

The effects and genetic diversity of
salmon gill poxvirus in freshwater
Atlantic salmon in Scotland

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Declaration

I, Rosa Allshire, submit this thesis for the degree of Doctor of Philosophy. I certify that this thesis has been written by me, that the work presented has been conducted independently by me and has not been submitted for any other degree.

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Abstract

Salmon gill poxvirus (SGPV) is an emerging pathogen causing significant losses in the Scottish Atlantic salmon farming industry. Clinical presentation varies widely, from sub-clinical infection to severe clinical signs and gill pathology. Understanding the causes for this variation in clinical presentation and mortality is key to mitigating the significant economic losses caused by this virus. To date, little research has been done in Scotland on the effects of SGPV infection, and why disease presentation is so unpredictable. In this thesis, a natural outbreak of SGPV disease in an enclosed freshwater system was studied. A range of SGPV positive samples from geographically distinct detections were sequenced to explore the genetic diversity of SGPV in Scotland.

During the naturally-occurring outbreak, histological findings showed the gills to be the primary target organ, with severe pathology correlating with high viral loads. Common pathologies associated with SGPV disease (SGPVD) were found in the gills, including severe hyperplasia, hypertrophy and widespread inflammation. However, epithelial apoptosis, described in many published studies as the pathognomonic clinical sign of SGPV infection, was only found in low levels during this study. Downregulation of anti-viral immune markers MDA-5 and Mx was observed in clinically diseased fish, when compared with sub-clinically infected fish, which suggests that the host antiviral immune response is being differentially modulated.

Samples positive for SGPV were collected from outbreaks and incidental detections across six geographically distinct sites in Scotland and used for whole-genome sequencing and phylogenetic analysis. Genetic diversity was found to be limited between these cases, with sequence similarity of $>85\%$ in near-complete genomes. Some areas of nucleotide variation were also identified, possibly representing targets for phylogenetic studies. This work provides an insight into the pathogenesis of a natural outbreak that will aid the control and monitoring of SGPVD on freshwater Atlantic salmon farms.

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Abbreviations

°C – Degrees Celsius

µl – Microgram

AB – Antibody

ACGD – Atypical cellular gill disease

AGD – Amoebic gill disease

AHPND – Acute hepatopancreatic necrosis disease

ATPase - Adenosine triphosphatase

ASG-10 - Atlantic salmon gill cell line 10

ASG-13 – Atlantic salmon gill cell line 13

ASK – Atlantic salmon kidney cell line

ASPV – Atlantic salmon paramyxovirus

bp – Base pair

cDNA – Complementary DNA

CEV – Carp edema virus

CGD - Complex gill disease

CGPV - Cod gill poxvirus

CHSE-214 – Chinook salmon embryo cell line 214

CMS – Cardiomyopathy syndrome

CO₂ – Carbon dioxide

CPE – Cytopathic effect

CPXV – Cowpox virus

Ct – Cycle threshold

CyHV3 – Cyprinid herpesvirus

DD – Degree days

dH₂O – Distilled water

DNA – Deoxyribonucleic acid

dNTP – Deoxynucleotide

dsDNA – Double stranded DNA

dsRNA – Double stranded RNA

e.g. - Exempli gratia (example)

ECTV – Ectromelia virus

EDTA - Ethylenediaminetetraacetic acid

EMEM - Eagles minimum essential media

EPC – Epithelioma papulosum cyprinid cell line

FAO – Food and Agriculture Organisation

FW - Freshwater

g - Gram

g - Gravitational force

GATU - Gene annotation transfer utility

GTP – Guanosine triphosphate

HIV – Human immunodeficiency virus 1

HSMI – Heart and skeletal muscle inflammation

IFN – Interferon

IHC – Immunohistochemistry

IHNV – Infectious hematopoietic necrosis virus

IL – Interleukin

IPN – Infectious pancreatic necrosis

IPNV – Infectious pancreatic necrosis virus

ISAV – Infectious salmon anaemia virus

ISG-15 – Interferon-stimulated gene 15

ISA – Infectious salmon anaemia

ISH – In situ hybridisation

kbp – Kilobase pairs

KSD – Koi sleepy disease

L - Litre

LB – Luria broth

M – Molar

MDA-5 – Melanoma differentiation-associated gene 5

mg – Microgram

ml – Millilitre

mM – Millimolar

MPXV – Monkeypox virus

MS-222 - Tricaine mesylate

MVLA – Multi-locus variable-number tandem-repeat

Mx – Myxovirus resistance gene

NA SGPV – North American SGPV

NaCl - Sodium chloride

NaOH - Sodium hydroxide

NCBI - National Centre for Biotechnology Information

ng – Nanogram

NKA - Na⁺/K⁺ ATPase

NKA - Na⁺/K⁺ ATPase

nM - Nanomolar

nm – Nanometre

ORF – Open reading frame

PaPV - *Plecoglossus altivelis* poxvirus-like virus

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PD – Pancreas disease

PGD - Proliferative gill disease

PGI - Proliferative gill inflammation

pH - -log₁₀ (Hydrogen ion concentration)

PMCV – Piscine myocarditis virus

ppm – Parts per million

PRV – Piscine orthoreovirus

QC – Quality control

qPCR – Quantitative polymerase chain reaction

RAS – Recirculating aquaculture system

RNA – Ribonucleic acid

RT – Room temperature

RT-PCR – Reverse transcription PCR

S0 – Out-of-season salmon

S1 – In-season salmon

SAV – Salmonid alphavirus

SGPV - Salmon gill poxvirus

SGPVD - Salmon gill poxvirus disease

SHPV – Seahorse poxvirus

SISPA – Sequence independent single primer amplification

SW - Seawater

TAN – Total ammonia nitrogen

TEM – Transmission electron microscopy

TiLV – Tilapia lake virus

TUNEL - Terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling

U – Units

V - Volts

VACV – Vaccinia virus

VARV – Variola virus

VZV – Varicella zoster virus

W - Watts

WSI – Whole slide imaging

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Chapter 1

General Introduction

1.1 Aquaculture

Aquaculture is the fastest-growing and one of the most valuable food production sectors globally, providing an increasingly important source of food for human consumption (FAO, 2024). With the global human population predicted to approach 10 billion by 2050, alongside the continued decline of many wild fish stocks, ensuring adequate and sustainable nutrition for future populations represents a growing challenge (UNDP, 2015). Fish and other aquatic products are a vital source of high-quality protein and essential micronutrients, contributing significantly to global food security (Liu et al., 2018). In 2022, global aquatic animal aquaculture production reached 94.4 million tonnes, with an estimated value exceeding USD 295.7 billion, and now accounts for more than half of the world’s total seafood supply (FAO, 2024). Within this sector, salmonids have been the most valuable aquatic species group since 2013, representing approximately 20% of the total global value of farmed aquatic animal products in 2022 (FAO, 2024). The continued expansion of aquaculture reflects its critical role in meeting rising seafood demand as capture fisheries plateau or decline.

Aquaculture production encompasses a range of farming systems that differ in their level of environmental control, intensity, and interaction with surrounding ecosystems (Lekang et al., 2016). These systems broadly include open-water production, such as marine net pens or sea cages, as well as land-based systems, including flow-through and recirculating aquaculture systems (RAS) (Lekang et al., 2016). In many species, production is divided across multiple

stages, with early life stages typically reared in controlled freshwater hatcheries before transfer to larger grow-out systems. Open-water sea cage farming remains the dominant method for producing many marine finfish species due to its relatively low capital costs and scalability. However, this approach is associated with environmental challenges, including nutrient discharge, disease transmission, and exposure to fluctuating environmental conditions (Price et al., 2015; Carballeira Braña et al., 2021). In contrast, land-based aquaculture systems allow for greater control over water quality, biosecurity, and environmental variables, albeit at higher operational and energy costs (Mota et al., 2022). Among land-based approaches, RAS has gained increasing attention as a means of improving production efficiency and sustainability through water reuse and enhanced system control.

1.1.1 Atlantic salmon aquaculture

Among cultured fish, Atlantic salmon (*Salmo salar*, Linnaeus, 1758) is one of the most intensively farmed and highest-value species worldwide (FAO, 2024). Although commercial production began less than 50 years ago, Atlantic salmon aquaculture has developed into the most valuable marine or brackish-water finfish farming industry, with a global value exceeding USD 38 billion (FAO, 2024). Global production of farmed Atlantic salmon now exceeds 4.2 million tonnes annually and surpassed wild-caught salmon production as early as 2004 (FAO, 2024). Farmed Atlantic salmon currently accounts for approximately 49% of the total global salmon market (FAO, 2022). The principal producing countries include Norway, Scotland, Chile, Australia, and Canada (FAO, 2024). Atlantic salmon aquaculture is characterised by a complex, multi-stage production cycle involving both freshwater and marine environments (Klemetsen et al., 2003). Production typically begins in land-based freshwater hatcheries, where eggs are incubated and juveniles reared through the alevin and parr stages. Juvenile salmon are subsequently transferred to either flow-through or recirculating freshwater systems to produce smolts, which undergo physiological changes that enable their transition to seawater (Klemetsen et al., 2003; McCormick et al., 1998). Following smoltification, fish are transferred to marine grow-out facilities, most commonly open-water sea cages, where they are reared to market size (Hoar, 1988). In Scotland, Atlantic salmon aquaculture represents one of the nation’s most economically significant industries, accounting for more than 50% of total food export revenues. Scotland is the largest producer of farmed Atlantic salmon in the UK, and among the leading producers in Europe, with production approaching 138,000 tonnes in 2023 (Salmon Scotland, 2024). Over the past three

decades, the sector has become a cornerstone of economic development in the Highlands and Islands, directly employing approximately 2,500 people and supporting over 10,000 jobs across the wider supply chain (Salmon Scotland, 2024). As global demand for farmed fish continues to rise due to population growth and declining capture fisheries, threats to Atlantic salmon aquaculture pose substantial economic and food-security risks. One of the most significant ongoing challenges facing the industry is the prevalence of gill diseases, which negatively impact fish welfare, productivity, and profitability (Matthews et al., 2013; Boerlage et al., 2020).

1.1.2 Recirculating aquaculture systems

Recirculating aquaculture systems (RAS) are intensive land-based farming systems that treat and reuse water through a series of mechanical, biological, and chemical filtration processes. Unlike flow-through systems, which rely on a continuous supply of new water, RAS minimise water use by recirculating up to 90–99% of system water, thereby reducing freshwater demand and effluent discharge (Crouse et al., 2021; Gichana et al., 2018). Key system components typically include mechanical filters for solid waste removal, biofilters for nitrification, degassing units, oxygenation systems, and disinfection technologies such as ultraviolet or ozone treatment (Gichana et al., 2018). The primary advantage of RAS lies in the high degree of control it provides over environmental conditions, including temperature, dissolved oxygen, photoperiod, and water quality parameters (Mota et al., 2022). This level of control can enhance growth performance, improve feed efficiency, and reduce exposure to pathogens and parasites commonly encountered in open-water systems (Kolarevic et al., 2014). As a result, RAS has become widely adopted for the early freshwater stages of Atlantic salmon production, where maintaining stable conditions is critical for survival, development, and welfare. While recent industry developments have explored the use of RAS for post-smolt and even full grow-out production, these applications remain limited and are associated with substantial technical, energetic and economic challenges (Brown et al., 2025). High capital investment costs, increased energy demand, and the need for precise system management to maintain optimal water quality have constrained widespread adoption of RAS beyond freshwater production stages (Badiola et al., 2018). Consequently, in contemporary Atlantic salmon aquaculture, RAS is most commonly integrated into the production cycle during the fry and parr stages, prior to smoltification and subsequent transfer to seawater (Brown et al., 2025). At this stage, RAS offers clear advantages in terms of biosecurity, environmental control, and efficient resource use, while allowing producers to optimise juvenile quality before

fish enter marine grow-out systems, typically in open-water sea cages (Hashmi et al., 2025). Understanding fish health and physiological responses within these early-stage RAS environments is therefore fundamental to improving overall production performance and welfare in Atlantic salmon aquaculture.

1.2 Gill disease

Gill diseases are a substantial and increasing problem in Atlantic salmon aquaculture globally (Herrero et al., 2018; Boerlage et al., 2020; Foyle et al., 2020). The gills play a number of key roles, providing not only the function of gas exchange but also osmotic and ionic regulation and excretion of nitrogenous waste. Having a high surface area and needing both to mediate physiological exchanges while acting as a key physical and immunological barrier to pathogen and toxicant entry, the gills are very vulnerable as they provide a direct interface with the external environment (Evans et al., 2005). Hence the gills are exposed to ambient chemical and physical water conditions as well as to any water-borne pathogens directly (Mitchell & Rodger, 2011). Additionally, during smoltification the gills undergo pronounced structural and physiological changes to facilitate adaptation to seawater, including thinning of the epithelium, increased numbers of chloride cells and reorganisation of tight junctions to support ion secretion (Laurent & Perry, 1991). While essential for successful osmoregulation, this restructuring can temporarily reduce gill barrier integrity by increasing epithelial permeability and affecting local immune defences (Johansson et al., 2016). Most mortalities associated with gill disease are reported to occur in the late summer or autumn when a few months after S1 fish are transferred to seawater (Herrero et al., 2018). The reason for this increased susceptibility in S1 smolts in comparison to their S0 counterparts is not clear, however it is likely due to environmental insults like phytoplankton and zooplankton levels at this time (Gunnarsson et al., 2017; Herrero et al., 2018; Gjessing et al., 2019). Additionally, S0 smolts have been shown to have better hypo-osmoregulatory abilities and higher body fat deposits, which may affect their resilience (Lysfjord et al., 2004; Kristensen et al., 2012). While losses through direct mortality are significant, reduced growth rates, poor feed conversion and compromised immunity all contribute to the economic damage caused by gill diseases (Rodger et al., 2011; Foyle et al., 2020). Many parameters have been suggested as risk factors for gill diseases including individual pathogens, environmental factors like increased salinity and relatively higher water temperatures and physical irritants like plankton (Kvellestad et al., 2005; Mitchell & Rodger, 2011; Nylund et al., 2011; Matthews et al., 2013; Boerlage et al., 2020), however it is now commonly accepted that many gill disease are multifactorial (Steinum et al., 2010).

Complex gill disease (CGD), or complex gill disorder, is an umbrella term used to encompass a wide range of multifactorial gill disorders, including proliferative gill disease (PGD) and pro-

liferative gill inflammation (PGI) (Herrero et al., 2018; Noguera et al., 2019). Though varying presentations of CGD, with many different pathogens detected have been reported (Kvellestad et al., 2003; Herrero et al., 2018), common general histological signs include inflammation, hyperplasia and apoptosis (Kvellestad et al., 2005; Steinum et al., 2010; Nylund et al., 2011). Clinical signs associated with CGD include general respiratory distress, lethargy, erratic swimming and reduced feed conversion (Herrero et al., 2018; Mitchell & Rodger, 2011). Many pathogens have been isolated from fish displayed signs of CGD, including parasites (Matthews et al., 2013; Weli et al., 2017), bacteria (Steinum et al., 2010; Gjessing et al., 2021) and viruses (Kvellestad et al., 2003; Nylund et al., 2008), however in recent years three pathogens have emerged as the most consistent infectious agents associated with CGD- *Candidatus* Branchiomonas cysticola, *Desmozoon lepeophtherii* and salmon gill poxvirus (SGPV) (Gjessing et al., 2017; Gjessing et al., 2019; Gjessing et al., 2021).

Although no treatment is 100% effective against most gill diseases, several common management approaches are used. These include improving aeration, the use of functional feeds, administration of antibiotics via feed, freshwater bathing and hydrogen peroxide baths (Florent et al., 2007; Kierner & Black, 1997; Parsons et al., 2001; Rodger et al., 2011). Each of these methods has specific benefits and limitations. While antibiotics can target bacterial infections, their use is strictly regulated and they often fail to fully resolve the condition. Freshwater bathing and hydrogen peroxide have historically been used to treat amoebic gill disease (AGD) and have been shown to reduce parasite loads to some extent (Powell et al., 2015), however, their effectiveness is limited against multifactorial conditions, such as complex gill disease, and treatments must be repeated regularly (Parsons et al., 2001; Clark et al., 2003). In addition, hydrogen peroxide can cause significant damage to gill tissue depending on the concentration used and the frequency of use (Veltman et al., 2025).

1.3 Poxviruses

1.3.1 *Poxviridae*

The Poxviridae family is a family of large, double-stranded, enveloped DNA viruses, with genomes ranging in size from 127 to 360 kb in length (Tulman et al., 2004; Jacob et al., 2018). Poxviruses are complex DNA viruses with an ovoid or brick shape and linear double stranded genomes, with termini that form covalently closed hairpin loops (Moss, 2013; Upton et al., 2003). They can be distinguished from other viruses by their ability to replicate entirely in the cytoplasm of their hosts cells (Moss, 2013). After uncoating, the virion produces early enzymes and virion proteins as well as late enzymes and virion proteins. This is done independently from the host cell nucleus and can be seen through light microscopy as basophilic B-type inclusion bodies. Poxviruses have toxic effects on cells, causing them to round and clump and triggering degeneration of cell ultrastructure, causing disease in a wide variety of species (Diven, 2001). The host range of poxviruses varies from extremely narrow, e.g. variola virus (VARV) which causes smallpox and only replicates in humans, to very wide, e.g. cowpox (CPXV) or monkeypox (MPXV) can infect a wide range of mammalian species (Haller et al., 2014).

The *Poxviridae* family is traditionally divided into two subfamilies- *Entomopoxvirinae* which infect insects, and the *Chordopoxvirinae* which infect vertebrates (Hughes et al., 2010). Poxviruses can be extremely dangerous for their hosts. Myxoma virus (MYXV), which causes myxomatosis in European rabbits, may elicit a mortality rate of up to 99% (Fenner & Woodroffe, 1953). Smallpox, caused by VARV, has been estimated to have killed more than a billion people (Smith & Kotwa, 2002). As such large and often dangerous viruses, poxviruses were some of the first viruses to be studied (Fenner, 2000). Some of the most well-known poxviruses include vaccinia virus (VACV), which was the first virus to be visualised microscopically in 1886 and is the prototypic poxvirus used in many studies (Moss, 2020), and VARV, the causative agent for smallpox (Smith & Kotwa, 2002).

The first record believed to relate to a smallpox outbreak was in 1350 BCE during the Egyptian Hittite war. The virus was subsequently widespread throughout the world, causing millions of mortalities worldwide, until it was declared eradicated by the World Health Organisation in 1980 (Fenner et al., 1988; Barquet, 1997; WHO, 1979). VARV and CPXV were the first viruses to be used to develop the concept of ‘vaccination’ (Fenner et al., 1988). Initially, the VARV was used to immunise through what was known as variolation, where material from a patient

suffering from smallpox was introduced subcutaneously into a naïve patient to immunise them and which, while effective, also had a risk of severe and fatal infection with not only smallpox, but with other infectious diseases transferred from the original patient (Willis, 1997; Haller et al., 2014). A much less dangerous alternative was developed when Edward Jenner noticed that milkmaids who had been exposed to CPXV did not contract smallpox and so began to infect people with the less dangerous cowpox in order to protect them from smallpox endemics (Barquet, 1997). This approach laid the foundation for modern vaccination and led to the development of live attenuated vaccines which remain the basis for effective immunisation against poxviruses. Live attenuated vaccines are most commonly used against poxviruses as they trigger strong cell-mediated immunity in addition to antibody response (Chaudhri et al., 2004; Minor, 2015). In contrast, inactivated vaccines do not involve replication within the host and primarily stimulate the antibody response, providing more limited and potentially short-term protection (Boumart et al., 2016).

Although poxvirus hosts include a huge variety of insects, reptiles, birds and mammals (Payne, 2017), only five poxviruses infecting fish have yet been described, carp edema virus (CEV), salmon gill poxvirus (SGPV), *Plecoglossus altivelis* poxvirus-like virus (PaPV), cod gill poxvirus (CGPV) and seahorse poxvirus (SHPV), four of which show a high affinity for the gills (Gjessing et al., 2015; Miyazaki et al., 2005; Wada et al., 2011; Gjessing et al., 2025).

1.3.2 Carp Edema Virus

Carp edema virus (CEV) is a highly infectious virus which infects both common carp and koi carp. Originally described in juvenile koi carp in Japan in 1974 (Miyazaki et al., 2005), CEV has now been reported in carp elsewhere in Asia (Zhang et al., 2017), Europe (Adamek et al., 2022) and Northern America (Tolo et al., 2021).

CEV is the causative agent for Koi Sleepy disease (KSD) which affects both common and koi carp (Miyazaki et al., 2005). Clinical signs of disease include extreme lethargy and unresponsiveness in addition to anorexia, pale gills with swelling and necrosis, skin erosion and tissue oedema (Miyazaki et al., 2005; Zhang et al., 2017). KSD has been reported to causes very high mortality rates (75% - 100%), particularly in juvenile koi carp (Miyazaki et al., 2005; Jung-Schroers et al., 2015), whereas in common carp the disease is more protracted and has lower mortality rates (Lewisch et al., 2015). Mortality is strongly correlated with water temperature and with stress, with most disease outbreaks occurring in the week following fish transfer (Lewisch et al., 2015).

CEV has the largest genome of all poxviruses currently known, with 456 kbp encoding a predicted 392 genes (Mekata et al., 2021). Three main genogroups of CEV have been identified, each with distinct geographical distributions. The European genogroup (I) originated in farmed and wild common carp in Europe but has since spread to the USA (Matras et al., 2017). The Asian genogroup (II) originated in common carp in Asia and has now spread globally through the koi carp trade and a third genogroup (III) has been identified more recently, primarily in Austria, and appears to be genetically distinct from both genogroups I and II, indicating further diversification of the virus.(Adamek et al., 2018).

1.3.3 Cod gill poxvirus

One of the few other characterised aquatic poxviruses is the recently discovered cod gill poxvirus (CGPV). CGPV was first identified during studies on gill disease in farmed Atlantic cod, where affected fish exhibited respiratory distress, reduced growth performance and increased mortality (Gjessing et al., 2025).

Histopathological examination of affected gills revealed the presence of cytoplasmic inclusion bodies within gill epithelial cells consistent with poxvirus infection (Gjessing et al., 2025). The classification of this pathogen as a poxvirus was further supported by transmission electron microscopy, which demonstrated large, brick-shaped virions replicating within the cytoplasm of infected cells. Using methods like PCR, transmission electron microscopy and *in-situ* hybridisation, as well as next generation sequencing, CGPV was confirmed to be a novel member of the poxviridae family.

Clinical signs of CGPV include respiratory distress, lethargy, reduced feeding activity, decreased growth and increased mortality (Gjessing et al., 2025). Although the most severe pathology seems to be in the gills, where epithelial hyperplasia, hypertrophy and inflammation have been observed, and in the heart, where inflammation and circulatory disturbances were also observed, some minor pathology has also been found in the spleen kidney and liver. In the same samples, *Candidatus branchiomonas cysticola* and *Trichodina*-like parasites were identified, suggesting the possibility that CGPV may occur as part of multifactorial infections.

CGPV shows a high degree of sequence homology to SGPV and groups within the same clade in the phylogenetic tree (Gjessing et al., 2025), however further studies investigating CGPV epidemiology, host–pathogen interactions and disease progression will be essential to understand its role in cod health and to place it more fully within the broader diversity of aquatic poxviruses.

1.3.4 *Plecoglossus altivelis* poxvirus-like virus

Plecoglossus altivelis poxvirus-like virus (PaPV) was first isolated from cultured ayu (*Plecoglossus altivelis*), also known as sweetfish, with atypical cellular gill disease (ACGD), or ‘Boke’, in Japan in 2010 (Wada et al., 2011) and has since been found in both wild and cultured ayu populations as well as in wild sardines (Nakayama et al., 2016). Clinical presentation of ACGD includes a loss of appetite and condition, abnormal swimming patterns or ‘spinning’ and elevated positioning in the tank, which often very quickly leads to mortality of up to 90% (Wada et al., 2008, 2011). Similarly to KSD, ACGD outbreaks are reported to be closely correlated to water temperature (Ishikawa et al., 2022).

1.3.5 Salmon Gill Poxvirus

Salmon gill poxvirus was first isolated from the gills of a farmed Atlantic salmon diagnosed with proliferative gill disease in northern Norway in 2006 (Nylund et al., 2008). It was clear from morphogenesis that this newly discovered virus was a member of the family *Poxviridae* and so was named salmon gill poxvirus (SGPV). All farms where SGPV was confirmed suffered significant mortalities which peak in the months following transfer of smolts from freshwater to sea cages, but also affected fish during the freshwater production stages (Nylund et al., 2008). Though discovered and classified as a member of the *Poxviridae* family in 2006, the entire SGPV genome was not sequenced until 2015 when Gjessing and colleagues isolated and sequenced the virus found in the gills of infected fish (Gjessing et al., 2015). Phylogenetic analysis of the full genome then showed that not only was SGPV indeed a poxvirus, but it was found to be the deepest representative of the chordopoxviruses which infect vertebrates, belonging at the base of the *Chordopoxvirinae* phylogenetic tree (Figures 1.1 1.2)(Gjessing et al., 2015). The phylogenetic study found that the SGPV genome contains almost all of the key chordopoxvirus genes that are required for replication and expression, but in a substantially different order.

The same group also developed both qPCR and immunohistochemistry methods to detect the virus and analysed both current and historic samples for SGPV presence. They found that, although not described until 2006, PCR analysis of archival materials show that salmon gill poxvirus has been present on Norwegian salmon farms since at least 1995 (Gjessing et al., 2015).

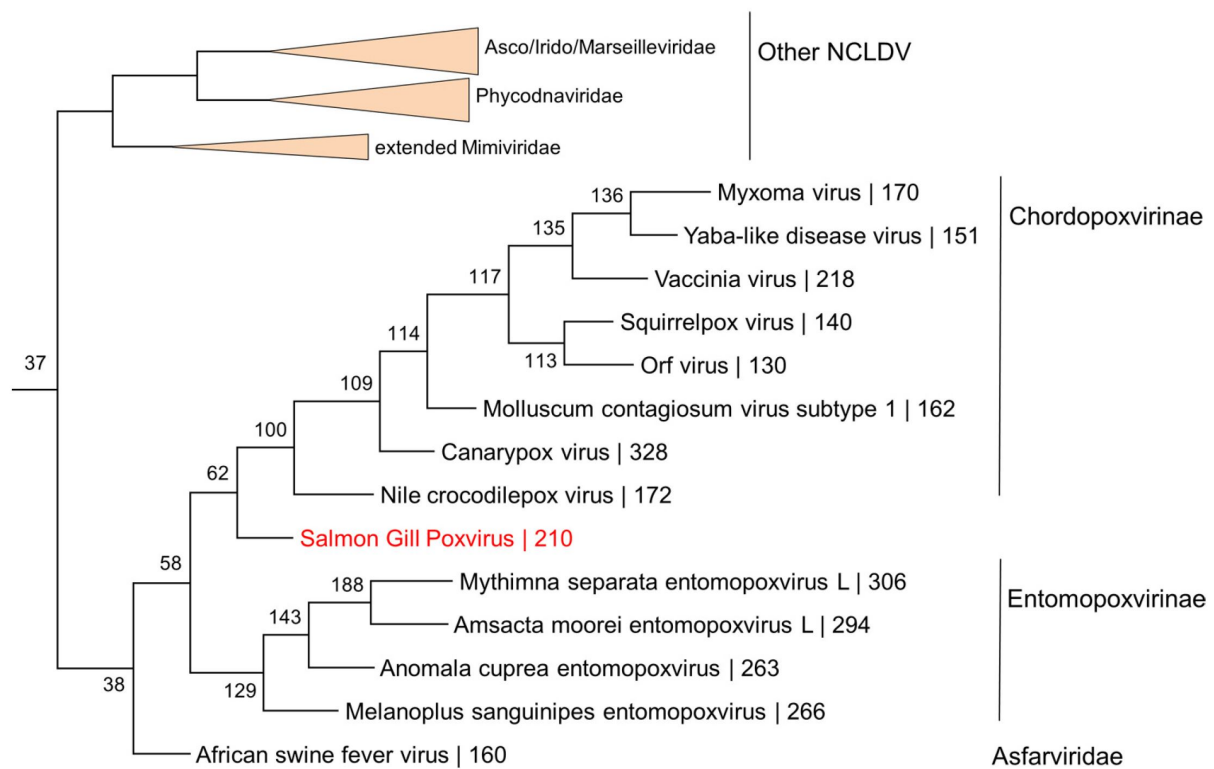


Figure 1.1: Phylogenetic tree of the family *Poxviridae* including salmon gill poxvirus at the base of the *Chordopoxvirinae* branch (Gjessing et al., 2015)

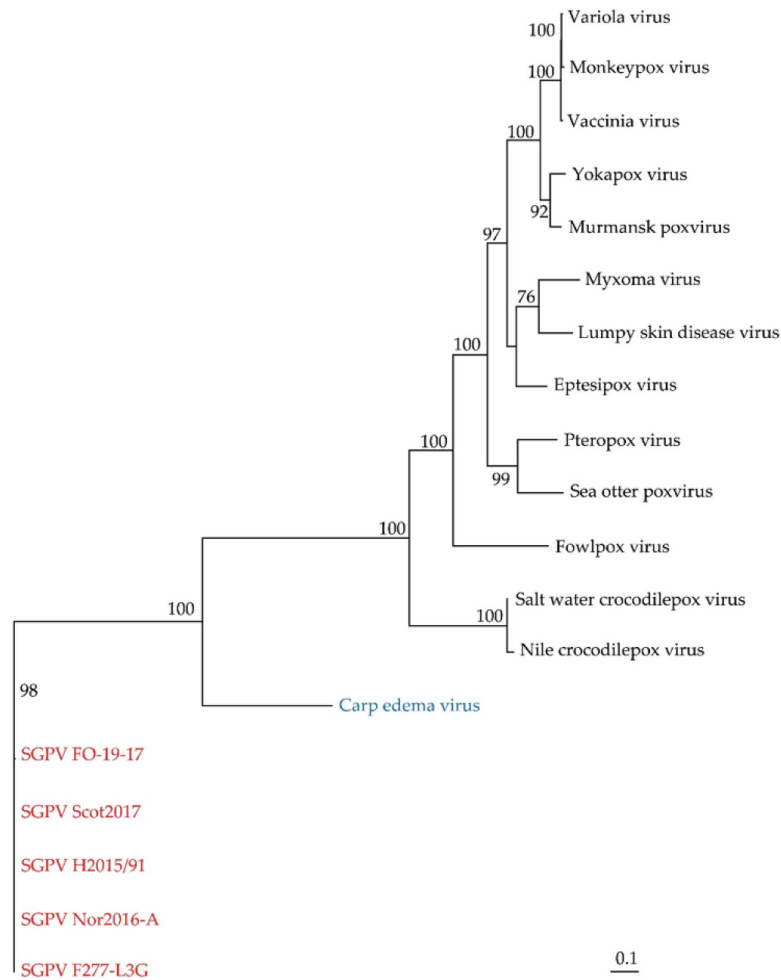


Figure 1.2: Updated phylogenetic tree of the Chordopoxviruses including carp edema virus and new SGPV samples analysed (Nylund et al., 2023)

1.4 Current knowledge

1.4.1 Salmon Gill Poxvirus Disease

Clinical presentation

Outbreaks of SGPV disease have been reported during all stages of Atlantic salmon production, though the most acute outbreaks with severe mortality rates generally occur during the freshwater phase (Gjessing et al., 2017; Tartor et al., 2022). While SGPV has been detected on seawater sites, it is commonly as a part of multi-factorial disease (Nylund et al., 2008; Gjessing et al., 2016; Herrero et al., 2018). However, when SGPV infections coincide with smoltification it often results in failed seawater transfer and high mortality rates (Gjessing et al., 2017). Presentation of SGPV infection varies widely from subclinical infections to those giving rise to severe clinical signs and mortality rates of up to 80% (Nylund et al., 2008). Clinical signs, when present, often include lethargy, lack of appetite, erratic swimming patterns, increased respiration, crowding on the bottom of the tank, and then later in the infection, high positioning in the tank and gaping (Gjessing et al., 2017; Gjessing, Aamelfot, et al., 2018; Wiik-Nielsen et al., 2017).

Additionally, a muscular rigidity or ‘live rigor’ has been reported both in Norway (Thoen et al., 2020; Tartor et al., 2022) and informally in Scotland. While the cause of these tonic seizures is not yet well understood, Thoen and colleagues (2020) hypothesise that it may be linked to the respiratory distress often observed in diseased fish. Respiratory distress has been shown to cause hypercapnia and respiratory acidosis in trout (Fievet et al., 1988) and respiratory acidosis is known to cause issues with metabolism in muscles (Cochran & Burnett, 1996), which may be linked to this characteristic “live rigor” of infected fish.

Subclinical infections

Far less well understood, although frequently occurring, are subclinical infections with SGPV. A low viral load in apparently healthy fish has been widely reported (Amundsen et al., 2021; Thoen et al., 2020; Tartor et al., 2022). An infection trial in 2022 showed that fish infected with SGPV did not develop clinical signs of disease until injected with cortisol to simulate stress (Tartor et al., 2022), suggesting that SGPV infection alone is not pathogenic but requires a trigger of some description to escalate into disease.

Histopathological effects of disease

The main target organ of SGPV is the gills, where most of the pathology is centred (Gjessing et al., 2015). The most common pathological effects reported in Atlantic salmon suffering from salmon gill poxvirus disease (SGPVD) are severe epithelial and mucous cell hyperplasia, defined as an increase in cell number due to enhanced cellular proliferation (Kumar et al., 2025), and hypertrophy, referring to an increase in individual cell size, together with inflammation, deformed chloride cells, and apoptotic epithelial cells, indicative of programmed cell death characterised by controlled cellular dismantling and removal (Nylund et al., 2008; Gjessing et al., 2015; Gjessing et al., 2016; Wiik-Nielsen et al., 2017; Gjessing et al., 2019; Kumar et al., 2025).

Historically SGPV has most often been found in fish with proliferative or multifactorial gill disease which can confound the histology observed (Nylund et al., 2008; Wiik-Nielsen et al., 2017). Epitheliocysts have also often been reported in cases of severe SGPVD on seawater production sites (Gjessing et al., 2017; Wiik-Nielsen et al., 2017), but both these studies also found bacterial co-infections in the diseased fish.

Additionally, the gills may not be the only organs affected by SGPV. Haemophagocytosis has been observed in both the kidney and spleen of severely infected fish, which Gjessing and colleagues (2015) hypothesise could be a signal of circulatory distress either caused by or contributing to the SGPV disease outbreak. Later, in a study in 2018, the group found SGPV in epithelial cells covering the thymus which also had significant epithelial apoptosis in the gills, as well as in the skin of infected fish. However in both these studies only very low or no viral copy numbers were found upon qPCR analysis of these organs, suggesting that these are secondary effects of the disease, while the gills remain the primary target (Gjessing, Christensen, et al., 2018).

1.4.2 Potential triggers of disease

As discussed above, multiple studies have demonstrated that the presence of SGPV does not necessarily result in clinical disease or associated mortality (Amundsen et al., 2021; Thoen et al., 2020; Tartor et al., 2022). This raises the important question of which factors may trigger the progression from subclinical infection to overt disease. Current evidence suggests that host immune suppression, particularly that associated with stress, plays a central role.

Stress

Several studies indicate that stress induced immune modulation is a key factor in the development of SGPVD. In a study of a natural outbreak, Gjessing and colleagues (2018) suggested that gill damage caused by SGPVD impairs the host's innate immune defences, potentially facilitating invasion by secondary pathogens. In a subsequent experimental study, the same group successfully induced SGPVD in subclinically infected fish through cortisol injection to simulate stress, whereas infected control fish remained clinically healthy (Thoen et al., 2020). In this trial, the expression of several immune markers was delayed in the cortisol injected group compared with controls, with peak expression occurring later in infection, suggesting stress-associated immune modulation (Amundsen et al., 2021). This finding is consistent with reports linking stressful events such as seawater transfer and smoltification to SGPVD outbreaks (Nylund et al., 2008; Gjessing et al., 2017; Thoen et al., 2020). Collectively, these studies indicate that stress-induced impairment of immune function is a critical trigger for disease development in SGPV infected fish.

Co-infections

The role of immune modulation in SGPVD aligns with what is known for other poxviruses, many of which have evolved mechanisms to interfere with host immune responses in order to facilitate infection and enhance viral replication (Kotwal, 1997). Members of the *Poxviridae* family have been shown to target key mediators of the innate immune response, including interferons (IFNs), interleukins (ILs), and chemokines (Seet et al., 2003). Well-studied poxviruses such as VACV, CPXV, MYXV, and CEV employ immune evasion strategies that block or delay chemokine, cytokine, and serine protease activity (Smith & Kotwa, 2002; Alzhanova & Früh, 2010; Zawisza et al., 2024). Similarly, Amundsen and colleagues (2021) observed delayed expression of several immune effectors in SGPV-infected fish, including Mx (an interferon-induced GTPase involved in viral inhibition), IFN γ (a cytokine essential for innate and adaptive immunity), and granzyme (a serine protease involved in apoptosis). Such immune suppression may explain why SGPV is frequently detected in multifactorial disease outbreaks, as impaired host defences can increase susceptibility to secondary infections (Nylund et al., 2008; Gjessing et al., 2015; Gjessing et al., 2017; Downes et al., 2018; Herrero et al., 2022).

1.4.3 Transmission

Despite the increasing number of studies on SGPV published in recent years, significant knowledge gaps remain regarding viral transmission pathways and the existence of potential reservoirs, particularly within closed freshwater recirculating aquaculture systems (RAS). SGPV infection has been detected throughout the salmon production cycle, affecting both S0 salmon, which are transferred to sea in the same year as hatching, and S1 salmon, which are transferred the following spring (Nylund et al., 2008; Gjessing et al., 2025; Garseth et al., 2018).

Horizontal transmission

Horizontal transmission of SGPV has been demonstrated in several experimental and field-based studies (Gjessing et al., 2017; Wiik-Nielsen et al., 2017; Garseth et al., 2018). Transmission via water has been confirmed, indicating that direct fish to fish contact is not required for infection (Wiik-Nielsen et al., 2017). This finding supports the hypothesis that water sources may act as an important route for viral introduction into freshwater farming systems.

Wild Atlantic salmon returning from marine migration into brackish rivers have been shown to carry SGPV, and low viral loads have also been detected in anadromous trout cohabiting with Atlantic salmon (Garseth et al., 2018). In contrast, SGPV was not detected in salmon or trout from freshwater rivers lacking anadromous fish. These observations suggest that the use of untreated water from rivers containing anadromous fish may pose a risk for SGPV introduction into freshwater aquaculture facilities.

Another potential mode of pathogen spread that is increasingly being explored in aquaculture is aerosolisation. Routine onsite activities like water pumping, aeration, degassing and high pressure cleaning methods can produce fine droplets capable of transporting viral particles between tanks or units (Wooster & Bowser, 1996; Roberts-Thomson et al., 2006; Shinn et al., 2025). Aerosolised transmission of SGPV has now been shown to occur in a study on a RAS in the Faroe islands, (Krishna et al., 2025), with the viral load detected correlating with that found in the fish and the water and the highest level of SGPV containing aerosols found in the room containing the system biofilter. Given that SGPV is shed into the water column and can be transmitted via water, aerosolisation represents an indirect form of horizontal transmission that may contribute to enclosed system dissemination. This route is of particular concern in RAS with shared airspaces or limited physical separation between production units, highlighting the importance of ventilation design and biosecurity measures in mitigating pathogen spread.

Vertical transmission

Vertical transmission of SGPV has been proposed as a possible route of infection, particularly in freshwater RAS facilities where the virus can persist despite treatment of incoming water. SGPV has been detected repeatedly in some RAS facilities across multiple production cycles, even following deep system disinfection (Gulla et al., 2020). This persistence suggests either long term environmental survival of the virus within the system or repeated reintroduction via infected fish or water source. A suspected case of vertical transmission was investigated by Gulla and colleagues (2020), in which two cohorts of fry originating from the same SGPV-positive broodstock developed disease. However, genomic analysis revealed that the viral variants differed between cohorts, indicating that vertical transmission had not occurred in this case. While current evidence suggests that vertical transmission is unlikely, it cannot be definitively excluded and warrants further investigation.

The persistence of SGPV in freshwater RAS highlights challenges associated with biosecurity and virus inactivation. Despite treatment of incoming water and comprehensive disinfection protocols, recurring outbreaks have been reported (Gulla et al., 2020), suggesting that standard biosecurity measures may be insufficient for complete viral elimination. The ability of poxviruses to persist in the environment is well documented, and resistance to disinfection has been reported for several members of the *Poxviridae* family. However, specific data on SGPV susceptibility to commonly used disinfectants and inactivation methods in aquaculture systems remain limited. Further studies evaluating effective disinfection strategies, environmental persistence and viral reservoirs within RAS infrastructure are needed to improve biosecurity and reduce the risk of repeated outbreaks.

1.4.4 Diagnostic approach

Currently the primary method of SGPV detection, in addition to clinical signs and characteristic pathology, is qPCR using a primer set designed to target the D13L open reading frame (ORF) in the SGPV genome, which is homologous to the D13L ORF in VACV (Gjessing et al., 2015). Moreover, both an immunohistochemistry assay targeting the L1R protein and an in situ hybridisation assay have also been developed for SGPV, and have been shown to successfully visualise SGPV in infected tissues (Gjessing et al., 2015; Gjessing, Christensen, et al., 2018; Thoen et al., 2020).

Koch's postulates have limited applicability to aquatic diseases as they were developed for

simple, single-host infections and do not account for the complexity of aquatic ecosystems (Hudson & Egan, 2024). In aquaculture, unlike in terrestrial pathology, many pathogens are ubiquitous in the environment or present even in healthy hosts, and disease expression is often strongly influenced by environmental factors such as temperature, stocking density and water quality (Boyd, 2017). Additionally, many aquatic diseases are multifactorial, involving interactions between multiple pathogens and host stress, as has been shown to be the case for SGPV (Tartor et al., 2022). The fact that SGPV is also so often present subclinically violates the first postulate. Consequently, the strict criteria of Koch’s postulates, that were originally developed for the study of terrestrial bacteria, are inappropriate and insufficient for establishing causality in SGPV and many aquatic diseases. However, it remains that the *in vitro* culture of SGPV is the next step required in the study of the disease. Although the applicability of Koch’s postulates is debatable, one of the remaining hurdles in the study of SGPV is *in-vitro* culture. Although a North American variant of SGPV has been cultured in chinook salmon embryo cells (CHSE-214) (LeBlanc et al., 2019), this result has not been replicated elsewhere despite several attempts (Gjessing, Christensen, et al., 2018; Tartor et al., 2022). To enable the further in depth study and understanding of SGPV, a reliable cultivation model is required.

1.4.5 Genetic characterisation of SGPV

Salmon gill poxvirus was first identified as a poxvirus following its detection in gill tissue from Atlantic salmon with proliferative gill inflammation (PGI) in Norway in 2006, where transmission electron microscopy revealed poxvirus-like particles (Nylund et al., 2008). The first complete genome sequence, published in 2015, enabled robust phylogenetic placement and showed that SGPV diverged early from other chordopoxviruses, clustering near the base of the *Chordopoxvirinae* branch within the *Poxviridae* (Gjessing et al., 2015). This basal phylogenetic placement suggests an early divergence of SGPV within the chordopoxviruses, consistent with a long and distinct evolutionary history (Gjessing et al., 2015). Comparative genomic analysis also highlighted substantial differences in gene content, with SGPV lacking 38 genes that are otherwise highly conserved among chordopoxviruses (Gjessing et al., 2021). Although many genes involved in viral gene expression, genome replication and virion morphogenesis were identified, most genes associated with viral membrane biogenesis were absent, raising the possibility that these functions evolved after SGPV diverged or were subsequently lost in the SGPV lineage (Gjessing et al., 2015). However, despite the lack of these genes, it appears that SGPV employs a similar

pathway, as recognisable poxvirus crescent membranes, immature and mature virion particles can be seen during SGPV replication (Gjessing et al., 2015). Unfortunately, the current absence of reliable culture techniques for SGPV restricts the scope for studies aiming at elucidating the mechanisms of membrane biogenesis in SGPV.

A subsequent study employed multi-locus variable-number tandem-repeat analysis (MVLA) to investigate the genetic diversity and population structure of SGPV across geographically distinct outbreaks (Gulla et al., 2020). Using this approach, isolates from multiple outbreaks in Norway along with single isolates from Scotland, the Faroe Islands and Canada were compared. Overall, the analysis revealed relatively low genetic diversity among the sampled viruses, with limited clustering according to country of origin. This suggests a high degree of genetic conservation within regional SGPV populations, particularly among European isolates.

Despite this overall conservation, a clear transatlantic divergence was observed, with European and Canadian isolates forming distinct groups (Gulla et al., 2020). This pattern may reflect long-term geographic separation, limited contemporary exchange of infected host material between continents, or historical divergence followed by regional circulation within Atlantic salmon populations. Notably, no association was identified between MVLA genotype and disease severity (Gulla et al., 2020). Together, these findings suggest that while SGPV exhibits some degree of geographic structuring, its genetic diversity is relatively constrained, consistent with a stable virus–host association and possibly long term coevolution with salmonid hosts.

1.5 Aims

Although SGPV causes considerable losses to the Atlantic salmon aquaculture industry both in Scotland and worldwide, there are still several pressing gaps in our knowledge of the virus and the disease it causes. The huge variation in presentation from subclinical to severe disease and mortality is concerning, as is our lack of understanding as to what causes this. Additionally, while SGPV has a significant impact on Scottish aquaculture, an outbreak of the disease in Scotland has not been studied in detail.

The overall aim of this project was to improve our understanding of salmon gill poxvirus by investigating the impacts of environmental factors and identifying potential risk factors for disease, studying the effects of SGPV internally and trying to identify any differences between outbreaks that may contribute to the wide range of presentation.

- **Chapter 2.** To investigate the influence of environment on subclinical SGPV infection and to identify what parameters may act as risk factors for disease outbreak.
- **Chapter 3.** To explore the physical effects of SGPV infection and disease in a Scottish outbreak, and study response to infection in the fish.
- **Chapter 4.** To sequence and phylogenetically analyse SGPV isolates from geographically distinct outbreaks to try to identify if genetic difference could account for the variation of presentation.

Chapter 2

Influence of environmental factors on salmon gill poxvirus infection

2.1 Introduction

Salmon gill poxvirus is a relatively recently discovered virus that has become increasingly problematic in Atlantic salmon culture in Scotland over the last 10 years. Though SGPV infection does not always lead to disease, when disease outbreaks occur, clinical presentation can be severe, resulting in extreme losses through both reduced growth and very high mortality rates (Nylund et al., 2008; Gjessing, Christensen, et al., 2018).

Despite SGPV being a very prevalent issue in Atlantic salmon aquaculture, with the potential to cause huge losses to the industry, it is still relatively poorly understood. One of the most pressing questions surrounding SGPV infection is the source of the virus, especially in enclosed freshwater re-circulating aquaculture systems (RAS). Infection with SGPV has been reported during almost all stages of production, and disease outbreaks are a common issue during the freshwater pre-smolt stage, often in fully enclosed RAS systems (Wiik-Nielsen et al., 2017; Garseth et al., 2018).

One of the reasons why identifying the source of SGPV in enclosed aquaculture systems such as RAS is critical is that, once infected, SGPV can persist for years, reappearing in each new production cycle (Gjessing et al., 2015). As systems are thoroughly cleaned between cycles, one theory is that SGPV may be persisting in biofilters, which are not always entirely restarted

between production cycles. Studies have shown that both bacteria and viruses can persist in biofilms (Noble & Summerfelt, 1996; Bullock & Schill, 2006; Michaud et al., 2009; Christiansen et al., 2021), and potentially even infect fish in subsequent cycles (Moresco et al., 2022). Given the potential of SGPV to incur high costs to the industry, there is an urgent need to reach a better understanding of the source of SGPV infection.

2.1.1 Sources of infection

Several possible sources of the SGPV have been suggested. One potential source of infection is the incoming water supplying recirculating aquaculture systems. In a study on SGPV prevalence in wild fish, the virus was found to be present not only in wild Atlantic salmon in rivers, but also at low levels in trout (*Salmo trutta*) in the same rivers (Garseth et al., 2018). Garseth and colleagues therefore suggested that the use of rivers like these, which contain wild anadromous fish, as water sources for enclosed RAS systems, may constitute a risk of SGPV infection despite treatment of incoming water. Another possible source of infection in these enclosed systems is vertical transmission. Horizontal transmission has been shown to occur in several studies (Nolsøe et al., 2015; Wiik-Nielsen et al., 2017), however the question of whether vertical transmission is possible has not yet been definitively answered. A study in 2020 investigated two suspected cases of vertical transmission of SGPV, where eggs from infected broodstock started to display clinical signs of infection in the fry stage on two separate sites after hatching (Gulla et al., 2020). However, Gulla and colleagues established through sequencing that the variants of SGPV in the fry and the broodstock were not the same, and concluded that it was not a case of vertical transmission but infection with SGPV from another source.

2.1.2 Clinical presentation and mortality

Another challenge of SGPV is the marked variation in clinical presentation in infected fish, which can range from severe clinical signs and related mortality of up to 70%, to no clinical signs of infection at all (Nylund et al., 2008; Gjessing et al., 2017). In fact many SGPV infections remain entirely subclinical (Amundsen et al., 2021; Thoen et al., 2020; Tartor et al., 2022). When present, clinical signs typically include lethargy, loss of appetite, erratic swimming and crowding at the bottom of the tank (Gjessing et al., 2017; Wiik-Nielsen et al., 2017; Gjessing, Christensen, et al., 2018). Later in the infection, high positioning in the water column and general respiratory distress, or gaping, is often observed (Gjessing et al., 2016; Wiik-Nielsen et al., 2017; Gjessing

et al., 2019). Mortality rate of between 2-70% have been reported when disease outbreaks do occur (Gjessing et al., 2017). More specific to SGPV perhaps in the “live rigor” widely reported in disease outbreaks in Norway, where the fish seize up immediately prior to mortality (Thoen et al., 2020; Tartor et al., 2022).

In addition to clinical signs, SGPV infection is generally confirmed by qPCR (Gjessing et al., 2015). High viral load correlates with the most severe clinical presentation of disease (Garseth et al., 2018), while viral load remains relatively low in subclinical infections (Gjessing et al., 2015). Prevalence of SGPV infection has been reported as extremely high, roughly 80% (Wiik-Nielsen et al., 2017; Garseth et al., 2018) in studies conducted in RAS systems, and disease outbreaks are reported to be very synchronised in fish within a system (Tartor et al., 2022). The vast variation in clinical presentation and the triggers for these fast and severe disease outbreaks are crucial questions to address if we are to mitigate the threat that SGPV poses to the production industry.

2.1.3 Potential triggers of disease outbreak

The widespread presence of sub-clinical SGPV infection, with only intermittent but severe disease outbreaks, suggests that some external factors may act as triggers for disease outbreak in infected fish. Stress has been shown to cause disease in sub-clinically infected fish (Thoen et al., 2020), however which stressors cause this escalation and under what circumstances are still poorly understood.

One potential stressor could be the presence of other pathogens. Infections involving SGPV are commonly multi-factorial, often referred to by the umbrella term complex gill disease (CGD). Gills come into direct contact with the external environment and, as a result, are highly vulnerable to water-borne pathogens. Many gill-related diseases are caused by a combination of aquatic pathogens. Biological agents isolated from fish diagnosed with CGD include bacteria, parasites, plankton and viruses, including SGPV (Herrero et al., 2018). The role of SGPV in cases of CGD is not yet well understood, but it has been shown that the physical gill damage caused by SGPV can leave fish vulnerable to secondary infections (Gjessing et al., 2017). Additionally, a study on the immune response to SGPV infection and stress found that SGPV infection may also suppress aspects of the immune response, which could lead to infection with opportunistic secondary pathogens (Amundsen et al., 2021). Alternatively, infection with other pathogens or the resulting stress may be the triggers that cause sub-clinical SGPV infections to evolve into

disease outbreaks (Tartor et al., 2022). The role of SGPV and any related immuno-suppression in these multi-factorial infections is certainly an important question to answer.

Other stressors that may affect sub-clinically infected fish are environmental parameters. In enclosed RAS systems, like the one in this study, water quality plays a key role in fish welfare and disease prevention (Yavuzcan Yildiz et al., 2017). Some of the key parameters that require continuous monitoring and control include water temperature, dissolved oxygen, pH, carbon dioxide and salinity, all of which directly influence fish metabolism, respiration and osmoregulation (Ytrestøyl et al., 2020; Lindholm-Lehto, 2023). Equally important are factors that have indirect effects on the salmon such as nitrogenous waste like ammonia, nitrite and nitrate which are produced and can lead to toxicity if not controlled (van Rijn, 2013). Ionic composition is also critical, with calcium and magnesium, together with overall water hardness, playing key roles in osmoregulation, bone development and gill health (Fivelstad et al., 2003; Kroglund et al., 2008). As many of these parameters are strongly interrelated, effective RAS operation relies on the close monitoring and precise control of water quality to maintain the health and welfare of the fish.

Although the interactions between environmental factors and SGPV are not yet elucidated, we know that water chemistry can impact a fish's response to pathogens and infections, and can be a source of stress to the fish. For example, outbreaks of Koi sleepy disease (KSD), caused by the poxvirus CEV, have been shown to be related to water temperature (Miyazaki et al., 2005; Way et al., 2017). Similarly, salinity has been shown to affect the severity of disease in cases of infectious haematopoietic necrosis disease, caused by infectious haematopoietic necrosis virus (IHNV) in Atlantic salmon (Lapatra, 1998). Which parameters affect SGPV infection and what combination of these parameters pose the highest risk of mortality is certainly an area that requires more study.

The research reported in this chapter aimed to investigate the impact of environmental factors with respect to a natural SGPVD outbreak, in particular looking to better understand what farming conditions might be associated with disease outbreaks when combined with SGPV infection. This was achieved by following two comparable cohorts of fish, considered from site history to be at risk of developing SGPVD, through freshwater production. To this end, the study involved monitoring of viral infection and recording of clinical signs, mortality and environmental factors through an outbreak of SGPVD.

2.2 Material and Methods

2.2.1 Ethical statement

Animal handling and collection in this study was carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 Amended Regulations (SI 2012/3039) and the work was approved by the University of Stirling Ethics Committee (Animal Welfare and Ethics Review Board) (AWERB 18 19 034 Non-ASPA). All animals were euthanised according to schedule 1 procedure.

2.2.2 Site location and experimental design

This study was performed on a commercial freshwater Atlantic salmon smolt production site located in central Scotland with a recurrent history of SGPV and associated disease outbreaks. Farm A (site name and location have been anonymised), has two large freshwater recirculation systems on-site, the C system and the G system. These two systems are fully separate, located in different buildings and with strict biosecurity measures at each, but share a common water source, fry source and husbandry practices by the shared staff.

The study began in October 2018 when a new intake of S0 (out of season) Atlantic salmon fry of the same batch were stocked from a hatchery into both systems on Farm A and continued over a four month period. The G system has a substantial and more recent history of SGPV infection and had experienced a disease outbreak during the previous cycle, while the C system had experienced clinical SGPV disease in the past but not in the three years prior to this study. These systems were on the same site but in different buildings as the C system building has been converted from a hatchery, however both systems share all husbandry practices and water sources. One tank of fish was selected from each system for the duration of the study, with the aim of sampling the same stock consistently, hereby referred to as the G tank and the C tank. These tanks were chosen as they contained fry from the same source (stock and location) and were not mixed with other stock throughout the study, as other tanks in the G system were mixed with incoming stock from a different hatchery soon after stocking. The fish sampled from C system were in a tank of 200,000 fish, while the fish from G system had to be separated into a smaller grading tank after the first fortnight of the study (80,000 fish) in order to ensure the fish sampled were not mixed with fry from other sources.

2.2.3 Clinical signs and mortality

Clinical signs of disease observed were reported by onsite staff. Mortality in each of the experimental tanks was recorded daily by staff by counting fish collected in the out box. The percentage mortality per day was then calculated compared with the initial population size in each tank.

2.2.4 Water chemistry

Water chemistry conditions in each of the experimental tank were measured and recorded daily by on-site staff as part of routine monitoring. Conditions recorded include incoming water temperature, tank water temperature, pH, alkalinity ($\text{HCO}_3^- - \text{CO}_3^{2-}$), unionised ammonia (NH_3), total ammonia nitrogen (TAN), calcium, CO_2 , hardness (Ca–Mg), magnesium, nitrite (NO_2^-), nitrate (NO_3^-), and salinity. Similarly, the amounts of bi-carbonate and salt added to regulate water chemistry were recorded.

2.2.5 Biofilter testing

The biofilters from each system (G and C) were tested daily for SGPV as part of routine on-site surveillance. Swabs were taken from the biofilters of each unit daily and qPCR was performed on-site for the presence of SGPV using the primers in Table 2.2.

2.2.6 Sampling

Epitools (<https://epitools.ausvet.com.au>; Sergeant, 2018) was used to calculate a sample size that would be representative of the population being studied, a conservative estimate of 20% prevalence, perfect test sensitivity and specificity was presumed and the larger tank size of 200,000 fish was used. Based on this calculation, a total of 14 fish were sampled from each tank every two weeks, with this increasing to twice-weekly for two weeks once any clinical signs (or an ‘outbreak’) were observed in the fish (appendix 5.1). The fish sampled and sample types collected at each timepoint are summarised in Tables 2.1 and 5.2.

Fish were selected at random by netting and euthanised with an overdose of MS-222 (500 ppm, PHARMAQ, Germany). Following euthanasia, samples were collected from 14 fish from each tank (appendix 5.1). The second left gill arch of each fish was taken and stored in 1 ml of RNAlater (0.45 M ammonium sulphate, 2 mM EDTA, and 25 mM sodium citrate, pH 5.2) at 2–8 °C for 24 hours before being transferred to -70 °C for long term storage before processing.

RNAlater was chosen as a preservative as it has been shown to effectively preserve both DNA and RNA and allow for the later extraction of high-quality nucleic acids (Wang et al., 2018).

Table 2.1: Samples used for the detection and quantification of SGPV and screening for background infections

Date	Tank	Gill tissue- DNA	Gill tissue- RNA
23.10.18	G	14	2
01.11.18	G & C	14 & 14	2 & 2
05.11.18	G & C	14 & 14	2 & 2
15.11.18	G & C	14 & 14	2 & 2
19.11.18	G & C	14 & 14	2 & 2
22.11.18	G & C	14 & 14	2 & 2
26.11.18	G & C	14 & 14	2 & 2
03.12.18	G & C	14 & 14	2 & 2
17.12.18	G & C	14 & 14	2 & 2
02.01.19	G & C	14 & 14	2 & 2
14.01.19	G & C	14 & 14	2 & 2
28.01.19	G & C	14 & 14	2 & 2
11.02.19	G & C	14 & 14	2 & 2
25.02.19	G	14	2

2.2.7 DNA extraction from gill tissue

As SGPV is a DNA virus, DNA was extracted from gill tissue to detect and quantify SGPV load. DNA extraction was performed on all gill tissue samples using the DNeasy Blood and Tissue kit (Qiagen, The Netherlands) as per the manufacturer's instructions. DNA quantity and purity were determined using the NanoDrop spectrophotometer (Thermo Fisher Scientific, USA), and DNA integrity was assessed by electrophoresis using 250 ng DNA in a 1% agarose gel stained with ethidium bromide before being stored at -20 °C.

2.2.8 Detection and quantification of SGPV

A quantitative PCR (qPCR) protocol to detect SGPV was established in the lab using the primers and probe previously published by Gjessing et al., 2015 targeting a homolog for the D13L open reading frame (ORF) in VACV (Table 2.2). A plasmid was designed and ordered using Invitrogen Life Technologies online tool with a pMA-T vector backbone containing the amplicon these primers select for (101 bp). The coiled plasmid (2475 bp) was then linearised using a *PvuII* restriction enzyme (Thermo Fisher Scientific, USA) and run alongside undigested plasmid in a 1% agarose gel to confirm it's identity. The band of linear plasmid was then extracted as per the manufacturer's instructions (Monarch DNA Gel Extraction Kit, New England Biolabs, USA) and

the DNA quantified by Qubit fluorometer (Invitrogen, USA), before being diluted appropriately for use as qPCR standards (10^7 - 10^1 DNA molecules/ μ l). qPCR reactions were run in duplicate on a Lightcycler 480 (Roche, Switzerland) using the Lightcycler 480 Probes Master kit (Roche, Switzerland) on 96-well plates (Roche, Switzerland) with a final concentration of 500 nM for both primers, 200 nM probe, labelled with 6-carboxyfluorescein and a minor groove binding nonfluorescence quencher (MGBNFQ), and 200 ng of template DNA in a total reaction volume of 20 μ l at the program listed in Table 2.3.

Table 2.2: Primers and probe used for detection and quantification of SGPV by qPCR (Gjessing et al., 2015).

Primer	Sequence (5'-3')	T_m	Product size (bp)
Forward	ATCCAAAATACGGAACATAAGCAAT	53.6 °C	106
Reverse	CAACGACAAGGAGATCAACGC	56.8 °C	
Probe	CTCAGAAACTTCAAAGGA	45.9 °C	

Table 2.3: The qPCR conditions for the detection and quantification of SGPV

Step	Temperature	Time	Cycles
Pre incubation	95 °C	10 min	1
Denaturation	94 °C	15 sec	40
Annealing	55 °C	30 sec	
Extension	72 °C	15 sec	
Melt curve	95 °C	10 sec	1
	60 °C	30 sec	
	95 °C	30 sec	
Cooling	40 °C	10 sec	1
Storage/holding	15 °C	∞	

2.2.9 Confirmation of SGPV presence

A series of primer sets to detect SGPV were designed using the SGPV complete genome on NCBI Genbank (NC_027707.1). Primers were designed to target highly conserved regions of the viral DNA including capsid proteins and two polymerase subunits, subunit 35 and subunit 18, using the Primer3 v2.6.1 and Primer3Plus online primer design tool, v3.3.0 (Koressaar & Remm, 2007; Untergasser et al., 2012). Seven sets of primers were designed – four targeting a major capsid protein, including two semi-nested, two targeting the polymerase subunit 35, one of which was semi-nested, and one targeting the polymerase subunit 18. These primers were used for PCR on 5 samples from Farm A which had very high levels of SGPV as per the qPCR in 2.2.8.

Table 2.4: Primer sets designed on Primer 3 targeting conserved regions of the published SGPV genome to confirm SGPV presence in the study samples

Set	Target gene	Primer name	Sequence (5'→3')	Annealing temperature	Product size (bp)
1	Capsid protein	SGPV FP1 SGPV RP1	TATCGAGGTCACGGTGATGA CCCTCATCGGACTTTGTGTT	55 °C	729
2	Capsid protein	SGPV FP2 SGPV RP1	AGCCTGTTCTGGGAATTGTG CCCTCATCGGACTTTGTGTT	55 °C	619
3	Capsid protein	SGPV FP3 SGPV RP3	ATGAAGGCCACGAAACAATC TCATCACCGTGACCTCGATA	54 °C	454
4	Capsid protein	SGPV FP4 SGPV RP1	GAGAGTCACCCGACGTCAAT CCCTCATCGGACTTTGTGTT	56 °C	340
5	Polymerase 35	SGPV plm35 FP1 SGPV plm35 RP1	GATGACGTGCGAGTGTTTTG TGAAACATGGCCATCAGAAA	53 °C	522
6	Polymerase 35	SGPV plm35 FP2 SGPV plm35 RP1	TCCGGAAACTGATCTGGAAG TGAAACATGGCCATCAGAAA	53 °C	409
7	Polymerase 18	SGPV plm18 FP1 SGPV plm18 RP1	GCTGCTTTGAAGCTAGCAGA GGTCCGGTCCTAATTCGTGT	56 °C	223

2.2.10 Vertical transmission

To test for vertical transmission, 59 eyed eggs from SGPV positive broodstock were obtained from a second salmon production company experiencing an SGPVD outbreak. The female and male broodstock were confirmed to be infected with SGPV by qPCR of gill tissue onsite at the hatchery. The ova were stripped from one female and fertilised with milt from two males onsite before being sampled at 257 degree-days (DD) and stored in absolute ethanol at 2-8 °C for processing.

DNA was extracted using an adapted HotShot protocol (Howard et al., 2023). In brief, eggs were individually soaked in a Tris-HCL buffer (1 M, pH 8) for 15 minutes to soften them before being removed, blotted dry, and placed into 2 ml screw cap tubes (Alpha Laboratories, UK). A sterile needle was then used to burst and disrupt the egg tissue as much as possible before 400 µl of alkaline lysis reagent (25 mM NaOH and 0.2 mM EDTA, pH 12) was added to each tube. Tubes were then inverted to mix and incubated for 30 minutes at 90 °C before being removed and placed on ice for 5 minutes. 400 µl of neutralisation buffer (Tris-HCL, pH 5) was then added, samples were centrifuged for 5 minutes at 20,000 *g* before the supernatant containing the DNA was removed into clean 1.5 ml tubes and stored at -20 °C.

The samples were then analysed in duplicate 50 µl reactions containing 25 µl MyTaq mix (BioLone, UK), 1 µl each of the forward and reverse primers previously described (Table 2.2), 200 ng DNA and nuclease-free water. PCR was carried out using the following conditions (Table 2.5).

Table 2.5: PCR conditions for the detection of SGPV

Step	Temperature	Time	Cycles
Initial denaturation	95 °C	1 min	1
Denaturation	95 °C	15 sec	35
Annealing	55 °C	15 sec	
Extension	72 °C	10 sec	
Final extension	72 °C	5 min	1
Storage/holding	15 °C	∞	1

2.2.11 RNA extraction from gill tissue and cDNA synthesis

To test for RNA viruses, total RNA was extracted from gill tissue using TRI-reagent (Sigma-Aldrich, USA) according to the methods of Chomczynski (Chomczynski, 1993) with minor modifications. A section of 100 mg of gill tissue from the second left gill arch was homogenised in 1 ml of TRI-reagent using a Mini-Beadbeater 24 (Biospec, USA) and total RNA was extracted using the method published by Chomczynski and colleagues (1993). After phase separation, the aqueous phase was transferred to a new tube and RNA was precipitated using 0.5 volume RNA precipitation solution (1.2 M NaCl, 0.8 M sodium citrate sesquihydrate) and 0.5 volume of 100% isopropanol. After incubation at room temperature (RT) for 10 minutes, samples were centrifuged at 20,000 g, 4 °C for a further 10 minutes before the supernatant was removed and the RNA pellet was washed with 75% ethanol. Total RNA was then resolubilised in 100 μ l of nuclease-free water. Total RNA quantity and purity was determined using the NanoDrop spectrophotometer (Thermo Fisher Scientific, USA), and RNA integrity was assessed by electrophoresis using 250 ng RNA in a 1% agarose gel stained with ethidium bromide. Total RNA was then stored at -70 °C.

cDNA was synthesised from 2 μ g total RNA extracted from gills using the SuperScript III kit (Invitrogen, USA) as per the manufacturer's instructions. Briefly, 1 μ l oligo (dT) (50 μ M) and 1 μ l of dNTP (10 μ M) were added to the total RNA and nuclease-free water to make reaction volumes of 10 μ l. Samples were then incubated at 65 °C for 5 minutes, then on ice for a further 1 minute. Then 10 μ l of the cDNA synthesis mix (2 μ l of 10X RT buffer, 4 μ l 25 mM MgCl₂, 2 μ l 0.1 MDTT, 1 μ l RNaseOUT and 1 μ l SuperScript III RT enzyme) was added to each sample and mixed. Samples were then incubated at 50 °C for 50 minutes, then at 85 °C for 5 minutes to terminate the reaction. Finally, 1 μ l of RNase H was added to each sample before cDNA samples were incubated at 37 °C for 20 minutes then stored at -20 °C.

2.2.12 Background infections

Two fish from each group at each sampling point were tested for concurrent viral infection of salmonid alphavirus (SAV), the causative agent of pancreas disease (PD), piscine orthoreovirus (PRV) which causes heart and skeletal muscle inflammation (HSMI) and piscine myocarditis virus (PMCV) which causes cardiomyopathy syndrome (CMS) in Atlantic salmon.

qPCRs were performed on the synthesised cDNA using Luminaris HiGreen qPCR mastermix (Thermo Fisher Scientific, USA) in duplicate on 96-well plates in a LightCycler 480 (Roche). The primers, designed by (McGowan. M et al., 2018) were used (Table 2.6). Standards were generated by synthesising plasmids containing an insert that corresponded to the primer set used, using the same method described in section 2.2.8 (Table 2.6).

Table 2.6: Primer sets used for detection and quantification of piscine orthoreovirus (PRV), piscine myocarditis virus (PMCV) and salmonid alphavirus (SAV) (McGowan. M et al., 2018)

Virus	Primer	Sequence (5'-3')	T_m	Product size (bp)
PRV	FP2	GGTTCAAACGACAGACCAGACAG	61 °C	78
	RP2	CATTATGCCACGCATATCGTCTC		
PMCV	FP2	GACCAACCCAGAACCAGCG	61 °C	82
	RP	TCAACACCCATCTGTCTTTGGTATG		
SAV	UP2	AAGAAATGTACCAGATTTTCCACTAC	60 °C	106
	RP4	GCATGTTTRCGACGGTGYTAATCTCTAC		

2.2.13 Machine learning approach

WEKA (Waikato Environment for Knowledge Analysis, v3.8.6; Witten et al., 2011), an open source machine learning workbench software, was used to assess association of different water quality parameters with mortality of fish in the tanks. Using the DecisionTable classifier (Kohavi, 1995), pH, tank water temperature and CO₂ were identified as the most significant predictors of mortality in the dataset. A training dataset was then generated using all combinations of these parameters to identify patterns in order to build a model. This model was then applied to the data using mortality as the outcome variable and pH, temperature and CO₂ as attributes to generate predictions for each combination of attributes.

2.2.14 Statistical analysis

As this study investigated a naturally occurring outbreak rather than experimentally manipulated environmental conditions, statistical correlation analysis was used to explore associations between

environmental parameters and disease metrics. The Shapiro-Wilk test was used to determine the normality of all data. To assess the statistical significance of the relationships between mortality and water chemistry parameters, SGPV level and SAV level, Spearman's test of correlation with Bonferroni's correction for multiple tests was used. The statistical difference between tanks was measured using the Mann-Whitney test. P-values ≤ 0.05 were considered significant. All statistical analysis was done using R v4.3.0 (R Core Team, 2023).

2.3 Results

2.3.1 Clinical presentation

Clinical signs of SGPVD were observed by on-site staff in the G tank within 3 days of stocking in October 2018. Signs noted in the G tank included abnormal swimming patterns, loss of appetite, lethargy, and signs of general respiratory distress like gulping and elevated positioning in the water column, particularly in the first 2 weeks of the study. Additionally, a condition referred to as “live rigor”, a tonic seizure and resulting immobility of the fish, was observed in diseased fish immediately prior to mortality. This tonic state in the late stages of infections has been widely reported anecdotally during SGPVD outbreaks in Scotland. No clinical signs were observed in the fish in the C tank throughout the study period.

2.3.2 Mortality

The mortality rate in the G tank was consistently and significantly higher than in the C tank ($P\text{-value} = 1.87 \cdot 10^{-10}$), which mirrored clinical signs observed, and viral load detected in the fish (Figure 2.1). In the G tank, two notable peaks in mortality rate were recorded, the first smaller peak (0.62% mortality) in mid-November and the second, much larger peak (4.5%) in mid-December. In the C tank, mortality remained near zero throughout the study period.

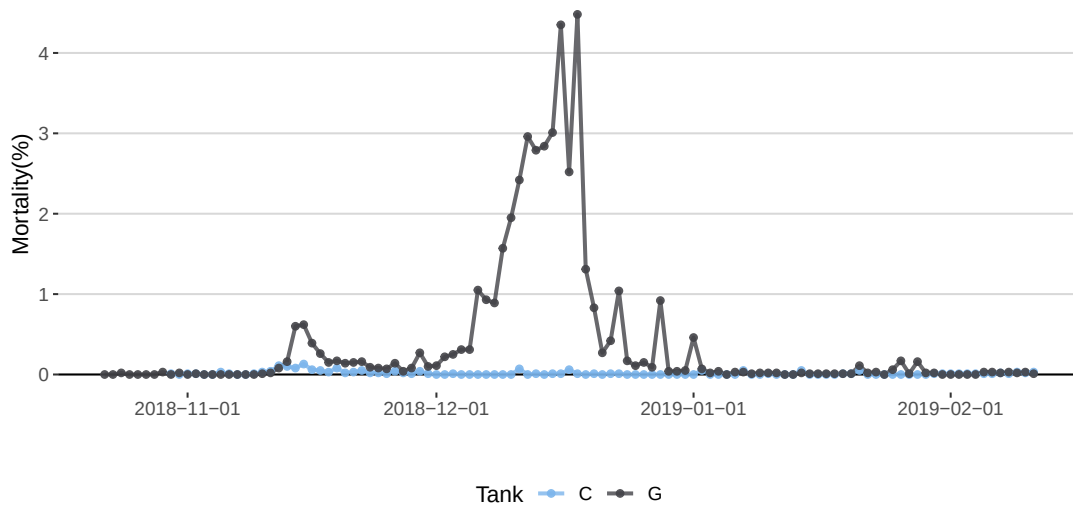


Figure 2.1: Percentage daily mortality recorded in the G tank and the C tank over the six-month study period

2.3.3 Detection and quantification of SGPV

Once the presence of SGPV was confirmed in the samples taken from Farm A, qPCR was done to quantify viral levels in each sample using the primers and method published by Gjessing and colleagues (Gjessing et al., 2015). Samples from both the G and the C tanks tested positive for SGPV, despite only the fish in the G tank displaying clinical signs of disease (Figure 2.2). The viral level peaked in both tanks on the November the 15th sampling point. This peak in viral level in G coincides strongly with the first, smaller peak in mortality rate, in addition to the clinical signs observed (Figure 2.3). However no significant relationship was found between SGPV level and mortality rate ($P\text{-value} = 0.11$). While detected in both tanks, the SGPV level in the G tank was much higher and fluctuated more than the viral level in the C tank, which remained low and consistent throughout the study period (Figure 2.3). The variation in viral load between tanks was at its greatest during the first month post stocking, mirroring the difference observed in clinical presentation.

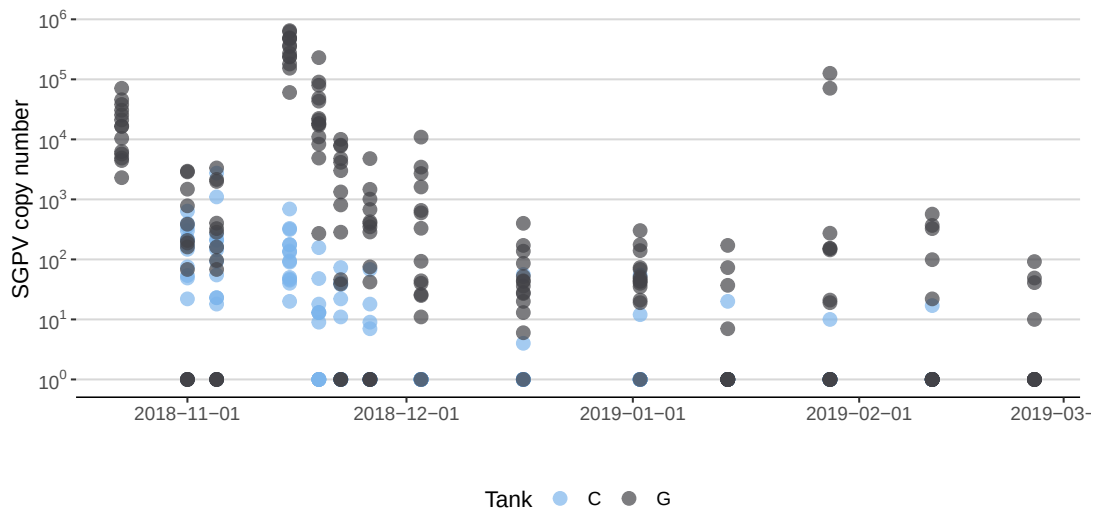


Figure 2.2: Viral load of SGPV detected in each of the 14 fish sampled from each tank at each sampling point

2.3.4 Confirmation of SGPV PCR

As many of the clinical signs observed in this study were not necessarily specific to SGPVD, and the "hallmark" apoptosis was widely missing, it was confirmed that the virus found matched the published SGPV genome (Gjessing et al., 2015). To do this, we designed seven new sets of primers. Highly conserved regions of the viral genome were targeted, four sets were designed to target a major capsid protein and three sets were designed (Table 2.4 to target two polymerases

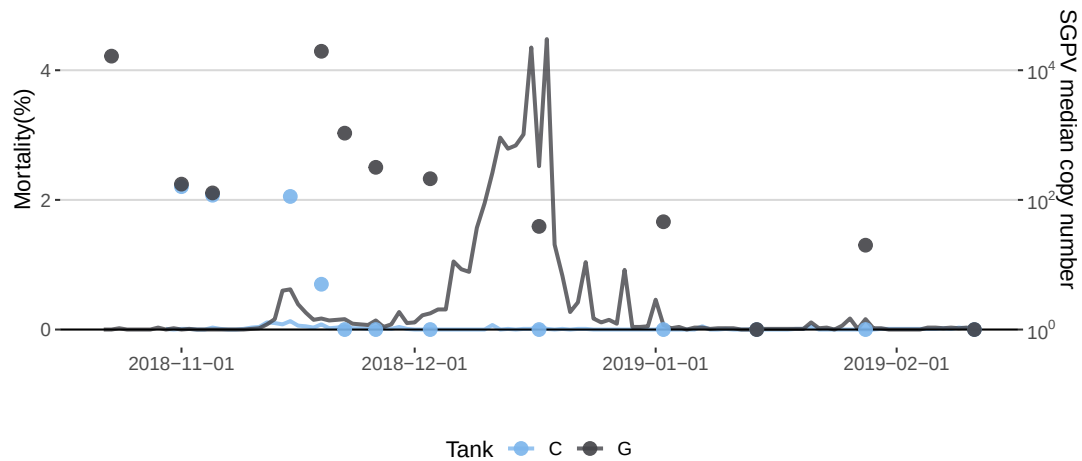


Figure 2.3: Percentage daily mortality (solid lines) and median level of SGPV (●) in each tank for the duration of the study period

present in the published SGPV genome (Gjessing et al., 2015)). Five samples with high SGPV load were then tested using all seven primer sets to confirm SGPV presence. All seven primers sets successfully produced the expected size amplicon in all samples, which was corroborated by electrophoresis, suggesting the presence of SGPV. As these results were fully concordant with the quantitative PCR data and served solely as confirmatory evidence, representative gel images are not shown.

2.3.5 Background infections

No samples were positive for PRV or PMCV in this study. However, SAV was found to be present in fish in both tanks throughout the course of the study (Figure 2.4). While copy numbers in both tanks were comparable for the first two weeks of the study, levels in the C tank then dropped and, although following a similar pattern to the G tank, remained comparatively low throughout the studied period. In the G tank however, copy numbers rose steadily from mid-November and peaked on the 3rd December, then dropped slightly but remained relatively high for the remainder of the study when compared with the C tank. This correlates with clinical presentation observed on Farm A, where an outbreak of PD was confirmed in the G unit in November 2018 and also coincides with the second larger peak in mortality recorded in the G tank in mid-December.

Though SAV was present in the C tank, no clinical disease was observed. The relationship between SAV level and mortality was not significant ($P\text{-value} = 0.32$). When SAV load is

compared with the SGPV load (Figure 2.5) and daily mortality rate in both tanks, there appear to be some interaction. Note that SGPV copy numbers in the G tank peaked on the 15th of November in unison with a small peak in mortality (Figure 2.3), SAV copy numbers peak shortly after on 3rd December and then the mortality rate rises sharply and peaks on the 16th and the 18th of December. The relationship between SGPV and SAV level was not statistically significant (P-value = 0.07).

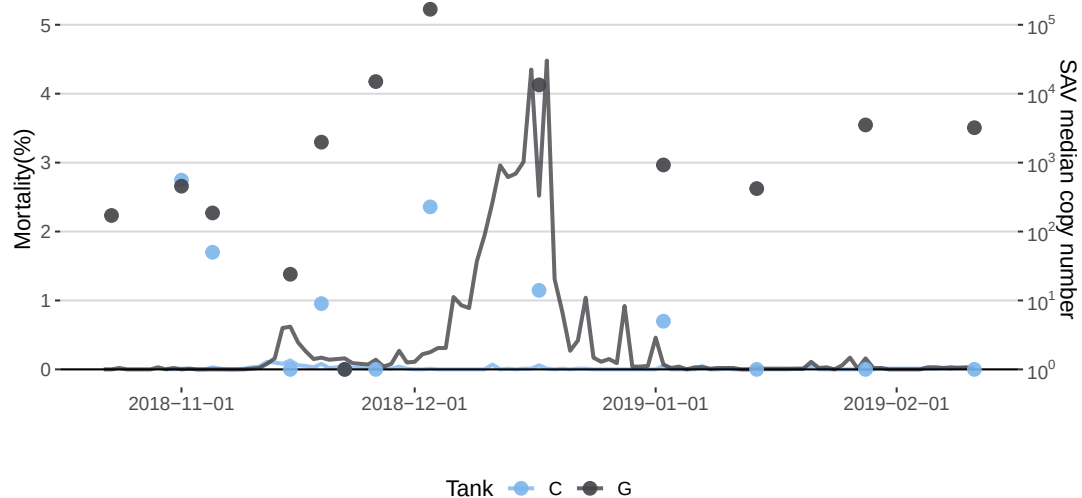


Figure 2.4: Percentage daily mortality (solid lines) and median level of SAV (●) in each tank for the duration of the study period

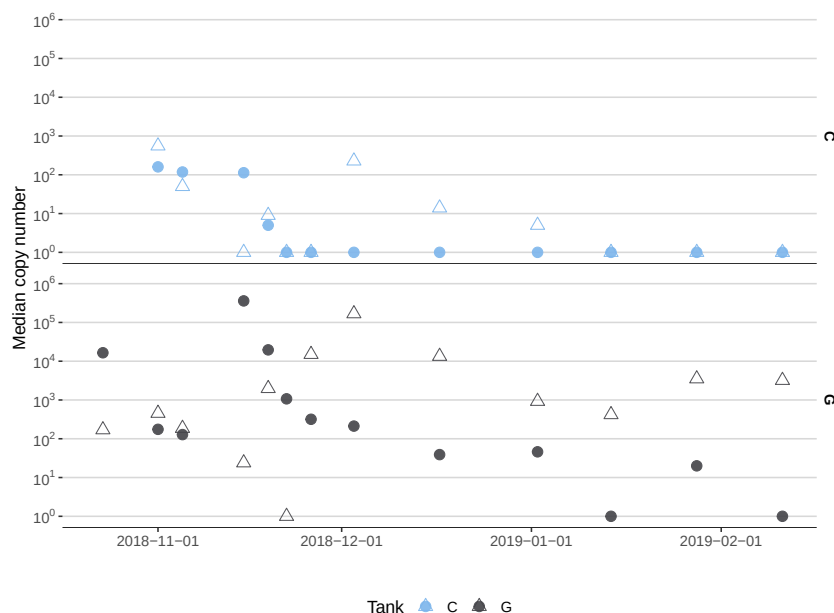


Figure 2.5: Median SGPV (○) and SAV (△) copy numbers detected in tank G and tank C at each sampling point during the study period

2.3.6 Biofilter qPCR

Very low levels of SGPV, bordering on the limits of detection, were found intermittently in the G system biofilter, the system in which clinical disease was present in the fish (Figure 2.6). No SGPV was detected in the C system biofilter, where fish never displayed any clinical signs. Although viral levels detected in the G system did increase slightly in mid-November, coinciding with the peak of SGPV detected in the fish in the G tank and the smaller mortality peak, the copy numbers detected in the biofilter were always much lower than in the fish, on the threshold of detection.

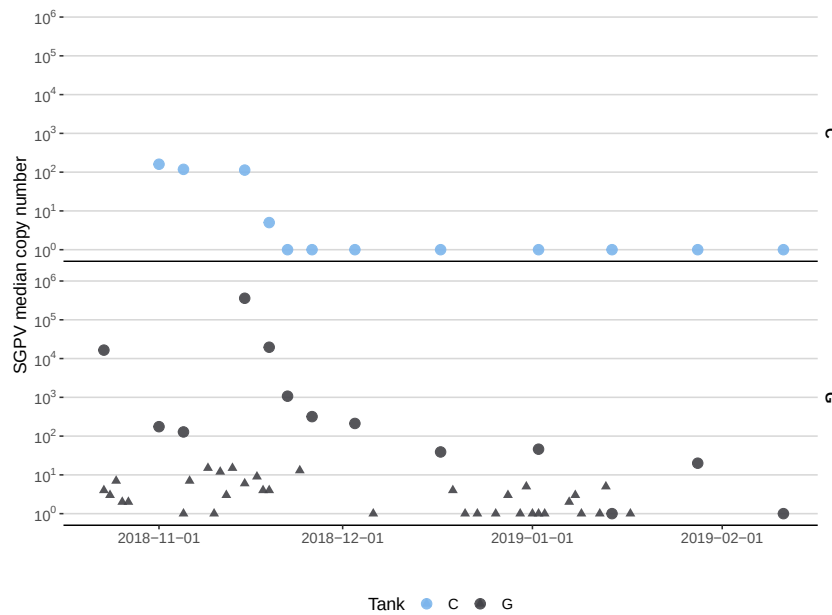


Figure 2.6: Viral load of SGPV found in the biofilters of each tank (Δ) by on-site testing compared with the SGPV load in the fish sampled ($\bigcirc$) at each sampling point

2.3.7 Egg PCR

To test for vertical transmission, DNA was extracted from 59 eyed eggs from naturally infected broodstock and tested for SGPV. Standard PCR was done using the primers previously described and the SGPV positive control developed in section 2.2.8 (Table 2.2). Standard PCR was used as the high level of lipids in DNA extracted from eggs using HotShot methods can interfere with fluorescence based techniques like qPCR. No SGPV was found in any of the eggs tested.

2.3.8 Water chemistry

The main environmental variables were generally comparable between the two systems throughout the study period (Table 2.7). Tanks C and G have similar water quality values with large standard deviations, however there was a significant difference between tanks in calcium (P-value = $3.60 \cdot 10^{-3}$), CO₂ (P-value = $2.55 \cdot 10^{-14}$), water hardness (P-value = $5.82 \cdot 10^{-3}$) and TAN (P-value = $8.95 \cdot 10^{-3}$), in which the G tank values were greater than the C tank, and in magnesium (P-value = $3.60 \cdot 10^{-3}$) which was higher in the C tank.

Table 2.7: Summary of the water chemistry parameters recorded over the four month study period, the mean and standard deviation in each tank as well as the ideal RAS parameters where appropriate (Timmons et al., 2018). For each value, mean and standard deviation (in brackets) are presented.

	G Tank	C Tank	Ideal
Incoming water temp. (°C)	6.11 (2.25)	5.90 (2.16)	
Tank water temp. (°C)	12.60 (1.15)	12.52 (0.94)	10-15
pH	7.30 (0.17)	7.34 (0.20)	6.5-8.5
Total alkalinity (mg/l)	66.87 (17.00)	63.81 (17.67)	50-200
Ammonia (NH ₃) (mg/l)	0.002 (0.001)	0.002 (0.001)	0.00125-0.03
Calcium hardness (mg CaCO ₃ /l)	13.50 (19.69)	0.24 (19.85)	
CO ₂ (mg/l)	6.03 (1.52)	4.37 (1.16)	<20
Total hardness (mg CaCO ₃ /l)	181.86 (37.67)	167.17 (90.94)	>50
Magnesium (mg/l)	13.50 (19.69)	20.24 (19.85)	
Nitrate (mg/l)	46.79 (14.97)	36.43 (11.24)	<100
Nitrite (mg/l)	0.06 (0.02)	0.06 (0.02)	<1.0
Salinity (‰)	1.96 (0.32)	1.90 (0.28)	2.0-3.0
TAN (mg/l)	0.40 (0.20)	0.35 (0.22)	<1.0
Added Bi-carbonate (kg)	72.57 (33.91)	75.24 (30.96)	
Added Salt (kg)	292.04 (330.77)	352.38 (337.53)	

A significant relationship was found between three of the environmental parameters and mortality rate in the G tank, and one in the C tank (Table 2.8). In the clinically affected G tank the TAN (Appendices Figure 5.3), CO₂ level (Appendices figure 5.4) and water hardness (Appendices figure 5.5) showed a significant correlation with mortality rate.

Table 2.8: Significance of the correlation between mortality and incoming water temperature, tank water temperature, pH, alkalinity, ammonia, bi-carbonate input, calcium, CO₂, water hardness, magnesium, nitrate, nitrite, salinity, salt input, and total ammonia (TAN), including 0-7 days offset to allow for potential delayed interactions. The correlation was determined using Spearman's test of correlation and Bonferroni's correction for multiple tests. Statistically significant P-values are indicated with a bullet point (●). Detailed P-values in appendix 5.3.

Days offset	C ₀	G ₀	C ₁	G ₁	C ₂	G ₂	C ₃	G ₃	C ₄	G ₄	C ₅	G ₅	C ₆	G ₆	C ₇	G ₇
Incoming water temp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tank water temp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Total Alkalinity	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ammonia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Calcium hardness	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CO ₂	●	●	-	●	●	●	●	●	●	●	-	●	-	●	-	●
Total Hardness	-	●	-	●	-	●	-	●	-	●	-	●	-	●	-	●
Magnesium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrite	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Salinity	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TAN	-	●	-	●	-	●	-	●	-	●	-	●	-	●	-	●
Added Bicarbonate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Added Salt	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

2.3.9 Machine learning analysis

Using a DecisionTable classifier (Kohavi, 1995), we identified pH, tank water temperature and CO₂ as the best predictors of mortality, with a correlation coefficient of 0.98. The predictions for each combination of these parameters were then calculated to identify the risk of mortality with each combination (Figure 2.7A) in this study. This analysis indicated that the highest risk of mortality was associated with a temperature of 12 °C when combined with a CO₂ level of 4 mg/l and a pH of 7.5 (Figures 2.7B, 2.7C and 2.7D). Using this data, the best combination of these three parameters to avoid mortality in the present study were also identified (Figure 5.6).

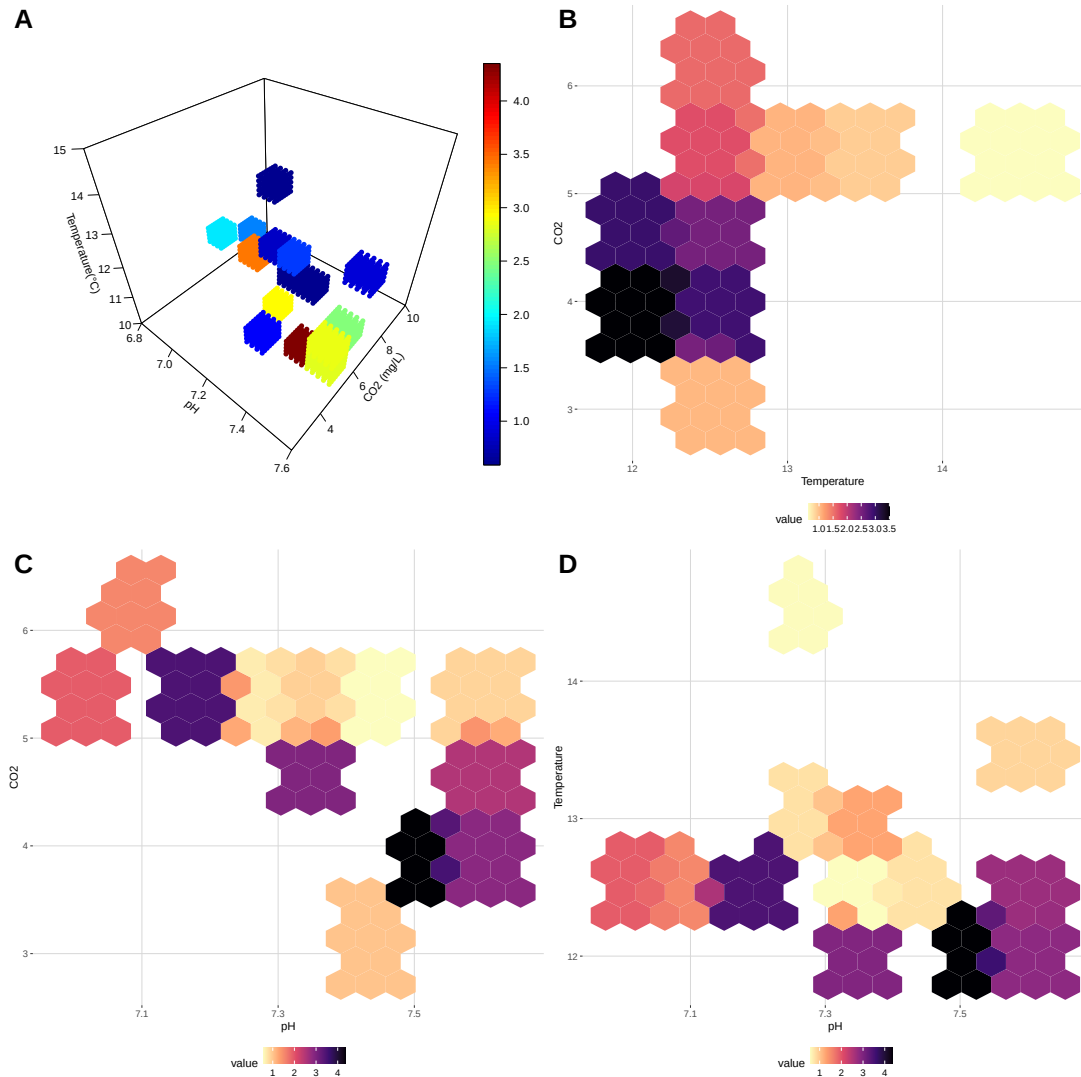


Figure 2.7: Combinations of pH, temperature and CO₂ predicted to be associated with high risk of mortality, with colour scales showing percentage related mortality. Only mortality >0.5% is shown. **A.** 3d visualisation. **B.** Combination of CO₂ and water temperature predicted to be associated with high risk of mortality. **C.** Combination of CO₂ and pH predicted to be associated with high risk of mortality. **D.** Combination of temperature and pH predicted to be associated with high risk of mortality.

2.4 Discussion

2.4.1 Source of infection

The source of SGPV in enclosed freshwater systems like Farm A has been under debate since the virus was first identified in freshwater systems (Nylund et al., 2008; Gjessing et al., 2015; Garseth et al., 2018). While evidence for horizontal transmission of the virus has been provided by several challenge experiments (Nolsøe et al., 2015; Gjessing et al., 2017; Wiik-Nielsen et al., 2017), clear evidence for vertical transmission is as yet lacking (Garseth et al., 2018). In this study, none of the eggs taken from SGPV positive broodstock tested positive for SGPV providing no evidence of vertical transmission. Similarly in a study by Gulla and colleagues (2020), where two groups of fry from the same SGPV infected broodstock experienced SGPVD outbreaks, analyses revealed that the variants of SGPV in the broodstock and the fry were not the same, therefore it was concluded that infection was not transmitted vertically.

Another potential source of infection in RAS systems is pathogens being brought in with water, fish or equipment (through failed biosecurity measures), persisting in the biofilters and reinfesting subsequent cycles of fish. It has been suggested that pathogens may accumulate more readily in the biofilters of RAS systems than in flow-through systems due to low dilution rate (Sharrer et al., 2005), which could explain the widespread and persistent presence of SGPV in enclosed RAS systems. Biofilters have been shown to accumulate bacteria (Bullock & Schill, 2006; Michaud et al., 2009) and viruses (Noble & Summerfelt, 1996; Christiansen et al., 2021). It has also been shown that enveloped viruses can persist in biofilms while retaining their infectivity (Moresco et al., 2022).

To test this, the biofilters in both the G and C systems were swabbed and tested for SGPV by on-site staff throughout the study period. The biofilter in the C system, in which the fish were sub-clinically infected with SGPV, tested negative consistently through the cycle. However, in the G systems, which did experience a disease outbreak, SGPV was found to be present in the biofilter at a consistent but very low level, peaking slightly several weeks into the study. However, it is important to note that the detection of SGPV by qPCR does not necessarily indicate the presence of infectious particles (Hamza et al., 2011). Moreover, the low but consistent detection of SGPV in the G system biofilter and the absence of SGPV in the biofilter of the C system does not necessarily mean that the biofilters are the source of infection, but merely indicate that the clinically infected fish are shedding viral particles that deposit in the biofilter. The coinciding

peaks in viral load in the G system biofilter and in the diseased fish would be in accordance with this interpretation.

2.4.2 Clinical presentation

Clinical signs observed in diseased fish in the G tank mirrored those typically described in SGPV outbreaks, such as lethargy, increased respiration, loss of appetite and abnormal positioning in the water column. In the later stages of infection, gaping at the surface of the tank and tonic seizure were also observed in the G tank, while no overt clinical signs were observed in the C tank. This general respiratory distress, poor food conversion rate and change in swimming patterns correlates with typical presentation reported during SGPVD outbreaks in both Scotland and Norway (Gjessing et al., 2017; Gjessing, Christensen, et al., 2018; Thoen et al., 2020), however many of these clinical signs have also been reported during PD outbreaks and so cannot be attributed directly to SGPV infection (McLoughlin et al., 2002). Conversely, the “live rigor” observed in the diseased fish immediately before mortality has not been reported during PD outbreaks but has been observed in other studies on SGPV (Gjessing et al., 2016; Thoen et al., 2020). The cause of this unusual presentation has not yet been identified, however Thoen and colleagues (2020) hypothesise that it is related to the respiratory distress observed in diseased fish. Respiratory distress has been shown to cause hypercapnia and acidosis in trout (Fievet et al., 1988), and respiratory acidosis is known to cause lactate build up and subsequent problems with metabolism in muscles (Cochran & Burnett, 1996), which may be causing this characteristic “live rigor”.

2.4.3 Co-infection

As this was a naturally occurring outbreak in a commercial system, the possibility of co-infections was considered. When samples taken for this study were screened, no PRV or PMCV was found in any fish sampled, however SAV was found in both the G tank and the C tank. Although SAV is a major viral disease in Atlantic salmon aquaculture, it is most commonly detected during the seawater phase of production and vaccination against SAV is usually not performed until close to seawater transfer (McLoughlin & Graham, 2007). Clinical signs of PD, caused by SAV, include many also observed during SGPVD outbreaks- lethargy and loss of appetite (McLoughlin et al., 2002). In the G tank which developed SGPVD, the SAV load was initially quite low after input, but then rose and peaked two weeks after the peak in SGPV. The fish in

this tank developed pancreas disease caused by the SAV, with associated mortality visible in the larger second mortality peak. In the C tank, which was sub-clinically infected with SGPV, a similar pattern of SAV viral load was seen, although the levels of both SAV and SGPV remained comparatively low. It is known that SAV induces a rapid IFN response during infection (Herath et al., 2012), however less is known about SAV as a secondary infection. In another study it was found that previous infection with PRV appeared to provide some protection against SAV (Røsæg et al., 2017), however that does not appear to be the case in this study, where the fish with SGPVD in tank G experienced more severe PD and associated mortality than the subclinical fish in tank C. Gjessing and colleagues (2017) hypothesise that SGPVD and the gill damage it causes may impair the immunity of infected fish, which would explain why the SAV load in the diseased G tank began to rise and cause PD in the weeks following the peak in SGPV infection. This is supported by another study in which the immune response to SGPV was quantified and found to be downregulated compared with healthy fish (Amundsen et al., 2021). Alternatively, it is possible that SGPV was acting as the opportunistic secondary pathogen in this case, as SAV was detected in the fish from the beginning of the study. In either case, the above observation correlates with the high occurrence of SGPV in multifactorial infections in many studies (Nylund et al., 2008; Gjessing et al., 2015; Wiik-Nielsen et al., 2017; Herrero et al., 2018; Østevik et al., 2022). However it is also important to note that only 2 fish from each sampling point were tested for SAV, and so viral load may not be representative of the viral load of the cohort as a whole.

2.4.4 Water chemistry

Another external factor that could contribute to or interact with SGPV infection is the water chemistry in the production system although it is difficult to distinguish which parameters influence disease progression and which parameters are influenced by the presence of diseased fish. The water chemistry in the two tanks was analysed with a view to identifying any variations that could account for the difference in presentation between the two tanks. The three factors that consistently had a significant correlation with mortality in the G tank were total ammonia nitrogen (TAN), water hardness (Ca-Mg) and CO₂. Total dissolved ammonia correlated strongly with mortality in the G tank, dropping initially after the peak in SGPV infection and related mortality, then slowly rising again later in the study. One likely explanation for this variation is the poor food conversion rate which is a result of the loss of appetite common in SGPVD infected fish leading to a reduction in TAN (Kajimura et al., 2004; Rubino et al., 2014). Additionally, drop

in TAN in tank water has been linked to increased susceptibility to disease in Atlantic salmon smolts (Ackerman et al., 2006).

The correlation between mortality and total water hardness in the G tank is not entirely surprising either. The significantly higher water hardness in the G tank is most likely a result of the disease in that tank compared to the clinically healthy C tank. In other gill disease outbreaks it has been shown that gill damage, particularly to the epithelial and chloride cells, can cause a reduction in ion uptake by the gills, leading to harder water than in tanks with healthy fish (Roberts & Powell, 2003; Zawisza et al., 2025).

In the diseased G tank, CO₂ levels rose substantially immediately prior to the SGPV related mortality peak, coinciding with the rise in viral load measured in the fish sampled from the same tank. While the CO₂ level in both tanks remained within even very conservative estimates of acceptable levels (<10 mg/l) (Fivelstad et al., 1998), increase in CO₂ levels in Atlantic salmon culture have been linked to increased blood cortisol levels (Fivelstad et al., 1999; Pirhonen & Schreck, 2003). As glucocorticoids like cortisol have been shown to downregulate the immune response (Espelid et al., 1996; Fast et al., 2008), this may explain why disease outbreak occurred in one tank but not in the other. Thoen et al., 2020, have shown that healthy fish exposed to SGPV and injected with cortisol developed clinical disease, while fish exposed to SGPV but not injected with cortisol did not display any clinical signs of disease.

This interaction between water parameters could have a substantial impact, as was shown using machine learning. Using this method, a combination of temperature, CO₂ and pH were identified as good predictors of mortality in this study. These water chemistry parameters are of course closely linked, with their correlation to mortality most likely indicating some degree of stress induced immunosuppression. Elevated tank water temperature has been shown to cause an increase in plasma cortisol and subsequently downregulation of the immune response (Downes et al., 2018; Herrero et al., 2018; Madaro et al., 2018), as has CO₂ as discussed above. This does not explain what causes SGPV infection to escalate into clinical disease, however it may identify controllable factors that may help limit mortality when SGPV infection occurs. While this model was developed using the relatively small dataset produced in this study, application of this approach applied to a much larger dataset gathered from multiple disease outbreaks on different sites could be extremely useful. Identifying specific combinations of environmental factors that are strong predictors of mortality would allow the appropriate management and mitigation needed to avoid mass mortality events that are so costly in the industry.

2.5 Conclusions

This study explored the environmental context of a natural outbreak of SGPV on a freshwater smolt production site in Scotland. While the source of infection could not be ascertained, we found no evidence for vertical transmission in this work. Although SGPV was found in one of the biofilters studied, it does not appear as though they were the source of infection in this case. Interestingly, we observed co-occurrence of SGPV and another pathogen, SAV. However, more research is needed to assess whether other pathogens, in this case SAV, may interact with SGPV to produce clinical disease, and to fully elucidate the nature of this interaction. While analysis of water quality parameters did not reveal a clear trigger of disease in this case, a combination of CO₂, temperature and pH were identified as good predictors of mortality in SGPV infections. This new insight could help to manage SGPV infections on production sites and to limit the resulting losses through mortality.

Chapter 3

Histological characterisation and immune response to SGPV infection

3.1 Introduction

Salmon gill poxvirus infection has been associated with severe gill pathology and, in some outbreaks, substantial mortality in Atlantic salmon (Nylund et al., 2008; Gjessing et al., 2017) since its initial identification in gill tissue from salmon affected by proliferative gill disease in Norway (Nylund et al., 2008), a growing body of work has described the histological features of SGPV infection and the associated host immune responses. SGPV is considered an epitheliotropic virus, replicating primarily in the respiratory epithelial cells of the gills, although it has also been detected in other epithelial tissues, including the skin and oral cavity (Gjessing, Christensen, et al., 2018). The presence of SGPV in multiple tissues raises the possibility of pathology beyond the gills, particularly in the context of frequent co-infections. As such, visualisation of SGPV in infected tissues is an important component of understanding viral distribution and disease progression. Together with histological characterisation, these approaches also provide essential context for interpreting the host immune responses associated with both clinical and sub-clinical SGPV infection.

3.1.1 Histology

Severe epithelial cell hyperplasia of the secondary lamellae is the most commonly observed pathology, often resulting in the fusion and sometimes loss of the secondary lamellae (Nylund et al., 2008; Nolsøe et al., 2015; Garseth et al., 2018; Gjessing et al., 2017; Wiik-Nielsen et al., 2017; Gjessing, Christensen, et al., 2018; Gjessing et al., 2019, 2020). It has been suggested that this is linked to the respiratory distress often displayed by disease fish prior to mortality (Gjessing et al., 2015), as a result of the reduction of surface area in the gills. However, another more recent study found that this hyperplasia in epithelial cells was only found in the late stages of infection, or the recovery period (Gjessing et al., 2020). Hyperplasia and hypertrophy of the epithelial cells in the gills is a common feature in many gill disorders and also occurs during infection with Carp edema virus (CEV), another poxvirus infecting fish (Oyamatsu et al., 1997; Miyazaki et al., 2005).

Hyperplasia has also been observed in the chloride cells of infected gill tissue, along with general malformation (Gjessing et al., 2015; Nolsøe et al., 2015; Gjessing et al., 2017). Chloride cells, or mitochondrion rich cells, are highly specialised ion transport cells present in the epithelium of the gills, which are the site of ion uptake in freshwater and of salt secretion in seawater (Perry, 1997). Changes in chloride cell location and morphology have been reported in studies on amoebic gill disease in Atlantic salmon (Chalmers et al., 2017; Chang et al., 2019). Chloride cell hyperplasia and malformation has also been found in SGPV infections, where abnormal chloride cell shape and localisation was seen in fish with severe SGPVD (Gjessing et al., 2017). While the effects of this hyperplasia or malformation of chloride cells on infected fish is not yet fully understood, it has been hypothesised that this may be linked to issues surrounding smoltification, a process in which chloride cells play an integral role (Gjessing et al., 2017).

The mucous cells in the gills are responsible for producing mucus which provides an important barrier between the gills and the external environment, and help to trap particles and pathogens present in the water (Amundsen et al., 2021). These cells have been shown to be highly responsive to the environment and any changes in it (Haddeland et al., 2021). In humans, proliferation of mucous, or goblet cells is one of the first responses to exogenous irritants in the respiratory system (Rogers, 1994; Whitsett & Alenghat, 2015). Similarly, in Atlantic salmon, mucous cell counts have long been used as a measure of gill health as mucous cell hyperplasia and hypertrophy are commonly found during gill disease outbreaks (Ferguson et al., 1992; Roberts & Powell, 2003; Ferguson, 2006; Foyle et al., 2020). Therefore, it is not surprising that mucous cell hyperplasia

has been reported in studies on SGPVD (Gjessing et al., 2019; Gjessing et al., 2021).

Apoptosis is a form of programmed cell death executed by a group of proteases called caspases (Hengartner, 2000). Though apoptosis is an intrinsic process which plays an important role in many biological processes, many pathogens utilise apoptosis to facilitate their survival and replication in host cells. This is done both through inhibiting and triggering apoptosis to evade the immune system (Favaloro et al., 2012; Singh et al., 2019). Many studies have found viruses that inhibit apoptosis in order to allow replication in host cells, including Epstein-Barr virus and Cytomegalovirus (Desbien et al., 2009; Pauleau et al., 2007). Several viruses have been shown to activate apoptosis in order to facilitate viral spread and to stunt immune response, including bovine herpesvirus (Hanon et al., 1999), poliovirus (Gosselin et al., 2003) and VACV (Ramsey-Ewing & Moss, 1998). Reports of epithelial apoptosis in the gills of fish infected with SGPV are varied, with some studies describing little to no apoptosis observed (Nylund et al., 2008; Wiik-Nielsen et al., 2017), while others go so far as to describe apoptosis as a hallmark sign of SGPV (Gjessing et al., 2015; Gjessing et al., 2017; Gjessing et al., 2020). The reason behind this difference in pathology is not yet understood, but could be attributed to the presence of other pathogens in most of these studies (Nylund et al., 2008; Garseth et al., 2018; Gjessing, Christensen, et al., 2018; Gjessing et al., 2019).

While the majority of SGPVD related pathology is found in the gills (Gjessing et al., 2015; Gjessing, Christensen, et al., 2018), signs of circulatory disturbance in the kidney and spleen, sometimes an enlarged spleen and generally pale organs have also been reported (Garseth et al., 2018; Gjessing, Christensen, et al., 2018), however these have been suggested to be as a result of secondary infections, which were present in all of these studies. (Gjessing et al., 2016, 2017; Garseth et al., 2018).

3.1.2 Visualisation of SGPV

Though the pathology caused by SGPVD is increasingly better understood, the pathways mediating between viral infection and severe clinical disease are still unclear. Salmon gill poxvirus was first discovered and identified as a poxvirus when visualised using TEM in 2008 (Nylund et al., 2008). For a time, diagnosis was only possible using this method, until the virus was first sequenced and a qPCR was established (Gjessing et al., 2015). As SGPV has been shown to be present in many multifactorial cases of CGD, the presence of SGPV and its potential role in disease outbreaks has been easy to overlook (Gjessing et al., 2017; Herrero et al., 2018;

Gjessing et al., 2019). The diagnosis of SGPVD is now much easier using more sensitive molecular methods, however the visualisation of SGPV in tissues is an important aspect of improving understanding of disease progression and the tissue tropism of SGPV. In 2018 Gjessing and colleagues generated an antibody raised in a rabbit immunised against a synthesised protein from the SGPV gene 97, a homologue of the L1R gene in VACV. Using this antibody, as well as a mouse monoclonal antibody which is homologous for the E3L VACV gene (Weaver et al., 2007), they positively stained for SGPV in the epithelial cells of infected gill tissue (Gjessing, Christensen, et al., 2018). In addition to this successful immunohistochemistry, a new method of *in-situ* hybridisation, RNAscope, has been successfully utilised to stain for SGPV (Thoen et al., 2020; Amundsen et al., 2021). This involved the design of an RNAscope probe targeting the SGPV 98 gene, a homologue of the D13L gene in VACV (Thoen et al., 2020). Visualisation of SGPV thus promises to be a useful tool in the study of SGPV, infection sites and disease progression in the gills.

3.1.3 Host immune response

The host immune response to SGPV infection has only recently begun to be studied, however it is already clear that there is some degree of immune modulation occurring during SGPV infections. Gjessing and colleagues (2020) have reported that mucosal protection was suppressed in infected fish, with not only the production of mucus in the gills being altered, but even the composition of the mucus itself. The authors reported that several immune effector genes involved in mucosal production were downregulated in infected fish, suggesting that SGPV compromises mucosal defence against pathogens. Several other genes were also found to be strongly upregulated including interferon regulated genes like Mx, ISG15 and Viperin, which is in agreement with the observed immune response to many other poxviruses (Smith & Kotwa, 2002). Thoen and colleagues (2020) looked at immune response during an SGPV infection through experimental challenge where two groups were infected with SGPV through cohabitation with infected dead salmon and one group was injected with hydrocortisone to simulate stress. This injected group developed clinical signs and experienced higher mortality, suggesting that stress can trigger disease outbreaks in sub-clinical infections of SGPV. This supports the findings of Espelid and colleagues (1996), who found that stress was one of the main factors that makes fish more vulnerable to disease in aquaculture. The authors found that there was a delay in the expression of several immune response genes including Mx until 10 days post exposure to SGPV, followed

by a strongly correlated relationship between viral load and Mx expression as mortality peaked. These findings strongly suggest that stress may well be the trigger that causes sub-clinical SGPV to evolve into clinical disease.

Another gene involved in the anti-viral response in poxviruses is melanoma differentiation associated protein 5 (MDA-5), which is a retinoic acid-inducible gene-I-like receptor involved in viral recognition. While MDA-5 is activated by RNA, and is traditionally measured in infection with RNA viruses, poxvirus replication involves the synthesis of viral complementary dsRNA which also initiates MDA-5 (Boone et al., 1979; Myskiw et al., 2011). This has been demonstrated in several studies on the immune response to VACV (Myskiw et al., 2011; Liu et al., 2015; Georgana et al., 2018), but has not yet been tested in SGPV infected hosts.

Though the effects of SGPV infection on smoltification are not yet well understood, issues surrounding smoltification and seawater transfer have been widely reported when they coincide with SGPV infection. Several studies have found that SGPV related mortality can reach severe levels during smoltification, both immediately before and after seawater transfer (Gjessing et al., 2017; Gjessing et al., 2020). One factor that could contribute to this extreme mortality during smoltification is the associated rise in plasma cortisol during this phase. It is well documented that cortisol is one of the major endocrine regulators of osmoregulatory processes during the transition to seawater (Culbert et al., 2022). This increase in cortisol has a number of functions including stimulating NKA and other ion transporters in the gills (Pelis & McCormick, 2001). However it has also recently been shown, as discussed above, that stress can cause sub-clinical SGPV infection to escalate into disease (Thoen et al., 2020) in a study where fish were injected with hydrocortisone to simulate stress, resulting in a marked increase in plasma cortisol. It is therefore very possible that this increase in plasma cortisol could be magnifying the effects of SGPV on infected fish during this production phase, causing this significant mortality observed.

Another factor that may be linked to these difficulties around smoltification is the deformation and dislocation of chloride cells in infected gills which, as previously mentioned, is a commonly reported effect of SGPVD (Gjessing et al., 2015; Gjessing et al., 2017; Gjessing et al., 2019, 2020). As mitochondria-rich cells that help secrete NaCl to counterbalance the loss of water in seawater, chloride cells play an integral part of the smoltification process (Morera et al., 2021). In some studies, this deformation in chloride cells also correlates with an SGPV associated regulation of Na⁺/K⁺-ATPase, a gene involved in ion exchange, typically upregulated during smoltification under normal conditions, however the precise cause for this difficulty in smoltification is not yet

well understood (Gjessing et al., 2020).

With limited studies published on the effects of SGPV in infected fish, there is a lack of understanding of the wider impact of infection in terms of pathology observed in major organs. The aim of the work described in this chapter is to investigate the physical effects of SGPV infection on key organs of diseased fish, and to analyse the immune response to infection in both clinical and sub-clinical infections. This was done by analysing the pathological effects on and immune response in the fish followed through SGPV infection and, in one cohort SGPVD outbreak, on Farm A.

3.2 Material and Methods

3.2.1 Ethical statement

Animal handling and collection in this study was carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 Amended Regulations (SI 2012/3039) and the work was approved by the University of Stirling Ethics Committee (Animal Welfare and Ethics Review Board)(AWERB 18 19 034 Non-ASPAs). All animals were euthanised according to schedule 1 procedure.

3.2.2 Sampling

To examine the effects SGPV infection, samples of organs were taken for histological analysis from the fish sampled from both tanks on Farm A. Samples taken included the second right gill arch, heart, spleen, liver, pyloric caeca, hindgut, head kidney, skin/muscle for each fish sampled. These tissue samples were fixed in 10% neutral buffered formalin (CellPath, UK) at RT for a year before processing and wax embedding of the tissues.

Samples were taken from both tanks during the case study for histopathology and for biomarker analysis (Table 3.1).

Table 3.1: Number, date and tank source identities for samples used for histological screening, immune marker detection and Na⁺/K⁺-ATPase quantification work

Date	Tank	Histology	Gill tissue- RNA immune markers	Gill tissue- RNA ATPase
23.10.18	G	6	6	-
01.11.18	G & C	0 & 4	0 & 6	-
05.11.18	G & C	6 & 4	6 & 6	-
15.11.18	G & C	6 & 4	6 & 6	-
26.11.18	G & C	6 & 4	6 & 6	-
17.12.18	G & C	6 & 4	-	-
02.01.19	G & C	6 & 4	-	-
11.02.19	G & C	6 & 4	-	6 & 6

3.2.3 Histology

To assess the pathology present in sampled fish, and to correlate it with viral load samples, formalin fixed tissues from all fish sampled were dehydrated through a series of alcohol baths (Shandon Citadel 2000, Thermo Fisher Scientific, USA), embedded in paraffin wax, sectioned at 5 µm and mounted on glass microscope slide. Slides were then de-waxed with xylene, stained

with haematoxylin and eosin, cover-slipped using Pertex (Pioneer Research Chemicals, UK) and examined using a light microscope (CX23, Olympus, Japan). Slides were scanned using a Axioscan (Zeiss, Germany).

3.2.4 TUNEL assay

To visualise apoptosis in the infected gill tissue, the *in-situ* cell death detection kit (Roche, Switzerland) was used on gill and skin/muscle slides. Slides with infected tissue based on qPCR results (Chapter 2, section 2.2.8) were de-waxed through two xylene washes (5 minutes per wash) and re-hydrated through ethanol washes (100% for 5 minutes and 70% for 3 minutes) before being placed in a slide holder of freshly made and pre-heated sodium citrate buffer (0.1 M) and microwaved at 750 W for 10–15 seconds, without allowing the buffer to boil. Proteinase K (100 µl, 20 µg/µl) was then added to each slide before incubating for 15 minutes at RT. The slides were then rinsed with phosphate buffered saline (PBS) and 50 µl DNase (10,000 U/µl) was added to the positive control to simulate the DNA degradation associated with apoptosis. A total of 50 µl of labelling solution, containing fluorescein, was added to the negative control, and 50 µl of a mixture of labelling solution and enzyme solution was added to the other slides. The tissues were then incubated at 37 °C in a dark humidifying chamber for 1 h before the fluorescence indicating apoptosis was observed under a fluorescent microscope (TH4-200, Olympus, Japan), fluorescence was excited at 488nm and green fluorescence was visualised at 530nm.

3.2.5 RNAscope *in situ* hybridisation

In order to visualise the SGPV in gill tissue an *in situ* hybridisation RNAscope assay was performed by MSc student Imanina Shahrin under the supervision of Rosa Allshire using the RNAscope 2.5 HD Duplex reagent kit (Advanced Cell Diagnostics, Bio-Techne, USA) as per the manufacturer's direction (322500-USM / Date 04092019) with a few alterations. Samples were chosen based on SGPV level as measured by qPCR (Chapter 2, section 2.2.8), sectioned and mounted on Superfrost Plus slides (Thermo Fisher Scientific, USA) to minimise tissue loss during the protocol. The samples were then de-waxed with xylene before being treated with hydrogen peroxide for 15 minutes to block endogenous peroxide activity then with target retrieval reagents for 15 minutes to undo cross-linking caused by the formalin fixation, and finally protease plus for a further 15 minutes to improve cell permeability. The slides were stored in 5× saline sodium citrate to prevent drying out before probe hybridisation.

Two experimental probes were used, one targeting the D13L gene in SGPV, V-SGPV-098 (accession no. KT159937), and one targeting the caspase-3 gene which is linked to apoptosis, Ssa-casp3-C2 (accession no. NM_001139921.1). A host (*S. salar*) probe (Ssa-ppib) and the RNAscope negative control (DapB-1ZZ) were used as controls. The probes were added to each slide and incubated at 40 °C for 2 hours in a HybEZ II hybridisation oven (Advanced Cell Diagnostics, Bio-Techne, USA).

The hybridisation signal was amplified using 10 different hybridisation amplifiers as per manufacturers directions. FastRed (red) and horseradish peroxidase (green) were used as chromogenic labels for Casapase 3 and for SGPV respectively. Finally, the slides were counter stained with 50% haematoxylin for better visualisation before being mounted with VectaMount (Vector Labs, USA).

3.2.6 qPCR for immune markers

To investigate any potential immune response in infected fish, two general antiviral genes were measured in 6 fish from each of the two units at 4 timepoints (Table 3.2) to analyse the antiviral response in infected fish. The markers chosen were MDA-5, *S. salar* melanoma differentiation associated protein which is a retinoic acid-inducible gene-I-like receptor involved in viral recognition and Mx- α , *S. salar* interferon-induced CTP-binding protein, which is a GTPase that inhibits viral replication. Absolute quantification using a standard curve was carried out in duplicate on the cDNA synthesised previously (Chapter 2, section 2.2.11) using SYBR green (Applied Biosystems, USA), 500 nM primers and 2 μ l template cDNA (1:10 dilution) in 96 well plates on the LightCycler 480 (Roche, Switzerland) as per manufacturer's instructions using the primers listed in Table 3.3.

Table 3.2: Sampling points chosen for analysis of immune response expression and description of sampled states

Timepoint	Date	Description
F0	2018-10-22	Earliest possible sampling point
F3	2018-11-05	Clinical signs onset
F4	2018-11-15	Peak of SGPV related mortality
F7	2018-11-26	Recovery period after mortality peak

Table 3.3: Primers used for the detection and quantification of MDA-5 and Mx- α expression. Primers were designed from the sequence accession number KU376486.1 and NM_001123693.1 for the genes MDA-5 and Mx- α respectively (McGowan. M et al., 2018)

Gene	Primer	Sequence (5'-3')	T_m	Product size (bp)
MDA-5	FP	AGAGCCCGTCCAAAGTGAAGT	60 °C	68
	RP	AACATCTTCCCCAGAGCAGACT		
Mx- α	FP	GATGCTGCACCTCAAGTCCTATTA	60 °C	72
	RP	CGGATCACCATGGGAATCTGA		

3.2.7 Na⁺/K⁺-ATPase quantification

As difficulties surrounding smoltification and seawater transfer have been widely reported in relation to SGPVD, DNA from 5 fish from each unit immediately before transfer were tested for Na⁺/K⁺-ATPase levels. A plasmid to be used as a positive control for Na⁺/K⁺-ATPase $\alpha 1\beta$ (NKA $\alpha 1\beta$) was made by PCR, ligation, cloning, and transformation. The RT-PCR was performed using gill cDNA (see Chapter 2, section 2.2.11) and the primers in Table 3.4 (Vargas-Lagos et al., 2018). *S. salar* DNA was used to extract the insert to be used in the plasmid. The reaction was done in a 0.2 ml tube with a final volume of 50 μ l and a final primer concentration of 400 nM using the 1 $\times$ MyTaq HS mix (Bioline, UK). The reaction was run on the following program (Table 3.5) on the thermocycler (Biometra, Germany). The product produced was then run in a 2% agarose gel stained with ethidium bromide. The PCR products were then purified using the DNA Clean and Concentrate kit (Zymo Research, USA), with a 5:1 ratio of binding buffer to PCR products. The TA Cloning Kit Dual promoter pCR II (Invitrogen, USA) was then used according to manufacturer's instructions. The ligation reactions were then incubated at RT for 1 hour before being stored at -20 °C. Competent cell transformation was performed by adding 5 μ l of the ligated DNA to 50 μ l One Shot TOP10f chemically competent *Escherichia coli* cells and incubating cells on ice for 30 minutes. A heat shock was then administered for 45 s at 42 °C before the cells were incubated on ice for a further 2 minutes. 300 μ l of LB medium was then added to each cell suspension and the tubes were incubated at 37 °C for 60 minutes in a shaking incubator. LB agar plates containing 100 μ g/ml ampicillin and spread with 80 μ l X-gal and 40 μ l IPTG for blue white colony screening were prepared. 150 μ l of each suspension was then spread on the plates before they were incubated overnight at 37 °C.

The following day plates were observed for bacterial growth, colonies were picked and placed in tubes containing broth overnight in a shaking incubator at 37 °C. The following day, the plasmid was extracted from the bacterial suspension using the High Pure Plasmid Isolation kit (Roche, Switzerland) as per manufacturer's instructions. The plasmid was quantified using the Qubit, and an aliquot was sent for Sanger sequencing to confirm the correct sequence. Once confirmed, the plasmid was diluted into the appropriate concentrations for qPCR standards (10^7 - 10^1 DNA molecules/ μ l).

Total gill RNA was extracted from 6 fish from each of the two units at the final timepoint prior to seawater transfer (11th February 2019) and cDNA was synthesised as described Chapter 2, section 2.2.11. The qPCR were then performed using the primers in Table 3.4, the generated

standards and cDNA per manufacturer’s directions.

Table 3.4: Primers used for the detection and quantification of NKA $\alpha 1\beta$ gene expression in fish from both tanks immediately prior to seawater transfer, as detected by qPCR. The reference sequence used for the primer design was AJ250810. Primers designed by (Vargas-Lagos et al., 2018)

Primer	Sequence (5’-3’)	T_m	Product size (bp)
Forward	GCTACATCTCAACCAACATTACAC	60 °C	90
Reverse	TGCAGCTGAGTGCACCAT		

Table 3.5: PCR program used for amplification of NKA $\alpha 1\beta$ using MyTaq

Step	Temperature	Time	Cycles
Initial denaturation	95 °C	1 min	1
Denaturation	95 °C	15 sec	
Annealing	60 °C	15 sec	35
Extension	72 °C	10 sec	
Final extension	72 °C	5 min	1
Storage/holding	4 °C	∞	1

3.2.8 Statistical analysis

The Shapiro-Wilk test was used to determine if the data were normal, then the associations between viral load (SGPV and SAV) and pathology scores were investigated using Spearman’s rank correlation. Correlation analyses were performed using individual fish pathology scores from all fish sampled during the study and corresponding viral loads. As fish were sampled randomly from tanks and individual clinical signs were not recorded, the analysis focused solely on histopathological severity rather than clinical disease presentation. The Mann-Whitney rank sum test was used to find which pathologies were consistently scored the highest, and which tank had the higher scores in each parameter. The statistical significance between tanks was measured using the Mann-Whitney test. P-values < 0.05 were considered significant. All statistical analysis was done using R v4.3.0 (R Core Team, 2023).

3.3 Results

3.3.1 Histopathology

To further study and understand the effects of SGPV infection, organs were sampled from 10 fish at each sampling point during the study for histopathological analysis, 6 from the G tank which experienced SGPVD and 4 from the sub clinically infected C tank. A semi-quantitative scoring system was developed by assessing a subset of samples across sampling points for the most common pathology observed in all organs. A quantification method for each of these parameters was then established by scoring each pathology as absent (0), mild (1), moderate (2) or severe (3) and creating a key for each of these scores (Figure 3.1). Apoptotic cells were scored per 10 lamellae, with 1-2 apoptotic cells given a score of 1 (mild), 3-4 given a score of 2 (moderate) and more than 4 given a score of 3 (severe). Similarly, mucous cell hyperplasia was scored 1 (mild) if 1-4 cells were observed per 10 lamellae, 2 (moderate) if 4-7 cells were found, and 3 (severe) if more than 7 hyperplastic mucous cells were observed. The slides were then read, and the pathology present quantified using this system.

Pathology was observed in the gills, liver, heart, skin and both the red and white muscles of diseased fish (G tank), with the gills and muscles being most severely affected (Figure 3.2 and Table 5.4). Histopathological changes were more frequent and more severe observed in fish from the G tank compared with the C tank. Gill pathology followed a similar pattern, with higher scores more commonly observed in fish from the G tank (Table 3.6).

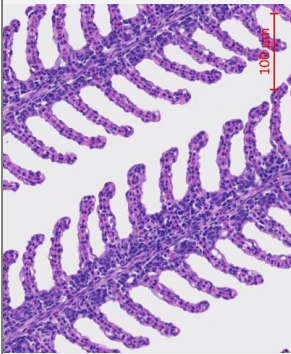
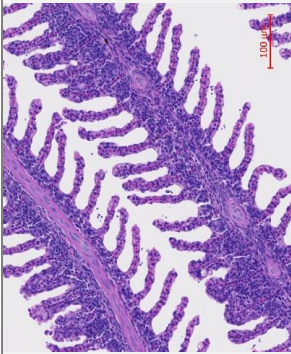
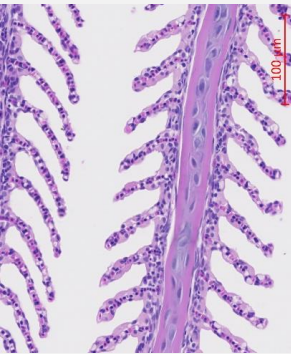
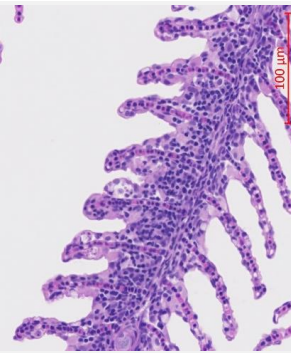
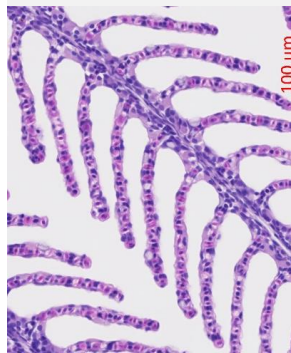
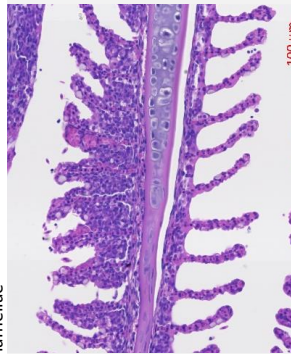
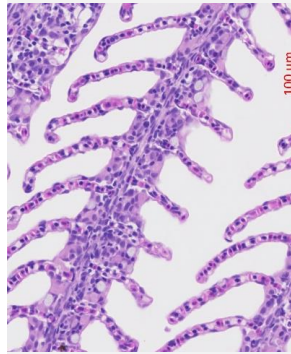
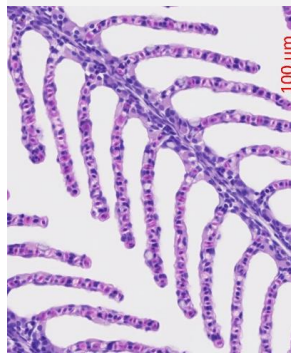
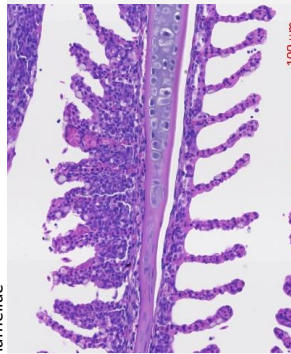
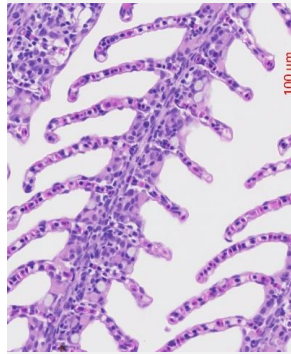
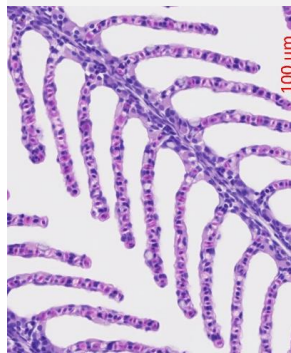
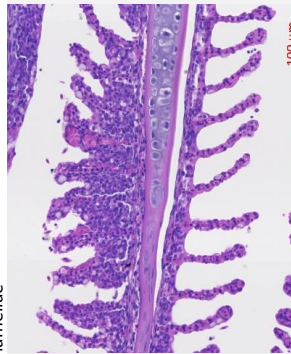
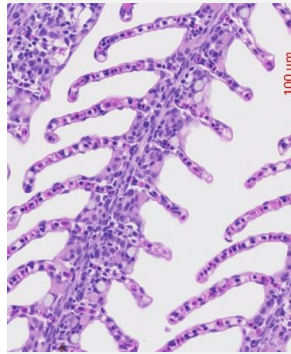
GILL PATHOLOGY	0				1				2				3			
	Normal appearance				Mild hyperplasia, low level of foci or widespread hyperplasia affecting <10% of gill tissue				Moderate, widespread of multifocal hyperplasia affecting 10-50% of gill tissue				Extensive lamellar hyperplasia with >50% of gill tissue affected			
Epithelial hyperplasia <i>Mitchell et al. 2012</i>																
	Normal appearance				Mild, with a slight increase in chloride cells at the base of the secondary lamellae				Moderate amounts of cells beginning to proliferate along the secondary lamellae				Severe hyperplasia, proliferating along the length of the secondary lamellae, altering their structure			
																
Chloride cell hyperplasia <i>Pacorig et al. 2022</i>																
Mucous cell hyperplasia																
	Normal appearance				Slight increase in mucous cells				Moderate increase in the number of mucous cells compared to healthy gills				Severe hyperplasia, substantial number of mucous cells			
																

Figure 3.1: The key for the semi-quantitative scoring system used to score fish pathology, including the papers from which a scoring method for that particular pathology was adapted. All corresponding photographs were taken from the present study (Mitchell et al., 2012; Pacorig et al., 2022).

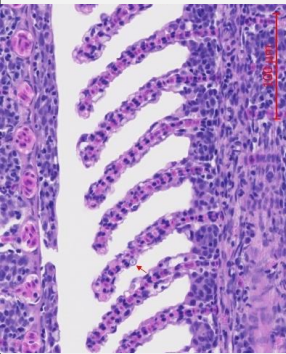
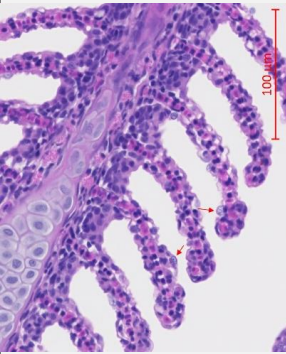
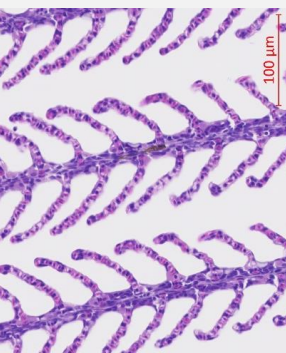
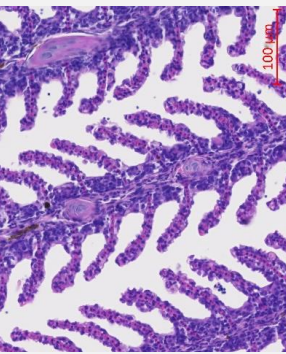
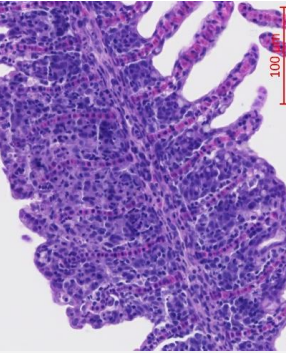
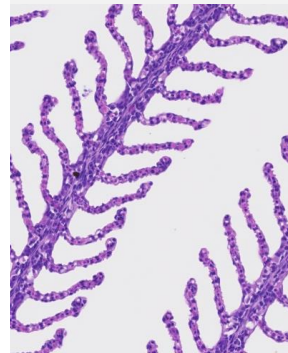
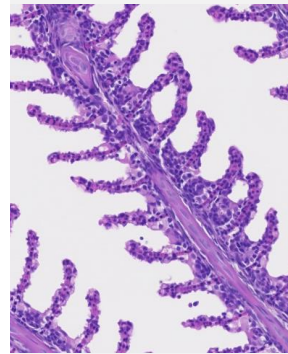
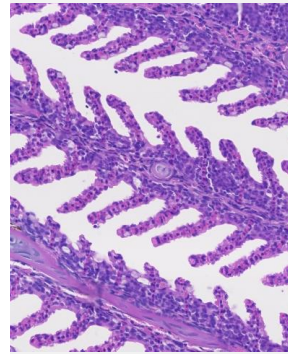
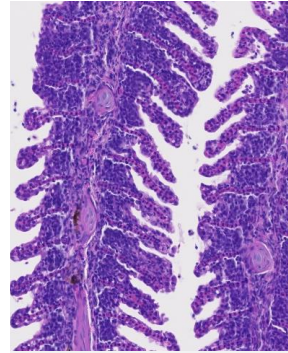
GILL	PATHOLOGY	0				1				2				3			
		Normal appearance				Sparse, a few apoptotic cells present				Obvious apoptosis but with some lamellae still free from apoptotic cells				Almost all of the respiratory epithelium affected			
	Apoptosis <i>Thoen et al. 2020</i>																
	Lamellar inflammation <i>Bradford et al. 2010</i>																
	Filament inflammation																

Figure 3.1: Key for the semi-quantitative scoring system developed to score pathology[The key for the semi-quantitative scoring system used to score fish pathology, including the papers from which a scoring method for that particular pathology was adapted. All corresponding photographs were taken from the present study (Bradford et al., 2010; Thoen et al., 2020).

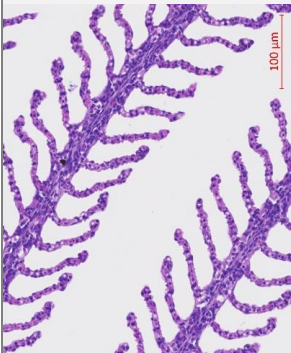
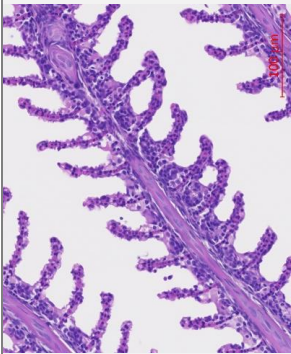
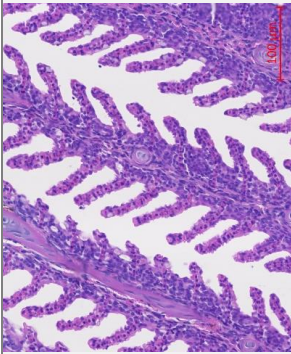
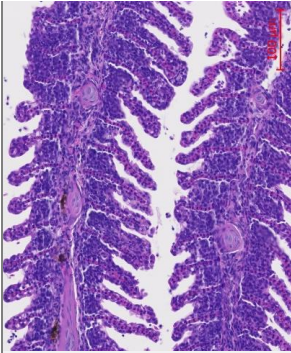
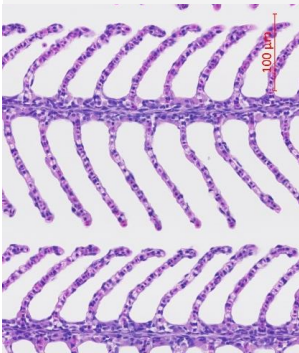
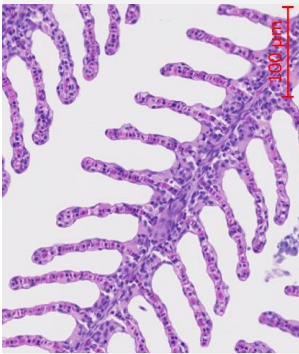
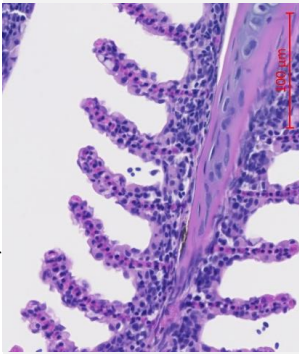
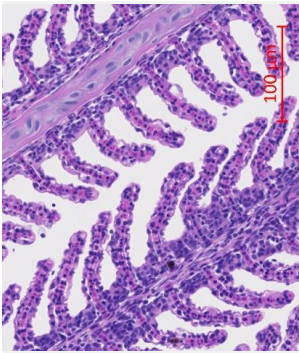
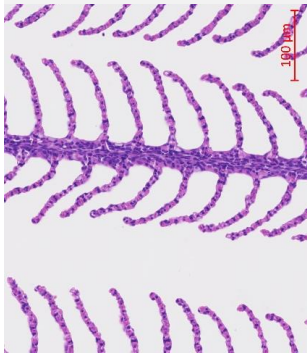
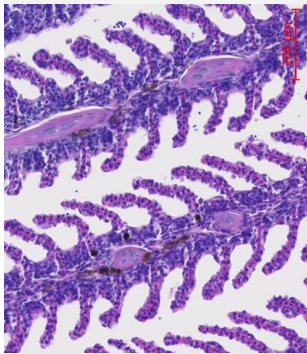
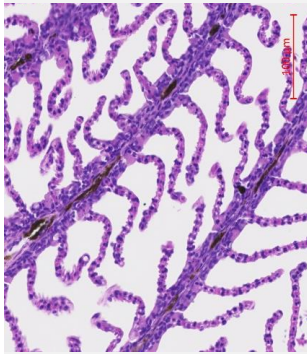
GILL PATHOLOGY	0				1				2				3			
	Normal appearance				Mild hypertrophy on <10% of lamellae				Moderate hypertrophy affecting 10-25% of secondary lamellae				Severe irreversible hypertrophy in >25% of lamellae			
Hypertrophy																
	Normal appearance				Mild hypertrophy on <10% of lamellae				Moderate hypertrophy affecting 10-25% of secondary lamellae				Severe irreversible hypertrophy in >25% of lamellae			
Clubbing																
	Normal appearance				Mild clubbing affecting <10% of lamellae				Moderate focal clubbing misshaping lamellae in 10-25% of lamellae				Severe clubbing, warping lamellae and affecting >25% of lamellae			
Melanin deposits																
	Normal appearance				One or two small deposits found on one lamella				Several small or one large melanin deposit on one or adjacent lamellae				Significant and widespread melanin deposits			

Figure 3.1: Key for the semi-quantitative scoring system developed to score pathology[The key for the semi-quantitative scoring system used to score fish pathology. All corresponding photographs were taken from the present study.

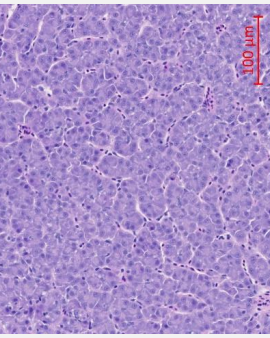
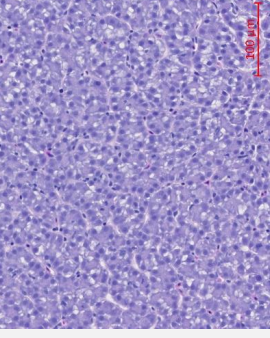
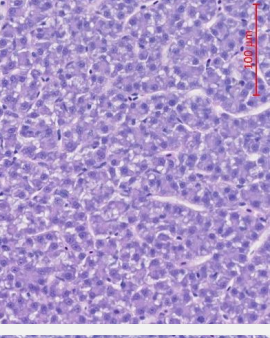
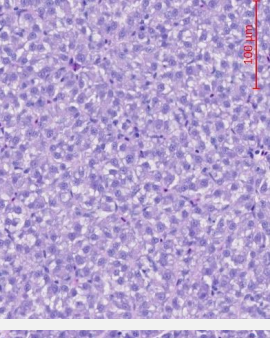
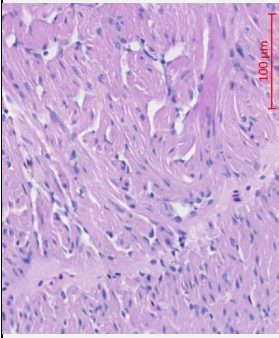
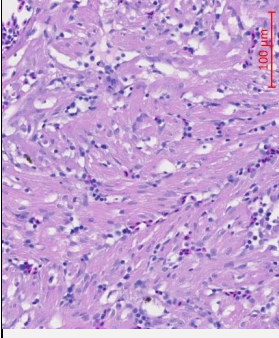
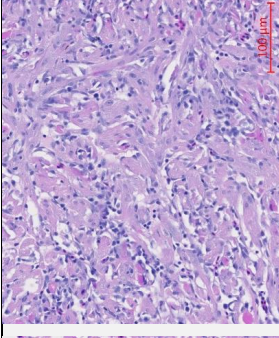
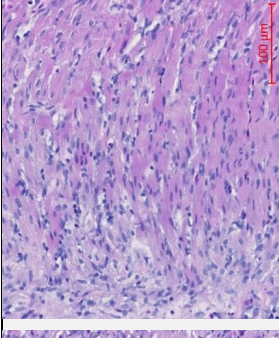
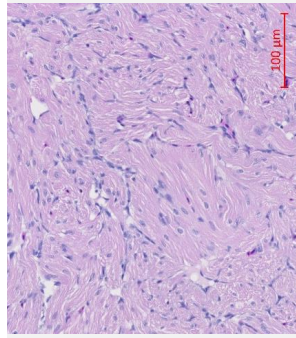
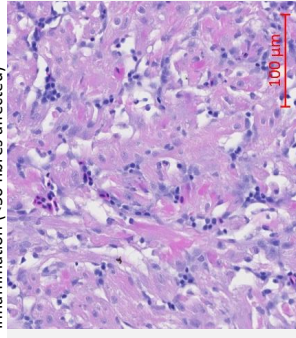
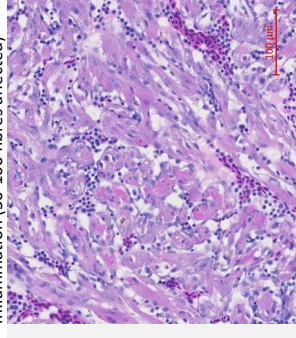
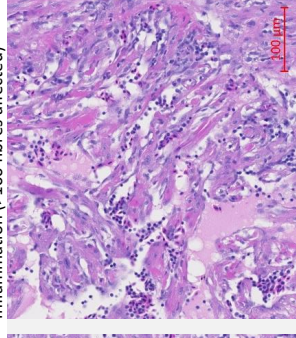
LIVER	PATHOLOGY	0				1				2				3			
		Normal appearance				Minor signs of fat accumulation affecting >25% of liver tissue				Moderate levels of lipidosis affecting >25%				Severe levels of lipidosis observed in >50% of the liver tissue			
HEART	Lipidosis																
		Normal appearance				Minor signs of fat accumulation affecting >25% of liver tissue				Moderate levels of lipidosis affecting >25%				Severe levels of lipidosis observed in >50% of the liver tissue			
		100 µm				200 µm				100 µm				100 µm			
HEART	Inflammation <i>Christie et al. 2007</i>																
		Normal appearance				Focal myocardial degeneration ± inflammation (<50 fibres affected)				Multifocal myocardial degeneration ± inflammation (50-100 fibres affected)				Severe diffuse myocardial degeneration ± inflammation (>100 fibres affected)			
		100 µm				100 µm				100 µm				100 µm			
HEART	Necrosis																
		Normal appearance				Minor focal necrosis observed				Moderate multifocal necrosis found				Severe diffuse and multifocal necrosis present			
		100 µm				100 µm				100 µm				100 µm			

Figure 3.1: Key for the semi-quantitative scoring system developed to score pathology]The key for the semi-quantitative scoring system used to score fish pathology, including the papers from which a scoring method for that particular pathology was adapted. All corresponding photographs were taken from the present study (Christie et al., 2007).

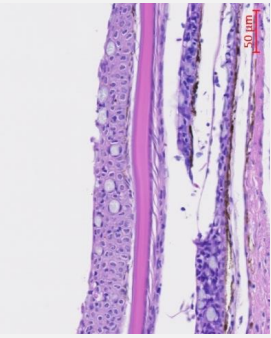
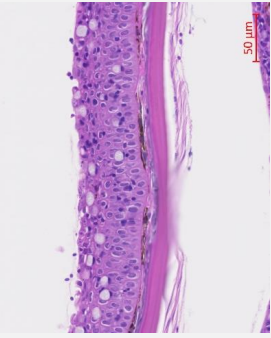
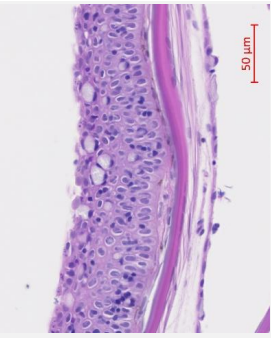
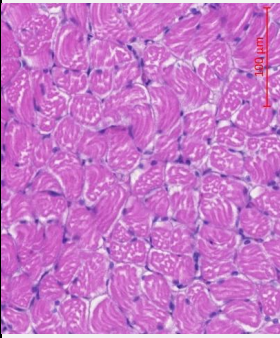
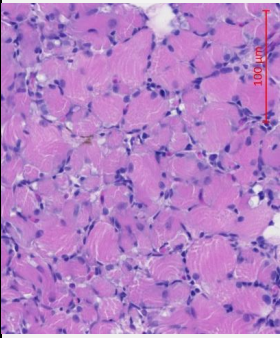
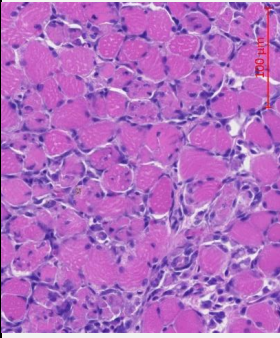
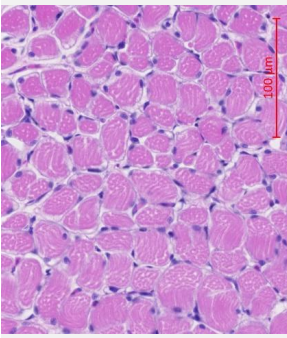
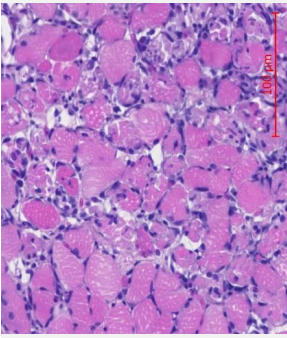
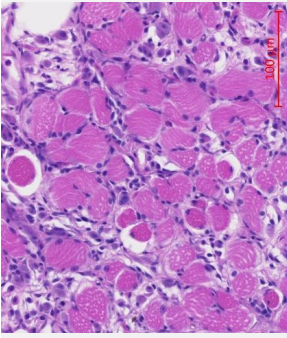
SKIN	PATHOLOGY			
	0	1	2	3
Inflammation <i>Hurty et al. 2002</i>	 Normal appearance	 Mild changes, with minimal inflammatory cells	 Moderate numbers of inflammatory infiltrates, including macrophages	 Severe changes, with marked inflammation and significant numbers of macrophages
RED MUSCLE	Inflammation <i>Christie et al. 2007</i>	 Normal appearance	 Focal inflammation	 Multifocal inflammation
	Degeneration <i>Christie et al. 2007</i>	 Normal appearance	 Multifocal degeneration	 Severe diffuse degeneration

Figure 3.1: Key for the semi-quantitative scoring system used to score fish pathology, including the papers from which a scoring method for that particular pathology was adapted. All corresponding photographs were taken from the present study (Hurty et al., 2002; Christie et al., 2007).

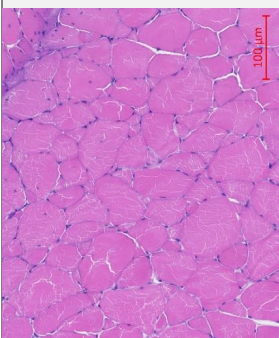
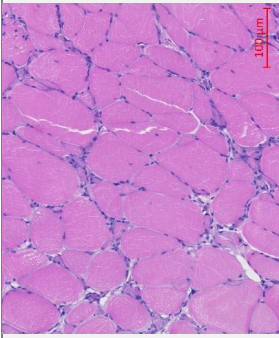
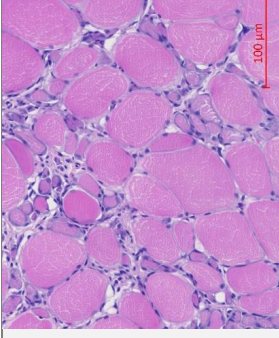
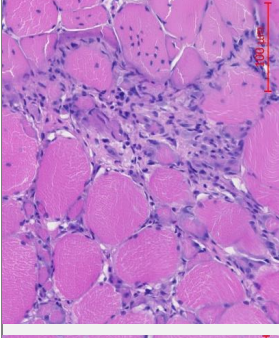
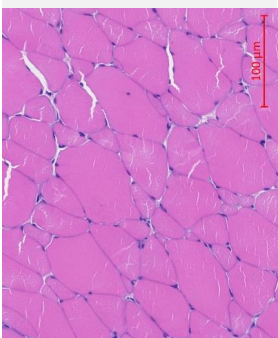
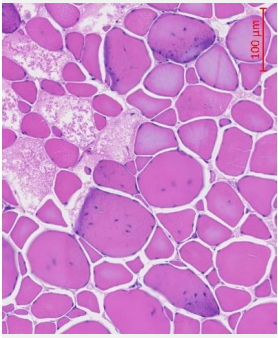
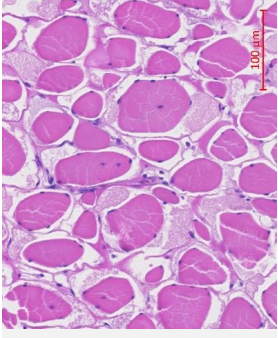
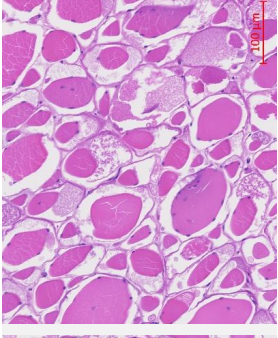
WHITE MUSCLE	PATHOLOGY Inflammation <i>Christie et al.</i> 2007	0	1	2	3
		 Normal appearance	 Focal inflammation	 Multifocal inflammation	 Severe diffuse inflammation
	Degeneration <i>Christie et al.</i> 2007	 Normal appearance	 Focal signs of early degeneration	 Multifocal degeneration	 Severe diffuse degeneration

Figure 3.1: Key for the semi-quantitative scoring system used to score fish pathology, including the papers from which a scoring method for that particular pathology was adapted. All corresponding photographs were taken from the present study (Christie et al., 2007).

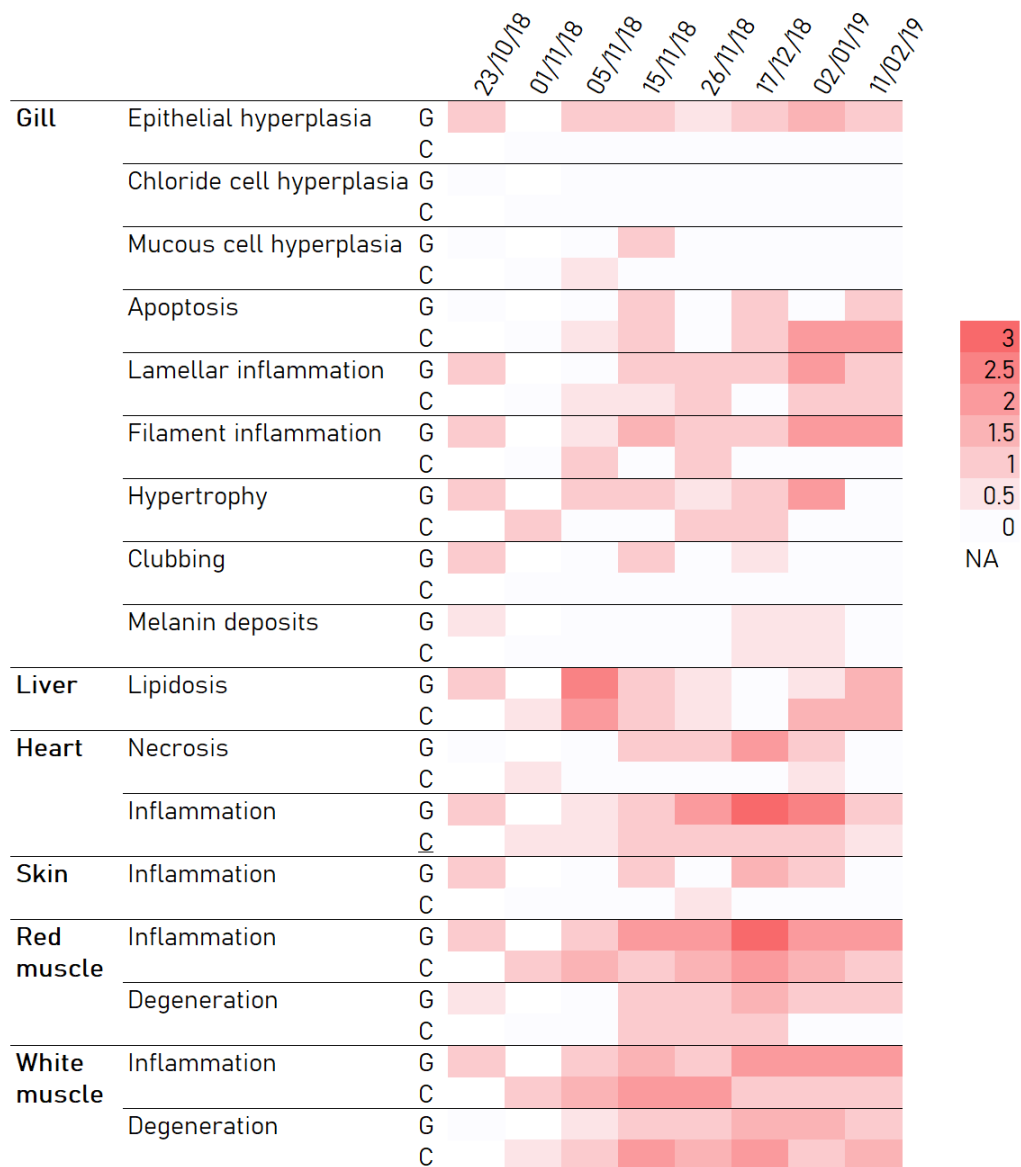


Figure 3.2: Median pathology scores for each histopathological feature scored in fish from tank C and G over the six-month study period. Scale shows histopathology scores ranging from "normal" (0) to severe (3). NA indicates sample not taken as only the G tank was sampled on 23.10.18 and only the C tank was sampled on 5.11.18 when each tank was first stocked

Table 3.6: Summary and results of the fish sampled and tested for SGPV, SAV and histopathology

Outcome	C tank	G tank	Total
Fish sampled	182	182	364
SGPV positive	61 (33.5%)	142 (78.0%)	203 (55.8%)
Fish tested for SAV	24	26	50
SAV positive [†]	18 (75.0%)	12 (46.2%)	30 (60.0%)
SGPV and SAV positive [†]	5 (20.8%)	12 (46.2%)	17 (34.0%)
Fish examined by histopathology	28	42	70
Histopathology score ≥ 2 (any tissue) [‡]	15 (53.6%)	38 (90.5%)	53 (75.7%)
Gill histopathology score ≥ 2 [‡]	8 (28.6%)	24 (57.1%)	32 (45.7%)

[†] Percentage of fish tested for SAV.

[‡] Percentage of fish examined by histopathology.

In the gills, mild to moderate epithelial hyperplasia was observed in fish from the diseased G tank (median score between 0.5 and 1.5 over the study period, range 0-2), while almost none was found in the C tank (median score 0, range 0-1). Apoptosis, widely considered the hallmark of SGPV infection, was observed at mild to moderate levels in fish from both tanks, being more severe in the C tank at the two final sampling points (2nd and 11th February 2019) with a median score of 2 (range 0-2). While the C tank showed a marginally higher level of apoptosis than the G tank, the absolute levels were minimal in both groups, suggesting that the observed difference is probably an artefact. Consistent levels of mild to moderate inflammation were observed both in gill filaments and lamellae in the G tank (median score from 0-2, range 0-3), while less was found in the C tank (median score from 0-1, range 0-2). Both epithelial hyperplasia and inflammation in the filament had a significant correlation with SAV load, as measured by qPCR (Chapter 2, section 2.2.8), (Tables 3.6 and 3.7). However, these correlations reflect population level trends and do not imply that all fish with detectable viral loads exhibited lesions to the same degree. Considerable variation was observed in pathology scores between individual fish sampled. Hypertrophy was observed in samples from both groups, although slightly more in the G tank, which experienced disease. While only mild clubbing was observed, clubbing was the only pathology found to have a significant correlation with SGPV level (Table 3.7).

Table 3.7: Spearman rank correlation analysis between the pathology scores recorded and SGPV and SAV viral loads. P-values are shown after adjustment for multiple comparisons using Bonferroni correction. Statistically significant correlations ($P < 0.05$) are indicated by an asterisk (*). NA indicates that correlation analysis was not applicable or could not be performed. All histopathological assessments were conducted at the level of the individual fish. Analyses included all sampled fish irrespective of infection status or tank of origin.

Organ	Pathology	SGPV	SAV
Gill	Epithelial hyperplasia	0.171	0.0126*
	Chloride cell hyperplasia	1	1
	Mucous cell hyperplasia	0.189	1
	Apoptosis	1	1
	Lamellar inflammation	1	1
	Filament inflammation	1	0.010*
	Thrombosis	0.679	1
	Epitheliocysts	0.561	NA
	Hypertrophy	1	1
	Clubbing	$3.62 \cdot 10^{-5}$ *	1
	Melanin deposits	1	1
Liver	Inflammation	0.141	1
	Lipidosis	1	1
Heart	Necrosis	1	1
	Inflammation	1	1
Skin	Inflammation	0.104	0.477
Red muscle	Inflammation	1	1
	Degeneration	1	0.178
White muscle	Inflammation	1	1
	Degeneration	0.157	1

In the liver, both groups showed mild to moderate vacuolation indicative of lipidosis. Moderate to severe inflammation was observed in the hearts of diseased fish in the G tank in addition to mild to moderate necrosis, while only mild inflammation was found in the C tank heart samples and almost no necrosis. Mild inflammation was found in the skin of fish from both groups, although slightly more in the diseased G tank. The most severe pathology observed was moderate to severe inflammation of both the white and red muscle of fish sampled from the G tank, though mild to moderate levels were also found in the C tank. Mild red muscle degeneration was seen in both groups. Mild to moderate degeneration in the white muscle was also found in both groups, although slightly more severe in the C tank, which did not experience an SGPVD outbreak. As these fish were also found to be infected with SAV, it is not possible to attribute any pathology to SGPV infection alone.

3.3.2 TUNEL assay

Since one of the mostly commonly reported pathologies associated with SGPVD is epithelial apoptosis in the gills, a cell death detection assay was performed to visualise apoptosis. The TUNEL assay was performed on gill and muscle tissue sections taken from the longitudinal study on Farm A. Sections were taken from 12 fish that had high levels of SGPV and from 8 fish from the study on Farm A that tested negative for SGPV via qPCR in order to establish the technique in the lab and produce reliable positive and negative controls before persisting with the technique. No fluorescence was observed in the positive control tissue prior to heat induced antigen retrieval. In contrast, following antigen retrieval, abundant non-specific fluorescence was observed, particularly in blood cells (Figure 3.3). Due to these difficulties and time constraints, this technique was not further used.

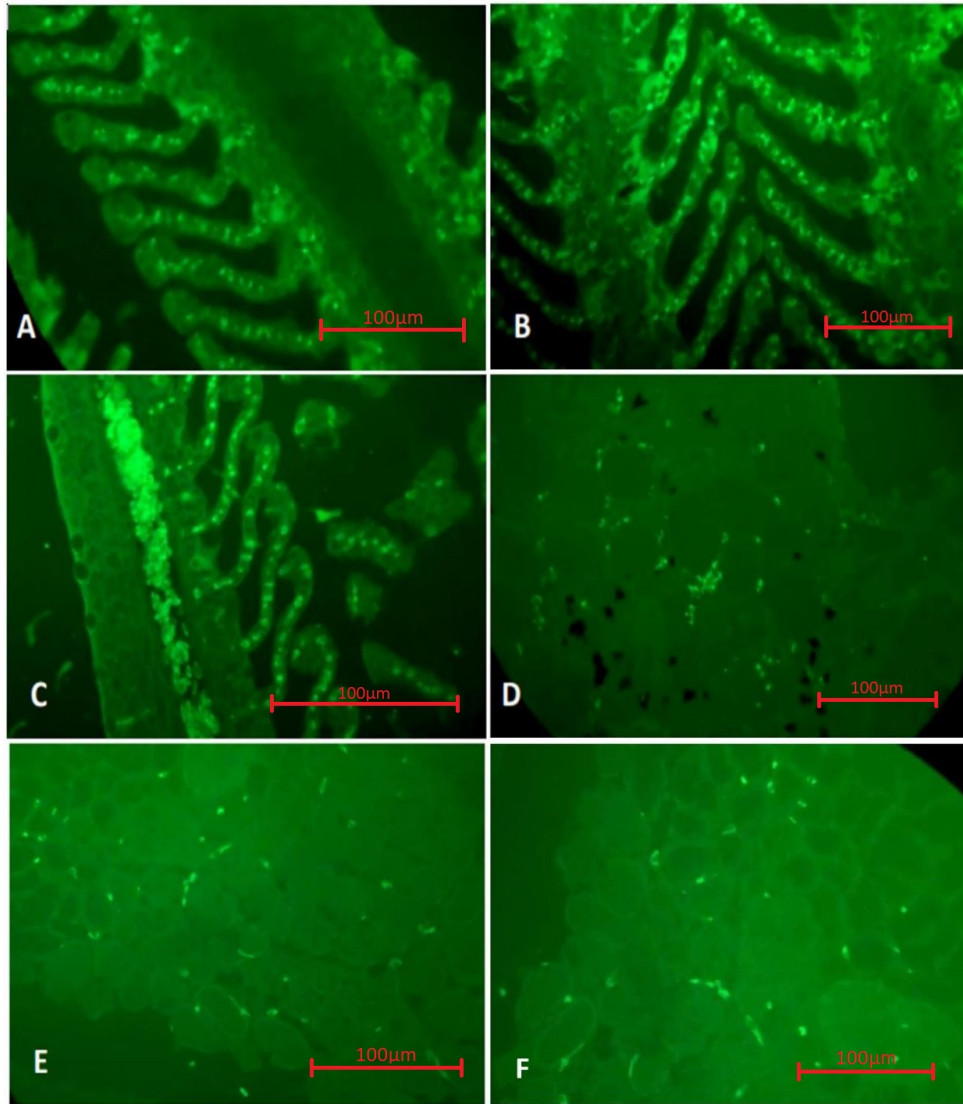


Figure 3.3: Non-specific fluorescence observed in gill (**A-C**) and muscle (**D-F**) sections following antigen retrieval while trying to optimise the TUNEL assay

3.3.3 RNAscope

Using the RNAscope duplex assay and counterstaining with H&E, SGPV was successfully visualised in gill tissue with high SGPV load as detected by qPCR (Figure 3.4), however no positive staining for Caspase 3 was observed in any of the slides.

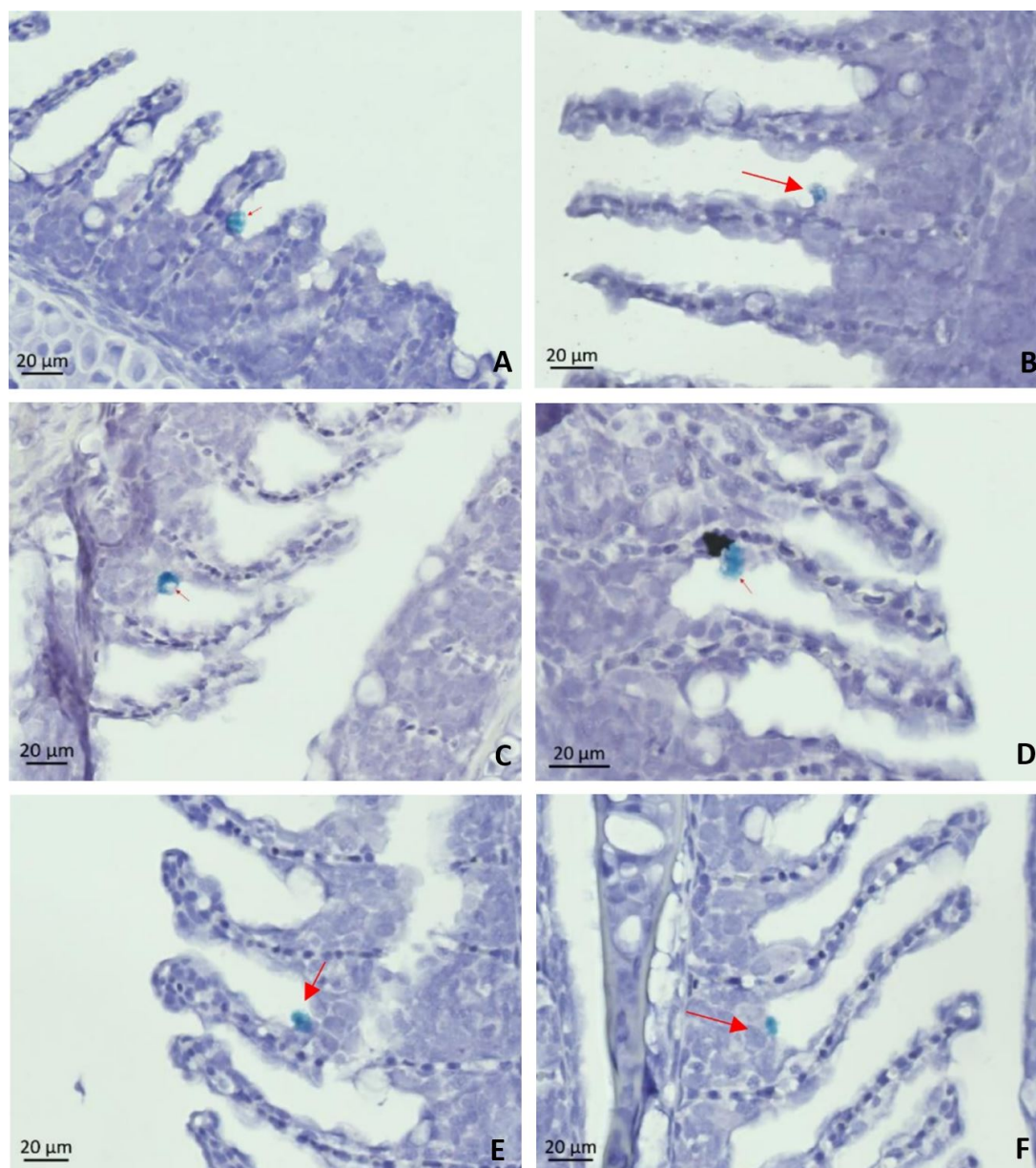


Figure 3.4: Example gill sections stained with *in situ* hybridisation (RNAscope) for salmon gill poxvirus (shown by arrows)

3.3.4 Immune response

A similar pattern of expression was found in both MDA-5 and Mx- α when measured (Figures 3.5 and 3.6), with expression in the fish from the C tank remaining somewhat consistent during all sampling points tested, while the expression in fish from the G tank dropped sharply in the third and fourth points tested, before rising again to its original level. This downregulation in Mx and MDA-5 expression in fish from the G tank also inversely correlates to SGPV level in the same samples, as well as the first of the two peaks in mortality observed.

A generalised linear model was run to compare these two antiviral marker levels between units at the 3 timepoints where both units were sampled (5th, 15th and 26th November 2018). While the decrease in both Mx and MDA-5 copy numbers in the diseased tank was not significant overall ($P = 0.72$ and $P = 0.809$ respectively), a significant difference was found between MDA-5 copy number in the G unit and the C unit on the 5th November ($P = 0.006$) and on the 15th November ($P = 0.003$). Similarly, a significant difference was found at the same sampling points between Mx copy number in each tank ($P = 0.018$, $P = 0.011$), which correspond to the clinical outbreak and peak in SGPV level in the fish in the G unit suggesting some response to SGPV infection was taking place.

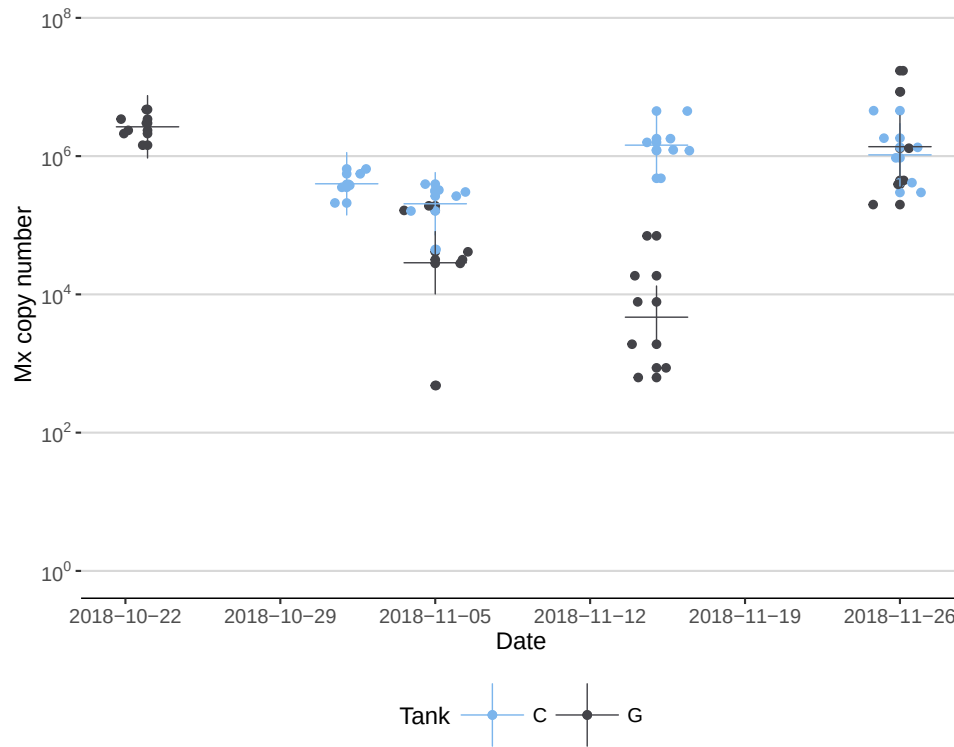


Figure 3.5: Response observed in Mx levels in both tanks during the lead up, peak and recovery after a naturally occurring SGPV infection. Mean values marked with a cross (+)

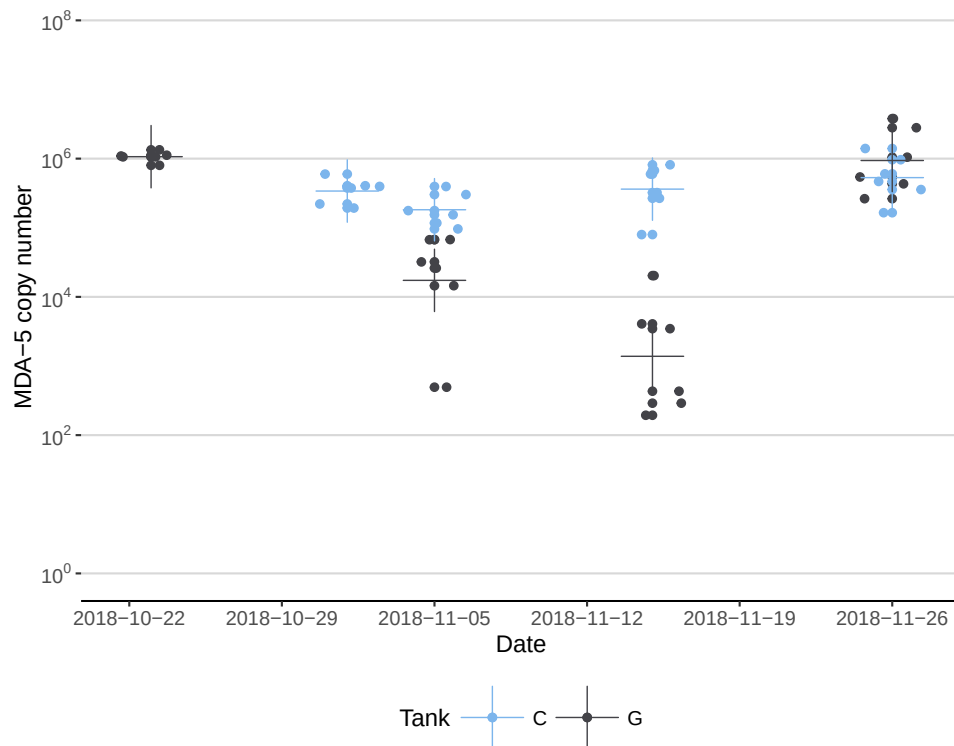


Figure 3.6: Response observed in MDA-5 levels throughout a naturally occurring SGPV infection in the G tank, which experienced clinical signs, and the C tank which did not. Mean values marked with a cross (+)

3.3.5 Na⁺/K⁺-ATPase quantification

NKA $\alpha 1\beta$ expression was marginally lower in fish sampled from the G tank which had a disease outbreak compared with the C tank which never showed any clinical signs of disease, but no significant difference was found (P-value = 0.615; Figure 3.7).

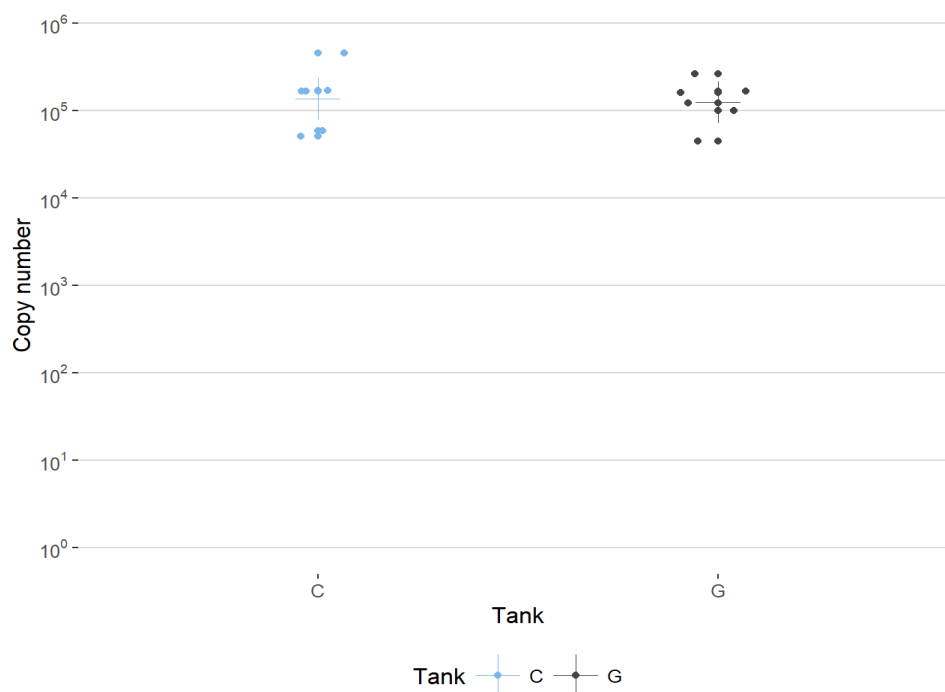


Figure 3.7: NKA $\alpha 1\beta$ expression in both groups of fish in the lead up to smoltification, at the final sampling point before transfer to sea. Cross represents the mean in each group (+).

3.4 Discussion

3.4.1 Histopathology

It is important to bear in mind that this study was of natural outbreak on a commercial site during which an outbreak of PD also occurred and so interpretation of the histopathology in the diseased group is complicated and any pathology observed in the G tank cannot be attributed solely to SGPV. Nonetheless, since PD is not known to affect the gills, any gill lesions identified are most plausibly the result of SGPV infection.

In this study, the gills were by far the most severely affected organs during the SGPV outbreak studied on Farm A. This concurs with other studies on the effects of SGPV, which report the gills as the target organ of infection (Gjessing et al., 2015; Gjessing et al., 2016; Thoen et al., 2020).

Widespread inflammation was found in both the lamellae and filaments of fish with high viral loads. This is not altogether surprising given that generalised inflammation is a very common sign of gill diseases (Kvellestad et al., 2005; Herrero et al., 2018). However, histological findings reported during SGPV infections are variable in the literature, for example no abnormal gill inflammation was reported in some studies (Gjessing et al., 2015; Gjessing, Christensen, et al., 2018). Conversely, Nylund and colleagues (2008) reported severe inflammation in both freshwater and seawater outbreaks of SGPVD. It is also worth noting that all of these studies, including that presented in the current manuscript, were observational studies of natural outbreaks of SGPVD, and so co-infections or other interactions may be confounding the histopathology observed.

As is common in many gill diseases, high levels of hyperplasia and hypertrophy were observed in the gills of diseased fish sampled from Farm A. This correlates closely to findings in several Norwegian studies where epithelial and mucous cell hyperplasia were prominent in the gills of SGPV infected fish (Nylund et al., 2008; Gjessing et al., 2015, 2020). Gjessing and colleagues (2015) note that in their study, hyperplasia was most severe in the later stages of infection and recovery, which mirrors the results found on Farm A where hyperplasia, in the gill epithelium in particular, increased after the peak of SGPV infection. Severe hyperplasia in the gills is known to cause fusion of the secondary lamellae and as a result reduce the gill surface area (Leef et al., 2005), which links to the general respiratory distress observed in diseased fish during this study. Unusual amounts of haemophagocytosis have been found in the haematopoietic organs of SGPV infected fish during observational studies in Norway (Gjessing et al., 2015; Gjessing,

Christensen, et al., 2018) which are generally attributed to circulatory disturbances caused by caused by co-infections (Gjessing et al., 2017). In the present study, however, no abnormal haemophagocytosis was found in the spleens or kidneys of diseased fish in the first screening of the histology slides and so haemophagocytosis was not scored. In 2015, Gjessing and colleagues reported haemophagocytosis in both the spleen and kidney of diseased fish however very little to no SGPV was found in these organs when tested with qPCR. Similarly, in their 2018 study, the same group identified SGPV particles and apoptotic cells in the epithelium covering the thymus of heavily infected Atlantic salmon fry. This could indicate immune depletion in severely infected fish, but has not been reported by other studies. These data support the theory that while SGPV may be found at low levels in other parts of the fish such as the spleen, kidney and oral cavity, the gills are the primary target organs of SGPV infection (Gjessing et al., 2015).

Outside of the gills, unexpectedly, the most common pathology found in this study was in the muscles of diseased fish. Moderate to severe inflammation was found in both the red and white muscle tissue of samples with high viral loads, accompanied by mild to moderate amounts of necrosis. Pathology in the muscles has not been reported in previous studies and so may be attributed to the pancreas disease (PD) observed in these fish, which causes characteristic red muscle inflammation and necrosis (McLoughlin et al., 2002). However, this could also be linked to the very distinct clinical sign of SGPV infection observed in this study, where severely infected fish become tonic, or experience “live rigor”, which has also been observed in many outbreaks in Scotland and in Norway (Thoen et al., 2020). The cause of this unusual presentation has not yet been identified, however Thoen and colleagues hypothesise that it is related to the reduction of gill surface and ensuing respiratory distress seen in diseased fish. Respiratory distress has been shown to cause hypercapnia and respiratory acidosis in trout (Fievet et al., 1988) and respiratory acidosis is known to cause issues with metabolism in muscles (Cochran & Burnett, 1996), which may be causing this characteristic “live rigor” of SGPV infected fish in the late stages of disease.

Perhaps the most surprising observation in the present study was that only low levels of epithelial apoptosis were observed in the gills, even in fish with high viral loads detected by qPCR. Similarly, when the RNAscope method of in situ hybridisation was used to stain for caspase-3, a marker of apoptosis, very little signal was detected. Although epithelial apoptosis in the gills has been described as a characteristic feature of SGPV infection (Gjessing et al., 2017), only mild to moderate epithelial cell apoptosis was observed in this study and was present in fish from both tanks. In contrast, pronounced epithelial apoptosis has been reported during

SGPV outbreaks in all phases of production in Norway (Nylund et al., 2008; Gjessing et al., 2015; Garseth et al., 2018; Gjessing, Christensen, et al., 2018). However, the findings of the present study resemble those reported by Gjessing et al. 2017, where little epithelial apoptosis was observed in salmon with high viral loads and severe clinical signs of disease. In that study, the authors hypothesised that during chronic disease, apoptosis may be masked by other gill pathologies or multifactorial infections, as the fish examined also developed amoebic gill disease (AGD). Similarly, Downes et al. 2018 found no detectable apoptosis in the gills of fish suffering from multifactorial gill disease that included SGPV. This explanation is unlikely to apply in the present study, however, as the secondary disease observed was pancreas disease (PD), which does not cause primary gill pathology.

Another possible explanation is that viral loads may have been lower in a proportion of the fish sampled. Reduced epithelial apoptosis has previously been associated with higher Ct values in qPCR assays, with only minor apoptosis observed with higher Ct values (Thoen et al., 2020). Alternatively, differences between SGPV strains circulating in Norway and those present in Scotland may influence pathogenicity and the extent of epithelial cell death. Such strain-dependent variation could potentially explain differences in clinical presentation between outbreaks in different geographic regions. Indeed, a similarly limited degree of epithelial apoptosis has also been reported during SGPVD outbreaks in Canada (LeBlanc et al., 2019), suggesting that the classical pathology described in Norwegian outbreaks may not be universally observed.

3.4.2 Visualisation of SGPV

Visualisation of the SGPV was severely limited due to prolonged storage of tissue samples in formalin, which likely led to RNA degradation and excessive crosslinking (Colburn et al., 2024). Detection of apoptotic cells using the TUNEL assay was severely limited by long-term formalin storage, as extensive crosslinking and DNA degradation likely interfered with enzyme access to DNA strand breaks. Similarly, this crosslinking reduces probe accessibility and signal quality during RNAscope analysis however, although extended formalin storage can severely compromise RNAscope by degrading RNA and masking targets, it does not typically generate false positives. Results can still be considered valid as long as the assay controls perform correctly. In this case, some SGPV infected cells were successfully visualised using the RNAscope ISH. The infected cells visualised tended to be at the base of the secondary lamellae of infected gills, correlating with epithelial cell hyperplasia causing the thickening of the lamellae. The visualisation of SGPV

has been previously published using both traditional IHC (Gjessing, Christensen, et al., 2018) and RNAscope ISH (Amundsen et al., 2021). Similarly to this work, Amundsen and colleagues detected SGPV infected cells primarily, although not exclusively, at the base of the secondary lamellae. This congregation of SGPV infected cells could be related to the density of mucus at the base of the secondary lamellae, where mucous cells are most abundant (Persson et al., 2021). As one of the functions of mucus in the gills is protection against pathogens, through the entrapment of microbes (Dash et al., 2018), this accumulation of SGPV particles is not altogether surprising. Other viruses found in the gills have been shown to collect in mucus (Eide et al., 2011), and it has even been shown that enveloped viruses like SGPV can persist and remain infectious for longer in mucus (Pease et al., 2022). This amassing of viral particles in mucus rich areas also suggests that the use of gill swabs could be an effective method of testing for SGPV. Thoen and colleagues (2020) tested this theory and found that swabs taken from the gills of infected fish had even lower Ct values than DNA extracted from gill tissue from the same fish, indicating that gill swabs may be the most accurate method of testing for SGPV, as well as being non-lethal.

It has even been shown that SGPV infection can alter the composition of the mucus in the gills (Gjessing et al., 2020), including the activation of proteases and antiviral peptides and the induction of cytokines and chemokines to activate immune cells such as T-cells and IgT and B cells. Gjessing and colleagues reported downregulation of several mucins, but the upregulation of Muc5, suggesting that SGPV infection interferes with the fish's mucosal defence against pathogens in some manner. Alteration in mucus composition is also seen during infections with the parasite *paramoecia perurans*, which causes amoebic gill disease (AGD) (Roberts & Powell, 2004). This aligns with what we know about the importance of mucus in fish as a first line of defence against pathogens, with immunologically critical functions (Benktander et al., 2020).

3.4.3 Anti-viral gene expression

In the present study, copy numbers of two markers involved with viral recognition and inhibition of viral replication (MDA-5 and Mx respectively) were measured by absolute quantification using the standard curve method. Absolute quantification using a standard curve was used, in lieu of relative qPCR and reference genes, as it provides precise and accurate measurements of the target nucleic acid as it does not depend on the expression levels of reference genes. This reduces

the variability and any potential errors associated with the choice and stability of reference genes. It is assumed that housekeeping genes expression is constant, however a variety of factors can cause fluctuation in expression, including viral infection (Lovoll et al., 2011). Several of the most commonly used reference genes in Atlantic salmon, including EF- α , β -actin, 18S and RPS20 have been shown to be significantly impacted by viral infections (Ingerslev et al., 2006; Julin et al., 2009; Lovoll et al., 2011). In gill tissue, particularly, several commonly used reference genes have been shown to be unstable (Ingerslev et al., 2006). As a result, the choice of reference gene can influence the statistical interpretation of the results. The use of absolute quantification by standard curve allows the exact quantification of copy numbers in the sample, given the method and RNA input is normalised.

The expression of both MDA-5 and Mx varied widely between groups studied. Both markers in the clinically diseased tank dropped during the peak of SGPV infection before rising to their original level, while in the sub-clinical tank they both stayed consistent throughout the study. This negative correlation between anti-viral markers and SGPV level supports the theory that SGPV is modulating the immune response in diseased fish. Poxviruses are one of only a few families of viruses that have been found to have evolved genes whose products directly interfere with various components of the host immune system to allow their successful replication and propagation (Seet et al., 2003; Cui et al., 2021). For example, many poxviruses have been found to encode virokines that block chemokine activity, such as the T1 and T7 proteins in MYXV (Seet et al., 2003) and the CPV-P35 protein in CPXV (Seet et al., 2001). It has also been reported that some poxviruses can cause an upregulation of both MDA5 and Mx (Guerra et al., 2007; Amundsen et al., 2021), although other poxviruses have been shown to downregulate these anti-viral markers (Gil et al., 2001; Guerra et al., 2007). Interestingly, SAV has been shown to upregulated Mx during disease (Herath et al., 2012), which is perhaps why we see a rise in Mx expression after the peak in SGPV, which correlates with the rise and peak of SAV load in the fish

Stress has been shown to trigger disease in many viral infections, including fish with sub-clinical SGPV (Amundsen et al., 2021). This has also been shown in a study on infectious pancreatic necrosis virus (IPNV) infection in Atlantic salmon where viral replication was much higher in infected fry when subjected to daily stress than in the control group (Gadan et al., 2012). In contrast with the findings in the present study, when the immune response was analysed in Amundsen's study, a positive correlation between Mx and SGPV level was found. However,

it was also found that change in Mx expression was delayed until 10 days post exposure when it began to rise, which could be what we are seeing in our study. Although it has been found that VAVC and VARV themselves do not affect Mx (Lorenzo et al., 2017), it has also been shown that high blood cortisol levels, and high viral titres of other viruses can suppress immune response, including Mx, in Atlantic salmon (Gadan et al., 2012; Gamil et al., 2020), therefore more in-depth study of this response is required to understand this interaction.

This apparent modulation of immune response may also explain the wide variation in clinical presentation and in mortality rates reported during SGPV infections. Visibly healthy fish that never show clinical signs have been found to be infected with SGPV, like the fish in the C system of this study, but has also been shown to cause severe clinical signs, like the G system fish, and, in other studies, mortality rates of up to 80% (Nylund et al., 2008; Thoen et al., 2020). This suggests that prevailing sub-clinical SGPV infections may be triggered under some circumstances to erupt into disease (Gulla et al., 2020). This theory is supported by the fact that SGPV has also been found in several cases of complex gill disease or proliferative gill disease, multifactorial diseases that cause significant losses in Atlantic salmon production, particularly during the seawater phase (Nylund et al., 2008; Wiik-Nielsen et al., 2017). Therefore, SGPV could either be a secondary pathogen that causes disease in fish that are already immunocompromised by other pathogens, or SGPV related modulation of the immune response allows other opportunistic pathogens to gain a foothold in the infected salmon. However, there are several limitations to the present study of immune response. Only four timepoints were studied which could allow for much missing information. Additionally, only two tanks of fish were sampled and so tank effect must be considered. As this was a natural outbreak, with unknown infection dates for each fish, testing only four times clearly limited the data output and shorter sampling windows would be advisable for future studies. Finally, we cannot account for the effects of other factors or pathogens on the immune response given that this study was held in a commercial setting, therefore further and more in depth study of the immune response is required to understand the immune response associated with SGPVD.

No significant difference was found in NKA $\alpha 1\beta$ expression levels between the tank which experienced SGPVD and the sub-clinically infected tank in the present study. Issues during seawater transfer and smoltification have been described in many reports of SGPVD, including poor saltwater adaptation and related high mortality (Nylund et al., 2008; Gjessing et al., 2017). These difficulties have been proposed to be linked to Na⁺/K⁺-ATPase (NKA) levels involved

in chloride secretion in seawater (Gjessing et al., 2017). A similar study on a natural outbreak in a freshwater smolt production site in Norway found an increase in ATPase level in fish that had survived SGPVD and recovered (Gjessing et al., 2020). The same study also reported high levels of malformed chloride cells in the gills, which they hypothesise may be linked to poor smoltification observed once the fish were transferred to sea cages. On the contrary, in the present study, no change in NKA $\alpha 1\beta$ or malformation of chloride cells was found, which suggests that the SGPV infection did not affect smoltification in this case. This is supported by the fact that these fish reportedly experienced no unusual issues or mortality when adjusting to seawater. The similarity in NKA $\alpha 1\beta$ expression between both tanks in the final sampling before seawater transfer suggests that SGPV infection did not have any impact on smoltification. There are several possible reasons for this difference in effect. The Norwegian study (Gjessing et al., 2020) followed a cohort of S2 (in season) fry that were stocked in the spring and transferred to sea in the summer while the present study followed an S0 (out of season) cohort from autumn to winter. It has been previously shown that S0 farmed Atlantic salmon have a better growth rate and experience less mortality when compared with S1 counterparts (Kristensen et al., 2012). But it has also been shown that S0 cohorts can experience more difficulties surrounding smoltification (Alne et al., 2011). Another, more likely, cause of this difference in Na⁺/K⁺-ATPase expression between studies is the length of the recovery period between SGPVD outbreak and seawater transfer. Although disease outbreaks occurred very early on in both studies, fish were transferred to sea only 6 weeks after the peak in SGPV level in the Norwegian study, while in the present study, transfer did not take place until 12 weeks after the peak of infection. It is possible that the fish in our study had more time to recover following SGPVD and so the outbreak had less or no impact on their ability to smoltify.

A shortcoming of both this study and the comparable study in Norway (Gjessing et al., 2020) is of course that they were both natural outbreaks on commercial sites, and therefore we cannot ignore the possibility of confounding factors in both cases. Additionally, the present study only measured one isoform of NKA (NKA $\alpha 1\beta$), when both NKA $\alpha 1\alpha$ and NKA $\alpha 1\beta$ have been shown to influence smoltification (Mackie et al., 2005), and so this work does not give a complete picture of any potential abnormalities.

3.5 Conclusions

This study aimed to provide a better understanding of the effects and impact that SGPV has on infected fish, both when it escalates to disease outbreak and when it remains subclinical. The histological effects, as measured by the semi-quantitative scoring system developed, yielded several interesting insights into SGPV infection. Aside from the expected gill pathology common to many gill diseases, the most common pathology observed was inflammation in the muscles of diseased fish, which may link to the "live rigor" observed in severely diseased fish. Another unexpected observation was a relative lack of apoptosis in the gills, previously described as a hallmark characteristic of SGPVD in the literature.

Two immune markers linked to viral infection were shown to have a very pronounced response in the diseased tank when compared with the subclinically infected tank, which supports the theory that SGPV modulates the immune response in some manner. No variation in NKA $\alpha 1\beta$ or difficulties during smoltification were reported in either of the groups studied, contrary to many published studies.

These new insights help to elucidate the impact of SGPV and especially to highlight the variation in response possible between infections.

Chapter 4

Genetic diversity of SGPV in freshwater Atlantic salmon in Scotland

4.1 Introduction

4.1.1 Viral culture

Tissue culture was first developed in the very early 1900's (Carrel & Burrows, 1911), but it was not until almost 50 years later that cell culture as we know it today began (Swim & Parker, 1953). The use of cell culture to grow viruses began when Weller and colleagues (1949) successfully cultured poliomyelitis virus in a human embryonic cell line. The first poliomyelitis vaccine was developed using a monkey kidney cell line several years later (Salk, 1955). Since then, cell culture has played a vital role in the study of virus pathogenesis, and in the development of vaccines.

While vaccine development and production have progressed significantly since then, cell culture is still a vital technique in the production of some types of vaccines including viral vector vaccines, like the AstraZeneca COVID-19 vaccine (Graham et al., 2020). In addition to vaccine production, the use of cell culture as a diagnostic technique still holds many advantages over molecular techniques. While PCR based techniques can detect the presence of viral DNA, it does not prove replication is taking place and so can not distinguish between infectious and non-infectious viral matter.

PCR also only detects a small section of the viral genome which can be problematic in the case of highly variable viral genomes, such as HIV (Zanini et al., 2017). This can also be an

issue due to the integration of viral genes into their hosts genomes, and vice versa, large DNA viruses have been found to acquire genes from their hosts over their evolution (Tidona & Darai, 2000; Arbuckle et al., 2010). Furthermore, only known viruses with sequenced genomes can be tested for, whereas a panel of cell lines can be used to study and help identify an unknown virus. Although cell culture alone is not a perfect method for detection and diagnosis, it certainly plays a key part in the study of viruses.

The pathogenesis of SGPV is still relatively unknown, largely due to difficulties surrounding virus culture. In a study published in 2018, LeBlanc and colleagues successfully cultured a Canadian variant of SGPV (NA SGPV) in chinook salmon embryo cells (CHSE-214) (LeBlanc et al., 2019). In this work, gills, kidney, spleen and pyloric caeca/pancreas from diseased fish were pooled and used to inoculate the cell line. Cytopathic effect (CPE) was observed 21 days post-inoculation, then the lysate was used to re-infect more CHSE-214 cells. The presence of SGPV was then confirmed by TEM and high-throughput sequencing.

In contrast, attempts to culture European isolates of SGPV have thus far been unsuccessful. In Norway, culture has also been attempted in several cell lines including Atlantic salmon kidney (ASK) and Atlantic salmon gill (ASG-10), in addition to CHSE-214 however no viral replication has been observed (Gjessing, Christensen, et al., 2018; Tartor et al., 2022).

4.1.2 Genetic study of SGPV

The *Poxviridae* family is a group of large, double stranded DNA viruses, subdivided into the Entomopoxvirinae, which infect invertebrates, and the Chordopoxvirinae, which infect vertebrates. Poxvirus genomes are among the largest viral genomes, ranging in size from 127 – 360 kbp (Tulman et al., 2004; Jacob et al., 2018). Salmon gill poxvirus was first isolated from the gills of infected fish in Norway in 2006 and subsequently identified as a poxvirus (Nylund et al., 2008). SGPV was sequenced for the first time in 2015, and was not only confirmed to be a poxvirus, but was found to be the most basal known member of the chordopoxviruses (Gjessing et al., 2015).

The SGPV genome was found to be 240 kbp in size, encoding 210 proteins. Many of these genes were found to be homologues of other chordopoxvirus genes, involved in genome replication and envelope formation, although ordered substantially differently (Gjessing et al., 2015). Additionally, 38 highly conserved genes in chordopoxviruses were found to be entirely missing, and several entirely new genes were identified, although their functions remain unknown (Gjessing et al., 2015). A partial genome was sequenced from a Canadian sample of the virus (NA SGPV)

in 2018, with 29% predicted genome coverage and a genetic similarity of 79% to the previously published Norwegian genome (Gjessing et al., 2015; LeBlanc et al., 2019).

Very few SGPV whole or partial genomes have been sequenced to date, largely due to the difficulties in doing so. One of these difficulties is the lack of an *in vitro* culture method available, meaning that metagenomic samples must be used. Within samples like these, the percentage of target DNA is relatively very low in comparison to the host DNA present. In this case, as the gills are the primary target organ for SGPV, DNA extracted from gill tissue is often used, and as the gills come into direct contact with the external environment, samples like these have high proportions of nucleic acid from not only the host, but also other organisms present in the gills (Boerlage et al., 2020), making sequencing of the target DNA challenging.

The study presented in this chapter aimed to culture and sequence SGPV in Scotland for the first time, and to use phylogenetic analysis to make comparisons with previously published genomes. To do this, SGPV samples from geographically distinct disease outbreaks in fresh and seawater farms around Scotland were sequenced and analysed to study any genetic diversity present.

4.2 Material and Methods

4.2.1 Ethical statement

Animal handling and collection in this study was carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 Amended Regulations (SI 2012/3039) and the work was approved by the University of Stirling Ethics Committee (Animal Welfare and Ethics Review Board)(AWERB 18 19 034 Non-ASPA). All animals were euthanised according to schedule 1 procedure.

4.2.2 Sampling

Samples positive for SGPV as tested by qPCR on site were provided by the health team at Cooke Aquaculture Scotland for viral culture and for sequencing from a variety of production sites around Scotland, including Farm A (Table 4.1).

Table 4.1: Date and number of samples used for sequencing / phylogenetic analysis and for attempted viral culture

Date	Farm	Sequencing	Viral culture
17.08.18	A	1	-
22.08.18	A	-	4
23.01.19	B	1	-
29.08.19	D	1	-
20.08.10	E	1	-
14.11.19	F	1	-
17.02.20	K	1	-

4.2.3 Viral culture

Culture of SGPV was attempted in CHSE-214 cells as successful culture had been reported using this cell line previously (LeBlanc et al. 2018). Eagles' minimum essential medium (EMEM)(GIBCO Life Technologies, USA) was supplemented with 10% foetal bovine serum (Life Technologies, USA), 1% L-glutamine (GIBCO Life Technologies, USA) and 1% non-essential amino acids (NEAA) (GIBCO Life Technologies, USA). Medium was re-supplemented with L-glutamine every 10 days once in use. Cell flasks were passaged once the cells became confluent (usually 7-10 days post subculture). To subculture, cells were washed twice with Dulbecco's buffered solution (Thermo Fisher Scientific, USA), then detached using Trypsin-EDTA (1×)(GIBCO Life Technologies, USA). Suspended cells were then aspirated to ensure a homogeneous suspension and

divided equally into three daughter flasks with fresh medium. Flasks were then kept in a 22 °C CO₂ incubator, with the lids loosened, until further sub-culturing or viral inoculation.

For viral inoculation, gill tissue was taken from fish showing clinical signs of SGPVD, confirmed on site as SGPV with qPCR, and stored in universal transport media (COPAN Diagnostics, USA) at 2-8 °C for 2 days prior to extraction and inoculation. The protocol used was provided by LeBlanc (personal communication, 2018) in Canada, who had reported viral growth using this method. To inoculate, a 2% homogenate was made by grinding the infected gill tissue with sterile sand, using a pestle and mortar and suspending it in a diluent made up of Hank's balanced saline solution (Thermo Fisher Scientific, USA), 2% foetal bovine serum (Life Technologies, USA) and penicillin/streptomycin (penicillin 100 IU/ml, streptomycin 100 mg/m)(GIBCO Life Technologies, USA). The mixture was then centrifuged at 2500 *g* for 15 minutes to pellet out the sterile sand and 5 ml of the gill tissue homogenate was then filtered through a 0.45 µm filter (Sartorius, Germany) to remove any bacterial contamination. The homogenate was then diluted (HBSS + 2% FBS + penicillin/streptomycin) to 0.4% before 150 µl was inoculated onto each well of a 24-well plate (Thermo Fisher Scientific, USA) of CHSE-214 cells at 70-90% confluence. Plates were sealed with parafilm (Bemis, USA), incubated at 15 °C with 1% CO₂ and observed daily for cytopathic effect (CPE).

4.2.4 Illumina whole genome sequencing

Samples were provided by Cooke Aquaculture and collected from six salmon production sites across Scotland during SGPVD outbreaks, including Farm A (sample A3). DNA was extracted from samples received as tissue samples (Table 4.2) and all samples were used for whole-genome sequencing . High-throughput sequencing was selected due to its suitability for large viral genomes and its favourable cost per base, which decreases substantially with increased genome size and sequencing depth as a result of massive parallelisation. In contrast, while Sanger sequencing is cost-effective for small numbers of short fragments, its cost increases linearly with genome size and coverage requirements, making it impractical for whole-genome or de novo sequencing of large viral genomes. Sanger sequencing therefore remains best suited for targeted validation of specific genomic regions rather than comprehensive genome analysis.

Illumina MiSeq sequencing was employed in this study due to its high base-calling accuracy and proven suitability for sequencing large viral genomes with complex terminal structures. Illumina technology was selected in preference to Oxford Nanopore sequencing because of its

superior read accuracy, which is critical for high-confidence read mapping and variant detection when a complete reference genome is available. Although Nanopore sequencing offers long reads that can facilitate the resolution of repetitive regions, read accuracy was prioritised for this study.

Library preparation was performed using the Nextera XT DNA Library Preparation Kit (Illumina, USA) according to the manufacturer’s instructions. Prepared libraries were sequenced in-house on an Illumina MiSeq platform using paired-end 300 bp reads (PE300).

Table 4.2: Sampling site locations, type, date, condition on arrival and Ct value (as measured by onsite staff) of material used for sequencing of SGPV

Sample	Location	Farm	FW or SW	Date taken	Condition	Ct value
A3	Mainland	A	FW	2018	DNA	14.17
B5	Shetland	B	SW	2019	Gill tissue	20.06
D6	Shetland	D	FW	2019	Gill tissue	19.76
E1	Shetland	E	SW	2019	DNA	24.66
F4	Shetland	F	FW	2019	DNA	25.61
K2	Mainland	K	FW	2020	DNA	24.01

4.2.5 Sequence independent single primer amplification

Sequence-independent single primer amplification (SISPA) was employed after initial sequencing attempts using just the Illumina library preparation and MiSeq protocol yielded insufficient viral genome coverage, likely due to low viral DNA abundance and the presence of complex terminal structures. SISPA enables non-targeted amplification without prior sequence knowledge, increasing the relative abundance of viral nucleic acids and improving the likelihood of recovering genome regions that were under-represented using standard library preparation methods.

The protocol used was developed by Dr Jessica Benkaroun (Benkaroun et al., 2021). Each reaction contained 1 µl of SISPA Primer A (5'-GTT TCC CAC TGG AGG ATA-(N₉)-3'), 3 µl NEBuffer (New England Biolabs, USA), 1 µL dNTPs (New England Biolabs, USA), 0.1 µl DNA polymerase (Invitrogen, USA) and 4.99 µl nuclease free water (Invitrogen, USA), and was run on the following incubation program in the thermocycler (Biometra) (Table 4.3). A mixture containing 25 µl 2× MyTaq buffer, 1 µl SISPA primer B (5'-GTT TCC CAC TGG AGG ATA-3') and 4 µl nuclease free water was then added to each reaction, before the following amplification program was run in the thermocycler (Table 4.4). Samples were then cleaned using magnetic beads (AMPure, Agencourt, USA) before library preparation and sequencing were carried out as per manufacturer’s directions.

Table 4.3: Incubation program for the first stage of sequence independent single primer amplification

Temperature	Time
25 °C	15 min
37 °C	60 min
75 °C	20 min
15 °C	∞

Table 4.4: Amplification program used in the second stage of sequence independent single primer amplification process

Step	Temperature	Time	Cycles
Initial denaturation	95 °C	2 min	1
Denaturation	95 °C	15 sec	
Annealing	50 °C	15 sec	35
Extension	72 °C	10 sec	
Final extension	72 °C	5 min	1
Storage/holding	15 °C	∞	

4.2.6 SureSelect targeted enrichment

Following two successive sequencing attempts that generated too few and very short reads, hybridisation capture-based targeted enrichment to select for SGPV DNA within the samples was performed (Figure 4.1). A SureSelect XT Target Enrichment System for the Illumina platform was ordered from Agilent with a probe designed to specifically select for SGPV DNA using the published genome (NC_027707.1) and to exclude host DNA (*S. salar*, NC_059442.1).

The SureSelect protocol published by the manufacturers did not initially produce DNA of a sufficient quantity and quality to proceed to sequencing, and therefore was optimised. To optimise the targeted enrichment protocol, each half of the protocol was done separately and DNA quantity and fragment size were assessed by electrophoresis following the second magnetic bead clean up stage before proceeding with the enrichment. If the DNA quantity was below what was expected (50-100 ng/ μ l) or fragment size was not optimum for sequencing (200-800 bp), the steps were further optimised and redone.

All steps were carried out on only 2-4 samples at a time, as timing in many steps such as fragmentation and cooling steps proved to be very important in maintaining DNA quality.

In the first AMPure bead clean up step, 180 μ l of magnetic beads were added to each sample containing 100 μ l of DNA instead of the 80 μ l in the Agilent protocol as large amounts of DNA were being lost at this step. Due to the fragment size being created in the enzymatic fragmentation step (150-250 bp), a higher ratio of AMPure beads to sample ratio was needed

to prevent the loss of these fragments during the ethanol washes. Once the targeted enrichment was performed, the DNA libraries were quantified using a Qubit fluorometer before being sent to Novogene for whole genome sequencing on an Illumina NovaSeq at PE150 read length.

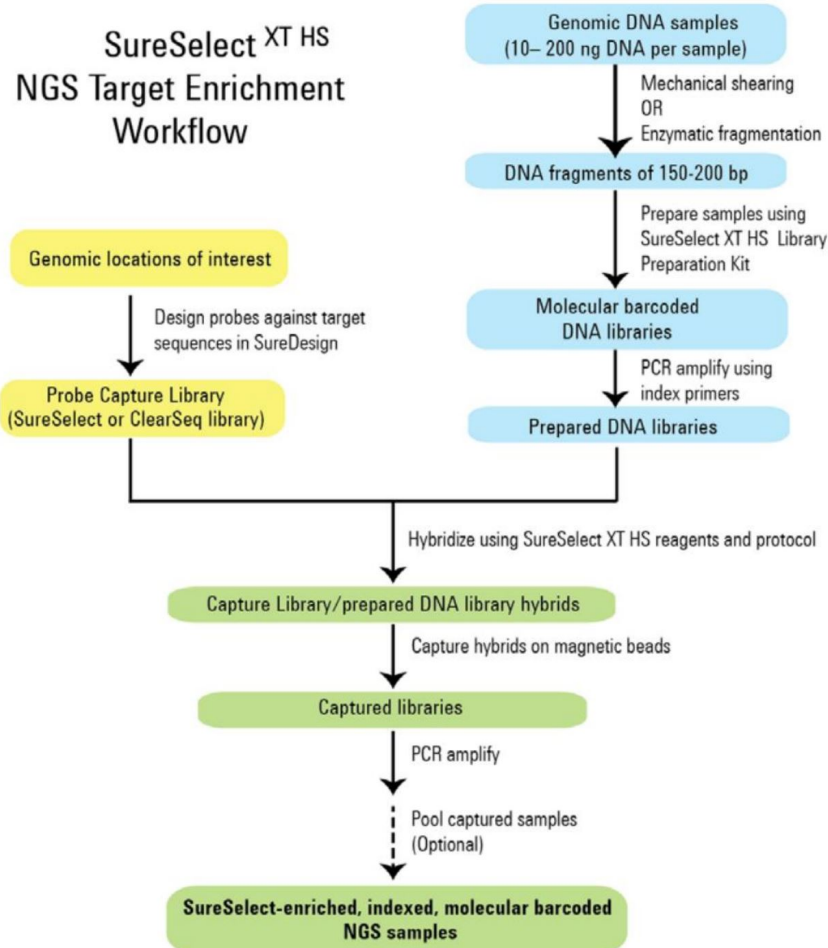


Figure 4.1: Workflow for sample preparation and targeted enrichment of the viral DNA using SureSelect technology (from Agilent SureSelect XTHS Brochure 5991-8165EN)

4.2.7 Genome assemblies

Assemblies and analysis were performed by Dr Michaël Bekaert. Reads from the sequencing libraries were used separately during the assembly process and reads were filtered for quality ($QC > 20$), length (150 nt), and absence of primers/adaptors and complexity (entropy > 15) using fastp (Chen et al., 2018). Raw data were aligned to the *S. salar* genome (Ensembl assembly ICSASG_v2) using bwa v0.7.17 (Li & Durbin, 2010). The remaining sequences, cleared from the host genome, were assembled using Spades v3.14.0 (Bankevich et al., 2012) and Megahit v1.2.9 (Li et al., 2017). The initial *de novo* output was re-aligned against the published SGPV genome

(assembly GCF_001271235.1), using CONTIGuator v2.7.5 (Galardini et al., 2011) to order the contigs when continuity was not complete. Finally, Pilon v1.23 (Walker et al., 2014) was used for polishing and correcting sequence errors. Subsequently, all genomes were annotated based on the SGPV published genome using GATU (Tcherepanov et al., 2006).

4.2.8 Genome comparison and phylogenetic trees

Genome sequences were aligned using GramAlign v3.0 (Russell, 2014). Further genome sequences of published poxviruses were downloaded from the NCBI. PIRATE v1.0.4 (Bayliss et al., 2019) was used to create a detailed pan-genome and to classify the core genes of all viruses. The core gene coding sequences were concatenated and aligned using kalign (Lassmann, 2020). The resulting alignment was used to generate an unrooted phylogenetic tree using FastTree v2.1.11 (Price et al., 2010).

4.2.9 Generation of novel primers

Once the 6 new whole genomes of SGPV were generated, some variation between samples from different geographical areas was noted. Tandem-rich regions with high variability were detected by alignment (shaded red areas Figure 4.2A), with highly conserved sections on either side, and identified as potential targets for the use of PCR to differentiate between genomes. Primers were then designed for two of these regions of suitable size for PCR (<1000 bp) (Table 4.5), as a more efficient and cheaper method of identifying viral strains using PCR and Sanger sequencing. Unfortunately due to time and sample restrictions, these primers have not yet been tested.

Table 4.5: New primer sets designed to target the variable regions in the SGPV genome

Set	Primer	Sequence (5'→3')	Location	Annealing temperature
1	SGPV2FP1	TAGGGTGAGGGTGAGTTCTGG	84,662	61 °C
	SGPV2RP1	TACTCATACCCGTCCGACCA	84,888	60 °C
2	SGPV2FP2	AAACGTAGAAGAGGTCTCTGACA	136,899	59 °C
	SGPV2RP2	ACAGGTGTATCCGTTGGAAAA	137,304	58 °C

4.3 Results

4.3.1 Viral Culture

No abnormal cytopathic effects (CPE) were observed in the CHSE-214 cells, indicating a lack of successful viral replication which was confirmed by qPCR. After 23 days, no CPE was observed and monolayer aging was seen in both the inoculated and negative control cells, as would be expected after 23 days without passage, therefore viral culture was deemed to be unsuccessful. In comparison, (LeBlanc et al., 2019) reported cytopathic effects (CPE) indicative of viral growth on day 21. Unfortunately, due a lack of SGPV related outbreaks and resulting access to fresh viral samples, and then to the COVID-19 pandemic, viral culture was not attempted again.

4.3.2 Sequencing

A mean of 8.9 million short reads were generated by genome sequencing of the 6 samples (Table 4.6). The genome sizes ranged between 120.7 kB and 234.9 kB, with GC ratios from 37.4% to 39.3% and were covalently closed hairpin in shape. Only F4 had significantly fewer reads, showed a substantial gap in its sequence of 120.7 kB, and also had far more contigs than the other 5 samples (Figure 4.2).

Table 4.6: Assembly statistics for the 6 new whole genomes sequenced from geographically distinct sites around Scotland compared with the previously published reference genome (Gjessing et al., 2015). The raw sequencing reads of all libraries and assembled genomes are available at the European Nucleotide Archive (Project PRJEB47286)

Sample	Raw reads	QC	SGPV	Assembly	Genes	Genes vs Reference	ENA Accession
A3	8,911,280	8,854,096 (99.4%)	8,770,054 (98.4%)	232,115	204	-5 (+61)	OY730730
B5	8,963,172	8,879,134 (99.1%)	7,763,452 (86.6%)	234,514	203	-7 (+64)	OY730733
D6	7,945,680	7,792,244 (98.1%)	4,859,598 (61.2%)	232,293	203	-7 (+69)	OY730732
E1	8,381,296	8,193,168 (97.8%)	2,888,012 (34.5%)	234,992	201	-8 (+65)	OY730731
F4	9,788,350	9,529,364 (97.4%)	345,440 (3.5%)	120,689	66	partial genome	OY730728
K2	9,331,058	7,997,028 (85.7%)	250,912 (2.7%)	231,118	202	-8 (+63)	OY730729

Phylogenetic analysis showed two principal branches on the phylogenetic tree. The upper branch, containing the B5 and the F4 samples, contains two samples from Shetland sites, one freshwater and one seawater. The second branch contains the samples from 2 mainland Scotland and 2 Shetland sites. A3 and K2 are both mainland freshwater production sites, while E1 is a seawater site on Shetland. D6, which is more distantly connected, is a freshwater site on Shetland.

The majority of genes identified were consistent between the published reference genome and the new genomes (Table 4.7). Using the five complete Scottish SGPV genomes and the reference genome, the pan-genome comprised approximately 210 genes, of which 198 were shared across all seven genomes. This corresponds to 94.3% of the pan-genome constituting the core genome, with the remaining 5.7% representing accessory genes. These results indicate a highly conserved genome structure across geographically distinct isolates. The partial genome F4 was excluded from this analysis due to incomplete gene representation. Gene length between genomes was compared and genes with variation were analysed. Two genes were identified to have significant differences, AL387-gp135 and AL387-gp167.

AL387-gp135 is a gene originally identified in the published reference genome and was also found to be present in all genomes sequenced in this study aside from the partial sequence F4, likely due to sequencing error rather than actual absence. Similarly, the gene is only partially present in the A3 genome. Multiple insertion-deletions are present in the E1, K2 and D6 genomes (Figures 4.3 and 4.4) compared with the reference genome. All variation is in the central region of the gene.

Using the Pfam database Interpro (Paysan-Lafosse et al., 2023) a coiled methyl-accepting chemotaxis protein-signalling domain was identified in the AL387-gp135 gene of all genomes apart from the partial gene in the A3 genome (Figure 4.5).

The AL387-gp167 gene was also found to be present in all genomes sequenced as well as the reference genome, although with some variation. Several insertion-deletions are present in the A3, E1 and F4 (Figures 4.6, 4.7 and 4.8) genomes when aligned against the reference genome. A non-cytoplasmic domain was identified using Interpro in the AL387-gp167 gene in the reference genome (Figure 4.9), however this motif was not identified in the AL387-gp167 gene in any of the newly sequenced Scottish genomes.

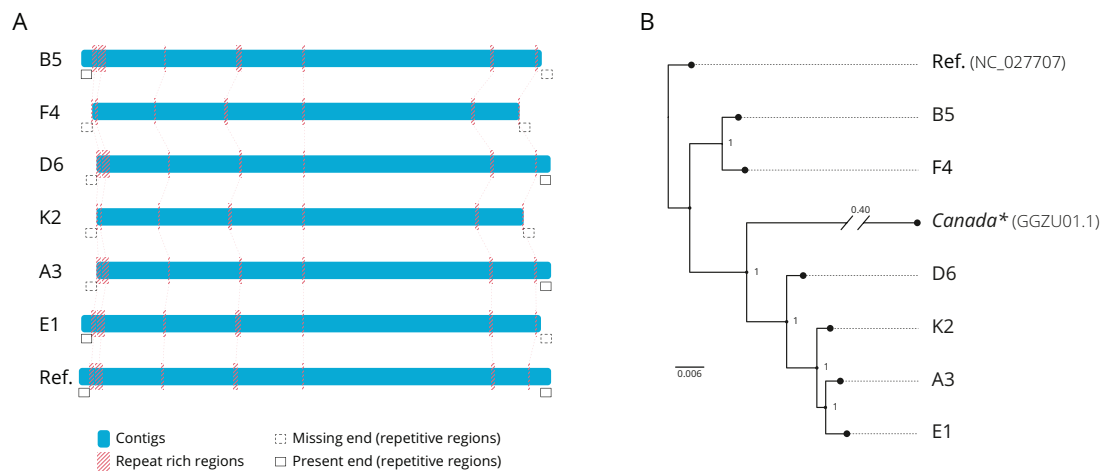


Figure 4.2: Summary of the genome sequences. **A.** Visual representation of the six novel genomes, with contigs and repeat rich sections marked. **B.** Phylogenetic tree showing the relationship between the six new Scottish genomes, the Norwegian reference genome and the Canadian partial genome.

Table 4.7: Genes present in each of the new Scottish genomes sequenced in comparison with the Norwegian reference genome. Coverage refers to how many genomes contained each gene and min and max refer to the size of the coding region in nucleotides

Gene	Annotation	Coverage	Min.	Max.	A3	B5	D6	E1	F4	K2	Ref.
AL387_gp167	hypothetical protein	7	863	1310	•	•	•	•	•	•	•
AL387_gp162	B22R family protein	7	2753	3905	•	•	•	•	•	•	•
AL387_gp178	hypothetical protein	7	908	1343	•	•	•	•	•	•	•
AL387_gp015	hypothetical protein	7	545	746	•	•	•	•	•	•	•
AL387_gp051	Peptidase M60-like family	7	2390	2717	•	•	•	•	•	•	•
AL387_gp195	hypothetical protein	7	1328	1634	•	•	•	•	•	•	•
AL387_gp072	hypothetical protein	7	455	767	•	•	•	•	•	•	•
AL387_gp080	DNA-binding virion core protein IIL	7	884	1142	•	•	•	•	•	•	•
AL387_gp091	D5-like helicase-primase	7	2159	2474	•	•	•	•	•	•	•
AL387_gp108	virion core protein P4a	7	2402	3524	•	•	•	•	•	•	•
AL387_gp123	NAD+ dependent DNA ligase	7	1484	2291	•	•	•	•	•	•	•
AL387_gp174	hypothetical protein	7	2333	2561	•	•	•	•	•	•	•
AL387_gp181	hypothetical protein	7	1160	1676	•	•	•	•	•	•	•
AL387_gp033	hypothetical protein	7	599	971	•	•	•	•	•	•	•
AL387_gp135	hypothetical protein	6	746	1730	•	•	•	•	•	-	•

Table 4.7: *Cont.*

Gene	Annotation	Coverage	Min.	Max.	A3	B5	D6	E1	F4	K2	Ref.
AL387_gp109	hypothetical protein	6	839	1199	•	•	•	•	-	•	•
AL387_gp206	hypothetical protein	6	518	998	•	•	•	•	-	•	•
AL387_gp089	hypothetical protein	5	3776	6146	•	-	•	•	-	•	•
SGPVD6-195	hypothetical protein	3	518	998	•	-	•	-	-	•	-
SGPVB5-110	S-rich repeats	3	410	626	•	•	•	-	-	-	-
AL387_gp089.p01	hypothetical protein	5	3776	6146	•	-	•	•	-	•	•
AL387_gp203	hypothetical protein	3	1187	1484	-	•	-	-	•	-	•
AL387_gp190	hypothetical protein	3	920	1118	-	•	-	-	•	-	•

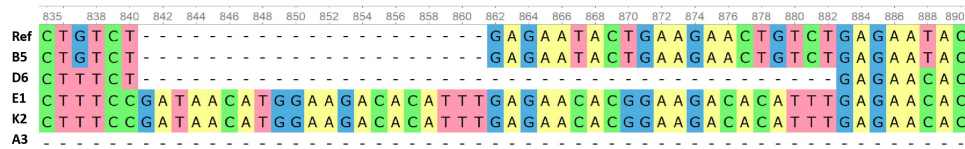


Figure 4.3: Insertion-deletion in the gene AL387-gp135 in the central regions of the E1 and K2 genomes

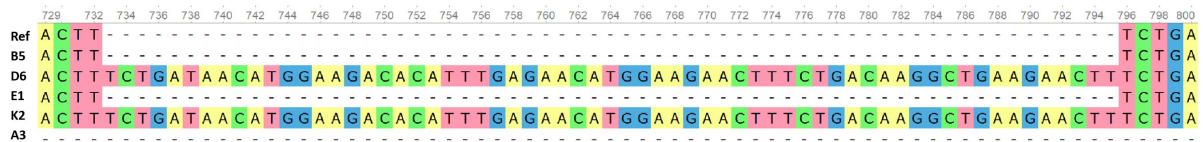


Figure 4.4: Insertion-deletion in the gene AL387-gp135 present in the D6 and K2 genomes sequenced



Figure 4.5: Methyl-accepting chemotaxis protein signalling domain found in the AL387-gp135 gene

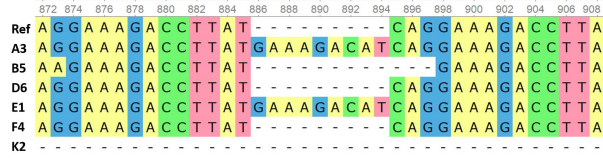


Figure 4.6: Insertion-deletion in the gene AL387-gp167 present in the A3 and E1 genomes sequenced

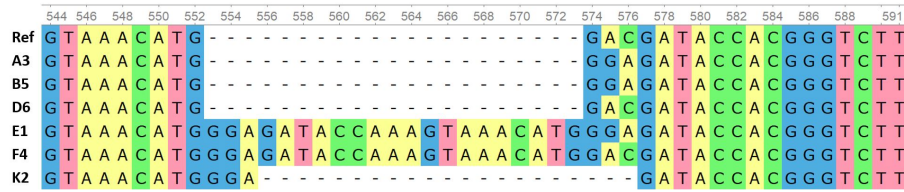


Figure 4.7: Insertion-deletion in the gene AL387-gp167 present in the E1 and F4 genomes

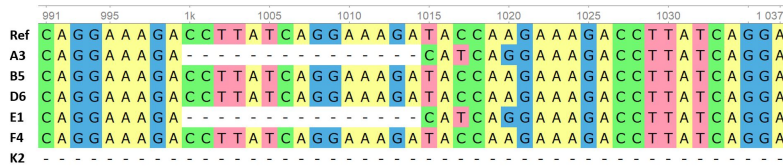


Figure 4.8: Insertion-deletion in the gene AL387-gp167 in the A3 and E1 genomes compared with the reference genome

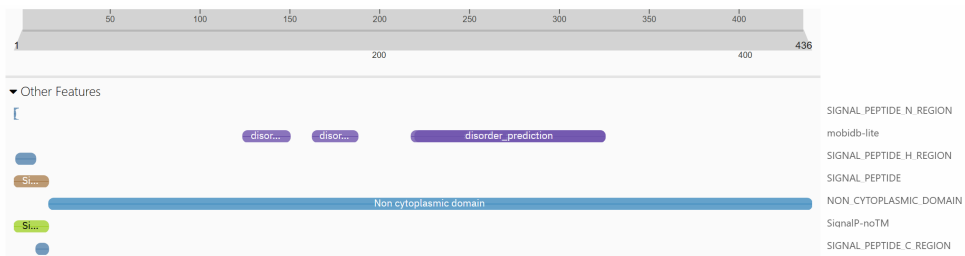


Figure 4.9: Non-cytoplasmic domain found in the protein AL387-gp167, identified only in the reference genome

4.4 Discussion

4.4.1 Viral culture

One of the biggest obstacles in the study of SGPV and its replication is the lack of a suitable cell culture model for its cultivation. Although North American SGPV (NA SGPV) has been cultured successfully in chinook salmon embryo cells (CHSE-214) (LeBlanc et al., 2019), this has as yet not been repeated and no studies have yet been published that report the culture of European SGPV isolates. Despite using the same protocol as LeBlanc and colleagues (2019), culture of SGPV in CHSE-214 cells in the present study was unsuccessful. This suggests either that this cell line and protocol are not well suited to the culture of SGPV, or that there is some variation between strains of SGPV.

In other studies, attempts to culture SGPV have been made in a wide variety of cell lines including CHSE-214 cells, epithelioma papulosum cyprinid cells (EPC), Atlantic salmon head kidney cells (ASK) and even Atlantic salmon gill cells (ASG-10) without success (LeBlanc et al., 2019; Tartor et al., 2022). Plans had been in place to attempt SGPV culture in primary cell lines, developed from Atlantic salmon gills. Unfortunately, due to the lack of SGPV disease outbreaks for a prolonged period, and then limited access to farms and laboratory facilities during the COVID-19 pandemic, only one attempt at culturing SGPV was made in this study. Culture on primary cell lines has since been unsuccessfully attempted by Gjessing and colleagues (2018).

The first successful experimental infection with SGPV was performed by Thoen and colleagues (2020) by exposing naïve fish to SGPV infected material and injecting them with hydrocortisone to simulate stress. The use of cortisol in cell lines has in some cases been shown to impact cell viability by affecting membrane integrity and therefore improving viral replication (Bushell and Hobson 1978, von Krogh et al. 2019), however in the study by Tartor and colleagues (2022), this too proved unsuccessful. The difference between strains of SGPV, their pathogenicity and ability to be cultured may be a key part of this puzzle. Exploring the culture of NA SGPV in existing Atlantic salmon gill cell lines, and the development of an Atlantic salmon gill cell line using North American Atlantic salmon would be interesting next steps for future studies.

4.4.2 Genetic study of SGPV

The present study has resulted in the description of the first six SGPV genomes derived from samples from Scottish farms, which is a valuable addition to the literature on salmon gill poxvirus.

Little variation between genomes in this study and the published Norwegian SGPV genome were found. This level of variation within a geographical area this size is not unusual, for example variants of VACV from Brazil were found to have a minimum shared identity of 97.5% (Drumond et al., 2008). Significant variation in poxvirus genomes can also be linked to a wide range of hosts (Oliveira et al., 2017), which does not appear to be the case with SGPV, whose only known natural host is Atlantic salmon (Garseth et al., 2018). Our results also align with a study in which multi-locus variable-number tandem repeat analysis was used, which found that found several European samples of SGPV had the same, conserved lineage, while a much larger variation was found between European and North American samples (Amundsen et al., 2021). While a full genome of the NA SGPV is not yet available, a partial sequence was obtained by LeBlanc and colleagues (2019) during their study , which bore only a 79.1% similarity to the Norwegian SGPV genome, indicating a clear transatlantic separation.

Although no link between genetic variation and virulence has been found (Gulla et al., 2020), many of the genes in SGPV and their functions remain unknown, with no clear homologues to those of other poxviruses (Gjessing et al., 2020). However, given the limited differences in genomes in this study, it is unlikely that the variation in clinical presentation can be tied to genetic diversity. Some of the most interesting genes found in these six novel SGPV genomes, as well as the published Norwegian genome, are homologues to the B22 family of genes found in VARV. These genes encode large transmembrane proteins and are involved with inhibition of the adaptive immune response, specifically the suppression of T-cells (Alzhanova et al., 2014). Yang and colleagues (2011) predicted early expression of these B22 genes based on their expression in VACV, and this was confirmed by Amundsen and her group (2021), who found that B22R1-3 was expressed in fish one day post exposure to SGPV. While it now appears unlikely that the variation in clinical presentation can be tied to genetic diversity as we first hypothesised, the expression of this B22 family of genes, which we have also found in the six novel Scottish genomes, could be a particularly useful early marker of SGPV infection.

Although the effects of the different strains of SGPV sequenced in the present study are not yet fully understood, five new whole and one partial genomes have been sequenced, with minor differences identified. The next step in understanding the significance of these differences would be direct gene comparison between genomes and to further investigate the early expression of the B22 genes. For this however, there is an urgent need for a cell culture model.

4.5 Conclusions

This work aimed to search for genetic variation in Scottish samples of SGPV. This was done by sequencing samples from outbreaks across Scotland on both freshwater and seawater sites, and by comparing these sequences to those previously published from Norway and Canada. Five new whole genomes and one partial genome were successfully sequenced from outbreaks in Scotland. While little variation between these new sequences was found, several tandem-rich, highly variable areas were identified as good targets for future analysis. Additionally, two sets of primers were designed to target these regions of high-variability.

Six new Scottish SGPV genomes have been sequenced, more than doubling the published SGPV genomes available worldwide. This will allow more in-depth study on the variation between strains of SGPV, as well as potentially provide some insight into the history and movement of the virus in Atlantic salmon aquaculture, which may allow the industry to mitigate future threats SGPV may pose.

Chapter 5

General Discussion

5.1 Introduction

Salmon gill poxvirus remains a significant challenge for freshwater Atlantic salmon aquaculture, both in Scotland and globally, due to its variable clinical presentation and the gaps in understanding of the virus and disease. This study was designed to address several of these knowledge gaps by examining environmental factors, potential risk factors and the biological effects of SGPV, as well as exploring differences between outbreaks that may underlie the wide spectrum of disease presentation. Through the present study, three key aspects of SGPV have been explored- the environmental conditions that influence disease expression, the variation in clinical and histopathological presentation during a natural outbreak in Scotland and the genetic diversity of the virus across Scottish outbreaks. By integrating these findings, this discussion seeks to evaluate the extent to which the study addresses the original research aims, situate the results within the existing literature and consider how technical or environmental factors may have influenced outcomes and interpretation.

5.2 Host-pathogen interactions and clinical variation

Understanding the factors that drive the wide variation in clinical presentation of SGPV infection remains one of the most pressing challenges in SGPV research. As a pathogen capable of existing in a seemingly benign state or causing severe pathology with mortality rates of up to 80% (Nylund et al., 2008), improved insight into the determinants of disease expression is essential. In the

present study, SGPV infection remained subclinical in one of two of the tanks studied throughout the sampling period, despite the fish sharing the same origin, husbandry practices, location and largely similar water parameters. This contrast highlights the complexity of host-pathogen-environment interactions underlying SGPV disease.

Subclinical infection is a well-recognised feature of poxviruses more broadly. Monkeypox virus (MPXV), for example, exhibits a wide clinical spectrum ranging from subclinical infection to severe disease and death (Stanford et al., 2007; Golden et al., 2024). Similarly, ectromelia virus (ECTV), which causes mousepox, and variola virus (VARV), the causative agent of smallpox, are both known to have subclinical forms (Briody, 1959; Heiner et al., 1971). These parallels suggest that latent or low-level infection may be a common strategy among poxviruses. One possible explanation for this variability lies in the evolutionary history of poxviruses. When a virus crosses into a novel host species, dramatic differences in pathogenicity can arise (Weiss, 2002). Myxoma virus (MYXV), for instance, is relatively non-pathogenic in its natural hosts, the South American tepeti (*Sylvilagus brasiliensis*) and the North American brush rabbit (*Sylvilagus bachmani*), but causes near complete mortality in the European rabbit (*Oryctolagus cuniculus*) (Fenner & Ratcliffe, 1965; Nash et al., 1999). However, this mechanism alone cannot explain the findings of the present study. SGPV has only been described in Atlantic salmon, and both study tanks originated from the same host population. The occurrence of clinical disease in only one tank therefore suggests that additional factors are involved.

Host physiological status and immune competence are likely to play a central role in determining whether SGPV infection remains subclinical or progresses to overt disease. Although only two tanks were examined, the stark difference in pathology between them suggests that one group may have been exposed to stressors absent from the subclinically infected tank. This interpretation is consistent with previous studies demonstrating that SGPV disease can be experimentally induced in subclinically infected fish through cortisol injection (Thoen et al., 2020). More broadly, stress induced disease reactivation is well documented across viral systems (Rector et al., 2014; Bauer & Fuente, 2016). For example, stress has been shown to trigger clinical disease in latent varicella zoster virus infections (Mehta et al., 2004), while in fish, latent cyprinid herpesvirus infections can be reactivated following stress exposure (Eide et al., 2011).

Viruses may exploit host immune modulation directly or take advantage of immunosuppression caused by external factors (Rouse & Horohov, 1986; Naniche & Oldstone, 2000). In poxviruses specifically, host immune responses are thought to be critical in determining whether

infection remains subclinical or progresses to disease (Buller & Palumbo, 1991; Müllbacher & Lobigs, 2001; Chaudhri et al., 2004). Several poxviruses, including vaccinia virus (VACV), MPXV, and ECTV, exhibit subclinical infections that can transition to clinical disease following immune perturbation (Jackson et al., 2001; Rehfeld et al., 2017; Golden et al., 2024). In the present study, several environmental parameters were identified as potential stressors associated with SGPV disease expression. Total ammonia nitrogen, water hardness and CO₂ concentrations were strongly correlated with mortality in the presence of SGPV infection. Furthermore, a machine learning model identified a combination of CO₂, temperature and pH as a strong predictor of mortality. Although none of these parameters exceeded commonly accepted thresholds for Atlantic salmon welfare, each has previously been shown to induce physiological stress under certain conditions (Ackerman et al., 2006; Kroglund et al., 2008; Kieffer et al., 2002; Vargas-Chacoff et al., 2018; Mota et al., 2020). Stress-induced elevation of cortisol may suppress immune function (Espelid et al., 1996; de Salles-Gomes et al., 1965; Fast et al., 2008), potentially allowing viral replication and disease emergence. Although in the present study both experimental groups shared the same origin, husbandry practices, site and broadly comparable water parameters, it is possible that some unmeasured or subtle tank-level differences could cause stress that could contribute to this difference in presentation.

5.3 Determinants of disease emergence and variation

An important factor influencing disease dynamics in the present study is the presence of salmonid alphavirus (SAV) in both study tanks throughout the sampling period. Co-infection, defined as simultaneous infection with two or more genetically distinct pathogens (Okon et al., 2023), is common in aquaculture settings and can profoundly affect disease outcome, immune responses and host survival. Despite their prevalence, the effects of co-infections in aquatic systems remain poorly understood. This knowledge gap reflects both the tendency to study pathogens in isolation under laboratory conditions and the technical challenges associated with detecting and characterising multiple pathogens within a single host (Kumar et al., 2016). As a result, multifactorial diseases such as complex gill disease (CGD) may be oversimplified, with emphasis placed on culturable or presumed primary pathogens (Kumar et al., 2018). Interactions between co-infecting pathogens may be direct, such as competition for host tissues, or indirect through modulation of host immune responses (Cox, 2001; Lello et al., 2004; Telfer et al., 2008).

In the present study, expression of immune markers was inversely correlated with SGPV load, with downregulation coinciding with peak viral load and pathology. Subsequently, SAV load increased and peaked only in the clinically affected tank. This temporal pattern is consistent with immune suppression facilitating secondary infection, a phenomenon widely reported in fish. For example, tilapia co-infected with tilapia lake virus (TiLV) and *Aeromonas hydrophila* experienced significantly higher mortality than fish infected with either pathogen alone (Nicholson et al., 2020). Conversely, antagonistic interactions have also been observed- Atlantic salmon infected with infectious pancreatic necrosis virus (IPNV) were found to be less susceptible to subsequent infectious salmon anaemia virus (ISAV) infection (Johansen & Sommer, 2001). These contrasting outcomes highlight the context-dependent nature of co-infection dynamics.

The frequent detection of multiple pathogens in naturally infected Atlantic salmon further underscores the importance of understanding these interactions. IPNV and IHNV have both been isolated alongside *Flavobacterium psychrophilum* in severe disease outbreaks (Evensen & Lorenzen, 1997; Ma et al., 2019), while Atlantic salmon paramyxovirus (ASPV) and SGPV have been detected together in CGD cases (Herrero et al., 2018). Given these observations, controlled studies examining SGPV co-infections with viral, bacterial and parasitic pathogens are essential to improve disease management strategies.

Several limitations of the present study must be acknowledged. The small sample size, comprising only two tanks, raises the possibility of tank effects, and the fact that this was a natural infection means that infection dose, timing, and source are unknown. Nonetheless, these constraints also reflect real-world farming conditions. Larger scale, controlled studies incorporating defined co-infection scenarios, varied exposure routes and immune profiling will be critical to disentangling the multifactorial drivers of SGPVD.

5.4 Viral characteristics and diversity

Genetic variation within SGPV represents another potential contributor to variation in disease presentation. The rapid expansion of Atlantic salmon aquaculture over the past five decades has imposed strong selective pressures on pathogens, often favouring increased virulence (Gandon et al., 2001; Sundberg et al., 2016). High host densities and shortened production cycles can accelerate pathogen evolution, as illustrated by ISAV (Rimstad & Markussen, 2020). Modern virulent ISAV strains are thought to have evolved from an avirulent wild-type ancestor (ISAV-

HPR0) following adaptation to intensive farming environments (Mennerat et al., 2010; Rimstad et al., 2011). Similar evolutionary dynamics may be occurring in SGPV.

Like other poxviruses, SGPV is a double-stranded DNA virus with covalently closed hairpin termini (Geshelin & Berns, 1974; Baroudy et al., 1982). The central genomic region encodes conserved genes involved in replication and transcription, whereas the terminal regions are thought to contain genes associated with host specificity and virulence (Gubser et al., 2004; Gjessing et al., 2015). In the present study, the most variable sections were located within these terminal regions, consistent with previous findings (Nylund et al., 2023). Although no definitive association between genetic variation and virulence has yet been demonstrated (Gulla et al., 2020), many SGPV genes remain functionally uncharacterised, limiting interpretation. Targeting these variable genomic regions may facilitate the development of strain typing tools for epidemiological investigations. Moreover, recent identification of a putative M60 family metalloprotease, potentially involved in virulence, highlights promising avenues for future research (Shon et al., 2021; Nylund et al., 2023). Functional studies, potentially employing gene editing approaches, will be essential to clarify the role of these genes. However, progress in this area remains constrained by the absence of a reliable *in vitro* cultivation system for SGPV.

5.5 Future approaches

One of the most significant remaining challenges in SGPV research is the development of a robust cultivation method. Although viral culture attempts in the present study were unsuccessful, this aligns with most European reports to date. The only published successful isolation involved a North American SGPV variant cultured in CHSE-214 cells (LeBlanc et al., 2019). The apparent disparity between North American and European SGPV isolates warrants further investigation, including comparative genomics and trials in additional Atlantic salmon gill cell lines.

Attempts to propagate SGPV in conventional fish cell lines, including Atlantic salmon kidney (ASK), Atlantic salmon gill (ASG-10) and Chinook salmon embryo (CHSE-214) cells, have been unsuccessful for the European variant of the virus. Under a range of incubation conditions, no evidence of viral replication or cytopathic effects was observed (Tartor et al., 2022). These findings highlight the fastidious nature of SGPV and the current lack of a permissive *in vitro* culture system. To date, true primary gill epithelial cell cultures have not been evaluated for SGPV and remain unexplored in the literature. Primary gill cells could provide a more physiologically

relevant environment for viral entry and replication, as they retain species-specific receptors and signalling pathways that are frequently altered or lost in immortalised cell lines. Indeed, primary gill epithelial cells from Atlantic salmon have been successfully used to investigate infection dynamics and cell tropism of other fish viruses, such as ISAV (Dannevig et al., 1995; Weli et al., 2013). Nonetheless, even primary monolayer cultures lack the three-dimensional tissue architecture and complex cell–cell interactions that may be critical for SGPV replication. For example, human papillomavirus (HPV) cannot be propagated in conventional monolayer cultures because it requires a living, stratified epithelium to complete its replication cycle, highlighting how certain viruses depend on intact tissue architecture for productive infection (Della Fera et al., 2021; Jin et al., 2024). Therefore, another avenue worth exploring in attempts to culture SGPV is the use of *ex vivo* organ explant systems. Explant cultures preserve native tissue organisation and cellular diversity that are lost in conventional monolayer systems (Anderson & Jenkinson, 1998; Antoni et al., 2015). This approach may be particularly suitable for SGPV, as available evidence suggests that both strict tissue tropism and host immune responses play a significant role in viral replication and disease progression. Support for this strategy is provided by the successful use of carp gill explants to culture CEV, another fish poxvirus, albeit with limited replication (Adamek et al., 2017). Moreover, explant systems allow controlled manipulation of environmental parameters such as temperature, oxygen availability, and CO₂ levels, facilitating direct investigation of how environmental stressors influence viral replication and host responses. A staged approach, in which gill explants are used to initiate or amplify infection prior to transfer into primary gill cell cultures, may therefore provide a pragmatic pathway toward establishing a reproducible *in vitro* model for SGPV and enable downstream studies of viral biology, immune modulation and virulence determinants.

Beyond virology, the application of machine learning represents a promising tool for SGPV disease management. In this study, combinations of temperature, CO₂, and pH were strongly associated with mortality in infected fish. Machine learning approaches are increasingly applied in aquaculture for monitoring fish size, weight, behaviour, and water quality (Xia et al., 2019; Li et al., 2020; Fernandes et al., 2020; Gladju et al., 2022). While environmental-based disease prediction remains relatively underexplored, similar models have successfully predicted disease outbreaks in shrimp and rainbow trout (Khiem et al., 2020; Yilmaz et al., 2022).

Although the predictive model developed here is limited by sample size and potential overfitting, its performance highlights the possibility of integrating environmental monitoring with

disease surveillance. Expansion of this approach across multiple sites and production cycles could provide a valuable decision support tool for mitigating SGPV outbreaks. Additionally, advances in digital pathology offer opportunities to enhance histopathological assessment of SGPV. Whole-slide imaging and machine learning-based image analysis have transformed human pathology and are increasingly applied in fish health research (Carmichael et al., 2023; Nam et al., 2020). In the present study, differences in gill pathology between Scottish and Norwegian outbreaks suggest that automated, standardised image analysis could improve comparative assessments and reduce observer bias. Integration of digital pathology with traditional scoring systems may represent a powerful approach for future SGPV research.

5.6 Conclusion

Salmon gill poxvirus represents a significant threat to Atlantic salmon aquaculture in Scotland, causing both high mortality and sub-lethal effects on growth and smoltification (Nylund et al., 2008; Gjessing et al., 2017). Despite its impact, understanding of the virus and the disease it causes remains limited. This study provides the first detailed investigation of a natural SGPV outbreak at a freshwater production site in Scotland, addressing key gaps in knowledge regarding factors influencing disease presentation. Our findings indicate that variation in clinical outcomes between two systems on the same site, with the same water and egg source, likely reflects differences in environmental parameters and their interactions. Several water quality measures were identified as potential risk factors for disease manifestation, whereas vertical transmission and biofilter-mediated spread appear unlikely in enclosed freshwater systems. Co-infection with other pathogens, particularly SAV, also appears to modulate SGPV disease, underscoring the multifactorial nature of outbreak dynamics.

Comparison of gill pathology between Scottish and Norwegian outbreaks revealed notable differences, particularly in the prevalence of epithelial apoptosis, suggesting that viral strain variation or host-specific factors may influence clinical outcomes. The sequencing of six new Scottish SGPV genomes, alongside the design of targeted primers, provides valuable tools to further investigate viral diversity and strain-specific effects. Observations of potential immune modulation in clinically affected fish also suggest that, like other poxviruses, SGPV may actively alter host immune responses, opening new avenues to explore viral pathogenesis.

Collectively, this study advances understanding of SGPV epidemiology, pathology, and host–virus interactions. By identifying both environmental and biological factors associated with disease variation, these findings provide a foundation for improved disease monitoring and management strategies in Scottish aquaculture, while highlighting key priorities for future research, including viral diversity, immune modulation, and the role of co-infections.

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Appendices

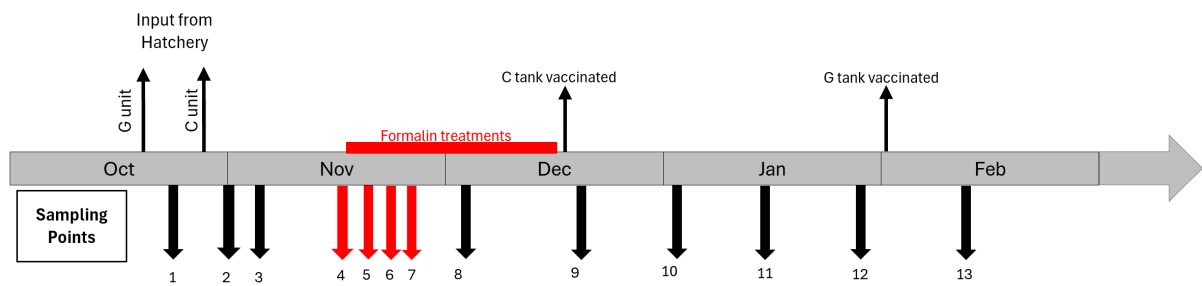


Figure 5.1: A timeline demonstrating the study period, notable events and sampling points. Red arrows indicate the intensive sampling period during clinical disease outbreak

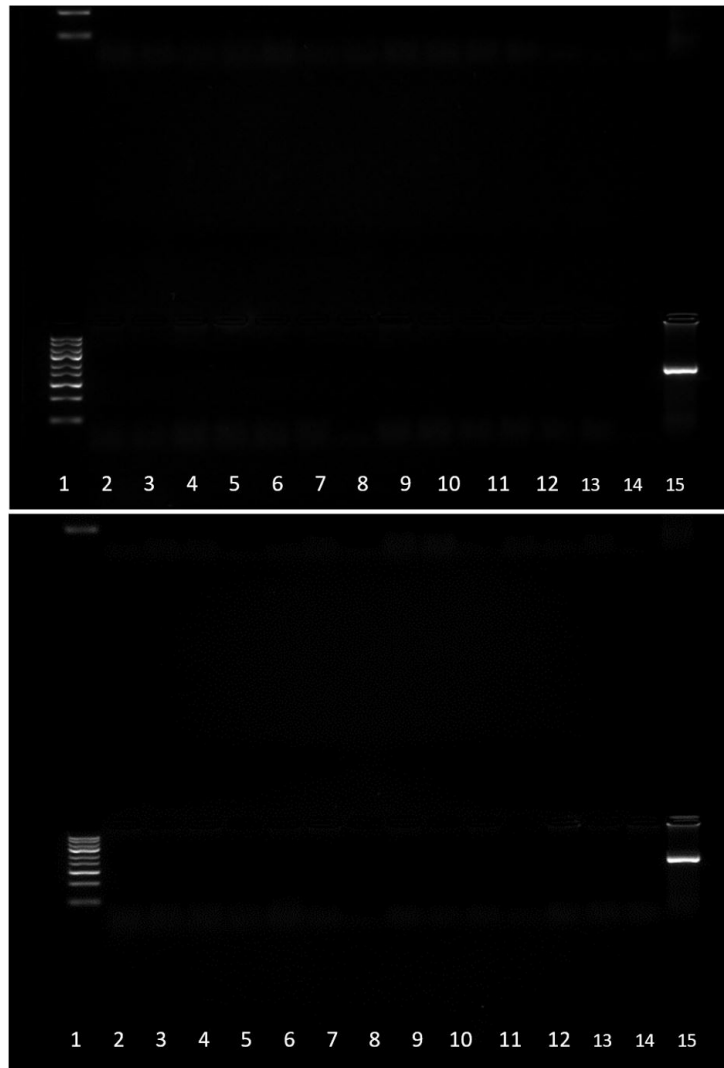


Figure 5.2: The agarose gels containing no amplification after PCR for SGPV detection was done on DNA from eggs produced by SGPV positive broodstock. DNA ladder in lane 1, the negative control in lane 8, and the positive control in lane 15 of each gel

Table 5.1: Date of each sampling point and the techniques that were applied to samples taken, with X indicating technique used

Sampling point	Date	Tank	SGPV qPCR	Background qPCR	Histology	Immune marker qPCR	NKA qPCR
1	23.10.18	G C	X	X	X	X	
2	1.11.10.18	G C	X	X	X	X	
3	5.11.18	G C	X X	X X	X X	X X	
4	15.11.18	G C	X X	X X	X X	X X	
5	19.11.18	G C	X X	X X			
6	22.11.18	G C	X X	X X			
7	26.11.18	G C	X X	X X	X X	X X	
8	3.12.18	G C	X X	X X			
9	17.12.18	G C	X X	X X	X X		
10	2.1.19	G C	X X	X X	X X		
11	14.1.19	G C	X X	X X			
12	28.1.19	G C	X X	X X			
13	11.2.19	G C	X X	X X	X X		X X

Table 5.2: Summary of samples, sources, and processing methods used

Sample type	Source	Processing method
Gill tissue samples	All fish sampled at each sampling point on Farm A	DNA and RNA extraction for qPCR
Histology samples	All fish sampled at each sampling point on Farm A	Formalin fixation, paraffin embedding and sectioning, for H&E staining, TUNEL assay and RNAscope
Geographical samples	SGPV Collected and submitted by Cooke Health staff	DNA extraction (where required), followed by SureSelect targeted enrichment prior to sequencing
SGPV positive eggs	Submitted by a commercial producer for diagnostic testing	HotShot-style DNA extraction followed by PCR
Gill tissue for viral culture	Collected and submitted by Cooke Health staff	Tissue homogenisation and inoculation onto CHSE-214 cell lines

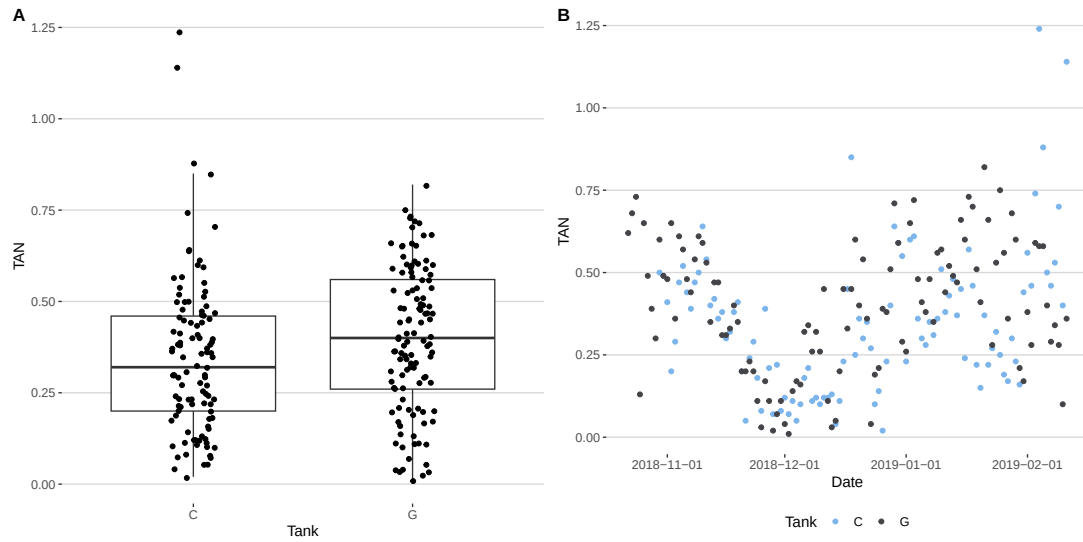


Figure 5.3: Summary of the TAN distribution **A.** per tank. **B.** over the study period.

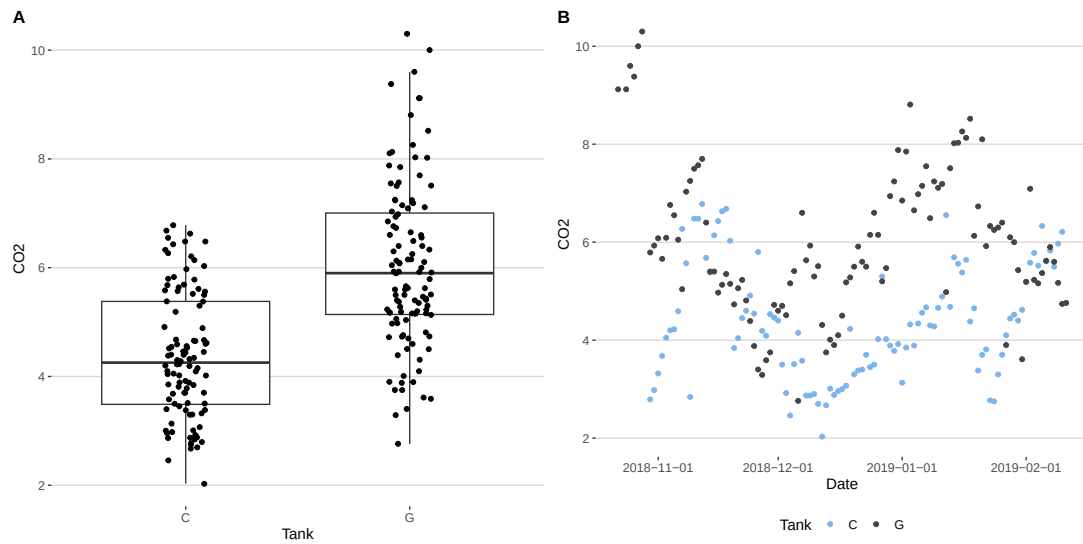


Figure 5.4: Summary of the CO₂ distribution **A.** per tank. **B.** over the study period

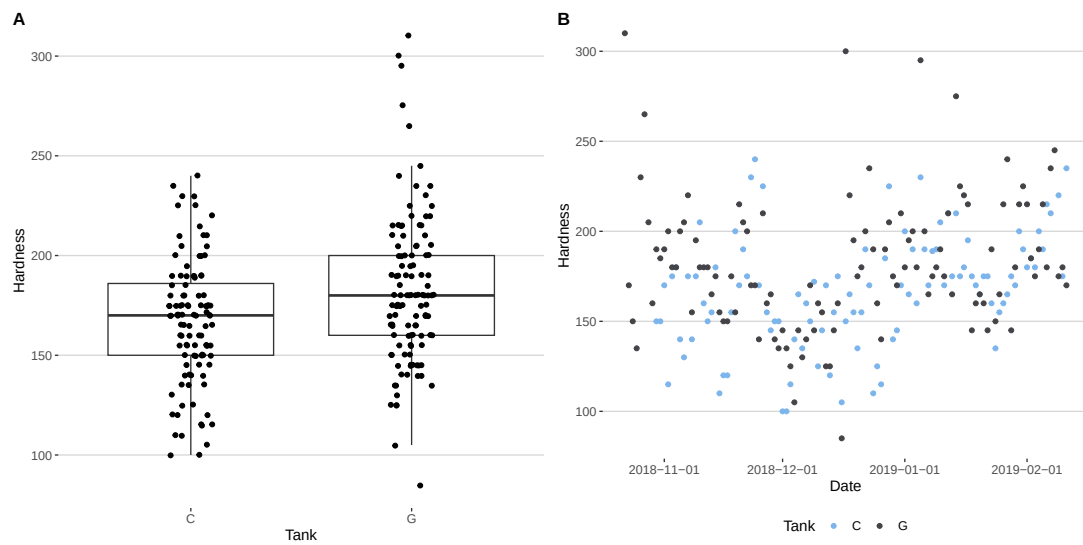


Figure 5.5: Summary of the water hardness distribution **A.** per tank. **B.** over the study period

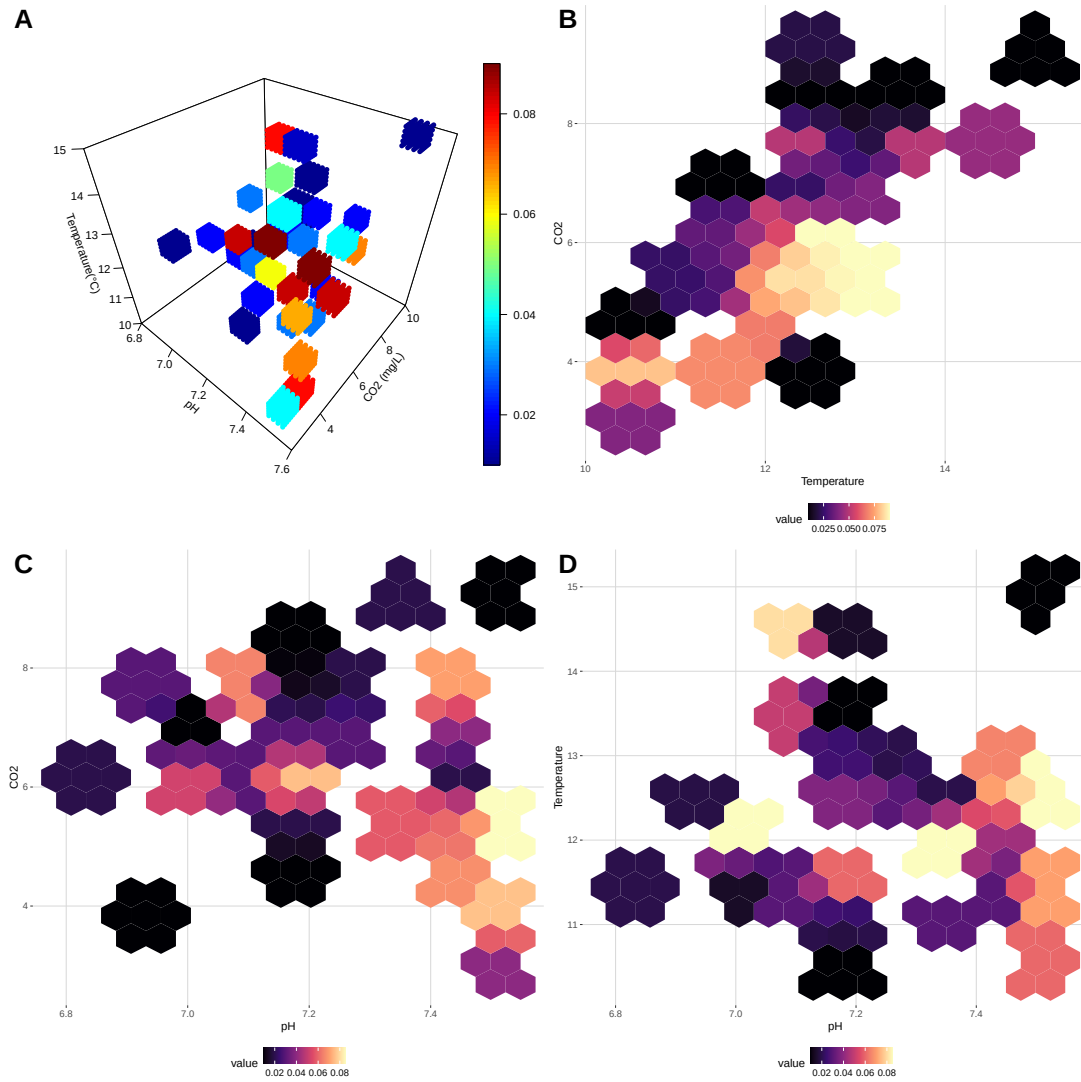


Figure 5.6: The combinations of pH, temperature and CO₂ predicted to be associated with least risk of mortality. **A.** 3D visualisation. **B.** The combination of CO₂ and water temperature predicted to be associated with least risk of mortality. **C.** The combination of CO₂ and pH predicted to be associated with least risk of mortality. **D.** The combination of Temperature and pH predicted to be associated with least risk of mortality

Table 5.3: The significance of the correlation between mortality and incoming water temperature, tank water temperature, pH, alkalinity, ammonia, bi-carbonate input, calcium, CO₂, water hardness, magnesium, nitrate, nitrite, salinity, salt input, and total ammonia (TAN). The correlation was determined using Spearman's test of correlation and Bonferroni's correction for multiple tests.

Days offset	C ₀	G ₀	C ₁	G ₁	C ₂	G ₂	C ₃	G ₃	C ₄	G ₄	C ₅	G ₅	C ₆	G ₆	C ₇	G ₇
Incoming water temp.	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Tank water temp.	1	1	1	1	1	1	1	1	1	1	1	1	0.58	1	1	1
pH	1	0.55	1	1	1	1	1	1	1	1	1	0.4	1	1	1	1
Total Alkalinity	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ammonia	1	1	1	0.83	1	0.32	1	0.55	1	1	1	0.69	1	0.09	-	0.38
Calcium hardness	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CO ₂	9.65·10 ⁻³	4.68·10 ⁻³	1.91·10 ⁻²	9.29·10 ⁻⁴	1.09·10 ⁻²	3.31·10 ⁻³	9.48·10 ⁻³	4.99·10 ⁻³	7.27·10 ⁻⁴	9.65·10 ⁻³	0.16056	1.56·10 ⁻³	1	3.07·10 ⁻⁴	1	2.66·10 ⁻³
Total Hardness	1	0.01	1	0.03	1	4.73·10 ⁻⁵	1	6.31·10 ⁻⁶	1	4.32·10 ⁻⁵	1	1.61·10 ⁻³	1	3.43·10 ⁻⁴	1	7.85·10 ⁻⁵
Magnesium	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Nitrate	1	1	1	1	1	1	1	1	1	1	1	0.01	1	0.67	1	1
Nitrite	1	1	1	1	0.34	1	1	1	0.46	1	1	1	1	1	1	1
Salinity	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.19
TAN	1	4.10·10 ⁻⁴	1	4.34·10 ⁻⁴	1	2.45·10 ⁻⁴	1	8.04·10 ⁻⁵	1	2.42·10 ⁻³	1	1.38·10 ⁻⁵	1	8.81·10 ⁻⁷	1	2.06·10 ⁻⁵
Added Bi-carbonate	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Added Salt	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Table 5.4: Detailed median histopathological scores for each features in the tanks C and G. N/A represents the timepoints that were not sampled for each tank.

Tissue	Feature	Tank	2018-10-23	2018-11-01	2018-11-05	2018-11-15	2018-11-26	2018-12-17	2019-01-02	2019-02-11
Gill	Epithelium hyperplasia	G	1	N.A.	1	1	0.5	1	1.5	1
	Epithelium hyperplasia	C	N.A.	0	0	0	0	0	0	0
	Mucous cell hyperplasia	G	0	N.A.	0	1	0	0	0	0
	Mucous cell hyperplasia	C	N.A.	0	0.5	0	0	0	0	0
	Apoptosis	G	0	N.A.	0	1	0	1	0	1
	Apoptosis	C	N.A.	0	0.5	1	0	1	2	2
	Lamellar inflammation	G	1	N.A.	0	1	1	1	2	1
	Lamellar inflammation	C	N.A.	0	0.5	0.5	1	0	1	1
	Filament inflammation	G	1	N.A.	0.5	1.5	1	1	2	2
	Filament inflammation	C	N.A.	0	1	0	1	0	0	0
	Hypertrophy	G	1	N.A.	1	1	0.5	1	2	0
	Hypertrophy	C	N.A.	1	0	0	1	1	0	0
	Clubbing	G	1	N.A.	0	1	0	0.5	0	0
	Clubbing	C	N.A.	0	0	0	0	0	0	0
	Melanin deposits	G	0.5	N.A.	0	0	0	0.5	0.5	0
	Melanin deposits	C	N.A.	0	0	0	0	0.5	0.5	0
Liver	Lipidosis	G	1	N.A.	2.5	1	0.5	0	0.5	1.5
	Lipidosis	C	N.A.	0.5	2	1	0.5	0	1.5	1.5
Heart	Necrosis	G	0	N.A.	0	1	1	2	1	0
	Necrosis	C	N.A.	0.5	0	0	0	0	0.5	0
	Inflammation	G	1	N.A.	0.5	1	2	3	2.5	1
	Inflammation	C	N.A.	0.5	0.5	1	1	1	1	0.5
Skin	Inflammation	G	1	N.A.	0	1	0	1.5	1	0
	Inflammation	C	N.A.	0	0	0	0.5	0	0	0
Red muscle	Inflammation	G	1	N.A.	1	2	2	3	2	2
	Inflammation	C	N.A.	1	1.5	1	1.5	2	1.5	1
	Degeneration	G	0.5	N.A.	0	1	1	1.5	1	1
	Degeneration	C	N.A.	0	0	1	1	1	0	0
White muscle	Inflammation	G	1	N.A.	1	1.5	1	2	2	2
	Inflammation	C	N.A.	1	1.5	2	2	1	1	1
	Degeneration	G	0	N.A.	0.5	1	1	1.5	1.5	1
	Degeneration	C	N.A.	0.5	1	2	1.5	2	1	1.5