

Molecular regulators of smoltification and viral infection management tools for salmon aquaculture

Thesis submitted for the degree of Doctor of Philosophy

**UNIVERSITY of
STIRLING**



By

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Declaration

The work presented in this thesis is all my own work, unless otherwise stated, and has not been submitted for any other degree except some of the work in Chapter 3, which builds upon work performed in my MSc thesis. All work was conducted at the Institute of Aquaculture, University of Stirling, Stirling unless otherwise stated.

All literature, quotations marks and sources of information used have been acknowledged.

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Abstract

Accurate smoltification and disease management in Atlantic salmon (*Salmo salar*) are key issues for the aquaculture industry. Due to their anadromous lifecycle the transfer of salmon from fresh water (FW) to seawater (SW) is crucial to their survival; too early can cause mortality, too late can cause desmoltification and long-term health problems. Both scenarios can increase susceptibility to four viral diseases: Salmon alphavirus (SAV), Infectious salmon anemia virus (ISAV), orthoreovirus (PRV), and Piscine myocarditis virus (PMCV). They all show similar clinical and histopathological symptoms and can easily spread throughout farms. Understanding the initial innate immune response to these viruses may provide biomarkers that could help identify and monitor infections.

An in house and onsite Na^+/K^+ ATPase (NKA) qRT-PCR assay was developed for the salmon biomarker ATPase to test smoltification readiness in salmon smolts. Tested against NKA enzymatic assays it showed a similar success rate over 3 years: NKA qRT-PCR (57%), NKA activity assay (60%). Onsite tests confirmed that the ATPase mRNA transcript is a useful biomarker for smoltification detection.

An in-lab and mobile multiplex qRT-PCR assay was developed for detection of SAV, PRV and PMCV. The analytical sensitivity of the SAV (86.5% SE 0.11), PRV (90.94%, SE 0.09) and PMCV (100.46%, SE 0.19) assays was 10^2 copies for PMCV and 10^3 for SAV and PRV. Initial results suggest individual assays could be run on site at farms. Addition of an internal control, probit analysis and viral positive tests are still required for multiplex assay integration.

Salmon erythrocytes were infected with ISAV, SAV and Poly I:C to investigate whether they induce and up-regulate innate immune response genes. All genes were expressed at low levels in all parameters investigated including non-infected control erythrocytes. These findings suggest erythrocytes act as an initial buffer to viral infections and may help stimulate the innate immune response.

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Abbreviations

AI – Immune adherence

AS – Atlantic salmon cells

AMPS – Antimicrobial peptides

ATP – Adenosine 5'triphosphate disodium triphosphate

BCP – 1-Bromo-3-chloropropane

BF2 – Bluegill fry cells

°C – Degree Celsius

CARD – caspase recruitment domain

CC – Chloride cells

cDNA – complimentary deoxyribose nucleic acid

CFTR – Cystic fibrosis transmembrane conductance regulator

CHSE-214 cells – Chinook salmon embryo-214

CLRs – C-type lectin receptors

cm – centimetre

CMS – Cardio myopathy syndrome

CO₂ – Carbon dioxide

CPE – Cytopathic effect

CS – Chicken serum

CSV – Chum salmon reovirus

Ct – Cycle threshold

CTD – C-terminal repressor domain

dd – Degree days

DEPC-treated water – 0.1% diethylpyrocarbonate treated water

DMEM – Dulbecco's Modified Eagle Medium

DNA – Deoxyribose nucleic acid

dNTP – Deoxynucleotide

ds – double stranded

dsRNA – double stranded RNA

dH₂O – distilled water

Ef1a – Elongation factor 1 alpha 1

eIF-2 α – Eukaryotic initiation factor 2

EMEM – Eagles minimum essential media

ELISA – enzyme linked immunosorbant assay

EPC – epithelioma papulosorum cyprinid cells

FHM – Fat head minnow cells

FCS – Fetal bovine serum

g – Grams

g – Gravitational force

GBP – Guanylate binding protein

GH – Growth hormone

GH-R – Growth hormone receptor

H₂O – Water

HBSS – Hanks balanced salt solution

HE – Haemagglutinin-esterase

hr – Hour

HSC – Hematopoietic stem cells

HSMI – Heart and skeletal muscle inflammation

IFIT5 – Interferon-induced protein 5

IFNa – Interferon alpha 1

IFNb – Interferon beta 1a

IFNc – Interferon alpha c

IFN- γ – Interferon gamma

IFN γ rel-2 – Interferon gamma related 2

IGF-1 – Insulin-like growth factor 1

IHNV – Infectious hematopoietic necrosis virus

IKK α – I κ B kinase α

IL-1 β – Interleukin 1 beta

IP – intraperitoneal injection

IP-10 – Interferon gamma-induced protein 10

IRAK1 – interleukin-1 receptor-associated kinase 1

ISA – Infectious salmon anemia

ISAV – Infectious salmon anemia virus

ISGs – Interferon stimulated genes

ISG15 – Interferon-stimulated gene 15

ISRE – IFN stimulated response element

JAK-STAT – Janus kinase – signal transduction and activator of transcription signalling pathway

kb – Kilobase

kg – Kilogram

l – Litre

LDH – L-lactate dehydrogenase

LGP2 – Laboratory of genetic and physiology 2

LPS – Lipopolysaccharides

LRR – Leucine rich repeats

MAVs – Mitochondrial antiviral signalling protein

MDA5 – Melanoma differentiation-associated gene 5

mg – Milligram

MgCl₂ – Magnesium chloride

MHC – major histocompatibility complexes

ml – Millilitre

mm – Millimetre

mM – Millimolar

MRC – Mitochondrion rich cells

mRNA – messenger ribonucleic acid

Mx – Interferon-induced GTP-binding protein Mx1

MyD88 – Myeloid differentiation primary response 88

NAD⁺/NADH – Nicotinamide adenine dinucleotide

NCCs – Non-specific cytotoxic cells

NF – κβ activating kinase – nuclear factor kappa-light-chain-enhancer of activated B cells

NK – Natural killer cells

NKA – Na⁺/K⁺ ATPase

NKCC – Na⁺/K⁺2Cl⁻ co-transporter

Nkl – Antimicrobial peptide Nk-lysin

NOAA – National Oceanic and Atmosphere Administration

ND-40/INGEPAL CA-630 – octylphenoxyethoxyethanol

Nkl – Nk-lysin

O₂ – Oxygen

OISST – Optimum Interpolation Sea Surface Temperature

ORF – Open reading framework

PAMPS – Pathogen associated molecular pattern

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PD – pancreas disease

PEP – Phosphoenolpyruvate monopotassium salt

pH - $-\log_{10}$ (hydrogen ion concentration)

PK – Pyruvate kinase

PKR – Protein kinase R

PMCV – Piscine myocarditis virus

PNV – Piscine nodavirus

Poly I:C – Polyinosinic:polycytidylic acid

PRV – Piscine reovirus

PRR – Pattern recognition receptors

RT-PCR – Reverse transcription polymerase chain reaction

qRT-PCR – Quantitative real time polymerase chain reaction

RdRP – RNA-dependant RNA polymerase sequence

RIG-I – retinoic-acid-inducible gene I

RFLP – Restriction fragment length polymerisation analysis

RLRs – Retinoic acid – inducible gene I – RIG I

ROS – Reactive oxygen species

RNA – Ribonucleic acid

RT – Reverse transcriptase

RTG-2 – Rainbow trout gonad cells

SAV – Salmon alpha virus

SD – Sleeping disease

SE – Standard error

SEID – Sucrose ethylenediaminetetraacetic (EDTA) imidazole deoxycholate

SHK-1 – Salmon head kidney cell line

SOCs – Suppressor of cytokine signalling

ssRNA – Single stranded RNA

StD – Standard deviation

TCID₅₀ – Tissue culture infective dose TIR – Toll/IL-IR

TLRs – Toll like receptors

TNF- α – Tumour necrosis factor- α

TRAF3 – TNF receptor-associated factor 3

TRIF – TIR domain-containing adaptor inducing interferon- β

TO – Cell line derived from Atlantic salmon head kidney leucocytes

TRIS-HCl – Tris hydroxymethyl amino methane hydrochloride

VHSV – Viral hemorrhagic septicaemia virus

VIG-1 – VHSV-induced gene

101 – Beta defensin 1a

Chapter 1: General Introduction

1.1 General background

The Atlantic salmon (*Salmo salar*) is a key species in the aquaculture industry. Currently it is the highest valued (US \$) farmed finfish species globally, accounting for 16.5% of the total value of fish products traded in 2015, with over 2.3 million tonnes produced (FAO 2015). Atlantic salmon are produced in the UK, Ireland, Norway, Faroe Islands, USA, Canada, Chile and Australia. Although in high demand, the production of Atlantic salmon has been hindered due to difficulties in husbandry management of large density populations. This is mainly due to the demands of physiological adaptations (smoltification), environmental (temperature, light and salinity) and biotic factors (bacteria, virus and parasites) that influence health and susceptibility to disease throughout its rearing time (Murray and Edmund. 2005; Krkosek. 2010).

Smoltification is a key biological process that all salmon parr must undertake before transitioning from fresh water (FW) to seawater (SW). The endocrine system drives morphological, physiological, behavioural and biochemical changes preparing the fish for SW (Hoar 1976; Folmar and Dickhoff. 1980; Barron 1986; McCormick 1987; Dickhoff 1997; McCormick 2001; Ebbesson et al 2008; McCormick. 2009a; Stefansson 2012). The timing of transfer from FW tanks to open water SW pens in aquaculture is of particular importance. Transferring too early will lead to poor performance, mortality and increased likelihood of disease outbreaks. Transferring too late can lead to desmoltification. This is a process where the fish begin to regress back to a FW adapted state, impacting fish health, survival and eventual successfully transfer to SW. The most common methods used to follow development of smoltification are the smolt index, SW bath tests, plasma chloride measurements and Na^+/K^+ -ATPase (NKA) enzymatic activity assays (Zaugg 1982). Although these techniques are generally accepted as reliable indicators of smoltification they have their drawbacks (see section 2.1). Following the success of NKA assays, molecular tests quantifying one of more mRNA isoforms of NKA with current quantitative real time polymerase chain reaction (qRT-PCR) techniques to detect smoltification would allow for rapid screening and onsite tests to be applied. This impacts husbandry and health management by reducing time and increasing reliability and reproducibility of smoltification biomarkers to ensure successful transfer of smolts leading to optimised performance.

Disease outbreaks can be devastating for farms leading to significant loss if not complete collapse of a population. There are multiple viral infections that can afflict cultured salmon during their lifetime. Salmon alpha virus (SAV) is the causative agent of pancreas disease (PD) in the UK (Fringuelli et al., 2008; Graham et al., 2012; Hjortaas et al., 2013) and Norway (Hodneland et al., 2005) as well as sleeping disease (SD) in Europe in rainbow trout (*Oncorhynchus mykiss*) (Castric et al., 1997; Graham et al., 2003, 2007). Piscine reovirus (PRV) is an opportunistic virus, causing heart and skeletal muscle myopathy (HSMI) (Palacios et al., 2010; Lovoll et al., 2010; Haugland et al., 2011; Finstad et al., 2012; Garseth et al., 2013; Wessel et al., 2017). Piscine myocarditis virus (PMCV) is the causative agent of cardiomyopathy syndrome (CMS) (Haugland et al., 2011). Infectious salmon anemia virus (ISAV) is the causative agent of infectious salmon anemia (ISA) (Kibenge et al., 2004) across Europe (Roger et al., 1998; Nylund et al., 2003; Plarre et al., 2005), Canada (Mullins et al., 1998), USA (Bouchard et al., 2001) and Chile (Godoy et al., 2008). The morphological and histopathological similarities between the four viruses makes it difficult to ascertain which virus or combination of the viruses the fish are infected with. Development of singleplex and multiplex qRT-PCR assays for diagnostic detection of these viruses could ensure a fast and clear indication of which virus is present and at what concentration. This would then allow for the best advice on the next steps needed to reduce or prevent an outbreak, improving overall health management.

All Ectothermic vertebrate erythrocytes vary from mammalian and avian counterparts in that they are nucleated and thus contain organelles with the potential to actively produce responses to their environment. Immune based responses of erythrocytes to pathogenic infections were originally suggested by Morera and MacKenzie. (2011), and have since been reported in various fish species (Workenhe et al., 2007, 2008; Morera et al., 2011; Dahle et al., 2015; Wessel et al., 2015; Pereiro et al., 2017). Further evidence has shown that ISAV and PRV can infect salmon erythrocytes (Workenhe et al., 2007; Finstad et al., 2014) and replicate within them (Workenhe et al., 2008; Wessel et al., 2015). Insight into the mechanisms that trigger these responses in erythrocytes would allow us to better understand their role in the immune response.

1.2 Life cycle of Atlantic salmon

Wild Atlantic salmon are anadromous salmonidae that can spend the first 1 – 2 years of their life developing in FW after hatching in rivers. Once reaching a specific weight (60 – 120g) and body size (10 – 15cm) (McCormick et al., 1987) the process of smoltification then occurs in the subsequent spring. Salmon will normally reach this critical weight within the first year and begin smoltification. Smoltification is the physiological and morphological process in which salmon parr adapt their biology from FW to SW before migrating to sea. The period spent at sea varies dependant on growth but can vary from 1 – 4 years with an average of around 1 – 2 years (Klemesten et al., 2003). When at an adequate size the salmon then return to their natal rivers to spawn (Hoar 1976; Folmar 1980) (Figure 1.1A). This natural lifecycle has been adapted for farmed salmon. Broodstocks are selected and eggs stripped and incubated until hatched. The fry are then grown in tanks often under light and temperature manipulation until developing into salmon parr, where smoltification starts either naturally or is artificially induced (8 – 16 months). The smolts are then transferred to sea cages where the final growth stage is carried out for up to 2 years until a suitable harvest weight is achieved (2 kg+) (Figure 1.1B).

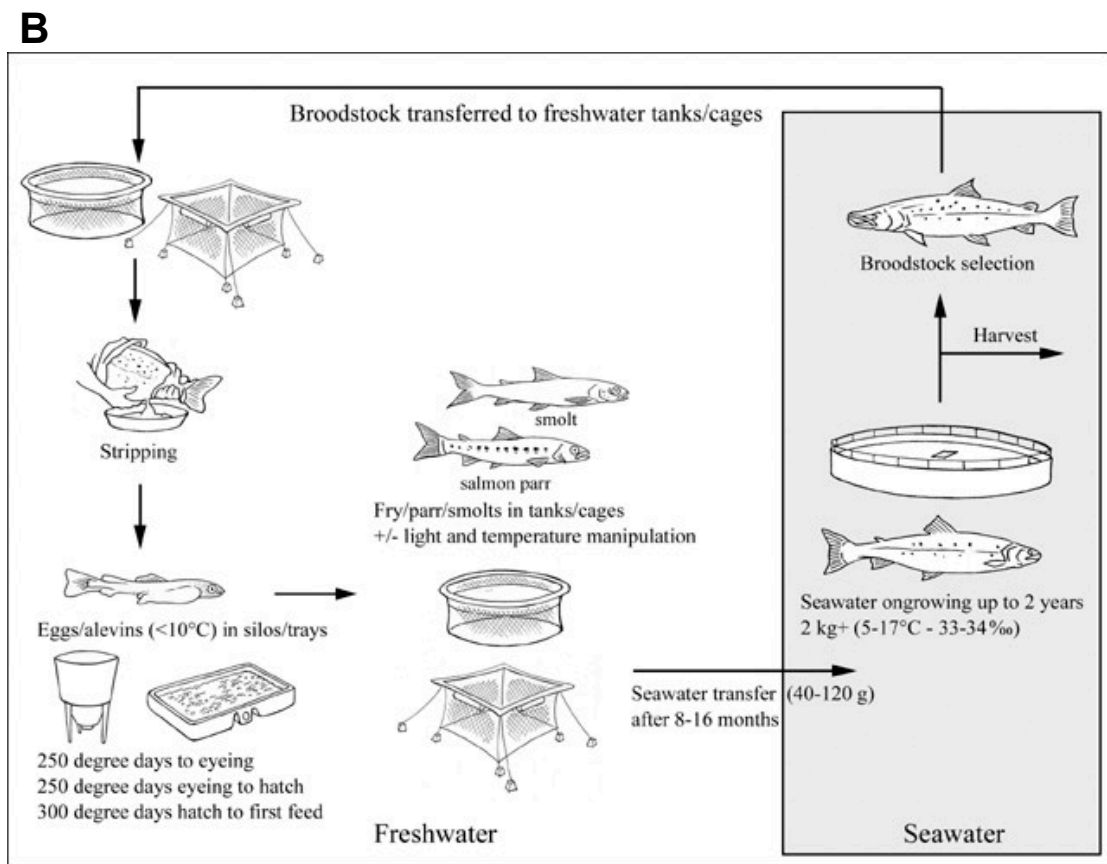
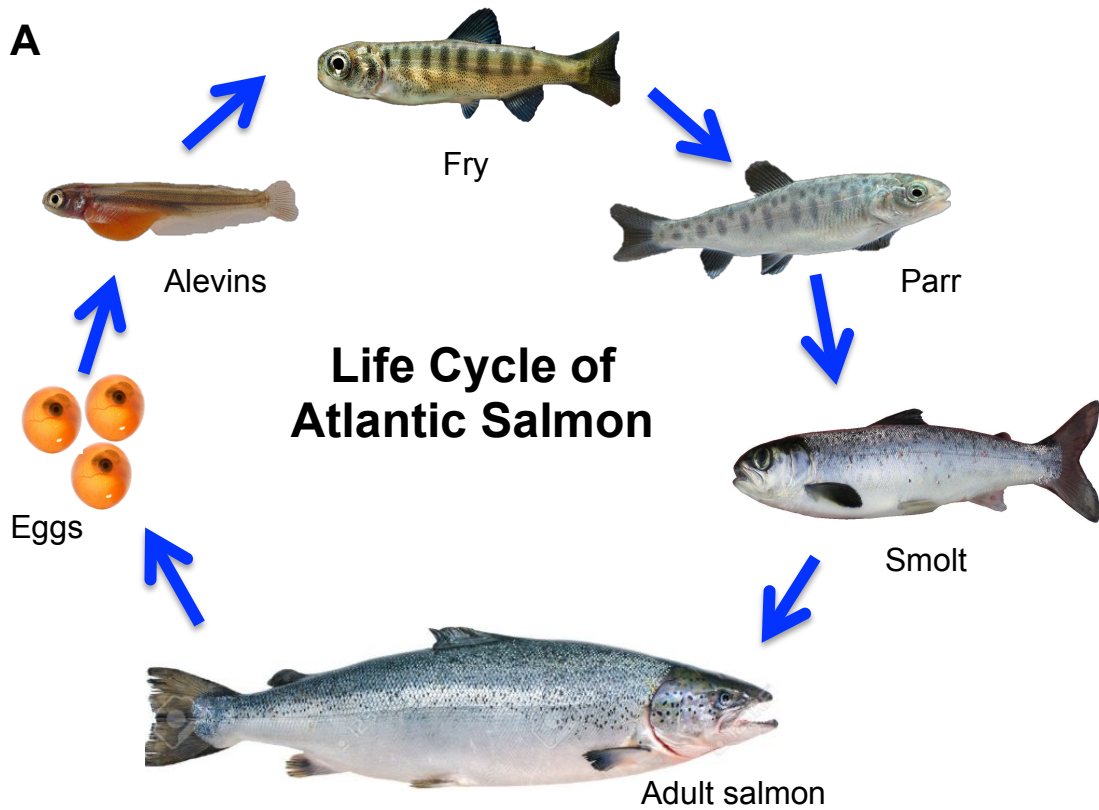


Figure 1.1 A. The lifecycle of Atlantic salmon (*Salmo salar*). B. The production cycle of Atlantic salmon from broodstock to harvest. Source: www.fao.org.

1.3 Smoltification

1.3.1 Osmoregulation

Atlantic salmon have adapted their anadromous strategy to utilise FW areas for reproduction and the oceans for a rich food supply to promote growth, before returning to natal rivers to produce offspring (Hoar 1976; Folmar 1980). There are many physiological changes and adaptations that juvenile salmon must undertake to cope with the high salinity differences between FW and SW, and for adults' returning to their spawning grounds from SW to FW. Arguably the most critical is the development of its osmoregulatory ability.

Plasma osmotic balance must be maintained in teleost fish at all times to ensure survival in its environment (Edwards and Marshall, 2013). In FW, the balance is maintained through the active uptake of ions (Na^+ , Cl^- , Ca^{2+}) through the gills by the use of ionocytes (formerly known as chloride cells (CC) or mitochondrion-rich cells (MRC)) and secretion of dilute urine to counter the loss of ions through the passive gains of water. Uptake of salts from food can also be absorbed through the intestine. In SW, fish must actively drink SW to absorb water and monovalent ions across the gut, whilst ionocytes in the gills actively sequester ions (Na^+ and Cl^-) from the blood and pump them back into SW. Divalent ions are excreted through the gut and kidney. An osmotic pressure around one third of SW must always be maintained within teleost fish regardless of whether they reside in FW or SW (Evans et al., 2005).

1.3.2. Gills/Ionocytes

Ionocytes are oval shaped cells that are generally located in the secondary lamella and opercular epithelium of teleost gills. They contain numerous mitochondria and have a network of tubular systems formed in the basolateral membrane forming an apical crypt, providing a large surface area for ion exchange (Figure 1.2) (McCormick 2001). They were formerly known as CCs when initially described by Keys and Willmer. (1932) in the gills of the eel *Anguilla vulgaris*. They suggested that the cells had some form of active chloride activity within the gills. This was later confirmed by Foskett and Scheffy (1982) in the Mozambique tilapia (*Sarotherodon mossambicus*) where they demonstrated ionocytes to be the salt (NaCl) secretory cell. These findings were further described and confirmed in other teleosts (Foskett et al., 1983; Zadunaisky 1984; Karnaky 1986). Further examination of the cells uncovered other functions such as acid-base regulation (Evans et al., 2005; Evans 2008; Perry and Gilmour. 2006; Gilmour and Perry. 2009; Hwang and Perry. 2010)

and ammonium secretion (Wilkie 2002; Evans 2005; Weihrauch et al., 2009). Although crucial for homeostasis, we will focus on the ionocytes role in ion regulation in Atlantic salmon.

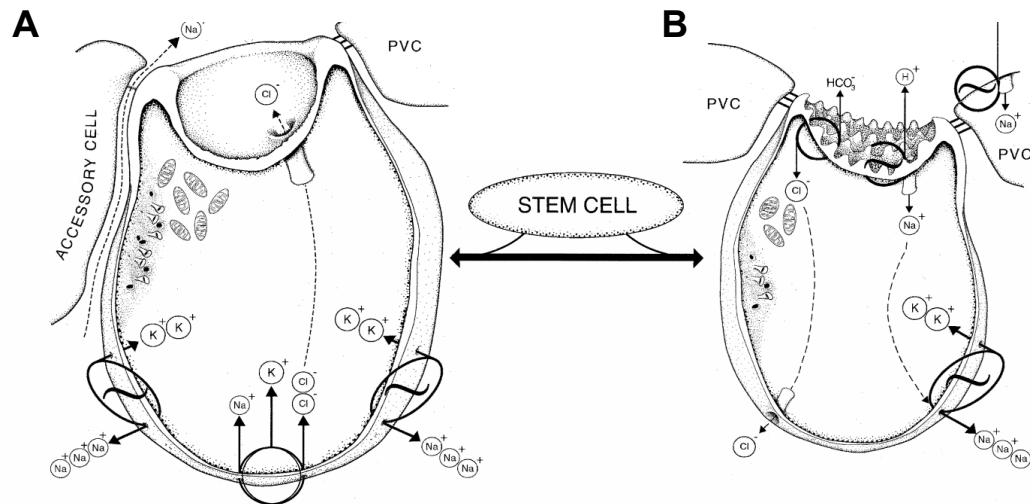


Figure 1.2 Structural and morphological differences between the saltwater (SW) adapted ionocyte (A) and the fresh water (FW) adapted ionocyte (B). SW adapted ionocytes (A) are larger and contain a distinct apical crypt in their structure whereas FW adapted ionocytes (B) are generally smaller and have a broad apical surface. Adapted from McCormick. (2001).

Initial research into Atlantic salmon ionocytes cells focused on the three key life stages – parr, FW smolt and SW smolts. A change in structure and increase in ionocyte cell size and abundance were observed in juvenile and FW smolt Atlantic salmon whilst still in FW (Langdon & Thorpe. 1984; Langdon 1985; Pisam et al., 1988). No change was evident in parr however, giving evidence towards adaption of ionocytes in FW in preparation for a SW environment. The role of NKA was suggested to be a major contributor towards the adaption of the gill epithelium from ion absorbing to secretion in both these papers and in previous research (Zaugg and McLain. 1970; Saunders and Henderson. 1978). Based on this evidence it was proposed by McCormick and Saunders (1987) that increases in NKA in the gills of salmon was directly responsible for the development of smolt ionocytes from FW adaption to SW adaption. This hypothesis was proven correct when NKA concentrations were shown to consistently increase in gill ionocytes during smolt SW acclimatisation (Prunet et al., 1989; McCormick 1995,1996, 2000, 2001; Bystriansky and Schulte. 2011; Handeland 2013). Further analysis linked growth hormone (GH) along with insulin-like growth factor 1 (IGF-1) and cortisol to regulatory roles in osmoregulation in salmon ionocytes (see section 1.3.4). Subsequently two other major ion transport proteins were shown to be involved in

assisting with the secretion of sodium and chloride ions, $\text{Na}^+/\text{K}^+2\text{Cl}^-$ co-transporter 1 (NKCC1) (Pelis et al., 2001) and a homolog of cystic fibrosis transmembrane conductance regulator (CFTR) (Marshall and Singer 2002). In SW the system involves basolateral NKA pumping 3 Na^+ ions out of the cell whilst 2 K^+ ions are pumped in. The subsequent negatively charged cell creates an electrochemical gradient that is utilised by NKCC to uptake Cl^- into the cell. Chloride then leaves the cell through an apical chloride channels known as CFTR based on the electrical gradients. Sodium is excreted out the epithelium by the paracellular pathway (Evans et al., 2005).

1.3.3 Key transporter proteins

1.3.3.1 $\text{Na}^+/\text{K}^+ \text{-ATPase}$ (NKA) Sodium-Potassium Pump

NKA, known as the sodium-potassium pump, is the enzyme responsible for active transport of sodium out of the cell and potassium into the cell. NKA is found in all animal cells and contains protein sub units α and β . The α subunits are the catalytic drivers and transporters containing the binding sites for ATP, Na^+ , K^+ and its inhibitor ouabain (Lingrel and Kuntzweiler, 1994). The β -subunits are glycoproteins that stimulate the structure and folding of the protein in the basolateral membrane. Both subunits work together to drive the electrochemical gradient required for ion uptake, secretion and overall osmotic balance within the cell. A third subunit γ (also referred to as FXD) was identified however its direct influence on the NKA process is still not entirely understood. It is currently believed to adapt the kinetic function of other cells in terms of Na^+ and K^+ transport (Garty and Karlish. 2006; Geering 2006). Recent studies however have shown that it may contribute to the pumps kinetic properties and assist in transmission of external signals that regulate NKA (Tipsmark et al., 2010). The number of α and β subunits varies between species, however it is generally accepted that four α (1 – 4) and three β (1 – 3) are present in mammals (Takeyasu et al 1990; Pressley. 1992) and four α (1 – 4) and three β (1 – 3) in vertebrates (Blanco and Mercer. 1998). When examined in fish however, up to 9 α subunits and 5 β subunits were found in zebra fish (Rajaro et al., 2001; Liao et al., 2009) and up to 5 α subtypes and 4 β subunits in rainbow trout (Richards et al., 2003; Gharbi et al., 2004).

The functional significance of the isoforms were poorly understood in fish. Research into seawater exposure in Atlantic salmon by Singer *et al.* (2002) observed a significant increase in mRNA levels of the α subunit over a 2-week period. A following study on rainbow trout during salinity transfer by Richards *et al.* (2003)

identified 5 isoforms of α (α 1a, α 1b, α 1c α 2a and α 3), but more significantly showed that the isoform α 1a was down regulated and α 1b up regulated when transferred to high salinity SW. Two other isoforms, α 1c and α 3, were expressed but showed no variation in expression when transferred. This led to the suggestion that α 1a and α 1b could play a significant role in FW and SW acclimatisation. This hypothesis was verified in several studies in both Atlantic salmon (Mackie et al., 2005; Bystriansky et al., 2006; Nilsen et al., 2007; Madsen et al. 2009; McCormick et al., 2009b; Bystriansky et al., 2011; Stefansson et al., 2012; McCormick et al., 2013a) and other salmonid species (Shrimpton et al., 2005; Bystriansky et al., 2007a,b; Larsen et al., 2008). It was found that α 1a was consistently expressed in high concentrations and α 1b in low concentrations in FW, but within 24 hours of exposure to SW the expression of both isoforms reversed. McCormick *et al.*, (2009b) and Madsen *et al.*, (2009) showed that α 1a was the FW isoform and α 1b the SW isoform, and that they are present in distinct ionocyte cells. High concentrations of α 1a are located in the filamental and lamellar ionocytes in FW with low concentrations of α 1b in filamental ionocytes. The opposite expression was found once salmon are transferred to SW. During smolt development, α 1a and α 1b mRNA levels were transcribed at different abundance (Nilsen et al., 2007), with α 1b gradually increasing as smolts developed salinity tolerance. A decrease of 98% was seen in α 1a after SW exposure (McCormick et al., 2013a). This suggests that α 1a ionocytes are transforming to α 1b ionocytes gradually during the smolt development process before rapid transformation occurs following SW transfer (Figure 1.3). This provided a large body of evidence that both isoforms play key roles in the osmoregulation, adaptation and acclimatisation of ionocytes in salmonids and other euryhaline teleosts. Their consistent and reliable expression in FW and SW would suggest they are potential biomarkers for smoltification detection in Atlantic salmon.

1.3.3.2 $\text{Na}^+/\text{K}^+2\text{Cl}^-$ co-transporter (NKCC)

$\text{Na}^+/\text{K}^+ 2\text{Cl}^-$ cotransporters (NKCC) are a group of proteins that are widely distributed throughout the cells and tissues of many animal species (Hass 1994; Hass and Forbush. 1998; Mount et al., 1998; Russel 2000). Cotransport of Na^+ , K^+ and Cl^- was first described by Wiley and Cooper (1974) where they showed net movement of Na^+ and K^+ ions were dependant on concentrations of the ions inside or outside of red blood cells. This was later followed by work by Geck *et al.* (1980) where they showed that movement of Na^+ , K^+ and Cl^- ions was based on electrical concentration gradients towards a neutral balance of ions within and outside ehrlich cells (tumour cells). Subsequent research discovered two distinct isoforms of NKCC;

NKCC1 cloned from elasmobranch rectal glands (Xu et al., 1994) and human kidney (Gamba et al., 1994) and NKCC-2 from human kidney (Gamba et al., 1994) and rabbit kidney (Payne and Forbush. 1994). NKCC1 was subsequently found in almost all other cell types and is regarded as the secretory isoform when found in the basolateral membranes of secretory epithelia. NKCC2 is exclusively found within the apical surface of the kidneys and is regarded as the absorptive isoform (Haas and Forbush. 2000; Russel 2000).

In teleost fish NKCC1 is one of the major ion transport proteins utilised for salt secretion and its crucial role in uptake of Cl^- in SW (Figure 1.3). It utilises low Na^+ concentrations to recruit Cl^- ions into ionocytes (see section 1.3.2). NKCC1 was found localised in the basolateral membranes of ionocytes in Atlantic salmon (Pelis et al., 2001), killfish (*Fundulus heteroclitus*) (Marshall et al., 2002) giant mudskipper (*Periophthalmodon schlosseri*) (Wilson et al., 2000) and other species (Cutler and Cramb. 2002; McCormick et al., 2003); suggesting that NKCC1 is ubiquitous in the ionocytes of euryhaline species. On examination of Atlantic salmon during smoltification, Pelis *et al.* (2001) showed that NKCC1 positive ionocytes increased during the smolting period alongside NKA activity, with 3x increase in NKCC1 from pre-smolts to smolts. They used the T4 monoclonal antibody developed by Lytle *et al.* (1995) to distinguish between both isoforms of NKCC. Similar findings were found in anadromous and landlocked salmon with a 5-fold increase in mRNA levels of NKCC1 (Nilsen et al., 2007). These findings suggest a strong correlation between NKCC1 and the adaption of Atlantic salmon to SW. Other studies on brown trout (*Salmo trutta*) (Tipsmark et al., 2002), southern flounder (*Paralichthys lethostigma*) (Tipsmark et al., 2008) green sturgeon (*Acipenser medirostris*) (Sardella and Kultz. 2009) and brackish medaka (*Oryzias dancena*) (Kang et al., 2010) showed a similar increase in NKCC1 during SW adaption. This further evidenced the link between NKCC1 concentration and SW adaption, and suggested a significant increase in NKCC1 in FW smolts and other teleost species may indicate SW readiness of the animal. This is further supported by other research on teleosts transferred from SW to FW, where a marked decrease in the expression of NKCC1 has been observed (Tipsmark et al., 2004; Lorin et al., 2006; Tipsmark et al., 2008).

1.3.3.3 Cystic fibrosis transmembrane conductance regulator (CFTR)

Cystic fibrosis transmembrane conductance regulator (CFTR) is another ion transport protein that acts as a Cl^- channel across epithelial cell membranes (Figure 1.3). It was first discovered when gene sequences for cystic fibrosis were conducted in humans (Rommens et al., 1989; Riordan et al., 1989; Kerem et al., 1989).

Subsequently, Bear *et al.* (1992) showed that a recombinant CFTR protein regulated concentration of Cl^- in the cells and suggested that CFTR was the Cl^- channel. The CFTR protein was found to be expressed in the airway submucosal glands of humans (Jacquot *et al.*, 1993). More specifically in the basal and apical membranes, secretory granules and cell cytoplasm. This led to research into CFTRs presence and function in other animals. Through electrophysiological measurements the CFTR anion channel was identified to be in the apical crypt membrane of ionocytes in killfish, and suggested as an active secretor of Cl^- in SW teleosts (Marshall *et al.*, 1995). Immunolocalisation studies later confirmed the location of CFTR in the apical crypt in killfish (Mickle *et al.*, 2000) and mudskippers (*Periophthalmus schlosseri*) (Wilson *et al.*, 2000). Additional work on killfish transferred from FW to SW revealed that CFTR was rapidly expressed and increased after 1 hr, and continued to increase up until 24 hrs (Singer *et al.*, 1998; Marshall *et al.*, 1999). It also increased in parallel with NaCl secretion in ionocytes. This suggested that CFTR had a significant role in SW adaptation, and due to its fast regulation it could be the initial protein expressed to buffer the immediate effects of SW before the rest of the osmoregulatory factors are initiated.

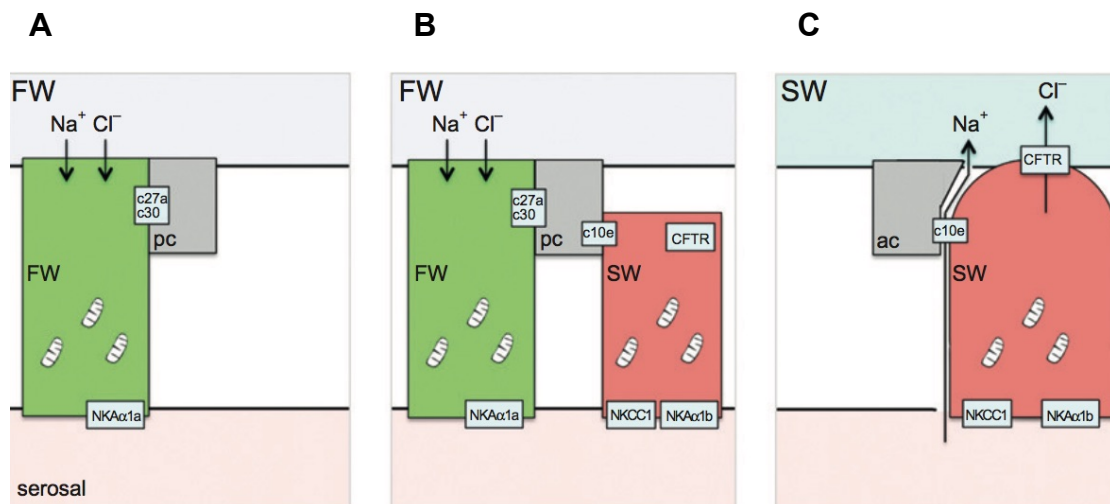


Figure 1.3. The changes in gill ionocytes from $\alpha1a$ dominant in fresh water (FW) to $\alpha1b$ dominate in seawater (SW). In FW parr (A) only $\alpha1a$ ionocytes are present. During smoltification (B) $\alpha1a$ ionocytes still dominate with development of $\alpha1b$ ionocytes beginning. The $\alpha1b$ are small and do not extend out above pavement cells in to the open environment and are therefore mainly dormant and inactive. (C) During SW transfer $\alpha1a$ ionocytes completely disappear and $\alpha1b$ ionocytes increase in size, breaking through the pavement cells, exposing them to the environment and becoming active. Upregulation of cystic fibrosis transmembrane conductance regulator (CFTR) and $\text{Na}^+/\text{K}^+ 2\text{Cl}^-$ cotransporters 1 (NKCC1) begin during smoltification and increase substantially after SW exposure. Adapted from McCormick (2013b).

Two isoforms of CFTR were isolated and cloned from Atlantic salmon (Chen et al., 2001) termed CFTR1 and CFTR2. Initial tests on Atlantic salmon FW to SW exposure indicated CFTR1 mRNA was significantly higher than basal levels over a 2-week period, whereas CFTR2 was only temporarily expressed at a high rate for the first 24 hrs (Singer et al., 2002). Further analysis supported this when Nilsen *et al.* (2007) monitored both isoforms in land locked and anadromous juvenile Atlantic salmon. They found CFTR1 mRNA levels continually increased over smolting months, however CFTR2 mRNA levels did not. This suggested CFTR1 is the dominant isoform associated with SW adaption, however they questioned the role of CFTR2 due to its stability in FW. Further research by Stefansson *et al.* (2012) contradicted those of Singer *et al.* (2002) with CFTR1 findings showing a significant reduction in mRNA levels of both isoforms of CFTR post SW transfer. Although some contrasting data was found in Atlantic salmon on the expression of each isoform pre and post SW transfer, the evidence gathered on the high numbers of CFTR found within the apical membrane of ionocytes in other euryhaline species during FW to SW transfer (Singer et al., 1998; Marshall et al., 1999; Marshall et al., 2002; McCormick et al., 2003; Tang and Lee. 2007) suggests CFTR plays an important roll in conjunction with NKA and NKCC in SW acclimatisation (McCormick et al., 2013b).

1.3.4 Endocrine control in smoltification

The endocrine system is a key component in regulating environmental stimulus and signalling the development and adaption of most organisms. It has the intrinsic ability to co-ordinate responses to all or specific parts of the body through interpretation of internal stimuli, such as body development and/or adaption in regards to external stimuli such as temperature, day length or salinity. These stimuli can result in one or likely more changes in physiology, morphology, biochemistry and behaviour that will have a major impact on the performance and survival of the animal (Figure 1.4).

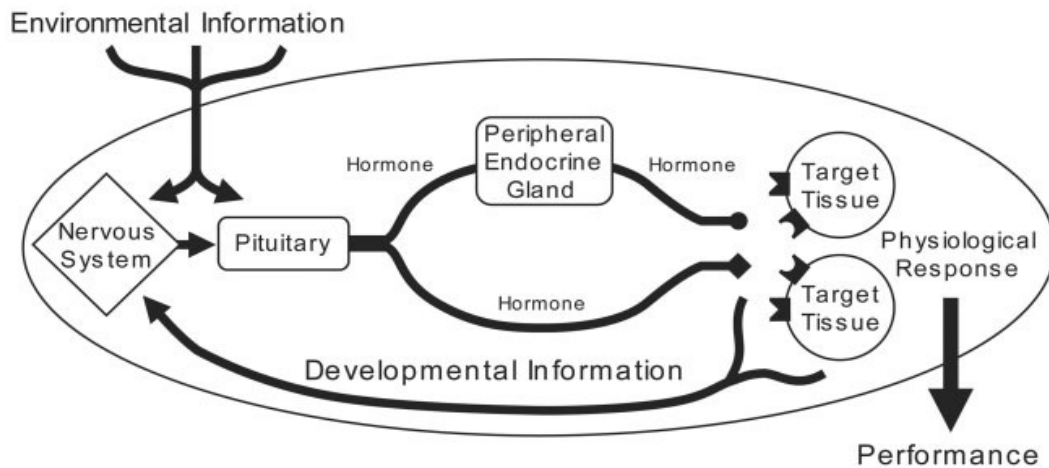


Figure 1.4. The process of the endocrine system in regards to external environmental stimuli and the subsequent internal system pathways that result in changes in behaviour, physiology and morphology in the animal. Adapted from McCormick. (2009a)

Understanding the complex systems involved in stimulation of specific target tissues in teleosts is particularly difficult due to their divergent evolutionary histories producing large variations in their morphology, physiology, behaviour and environment (Bern. 1967). A large body of research has been undertaken to understand the endocrinology of salmonids, specifically in their ability to osmoregulate. The primary endocrine systems focused on were growth hormones (GH), Insulin-like growth factor 1 (IGF-1), cortisol and prolactin.

1.3.4.1 Growth hormone (GH) and Insulin-like growth factor 1 (IGF-1)

Initial experiments on stimulation of GH in salmonids indicated that GH promoted both growth and SW tolerance (Smith. 1956; Komourdjian et al., 1976; Clarke et al., 1977; Miwa and Inui. 1985). Due to GH promoting growth in salmonids, and with the knowledge that a greater size is associated with salinity tolerance (McCormick and Saunders. 1987; Hoar. 1988) tests were carried out on large juveniles to see if GH still promoted SW adaption independently of this (Bolton et al., 1987; Saunders et al., 1998, Devlin et al., 2000). This research confirmed that GH had an influence on SW tolerance independent of fish size. A subsequent review by Sakamoto *et al.* (1993) highlighted the many salmonid species that showed correlation between salinity tolerance and an increase in plasma GH. Further research has shown that an increase in plasma GH is associated with indicators of smolt development in Atlantic salmon, with parr showing low levels of plasma GH as opposed to smolts that present large increases in plasma GH (Bjornsson et al., 1997; McCormick et al., 2000, 2001, 2007; Bjornsson et al., 2011). Agustsson *et al.* 2001, showed the mechanisms involved during smoltification within the GH system in Atlantic salmon.

Increases in GH secretion and synthesis increased plasma GH levels whilst GH clearance rates increased with GH receptor binding. This promotes the activation of the GH-IGF-1 axis during peak smolt SW adaption. Studies by Nilsen *et al.* (2008) on landlocked salmon showed no increase in GH level during the normal smolting periods in anadromous salmon, again further highlighting the critical role of endocrine control during SW adaption.

Studies into increased plasma levels of IGF-1 showed a similar trend to GH in salmonids with increased SW tolerance (McCormick *et al.*, 1991; McCormick 1996, 2000, 2002). IGF-1 levels were also shown to increase in parr, and to a higher degree smolts during spring (McCormick *et al.*, 2007). This trend has not been shown in all studies however, with IGF-1 plasma levels from FW to SW showing no change in expression (Nilsen *et al.*, 2008). IGF-1 was suggested to mediate and control the expression level of GH in promoting salmon SW adaption during smoltification.

GH has been shown to be the main activator for the stimulation of IGF-1 gene expression in the liver and gills of salmonids (Cao *et al.*, 1989; Duan and Plisetskaya. 1993; Sakamoto *et al.*, 1995) as well as growth (McLean and Donaldson. 1993; Bjornsson *et al.*, 1997). This in theory agrees with the dual effector theory of action proposed by Green *et al.* (1985) stating that GH and IGF-1 work as a GH/IGF-1 axis. However, GH levels do not systematically control IGF-1 as IGF-1 has been shown at high levels during low levels of GH under varying environmental factors and in SW acclimatised fish (Laresen *et al.*, 2001; Pierce *et al.*, 2002; Stefansson *et al.*, 2003, 2008). Two studies focussing on endocrine changes during migration of Atlantic salmon indicated that GH levels significantly dropped after several weeks at sea, but IGF-1 remained at higher levels (Stefansson *et al.*, 2003; McCormick *et al.*, 2013c). Steffanson *et al.* (2008) however suggested that a high expression of IGF-1 is maintained through stimulation of the hepatic growth hormone receptor (GH-R). GH and IGF-1 have been shown to increase the number and size of ionocytes in salmonids (Sakamoto *et al.*, 1993; Pruent *et al.*, 1994; Xu *et al.*, 1997) as well as increase NKA activity (Madsen *et al.*, 1995; McCormick *et al.*, 2000; McCormick, 2001). GH has also been shown to up regulate NKCC (Pelis and McCormick, 2001). However these hormones do not exclusively control smoltification, and the impact of the steroid hormone, cortisol, upon SW adaptation also plays a critical role.

1.3.4.2 Cortisol

Cortisol, the major corticosteroid in teleost fish, was the original hormone associated with a role in SW acclimatisation due to its role in salt secretion. It was first discovered to increase during SW acclimation in Atlantic salmon smolts (Fontaine and Hately. 1954), and subsequently in Mozambique tilapia (Assem and Hanke. 1981) and coho salmon (*Oncorhynchus kisutch*) (Specker and Schreck. 1982). Ionocytes in Atlantic salmon parr and smolts showed a large increase in their cortisol receptors during spring with a 10 fold increase in plasma cortisol levels in smolts compared to parr (Shrimpton and McCormick. 1998a; McCormick et al., 2007). Similar increases were observed in anadromous but not landlocked salmon (Nilsen et al., 2008). An increase in NKA activity and NKCC with increased cortisol has also been observed (Madsen et al., 1995; Pelis and McCormick. 2001; McCormick et al., 2000, 2008; Tipsmark and Madsen. 2009) similar to those observed by GH and IGF-1, suggesting a GH-IGF-1-Cortisol axis in SW acclimatisation. Early examination of this complex in Atlantic salmon revealed that a combination of both cortisol and GH injections increased NKA activity and SW tolerance more than individual injections of each hormone (Madsen. 1990; McCormick 1996) indicating a combined action between them. A combined IGF-1 cortisol injection was found to be no different than individual injections of IGF-1 and cortisol however (Seidelin et al., 1999). The interactions were further shown when injections of cortisol into FW Atlantic salmon promoted higher mRNA abundance of the $\alpha 1a$ isoform of NKA, but injection of GH alone had no effect on this isoform. However, both GH and cortisol combined injections increased $\alpha 1b$ mRNA abundance (Tipsmark and Madsen. 2009). In the presence of cortisol both GH and IGF-1 gill receptor expression increased. Recent research by McCormick *et al.*, (2013a) supports this and further states that GH may act as a switch for cortisol from ion secreting (FW) to ion uptake (SW). This is due to co-injections of GH and cortisol decreasing the mRNA abundance of the $\alpha 1a$ isoform and increasing the mRNA abundance of the $\alpha 1b$ isoform in juvenile Atlantic salmon. This body of research suggests that GH and IGF-1 work in synergy with cortisol to promote the adaption of osmoregulation in the gills from FW to SW in most euryhaline teleosts (Figure 1.5).

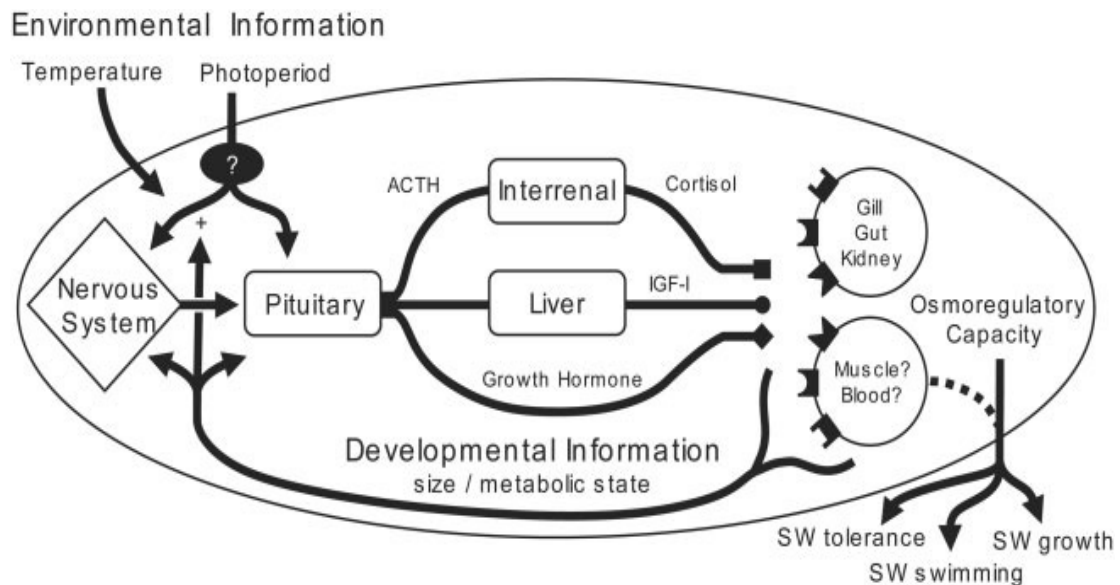


Figure 1.5. The endocrine control of juvenile Atlantic salmon in seawater (SW). Growth hormone (GH), Insulin like growth hormone 1 (IGF-1) and cortisol interact to promote the physical changes in osmoregulatory organs required for SW adaption. Adapted from McCormick. (2009a).

1.3.4.3 Prolactin

Prolactin has been shown over many studies to be the promoter of ion uptake and inhibitor of ion secretion in many teleost species, increasing and promoting retention of Na^+ and Cl^- ions (Foskett et al., 1983), and is thought to be the FW adapting hormone in teleosts (Manzon. 2002). In several salmonid species plasma prolactin levels were found to increase over the winter and early spring before a decrease during the smolting periods of April and May, and after SW exposure (Prunet et al., 1986; Young et al., 1989; Yada et al., 1991). Prolactin receptor mRNA has also been found to decrease during smoltification and after 1 month of SW exposure in anadromous Atlantic salmon, with prolactin ionocyte receptors also decreasing or remaining stable (Kiilerich et al., 2007; Nilsen et al., 2008). Its effect on NKA activity in salmonids is still to be determined with conflicting research indicating decreases (Madsen and Bern. 1992; Shrimpton and McCormick. 1998b) and no effect (Madsen et al., 1995; Seidelin and Madsen. 1997; 1999; Tipsmark and Madsen. 2009). It has also been shown to reduce the size and stimulate formation of smaller ionocytes (Evans et al., 2005). Although there is conflicting evidence with NKA activity, the evidence gained so far indicates that prolactin is a prominent hormone in FW acclimatisation. It has been proposed that prolactin and cortisol work together in controlling both FW and SW adaption dependant on the concentration of GH and prolactin present (McCormick. 2001; Sakamoto and McCormick. 2006). As prolactin and GH are antagonists to one and other (Madsen and Bern. 1992; Seidelin and

Madsen. 1997) it appears whichever is in higher concentrations will stimulate an ion absorption (FW) or ion secreting (SW) effect on fish. It has also been suggested that there may be a distinct receptor of cortisol that is the FW acclimatiser and SW acclimatiser. In teleosts, recent molecular data has shown that there are two different receptors which mediate cortisol; glucocorticoid receptors (GR) and mineralocorticoid-like receptor (MR). GR receptors were originally thought of as the primary mediator of cortisol (Mommsen et al., 1999), however in salmonids MR was revealed to have an equal affinity to cortisol (Strum et al., 2005). Subsequent research has indicated that GRs regulate ion balance (Prunet et al., 2006) and suggests that cortisol plays a dual role in FW and SW acclimatisation, as GR was shown to have a distinctive role in hypo-osmoregulation during smoltification and MR in hyper-osmoregulation in desmoltification of Atlantic salmon (Kiilerich et al., 2007). This suggests that the MR and prolactin may work in synergy as the controllers of FW adaptation in salmonids.

1.3.5 Aquaculture control

In Atlantic salmon the development of parr-smolt transformation consists of physiological, morphological and behavioural changes in preparation for SW acclimatisation (McCormick and Saunders. 1987; Hoar. 1988). Many environmental factors are essential for the development of salmon leading up to and during the smoltification period. The most important of these is photoperiod. In migratory salmon smolts photoperiod is the key influencer of development (Hoar. 1976). Increased periods of light have been shown to promote out of season (winter – early spring) and advanced parr-smolt development in Atlantic salmon (McCormick et al., 1987; Duston and Saunders. 1990, 1995), proposing the feasibility of controlling parr-smolt smoltification development throughout the year and producing a second production group of off-season salmon in winter. Continuous light however inhibits normal smolt characteristics such as salinity tolerance, gill NKA activity and critical parts of the endocrine system (McCormick et al., 1987; Solbakken et al., 1994; Steffanson et al., 2007). Endocrine factors such as GH, IGF-1 and cortisol have all been shown to be influenced by photoperiod, which works as a trigger in the light-brain-pituitary axis (Nilsen et al., 2008; Bjornsson et al., 2011). GH specifically appears to be the most responsive, increasing in concentration days after an advanced photoperiod (McCormick et al., 1995) but remaining low in continuous light (Bjornsson et al., 1995; Bjornsson et al., 2000). To respond to increased photoperiods a 6-week short day photoperiod was suggested as a minimum

requirement for parr-smolt transformation success (Duncan and Bromage. 1998; Handeland and Stefansson. 2001).

Photoperiod is widely regarded as the seasonal Zeitgeber that triggers the development and preparation of Atlantic salmon for migration and is suggested to increase the sensitivity of the fish to other environmental cues (McCormick et al., 1998). One of these cues is temperature, which plays a significant role in the parr-smolt adaption. Increases in temperature up to 16°C have been shown to promote early smolt development including increased NKA activity in Atlantic salmon, as well as controlling physiological, morphological and behavioural development (Johnston and Saunders. 1981; Solbakken et al., 1994; Handeland et al., 2000, 2013; McCormick et al., 2000). However, McCormick *et al.* (2002) tested both a normal and short day length on a normal and advanced increased temperature regime, and found that although NKA levels increased faster in the increased temperature regime and normal day length, the overall NKA activity peaked at the same time as the normal temperature and normal day length regime. Full smoltification was also not achieved under short day length and increased temperature regime, suggesting that increased temperature can influence smolt development but is not a zeitgeber like photoperiod. Following on from this research, Handeland *et al.* (2004) showed that peak NKA activity was seen around 350 degree days (dd) regardless of the temperature Atlantic salmon were reared in. Although increases in NKA were seen in higher temperatures, all fish groups NKA peaks were obtained around the same time frame. Further research by Zydlewski *et al.* (2005) appeared to support this as they found that the effects of temperature were based on temperature experience over time, as opposed to meeting a particular temperature threshold to begin and terminate smoltification. McCormick *et al.* (2000) also indicated that low temperatures limit the response of Atlantic salmon to increased day length by suppressing the response of the endocrine system. Suggesting the smolt window is controlled more predominately by the endocrine system's response to photoperiod than temperature. From this it can be established that photoperiod initiates the time window of the parr-smolt adaption to SW, but other factors such as temperature work to influence the successful adaptations and transition into SW.

During early aquaculture farming production of Atlantic salmon, the natural lifecycle of the fish was followed to produce SW ready smolts after 12 – 18 months post hatching. These in season smolts were termed S1 smolts. The process involved selecting the broodstock in early spring/summer and holding them on site prior to stripping of the eggs in autumn/winter. The eggs were then fertilised and left to

hatch. Generally after 12 weeks alevins will hatch and grow into parr over the subsequent year. In the following year the parr were stimulated by the natural seasonal patterns of longer light and rising temperature during spring. The parr would then undergo smoltification and were transferred to SW pens. Since then, subsequent research into photoperiod and temperature manipulation was carried out (as mentioned previously) to produce a faster protocol for off-season SW ready smolts from as little as 6 months post hatching. Smolts produced through this process were originally named S^{1/2}, but are more commonly referred to as S0 smolts. These smolts vary from their S1 counterparts as they can be produced out of season, usually between July – November. This process involves the artificial increase of water temperature and manipulation of photoperiod utilising long day and short day regimes to promote faster growth and simulating a full year life cycle within a 6-month period. The process of smoltification can be further manipulated through the use of special feeds and salt treatments. This can help promote the healthy growth and development of the fish during smoltification to limit mortality during SW transfer. In reference to the study conducted in Chapter 2, a special feed called SuperSmolt® was used. The SuperSmolt® feed helps promote and stimulate the hyposmoregulatory changes in salmon during the smolt window. The feeding regime involved the use of 24-hour light, which allowed for 24-hour feeding. This feed was generally administered over a 4 – 6 week period at each farm. The feed period could be extended or reduced at each farm's discretion. The varying factors that increased or decreased the feeding period were based on the time of year the feed was first administered and on the results of SW readiness testing. Fish were assessed at time points throughout this period and transferred based on their SW readiness.

The crucial period in which smolts adapt themselves morphologically and physiologically in preparation for SW is known as the smolt window. This window can vary but is primarily dependant on the environmental factors of photoperiod and temperature (as mentioned previously). If the fish remain within a FW environment for an extended period of time (~150dd) during this window a process known as desmoltification can occur. Desmoltification is the process in which most of the vital development aspects for SW survival are lost, such as salinity tolerance and regression of morphology back to a parr like state (Folmar. 1982; Hoar. 1988).

Other environmental factors such as acid rain/run off, and the subsequent increase of inorganic aluminium (Al) in water can cause debilitating conditions and mortality in fish (Hesthagen, 1989; Campbell et al., 1992; Kroglund and Finstad. 2003; Scott

and Sloman. 2004; Kroglund et al., 2007, 2008; Monette and McCormick. 2008; Nilsen et al., 2010; Nilsen et al., 2013). Inorganic Al accumulates in the gills disrupting their structure and function. Moderate exposure to smolts lead to poor osmoregulation and poor physiological adaptations, and high concentrations lead to mortality (Kroglund and Finstad. 2003; Kroglund et al., 2008; Monette and McCormick. 2008). These acidic episodes during smoltification were shown to have a significant impact on their survival after SW migration and overall adult return rates (Kroglund et al., 2007; Nilsen et al., 2010). It was shown to take up to 2 weeks for smolts to recover in FW after exposure to 2 – 7 days of high pH and Al (Nilsen et al., 2013). Reduction in NKA activity has been identified with high concentrations of Al and pH (Kroglund et al., 2007; Nilsen et al., 2010) however in contrast to this other research has shown no change in NKA activity (Monette and McCormick. 2008; Nilsen et al., 2013). Isoform mRNA abundance of α 1a and α 1b were found not to be altered during exposure, however after transfer back to FW α 1a was upregulated at an increased rate and α 1b down regulated (Nilsen et al., 2013), suggesting Al and high pH delay the preparatory secretion of the α -subunits.

1.4 Biomarkers for Atlantic salmon production

A biomarker or biological marker is generally defined as a measurable indicator of normal biological processes or condition within an organism. They can be used to indicate changes in biological processes that impact the physiological state of an organism, such as a disease state. The National Institute of Health defined it as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention (Atkinson et al., 2001). Consistent and reliable biological expression is the most important pre-requisite for a reliable biomarker. A biomarker widely used in most eukaryotes including Atlantic salmon is elongation factor-1a gene (Ef1a). This is due to its production being ubiquitous across all eukaryotes (Sasikumar et al., 2012). It was originally suggested by Olsvik *et al.* (2005) and is now currently used as an Atlantic salmon biomarker for gene expression to identify good quality RNA/cDNA due to its crucial role in translation during protein synthesis.

As detailed in section 1.3, NKA activities consistently increase across normal parr-smolt development. This is a good starting parameter for isolating a biomarker for smoltification, as this is a consistent biological process that occurs in all developing Atlantic salmon (see section 1.3). The identification of the isoforms α 1a as the FW isoform and α 1b as the SW isoform, and their changes in mRNA abundance during

smoltification (see section 1.3.3.1) allows the utilisation of these isoforms as potential biomarkers due to the consistency of their up and downregulations during this period. The $\alpha 1a$ isoform could be used to monitor the full smoltification process following its high mRNA abundance at the initial stages of smoltification to a gradual decrease over time as they adapt into SW ready smolts (see section 1.3.3.1). Expression levels of $\alpha 1b$ mRNA could be monitored during and after SW transfers to ensure fish are fully adapted by observing whether $\alpha 1b$ mRNA is significantly upregulated post transfer.

Other molecular biomarkers have been suggested for Atlantic salmon focusing on gene expression during bacterial and viral infections. Salmon macrophages infected with *Piscirickettsia salmonis* indicate 71 gene transcripts that were either upregulated or downregulated during infection (Rise et al., 2004). Macrophage and dendritic cells infected with ISAV indicated up to 24 genes highly expressed during infection (Workenhe et al., 2009). In both these cases a mixture of functional and immune genes were either upregulated or downregulated. Focusing on a number of these genes showing high and consistent expression changes would potentially allow for viral infection monitoring of these diseases. The potential of immune genes will be touched upon in the following section on antiviral immunity (section 1.5) with focus on erythrocytes as potential biomarkers for innate immunity.

1.5 Antiviral immune response

1.5.1 Overview of the immune system

In vertebrates there are two types of defence mechanisms: innate and adaptive. The innate immune system is controlled by germline encoded pattern recognition receptors (PRRs), which detect specific parts of invading pathogens known as pathogen-associated molecular patterns (PAMPs) (Akira et al., 2006). The adaptive immune system is controlled by two types of antigen receptors, T-cell and B-cells that respond to specific antigens presented by infected cells. These cells generate a fast and specific response to eliminate the pathogen and produce immunological memory for quicker response during secondary infections of the pathogen (Banchereau et al., 2000). The innate and adaptive immune system work together to produce an effective immune response (Carrol and Prodeus. 1998; Carrol. 2004; Clark and Kupper. 2005). An example of this is the innate complement system that can be activated through the adaptive immune response (Carrol. 2004; Sarma and Ward. 2011). The innate immune system is the more primitive of the two systems. The evolution of innate and adaptive systems began from basic unicellular amoebae

that could phagocytose (Desjardins et al., 2005), to recognition of self and non-self in invertebrates (Cooper. 2010), to basic innate and partial adaptive immune function supported by leucine rich repeats (LRRs) in jawless vertebrates (Litman et al., 2010). It wasn't until jawed cartilaginous teleosts that adaptive immunity evolved with major histocompatibility complexes (MHC), T cell receptors and immunoglobulins present. Further sophisticated evolutionary adaptations and complexities were seen in the adaptive immune system in higher vertebrates (Cooper and Alder. 2006; Buchmann. 2014; Zimmerman et al., 2014). This evolutionary adaptation has led to differences in the immune system structure between different vertebrate species. A notable evolutionary difference is between mammals and teleosts; in mammals bone marrow is crucial for maintaining a functional immune system, but an equivalent is absent in fish. Head kidney in fish however have been shown to contain similar more primitive functioning haematopoiesis as seen in bone marrow, as well as being the primary site for antibody production (Tian et al., 2009; Secombes and Wang. 2012). Although immune structures can vary germ-line encoded PRRs are generally evolutionary conserved across vertebrates and invertebrates (Buchmann et al., 2014). The immune system has a series of cellular and humoral components, which control the immune response. The cellular components are primarily leucocytes, which consist of macrophages, monocytes, neutrophils, eosinophils, lymphocytes (T cells, B cells and large granular lymphocytes) and auxiliary cells (basophils, mast cells and platelets). Humoral components consist of antimicrobial proteins (AMPs), antibodies and complement proteins such as cytokines, which signal the pathways and activate the innate and adaptive immune response.

1.5.2 Cellular components of the innate immune response

1.5.2.1 Macrophages

Macrophages are specialised cells that act as a first response to pathogenic infections and orchestrate the subsequent development of the specific immune response. They are free roaming cells that are present within most tissues. These cells recognise PAMPs through PRRs located on their cell membrane or phagosomes (Mosser. 2003; Gordon. 2007; Dale et al., 2008). These can consist of TLRs, C-type lectin receptors (CLRs), and complement receptors (See section 1.5.3 for more detail). Once PRR-PAMP recognition has occurred the macrophage engulfs the pathogen by phagocytosis and breaks it down with digestive enzymes in the lysosome. Antigens of the pathogen are then presented for CD4 cells (T-helper cells) of the adaptive immune response by MHC class II proteins on the cell surface.

The active macrophage also produces pro-inflammatory cytokines such as tumour necrosis factor- α (TNF- α), Interleukin-1 β (IL-1 β), IL-6, IL-12 and IL-18 that activate other chemokine's in recruiting inflammatory leukocytes (neutrophils and monocytes) to the site of infection, and produce nitric oxide through induction of the NO synthase gene (Mosser. 2003; Gordon. 2007; Dale et al., 2008). Once an infection has occurred more macrophages can be differentiated and recruited from monocytes to respond to the pathogens, and increase the innate and adaptive response. These responses work together to promote a coordinated local and systematic immune response. Research into macrophage activity to pathogenic infections in teleosts has shown that the interactions are the same as those found in mammals (Hodgkinson et al., 2015). The activation and interactions between PRR-PAMPs and subsequent innate immune system response have been documented in bacterial stimulated salmonid macrophages (MacKenzie et al., 2003; Rise et al., 2004; Iliev et al., 2005, 2006; Ewart et al., 2008; Boltaña et al., 2011) and virally stimulated (Falk et al., 1995; Iliev et al., 2005, 2006; Xu et al., 2016).

1.5.2.2 Neutrophils

Neutrophils are phagocytosing cells that are one of the first recruited to an inflammatory site and work together with macrophages to combat initial pathogen infections (Silva. 2010). They locate sites of infection through chemotaxis following chemical gradients of chemokines released by macrophages such as IL-8 and interferon gamma (IFN- γ), or by the complement system. (Mayadas et al., 2014) Activated neutrophils can kill invading pathogens by phagocytosis, production of reactive oxygen species (ROS), or by degranulating to release cytotoxic granules and generating neutrophil extracellular traps (NETs) (Brinkmann et al., 2004; Nahthan. 2006; Mayadas et al., 2014). These characteristics are shared in teleosts, however mammalian neutrophils account for the majority of leucocyte cells present in the body (30 – 70%) whereas around <5% of leukocytes are neutrophils in most teleosts (Silva. 2010; Havixbeck et al., 2015). There is limited research on the role of salmonid neutrophils during viral infections (Rønneseth et al., 2006; Montero et al., 2009) however it is assumed that due to the functional similarities to mammalian neutrophils that they play a similar role during viral infections.

1.5.2.3 Natural Killer (NK) cells

Natural killer (NK) cells are lymphocytes that act as an alternate form of innate immunity by targeting and destroying invading pathogens through detection of abnormalities on cell surfaces, such as glycoproteins and bacterial or viral molecules by its cell receptors (Vivier et al., 2008). NK cells are activated by Type I

interferons (IFN I) from viral compromised cells and/or macrophage-derived cytokines IL-2, IL-12, IL-18 and IL-15. In turn the NK cells induce Type II IFNs (IFN II) and TNF- α when exposed to TLR ligands, triggering other parts of the innate immune response (Savori et al., 2004; Vivier et al., 2008). Once bound to a target pathogenic cell, NK cells secrete perforin, a cytolytic protein, that forms pores in the cytoplasm, that in turn facilitate the release of secretory granules (granule serine protease, granzymes) into the cell inducing apoptosis (Trapani and Smyth. 2002; Voskoboinik et al., 2010).

In several teleost species NK like cells and non-specific cytotoxic cells (NCCs) have been described (Nakanishi et al., 2011). NCCs are thought to be the evolutionary precursor to mammalian NK cells (Evans and Jaso-Friedmann. 1992). NCCs act in a similar manner to mammalian NK cells by targeting and destroying pathogenic cells using granule exocytosis pathways (Praveen et al., 2004). In salmonids limited research has been conducted, however NCCs were shown to have variable effects on IPNV in rainbow trout (Yoshinaga et al., 1994) but have a positive response to viral hemorrhagic septicaemia virus (VHSV) infections (Utke et al., 2008).

1.5.2.4 Antimicrobial peptides (AMPs)

Antimicrobial peptides (AMPs) are small molecules (~10 – 50 amino acids) of highly conserved peptides divided into several subgroups based on their amino acid composition and structure. They have been shown to assist in the immune response against bacterial and viral pathogens in most vertebrates and invertebrate species (Diamond et al., 2009). In mammals AMPs are produced and synthesised by epithelial cells, macrophages, neutrophils, monocytes and NK cells upon phagocytosis of the pathogen and through pathogen – receptor binding (Ganz. 2003). In phagocytosed pathogens the AMPs migrate and fuse to the pathogen and begin the synthesis and release of highly concentrated defensins, enhancing the antimicrobial ability of the cell (Ganz 2003; Klotman and Chang. 2006; Diamond et al., 2009). AMPs have also been shown to initiate chemotaxis activity to recruit more neutrophils to the site of infection (Diamond et al., 2009).

In teleosts over 90 AMPs have been identified and classified into five major families of AMPs; β -defensins, cathelicidins, hepcidins, histone-derived peptides and the teleost specific piscidins (Wang et al., 2009; Masso-Silva and Diamond. 2014). AMPs are mainly secreted in mucosal tissues as first points of defence but can also be expressed from liver kidney and spleen (Masso-Silva and Diamond. 2014). They have been shown to play a similar antimicrobial role in the innate immune response

against pathogenic infections as in mammals, (Smith et al., 2010) as well as contributing to the inflammatory response through neutrophil recruitment and enhancement of phagocytosis (Masso-Silva and Diamond. 2014). In salmonids all five families have been discovered over several different species (Masso-Silva and Diamond. 2014). *In vitro* testing of a rainbow trout β -defensin displayed antiviral action against VHSV (Falco et al., 2008). Following this an *in vivo* test using head kidney leucocytes of rainbow trout stimulated with Poly I:C showed upregulation of four β -defensins (Casadei et al., 2009). Hepcidin has also been shown to be upregulated in rainbow trout macrophages during Poly I:C stimulation and injection of infectious hematopoietic necrosis virus (IHNV)(Chiou et al., 2007). It appeared to be a first response to infection as the abundance of hepcidin decreases as the infection progresses and IFNs and interferon stimulated genes (ISGs) abundance increase.

1.5.2.5 Cytokines

Cytokines are small proteins that are critical in cell signalling during the immune response. They are produced by numerous immune cells such as macrophages in response to PAMP-PRR recognition of an invading pathogen. The initial cytokines stimulated by TLRs such as pro inflammatory cytokines TNF alpha, IL-1 β and IL-16 cause a cascade effect stimulating other immune responsive cytokines (e.g. IFNs) promoting both the innate and adaptive immune response (Dinarello 2007). The major cytokine families found in mammals (IL-1s, TNF, Cysteine knot cytokines, Type I and Type II α helical cytokines) have also been discovered in teleosts (Zou and Secombes. 2016). This implies that the teleost and mammalian innate immune responses contain similar structural properties in combating pathogenic infections, although the subsets and individual cytokines within these families may have evolved differently to one another. The following section on innate immune response (1.5.3) will cover some of the cytokines involved in viral infections of both mammals and teleosts.

1.5.3. The innate immune response

1.5.3.1 Pattern recognition receptors (PRR) and pathogen associated molecular patterns (PAMP)

As previously mentioned, the innate immune response is initiated by the recognition of PAMPS of invading pathogens by PRRs. PRRs can then either present at the cell surface or signal to the host through intracellular mechanisms by using a multitude of signalling pathways through utilisation of adaptor molecules, kinases and

transcription factors (Akira and Takeda. 2004). These pathways signal the activation, synthesis and reprogramming of the transcriptome resulting in the production of antiviral and inflammatory cytokines, other immune signalling proteins and immunoreceptors which work together as the early host response to infection (Akira et al., 2006). PRRs are not highly specific and can potentially bind to numerous molecules with similar recurring patterns related to pathogenic and non-pathogenic origin. Invading pathogens that can signal PRR-PAMP recognition can be bacterial, fungal and viral. The most common bacterial PAMPs that stimulate PRRs are from components of their cell wall such as lipopolysaccharides (LPS), peptidoglycan and RNA and DNA from the unique structures of the invading bacterium (Boltaña et al., 2011). For fungal infection β -glucan from the cell wall is the most common PAMP (Brown and Gordon. 2001). Viral PAMPs are detected by either viral motifs on the cell surface by transmembrane PRRs or by cytosolic recognition of conserved parts of double stranded (dsRNA) or single stranded RNA (ssRNA) and DNA (Wilkin and Gale. 2010). The main class of PRRs that detect viral PAMPs include the transmembrane Toll like receptors (TLRs) and cytosolic RIG-I like receptors (RLRs: Retinoic acid-inducible gene 1, RIG I; melanoma differentiation-associated gene 5, MDA5; Laboratory of genetic and physiology 2, LGP2). As viral infections are the motivation for part of this research we will focus on these exclusively.

1.5.3.2 Toll like receptors (TLRs)

TLRs are type 1 transmembrane proteins characterised by 3 major domains consisting of LRRs, transmembrane and intracellular. TLRs function as both an antimicrobial defence promoting the expression of type I IFNs, and as an inflammatory response promoting the inflammatory cytokines IL-1 β , IL-6 and TNF- α . Currently 12 types of TLRs are known for mammals (Akira and Takeuchi. 2006) with two identified as PRRs for ssRNA (TLR7 and TLR8) (Heil et al., 2004) and one for dsRNA (TLR3) (Alexopoulou et al., 2001). TLR signalling is initiated by dimerisation of the TLRs after binding to their PAMP. This sends a signal for the recruitment of one of four Toll/IL-IR (TIR) receptor domain cytosolic adapter molecules specific to the TLR that began signalling. For TLR3 the adaptor molecule utilised is TIR domain-containing adaptor inducing interferon- β (TRIF) that recruits and initiates phosphorylation of NF- κ B activating kinase and interferon regulator transcription factor 3 (IRF-3) which translocate to the nucleus and induces production of IFN β and type I IFNs (Kawai and Akira. 2009). For TLR7, myeloid differentiation primary response 88 (MyD88) is the adaptor molecule used which activates interleukin-1

receptor-associated kinase 1 (IRAK1) and $\text{I}\kappa\text{B}$ kinase α ($\text{IKK}\alpha$) to promote phosphorylation and transfer of IRF7 to the nucleus to induce type I IFN production. TLR8 follows a similar pathway activating NF- κB and signaling an antiviral response. (Kawai and Akira. 2009). TLR7 has been shown to be the essential TLR for overall IFN production in mammals (Honda et al., 2005) however the same is not seen in teleosts.

There are 3 primary TLRs that are responsible for viral PAMP detection in teleosts. TLR3 in fish is an ortholog of the human TLR3 that is also located intracellularly and is one of the two TLRs responsible for identifying dsRNA viruses (Pietretti and Wiegertjes. 2014). TLR 22 is an aquatic exclusive TLR that has been discovered in various teleost species (Hirono et al., 2004; Meijer et al., 2004; Rebl et al., 2007; Matsuo et al., 2008). It is found on the cell membrane surface and induces an IFN response by Poly I:C stimulation (Hirono et al., 2004) and in the presence of dsRNA virus (Matsuo et al., 2008). TLR3 and TLR22 are distinct from one another but appear to play a dual role in the recognition of dsRNA. TLR3 appears to recognize shorter dsRNA and TLR22 longer dsRNA and implies that they both have a functional role in the type I IFN induction pathway (Matsuo et al., 2008; Pietretti and Wiegertjes. 2014). TLR7 appears to be the PRR for viral ssRNA and promotes both the production of type I interferons and pro inflammatory cytokines (Pietretti and Wiegertjes. 2014).

1.5.3.3 Retinoic-acid-inducible gene 1 (RIG-I) and RIG-I like receptors (RLR)

The RLRs are cytosolic PRRs that detect replicating viral RNA in the cytoplasm during replication in the infected host cell, contrary to TLRs, which detect invading virus in the endosome (Kawai and Akira 2006). This occurs in most cell types and instigates an anti-viral response by initiating type I IFN production (Akira et al., 2006; Yoneyama and Fujita. 2008; Kawai and Akira. 2009). The RLRs consist of 3 members that are all structurally related, RIG-I, MDA5 and LGP2. All 3 contain a DExD/H helicase domain, which is involved in unwinding DNA or RNA, and C-terminal repressor domain (CTD), which is used to bind to viral RNA (Cui et al., 2008; Takahasi et al., 2009). RIG-I and LGP2 have been shown to recognise both ssRNA and dsRNA with MDA5 recognising short dsRNA and preferentially Poly I:C (Kato et al., 2005, 2006; Takahasi et al., 2009). Both RIG-I and MDA5 contain a tandem caspase recruitment domain (CARD) – like regions, which are essential for interactions with adaptor molecules in antiviral response (Chen et al., 2017). RLRs have been identified in some teleost species (Zou et al., 2010; Chang et al., 2011;

Ohtani et al., 2010, 2011; Xu et al., 2016) and have been shown to have the same structures as mammals. During viral infection in fish RIG-I and MDA5 are bound to viral RNA where they recruit mitochondrial antiviral signalling protein (MAVs) and begin phosphorylation of IRF-3/IRF7, with those transcriptional factors translocated to the nucleus to induce the transcription of type I IFNs, ISGs and inflammatory cytokines (Figure 1.6) (Chen et al., 2017).

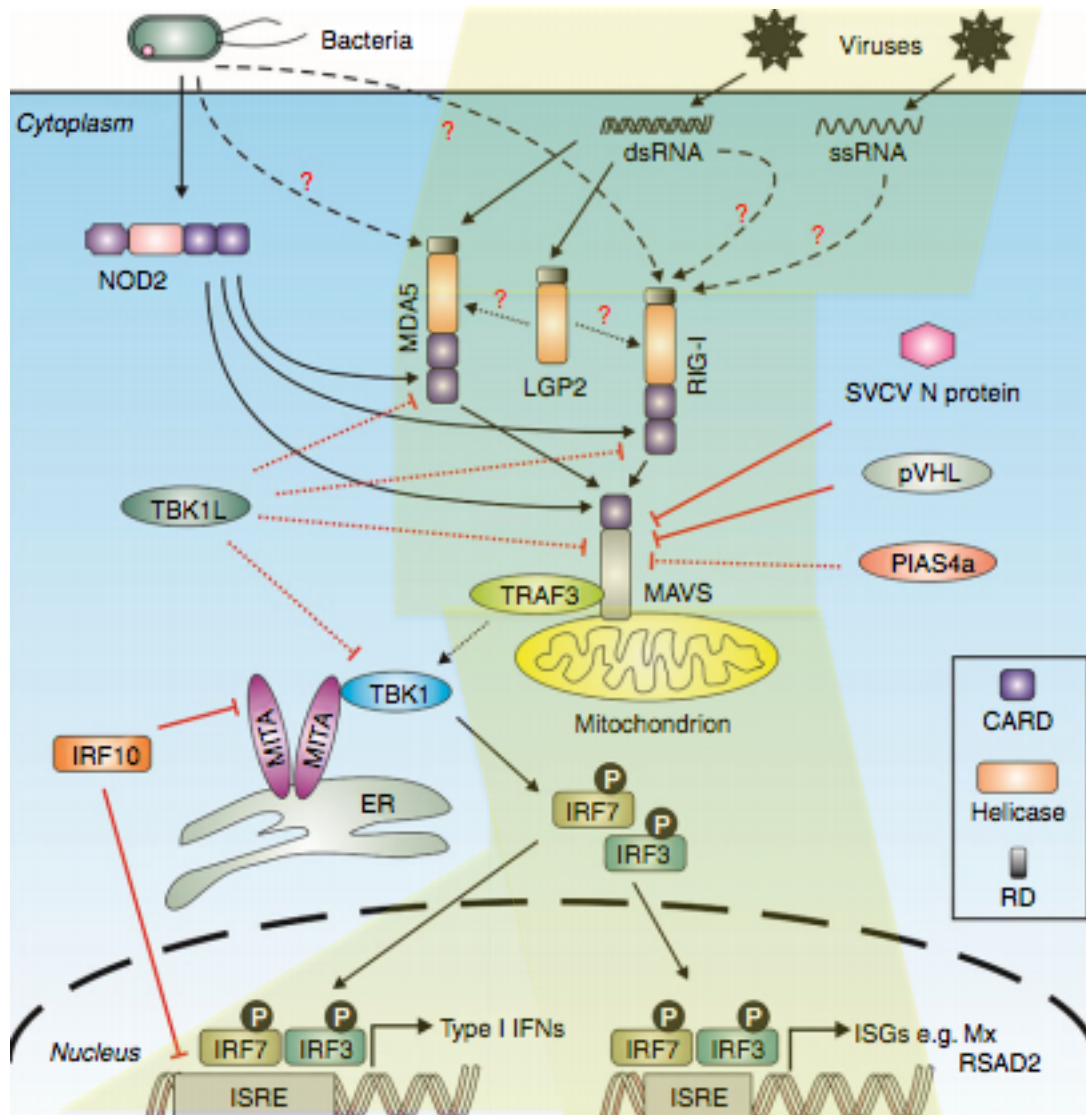


Figure 1.6 Teleost retinoic acid-inducible gene I (RIG-I) and RIG-I like receptor (RLR) – signalling pathway to viral infection (Highlighted in yellow). Binding of MDA5 or RIG-I to single or double stranded RNA through pattern recognition receptor (PRR) pathogen associated molecular patterns (PAMP) recognition occurs. Recruitment of mitochondrial antiviral signalling protein (MAVs) and association of TRAF3 instigates phosphorylation and activation of interferon (IFN) regulatory factor 3 (IRF3) and or 7 (IRF7). These transcriptional factors are then translocated into the nucleus and bind with the IFN stimulated response element (ISRE) motif that signals the production of type I IFNs and IFN-stimulated genes (ISGs). Adapted from Chen *et al.*, (2017).

1.5.3.4 Interferons (IFN)

IFNs are a part of the class II helical cytokine family and are a crucial part of the innate immune response in jawed vertebrates. They were first discovered in 1957 (Isaac and Lindenmann, 1957) and subsequently were found to exist in early jawed vertebrates (Gnathostomes) and preserved throughout the evolutionary changes and gene duplications of teleosts as they branched off and evolved into amphibians, reptiles, birds and mammals (Secombes and Zou, 2017). They are grouped into type I, II and III based on structure, function and receptors. Type I and III have been shown to be the main instigators of antiviral response through specific signalling pathways (Samuel, 2001; Kolenko et al., 2003; Randall and Goodbourn, 2008) whereas type II (IFN- γ) are involved in both the innate and adaptive immune response to both viral, bacterial and protozoal infections (Schoenborn and Wilson, 2007). In teleosts type I IFN has been phylogenetically classified into six groups (IFN a - f) (Aggad et al., 2009; Zou et al., 2007, 2014, 2018; Chang et al., 2009; Sun et al., 2009). Group I IFNs (a, d and e) are present within all teleosts and in most cell types and tissues, whereas group II IFNs (b, c and f) have currently only been found within salmonids, cyprinids and Pleuronectiformes (Aggad et al., 2009; Chang et al., 2009; Zou et al., 2014; Pereiro et al., 2014) and are predominately expressed in primary leukocytes and the head kidney (Zou et al., 2007; Sun et al., 2009). Salmonids at present have the highest gene copy number of IFNs in jawed invertebrates containing all known teleost type I IFNs (a, b, c, d, e, f) (Zou et al., 2014; Sun et al., 2009). This is likely due to ancient IFNs being conserved during the genome duplication event in teleosts.

Initial tests into Atlantic salmon type I IFNs showed that salmonid cell lines containing IFNa were protected against IPNV and induced the ISG Mx protein as well as stimulating IFN transcripts in the head kidney from Poly I:C stimulation. Subsequent work on Atlantic salmon showed that IFNa was the main subtype induced through the RLR pathway, and IFNb and c through the TLR3/TLR22 pathway (Svingerud et al., 2012). The ISGs Mx, viperin, Interferon-stimulated gene 15 (ISG15) and Interferon-induced protein 5 (IFIT5) were all shown to be induced by IFNa, b and c expressing plasmids through intramuscular injections. IFNa was shown to only induce ISGs at the site of injection, however IFNb and c systematically induced ISGs in the head kidney heart and liver (Chang et al., 2014). The importance of IFNc in long-term antiviral protection was further highlighted by its ability to protect fish from ISAV 8 weeks post injection (Chang et al., 2014). This was also supported in previous ISAV infections where IFNc showed inhibition of the virus

along with IFN α 1, which induced Mx and ISG15 (Svingeruld et al., 2013). Similar mRNA expression patterns have been found in rainbow trout spleen IgM⁺ cells, where VHSV and Poly I:C upregulated TLR3 and type I IFNs (Abos et al., 2015).

In teleosts two members of type II IFNs have been identified in salmonids, IFN- γ and IFN- γ related molecules (IFN γ rel) (Zou et al., 2005; Robertsen. 2006; Sun et al., 2011). The antiviral activities of these IFNs in salmonids are still not fully understood, however IFN- γ were shown to weakly induce Mx expression in rainbow trout cells (Zou et al., 2005) and protected Atlantic salmon cells against IPNV and SAV 3 (Sun et al., 2011). IFN- γ was shown to have a weaker effect than IFN α in protection against IPNV and SAV 3 but was more effective at inducing guanylate binding protein (GBP), IRFs and Interferon gamma-induce protein 10 (IP-10). IFN- γ was suggested to have a direct role in induction of Mx and ISG15 as IFN α antibodies could not completely stop IFN- γ upregulation of these ISGs, indicating IFN α and IFN- γ had a dual role in ISG induction (Sun et al., 2011). Further evidence suggesting IFN- γ plays a lesser but likely dual role in upregulation of ISGs with IFN α has been shown in SAV 3 infected salmonids. IFN α was shown to upregulate ISGs and induce an antiviral state preventing virus replication, whereas IFN- γ was able to upregulate ISGs to a lesser extent and was unable to prevent virus replication (Xu et al., 2010).

Type III IFNs have yet to be identified in teleosts (Boudinot et al., 2016).

The initiations of IFN signalling through TLRs and RLRs to a viral infection, and the IRFs produced have been outlined in the two previous sections (1.5.3.1 and 1.5.3.2). However IFNs actions are further mediated through IRFs interaction in the Janus kinase – signal transduction and activator of transcription signalling pathway (JAK-STAT) to produce antiviral ISGs. This involves the IFNs binding to the JAK1 and 2 proteins, then recruitment and phosphorylation of STAT, which is then translocated to the nucleus and initiates or inhibits the transcription of ISGs (Aaronson and Horvath. 2002; Rawling et al., 2004; Yoshimura et al., 2007). The JAK-STAT pathway controls the IFN response through a negative feedback loop using the ISG suppressor of cytokine signalling (SOCs) (Yoshimura et al., 2007). This ISG can increase and decrease cellular signalling of IFN during infections and has been shown to negatively effect the IFN pathways in salmonids (Wang and Secombes. 2008, Wang et al., 2010; Skjesol et al., 2014).

1.5.3.5 Interferon stimulated genes (ISGs)

As mentioned in the previous sections ISGs are antiviral genes that are stimulated by IFNs. Currently a large number of ISGs have been described in mammals, which has led to investigations into the evolutionary development of ISGs produced in other vertebrate species. A genomic survey into Atlantic salmon ISGs indicated up to 117 genes associated with responsiveness to viral infection (Krasnov et al., 2011a). The biological effects of many of these genes are still to be determined, however a core set of genes that are induced in most viral infections have been studied in more depth (Verrier et al., 2011) of which we will briefly summarise below.

Mx genes are almost ubiquitous among vertebrates and are evolutionary conserved large GTPases. They are controlled and stimulated by type I and III IFNs against mainly ssRNA and dsRNA infections. They do this by inhibiting the transcription of viruses during replication in the cytoplasm (Verhelst et al., 2013). Antiviral activity of Mx has been shown against multiple viruses in salmonids. Atlantic salmon cell lines infected with ISAV showed an increase in Mx production, however resistance to the virus and reduction in replication varied (Jensen and Robertsen. 2002; Larsen et al., 2004; Kibenge et al., 2005; Sun et al., 2011). Similar results were shown for IPNV and SAV with upregulation of Mx, but varied resistance to the virus was observed in some cell lines and fish compared to others (Jensen and Robertsen. 2002; Larsen et al., 2004; Strandskog et al., 2011; Xu et al., 2012; Grove et al., 2013). It appears Mx does contribute to the innate immune response, but its effectiveness appears to vary between viruses.

Protein kinase R (PKR) is a protein that is involved in cell proliferation and apoptosis. It is activated by binding to dsRNA where it undertakes autophosphorylation of eukaryotic initiation factor 2 (eIF-2 α), which impedes protein synthesis, and translation of viral mRNA (Garcia et al., 2006). Initial studies on teleosts found that human PKR could phosphorylate eIF-2 α of rainbow trout and zebrafish and that IPNV infection promoted an increase in eIF-2 α . This suggested a fish PKR was present and had similar antiviral properties as in humans (Garner et al., 2003). Several PKR like proteins were discovered in other teleosts including a variant named PKZ that specifically binds to dsDNA as well as dsRNA (Verrier et al., 2011). Its role in assisting in viral defence was shown in Atlantic salmon TO cells and Chinook embryo cells (Bergan et al., 2008).

ISG15 is an ubiquitous molecule that conjugates to target proteins in a process known as isgylation (Morales and Lenschow. 2013). It appears to have a broad

range of targets and will specifically target newly translated proteins (Durfee et al., 2010). Research on ISG15 has shown it has antiviral properties inhibiting several human and mice viruses (Morales and Lenschow. 2013). The isgylation of IRF3 by ISG15 was shown to retain activated IRF3 longer and therefore increase the type I IFN response creating a positive feedback loop (Lu et al., 2006; Shi et al., 2010). In salmonids an ISG15 homologue was first discovered in rainbow trout leukocytes infected with VHSV (O'Farrell et al., 2002). Further research on Atlantic salmon indicated that ISG15 is upregulated by Poly I:C, ISAV, SAV 3 and IPNV and found to actively bind to ISAV and type I IFN α and β proteins This suggests ISG15 acts as an antiviral constituent and promotes the type I IFN response in teleosts in a similar way to higher vertebrates (Røkenes et al., 2007; Xu et al. 2010)..

Vig-1 (VHSV-induced gene), later known as Viperin (virus inhibitory protein endoplasmic reticulum-associated, IFN-inducible), was first discovered to be highly upregulated in rainbow trout leucocytes induced with VHSV (Boundinot et al., 1999). The gene and its homologs were subsequently discovered in other mammals and vertebrates. In mammals, viperin interacts with numerous RNA and DNA viruses and is induced by all 3 IFNs types (I II and III). It inhibits protein secretion, preventing replicating viruses from budding and releasing from infected cells (Hinson and Cresswell. 2009; Fitzgerald et al., 2011). In salmonids further research on rainbow trout macrophages and fibroblast cell lines showed an upregulation of viperin against chum salmon (*Oncorhynchus keta*) reovirus (CSV), dsRNA and Poly I:C (DeWitte-Orr et al., 2007). A similar induction of Viperin was seen in Atlantic salmon macrophages infected with ISAV (Workenhe et al., 2009) and experimentally challenged salmon with SAV (Grove et al., 2013).

1.5.4 Erythrocytes in immune response

Erythrocytes are the most abundant cell type circulating in the blood of vertebrates. They are characterised as oval shaped and contain respiratory globin pigments. Of these respiratory pigments, the haemoglobins are the most common proteins and give the cells their distinct red colour. They vary in cell structure with erythrocytes of non-mammalian species containing a nucleus and cytoplasm bound organelles (Claver and Quagila. 2009). The life span of erythrocytes varies between vertebrate groups, with human cells having a half-life of ~120 days and teleosts varying between species from 50 – 500 days (Fischer et al., 1998; Morera and MacKenzie. 2011; Witeska. 2013). The effects of cell ageing in teleosts have been shown in rainbow trout with reduction in cellular components and total RNA levels, as well as a reduction in aerobic energy production as the cells age (Lund et al., 2000; Phillips

et al., 2000). The number of erythrocytes varies in vertebrates from $1 - 5 \times 10^6 \text{ mm}^3$ and appears to increase in number on an evolutionary scale, with teleosts having the least and mammals having the highest (Claver and Quaglia. 2009; Morera and MacKenzie. 2011).

Erythrocytes develop from hematopoietic stem cells (HSCs) through the blood cell formation process of haematopoiesis followed by erythropoiesis. Both processes are highly conserved between teleosts and other vertebrates (Paffet-Lugassay et al., 2007). The principally accepted basic function of erythrocytes is the transport of oxygen (O_2) and carbon dioxide (CO_2) throughout the body utilizing the respiratory globin pigments for gas exchange to and from cells. However, several other functions have been attributed to erythrocytes including sugar transport, cell proliferation, redox homeostasis, antimicrobial activities and antiviral response (Morera and Simon. 2011). Nelson. (1953) first suggested erythrocytes might be involved in the innate immune response. He found erythrocyte receptors bound to the immune complexes of invading bacteria and proposed the term immune adherence (AI). Subsequent research reported that human erythrocytes had numerous receptors on their surface (Hess and Schifferli. 2003) with further evidence showing erythrocytes assisting in T cell proliferation, cytokine secretion and IL-2 expression in humans (Kalechman et al., 1993; Porto et al., 2001). Due to nucleated erythrocyte cells being derived from hematopoietic origin, and containing similar cellular components and organelles, it would suggest that they could have the ability to contribute to the immune response.

Several studies on teleost erythrocytes have been undertaken over the years showing different immune based attributes. One of the first detected was the phenomenon known as erythrocyte rosetting. Erythrocyte cells attach by cell receptors and fully surround an invading pathogen. This was shown to occur in rainbow trout erythrocytes (Passantino et al., 2002), with a subsequent follow up study indicating the erythrocytes also secreted cytokine-like factors to influence macrophage functions (Passantino et al., 2004). Since then AMPs have been isolated from rainbow trout erythrocytes (Fernandes and Smith. 2004) where they were shown to have antibacterial proinaceous activity against gram-positive bacteria. They were also found in the haemoglobin of channel catfish (*Ictalurus punctatus*, Rafinesque) (Ullall et al., 2008) where they showed antimicrobial activity against white spot disease (*Ichthyophthirius multifiliis*). More recently antimicrobial peptide Nk-lysin (Nkl) were found to be expressed in turbot erythrocytes

(*Scophthalmus maximus*) and contributed to the antiviral defence against VHSV (Piereiro et al., 2017).

Specific research into the viral response of teleost erythrocytes provided more evidence of their function and contribution in the innate immune response. Salmonids erythrocytes stimulated with Poly I:C were shown to upregulate type I IFNs, TLRs and ISGs (Workenhe et al., 2008; Morera et al., 2011). Viral infections of Atlantic salmon erythrocytes with ISAV and PRV also showed production and increases of TLRs, RLRs IFNs and ISGs at varying degrees of abundance (Workenhe et al., 2008; Finstad et al., 2014; Wessel et al 2015; Dahle et al., 2015). A recent study in rainbow trout contrastingly showed either no change or a slight downregulation in IFNs and ISGs with erythrocytes infected with VHSV (Nombela et al., 2018). These findings suggest that teleost erythrocytes have a much more complex series of functions than first thought and that there is a reliable amount of information from both teleosts and other vertebrate erythrocytes indicating, that they contribute in some way to the immune response.

1.6 Atlantic salmon viruses and disease

1.6.1 Salmon alpha virus (SAV) pancreas disease and sleeping disease

Salmonid alpha virus (SAV) is a member of the *Togaviridae* family in the genus *Alphavirus*. It is a small spherical shaped virus consisting of a viral envelope and nucleosapide. Within the nucleosapide is a single-strand + sense strand RNA genome which contains two open reading frameworks (ORF). The 3' end of the genome codes for structural proteins (capsid E1, E2, E3 and 6k) and the 5' end for non-structural proteins (nsP1, P2, P3 and P4) (Strauss and Strauss. 1994; Powers et al., 2001). There are currently 6 known subtypes of SAV (1 – 6) that have been characterised (Fringuelli et al., 2008). The virus causes pancreas disease (PD) in Atlantic salmon in the British Isles (SAV 1, 3 – 6) (Fringuelli et al., 2008; Graham et al., 2012) and in both Atlantic salmon and rainbow trout in Norway (SAV 3) (Hodneland et al., 2005). The virus also causes a different disease in FW reared rainbow trout called sleeping disease (SD) (SAV2) throughout Europe (Castric et al., 1997; Graham et al., 2003; McLoughlin and Graham et al., 2007). SAV 2 was thought to be exclusive to rainbow trout in FW, but has also been described in Atlantic salmon causing PD in the UK (Fringuelli et al., 2008) and more recently in Norway (Hjortaas et al., 2013). This shows the variation and range of the virus, as different subtypes infect different fish based on species, habitat and location (Figure 1.7).

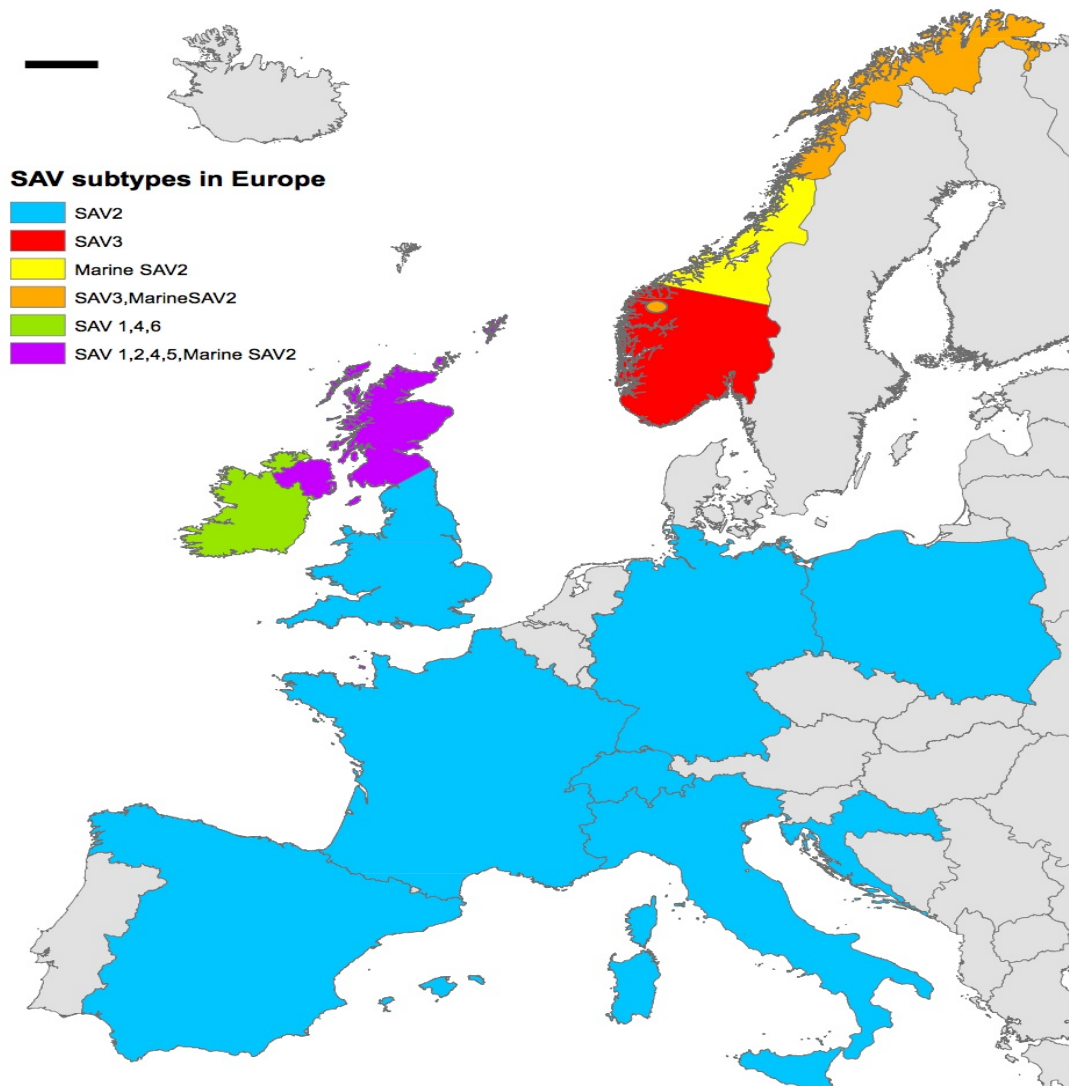


Figure 1.7 Map showing the distribution of all 6 types of *Salmonid alphavirus* (SAV) across Europe. Adapted from Jansen et al., (2017).

PD was first described in Scotland in 1976 (Munro et al., 1984) and subsequently in Ireland in 1984 (McArdle and Crummy. 1985) and Norway in 1989 (Poppe et al., 1989). More outbreaks were recorded in the following years in Ireland (Crockford et al., 1999) and Norway, however records for prevalence in Scotland were not available due to it not being classed as a notifiable disease at the time. A recent survey on 104 active sites in Scotland showed SAV prevalence at 18% (Lester et al., 2011) though not all fish infected with SAV showed clinical signs of PD, indicating PD prevalence at <18%. PD generally occurs after 1 – 2 years post SW transfer. Although numerous subtypes exist, histopathological studies have shown similar pathological and morphological similarities for both salmon and rainbow trout (McLoughlin and Graham. 2007; Taksdal et al., 2015). External PD symptoms are limited but include low or non-feeding fish and abnormal swimming behaviour.

Internal symptoms are characterised by severe skeletal and muscle lesions, inflammation and degradation, leading to muscle myopathy, complete loss of exocrine pancreatic tissue and petechial haemorrhages (McLoughlin et al., 2002; McLoughlin and Graham. 2007; Taksadal et al., 2007). A detailed sequential study by McLoughlin *et al.* (2002) observed that the major organs with detrimental lesions were the pancreas, heart and skeletal muscles. Acute and chronic pancreatic lesions along with muscle and heart myopathy are most commonly found within infected fish.

SD was first recorded in rainbow trout in France (Boucher et al., 1994) and is characterised by lethargic fish with swollen abdomens lying on their side at the bottom of the tank due to necrosis of skeletal red muscle. Its internal symptoms are similar to that of PD with lesions in the exocrine of pancreas, heart and skeletal muscle (Boscher et al., 2006).

1.6.2 Piscine orthoreovirus (PRV) and heart and skeletal muscle inflammation (HSMI)

Piscine orthoreovirus (PRV) is a double stranded (ds) RNA virus in the family *Reoviridae*. It has a genome of 10 RNA segments consisting of 3 size classes, 3 large (L1, L2 and L3), 3 medium (M1, M2 and M3) and 4 small (S1, S2, S3, and S4) where the 5' end of the S1 and S2 segments are postulated to be the ORF (Palacios et al., 2010). PRV was first designated as piscine reovirus but has since been re-classified as an orthoreovirus due to its closer association to this genus (Markussen et al., 2013; Kibenge et al., 2013). PRV has been described in both wild and farmed Atlantic salmon in Norway (Palacios et al., 2010; Lovoll et al., 2012; Garseth et al., 2013) and farmed Atlantic salmon in Ireland (Rodger et al., 2014). PRV or PRV like viruses have been detected in both farmed and wild rainbow trout, Coho salmon, Sockeye salmon (*Oncorhynchus nerka*), Chinook salmon (*Oncorhynchus tshawytscha*) and Sea trout (*Salmon trutta*) in the US and Canada (Marty et al., 2015; Siah et al., 2015; Garver et al., 2016), Chile (Siah et al., 2015), Japan (Takano et al., 2016) and Norway (Garseth et al., 2013).

PRV is the suspected infection agent for heart and skeletal muscle inflammation (HSMI) in Atlantic salmon (Palacios et al., 2010). HSMI was first detected in Atlantic salmon in a Norwegian farm in 1999 (Kongtrop et al., 2004a). The disease usually occurs in month 5 - 9 post SW transfer of salmon, but has been found to effect some fish within a few weeks of SW transfer (Kongtrop et al., 2004b) and in pre-smolt FW farms (Lovoll et al., 2012). The disease is characterised by lesions in the heart and

red skeletal muscle causing inflammation, and chronic deterioration and failure of the heart and muscles (myocarditis and myopathy), leading to mortality rates as high as 20% (Kongtrop et al., 2004a, 2004b). External symptoms are limited, but are associated with abnormal swimming and low to non-feeding fish. The similarities between internal and external symptoms are closely related to both CMS and PD (Table 1.1).

Tissue/organ	Lesions description	CMS	HSMI	PD
Heart	Epicarditis	+	+	+
	Compact-myocarditis and degeneration	-	+	+
	Spongy-myocarditis and degeneration	+	+	+
Skeletal muscle	Inflammation and degradation	-	+	+
Liver	Necrosis of hepatocytes	-	-	+
Pancreas	Necrosis of exocrine tissue	-	-	+

Table 1.1 Comparison of the histopathological lesions in Atlantic salmon infected with Cardio myopathy syndrome (CMS), Heart and skeletal muscle inflammation (HSMI) and Pancreas disease (PD) (Modified from Yousaf et al., 2013)

These similarities in symptoms led to PRV being proposed as the infectious agent of CMS. However, research by Haugland *et al.* (2011) indicated PMCV as the causative agent of CMS. PRV was still seen in high titres in CMS infected fish suggesting it was acting as a possible opportunist (Lovoll et al., 2010; Haugland et al., 2011). Further evidence by Garseth *et al.* (2013) proposed that PRV may not be the direct cause of HSMI but acts an opportunist in HSMI outbreaks, as high titres were found in wild Atlantic salmon but caused no symptoms associated with HSMI. PRV was also found ubiquitously in viral co-infection research by Wiik-Nielsen *et al.* (2016), further suggesting the virus is present in varying titres in Atlantic salmon but does not seem to cause disease. Currently there is still no definitive evidence associating PRV as the primary cause of HSMI outbreaks, but due to its high titres and presence within infected fish during outbreaks it is likely it plays a key role. In 2017 PRV was experimentally shown to cause HSMI (Wessel. 2017).

PRV infects Atlantic salmon erythrocytes, with more than 50% of erythrocytes PRV positive during trials (Finstad et al., 2014). This was also successfully conducted in erythrocytes *ex vivo* (Wessel et al., 2015). Genes associated with the innate immune system were shown to be up regulated by erythrocytes during infections of PRV and non-immune genes suppressed (Wessel et al., 2015; Dahle et al., 2015).

This suggests that salmon erythrocytes have the ability to mount an innate immune response towards PRV and also signal a change in transcription and translation of other non-immune genes.

1.6.3 Piscine myocarditis virus (PMCV) and cardio myopathy syndrome (CMS)

PMCV is a recently discovered double stranded RNA virus designated to the family *Totiviridae*. The virus contains 3 ORFs; ORF1 is thought to encode the major capsid proteins, ORF2 encodes RNA-dependant RNA polymerase sequence (RdRP) and ORF3 appears to be translated as a separate novel protein that is unseen in any currently described *Totiviridae* family, however it has been shown to have some similarities to a chemokine superfamily motif (Haugland et al., 2011).

PMCV is regarded as the causative agent of CMS (Haugland et al., 2011) and currently only affects Atlantic salmon generally between 12 – 18 months post SW transfer (Brun et al., 2003). There has been recent research to show that fish as early as 5 – 6 months post SW transfer have been susceptible to the disease as well (Wiik-Nielsen et al., 2016). The disease was discovered in Norway in 1985 (Armin and Trasti. 1988, Ferguson et al., 1990) and later in the Faroe Islands (Pope and Sande. 1994), Scotland (Rodger and Turnbull. 2000) and in migrating salmon in Norway (Pope and Seierstad. 2003). CMS is characterised by lesion promoting inflammation and degradation of the ventricular spongy myocardium and atrium tissue within the heart. This can lead to pericardial tamponade, blood clots in the liver and heart followed by sudden heart failure and mortality (Ferguson et al., 1990; Pope and Seierstad. 2003). Like HSMI, external disease symptoms are difficult to identify with healthy looking fish suffering sudden mortality. Disease symptoms can include swelling and skin haemorrhages, decreased feeding and slow or erratic swimming (Roger and Turnbull. 2000).

CMS was shown to be transmissible between Atlantic salmon in Scotland (Bruno and Noguera. 2009) and Norway (Fritsvold et al., 2009) after infections of tissue homogenates of CMS infected fish into naïve fish. Following this, infections of PMCV through intraperitoneal injection (IP) were shown to cause cardiac pathology similar to CMS in posts smolts (Timmerhaus et al., 2011). The disease is able to spread and infect cohabiting fish in open water (Haugland et al., 2011) as well as spread horizontally in SW between farms (Jensen et al., 2013). PMCV was also discovered in wild Atlantic salmon in 2012 by Garseth *et al.* (2012). Annual outbreaks of CMS reported in Norway have ranged between 49 and 89 (Jensen et al., 2013) between 2005 – 2012, indicating the financial impacts this can have on

fish farm production, particularly as this disease is prevalent nearer the time of harvest.

1.6.4 Infectious salmon anemia virus (ISAV) and infectious salmon anemia (ISA)

Infectious salmon anemia virus (ISAV) is a highly infectious orthomyxovirus assigned to the genus *isavirus* of which it is the only member. The virus is enveloped and spherical in shape, consisting of 8 negative sense single stranded RNA segments, which encode 10 proteins (Falk et al., 1997; Mjaaland et al., 1997; Clouthier et al., 2002). Nine of these proteins are structural and consist of two surface glycoproteins, a haemagglutinin-esterase (HE) protein, fusion proteins, a matrix protein, a nucleoprotein and accessory proteins (RNA polymerase PB1, PB2 and PA) (Biering et al., 2002; Falk et al., 2004; Aspehaug et al., 2005; Kibenge et al., 2007); see Aamelfot and Falk. (2014) for an in-depth review on structure and function. It is the causative agent of infectious salmon anemia virus (ISA) in Atlantic salmon (Falk et al., 1997; Kibenge et al., 2004) and was first discovered in Norway in 1984 (Thorud and Djupvik. 1988). Since then disease outbreaks have been reported in salmonids across Europe (Roger et al., 1998; Nylund et al., 2003; Plarre et al., 2005), Canada (Mullins et al., 1998; Bouchard et al., 1999; Lovely et al., 1999), USA (Bouchard et al., 2001) and Chile (Godoy et al., 2008; Mardones et al., 2009). Fish inflicted with the disease can suffer severe anemia and structural damage to internal tissues that can lead to mortality.

Clinical signs of ISA are similar to that of the 3 viruses mentioned previously with lethargic sluggish swimming behaviour near the surface, low to non-feeding fish, darkening and haemorrhage of the skin, and pale gills and fins. Internal symptoms can be variable but usually involve swollen spleens; petechial haemorrhage in swim bladders and muscles; and haemorrhages and necrosis of the kidney, liver and renal intestine (Evensen et al., 1991; Mullins et al., 1998, Roger et al., 1998, Bouchard et al., 2001, Simko et al., 2000). Controlling the spread of ISAV is problematic, as replication of ISAV has been experimentally shown in brown trout (Nylund and Jakobsen. 1995) and rainbow trout (Nylund et al., 1997) without any disease outbreaks or symptoms, as well as in wild populations (Plarre et al., 2005); suggesting that some salmonids may be dormant carriers of the disease. Other species of fish may be carriers of ISAV as it has been shown to survive and replicate within herring (*Clupea harengus*) (Nylund et al., 2002) and Atlantic cod (*Gadus morhua*)(Grove et al., 2007) without causing any clinical or disease symptoms.

ISAV was shown to replicate within erythrocytes and induce IFN α production (Workenhe et al., 2008). No subsequent research on ISAV erythrocyte interactions has been conducted at this time however.

1.7 Detection systems for Atlantic salmon aquaculture management

With the abundance and variability of viruses that can potentially infect and cause mortality on a large scale in salmon aquaculture systems, it is of utmost importance that robust and reliable detection platforms are created. Development of qRT-PCR assays has been shown to improve sensitivity in detection of numerous fish viruses; SAV (1 – 3) (Hodneland and Endressen. 2006), ISAV (Munir and Kibenge. 2004) and piscine nodavirus (PNV) (Hick and Whittington, 2010; Panzarin et al., 2010). Although qRT-PCR assays have been developed for PRV (Palacios et al., 2010) and PMCV (Løvoll et al., 2010; Haugland et al., 2011), both assays were based on initial sequencing information and not as a detection tool. SAV currently has no detection assays for SAV 4, 5 and 6, however a recent multiplex assay has attempted to cover all subtypes in a multiplex assay, but was only able to detect 1, 2 and 5 (Shi et al., 2017). The similar external and internal symptoms associated with SAV, PRV and PMCV as well as evidence showing co-infection of 2 or all 3 of the viruses (Lovoll et al., 2010; Haugland et al., 2011; Wiik-Nielsen et al., 2016) shows the difficulties in determining which virus Atlantic salmon are infected with. Having a diagnostic tool that can distinguish between viruses and show the quantities of each virus present within the animal would help improve management greatly, by ensuring the correct steps are taken once results are known to mitigate and control the viral loads present on the farm. This idea can expand to non-viral detection with biomarkers and other genes for rapid detection of other biological processes in Atlantic salmon.

Multiplex diagnostics is a possible route incorporating multiple virus assays into one detection platform. Several marine based multiplex assays have been developed (Khawsak et al., 2008; Kou et al., 2008; Panichareon et al., 2010), however non-specific binding and competition for resources can make multiplex development difficult (Henegariu et al., 1997; Brownie et al., 1997; Polz et al., 1998), and is likely one of the limiting factors in development of these in the marine field.

Mobile platforms have been successfully developed for rapid screening of avian influenza virus (Wahed et al., 2015) and Ebola virus (Faye et al., 2015). Introducing mobile onsite detection platforms for viruses in aquaculture could reduce time to

result, improve response times and overall control outbreaks on farms. Several days could be saved from the sample being sent from the fish farm to the laboratory, and the following analysis and return of the result. This again could be expanded for the use of biomarkers and other gene based analysis in management of Atlantic salmon.

This thesis aims to research, develop and answer the following objectives:

1. To develop and validate a novel NKA qRT-PCR assay utilising the $\alpha 1a$ mRNA marker for determination of smoltification and compare it to the current industry standard NKA activity assay.
2. To develop and validate a multiplex qRT-PCR assay for in lab and onsite detection of SAV, PRV and PMCV
3. To characterise erythrocyte innate immune response to viral infection of ISAV and SAV.

Chapter 2: Na K⁺ ATPase smoltification

2.1 Introduction

The Atlantic salmon like many other salmonid species, exhibits an anadromous lifecycle, where hatching and early development are confined to FW, followed by a migratory phase into SW. During this period fish will have a significant growth in size before completing their adult lifecycle by returning to spawn in FW (Folmar. 1980; Hoar. 1976). Due to the high salinity differences between FW and SW, salmon must adapt their physiology to cope with this osmotic challenge. During the juvenile development stage of salmon, many factors and adaptations take place within the fish due to physical and environmental cues. Throughout this developmental stage environmental factors, mainly seasonal photoperiod and temperature will begin initiation of the smoltification process, preparing the fish for transition into the marine environment (Duston and Saunders. 1990; McCormick et al., 2000; Bjornsson. 2007; Handeland. 2013).

Smoltification is a biological process driven by the endocrine system in which salmon parr are able to adapt into SW ready smolts through a number of morphological, physiological, behavioural and biochemical changes (Hoar 1976; Folmar and Dickhoff. 1980; Barron. 1986; McCormick. 1987; Dickhoff. 1997; McCormick. 2001; Ebbesson et al., 2008; McCormick. 2009a Stefansson. 2012). Many studies have been undertaken to examine the physiological, behavioural and morphological changes that occur in salmon during the smoltification period (McCormick et al., 2013b). These have primarily focused on several endocrine factors such as growth hormones (GH) and thyroid hormones (Prunet et al 1989; McCormick et al 1995, 1996, 2000 2001; Agustsson et al., 2001; Ebbesson et al., 2008; Bjornsson et al., 2011), insulin-like growth factor 1 (IGF-1) (McCormick et al., 1995, 1996, 2000; Dickhoff et al., 1997; Agustsson et al., 2001) and cortisol (McCormick. 1996, 2000, 2001, Kiillerich et al., 2007, Ebbesson et al., 2008). From this research, it was found that GH and cortisol play a significant role in osmoregulation in salmon by inducing development of ionocytes for SW regulation in the gills. The gill epithelium changes from ion absorbing to ion secreting from the migratory stage of FW to SW. McCormick and Saunders (1987) suggested that the transport protein NKA was responsible for salt regulation and seawater acclimatisation in salmon.

NKA concentration has been shown to consistently increase in the gills during smolt SW acclimatisation (Prunet et al., 1989; McCormick. 1995, 1996, 2000, 2001;

Bystriansky and Schulte. 2011; Handeland. 2013). It also appeared to work in conjunction with two other ion transport proteins, NKCC, which assists in salt secretion through gill chloride cells (Pelis et al., 2001); and CFTR, where chloride leaves through an apical channel based on favourable electrical gradients (Marshall and Singer. 2002). This led to more research into the effects of NKA expression in salmon and its potential use as an indicator for smoltification. Five isoforms of NKA were identified and characterised in rainbow trout; $\alpha 1a$, $\alpha 1b$, $\alpha 1c$, $\alpha 2a$ and $\alpha 3$ (Richards et al., 2003). Of the five isoforms, $\alpha 1a$ and $\alpha 1b$ were directly expressed following SW transfers. It was found that $\alpha 1a$ expression decreased sharply when transferred to SW whilst $\alpha 1b$ increased sharply. These isoforms and expression patterns were later confirmed in Atlantic salmon (Mackie et al., 2005; Bystriansky et al., 2006; Nilsen et al., 2007; Madsen et al., 2009). Further data on exposure of mature SW living salmon to FW showed a 7 fold increase in expression of $\alpha 1a$ and 60% decrease in $\alpha 1b$ (Bystriansky and Schulte. 2011). This trend was also confirmed in wild salmon (Stefansson et al., 2012). It was found that $\alpha 1a$ is the FW isoform and $\alpha 1b$ the SW isoform (McCormick et al., 2009b; Madsen et al., 2009). This provides growing evidence towards the importance of the two isoforms in SW regulation and acclimatisation and their potential use as biomarkers for smoltification detection in salmon.

Currently the most common use for smoltification detection in Atlantic salmon is through the smolt index, ATPase enzymatic activity assays (NKA activity assays), plasma chloride measurements and SW bath tests. The smolt index is often used, but is limited in its approach due to it not being an objective test. It is based on a 3 point scoring system judged solely on visible morphological changes that is subjective to the interpretations of the human observer.

Gill based NKA activity assays were developed to focus on the measurement of gill Na^+, K^+ ATPase activity using salmon gill tissue throughout the smolt period (Zaugg 1982). The gill arch is removed and the ATPase enzyme activity measured by testing of the protein. This provides a final NKA activity ($\mu\text{mol ADP}/\text{mg protein}^{-1} \text{h}^{-1}$). A threshold of activity for determining if smoltification had occurred is then used based on previous research or data. Once this threshold is crossed the fish are deemed ready for SW transfer (see section 2.2.5 for more detail). This is currently regarded as a relatively reliable indicator of smoltification due to the tests being objective and quantifiable. However, due to the different roles $\alpha 1a$ and $\alpha 1b$ play in SW acclimatization this assay may not provide a completely accurate picture on the overall role of the protein (Richards et al., 2003). Other factors limiting this technique

are its inability to be conducted in the field due to toxicity and its time consuming process. In addition its statistical solidity is debatable due to inherently high variation in results. However, NKA activity assays are currently the only ones that have been widely applied and accepted on an industrial scale.

SW bath tests (survival tests) were the main standard test used for identification of SW ready smolts before the development of NKA activity assays. They are still currently used today, sometimes in conjunction with NKA activity assays, however they are limited in their indication of SW readiness in salmon. Fish are placed in a SW bath with sea readiness based on survival rate. High mortalities in SW bath tests indicate the fish are not fully adapted for SW conditions with low mortalities indicating they are. This is a welfare issue for salmon due to the stressors associated with the capture handling and exposure to air as well as the potential mortality for underdeveloped smolts exposed to SW. Rises in plasma cortisol level are common and it can take days to weeks for fish to recover back to their baseline levels (Ashley et al., 2007; Stien et al., 2013). Those that have not fully adapted and subsequently die suffer throughout the entire process. Those that do survive are still submitted to a stressful and harmful test that can lead to mortality in the days and weeks after.

A similar test to this is the observation of plasma chlorides in smolts. Studies into SW acclimatisation have shown that plasma chlorides closely mirrored the osmotic changes in Atlantic salmon (Solbakken et al., 1994). Smolts are placed in SW baths for a period of time (12 - 24hrs+) and blood taken to assess their plasma chloride levels. A FW smolt will have a high plasma chloride level (160 – 200 Cl mM) with a SW ready smolt averaging around 150 Cl mM. Smolts can also be left in varying concentrations of SW and assessed over time. SW ready smolts will stabilise their levels to ~150 Cl mM after ~24hrs. A smolt that is not able to regulate its plasma chloride levels will likely suffer mortality with long SW exposure due to the underdevelopment of its gills from FW adaption to SW adaption. This test also suffers from similar welfare issues stated in the SW bath tests above.

Ion regulatory tests have also been used to assess the SW readiness of smolts. The ions normally tested are the key ones associated with ion regulation in the gills (Na^+ , K^+ and Cl^-). Monitoring of the ion concentrations has been shown to indicate when a smolt is adapting to SW conditions. Increased uptake of Cl^- ions through recruitment of Na^+ occurs in smolts both in preparation for and whilst in a SW environment. This is mitigated through the major ion transport protein NKCC1 (see section 1.3.2).

Monitoring of these ion concentrations can help assess how far developed a smolt currently is and whether it is ready for SW transfer. Blood is taken from the fish and ion plasma assessed through ion chromatography.

In this study S0 fish will be examined, however, unlike standard S0 fish they will be on a special feed called Supersmolt[®] for a period of 4 – 6 weeks under 24hr light. The Supersmolt[®] feed helps promote and stimulate the hypo-osmoregulatory changes in the salmon during the smolt window to align together (see section 1.3.5 for more details). This is to promote a higher percentage of the population to enter the SW transfer window at the same time.

Very few developments of qRT-PCR assays utilizing potential biomarkers within salmon during smoltification have been described (Olsvik et al., 2005). Due to the recent development of mobile qRT-PCR platforms and the ease, speed and relative cheapness of the technique, an assay that is developed to successfully track and detect the point of optimal SW transfer of Atlantic salmon would be highly desirable for the industry. With numerous indicators of smoltification available (T-GH, GH, cortisol and IGF-1), testing and development of qRT-PCR assays could provide a fast and effective technique for smoltification analysis.

Therefore the aims of this research were to develop and validate a novel NKA qRT-PCR assay for the marker $\alpha 1a$ mRNA. In order to do this we would:

1. Develop and validate an in lab qRT-PCR assay for the biomarker $\alpha 1a$
2. Conduct a large initial study over 3 years on active hatcheries across Scotland where we would compare the efficiency of our NKA qRT-PCR assay with the industry standard NKA activity assay at detecting smoltification
3. Develop a mobile diagnostic platform, transfer the assay on to the mobile device and conduct field tests onsite at hatcheries.

2.2 Materials and methods

2.2.1 NKA qRT-PCR assay development

2.2.1.1 Primer design

Primers and probe were adapted from Nilsen *et al.* (2007) and Madsen *et al.* (2008) using multiple phylogenetic approaches with different salmoind targets*

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2.2.1.2 Generation of Plasmid standard

The assays were designed for the α 1a region (ATPase) of *S. salar*. The target region was ligated into plasmid pGEM3 and a quantitative DNA standard was derived as described by Weidmann *et al.* (2003).

2.2.1.3 qRT-PCR tests of individual standards

For assay development NKA qRT-PCR was performed in 96 well skirted qPCR plates (20 μ l) containing 1x LightCycler[®]480 RNA Master Hydrolysis Probes, 3.25mM activator Mn(OAc)₂, 500nM primers, 200nM probe and 1 μ l plasmid DNA as template on an Agilent Technologies Stratagene MX3005P (version 4.10, build 389). qRT-PCR reactions were ran in triplicate from 10⁸ - 10¹ DNA molecules per reaction as follows: reverse transcription for 3 min at 63°C, activation for 30 s at 95°C, followed by 45 cycles consisting of amplification for 5 s at 95°C and 15 s at 60°C and a cooling step of 40 s at 40°C.

For transfer of the NKA qRT-PCR assay onto the mobile SmartCycler[™] system, qRT-PCRs were performed using the LightCycler 480 RNA master hydrolysis probes (Roche) in 25 μ l SmartCycler[™] tubes. Due to the increase in tube volume concentrations were kept the same as described above, but volume was increased for all components including template DNA (2.5 μ l). Dried primer, probe and quantitative RNA standard were tested on the SmartCycler[™] in triplicate to ensure sensitivity was not lost between devices.

2.2.1.4 Dried standard, primers and probe

For optimal long term storage and stability, DNASTable[®] plus (Biomatrix) was used to dry quantitative DNA standard, primers and probes using a ratio of 1:4 DNA stable solution to sample volume. The solutions were mixed by pipetting every 2 - 5 min for 15 min and then dried in a DNA speed Vac – DNA110 (Savant). All samples were performed in triplicate. Samples were then rehydrated by adding the same volume of H₂O as the original sample volume. The rehydrated samples were then

tested in a qRT-PCR using LightCycler 480 RNA master hydrolysis probes (Roche) following the qRT-PCR protocol described above (section 2.2.1.3). Some samples were left in a dried condition for 2 weeks then rehydrated and tested by qRT-PCR to test long-term storage.

2.2.2 RNA extraction

RNA extractions were carried out using the QuickGene Mini80 RNA extraction robot. Gill tissue samples were cut and weighed to a specified weight (10-20mg). These were then placed in 1.5ml tubes containing 500µl of lysis buffer, three 3.5mm glass beads and 0.1mm zirconia beads. The samples were then vortexed for 30 s to breakdown the gill tissue followed by centrifugation for 3 mins at 16 800g in a 5418R centrifuge (Eppendorf). This was then repeated. Supernatant (350µl) were removed from each sample and transferred to new 1.5ml tubes and 175µl of solubilisation buffer added. The tubes were briefly vortexed and 175ml of ethanol then added. The tubes were then vortexed for 1 minute and briefly spun down in the centrifuge. The supernatants of each sample were transferred into the individual microtube columns of the Mini80. The supernatant was washed through the column with a further 3 wash steps of 750µl of wash buffer for each sample. To ensure high RNA yield 50µl of elution buffer was used per sample. Elution buffer was left in the column for 4 minutes before being washed through into empty 1.5ml tubes. RNA concentration was determined using the ND-1000 system (NanoDrop, Thermo Scientific) and ND-1000 version 3.8.1 software. Extracted RNA was then diluted (200-400ng/µl) and frozen at -70°C.

2.2.3 Fish handling and sampling

Gill tissue samples were collected from 11 farms across Scotland over a 3-year sampling period (2015 – 2017). All farms were located on the Western Coast of Scotland and used FW tanks for smolt production, except for Loch Shin, which used FW sea cages (Table 2.1). The farms were spread from the Mid Western Coast beginning at Ormsary, up to the North Western coast and across to the Isle of Harris, with the most Northern farm located on the Shetland Islands (Girista) (Figure 2.1). The farms varied in size and production with Russel burn and Ormsary the largest producers, and Barvas and Mingary with significantly lower production. For each year's analysis the secondary gill arch was collected at 16 sites (2015), 13 sites (2016) and 9 sites (2017) for NKA activity assay (n=25) and NKA qRT-PCR (n=8) from S0 smolts. Both left and right gill arches were collected from the same fish at each time point for NKA qRT-PCR and NKA activity analysis. Gill samples for NKA activity assays analysis were collected in 1.5 ml tubes containing a 1%

Sucrose ethylenediaminetetraacetic (EDTA) imidazole deoxycholate (SEID) solution, and for NKA qRT-PCR analysis in 1.5ml tubes containing RNA later. Three time points of initial (I), mid (M) and final (F) were pre-determined with a window of 1-3 weeks between each point. I points corresponded with the beginning of feeding of salmon with SuperSmolt® feed only, and F points with the ending of feeding before SW transfer. M points were taken between 2 - 3 weeks after initial feeding. For more detail on start and end dates, average fish size and k-factor see appendix C2.13. SuperSmolt® is a feed provided by Europharma Scotland Ltd that homogenises the osmoregulatory development of salmon during smoltification to ensure all salmon are ready for SW transfer at the same time. A constant light photoperiod regime (24h) was utilised throughout the testing period (I – F points) at each site over the 3-year study. Analysis of NKA enzyme activity assays were conducted at the lab at Europharma Scotland Ltd. NKA qRT-PCR analysis were carried out at the Institute of Aquaculture, University of Stirling. Sites sampled, as well as number of tanks varied from year to year with some only being sampled over 1 or 2 years.

Table 2.1. Table showing basic information on location, tanks, production and owners of all the farms where samples were gathered for the 3 year investigation of NKA activity assays and NKA qRT-PCR assays. * Represents the farms visited for onsite analysis of the mobile NKA qRT-PCR.

Site	Acronym	Location	No. of Tanks	Fish produced	Company
Russel Burn	RB	Russel burn, Strathcarron	6 G tanks (340m ³), 20 A and B tanks (40m ³)	Up to 3.2million	The Scottish Salmon Company (SSC)
Ormsary*	OR	Ormsary, Lochgilphead Argyll	Range of tanks of variable size (95m ³ and 31m ³)	Up to 2.4 million	Landcatch Natural Selection Ltd
Geocrab	GC	Geocrab Mill, Isle of Harris, South Harris	6 tanks (180m ³)	Up to 1 million	The Scottish Salmon Company
Gairloch	GL	Inver Kerry, Gairloch, Ross-shire	18 (25m ³)	Up to 570k	Landcatch Natural Selection Ltd
Loch Damph*	LD	Loch damph, Kishorn, Strathcarron	12 loch cages	Up to 800k	Scottish Sea Farms Ltd
Loch Shin	LS	Loch Shin, Overscaig, Sutherland	31 fresh water cages	> 1 million	Migdale Smolts Ltd
Barvas	BAR	Barvas, Isle of Lewis	18 (20m ³) tanks	Up to 330k	The Scottish Salmon Company
Girlsta	GIR	Girlsta, Shetland	No information	No information	Grieg Seafood Shetland Ltd (Hatchery)
Mingarry*	MIN	Mingarry, Milton, South Uist	42 (10m ³) tanks	Up to 340k	Hebridean Smolts Ltd
Clachbreac	CLA	Clachbreac, Ormsary, Lochgilphead, Argyll	7 (95m ³) tanks	Up to 500k	Landcatch Natural Selection Ltd
Kinlochmoidart	KLM	Kinlochmoidart Lochailort, Inverness-shire	7 tanks and 2 RAS systems (total vol 770m ³)	Up to 600k	The Scottish Salmon Company



Figure 2.1. Locations of the 11 farms sampled in the 3-year study.

2.2.4 NKA qRT-PCR assay S0 trials

NKA qRT-PCR were conducted using Brilliant III ultra-Fast SYBR[®] Green qRT-PCR Master Mix. Reactions were performed in a 20µl volume containing 2x SYBR green qRT-PCR master mix, 500nm primers and 1µl RNA as template. RNA samples were grouped into I, M and F point plates. Two standard curves were ran 10^8 - 10^1 as well as 4 negative controls (NTCs) per plate. The temperature profile was adapted in the reverse transcription step for 10 min at 50°C, activation for 3min at 95°C, followed by 45 PCR cycles as previously described (section 2.2.1.3).

2.2.5 NKA activity assay

ADP standards were checked everyday to ensure measured conversion of NADH to NAD⁺ was within a kinetic correlation coefficient of >0.99% with an optical density (OD) of between 0.6 - 0.8 between the time points of 0 - 150 seconds. The ADP standard curve used ranged from 1nM - 25nM. ADP standards were defrosted, vortexed and a 10µl volume pipetted into a 96 well flat bottom non-sterile ELISA

plate in duplicate for each value range. 200µl of Na⁺ K⁺ ATPase inhibitor –ve ouabain was added to each well and ran in a Versa max microplate reader (SoftMax 4.5.4) for 5 mins at 25°C. If the standards adhered to the acceptable range of NADH conversions then the next step was carried out. If the standards were outside the acceptable range the plates were re-run and/or new standards of –ve ouabain were made.

For each day of analysis, three concentrations of SEID solution were made (0.5%, 1% and 7:3) along with an assay mix containing 0.23nm NADH, 1M imidazole, salt solution, 0.53mM adenosine 5'triphosphate disodium triphosphate (ATP), 2.1mM phosphoenolpyruvate monopotassium salt (PEP), b-nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADH), 3.75mM L-lactate dehydrogenase from rabbit muscle, (LDH) pyruvate kinase from rabbit muscle (PK) and distilled water (dH₂O). Twenty-five gill samples from farms and 2 quality control gill samples of known activity were prepared per run. Once defrosted, gills were placed in a 5ml tissue grinder tube containing 1ml of 7:3 SEID solution. Gills were then manually crushed for 1 minute. The gills were graded against a dilution factor based on fish weight, and volumes of gill homogenate transferred into 1.5 ml tubes in the corresponding volume of 0.1% SEID solution. All samples were then centrifuged at 350g for 5 minutes and the supernatants transferred to 1.5ml tubes and placed on ice. Bradford protein assays were performed at 25°C for 5 minutes on all samples to determine if the proteins fell between a range of 500-1500ug/ml. If proteins fell outside this range the dilution of supernatant to H₂O was recalculated. 80µl of each sample were transferred into a new 1.5ml tube containing 320µl of H₂O. 10 µl of each sample and a protein standard curve were pipetted onto a 96 well plate in quadruplicate and 200µl of –ve ouabain was then added to each well and analysed. Once the protein analysis was verified, 10µl of each sample was pipetted into a 96 well plate in octuplicate. 200µl of –ve ouabain was added to one half of the plate containing 4 of the 8 repeated samples, with 200µl of +ve ouabain added to the other half. NKA activity assay analysis was then performed at 25°C for 20 minutes. NADH disappearance was measured at 340 nm and NKA activity (µmol ADP mg protein⁻¹ h⁻¹) generated based on the variance between the absence and presence of ouabain on ATP hydrolysis. A threshold for determining if smoltification had occurred was used at an activity level of 10 µmol ADP/mg protein⁻¹ h⁻¹ and above for the protein. This threshold is based on previous successful transfer data obtained from Europharma over several years. Known quantitated samples from the

previous S0 season were used as quality controls for both the NKA activity assay and protein readings alongside the unknown samples.

2.2.6. 3-year NKA activity and NKA qRT-PCR statistical analysis

Unpaired T-tests were conducted on NKA qRT-PCR results and NKA activity assays for sites sampled at I and F testing points only. For sites sampled at I, M and F point's one-way ANOVAs were used. We compared copy number fold change (fc) between sampling time frames on sites for NKA qRT-PCR and NKA activity fc for NKA activity assays.

2.2.7 Smolt index analysis

Smolt indexes were taken for each fish at each site using a 3-point scoring system (Sigholt et al., 1995) based on silvering of fish, presence or lack of parr marks and changes in fin morphology. A number range from 1 – 4 was used, with 1 referring to parr like morphology and 4 to fully smoltified salmon morphology. Each score was totalled and averaged to give the final smolt index of the fish.

2.2.8 Three year sea water temperature analysis

Average sea surface temperatures were obtained from the National Oceanic and Atmosphere Administration (NOAA) website using 1/4° Optimum Interpolation Sea Surface Temperature (daily OISST) data for 2015 – 2017 at 3 locations on the West coast of Scotland (South to North). Each fish farm was assigned one of the 3 location temperatures based on closest proximity. Daily temperatures between June and September during a 3-year period were analysed and data from each site were compared.

2.2.9 Three year Degree day analysis

Daily average temperatures were obtained from the Scottish Salmon company (SSC) at sites Russel burn and Geocrab for all 3 years of testing. Degree-days were calculated based on the formula $DD = (\bar{T}_{Max} + \bar{T}_{Min}/2) - T_0$ from temperatures recorded on site against a temperature threshold (T_0) of 0°C. \bar{T}_{Max} and \bar{T}_{Min} are the maximum and minimum daily ambient temperatures recorded. All I points for each site started at 0 dd with subsequent days calculated based on daily temperature records up until each F point. For sites that included M points these were also recorded. The data obtained was then analysed and compared between sites and years.

2.2.10 Mobile NKA qRT-PCR testing

2.2.10.1 SmartCycler™ system

To test the NKA qRT-PCR onsite a suitcase laboratory was assembled based on experiences with a mobile laboratory developed for the detection of haemorrhagic fever viruses (Wahed et al., 2015; Faye et al., 2015). The mobile laboratory consisted of one suitcase containing everything needed for RNA extraction of gill arches, one suitcase containing a PCR flow workbench, and one suitcase containing the mobile SmartCycler™. A small onsite test was conducted on salmon smolts (n=32) at Clachbreac, Scotland (Landcatch Natural Selection Ltd). Gill arches were removed and RNA extracted (see section 2.2.2) and tested by qRT-PCR using dried ATPase RNA standard, primers and probes. All extracted RNA was transported back to the Institute of Aquaculture on ice where it was re-tested on the SmartCycler™.

2.2.10.2 Genesig Q16 system

Tests were conducted onsite at 3 fish farms in Scotland (Ormsary, Landcatch Natural Selection Ltd; Clachan, Hebridean smolts Ltd; and Loch Damph, Scottish Sea Farms) at two different time points. This was to refine the mobility of the onsite NKA qRT-PCR platform as well as validate it on a new PCR platform (Genesig Q16 qRT-PCR system). We tested 28 salmon smolts per site per visit (n = 28). Gill arches were removed, and RNA extracted (see section 2.2.2) and tested using an ATPase kit (PrimerDesign Ltd) containing 2x Oasig master mix, dried primers and probe, positive RNA extraction control and positive ATPase control. The ATPase kit was developed using the same primers and probe used for the 3-year analysis and SmartCycler™ tests.

2.3 Results

2.3.1 NKA qRT-PCR assay development

To validate our new primer and probe, qRT-PCR reactions were carried out in triplicates on the MX3005 cycler using a decimal dilution range of 10^7 - 10^1 RNA molecules per reaction. The NKA assay yielded a standard curve of high efficiency (98.9%, standard error (SE) 0.24) and sensitivity, detecting down to 10^2 copies (Figure 2.2).

2.3.2 Dried primer, probe and standards tests

Primers, probe and standards were successfully dried and rehydrated after daily and long-term storage (2 weeks). Primers and probe were tested against both normal non-dried standard (10^5) and rehydrated dry standard (10^5) to check for any loss of efficiency or sensitivity. Both tests showed no loss of sensitivity to non-dried (C_T 24.3 ± 0.3) and dried standard, primer and probe (C_T 24.7 ± 0.6).

2.3.3 Transfer of NKA qRT-PCR assay to SmartCycler™

The assay was successfully transferred to the SmartCycler™ system, tested in triplicate and yielded a robust standard curve showing high efficiency (93.43%, SE 0.119) (Figure 2.2). The assay detected down to 10^2 copies indicating no loss of sensitivity between devices. There was however a minor loss in efficiency of ~5%.

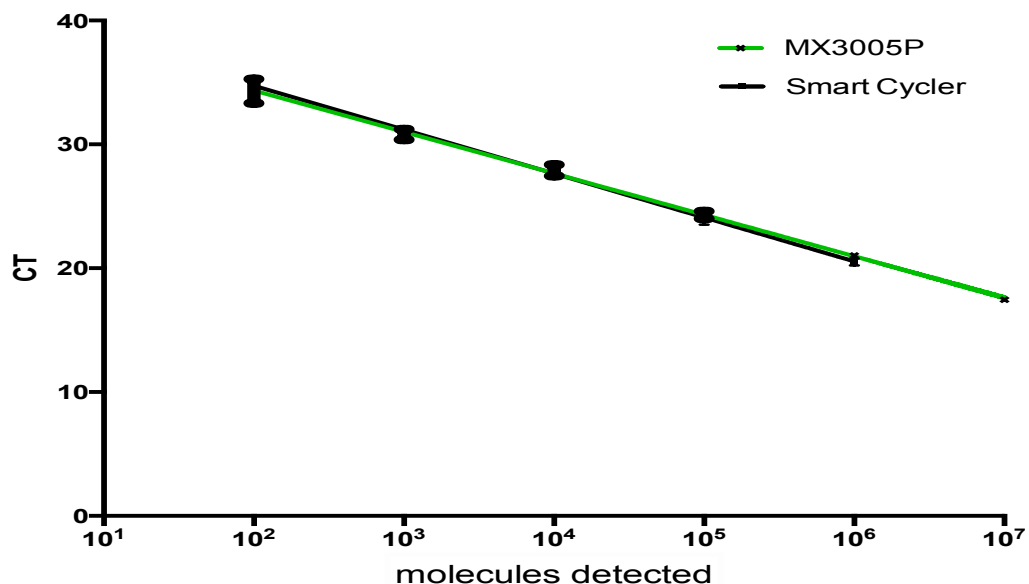


Figure 2.2. Standard curve results for MX3005P NKA qRT-PCR assay (98.9%, SE 0.24) and SmartCycler™ assay (93.43%, SE 0.119). Each dilution ranged from 10^7 - 10^1 RNA molecules. These were tested in triplicate with their mean values plotted as the C_T value points along with SE (not all visible).

2.3.4 Comparative ATPase assay validation

2.3.4.1 Analysis of sites sampled at I and F time points 2015

2.3.4.1.1 NKA activity results

Of the 16 sites tested by NKA activity assays, 12 showed a significant increase ($P < 0.05$) in NKA activity (Figure 2.3, appendix C2.1). Gairloch A and B had the largest fold change (fc) (tank A, 3.77; tank B, 3.7). The lowest fold change was recorded at Russel Burn E (1.19). No other sites with multi-tank sampling showed a universal increase in NKA activity, with Geocrab, Russel burn and Ormsary each having one tank with no significant fold change in NKA activity.

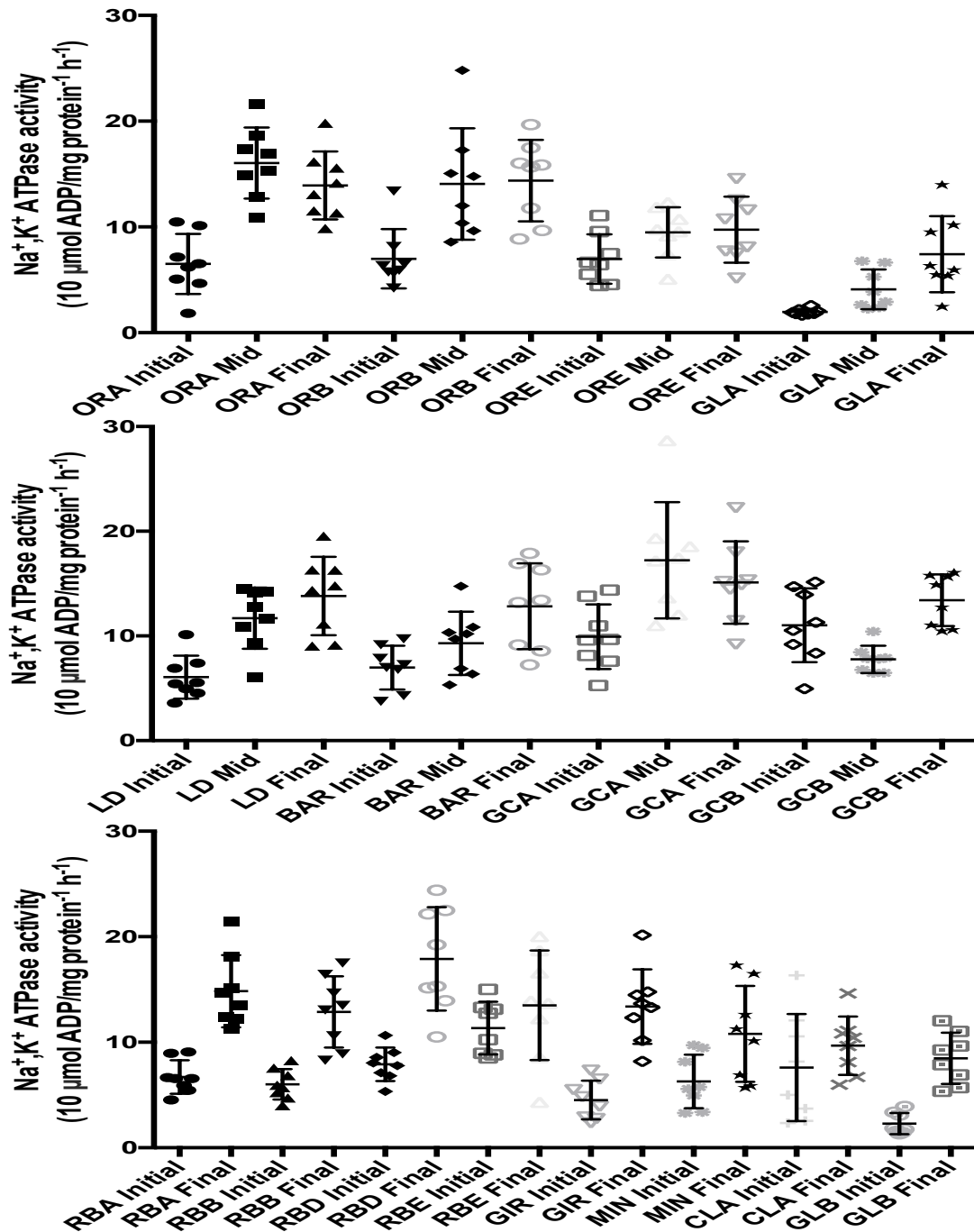


Figure 2.3. Mean NKA activity assays for I, M and F points for 16 individual sites in Scotland (2015). Ormsary A, B and E (ORA, ORB and ORE), Gairloch A and B (GLA and GLB); Loch damp (LD); Barvas (BAR); Geocrab A and B (GCA and GCB); Russel burn A, B, D and E (RBA, RBB, RBD, RBE); Girlsta (GIR); Mingary (MIN); and Clachbreac (CLA). N = 25, NKA enzymatic activity expressed as mean \pm standard deviation (StD). The red dotted line indicates the enzymatic activity value used to identify when smoltification has occurred and fish are safe for SW transfer. This has been determined by historical data of successful transfers by Europharma Ltd.

2.3.4.1.2 NKA qRT-PCR results

A significant decrease in NKA mRNA abundance was observed at 9 of the 16 sites (Figure 2.4 appendix C2.4). The largest significant decrease was found at Girlsta (3.26 fc) and lowest at Gairloch A (2.11 fc). Of the 6 remaining sites, 4 showed no significant decrease in NKA copy number. Russel burn D (2.75 fc) and Clachbreac (1.67 fc) were the only tanks to show a significant increase in NKA copy number. Ormsary showed a universal decrease in NKA copy number over multi-tank sampling.

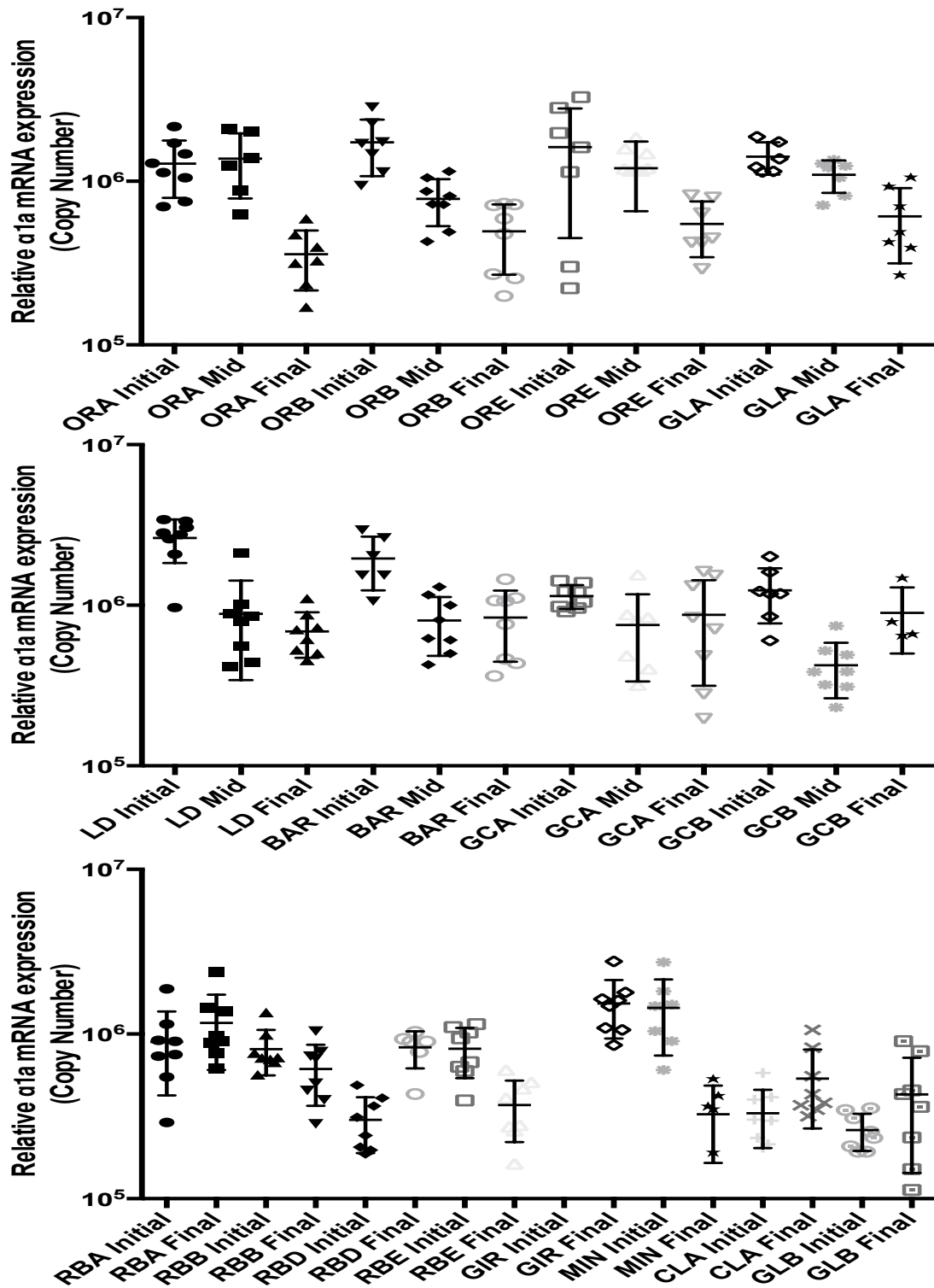


Figure 2.4. Mean NKA qRT-PCR for I, M and F points for 16 individual sites in Scotland (2015). Ormsary A, B and E (ORA, ORB and ORE); Gairloch A and B (GLA and GLB); Loch damp (LD); Barvas (BAR); Geocrab A and B (GCA and GCB); Russel burn A, B, D and E (RBA, RBB, RBD, RBE); Girlsta (GIR); Mingary (MIN); and Clachbreac (CLA). N = 8, NKA copy number expressed as mean \pm StD.

2.3.4.2 Analysis of sites sampled at M points 2015

2.3.4.2.1 NKA activity results

Eight sites were tested for significance between I - M and M - F. For NKA activity assays 4 showed a significant increase between I - M points and 2 between M - F points (Appendix C2.7). Ormsary E was the only tank to show no significance between all 3-time points.

2.3.4.2.2 NKA qRT-PCR results

Of the 8 sites tested for I, M and F points, 5 showed significant decreases between I – M points and 2 between M – F points for NKA qRT-PCR (Appendix C2.10). There appeared to be no correlation between individual sites with Geocrab and Ormsary sites showing opposite results for different tanks. Geocrab A was the only site to show no change in NKA copy number over all 3-time points.

2.3.4.3 Analysis of sites sampled at I and F points 2016

2.3.4.3.1 NKA activity results

From the 13 sites sampled, 10 were found to have significant increases in activity (Fig 2.5, appendix C2.2). The largest fc was found at Ormsary E (3.73) and lowest at Ormsary A (1.41). One site showed a significant decrease in activity (Loch Shin 1.34).

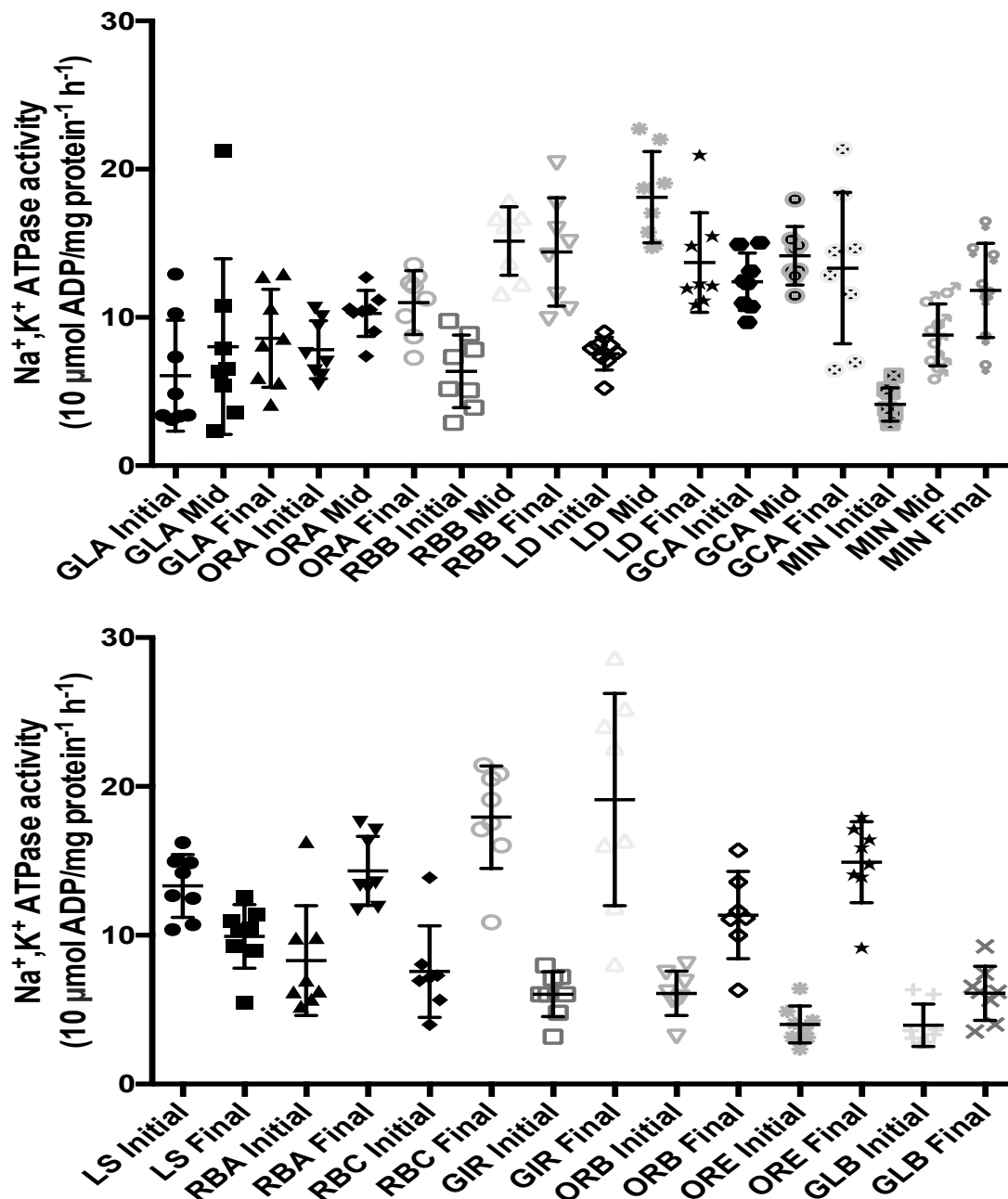


Figure 2.5. Mean NKA activity assays for I, M and F points for 13 individual sites in Scotland (2016). Ormsary A, B and E (ORA, ORB and ORE); Gairloch A and B (GLA and GLB); Loch damp (LD); Geocrab A (GCA); Russel burn A, B, C (RBA, RBB, RBC); Mingary (MIN); Girlsta (GIR); and Loch Shin (LS). N = 25, NKA enzymatic activity expressed as mean \pm StD. The red dotted line indicates the enzymatic activity value used to identify when smoltification has occurred and fish are safe for SW transfer. This has been determined by historical data of successful transfers by Europharma Ltd.

2.3.4.3.2 NKA qRT-PCR results

For NKA qRT-PCR a significant decrease in NKA copy number was found in 7 of the 13 sites (Figure 2.6, appendix C2.5). The largest fc was found at Gairloch A (2.4) and lowest at Girlsta (1.5). Four sites showed significant increases in NKA copy number; Russel burn A (2.66), B (1.3) and C (1.92), and Loch Shin (1.53).

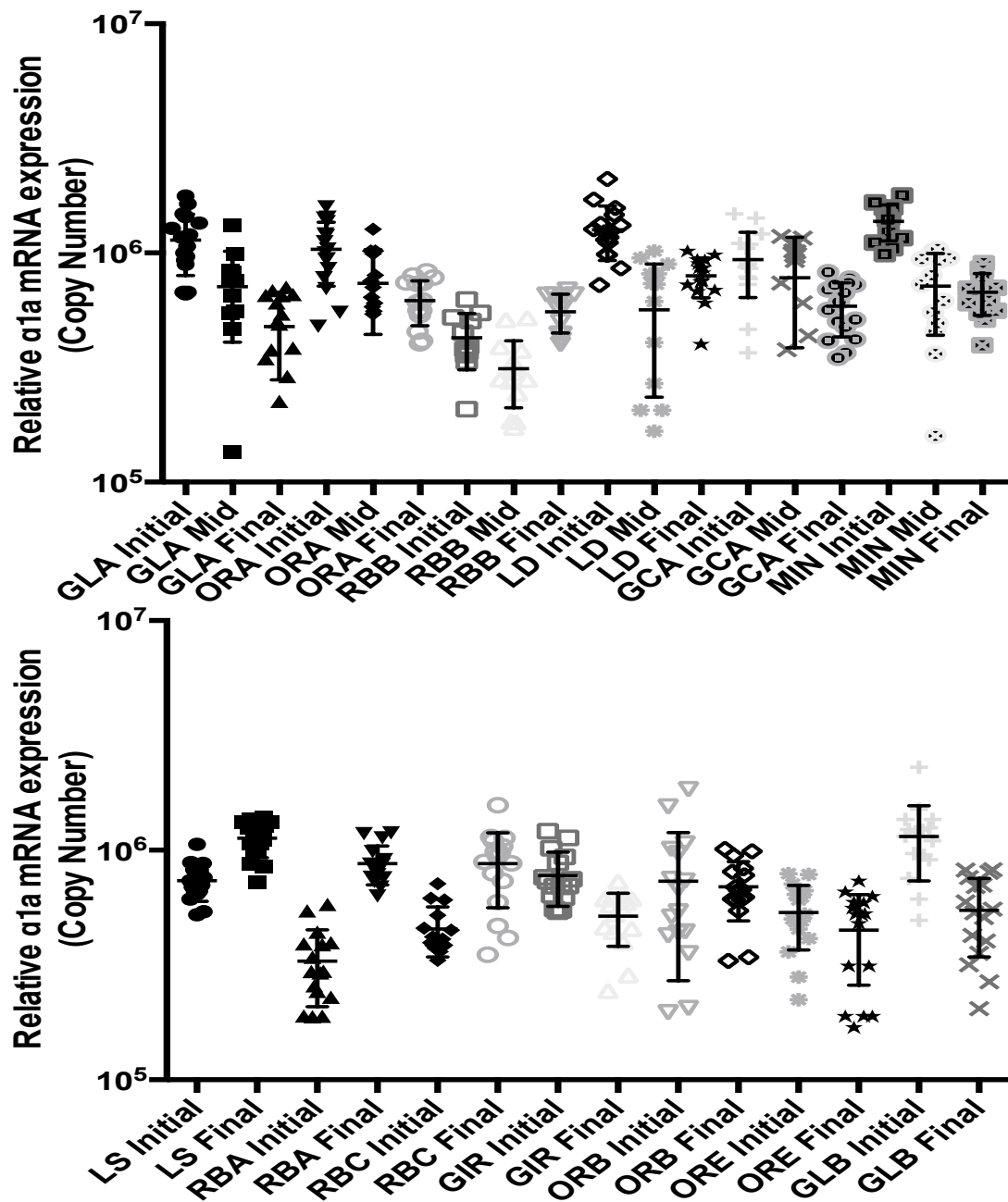


Figure 2.6. Mean NKA qRT-PCR for I, M and F points for 13 individual sites in Scotland (2016). Ormsary A, B and E (ORA, ORB and ORE); Gairloch A and B (GLA and GLB); Loch damph (LD); Geocrab A (GCA); Russel burn A, B, C (RBA, RBB, RBC); Mingary (MIN); Girlsta (GIR); and Loch Shin (LS). N = 8, NKA copy number expressed as \pm StD.

2.3.4.4 Analysis of sites sampled at M points 2016

2.3.4.4.1 NKA activity results

NKA activity showed a significant increase between I and M for 4 of the 6 sites and for 2 sites between M and F (Appendix C2.8). Only two sites (Loch Damph and Mingary) showed significant increases between I - M - F points.

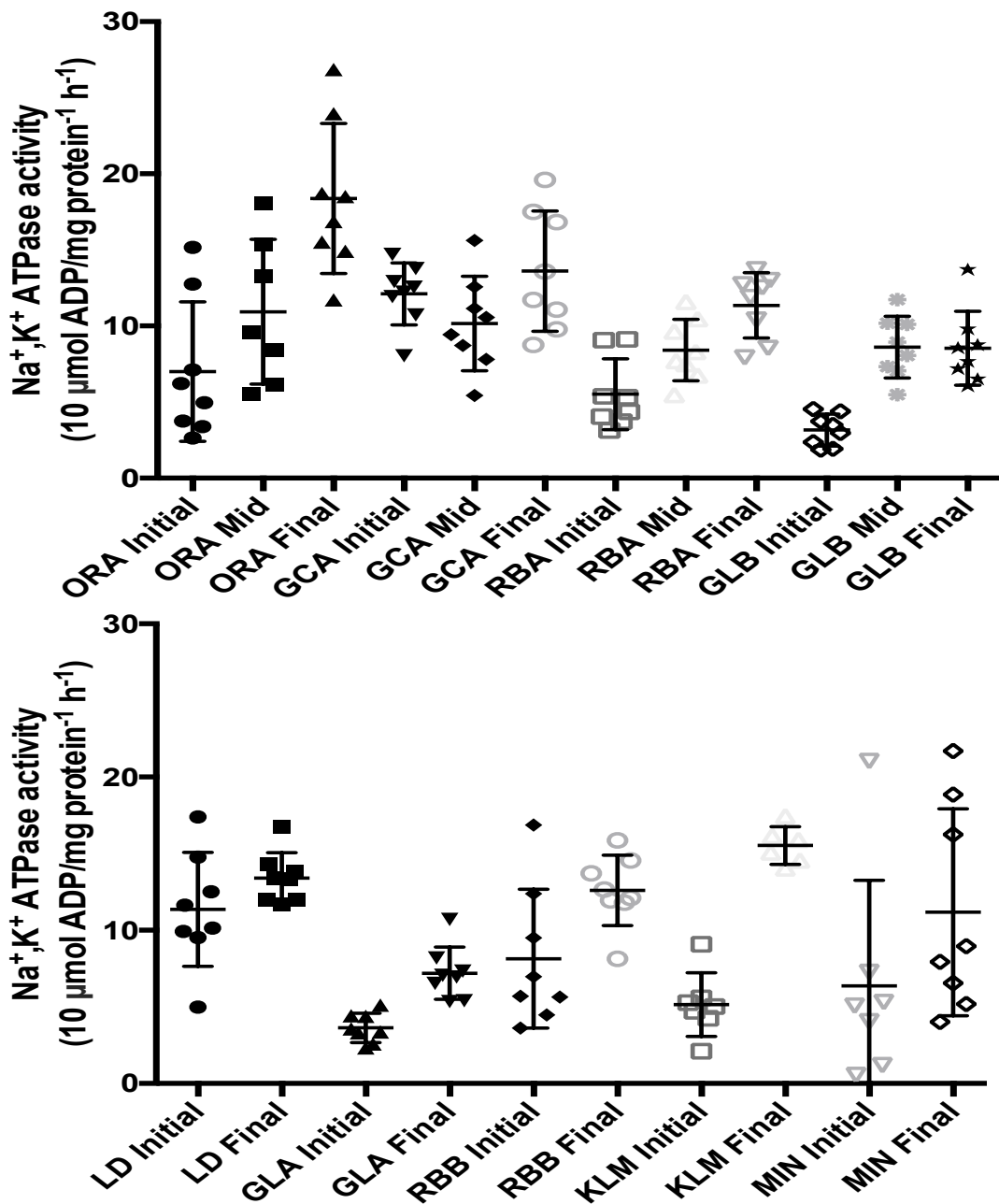


Figure 2.7. Mean NKA activity assays for I, M and F points at 9 individual sites in Scotland (2017). Ormsary A (ORA); Gairloch A and B (GLA and GLB); Loch damph (LD); Geocrab A (GCA); Russel burn A and B (RBA, RBB); Mingary (MIN); and Kinlochmoidart (KLM). N = 25, NKA enzymatic activity expressed as mean \pm StD. The red dotted line indicates the enzymatic activity value used to identify when smoltification has occurred and fish are safe for SW transfer. This has been determined by historical data of successful transfers by Europharma Ltd.

2.3.4.4.2 NKA qRT-PCR results

NKA qRT-PCR showed a significant increase between initial – mid for 5 of the 6 sites and 1 site mid final (Russel burn B) (Appendix 2.11). Only one site (Russel burn B) showed a decrease between I - M - F points.

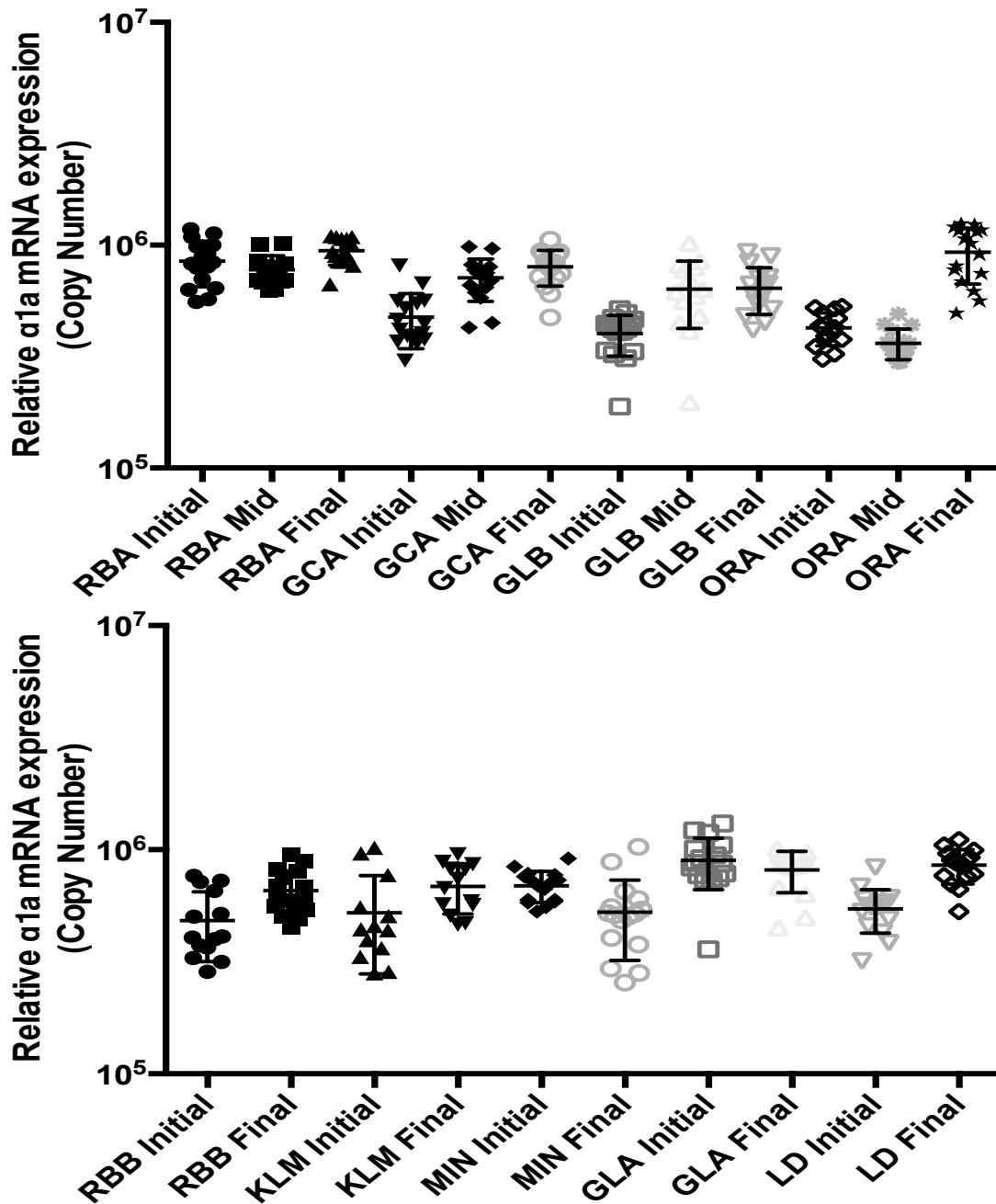


Figure 2.8. Mean NKA qRT-PCR for I, M and F points for 9 individual sites in Scotland (2017). Ormsary A (ORA); Gairloch A and B (GLA and GLB); Loch damp (LD); Geocrab A (GCA); Russel burn A and B (RBA and RBB); Mingary (MIN); and Kinlochmoidart (KLM) N = 8, NKA copy number expressed as mean ± StD.

2.3.4.5 Analysis of sites sampled at I and F points 2017

2.3.4.5.1 NKA activity results

Overall 9 sites were examined of which 6 of the 9 sites showed a significant increase in NKA activity between I and F points (Figure 2.7, appendix C2.3). Two of these were below the threshold of acceptable levels of NKA for safe transfer to SW (Gairloch A and B). The largest fc was at Kinlochmoidart (3.02) and the lowest at Geocrab (1.12).

2.3.4.5.2 NKA qRT-PCR results

NKA qRT-PCR had only one site where NKA copy number decreased between I and F points (Mingary)(Figure 2.8, appendix C2.6). Six sites showed an increase in copy number between I and F points. The largest fc was at Ormsary (2.19) and lowest at Gairloch A and Russel burn A (1.1).

2.3.4.6 Analysis of sites sampled at M points 2017

2.3.4.6.1 NKA activity results

NKA activity showed a significant increase between I and M for 4 of the 6 sites and for 2 sites between M and F (Appendix C2.9). Only two sites (Loch damph and Mingary) showed significant increases between initial – mid – final points

2.3.4.6.2 NKA qRT-PCR results

NKA qRT-PCR showed a significant increase between I and M for 2 of the 4 sites (Gairloch B and Geocrab) and for 2 sites between M and F (Ormsary and Russel Burn A)(Appendix C2.12).

2.3.5 Three year sea water temperature analysis

Average sea temperatures for all 3 years were split into 3 sites based on closest location to each farm. Site 1 consisted of Ormsary and Clachbreac. On average 2017 was warmer from Jun – Sep against 2015 (0.77 – 1.4°C) and Jul – Aug against 2016 (0.1 – 0.69°C). Site 2 included Russel Burn, Geocrab, Kinlochmoidart and Mingarry. On average 2017 was warmer than 2015 between Jun – Aug (1.12 – 1.79°C) and 2016 between Jun – Aug (0.29 – 1.05°C). Site 3 included Gairloch, Loch shin, Barvas and Loch Damph. Overall 2016 and 2017 were warmer on average than 2015 between Jun and September (0.41 – 1.57°C) with 2016 and 2017 on average within 0.1 – 0.3°C between Jun – Oct.

2.3.6 Three year Degree day analysis

Overall 2 sites were examined over the 3-year period. The dd between I and F points were fairly consistent for Russel burn across the 3-year study (327 – 392dd) as were the feeding regime period (FRP) (26 – 29 days) (Figure 2.9). Russel burn C 2016 was the only site to have a longer FRP of 35 days resulting in 504dd at F point. For Geocrab there was a much higher variation in dd (255 – 706dd) and FRP (23 – 43 days). The shortest FRP and DD were observed at Geocrab B 2017 and longest at Geocrab A 2016. Five of the 14 sites tested were observed to have FRP >400dd. This is past the generally accepted SW transfer window point.

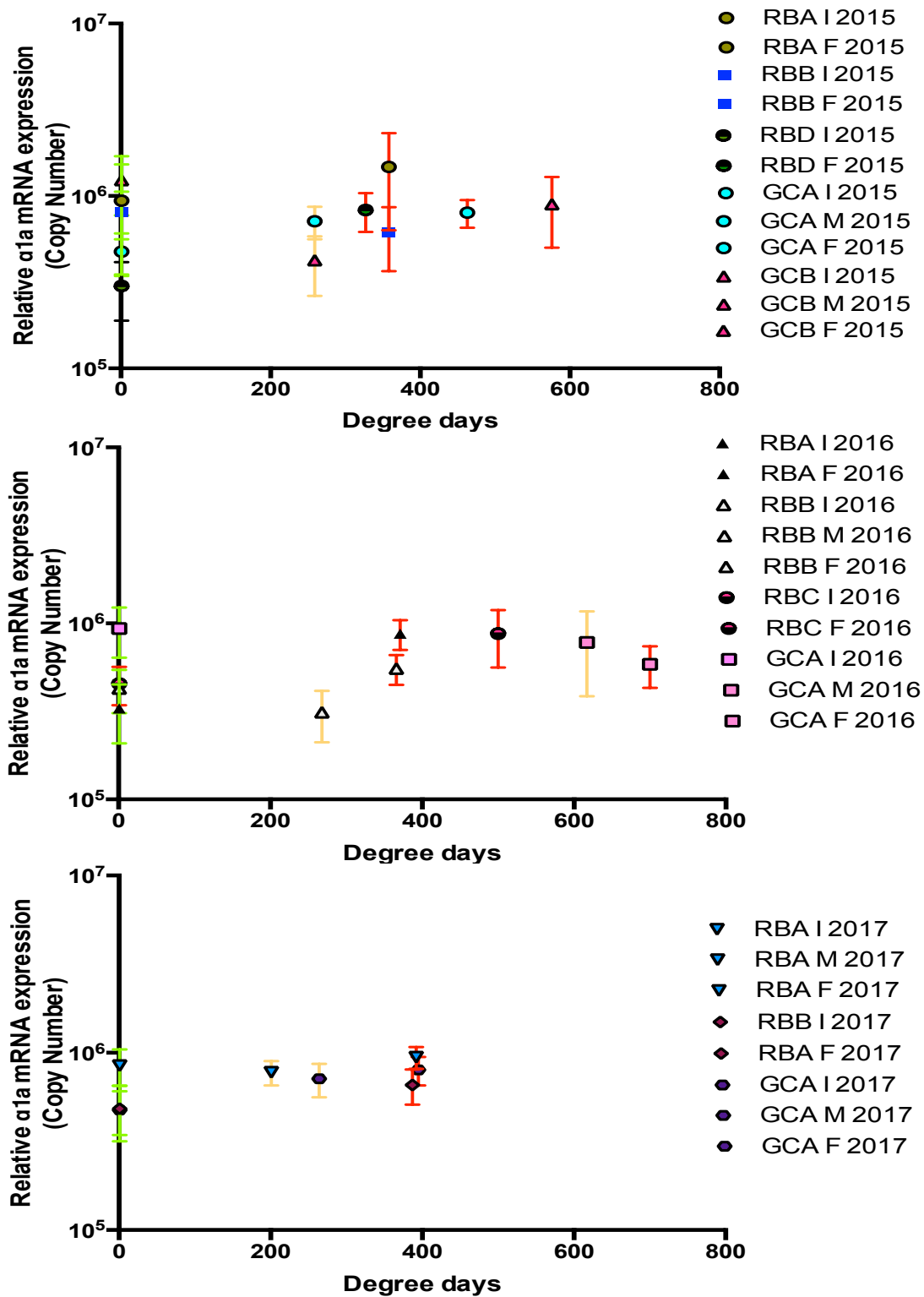


Figure 2.9 Mean NKA qRT-PCR mRNA copies expressed for I, M and F points for sites Russel burn (RBA, RBB, RBC and RBD) and Geocrab (GCA and GCB) against degree days (dd) over the 3-year study period. N = 8, NKA copy number expressed as mean \pm StD.

2.3.7 Onsite versus Lab testing SmartCycler™

On site RNA extraction and qRT-PCR were successfully carried out using the onsite mobile lab at one site. From the 32 samples tested on site an average C_T of 26.49 ± 1.69 SD was found. We then re-tested 16 of the extracted RNA samples a day later in the lab with an average C_T of 26.96 ± 2.1 SD. No statistically significant difference was found between on site testing and in lab testing ($p = 0.338$).

2.3.8 Onsite testing Q16 qRT-PCR mobile lab

On site RNA extraction and qRT-PCR on the Q16 device were successful carried out at all 3 sites (Figure 2.9). There was a significant increase in NKA copy number at Ormsary between I and F ($p = 0.03$). Both remaining sites showed no significant change in NKA copy number (Clachan: $p = 0.59$, Loch Damph: $p = 0.08$). There were notable issues with the positive control for both F points at Ormsary and Clachan. Positive control variation ranged from 13 – 17 C_T where a good positive control was expected to range from 17 ± 1.4 C_T . Due to these issues we were unable to reliably compare copy numbers between sites. The mobile equipment used was reduced from our initial tests with the SmartCycler™ system with reduction in the size of the cases used and reduction in equipment taken.

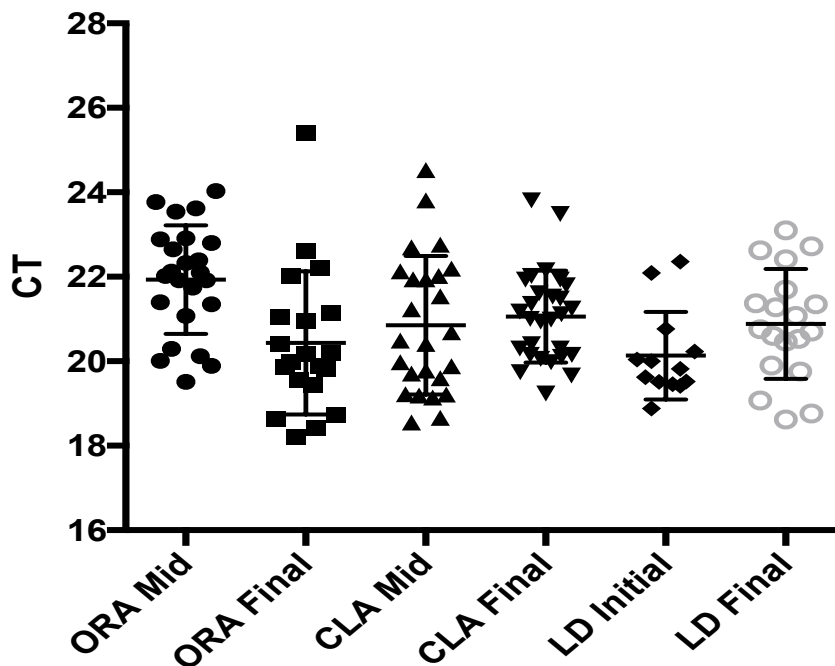


Figure 2.10. Onsite testing of the NKA qRT-PCR assay on the Q16 system at sites Ormsary (ORA), Clachan (CLA) and Loch Damph (LD). Tests were conducted at two different time points at 3 different sites. $N = 28$, NKA C_T expressed as mean \pm StD

2.4 Discussion

Analysis of the initial and final points of all three years showed a significant increase in NKA activity at 28 of the 38 sites tested and a significant decrease at 1 site. The NKA activity threshold of 10 for SW readiness was not achieved in 8 of the 38 sites and 5 sites showed SW readiness already at the initial point of testing. The NKA threshold of 10 was determined by Europharma Scotland Ltd based on historical data of successful transfers of fish. NKA qRT-PCR assay expression significantly decreased at 17 out of the 38 sites. In contrast, a significant increase was seen at 7 of the 38 sites. The NKA activity assays results were comparable with the NKA qRT-PCR assay results at 15 out of the 38 sites. As a secondary reference these results were compared with the smolt index recorded at each site to try and better understand variations in the results of the two tests. For 2015 and 2016, both NKA activity assays and NKA qRT-PCR showed similar agreement with the smolt index, although not at all the same sites (Table 2.2). In 2017 there was higher agreement between NKA activity assays and smolt index (4/9 sites) than NKA qRT-PCR (1/9 sites). Overall only 27% of all sites showed unanimous agreement between the 3 tests over the 3-year period.

Table 2.2. Comparison of NKA enzymatic activity and NKA α 1a qRT-PCR expression to smolt index over the 3-year sampling period. It was found that in 2015 and 2016 both tests showed no significantly higher correlation with the smolt index. In 2017, NKA activity assays were shown to correlate higher (4/9) with smolt index than NKA qRT-PCR (1/9). Green tiles indicate significant increases for NKA activity assays and smolt index, and significant decrease in NKA qRT-PCR between I and F points. Red tiles show no significant change between I and F points. Blue tiles signify a decrease in expression of NKA activity assays and smolt index, and an increase in expression of NKA qRT-PCR. Orange tiles indicate where NKA activity assays did not reach the threshold of 10 for safe SW transfer.

2015	RBA	RBB	RBC	RBD	RBE	GCA	GCB	LD	ORA	ORB	ORE	BAR	GLA	GLB	CLA	MIN	Copy number and Smolt Index Agreement	NKA and Smolt Index agreement	All 3 agreement
α1a qRT-PCR	Red	Red	N/A			Red	Red						Green	Red	Red		8/16 sites	9/16 sites	6/16 sites
NKA	Green	Green	N/A		Red	Red	Red				Red	Green	Orange	Orange	Red				
Smolt index	Green	Green	N/A											Red	Red				
2016	RBA	RBB	RBC	RBD	RBE	GCA	GCB	LD	ORA	ORB	ORE	BAR	GLA	GLB	CLA	MIN			
α1a qRT-PCR	Blue	Blue		Blue		Green	Blue				Red	N/A			N/A		7/13 sites	8/13 sites	4/13 sites
NKA	Green	Green				Red	Blue					N/A		Red	N/A				
Smolt index	Red	Green				Green	Red					N/A		Green	N/A				
2017	RBA	RBB	RBD	RBE	GCA	GCB	LD	ORA	ORB	ORE	BAR	GLA	GLB	KLM	MIN				
α1a qRT-PCR	Red	Blue	N/A	N/A	N/A	Blue	N/A	Blue	Blue	N/A	N/A	N/A	Red	Blue	Blue		1/9 sites	4/9 sites	0/9 sites
NKA	Green	Green	N/A	N/A	N/A	Red	N/A	Green		N/A	N/A	N/A	Orange	Orange	Green	Red			
Smolt index	Green	Green	N/A	N/A	N/A	Green	N/A	Green		N/A	N/A	N/A			Green	Green			

Russel Burn

Due to the contrasting results between the tests obtained from the Russel burn site, these were analysed in more detail. The site is located on the Western coast of Scotland, near Strachcarron and on the edge of Loch Kishorn. Its surrounding topography is very rocky with many run off areas from the surrounding hills. There is a high concentration of limestone, calcareous tufa, dolomite and colomite with an old disused copper mine further up stream. It is a large farm with up to 3.2 million fish produced in the S0 season (July – October) and contains 20, 340m³ tanks and 20, 40m³ tanks for Atlantic salmon smolt production (Table 2.1).

2015

For 2015 NKA qRT-PCR showed no significant decrease in NKA mRNA abundance at site A (1.57fc) and B (1.32fc). In contrast both NKA activity assays indicated fish were ready for SW transfer with NKA activity > 10 NKA (A 2.22 fc, B 2.14 fc). Smolt indexes signified a shift towards, but not complete smoltification, having increased significantly from the initial test points (A 1.4 – 3, B 1.6 – 3). Degree days were at or close to the appropriate SW transfer time of 350 - 400dd at A and B (358dd) and D (327dd) suggesting the fish were at the point of SW transfer. Saltwater bath tests however were unsuccessful; supporting the NKA qRT-PCR assay results indicating that smoltification had still not occurred. At Russel Burn D both NKA activity assays (2.26 fc) and NKA qRT-PCR mRNA abundance (2.76 fc) increased significantly from initial to final points, however a relatively low smolt index increase (1.75 – 2.7) was recorded. At Russel Burn site E NKA activity assays remained stationary over both initial and final points (1.1 fc), with NKA qRT-PCR mRNA abundance decreasing significantly (2.5 fc), and smolt index increasing (2.2 – 3.6). Although the smolt index was on the mid-range scale of 2.2 at the initial point, the final point of 3.6 is a significant shift towards complete smoltification.

There was confirmed aluminium (Al) contamination at all sites. The highest contamination occurred at sites A and B with low to minimal contamination at sites D and E. It is known that metal contaminants affect the behaviour and physiology of fish (Scott and Sloman. 2004). Research into aluminium and acidic water exposure has shown that in moderate to high levels of exposure, NKA activity can be effected (Nilsen et al., 2010) and that recovery period for smolts can be up to two weeks or longer (Nilsen et al., 2013). Transfer of these exposed fish back to good quality FW showed an up regulation of α 1a mRNA and reduction in α 1b as expected in normal conditions (Richards et al., 2003; Nilsen et al., 2007; Madsen et al., 2009;

Bystriansky and Schulte. 2011). The increase at the final point of site B for the NKA qRT-PCR assay may have indicated the fish had recovered from metal contamination and were back into full smoltification development (Nilsen et al., 2013). This may explain the lower $\alpha 1a$ expression levels at all 4 sites initial points and could be the cause of the increase in expression at the final points of sites A and B due to fish health recovery.

All tanks required a further 3 weeks before fish were successfully transferred to sea suggesting the NKA qRT-PCR assay had indicated a failed smoltification process at sites A, B and D in contrast to the NKA activity assays which had erroneously indicated that the fish were ready for SW transfer at all 4 sites (A, B, D and E). The high readings for the NKA activity assay recorded during this period of metal contamination are supported by work by Nilsen *et al.* (2010, 2013). They found NKA activity was only slightly, or not at all, affected by aluminium contamination treatments; however $\alpha 1a$ mRNA levels after 7 days were reduced and after transfer back to FW increased substantially, potentially as a compensatory mechanism.

2016

A similar trend was observed in the 2016 sampling of Russel Burn sites A, B and C where there were again significant increases in NKA qRT-PCR mRNA abundance (A: 2.66 fc, B: 1.3 fc and C: 1.92 fc) and NKA activity (A: 1.73 fc, B: 2.27 fc and C: 2.37 fc). Smolt indexes were stable at site A (2.2 – 2.4), but increased from I to F at sites B (2.2 – 2.9) and C (1.74 – 3). Final points with smolt indexes of 3 and below would suggest full smoltification had not occurred yet. Degree Days for site A (371) and B (366) were within the appropriate SW transfer window of 350 – 400dd suggesting the fish were ready for SW transfer. Site C (504) however was overdue, and within the window for potential desmoltification due to the fish being kept within FW for an extra 3 weeks at temperatures $> 12^{\circ}\text{C}$ (Stefansson et al., 1998; Handeland et al., 2004). There were no reports of metal contamination in 2016. It is known that the surrounding area around Russel Burn suffers from Al and other deposit run off from the rocky environment. The surrounding rocky area consists of high amounts of limestone (CaCO_3) and dolomite ($\text{CaMg}(\text{CO}_3)$) and is located down river of a now disused copper mine (Cu_2). Without confirmation of metal contamination it is difficult to ascertain why the NKA activity assays were showing such a strong signal for SW readiness whilst the NKA qRT-PCR was suggesting the fish were under some physiological stress before recovery at the first final points. The dd recorded at F points for all 3 sites would support the NKA activity assay

results suggesting the fish were ready or within the SW transfer window. It should be noted however that further final points were conducted at sites A and B before successful transfer to sea. This could suggest NKA assays had pre-emptively indicated transfer to SW too early with NKA qRT-PCR and smolt indices suggesting the fish were not ready. Although data wasn't available for dd for these further 3 weeks, it is clear to estimate that the dd would be > 400dd for the majority of this time. This suggests the fish would have entered the window where desmoltification can occur (Stefansson et al., 1998; Handeland et al., 2004). With conflicting data from these 4 data sets and the delay in transfer of the fish it is difficult to ascertain which data set was the most reliable at predicting successful SW transfer.

2017

For Russel Burn 2017 sampling no significant increases in NKA qRT-PCR mRNA abundance were observed (A: 1.11fc and B: 1.36 fc), with NKA activity increasing significantly at both sites (A: 2.06 fc and B: 1.55 fc). Smolt indexes for both sites increased from I to F (A: 2.2 – 3.15 and B: 2.4 – 3.5) although site A's final smolt index of 3.15 is on the outer range for a final point reading. There were no reports of metal contamination during this year. Both sites dd (A: 392 and B 388) were at the optimum time for SW transfer supporting the NKA activity assay results and high smolt index for site B. This would suggest that there was an issue with the NKA qRT-PCR assay during this year, possibly due to an error during the running of the sample, sample handling or another environmental factor not detected in NKA activity assays and smolt index.

After 3 years of data where both tests never agreed fully it is likely a combination of environmental factors potentially based on location affected both tests to a different degree each year. This is highlighted with the outbreak of metal contamination in 2015, and irregular smolt indices that vary on degrees of agreement with each assay over this 3-year period. Although no metal contamination was recorded in 2016 and 2017, due to the topography of the site it is possible low levels have been present, particularly during times of heavy rainfall. Short periods of limited metal contamination could lead to small delays in smolt development than those shown in previous research into moderate and heavy contamination (Kroglund and Finstad. 2003; Kroglund et al., 2007, 2008; Monette and McCormick, 2008; Nilsen et al., 2010; Nilsen et al., 2013). Daily or weekly testing would need to have been carried out to validate or refute this, however. It should be taken into consideration however, that the dd recorded at F points for each of the 3-years were around the optimum

time for SW transfer in regards to the literature (Stefansson et al., 1998, Handeland et al., 2004). Temperature is another possibility for the variations, and this will be addressed later in this discussion.

Other sites

Only one site, Loch Shin, had both a decrease in NKA activity assay (1.34 fc) and increase in NKA qRT-PCR (1.53 fc). Both assays showed the opposite result of what we would be expected from each assay during a normal smolting period. The decrease in NKA activity (1.34 fc) below the threshold of 10 at the final point, and the increase in copy number of NKA qRT-PCR could indicate the salmon were undergoing desmoltification. It is well documented that salmon smolts that remain in FW past the stage of full smolt development begin to regress back to a parr-like appearance and partial morphology (Folmar. 1982; Hoar. 1988). Although limited research has been carried out on the endocrine system's involvement in loss of smolt characteristics over time, research into GH (Agustsson et al., 2001) and IGF-1 (Shimomura et al., 2012) have indicated they play a role and indicate a loss in in NKA activity as a potential sign of desmoltification. The smolt index for the initial point was relatively high compared to most sites standards (2.85) and peaked at a mid point of (3.5), and remained similar at the final point (3.49). We would expect fish going through desmoltification to have had a very high smolt index for a prolonged period of time in FW before reverting back to their pre-smolt stage (Hoar. 1988), however the time points between initial and final were only 2.5 weeks apart. Without further data previous to the first initial point and without the correct tools to characterise de-smoltification it is difficult to say for certain whether this is what occurred.

The 2017 data also showed a unique occurrence in regards to the NKA qRT-PCR where 6 of the 9 sites had a significant increase in mRNA abundance (appendix C1.6) and 2 where a small but non-significant increase in mRNA abundance was recorded. Only one site (Mingary) showed a significant decrease as would be expected for the assay. This year also had 5 of the 9 NKA activity assays indicating no smoltification at final points. Although points made about desmoltification, metal contamination and other factors have been discussed above and may account for some of the results, other environmental factors may be able to explain this year's high variation in assay failures, particularly that of temperature. The influence of temperature on full smolt development is well documented as a crucial environmental factor (Solbakken et al., 1994; McCormick et al., 2000; Handeland et

al., 2000, 2004, 2013). Research into the effects of rearing smolts at different temperatures has shown that those raised at higher temperatures (12 – 12.7°C) will exhibit maximum levels of NKA activity 4 to 6 weeks earlier than those raised at lower temperatures (8.3 – 8.9°C), and thus advance the smolt process (Handeland et al., 2000; Handeland et al., 2013). Evidence has also shown that smolts maintained in FW past their SW transfer window (>400dd) at elevated temperatures (12 – 14°C) showed significant down regulation in NKA generally after a 4 – 6 week period (Stefansson et al., 1998; Handeland et al., 2004) indicating desmoltification. The development of normal smolt morphological changes such as loss of parr marks, silvering of the scales and increase in body mass have been shown to develop faster in higher rearing temperatures in hatcheries (Handeland. 2004). Taking the evidence into account we looked at average sea temperatures for all 3 years were analysed and temperature profiles split into 3 sites based on closest location to each farm to ascertain whether temperature could have had some effect on smolt development (Figure 2.10).

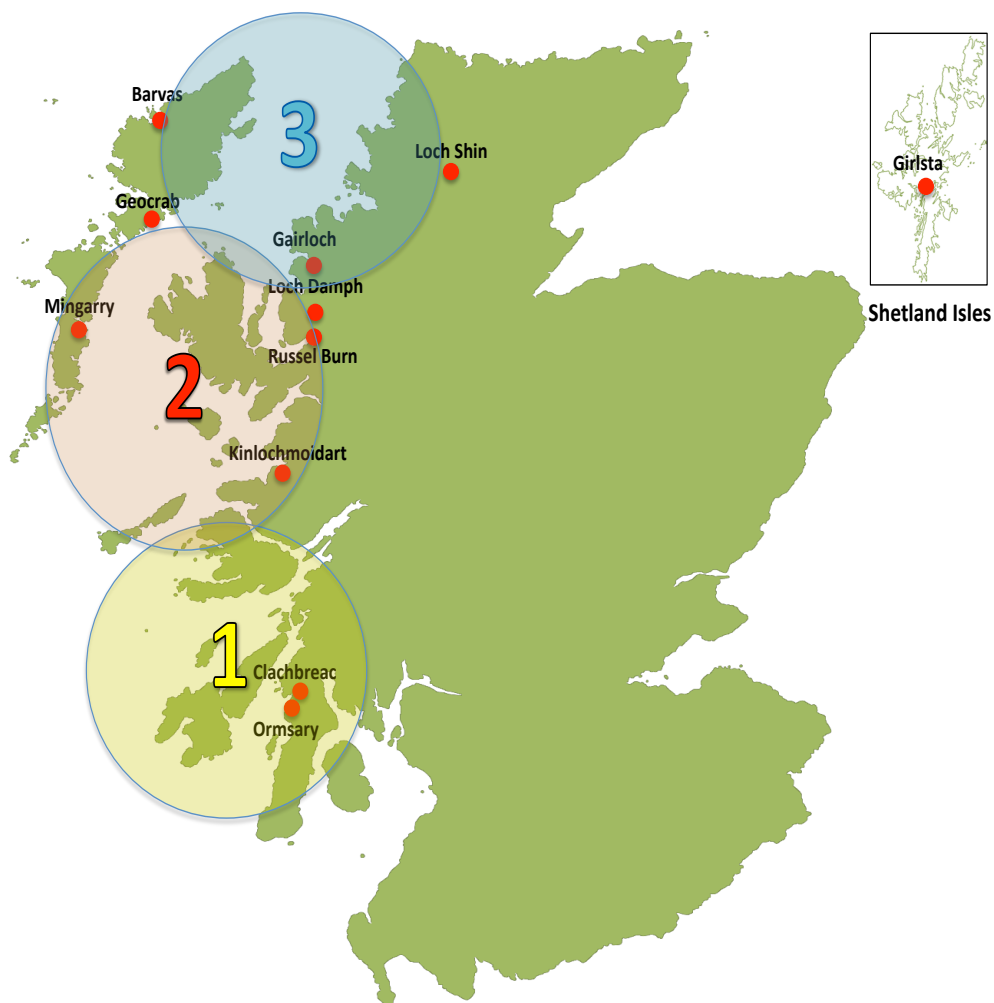


Figure 2.11. Map indicating temperature profile locations of 11 sites sampled over the 3-year study.

Mingary is one of the few sites where 100% success with the NKA qRT-PCR was observed over the 3 years. The sampling times over the 3 years were generally consistent, starting in September and ending in October. For Ormsary, sampling times took place earlier at 2 of the 3 tested sites in 2016 (July – Aug), therefore it is difficult to associate the failures of the assays with temperature influence due to the limited variation over all years. Sample periods for Russel Burn have been consistent over all 3 years. Most of the NKA qRT-PCR assays were tested during the months with highest variation in average temperatures between 2015 and 2017. However, the two assays, which were successful in 2015, were tested later in the year when temperatures across all 3 years were similar (Sep – Oct); implying that if temperature was having an effect then we should see a similar result should have been observed across 3 years.

Geocrab samplings were taken at similar time points between Jun – Aug. No NKA activity assays successfully indicated smoltification over the 3 years, however NKA qRT-PCR assays did indicate smoltification at one point in both 2015 and 2016. Both Jun 2015 and 2016 were on average 1 – 1.3°C cooler than 2017; conversely Aug 2016 was warmer than 2015 and close to 2017 levels. Sampling times varied per year for Gairloch with tests starting late Sep – Oct 2015 towards Jun – Sep 2017. There appears to be no correlation between temperature and NKA activity assays across 3 years as all assays failed every year. There was also no correlation between successful and unsuccessful indications of smoltification for the NKA qRT-PCR assays during the 3 years, as tests showed decreases and increases of NKA mRNA abundance during cooler temperatures in 2015, and warmer temperatures in 2016.

For Loch Damph, both assays were effective in both 2016 and 2017 where sampling was conducted during September. The variation in average temperature between 2015 and 2016 was 0.89°C in September and 0.74°C in August, suggesting temperature had no influence on the assays. The 2017 sampling took place earlier in August, when average temperatures were 1.12°C higher than 2015. It is difficult to incorporate the 2017 result as I to F testing was taken over 10 days. A key point should be made that although most temperature variations are quite minimal over the 3 years of testing the average temperatures for all years between July and September were above 12°C and had highs of 15°C in some months for some years. This is already in the range shown to increase the speed of smolt development through elevated NKA activity (Handeland et al., 2000, 2013) and within the window of smolts becoming susceptible to desmoltification when held in

FW once full development has occurred (Stefansson et al., 1998 and Handeland et al., 2003). Due to the influence that longer rearing times have at these temperatures on fish NKA activity, records into total hatchery rearing times at each farm may indicate those that have been reared for a longer period of time may be the groups that failed for both the NKA activity and NKA qRT-PCR assays. It would appear in our case that it was not the fundamental factor in the failures of NKA activity and NKA qRT-PCR assays during the 3 years of testing but could have had some influence based on rearing times at individual farms.

A further way to examine the influence of temperature on smolt development was to examine dd between sites and years. Unfortunately onsite temperature data was only available for 2 sites, Russel Burn and Geocrab, for the full 3-year test period. Degree-days were calculated based on the formula $DD = (T_{Max} + T_{Min}/2) - T_0$ where $T_0 = 0^{\circ}C$. Examination of the dd allows for a better spatial observation of where the fish are development wise at each time point assessed. As mentioned previously, temperature is a crucial environmental factor for successful smolt development (Solbakken et al., 1994; McCormick et al., 2000; Handeland et al., 2000, 2004, 2013) and can have inhibitory effects at elevated temperatures where smolts pass their SW transfer window (>400dd) (Stefansson et al., 1998; Handeland et al., 2004). When comparing dd between sites, it was found that Russel burn had kept within the optimal smolt transfer window at all but one of its F points (RBC 2016, 504dd). Geocrab in contrast had only one site (GCA 2017, 395dd) within this window at its F points with the remaining sites ranging from 460 – 700dd+. At the sites where Russel burn (RBA, B, D 2015, RBA, B 2016 and RBA B 2017) and Geocrab (GCA 2017) were within the optimal smolt transfer window at F points for dd, it would be expected that the NKA activity, NKA qRT-PCR and smolt index would all point towards an almost fully developed SW ready smolt. For all 3-years NKA activity assays agreed with this as did smolt indices, showing a general move towards full smoltification characteristics in 5 out of the 7 sites tested with optimal dd for SW transfer. NKA qRT-PCR generally showed the opposite, with no significant change or an increase in copy number. It is known that metal contamination affected smolt development at Russel burn in 2015, however dd are only a measurement of potential growth due to temperature and therefore would not be affected by other environmental factors such as metal contamination. For the subsequent 2 years however, dd would support the NKA activity assay results indicating the fish were ready for SW transfer. For comparison, fish at Geocrab sites were held for a much longer period of time (>700dd at GCA 2016). NKA activity for all these sites began

at, or above the threshold used to indicate when fish are ready for SW transfer (NKA > 10) and increased throughout the testing period. NKA qRT-PCR also reported a significant increase ($p < 0.001$) in $\alpha 1a$ in 2017 between I and M points. The extended dd would suggest the fish would have moved into a desmoltification state during the last 2 weeks of testing, however with the NKA qRT-PCR and NKA activity results observed, it is possible the fish may have been in a desmoltification state before this. Signs of this were not observed in the smolt indices where they were reported within normal I point range (1.1 – 2) and F points (3 – 3.3). NKA qRT-PCR also supported this, as the mRNA levels remained stable in 2015 ($p = 0.288$) and decreased as expected in 2016 ($p = 0.005$). No losses of fish were reported due to desmoltification either.

Due to the conflicting data at both Russel burn and Geocrab, it is hard to pin point a particular cause for the variation in the results. This is particularly apparent in the Geocrab results as the temperatures remained over 13°C for all sites with >400dd. It is possible that fish at these sites were subjected to desmoltification due to the $\alpha 1a$ mRNA observed increasing at I to M points (GCA 2017) and M to F points (GCA 2015), and most interesting of all a decrease from I to M and increase from M to F (GCA 2015). The trend shown at GCA 2015 is a common trait found for the $\alpha 1a$ mRNA marker (McCormick et al., 2009b; Madsen et al., 2009; McCormick et al., 2009b; Madsen et al., 2009; Nilsen et al 2013). The marker slowly decreases to a point where the fish are ready for SW transfer, and if the fish are not transferred the marker then increases again due to prolonged FW stress in an attempt to revert back to FW salmon traits. This is further supported by the high NKA activity recorded at Geocrab throughout testing. Due to the lack of extra sample points throughout the testing period it is difficult to assess whether this is true. There are only two sites (GCB 2015 and RBB 2016), which showed a similar trend of decreasing $\alpha 1a$ mRNA from I to M points (GCB 2015 260dd, RBB 2016 270dds) followed by an increase at F points (GCB 2015 575dd, RBB 365dd). It is possible that if more sampling had been carried out in this dd range of 250 – 400, or more dd data was available from other sites, that a trend may have been observed. Due to the vast differences in dd between both sites over the 3-year period it would be expected that they would look significantly different in comparison with NKA qRT-PCR activity. On cross-examination however, there is little variation in the I, M and F points. Further observation and testing based on dd instead of calendar days would allow for closer monitoring of the NKA qRT-PCR and NKA activity. It could also allow for trends to be observed for pre and post optimal SW transfer windows based

on a dd window. This could also be further enhanced to focus on site-specific optimal dd SW transfer ranges.

To observe any variations in expression between I and F points, a selection of sites were also sampled at M points. This was to determine whether the population shifted in a particular direction regarding NKA activity assays and NKA qRT-PCR mRNA abundance. It is important to monitor the normal full smolt window of 4 – 6 weeks between I, M and F to ensure a clear depiction of expression is recorded throughout the full smolting period. Those farms that sample outside of this window risk the validity of both tests as the fish remain in FW potentially past their optimum time window. Therefore those sampling at longer time ranges risk the wrong interpretation of where the fish are biologically in regards to smoltification. Regarding current literature, it would be expected that M points would have a relatively high abundance of $\alpha 1a$ mRNA similar to those at I points due to the high expression of this isoform in its osmoregulatory role in a FW environment (Mackie et al., 2005; Bystriansky et al., 2006; Nilsen et al., 2007; Madsen et al., 2009; McCormick et al., 2009b, 2013a; Bystriansky et al., 2011; Stefansson et al., 2012). However, due to the switching of ionocytes to the $\alpha 1b$ isoform in preparation for a SW environment it is possible that a small reduction in expression of $\alpha 1a$ may be observed (Nilsen et al., 2007 and McCormick et al., 2013a). For the NKA activity assay a constant increase in activity from I, M and F point would be expected as the fish adapt to fully smolted salmon (Prunet et al., 1989; McCormick. 1995,1996, 2000, 2001; Bystriansky and Schulte. 2011, Handeland. 2013).

For NKA activity over the 3-year period, a significant increase between I and M was found in the majority of sites sampled (13 / 18), but only a significant increase in a minority of sites between M and F (5 / 18). The trend of increasing NKA activity is as would be expected from previous literature (Prunet et al., 1989; McCormick. 1995,1996, 2000, 2001; Bystriansky and Schulte. 2011; Handeland. 2013), however the limited increase from M – F shows the high variation that is present in NKA activity between the population of fish sampled, and suggests that the largest increase in NKA activity is within the first few weeks of smoltification development. Higher variations in StD were observed in most M and F points when compared to I points, suggesting high variation in expression of NKA activity within the population. There were two sites where the M point was lower than both the I and F points (GCB 2015) and GCA 2017)), and the M point was higher than both I and F points (ORA and GCA 2015) and LD and GCA 2016). This could be due to site-specific issues with GCA and B during these years and possibly other factors for LD such as

environmental. There was no noticeable association with variation in length of the sampling period between I, M and F that may have affected this. Other sites as well as the same sites, on different years such as LD with the same time scale have not shown the same trend.

For NKA qRT-PCR analysis over the 3-year period there was a significant decrease in I and M points for the majority of sites (12 / 18), but only one site (ORA – 2015) showed a significant decrease between M and F points. This would again suggest that, similar to NKA activity assays that, the largest decrease in $\alpha 1a$ mRNA abundance was in the first few weeks of smoltification, with a slower decrease in the subsequent 2 – 4 weeks. This appears to partly go against the established literature, indicating high levels of $\alpha 1a$ are present before a rapid changing of ionocytes to $\alpha 1b$ (Mackie et al., 2005; Bystriansky et al., 2006; Nilsen et al., 2007; Madsen et al., 2009; McCormick et al., 2009b, 2013a; Bystriansky et al., 2011; Stefansson et al., 2012), however most F points do show $\alpha 1a$ mRNA in excess of 1×10^5 copies before transfer, before declining rapidly again to background levels in SW transferred smolts.

From the data gathered over this 3-year study, it would appear that there are two distinct drops in $\alpha 1a$ expression, with an initial drop from the I to M phase of smoltification with a slower reduction in expression towards a baseline level of 1×10^5 mRNA copies for SW ready smolts at the F phase. This is then followed by the rapid decrease in $\alpha 1a$ down to low background levels in SW transferred smolts. A similar trend can be attributed to the NKA assays, with smolts reaching high levels of NKA expression near the M phase followed by a slow increases in NKA towards F phase SW ready smolts.

It is possible that in some or even all RNA samples extracted on site, mRNA degradation may have occurred despite being placed in RNA later for NKA qRT-PCR assays and SEID solution for NKA activity assays. Research on degradation of salmon gill tissue over time in RNA later on brain, kidney, liver and muscle indicates degradation of mRNA within 4-8 hours at room temperature in RNA later (Seear et al., 2008). It also states that all tissues are generally stable for 1-hour post mortem. For our onsite tests, the overall process from killing of the fish to running of the PCR is approximately done within this 1-hour time frame, reducing the chance of mRNA degradation and producing a potentially more reliable result on $\alpha 1a$ mRNA levels within the gill tissue. In contrast, the collection of gill tissue for lab analysis of both NKA qRT-PCR and NKA activity assays were carried out as quickly as possible after

killing of the fish with each tissue transferred into RNAlater (NKA qRT-PCR assay) and SEID (NKA activity assay), and placed in dry ice and or ice packs. It can then take up to or >24 hrs for the samples to reach the lab. The use of dry ice for transport was not used universally over all sites and between labs, increasing the possibility of degradation during transport. To account for the possibility of mRNA degradation, it could be possible to test storage of gill tissue in RNA later and SEID at room temperature, chilled (2-8°C) and frozen, and compare to fresh sample analysis following such examples in human tissue analysis (Mutter et al., 2004; Florrel et al., 2001). We did however find no statistical difference between our on site test results and in lab results. These samples were transported in RNAlater in a cool box maintained at -5°C and then transferred into a -70°C freezer within 10 hours of gill extraction. The natural variations of qRT-PCR assays have been determined at ± 1.4 CT's (Niesters. 2001). All the comparative data sets from all our sites fell within this range.

It should also be taken into consideration the different efficiencies calculated from the samples when compared to the NKA qRT-PCR plasmid standard. The samples were extracted as pure RNA, however within the extracted RNA there would have been potentially thousands of other mRNA transcripts from different targets. The plasmid standard in comparison is specific only to the $\alpha 1a$ mRNA target of ATPase. The integrity of the RNA samples would also be more susceptible to degradation, increasing the chances of target sample breaking down and therefore potentially not being amplified by the specific PCR primers and probes. Plasmid DNA standards are well known for their robustness, even at room temperature without losing any efficiency, producing consistent and reproducible results (Pfaffl, 2004). Therefore, it is possible that the samples tested were not always showing the true mRNA abundance that was present within fish tested at some sites and times points. This may potentially explain some of the unexpected increases and low abundance of mRNA at time points where it would have been expected to be the opposite. Converting the mRNA extracted to cDNA could help mitigate the potential breakdown in integrity of the target and allow easier storage and sampling.

An in lab NKA qRT-PCR assay was successfully tested with samples from multiple fish farms over a 3-year period against NKA activity assays. For the first two years although not agreeing at all sites both assays had similar successes and failures in detecting smoltification (qRT-PCR 57%, NKA activity 60%). The final year (2017) was the only variation to this, as all but one NKA qRT-PCR assay failed to show downregulation of $\alpha 1a$ mRNA associated with succesful smoltification, with 4 of the

NKA activity assays indicating successful smoltification. It is also interesting to note that NKA activity assays did not seem to be influenced by metal contamination according to our results from 2015 at Russel Burn. This is in line with previous research on metal and acid contamination indicating no effect on NKA activity (Monette and McCormick. 2008; Nilsen et al., 2013). Results from those studies showed that the NKA activity assays gave a false positive signal for SW transfer with NKA qRT-PCR assays and salt-water bath treatments indicating the fish were not ready for transfer. Further analysis would need to be conducted on NKA activity assays with metal contaminated salmon to ascertain the reason as to why this has occurred.

Although various factors including temperature, metal contamination, RNA quality, dd and rearing times have been discussed, it has not been possible to address all the failures of each assay within the test periods. Given the variety of factors, it would appear that some, if not all could play a role in influencing the results of both our assays, or that at times it was down to the stock of fish reared that year. It is possible that through the use of Supersmolt[®], pseudo smolts are produced out with standard feed smolts. This could potentially produce smolts that develop faster and need to be transferred earlier. Comparissons between fish fed on standard feed and Supersmolt[®] would need to be performed in tandem to asses whether this could be true. The use of dd instead of calendar days would also be a benifit for closer assessment of the fish during development to observe whether Supersmolt[®] fed fish are developing at a faster rate that was not observable in this study. A standardisation of smoltification procedures on each farm for rearing times, temperatures etc. would be the optimal way to fine tune both these tests and discover the biggest environmental and physical problems effecting them. This however is completely impractical from any farms point of view.

A mobile diagnostic kit for onsite detection of the NKA isoform $\alpha 1a$ in salmon gills was successfully designed and trialled. Low levels of expression were detected (1×10^4 mRNA copies) from all extracted gill samples from smoltified salmon. These results correlated with previous research stating a fall and low adundance of $\alpha 1a$ mRNA post smoltification (Richard et al., 2003; Bystriansky et al., 2006; Bystriansky and Schulte 2011. Stefansson et al., 2012).

A modified version of the mobile diagnostic kit containing less equipment for easier transport and operation on site, and changing to a new qRT-PCR device (Q16), was successfully tested. The extraction and assay were performed successfully at most

sites with some issues recorded for internal positive controls, which limited our analysis. Positive control C_T varied beyond the normally accepted variation of 1.4 C_T at 3 of the time points tested (Ormsary F and Uist F). This gave incorrect copy numbers for the resulting samples as the positive was off by ~ 1 log. No definitive cause for this variation was discovered, however a new buffer solution has been developed specifically for the positive control, which may alleviate this issue. We saw no significant decrease in ATPase C_T for all 3 sites, but an increase at Ormsary. This could be due to the varying time ranges of when we started sampling (mid smolt for Ormsary and Clachan), and the time between sample points (Loch Damph - 9 weeks, Clachan - 2 weeks and Ormsary - 4 weeks). In particular, Loch Damph smolts were to be held for another 2 weeks post final point sampling leading to a total smolt period of 11 weeks. Supersmolt[®] feed used to induce smoltification at this farm was only administered to fish after the second sampling indicating both were I points.

There are various factors including morphological and environmental which may have played a part in these results that have been highlighted previously in this discussion. These experiences however also indicate that planning visits is crucial in applying the mobile qRT-PCR platform in order to efficiently test the I – M and F points reflecting the protocol and timeline implemented at each hatchery. The deployment study showed that we were able to optimise the suitcase lab by reducing its size and materials needed as well as optimise the RNA and qRT-PCR protocols. The Q16 was also successfully able to run whilst being transported in a car, which opens up the possibilities for multiple site visits as tests from one farm can be conducted whilst travelling to the next.

To identify smoltification in salmon we have successfully developed an in lab and onsite qRT-PCR platform for the detection of the NKA transcript $\alpha 1a$. High sensitivity was shown for the in lab (98.9%, SE 0.24) and mobile (93.43%, SE 0.119) assays when tested using a quantitative RNA standard (both within optimal efficiency range of 90 – 105%).

Multiple qRT-PCR assays have been developed both academically and industrially for detection of ATPase $\alpha 1a$ for in lab testing. To our knowledge, this is the first assay developed in the lab that has been specifically modified to work on a mobile platform on site at fish farms. Both onsite assays were successfully tested on multiple farms. These tests have proven it is possible to test during a smolt period on site and provide results on the same day. This leaves the possibility for further

refinement of the assay, development of other biomarkers and transferring viral assays targeting salmon disease for onsite testing.

Chapter 3: Multiplex Viral Diagnostics

3.1 Introduction

Salmon alphavirus (SAV), Piscine reovirus (PRV) and Piscine myocarditis virus (PMCV) are 3 of the most prominent viruses causing major detrimental impacts on salmon health and production within the salmon aquaculture industry in Europe. The most prominent of these pathogens is salmonid alphaviruses (SAVs). There are currently 6 known subtypes of SAV that have been characterised (Fringuelli et al., 2008). The virus causes PD in Atlantic salmon in the UK (SAV 1 - 6) (Fringuelli et al., 2008; Graham et al., 2012; Hjortaa et al., 2013), and both Atlantic salmon and rainbow trout in Norway (SAV 3) (Hodneland et al., 2005). The virus also causes SD, which afflicts rainbow trout across Europe (Graham et al., 2003, 2007; Castric et al., 1997; Jansen et al., 2017). All subtypes show similar pathological and morphological effects (muscle and heart myopathy, pancreatic lesions, non feeding fish and abnormal swimming behaviour)(McLoughlin et al., 2007) and cannot be distinguished by histopathological analyses.

PRV, a recently discovered double stranded RNA virus has been strongly associated as the infection agent of heart and skeletal muscle inflammation (HSMI) in Atlantic salmon (Palacios et al., 2010; Finstad et al., 2012), and recently has been shown to be the causative agent of the disease (Wessel et al., 2017). HSMI is characterised by lesions in the heart and red skeletal muscle, causing moderate to severe inflammation of the tissues, which can then lead to heart and muscle failure (myocarditis and myopathy). External symptoms are low, with non-feeding and abnormal swimming being the most commonly observed. The virus was considered to be the cause of cardiomyopathy syndrome (CMS) in salmon but research by Lovoll *et al.* 2010 and Haugland *et al.* 2011 showed the virus was acting as a potential opportunistic secondary infection in CMS infected fish. Further evidence of its opportunistic nature was shown by Garseth *et al.* (2013) where high titres were observed in Atlantic salmon with no HSMI symptoms. It was also shown that PRV can stop secondary infections of SAV (Lund et al., 2016). Recent findings by Wessel *et al.* (2017) now suggest that PRV is in fact the etiological agent, however not all strains can induce HSMI. As more evidence is gathered on PRV it is clear that due to its abundance, it plays a key role in disease outbreaks in salmon.

PMCV is another recently discovered double stranded RNA virus (Haugland et al., 2011) that is the causative agent of CMS in Atlantic salmon. It generally affects

salmon between 12 – 18 months after SW transfer (Brun et al., 2003) and is characterised by lesions on the heart, causing inflammation and degradation of the atrium and ventricular spongy myocardium. This can lead to heart failure and mortality of the fish if infection is severe enough (Ferguson et al., 1990; Poppe and Seierstad. 2003). External observations of the disease are similar to both PRV and SAV with decreased feeding, abnormal or erratic swimming behavior, swelling and skin hemorrhages (Rodger and Turnbull. 2000). These symptoms are not always present, with healthy looking fish displaying sudden mortality. The virus is able to infect cohabiting fish and spread into the water column, and though it is not yet known how this is achieved it has been postulated (Haugland et al., 2011). Due to the similarity of the internal and external morphological impacts these 3 viruses have on Atlantic salmon, coupled with potential opportunistic secondary infections after one has caused primary infection, demonstrates why a fast and effective detection platform would greatly help in the detection of each virus. This could help mitigate and limit the outbreaks of disease and in turn reduce mortality and loss of profit for fish farm production.

The introduction of qRT-PCR has produced the most popularly and sensitive platform for detection of mRNA (Bustin. 2000). The development of this technique allowed real time viewing of amplification at each cycle threshold (C_t) as well as the use of target amplicon specific primers and probes. When comparing reverse transcription polymerase chain reaction (RT-PCR) to qRT-PCR, increases in detection and sensitivity are generally found (Bustin and Mueller. 2005). This has been shown to be the case in numerous fish viruses such as SAV (Hodneland and Endresen. 2006) and ISAV (Munir and Kibenge. 2004). One of the most effective and commonly used probe formats for qRT-PCR is dual-labeled fluorogenic probes (TaqMan probes). This assay was originally proposed and developed by Holland *et al.* (1991) and was tested and finalised by Heid *et al.* (1996). TaqMan probes utilise two reporter dyes, one at the 5' end containing the fluorescent reporter dye that is quenched by the other at the 3' end. Detection of the reporter dye is only possible when correct hybridisation, polymerisation and cleaving of the probe occur. Therefore at each cycle the fluorescence detected is directly proportional to the concentration of DNA template present (Holland et al., 1991; Heid et al., 1996; Bustin. 2000). The advantage of this probe was the increase in speed, sensitivity, real time viewing of quantification and reduction in contamination due to its closed tube system (Heid et al., 1996).

The development of specific mRNA target probes in qRT-PCR assays has provided the ability to run multiplex assays. These can contain multiple mRNA target specific primers and probes aimed at different targets within the same reactions and protocol. This can lead to decreased assay preparation and running time. Multiplex assays have been successfully developed and used for a multitude of human viruses; gastroenteritis (Pang et al., 2005), human influenza (Suwannakarn et al., 2008) and respiratory tract infection (Brittain-Long et al., 2008) to name a few. Developments of marine based multiplexes have been produced for viral (Khawsak et al., 2008; Kou et al., 2008; Panichareon et al., 2011; and Hoferer et al., 2017), bacterial (Cerro et al., 2002) and parasitic (Li et al., 2017) assays. These have been developed at a slower rate using a variety of different multiplex techniques. Inherently there are fundamental problems that must be addressed and adhered to when using qRT-PCR to ensure that the assays produced provide accurate technological and biological results that can be interpreted and compared correctly. Specificity of the primers and probe to amplify the correct target region, quality of RNA used (both standards and samples), operator variability and data analysis are just some of the aspects that can have a detrimental effect if not adhered to (Bustin and Nolan. 2004). These factors resulted in the publication of a set of minimum information for publication guidelines for qRT-PCR assays (MIQE) (Bustin et al., 2009). These issues, particularly those of specificity, are amplified during multiplex development due to the increasingly complex nature of the mix as more assays are added (Elnifro et al., 2000).

Following these guidelines we aimed

1. To produce 3 individual qRT-PCR assays for the detection and rapid screening of all subtypes of SAV, PRV and PMCV.
2. To incorporate these 3 assays into a multiplex qRT-PCR assay with an internal control (IC) using Taqman probes.
3. To transfer the multiplex assay to a mobile qRT-PCR device (SmartCycler™ system).
4. To test this assay against positive samples of SAV, PRV and PMCV.
5. Overall we aimed at producing a sensitive and specific multiplex assay that would be able to detect SAV, PRV and PMCV both in lab and on site at fish farms.

3.2 Materials and Methods

3.2.1 Design of primers

Primer design for the detection of SAV, PRV, PMCV and SIGV were designed by aligning all available sequences from Genebank (SAV (147), PRV (81), PMCV (94) and SIGV (6)) in a clustalV alignment using Lasergene software MEGALIGNDNASTAR[®]. Primers and probes were placed into conserved regions (Table 3.1). To cover the variability of the SAV genotypes, several combinations of primers and probes were originally designed. Norwegian, Scottish and Irish SAV genotypes (SAV 1, 3-6) were detected by two 5' primers (SAV FP, SAV FP3), one probe (SAV LNA), and one 3' primer (SAV RP2). Sleeping disease virus (SAV 2) was detected by one of the two 5' primers already mentioned (SAV FP), one probe (SAV SD LNA P) and one 3' primer (SAV SD RP). All primers were additionally tested in silico for any secondary structures and primer-dimer formation with VisualOMP Version 7.2.48.0 (DNA software, Inc.).

Subsequently, when combining the selected assays in to a multiplex reaction the primers and probes used for SAV were refined and reduced to two primers that covered all 6 SAV genotypes (SAV UP2, RP4) and one probe (SAV LNA P). In all cases LNA nucleotides were used in the probes to reduce probe length and to allow the use of shorter conserved sequence signatures.

Additionally, different sets of primers were designed to amplify target regions of the viral genomes (Table 3.2). These amplified fragments were used to develop the respective quantitative RNA standards. Again all primers were additionally tested in silico for any secondary structures and primer-dimer formation with VisualOMP Version 7.2.48.0 (DNA Software, Inc.).

Table 3.1. Primers and probes designed for detection and amplification of SAV, PRV PMCV and SIGV RNA standards in qRT-PCR tests.

Virus	Primer or probe	Sequence (5' -> 3')	TM (°C)	Product size (bp)
SAV	SAV UP2	AAGAAATGTACCAGATTTTCCACTAC	59.1	106
	SAV RP4	gCATgTTRCgACggTgYTAATCTCTAC	60.9	
	SAV LNA	6FAM-AATCGGCA+GA+GC+GTC--BBQ	46.6	
PRV	PRV FP2	GGTTCAAACGACAGACCAGACAG	62.4	78
	PRV RP2	CATTATGCCACGCATATCGTCTC	60.6	
	PRV LNA	CY5-ATTgAAgCTAAgCgACg—BBQ	42.3	
PMCV	PMCV FP2	GACCAACCCAGAACCAGCG	60.7	82
	PMCV RP	TCAACACCCATCTGTCTTTGGTATG	61.3	
	PMCV2 LNA P3	LC610-ATTgAAgCTAAgCgACg--BBQ	41.8	
Sigma	FP	gTgACATTCCAAgTAACTgATT	49.6	91
	RP	CAACggCAgTTTggATA	50.0	
	LNA	YAK-CCCTCCgTgTCCTCCCggTACC-BHQ2	68.3	

Table 3.2. List of all primers and probes used for amplification of target regions, plasmid standards and sequence analysis (M13 primers (MCS = multiple cloning site of pCR™II)) of SAV, PRV and PMCV.

Virus	Primer or probe	Sequence (5' -> 3')	TM (°C)	Product size (bp)
SAV	SAV UP2	AAGAAATGTACCAGATTTTCCACTA C	59.1	317
	SAV DP2	CTAGTGGAGGCAAGGAAGTGAA	60.4	
PRV	PRV UP	TTCTATGACGACCAACATAACACTT CA	60.4	835
	PRV DP	AGGGATTGTGTGACCAGAGTGC	62.1	
PMCV	PMCV UP	TGTCAAACAAGATGAAGAGTTTTTT ACTG	59.6	910
	PMCV DP	CTAGACTCCTACTCTGAATCCCTCT TCAT	65.3	
M13	M13 UP	GTAAAACGACGGCCAG	51.7	MCS
	M13 DP	CAGGAAACAGCTATGAC	50.4	

3.2.2 PCR

PCR reactions were carried out for SAV, PRV and PMCV in 0.2ml tubes containing a final volume of 50 µl per reaction, using a final concentration of 400 nM primers (Table 3.2), and 1x MyTaq™ HS Mix (Bioline). Template plasmid DNA containing the region of interest of the SAV genome was kindly provided by Dr. Michael Leaver (Institute of Aquaculture, University of Stirling), whilst plasmid DNA containing regions of PRV and PMCV genome were ordered from, and synthesized by Life Technologies. Several 10-fold dilutions for each plasmid DNA were prepared and 1 µl of each dilution was used as template for the PCR reactions. All reactions were run in a T gradient thermocycler (Biometra) using the following conditions: activation for 3 min at 95°C, followed by 35 cycles of denaturing for 15 s at 95°C, annealing for 15 s at 55°C, and extension for 30 s at 72°C. The PCR products were run on a 2% agarose gel stained with ethidium bromide and analysed using GeneSnap version 7.12 (Syngene).

3.2.3 Purification of PCR products

PCR products were purified using the DNA Clean & Concentrator™ 5 Kit (Zymo Research), according to manufacturer's instructions, using a 5:1 ratio between the DNA binding buffer and the PCR products. Purified DNA was eluted into 20µl DNA elution buffer. DNA concentration was determined using the ND-1000 system

(NanoDrop, Thermo Scientific) and ND-1000 version 3.8.1 software. Purified PCR products were directly used for the ligation procedure, and then kept at -20°C for long-term storage.

3.2.4 Ligation, cloning and transformation

This procedure was carried out using the TA Cloning Kit Dual promoter pCR™II kit (Invitrogen), according to manufacturer's instructions, using a vector to PCR insert ratio of 1:3. The ligation mixtures consisted of the purified PCR products (SAV: 12ng, PRV: 32.1ng, PMCV: 35ng), 1x Express Link™ T4 DNA Ligase Buffer, 50 ng pCR™II vector and 5U ExpressLink™ DNA Ligase to a final volume of 10µl. The ligations were incubated for 1 h at room temperature and then were stored at -20°C.

After the ligations were carried out, 2ul of each ligation reaction was transferred to separate vials containing 50 µL One Shot® TOP10F' chemically competent *Escherichia coli*, provided with the TA Cloning Kit Dual promoter pCR™II kit (Invitrogen). Transformation was carried out according to manufacturer's instructions. From each transformation vial 50µl, 100µl and ~150-200µl were spread on separate Luria-Bertani (LB) agar plates containing 100µg/ml ampicillin. As TOP10F' cells were used, the LB plates needed to be equilibrated by pre warming them for 30 min at 37°C before addition of the spread from each transformation vial. The plates were then incubated overnight at 37°C.

The following day, plates were analysed for growth. Ten single colonies were picked per transformation reaction and placed in separate falcon tubes containing 5 ml of LB broth supplemented with 100µg/ml ampicillin. The tubes were then incubated overnight at 37°C in a Maxq 2000 shaker (Barnstead lab line) at ~ 2g.

The cultures obtained for each transformation reaction were split into two tubes. One tube contained 4.5 ml bacterial suspension and was used to perform the plasmid DNA extraction, whereas the remaining volume was transferred to a tube containing 0.5 ml sterile glycerol (consisted of same volumes of LB broth and glycerol). The bacterial cultures mixed with the glycerol were kept at -70°C, as reserve stocks. Plasmid DNA extraction was then conducted following the protocol outlined in the High Pure Plasmid Isolation Kit (Roche). Plasmid DNA was eluted in 100µl elution buffer (10mM Tris-HCl, pH 8.5) and the concentrations were determined in the ND-1000 system (as described in section 3.2.3). Plasmid DNAs were stored at -20°C.

3.2.5 Recombinant plasmid analysis

3.2.5.1 Restriction fragment length polymorphism analysis

Restriction fragment length polymorphism (RFLP) reactions were carried out for each plasmid DNA. SAV was digested using the restriction enzymes *Bam*HI-HF and *Bse*RI, PRV with *Not*I and PMCV with *Bam*HI-HF and *Mfe*I. All restriction enzymes were purchased from New England BioLabs. The different reactions consisted of 50 µl solutions containing 1µg plasmid DNA, 10 U of each restriction enzyme, and 1x buffer solutions (CutSmart™ for SAV and PMCV digestion reactions; NEBuffer 3.1 for PRV digestion reaction). All reactions were incubated at 37°C for 1 h in a T gradient thermocycler (Biometra). 250ng of each reaction was then loaded onto a 1% agarose gel stained with ethidium bromide alongside the same amount of non-digested SAV, PRV and PMCV plasmids for comparison. Gel analysis was conducted using GeneSnap version 7.12 (Syngene).

3.2.5.2 Sequencing

Plasmid DNAs obtained in section 3.2.4 were sent for sequencing to GATC Biotech, using LIGHTrun sequencing. LIGHTrun sequencing was prepared in tubes containing a mixture of 5µl plasmid DNA (80-100 ng/µl) and 5µl primer (5 µM). Primers used for sequencing were M13-FP (5'-GTAAAACGACGGCCAG-3') and M13-RP (5'-CAGGAAACAGCTATGAC-3'). When sequences were obtained, they were analysed using the Lasergene software 7 (DNASTAR®) to determine correct orientation and insertion into the vector pCR™II. The promoter (SP6, T7) used in the following transcription steps was dependant on whether the inserts were in a forward or reverse orientation.

3.2.6 In vitro transcription

3.2.6.1 PCR M13 FP-RP primers

Sigma virus (SIGV) was provided from the Institute of Pasteur Paris, and a plasmid with the SIGV G gene ligated into pCR™II was provided by supervisor Manfred Weidmann. For SAV, PRV, PMCV and SIGV a further set of PCR reactions were conducted to ensure the inserts were present in each of the eluted plasmid DNA, as well as to obtain PCR products containing T7 and SP6 promoter sites. PCR solutions (50µl final volume) contained 400mM M13 FP-RP primers, 1x MyTaq™ HS Mix (Bioline), and several dilutions of plasmid DNA. The reactions were then run in a T gradient thermocycler (Biometra) using the following protocol: activation for 1 min at 95°C, followed by 35 cycles of denaturing for 15 s at 95°C, annealing for 15 s

at 60°C and extension for 15 s at 72°C. PCR products were ran on a 1% agarose gel (2% agarose gel in the case of SAV) stained with ethidium bromide.

3.2.6.2 Purification of PCR products

For SAV and PMCV PCR products, a further purification step was performed, as it was not possible to obtain a single band. The target bands were identified using a white/UV transilluminator, cut from the gels using sterile blades, and then placed in separate 1.5ml tubes. Purification of the PCR products by centrifugation was conducted using the Wizard® SV Gel and PCR Clean-Up System (Promega), according to manufacturer's instructions. Purified DNA was eluted in 30µl nuclease-free water and the concentrations were estimated using the NanoDrop ND-1000.

3.2.6.3 In vitro transcription

For each transcription reaction (40µl final volume), 0.2-ml tubes containing 1x Transcription Buffer solution, 2.5mM rNTP's, 40 U T7 RNA polymerase (Roche) and 1 µg M13 PCR product were placed in a T gradient thermocycler at 37°C for 2 h followed by a 2 min inactivation phase at 65°C.

3.2.6.4 DNase treatment

Removal of remaining plasmid DNA from the transcription reaction was carried out by DNase treatments with the DNA-free™ kit (Ambion). First of all, a routine DNase treatment was performed in a 50µl solution containing each of the initial transcription volumes, 1x DNase I Buffer, and 2 U rDNase I in a 1.5ml tube. The reaction was incubated at 37°C for 30 min. 5 µl DNase inactivation reagent were then added and incubated at room temperature for 2 min. The tubes were then centrifuged at 14500g for 1.5 min and the supernatant containing the RNA was transferred to a new 1.5ml tube.

In some cases a further rigorous DNase treatment was conducted using the same kit, with some modifications. The following protocol was repeated twice. Tubes containing the previously DNase-treated RNAs were initially heated at 95°C for 5 min and then cooled on ice for 3 min. Then, these tubes containing 1x DNase I Buffer and 2 U rDNase I were incubated for 30 min at 37°C. After this incubation, a second addition of 2 U rDNase I was added to the tubes and incubated for further 30 min at 37°C. Once this incubation was complete, 0.2 volumes of DNase inactivation reagent were added to the tubes and incubated at room temperature for 2 min. The tubes were then centrifuged at 14 500g for 1.5 min and the supernatant transferred to a new 1.5ml tube.

The volumes of 10x DNase I Buffer and DNase Inactivation Reagent used for each treatment was calculated based on the current remaining volume for each RNA after performing a DNase I treatment.

3.2.7 qRT-PCR test for remaining plasmid DNA

The DNase-treated RNA samples were analysed by real time one-step RT-PCR using the QuantiTect® Probe RT-PCR Kit (Qiagen) to check for any remaining plasmid DNA and their respective CP values. Reactions were run in LightCycler® capillary tubes (20µl) (Roche) containing 1x QuantiTect Probe RT-PCR Master Mix, 500 nM primers, 200 nM probes (Table 3.1), 0.2µl QuantiTect RT Mix and 1µl transcribed RNA. In addition, the same reaction was performed omitting the QuantiTect RT Mix (RT- reaction). Non-template controls (NTC) were also included and consisted of the same reaction volumes, with nuclease-free water added instead of transcribed RNA.

Capillary tubes were then placed in a LightCycler 2.0 thermocycler (Roche). The same temperature profile was used for all samples: reverse transcription for 20 min at 50°C, activation for 15 min at 95°C, followed by 45 cycles consisting of amplification for 0 s at 95°C and 1 min at 60°C, and a final cooling stage for 5 min at 40°C. Analysis was conducted using LightCycler® software version 4.1.1.21 (Roche), with the C_T values obtained for both the RNA and RT- reactions. Based on the difference of the C_T values between the RNA and RT- reactions, a decision was made on whether further DNase treatments were required (see section 3.2.6.4) to remove any remaining plasmid DNA.

3.2.8 Quantification of transcribed RNA

Quantification of each transcribed RNA sample was conducted using the Quant-iT™ RiboGreen® RNA Reagent Kit (Invitrogen), according to manufacturer's instructions. A high-range assay was conducted, using different dilutions of RNA standard from 1 µg/ml to 20ng/ml. The RNA standard contained 16S and 23S rRNA from *E. coli*. RNA standards, controls and samples were prepared in 1.5ml tubes. Each solution was transferred onto 4 separate wells on a 96 well polystyrene ELISA microplate (F-bottom, chimney well, Fluotrac™ 600 high binding, Greiner bio-one). The analysis was performed in a Synergy HT Multi-Mode Microplate Reader (BioTek) and the quantification was conducted with Gen5 2.04 software. Calculations were then made between the samples and negative control to determine the concentration of RNA present in each of the transcribed RNA samples. The samples were then diluted in

tRNA solution (100ng/μl) (Sigma-Aldrich, UK) to 10^9 - 10^1 molecules/μl for SAV, PRV and SIGV and 10^8 - 10^1 molecules/μl for PMCV.

3.2.9 qRT-PCR tests of individual standard curves

Analysis of the RNA standards for SAV, PRV, PMCV and SIGV were conducted using LightCycler® 480 RNA Master Hydrolysis Probes (Roche). Reactions were run in LightCycler® capillary tubes (20μl) (Roche) containing 1x LightCycler®480 RNA Master Hydrolysis Probes, 3.25mM activator $Mn(OAc)_2$, 500nM primers, 200nM probes (Table 3.1), and 1μl RNA as template. Concerning the RNA templates, serial dilutions of SAV, PRV and PMCV *in vitro* transcribed RNA from 10^7 - 10^1 molecules were included in the reaction to gauge an initial standard curve. The qRT-PCR reactions were run in the LightCycler 2.0, as follows: reverse transcription for 3 min at 63°C, activation for 30 s at 95°C, followed by 45 cycles consisting of amplification for 5 s at 95°C and 15 s 60°C and finally, a cooling step of 40 s at 40°C. Analysis of the reactions was conducted using LightCycler® software version 4.1.1.21 (Roche).

This process was then repeated using triplicates of each dilution to produce standard curves. Standard curve efficiencies were calculated using a standard qPCR efficiency formula: $E = 10^{(-1/Slope)}$. Full process, protocol and analysis were conducted in the same procedure as stated above.

3.2.10 Transfer of individual assays to Smart Cycler system

After validation of each of the three viral standards (SAV, PRV and PMCV) on the LightCycler® 2.0 we then transferred each of these assays onto the SmartCycler™ system (Cepheid). The same 480 RNA Master Hydrolysis probe kit and temperature protocol was used as before (section 3.2.9). The total volume had to be corrected as the SmartCycler™® requires 2.5μl of RNA in a final mix volume of 25μl per reaction. Concentrations were kept the same for each component of the master mix.

The process was again repeated in triplicate for each assay to produce a standard curve indicating the efficiency and copy number detection threshold.

3.2.11 Incorporation of individual assays into multiplex assay

3.2.11.1 Assay optimisation

To optimise each individual assay within the complete multiplex assay, the efficiencies of each of the 3 RNA standards within the multiplex mix tested using the QuantiTect Multiplex RT-PCR kit (Qiagen). Reactions were run in LightCycler® capillary tubes (20μl) (Roche) containing 2x QuantiTect Multiplex RT-PCR NoROX Master mix, 200μm primers and probes (Table 3.1), 0.2ul/reaction QuantiTect

Multiplex RT Mix and 1ul RNA as a template. A serial dilution of 10^7 - 10^1 molecules was used for each RNA standard. The qRT-PCR reactions were ran in the LightCycler[®] 2.0 system as follows: reverse transcription 20 min at 50°C, activation 15 min at 95°C, followed by 45 cycles consisting of amplification at 94°C for 45 s and 75s for 60°C followed by a 10 min cooling step at 40°C. Subsequent alterations to the working concentration as well as the removal and replacement of primers and probes, primer dimer evaluation using syber green qRT-PCR, asymmetric PCR and visual OMP analysis were conducted. The new concentrations and combinations were re-tested following the protocol above to find the optimal set-up for each assay that would produce the most efficient and sensitive assays within the multiplex as possible. Concentrations and primers used in the final multiplex assay can be found in (Table 3.1).

3.2.11.2 Sigma virus positive control assay

After successful incorporation of the viral assays into a multiplex assay the procedure was repeated for the addition of the SIGV assay as the positive control. The sigma assay was tested as detailed above (3.2.11.1).

3.2.11.3 Mimic PCR Sigma development, testing and incorporation into the multiplex assay

Due to issues during incorporation of the SIGV assay into the multiplex mix, a switch from the originally developed assay (competitive) to a mimic PCR assay (non-competitive) was proposed and developed. The mimic assay was designed by selecting the front and reverse primers from the PMCV assay and the probe from the SIGV assay (Table 3.1) to form a hybrid target incorporating all 3-target sequences. This was then synthesised and inserted into a pcDNA 3.1 (+) plasmid (GeneArt AG, Regensburg, Germany). A restriction digest of the plasmid was then conducted using the restriction enzyme PME1 to linearise the plasmid. *In vitro* transcription was then undertaken as stated in section 3.2.6.

3.2.12 Multiplex assay triplicate tests

SAV, PRV and PMCV RNA standards were ran on the SmartCycler™ in triplicate to assess the sensitivity and specificity of the multiplex assay on the mobile platform. The procedure was carried out using the optimised protocol described in section 3.2.11.

3.2.13 Positive SAV viral RNA testing on SAV assay

To examine the coverage and specificity of the SAV primers and probes they were tested against all 6 SAV subtypes. One μ l (0.025 Tissue culture infective dose

(TCID₅₀) of SAV 1 – 6 RNA (1.25 TCID₅₀/100ul) were ran in triplicate following our standard SAV singleplex protocol (see section 3.2.9). As AFBI laboratories own subtype SAV 6, primers and probe were sent there for analysis.

3.3 Results

3.3.1 Generation of DNA Standards

3.3.1.1 Initial RT PCR, purification and ligation of plasmid DNA

The target regions for SAV, PRV and PMCV were amplified from the originally supplied plasmids using RT-PCR and specified designed primers (Table 3.2) to allow purification of the PCR product, followed by ligation into pCR™II vectors containing the T7 and Sp6 promoters. This was successfully completed for all 3 viruses with PCR product size fragments identified for SAV (316 bp), PRV (834 bp) and PMCV (907 bp) (Appendix C3.1)

3.3.1.2 Transformation of competent cells

To obtain multiple clones of each pCR™II vector, each ligation for SAV, PRV and PMCV was transformed into *E. coli* cells. This was successfully carried out through transformation of the ligation mixtures into competent TOP10F *E. coli* cells using the TA cloning Dual promoter (pCR™II) kit (Invitrogen) and cultured on LB plates containing ampicillin for selection overnight.

3.3.1.3 Isolation of pCR™II plasmid from *E.coli* and sequencing

To isolate the cloned pCR™II vectors from *E. coli* a mini prep was conducted on 10 colonies from each of the transformed overnight cultures in LB broth using the High Pure Plasmid Isolation kit by Roche. Nano drop analysis was conducted on all isolated plasmids to check concentration and purity of plasmid DNA. Isolated plasmids showing a purity of less than 1.6 in the 260/280nm absorbance range in NanoDrop analysis were removed from further analysis. Analysis indicated all but one plasmid contained high purity and concentration levels within similar range of each other. One PMCV plasmid was removed due to low levels of purity. All remaining plasmids were subjected to RT PCR's using M13 FP-RP primers.

PCR products were then analysed on agarose gels to check that the target inserts were present. Bands forming at the target size for SAV (540bp), PRV (1078bp) and PMCV (1151bp) indicated successful insertion into the plasmid. Positive results for the target size for SAV were found in 4 out of 10 of the SAV plasmids, for PRV 6 out of 10 of the PRV plasmids and for PMCV 1 out of 10 in the PMCV plasmids. These plasmids were sent for sequencing by GATC Biotech, using LIGHTrun sequencing to determine orientation, length and check for any significant changes (mutations) in the genetic structure of the insert (Lasergene software 7 (DNASTAR®)). Analysis indicated that 2 out of the 4 SAV sequences, 4 out of the 6 PRV sequences and 1

out of the 1 PMCV sequences contained the target inserted in a negative orientation. Based on all these findings plasmid 10 SAV (p10SAV), plasmid 9 PRV (p9PRV) and plasmid 7 PMCV (p7PMCV) were selected for transcription.

3.3.1.4 Restriction fragment length polymerisation analysis

A restriction digestion was conducted on p10SAV, p9PRV and p7PMCV as a tertiary procedure to test for successful insertion and orientation of the target size for each virus. The gel indicated successful insertion and a negative orientation for each plasmid, with the 3 expected bands for negative orientation (SAV 120bp, PRV 550bp and PMCV 350bp) secreted in the digested plasmids (Appendix C3.1).

3.3.2 Transcription of plasmids

3.3.2.1 Transcription of plasmid DNA

M13 primer directed PCR *in vitro* transcription of each plasmid was carried out and all remaining plasmid DNA was removed to generate an RNA standard from each plasmid. This was conducted through initial transcription of each plasmid, followed by several DNase treatments to remove the remaining plasmid DNA. RNA and DNA in the transcription mixtures were analysed in RT-qPCR and qPCR using specific primers and probes (Table 3.1), and CP values were compared. Following several qRT-PCR/qPCR tests and DNase treatments, differences in the CP values between plasmid DNA and RNA were found to have significantly increased enough for all 3 transcribed RNA's to allow for dilution of quantitative RNA standards. Negative controls showed no signs of contamination.

3.3.2.2 Quantification of transcribed RNA

To produce the RNA standards, quantification of each transcribed RNA was analysed using ssRNA specific fluorescent dye RiboGreen. The unknown RNA samples were compared to a RNA standard with known concentration and negative controls. The readings of the unknown samples were analysed and the concentration of RNA calculated. RNA concentrations (quantified in molecules/ μ l) were calculated as 2.89×10^9 (SAV), 4.4×10^9 (PRV), 8.9×10^8 (PMCV) and 4.84×10^{10} (SIGV). Serial dilutions of the RNA standards for SAV, PRV and PMCV were successfully produced in dilutions from $10^9 - 10^1$ for SAV, PRV and SIGV, and $10^8 - 10^1$ for PMCV.

3.3.3 Determination of individual qRT-PCR assay sensitivities

3.3.3.1 LightCycler 2.0

To determine overall efficiency of the RNA standards for all 4 assays using the designed primers and probes, multiple qRT-PCR reactions were carried out using decimal dilutions ranging from 10^7 – 10^1 molecules per reaction. Initial qRT-PCR's containing one sample per dilution were undertaken to determine the dilution qualities and efficiency. The results indicated that all 4 assays were highly efficient (SAV 98.9% SE 0.136; PRV 93.2%, SE 0.057; PMCV 99%, SE 0.135 and SIGV 95.68% SE 0.068)(Figure 3.1), with CP values increasing by about 3 cycles between each dilution, as well as no contamination in the negative controls. SAV, PRV and Sigma assays detected down to 10 molecules of RNA, indicating high sensitivity with PMCV detecting down to 100 molecules of RNA. The assays were tested in triplicate to further test their efficiency and sensitivity. For PRV (98.8%, SE 0.027) efficiency was found to increase slightly and sensitivity remains the same. For SAV (95.95%, SE 0.061), PMCV (98.56%, SE 0.027) and SIGV (93.07%, SE 0.066) efficiency dropped slightly with sensitivity remaining the same.

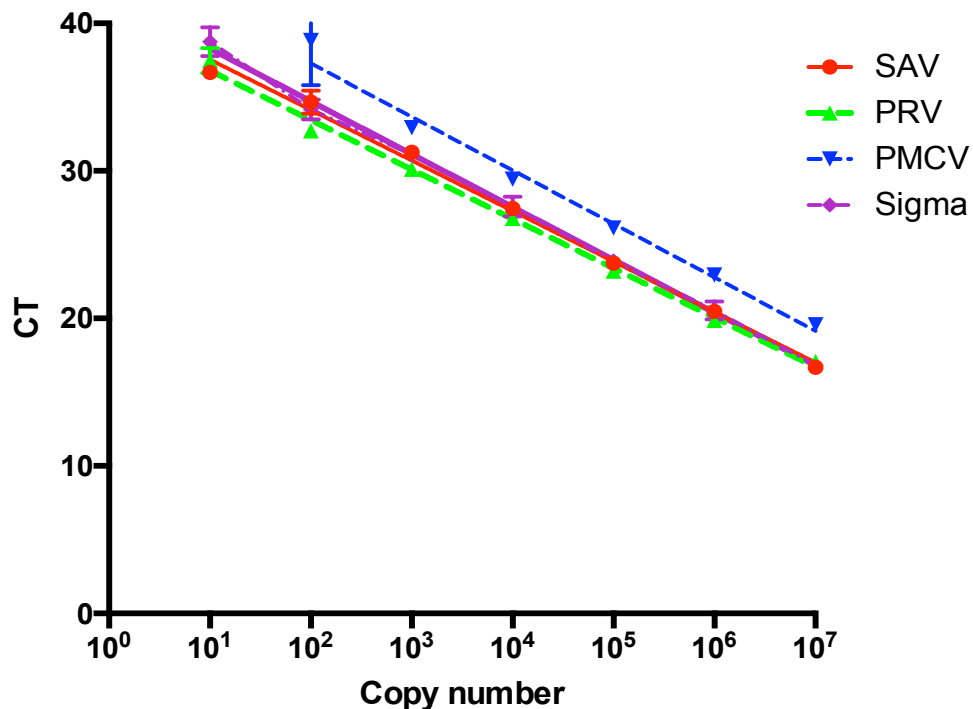


Figure 3.1. Standard curve results for individual SAV (98.9% SE 0.136), PRV (93.2%, SE 0.057), PMCV (99%, SE 0.135) and SIGV (95.68% SE 0.068) assays ran on the LightCycler 2.0 system. Dilution ranges from 10^7 - 10^1 RNA molecules were tested in triplicate and their mean values plotted as the C_T value points along with SE. SAV, PRV and SIGV assays were detected down to 10 molecules of RNA and PMCV down to 100 molecules of RNA.

3.3.3.2 SmartCycler™ System

To assess each of the 4 assays suitability for onsite testing we transferred and tested them in triplicate on the SmartCycler™. The qRT-PCR reactions were carried out using decimal dilutions of 10^7 - 10^1 molecules per reaction. A large increase in efficiency was found for SIGV (108.8%, SE 0.03) with a smaller increase seen in SAV (99.79%, SE 0.034). A small decrease in efficiency was found for PRV (96.49%, SE 0.04) however PMCV (85.73%, SE 0.08) was noticed to have dropped significantly between the two devices (Figure 3.2). All 4 assays showed no change in sensitivity between the two devices.

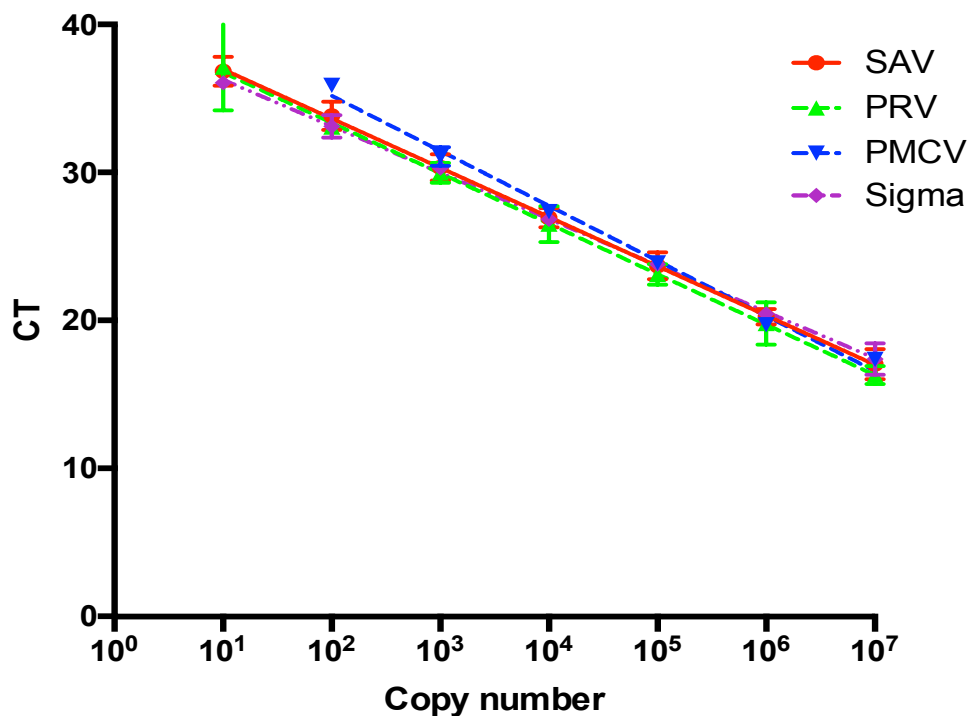


Figure 3.2. Standard curve results for individual SAV (99.79%, SE 0.034), PRV (96.49%, SE 0.04), PMCV (85.73%, SE 0.08) and SIGV (108.8%, SE 0.03) assays ran on the SmartCycler™ system. Dilution ranges from 10^7 - 10^1 RNA molecules were tested in triplicate and their mean values plotted as the C_T value points along with SE. SAV, PRV and SIGV assays were detected down to 10 molecules of RNA and PMCV down to 100 molecules of RNA.

3.3.4. Determination of Multiplex qRT-PCR assay sensitivity

To optimise the multiplex assay, different dilutions of primer and probe were tested along with short and longer temperature protocols. Once these were established the assay was tested in triplicate using the RNA standards of SAV, PRV and PMCV on the SmartCycler™ system to establish sensitivity and efficiency of each virus. A large decrease in efficiency was observed for SAV (86.5%, SE 0.11) and PRV (90.94%, SE 0.09) as well as a decrease in sensitivity from 10 to 1000 copies. A large

increase in efficiency for PMCV (100.46%, SE 0.19) was seen as well as an increase in sensitivity from 1000 to 100 copies (Figure 3.3).

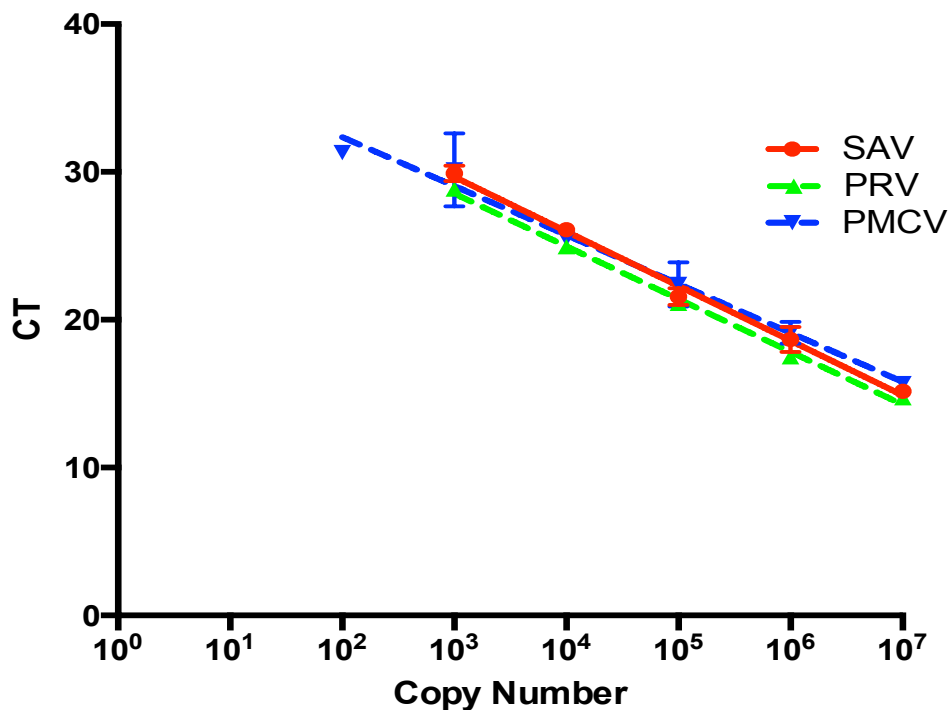


Figure 3.3. Standard curve results for multiplex assay against RNA standards of SAV (86.5%, SE 0.11), PRV (90.94%, SE 0.09) and PMCV (100.46%, SE 0.19) ran on the SmartCycler™ system. Dilution ranges from 10⁷-10¹ RNA molecules were tested in triplicate and their mean values plotted as the C_T value points along with SE. PMCV assay was detected down to 100 molecules of RNA and SAV, and PRV down to 1000 molecules of RNA.

3.3.5 Positive viral SAV RNA tests on SAV assay

The SAV assay detected all 6 subtypes of SAV at varying concentrations. SAV 1, 3 and 6 showed highest specificity with C_T ranging from 19 – 22 (10⁶ – 10⁵ copies), with SAV 2 and 5 showing much more specificity of C_T ranging from 29 – 33 (10⁴ – 10³ copies), and SAV 4 very low to no specificity with C_T of 37 – 38 (10¹ copies).

3.4 Discussion

To create molecular standards for SAV, PRV, PMCV and SIGV, qRT-PCR plasmids for each target region were created using PCR amplification, ligation and transformation. Orientation and successful insertion were confirmed through RFLP analysis and sequencing. All four derived individual RNA standards showed high efficiency on the LightCycler® 2.0 for SAV (95.95%, SE 0.061), PRV (98.8%, SE 0.027), PMCV (98.56%, SE 0.027) and SIGV (93.07%, SE 0.066) and sensitivity detecting down to 10^1 RNA molecules for SAV, PRV and SIGV and 10^2 for PMCV. The assays were transferred successfully to the mobile SmartCycler™ system where they showed high efficiency for SAV (99.79%, SE 0.034), PRV (96.49%, SE 0.04), PMCV (85.73%, SE 0.08) and SIGV (108.8%, SE 0.03); and sensitivity detecting down to 10^1 RNA molecules for SAV, PRV and SIGV and 10^2 for PMCV. The assays on the LightCycler® 2.0 were all a good pre-requisite for multiplexing as they were all within the optimal efficiency range of 90 – 105%. PMCV and SIGV, assays presented a potential problem when transferring the multiplex to the SmartCycler™ as they were both slightly out with the optimal efficiency range. High variation in efficiencies out with the optimal range in multiplex assays reduces the PCR efficiency drastically due to competition for resources in qRT-PCR reactions (Weidmann et al., 2008). Although an efficiency of >105% was recorded for SIGV, the standard deviation between individual Ct's are relatively small (0.1 – 0.8) and below the natural variation range for qPCR of +/- 1.4 (Niesters. 2001). Efficiencies higher than 100% are common in qRT-PCR possible due to an excessive amount of initial target RNA and overamplification due to residual synthetic activity of the reverse transcriptase enzyme (Suslov and Steindler. 2005) It is also known that the efficiency calculation using the formula $E = 10^{(-1/\text{slope})}$ is not optimal and is suggested that it overestimates the actual efficiency of the assay (Pfaffl. 2004).

Several PRV qRT-PCR assays have been developed prior to this one. Two of the assays were initially focused on the identification of the PRV viral genome (Palacios et al., 2010 and Haugland et al., 2011) and then subsequently used in field-testing on wild fish (Garseth et al., 2013) and genome analysis (Kibenge et al., 2013). Other assays focused predominately on immunological responses to PRV infections, primarily focusing on HSMI (Finstad et al., 2012; Wessel et al., 2017). This is the first assay specifically focused as a detection model for the virus, and that incorporates Taqman LNA probes technology. The primer design incorporates the largest number of sequences associated with PRV increasing its specificity and range. The high sensitivity of the assay should ensure it effectively detects and

quantifies viral genome from asymptomatic carriers during field-testing. Taking these factors into account and coupling it with its high efficiency, this may be the most sensitive and robust PRV assay currently developed.

Currently two qRT-PCR assays have been developed for PMCV (Løvoll et al., 2010, Haugland et al., 2011). Both assays were developed based on initial sequence information for an unknown virus, which led to the discovery of the family *Totiviridae* and the identification of PMCV. Subsequently, Løvoll's qRT-PCR protocol was used to identify the virus in wild Atlantic salmon (Garseth et al., 2012) and other fish species (Bockerman et al., 2011, Tengs and Bockerman, 2012) as well as test myocardial lesions (Wiik-Nielsen et al., 2012). The assay developed here is the only one utilizing TaqMan LNA probes and incorporates all available GenBank sequences available for PMCV in the development of the primers and probes used. The focus of this assay as a detection tool, its high sensitivity and specificity provides an up to date and highly robust PMCV detection assay.

SAV has had a vast array of studies conducted on it over the years on viable techniques to detect the virus, such as immunoperoxidase-based virus neutralization assays (Graham et al., 2003), antibody detection (Jewhurst et al., 2004, Graham et al., 2011) and two-step RT-PCR assays (Villoing et al. 2000b). Based on qRT-PCR detection platforms alone however, there is currently only one qRT-PCR assay that has been developed for subtypes 1, 2 and 3 (Hodneland and Endressen. 2006). This at the time was an adequate test for SAV until a further 3 subtypes were discovered (Fringuelli et al., 2008). A recent qRT-PCR study has attempted to detect all 6-sub types, but has so far only shown to detect 1, 2 and 5 (Shi et al., 2017). The individual SAV assay produced in this study was shown to detect SAV 1, 3 and 6 to a high specificity, but 2, 4 and 5 to a lower specificity than would be desirable for lower copy number detection. As an individual assay it would be possible to create and incorporate other SAV primers that cover subtypes 2, 4 and 5 to enhance it's specificity to these subtypes. Only two primers and one probe were used to cover all 6 subtypes in this assay. The reasoning for this was to reduce complexity when incorporated into the multiplex assay. Its potential in its current form could lead it to be used as the first qRT-PCR platform for detection of SAV 6. Further validation tests would need to be conducted before it could be reliably used.

Multiplex analysis requires the ability to show reliable and repeatable evidence demonstrating that the efficiency and sensitivity of the assays incorporated are not impaired from their singleplex results (Elnifro et al., 2000 Bustin et al., 2009). One of

the major problems with the addition of new primer sets is the unpredictability of non-specific binding (primer dimers) (Henegariu et al., 1997) and how to successfully reduce and remove them (Brownie et al., 1997). The nonspecific products can be amplified at an increased rate compared to the desired target due to their small size and out compete for resources within the reaction, impairing the amplification of the desired targets. Certain targets can also show a bias in template to product ratios by out competing and dominating the reaction at the beginning due to faster amplification leaving fewer resources for the remaining targets (Polz et al., 1998). Varying concentrations of other PCR components, such as buffers, enzymes and activators (Mn(OAc)) can also help overcome sensitivity and specificity problems (Elnifro et al., 2000).

The SAV, PRV and PMCV assays were initially incorporated into one multiplex assay. Triplicate runs on both the LightCycler[®] 2.0 and SmartCycler[™] system showed that efficiency and sensitivity had dropped for SAV (86.5%, SE 0.11) and PRV (90.94%, SE 0.09) from the singleplex assays. This was undesirable, however a small drop off in efficiency and sensitivity was expected due to the increased competition for resources in the multiplex mix. The drop off in efficiency for SAV was drastic and below the recommended 90% for an efficient multiplex assay (Weidmann et al., 2008). PRV was only reduced slightly, with PMCV (100.46%, SE 0.19) increasing slightly. Addition of the SIGV assay to the multiplex was attempted and tested against SAV. As SAV had the lowest efficiency within the multiplex at the time it would therefore likely be affected the most by the addition of the Sigma assay. SAV was drastically affected by the SIGV assay with delayed amplification and detection reduced to below 10⁴ copies. A mimic target driven by the PMCV primers and detected by the Sigma probe was synthesized to reduce the number of primers used in the multiplex reaction mix (Table 3.1). The synthesized sequence ligated in to plasmid pcDNA_3.1 (+), however caused issues with linearisation and transcription during the process to produce the mimic standard.

When the target PMCV primers were ran in a PCR with the mimic plasmid only a non-specific high bp band was observed. The plasmid was then linearised using the restriction enzymes PmeI to ensure the primers were able to bind more effectively to the target region and to reduce any potential inhibitory binding effects that supercoiled plasmid DNA would have on the primers. A PCR was performed again using the linearised plasmid but no bands were produced when the PCR product was ran on an agarose gel. Analysis of potential secondary structures of the

linearised DNA using visual OMP analysis may indicate problems with accessibility to the PCR primers. If no significant secondary structures are found utilising multiple restriction enzymes at opposite ends of the target region, including the T7 promoter site could be another option to re-test the primers and *in vitro* transcription by isolating a smaller target region. Failing all of this, selecting a different plasmid may solve the problem as the current plasmid is highly complex containing all known cut sites for restriction enzymes and including multiple different promoter sites. Using M13 primers as was done for SAV, PRV and PMCV to isolate the target would have been ideal however no plasmids were available that incorporated the T7 promoter, our PMCV and SIGV target and M13 primers within the one specific region on the plasmid. Redesigning the mimic based on the PRV primers could be a possibility, but as its efficiency is lower there may be even more competition for resources between the mimic and PRV assay during qRT-PCR.

Many problems still remain to be solved before this multiplex assay can be completed. Adjustments of the primer and probes sequences, concentration and targeted areas have been extensively explored in this study. Another possible direction to explore if the suggested mimic plasmid solutions were to fail would be to split the multiplex into two assays; one incorporating the PRV, PMCV and SIGV assay, and another with multiple SAV assays targeting specific subtypes of SAV more effectively. The problem with this approach would be the increase in the number of assays and therefore tests needed to screen all 3 viruses at once.

Successful development of the multiplex assay on both lab and mobile platforms would give huge advantages to fish farms affected by these 3 viruses across the UK, Norway and the rest of Europe. Identifying any virus that is present on the farm using one test instead of 3 would lead to earlier detection and subsequent faster reaction time to prevent, quarantine and manage positive viral detection on a farm. This in turn would save time and money for fish farmers, reducing their costs by using fewer tests, correct treatments and reducing overall loss of fish. The importance of having a test that can screen for all 3 viruses is highlighted by the evidence showing co-infections of the 3 viruses in salmon (Lovoll et al., 2010; Haugland et al., 2011; Wiik-Nielsen et al., 2016). As stated previously, the viruses generally show similar external symptoms so it would be difficult to tell what the fish has been infected with and whether co-infections have taken place without histopathology. This could then lead to one of the viruses being identified as the source and treated, only for the secondary co-infection to then take over after

treatment if not identified during histopathology.

In conclusion, highly efficient and sensitive qRT-PCR assays have been produced for SAV, PRV, PMCV and SIGV virus, and transferred onto a mobile platform for onsite testing. The SAV assay detected SAV subtypes 1, 3 and 6 efficiently. A preliminary multiplex assay was developed and optimised incorporating SAV, PRV and PMCV and transferred onto a mobile platform. Further adjustments to this assay and addition of an internal positive control are required before probit analysis and sample testing can be undertaken. Splitting of the multiplex into two assays was also suggested and is a possible path to follow to increase sensitivity and specificity.

Chapter 4: Do salmon erythrocytes elicit an immune response when infected with ISAV and SAV?

4.1 Introduction

The immune response is based on a linked network structure that begins with pattern recognition receptors (PRR) that bind to specific structural targets of pathogens, referred to as pathogen-associated molecular patterns (PAMPs). PRRs then trigger a cascade effect on activation of specific innate host defence modules such as phagocytes, inflammasomes, eosinophils, and basophils, dependant on the signalling proteins secreted e.g. toll like receptors (TLRs) and c-type lectin receptors (CLRs) (Medzhitov. 2007).

Erythrocytes are the most abundant circulating cell present in all vertebrates. Their principally accepted basic function is transport of oxygen and carbon dioxide throughout the body through gas exchange from their respiratory globin pigments. The process in which different vertebrates control this is similar, however teleost species possessing functional anion exchanges within their haemoglobin lack the ability to buffer extracellular acid loads as effectively as air breathing vertebrates (Nikinmaa. 1997).

As more research has been conducted, new and other potential functions have been discovered for human, mammalian, fish, reptile and bird erythrocytes; which have been described in an extensive review by Morera and MacKenzie (2011). One of the most interesting potential functions found is the ability for erythrocytes to directly influence the immune response. This was first described by Nelson. (1953) in human erythrocytes, where the term immune adherence (AI) was proposed. This involved the binding of immune complexes on bacteria to erythrocyte receptors, signifying a direct role in immune response. These findings were later verified where numerous complement receptors on human erythrocytes were discovered (Hess and Schifferli. 2003). Similar immune based responses have been found in bird erythrocytes with the production of cytokines (Passantino et al., 2007), TLR's and type I IFNs (St Paul et al., 2013). Piscine erythrocyte research has also now suggested that they play an active role in the immune response, and contain the transcriptional and translational functions to express genes and proteins that identify and respond to pathogens (Morera and MacKenzie. 2011).

Unlike mammalian erythrocytes, piscine erythrocytes are nucleated and contain organelles in their cytoplasm (Claver and Quagila. 2009). Antimicrobial peptides

(AMPS) such as antibacterial proteinaceous factors against *Planococcus citreus* have been isolated from rainbow trout erythrocytes (Fernandes and Smith, 2004). AMPs were also derived from a β -chain of the respiratory transport pigment haemoglobin in channel catfish (*Ictalurus punctatus*, Rafinesque) and shown to express antimicrobial properties against parasites (Ullal et al., 2008). The AMP Nk-lysin (Nkl) was also found to be expressed by turbot (*Scophthalmus maximus*) erythrocytes, suggesting an antiviral role against Rhabdovirus in the fish (Pereiro et al., 2017). Previous evolutionary studies have suggested that the AMPs derived from haemoglobin were an evolutionary innate immune precursor conserved within the current haemoglobin transport pigment today (Jiang et al., 2007).

Studies into rainbow trout erythrocytes identified PRR and PAMP responses to Poly I:C stimulation (Morera et al., 2011). These included upregulation of the expression of genes encoding IFN- α , TLR and the immune cell recruiting CC chemokine (CCL4). Infection of Atlantic salmon erythrocytes by salmon anaemia virus (ISAV) showed expression of IFN system genes in response to ISAV infection (Workenhe et al., 2008). This was also shown for the expression and up regulation of the antiviral immune genes IFN- α , RIG-I, Mx and PKR during PRV infection of Atlantic salmon erythrocytes (Finstad et al., 2014; Wessel et al., 2015; Dahle et al., 2015). These findings illustrate the potential complexity of piscine erythrocytes functions. The presence of PRRs and subsequent PAMP-PRR responses would suggest that piscine erythrocytes actively contribute to the active immune response. The data available is still lacking and has only been demonstrated in a small number of species and in regard to specific viruses and diseases.

ISAV and SAV are both highly infectious viruses causing severe economic loss to the salmon fish farming industry (see section 1.6 for more detail). ISAV is a fish *orthomyxovirus* assigned to the genus *isavirus* and is the causative agent of the highly infectious disease infectious salmon anaemia (ISA) (Kibenge et al., 2004). Disease outbreaks have occurred across Europe (Roger et al., 1998, Nylund et al., 2003 and Plarre et al., 2005), Canada (Mullins et al., 1998; Bouchard et al., 1999; Lovely et al., 1999), the USA (Bouchard et al., 2001) and Chile (Godoy et al., 2008; Mardones et al., 2009). Fish infected with the disease suffer severe anemia and damage to internal tissue usually leading to mortality. External symptoms consist of slow moving fish near the surface, darkened and haemorrhaging skin, and pale gills and fins. Internal histopathology generally shows haemorrhages and necrosis in the kidney, liver and renal intestine (Mullins et al., 1998, Roger et al., 1998, Bouchard et al., 2001, Simko et al., 2000). ISAV has been shown to actively enter and replicate

within erythrocytes, entering by endocytosis and inducing the interferon pathway immune response (Workenhe et al., 2007; Workenhe et al., 2008). No follow up studies at present have been undertaken.

Examining the potential immuno-based responses that erythrocytes exhibit and trigger when infected with viruses would give us a better understanding of how erythrocytes contribute to the innate immune response and systems in fish. ISAV is an ideal candidate to focus on as it has been shown in previous research to infect, replicate and induce an immune response within erythrocytes. Examining whether a similar response is found in erythrocytes infected with SAV would be greatly beneficial to understanding and combating the virus.

In order to verify if virus replication in erythrocytes induces the expression of innate immune response genes we aimed to:

1. Isolate and infect erythrocytes from Atlantic salmon with ISAV and SAV at MOIs (multiplicity of infection, see formula in section 3.4) 10, 1 and 0.1 alongside Poly I:C stimulated and non-infected control erythrocytes for a 24 hr period
2. Extract erythrocyte RNA and convert to complementary DNA (cDNA) to test against a set of viral response gene to observe if any immune responses were recorded.

4.2 Materials and Methods

4.2.1 Fish handling and sampling

Fish were sourced from the University of Stirling's Institute of Aquaculture fish farm, Buckieburn in May of 2017. Blood was harvested from six individual juvenile salmon (0.5 – 1ml) per visit. The blood from each fish was transferred into individual falcon tubes on ice containing 30ml of Dulbecco's Modified Eagle's medium high glucose (DMEM) mix containing 10% chicken serum (CS) and primocin (90µg/ml) and transported back to the lab on ice for immediate processing.

4.2.2 Processing of blood

All tubes were centrifuged at 700g for 10 mins. The supernatant was removed and the pellets resuspended in 30ml of DMEM and primocin (100µg/ml). The resuspended pellets were poured into 10ml of histopaque per tube. The tubes were centrifuged at 700g for 15 mins and the supernatant removed. The pellets were fully resuspended in 10ml of Dulbecco's phosphate-buffered saline (DPBS) and centrifuged at 700g for 15 mins. The supernatant was removed and the pellets resuspended in 30ml of DMEM and primocin (100µg/ml). Dilutions of 1:10 and 1:100 were made from the neat stocks of erythrocytes per fish to calculate each concentration. The 1:100 dilutions were observed under a microscope and cells counted using a haemocytometer. The six non-diluted erythrocyte preparations were diluted in DMEM, CS and primocin (90µg/ml) to a concentration of 2×10^6 cells/ml. These were plated in quadruplicates per fish in 12 well plates (6 x 4 x 12) and left to incubate at 15°C, 1% CO₂ for 24 hours to allow the cells time to acclimatise.

4.2.3 Growth and cultivation of viral supernatants

We used Chinook salmon embryo-214 (CHSE-214) cells for growth of both ISAV and SAV. CHSE-214 cells were passaged overnight in 25cm³ flasks at 22°C, 5% CO₂ to 80% confluence. ISAV and SAV were made up of 5ml stocks diluted at 1/100 and 1/1000 dilutions in Hank's balanced salt solution (HBSS), 2% fetal bovin serum (FCS). Media was removed from each flask and 1ml of each dilution of ISAV and SAV added to individual flasks with 1ml of 2% HBSS added to control flasks containing no virus. These were then incubated at 15°C, 1% CO₂ for 1 hour. The virus cultures were then removed from each flask and replaced with 5ml of Eagles minimum essential media (EMEM) and incubated at 15°C, 1% CO₂ with daily checks for cytopathic effect (CPE). ISAV and SAV supernatants were then harvested by transferring them to 15ml tube and centrifuging at 2500g for 15mins at 4°C. The supernatant was then transferred to fresh tubes, and the virus suspensions diluted

to 1×10^5 cells per ml in 2% HBSS. Media was removed from the flasks and 8 ml of ISAV added to one flask, and 8ml of SAV to the other for 1 hr at 15°C, 1% CO₂. Virus suspensions were then removed and replaced with 40mls of EMEM media. CPE was checked daily. Cultures when then harvested as before into 50ml tubes, and stored at 4°C for short term and -80°C long term.

4.2.4 Ultracentrifugation and quantification of ISAV and SAV

5ml of Optriprep cushions were layered into the base of ultracentrifugation tubes and 25ml of viral supernatant added on top, ensuring layers were not mixed. These were then centrifuged at 25 000g for 90mins. The supernatant was discarded and 100µl of 1x NTE (NaCl-Tris-EDTA buffer) added to each tube and chilled overnight at 4°C to resuspend concentrated viruses. All samples were pooled and diluted out in to 1/10, 1/100, 1/1000 and 1/10000 dilutions. The samples were quantified by virus specific qRT-PCR on the LightCycler® 480 thermocycler (Roche) using Lightcycler® 480 RNA master hydrolysis probes (Roche). The samples were ran in triplicate alongside known quantitative standard curves for each virus, which were ran in duplicate. The same temperature profile was used for all samples: reverse transcription for 3 mins at 60°C, activation for 30 s at 95°C; and PCR cycle of 95°C for 5 s, 60°C for 15 s repeated for 40 cycles.

4.2.5 “Infection” of erythrocytes with ISAV, SAV and Poly I:C

For each of the 6 fish erythrocytes samples that were processed plated and incubated; (see section 4.2.1 and 4.2.2) each plate was “infected” in triplicate with an MOI of 10, 1 and 0.1 of ISAV (6 plates) and SAV (6 plates) (see appendix C4.1). MOI for each virus was calculated from the average copy number obtained through qRT-PCR multiplied by 0.7 to account for cell viability: TCID₅₀ x 0.7 = Total viable cells. The MOI was calculated using this formula: $P(0) = 1 - e^{-\text{moi}}$. Concentrations of each MOI were then subsequently calculated for infection. We added Poly I:C at 10ug/ml per well. Each plate was centrifuged at 1000g for 1 min before infection with the virus and stimulation with Poly I:C. We removed 500µl from each well. Each well was then infected or stimulated with the virus or Poly I:C. The plates were then returned to the incubator for 1hr at 15°C, 1% CO₂. 500µl of DMEM, CS and primocin (100µg/ml) were added back to each well and incubated for 24 hrs at 15°C, 1% CO₂.

4.2.6 Harvesting and RNA extraction of erythrocytes

1.5ml of cells from each of the first wells were transferred into 2ml tubes and centrifuged at 200g for 1 min. The supernatant was removed from each tube. This was repeated with the two remaining wells at each MOI per fish. 500ul of PBS was

added to each pellet and resuspended by vortexing. Tubes were centrifuged at 200g for 1 min with the supernatant removed. We added 0.2% octylphenoxypolyethoxyethanol (NP-40) to each pellet, vortexed for 10 s and centrifuged at 16 800g for 1 min. The supernatant was transferred into Tri reagent. 1-Bromo-3-chloropropane (BCP) was added to each tube, vortexed for 10 s and then incubated on ice for 15 mins. The tubes were then centrifuged at 12 400g for 10 mins. The top layer of solution (RNA) was removed and placed in new 1.5ml tubes containing isopropanol. These were then mixed by inversion and placed at -80°C overnight. The samples were then centrifuged at 16 800g for 10 mins at 4°C to form a pellet. All liquid was removed from each samples and the pellet washed with 1ml of 75% ethanol and centrifuged at 5 500g for 5 mins. All liquid was removed from each tube and allowed to air dry. The pellets were resuspended in 20µl of H₂O and the RNA concentration checked on a NanoDrop™ 1000 spectrophotometer (ThermoFisher). A random selection of extracted RNA samples from ISAV and SAV controls as well as Poly I:C stimulated samples were ran on a 1% agarose gel to confirm 28s and 18s bands were present with no DNA contamination. All RNA was then stored at -80°C.

4.2.7 Generating cDNA of ISAV, SAV, Poly I:C and Control RNA

cDNA synthesis was conducted using Tetro reverse transcriptase (Bioline). Each reaction consisted of oligo (dt)₁₈ (5µm), random hexamers (20µm), 10mM dNTP mix, 5X RT buffer, Tetro Reverse Transcriptase (200µ/µl), DEPC-treated water and RNA (up to 5µg). The reactions were ran in a thermal cycler (Techne Flexigene) at 25°C for 10 mins, 45°C for 60 min for reverse transcription and 85°C for 5 min to terminate the reaction. Each cDNA reaction was diluted 1/10 in DEPC-treated water and stored at -20°C.

4.2.8 Preparation and testing of target specific primers

Primer pairs for 31 salmon immune genes primers (table 4.1) were diluted to a working concentration of 10mM in DEPC-treated water. All primer sets were ran against a positive salmon cDNA sample (SPL/IC) in triplicate to check primer efficiencies. We used a custom made in house Syber green containing 10 x immolase buffer (Bioline), 50mM MgCl₂, 25mM dNTP, 1/100 diluted sybr green (Invitrogen) and immolase (Bioline) 2500 units. These were ran in a LightCycler® 480 Instrument II (Roche) at 95°C for 10 mins and 95°C for 30s; followed by PCR at 62°C for 30s, 72°C for 30s and 80°C for 5s (x40); and a melt curve 95°C for 5s (ramp rate 4.8°C/s), 75°C for 60s (ramp rate 2.5°C/s). All triplicate samples were ran

on a 1.5% agarose gel and checked for target bands. Those that failed or showed double bands were removed from further testing.

4.2.9 Generation and testing of Atlantic salmon gene standards

4.2.9.1 PCR

All valid primer pairs for each gene from the previous step were re-run in triplicate using MyTaq HS mix (Bioline) to produce a larger volume of PCR product for purification and generation of each standard. Each reaction consisted of 5x MyTaq, primers (20µm) MyTaq HS DNA polymerase, DEPC-treated water and positive salmon cDNA template (SPL/LC) that covers all Atlantic salmon mRNAs. The reactions were ran on a thermocycler (Eppendorf MasterCycler) at 95°C for 1 min; and 95°C for 15s, 60°C for 15s and 72°C for 10s (x40). All reactions were ran on a 1.5% agarose gel.

4.2.9.2 PCR purification

PCR products were purified using PureLink® PCR purification kit (ThermoFisher). 4x binding buffer was added to 1x PCR product (50 -100µl) and added to PureLink® spin columns. The columns were centrifuged at 10 000g for 1 min and the flow through discarded. 650µl of wash buffer was added to each column and centrifuged at 10 000g for 1 min with flow through discarded. The columns were then centrifuged at maximum speed (12 000g) for 2 – 3 mins with the residual wash buffer discarded. The columns were then placed in 1.5ml collection tubes and 50ul of elution buffer added. The columns were incubated at room temperature for 1 min and then centrifuged at max speed (12 000g) for 2 mins. The purified PCR concentrations were quantified on a spectrophotometer (ND-1000). Any genes showing poor quality were removed from further testing. The genes were diluted to 10ng/µl and a 10 fold serial dilution made of each.

4.2.9.3 qRT-PCR test of standard curves

All standards were ran in duplicate on the LightCycler® 480 Instrument II (see section 4.2.8). Failed standards were removed from further analysis.

4.2.10 cDNA gene expression quality check

All cDNA from ISAV, SAV infected and Poly I:C stimulated cells and control cDNA were tested against the salmon gene elongation factor 1 alpha (Ef1α) first as it is suggested as a good reference gene for gene expression in qRT-PCR examination of Atlantic Salmon (Olsvik et al., 2005). All samples and Ef1α standards were ran in duplicate on the LightCycler® 480 Instrument II (see section 4.2.8). Low quality

samples were removed from further testing. We insured coverage of samples from at least 4 different fish across SAV, ISAV, Poly I:C and controls.

4.2.11. qRT-PCR tests of SAV, ISAV, Poly I:C and control cDNA against salmon immune genes

Using the remaining 16 genes (Table 4.1), qRT-PCR reactions were ran against 42 cDNA samples (15 ISAV, 13 SAV, 5 Poly I:C and 9 Controls) using the LightCycler® 480 Instrument II (see section 4.2.8). All samples and standard curves were run in triplicate. Due to the small volume of RNA obtained per sample from erythrocyte extractions, it was not possible to re-run any optimised standards for any of the other genes that had initially failed.

Table 4.1. Primers in 5' to 3' orientation, amplicon size and Genbank number of all salmon genes tested against erythrocyte cDNA

Primer	Sequences	Amplicon size (bp)	GenBank Acc. No.
EF1 α -F	CAAGGATATCCGTCGTGGCA		
EF1 α -R	ACAGCGAAACGACCAAGAG	327	AF321836
IFNa1-F	TGCAGTATGCAGAGCGTGTG		
IFNa1-R	TCTCCTCCATCTGGTCCAG	101	NM_001123710.1
IFNb-F	TGCATTGGAGGCTATGCGATAT		
IFNb-R	TTCCCAAACACCACCTACGACA	151	EU735552.1
IFNc-F	ATGTATGATGGGCAGTGTGG		
IFNc-R	CCAGGCGCAGTAACTGAAAT	118	JX524153.1
IFNe-F	TGGCCTTCATTTTCGCACT		
IFNe-R	GTTTTCATAGACTTTACGCATTG	157	AGKD0100007
IFNf-F	TGTTCTTTACCCGGTTGTCTATG		
IFNf-R	AAGCTTTTCCCCCTCCTGTA	184	AGKD01028711
IFNyrel-2-F	GAAAGGCCCTTTGGATCTGAACA		
IFNyrel-2-R	CAACAGCATCATTGGTCTTAATG	299	AGKD01091451
Mx-F	CCTCCTGAAATCCGCGAAGAC		
Mx-R	GAGTCTGAAGCATCTCCCTCTG	365	NM_001123693.1
RIG-I-F	ACTGATCGGGAGAGGACACAA		
RIG-I-R	CTTGACCACATTGCCAACGTAT	202	NM_001163699.1
MDA5-F	AGCTCAATGGGTTTCAGGAGAA		
MDA5-R	CTCTTCAGCCACTGTTGTTGC	181	KU376486.1
S02(TNF α 1)-F	ACTGGCAACGATGCAGGACAA		
S02(TNF α 1)-R	GCGGTAAGATTAGGATTGTATTCA CCCTCT	144	NM_001123589.1
IL-1 β -F	CCGTCCCATTGAGACTAAAG		
IL-1 β -R	TGTCGCTCTGCTGGCTGA	156	NM_001123582.1
gIP (CXCL_11)-F	TGCCGGAACATGGAGATCAT		
gIP (CXCL_11)-R	TTACTGCACACTCCTTTGGTT	127	XM_014143455.1
S27 (IL17C3)-F	CTGCAAGGTTTCATAATGAGCATCC		
S27 (IL17C3)-R	CCTCTTCTTGTCGGAATCTTCTGAG T	186	AGKD01040242
S32 Cathelicidin 1-F	TGTCCTCTGAAGAAAATGGGAAA CT		
S32 Cathelicidin 1-R	TCTTCTTGTCGGAATCTTCTGCAT	156	GQ870278
S33 Cathelicidin 2-F	CCTCTGAAGAAAATGGGAAACG		
S33 Cathelicidin 2-R	CCTCTTCTTGTCGGAATCTTCTGAG T	170	NM_001123573
S101 Beta- defensin-1a-F	GCAACTAGAATGTCTTGTCAACGT ATGG		
S101 Beta- defensin-1a-R	TGAGAAACACAGCACAGAATCCC T	200	*IP protected

4.3 Results

4.3.1 Erythrocyte processing, cultivation, infection and RNA extraction

Erythrocyte cells were successfully processed, plated and cultivated for 24 hrs from 12 individual Atlantic salmon at a concentration of 2×10^6 cells per ml. Infection of the erythrocyte cells with ISAV and SAV were successfully carried out on 6, 12 well plates each (1 per fish) at MOIs of 10, 1 and 0.1. RNA extractions were positive in 31 of the 36 ISAV extractions, 26 of 36 SAV extractions, 9 of 12 Poly I:C extractions and 23 of 24 control extractions (12 ISAV, 11 SAV). Quality and concentration was assessed through spectrophotometer measurements. Further RNA quality tests were conducted using RNA gel electrophoresis. Four samples from ISAV, 3 from SAV controls and 3 - 4 samples from Poly I:C stimulated erythrocytes showed RNA bands at 28s and 18s (Figure 4.1 and 4.2).

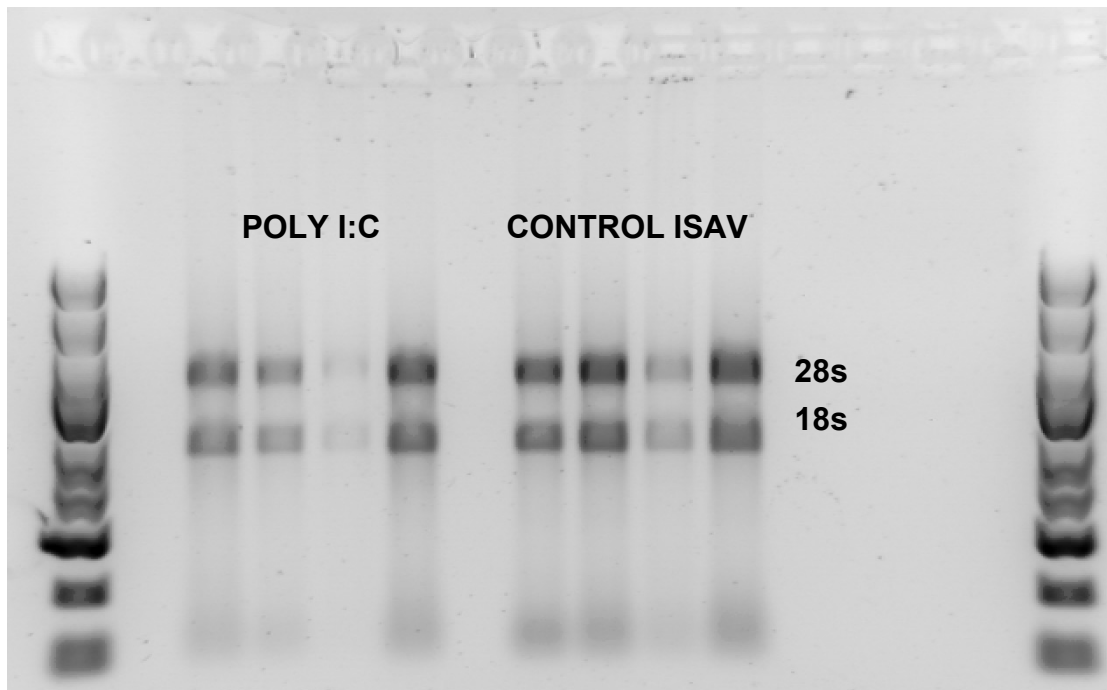


Figure 4.1. A 1% agarose gel run of 4 control RNA samples from ISAV stimulated erythrocytes and 4 Poly I:C stimulated erythrocytes. All but Poly I:C F3 showed RNA bands at 28s and 18s bands confirming pure RNA.

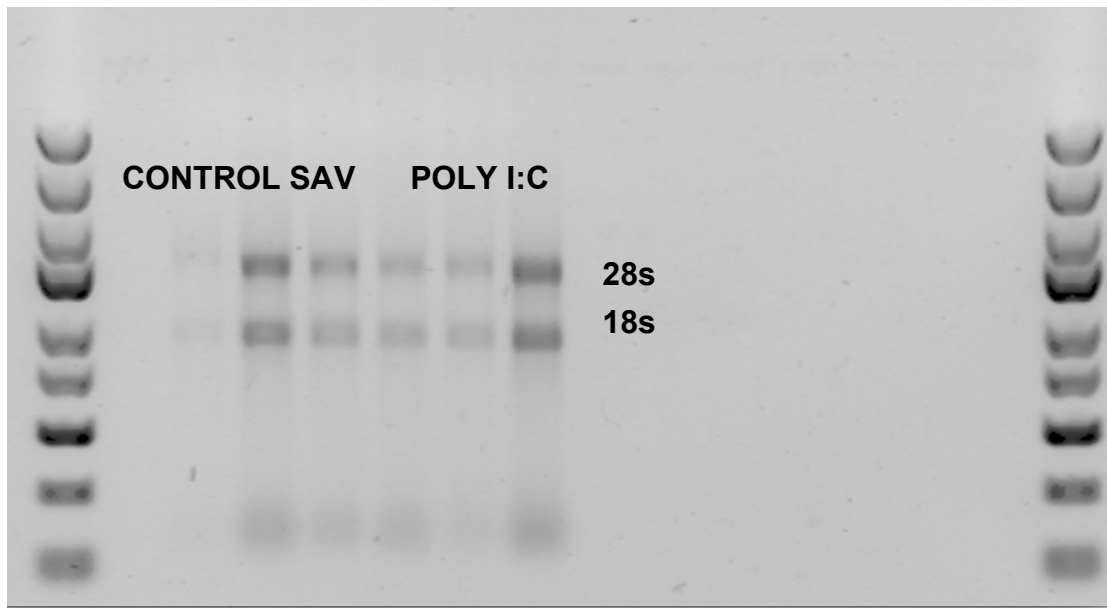


Figure 4.2. A 1% agarose gel run of 3 control RNA samples from SAV stimulated erythrocytes and 3 Poly I:C stimulated erythrocytes. All but Con F1 1 showed RNA bands at 28s and 18s bands confirming pure RNA.

4.3.2 cDNA generation of ISAV, SAV, Poly I:C and controls and Ef1 α qRT-PCR quality test.

All 54 RNA samples (18 ISAV, 18 SAV, 6 Poly I;C and 12 controls) were successfully converted to cDNA. The Ef1 α qRT-PCR confirmed good gene expression (Cts < 25) for 42 of the 54 samples (15 ISAV, 13 SAV, 5 Poly I;C and 9 Controls).

4.3.3 Primer target and qRT-PCR standard testing on salmon genes

Of the 31 primer combinations tested against positive salmon cDNA (SPL/IC), 21 produced the expected bands at their target size. The remaining 10 produced either double bands or no bands at all. These primer pairs were removed from further analysis. The remaining 21 mRNA targets were successfully purified and diluted into a working concentration of 10ng/ μ l. A further 3 genes were removed when they produced no standard curve during qRT-PCR runs against the positive salmon cDNA template (SPL/LC).

4.3.4 qRT- PCR tests of ISAV, SAV, Poly I:C and control erythrocyte cDNA tests against 18 salmon genes

4.3.4.1 Standard curves

Out of 18 of the genes tested 7-produced efficient standard curves (Table 4.2, Figure 4.3), 6 had efficiencies lower than 90% (GIP, S27, RIG I, MDA5, S2 and S32)

and 5 showed no standard curves (IFNe, IFNf, EF1 α , 105 and S33). Targets below 90% were not re-analysed.

Table 4.2. Standard curve results showing efficiency (%) and standard error (SE) for IFNa, b, c Mx, IL-1 β , IFN γ rel-2 and 101

Gene	Efficiency (%)	Standard error (SE)
IFNa	91.53	0.097
IFNb	98.84	0.128
IFNc	91.71	0.122
Mx	92.10	0.099
IL-1 β	93.11	0.055
IFN γ rel-2	96.57	0.206
101 beta defensin-1-a	89.57	0.125

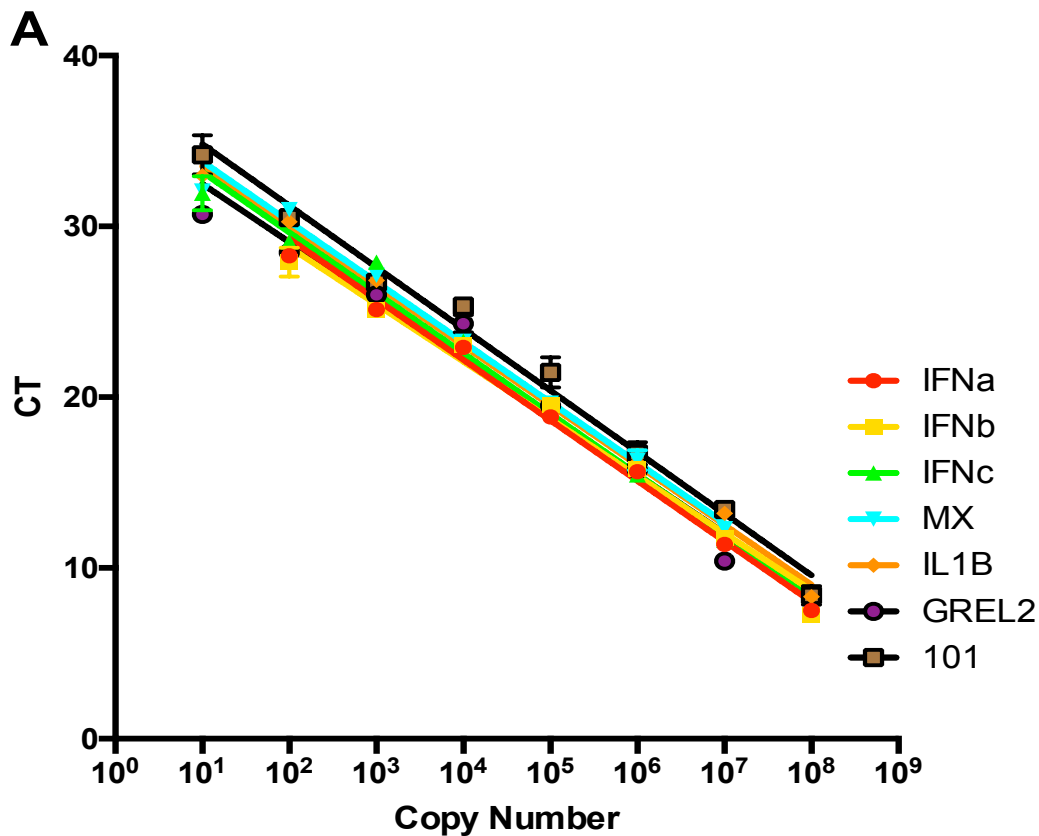


Figure 4.3. Standard curve results for individual IFNa (E: 91.53, SE 0.097), IFNb (E: 98.84%, SE 0.128), IFNc (E: 91.71%, SE 0.122), Mx (E: 92.10%, SE 0.099), IL-1 β (E: 93.11%, SE 0.055), IFN γ rel-2 (E: 96.57%, SE 0.206) and 101 (E: 89.57%, SE 0.125) assays ran on the LightCycler[®] 480 II system. Dilution ranges from 10^8 - 10^1 RNA molecules were tested in duplicate and their mean values plotted as the C_T value points along with SE. IFNc, Mx, IL-1 β , IFN γ rel-2 and 101 assays were detected down to 10 molecules of RNA, and IFNa and IFNb down to 100 molecules of RNA.

4.3.4.2 Comparison of SAV MOI, control and Poly I:C comparisons

For each of the 7 genes with efficient standards, the copy numbers expressed were compared from the MOIs, controls and Poly I:C cDNA for significant differences in expression. For all 7 genes, no significant differences were found between MOIs and controls. A significantly higher mean expression of Mx was observed in Poly I:C stimulated cells than in cells infected at all 3 MOIs (M10: $p = 0.028$, M1: $p = 0.016$, M0.1: $p = 0.025$). Significantly lower mean expression of 101 (beta defensin 1a) was recorded in MOI 10 ($p = 0.003$) and 1 ($p = 0.002$) compared to Poly I:C. Controls of IFNc ($p = 0.013$) and Mx ($p = 0.024$) showed significantly lower mRNA abundance than those observed in Poly I:C stimulated. High variations in copy number between samples were found in all MOIs of IFNa, b, Mx, IFN γ rel-2 and 101 and MOI 10 of IFNc (Table 4.3, Fig 4.4). IL-1 β showed the least variation across all MOIs, control and Poly I:C. There appeared to be a correlation of decreasing copy number with decreasing MOI in IFNb stimulated SAV ($r = 0.168$). This would suggest that for SAV infection IFNb is upregulated to slightly higher levels in high MOIs.

Table 4.3. Mean and standard deviation of SAV and ISAV infected erythrocyte cDNA samples against IFNa, b, c, Mx IL1B, IFN γ rel-2 and 101 genes ran on the LightCycler® 480 II system.

SAV				ISAV			
Gene	MOI	Mean	Std	Gene	MOI	Mean	Std
IFNa	10	319.8	273.7	IFNa	10	622.8	599.3
	1	597.5	539.7		1	782.8	557.0
	0.1	271.0	186.8		0.1	525.6	388.3
IFNb	10	739.3	1193.0	IFNb	10	5829.0	4296.0
	1	128.7	152.3		1	29.8	47.7
	0.1	28.4	19.3		0.1	217.3	37.7
IFNc	10	3668.0	3442.0	IFNc	10	4740.0	2456.0
	1	2760.0	1776.0		1	3296.0	2331.0
	0.1	1996.0	1317.0		0.1	3281.0	1779.0
Mx	10	498.9	806.3	Mx	10	498.9	806.3
	1	263.3	264.8		1	263.3	265.8
	0.1	472.3	457.7		0.1	472.3	457.7
IL1B	10	1031.0	475.5	IL1B	10	1679.0	803.8
	1	1300.0	474.2		1	1860.0	690.9
	0.1	816.1	379.6		0.1	1557.0	655.0
IFN γ rel2	10	10619	19591.0	IFN γ rel2	10	3523.0	6844.0
	1	2664.0	2210.0		1	1745.0	2044.0
	0.1	3819.0	2254.0		0.1	2978.0	5312.0
101	10	334.8	260.0	101	10	338.4	364.5
	1	258.1	155.7		1	456.6	305.3
	0.1	1548.0	2557.0		0.1	1104.0	1111.0

4.3.4.3 Comparison of ISAV MOIs, control and Poly I:C

For 6 of the 7 genes no significant differences were found between MOI and controls (Table 4.3). There was a significantly lower mean expression of Mx at MOI 1 ($p = 0.024$) than in the control. For 101, all 3 MOIs showed a significantly lower mean expression than those in Poly I:C (M10: $p = 0.002$, M1: $p = 0.003$, M0.1: $p = 0.0144$). Significantly higher expression of IFN α was found in controls compared to Poly I:C stimulated ($p = 0.03$), but a significantly lower expression found in Mx ($p = 0.013$) for the control compared to Poly I:C stimulated. High variations in copy numbers were observed across all genes at all MOIs (Table 4.3, Figure 4.4). IL-1 β again showed the least variation across MOIs, Poly I:C and controls. There appeared to be a correlation of increasing copy number with decreasing MOI in IFN β stimulated ISAV ($r = 0.159$). This would suggest that for ISAV infection IFN β is upregulated to slightly higher levels in low MOIs.

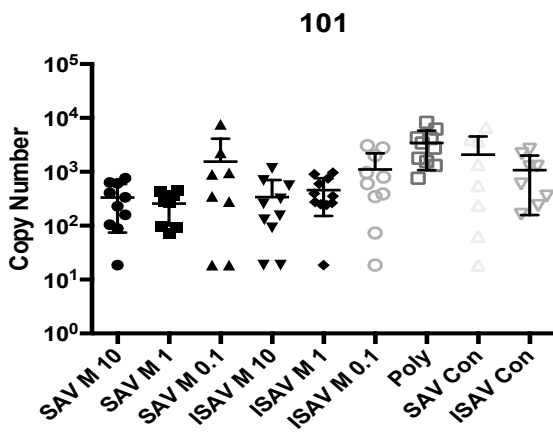
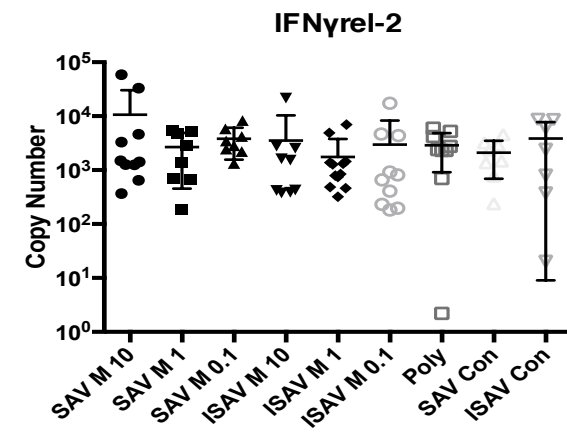
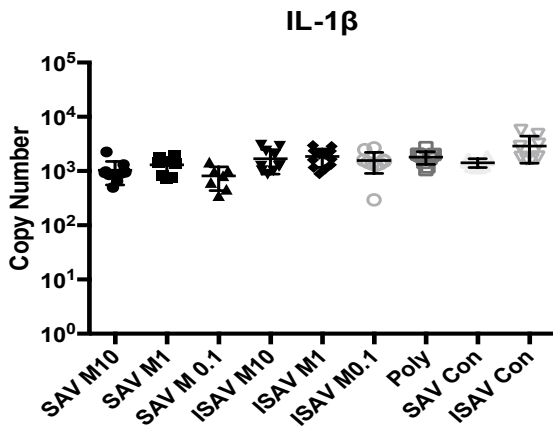
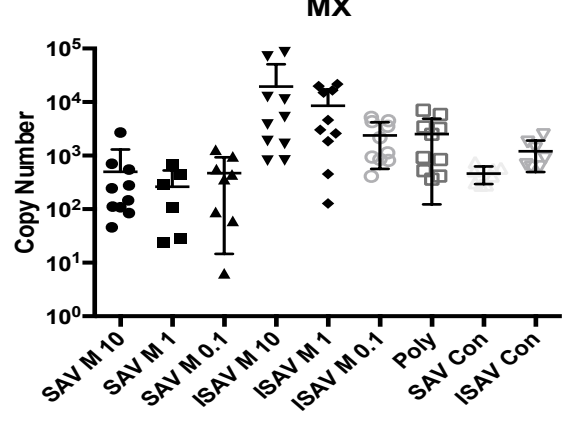
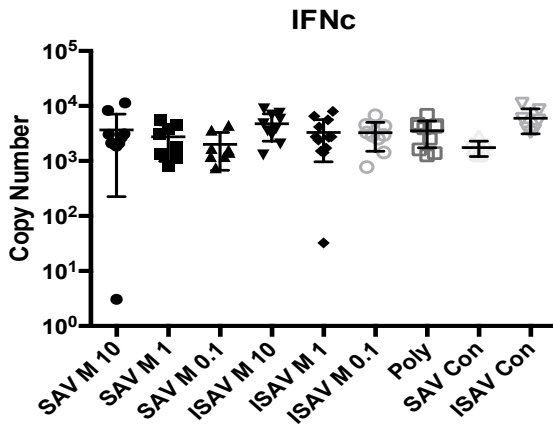
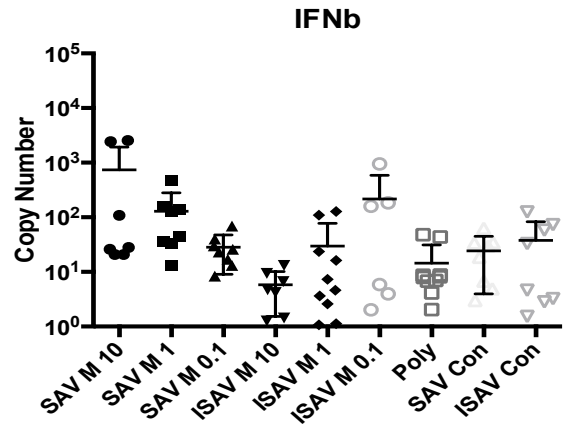
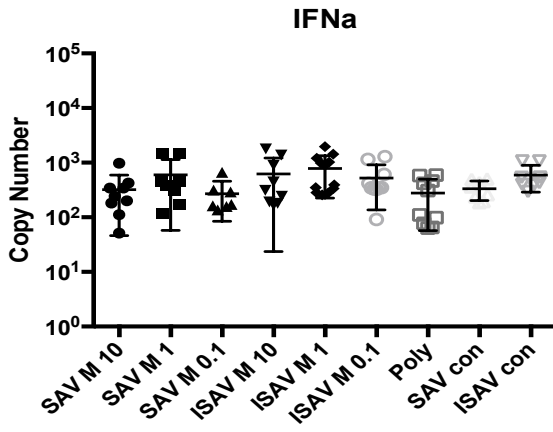


Figure 4.4. Erythrocyte cDNA generated from 24hr viral infection of SAV and ISAV at MOIs 10, 1 and 0.1, Poly I:C stimulated and control tested against 7 Atlantic salmon immune genes and defensins. Interferon (IFN) a, b and c, Interferon-induced GTP-binding protein Mx1 (Mx), Interleukin 1 beta (IL-1 β), Interferon gamma related 2 (IFN γ rel-2) and Beta defensin 1a (101). N = 8 – 12, copy numbers expressed as mean +/- StD.

4.4 Discussion

SAV and ISAV were successfully grown in CHSE-214 cells. There were numerous cell lines for each virus, which could be utilised, each with advantages and disadvantages. The CHSE-214 cell lines were chosen due to the extensive work carried out over the past 20 years showing consistent and competent growth of all SAV subtypes and ISAV (Table 4.4).

Table. 4.4. Comparison of cell lines used for growth of both ISAV and SAV over the past 20 years

Cell line	SAV	ISAV
CHSE-214	Nelson et al., 1995, Castric et al., 1997, Villoing et al., 2000, Graham et al., 2003, Hodneland et al., 2005, Boscher et al., 2006, Graham et al., 2008, Hearth et al., 2009 Pettersen et al., 2013, Graham et al., 2014	Bouchard et al., 1999, Kibenge et al., 2000, Bouchard et al., 2001, Grant and Smail. 2003, Godoy et al., 2008
TO	Graham et al., 2008	Wergeland and Jakobsen. 2001, Grant and Smail. 2003, Kibenge et al., 2006
RTG-2	Nelson et al., 1995, Villoing et al., 2000, Graham et al., 2003, Graham et al., 2008	N/A
CHH-1	Hearth et al., 2009, Pettersen et al., 2013	N/A
AS	Nelson et al., 1995,	N/A
BF-2	Nelson et al., 1995, Graham et al., 2008	Godoy et al., 2008
FHM	Nelson et al., 1995, Graham et al., 2008	N/A
EPC	Nelson et al., 1995, Graham et al., 2008	Godoy et al., 2008
SHK-1	Graham et al., 2008 Hearth et al., 2009	Dannevig et al., 1995, Falk et al., 1997, Kilbenge et al., 2000 Simko et al., 2000, Bouchard et al., 2001 Grant and Smail 2003, Falk et al., 2004 Godoy et al., 2008, Kilbenge et al., 2016
ASK	N/A	Devold et al., 2000, Falk et al., 2004, Plarre et al., 2005, Kilbenge et al., 2016

The original concentration of ISAV harvested was too low for an MOI of 10, so a density gradient ultracentrifugation was conducted to increase the concentration of ISAV. It has been shown that density gradient ultracentrifugation can increase and further purify virus particles (Fauquet et al., 2005). After ultracentrifugation ISAV concentration were found to have increased high enough to allow infection of ISAV at an MOI of 10.

To ensure no false positive results were obtained for gene expression, all leukocytes and immune-gene expressing entities were removed from the salmon blood obtained from the farm through a rigorous wash procedure. It is essential that these components are removed due to their immunological responses during viral infections (Medzhitov 2007; Tajeuchi and Akira. 2010). Although all steps were taken to remove leukocytes there is likely still a small <1% remaining. Due to the low numbers of leukocytes found within 1ml of untreated blood and the steps taken to remove them, any remaining leukocytes are unlikely to have contributed any noticeable expression of viral response genes. All erythrocytes from each fish were successfully concentrated and plated at 2×10^6 cells/ml and incubated overnight to allow for acclimatisation before viral infection and Poly I:C stimulation. Blood extraction, washing, concentrating and plating of the erythrocytes were carried out within 12 hours, reducing any variation in blood physiology from live to sacrificed fish (Clark et al., 2013). To ensure a homogenised culture for infection within the erythrocytes was achieved, an absorption based infection protocol was conducted. Tenfold dilutions of MOI for each virus were utilised to determine linear dependency between virus infection and innate immune gene expression.

RNA extraction was successfully carried out on all samples following our custom protocol involving wash steps including NP-40 and standard Tri-reagent extraction. The original procedure for RNA extraction using Tri-reagent alone failed to produce competent pure RNA without DNA contamination, so an extra wash step utilising NP-40 was implemented. NP-40 is a non-ionic detergent known for its ability to breakdown cytoplasmic cell membrane, but leave nuclear membranes untouched. Using this reagent isolated the nucleus for RNA extraction and removed all other DNA contamination from within the cytoplasm of the cell. This was adapted from a previous method used to extract rainbow trout erythrocytes (Morera et al., 2011). This reagent has been shown to be useful when working with nucleated cells in gene expression studies of low cell numbers (Le et al., 2015). The use of NP-40 helped to reduce DNA and other cytoplasmic contamination, increase the quality of extracted RNA and ensure that only mature cytoplasmic RNA was acquired from the

salmon erythrocytes. This would suggest NP-40 as a suitable addition to the production of an optimised and homogenised RNA extraction procedure for salmon erythrocytes.

The 54-erythrocyte RNA samples (18 ISAV, 18 SAV, 6 Poly I:C and 12 controls) were successfully converted to cDNA, to increase the stability and reduce the loss of RNA quality due to degradation. To assess the quality of the cDNA produced from each of the 54-erythrocyte samples they were tested for Ef1 α mRNA by qRT-PCR. Ef1 α mRNA has been shown to have many roles and is produced ubiquitously across all eukaryotes (Sasikumar et al., 2012). Ef1 α 's specific role in the successful translation during protein synthesis suggests that it would be a useful reference/housekeeping gene for RNA quality. This has been shown in Atlantic salmon where Ef1 α mRNA was suggested as a reference gene for assessing RNA in gene expression studies (Olsvik et al., 2005) and it is commonly used in gene expression studies in many other fish species (Williams et al., 2003; Tang et al., 2007; Zheng and Sun, 2011). On this basis the results from the qRT-PCR tests led to the removal of 12 lower quality samples. These samples had originally shown relatively good RNA quality on the ND-1000 spectrophotometer. This helped reduced any variations that non-specific amplification, low quality and contaminated cDNA samples would have on our final results.

Research on the salmon erythrocyte immune response to virus infection is currently limited to PRV and ISAV. PRV has been shown to infect erythrocytes and promote expression of IFNs, RIG-I, Mx and PKR whilst suppressing other mRNAs (Finstad et al., 2014; Wessel et al., 2015; Dahle et al., 2015). Contrasting data has come from these two results with Wessel et al. (2015) showing large up regulation of IFN after 1 day compared to low up regulation of Mx, RIG-I and PKR, before dropping significantly by day 7 where Mx, RIG-I and PKR had peaked. Whereas Dahle *et al.* (2015) showed a limited increase in expression of these 4 genes after 1 week with significantly higher fold changes observed after 4 – 5 weeks. It should be noted that all genes were expressed at higher levels than control erythrocytes at this time, indicating at least a small increase in expression was observed.

High variation in expression of each gene is also noticeable per sample in both experiments; showing upregulation of these genes can be irregular and highly variable to the individual. Other factors that may explain this are low expression and variation in degradation between cells, or cell life cycle stages and reduced activity due to older cells. This has been shown in rainbow trout, with erythrocytes showing

less activity with reduced cellular components and total RNA levels with increasing age (Lund et al., 2000; Phillips et al., 2000). When we compare these results, there were similarities in low mRNA abundance and high variation of Mx, and IFN with Dahle *et al.* (2015); however as stated previously our virus positives are generally not significantly higher in gene expression than our erythrocyte controls and Poly I:C stimulated cells. An explanation for the higher expression seen by Dahle *et al.* (2015) could be due to the research being carried out *in vivo*. Other cells and tissues, and in particular contamination of leukocytes may have stimulated the expression of the immune genes in these erythrocytes.

Similar high-level expressions have been shown in ISAV infected salmon erythrocytes for IFN α 1, IFN α 2 and Mx (Workenhe et al., 2008). Mx and IFN increased by up to 10 fold over 24 hrs, and remained above 7 fold for the remaining 4 days. A recent publication on rainbow trout erythrocytes exposed to viral haemorrhagic septicaemia virus (VHS) showed similar results to the results presented here with no significant upregulation in Mx or any other immune genes when compared to non infected control erythrocytes. (Nombela et al., 2018). Previous research on Poly I:C stimulation in rainbow trout revealed that IFN α and CCL4 were significantly up regulated over a 24 hrs period, with Mx slightly but not significantly upregulated. Our findings highlight that Mx was significantly higher than all SAV MOIs, but were similar for ISAV. The results found in this study for ISAV and Poly I:C stimulation are further supported by Workenhe *et al.* (2008) who observed similar results between IFN and Mx over a 24 hr period. They did, however find a significant upregulation of Mx and down regulation of IFN after 72 hrs. These results suggests that erythrocytes may up or down regulate certain immune genes based on the type of virus, the PAMP it is stimulated with and for how long it has been infected or stimulated for. Due to the base functionality of leukocytes as active substantial immune responders to invading pathogens, it is not reasonable to assume a similar functional mechanism and response via PAMP stimulation of erythrocytes to PAMP stimulation of leukocytes.

The innate immune system is mediated through a collection of distinct modular structures, formed of subsets of leukocytes that are stimulated by their intra and/or extracellular PRRs responding to PAMPS of invading pathogens (Medzhitov. 2007). Combinations of PAMP-PRR interactions lead to the activation of distinct immunological pathways. A key promoter in viral detection is TLR3, which signals the production of IFNs and inflammatory cytokines (IL-1 β and IL-16) from tissue resident macrophages in recognition of viral nucleic acid and other viral components

(Takeuchi and Akira. 2010). Induction of anti viral immunity is also promoted through intracellular proteins RIG-I and MDA5, which stimulate type I IFNs by recognition of both single and double stranded viral RNA (Gitlin et al., 2006; Kato et al., 2006; Pichlmair et al., 2006).

Type I and II IFNs were originally shown to be essential for immunity in mice (Muller et al., 1994) and were subsequently shown in other vertebrates (Samuel. 2001). Viral infected leukocytes in mammals drive IFN- α expression, whilst IFN β is produced predominantly in fibroblasts (Derynck et al., 1980; Goeddel et al., 1981). More specifically, the majority of IFN- α production is from a subtype of dendritic cells (DC) called plasmacytoid DCs. These are the main promoters of IFN- α and can produce >200 fold IFN- α than any other cell type (Siegal et a., 1999; Colonna et al., 2004). Evidence suggests these cells are present within teleosts (Ohta et al., 2004; Pettersen et al., 2008; Lovy et al., 2008, 2009) with functional identification recently reported in rainbow trout (Bassity and Clark. 2012) and zebra fish (*Danio rerio*) (Shao et al., 2015). Further research is needed to confirm functional similarities and differences between teleost and mammalian dendritic cells.

IFN expressions in teleost leukocytes have shown that stimulation with the PAMP Poly I:C produced a large up regulation of IFN type I and II in rainbow trout (Zou et al., 2007) and Atlantic salmon (Sun et al., 2009). Previous to this, human embryonic kidney 293 cell transfected with Atlantic salmon IFN genes indicated high expression of IFN transcripts and induction of the antiviral protein Mx that protected the fish from IPNV (Robertsen et al., 2003). Mx is an important antiviral protein known as an interferon stimulated gene (ISG) that is stimulated by type I and III IFNs in most vertebrates (Verhelst et al., 2013), and has been shown to promote resistance to ISAV and IPNV in Atlantic salmon (Jensen and Robertsen. 2002; Larsen et al., 2004; Kibenge et al., 2005; McBeath et al., 2007). This system shows the major influence leukocytes have on the anti viral response to invading pathogens in vertebrates.

Although the full system of genes, proteins, pathways and the types of leukocytes that activate and control the innate immune response is not yet entirely understood in salmonids, there is substantial evidence of its key role in the innate immune response to resistance and suppression of pathogens that infect them. The system appears highly conserved across all vertebrates with the IFN systems initiation of antiviral ISGs clearly present within many fish species, indicating it plays a similar role to other higher vertebrates. This would suggest that although certain

components may vary in teleosts the final result is a system that promotes resistance to viral infection.

When we compare the leukocyte response during viral infection and PAMP stimulation to our erythrocyte results, we consistently observe a switch of the cells of leukocytes from a neutral to a highly active antiviral state represented by a large increase in mRNA transcripts related to the antiviral response. This then triggers a cascade of expression of other cytokines and anti viral proteins driving the development of a systemic antiviral response. In leukocytes, the pathways involved have been extensively studied in the vertebrates, with increased progress being made in identification and expression of Atlantic salmon peripheral blood leukocytes (PBL) with the development of an oligonucleotide microarray (OMN) (Krasnov et al., 2011b). In contrast, most immune genes tested in erythrocytes in this work showed consistent low mRNA abundance regardless of virus, MOI, POLY I:C stimulation and controls.

For Wessel *et al.* (2015) erythrocyte mRNA abundance did appear to follow a similar trend as the leukocytes system with high IFN- α mRNA abundance early, followed by high mRNA abundance of Mx and PKR later. An increase in RIG-I mRNA abundance was also recorded early, but showed a much lower relative increase (~5 to 20 fold) above basal levels compared to IFN (~5 to 150 fold). After 7 days, this trend swapped with RIG-I mRNA abundance increasing up to 90 fold and IFN decreasing to ~ 0 – 40 fold. Following a standard viral PRR-PAMPs response of a leukocyte, RIG-I should be stimulated and expressed in higher abundance before IFN followed by ISG. It may be that at the first time point of 1 day, that RIG-I had signalled a high production of IFN transcripts and therefore explains the high abundance of these transcripts over RIG-I.

High expressions of IFN and Mx and gradual increases in IL-1 β to ISAV injections have been shown in Atlantic salmon (McBeath et al., 2007). Other studies, including those presented here, appear to show that erythrocytes do not have the same high-level expression and systematic control of immune response as leukocytes. However, low-level expression of immune genes in erythrocytes does not mean that they don't play an important role in regulating homeostatic balance. Morera *et al.* (2011) hypothesised that although expression of mRNA in PAMP-activated leukocytes is far greater than that seen in erythrocytes, the high abundance of erythrocytes in circulation could lead to millions of mRNA transcripts/ml of blood. The numbers of leukocytes present in circulating blood of fish is ~2% whereas

erythrocytes are over 90% (Witeska. 2013). So although activated leukocytes express high levels of mRNA, their relatively low numbers per ml of blood question whether the overall mRNA levels expressed are significantly higher than those expressed in low concentration by ~ 50 times more abundant erythrocyte cells per ml. Thus the effects of erythrocyte mRNA transcripts could be more significant in regulating homeostatic balance than first thought.

Varying degrees of expression were reported between genes based on virus type, PAMPs and exposure time. A common trend has appeared throughout all these studies indicating that non-infected erythrocytes appear to exhibit low-level expressions of innate immune genes. Based on the evidence shown in this research and previous work, it is unlikely that erythrocytes upregulate innate immune genes to the levels that leukocytes and other immune tissues do. It is more likely that they have a stable background of genes expressed that act as a barrier against initial viral infection and contribute to the PRR driven response.

Evidence of erythrocyte functional response has been shown in other organisms. In mammalian erythrocytes, surface glycoproteins were shown to act as decoy receptors for pathogens creating a pathogen sink (Baum et al., 2002). Human erythrocytes showed specific binding of immunodeficiency virus (HIV)-1 (Beck et al., 2009), and binding of immune complexes (IC) that act as a passive transporter to macrophages (Hess and Schifferli. 2003). Bird erythrocytes have been shown to up regulate cytokine transcripts, TLR and type I IFNs (Passantino et al., 2007; Morera et al., 2011; St Paul et al., 2013). Antimicrobial production from haemoglobin was found in both vertebrates and invertebrates that produced respiratory proteins of highly toxic reactive oxygen species (ROS) (Jiang et al., 2007). The antimicrobial action appears to be an ancient innate immunity mechanism conserved from over 500 million years ago, suggesting that this was the evolutionary origin of current haemoglobin activity (Jiang et al., 2007). Further antimicrobial activity has been shown in rainbow trout, where AMPs showing antibacterial action were isolated from erythrocytes (Fernandes and Smith. 2004), and were similarly found within the haemoglobin of channel catfish (Ullal et al., 2008). Further research into nucleated erythrocytes will contribute to our understanding of their role in immune and anti microbial response.

We have demonstrated that nucleated erythrocytes of Atlantic salmon express low levels of certain immune genes, suggesting a constitutive expression of these proteins in circulating erythrocytes. Infection with varying MOIs of ISAV and SAV

and stimulation of Poly I:C showed no clear increase in expression of any of the genes tested. From this and previous research, it appears that piscine erythrocytes exhibit an active immune response by acting as a barrier and as a possible first line of defence to viral infection, and promoting a PRR driven response. To further analyse this a longer time series over similar periods to previous work, as well as testing more immune genes, viruses and samples, would help in our understanding to the extent to which erythrocytes interact with the PRR-driven response during infection in Atlantic salmon. It may be the case that 24 hrs was not may not long enough for ISAV and SAV to attach, infect or stimulate the erythrocytes into signalling the PRR system within erythrocytes.

The *in vitro* culture could also be lacking key activating factors produced by leukocytes. To test this, whole blood cultures could be run and stimulated in a similar manner to this experiment's protocol. Erythrocytes and leukocytes could then be purified from these culture and their expression levels measured. The controls for all 7 genes showed very little variation in expression except for a few outliers in IFN β , IFN λ -rel2 and 101 indicating overall stability in the levels of expression often irrespective of treatment. This along with previous research supports the case that erythrocytes at minimum are maintaining a constant low-level expression of these genes, and specific genes may fluctuate from low to high over a period of time.

Chapter 5: Future Prospective

To improve husbandry management of Atlantic salmon in the Aquaculture industry worldwide, fast, reliable and effective diagnostic tools for smoltification and viral detection are needed. Understanding the immunological processes and cellular components involved in first responses to viral infections in the innate immune system could open up the potential to utilise biomarkers associated with infection to monitor and assess salmon health throughout their lifecycle, from hatcheries to open SW pens.

In Chapter 2 we developed an in lab and onsite NKA qRT-PCR assay for the $\alpha 1a$ mRNA transcript ATPase, which was shown to be as effective at smoltification detection as industry standard NKA activity assays. Further in lab NKA qRT-PCR and NKA activity assays comparison could be continued to give a larger database of yearly variation in success of each assay, and provide more site specific data. This would allow known environmental factors, fish population specifics and site-specific factors to be taken into account based on each site, and applied to the analysis of future tests. These tests could also include fish farms that do not use the supersmolt feed to verify that the NKA qRT-PCR assays work as effectively as those that do and in comparison to NKA activity assays. The tests could also be expanded to monitor S1 fish to assess whether a similar trend or different trend to S0 fish is found. It would be interesting to see if this applies to the UK as well, and if so the expansion into this area would likely require study on other smoltification biomarkers that could be utilised as effectively. Changing the measurement times of I, M and F points from calendar days to dd could also open up better spatial monitoring of the NKA activity assay and NKA qRT-PCR. Through analysis of the data no trends were found, however the potential to monitor both assays to a closer degree by switching test periods over dd (e.g. 0dd, 100dd, 200dd, 300dd etc.) would allow for a more reliable interpretation of how the $\alpha 1a$ marker and NKA activity changes throughout the smolt period. Utilising dd would be a better indicator over calendar days as the developmental period before during and after smoltification has been heavily researched (see section 1.3), providing a good basis to plan and estimate dd time points to sample at leading up to when fish should be at the point of SW transfer (350 – 400dd) and when they begin to enter desmoltification (400dd>). This could help answer some of the questions about the abnormal results seen throughout the years for both assays and potentially show how the $\alpha 1a$ mRNA abundance changes throughout the smolt period.

Further testing of the mobile assay should be pursued to increase the robustness of the α 1a mRNA transcript as a smoltification biomarker for onsite detection. This would provide a preliminary onsite qRT-PCR test that is faster, provides results on the day, and is cleaner and potentially cheaper than NKA activity assays. Alongside this, further assays utilising smoltification biomarkers could be developed in lab and transferred onto the same mobile diagnostic platforms. Originally this project incorporated a second biomarker, GAPDH. The assay was developed and tested on 2015 samples, however all test points for all sites showed no statistically significant variation between them. Due to this we did not use the assay for the subsequent two years.

There are numerous other biomarkers that could be utilised and tested for smoltification monitoring. Developing several biomarker assays would give a greater overall picture on the progress salmon are making during smoltification, improve reliability, help reduce false positives and identify compromised assays. Farms could make a judgement call more easily when observing 3 or more biomarker results over just the current one that has been developed at the moment. A previous masters thesis conducted in 2014 at the University of Stirling (Kelly Wood, Masters thesis, 2014) utilised a CCL4-like chemokine as a potential biomarker. The idea behind using this biomarker was that CCL4 mRNA abundance would increase during smoltification. This would be due to increased inflammation from damage sustained in the gills whilst adapting to a SW environment during smoltification development. The preliminary study however only indicated a significant increase at 1 of 4 sites, suggesting the marker was not a good indicator for smoltification. Following on from this idea, the testing of other mRNA transcripts involved in the inflammatory response (see section 1.5.3) as well as re-testing CCL4 at more sites could help produce several reliable biomarkers for monitoring smoltification.

The monitoring of abundance and ratio changes between α 1a and α 1b over a smolt period could allow for a better indication of the development process as α 1a ionocytes switch to α 1b ionocytes. The use of biomarkers that are affected by environmental factors such as metal contamination, but remain at stable levels throughout the smolt period could also help to monitor and ascertain when these outbreaks occur and act quickly to remedy the situation. In Atlantic salmon and other species metallothioneins (MT) (Fabrin et al., 2018; Stankeviciute et al., 2018) and hepatic glutamic oxaloacetate transaminase (GOT) (de la torre et al., 1998) have been shown as a reliable indicator of metal contamination in FW environments. Utilising a ubiquitous biomarker such as EF1a that remains stable throughout the

salmon lifecycle could be used as a baseline control to compare against the results from the other biomarkers tested and potentially indicate abnormal sample results.

Optimisation of the mobile lab to reduce the size, weight and amount of equipment required on farms could be addressed before more onsite tests are conducted. Reducing and replacing basic equipment such as centrifuges and vortexes with smaller portable versions would increase the portability and ease of transport on and off sites. The boxes could be professionally re-designed to a higher quality product to increase the ease of usability and reduce contamination in the case of spill solutions. To do this, another industrial partner or industrial funding would need to be acquired due to the current partner stepping down following this project's conclusion.

The results also showed that the NKA activity assay was susceptible to metal contamination giving a false positive result on numerous occasions; whereas NKA qRT-PCR indicated that smoltification was not proceeding as normal. This suggests the qRT-PCR assay is more reliable in compromised environmental conditions. We would have to acquire data on metal run off pumped into any farms affected by metal contamination to be able to compare it with the smoltification data of both the NKA activity and NKA qRT-PCR assays. With this data we would be able to obtain a better understanding of how smoltification is influenced, and then to advise how the farms should respond to metal influx.

Viral outbreaks of SAV, PRV, PMCV and ISAV are routinely reported yearly with varying degrees of disease outbreak amongst fish farms in Europe, Canada, the USA and Chile (see section 1.6). Production of a reliable qRT-PCR assay for detection of these diseases would be highly valuable to the industry, particularly due to the clinical and histopathological similarities between SAV, PRV and PMCV making diagnosis difficult, delaying treatment and preventing loss of fish. The development in this study of a multiplex assay for both in lab and on site detecting of SAV (1 - 6), PRV and PMCV provides a significant tool which could be used by farms to monitor, determine and treat viral infections throughout the year. On site analysis could be conducted in emergency cases, or lab based analysis for routine checks.

Currently the multiplex assay is incomplete, with an internal positive still to be incorporated as well as probit analysis, positive sample tests for PRV and PMCV and blind samples for all 3 viruses to fully verify it. As suggested in the discussion of Chapter 3, failing the incorporation of the 3 viruses and the internal control the assay

could be split into two duplex assays containing PRV-PMCV and a dedicated SAV (1 - 6) assay. As ISAV is an important detrimental virus, this too could be incorporated into a duplex assay with any of the 3-original viruses.

The multiplex assay could also be used in a future study monitoring viral infections of all four viruses. In doing so, the viral infections could be monitored on a daily or weekly basis through qRT-PCR tests of infected fish to observe at what viral abundance the infection triggers disease within the fish. This could be done experimentally by infecting smolts and post smolts with each of the viruses and monitoring them alongside uninfected controls over a 6 – 8 week period, or until clinical signs of disease are observed. This would generate a data set that could provide a viral abundance threshold point where the fish switch from an infected to a disease state. This would enhance the ability of the original assays from purely detection of the virus to detection, monitoring and assessment. Based on the viral abundance recorded an estimated assessment on how long the fish is likely to become susceptible to disease outbreak could be made. This would not only alert farmers to the virus being present within their farms, but would also give an estimation of how bad the infection is and how long they have to prevent further outbreaks within their farm. This could significantly help the industry reduce loss of fish and profit by not only stopping further outbreaks but also treating fish that have not yet shown clinical signs of disease but have been infected with the virus. It would also help in the case of PRV, which is known to be an opportunistic virus (see section 1.6.2). Detecting low to moderate levels of this virus would alert farms to the susceptibility of those fish as they have been immunocompromised and likely more vulnerable to primary viral infections such as SAV, PMCV and ISAV.

We have shown in Chapter 4 that erythrocytes appear to have a significant constitutive level of antiviral mRNA abundance in Atlantic salmon. This suggests that a “barrier” is in place that does not require significant transcriptomic reprogramming. This is supported by recent studies focusing on infections of erythrocytes with the same or similar virus (see section 4.4). Following on from this project, a future study using a similar methodology but focusing on analysis over a longer time series would give a better understanding of whether there is variation in up or downregulation of these genes in viral infected erythrocytes. It has been shown in teleost that as erythrocytes age there is a reduction in cellular components and total RNA levels, as well as a reduction in aerobic energy production (see section 1.5.4). Conducting a longer time series test would allow us to view how erythrocytes react to viral infection as they age. A separate time series where

erythrocytes are cultivated for 2, 4, 6, 8 weeks etc. before infection with the virus is another approach that could be applied. It would also allow for a more robust comparison with recent studies that have focused on longer time series.

As stated in the discussion of Chapter 4, it may be the case that 24 hrs is not long enough for the virus to attach and infect the erythrocyte cells, and therefore may not have initiated any PRR-driven response. Setting up a time series of 5 – 7 days should give enough time for the virus to interact with the erythrocyte and to monitor a response, however this time period could be extended as in other erythrocyte studies (Dahle et al., 2015) to observe any delayed up or downregulation of genes. This would be of particular interest for PRV infections as they are known to remain dormant in salmon (Lovoll et al., 2010; Haugland et al., 2011; Garseth et al., 2013; Wiik-Nielsen et al., 2016), but are known to infect and replicate within erythrocytes (Finstad et al., 2014; Wessel et al., 2015). Following a time series of 1 – 6 weeks of a PRV infection could shed light on whether the virus can go undetected during initial infections by erythrocytes and whether the erythrocytes are eventually stimulated into responding many weeks after infection. This would also be interesting with a dual infection assay where PRV is initially infected and another virus is infected at different time points after initial infection. PRV has been shown to act as an opportunistic secondary infection after salmon are infected with another virus, and even preventing other secondary infections from occurring (Lund et al., 2016). Studying how PRV and secondary viral infections influence erythrocytes in detecting, being infected and responding to this dynamic could provide good insight into this infection model in erythrocyte innate immune response.

Other viruses (PRV and PMCV) could be tested in the same manner, and monitored to see if any variations in mRNA levels are observed and are potentially viral specific. A study focused on observations into whether all 4 viruses can infect and replicate within erythrocytes would also be beneficial to our understanding of how erythrocytes can initiate a primary defence, buffer initial infection or are compromised on a virus specific basis. In doing these tests, any genes that may be upregulated on a consistent basis could be further utilised as biomarkers to detect infections within fish before the virus has time to spread throughout the fish farm and cause disease. This could be utilised through the development of a qRT-PCR assay as demonstrated in Chapter 2 and 3 of this thesis. Understanding the viral infection model of erythrocytes and potentially showing that it is a first point of infection could open up new developments for rapid molecular diagnostics in salmon, and potentially other aquaculture species. Antibodies could be developed to monitor

erythrocyte responses or used to monitor levels of antiviral proteins. Blood samples could be taken to diagnose and monitor fish for viral infection and load present within erythrocyte cells. This would help benefit welfare by not having to sacrifice the fish during sampling by only taking a small sample of blood before returning fish to their tanks or pens. This would also benefit the industry by providing more quick detection tools to identify, treat and reduce infections within fish farms.

As stated in Chapter 4 there is growing evidence in both teleosts and other vertebrates that not only do nucleated erythrocytes appear to participate in the innate immune response, but also non-nucleated erythrocytes (see section 4.4). The evidence shown across both vertebrates and invertebrates on erythrocytes containing haemoglobin have been shown to actively secrete AMPs in the presence of infectious pathogens and promote the release of ROS. Not only should there be more focus on further studies of teleost erythrocytes, but a similar increase in focus should be given to the immunological response and capabilities within vertebrates and invertebrates erythrocytes. Combining our understanding of teleost erythrocytes with higher vertebrates could provide us with the evolutionary links to the immunological variations and development of erythrocytes, and how they have changed in the innate and potentially active immune response.

The qRT-PCR assays described in this thesis provide novel tools for the detection of both smoltification and viral infection in Atlantic salmon, which could be of great advantage to fish farms in the future. Additionally, this research has examined how viral infection of erythrocytes leads to stimulation of the innate immune response. The results presented here provide a platform for future studies to further develop and refine these assays for use in the aquaculture industry.

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Appendices

Chapter 2

Table C2.1 Details of the mean NKA activity assays, fold change and p-value for initial and final points for 16 sites sampled in 2015.

Site	Initial Activity (Mean)	Final Activity (Mean)	Fold change	Significant (p<0.05)
GLA	1.97 ± 0.30	7.42 ± 3.60	3.77	<0.001
GLB	2.29 ± 1.00	8.48 ± 2.44	3.70	<0.001
LD	6.06 ± 2.05	13.81 ± 3.74	2.27	<0.001
RBD	7.91 ± 1.59	17.90 ± 4.90	2.26	<0.001
RBA	6.71 ± 1.59	14.84 ± 3.43	2.22	<0.001
ORA	6.50 ± 2.84	13.93 ± 3.21	2.14	<0.001
RBB	6.02 ± 1.44	12.87 ± 3.37	2.14	<0.001
ORB	6.99 ± 2.80	14.38 ± 3.85	2.05	<0.001
BAR	6.98 ± 2.09	12.84 ± 4.10	1.84	0.004
MIN	6.28 ± 2.54	10.80 ± 4.54	1.71	0.032
GCA	9.92 ± 3.08	15.10 ± 3.93	1.52	0.064
ORE	6.97 ± 2.34	9.74 ± 3.121	1.39	0.113
CLA	7.60 ± 5.08	9.68 ± 2.76	1.27	0.331
GCB	11.00 ± 3.52	13.41 ± 2.46	1.21	0.1798
RBE	11.30 ± 2.50	13.50 ± 5.19	1.19	0.314
GIR	4.52 ± 1.936	13.38 ± 3.54	2.96	<0.001

Table C2.2 Details of the mean NKA activity assays, fold change and p-value for initial and final points for 13 sites sampled in 2016.

Site	Initial Activity (Mean)	Final Activity (Mean)	Fold change	Significant (p<0.05)
GLA	6.08 ± 3.74	8.59 ± 3.31	1.41	0.511
GLB	3.95 ± 1.42	6.10 ± 1.81	1.54	0.020
ORA	7.83 ± 1.95	11.01 ± 2.16	1.41	0.008
ORB	6.10 ± 1.49	11.35 ± 2.93	1.86	0.002
ORE	4.00 ± 1.25	14.91 ± 2.73	3.73	<0.001
MIN	4.13 ± 1.12	11.83 ± 3.17	2.86	<0.001
LS	13.31 ± 2.10	9.92 ± 2.14	1.34	0.007
RBA	8.29 ± 3.69	14.33 ± 2.32	1.73	0.002
RBB	6.36 ± 2.45	14.42 ± 3.65	2.27	<0.001
RBC	7.561 ± 3.08	17.93 ± 3.44	2.37	<0.001
LD	7.56 ± 1.10	13.70 ± 3.37	1.81	<0.001
GIR	6.04 ± 1.50	19.11 ± 7.13	3.16	0.001
GCA	12.40 ± 1.93	13.33 ± 5.11	1.08	0.846

Table C2.3 Details of the mean NKA activity assays, fold change and p-value for initial and final points for 10 sites sampled in 2017.

Site	Initial Activity (Mean)	Final Activity (Mean)	Fold change	Significant (p<0.05)
LD	11.36 ± 3.72	13.41 ± 1.65	1.18	0.177
ORA	6.99 ± 4.58	18.38 ± 4.92	2.60	<0.001
GLA	3.16 ± 1.05	8.55 ± 2.43	2.70	0.001
GLB	3.63 ± 0.96	7.20 ± 1.71	1.98	<0.001
GCA	12.11 ± 2.03	13.6 ± 3.96	1.12	0.612
RBA	5.52 ± 2.33	11.35 ± 2.15	2.06	<0.001
RBB	8.15 ± 4.53	12.6 ± 2.30	1.55	0.026
KLM	5.15 ± 2.08	15.53 ± 1.23	3.02	<0.001
MIN	5.54 ± 6.79	11.18 ± 6.75	2.02	0.118

Table C2.4 Details of the mean Na⁺ K- qRT-PCR assays, fold change and p-value for initial and final points for 16 sites sampled in 2015.

Site	Initial Copy number (Mean)	Final Copy Number (Mean)	Fold change	Significant (p<0.05)
GIR	2143000 ± 853292	656857 ± 449975	3.26	<0.001
MIN	982143 ± 482088	337417 ± 131421	2.91	0.012
ORE	1458000 ± 508203	532857 ± 244397	2.74	0.001
ORA	1176000 ± 460046	399625 ± 191597	3.02	<0.001
ORB	1319000 ± 844766	456375 ± 215721	2.89	<0.001
LD	2453000 ± 711874	670875 ± 295712	3.65	<0.008
BAR	1917000 ± 667712	888375 ± 473250	2.16	0.001
GLA	1195000 ± 299151	567571 ± 313763	2.11	<0.001
RBE	838375 ± 289400	334143 ± 226982	2.51	0.023
GCB	1502000 ± 762587	991333 ± 559937	1.51	0.220
RBB	809625 ± 248795	613250 ± 246717	1.32	0.135
GCA	1135000 ± 280587	846375 ± 514960	1.31	0.288
RBA	938125 ± 587866	1475000 ± 841963	1.57	0.164
GLB	230375 ± 52312	417875 ± 307338	1.81	0.130
CLA	607500 ± 636633	540125 ± 295695	1.12	0.791
RBD	300875 ± 111831	829333 ± 210599	2.76	<0.001

Table C2.5 Details of the mean Na⁺ K- qRT-PCR assays, fold change and p-value for initial and final points for 13 sites sampled in 2016.

Site	Initial Copy number (Mean)	Final Copy Number (Mean)	Fold change	Significant (p<0.05)
GLA	1137000 ± 341164	476587 ± 197507	2.40	<0.001
GLB	1148000 ± 414213	547688 ± 205257	2.10	<0.001
ORA	1038000 ± 322637	618688 ± 137320	1.68	<0.001
ORB	732563 ± 462127	691750 ± 199407	1.63	0.749
ORE	535375 ± 167498	449125 ± 191111	1.20	0.185
MIN	1373000 ± 246014	673000 ± 138819	2.04	<0.001
LS	736938 ± 137903	1128000 ± 201630	1.53	<0.001
RBA	328938 ± 120884	875000 ± 168491	2.66	<0.001
RBB	426636 ± 117626	553571 ± 106833	1.30	0.015
RBC	454000 ± 111541	874875 ± 314199	1.92	<0.001
LD	1264000 ± 337435	794188 ± 156557	1.59	<0.001
GIR	775250 ± 205355	515533 ± 133851	1.50	<0.001
GCA	935125 ± 296623	585375 ± 154943	1.60	0.005

Table C2.6 Details of the mean Na⁺ K- qRT-PCR assays, fold change and p-value for initial and final points for 9 sites sampled in 2017.

Site	Initial copy number (Mean)	Final copy number (Mean)	Fold change	Significant (p<0.05)
LD	544125 ± 119475	852188 ± 148516	1.57	<0.001
ORA	425467 ± 71487	929813 ± 259999	2.19	<0.001
GLA	895375 ± 231684	812867 ± 169952	1.10	0.266
GLB	400938 ± 83463	641063 ± 152740	1.59	<0.001
GCA	474938 ± 131467	800438 ± 145754	1.68	<0.001
RBA	847500 ± 195862	945214 ± 132438	1.11	0.213
RBB	483357 ± 166367	657063 ± 147710	1.36	0.006
KLM	523462 ± 243882	686077 ± 169909	1.31	0.062
MIN	690563 ± 109574	525688 ± 204994	1.31	0.009

Table C2.7 Details of the mean NKA activity assays, p-value and significance (yes/no) for initial against mid and mid against final points for 8 sites sampled in 2015

Site	Initial vs Mid		Mid vs Final	
	Significance	p-value	Significance	p-value
GLA	No	0.663	No	0.966
ORA	Yes	0.046	No	0.723
ORB	Yes	0.006	No	0.987
ORE	No	0.162	No	0.978
BAR	No	0.331	No	0.09
LD	Yes	<0.001	Yes	0.01
GCA	No	0.550	No	0.870
MIN	Yes	0.001	Yes	0.040

Table C2.8 Details of the mean NKA activity assays, p-value and significance (yes/no) for initial against mid and mid against final points for 6 sites sampled in 2016

Site	Initial vs Mid		Mid vs Final	
	Significance	p-value	Significance	p-value
ORA	Yes	0.046	No	0.723
GLA	No	0.663	No	0.966
MIN	Yes	<0.001	Yes	0.040
LD	Yes	<0.001	Yes	0.010
GCA	No	0.555	No	0.874
RBB	Yes	<0.001	No	0.866

Table C2.9 Details of the mean NKA activity assays, p-value and significance (yes/no) for initial against mid and mid against final points for 4 sites sampled in 2017

Site	Initial vs Mid		Mid vs Final	
	Significance	p-value	Significance	p-value
ORM	No	0.27	Yes	0.018
GLB	Yes	<0.001	No	0.997
GCA	No	0.443	No	0.095
RBA	Yes	0.037	Yes	0.034

Table C2.10 Details of the mean Na⁺ K- qRT-PCR assays, p-value and significance (yes/no) for initial against mid and mid against final points for 8 sites sampled in 2015

Site	Initial vs Mid		Mid vs Final	
	Significance	p-value	Significance	p-value
GLA	No	0.096	No	0.058
ORA	No	0.443	Yes	0.042
ORB	Yes	0.043	No	0.733
ORE	No	0.539	No	0.338
BAR	Yes	<0.001	No	0.788
LD	Yes	<0.001	No	0.999
GCA	Yes	0.028	No	0.403
MIN	Yes	0.001	Yes	0.040

Table C2.11 Details of the mean Na⁺ K- qRT-PCR assays, p-value and significance (yes/no) for initial against mid and mid against final points for 6 sites sampled in 2016

Site	Initial vs Mid		Mid vs Final	
	Significance	p-value	Significance	p-value
ORA	Yes	0.011	No	0.455
GLA	Yes	<0.001	No	0.979
MIN	Yes	<0.001	No	0.860
LD	Yes	<0.001	No	0.081
GCA	No	0.311	No	0.176
RBB	Yes	0.027	Yes	<0.001

Table C2.12 Details of the mean Na⁺ K- qRT-PCR assays, p-value and significance (yes/no) for initial against mid and mid against final points for 4 sites sampled in 2017

Site	Initial vs Mid		Mid vs Final	
	Significance	p-value	Significance	p-value
ORM	No	0.530	Yes	<0.001
GLB	Yes	<0.001	No	0.994
GCA	Yes	<0.001	No	0.209
RBA	No	0.421	Yes	0.017

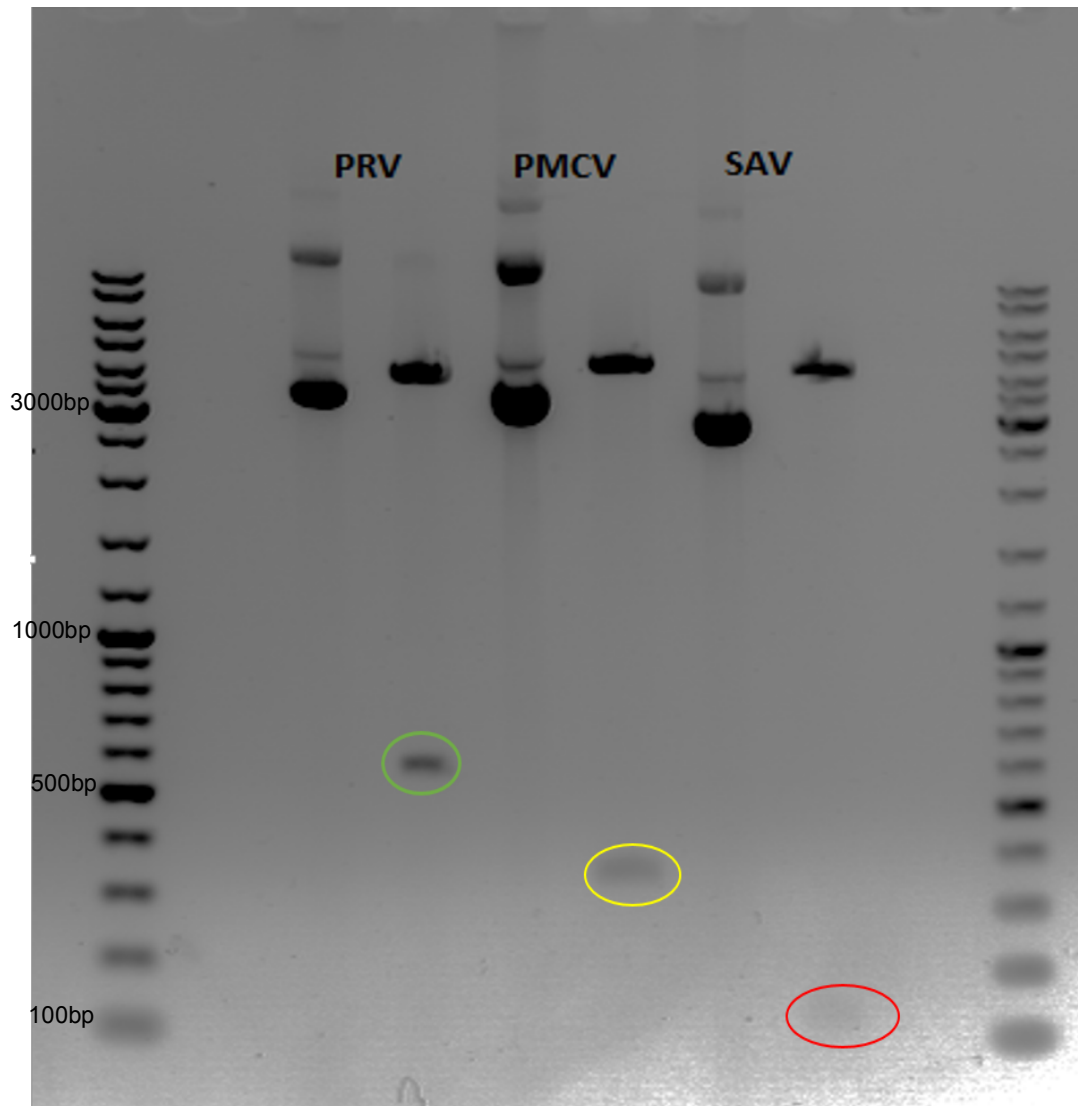
Table C2.13 Details of the start and sea transfer dates, average size and k-factor of the fish during the Supersmolt® feeding regime at all sites over the 3-year testing period. *Degree days (dd) for sea transfer are only recorded for sites Russel burn and Geocrab due to no onsite temperature data availability from all other sites.

Site	Year	Supers molt feed (SS) start date	Avg size SS feed start (cm)	Avg size SS feed end (cm)	K-Factor (range)	Sea transfer date and degree days (dd)*
Russel Burn A	2015	28/07/15	13.73 ± 0.67	15.65 ± 0.87	1.29 – 1.42	25/08/15 359dd
	2016	22/07/16	14.38 ± 1.07	16.86 ± 1.20	1.29 – 1.38	16/08/16 371dd
	2017	26/07/17	15.96 ± 0.96	17.86 ± 1.07	1.23 – 1.40	22/08/17 392dd
Russel Burn B	2015	28/07/15	14.36 ± 1.04	15.78 ± 0.97	1.31 – 1.38	25/08/15 359dd
	2016	28/07/16	15.14 ± 1.07	16.03 ± 0.83	1.18 – 1.47	23/08/16 366dd
	2017	02/08/17	16.14 ± 0.85	19.49 ± 1.24	1.17 – 1.39	29/08/17 388dd
Russel Burn C	2016	28/07/16	14.74 ± 1.22	16.18 ± 1.04	1.17 – 1.40	31/08/16 504dd
Russel Burn D	2015	06/08/15	14.08 ± 0.74	15.66 ± 0.98	1.23 – 1.42	01/09/15 328dd
Ormsary A	2015	07/08/15	15.54 ± 1.24	18.98 ± 1.24	1.22 - 1.35	02/09/15
	2016	29/07/16	14.75 ± 0.80	17.15 ± 0.88	1.21 – 1.26	05/09/16
	2017	21/08/17	15.72 ± 1.05	18.55 ± 1.56	1.16 – 1.27	19/09/17
Ormsary B	2015	14/08/15	15.08 ± 0.76	16.93 ± 1.36	1.16 – 1.27	07/09/15
	2016	29/07/16	14.66 ± 0.69	18.24 ± 1.86	1.23 – 1.31	05/09/16
Ormsary E	2015	15/09/15	15.83 ± 0.91	17.66 ± 0.88	1.21 – 1.25	07/10/15
	2016	09/08/16	13.72 ± 1.16	18.15 ± 1.08	1.2 – 1.31	22/09/16
Geocrab A	2015	27/07/15	14.01 ± 0.69	17.15 ± 0.76	1.24 – 1.29	26/08/15 463dd

	2016	04/07/16	14.24 ± 0.44	17.90 ± 1.19	1.11 – 1.36	15/08/16 706dd
	2017	19/07/17	15.95 ± 0.96	17.33 ± 0.75	1.22 – 1.28	09/08/17 395dd
Geocrab B	2015	27/07/15	13.05 ± 0.48	16.97 ± 1.05	1.18 – 1.31	03/09/15 576.5dd
Gairloch A	2015	22/09/15	15.96 ± 0.92	17.83 ± 1.34	1.20 – 1.23	21/10/15
	2016	03/08/16	15.11 ± 0.88	17.56 ± 1.62	1.20 – 1.30	12/09/16
	2017	27/07/17	14.85 ± 0.68	19.07 ± 0.83	1.15 – 1.31	19/09/17
Gairloch B	2015	22/09/15	15.86 ± 1.06	17.40 ± 1.06	1.21 – 1.31	29/10/15
	2016	17/08/16	14.19 ± 0.77	16.27 ± 0.89	1.21 – 1.30	19/09/16
	2017	16/08/17	15.01 ± 0.99	18.30 ± 1.2	1.13 – 1.27	25/09/17
Loch Damph	2015	09/09/15	17.04 ± 0.87	19.31 ± 1	1.07 – 1.2	30/09/15
	2016	02/09/16	16.24 ± 0.96	19.20 ± 0.9	1.17 – 1.19	21/09/16
	2017	05/09/17	17.26 ± 1.12	17.69 ± 1.35	1.13 – 1.26	18/09/17
Loch Shin	2016	02/08/16	17.01 ± 0.98	18.28 ± 1.26	1.19 – 1.28	24/08/16
Barvas	2015	16/09/15	16.55 ± 1.4	18.98 ± 0.99	1.15 – 1.21	22/10/15
Girista	2015	26/08/15	16.78 ± 1.52	20.9 ± 1.84	1.12 – 1.24	20/10/15
	2016	30/08/16	19.06 ± 1.29	21.38 ± 1.35	1.15 – 1.28	28/09/16
Mingarry	2015	02/09/15	15.98 ± 1.16	19.45 ± 1.26	1.13 – 1.21	08/10/15
	2016	31/08/16	16.1 ± 1.23	19.55 ± 1.18	1.19 – 1.24	06/10/16
	2017	14/09/17	18.08 ± 1.28	19.35 ± 0.92	1.19 – 1.21	04/10/17
Clachbreac	2015	07/08/15	16.1 ± 1.19	18.92 ± 1.18	1.11 – 1.23	14/09/15
Kinlochmoid- art	2017	24/08/17	16.68 ± 1.13	19.29 ± 1.00	0.95 – 1.07	29/09/17

Chapter 3

Figure C3.1 Agarose gel results showing the restriction fragment polymorphism of digested and undigested SAV, PRV and PMCV plasmids. Digested plasmids showed the expected target bands of 550 bp PRV (green circle), 350 bp PMCV (yellow circle) and 120 bp SAV (red circle) and the respective plasmid vector. Undigested plasmids (columns 1, 3 and 5) only the undigested plasmid. A 10 kb ladder (0.1 $\mu\text{g}/\mu\text{l}$) was used for measurement.



Chapter 4

Figure C4.1 Layout of erythrocyte infection assay for ISAV and SAV infection at 3 MOIs (10, 1 and 0.1)

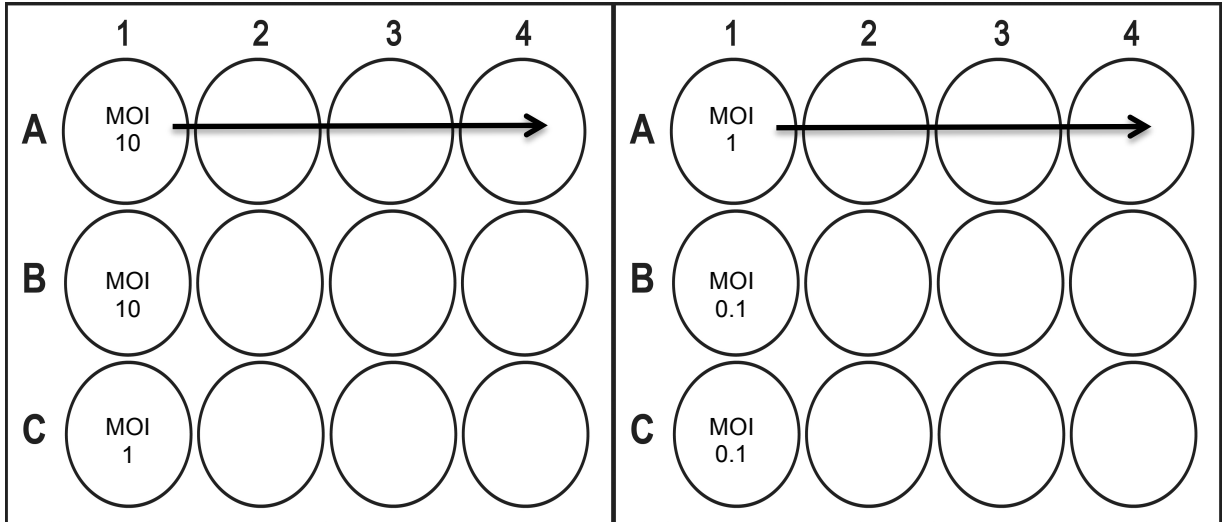


Figure C4.2 Layout of erythrocyte infection assay for Poly I:C stimulated wells.

