chr 3	67370154	35.2	0.00020	Intergenic	17256	*LOC106601288*	RAVER1	Ribonucleoprotein, PTB Binding 1
chr 3	80155278	35.8	0.00190	intron (2 of 3)	4840	*LOC106601757*	SAMD9	Sterile Alpha Motif Domain Containing 9
chr 16	12223363	36.2	0.00281	intron (1 of 9)	20010	*LOC106573212*	GALNT18	Polypeptide N-Acetylgalactosaminyltransferase 18
chr 12	28957113	36.5	0.00007	intron (10 of 16)	9408	*LOC106564799*	PEX6	peroxisome activity specific
chr 17	4486060	37.5	0.00004	Intergenic	-2753	*spp2*	SPP2	Secreted Phosphoprotein 2
chr 15	23255432	37.8	0.00000	exon (25 of 29)	13950	*LOC100136579*	APOBR	Macrophage relevant expressed in heart
chr 13	73842949	38.0	0.00012	TTS	11272	*LOC106567964*	RPS6KB2	Ribosomal Protein S6 Kinase B2
chr 12	40448976	38.4	0.00005	intron (1 of 2)	13214	*LOC106565240*	CHST11	Carbohydrate Sulfotransferase 11
chr 25	7720228	41.9	0.00000	Intergenic	-1967	*LOC106586112*	ARPC3	Actin Related Protein 2/3 Complex Subunit 3
chr 21	12911078	42.3	0.00000	intron (3 of 3)	13506	*LOC106581835*	WNT10A	Wnt Family Member 10A
chr 10	99677626	42.3	0.00000	Intergenic	-10999	*LOC106561572*		Stonustoxin subunit beta [Salmo salar (Atlantic salmon)]
chr 9	99739654	43.5	0.00000	Intergenic	40517	*clptm1*	CLPTM1	Regulator Of GABA Type A Receptor Forward Trafficking
chr 1	28959324	47.9	0.00000	Intergenic	10097	*gnpi*	GNPI1	Glucosamine-6-Phosphate Deaminase 1
chr 6	9427656	48.3	0.00000	intron (3 of 15)	11764	*usp36*	USP36	Ubiquitin Specific Peptidase 36
chr 6	44751660	54.7	0.00000	exon (3 of 3)	5310	*LOC106607551*	MYCN	Glucosamine-6-Phosphate Deaminase 1
chr 17	57307590	55.7	0.00000	Intergenic	-15949	*LOC106576667*	CACNA1C	Calcium Voltage-Gated Channel Subunit Alpha1 C
chr 6	69309332	55.8	0.00000	Intergenic	-29938	*LOC106608057*	GZF1	GDNF Inducible Zinc Finger Protein 1

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