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*IN VIVO AND IN VITRO STUDIES ON
SALMON PANCREAS DISEASE VIRUS (SPDV)
IN ATLANTIC SALMON (SALMO SALAR L.)*

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Thesis submitted for the degree of Doctor of Philosophy

University of Edinburgh

2017

DECLARATION

I declare that this thesis was composed by myself, that the work contained herein is my own and where collaboration was received this is explicitly stated in the text. This work has not been submitted for any other degree or professional qualification.

Two publications as senior author are included in this thesis. The work presented in Chapter 3 corresponds to Paper 1: *An in vitro model to study viral host pathogen interactions and pathogenesis*, Patricia A. Noguera, Bianka Grunow, Matthias Klinger, Katherine Lester, Bertrand Collet and Jorge del Pozo. PLOS ONE (2017), Vol:12 (7) pp:22.

The work presented in Chapter 4 corresponds to Paper 2: Use of Salmon Cardiac Primary Cultures (SCPCs) of different genotypes for comparative kinetics of mx expression, viral load and ultrastructure pathology, after infection with Salmon Pancreas Disease Virus (SPDV), Patricia Noguera, Bertrand Collet, Matthias Klinger, Hristo Örün, Jorge del Pozo. *Fish and Shellfish Immunology* 72 (2018) 181–186.

They both are my own work, I carried out the experiments, data analysis and the writing of the final papers; co-author's contributions are specified and acknowledged.

Additionally, 2 publications as co-author are added under the Annex as A and B. They represent collaborative work with parallel projects related to the subject of this thesis.

- A method to measure an indicator of viraemia in Atlantic salmon using a reporter cell line. Bertrand Collet, Katy Urquhart, Patricia Noguera, Katrine H. Larsen, Katherine Lester, David Smail, David Bruno, *Journal of Virological Methods* Vol.191 (2013) 11t– 117.

I was responsible for the design and carried out one of the two experiments from where data was collected, and contributed to data analysis and on the writing of the final paper.

- Identification of a wild reservoir of Salmonid alphavirus in common dab *Limanda limanda*. with emphasis on virus culture and sequencing, D. W. Bruno, P. A. Noguera, J. Black, W. Murray, D. J. Macqueen, I. Matejusova, *Aquaculture Environment Interactions* Vol. 5: 89–98 (2014). I collaborated with the data and material collection and was responsible for histopathological assessment of the tissue samples and contributed as well in the writing of the final paper.

In both cases permission to include these papers on the current thesis has been obtained before its submission.

Patricia A Noguera

ACKNOWLEDGEMENTS

My expressed gratitude to Marine Scotland Science (MSS) who supported this thesis framed under a research project on Salmonid Alphavirus, performed at large at the Marine Laboratory in Aberdeen.

Many people deserve credit for helping me conduct and complete this PhD, but first of all I want to acknowledge the encouragement and support received by my former colleague at MSS, line manager for years and good friend, Dr David Bruno. As the leader of the research project that framed this thesis, David played a fundamental role and I am grateful for his open mind attitude and interest on developing different and novel approaches which translated on the support for this thesis.

I am also very grateful to my MSS supervisor Dr Bertrand Collet, whose wide knowledge on fish immunology was particularly helpful for the work. My gratitude extends to former and current MSS colleagues Katherine Lester, Dr Catherine Collins, Dr Iveta Matejusova, Dr Una McCarthy and Dr Malcolm Hall, who contributed on different aspects and circumstances, some sharing their knowledge and expertise, others with fruitful discussions and guidance. I am also especially thankful for the laboratory and technical support provided by Julia Black, Nichola Still, Judy Simons and Mark Fordyce. An extended thanks goes to a number of other people who simply but importantly, were there with their encouraging support, patience and friendship!

Professor John Fazakerley, formerly University of Edinburgh, provided valuable initial guidance as my first principal supervisor until assuming a new role as director of the Pirbright Institute. Late Professor Stephen Bishop continued in the role and became a most supportive supervisor during the unfortunately limited time until his unexpected far too early departure. I am also especially grateful to Professor Bernadette Dutia who was incredible supportive, had always an open door and a smile in spite of the many difficult times along these years. And finally, I am most definitely in deep gratitude to my last designated principal supervisor Dr Jorge del Pozo. Jorge's involvement, commitment and responsibility to the role has been remarkable and made all the difference. A massive thank you Jorge, without you this simply would have not happened!

I dedicate this work to my extraordinary family, and very specially to my wonderful sons Martin and Antu ;-)

LAY SUMMARY

Farmed Atlantic salmon (*Salmo salar* L.) produced across 4 continents contributes globally to food security and regionally to social and economic development. Salmon is the biggest selling seafood product in the UK and particularly for Scotland, its economical relevance is highlighted by the job opportunities and prosperity the farming activity brings to the country's most remote coastal areas and rural communities.

A major constrain to profitability and expansion of a sustainable production is the impact of disease, with viral conditions among the most serious.

Historically, Pancreas disease (PD) and Sleeping disease (SD) were recognised independently affecting Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*), respectively. Both were found to be induced by closely related isolates of the same virus species, Salmon Pancreas Disease Virus (SPDV). The aetiological agent belongs to the genus Alphavirus within the family Togaviridae and infection can lead to clinical disease with characteristic acinar pancreatic necrosis and a range of myopathies (skeletal and heart muscle). Mortality is not a necessary outcome and it is frequently not significant. However, severely affected fish stop eating and therefore have reduced growth rate and fish can also present visible lesions at the fillet level leading to downgrading at time of slaughter.

The current thesis developed at Marine Scotland Science (MSS), the Scottish National Reference Laboratory, was policy driven to produce work which would contribute with knowledge gaps identified by the industry and research communities.

The focus of the thesis was to improve *in vivo* and develop *in vitro* infection models, aiming to assist with studies on the host pathogen interaction, primarily of SPDV but also other cardiotropic conditions affecting Atlantic salmon.

The *in vivo* work resulted in the first successful co-habitation challenge model applied by MSS, working in sea water with Atlantic salmon post smolts, the developmental stage predominantly affected by the disease. The *in vitro* work accounted for the more innovative part of this thesis. An *ex vivo* cardiac tissue culture originated from Atlantic salmon embryos were explored as a species-specific model for studies of viral host -pathogen interactions. While *in vitro* fish based models had been previously developed and extensively applied in biomedical research, paradoxically they have been much less explored as disease model for fish health issues. The work involved an adaptation and refinement to produce salmon cardiac primary cultures (SCPCs). To test permissiveness to viral infections, SCPCs were successfully challenged with SPDV as well as other cardiotropic viral agents. The kinetics of viral infection and some aspects of the immune response could also be studied. SCPCs were maintained under laboratory conditions with minimal support for a period up to 6 months. Moreover, their potential to examine genotype-based differences linked to their embryo of origin was explored, and the model was also used to gain insights on the ultrastructural morphology of SPDV replication cycle by transmission electron microscopy examination.

With SPDV and other virus associated myocarditis affecting Atlantic salmon aquaculture, the SCPCs model represents the most relevant contribution of this PhD.

ABSTRACT

Salmon Pancreas Disease Virus (SPDV) is the only viral species of the genus *Alphavirus*, family *Togaviridae*, affecting fish.

SPDV induces two conditions historically recognised independently as Pancreas disease (PD) and Sleeping disease (SD), affecting Atlantic salmon (*Salmo salar* L) and rainbow trout (*Oncorhynchus mykiss*), respectively. Infection by SPDV can lead to clinical disease with characteristic acinar pancreatic necrosis and a range of myopathies of the skeletal and heart muscle. Mortality is not a necessary outcome of the disease and usually is not significant. However, affected fish stop eating and therefore present a reduced growth rate and the disease can also leave visible lesions at the fillet level that lead to downgrading at slaughter. SPDV can affect in the fresh and sea water environments, but a higher and most relevant impact reported in the latter. Historically, PD has posed a significant challenge to the Atlantic salmon farming industry in the UK, as well as in other salmon producing countries.

This thesis was developed and conducted at Marine Scotland Science (MSS), the Scottish National Reference Laboratory, with the aim to contribute to knowledge gaps identified by the industry and research communities. The focus was on development and improvement of *in vivo* and *in vitro* infection models to assist with host pathogen interaction studies.

In vivo work was to establish an experimental challenge model to induce SPDV infection in a more natural way than by intra-peritoneal (IP) injection. The first step involved selection of an infective SPDV isolate through a comparative IP challenge study. An infective isolate was then used to establish a co-habitation challenge model in “post smolts”, the sea-water stage predominantly affected by PD. Additionally, during this experiment assessment of viral tissue tropism along time and potential intra-subtype differences in infectivity was undertaken.

In vitro work accounted for the more innovative part of this thesis with the development, optimization and application of an *ex vivo* cardiac primary culture originated from Atlantic salmon embryos. While fish origin aggregates of self-contracting cardiomyocytes had been previously isolated and suggested as a robust tool on human biomedical research and pharmacological and toxicology testing, paradoxically very little has been done to explore the approach of *ex vivo* primary cultures as a disease model with the specific goal for health issues affecting fish. The work involved an adaptation and refinement to produce salmon cardiac primary cultures (SCPCs). Once this was achieved, SCPCs could be kept under laboratory conditions with minimal maintenance for periods up to 6 months. Following this work, SCPCs were successfully challenged with different SPDV isolates as well as another cardiotropic viral agent (Infectious Salmon Anaemia, ISA). The kinetics of SPDV and ISA viral infection and one element of the immune response (i.e. expression of *mx* gene) were studied. As part of this study, the comparative response of SCPCs of diverse genetic backgrounds (i.e. IPN resistant vs. IPN sensitive) was also assessed. Differences were observed, which highlights potential usefulness of SCPCs to examine genotype-based differences in response to viral disease. Finally, SCPCs were used to examine the SPDV infection cycle ultrastructure by transmission electron microscopy (TEM). This work resulted in novel insights on the replication cycle of SPDV, drawing from the extensive literature in mammalian alphavirus work.

With SPDV and other virus associated myocarditis severely affecting Atlantic salmon aquaculture at present, I believe that the SCPCs model represents the most relevant contribution of this PhD.

Table of Contents

Declaration	i
Acknowledgements	ii
Lay Summary	iii
Abstract	v
Chapter 1: Introduction	3
Aquaculture and food security	3
Atlantic salmon.....	5
Farming of Atlantic salmon	7
Industry challenges: health management.....	8
Intrinsic factors	8
Extrinsic factors	10
Nutritional	10
Environmental	10
Husbandry	11
Diseases.....	11
Alphavirus: the agent.....	12
Alphavirus structure.....	13
Alphavirus replication cycle	15
Salmon pancreas disease virus (SPDV)	19
SPDV subtypes and serology.....	19
SPDV: the disease	21
Clinical signs and gross pathology.....	23
Histopathology.....	25
Disease diagnosis	30
Prognosis	30
Differential diagnoses.....	31
Disease control.....	31
Viral tissue tropism, pathogenesis and host response.....	33
Knowledge gaps, the future and thesis objectives	36
References.....	39
Chapter 2: SPDV virulence and tissue tropism	54
Introduction	54
Materials & Methods	56
Results	58
Discussion.....	63
References.....	66

Chapter 3: Experimental comparison of SPDV-1 using a co habitation sea water model.....	70
Introduction	70
Materials and methods	72
Results	74
Discussion.....	78
References.....	81
Chapter 4 : Salmon cardiac primary cultures, a species-specific <i>in vitro</i> model to study Atlantic salmon cardiotropic conditions	85
Introduction	86
Paper 1	86
Conclusions	108
References.....	109
Chapter 5: Exploring applications of scpc_s: assessment of embryo genotype influence and description of ultrastructure pathology after infection with spd_v	110
Introduction	111
Paper 2	111
Conclusion	118
References.....	118
Chapter 6: Ultrastructural insights into the replication cycle of spd_v using scpc_s.....	119
Introduction	119
Material and Methods	121
Results	122
Discussion.....	124
Entry mechanisms	125
Post internalization events	128
Viral assembly and budding	129
Additional observations	130
Conclusions	131
References.....	138
Final discussion.....	146
Annex	149
For chapter 1.....	149
Attendance and presentation of work related to this thesis along the period of study: ...	150
Additional papers	150

CHAPTER 1: INTRODUCTION

Aquaculture and food security

With a general decline in the world's marine fisheries since the late 1990's, farmed fish and shellfish production has been called to play a bigger role in the supply of an increasing demand for fish and other aquatic food species. World fish consumption per capita reached over 20kg a year in 2014, a record influenced by the combined effect of management improvement on some stock fisheries but significantly, by the vigorous growth and contribution of aquaculture which now provides 50% of all fish for human consumption [1]. Moreover, the actual share of wild fish stocks exploited within biologically sustainable levels adds a further concern, as it has declined from 90% in the 1970s, to 68.6 % by 2013 [1] resulting in over 30% of the stocks effectively being overfished. In this context, aquaculture has become the world's fastest-growing agricultural business [2] (Fig. 1).

The production of salmonids by aquaculture has markedly increased in the past 30 years at the time their capture has declined steadily (Fig 2) and while not the largest by biomass within world fish global production, salmonids are a highly valued commodity due their important input to the regional economy of several countries. This is reflected by the strong increase of world trade in salmonids, becoming the largest fish commodity (by value) in 2013 [1].

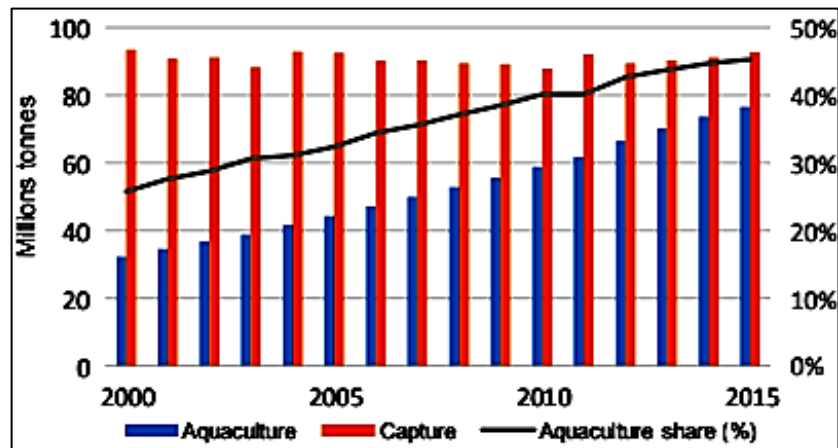


Fig 1: Current status of the contribution from capture and aquaculture to the global world totals of aquatic animals. Source: <http://www.fao.org/3/a-bs235e.pdf>

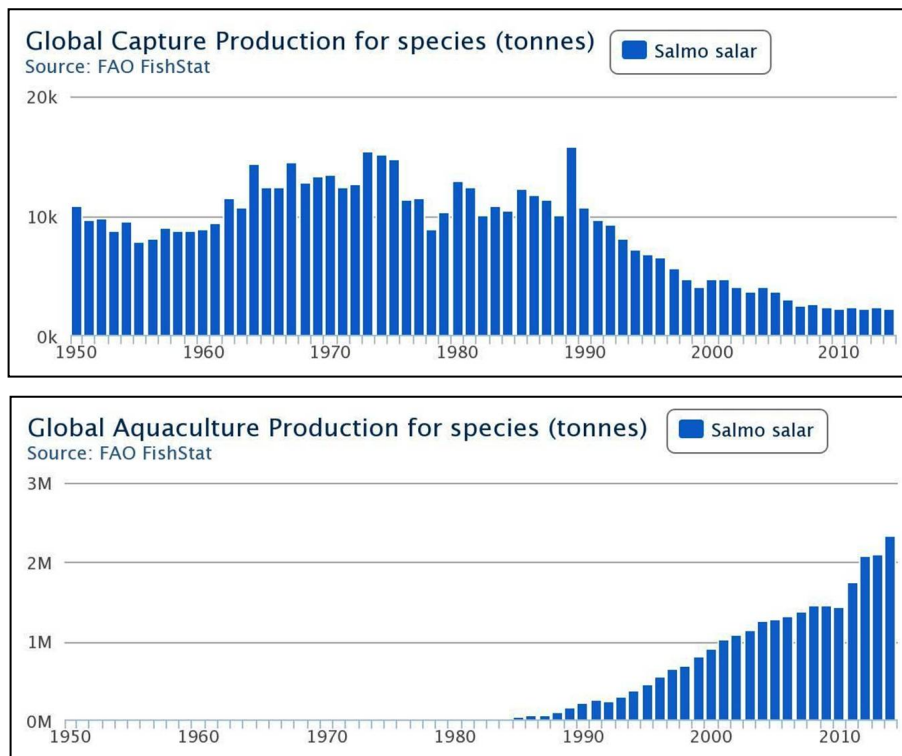


Fig 2: Capture versus production of Atlantic salmon. Source: FAO Species Fact Sheets <http://www.fao.org/fishery/species/2929/en>

In Scotland, the aquaculture industry is dominated by the production of Atlantic salmon (*Salmo salar*, Linnaeus, 1758). This historically iconic fish is a pride for a country that is home to some of the finest salmon rivers in the world (see <http://www.salmonatlas.com/>; <http://www.salmonfishingscotland.com/>). Farming of Atlantic salmon has also its origin in the UK, with the early work in the 19th century focusing on the freshwater production of juvenile fish to re-stock rivers aiming to enhance the returns. In Scotland Atlantic salmon is highly valued in terms of its ecological role as central to aquatic biodiversity, water quality and environmental changes indicator and a key stone species for recreational fisheries [3].

Years later, salmon production in Scotland pioneered the aquaculture industry with 50 tonnes harvested in 1973, rising to 28,000 tonnes 15 years later and reaching 152,000 tonnes by 2013 [4]. Farmed Atlantic salmon is currently the biggest-selling seafood in the UK, making a substantial contribution to the Scottish economy estimated at £670m in 2015 [5]. Its economical relevance is compounded by the job opportunities and prosperity aquaculture has brought to many of the country's most remote coastal areas and rural communities [6].

Salmon is currently produced in countries across 4 continents and is estimated to provide directly and indirectly over 120,000 jobs and up to \$10 billion US dollars globally [4]. The

main producers are Norway, Chile, Scotland and Canada, followed by Ireland, USA, New Zealand, Australia (Tasmania), Denmark (Faroe Islands) and Iceland (Fig 3).

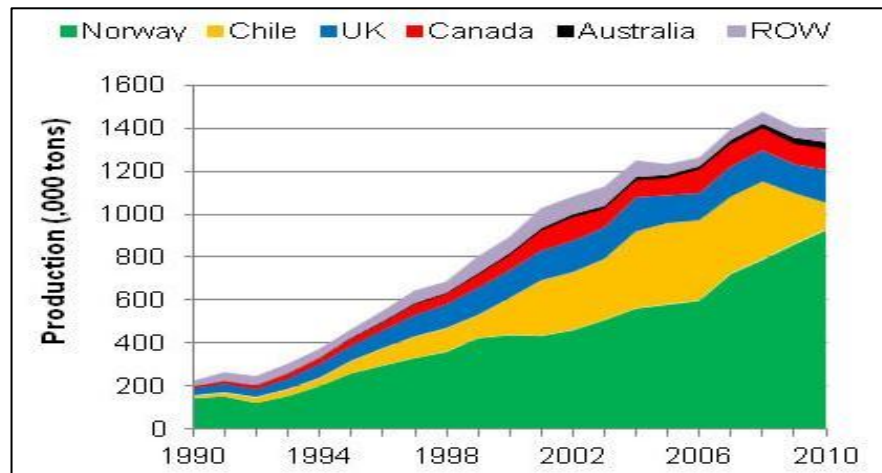


Fig 3: Total Atlantic salmon production by country from 1990 to 2010. ROW: rest of the world. Source: FAO Fishstat.

In order to support the growth of salmon aquaculture, reliable research data is essential for the implementation of evidence-based policies to balance the environmental sustainability with the socioeconomic management of the resources available [7].

Atlantic salmon

Atlantic salmon belongs to the family Salmonidae within the order Salmoniformes. They are diadromous fish (migratory between fresh and sea water), specifically anadromous, therefore adults live in the sea and migrate to fresh waters to breed [8].

The life cycle of Atlantic salmon starts with spawning in fresh water. Salmon will migrate up river to different locations depending of the time they enter the fresh water. The mature females build nests in the gravel where her eggs will be deposited and fertilised by the sperm of one or several males. The eggs are then covered with layers of gravel forming the "redd" where incubation takes place. The location for spawning along the river varies and will occur first in the higher reaches of the rivers and, progressively, at lower altitudes later on the season. The average winter temperatures are higher at lower altitudes and consequently, and in spite of the later spawning, hatching takes place first at low altitudes and progressively at higher altitude locations later [9]. This suggests that spawners match their spawning time with the winter temperature characteristics of their location and as a result, the timing of fry emergence tends to match the advent in late spring of local stream conditions that are favourable for their survival [9].

The young fish, known as “alevin” or “sac fry”, retain a yolk sac from where they feed for a few weeks while remaining in the protected environment of the redd. With the sac fully absorbed and approximately 3cm in length, they emerge from the gravel as “fry” to start independent feeding. Once more, temperature determines not only the rate of development and the hatching time, but also the date at which the yolk-sac is fully reabsorbed and therefore the beginning of external feeding. The fry grows up to become a “parr”, identifiable by the development of conspicuous flank marks. Parr continue feeding and growing for one or more years until they are ready for “smoltification”. This is a complex adaptive process involving coordinated changes in the morphology, biochemistry, physiology and behaviour, that prepares parr for downstream migration from the river into the sea [10]. As fish become “smolts”, significant changes are observed in their appearance, turning silvery and slender, losing positive rheotaxis (facing the water current) and abandoning the bottom dwelling behaviour to start schooling downstream [11]. The duration of this process depends on water temperature and food availability, and usually occurs at 2-3 years of age in Scotland. Interestingly, if a smolt fails to enter the marine environment within a certain period they will undergo a relatively quick “de-smoltification” process until the following season [11,12]. Overall, the most common river age (time spent in this environment) for salmon is 2–4 years, and most common sea age at first spawning is 1–3 years [13].

Life at sea is challenging with growth and survival depending on factors including sea surface temperature, competition for food, predation, fishing, pollution and infection by parasites and other disease agents. The marine phase is effectively a serious challenge that drastically reduces the numbers of fish that will reach adulthood and return to the rivers to start the cycle again [14].

During oceanic life, adult salmon grow rapidly on a diet of crustaceans and small fish. Adult salmon undertake extensive migrations which, depending on where they start their journey, can take them to the coasts of Norway, the Faroe Islands or south-western Greenland [15]. By the time they become sexually mature, they will have spent one or more years at sea. At this point, salmon migrate back to the river where they originated re-entering at different times the year, but mainly between April and November. This depends mainly on whether they return after one year (“grilse” with average weights of 2-3kg and summer entry), or have been several years at sea (“Multi-Sea-Winter fish” or “salmon”, which returns earlier in the spring). The latter have been longer at the feeding grounds and are much larger animals, reaching up to 15kg [12,16]. Once in fresh water, adults do not feed and live on accumulated reserves until spawning in the autumn. Unlike their Pacific relatives (*Oncorhynchus spp.*), Atlantic salmon is iteroparous, meaning a proportion can survive reproduction and go back to the ocean to feed the following spring, returning again to spawn the following year [8]. The above life cycle is illustrated in Fig 4.

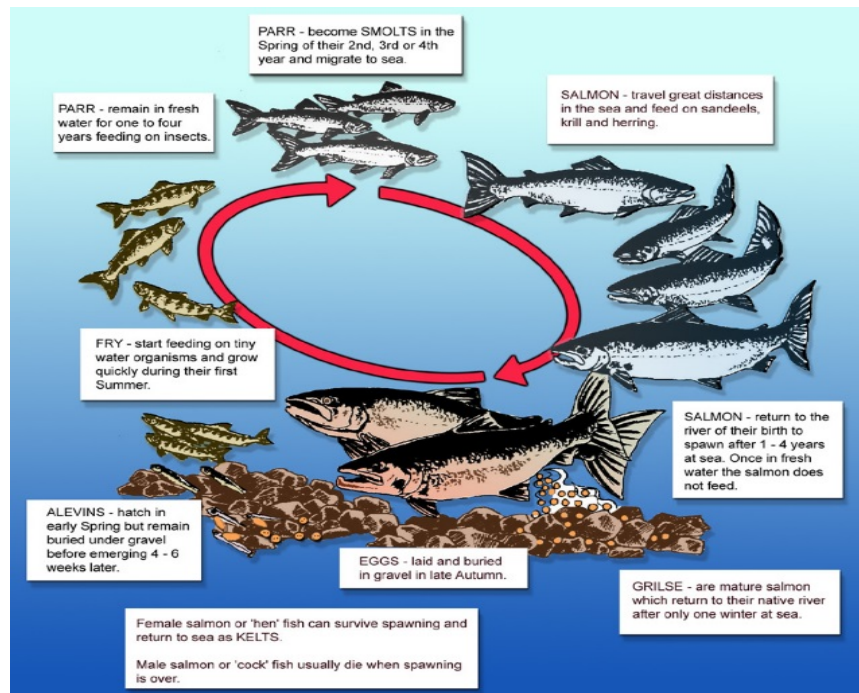


Fig 4: Atlantic salmon life cycle. Sourced at “Salmon in the classroom”.
http://www.snh.org.uk/SalmonintheClassroom/salmon_lifecycle.shtml

In summary, wild Atlantic salmon is a highly valued biological and economic resource which faces many challenges and uncertainties, particularly those that compromise survival during their oceanic life. Therefore, the species is the focus of considerable efforts, commitment and determination by the international community to support their conservation and the preservation of their habitats [14,17]. Additionally, as mentioned above Atlantic salmon is one of the main aquaculture species worldwide.

Farming of Atlantic salmon

The production of farmed Atlantic salmon aims to simulate the natural lifecycle up to the adult stage. In this respect, farmed fish are hatched, raised and harvested under controlled intensive rearing systems. Production still requires both fresh and sea water phases. The former is performed in land-based hatcheries from egg incubation to pre-smolt stage, and the latter in sea water enclosures starting from transfer of the pre-smolts to sea until harvest between 18 and 22 months later [18]. The majority of the industry transfers smolts to cages in open water systems for on-growing to harvest weight. In recent years, some companies in Scotland, Norway and Chile are investing into Recirculation Aquaculture Systems (RAS), enabling land-based production of smolts. The rationale behind the RAS includes the reduction in the mortality typically associated with smolt transfer to sea which can account

for up to 20% of the stock [19]. Farmed smolts show high vulnerability when they first encounter the marine environment and the right timing for transfer to full sea water is crucial. Transfer is frequently accompanied by increased disease susceptibility, mortality and a period of reduced feed intake and growth rates [20]. Therefore, the use of RAS expects to achieve stronger and larger smolts before they are put into sea water and a shorter sea growing stage.

The salmon industry is tightly controlled, and companies normally have in place strict health management, bio-security and emergencies contingency plans. In-house veterinary support, regular disinfection procedures and disease mitigation programs are the norm. Many also voluntarily comply with government managed monitoring and surveillance schemes for the prevention of specific diseases and coordinate synchronised zone/area management programs [21,22].

Several factors challenge the sustainable growth of the salmon farming industry including economic, environmental, nutritional and health issues. In this thesis I focus on a specific fish health related matter and therefore, this overview will also focus on fish health factors.

Industry challenges: health management

Unfortunately, one of the major constraints to the successful expansion of a sustainable salmon farming industry is still the management of health issues and disease control [23,24]. This hampers development due to significant economic implications, but it also poses a serious animal welfare concern [25,26].

Health challenges can arise and be influenced by different factors, which may be intrinsic or extrinsic to the fish.

Intrinsic factors

The inherent characteristics of fish (species, sex, reproductive stage, genome, etc) are the most obvious and relevant intrinsic factors. In the selection of a new species for production, one of the initial most relevant factors is the capacity to adapt to the farming condition where selective pressures comes into play, favouring the developing of those organisms that are better suited for it. This process is referred to as “domestication” [27]. Additionally, some commercial production systems involve the use of monosex and sterile stocks, benefiting from the optimization of production strategies and reproduction containment. In some species, one sex grows faster, matures later or is of higher commercial value. For

example, male salmonids normally mature a year earlier than the females, and therefore the combination of monosex and sterility serves the dual purpose of preventing the growth loss associated to the development of reproductive structures and avoids the risk of potential interbreeding of escapes with wild populations [28]. However, production of sterile triploid Atlantic salmon has also health issues to overcome, such as a higher incidence of cataract and heart and skeletal deformities [29,30].

On the other hand improving the genomic background through selective breeding for enhanced performance has been for years the focus of both the producers and researchers alike [31]. Part of the work presented here is focused on the effect of genomic background on disease; hence the following expanded review of this subject.

Compared to that of terrestrial livestock, the selective breeding of Atlantic salmon is relatively new [32]. However, major and accelerated advances have taken place in the past ~20 years and today specialised professional breeding companies offer embryonated ova with desirable trait/traits that are tailored to the challenges encountered by the industry. The selection of candidate brood stocks is performed by different approaches, usually assisted by identification of suitable individuals by detection of genetic markers associated with the selected trait [33].

From the breeder's perspective, desirable traits include improved robustness, fillet colour and growth, delayed sexual maturation, production of sterile fish and importantly, resistance to disease [34]. Many characteristics are usually affected by a large number of genes that individually have a small effect on the trait, but the combined effect explains a large part of genetic variation. One of the genetic markers used is the QTL (quantitative trait locus) a genetic locus, the alleles of which affect a given variation. Generally, quantitative traits (a measurable phenotypic variation) are multifactorial and influenced by several polymorphic genes and environmental conditions. Therefore one or many QTLs can influence a trait or a phenotype [35]. The identification of a QTL (and occasionally its functional analysis) allows to link two types of information, the phenotypic data (the measure of the trait) with the genotypic data (one QTL or a combination of QTLs), in order to explain the genetic basis of variation [36].

A quite rare and successful case has been the discovery of the QTL associated with resistance against a viral disease known as Infectious Pancreatic Necrosis (IPN). IPN resistance was found not only to be heritable, but that the trait was almost entirely explained by the variations in a single QTL located on linkage group 21 (LG 21) with significance at the genome-wide level [32,37]. This soon resulted in breeding companies implementing this QTL in marker-assisted selection (MAS) and the production of ova leading to fish with a high resistance for IPN [37]. Similarly, markers associated with a major QTL affecting resistance

to Pancreas disease (PD) induced by *Salmon pancreas disease virus* (SPDV) and focus of this thesis have been recently reported to being incorporated into selective breeding programs to improve PD resistance [38].

Recently, another major step forward has been the development of a powerful exploratory tool that enables the efficient capture of a high numbers of QTLs associated with a given trait: SNP chips. This technology involves the use of a high-density map made of Single Nucleotide Polymorphisms (SNP) markers. The use of SNP chips enables the correlation between the predisposition to disease and the genotype at hundreds of thousands of loci per fish [39]. This procedure is called Genomic Selection (GS), and it overcomes the challenge of the traditional method of identification of QTL analyses based on low number of markers, where only a limited proportion of the total genetic variance is captured by a single marker.

Selection of broodstock based on genetic markers has effectively started a new era within the field of salmon breeding and currently, ova selected for several traits including resistance to viral, bacterial and parasitic disease are commercially available [40].

Extrinsic factors

Extrinsic factors influencing cultured fish health arise from a wider spectrum of variables, including nutritional, environmental, husbandry, and the presence/absence of specific pathological agents. These are briefly outlined below.

Nutritional

As with any livestock, nutrition has strong effects on fish stress tolerance and general performance. An adequate supply of balanced nutrients is paramount for normal growth, as well as for maintenance of an efficient immune system. This encompasses the mechanisms to fight against potential pathogens, linking nutrition with health status directly [41]. There are also pathological conditions that are the direct result of nutritional deficiency/imbalance such as hepatic lipoidosis, steatitis, enteritis or skeletal deformities associated to vitamin deficiencies, for example [42].

Environmental

Among the environment factors, water quality plays a most critical role and can significantly affect fish health. Variation of key water quality parameters outside acceptable ranges for the species can lead to stress, distress, impaired health and mortality [43]. Water quality is usually more stable in the marine environment, and is more critical during the fresh water phase, especially in recirculation (closed) systems. Relevant parameters include temperature, dissolved oxygen, pH, alkalinity, salinity, water hardness and dissolved organic

matter. All of these need to be monitored and maintained in accordance to the species requirements and the culture conditions. For example, a most common problem in farmed fish is associated with oxygen, where both hypoxia and hyperoxia can result in vascular and metabolic disorders that lead to acidosis and death [42].

Husbandry

Husbandry can have a major influence on the fish and their welfare. Practices such as grading, transport, strategies for predator's control, vaccination, following periods (or lack thereof) are some examples. Another major example is associated to inappropriate stocking densities, which can lead to acute welfare infringements or to a chronic reduction in welfare status, as well as facilitating horizontal transmission of disease [44].

Diseases

Many of the factors above described have been optimized and minimized during the development of aquaculture practices but the presence of bacterial, viral and parasitic diseases still remains at the top of the production challenges, generating the largest economic losses in salmon farming. An unfortunate and spectacular example for the salmon industry has been a viral disease, infectious salmon anaemia (ISA). Outbreaks, in Norway (1984), Scotland (1998), and Chile between 2007 and 2009, resulting in unprecedented economic losses and severe social impact [45].

Among diseases, those of parasitic and viral aetiology are the major concerns. Parasitism by sea lice (*Lepeophtheirus salmonis*) is the main problem for the Atlantic salmon industry, causing economic and welfare losses, leading to environmental impact, and having a detrimental effect on the public perception of the industry [46,47]. Viruses remain important pathogenic threats to the aquaculture industry in general, and to farmed salmonid species in particular [48]. Several viral conditions affecting Atlantic salmon include infectious pancreatic necrosis (IPN), infectious salmon anaemia (ISA), infectious haemorrhagic septicaemia (IHN), viral haemorrhagic septicaemia (VHS), heart and skeletal muscle inflammation (HSMI), cardiomyopathy syndrome (CMS) and pancreas disease (PD/SD). These diseases cause massive associated losses to the global salmonid aquaculture industry [48].

With no efficient available treatment for piscine viral diseases, their control relies on either costly vaccination strategies, preventing the introduction of the causal agents or, once in the system, implementation of comprehensive management strategies.

With few historical exceptions, preventing introduction is very challenging due the nature of the system of intensive farming itself. Once the pathogen has entered the farm, the aquaculture environment can facilitate disease, mostly due to the high concentrations of

available hosts. This leads to easier pathogen load amplification through horizontal transmission. Farmed fish can act as disease vectors for wild fish if they escape [49]. Additionally, specific infectious viral agents can survive outside the host for some days and even longer when adsorbed to other substances like clay, which extends the survival in the environment to weeks. This favours maintenance and spread of virus in the environment [50]. As a result, the impact of viral disease is not only to other hosts in the immediate vicinity, but further within the inter-connected water systems through current and tidal flows; common features in the coastal areas, lochs and fjords where most salmon farming sites are located [51].

Among viral conditions affecting farmed salmonid, *Salmon Pancreas Disease Virus* (SPDV), is responsible for the diseases known as Pancreas disease (PD) in Atlantic salmon, and Sleeping disease (SD) in rainbow trout (*Oncorhynchus mykiss*). SPDV has represented one of the most significant health issues faced by the farming industry for an extensive period of time [52], and is the focus of this thesis.

Although SPDV is also widely referred to as Salmonid alphavirus (SAV), in this thesis SPDV will be preferred as the only currently approved name by the International Committee of Taxonomy of Viruses (ICTV, 2013).

Alphavirus: the agent

Alphavirus is one of the two genera within the family *Togaviridae* containing over 40 members present worldwide in a wide range of invertebrate and vertebrate hosts [53,54]. The vast majority are arboviruses (transmitted by arthropod vectors), with their most common vector being mosquito species, although ticks, biting flies and lice can also be vectors for specific alphavirus species [55]. Vertebrate hosts include primates (humans and nonhuman), horses, pigs, rodents, birds, reptiles, amphibians and fish.

Many Alphaviruses around the world are responsible for a number of diseases of major relevance for human and veterinary medicine. Examples of these are infections associated with arthritis, rashes, fever and encephalitis. Viral maintenance in the environment is largely based in transmission between vectors and vertebrate hosts [56]. This is coupled with replication efficacy in many members of the genus, and a broad range of susceptible hosts. These characteristics has made them useful vector tools, and some alphaviruses have been patented as viral vectors (Semliki Forest virus (SFV), Sindbis virus (SIN) and Venezuelan equine encephalitis virus (VEEV)) and used for applications such as vaccine construction and therapy for cancer and central nervous system diseases [57,58]. Finally, in what could

be considered quite an opposite application, Alphavirus are also of biological warfare importance, due to the capability for airborne transmission of some species [59,60].

Overall, due to their relevance as a disease agent for humans and domestic animals, the genus alphavirus has been extensively studied and is one of the best-characterized enveloped viruses to date [56].

Alphavirus structure

Alphaviruses are small, enveloped viruses with icosahedral spherical morphology, 65~70 nm in diameter, molecular mass of 5.2×10^6 Da, density of 1.22 g/cm^3 , and a single copy of positive stranded genomic RNA of approximately 11.5 kb in length. This RNA has a type 0 cap (7-methylguanosine -m⁷G- cap structure) at the 5' end and a poly (A) tail at the 3' end [54,61]. The latter characteristic makes the alphavirus genome present itself to the host cell as a messenger RNA (mRNA) for immediate translation upon entry into the cytoplasm [56]. There are two open reading frames (ORFs) in alphavirus genomes, encoding four non-structural proteins (nsP1-4) involved in replication and pathogenicity, and five structural proteins (C, E3, E2 6K and E1) composing the virion [56]. Untranslated regions (UTRs) at each side of the ORFs are located at the 5' and 3' ends of the genome and between the ORFs (the subgenomic 5' UTR) (Fig 5). Beyond the traditional areas of viral promoter function and translational regulation, the 5' and 3' UTRs in alphavirus have other important and diverse roles related to immune evasion, host cell tropism, and viral pathogenesis which are only recently coming to be known [61].

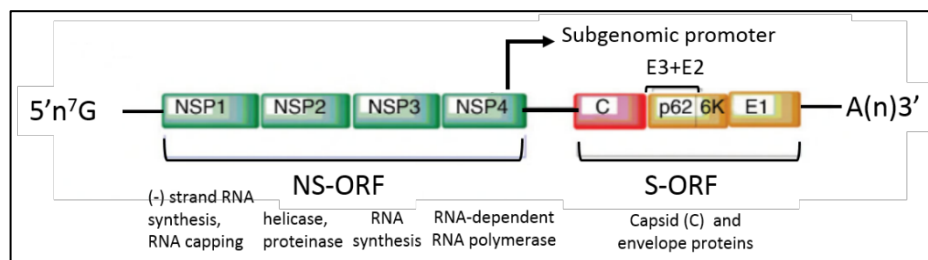


Fig. 5: schematic representation of the alphavirus genomic RNA. Open boxes =NS-ORF (non-structural protein ORF) or S-ORF (structural protein ORF), solid black lines=UTRs, P62= precursor of E3+E2, 5' m⁷G= 7-methylguanosine cap structure (type 0 cap), A(n)3'= poly adenylated tail. (adapted from [62]).

However, some key information on UTRs remains obscure, including the evolutionary forces that shape the genome heterogeneity among viral species and strains (or subtypes), which can vary greatly in length and sequence structure. For instance, SPDV, the first member of the group isolated from a fish, is the species with the shortest UTRs (5'-: 27 nucleotides; 3'-: 87–130 nucleotides) within the genus, while the Semliki Forest (SF) complex is the most

divergent and contains the longest UTRs with the greatest diversity in length (5'-: 76–85 nucleotides; 3'-: 227–713 nucleotides) [61].

The Alphavirus genomic RNA (R) is enclosed by 240 copies of capsid proteins C, arranged in a T4 symmetry (triangulation T=4) forming an icosahedral shaped nucleocapsid (NC) measuring 40nm (Fig 6). The NC is enveloped by a host acquired lipid bilayer membrane (M), in which 80 trimeric shaped spikes (S) are embedded (Fig 7). The spikes are formed by 240 copies of each E glycoprotein that are also icosahedrally ordered forming a scaffold that surrounds the viral membrane [54,63,64]. Each spike is a heterotrimer consisting of two transmembrane glycoproteins (E1 and E2) and an extrinsic membrane protein (E3) [63], (Fig 6).

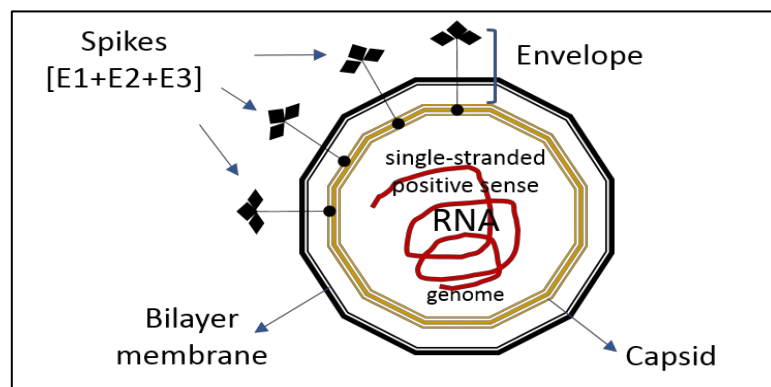


Fig 6: Schematic representation of an alphavirus virion demonstrating the spherical icosahedral shaped particle, with genomic RNA encased by the nucleocapsid and surrounded by an envelope conformed by host acquired lipid bilayer membrane embedded with (80) trimeric shaped spikes protruding from the surface.

From the outside in, three distinct segments of each the spike can be identified; a projecting domain that extends from the surface budded into a three-lobed structure (S), a thin plate domain covering the bilayer membrane (the skirt, turquoise coloured in Fig 6-B), and a transmembrane domain that penetrates the lipid bilayer connecting the projecting portions of the spikes, directly with the NC [63,65], (Fig 7).

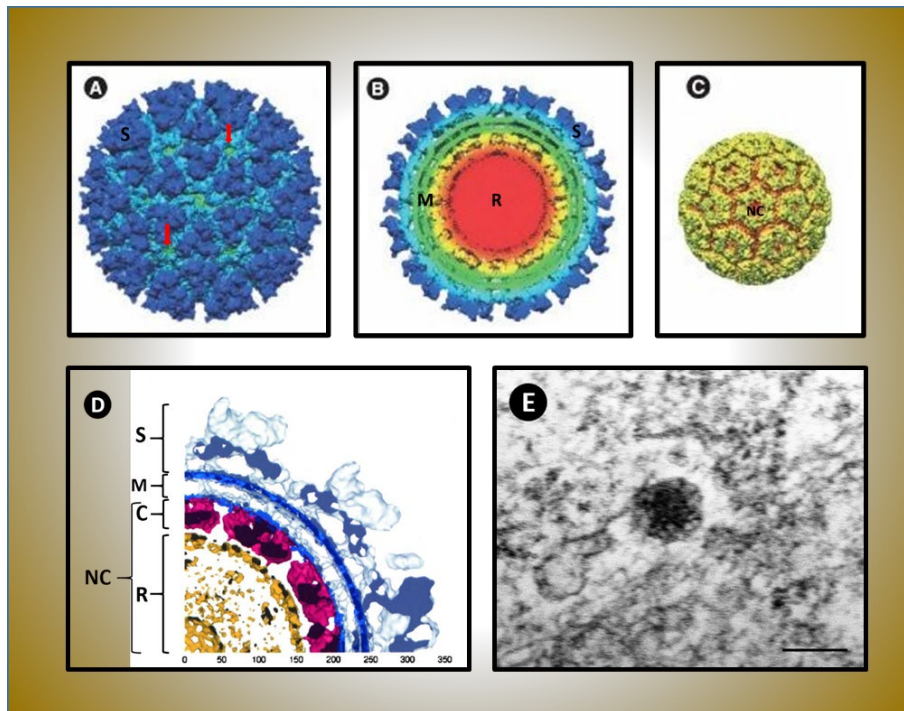


Fig 7: (A-D) Alphavirus structure based on Sindbis virus. A) Surface view down at the icosahedral twofold axis. Trimeric spikes are seen as protruding flowers in blue. Red arrows point to visible small portion of the lipid bilayer of the membrane underneath. B) cross-section of the virus showing the organization of the particle with the glycoproteins (blue), skirt region of the envelope (turquoise), the lipid bilayer (green) penetrated by the transmembrane helices of glycoproteins, the protease domain of the capsid protein (yellow), and protein–RNA region (orange). C) Radial section of the nucleocapsid core viewed down at the icosahedral twofold axis. D) Graphical representation of genomic RNA (R) and capsid proteins (C) conforming the nucleocapsid (NC), lipid bilayer membrane (M) and spikes (S). E) complete viral intra cytoplasmic particle as observed by TEM, scale bar 100nm (A-C adapted from [54], D from [63], and E from [66].

Alphavirus replication cycle

Due a wide range of susceptible hosts, the majority of Alphaviruses are capable of replicating in a range of vertebrate and invertebrate cell lines [67]. The alphavirus replication cycle has been thoroughly studied, mostly using reference viruses in mammalian or mosquito cell lines, respectively (SINV, SFV and Chikungunya virus -CHIKV-). These two approaches have shown to have different outcomes, normally with clear cytopathic effects in vertebrate cells, and with persistent infections with only partial cytopathic effects in mosquito cells [68–71].

The widely accepted current replication cycle model for how Alphavirus transfers its RNA into the cell starts with the virion binding to a cell receptor where the envelop E2 glycoproteins are believed to be responsible for the attachment. This is followed by clathrin-mediated endocytosis and internalisation into an endosomal compartment [72]. Within the endosome, the mildly acidic pH triggers the dissociation of the transmembrane glycoproteins

(E2/E1 heterodimer) and a structural rearrangement of the virion. The latter releases E1 from its regulation by E2, exposing the fusion peptide of E1 responsible for membrane fusion, which inserts into the endosomal membrane bringing the virus and the endosome membranes together, forming a fusion pore that allows the nucleocapsid (NC) to enter the cytoplasm where it undergoes disassembly, exposing the viral RNA for translation [54,73].

For the purpose of this literature review, it is important to note that although it is well established that a protein receptor on the host membrane must be an entry requirement, an accurate identification of such receptor remains obscure [74,75]. Moreover, with better purification protocols and improved methods of analysis, the mechanism has recently come under scrutiny and an alternative pathway of entry, without involvement of endocytosis, involving exposure to acid pH and/or membrane fusion has been suggested [75]. These authors propose a direct release of the virus genome into the cytoplasm by a pore complex made from viral and host proteins. The hypothesis is supported by alphavirus cell entry having been shown to be a leaky process that allows passage of ions and small molecules across compromised plasma membranes [73]. Although the authors do not deny the existence of the aforementioned mechanism of endosome formation and acidification, they suggest that these events take place later on and away from the process of entry, and may occur only under laboratory conditions [75].

Nevertheless, the subsequent steps are not a matter of controversy and once the viral RNA is released, replication occurs in the cytoplasm within vacuoles derived from endosome and lysosome membranes. The first proteins to translate from the full-length viral RNA are the non-structural proteins, allowing the assembly of viral replicase complexes at membranes. The synthesis of the minus strand RNA predominates early in the infection whilst the plus strand and subgenomic RNA synthesis occurs later in the infection [54]. The structural proteins are then translated from the subgenomic RNA as a single polyprotein (capsid (C)-E3-E2-6K-E1). The virus assembly is a highly regulated process comprising multiple cleavage and oligomerization events. The capsid proteins (C) are autocatalytically cleaved off from the structural polyprotein and remain in the cytoplasm to encapsidate newly synthesized genomic RNA forming a new NC [76]. The resulting polyprotein (E3-E2-6K-E1) is subsequently translocated into the endoplasmic reticulum (ER) where it is processed by the host signalases yielding E3-E2 (known also as pE2), the precursor to E2:E3-E2, 6K and E1. The association as heterodimers between pE2 and E1 takes place in the ER. Each component is glycosylated and heterodimers eventually oligomerize to assemble into trimers of heterodimers, to form a spike (S). They are transported through the Golgi to the trans-Golgi network where pE2 undergoes furin-like protease dependent maturation, releasing E3 from E2 to the plasma membrane (PM) via the cell's secretory pathway. The presence of E3 within the heterotrimers provides resistance to the acidic environment preventing premature

activation. At the PM the trimers are arranged in an icosahedral array morphologically appearing as 80 flower-like spikes in the surface of mature virions [56]. When the translocated NC interacts with the endoplasmic domain of E2, the NC becomes enveloped by the E1-E2 icosahedral scaffold as it initiates the budding process [54,76,77], (Fig 8).

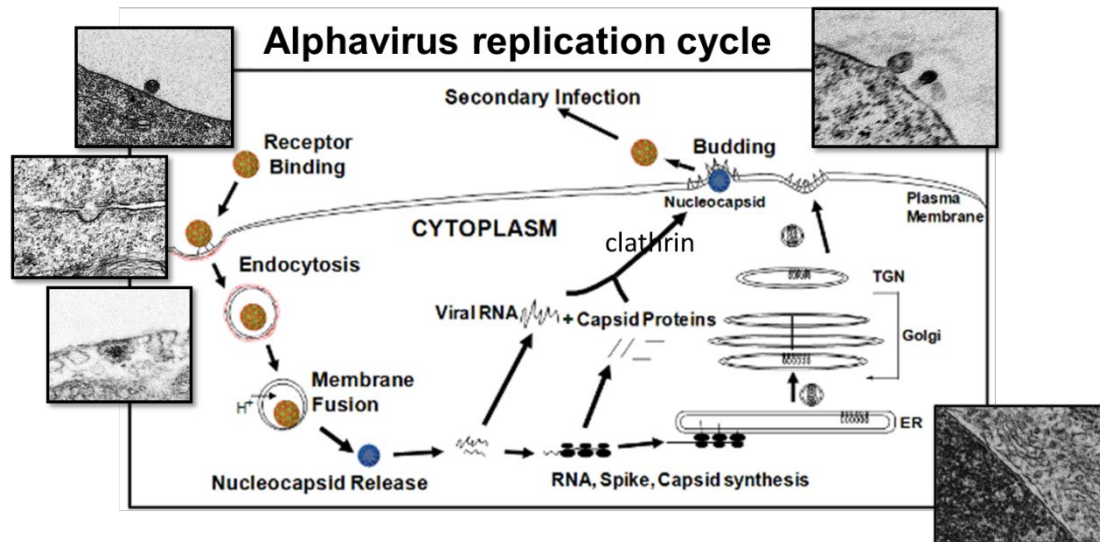


Fig 8: Alphavirus replication cycle. Adapted from Kielian Lab <https://sites.google.com/site/kielianlab/research>

Most of Alphavirus life cycle insights come from morphological and structural characterisations in vertebrate hosts, while the mechanisms of replication and assembly in the invertebrate hosts until very recently, remained relatively poorly understood. However, new research applying a variety of techniques including electron microscopy, live cell imaging and fluorescent dually labelled protein-tagged viruses, has provided strong evidence of fundamental mechanistic differences in the virus-host interaction between the insect vector and the mammalian hosts [71].

Based on this, a model was proposed for the spatial and temporal regulation of the alphavirus life cycle (Fig 9) in the mammalian host cell (Baby hamster kidney -BHK-15-) (A) or the insect vector (*Aedes albopictus* mosquito, Asian tiger) cells (B).

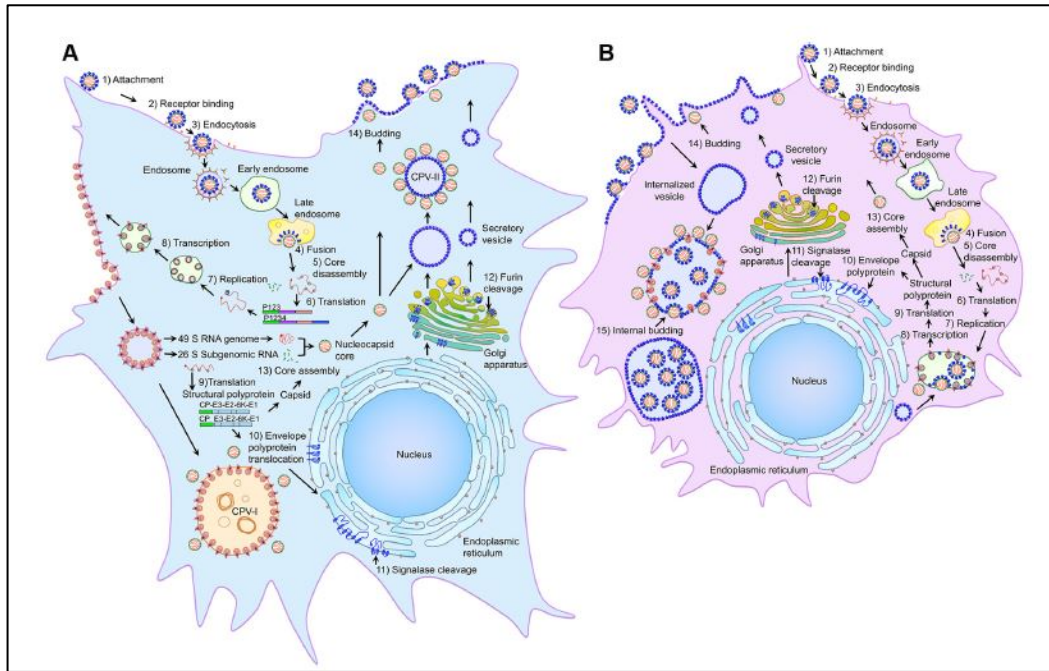


Fig 9: Model suggested for the alphavirus life cycle and the virus-induced structures in mammalian (A) and insect (B) cells. After [71] based on infection by Sindbis virus (SINV).

In essence, it has been shown that the replication and assembly machinery of alphaviruses is highly modified in mosquito cells, allowing continuous production of viruses at a reduced level (consistent with persistent infections). The non structural proteins and viral RNA in the replication complex (RCs) of mosquito cells were observed to have a distinct spacial organization in cytopathic vacuoles, compared to RCs in the mammalian cells. Specifically, through live imaging, ultrastructure (TEM) and immunofluorescence, evidence was provided that the mechanism for RC formation in mosquito cells is based around vesicles containing glycoproteins, with a proposed novel mechanism for replication complex formation capable of producing internally released particles by budding of complete viral particles into them. These may eventually be secreted as individual virions out of the cell through the secretory pathway [71]. This contrasts with the RCs in mammalian cell lines which are characterized by the induction of bulb-shaped membrane invaginations (spherules) limiting membranes of large cytoplasmic vacuolar structures (CPV type I) where viral RNA synthesis occurs. CPV-I are the proposed site of viral RNA synthesis, in which nsPs accumulate at the cytoplasmic neck of the spherules and the newly synthesized RNA diffuses into the cytoplasm through the spherule pore (ad Forshauer et al 1988, Jose etl 2017).

These different mechanisms may have relevance for SPDV (the focus of this thesis), where there is no identified arthropode vector and direct horizontal transmission between fish is

possible, and where some events of the virus life cycle and transmission have not been observed or remain unclear [78].

Salmon pancreas disease virus (SPDV)

When a new member of the alphavirus group is described, as has been SPDV, it benefits from the extraordinary baseline of information due the prolific work performed worldwide on Alphaviruses in general and on the many different host organisms/cells.

SPDV was the first member of the group with a fish species host and one of the few exceptions to an arthropod vector requirement [79–82]. It was found to be responsible for two major disease conditions in salmonids, known as Pancreas disease (PD) and Sleeping disease (SD) [52]. SPDV can live in fresh, brackish and marine water where the reservoirs are clinically diseased or covertly infected fish [83]. It primarily affects salmonids (Atlantic salmon *Salmo salar*, rainbow trout *Onchorhynchus mykiss*, and brown trout (*Salmo trutta*) [52,84,85]. SPDV has also been found in some non-salmonid marine species such as the common dab (*Limanda limanda*) without development of pathological changes [86–89]. The presence of SPDV in the common dab from regions not associated with salmon farming, supports a hypothesis that the dab represents a *bone fide* wild reservoir of the virus, independent of aquaculture activity [87]. Additionally, SPDV viral RNA has been detected by RT–PCR in sea lice (*Lepeophtheirus salmonis*) collected from Atlantic salmon simultaneously infected by the parasitic arthropod and SPDV. This led to the suggestion of sea lice as hypothetical vectors for the virus. However, there has been no proof of replication in the lice or certainty that the identification could have originated from non-lice tissues such as poorly digested blood, or remnants of host tissues. Therefore the role of the sea lice in SPDV infection transmission remains speculative [90,91].

There is limited published data regarding the life cycle of SPDV in the fish host. The limited published work include studies of the ultrastructural morphogenesis of SPDV *in vitro* (using Chinook salmon embryo -CHSE-214-,) and a thorough *in vivo* description of the pathogenesis by ultrastructure after experimental *in vivo* infection [78,92].

SPDV subtypes and serology

A phylogenetic analysis of the SPDV viral sequence (partial E2 and nsP3 genes) demonstrated that isolates recovered along a 18 years period from clinical cases, rather than being homogenously distributed were grouped in clusters, and six subtypes were suggested [93] (note SAV 1-6 = SPDV 1-6). These subtypes were shown to correspond to

geographic areas [94] with some subtypes showing a more restricted distribution, like SAV 3 (only recorded in Norway), SAV 5 (only in Scotland) or SAV 6 (infrequently present in Ireland). SAV 1 and SAV 4 are reported in both Scotland and Ireland and SAV 2 –the only strain present in fresh water- is reported in the UK and in continental Europe (Fig 10). A sea water strain of SAV 2 exists, and has been long known to exist in Scotland and has been more recently reported in Norway [94–96]. In general terms, infection by SPDV in fresh water is referred to as SD and normally affects rainbow trout, while in sea water is known as PD irrespective of whether it affects the most usual host Atlantic salmon, or rainbow trout reared in sea water.

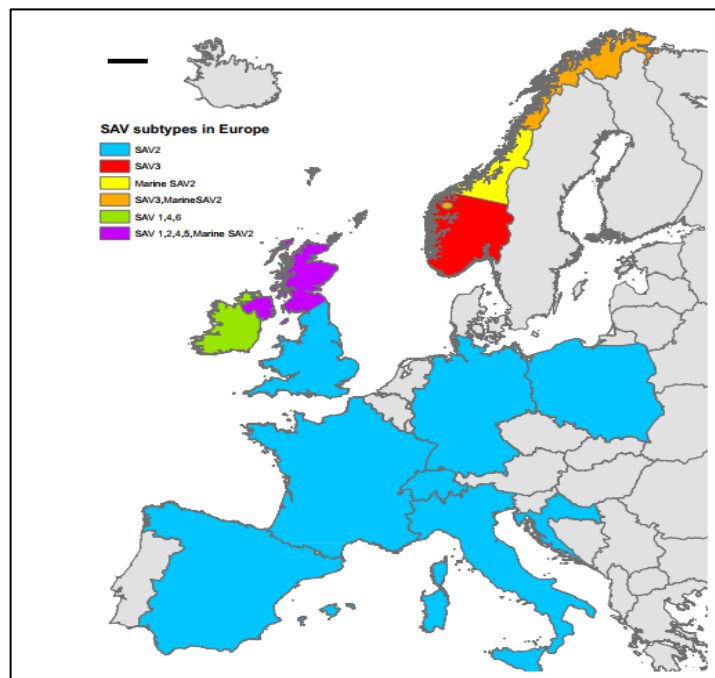


Fig 10: SPDV subtype distribution in Europe (source Jansen et al., 2017, Journal of Fish Diseases 2017, 40, 141–155).

Since the early descriptions of PD and SD and before the current subtype assignment, the virus causing these diseases were already recognised as different strains [97]. The cases affecting rainbow trout that were being reported in England and Scotland as well as in continental Europe were all considered to be caused by a SPDV subtype that is exclusive to fresh water. In light of the much wider information brought by both improved detection methods and increased surveillance in the last decade, the particular situation with SPDV 2 became clearer. It is now understood that there are two distinct branches of SPDV 2, namely the original one reported in France in rainbow trout (i.e. the aetiology of SD), and another, genetically distinguishable, SPDV 2 strain causing disease in Atlantic salmon at the marine phase. Interestingly, the marine field isolate P42p which had been isolated in CHSE-214 cells from farmed Atlantic salmon in Scotland [98] and historically had been considered as the Scottish type strain, was identified later as a marine SPDV 2 by sequence analysis

[82,93,94] . This confirms that marine SPDV 2 was present in Scotland from the initial PD descriptions. SPDV 2 is actually a very frequent, if not the most frequent, subtype in certain marine farming areas in Scotland, Shetland Islands and Orkney islands [94] (and internal MSS diagnostic records). Moreover, the marine SPDV 2 has been recently reported in the Norwegian industry [95], after years of reporting only a genetically homogenous and distinct type (recognised now as SPDV 3) [90].

Virulence and pathogenicity variations have been known for PD but since the assignation of subtypes, it has become apparent that this variation happens both between and within subtypes. Different isolates representing all 6 subtypes (F07-220 SPDV 1 from Ireland, F06-290 SPDV 2 from Scotland, PD03-13 SPDV 3 from Norway, F04-44 SPDV 4 from Scotland, F07-192 SPDV 5 from Scotland and F02-9 SPDV 6 from Ireland) were shown to induce different level of lesions, mortality or have a different pace of disease development [99]. More recently, intra subtype difference in virulence was also confirmed [100].

Recently, the serological reactivity between strains of each of the six currently genetically defined subtypes was examined [101]. When these authors compared the homologous and heterologous virus neutralization titres on sera from field cases and from experimentally infected fish, none of the viral subtypes consistently met the old serological criteria (Sub-committee on Inter-relationships among Catalogued Alphaviruses). This means that the current subtypes proposed on the basis of genetic work cannot actually be considered to separate subtypes within an alphavirus species using serology [101]. Earlier reports had already pointed towards this observation through experimental studies. For example, antisera raised against each of the six subtypes from different regions of Scotland, Ireland and Norway were used in neutralization assays and viral neutralization activity was detected in all when using isolate F93-125, the reference SPDV subtype 1 [99]. Despite the above described, the SPDV subtype division is widely accepted, used by the both industry and researchers and has been suggested to be maintained [101].

The significance of this cross-neutralization study is the awareness that, independently of their subtype grouping, all different subtype strains are highly similar and serologically identical which, in essence, is a very positive aspect for vaccine development purposes.

SPDV: the disease

The disease caused by SPDV in Atlantic salmon was initially recognised in Scotland, and reported as “Pancreas Disease” (PD) [102]. The empirical evidence to support a viral aetiology took more than a decade to be established [81], and its characterisation as an alphavirus induced condition even longer [103]. SPDV also affects other salmonid, namely the rainbow trout *Oncorhynchus mykiss* [104], but for historical reasons and in spite of the

aetiological agent been found to be a closely related strain of the same virus, the disease affecting rainbow trout in fresh water is known as “Sleeping Disease” (SD) [52,82].

SPDV affects the UK (England, Scotland and Northern Ireland) as well as other important salmon producing countries, such as Norway [105] and Ireland [106]. It has also been recorded in the USA [107], France [104], Spain, Italy [108], Germany [109], and recently Croatia [110], Poland [111], and Switzerland [112]. The Chilean salmon industry has reported both the disease and the agent to be absent in Chile [113] (and Marcela Lara, Head Animal Health Unit, Sernapesca, oral communication at Tri Nation meeting, Trondheim, Norway 2014).

Although PD morbidity can be very high, the disease does not always induce acute mortality. An important aspect of PD impact on Atlantic salmon aquaculture is that survivors may recuperate but a proportion undergoes either reduced growth and weight loss (“runts”), or show few or no clinical and external signs. However, runts are notoriously thinner individuals (see Fig 12 A-B) and therefore the flesh quality is markedly reduced. Runts are also prone to other infections accounting for high cumulative mortality of up to 10-50% during the entire production cycle. The reduced flesh quality found at harvest time accounts for the overall cost of PD due to carcass rejection/downgrading. Atlantic salmon PD outbreaks can extend for several months and associated mortality varies widely, from 2-3% up to a record 80% reported in Norway following a traumatic sea water transfer [114]. A country wide study in Norway showed that specific mortality due to PD between 1999 and 2002 was over 5% of the cumulative mortality in 80% of the farm sites and exceeded 15% in 33% of the production sites [115]. Ireland reported a 50% cumulative mortality to be related to PD in the period from 1988 to 1992 [116], which was then followed by a significant reduction to 9-15% by 2003-2004 [117]. There is no official information on the prevalence PD in Scotland as it was never made a notifiable disease in the country as it occurs in Norway and therefore, records available represent fragmented data. An 18% prevalence of affected farms was recorded based on a nationwide survey during 2006-2007, using data from 74 out of 250 active marine sites which were tested by real-time RT-PCR [118].

The overall cost of PD includes all those estimated in association with prevention, mortality, loss on growth rate, feed (due to poor feed conversion), additional staff time (required for treatments), and reduced carcass quality. This adds up to result in PD being a disease of high economic impact (Fig 11). For this reason, PD is considered the second most serious health risk to the salmon industry after sea lice [119].

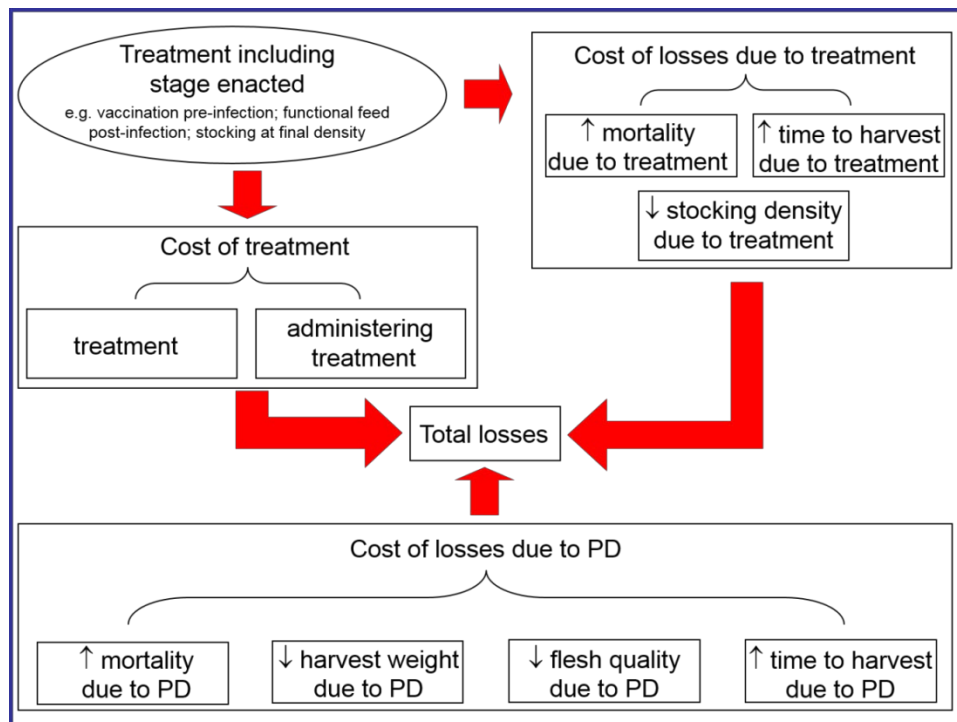


Fig 11: Convergence of all factors adding to the total losses of costs associated with prevention and control of PD. Source: adapted from MSS AQ003 project “Understanding the pathogenesis of salmonid alphavirus infection and improving strategies for management” report, with permission from Dr Malcolm Hall.

Clinical signs and gross pathology

PD outbreaks occur predominantly at the marine stage [117] and were typically seen in post-smolts soon after transfer to the sea water, however can also affect later the bigger growing fish [120]. Common clinical features of PD include lethargy and lack of appetite which starts around two weeks before the outbreak; affected fish also display abnormal swimming, are unable to maintain normal position in the water column remaining close to surface, siding towards edges or corners, or conversely lying at the bottom of the cages [102,114,120,121]. The latter signs have been associated with skeletal muscle damage, which predisposes the infected animals to mechanical erosion and ulceration of the skin and fins [120,122]. Severe skeletal muscle damage has been confirmed to affect some aspects of the fillet quality [123] with changes on the colour when raw or after smoking process. Recently it has also been shown that fish undergoing SPDV infection develop skin dysbiosis (i.e. changes to the microflora), likely rendering them more susceptible to secondary bacterial infections [124]. At the end of an outbreak, affected survivors are dramatically thinner than non-affected fish (Fig 12 A-B).

At necropsy, marked reduction of body fat (especially in the region around the pyloric caeca), absence of food in the gastrointestinal tract, and frequent presence of mucoid yellow casts

are observed [122] (Fig 12 C). These signs, although consistently present, are not pathognomonic for PD. This is also the case with other, more inconsistently reported signs and observations, such as scale pocket oedema, exophthalmia, ascites, petechial haemorrhage, pale heart and pericardial blood (Fig 13). In fact, many of the latter signs are characteristic of other viral diseases, like petechial haemorrhage in IPN , pale hearts in HSMI (induced by Piscine Reovirus –PRV-) or pericardial blood in CMS (induced by Piscine myocarditis virus –PMCV-) [114].

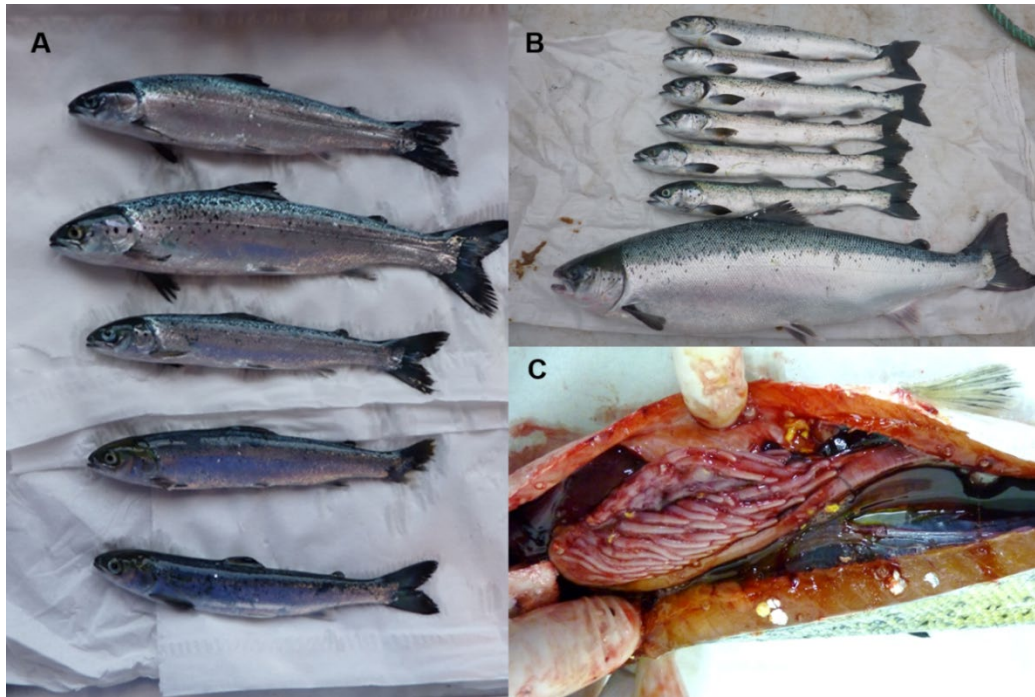


Fig 12: Gross pathology of SPDV infected Atlantic salmon. A) Marked reduced size and condition among post smolt affected at their first summer at sea (noticeable at bottom 3 individuals). B) runs above a non-affected grown fish after over a year at sea. C) total lack of visceral fat.

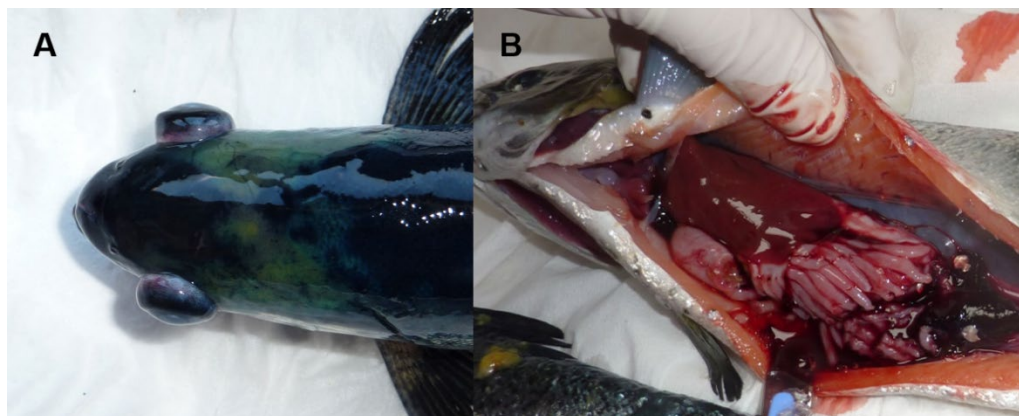


Fig 13: Gross pathology of SPDV infected Atlantic salmon. Less frequent observations in SPDV infected fish A) marked exophthalmia and B) pale heart and pericardial blood (clot). Note also total absence of visceral fat and haemorrhagic ascites.

Histopathology

The name PD came about as a result of the description in early reports of extensive exocrine pancreas lesions; total loss of acinar tissue and reduced peri-pancreatic fat [102]. These lesions were initially considered the hallmark of the disease [125]. However, further studies soon showed that striated muscle was also involved and that PD severely affected the heart and skeletal muscle tissues [105,106,126]. Degeneration, necrosis and inflammatory changes of the heart spongy ventricle and the white and red skeletal muscles were included in the description to conform the complete histological picture of this debilitating condition [106,114,120,121,127].

Overall, PD disease has been shown to have a complex presentation, with variable severity and lesion distribution. However, there is consistency in the timing of lesion development during the time course of outbreaks (Fig 14). The evidence that PD histopathological lesions developed in a sequential manner came from both experimental infections and natural outbreaks [106,120,121], following fish cohorts on different farms from sea water introduction to up to 6 months post introduction. These studies helped to explain early reports of natural outbreaks with different histopathological presentations, revealing that these differences were actually dependent on the time post infection correspond to acute, subacute, chronic and recovery stages.

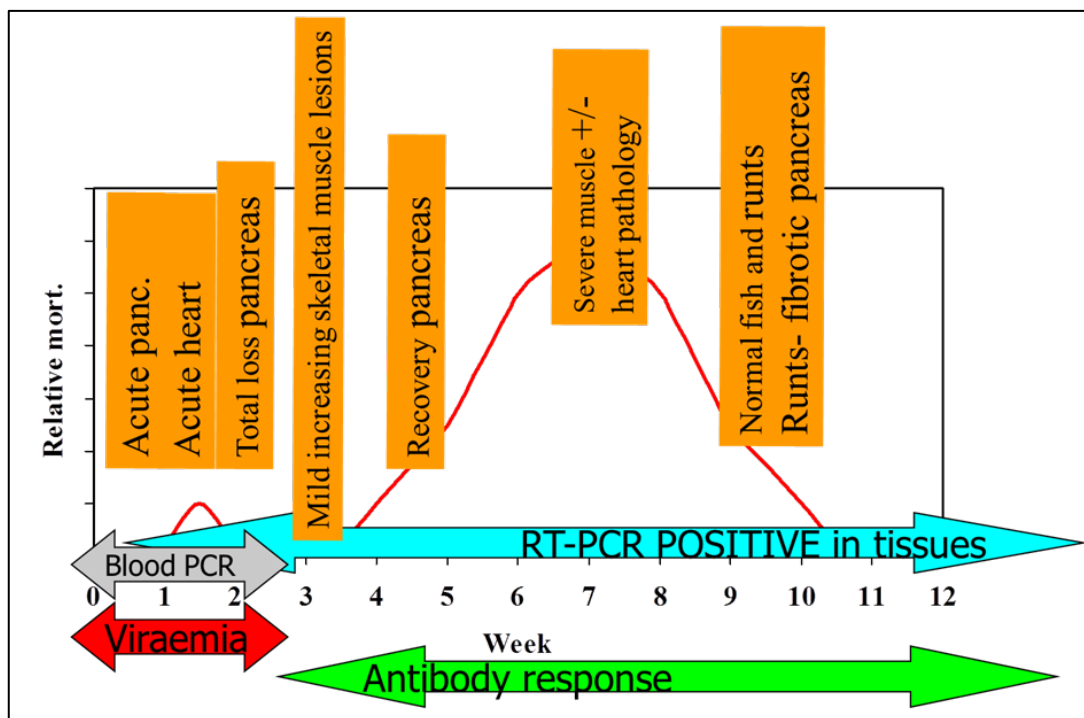


Fig 14: Pancreas disease: sequence of classical pathology alongside with molecular, virological and serological testing results at 12-14°C (image by Mariam McLoughlin, with permission from author)

Although the primary target cell type/s at SPDV first entry into the host has not yet been identified, entry through gills, skin and intestine is assumed [96]. In any case, this has not been associated with detectable histopathological changes. The sequential pathology studies show that the first organ with histopathological changes is the pancreas, with a relatively short-lived acute phase occurring within the first week post infection within the viraemic phase which last between 2 to 3 weeks, peaking half way this period. Early changes include individual pyknosis of exocrine pancreatic acinar cells, cell rounding, fine vacuolation and with features of apoptosis present, with occasional mild to moderate mononuclear cell infiltration. Extensive to total loss of acinar cells follows towards the end of the viraemic phase, associated with a variable peripancreatic inflammatory response including steatitis and serositis (Fig 15). Pancreatic changes are followed immediately by focal and acute lesions in the heart between 1-2 wpi, with hyper eosinophilia of scattered pyknotic cardiomyocytes (necrosis) and mild focal inflammation (Fig 16). This is followed by early skeletal muscle lesions (starting approximately 3wpi) where individual fibre necrosis and degeneration in white muscle can be observed (Fig 17). While the pancreas can start recovering between 4-5 wpi, the striated muscle lesions progress and advanced changes in heart and skeletal muscle peak between 6-8 wpi. These are seen as multifocal cardiomyocyte necrosis and subendocardial and myocardial focal to diffuse inflammatory cell infiltration in the heart (Fig 16), which in some cases also show macrophage infiltration of necrotic fibres, vacuolation of the spongy trabecular ventricular myocardium, as well as proliferation of endocardial cells. In the skeletal muscle, the myofibers are degenerated and inflamed with endomysial fibrosis, which is most severe in the red muscle, but also affects the white muscle (Fig 17).

At the later stages (by 9-10 wpi) pancreas fibrosis can be observed, particularly in fish that became runts [114]. In clinically healthy fish at 9-10 wpi, the pancreas has been observed to have very mild or no fibrosis, and it has been suggested that these differences depend on the level of inflammation during the acute phase. In addition, the striated muscle may also show regeneration capacity in recovered fish [126–128]. At the later stage, SPDV can develop into a chronic condition characterised by a severe reduction in the peripancreatic fat, the inflammation at the junction between ventricular compact and spongy myocardial layers and skeletal muscle endomysial fibrosis. A significant sub-endocardial fibrosis has been reported which may potentially indicate susceptibility to stress-related heart-failure [123]. This finding may relate to earlier descriptions of chronic PD been previously known as 'sudden death syndrome' (SDS) [127].

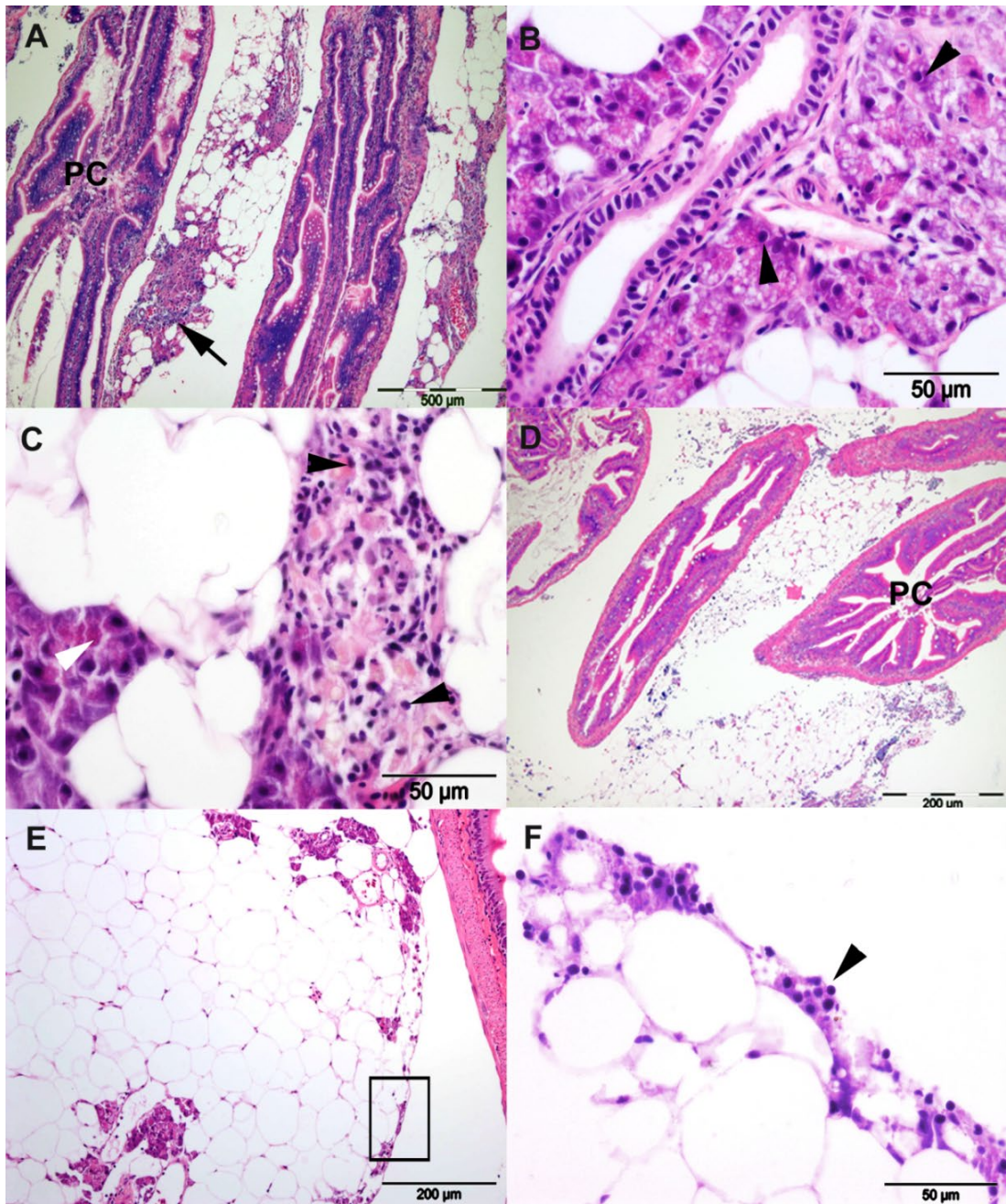


Fig 15: Atlantic salmon affected by SPDV. Light microscopy of pancreas stained with H&E. A) Overview at low magnification of the pancreatic tissue interspersed among the pyloric caeca (PC). Note loss of pancreatic architecture, reduced peri-visceral fat and patches of intense basophilic cells (arrow) suggestive of periacinar infiltration. B) Early changes in exocrine cells with degeneration and scattered pyknotic nuclei (arrow heads). Note foamy aspect of the cytoplasm rendering a vacuolated appearance. C) A patch of necrotic tissue to the right of the image shows a poor stain uptake of the degenerated cells, with higher eosinophilia (bright pink –orange colour) and pyknotic nuclei (arrow heads) in the absence of inflammatory cells. Note the adjacent area to the left shows normal acinar tissue containing zymogen granules (white arrow head). D) Total loss of exocrine pancreas noted in the peri-visceral fat tissue, a characteristic of chronic PD. E) Few patches of acinar cells can be observed in a recovering pancreas. Note a reduced cell size in the periphery. F) Higher magnification of the peripheral edge showing complete degeneration and pyknotic nuclei. Light microscopy of H&E stained sections

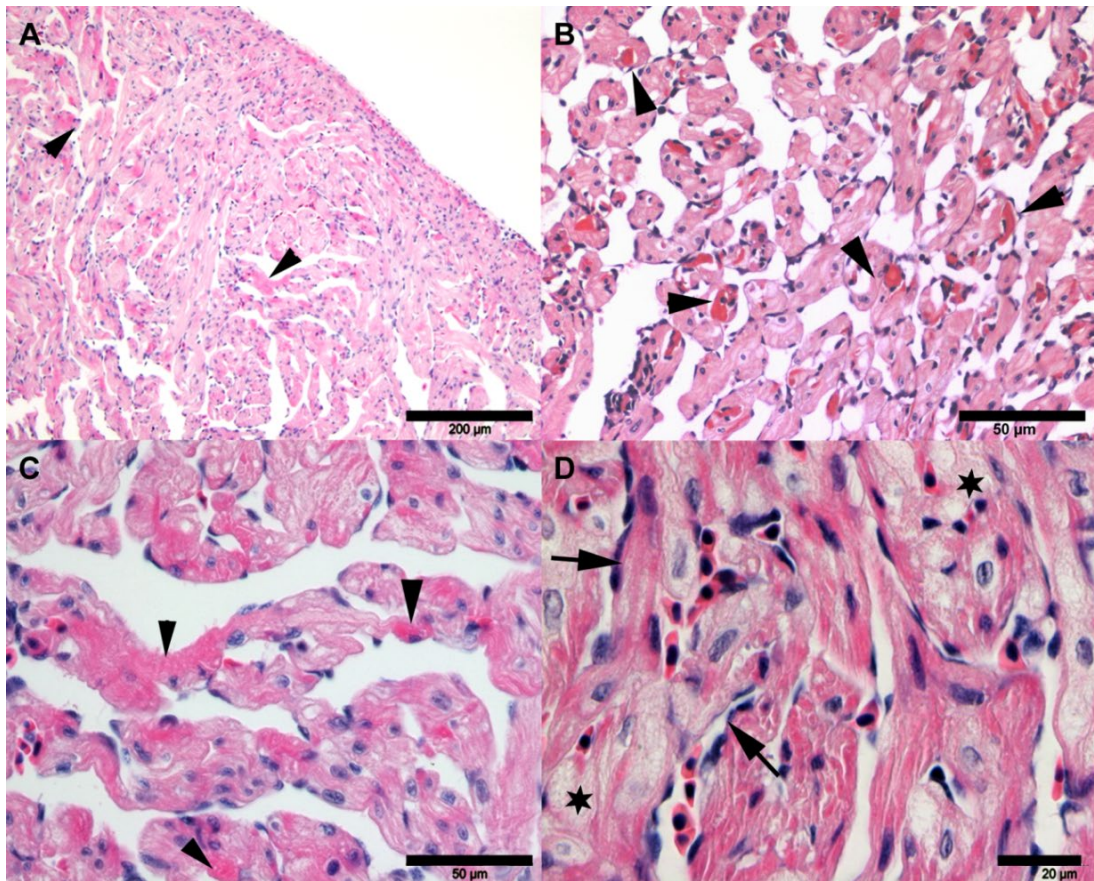


Fig 16: Atlantic salmon affected by SPDV. Light microscopy of the heart stained with H&E. A) Ventricular spongy myocardium showing scattered, strongly eosinophilic shrunken, cardiomyocytes (hyaline necrosis). B) Higher magnification shows the nature of individual cells necrosis (arrow heads) of cardiac muscle with very mild infiltration (lower left corner). C) Multifocal myocardial necrosis and formation of a larger coalescence lesion in ventricular spongiosum. D) Ventricular spongiosum showing slight thickening of the endocardium (arrows) and intra myofiber pale areas (stars) due loss of striation and vacuolation.

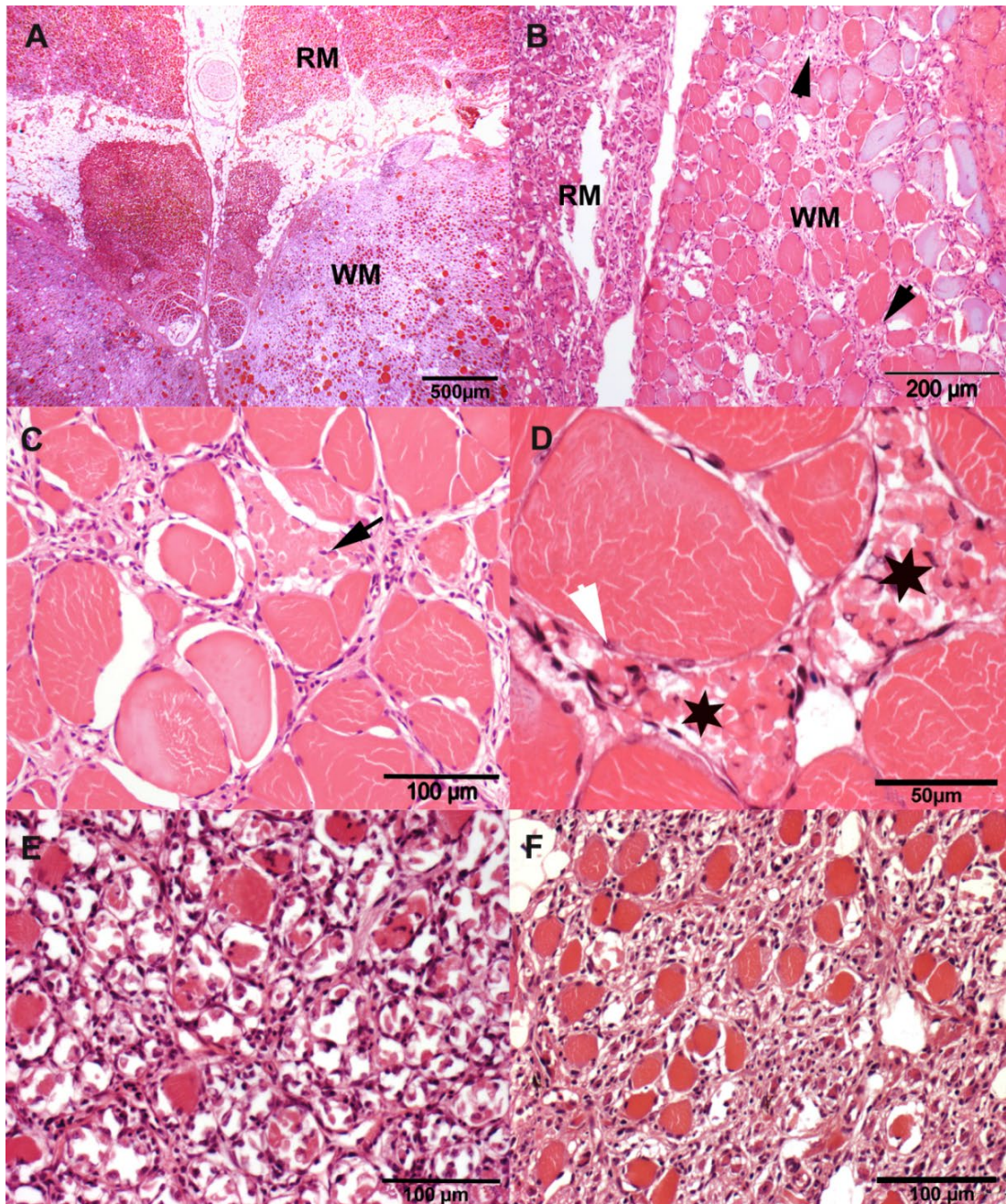


Fig 17: Atlantic salmon affected by SPDV. Light microscopy of the skeletal muscle stained with H&E. A) Low magnification overview of a section at the lateral line level shows evidence of severe degeneration and necrosis of both white and red skeletal muscle. B) Higher magnification showing the severe degeneration and necrosis affecting both red muscle (left) and white (right) skeletal muscle. Influx of inflammatory cells can be noted. C) High magnification showing necrosis of a single myofiber, with presence of phagocytic cell within (arrow). Note rounding of neighbouring, focal inflammation and mild thickening of the endomysium (upper left). D) High magnification of two individual WM fibers undergoing complete degeneration and necrosis (stars). E) Red muscle severe necrosis and inflammation. Note nearly hollow myofibers where only the endomysium is still visible. F) Severe thickening of the red muscle endomysium (fibrosis).

Disease diagnosis

Preliminary PD diagnosis is based on clinical signs and histopathology. This is followed by the isolation of the virus, serology, immunofluorescence and molecular RT-PCR detection, which are used for confirmation [122].

Although SPDV can be grown in culture, the virus proved historically challenging to isolate from field cases [52,98,129,130]. It is not uncommon that samples from fish with overt consistent PD clinical and histopathological signs and reliable PCR positive results, do not result necessarily in positive viral isolation. Isolation has been attempted by different groups/laboratories using a range of cell lines including Chinook Salmon Embryo (CHSE-214), Atlantic salmon fibroblast (AS-6), bluegill fry (BF-2), epithelioma papillosum carpio (EPC), rainbow trout gonad (RTG-2), Asian Grouper Kidney (AGK) and Chum salmon heart (CHH-1) cells [98,131,132]. Results point to a consensus that CHSE-214 yields the best results as a compromise of titres and detection of cytopathic effect (CPE). Variable success in general has been assigned to several factors including initial low viral loads and potential presence of virus neutralizing antibodies on the tissue samples [133]. Also, the tissue of origin for sampling has been reconsidered after work on virus tropism. This work determined that the traditionally used head kidney sample was not the most suitable, and shifting sampling towards the heart clearly improved successful isolation rates [134] (and internal MSS diagnostic reports). Finally, a recent investigation gives a reasonable hypothesis to explain the difficulties in viral isolation. Based on sequencing of SPDV subtype-3 isolates from natural infections in Norway, it was found that numerous deleted mutants (defective viruses containing genome deletions) are generated during the natural infection cycles. The presence of these “defective” viruses within infected populations/tissues, has been suggested as the possible explanation for the difficulty for cell culture isolation[132] and could relate to previous reports on culture adaptation requirements[135] and laboratory adapted cultures [82,85,134].

Prognosis

Factors that may influence the disease prognosis include water temperature and season that can accelerate the infection dynamics (duration and mortality rates), differences in virulence of subtypes/isolates [99] and host differential susceptibility [136]. Additionally, any stressful husbandry practices such as lice treatments, crowding or any other practice triggering severe or prolonged stress, will also have negative effects on the outcome of the infection [137].

Differential diagnoses

The differential diagnoses of PD include nutritional myopathies and specially other viral conditions of Atlantic salmon, such as IPN at the pancreatic level and CMS and HSMI. Clinical presentation of PD has been described above (5.1) and for CMS and HSMI autopsy findings contribute to differentiation as they point towards circulatory failure, including sometimes the presence of blood clots in the heart cavity. There may also be congested liver, splenomegaly and petechial (pin-prick) haemorrhages in the adipose tissue. Histologically, while the heart is the common organ involved in all 3 conditions and some lesions can be very similar depending of the time course of the disease, the presence of myopathy also in the red and white skeletal muscle allows differentiation of PD from CMS for example. Presence of multifocal zonal or patchy liver necrosis as a secondary lesion correlates with heart failure in HSMI, and this occurs while the pancreas remains unaffected by any inflammatory or degenerative reaction [122,138,139].

Disease control

Prophylaxis remains a first goal on the health maintenance of any livestock production. However, for the majority of farming sites located in endemic areas it is difficult to avoid the infection. SPDV viraemic phase is known to occur early in the infection cycle and correlates with the peak of shedding [99,140] and therefore it has high potential for water-borne spreading. Additionally, fat droplets in the water surface, originating from infected dead salmon [25,140], faeces and mucus [141] have been also shown to carry viable viral particles.

Among the factors that regulate the onset of a PD outbreak temperature influence has been shown to be inversely related the disease incubation period, with shorter incubation at higher temperatures [51]. Fish movements are well identified risk factors for introduction into a site, and both site to site transfers during production or from farming site to harvest can be involved [122]. Additionally, there is a potential for fish carrying the virus for up to 71 wpi prior to clinical disease outbreak [142], and so the risk from movements of infected fish that present no signs of PD is high.

A thorough and updated review on the epidemiology of PD has been recently published [96]. It showed that, in spite of the impressive amount of information generated after decades of study of the condition, there has been yet no scientific evaluation of current on-site bio-security measures, and information on the impact of available mitigation and control

strategies is limited, particularly for Ireland and Scotland. A notable exception is in Norway, where a project by the industry (“PD free”) aiming to reduce PD outbreaks, associated losses and spreading of the disease was started in 2007. Briefly, from 2008 the Norwegian coastline was divided into 2 administrative zones separated by a production-free area of 10 nautical miles and at the same time, industry then initiated an extensive vaccination program involving most marine salmonid farms. This was associated with a 24% reduction in outbreaks from 2007 to 2009 and an overall reduction of 10% for the period 2007–2010. Overall the synergic approach showed it had a positive effect in reducing the number of outbreaks and also in decreasing cumulative mortality and the number of fish discarded at slaughter [143].

A common practice in controlling PD to reduce risk is to use PD resistant ova generated from breeding programs and/or the implementation of vaccination schemes. Vaccination has been seen as a promising strategy since the early years of PD when it was already shown that pre-exposed fish became resistant to subsequent challenges [144] and that 100% of the fish were seroconverting by 3 weeks after experimental infection [121]. Also, passive immunisation by administration of antisera from naturally and experimentally infected fish, resulted in protection against subsequent challenge[145]. Soon after these studies, the first reported vaccine trial was performed using a simple formalin-inactivated virus tested by IP injection in fresh water parr [122]. Fish were resistant to infection when challenged 4 weeks later. Subsequent studies indicated that development of a commercial vaccine was possible. Indeed, in 2007 a commercial vaccine based on inactivated SPDV became available (Norvax® Compact PD, Intervet International B.V, currently MSD Animal Health)[143] and was used for the extensive vaccination program associated to the PD free project. While it was shown the vaccine had positive effects, controversy remains as to the overall effectiveness as PD cases in Norway did not reduce as much as expected.

For many years, there was a monopoly on the commercial supply of vaccine due a patent filed by Intervet (currently part of MSD Animal Health) that prevented the commercialization of alternative formulations. This was an outstanding issue for many years until 2017, when a decision by the Norwegian Supreme Court approved a vaccine formulation by another company (<https://www.pharmaq.no/updates/pharmaq-s-pd-val/>). This has opened the door for other companies to offer alternative options. For example, the vaccine above mentioned confers improved protection to PD, both when administered alone or as a component in a polyvalent vaccine [146].

This has enabled the investigation of other approaches, such as a recombinant (fully attenuated) salmonid alphavirus vaccine [147], as well as DNA vaccines. Two formulations using the latter approach has been recently reported to provide strong protection against

SAV3 infection without addition of IFN plasmid as adjuvant [148]. Progress towards commercialization has been made [149,150] and recently, the European Medicines Agency (EMA) recommended granting a marketing authorisation in the European Union (EU) for Clynav, a DNA vaccine to protect Atlantic salmon against PD caused by salmon alphavirus subtype 3 [151].

Other approaches to vaccination may include the use of replicons. Alphaviruses have been widely used in reverse genetics and protein expression systems, and self-amplifying RNA (replicon) vectors have been developed from different alphaviruses including Semliki Forest virus (SFV) [56,152]. In these alphavirus-based replicons, the subgenomic second open reading frame (ORF) is replaced with that of the gene of interest (GOI) and its expression is then executed by the alphavirus replication apparatus. Replicons are useful for basic studies and *in vivo* expression of GOI but can also be used as vector system for vaccination [153]. Recently, a SPDV replicon was shown to be active in cells from fish, mammals, insects and crustaceans, producing GOIs over a wide temperature range (4–37 °C) [79]. Moreover, a successful precedent of immunization against another important salmon viral disease, namely ISA, was recently reported using a Salmonid alphavirus-based replicon vaccine [154]. The use of this approach clearly opens new perspectives for the development of viral fish vaccines, together with the now approved DNA vaccines approach.

Effective vaccination is useful in limiting PD damage within farms, and also limits its spread to other farms. Infection pressure has been shown to have a strong effect on the probability of a PD outbreak at neighbouring farms, and success with vaccination approaches is an important tool to reduce the risk. Effects are due to a diminished infection pressure within and between farms, by reduction of the severity and mortality resulting in a significant decrease in virus shedding, even if vaccinated fish become infected [155,156].

Viral tissue tropism, pathogenesis and host response

In the mosquito host (*Aedes albopictus*), virus has been observed to reach the head ganglia, salivary glands, anterior and posterior midgut and thoracic muscle, being more persistent in the fat bodies; haemolymph, the hindgut, and the tracheole-associated cells [157]. In the vertebrate hosts, on the other hand, initial sites of detection include skeletal muscle, liver, lungs mesenchymal cells, fat cells, chondrocytes, fibroblasts, osteoblasts, neural and glial cells and macrophages in the skin. It often leads to infection of the draining lymph node and probably of other tissues and organs through viraemia as the main form of dissemination [158,159].

SPDV, has also a wide range of organ/tissue tropism including the pseudobranch and gills, heart muscle (atrium and ventricle), head kidney, pyloric caeca (specifically the fat associated pancreatic tissue), white and red skeletal muscle, brain, liver, spleen, head kidney leucocytes and transiently, in the serum [92,133,134,160–162]. From all these, the most suitable tissue for molecular diagnosis in Atlantic salmon, irrespective of the fish disease status, was found to be the heart and the pseudobranch [134], with brain tissue coming up also consistently positive although this is not a tissue that is frequently or consistently sampled. In a Norwegian study, A salmon infected with SPDV 3 viral RNA was detected using real-time RT-PCR in surviving experimental animals six months after infection [134]. Authors noted that such detection might not necessarily reflect the presence of active virus replication as the assays cannot estimate the precise amount of viable, infectious virus particles. However they consider that this raises the possibility of survivors becoming life-long asymptomatic carriers.

The manifestation of alphavirus infections, particularly in the several conditions affecting humans, is associated with the local effects of the virus replication in the target organs/tissues, such as arthritis when replicating in joints or encephalitis when replicating in the brain tissue. The associated local inflammatory response is believed to contribute to some of the tissue damage and disease symptoms [160,163,164]. This however may vary in the different alphavirus. In SPDV for example, the recognised main target tissues such as exocrine pancreas, striated heart and skeletal muscle, often show severe lesions but a range of inflammatory responses. Other infected tissues, such as gills, pseudobranch or kidney, often do not have observable damage despite harbouring the virus for extended periods of time during infection [92,162].

Virally induced cell death is an important aspect of viral pathogenesis. Apoptosis is frequently involved [165] and has been reported in A salmon for other viral diseases, such as ISAV [166] or IPNV [167]. With some exceptions, alphavirus infection of vertebrate hosts is known to result in cell death by induction of apoptosis [56]. Similarly, SPDV-associated apoptosis has been reported in pancreatic [168] and cardiac tissues [169]. However, the role and relevance of apoptosis in SPDV *in vivo* infections, its pathogenesis, its effect on virus competence and its replication efficiency still require further investigation [170].

Fish are known to be capable of mounting a strong and protective innate and acquired immune response to many viruses, as evidenced by the presence of a potent and functional type I interferon (IFN) pathway, and by the effective outcome of a number of vaccines that elicit a strong antigen-specific memory humoral response [171,172].

The type I IFN response is considered a robust first line of antiviral innate response in vertebrates [173]. Type I interferons (IFN α/β) and the corresponding interferon-stimulated genes (ISGs) such as *mx* 1,2 and 3 are known to play a pivotal role in the defence against virus, and their role is well described in alphavirus *in vivo* infections [174]. The host cell recognizes viral RNA by various receptors, starting a signal process that can lead to the synthesis and secretion of IFN. All nucleated cells have this capacity to respond and produce type I IFN. In brief, the engagement post recognition between pathogen-associated molecular patterns (PAMPs) and pattern recognition receptors (PRRs), triggers the activation of transcription factors (TFs), the subsequent expression of IFN inducing in turn the ISGs [175]. Once secreted, IFNs act both in auto- and paracrine manners, inducing an antiviral state inhibiting viral replication and preventing the infection in infected and un-infected cells, respectively [173].

Although there are relatively fewer studies in fish, there is abundant evidence of the role played by IFNs in protecting against viral infections including against SPDV, both *in vivo* and *in vitro* [168,176,177]. Recently, in addition to secreted IFNs, fish were found to possess a functional intracellular IFN (iIFN) [178] but its involvement in the immunity to viruses remains unclear. For a full review of the innate immune responses of salmonid fish to viral infections see review by Collet 2014 [48].

In mammalian hosts, a strong inhibition of the IFN response has been described as essential for alphaviruses to establish productive infections, such as Sindbis (SINV) infections triggering a general host cell shut-off affecting the induction and expression of IFNs and ISGs [179,180]. However, if the host cells are IFN treated prior to infection, ISGs were readily expressed and virus replication is severely reduced [179]. The former case has been shown for SPDV *in vitro* infections of an Atlantic salmon head kidney cell line (TO), where IFN α showed to induce protection against a subtype 3 SPDV in a time dependant manner related to exposure to IFN prior to infection [181]. Induction and expression of the IFN inducible gene *Mx* has been widely used in studies of fish immune response [176,182]. In Atlantic salmon both naturally and experimentally infected salmon, positive correlation has been demonstrated between IFN α -stimulated gene *mx* expression and cell protection against SAV-induced CPE [181].

In terms of the humoral response, antibodies (Ab) were found to be a relevant part of protection against PD since the early experimental infections. During these, effective SPDV neutralizing Abs were detected after 10 days post inoculation and 11 days later in the cohabitant fish [121]. The proportion of fish seroconverting increased from 60% 2 wpi to 100% at 3 wpi. From this point onwards, fish displayed an overall increasing titre in both

inoculated and cohabitants until termination of the experiment at 8 wpi. Even 12 weeks post inoculation, fish have been shown still to retain a good level of Ab titres (see Fig 14).

Finally, in a very different approach to examine the response in Atlantic salmon to infection with an SAV 3, the virus induced an important shift in the skin bacterial community composition [124] and the resulting unbalanced microbiota has a decreased abundance of Proteobacteria while opportunistic taxa increased (Flavobacteriaceae, Streptococcaceae and *Tenacibaculum sp.*). This can be directly related with infected fish susceptible to secondary bacterial infections.

Knowledge gaps, the future and thesis objectives

Although PD has been recognised for over 30 years, it remains a significant problem for the salmon farming industry in terms of both profitability and welfare. To assist industry in Scotland and Norway in meeting the policy objective of increased growth over the next ten years, a continued support based on sound scientific evidence is required.

Knowledge gaps in relation to disease control are regularly identified and discussed at meetings such as the “PD Tri-nation”, a forum started over a decade ago that brings together interested parties from the salmon farming countries (initially Norway, Scotland and Ireland) and includes industry, science-academia and policy representatives. Since it was established in 2005, it has provided an open forum for rapid dissemination of experience, new knowledge and efforts to help improve control strategies and, importantly, to identify the new or the permanent outstanding challenges.

Marine Scotland Science (MSS) supported a research programme on salmonid alphavirus for several years and this thesis project was initiated as a consequence of a Tri Nation meeting in 2009 where among others, the following knowledge gaps were identified and became the framework for this PhD project:

1. Increased knowledge on the virulence/pathogenicity of the virus subtypes
2. Improved experimental models for transmission and disease mechanisms
3. Improved knowledge on the virus and on possible natural hosts

Therefore, with the general objective to improve or develop new experiment approaches especially applicable to Atlantic salmon, the current thesis presents the results of the work undertaken combining *in vivo* (chapters 1 and 2) and *in vitro* approaches (chapters 2-5).

To contribute with knowledge gap 1 (“Increased knowledge on the virulence/pathogenicity of the virus subtypes”), I initially used an *in vivo* classical intra peritoneal infection with a single sampling time point at 35 dpi to establish the virulence of MSS available different subtypes as well as isolates within subtypes. These data were used to determine the most appropriate isolate for further work based on virulence, assessed through histopathology and molecular results. It also provided for the preliminary assessment and comparison of tissue tropism which became the basis for the selection of heart, brain and skeletal muscle among the 7 tissues examined (heart, brain, skeletal muscle, kidney, liver, pancreas and spleen) for testing in further *in vivo* challenges.

In relation to knowledge gap 2 (“improved experimental models for transmission and disease mechanisms”), I developed a working cohabitation model which at the time, was not available at MSS or had been reported to the best of my knowledge. Experimental work was performed at VESO –Vikan (Norway) and the model applied successfully established infection in the co habitant fish. The experiment was followed for 2 months with 8 sampling time points, and allowed to measure the expression of the viral and *mx* (component of the immune response) genes at different tissue levels. Additionally, this experiment allowed me to contribute with a contemporary MSS project working on viral assessment by non-lethal sampling. Extra samples (blood) taken for it were used to validate the capacity of a reported cell line to detect the viral infection. The work demonstrated that results obtained from the blood of infected fish closely reflect those from sampling at the organ level, validating the option for a non-lethal approach, in line with the 3R principles (reducing, refining, replacing) in animal experimentation (collaborative published paper as co-author, under Annex).

After confirming the relevance of the heart during the *in vivo* infections, the *in vitro* part of the work started, focused on introducing a cellular model based on embryological tissue of Atlantic salmon, to establish an *in vitro* species-specific, cardiac model.

The Salmon cardiac primary cultures (SCPCs) was characterised, different tools to work with the model were explored and importantly, viral infection permissiveness was tested using different cardiotropic viral agents (chapter 3 Paper 1). The model was further used to assess potential influences of the embryo genotype on the outcome of viral infection, which showed measurable differences in the viral and the *mx* gene expression between SCPCs originated from ova of different traits (chapter 4 Paper 2). This later experiment also included the description of associated pathological changes by ultrastructure examination using transmission electron microscopy (TEM).

Finally, to contribute to knowledge gap 3 (“improved knowledge on the virus and potential natural host”), by drawing from the extensive literature in mammalian alphavirus work the

SCPC model was used to examine and describe the SPDV infection cycle by ultrastructural by TEM. This resulted in novel insights on the virus replication cycle that had not been previously reported (chapter 5, paper in preparation).

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CHAPTER 2: SPDV VIRULENCE AND TISSUE TROPISM

Introduction

Historically, two new salmonid conditions, Pancreas disease (PD) and Sleeping disease (SD) were independently reported in Scotland and France affecting Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*), respectively (1,2). Evidence of their viral aetiology came between 1995 and 1997, when both diseases were associated with an enveloped virus, morphologically consistent with a member of the Togaviridae family (3,4), and later classified within the genus Alphavirus (5,6). It took a few more years -until genomic sequencing became widely used- for recognition that PD and SD are induced by closely related isolates of the same virus species (7).

While SPDV infections may affect Atlantic salmon both in fresh and sea water, it is recognised that the most serious effects are predominantly during the sea water phase (8,9). An epidemiological study performed in Norway followed fish cohorts along the production cycle from the fresh water, transfer to sea water and sea water growing phases (9). Their results showed that fish from sites with no detectable viral infection by RT-PCR, antibodies or pathological changes consistent with PD while in the fresh water, after transfer to sea water ~64% of the sites were positive. It is believed that SPDV challenge in sea water occurs from soon after fish are transferred as “post smolts” (developmental stage after smoltification process is completed and the fish has moved or been transferred to full sea water) and throughout the production cycle. PD can therefore affect all sizes at sea water and has been reported in Atlantic salmon up to ~6 kg (9).

Among the isolates recovered from Scottish salmon farms through diagnostic work at the Scottish national reference laboratory (Marine Scotland Science –MSS-), subtypes 1 and 2 appear as the most prevalent followed by 4 and 5 (10), and some of these isolates were available for experimental work. MSS had long experience with SPDV *in vivo* challenges however, they predominantly had been performed by intraperitoneal (ip) injection in parr fish (fresh water stage), either with infected tissue homogenates (11) or with purified virus isolates (12). At the time this PhD project started a cohabitation experimental model that would reproduce the infection in post smolts, in sea water - the developmental stage and environment when predominantly salmon are challenged in the field- had not yet been established at MSS.

A pantropic tissue distribution for SPDV had been previously suggested (13) based on experimentally ip infected Atlantic salmon with wild-type subtype 3 infectious material (tissue homogenates) derived from natural infections, and with isolate F93-125 (founder of subtype 1 and derived from the original PD case) (3). All the tissues examined (pseudobranch, gill, atrium, ventricle, head kidney, pyloric caeca and pancreas (hereafter refer as pancreas), and skeletal muscle), became RT-PCR positive at some point during the examined period of up to 190 dpi. Peak of viral detection for the homogenate inoculated fish was between days 21 to 60 and these fish showed a higher and earlier detection than those injected with F93-125 (13).

As referred to in the Introduction, six SPDV subtypes had been recognised (14) and their geographical distribution been reported (15,16) and summarised in Table 1. Early on it was noted that subtype clustering did not necessarily reflect infectiveness, mortality, virulence, or pathogenicity and to compound on this, intra-subtype differences has also been suspected (17,18).

SPDV subtype/disease	Region/country	Host	Environment
Sub 1 (PD)	UK (Scotland-Northern Ireland), Ireland	Atlantic salmon * Rainbow trout **	sea water* fresh water**
Sub 2 (SD)	UK-Continental Europe	Rainbow trout	fresh water
Sub 2 (PD)	Scotland -Norway	Atlantic salmon	sea water
Sub 3 (PD)	Norway	Atlantic salmon Rainbow trout	sea water
Sub 4 (PD)	UK(Scotland-Northern Ireland), Ireland	Atlantic salmon	sea water
Sub 5 (PD)	Scotland	Atlantic salmon	sea water
Sub 6 (PD)	Ireland	Atlantic salmon	sea water

Table 1: SPDV subtypes and their geographical distributions affected species and environment (adapted from OIE Manual of Diagnostic Tests for Aquatic Animals, chapter 2.3.6).

For this thesis work there was an initial attempt to set infection by co-habitation at MSS using isolate 4640, an MSS subtype 1 SPDV. In brief, 128 post smolts (~150 g) distributed in four

tanks were allocated to x 3 replicates for treatment and x1 for controls. Half of the fish on each tank (16/32) were ip (0.2 ml of 2×10^6 TCID₅₀ /ml) for the treatment tanks and 0.2 ml of culture media CHSE-214, for control fish). Based on previous reports (19) the expected time of infection of cohabitants (ch) was estimated around 14 days post infection (dpi) therefore a window between days 10 and 20 was set to be thoroughly covered by five sampling points at days 5, 8, 10, 15, 20, followed by additional three time points at 35, 45 and 60 dpi. At each point 2 ch fish per tank were sampled (6 x treatment and 2 controls). Seven tissues were sampled (pyloric caeca for pancreas, skeletal muscle, heart, liver, brain, kidney and spleen) and testing performed by quantitative RT-PCR. However, the experiment was stopped at 45 dpi as results from point 35 dpi became available showing there was still no viral signal at any fish or tissue, and all remaining ip fish were sampled. The ip fish testing showed ~70% of this them were negative for SPDV by RT-PCR. It was as results of this failed attempt that, to account for the potential effect of hypothetical differences in isolate infectiveness, the following step towards developing a successful cohabitation model was to do a previous selection of a highly infective isolate from those available in-house, by means of a comparative ip challenge.

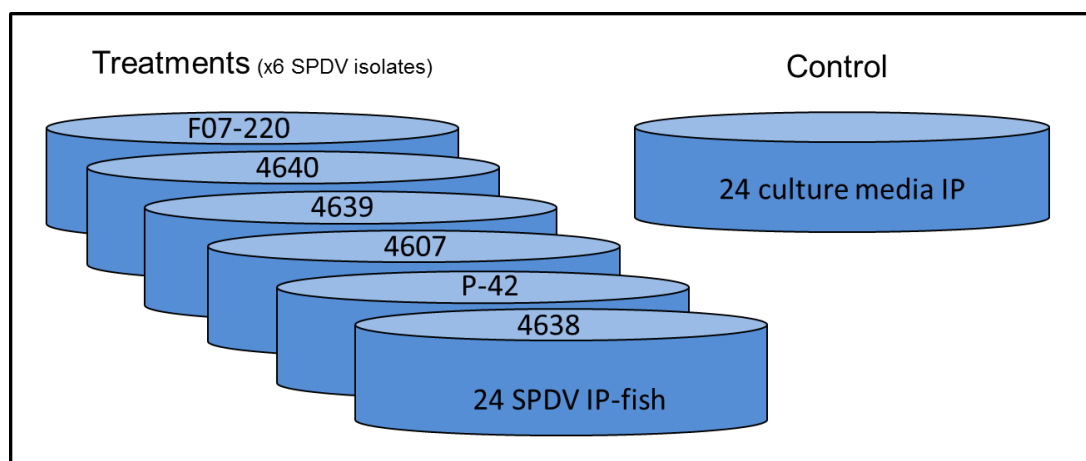
The aim for the work in this chapter was therefore to select candidates among 6 SPDV isolates representing 3 different subtypes from Irish and Scottish salmon farming cases. The challenge methodology was by ip and candidates would be chosen based on the frequency of infection and viral load among seven tissues (heart, kidney, liver, pancreas, spleen, brain and skeletal muscle) as measured by quantitative RT-PCR at a single time point (30 dpi).

Hypothesis: inter and /or intra subtype differences on infectiveness and tissue tropism among SPDV isolates can be assessed by RT-PCR viral load measurement on ip injected fish after 30 days post infection.

Materials & Methods

This study was carried out in strict accordance with the UK Animals (Scientific Procedures) Act 1986 (ASPA) under the project licence PPL3965. The protocol was approved by the Marine Scotland Ethical Review Committee. The challenge used 192 Atlantic salmon post smolts of an average weight of 200 ± 10 g already adapted at water full salinity. A single terminal sampling time point was set at 30 dpi. Fish were sourced from a farm with a history of freedom from SPDV. The SPDV infection-free status of the stock was further confirmed by pre-screening a subset of 30 fish using the same methodology described below for the rest of the samples.

The experimental design included eight tanks of 1 x 0.8 m where fish were equally distributed (x24/tank) and allocated either to as “Control” (culture media) or “Treatment” (SPDV isolates) tanks (Fig 1) at a stocking density of ~8 Kg/m³. Isolates tested represented SPDV subtypes 1 (x3), 2 (x2) and 5 (x1) (Fig 1). Subtypes 1 and 2 were chosen due their frequent presence in Scottish field cases and subtype 5 due the additional contemporary reporting of a “subtype 5 like” isolate in the common dab *Limanda limanda*, a wild fish suggested as a potential wild reservoir for the virus (20). These isolates were propagated at the MSS laboratory using Chinook salmon (*Oncorhynchus tshawytscha*) embryo cells (CHSE-214, ATCC CRL 1681) for 7 days (12).



Isolate ID/ subtype	Dose	Origin
F07-220 (1)	3.06x10 ³	Irish SW salmon, mortality reported
4640 (1)	1.55x10 ⁶	Scottish SW salmon, mortality reported
4639 (1)	1.29x10 ⁴	Scottish FW rainbow trout, mortality unknown
4607 (2)	4.4x10 ⁶	Scottish FW rainbow trout, mortality reported
P42 (2)	3.5x10 ⁷	Scottish SW salmon, “PD type” reference isolate
4638 (5)	1.93x10 ⁶	Scottish SW salmon mortality reported

Fig 1: Experimental setting, subtype identification, dose and origin of the 6 SPDV isolates used for the intra and inter-subtype comparison. Dose in TCID₅₀, SW= sea water, FW= fresh water.

Anaesthesia was performed in a solution of tricaine methanesulphonate (MS222 Sigma, Irvine, UK) at a dose of 80 mg/L before proceeding to ip inject fish with a dose of 0.2 ml of inoculums at doses contained in Table 1. Water temperature was maintained at 14° ± 1°C, flow at 300 L/h , and fish were fed commercial pellet *ad libitum* over the 30 days.

At termination of the experiment all fish were euthanized by an overdose of anaesthesia and weighed before necropsy. Sampling procedure was performed by immediately dissecting

open of the coelomic and pericardial cavities to collect samples into RNA later from pyloric caeca (PC), skeletal muscle (SM), heart (H), liver (L), brain (B), kidney (K), and spleen (S).

The viral load was measured as transcription of the nsP1 gene and the antiviral response was measured as expression of the *mx* gene, and normalized against elongation factor α (*elf* α) (21). In brief, from each sample 9.6 μ l of total RNA and 1.25 μ l 50 μ M oligo-d (T)₁₆ were mixed and heated to 70 °C for 10 min and chilled on ice. A final volume adjusted to 25 μ l was done by adding a master mix comprised of 1x RT buffer (25 mM Tris-HCl pH 8.3, 37.5 mM KCl, 5.5 mM MgCl₂), 0.5 mM each dNTP, 0.4 U RNase inhibitor and 1.25 U Multiscribe Reverse Transcriptase. The reaction was incubated at 48 °C for 90 min, heat inactivated at 95 °C for 5 min and stored at -80 °C until use. Real time qPCR assay was performed using a Roche LC480 System (Roche). The TaqMan probes and primers used to amplify the *elf*, *mx* and *nsP1* genes have been previously reported (22). One μ l cDNA was added to the mix on each of a 96-well optical plate (Roche): 10 μ l of TaqMan 2x PCR mix with UNG (Applied Biosystems), 8 μ l of dH₂O and 1 μ l of a 20x mix containing forward primer (18 mM), reverse primer (18 mM) and probe (5 mM). The standard cycling conditions were 50 °C for 2 min, 95 °C for 10 min followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. The approach to estimate the amplification efficiency was the slope-derived calculation from the standard curve method using the cycle threshold (Ct) value determined by the maximum secondary derivative method; values were converted into expression levels normalised against the housekeeping reference gene (*elf*), using a standard curve. Quantitative RT-PCR was performed in triplicates on the same sample and the average result presented. Elongation factor α (*elf* α) expression was used as an internal control to normalise gene expression levels across different samples.

Results

Using an ip injection route, all isolates successfully established some level of infection by 30 dpi (i.e. detectable virus in tissue). Viral presence as measured by RT-qPCR varied between the seven tissues examined. Overall, irrespective of the isolate the heart had the higher number of positive results, followed by skeletal muscle and brain (Fig 2). When summarizing the frequency data, isolate F07-220 (subtype 1) showed the overall higher frequency of positive individuals in all tissues, followed with equal percentages by isolates 4640 (subtype 1) and 4607 (subtype 2) (Table 2).

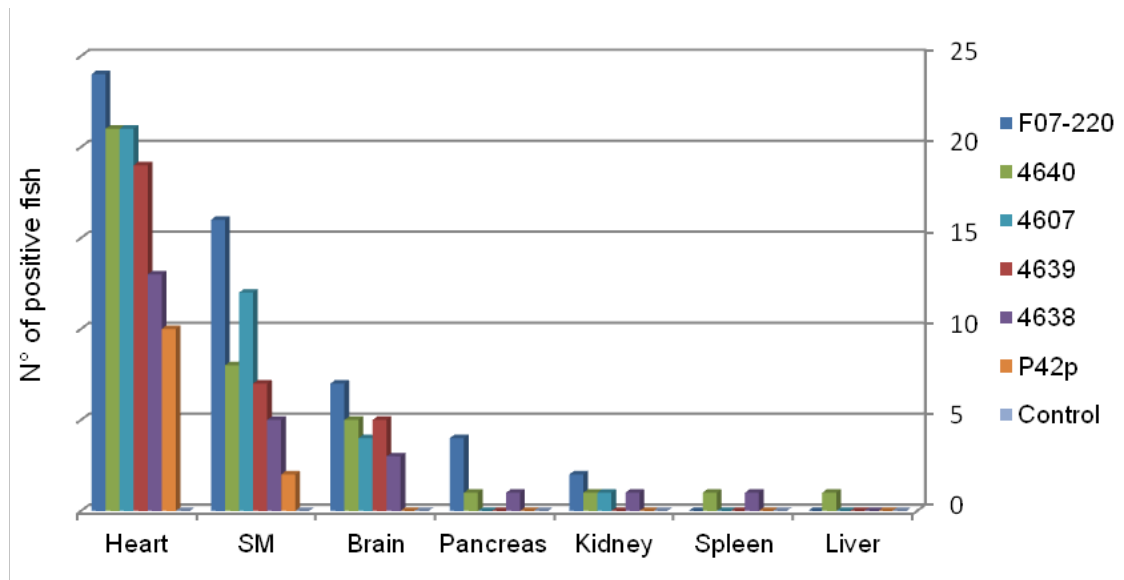


Fig 2: SPDV viral distribution frequency per tissue and isolate at 30 dpi (after PI challenge).

Isolate	Heart	SM	Brain	Pancreas	Kidney	Spleen	Liver	% infected tissues/isolate
F07-220 (1)	24/24	16/24	7/24	4/24	2/24	0/23	0/24	31.7%
4640 (1)	21/24	8/24	5/24	1/24	1/24	1/20	1/23	23.3%
4607 (2)	21/24	12/24	4/23	0/24	1/23	0/22	0/23	23.3%
4639 (1)	19/24	7/24	5/24	0/23	0/24	0/21	0/24	18.9%
4638 (5)	13/24	5/24	3/24	1/24	1/24	1/22	0/24	14.5%
P42p (2)	10/24	2/24	0/24	0/24	0/24	0/21	0/24	7.3%
Control	0/24	0/24	0/24	0/23	0/24	0/24	0/24	0.0%
% isolates/tissue	75.0%	34.7%	16.8%	4.2%	3.5%	1.6%	0.7%	

Table 2: Frequency of (+) fish by tissues and isolate measured at 30 dpi. Reduced number of total samples for some tissues is due to some samples giving undetermined PCR results (suggesting sample was too small or lost). The % of total (+) samples by tissue in all isolates is given in the lower horizontal row and the % of total (+) samples by isolate from all tissues, in the far-right column.

For the 4 isolates with the higher percentage of total infected samples from all tissues (F07-220, 4640, 4639 and 4607), the individual Ct values of all positives recorded in the three main tissues (heart, brain and skeletal muscle) are presented in Fig 3.

The average Ct value of all positive samples by tissue for the same 4 isolates is presented in Fig 4 and data represented as ratios of the viral nsP1 expression relative to the *elf* (average of all positive samples), in Fig 5.

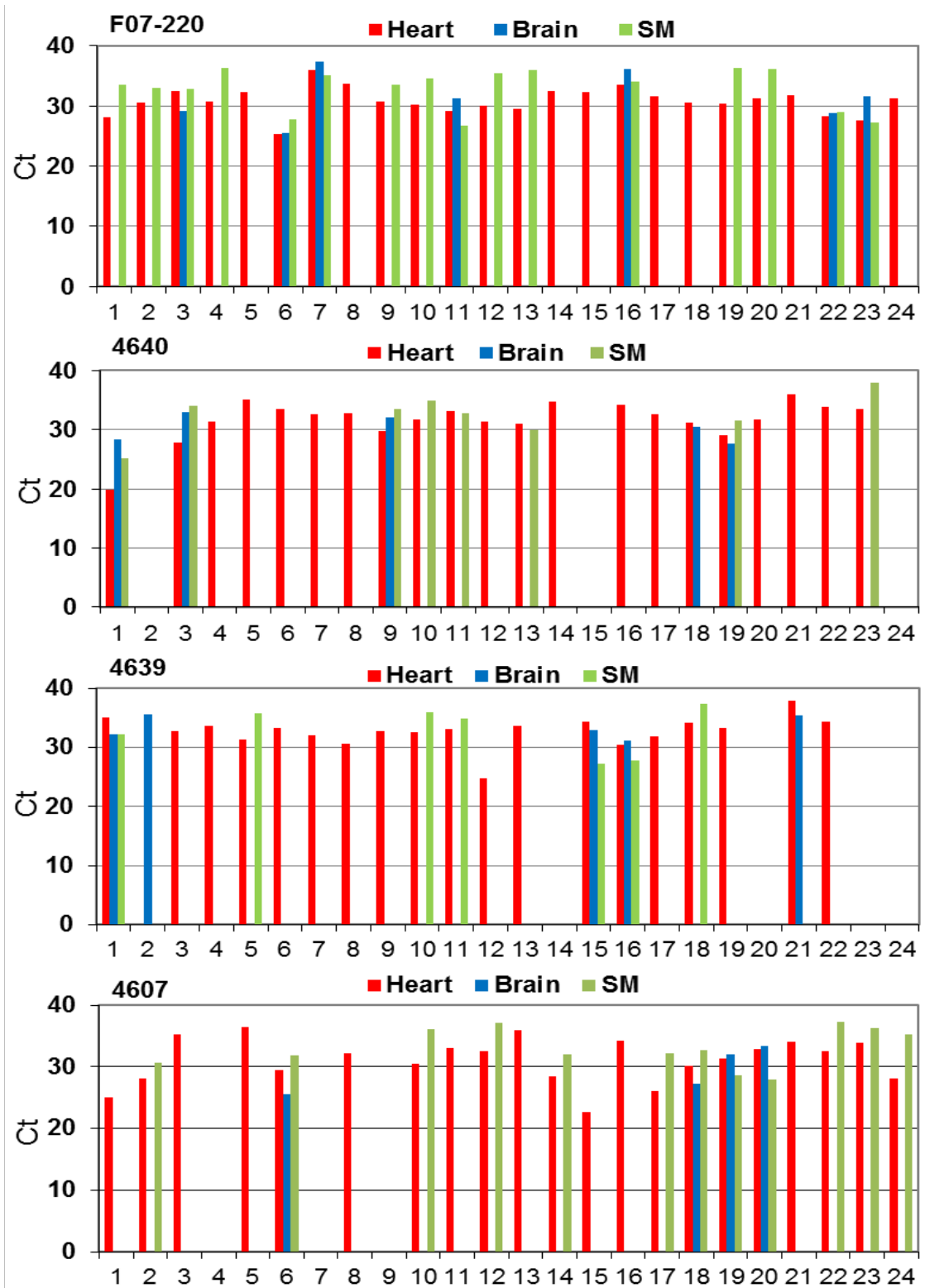


Fig 3: Heart, brain and skeletal muscle individual Ct values of all fish from the 4 isolates with higher than 18% of infected tissues.

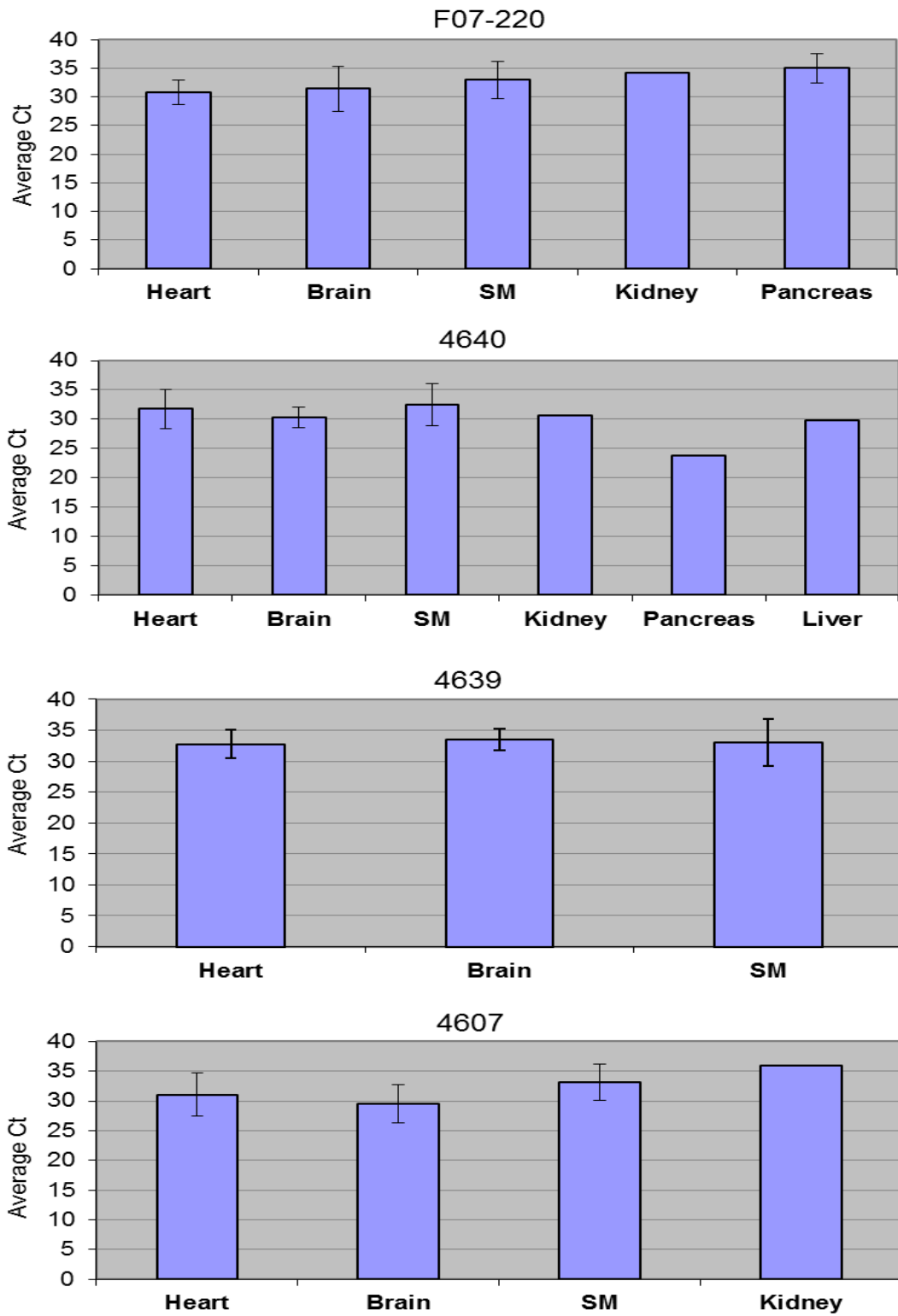


Fig 4: Average Ct values from all the positive tissues in the 4 isolates with higher than 18 % total infected samples. . Errors bars represent standard deviation; where not visible this is due the low sample number of positives for the particular tissue.

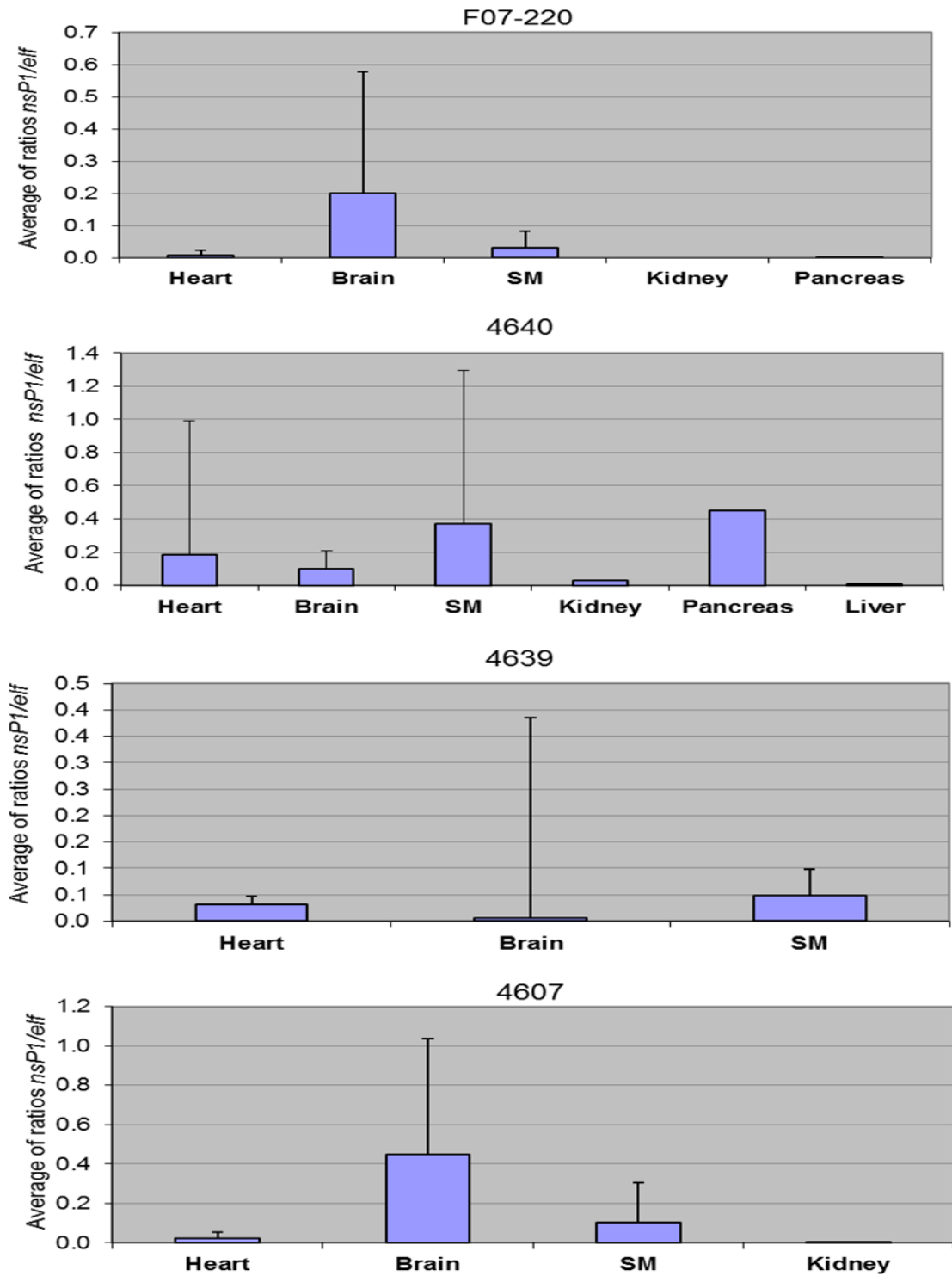


Fig 5: Average of the relative expression of viral nsP1 gene in all positive fish per tissue, in the 4 isolates with higher than 18% of infected tissues. Errors bars represent standard deviation, where not visible this is due the low sample number of positives for the particular tissue.

The average in weight gain from all fish per tank after 30 dpi relative to average weight at start is presented in Fig 6. All infected groups showed relatively decreased weight gain compared to the control group. Among infected fish isolates F07-220 and 4640 (subtype 1) had comparable and relatively lower weight gains. Isolates 4639 (subtype 1), P42 and 4607 (subtype 2) had intermediate relative weight gains, and isolate 4638 (subtype 5) had the highest relative weight gain.

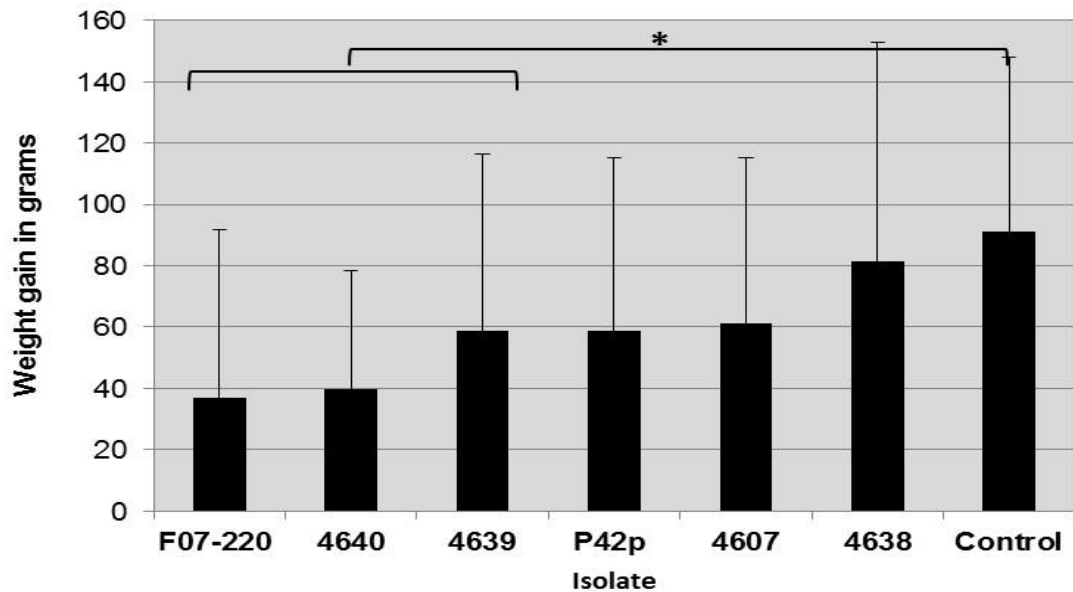


Fig 6 Average and standard deviation of total body weight gain per isolate from the beginning of the challenge to 30dpi. Number of fish per group = 24, * significantly different (Kruskal-Wallis $P < 0.05$)

Discussion

While currently inter and intra subtype variations on SPDV infectivity and pathogenicity are acknowledged and reported (18), at the time this work was carried out it was essentially suspected from diagnostic observations, preliminary experimental work and a subject of discussions among colleagues working in the discipline.

At the initial attempt of establishing infection by cohabitation with isolate 4640, a 50/50 ratio ip to ch fish failed to establish infection after 35 days. The ip fish examined at terminal sampling 45 dpi showed only ~30% had a positive SPDV signal at one or more of the heart, brain and /or skeletal muscle samples (data not shown).

Different factors may have determined the failure on transmission on that experiment, including an insufficient number of ip shedders, the effect of potential procedural errors on dose preparation or deliver, changes in virulence due passages and culture adaptation (18,23,24) or a potential variable susceptibility of the fish stock as previously reported (25). However the negative result could also have been due, as here hypothesized, to differences in infectivity between isolates for which there was no published evidence at the time. The

reported experiment in this chapter was therefore designed to assess these differences in a range of isolates available at the MSS laboratory.

The experimental comparison using purified isolates from outbreaks in Scotland (x5 isolates) or Ireland (x1), used detection of SPDV RNA in tissues after 30 dpi and differences in weight gain data to decide on best candidates. The 30 dpi time point for terminal sampling was chosen based on results from previous reports on ip infection challenges (13), and although earlier time points could have been chosen, it was meant to cover a window to allow detection of viral RNA in the skeletal muscle, which was reported to occur later during the infection.

Results pointed to 4 isolates with a better potential i.e the three subtype 1 isolates (F07-220, 4640 and 4607) and one of the subtypes 2 (4607). It is necessary to acknowledge the inoculum doses were different for each isolate, particularly those of F07-220 and 4639 lagging behind ~2 orders of magnitude compared to the others. This was indeed an unfortunate mistake due to the eagerness to initiate the experimental work and where information on titration became available only after the experiment started. Interestingly however, in spite of receiving the highest dose, isolate P42 in this experiment had the lowest viral level overall, while isolates F07-202 and 4639 with the lowest relative dose of infection, were among the highest.

Isolates 4638 and P42 presented the lowest percentage of infected tissues with 14.5 and 7.3 % respectively. The total weight gain used as additional criteria for selection showed that both 4638 and P42 were among the isolates with no significant differences against controls, along with 4607. For these reasons there was no further focus on these 2 isolates for the present work.

Isolate F07-220 showed the highest overall relative frequency of viral detection in all tissues, as well as the relatively lowest weight gain. Isolate 4640 and 4607 had similar frequency of detection in the main 3 tissues (heart, skeletal muscle and brain), however 4640 was also detected in other 3 additional tissues (liver, spleen and pancreas) although in single individuals.

Even though the actual target cell/cells for the virus has/have not yet been clearly identified (26), SPDV has been detected in all the tissues or organs examined and hence is being reported as a pantropic virus (13,27). More recently it was shown that it is also detectable in mucus and faeces (28).

General viral screening by PCR for diagnostic purposes at the Marine Laboratory was traditionally done from kidney samples. Kidney had proven successful for detection of several other salmon viral agents of Atlantic salmon such as Infectious Pancreatic Necrosis virus (IPNV), Viral Haemorrhagic Septicaemia virus (VHSV), Infectious haematopoietic necrosis (IHN) and Infectious Salmon Anaemia virus (SAV) (29). For SPDV however, it was becoming recognised that the inclusion of heart tissue was relevant to improve detection, particularly during the chronic stages where RNA had been shown to persist in the heart for ≥ 140 dpi (30). The results of this study, which examined a wider set of tissues for SPDV detection, further provided evidence to support the inclusion of heart when screening for SPDV, and a mix of kidney and heart tissues became the norm for diagnostic samples intended for SPDV at MSS (MSS internal protocols).

In this study at 30 dpi all fish had at least one tissue with a positive SPDV detection. However, with 75% of samples positive across all isolates, the most efficient tissue for SPDV detection was the heart, with more than double the frequency of detection when compared with the next most efficient (skeletal muscle) followed by the brain. In this study the four other tissues examined showed hardly any detection. The results from heart, skeletal muscle and brain are in line with SPDV detection by virus isolation or PCR from previous reports, including although less frequently, that of the brain (13,31,32). Interestingly, in this comparison, although frequency of detection and Ct values from the brain were not high, in the case of two isolates (F07-220 and 4607) the viral RNA relative expression level to housekeeping gene (*elf*), showed instead more relevant than heart or skeletal muscle. Brain viral RNA signal was reported to remain detectable up to 190 dpi in a Norwegian tissue tropism experiment (13). Considering the role of the brain as target tissue in alphavirus infections of other vertebrate hosts (33), relevance during SPDV infection is a subject that deserves yet further investigation.

Based on the current results two subtype 1 isolates (F07-220, 4640) were chosen for further work. Isolate 4607 also showed promising results with comparable frequencies of detection in the heart and skeletal muscle. In terms of weight gain however, it was the second best after the controls. As a subtype 2 isolate and associated with fresh water sleeping disease, 4607 was considered less suitable also because work in this project was intended to continue specifically in Atlantic salmon.

Incidentally, the choice for including 4640 was reinforced by additional information from an *in vitro* comparison exercise that was conducted contemporary at MSS. The *in vitro* cell culture comparison used TO cells (cell line originated from Atlantic salmon head kidney leucocytes) (34) and included all but one (F07-220) of the isolates used in the here reported *in vivo* work.

The study measured viral *nsP1* gene expression relative to *elf* along 14 days and results highlighted the performance of isolate 4640 (summary figure from that study provided under Annex as Chapter 1, annex figure 1). Although the infectivity and/or virulence of viral isolates may differ between *in vivo* and *in vitro* work, the additional information of 4640 *in vitro* infectivity reinforced the decision for its inclusion.

In regards to tissue sample for further studies, the heart, skeletal muscle and brain were chosen for further work. Although is not certain if the molecular signal represents a true carrier state, the results specially for the heart coincides with the organ being one of the most relevant during the disease with pathological changes characteristic of PD. The heart in PD infections as a theme has been maintained as a focus during the current PhD studies.

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CHAPTER 3: EXPERIMENTAL COMPARISON OF SPDV-1 USING A CO HABITATION SEA WATER MODEL

Introduction

The establishment of a cohabitation sea water infection model that mimics closely a natural infection on Atlantic salmon post smolts had been set as a requirement for the study of SPDV infection strategies in the first section of this PhD project.

Experimental transmission of SPDV had been successfully achieved soon after the disease was recognised, initially by intraperitoneal (ip) injection of tissue homogenates (1,2) and later with purified virus (3–6). This challenge approach by ip infection has been extensively used to study the disease including aspects of host immune response.

As it was found that SPDV can induce both Pancreas disease (PD) in Atlantic salmon (*Salmo salar* L.) as well as Sleeping disease (SD) in rainbow trout (*Oncorhynchus mykiss*) both in fresh and salt water, experimental infections have been frequently performed in fresh water using either fish hosts (7,8). In the case of Atlantic salmon, most experiments have used fresh water developmental stages: fry, parr or pre-smolt (4,6,9–11). Although an early report of successful transmission by co habitation (reported as “in contact” fish) using a 2:1 ratio of ip to cohabitant (ch) fish in sea water Atlantic salmon post smolts was reported (3) fresh water challenges remained a very common choice. The approach kept being popular even in co-habitation challenge performed more recently, such as the comparison of several sea water origin isolates representing the different subtypes (12).

This approach may have had a strong influence in the results of these studies, considering the profound adaptations of the fish’s endocrine, osmoregulatory, and immune system known to occur during the smoltification process preparing salmon for their sea water life (13,14). It is important to consider the influence that physiological, morphological and behavioural changes associated to smoltification could have on salmon susceptibility and response to SPDV infection. For example, substantial changes in immunocompetence, such as decreased levels of serum protein and IgM during the parr-smolt transformation (15) or the compositions of leukocyte populations (16) have been reported.

Although currently the above has been fully recognised and recently, more complex models of bath immersion (BI) in sea water have been proposed (17), at the time this thesis project was being designed a co habitation sea water model had not been established at Marine Scotland Science (MSS).

Teleost fish as other vertebrate hosts, present an innate, early line of defence against viral infections mediated by the interferon (IFN) system (18,19). Considered the most important component, type I interferon (IFN-I) is present and well developed in Atlantic salmon (20) and among the several IFN stimulated genes (ISGs), *mx* in salmonids has been profusely studied and shown to be only one with relevant antiviral properties (21). They induce the Mx proteins, members of the dynamin superfamily of large GTPases responsible for rendering the antiviral state within the cell.

Contemporary *in vitro* studies at MSS on the host response to SPDV of a novel genetically modified CHSE cell line over-expressing the Interferon (IFN) inducible *mx* gene, it was shown that the Irish type reference F93-125 was more sensitive to the Mx protein than isolate 4640 (Marine Scotland Science isolate, originated on sea water salmon from Scotland) (22). Additionally, these two same isolates despite showing a 99.4 % similarity in amino acid sequences over 9 coding regions, were reported to elicit different kinetics of viral *nsP1* and host *mx* genes expression (23), when infecting T0 cells, a line originated from Atlantic salmon head kidney leucocytes (24). While isolate F93-125 induced a fast and high level of viral gene expression leading to cytopathic effect, isolate 4640 induced low levels of viral gene expression and the culture did not show CPE. Additionally the expression level of the *mx* gene was considerably higher in cells infected with isolate 4640 than with F93-125 (23). All these suggest a fundamental difference in virulence strategy between highly similar SPDV 1 subtypes isolates, potentially involving IFN evasion mechanisms.

An additional factor to consider for results interpretation is the previously mentioned potential of cell culture adaptation (see Chapter 1). Similarly as with viral pathogen in other hosts, a low passage number of laboratory cultures to be used for *in vivo* infections is important to avoid or reduce the potential attenuation due culture adaptation, Such situation had been already suggested for SPDV (25,26) where isolate F93-125 had been reported to have lost virulence *in vivo*, potentially as the results of this culture induced adaptation (6).

The previous work had pointed to isolate 4640 as one of the more appropriate candidates for further experiments (chapter 1). This chapter's objectives were therefore to:

- Establish a working sea water challenge model by cohabitation
- Apply the model to compare *in vivo* putative intra subtype infectivity differences between F93-125 and 4640
- Identify viral tropism patterns during the course of infection (by quantitative RT-PCR)
- Evaluate viral *nSP1* and host *mx* genes expression along time.

Hypothesis: the dynamics of infection of two SPDV-1 isolates on Atlantic salmon smolts in a sea water, can be examined through a longitudinal time course experiment after natural infection by cohabitation

By challenging Atlantic salmon post smolts through a cohabitation model in sea water, this section of the work aimed to assess the kinetics of infection of two SPDV 1 isolates in sea water post smolts. Previous *in vitro* information of the two isolates supports comparison of intra subtype differences, using the kinetics of the viral expression and host *mx* response and evaluating the putative loss of *in vivo* infectivity of F93-125. Additionally, a virus distribution among three tissues (heart, brain and skeletal muscle) over 8 weeks post infection could be examined.

As the current experiment was to go ahead, unexpectedly the aquarium facility at MSS became unavailable. To compensate for this, a bid for external funding was developed through the Network of Animal Disease Infectiology Research (NADIR). The successful bid made funds available to take it to VESO-VIKAN in Namsos (Norway), finally allowing for the experiment here reported to be completed.

Materials and methods

Unvaccinated Atlantic salmon smolts (390) with an average weight of 160 g \pm 10 g were obtained from a single source. A sub sample (n=30) was pre-screened by RT-qPCR to check viral free status from SPDV, and other prevalent infections such as Infectious Pancreatic Necrosis virus (IPNV), Piscine myocarditis virus (CMSV) and Piscine Reovirus (PRV). Fish were acclimatized to seawater at 14° C for 2 weeks prior to the challenge. They were randomly distributed into groups of 72 fish into 5 separate 1 m diameter, 500 L tanks. Four tanks were allocated to “treatment” (replicates per SPDV isolate) and one to “control”. Water flow was maintained at 10.8 l/min and oxygen saturation at 70%. The rate of (ip) injected shedder fish to cohabitant (ch) fish was set at 1.25:1.

From each tank, forty fish were anaesthetised in a solution of tricaine methanesulphonate (MS222 Sigma, Irvine, UK) at a dose of 80 mg/L. Each fish had its adipose fin clipped using a VESO approved protocol for individual fish identification.

The virus isolates had been propagated at the MSS laboratory using Chinook salmon (*Oncorhynchus tshawytscha*) embryo cells (CHSE-214, ATCC CRL 1681) for 7 days (4), they were titrated in the same cell line and material sent frozen to VESO. Prior to the challenge, virus stock was thawed at room temperature and doses prepared. Shedder fish from replicate treatment tanks were ip injected with 200 µl at 6.25×10^5 TCID₅₀ for isolate 4640, and at 6×10^5 TCID₅₀ of isolate F93-125. Control tank shedder fish were ip injected with 200 µl of uninfected tissue culture media (CHSE-214). Sheddens were then released back to the tank, which they shared with thirty-two ch fish, where they fully recovered from anaesthesia within minutes. Water flow was reduced to 200 l/h (approximately 1/3) between days 5-10 post shedder infection. The experiment was run for 61 days post shedder infection. Fish were fed to appetite throughout, although food was withdrawn 12–24 h before handling for sampling.

Sequential sampling points of ch fish were pre-established for 5, 8, 12, 16, 21, 30, 44 and 61 days post shedder infection (dpi). At each time point an overdose of anaesthesia (MS222 as above) was used to euthanize a sample of 4 ch fish (identified by the absence of fin clipping) per tank, adding to a total of 20 fish per time point (8 per treatment and 4 controls). All fish were weighed and had a blood sample of ~2-4 ml taken through caudal vein puncture into vacutainers tubes containing sodium citrate (Greiner). These blood samples were taken for a collaborative contemporary project carried out by colleagues of the MSS research group. The work intended to develop and validate a method to measure viraemia indirectly using an Atlantic salmon reporter cell line. For RT-PCR, small portions of skeletal muscle (SM), heart (H), and brain (B) were collected into RNA later. At the termination of the experiment all remaining fish (ip shedders) were euthanized by terminal anaesthesia, and blood and heart tissue samples collected from 10 such fish from each treatment tank.

The viral load was measured as transcription of the *nsP1* gene and host response as expression of the *mx* gene, normalized against elongation factor (*elf α*) following the same procedures as described in chapter 1, but using duplicates instead of triplicates for the quantitative RT-PCR. Expression of *mx* from control fish was only performed in the heart samples. For quantitative assessment of the gene expression, delta Cts relative to *elf* was used. The weight average of all fish sampled per group (treatments and control) was calculated at each time point, and total weight gain per isolate was calculated as the difference between the average weights at the last time point relative to the average weight at the start of the experiment.

Results

No mortality was recorded among ch fish or any of the control fish during the length of the experiment. Only two individuals from each replicate tank of isolate 4640 were found dead on days 27 and 33 post infection, corresponding to ip (shedder) fish.

Isolate F93-125 failed to establish a measurable infection on the ch fish with no detection in any of the three tissues analysed along the 8 sampling time points. Results from ip fish at terminal sampling showed none of the F93-125 ip injected fish were positive after 61 dpi.

Isolate 4640 was successfully transmitted to ch fish. The first positive signal was recorded at 12 dpi in 2/8 fish in the heart and brain tissues. The peak of detection was at 30 dpi when 8/8 heart samples, 5/8 brain samples and 6/8 skeletal muscle samples tested positive. The peak was followed by a decrease by day 44 with 4/4 detection only on the heart and brain and by day 61 detection in heart decreased to 3/8 while skeletal muscle showed 5/8 positive fish (Fig. 1).

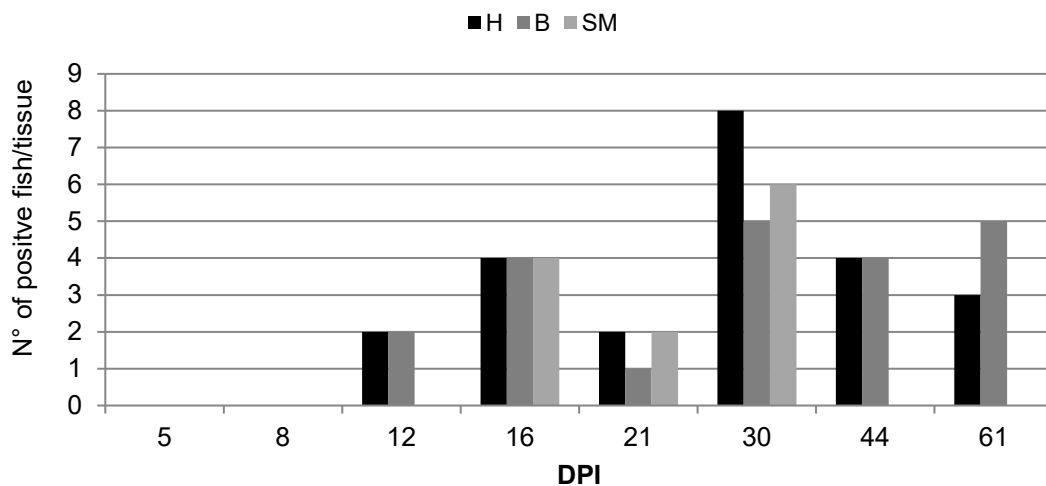


Fig. 1: Isolate 4640 *nsP-1* viral signal per tissues and time point. H=heart, B=brain, SM= skeletal muscle.

The viral *nsP-1* and *mx* expression levels relative to housekeeping gene *elf* are shown by tissue in figures 2-4. Briefly, in the heart viral *nsP-1* signal was first detected at 12 dpi and peaked by day 16, coinciding with the strongest expression of *mx* (Fig. 2). From that point viral signal decreased reaching lower levels than initial signal at day 12, by 61 dpi. The *mx* expression, particularly from infected fish only (purple curve in graphic), followed a similar projection to end at same levels than controls by the end of the 8 weeks period.

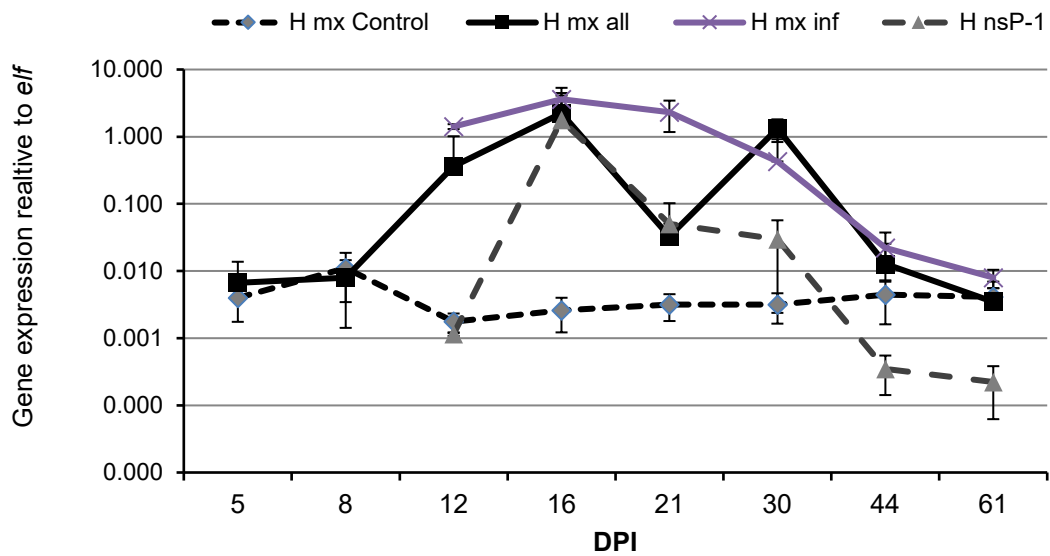


Fig. 2: Kinetics of viral *nsP1* and *mx* gene expressions of heart (H) tissue of Atlantic salmon post smolts, after experimental infection with SPDV subtype 1 (4640) by co habitation. The *mx* expression of infected fish only is indicated in purple. Vertical bars indicate standard deviation, sample size = 8 fish per time point.

Brain viral *nsP1* signal was first detected at 12 dpi, increasing by 16 dpi but describing a sharp decline by 21 dpi. After that the peak expression on this tissue was recorded by 30 dpi, declining thereafter. The *mx* expression showed a slow steady increase from 5 dpi on and peaking by 30 dpi. Note that when only viral infected fish are considered (purple line) the peak was recorded instead at 16 dpi. By 44 dpi viral and *mx* genes expression started to decline and continued so until terminal sampling. The infected fish only showed a decline at 44 dpi but increased again by 61 dpi terminal sampling.

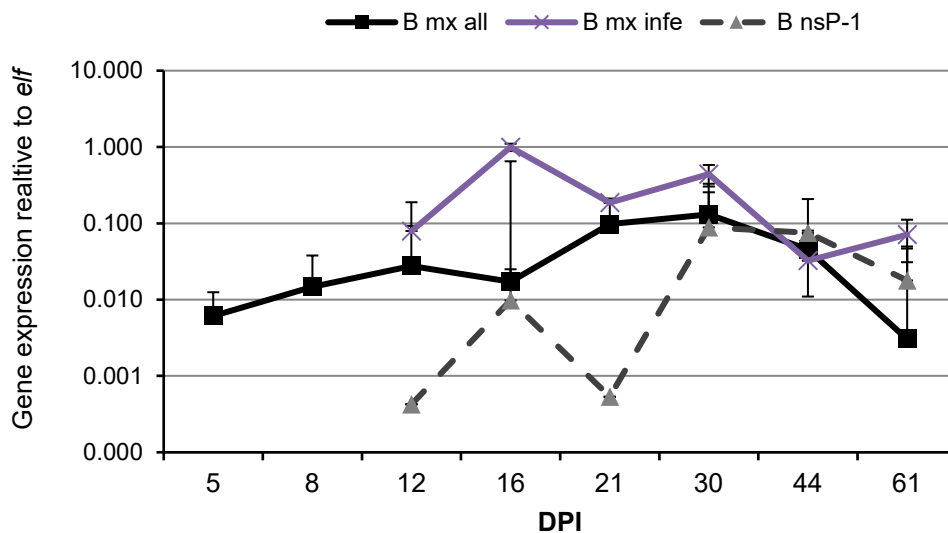


Fig. 3: Kinetics of viral *nsP1* and *mx* gene expression of brain (B) tissue of Atlantic salmon post smolts, after experimental infection with SPDV subtype 1 (4640) by co habitation. The *mx* expression of infected fish only is indicated in purple. Vertical bars indicate standard deviation, sample size = 8 fish per time point.

The skeletal muscle viral signal was detected only at 3 time points and started with its peak expression at 16 dpi, declining thereafter. The *mx* response peaked at 12 dpi and sharply decline by 16 dpi. Signal detection returned to peak level by 21 dpi and decreasing thereafter without reaching the low levels from 5 and 8 dpi. Note infected fish only (purple line) described the peak expression at 16 dpi in coincidence with viral signal.

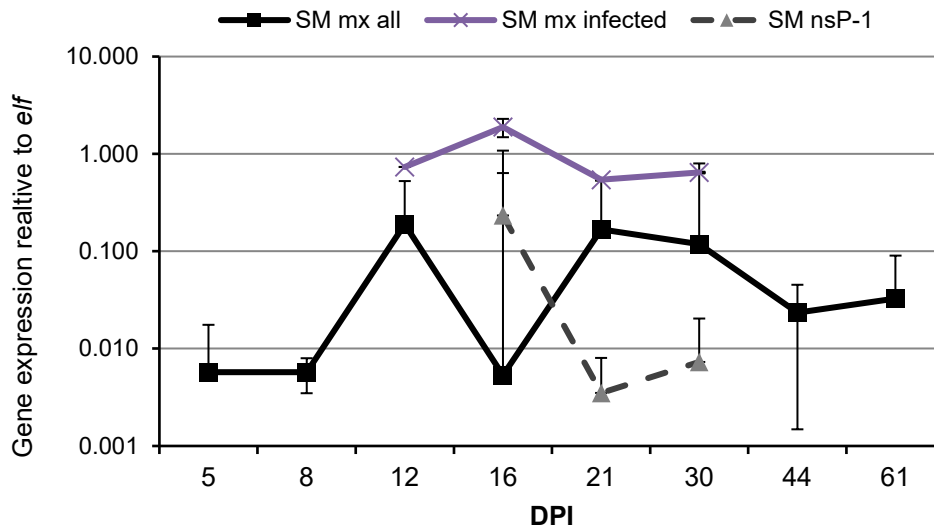


Fig. 4: Kinetics of viral *nsP1* and *mx* gene expression of skeletal muscle tissue of Atlantic salmon post smolts, after experimental infection with SPDV subtype 1 (4640) by co habitation. Vertical bars indicate standard deviation, sample size = 8 fish per time point.

No viral RNA signal in F93-125 ch fish was detected, however the heart *mx* gene expression after a steady decline along the 6 first time points showed a mild relative increase by 44 dpi, ending higher than controls fish by terminal sampling point (Fig. 5).

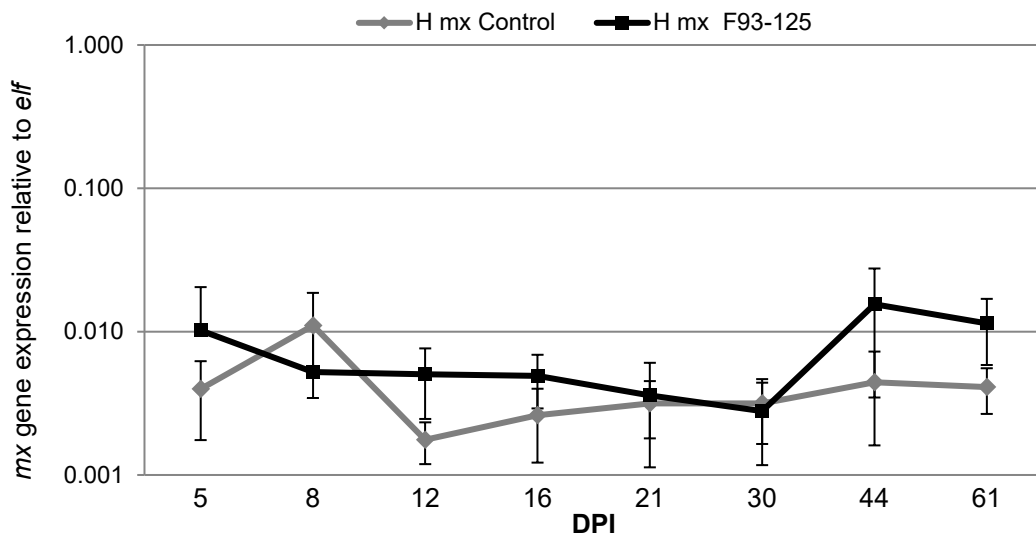


Fig. 5: Kinetics of *mx* gene expression of heart (H) tissue of Atlantic salmon post smolts, after experimental infection with SPDV subtype 1 (F93-125) through co habitation.

The average weight per group at each time point showed both infected groups lagged behind control fish the differentiation starting from 21 dpi on (Fig. 6).

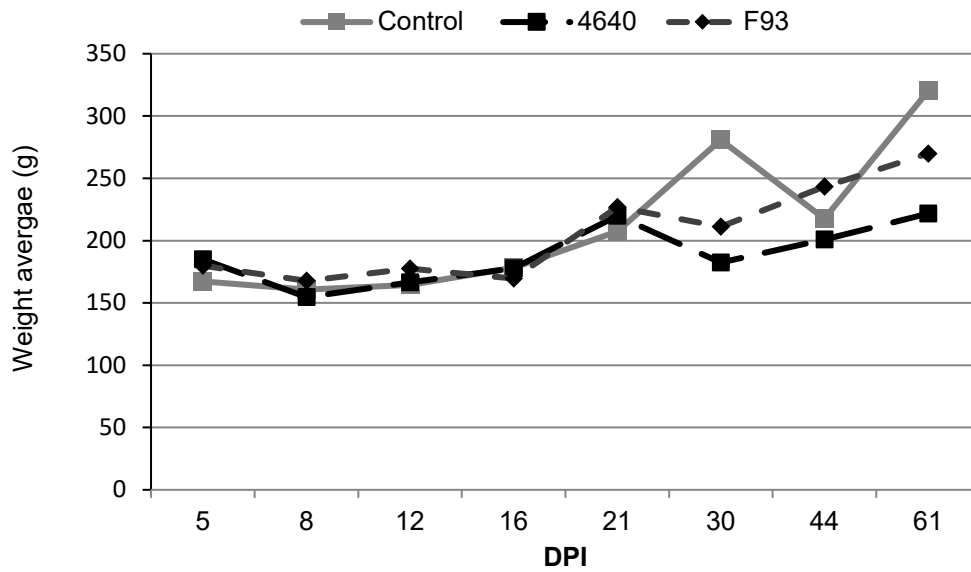


Fig. 6: co-habitant fish average weight by sampling time point.

Weight gain of ch fish challenged with isolate F93-125 was 59.2% that of control ch fish while ch fish challenged with isolate 4640 showed the lowest weight gain, reaching only 23.9% of that from controls and 41% of the gain by isolate F93-125 (Fig. 7).

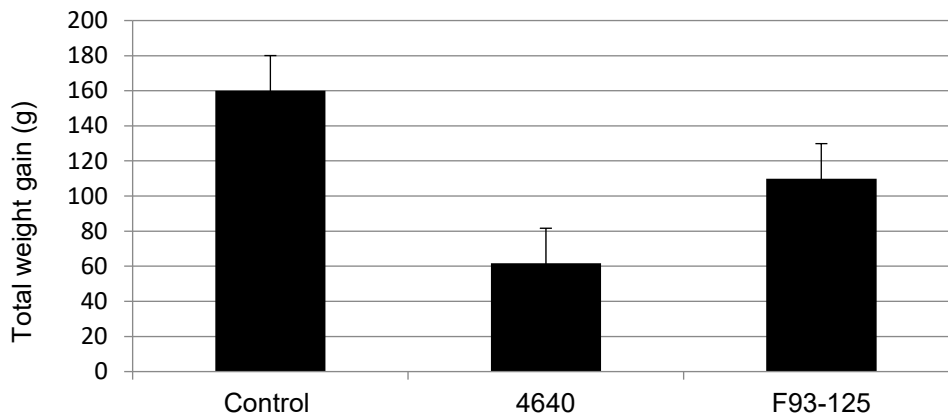


Fig. 7: Cumulative weight gain by co habitant fish on treatments and controls groups.

At terminal sampling of isolate 4640 ip fish, from only 3/20 heart tissue samples showed a low positive viral signal.

Discussion

The current experimental infection aimed to explore potential SPDV intra-subtype differences *in vivo*, by comparing two SPDV-1 isolates with reported *in vitro* differential performance in terms of infectivity. To mimic as close as possible natural infection in sea water at the developmental stage more vulnerable to the disease (17), the experimental setting aimed to achieve transmission by co habitation using salmon post smolts.

The reasons behind the very variable mortality and pathology observed after SPDV infection, both at the farm level and experimentally, are not fully understood but suggestions include the single or combined effect of co-infections, environmental factors and potential differences among subtypes (12,27).

Based on sequencing data of SPDV isolates collected over a 20 years period from marine farmed Atlantic salmon (28), analysis of the geographical distribution of SPDV showed that subtypes 1, 2, 4 and 5 can be found in Scotland. However, consistent clusters of a more dominant subtype were also shown to exist, such as subtype 1 in the region of Argyll and Bute or subtype 2 in the Shetland and Orkney islands. Albeit only descriptive, observations from field cases arriving at the MSS diagnostic laboratory pointed as well to considerable variations in the mortality, clinical presentation and pathological changes between cases associated with isolates belonging to the same subtype (personal observations, MSS diagnostic internal reports). Observed variations of the clinical and pathological presentation can be related to several other factors as previously suggested, as well as confounding effects of the disease stage at the sampling time. However, the observations also suggest intra-subtype or isolate-related variation.

Differences in the dynamics of infection between subtypes have been observed during the comparison of isolates originated in marine cases representing all 6 subtypes (12). In their experimental infection by co-habitation in fresh water, transmission was achieved with all isolates. However, there were differences in transmission efficiency and pathological, serological and virological levels were also highly variable. For instance, subtypes 1 and 3 had consistently higher virus loads and associated pathology, coinciding with a synchronous ch fish infection and differentiating these two subtypes from the performance of subtypes 2 and 6.

In the current experiment isolate F93-125 showed no detectable viral signal at any tissue along the 8 weeks of the experiment, this is in agreement with previously suggested infectivity attenuation of the isolate (6,25). The heart *mx* gene expression for F93-125 showed a slight increase relative to controls by terminal sampling (Fig. 5), the average

weight gain was intermediate between the controls and isolate 4640 (Fig. 6) and the cumulative weight gain showed to be ~40% lower to that of controls (Fig. 7). Altogether, this seems to indicate that while not detectable with the here applied approach, there could have been a mild effect of F93-125.

Results from previous Norwegian work on tissue tropism of different SPDV subtypes concluded in a similar hypothesis after using F93-125 as a representative of SPDV subtype 1 (26). Isolate F93-125 as the founder of subtype 1 (original PD case reported) (29) had been widely used for experimental infections until the suggested loss of virulence started to be reported. In the Norwegian experiment, no clinical signs or macroscopic lesions were observed but 40% from a total of 303 samples from 7 different tissues were positive for the virus by RT-PCR. The highest RNA loads and percentage of positive samples was observed within 7 dpi, however, the F93-125 viral loads were ~ 5000 folds lower than those from fish inoculated with the subtype 3 being compared (derived from infectious tissue homogenates). The authors' conclusion was that while F93-125 may have still be infectious, a culture adaptation process (or an adaptation to British salmon strains) could be behind the lower viral loads and a faster clearance by host immune response noted for the isolate (26).

The current experiment achieved the goal of cohabitation transmission with isolate 4640, with viral detection starting in one or more fish and tissues at 12 dpi and remaining detectable until the end of the experiment 61 dpi. Detection peaked at 30 dpi with all three examined tissues positive and the heart and the brain samples remaining positive by 61 dpi. No mortality was recorded but average weight among ch fish lagged behind control and of isolate F93-125 (Fig. 6), the total cumulative weight gain relative to controls was only ~24% (Fig. 7).

In the current experiment the kinetics of expression of the *mx* gene described a good alignment with the viral signal, particularly in the heart tissue (Fig. 2), followed by the brain (Fig. 3). From 12 dpi and until 44 dpi the heart *mx* expression was at all times higher relative to controls, which coincide with the increased on viral signal peaking by 16 dpi. When viral signal decreased to levels below those of the initial detection, the *mx* expression decreased too (44 dpi) and continued decreasing to reach control levels by 61 dpi. The brain tissue kinetics followed a similar pattern although viral signal was initially detected and peak with a week delay. For the skeletal muscle the signal of viral and *mx* gene expressions were not as neat, most likely related to the low number of positive fish. Particularly, the signal for the virus was fragmented but still showed a response of *mx* that remained induced even when virus signal was no longer detected in the tissue by 30 dpi (Fig. 4).

Interferon stimulated genes (ISGs) are essential for host antiviral defence and among them, *mx* is the most widely studied in teleost fish (30). Differences in the expression pattern(s) of the *mx* gene induction have extensively been used to assess host response during viral infections of fish, including Atlantic salmon (31,32). For comprehensive reviews on the subject see (20,30,33,34).

In all three tissues examined in this experiment, when the average *mx* expression of infected fish only (purple line in figures 2-4) was outlined, it showed always to be higher than the all fish average. This is not surprising and suggests that although *mx* induction becomes systemically detectable (as shown with these same set of samples when blood was tested (35)) the individual contributions of the specific infected tissues are clearly important. In this sense it highlighted the mistake of the current experimental design when choosing to measure from control fish only the heart *mx* rather than from all three tissues examined.

In principle, this *in vivo* comparison seemed not to align with previously reported *in vitro* results (23). However, it is to notice that host response to isolate 4640 at the heart tissue (as *mx* expression therefore the same parameter used for the *in vitro* work), may have comparatively managed to successfully restrict virus replication within the period analysed (Fig. 2), and *mx* levels returned to normal by the end of the experiment. In that way results are a similar outcome to what occurred *in vitro*, however in a much-extended time period of 8 weeks. The 4640 ip fish results indicating the virus had been cleared, would possible reflect the host response capacity suggesting that, although isolate 4640 can clearly infect and replicate for transmission to occur, it can also be cleared by the host within a period of 8 weeks.

As a parallel useful outcome of this experiment, the blood samples collected from isolate 4640 were used for a contemporary project that examined the capacity of a reporter cell line to detect viral infection. This resulted in the proof of concept of the successful detection of viraemia by monitoring blood from infected fish (35), with comparable results to those obtained with the here reported data from tissues. The parallel project resulted in a publication in the *Journal of Virological Methods* 191 (2013) 113– 117 (provided under Annex, Chapter 2, paper 1).

SPDV has unfortunately been around the aquaculture industry for decades; however, lots have been learnt since the early reports of the disease. Experimental models have experienced an important refinement and although ip and cohabitation models would remain as useful tools, since the work here reported was performed other very interesting and more complex approaches have been developed, such as the recently proposed infection by bath immersion used to demonstrate the differential susceptibility of smolts depending of time

after transfer to sea water (36) . This later approach where the exposure time and control of the viral dose can be accurately estimated would likely be the way forward to further exploring *in vivo* the remaining challenges SPDV impose to the sustainable growth of the salmon farming industry.

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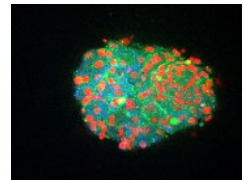
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CHAPTER 4: SALMON CARDIAC PRIMARY CULTURES, A SPECIES-SPECIFIC *IN VITRO* MODEL TO STUDY ATLANTIC SALMON CARDIOTROPIC CONDITIONS

The content of the following chapter is presented in the form of a published paper: Atlantic salmon cardiac primary cultures: An in vitro model to study viral host pathogen interactions and pathogenesis, Patricia A. Noguera, Bianka Grunow, Matthias Klinger, Katherine Lester, Bertrand Collet, Jorge del-Pozo. **PLOS ONE 2017, Vol:12 (7) pp:22.**



Introduction

Atlantic salmon (*Salmo salar*) production has increased in the past 30 years to become the world's largest by volume among salmonids. Inevitably, as any other intensively farmed species, infectious diseases in aquaculture are a major constraint to the industry and salmon is vulnerable to several viral agents that target the heart tissue, such as those induced by Salmon Pancreas Disease Virus (SPDV).

In order to help support the development of disease control and management strategies, the knowledge gaps identified by the industry and research communities were at the centre of this thesis project. One of such gaps was the improvement of experimental models for the study of transmission and disease mechanisms in SPDV infections.

In spite of a pantropic tissue distribution (1) infections by SPDV have three main target tissues recognised as most severely affected, e.g. the pancreas, the skeletal muscle and the heart (2), tissues traditionally examined for diagnosis by histopathology. Viral screening by molecular approaches (PCR) has shown the relevance of the heart tissue for detection and similarly, observed during the previous *in vivo* work of this thesis (chapters 1 and 2).

Understanding the basis of cardiac diseases in fish is limited by the lack of models that would better reflect the complexity of the heart (3). Research in fish disease seems to lag behind with approaches and technologies applied long used in veterinary and human medicine, such as cellular models. Interestingly, fish has become a widely used model for applied research on non-piscine species. This includes the development self-contracting cardiomyocyte aggregates isolated from rainbow trout and intended for human's drug pharmacological testing (4,5). It was the awareness of that type of approach that triggered the interest in adapting and establishing a model based on the species of interest –Atlantic salmon- and to evaluate their potential as an *in vitro* model specifically intended for fish disease research.

The content of this section presented as paper 1 represents my own work where I was responsible of the conceptualization, performing the experimental work, data curation, formal analysis, writing the original draft, collecting co-authors feedback, review and final editing. Paper co-author's specific contributions are fully acknowledged and details accessible through the on-line open access of this paper.

Paper 1

Please visit: <https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0181058>

RESEARCH ARTICLE

Atlantic salmon cardiac primary cultures: An in vitro model to study viral host pathogen interactions and pathogenesis

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OPEN ACCESS

Citation: Noguera PA, Grunow B, Klinger M, Lester K, Collet B, del-Pozo J (2017) Atlantic salmon cardiac primary cultures: An in vitro model to study viral host pathogen interactions and pathogenesis. PLoS ONE 12(7): e0181058. <https://doi.org/10.1371/journal.pone.0181058>

Editor: Lloyd Vaughan, Veterinary Pathology, SWITZERLAND

Received: November 5, 2016

Accepted: June 26, 2017

Published: July 20, 2017

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was funded by the Scottish Government under project AQ0030. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Abstract

Development of Salmon Cardiac Primary Cultures (SCPCs) from Atlantic salmon pre-hatch embryos and their application as in vitro model for cardiotropic viral infection research are described. Producing SCPCs requires plating of trypsin dissociated embryos with subsequent targeted harvest from 24h up to 3 weeks, of relevant tissues after visual identification. SCPCs are then transferred individually to chambered wells for culture in isolation, with incubation at 15–22°. SCPCs production efficiency was not influenced by embryo's origin (0.75/ farmed or wild embryo), but mildly influenced by embryonic developmental stage (0.3 decline between 380 and 445 accumulated thermal units), and strongly influenced by time of harvest post-plating (0.6 decline if harvested after 72 hours). Beating rate was not significantly influenced by temperature (15–22°) or age (2–4 weeks), but was significantly lower on SCPCs originated from farmed embryos with a disease resistant genotype ($F = 5.3, p < 0.05$). Two distinct morphologies suggestive of an *ex vivo* embryonic heart and a *de novo* formation were observed sub-grossly, histologically, ultra-structurally and with confocal microscopy. Both types contained cells consistent with cardiomyocytes, endothelium, and fibroblasts. Ageing of SCPCs in culture was observed with increased auto fluorescence in live imaging, and as myelin figures and cellular degeneration ultra-structurally. The SCPCs model was challenged with cardiotropic viruses and both the viral load and the *mx* gene expression were measurable along time by qPCR. In summary, SCPCs represent a step forward in salmon cardiac disease research as an in vitro model that partially incorporates the functional complexity of the fish heart.

Introduction

Farmed Atlantic salmon (*Salmo salar*) is one of the most important cold water diadromous species produced by aquaculture worldwide, exceeding 2.3 million tonnes in 2014 [1]. In the

UK, Scotland's record production in 2014 was associated with revenues of over £1.8 billion [2]. As with most species under intensive farming, disease management is one of the major challenges faced by the industry. Consequently, fish diseases and host-pathogen interaction remains among the top priorities for aquaculture research in Europe [3] and the UK [4].

Research in fin-fish infectious and emerging diseases has widely been performed in vivo, a costly approach requiring large numbers of fish and subject to variations between individuals [5]. Experimental work in aquaculture research involving live vertebrates has adopted the 3R principles (Replacement, Reduction and Refinement) as the framework for quality research. This is now incorporated into the European Directive 2010/63 and the amended UK Animals (Scientific Procedures) Act, 1986 (ASPA). It has also become a core requirement in the project authorisation process for fish experimental research, extended to the breeding, accommodation and care of fish as protected animals [6]. In this context, fish research is shifting the focus towards alternative approaches and procedures, such as *in vivo* non-lethal sampling [5,7] and *in vitro* models. All of these are capable of producing reliable results which can reduce the need for, or even replace, *in vivo* challenges.

Work on cell lines is a traditional *in vitro* approach, and lines derived from fish including salmonids, have been available and routinely used in diagnostics and research for many years [8]. Moreover, an *in vitro* model of rainbow trout (*Oncorhynchus mykiss*) satellite cells has recently been applied to study viral induced muscle atrophy due to *Salmonid alphavirus* (SAV) [9]. However, to understand some disease mechanisms, especially regarding host-pathogen interactions, cell monolayers are limited due to their lack of complexity [10–12].

Pathogenesis studies of Atlantic salmon disease potentially could be undertaken *in vivo* in other fish species; a commonly used approach in mammals. For example, the zebrafish (*Danio rerio*) has become a well-characterized and established vertebrate model [13–16]. Work on zebrafish has contributed enormously to the fields of genetics, developmental biology, toxicology, and biomedical research, specifically including cardiovascular studies [17–21]. The limitation of this approach for the study of salmonid disease is that zebrafish is a tropical fresh water species with optimum temperature at ~28°C, which makes it rather unsuitable for study of cold water piscine pathogens. This is also the case for other potential models such as medaka (*Oryzias latipes*) or fathead minnow (*Pimephales promelas*), among others.

Farmed Atlantic salmon is prone to numerous cardiotropic viral diseases, such as Cardiomyopathy Syndrome (CMS, induced by *Piscine myocarditis virus*), Heart and Skeletal Muscle Inflammation (HSMI, induced by *Piscine reovirus*), Infectious Salmon Anaemia -ISA- (ISAV), and Pancreas Disease (PD, induced by *Salmonid alphavirus* also known as SPDV) [22–24]; the later also induces Sleeping disease (SD) in rainbow trout. Mortality due these conditions varies, but all of them can also cause severe growth rate reduction and downgrading at harvest, with consequential impact on commercial value. These infections exhibit differences in cardiac immunopathological responses [25,26] and some important insights into host pathogen interactions have been gained through *in vitro* studies on non-cardiac cell lines [12,27,28]. However, understanding the basis of cardiac diseases in fish, as with other hosts, has been hampered by the lack of appropriate models that mimic the complexity of the organ [29]. Paradoxically, despite extensive use of fish as models for applied research on non-piscine species, there is a relative lack of fish models for research on diseases of fish in commercial production.

Previously, self-contracting cardiomyocyte aggregates have been isolated from rainbow trout [30–32] and successfully used for human's drug pharmacological testing [33]. We postulate that a similar model may be used for the study of salmon cardiotropic viruses. The primary aims of the present work were to establish, characterize and optimize the production of salmon cardiac primary cultures (SCPCs), using pre-hatch *S. salar* embryos and to evaluate their

potential as an in vitro model for disease research, through challenges with fish viral agents of known cardiac tropism.

Materials and methods

Embryo sources and husbandry

Wild (W) and farmed (F) fertilized salmon eggs were sourced from a native Scottish river population (Don District Salmon Fishery Board hatchery), and from a breeding program routinely used by the aquaculture industry (AquaGen-Norway). Eggs were received at different maturation stages, recorded in accumulated thermal units (ATUs), a measurement system that incorporates the cumulative effect of temperature over time (each unit equals 1 °C for 1 day). Batches of approximately 300 eggs were incubated at 5 °C +/- 0.3 °C in a 20L container with sterilised, aerated fresh water, replaced every second day.

Egg processing and SCPCs isolation

Processing was initiated 24h from reception and continued until hatching, which takes place at approximately 480 ATUs. Briefly, eggs were removed from incubation in batches of 6 to minimise temperature-induced stress, and transferred, in water from the bath, to a laminar flow cabinet for processing. Whole embryos were swiftly dissected out of their chorion with sterilized scissors and tweezers. Embryos were immediately transferred to 1.5 ml Eppendorf tubes containing 0.5 ml Trypsin (2.5% Trypsin 10x Gibco by Life Technologies-NZ), were finely cut with scissors to facilitate digestion, and incubated for 1 minute. The embryos were further dissociated by vigorous pipetting. The tissue lysate was then transferred into 12 ml centrifuge tubes with 2 ml culture medium. This was composed of L15 medium (Lonza, UK) supplemented with 10% foetal bovine serum (Sigma-Aldrich, USA), 100 U/ML Penicillin and 0.1 mg/ml Streptomycin (Fisher Scientific, UK) (modified L15). The complete process lasted ~2 min.

The tubes were centrifuged for 5 minutes at 565g and 15 °C, the culture medium removed and the pellet re-suspended in 2ml fresh modified L15 medium for plating. Material from each embryo was plated individually in x12 tissue culture plates (Cellstar-Greiner Bio-one-UK) and incubated at 22 °C. Plates were examined daily for the following 3 weeks under a dissecting microscope (Motic-SMZ 168). Culture medium was renewed and non-attached debris was removed after 24h and weekly thereafter.

Developing SCPCs were recognized by their beating, and were harvested as soon as observed by dissecting them out using the tip of a 20 μ l pipette. They were individually transferred into x4 wells chambered glass slide or x8 chambered coverglass (Nunc[®] Lab-Tek II, Thermo-Fisher-USA) for further culture. Modified L15 medium, refreshed weekly, was used for culture, and unless stated otherwise, SCPCs were cultured at room temperature (22 °C). The culture time varied between experiments, as detailed below. The maximum survival time in culture was of 5 months, noted in SCPCs that were not used in any experiments.

Histological and ultrastructural examination

For histological examination, SCPCs were fixed for 30 min with 10% formalin within the chambered slide. Addition of a drop of neutral red during fixation assisted with identifying the SCPCs during the process. Fixed SCPCs were collected and embedded in a pearl of HistoGel (Thermo-Scientific, USA), following the manufacturer's instructions. Once the pellet was solidified, it was processed routinely within a biopsy bag inside a disposable CellSafe biopsy insert (Cellpath Ltd, UK). Processed material was embedded in wax, sectioned at 3 μ and

stained with Harris haematoxylin and eosin (H&E) or Ziehl Neelsen (ZN) by routine procedures. Slides were examined using an Olympus BX60 microscope.

For EM observation, SCPCs were fixed *in situ* by adding 0.5 ml cold Karnovsky's fixative containing 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M phosphate buffer pH 7.4 (Electron Microscopy Sciences, USA) to the wells for 30 minutes at room temperature. Fixative was then replaced with 0.1M Phosphate buffer saline pH 7.4 (PBS), the sample transferred to a 1.5 ml Eppendorf tube and placed on a rocker for 15 min. This procedure was repeated twice followed by a further transfer to fresh buffer for short-term storage at 4°C. Post fixation was performed with 1% OsO₄ in 0.1 M cacodylate buffer for 2 h at 4°C. Samples were then dehydrated through a graded series of ethanol and embedded in araldite resin (Fluka, Switzerland). Ultrathin sections were cut on an Ultracut E (LEICA, Germany) and stained with 0.5% uranyl acetate (Laurylab, France) and 3% lead citrate (Laurylab) in an Ultrastainer AC20 (LEICA, Austria). Grids were examined with a JEOL electron microscope JEM 1011 at 60 kV or JEM-1400 Plus at 120kV (JEOL, Japan).

Fluorescent imaging

SCPCs were fixed *in situ* with 4% paraformaldehyde solution (pH 7.4) for 15 min at room temperature, washed twice with (PBS) and then incubated with Alexa Fluor[®] 488 Phalloidin (Life Technologies, USA) for filamentous actin (F-actin) according to manufacturer's instructions. Chambers were dislocated from slides and coverslipped using Vectashield antifade mounting medium with DAPI for nuclear staining (VECTOR Laboratories Ltd. UK). Samples were visualised and imaged with a Zeiss confocal LSM700 or a Zeiss Axio Imager M2 upright microscope with fluorescence attachment.

Viral infection and gene expression

Different virus isolates were used to test infectivity on the SCPCs model. One isolate of Infectious salmon anaemia virus (ISA 390–98 passage 8, 1.11×10^8 TCID₅₀) and three isolates of *Salmonid alphavirus* subtype 1 (SAV-1) identified as F07-220 (passage 2, 3.5×10^5 TCID₅₀, kindly donated by AFBI-Ireland), MS4640 (passage 7, 1.4×10^6 TCID₅₀) and F93-125 (passage 13, 4.6×10^7 TCID₅₀), representing agents with observed high, medium or no virulence on *in vivo* preliminary challenges. SCPCs were allowed to re-attach and acclimate for at least 48h before challenge. The final dose of infection was achieved by adding 1 or 0.5 ml of the virus aliquot directly into the chambered wells (100 or 50 ml respectively) to reach a hundred fold reduction of the inoculate concentration. To assess the effect of challenge dose, isolate F07-220 was also inoculated at a thousand fold reduction of the initial inoculate. After a 2 h absorption step, the culture medium was replaced in all chambers including the SCPCs exposed to culture medium only as negative controls. Single infected SCPCs or triplicates for each isolate were used in the evaluations of preliminary infection (7 days) and longitudinal infection (3 weeks), respectively. Incubation was performed at 15°C. At the end of the experiment, SCPCs were collected directly into 100µl lysis/binding buffer from the RNA extraction kit (see below), and immediately frozen at -80°C. For RNA extraction, samples were individually processed using a Dynabeads[®] mRNA DIRECT[™] Micro Kit (Catalog Number 61021, Ambion -Life technologies, USA) according to manufacturer's instructions.

For quantification of *mx* and viral gene expression by qPCR, total RNA was first reverse transcribed into cDNA using the TaqMan Reverse Transcription Reagent kit (Applied Biosystems) as follows: 9.6 µl of total RNA and 1.25 µl 50 µM oligo-d(T)₁₆ were mixed and heated to 70°C for 10 min and chilled on ice. The final volume was adjusted to 25 µl by adding Master mix comprised: 1x RT buffer (25 mM Tris-HCl pH 8.3, 37.5 mM KCl, 5.5 mM MgCl₂),

0.5 mM each dNTP, 0.4 U RNase inhibitor and 1.25 U Multiscribe Reverse Transcriptase. The reaction was incubated at 48°C for 90 min, heat inactivated at 95°C for 5 min and stored at -80°C until use. Real-time PCR (RT-qPCR) assays were performed on a Roche LC480 System (Roche). TaqMan probes and primers to amplify the elongation factor 1 α gene (*elf*), mx, SAV non-structural protein P1 (nsP1) and ISAV segment 8, are given in [12] and in [34]. One μ l cDNA was added to the following mix contained in individual wells of a 96-well optical plate (Roche): 10 μ l of TaqMan 2x PCR mix with UNG (Applied Biosystems), 8 μ l of dH₂O and 1 μ l of a 20x mix containing forward primer (18 mM), reverse primer (18 mM) and probe (5 mM). The standard cycling conditions were 50°C for 2 min, 95°C for 10 min followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. The crossing point (Cp) was determined by the maximum secondary derivative method and the values were converted into expression levels normalised against the reference gene, *elf*, using a standard curve. For preliminary infection, RT-qPCR was performed in triplicates on the same sample and the average result presented. For analysis of the kinetics of infection in longitudinal studies, the qPCR was performed on three infected SCPCs per time point (biological replicates). The sampling time points were 1, 7, 15, and 21 days post infection (dpi).

Statistical analyses

The total efficiency of generation of SCPCs, expressed as the number of SCPCs isolated per embryo, was compared between different egg sources (wild vs. farmed). The SCPCs harvest efficiency variability due to embryological stage was assessed by comparing the numbers harvested at 380, 395, 405, 410, 415, 445 ATU, and the time of harvest post plating by comparison between 24-72h, 96-120h, >120h. Beating rates were expressed as the number of beats per minute. The effect of temperature and age on beating rate of cultured SCPCs was assessed by comparison between SCPCs kept at 15°C and 22°C, and between 2 week and 4 week old SCPCs. Beating rate differences were also assessed between two types of farmed eggs (IPN sensitive and resistant, Aquagen, UK). Most of the above described data were not normally distributed (Kolmogorov Smirnov test), and Kruskal-Wallis (KW) test was used for evaluation of the differences between the medians of each group, with a threshold of significance of $p = 0.05$. Exceptions to this were the evaluation of the effect of temperature on SCPCs beating rate, and simultaneous analysis of egg type and culture age, where the data were normally distributed. In this instance, a two sample t-test was used, with a threshold of significance of $p = 0.05$. A two-way ANOVA was used to analyse simultaneously the differences between IPN resistant vs. susceptible genotype egg source and culture age in a sample subset.

Results

Salmon cardiac primary cultures (SCPCs) isolation

Primary cultures of autonomously beating, three-dimensional heart tissue were successfully isolated from Atlantic salmon pre-hatch embryos and were maintained under laboratory conditions with minimal support up to 5 months post isolation. The method applied was repeatable and robust and several factors affecting the efficiency of this protocol were evaluated.

Eggs of farmed and wild origin resulted in similar total production efficiency (Fig 1) with no significant differences noted (KW, $H = 0.11$, $p = 0.74$).

Embryonic developmental stage and timing of harvest after plating influenced SCPCs production efficiency for both farmed and wild eggs (Fig 2). SCPCs could be harvested from the isolation plate from 24h to 3 weeks from initiation of culture. Briefly, there was a small decline of up to 0.3 in efficiency with increasing ATU from ~380 to 445 ATUs (*i.e.* from reception and up to initiation of hatching), and a strong decline in efficiency (up to 0.6), when harvesting

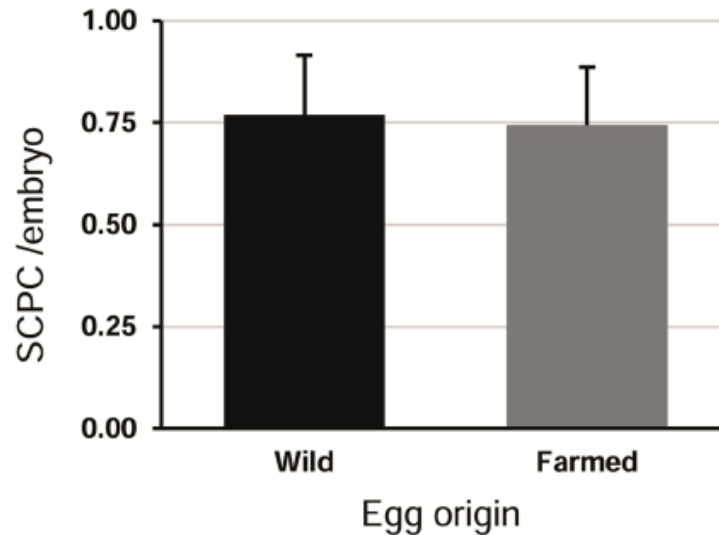


Fig 1. SCPCs generation efficiency by egg source. Generation efficiency was calculated as the number of SCPCs isolated per processed embryo. Wild n = 78, farmed n = 78; error bars = standard deviation; KW: H = 0.11, p = 0.74.

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after 72 hours post-plating. It was noted that most SCPCs were visible and could be harvested before 36h.

Several factors potentially influencing SCPCs beating rate were investigated. There was no significant difference in beating rate between SCPCs cultured at 15°C (51 ± 14.5 SD) and 22°C (50 ± 23 SD) (t-test: T = 0.12, p = 0.9), or between SCPCs at 2 weeks and 4 weeks post-isolation (KW, H = 0.03, p = 0.8). A two-way ANOVA was used to analyse simultaneously the differences between IPN resistant vs. IPN susceptible genotype egg source and culture age in a

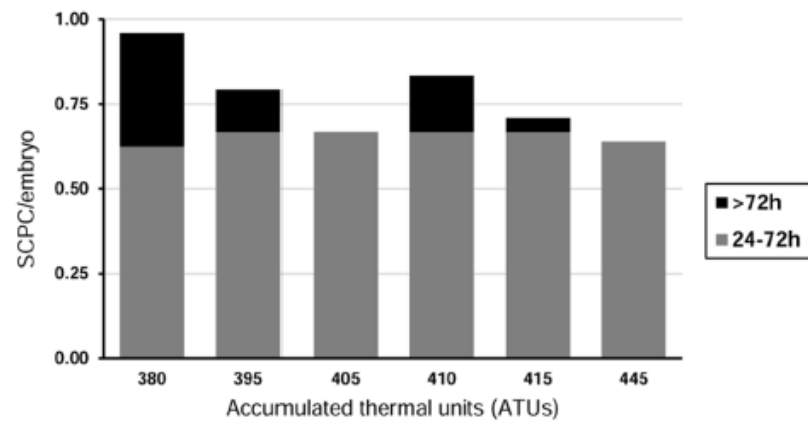


Fig 2. SCPCs generation efficiency by accumulated thermal units (ATUs) at processing and harvest timing (n_{380-415ATU} = 24; n_{445ATU} = 36). There is a small decrease in SCPC/embryo with increasing ATUs (descriptive only), while there is a large decrease when harvesting after 72h (KW: H = 8.64, p < 0.003).

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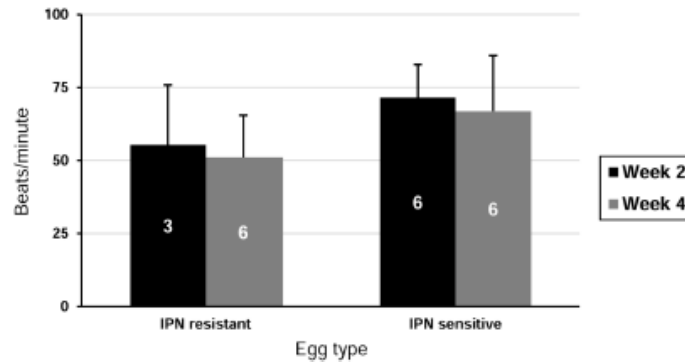


Fig 3. Effect of culture age and egg genotype on beating rate of SCPCs. Numbers within columns indicate total number of cultures used for each group. There is a significant difference in beating rate between IPN resistant and IPN sensitive eggs (two way ANOVA, $F = 5.3$, $p < 0.05$), but not between week 2 and week 4 (two way ANOVA, $F = 0.42$, $p = 0.53$).

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sample subset (Fig 3). Beating rate was significantly lower in SCPCs from eggs with an IPN resistant genotype ($F = 5.3$, $p < 0.05$), with no differences due to culture age ($F = 0.42$, $p = 0.53$).

Several technical details were noted during the development of the protocol. The use of sterilized water for egg incubation helped to minimize fungal contamination during the incubation and, importantly, the subsequent plating of the embryonic tissues, thus reducing the need of antifungal agents in the culture medium. Occasionally it was noticed during isolation that if SCPCs-like cell aggregates were not beating, a gentle disturbance with a pipette tip or a needle could initiate beating activity. Post transfer survival was dependent on the capacity of the SCPCs for prompt re-attachment. Attachment was improved by forcing the SCPCs directly into the bottom of the well at transfer. This prevented it from being trapped at the surface of the culture medium by surface tension.

Light and confocal microscopy

Two distinct SCPCs morphologies were recognised. The most common was suggestive of an *ex vivo* embryonic heart, while the less common was suggestive of *de novo* formation of discrete aggregates of self-contracting cells.

Features of the embryonic heart, such as a double chambered structure filled with blood, are present in the *ex vivo* morphology, especially during the initial stages of culture. *Ex vivo* SCPCs are the first recognisable beating tissue after isolation (within the first hours), although the beating rate may take approximately 1 to 3 days to become established. After transfer to the culture plate from the isolation plate, *ex vivo* SCPCs develop a peripheral cell monolayer, with strong attachment to the well 1–2 days after transfer (Fig 4A–4C and S1 Video. Live SCPCs, *ex vivo* type).

The *de novo* morphology is pleomorphic and lacks blood-filled chambers. *De novo* SCPCs are most frequently flattened, beating, round to ovoid aggregates (Fig 4D and 4E and S2 Video. Live SCPCs, *de novo* type) but there are also less frequent, large elongated, solid, tubular forms (Fig 4F). The latter pattern features irregular attachment, and can display arrhythmic beating pace. Their occurrence is less frequent and characteristically harvested at later stages of incubation.

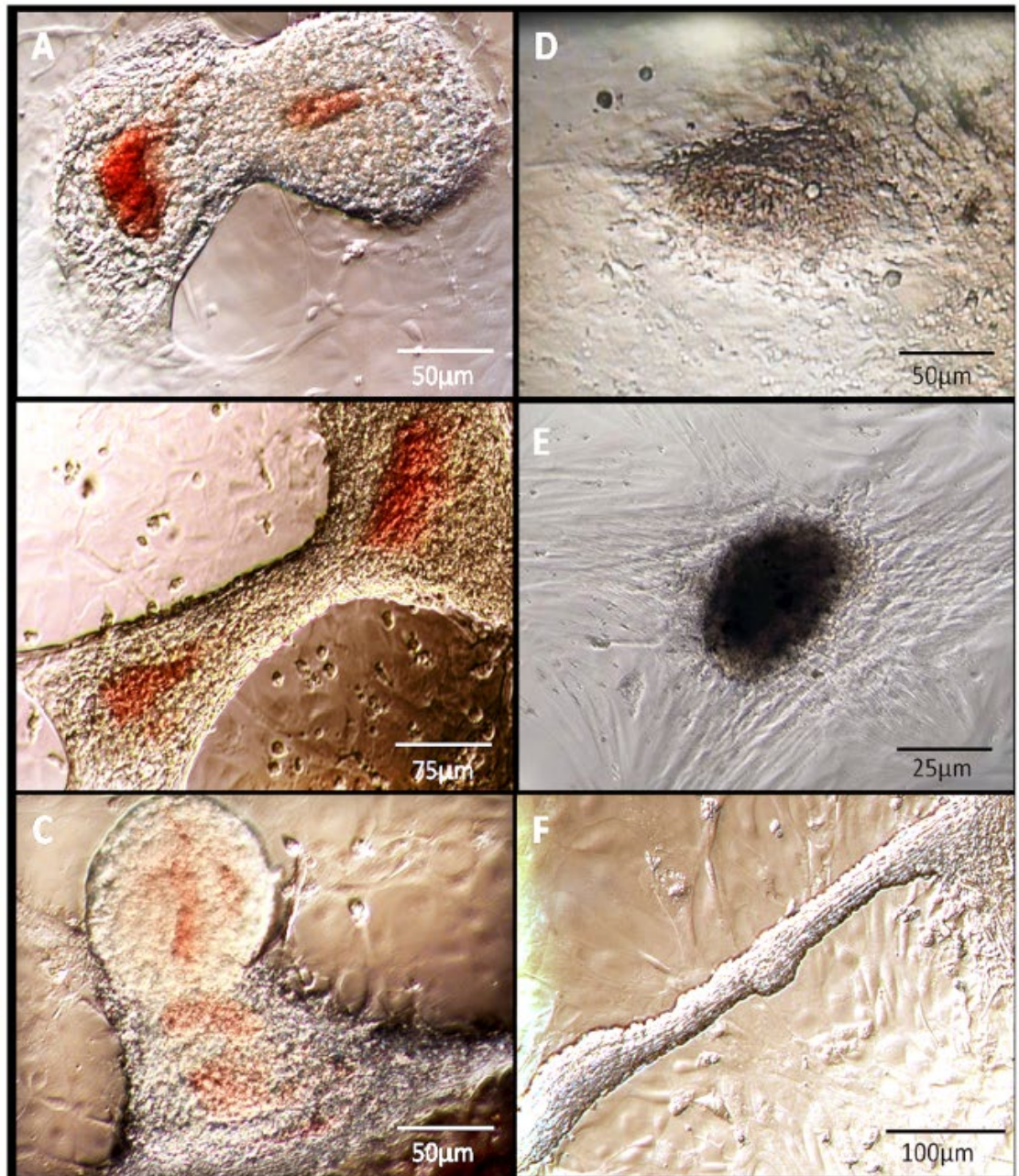


Fig 4. SCPCs morphological types as observed live under light microscopy: *Ex vivo* (A-C) and *de novo* (D-F). The *ex vivo* morphology features a characteristic double chambered structure containing blood cells (A), which may be retained during later culture stages (B-C). The *de novo* type is pleomorphic, commonly round to ovoid (D-E), and occasionally very elongated figures with a central non-attached section (F). Note a confluent surrounding cell monolayer is present in all types. Still images from video of unstained live SCPCs. Bright field microscopy with phase contrast.

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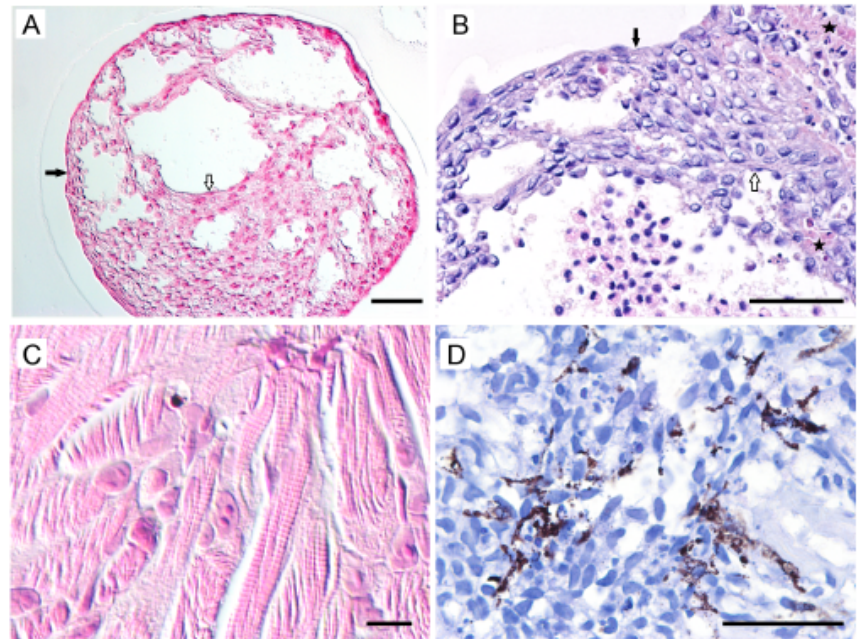


Fig 5. Histological sections of SCPCs. (A) Early stages of the *ex vivo* morphology shows a chambered structure with a trabecular arrangement of the myocardium, overlaid by a thin endocardial (white arrows)/epicardial layer (black arrows). (B) Note presence of pre-existing blood within chamber and areas of focal necrosis (stars). (C) In mature cultures of both *ex vivo* and *de novo* types the striation of the cardiac muscle becomes visible. (D) Non-acid fast, black, fine, extracytoplasmic and intracytoplasmic pigment granules are occasionally present (melanin). Haematoxylin and Eosin (A-C), Ziehl-Neelsen (D). Scale bar = 50 μ m (A, B, D), 10 μ m (C).

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SCPCs size varies in relation to morphology and age. The *ex vivo* type is more consistent in size, measuring between ~300 to 500 μ m in the longest axis of the beating region at early development. The round to ovoid *de novo* SCPCs type is usually smaller at the same stage, while the elongated forms could be considerably larger (Fig 4F). After several weeks in culture, the *ex vivo* type can lose the initial morphological likeness to the embryonic heart (Fig 4B and 4C).

The attachment of SCPCs to the plate, dependent on the surrounding monolayer (composed of fibroblast-like and epithelioid cells), appears to be essential for the survival of the primary culture. All non-attached, floating SCPCs died within a week of culture.

Histologically (Fig 5), *ex vivo* SCPCs show a characteristic hollow structure with a lumen frequently -but not always- filled with erythrocytes and leukocytes (pre-existing blood). The wall is made of loose anastomosing trabeculae resembling spongy myocardium, which are mostly composed of spindle cells with moderately defined cell borders, a central oval nucleus with watery to finely stippled chromatin, and a small amount of pale eosinophilic, wispy cytoplasm (myocytes). Striation is not visible histologically in early cultures, but becomes visible in mature cultures (4-8 weeks post isolation). Intercalating disks are not visible with light microscopy, but are visible ultrastructurally (image provided as S1 Fig. TEM of cardiomyocyte intercalated disc). The tissue resembling spongy myocardium is overlaid internally and externally by a thin layer of flattened cells (endocardium and epicardium, respectively). Histologically, *de novo* SCPC morphological type is structurally less defined, frequently

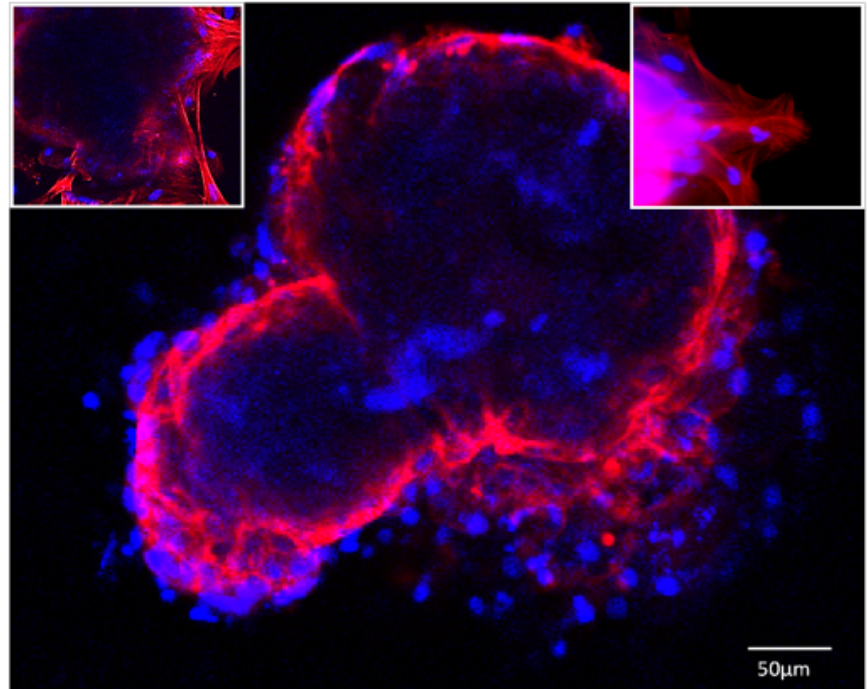


Fig 6. SCPCs *ex vivo* type under fluorescent confocal microscopy. The tridimensional morphology highlights a 2 chambered structure corresponding to the atrium and ventricle from the developing embryonic heart. Upper right and left corners inserts detail the edge zone where attachment occurs. Note elongated fibroblastic type cells anchoring the bulk of the SCPCs to the well bottom. Cytoskeleton F-actin filaments stained with Phalloidin (red) and nuclei with DAPI (blue).

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forming solid, concentric arrangements where an inner region resembling a lumen is only rarely observed.

Focal cellular degeneration and necrosis were observed in some SCPCs in early young cultures. Similar degenerative changes, as well as accumulation of pale brown intra-cytoplasmic granular deposits, were observed in both early and “aging” SCPCs. With Ziehl Nielsen staining in an early culture, these granules were not acid fast (consistent with melanin granules).

Ex vivo SCPCs were examined with confocal microscopy (Fig 6), which revealed a bichambered tridimensional structure surrounded by a population of elongated, mesenchymal cells (possibly fibroblasts) at the attachment region and surrounding monolayer.

There was evidence of cellular degeneration during culture. Non-stained mature cultures (>40 days old) showed increased autofluorescence suspected to be due to accumulation of lipofuscins during cellular aging (Fig 7).

Electron microscopy

Ultrastructural analysis performed in *ex vivo* SCPCs further confirmed the chambered structure with peripheral concentric cell layers and central lumen (image not shown). Both *ex vivo* and *de novo* morphologies feature cardiomyocytes with well-defined sarcomeres, abundance of mitochondria and tight junctions (macula adherens and fascia adherens; Fig 8B and 8C).

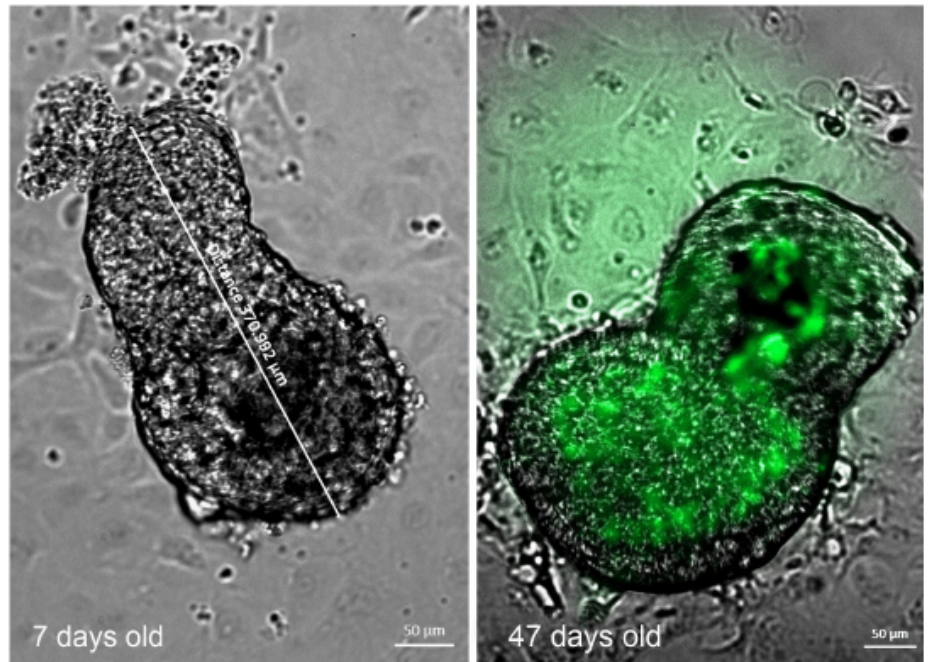


Fig 7. Live imaging of unstained SCPCs with fluorescent microscopy. Note increased auto fluorescence of SCPCs with increased culture age. Photographed with an Axio Imager M2, DIC superimposed fluorescence Green (Reflector 38HE Green fluorescent Protein with objective EC Plan-Neofluar 10x/ 0.30 M27).

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There are cells ultrastructurally consistent with endothelium as suggested by the lack of intracytoplasmic sarcomeres and abundant presence of caveolae on the cytoplasmic membrane. At 141 days, features of stress, aging and degeneration were present, such as pyknotic nuclei, intracytoplasmic myelin figures (consistent with lipofuscin—Fig 8D).

Preliminary infections

It was possible to infect SCPCs with a range of viral SAV isolates (both culture adapted and pathogenic during *in vivo* studies) as well as with one ISAV isolate.

The infection dose seems to have an effect on the amount of viral RNA detected, as observed in F07-220 challenge at two dilutions (Fig 9).

Longitudinal infection studies

During longer term infections the variables associated with the kinetics of infection (viral RNA and *mx* gene expressions) showed to be measurable along time. A varying response in both variables was observed between isolates. The individual data at 0, 7, 14 and 21 days post infection for the viral protein nsP-1 and the expression of *mx*, relative to *elf* is shown in Fig 10. Data on isolate MS4640 covers 14 days post infection only as culture for day 21 was accidentally lost. Source data file is provided as supportive information (S1 OPCR data. Source for graphics of the kinetics of infection).

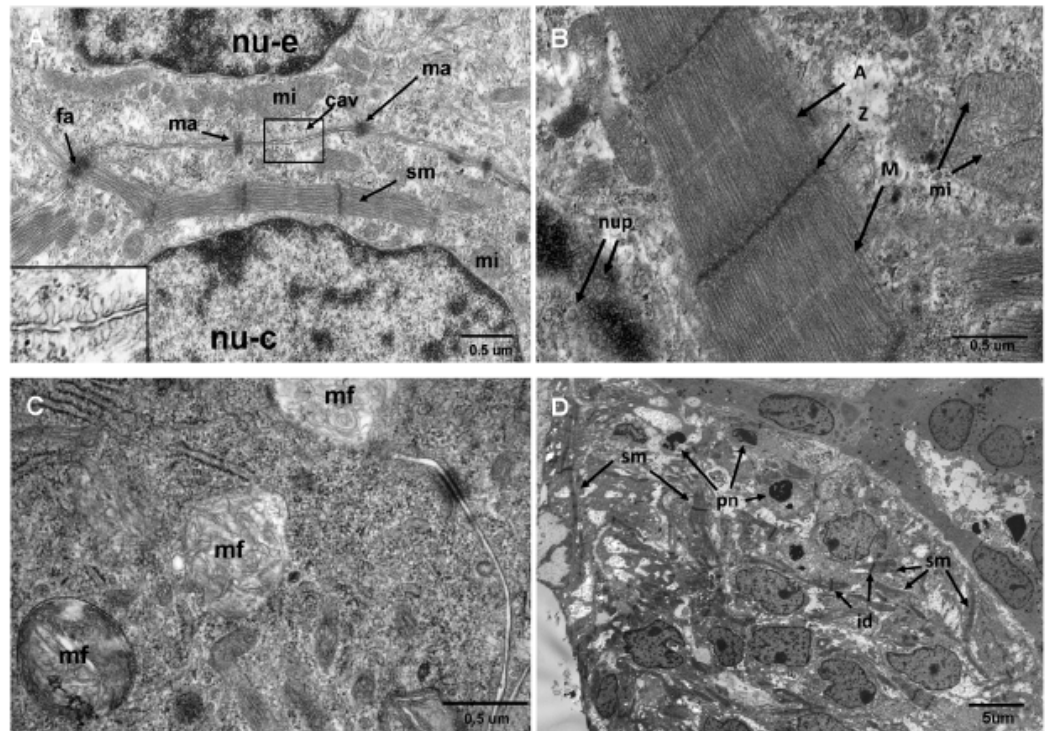


Fig 8. Ultrastructure of a beating 141 day old SCPCs. (A) an endothelial cell (top) overlays a myocyte (down); presence of fascia adherens (fa), desmosomes (macula adherens- ma) and cell membrane with caveolae (cav). Insert: detail of caveolae which are more numerous in the endothelial cell. (B) A sarcomere at maximum contraction shows the A-band, Z- and M-lines (A, Z, M), note also mitochondria (mi) and nucleus with pores (nup). (C) Abundance of intracytoplasmic myelin figures (mf). (D) There is evidence of focal degeneration, with widespread pyknotic nuclei (pn), sarcoplasmic rarefaction and fragmentation. Note that sarcomeres (sm) and intercalated discs (id) are retained (cardiomyocytes). Nuclei of endothelial cells (nu-e), nuclei of myocyte (nu-c), myofiber (mf), mitochondria (mi). TEM micrograph with a JEM 1011 at 60 kV and JEM-1400 Plus at 120kV.

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Discussion

The reduced availability of wild-capture fish in the context of increased global demand for sea-food, has resulted in continuous growth of the aquaculture industry worldwide [35]. In this context, the study of diseases affecting salmon, one of the most important species in cold water aquaculture, requires methodologies in agreement with bioethical principles seeking to investigate, facilitate and promote the advancement of alternative approaches [36]. The SCPCs model presented here is in line with the aim of this Directive to minimise the number of animals used in research while providing reliable results and allowing for optimal extrapolation into target species. Furthermore, non-hatched fish embryos are not classified as protected animals under UK regulations [37] and therefore, the approach poses no ethical conflict with regulations under the Animals (Scientific Procedures) Act 1986 (ASPAs). On our work the SCPCs production efficiency per embryonated egg varied but on average, was not higher than 1. This compares favourably yet with egg to smolt survival of 70–80% under the greatly improved farmed conditions and only 0.15% to 3.2% among wild salmon [38–41]. Moreover, the cost of

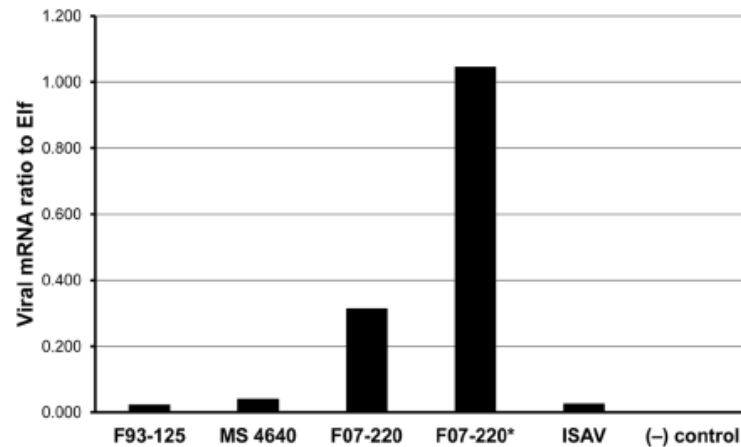


Fig 9. SCPC preliminary infection. Viral expression at 7 days post challenge with three different SAV and one ISAV isolate, expressed as the ratio of viral mRNA to *eif*. Values are average of triplicate readings of single infected SCPCs.

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embryonated eggs compared to that of fry/parr or smolts, also contributes to a significant economical saving of the SCPCs approach.

Model organisms and in vitro approaches have made an enormous contribution to the basic understanding of the fundamental properties of all living cells [42] and currently, in vitro tissue and biological models based on fish are a well-established practice in human and veterinary medicine [43].

Since the early development of fish cell lines, fish research has benefited from adopting and adapting human and veterinary medicine approaches [44] and similar benefits can be expected for studies on disease mechanisms and host-pathogen interactions. As acknowledged for human medicine studies however, the genetic, molecular, cellular and immunological differences between target organisms and models can limit the effectiveness [45]. To closer replicate the events and processes taking place during infection and disease in a targeted host and organ, closer taxonomic proximity and higher levels of biological complexity can only be beneficial, i.e. using species-specific fish models.

In this scenario the establishment of fish as a widely used vertebrate model for human biomedical research, including cardiovascular studies [15,19], only occurred as the result of the cumulative evidence that, despite anatomical differences, there was a high degree of conservation of the molecular and cellular basis of relevant processes and mechanisms. In particular, fish heart models have been found to have a higher similarity to the characteristics of the human heart than the traditional murine models and have been therefore suggested as a functional substitute for in vitro toxicological testing [15,19–21,32].

In order to establish if the SCPCs approach was a suitable infection and disease model, it was necessary to streamline SCPCs production and establish a methodology for viral infection. Previous work with rainbow trout [30–32] showed the potential of the in vitro heart culture approach. It is generally recognised however that trout is a hardier species than salmon; the wider temperature tolerance of trout might contribute to its higher robustness [46].

Building on previous, successful work with rainbow trout, our results showed that Atlantic salmon embryos of wild and farmed origin could also be successfully used to generate

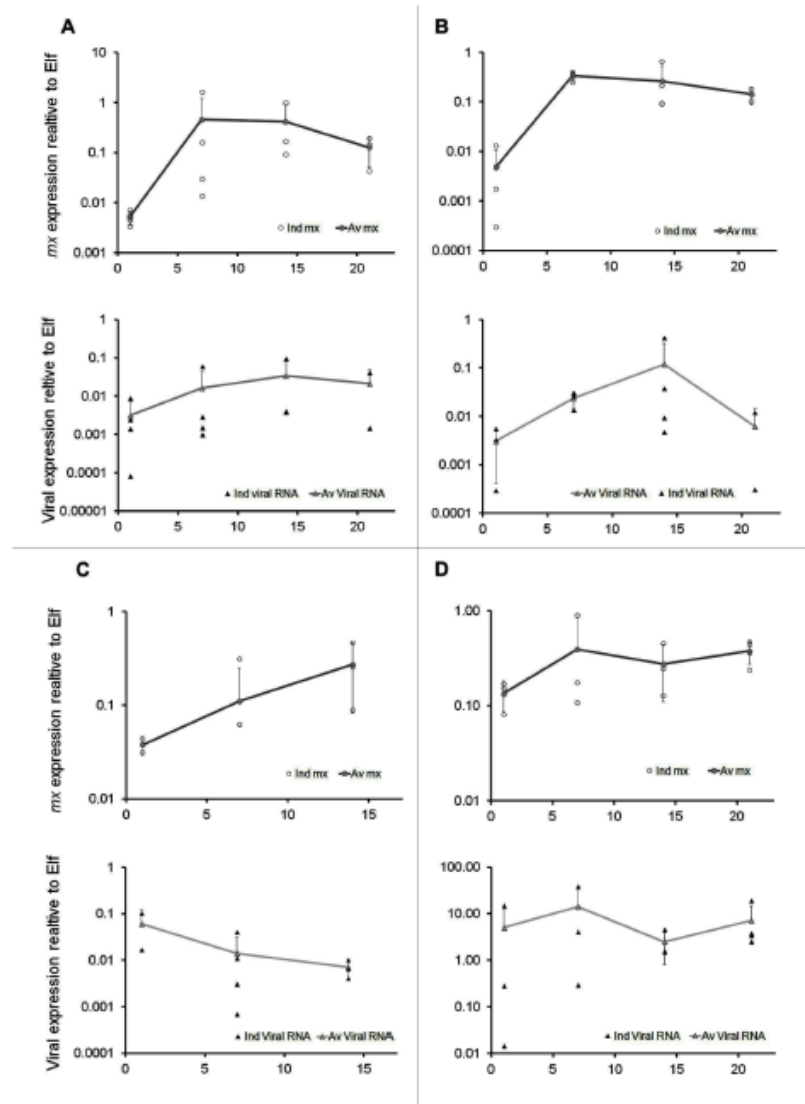


Fig 10. Kinetics of viral RNA and *mx* gene expression, for 4 different virus isolates in longer term challenge. A: F07-220 at 3.5×10^7 TCID₅₀ (SAV), B: F93-125 at 4.6×10^7 TCID₅₀ (SAV), C: MS4640 at 1.4×10^8 TCID₅₀ (SAV), D: ISA 390-98 at 1.11×10^8 TCID₅₀ (ISAV). Biological replicates (x3) average at each time point, individual data and SD, expressed as a ratio to the elongated factor (*elf*).

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tridimensional primary cultures of spontaneously beating heart tissues. SCPCs can be used as a species-specific model for host-pathogen interaction studies of Atlantic salmon cardiotropic agents.

The analysis of production efficiency indicators such as embryo development and SCPCs harvest time resulted in information to optimize the production of SCPCs. By focusing efforts in a specific embryo developmental period (initial 72h), it is possible to generate the numbers

of SCPCs required for experimental work while reducing processing time and, importantly, produced homogenous and synchronously developing batches of SCPCs.

ATU values is a commonly used metric to track progress of incubating eggs and estimate the window of different developmental landmarks, such as “eyed egg” (embryo eye spots become visible), hatching point or embryo emergence [39]. ATU values at particular developmental stages are dependent upon temperature, differs between fish species and are further influenced by environmental factors [41]. Consequently, there can be variation between batches in the exact number of ATUs required to reach different landmarks. Therefore, the most efficient SCPCs production window (expressed in ATUs) for each batch of eggs will depend upon embryo maturity at the initiation of laboratory work, and the incubation conditions provided. In our work, we detected that eggs incubated at 5°C, demonstrated a slight steady decline in production efficiency with increasing embryo maturation between 380 and 445 ATUs, i.e. the last 2 weeks prior to hatching. This decline has minimal impact in the efficiency of SCPCs production while the timing of harvest post plating was a more important factor, with a significantly improved efficiency during the first 24–72h from plating (Fig 2). Although SCPCs could develop and be harvested up to 3 weeks from plating, we recommend focussing on the early window detailed above in order to obtain synchronously developing SCPCs of similar age.

Furthermore, the more structurally predictable *ex vivo* morphological type is the most common form in this initial period, further contributing to the general homogeneity even when they continue further development in vitro.

It is important to note that awareness of the *de novo* type being more frequently encountered during later stages of incubation, although not used for this particular study, could contribute to experimental planning as they may be suitable for other type of studies.

SCPC beating rate may be useful as a proxy for cellular viability or degeneration during challenge studies. In our work, the beating rate varied between individual SCPCs, but, overall, there was only a significant effect of egg source (ANOVA, $F = 5.3$, $p < 0.05$), while age (weeks) did not have a significant effect (ANOVA, $F = 0.42$, $p = 0.53$) on beating rate. The SCPCs obtained from eggs without a QTL for resistance to IPN disease (labelled S = sensible to disease), beat at a higher rate, irrespective of the age of the culture. This suggests that the batch/egg source among farmed origin ones, significantly influences the variability of beating rate which should be taking into account in the design of studies planning to use this variable.

The ability to maintain SCPCs under laboratory conditions for several months with minimal support is a key feature of the model considering the flexibility it provides to experimental timetables. Similar longevity had been reported for rainbow trout self-contracting cardiomyocyte cultures [30,31,33], evaluated on the retention of the beating capacity. The persistence of beating capacity in aging SCPCs suggests that “beating” is a resilient feature that can be relied upon during long term studies. For example, age-dependent susceptibility is a well-known characteristic in mammalian Alpha virus infections whereby more severe disease is observed in younger hosts [47,48], and this may be replicated by the model. Similarly, in Sindbis virus (SINV) infections in mice, the mechanism of age-dependent virulence is due to the induction of neuronal apoptosis [49] and this feature has been found to be reflected also in cell culture infections [50]. The SCPCs culture ageing could therefore represent a positive feature, widening its applications into testing potential age-dependent susceptibility to infection.

SCPC ageing occurs during culture. The results of the present study with SCPCs derived from *S. salar*, shows that a process of degeneration develops during culture, despite the fact that beating capacity is retained for several months. This is supported by increased auto-fluorescence (AF) of live SCPCs recorded during culture (Fig 7) and ultra-structural observations

on 141 days old SCPCs showing increased myelin figures and other cell degenerative changes (Fig 8). These findings are consistent with lipofuscin accumulation within SCPCs.

Lipofuscins are the most commonly described AF compounds, frequently referred as “age pigments” [51]. They are due to the accumulation in endo-cytoplasmic granules of undigested cell materials resulting from phagocytosis and autophagy processes [52]. Several mechanisms apart from ageing can be involved in lipofuscin accumulation, such as disruptions in autophagy, lysosomal alteration or oxidative damage [53]. Other storage bodies referred to as ceroid pigments have similar AF properties and accumulate as a result of specific pathological conditions or experimental manipulations [51].

AF is frequently seen as a complication, in experiments especially when labelling with exogenous fluorochromes are required (lipofuscin is auto auto-fluorescent). However, AF also represents a useful biological tool as the endogenous fluorophores act as intrinsic biomarkers, strictly influenced by the morphological and metabolic conditions of the cells [54]. Therefore, the AF signal can be used for monitoring altered physiological or morpho-functional properties. Overall, the influence of SCPCs degenerative changes during culture and the resilience of their beating capacity, require further investigation to explore their potential usefulness, for example, its application in long term longitudinal studies. Occasionally, black, intracytoplasmic and extracytoplasmic pigment granules were noted histologically. These granules were not acid fast and are consistent with melanin granules.

SCPCs can be used in controlled viral challenges. The model was tested with different cardiotropic viral agents resulting in infection and replication. In spite of the small size of the SCPCs, the immune gene expression (*mx* gene) of infected SCPCs and the virus marker could be measured simultaneously by molecular approaches (Fig 9). Challenge using different virus types and isolates induced different responses, with all triggering an immediate increase of the expression of the *mx* gene, resulting in different outcomes in the viral expression response. Among the SAV isolates, only MS4640, showed a constant decreasing trend in the viral protein expression over a 2 weeks period. In contrast, F93-125 (a culture adapted isolate), demonstrated an increase before returning to initial levels by week 3 post challenge and F07-220 (a highly virulent isolate *in vivo*), showed a steady increasing trend in spite of the initial *mx* gene induction, which decreased after a week.

ISAV is a notifiable agent for the World Organization of Animal Health (OIE) and represent a very different virus type. SCPCs infection with ISAV led to an increase in the viral RNA expression during the first week, followed by a lower but maintained level of expression for up to 3 weeks in the presence of a stable *mx* gene induction.

When exploring pathogenesis, virus tropism is a very important factor and currently available fish cell lines are not permissive to infection with all ISAV strains. In vitro replication has been demonstrated in only a few cell lines (derived mainly from Atlantic salmon), such as salmon head kidney (SHK-1), Atlantic salmon kidney (ASK), Atlantic salmon (AS) and in head kidney leukocytes (TO) [55], while other routinely used cell lines tested appearing refractory to ISAV [56]. ISAV induces circulatory disturbances and has been shown to infect gill epithelial cells [55] and have a non cytolitic endothelio-tropism [57]. The SCPCs model was sensitive to ISAV (ISA 390–98 isolate) infection and the presence of endothelial tissue further suggest that the model could be used for the studies on ISAV pathogenesis. Visualization of this virus in the endothelia by immunofluorescence constitutes an area for future research.

The histopathological changes associated with infection by *Salmonid alphavirus* have been well described. Specifically, the heart develops focal to diffuse cardiomyocyte necrosis affecting both the compact and spongy ventricular and atrial muscle, with pyknotic nuclei, shrunken cells and hyaline degeneration. Infiltration and endocardial proliferation can also be observed [22,58,59]. However, the pathogenesis of the lesion has not been fully characterised, neither

the involvement of the heart in the outcome of the disease been fully elucidated. The SCPCs model proposed here would be a valuable tool to gain insights in the mechanisms of cell death involvement in the development of these lesions.

Further immunological and histochemical characterisation of the SCPCs would be helpful to provide, for example, the proportion of myocytes to other cell types undoubtedly present in the culture, as well as their interactions and the degree of differentiation over the duration of cultivation [60].

In summary, the heart is a complex organ with unique characteristics and in order to study the mechanisms of infectious disease affecting it, the closer we can get to the species and tissues involved the more likely that the results will reflect events occurring in the whole animal. The *S. salar* SCPCs model proposed here represents a step forward in fish disease research by enabling the aforementioned complexity to allow host–pathogen interactions studies at the organ level under controlled conditions. Furthermore, SCPCs represent a bioethical and cost effective approach to improve our understanding of early events in the interaction between the host and a viral pathogen affecting the cardiac tissue. It can also be used to gather preliminary data to help design specific *in vivo* challenges, as well as providing platforms for testing potential chemotherapeutants. Successful cryopreservation of SCPCs, as well as further typing of the cell types and their evolution over time would present an obvious advantage and are a clear priority for further research.

Supporting information

S1 Video. Live SCPCs, *ex vivo* type.

(MP4)

S2 Video. Live SCPCs, *de novo* type.

(MP4)

S1 QPCR data. Source for graphics of the kinetics of infection.

(XLSX)

S1 Fig. TEM of cardiomyocyte intercalated disc. Direct magnification 8000x. Scale bar = 500nm.

(TIF)

Acknowledgments

The authors would like to thank David Bruno for the initial encouragement and discussions that initiated this work, to Nicola Still, Mark Fordyce and Maria Campbell for support in histology processing and imaging, Julia Black in molecular biology and Warren Murray in virology. We are also grateful to Jutta Endler († April 2016) and Histro Örün (University of Lübeck) for their help with the TEM processing and imaging. Dr. David Graham (formerly at AFBI- Ireland) kindly provided us with virus isolate F07-220. Additional gratitude goes to Dr. Ian Davies (MSS) for his critical reading and suggestions for improvement of this manuscript.

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Conclusions

It was possible to isolate cardiac primary cultures originated from salmon embryos which were viable in culture for several months. By characterizing the production efficiency factors reported on paper 1 it was possible to establish a system to generate sufficient SCPCs numbers to allow for experimental work.

From the two morphologies the heart cultures developed, the *ex vivo* type, is likely the one with more potential for future work as it essentially represents the embryonic heart further maintained in culture and therefore, the closest to the heart as a functional organ.

Further work will be required to address important aspects with potential impact on the application of the model, such as the effect of aging during culture. Additionally, a thorough characterization through immunohistochemistry would be ideal and something missing during the current work.

The SCPCs model was challenged with different SPDV isolates and the viral load and the *mx* gene expression were measurable along time by qPCR. Applying the model to study other relevant viral cardio tropic agents such as *Piscine reovirus* (PRV, inducing Heart and Skeletal Muscle Inflammation) and *Piscine myocarditis virus* (PMCV, responsible for Cardiomyopathy Syndrome) would be ideal to further assess the model's suitability. The limitation for this has been the difficulty to obtain purified PRV and PMCV isolates.

The SCPCs model represents a step forward in salmon cardiac disease research as an *in vitro* approach that partially incorporates the functional complexity of the fish heart.

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CHAPTER 5: EXPLORING APPLICATIONS OF SCPC_s, ASSESSMENT OF EMBRYO GENOTYPE INFLUENCE AND DESCRIPTION OF ULTRASTRUCTURE PATHOLOGY AFTER INFECTION WITH SPDV

The content of the following chapter is presented in the form of a published paper: Use of Salmon Cardiac Primary Cultures (SCPCs) of different genotypes for comparative kinetics of *mx* expression, viral load and ultrastructure pathology, after infection with Salmon Pancreas Disease Virus (SPDV), Patricia Noguera, Bertrand Collet, Matthias Klinger, Hristo Örün, Jorge del Pozo. **Fish and Shellfish Immunology 72 (2018) 181–186.**

Introduction

Cellular *in vitro* models are long standing tools for medical and veterinary research. They include some based on fish species, primarily the zebra fish (*Danio rerio*) (1–3), but also others such as guppies (*Poecilia reticulata*) and rainbow trout (*Oncorhynchus mykiss*) (4–6) and extensively used in morphological studies, cellular and molecular processes, regenerative biology and pharmacological testing. Unfortunately, not many models based on fish are meant specifically to study fish issues (e.g. diseases, pathogen virulence and pathogenesis or cell tropisms).

During the work presented in chapter 3 to establish the Salmon Cardiac Primary cultures (SCPCs) as a study model, the question soon emerged as to whether the genetic background (egg origin) of the embryos used to produce SCPCs, could influence the outcomes after infection: does the embryo genetic trait manifests at the SCPCs model?

Using commercially available eggs (AquaGen® Norway) of two different genotypes (Atlantic Ova IPN sensitive” and Atlantic QTL-innOva® IPN/PD), the content of paper 2 here presented as chapter 4 was developed with the goal to respond to that question. The viral load, a component of the host immune response (*mx* expression) and the ultrastructure pathology were used for comparison.

The content of this section represents my own work having been responsible of the conceptualization, performing the experimental work, data curation, formal analysis, writing the original draft, collecting co-author’s feedback, review and final editing. Paper co-author’s specific contributions are fully acknowledged.

Paper 2

Please visit: <https://www.ncbi.nlm.nih.gov/pubmed/29102629>



Short communication

Use of Salmon Cardiac Primary Cultures (SCPCs) of different genotypes for comparative kinetics of *mx* expression, viral load and ultrastructure pathology, after infection with *Salmon Pancreas Disease Virus* (SPDV)



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ARTICLE INFO

Keywords:

Infection model
Salmon Cardiac Primary Cultures (SCPCs)
Salmon Pancreas Disease Virus (SPDV)
Host genotype
Ultra-structure pathology

ABSTRACT

In vitro fish based models have been extensively applied in human biomedical research but, paradoxically, less frequently in the research of fish health issues. Farmed Atlantic salmon can suffer from several viral conditions affecting the heart. Therefore, species-specific, cardiac *in vitro* models may represent a useful tool to help further understanding and management of these diseases. The mechanisms underlying genotype based resistance are complex and usually rely on a combined effect of elements from both the innate and adaptive immune response, which are further complicated by external environmental factors. Here we propose that Salmon Cardiac Primary Cultures (SCPCs) are a useful tool to investigate these mechanisms as the basis for genotypic differences between Atlantic salmon families in susceptibility to cardiotropic viral disease.

Using SCPCs produced from two different commercially available Atlantic salmon embryonated ova (Atlantic Ova IPN sensitive[®] (S) and "Atlantic QTL-innOva[®] IPN/PD" (R)), the influence of host genotype on the viral load and *mx* expression following *Salmon Pancreas Disease Virus* infection was assessed over a 15 day period. Both R and S SCPCs groups were successfully infected. A measurable difference between groups of viral *nsP1* and host antiviral *mx* gene expression was observed (i.e. a later, but larger onset of *mx* expression in the R group). *Mx* expression peaks were followed by a decrease in viral *nsP1* in both groups. Additionally, ultrastructural examination of infected SCPCs allowed the description of degenerative changes at the individual cell level. The SCPC model presents some advantages, over current fish cell culture monolayers and *in vivo* material, such as the presence of different cell components normally present in the target organ, as well as the removal of a layer of functional complexity (acquired immunity), making it possible to focus on tissue specific, early innate immune mechanisms. These preliminary results highlight the importance of considering genetic origin when selecting the fish source for the production of SCPCs, as well as their usefulness as screening tools for assessment of genotypic differences in disease resistance.

1. Introduction

In the last decade an increased number of viral conditions associated with severe cardiomyopathy have impacted farmed salmonids, such as those caused by Piscine Cardiomyopathy Virus (PCMV), Piscine Reo Virus (PRV) and *Salmon Pancreas Disease Virus* (SPDV), also commonly referred to as *Salmonid Alphavirus* (SAV) [1–4]. For consistency in this work and given that the first is yet the only accepted species name approved by the International Committee on Taxonomy of Viruses, we will refer to it as SPDV (ICTV Feb. 2013).

The focusing of this work on SPDV arises from the severe economic

and animal welfare impact this virus has had for over 30 years, threatening the sustainability of Atlantic salmon aquaculture [5,6]. Variability in the outcome of the SPDV infection is provided by differences in virulence between SPDV subtypes and strains [7]. However, differences in host susceptibility had long been suspected and were eventually confirmed through an epidemiological survey in Ireland, which indicated significant differences between diverse fish stocks, evaluated by levels of clinical disease and mortality. These differences were further confirmed through experimental challenges [8].

The concept of host natural resistance is the basis for development of selection-based approaches to disease control. As is seen with

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<https://doi.org/10.1016/j.fsi.2017.10.059>

Received 27 June 2017; Received in revised form 29 October 2017; Accepted 31 October 2017

Available online 02 November 2017

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humans and other animals, the wide variation in outcomes of a host pathogen interaction reflects a complex interplay of environmental and host factors. Among the most important intrinsic determinants of resistant/susceptibility to infection are the host genetics, and the functional diversity of immune response [9,10]. For example, differences in the innate antiviral response measured as interferon (IFN) induced *mx* gene expression, were found between different cell lines of salmon origin, correlating with their resistance and susceptibility [11,12].

In vivo and *in vitro* fish models have been extensively used in human biomedical research in a wide range of fields, and notably the zebra fish (*Danio rerio*) is today a strongly established vertebrate model for the study of human disease [13–15]. *In vivo* models are widely used in disease research in production fish. Paradoxically, in comparison the application of *in vitro* fish based models to study disease in production fish is relatively limited, although the potential is high. Traditional *in vitro* work using continuous fish cell lines has historically provided important insights; however it does pose certain limitations and risks. For example, contamination and replacement of the original cell type has been reported in EPC (Epithelioma papulosum cyprini), where currently used cultures were found to be contaminated by fathead minnow (*Pimephales promelas*) cells [16]. There is also the possibility that mechanisms observed *in vitro* are not translatable to the live host, for instance adaptation of a virus after repetitive passage resulting in tissue culture adaptation and consequent loss of virulence in host challenge studies [17–19]. Difference *in vivo* versus *in vitro* outcomes can also be related to immune cell interactions, giving rise to potential for adaptive immunity in the former versus predominantly innate immune responses in the latter.

While *in vitro* models are a simplified version of the host, they are desirable tools that could help replace or reduce the number of live animals required in experimental work [20,21]. A major drawback is the limited number of cell lines of the target species available. One critical aspect required to translate output from cell line models to animal is the availability of cell lines corresponding to the cell type present in the target organ. Using SCPCs it is possible to introduce several desirable factors that contribute to its translatability. Two such factors are increased complexity by inclusion of a mixture of cell types mimicking those present in the target organ, and isolation from the host species to which the model applies. For instance, working directly with rainbow trout tissues proved relevant when studying Sleeping Disease (SD), a condition in freshwater rainbow trout (*Oncorhynchus mykiss*) caused by a SPDV subtype. The use of primary cultures of muscle satellite cells from rainbow trout provided novel evidence that these cells were targeted during infection [22]. In the above scenario, development of *in vitro* salmonid cardiac models for the study of the mechanisms underlying cardiotoxic viral diseases of salmon represents an area of interest.

We developed an *in vitro* 3D heart primary culture model, the salmon cardiac primary culture (SCPC), from embryonic Atlantic salmon (*Salmo salar*). SCPCs contain several of the basic components of the fish heart, *i.e.* myocardium, epicardium, endocardium and fibroblast cells, and can be kept up to 5 months in culture with low maintenance, can be processed for histology, electron microscopy, and molecular techniques, and are permissive to viral infection and replication [23].

We hypothesized that the origin of salmon eggs used to generate the SCPCs can result in differences in the susceptibility of the SCPC model to SPDV. To assess this, we compared the kinetics of infection with a virulent SPDV subtype 1 isolate (SPDV) between SCPCs isolated from embryos of two different, commercially available Atlantic salmon stocks with different genetic traits. The possibility of assessing potential differences in host response due to genomic variability can widen the SCPCs application in genomic selection studies.

2. Material and methods

2.1. Embryo origin

Commercially available embryonated eggs “Atlantic Ova IPN sensitive” (S) and “Atlantic QTL-innOva⁺ IPN/PD” (R) were obtained from AquaGen[®] Norway (through AquaGen Scotland Ltd, Stirling University Innovation Park, Stirling, UK). The former has a genotype sensitive to Infectious Pancreatic Necrosis Virus (IPNV) and is usually sourced for use in vaccine trials. The Atlantic QTL-innOva⁺ IPN/PD is a genotype with a combined increased resistance to both IPNV and to SPDV (pancreas disease – PD), as described by the manufacturer.

2.2. SCPC production

SCPCs were produced following protocols previously described [23]. In brief, eggs from both genotypes, described above, were incubated separately in aerated, sterile sea water at 5 °C with primary isolation and harvesting of the identified cardiac tissue performed over a 2 week period prior to hatching. Embryos were stripped from their shell using sterile tweezers and scissors in a laminar flow cabinet. They were then transferred into 0.5 ml Trypsin (2.5% Trypsin 10x Gibco by Life Technologies-NZ), and mechanically dissociated to facilitate chemical digestion. The lysate was transferred into 2 ml of culture medium (L15 (Lonza, UK) supplemented with 10% foetal bovine serum (Sigma-Aldrich, USA), 100 U/ml Penicillin and 0.1 mg/ml Streptomycin (Fisher Scientific, UK)), and then centrifuged for 5 min at 565 g at 15 °C. The medium was then removed and 2 ml of fresh medium was added pipetting vigorously to re-suspend the tissue pellet. Each whole embryo lysate was then plated individually into 12 well tissue culture plates (Cellstar-Greiner Bio-one-UK) and incubated at 20 °C. Culture medium was then renewed at 24 h simultaneously removing the non-attached cell debris. Plates were examined daily under a dissecting microscope (Motic-SMZ 168) to collect developing SCPCs. These were recognised by their morphology and their beating capacity, and were carefully pipetted out using 100 or 200 µl tips. SCPCs were then individually transferred to x8 well chambered glass slides (Nunc[®] Lab-Tek II, Thermo-Fisher-USA) with 0.5 ml fresh culture media where they re-attached spontaneously and were incubated at 20 °C. SCPCs for both genotype groups were isolated and cultured in parallel to ensure synchronous development during the experiments.

2.3. Experimental design

SCPCs between 2 and 3 weeks old were identified as “S” (sensitive) or “R” (resistant) as per genotype of origin, and were challenged with SPDV virus. SPDV-1 isolate F07-220 originating from a field outbreak in Ireland, was used for the challenge. The virus was at passage 4 and was cultured in Chinook salmon (*Oncorhynchus tshawytscha*) cells (CHSE-214, ATCC CRL 1681) for 7 days. Its virulence and pathogenicity had been tested through an *in vivo* challenge study (unpublished data), where it produced moderate to severe PD histopathological changes. SCPCs were infected synchronously with identical viral dose by adding 50 µl of a SPDV-1 F07-220 virus stock solution directly into each SCPCs well to achieve a final dilution of 2.4×10^4 plaque forming units (PFU)/ml. After 2 h absorption at 15 °C, the culture media was exchanged by fresh media both in the infected treatment and control wells and incubation continued at 15 °C.

The experiment was run for 2 weeks using a total of 48 individual SCPCs distributed to have x4 infected and x2 controls per time point and genotype. Samples were taken individually at days 1, 10 and 15 days post infection (dpi), where 0dpi was considered the point of medium exchange after a 2 h viral absorption for infected and uninfected medium exchange for controls, respectively.

2.4. Quantitative PCR

Extraction of mRNA was performed from individual SCPCs using Dynabeads[®] mRNA Direct[™] Micro Purification Kit (Catalog N^o 61021, Ambion-ThermoFisher Scientific, UK) as per manufacturer's instructions. The kit is designed to isolate highly purified mRNA directly from crude lysates and is especially suitable for micro-samples. Elution was performed in a final volume of 10 μ l of elution buffer (10 nM Tris-HCl pH 7.5) from which an aliquot of 7.7 μ l mRNA was used for reverse transcription (RT) using a Taqman Reverse Transcription Reagent Kit (Applied Biosystems-ThermoFisher Scientific, UK) as follows: 7.7 μ l of mRNA and 1 μ l of 50 μ M Oligo d(T) [16] (Invitrogen[®], ThermoFisher Scientific, UK) were mixed and heated to 70 °C for 10 min and then chilled on ice. The final volume was adjusted to 20 μ l by adding 11.3 μ l of Master mix, comprised of 10x RT buffer (25 mM Tris-HCl pH 8.3, 37.5 mM KCl, 5.5 mM MgCl₂), 0.5 mM dNTP, 0.4 U RNase inhibitor and 1.25 U Multiscribe Reverse Transcriptase. The reaction was incubated at 48 °C for 90 min, heat inactivated at 95 °C for 5 min, and stored at -80 °C until use. Quantitative PCR (QPCR) assays were performed on a Roche LC480 System (Roche). TaqMan probes and primers to amplify the elongation factor *elf*, *mx* and SPDV non-structural protein P1 (*nsP1*) are given in Gahlawat et al., 2009. Experimental negative controls were routinely included at the stages of extraction, reverse transcription and QPCR.

The Ct (cycle threshold) was determined by the maximum secondary derivative method where the values were converted into expression levels using a standard curve [24]. The relative expression level of target genes (*mx* and *nsP1*) were then normalised to the level of expression of elongation factor 1 alpha (*elf*), allowing for comparison within and between genotypes. The errors of normalised gene expression are unlikely to be normally distributed and the most appropriate statistical analysis would utilise a permutation test. However, the combined effect of small group sizes and the need to correct for multiple comparisons mean that such analyses are unlikely to generate statistically significant p-values even if substantial biological differences are present. We have represented differences expressed in terms of "fold of increase", providing also both points estimates (means) and estimates of variance (standard deviation). Fold changes were calculated by comparison to the corresponding non infected controls (*mx*) or relative to day 1 post infection (*nsP1*).

2.5. Ultrastructural studies

For ultrastructural analysis of viral tropism, an identical infection protocol was applied using x2 SCPCs per time point/genotype and sampling at 2, 3 and 24 h post infection (hpi). Samples were fixed *in situ* by adding 0.5 ml cold Karnovsky's fixative 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M phosphate buffer pH 7.4 (Electron Microscopy Sciences, USA) to the well and left for 30 min at room temperature. The fixative contained Fixed SCPCs were then transferred to a 1.5 ml Eppendorf tube with 0.1M Phosphate buffer saline pH 7.4 (PBS) and washed for 15 min on a rotator disk placed on a rocker. The procedure was repeated twice followed by transfer to fresh buffer for short-term storage at 4 °C. The post fixation used 1% OsO₄ in 0.1 M Cacodylate buffer for 2 h at 4 °C followed by dehydration using a graded series of ethanol. Samples were then embedded in araldite resin (Fluka, Switzerland) and ultrathin sections cut on an Ultracut E (LEICA, Germany). Staining was performed with 0.5% uranyl acetate (Laurylab, France) and 3% lead citrate (Laurylab) in an Ultrastainer AC20 (LEICA, Austria). Examination of stained sections was performed on/with a JEOL electron microscope JEM 1011 at 60 kV or JEM-1400 Plus at 120 kV (JEOL, Japan).

3. Results and discussion

SCPCs, a species-specific cardiac model developed from *A. salmon*

embryos, has been proposed as an alternative approach to traditional *in vitro* work using cell lines with the aim to support studies of host pathogen interactions of cardiotropic viral agents, such as SPDV affecting *A. salmon*. The current work used SCPCs isolated from commercial *A. salmon* strains resistant to PD/IPN (R), and susceptible to IPN (S), as representatives of different genetic traits. While IPNV resistance has been found to represent a rare case where a single major gene or quantitative trait loci (QTL) explains the bulk of variation in resistance due to the genotype [25], the biological basis of genetic resistance to SPDV is more complex (*i.e.* polygenic) [26]. This work constitutes the first assessment of the usefulness of the SCPC model to evaluate the potential influence of the host genotype in the outcome of SPDV infections. SCPCs were infected synchronously with identical viral dose and the QPCR Ct values were normalised against the individual housekeeping gene (*elf*), therefore it is possible to compare the relative expression within and between genotypes.

3.1. Kinetics of *mx* and SPDV-*nsP1*

Both SCPCs of R or S origin showed an *mx* gene induction after infection with isolate SPDV-1 F07-220 over a 15 day period.

At 1dpi the *mx* expression in the S group showed a 24.7 fold increase relative to its control, compared to only a 1.6 fold increase in the R group. This was followed by a substantial induction of *mx* in the R group by 5dpi that with a magnitude of 5700 fold increase represents its peak at the time the S group with a 7.7-fold increase showed a transient decline compared to day 1. At 10dpi the R group had a 110 fold increase relative to control, a decrease from its peak expression of day 5, which is then maintained at similar values by 15 dpi with a 221 fold increase. In the S group the *mx* gene expression at 10 dpi showed a 812-fold increase representing the peak for this group and it also remained at a similar magnitude by 15dpi with a 219-fold increase relative to controls (Fig. 1a–b).

The viral transcript (SPDV-*nsP1*) in both groups had similar expression at 1dpi but afterwards differences between R and S groups were observed, both in magnitude and in kinetics. The earliest increase in *nsP1* was noted in the R group at 5dpi (2266-fold relative to day 1) which corresponded to the peak of *mx* gene expression for this group. This was followed by a transient decrease at 10dpi (31-fold) and a second peak of expression at 15dpi, with a 1619-fold increase, reaching similar levels as that of 5dpi.

Conversely in the S group *nsP1* remained similar at the 2 first time points with only a slight increase at 5dpi, (1.4 fold) coinciding with a decreased *mx* expression on that day. This was followed by a delayed but strong increase at 10dpi (289 fold) simultaneously with the increase in *mx* expression. Similar to observations on the R group, the increased and sustained level of *mx* expression seemed to induce the subsequent marked reduction on viral gene expression observed by 15dpi (5.6 fold) (Fig. 1a–b).

In this study overall the *mx* expression of infected SCPCs after viral induction remained higher than control SCPCs in both groups and at all-time points.

The baseline of *mx* expression on the control groups fluctuated slightly over time. The *mx* average of the controls were 0.00049 (R) and 0.00021(S). Differences in the basal gene expression in the unchallenged SCPCs may have been due to individual variation and given the small sample size in this case, further work is required to verify whether they are determined or not by the origin/genotype of the SCPCs.

Among infected cultures, after induction both groups maintained an *mx* expression level above the base line for the length of the experiment. The S group showed an early *mx* induction followed by a transient decrease before a peak expression for the group at day 10pi, a delayed response compared to the peak on the R group at 5dpi. The magnitude of *mx* expression of the R group was consistently higher from day 5 onwards than in group S, with a general average of 0.2208

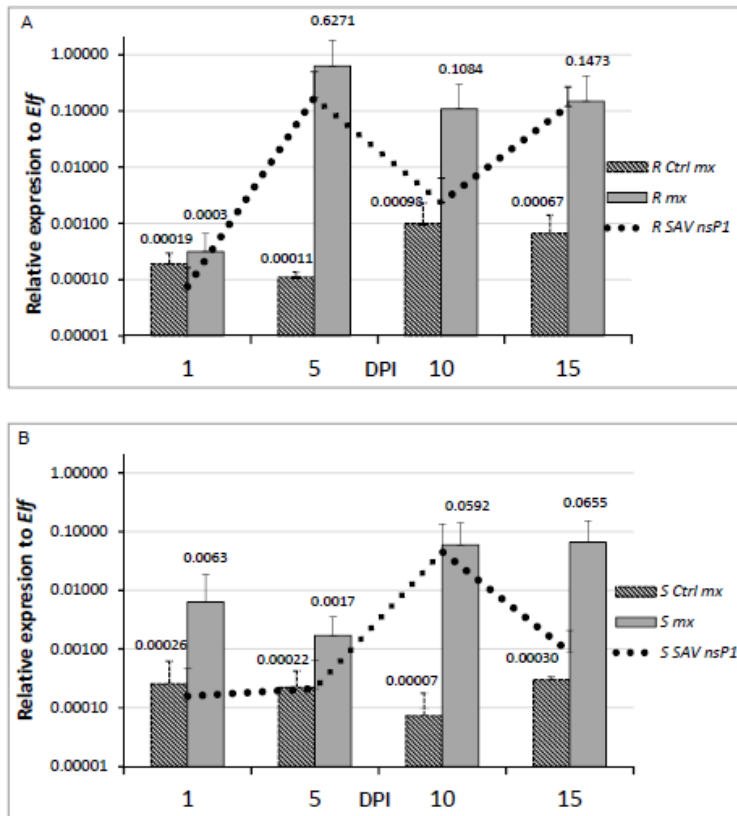


Fig. 1. Kinetics of *mx* and SPDV-*nsP1* gene expression in "R" (A) and "S" (B) genotype SCPCs after infection with isolate SPDV-1 P07-220. Values are normalised against the host reference gene *Elf* and expressed in a logarithmic scale. There are 4 biological replicates/time point. DPI: days post infection.

and 0.0332, respectively.

Conversely, on average, the total viral RNA expression level was higher in the R group (0.0731) than the S group (0.117), suggesting a relation to the expression of *mx* on each group.

Slow or reduced *mx* gene induction has been observed and associated with a strong viral replication in fish cell lines infected with IPNV [27] and SPDV [19]. Additionally, the amplitude of *mx* gene induction after infection with SPDV was negatively correlated with the viral gene expression in two salmonid cell lines that may have had different levels of resistance to this virus [11,12].

There were fluctuations in the expression of viral genome level in both groups. As experimental settings involved terminal sampling of all replicates at each time point, the expression indicates the cumulative effect and consequently, a peak in viral load at terminal sampling may represent a re-infection cycle of those samples after the 2 weeks period.

Factors other than the genetic trait itself may have influenced this outcome, including for example the models cell's composition, with a higher level of complexity than standard cell lines. Temperature and the cell line have been reported to influence the culture characteristics of SPDV *in vitro* [28], but these factors were constant in the present study. The genotype R has been commercially selected for SPDV resistance (AquaGen, Norway) and this may explain the relatively overall stronger *mx* response noted in our study when compared with genotype S, which has not been selected for SPDV resistance. These results parallel previous observations.

The kinetics of *mx* expression observed in the R group suggests a possible re-infection event. However if a second viral peak as occurred in the R group, would eventually also happened in the S group over

time (after the delay on the peak response) cannot be ruled out with the experimental setting currently used.

3.2. Ultrastructure

Ultrastructural examination confirmed both SCPCs of R and S origin are permissive to infection and viral replication. Qualitative, quantitative or temporal differences in the viral cycle or tropism were not noted between groups with this experimental design (i.e. $\times 2$ SCPCs samples per group at each of 2, 3 and 24hpi).

All SCPCs were alive and beating at the time of fixation. However, degenerative changes were observed at the individual cell level (Fig. 2 A). Changes included mitochondrial swelling and cristolysis (Fig. 2 B), cytosol dilution, hydropic degeneration, development of myelin figures and nuclear pyknosis (Fig. 2 C–D). Intracytoplasmic viral like particles (Fig. 2 D) were observed in cardiomyocytes, endothelial cells, fibroblast and occasional intraluminal leukocytes. Abundant cytoplasmic vacuolar structures such as cytopathic vacuoles, phagosomes and autophagosomes were observed in both cardiomyocyte and endothelial cells (Fig. 2 D and F). Sarcomere structural loss was also noted. Endothelial cells seemingly developed degenerative changes earlier (3hpi) than cardiomyocytes (24hpi onwards) and occasionally endothelial cells were seen detaching (Fig. 2 F).

Whereas the ultrastructural study did not reveal differences between the R and S groups, it confirmed the permissiveness to infection of both genotypes. It also revealed pathological changes similar to those previously reported during *in vivo* studies. SPDV is known to establish persistent but frequently non-lethal infections, with early

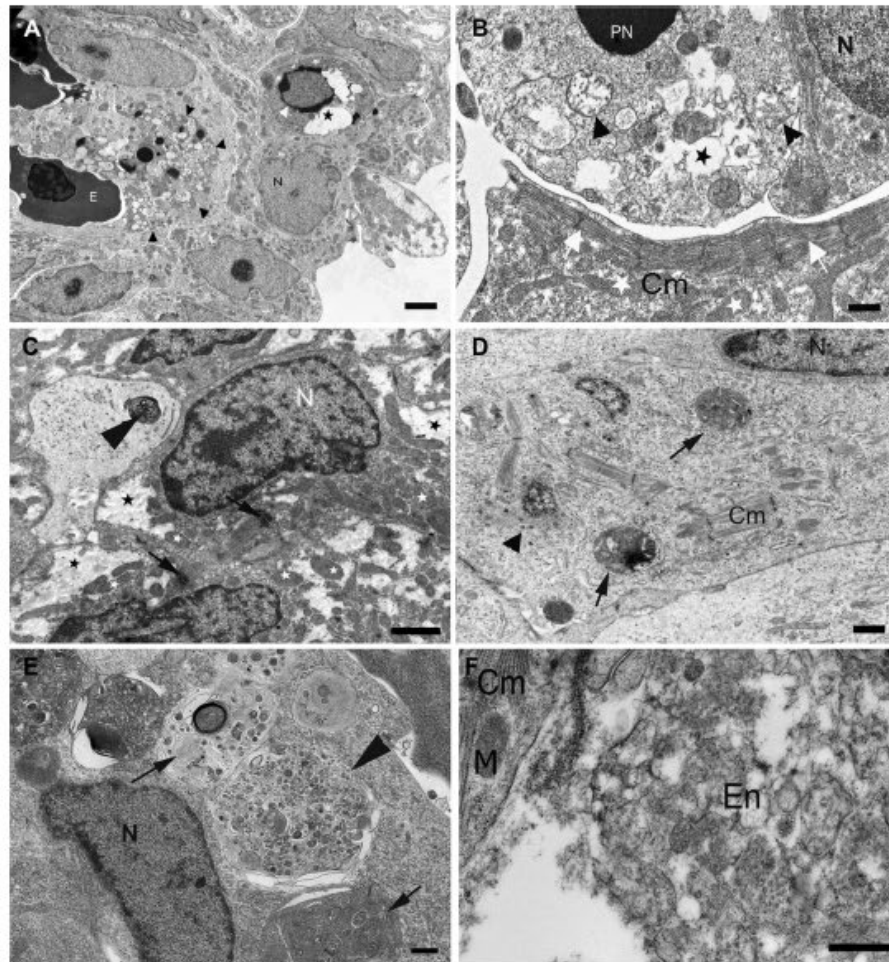


Fig. 2. Transmission electron micrograph of SPDV infected Salmon cardiac primary cultures at 3-24hpi. (A). Multifocal, single cell degeneration. A necrotic endothelial cell (black arrow heads) and a single cell with nuclear chromatin margination (white arrow head) and cytoplasmic hydropic degeneration (*) are among intact cells. Bar = 2 μ m. (B). Single cell showing chromatin condensation (pyknosis - PN), swollen mitochondria, cristolysis (black arrowheads) and cytoplasmic hydropic degeneration (stars). Bar = 500 nm (C). Early formation of intracytoplasmic myelin figures (autophagosome - arrowhead) in cell with marked cytosol dilution and hydropic degeneration (black stars). Adjacent normal cardiomyocyte shows abundant mitochondria (white stars) and intact intercalated disks (black arrows). Bar = 500 nm (D). Degeneration, sarcomere loss and cytosol dilution in a cardiomyocyte with large cytoplasmic vacuole (arrows) and free virus like particles (arrow heads). Bar = 500 nm (E). Large multi-vacuolar structures (arrowhead) containing autophagosomes figures and electron-dense intraluminal vesicles, consistent with autophagolysosomes. Multilamellar bodies with whorls of membranes forming myelin type figures (arrows). Bar = 500 nm. (F) Abundant cytoplasmic vesicular structures and degenerated mitochondria in a detaching degenerating endothelial cell. Bar = 500 nm. N = nucleus, Pn = pyknotic nuclei, Cm cardiomyocyte, E = erythrocyte, En = endothelial cell.

histopathological changes described as focalised individual myocardial necrosis [5,29]. Additionally, this work showed the involvement of endothelial cells and a potential role for them in SPDV virus entry. Endothelial cells from liver and brain have been reported as one of the pivotal target cells for viral replication in other alphaviral infections, such as Chikungunya virus (CHIKV) [30] and previous *in vivo* infections in salmon and trout with SPDV have shown infection of cells resembling hypertrophied scavenger endothelium-like cells in the kidney [31], and their potential role in the disease was speculated upon, suggesting the current observation of early involvement of cardiac endothelium in the SPDV infection would deserve further attention.

4. Conclusions

This work used SCPCs as representative of different commercially

available A. salmon genotypes to assess the potential of the SCPCs as a model to investigate the influence of host origin on the outcome of SPDV infection. Results show that after SPDV infection the two groups display a measurable difference on viral *nsP1* and host antiviral *mx* gene expressions.

The SPCCs model does not have influence of a systemic, acquired immune response. This can be seen as a missing element therefore a disadvantage, however it could also represent a useful feature as the removal of a layer of functional complexity enables the focus on the study of tissue specific, early antiviral innate immune mechanisms.

Despite the scope of this prospective project was limited and the fact that the mechanisms of genotype based host resistance for SPDV are likely to rely on a combined effect of host and other factors (i.e. not exclusively dependant on *mx* activation), these results indicate that the SCPC model can be used for screening host response differences to viral

infection. Moreover, the model showed to be suitable for ultrastructure examination bringing the benefit of the cell component of the heart to be included in the analysis. This would open the opportunity for more comprehensive SPDV viral cycle studies based on the tissue of one of the most affected organs during the disease condition.

With several viral conditions affecting the heart of Atlantic salmon the development of species-specific *in vitro* models represents a step forwards in line with current bioethics standards. Whilst more information is required in order to establish the SCPCs model as a tool for host genotypic screening, the present results showing measurable differences using the trait of disease resistance are promising. Results have also highlighted that, depending of the scopes, there will be a need to consider the consistency of genetic origin when selecting source of live material for the production of SCPCs. Further work to confirm the robustness of the model should include increasing the sample size, the comparison of using single individual samples versus multiple SCPCs (pooling), and exploring the potential of non-destructive approaches, such as sequential supernatant collection. The generation of assessment panels of biomarkers including other relevant genes, the application of tools such as RNAseq and microarray hybridization as well as immunocytochemistry ad live cell imaging, could further enrich the applicability of the model.

Acknowledgements

Funding by Marine Scotland Science (MSS), project AQ2000 is greatly acknowledged.

We are grateful to Dr. David Graham (formerly Agri-Food and Bioscience Institute, Belfast-Northern Ireland) who supplied the virus isolate used in this work. Our thanks go to Katherine Lester (MSS) for help and support with virus culture and maintenance and to Professor Dr Bernadette Dutia (University of Edinburgh), for excellent support at the Edinburgh University campus and for constructive feedback during the revision of this MS.

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Conclusion

Using SCPCs produced from two commercially available Atlantic salmon embryonated ova of different genotypes, the potential influence on the outcomes of infection with Salmon Pancreas Disease Virus (SPDV) was assessed.

Results showed that while both genotypes SCPCs got infected, a measurable difference could be observed in the kinetics of viral and *mx* gene expressions. The ultrastructural examination by transmission electron microscopy (TEM) allowed the description of degenerative changes associated with the infection at individual cell level, however they were not distinguishable between the groups.

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CHAPTER 6: ULTRASTRUCTURAL INSIGHTS INTO THE REPLICATION CYCLE OF SPDV USING SCPC_s

Note: the current chapter was prepared and written as a paper although it had not been submitted at the time this thesis was printed.

Introduction

The genus Alphavirus (*Togaviridae*) is a diverse group of viruses recorded worldwide from a wide range of invertebrate and vertebrate hosts [1]. Alphaviruses are predominantly arboviruses (transmitted by arthropod vectors), with mosquito species being the most common invertebrate host, along with ticks, biting fly and lice [2,3]. Vertebrate hosts include humans and nonhuman primates, equines, pigs, rodents, birds, reptiles, amphibians and fish. The majority of alphaviruses are capable of replicating in a range of insect and vertebrate cell lines [4].

Alphaviruses are small, enveloped viruses with a single copy of positive-stranded genomic RNA. The mature alphaviral particle has an icosahedral morphology of ~70 nm in diameter [5]. The genomic material is encased in an icosahedral nucleocapsid (NC), which in turn is enclosed within a lipid bilayer (envelope) where glycoproteins are inserted [6]. The envelope glycoproteins form an icosahedrally ordered shell or scaffold that surrounds the viral membrane [7], with its surface bearing characteristic trimeric spikes [5,8].

Several members of the genus Alphavirus are responsible for a wide range of debilitating conditions in humans and animals [3]. *Salmon Pancreas Disease Virus* (SPDV), also referred to as *Salmonid Alphavirus* (SAV), is the first member of the group isolated from a fish species and an exception in the genus in that it does not require an arthropod vector [9–12]. SPDV has been reported for over 3 decades in salmonids (Atlantic salmon *Salmo salar*, rainbow trout *Oncorhynchus mykiss*, and brown trout *Salmo trutta*) [13–15], and recently in some non-salmonid marine species such as the common dab (*Limanda limanda*), which may act as a wild reservoir [16–18]. The relatively more restricted host range of SPDV compared to that of terrestrial alphaviruses suggests temperature sensitivity is likely to play a role in virus replication [19].

SPDV is associated with two diseases affecting the salmonid farming industry [10,20]. Pancreas disease (PD) of Atlantic salmon was first recognised in 1976 and described by 1984 [21]. A similar condition in rainbow trout was reported initially in France in 1994 and was named Sleeping Disease (SD) [22]. The confirmation of their viral aetiology and evidence of being the same agent (SPDV) followed soon after the first reports of both diseases [11,20].

At present SPDV is divided into 6 groups, referred to as “subtypes”, based on analysis of the viral sequence [23]. Subtypes 1 and 2 are the most commonly found in all affected SPDV regions. Subtype 2 presents two forms, a freshwater one responsible for SD in the UK and continental Europe, and a sea water one which has been recorded in Scotland and more recently, in Norway [24–26]. Some subtypes show a more restricted geographical distribution, as subtype-3 recorded only in Norway and subtype-5 in Scotland [25,27].

SPDV has been detected in heart and skeletal muscle, pancreas, pseudobranch, gills, brain, liver, spleen, head kidney leukocytes and transiently, the serum [28–33].

Mortality rates vary widely between SPDV outbreaks, infected animals may experience chronic loss of appetite with consequent weight loss. Additionally, a proportion of the survivors never recuperate and remain extremely thin (“runts”), rendering them unsuitable for the market [13]. This is partly similar to many mammalian alphaviral infections that present a low mortality rate (Chikungunya, Venezuelan, Western, and Eastern equine encephalitis viruses (CHYKV, VEEV, WEEV, and EEEV, respectively), however the associated disease conditions can have long term sequelae [34,35].

Direct horizontal transmission of SPDV is well documented (see review by Atkins) [36], demonstrating that an arthropod vector is not mandatory for its spread. However, given all other alphavirus are arboviruses and because Atlantic salmon is highly susceptible to infections by the sea louse *Lepeophtheirus salmonis*, the possibility of a role of the parasite on SPDV transmission has been investigated. Despite positive RT–PCR detection in the lice, there was no proof of replication of the virus in that host [37], therefore, any potential involvement of sea lice remains conjectural.

Due to their pathogenic nature, several alphaviruses have been widely studied. Particularly the type reference Sindbis (SINV), the Semliki-Forst virus (SFV), the aforementioned VEEV, the Ross River virus (RRV) and recently the CHYKV species [5,38]. The advances achieved in the wider field of alphavirus research provide a profuse source for comparative analysis of the relatively newer and less studied SPDV, where several knowledge gaps are still recognised [13,25].

Recently, the morphogenesis of SPDV *in vitro* (as well as aspects of the pathogenesis *in vivo* have been reported by ultrastructural examination (in CHSE-214 cell line and Atlantic salmon parr, respectively) [33,39]. These studies showed some of the events after viral internalization, including endosome formation, features related to cytoplasmic replication, presence of spherules and virus budding at the plasma membrane. However, when compared to any other member of the genus Alphavirus, there is still a marked scarcity of further insights into the replication cycle of SPDV.

With the salmon heart being one of the most relevant affected organs during SPDV *in vivo* infections, the current work aimed to contribute to the understanding of the virus replication cycle using a salmon cardiac primary culture model [91,92]. The ultrastructure morphology of features observed from 2 up to 72 hpi are described and discussed by comparison with those reported for SPDV and for other alphaviral infections in mammalian and arthropods hosts.

Material and Methods

Commercially available embryonated Atlantic salmon eggs were obtained from AquaGen® and SCPCs generated following protocols previously described [40]. In brief, embryos were swiftly removed from the shell, mechanically and enzymatically disaggregated, tissues centrifuged in culture media and the final pellet re-suspended in fresh media for plating. Incubation was performed at room temperature and relevant tissue harvested from 24hs onwards, transferred as individual cultures into x8 well chambered glass slides (Nunc® Lab-Tek II, Thermo-Fisher-USA) for further incubation. SCPCs were maintained with 0.5 ml culture medium (L15, Lonza, UK) supplemented with 10 % foetal bovine serum (Sigma-Aldrich, USA), 100 U/ml Penicillin and 0.1 mg/ml Streptomycin (Fisher Scientific, UK)).

Infection was performed *in situ* at a dose of 2.4×10^4 plaque forming units (PFU)/ml, using a virulent SPDV subtype -1 isolate that originated from an outbreak in Ireland (F07-220 provided in kind by Dr David Graham, formerly at Agri-Food and Bioscience Institute, Belfast). After absorption at 15°C for 2h, the infected culture medium was exchanged for fresh medium and similarly, culture media was exchange on control wells with non-infected SCPCs. Incubation continued at 15°C and two to four infected SCPCs were sampled at 2, 3, 24, 48 and 72 hours post incubation (hpi), i.e. from end of absorption time and culture media exchange.

For transmission electron microscopy (TEM) SCPCs were fixed *in situ* by adding 0.5 ml cold Karnovsky's fixative (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate

buffer pH 7.4) to each well and left for 30 min at room temperature. Fixed SCPCs were transferred to a 1.5 ml Eppendorf tube with 0.1 M Phosphate buffer saline pH 7.4 (PBS) and washed for 15 min on a rotator disk. This procedure was repeated twice, followed by transfer to fresh buffer for short-term storage at 4°C. For post fixation, 1% OsO₄ in 0.1 M Cacodylate buffer was applied for 2 h at 4°C followed by dehydration using a graded series of ethanol. Samples were then embedded in araldite resin (Fluka, Switzerland) and ultrathin sections cut on an Ultracut E (LEICA, Germany). Samples from time points 2, 3 and 24 hpi were processed separately and the 48 and 72 hpi were pooled as a single sample for processing. Staining was performed with 0.5% uranyl acetate (Laurylab, France) and 3% lead citrate (Laurylab) in an Ultrastainer AC20 (LEICA, Germany). Examination was conducted in a JEOL electron microscope JEM 1011 at 60 kV or JEM-1400 Plus at 120kV (JEOL, Japan) and all morphological descriptions here presented correspond to observations on infected material, with no similar changes observed on non-infected controls.

Results

Ultrastructural examination confirmed the permissiveness of SCPCs to SPDV-1 infection and replication, with viral particles noted within cardiomyocytes, endothelial cells, fibroblasts and intraluminal leukocytes. Several features of the viral replication cycle were observed, primarily in the first two cell types.

At 2 and 3 hpi, the virus was seen undergoing internalization process or had already been internalized, with abundant endosomes and trafficking-like vesicles containing viral particles observed in the cytoplasm. Viral particles were observed in the proximity of the plasma membrane (PM) or attached to it. Two morphological types of internalization processes were apparent by TEM ultrastructure examination. A suspected clathrin-independent entrance which possibly involves attachment through an electron dense cloud between the viral particle and the PM (Figs 1 A) and internalization by an invagination of an electron-dense area of the cell surface resembling caveolae, (Fig 1 A, B), and internalization through the more widely accepted clathrin-mediated endocytosis (CCP), with formation of invaginations bearing a sub-membranous, spiky electron-dense band (Fig 1 C, D). In samples after 24 hpi both viral attachment and internalization by both apparent processes were still present. (Fig 6 A, B).

Internalization resulted in cytoplasmic membrane-bound vesicles containing viral particles consistent with endosomes (Fig 1 E). Free viral particles were also present in the cytoplasm (Fig 1 F) possibly after release from the endosome (Fig 1F insert). Virus-containing vesicles of different morphology were observed, occasionally within the same cell. These were

consistent with early (EE) and late (LE) endosomes (Fig 2). An electron-dense layer was noted lining early endosome membranes (Fig 2 A, C). To note was a tubular or neck structure observed connecting a pit coated vesicle to larger endosome-like compartment, containing

multiple intact virus-like particles (Fig 2 B). Internalised vesicles with single viral particles within larger endosome were also seen releasing their content (Fig 2 C), with the particles detaching from membrane in a similar manner as that described for cytoplasmic particle release (Fig 1 F, insert). Large vesicles consistent with LE containing numerous, densely packed, viral particles admixed with loose debris, were observed as early as 3 hpi (Fig 2 D).

Formation of spherules was observed at 3 hpi. These characteristically bulb-shaped convoluted invaginations, have been previously described in the replication cycle of alphavirus [41,42]. In our study, these were seen on the PM and within cytoplasmic vacuoles (Fig 3 A, B, C, D). These spherule-containing vacuoles were morphologically consistent with previously described type I cytopathic vacuoles (CPV-I). In our study, CPV-I were ≥ 200 nm in diameter, frequently located in the peri-nuclear region and in the proximity of the RER (Fig 3 E), and occasionally contained ~ 50 nm diameter, electron dense particles within (Fig 3 F). Additionally, spherule containing vacuoles occasionally had a prominent neck-like structure (Fig 3 C, D).

Characteristic type II cytoplasmic vacuoles (CPV-II), as those described for other alphavirus in vertebrate hosts, were not observed in this experiment, but cytoplasmic structures with features similar to CPV-II as more recently described for SINV *in vitro* infected mammalian cell (baby hamster kidney BHK-15 [42] were observed from 24 hpi onwards. They are seen as pleomorphic, round to ovoid structure with a darker periphery due its surface being covered by evenly distributed, ~ 25 - 30 nm electron dense particles (Fig. 4). The centre of these structures could either be mildly to highly electron dense, and individual electron dense particles were visible in some of them (Fig 4 B).

Viral budding at the PM was seen occasionally in samples over 48 hpi (Fig 5 A, B). Additionally, features resembling intra-cytoplasmic budding were seen (Fig 5 C, D). Extra or intracellular budding seemed associated with presence of electron dense sections of the plasma membrane and occasionally with e loos continuity (fragmented membrane) (Fig 5 A, C, D).

Two additional relevant features in infected SCPCs were observed. One consisted of cytoplasmic moderate electron dense, loosely organised granular aggregates, consistent with previously described ribo-nucleoprotein stress granules (SG) [43] (Fig 6 C and D) which

were always associated with viral like particles in the vicinity displaying a regular spiky surface (Fig 6 B,C and D). The second observation were the filopoid extensions of the PM, also frequent after 24 hpi, particularly on endothelial cells and in intraluminal leukocytes (Fig 6 E, F and insert).

Signs of mild to moderate degenerative changes associated with the infection were recorded from 24 hpi, they included mitochondrial swelling, loss of cristae, multiple-membrane vesicles, myelin figures, peroxisomes, nuclear apoptosis and cell detachment. These features are not shown here as they have been previously reported [40,44].

Discussion

Understanding replication cycle events and the underlying mechanisms used by successful viral pathogens is of great importance for the studies of host–virus interactions, and a requirement for the development and implementation of preventive measures such as vaccines and other targeted therapeutic strategies [45,46].

Biological *in vitro* experiments are often performed based on immortalized cell lines and several cell lines of fish origin have shown to be permissive to SPDV infection [19,47]. The value of the use of continuous cell lines versus primary cells remains debatable, as a compromise weighing the practicality of using a readily available tool (which may differ from the *in vivo* behaviour in important aspects), against the benefit ascribed to primary cells of having relatively higher biological relevance [48]. Indeed, care is required in interpreting results from the use of continuous cell lines as they do not always replicate accurately the same events noted in primary cell lines [49]. Further, the continuous serial passages required for cell line maintenance have been reported to cause genotypic and phenotypic variations [48]. The current work uses salmon cardiac primary cultures developed from Atlantic salmon embryos, representing one of the SPDV targeted organs on the most relevant affected host species.

Different alphaviruses such as CHIKV, SINV and SFV have been used to describe the viral replication cycle in affected vertebrates and invertebrate hosts. As zoonotic viruses, they have been the focus of extensive studies and several characteristics have shown to be shared among them. However, overall aspects of the traffic and assembly of viral components and the exit of the virions from host cells are yet not fully understood [50]. Moreover, given the variety of virus and host species, some mechanisms and a level of temporal or spatial variation among alphavirus virus species or between vertebrate and invertebrate hosts, is to be expected. Recently for example, differences between SINV

infected mammalian and insect cell lines (baby hamster kidney BHK-15 and mosquito *Aedes albopictus* C6/36 respectively) have been described [42].

Features of SPDV replication cycle and infection at the ultrastructure level have been previously described through studies on the morphogenesis *in vitro* using Chinook salmon embryo cells (CHSE-214) and the pathogenesis *in vivo*, using Atlantic salmon juveniles in fresh water [33,39]. These studies used two Irish subtype-1 isolates, the type reference F93-125 suggested to be culture adapted [15,30] for the *in vitro* work, and isolate F02-143 originated from a 2002 outbreak for the *in vivo* work [33,39]. The here reported work used isolate F07-220, a subtype-1 isolate originated from an outbreak in Ireland. The virulence and pathogenicity of the isolate had been tested previously through *in vivo* challenges [32] and also used to test infectivity during the development and application of the SCPCs model [40,44].

Based on the interpretation of morphological features at the ultrastructural level of SPDV infected SCPCs, our results generally align with those reported for other alphavirus species [36,42]. Features such as the viral attachment to the cell surface is consistent with the reported binding to host cell receptors for alphavirus [51,52], as is the formation of early (EE) and late (LE) endosomes after internalization, or the presence of replication complexes involving membrane re-assembly and formation of spherules, all similar or equivalent to previously described features of alphavirus on vertebrate cell models [5]. There is also alignment with previous ultrastructural descriptions of SPDV in cell culture, such as vacuole associated spherules [39]. Interestingly, while several organs were examined in this aforementioned mentioned *in vivo* infection work, the authors reported that although features of replication could be seen in heart, kidney and gills, virus-like particles were observed only in the heart [33] but so far, no model system was available for examining virus-like particles and especially replication/ re-infection *in vitro*.

Entry mechanisms

For this early step of the virus life cycle, the current work builds on previous work reported for SPDV *in vitro* infection [39] by providing a thorough description of the process of viral attachment and internalization. This step had not been observed before. This may have been due to differences on host cells and tissues used, potential difference on isolates virulence or the effect of differences on experimental settings, e.g. double the adsorption time -2 h- in the current experiment. In this study, viral particles were observed during the process of internalization at 2 and 3 hpi, and again, at 24 hpi, simultaneously to the presence of structures related to replication, suggesting a process of re-infection.

The initiation of infection was observed to occur through a close attachment of the virus with the cell, a contact that morphologically was observed as a mild electron dense cloudy region (Fig 1 A and B and insert). This type of attachment is very similar to that reported for SFV [53].

As intracellular parasites, viruses take advantage of the host cellular machinery for replication, requiring first to successfully deliver their genome into the cell. Most of the understanding of alphavirus entry has been obtained from studies focused on alphaviruses regarded as “Old World”, such as SINV and SFV. Comparably much less attention has been received by other species, although fundamental differences on the entry mechanisms have been suggested for other genus members such as VEEV [54].

Conventionally, enveloped RNA viruses exhibit a two-step entry mechanism involving a receptor-mediated binding to host cell surface (likely represented in our study by the previously described attachment features), followed by a low pH-triggered membrane fusion of the viral and cell membranes that delivers the RNA into the cytoplasm [51,55]. These two steps may occur at the cell surface or after internalization of the virus by endocytosis or some other route.

Even for the reference prototypical alphaviruses, the precise mechanisms of internalization are still not totally clear [56]. Currently, CCP endocytosis is thought to be the most common route and this is consistently reported for SFV [51] and CHIKV [57]. However, endocytosis independent of CCP has been shown to occur in CHIKV [58] as well as in the absence of caveolar vesicles [59]. Even for the reference species SFV, the canonical CCP entrance may not necessarily be the only route. Early work using transmission electron micrographs showed that while the majority of the viral particles were internalised by the CCP endocytosis, a small number were observed and depicted using uncoated membrane invaginations [53]. Additionally, alternative paths, as distinct as virus genome injection directly through the cell plasma membrane, may occur in SINV virus [60,61]. Seemly, under non-permissive conditions for endocytosis or any vesicular transport, this mechanism allows entry of the viral genome by direct penetration of the plasma membranes through a pore formed by viral, and possibly host, proteins; a process suggested to be time- and temperature-dependent [62,63].

Morphologically, two types of entry features suggesting different mechanisms were apparent in SPDV infected SCPCs. One in line with the classic mechanism of clathrin dependent endocytic pathway (CCP), well-established and described for alphavirus [51], and another seemly clathrin independent (CI) mechanism using a caveolar or lipid raft-like structure. Pathways of clathrin-independent endocytosis for different molecules including viruses have

been previously reported, as well as specifically for Chikungunya, a human affecting alphavirus [58,64].

The possibility that the feature described as CI mechanism may represent an artefact however, cannot be ruled out, as is based exclusively on the morphological observation of the absence of the characteristic clathrin spiky coat. It has been reported that once endocytosed, the clathrin coat on the virus-containing vesicles is rapidly removed and the virus delivered to the early endosome compartment [51].

In mammalian cells, the endocytic internalization of virus particles is possible through many different mechanisms, some on-going processes such as the CCP, and others ligand or cargo induced, such as the use of caveola [65]. Other mechanisms include macropinocytosis (adenoviruses), CI pathway from the PM (influenza and arenaviruses), caveolar pathway (one of the several cholesterol dependant used by SV40 or mouse polyoma) or a cholesterol-dependant endocytosis devoid of clathrin and caveolin-1 (used by polyomavirus and SV40) [65].

The clathrin-mediated pathway being the most commonly observed among viruses and the parallel observations in the infected SPCS of transport-like vesicles consistent with early and late endosomes and eventually lysosomes, would suggest the CCP mechanism is also the case for SPDV.

However, it is not yet known for certain whether some pathogens requiring entrance to the host cell such as viruses follow pre-existing cell's trafficking pathways or if they actually induce their own endocytic itinerary [64]. Important differences and variations are described for alphavirus in general, suggesting that there might not be a single model that can be used to compare or to fully describe the entry process in SPDV infection.

The SCPCs model represents therefore a useful tool to examine in more detail this important aspect of the virus cycle. Further work could contribute to a better understanding of the entry mechanism and elucidate if artefacts are responsible for some of the current observations or if indeed, different mechanisms could potentially occur simultaneously or under different conditions. The model can also be used to explore the hypothetical re-infection process suggested by the re-internalization features observed after 24 hpi.

Full understanding of the entry mechanisms of SPDV would not only be relevant for the elucidation of steps of the viral replication cycle, but could also play an important role for the potential development of rational antiviral therapies, for example exploiting compounds which could block critical steps of early infection events [56,62] .

Post internalization events

Following internalization, abundant trafficking vesicles were observed consistent with cytoplasmic translocation of virus particles. Newly internalised virus containing vesicles could be seen fused with endocytic vacuoles to form early endosomes (EE), with almost intact viral particles within. Free cytoplasmic virus-like particles were also observed. Late endosomes are larger vacuolar structures containing a mix of degenerating material (residual bodies) simultaneously with still visible viral particles. Both EE and LE were seen in samples at 2 and 3 hpi. For alphavirus, a low pH mediated process in the LE has been described to be necessary to allow the fusion of the viral and the endosome membranes to release the nucleocapsid core (NC) into the cytoplasm, where it will be disassembled [42].

In general, positive-strand RNA viruses replicate their genomes in association with remodelled, intracellular, membrane arrangements such as single or double-membrane vesicles. However, even in very thoroughly studied viruses the exact sites of RNA synthesis, the mechanisms involved or the topological relationships between membranes vesicle content and cytoplasm, are frequently poorly defined [66].

Consistent though with description for other alphavirus in mammalian and insect cell models, SPDV infection showed to induce the re-arrangement and remodelling of cell membranes. The alphavirus RNA is known to replicate and transcribe in “replication complexes” (RC) formed soon after infection on endosomal and lysosomal membranes by host and viral RNA proteins [5,67]. The replication and transcription induce the formation of spherules, visualised as multiple endocytic processes resembling caveolae and multi-caveolar-like vesicles. These structures are derived from host cell membranes, with PM, mitochondria and ER being frequently hijacked for that purpose by positive-strand RNA viruses [68]. The spherules can be re-localised by trafficking through the cytoplasm to the peri-nuclear region. A cytopathic vacuole type I (CPV-I) is formed as the replication spherules are internalized and the vesicles are fused with lysosomes.

The size of spherules has been reported to be closely connected to the length of the replicating RNA template [69]. The SCPCs infected with SPDV showed spherule-like structures forming at membrane surfaces and within vacuoles in the peri-nuclear region, with a size range of 35-55 nm. This is consistent with spherule formation of ~50 nm reported in SINV infection [42] and in SFV, where a viral-genome template of 11.5 kb induced spherules of ~58 nm diameter, and a template of 6 kb yielded ~39 nm spherules; the two size classes in the same cell [69]. In the current work a connecting tube or neck-like structure was observed in some spherules containing vacuoles, suggestive of trafficking of spherules into

the vacuoles located at the peri-nuclear region. Additionally, spherules themselves showed a connection similar to a neck opening into the cytoplasm. The latter has been reported to allow export of progeny RNA destined for translation or packaging and import of metabolites required for replication [70].

Naturally, many of the viral synthesis events that use the host cell machinery cannot be demonstrated or confirmed by ultrastructural examination. These include the RNA synthesis reported to occur with the non-structural proteins (nsP) accumulating at the cytoplasmic neck of the spherules, followed by newly synthesized RNA diffusing into the cytoplasm [42,67]. The structural capsid proteins (CP) are translated from the sub-genomic RNA and this polyprotein is then autocatalytically processed, resulting in the release of CP into the cytoplasm. The cellular secretory pathway plays an important role in the virus replication and assembly by transporting CPV-I and CPV-II to the PM, as demonstrated with SINV by live imaging experiments [42]. It was also shown that CPV-II originates from the *trans*-Golgi network ~4 hpi in mammalian cell lines. CPs in the cytoplasm encapsidate single genomic RNA to form the nucleocapsid core (NC), which will then bind to the glycoprotein spikes, already located at PM forming the envelope during virus budding [42].

Unequivocal CPV-II structures have not been observed in the current SCPCs infection. However, structures interpreted as hypothetically “functionally equivalent”, were observed and described. They are in line with the most recent description for the type of structure in a SINV infected vertebrate cell line [42]. A mild or dense appearance of the inner or core centre of the vacuoles is interpreted as possibly cross sections at different planes as has been described for CPV-II from another alphavirus *in vitro* (SFV infections on BHK-21 cells) [71]. CPV-II structures in SPDV *in vitro* infection had been reported [39], however different to the here described.

Viral assembly and budding

Alphavirus in vertebrate host and mammalian cell lines have been shown to assemble by budding at the PM of the infected cell when the ensemble NC core acquires a host derived envelope of phospholipid membrane, and an outer glycoprotein layer [72]. In our study particles budding from cell PM were observed at 48 hpi. Electron dense segments of PM were observed at or close to points of budding, which can be interpreted as the display of such envelope proteins. The rationale for this is based in electron tomography studies on SFV, where it was shown that the envelope E1/E2 glycoproteins are arranged in the CPV-II and introduced at the PM budding sites as a lattice, resembling already their organization on the viral envelope [71]. Interestingly, we noted cytoplasmic small vesicle structures of a similar size to complete virions but with an empty core bearing an external spiky surface

resembling those on the viral envelope. In previous SPDV morphogenesis *in vitro* work [39], a Golgi apparatus and vacuoles characterized by 'fuzzy'-coated membranes were incidentally reported as more frequent in the infected cells. In addition, with the current observations and the evidence for SFV, a potential interpretation of such structures may be that these vesicles represent envelope proteins before translocation to the PM through the secretory pathway.

Additional observations

While the focus of the current work was describing features believe to correspond to events of the SPDV replication cycle, other observations which were consider relevant were also noted and described. For example, the presence of clustered granular structures consistent with stress granules (SG), which to the best of the authors' knowledge, have not previously been reported for SPDV.

Development of SG in mammalian cells is not a rare event, as translational attenuation is known to result in the formation of stress granules which contain ribonucleoproteins (mRNPs) and stalled translation initiation complexes. The packaging of cytoplasmic mRNA into discrete RNA granules regulates the gene expression, by delaying the translation of specific transcripts [43]. SG appear to be associated with stress events that severely repress translation, such as heat shock, oxidative stress or UV radiation [73]. Additionally, viral infection causes stress at multiple levels which can also reduce host translation, and through a complex series of events, lead to the formation of stress granules [74]. Arboviruses such as the majority of alphavirus, moreover, have been speculated to be capable to modulate the SG response in invertebrate hosts, decreasing but not completely repressing the effects of antiviral pathways. This balances the levels of viral replication and the fitness of the vector host [75]. In SFV, SG has been reported to occur transiently and later disassemble with the ongoing viral replication. Many viruses can effectively counteract the assembly of SGs, suggesting their involvement in antiviral activity [76].

Another observed feature that represent an event not described for SPDV and differing from alphavirus in other vertebrate hosts, are the filopodial projections seen at the PM in endothelial cells and leukocytes. Similarly, the membrane bounded cytoplasmic vacuoles morphological consistent with internal budding (see Fig 5 and 6). Interestingly, the SCPCs model benefits from the presence of endothelial cells [40], a cell type which has recently been highlighted on its importance for the early events of Chikungunya infections [77]. Filopodial projections and the putative internal budding observed here have only recently been described associated with alphavirus in their invertebrate hosts (mosquito cell lines) [42]. Filopodial extensions associated with viral dissemination were shown to develop in a mosquito cell line (C6/36, originally known as the ATC-15) [78] and where the viral

glycoproteins and the virus budding also occurred both at the PM as well as into internal vesicles. Recent work lead to the hypothesis that, in the mosquito cell line model, alphavirus shows a characteristic that favours a transition towards persistent infections, and which would allow the virus to thrive in nature [42].

Conclusions

The observations gathered in our study of SPDV infected SCPCs, while in line with previous studies, also indicate the potential simultaneous occurrence of events described for mammalian alphaviruses infecting vertebrate and invertebrate cell lines. It is noteworthy that in mammalian cell lines alphavirus frequently results in acute infections and cytolysis, while in insect cell lines, the host cells survive infections and may later spread the disease through a biological process known as RNA interference (RNAi), a major antiviral pathway that modulates arbovirus infections by a gene silencing conserved mechanism [79–81]. This may be related to the capacity to establish persistent infection with no cytopathic effects [42,82]. Based on the current study, it may be suggested that SPDV may have the capacity to establish both cytolytic and non-cytolytic persistent infections in the same host. In this hypothetical scenario, the capacity to alternate between cytolytic and non-cytolytic persistent infections could relate to the clinical presentation of the disease. Mortality on SPDV infection is the outcome for a variable proportion of infected fish, whereas the condition persists without killing the majority of the host population. Also, clinical recurrence has been reported regardless of rest periods between introductions of new generations into vacant enclosures. Both of these observations may be suggestive of viral persistence in the host in the absence of clinical disease. Longitudinal studies in Scotland [83] and to a lesser extent in Norway, have reported fish from sites never diagnosed with PD that were found to be SPDV-positive at slaughter [84].

On one side, non-lethal SPDV infection of at least one non-salmonid species (common dab *Limanda limanda*) has been reported as a wild reservoir which presented no clinical signs or characteristic PD histopathological lesions [16–18]. On the other side, the members of *Alphaviridae* have been suggested to have originated in the aquatic environment [2], acquiring the ability to infect warm-blooded vertebrates and mosquito vectors secondarily, a characteristic described as a safeguard of the maintenance of the agent in the environment. Additionally, it has been hypothesized that arboviruses are more constrained than single-host RNA viruses in adaptability [85] by alternating transmission between vertebrate and invertebrate hosts. Together these would support a hypothesis that SPDV, as an aquatic and older alphavirus, may have retained a capacity to establish cytopathic as well as persistent infections in the same host before the group became terrestrial arbovirus. This could at least

partially explain the observations in Atlantic salmon aquaculture and the non-pathological infections noted in reservoir species in the marine environment.

Recent studies have pointed to fundamental mechanistic differences between insect and mammalian cell lines in the interaction of alphavirus replication and structural components, showing how viruses can exploit the resources and environment of their specific hosts [42]. Additionally, the involvement and potential role of host RNA-binding protein interactions during viral replication has been also explored [86]. This interaction with cell-type-specific factors is now recognised as an area requiring further research, to improve understanding of both the regulation of viral replication and gene expression and of virus species-specific pathogenesis [82]. The use of a species-specific model as in the present work, containing different cell types from a fish organ known to be affected by the virus, might prove of further practicality as it would support exploration of potential cell-type-specific factors in SPDV infections.

In conclusion, SPDV has been affecting the salmon farming industry for over 30 years but in spite the substantial amount of studies along these years, there are still a number of recognised knowledge gaps requiring further research, among them the transmission of the virus [25,87]. It will only be through a deeper and more thorough understanding of the mechanisms used by SPDV and its pathogenesis that effective targeted therapeutics can be developed. The current study provides information extracted from an *in vitro* model system which has given new insights into several aspects of the viral replication cycle, bringing hypothesis and opening new research avenues such as a putative capacity for non-cytolytic persistent infections by SPDV, which may be a result of evolution within *Alphaviridae*.

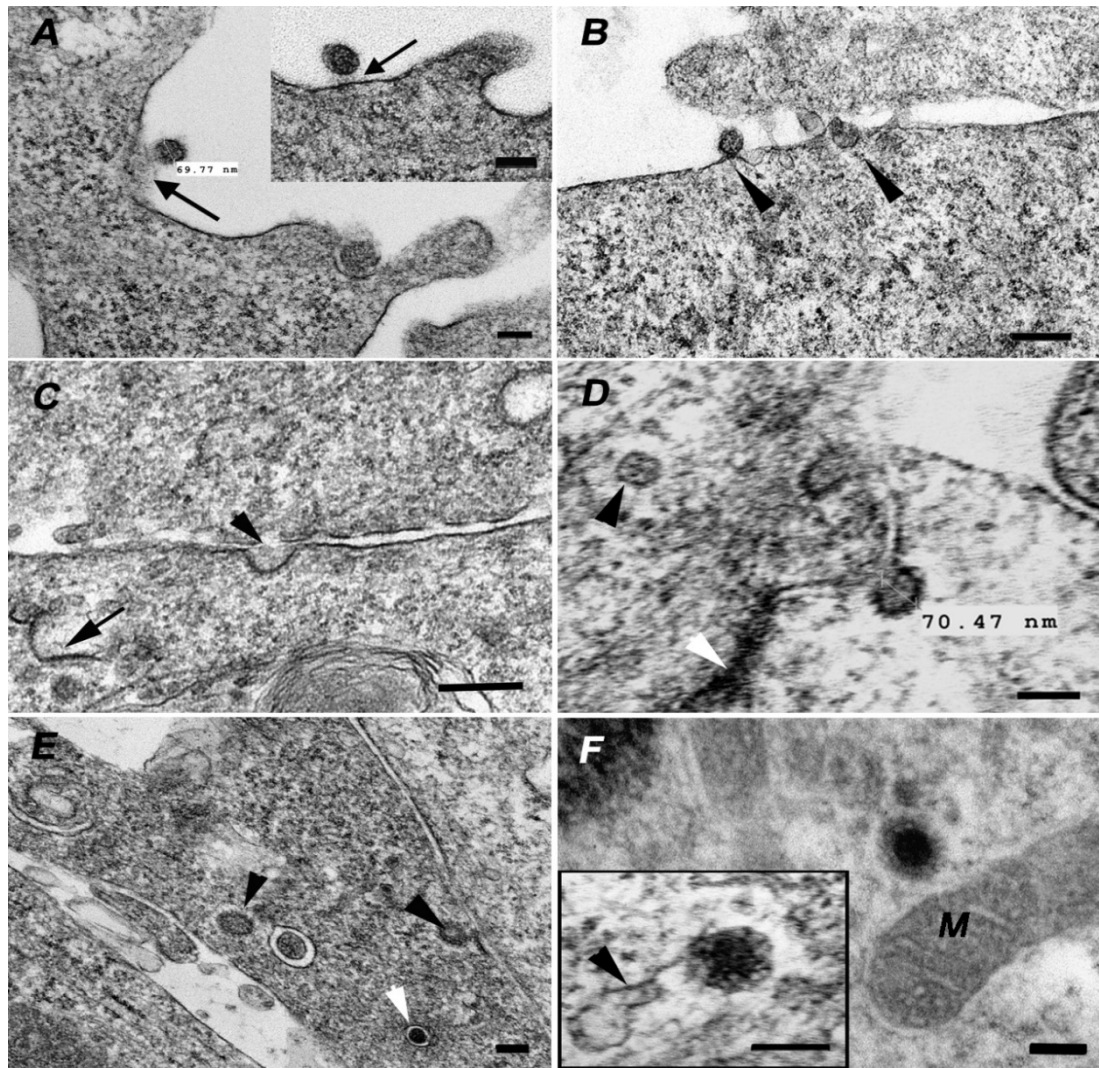


Fig 1: Transmission electron micrograph of Salmon cardiac primary cultures infected with SPDV subtype-1 at 2.4×10^4 plaque forming units (PFU)/ml and fixed for analysis at 2-3 hpi. Features of the viral entry. (A) viral particles at sequential steps of the entry process. Note contact established through an electron dense cloud (arrows) between the virion and the cell surface (top particle and insert) and initiation of internalization of a second virion below, already within the invaginating plasma membrane. (B) Extracellular virion attached to cell surface and formation of concavities at the plasma membrane adjacent or just below the point of contact (arrow heads). (C) Detail of a clathrin coated pit (CCP) at the cell membrane (arrow head) and seemingly remnants of a CCP membrane fragment (arrow) in the cytoplasm, likely post particle release. (D) Detail of CCP formation with an almost fully close and another CCP developing on the opposite adjacent cell. Note increased electron dense section of plasma membrane (white arrow) in the proximity of CCP. A possible internalised virion is observed within a fainting vacuolar structure (black arrowhead). (E) A cell where CCP-dependent (black arrowheads) are clearly observed while an internalised viral-like particle within a non-spiky-coated vesicle (white arrowhead) is seen simultaneously within the same cell. (F) Intact virion free in the cytoplasm above mitochondria (M). Insert shows a particle releasing from vesicle membrane still attached at a single point. Scale bars on A, C= 200 nm and B, D, E, F =100 nm on all other images.

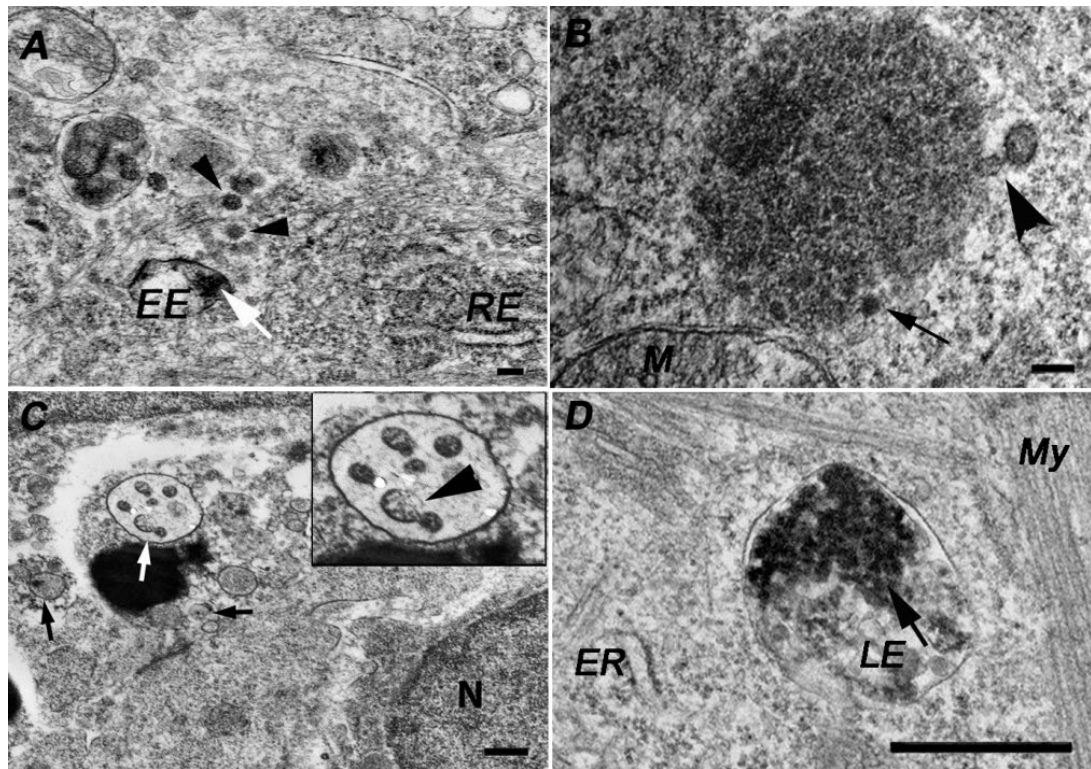


Fig 2: Transmission electron micrograph of Salmon cardiac primary cultures infected with SPDV subtype-1 at 2.4×10^4 plaque forming units (PFU)/ml and fixed for TEM analysis at 2 -3 hpi. Features of cytoplasmic events post viral internalization. (A) Recently internalized viral particles within early endosome (EE). An early endosome (EE) shows particles still partially linked to vesicles membrane (white arrow) and several free particles in the cytoplasm (arrow heads). Note active endoplasmic reticulum (RE). (B) Connection established between single particle pit coated vesicle and a larger endosome-like compartments, through a tubular /neck structure (arrow head). Note multiple intact virus particles (arrow) within the endosome (arrow). (C) Larger endosomal structure containing multiple vesicles (white arrow) with some viral particles yet being released from EE (insert enlargement). Note electron-dense layer lining endosome membranes. (D) Large vesicle consistent with LE containing numerous packed viral particles (arrow) mixed with debris. Note myofiber (My) and active ER. Bars: A, B, = 100 nm, C = 400 nm, D = 500 nm.

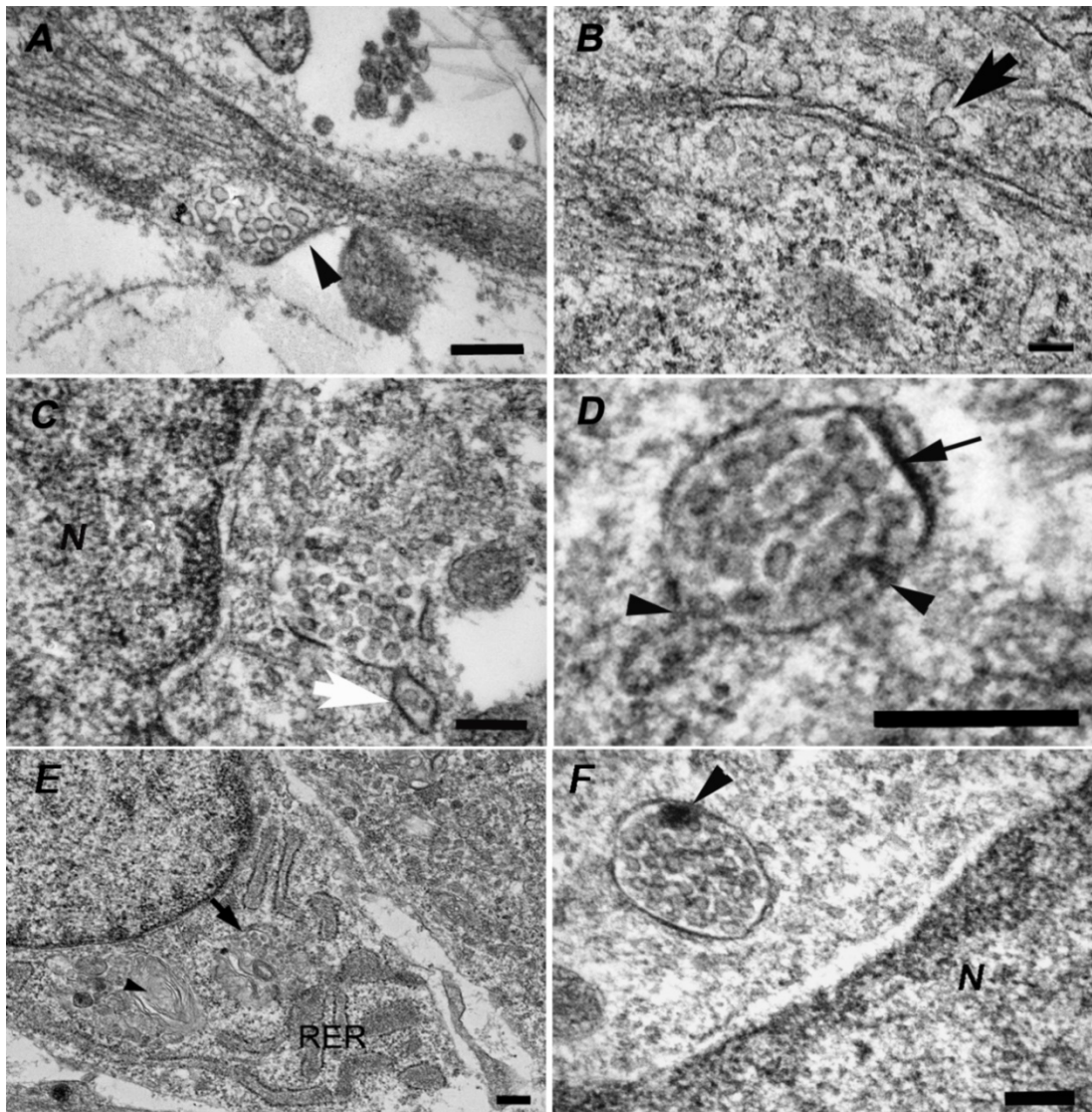


Fig 3: Transmission electron micrograph of Salmon cardiac primary cultures infected with SPDV subtype-1 at 2.4×10^4 plaque forming units (PFU)/ml and fixed for TEM analysis at 3 hpi. Features of membrane remodelling and formation of spherules and cytopathic vacuoles type I (CPV-I). (A) Spherules observed in the nearby of plasma membrane within a loose vacuolar structure. Note the connection between single spherule with membrane by a neck structure (arrowhead). (B) Higher magnification of spherules formation. (C) Spherules contained within a cytoplasmic vacuole nearby the nucleus (N). Note prominent tubular connecting structure (white arrow) also containing spherules. (D) a close up of a similar structure as shown in C, note spherule close contact with vacuoles membranes (arrowheads) and a high electron dense region of the later (arrow). (E) Spherules containing vacuoles morphologically consistent with cytopathic vacuoles type I (CPV-I) located in the perinuclear region (arrow) and in the proximity of the rough endoplasmic reticulum (RER). (F) CPV-I containing single electron dense (~50 nm diameter) particle. Bars: A, C, D, E, F = 200 nm, B = 100 nm.

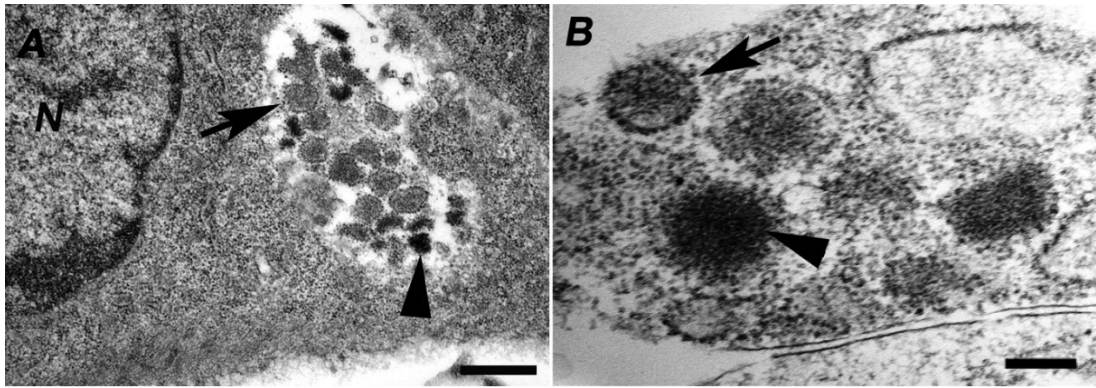


Fig 4: Transmission electron micrograph of Salmon cardiac primary cultures infected with SPDV subtype-1 at 2.4×10^4 plaque forming units (PFU)/ml and fixed for TEM analysis at >24 hpi. Features of cytoplasmic vacuoles type II (CPV-II) putative structures. (A) Numerous pleomorphic, round to ovoid CPV-II like complexes, clustered near the cell membrane. Note those with a mild electron dense centre and visible defined darker periphery (arrow), and those that appear as a dark, high electron dense vacuole (arrowheads). (B) As in A, at higher magnification both mild and high electron dense vacuoles are interpreted as sections at different levels of identical structures. Both present evenly distributed, ~25-30 nm nucleocapsid like particles arranged on their surface. Bars: A = 500 nm, B = 200 nm.

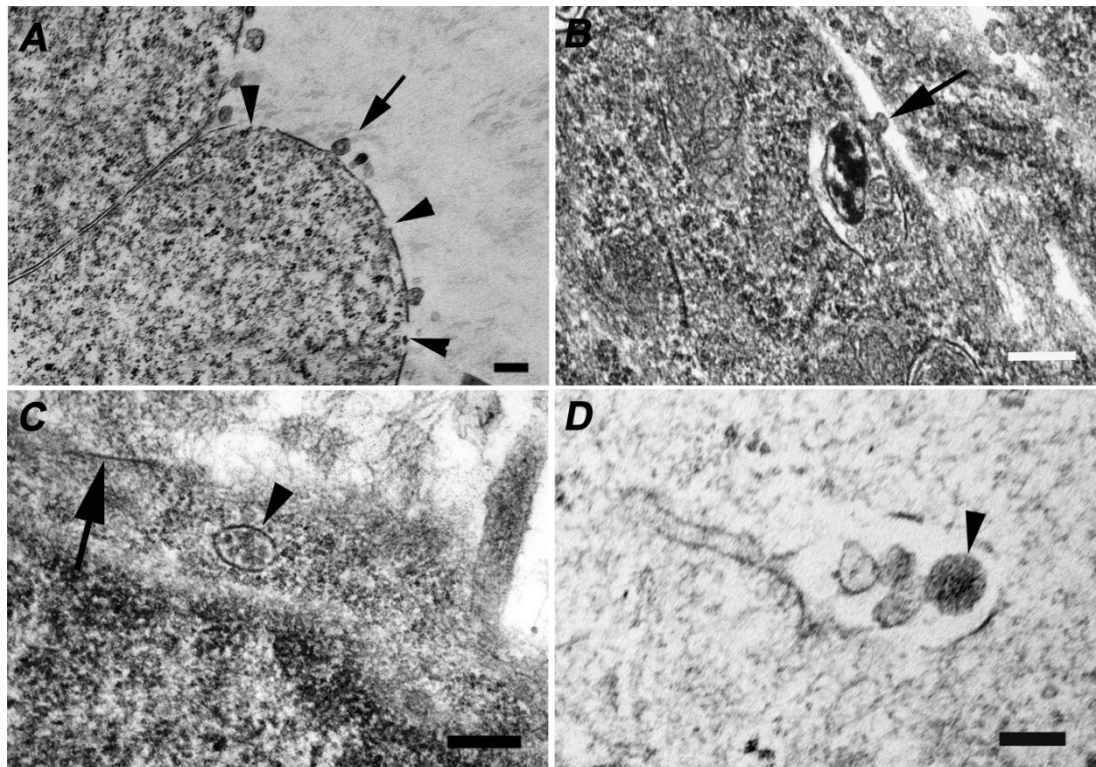


Fig 5: Transmission electron micrograph of Salmon cardiac primary cultures infected with SPDV subtype-1 at 2.4×10^4 plaque forming units (PFU)/ml and fixed for TEM analysis at 24 and >48 hpi. Features of extra and intra cytoplasmic budding. (A) Budding at plasma membrane (arrow). Note broken continuity of plasma membrane due small missing portions (arrowheads). (B) Budding particle where nucleocapsid core can be seen within as cell membrane envelope is being formed (arrow). (C) A cytoplasmic vacuolar structure containing viral like particles (arrowhead), hypothesised as a feature of putative intra cytoplasmic budding. Arrow indicates a distinctly darker section of plasma membrane resembling accumulation of envelope glycoproteins in preparation of the budding process. (D) A cytoplasm vacuole contained a viral particle. Note vacuole interrupted membrane and the tubular funnel. Bars: A, B, C =200 nm, D = 100 nm.

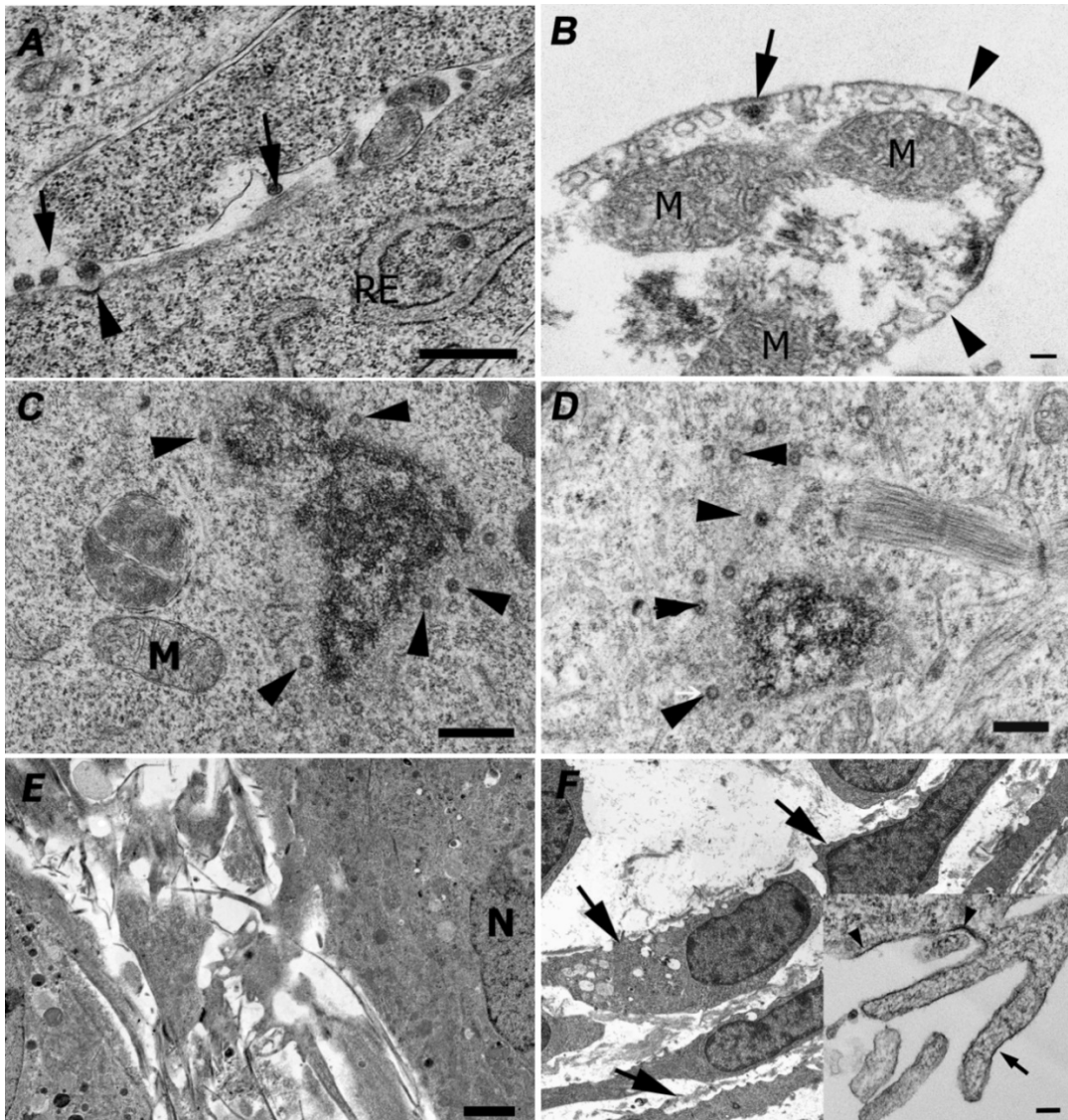


Fig 6: Transmission electron micrograph of Salmon cardiac primary cultures infected with SPDV subtype-1 at 2.4×10^4 plaque forming units (PFU)/ml. Additional observations at > 24 hpi. (A) Putative re-infection process. Attaching virion at plasma membrane (arrow), and a virion internalized by clathrin-mediated endocytosis (CCP) (arrow head). Note sub-membranous electron dense band. (B) Putative re-infection process by formation of caveolar type invaginations at cell surface (arrowheads) and virion internalization by a CCP-independent process (arrow). (C and D) Intra cytoplasmic clusters of loosely organised electron dense particles into aggregates consistent with ribonucleoprotein stress granules (SG). Note clear areas of fibrillar patches within the SG and presence in the surrounding area of viral like particles displaying a regular spiky surface (arrowheads). (E) A scaffold of filopodial extensions between two neighbouring cells. (F) Filopodial extension in an endothelial cell lining SCPCs internal chamber, and extending from intraluminal leukocyte. Insert shows branching filopodial extension with darker, electron dense section of their plasma membrane.

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FINAL DISCUSSION

Infections by Salmon Pancreas Disease Virus (SPDV), primarily in Atlantic salmon (*Salmo salar* L.) but also rainbow trout (*Oncorhynchus mykiss*), have been affecting the farmed salmonids industry in the UK, Ireland, Norway and continental Europe for over 30 years.

This thesis was framed within a project in accordance with governmental aquaculture policies at Marine Scotland Science (MSS), the Scottish National Reference Laboratory. At the heart of it was to address some of the SPDV knowledge gaps identified by the industry and the research communities. The general aim therefore was to improve and develop new approaches on experimental models, especially applicable to the study of SPDV in Atlantic salmon. The work was approached through *in vivo* and *in vitro* studies.

For the first part, the *in vivo* work aimed to move from the traditional intra peritoneal (ip) infections of salmon par in fresh water, to the implementation of sea water co-habitation challenges with salmon post smolts.

Through evaluation and selection among SPDV isolates of different subtypes, chapter 1 provided information on suitable isolates based on their intraperitoneal infectiveness at several tissue levels. Two isolates, namely 4640 and F07-220 were identified and results also provide information on virus tissue tropism, which later allowed narrowing the number of analysed tissues during the viral load assessment during co habitation.

A second part of the *in vivo* section used selected isolate 4640 along the subtype 1 reference F93-125 to successfully establish a viral infection in sea water by transmission through co habitation using Atlantic salmon post smolts (chapter 2). This experimental approach represents a more natural transmission route within the environment and with fish at the developmental stage predominantly affected by the disease. The experimental setting has since been used at MSS for subsequent disease studies, fulfilling the objectives of *in vivo* work established for this thesis.

Selected isolate F07-220 was the main one used for the experiments of the *in vitro* sections of this thesis. (Chapters 3-5) similarly aimed to bring an alternative approach and introduce a higher level of complexity to laboratory-based studies. The development, optimization and application of an *ex vivo* cardiac primary culture (SCPCs) originated from Atlantic salmon embryos, was set as a goal in order to study cardiotropic agents in Atlantic salmon (Chapter

3). The choice of the heart as target tissue for this section was partially based on the observations during the *in vivo* work where tissue tropism studies pointed to the relevance of the heart during SPDV infections. It was also influenced by the awareness that heart is one of the organs that describes major histopathological changes during the course of SPDV infections. Additionally, it was becoming very evident that besides SPDV, other viral agents such as Piscine Reovirus (PRV) and Piscine Myocarditis Virus (PMCV), responsible for the conditions known as heart and skeletal muscle inflammation (HSMI) and cardiomyopathy syndrome (CMS) respectively, also affected the salmon heart and were becoming increasingly a concern for the industry.

The results obtained for the *in vitro* section, the SCPCs, accounted for the more innovative part of this thesis. A relative lengthy process was required at start to refine the SCPCs production and achieve efficiency at synchronously obtaining sufficient numbers of cultures to allow for experimentation including longitudinal trials. During this process tools that could be applied, particularly related to SCPCs very small size, were explored and tested, including molecular approaches (RT-PCR), histology, histochemistry and electron microscopy. SCPCs could be kept viable under laboratory conditions with minimal maintenance for periods up to 6 months. The model was successfully challenged with different SPDV isolates and other cardiotropic viral agent (e.g. Infectious Salmon Anaemia, ISA,) to prove the model permissiveness to infection. The results showed the kinetics of viral infection and a relevant element of the immune response (i.e. expression of *mx* gene) could be studied along time (Chapter 3, paper 1).

Another explored application of the model was to compare the viral load and *mx* kinetics after SPDV challenge between SCPCs of different genetic backgrounds (i.e. IPNV resistant vs. IPNV sensitive). Results showed measurable differences between the groups after infection, highlighting the potential use of SCPCs to examine genotype-based differences in response to viral disease (Chapter 4, paper 2).

A further and last application of the SCPCs model within this project was to study the SPDV replication cycle using transmission electron microscopy (TEM) (Chapter 5). Profound a comprehensive information on virus replication cycle and its interactions with the host will always be a requisite in order to develop control strategies. This part of the work resulted in novel insights on the replication cycle of SPDV, drawing from the extensive literature in mammalian alphavirus work. Observed features included some not previously reported for SPFD, such as presence of filopodial extensions and “stress granule” like structures and particularly, putative intracytoplasmic budding capacity that underpins a suggested hypothesis that SPDV may be able to establish both cytolytic and non-cytolytic infections in the same host.

SPDV and other virus associated cardiomyopathies remain a serious issue affecting the Atlantic salmon aquaculture industry. Further work on this and other cardiotropic agents will still be required to help support the development of disease control and management strategies, including host genetic increased resistance and /or vaccine development.

The SCPCs model generated during this thesis has the potential to contribute on its present form, in aspects such as examining intra organ cell tropism or assess relevant replication cycle events such as the initial viral fusion, relevant to the understanding of potential inhibitors and extremely promising for both research and therapeutic uses. For other uses such as the host genotypic screening, further characterisation would be required to establish the model as a robust platform, including increasing experimental sample size, testing the use of single (individual) versus multiple (pooling) SCPCs, or exploring the potential of non-destructive approaches by using sequential culture supernatant collection. The “gold standard” required to verify the results would be provided by the comparison of results *in vitro* obtained with SCPCs of a given origin, with those further on time from *in vivo* challenges results, using fish originated in the same stock and using a combination of structural, biochemical and molecular approaches for the analysis.

ANNEX

For chapter 1

All isolates added to TO cells and incubated at 15°C. Samples were taken at days 1, 2, 3, 4, 6, 7, 10, 12 and 14 after inoculation (x3 infected and un-infected controls wells). RNA was extracted from the infected cell cultures using the RNA/DNA/Protein purification Kit (Qiagen) according to manufacturer's instructions and reverse transcribed to cDNA using the TaqMan® Reverse Transcription Reagent kit (ABI) with random hexamers. Real-time PCR assays were performed on an ABI 7000 Sequence Detection System (Applied Biosystems). TaqMan® probes to amplify the Elongation factor 1 a gene (*elf*), *mx*, and SPDV nsP1 genes.

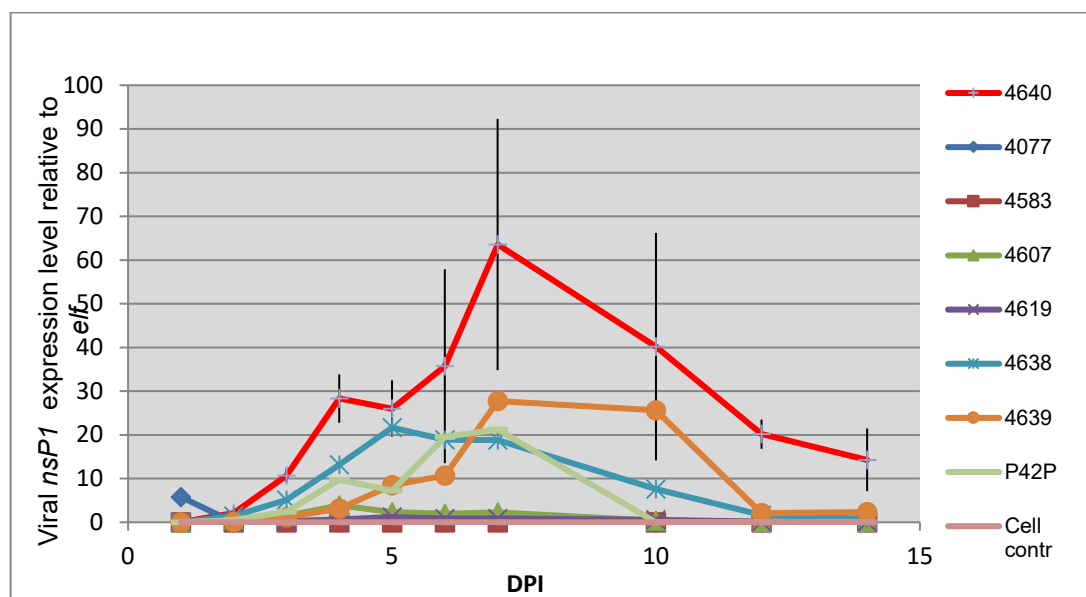


Figure 1: Kinetics of expression of *nsP1* in TO cells infected with different SPDV isolates. Viral RNA expressed as relative to reference housekeeping gene (*elf*). Data represent mean values \pm SE ($N = 3$). Note a group of higher responders including isolates 4640, 4639 and 4638. Peak expression of isolate 4640 (red) at 7 days post infection represents a 3325 fold increase relative to day 1.

Source: MSS internal reports (Collet B. 2010: Comparative analysis of five Salmon Alphavirus isolates in TO, an Atlantic salmon macrophage-like cell line).

Attendance and presentation of work related to this thesis along the period of study:

-15th International conference on Diseases of Fish and Shellfish of the European Association of Fish Pathologists (EAFF) Split, Croatia 2011 (poster) on initial development of SCPCs.

-Tri Nation PD meeting 2012 (oral presentation) Characterization of the model.

-16th International conference on Diseases of Fish and Shellfish of the European Association of Fish Pathologists (EAFF) Tampere , Finland 2013 (oral paper) permissiveness of infection of SCPCs.

- 9th International Conference on Viruses of Lower Vertebrates, Malaga, Spain 2014 (oral paper) application as in medium term trial, viral and *mx* gene expression. Selected as one of the 12 best scientific presentations.

-University of Edinburgh Student day 2015 (poster), Runner up price.

- 18th International conference on Diseases of Fish and Shellfish of the European Association of Fish Pathologists (EAFF) Belfast, Dublin 2017 (oral paper) use of the model for embryo genotype assessment.

Additional papers

A) *A method to measure an indicator of viraemia in Atlantic salmon using a reporter cell line*, Bertrand Collet, Katy Urquhart, Patricia Noguera, Katrine H. Larsen, Katherine Lester, David Smail, David Bruno.

B) *Identification of a wild reservoir of Salmonid alphavirus in common dab *Limanda limanda*, with emphasis on virus culture and sequencing*, D. W. Bruno, P. A. Noguera, J. Black, W. Murray, D. J. Macqueen, I. Matejusova.



A method to measure an indicator of viraemia in Atlantic salmon using a reporter cell line

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ABSTRACT

Article history:

Received 6 December 2012
Received in revised form 26 March 2013
Accepted 10 April 2013
Available online 18 April 2013

Keywords:

SAV
Atlantic salmon
Interferon
RTG-P1
Viral replication
Rainbow trout
Viral evasion
Luciferase
Viral diagnostic

RTG-P1 is a transgenic fish cell line producing luciferase under the control of the IFN-induced Mx rainbow trout gene promoter. This cell line was used to measure viraemia of Salmonid alphavirus (SAV), the cause of Salmon Pancreas Disease (SPD), a serious disease in farmed Atlantic salmon. Two SAV genotype 1 (SAV1) isolates were used in this study, F93-125 (tissue-culture adapted, from Ireland) and 4640 (from a field case in Scotland). The kinetics and magnitude of luciferase activity were monitored versus the time of infection. During a direct infection experiment, the induction of luciferase significantly increased 16- and 4-fold after incubation for 6 days with F93-125 at 15 and 20 °C, respectively. Filtration and heat treatment experiments demonstrated that the luciferase induction in RTG-P1 was dependent on viral replication. Unlike many cell lines used in fish viral diagnostic, RTG-P1 is not sensitive to salmonid serum, therefore, viraemia could be successfully monitored on serum collected from fish during a cohabitation challenge with 4640 isolate. A peak of viraemia could be detected 16 days post IP inoculation of the shedders. This novel cost-effective method to measure viraemia does not rely on development of cytopathic effect (CPE) in culture, is compatible with non-lethal blood collections in fish and can be used to assign emerging diseases to a viral aetiology.

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1. Introduction

Pancreas disease (PD) in farmed Atlantic salmon (*Salmo salar* L.) was recognised in Scotland in 1976 (Munro et al., 1984; McVicar, 1987; Ferguson et al., 1986; Rodger et al., 1994), and has continued to cause serious economic losses in Europe. The disease is caused by Salmonid AlphaVirus (SAV) of genotypes 1–6 (Weston et al., 2005; Graham et al., 2008). Pathology is characterised by intense necrosis of the pancreatic acinar tissue (Munro et al., 1984), as well as lesions in the heart and muscle (McLoughlin et al., 2002). Fish that survive a SAV infection, develop a strong and long lasting immunity to the disease (Houghton and Ellis, 1996). Following exposure to SAV, neutralising antibodies have been detected in salmonids (Graham et al., 2006) and induction of immune genes has been investigated (Herath et al., 2012).

Teleost fish are armed with an innate antiviral defense system based on interferon (IFN) production (Ellis, 2001), which represents the first line of defense against viral infective agents such as Infectious Pancreatic Necrosis Virus (IPNV) and Infectious Haematopoietic Necrosis Virus (IHNV) (Robertson, 2006). Interferon molecules are typically secreted by cells in response to a viral

infection. A rapid signalling pathway induces the expression of a number of proteins, including Mx, with direct and indirect antiviral properties (Collet and Secombes, 2002). There are three classes of IFNs; type I/III (α/β IFN) produced by most nucleated cell types, and type II (γ IFN) produced by more specialised immune cells. In higher vertebrates, type II IFN is generally accepted to be part of the adaptive response, whereas type I/III IFN is a mediator of the innate response. Most viruses are equipped with a diverse array of strategies to evade this powerful early cellular defense mechanism (Katze et al., 2002), often involving specially encoded proteins such as the non-structural (NS) protein of influenza A virus. In fish, it has been well demonstrated that type I IFN can rapidly induce expression of Mx which has been shown to have direct antiviral activity against IPNV or SAV (Larsen et al., 2004; Lester et al., 2012).

In 2003, a transgenic rainbow trout cell line based on the Rainbow Trout Gonadic cell line (RTG-2) was generated and submitted to the ATCC collection (Collet et al., 2004). This cell line expresses the firefly Luciferase gene under the control of a Mx regulatory sequence (Collet and Secombes, 2001). It was characterised with artificial viral mimics such as the double stranded molecule polyinosinic:polycytidylic acid (poly I:C) but very little work has been carried out with live viruses besides studies on pathogen-host interactions in salmonids (Collet et al., 2007). The purpose of the present work is to develop a method to measure SAV viraemia in fish.

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2. Material and methods

2.1. Virus isolates

Two SAV genotype 1 (SAV1) isolates were used in the current study, SAV1-F93-125 is tissue culture-adapted (Weston et al., 2005; Fringuelli et al., 2008; Matejusova et al., 2013) and was used for *in vitro* studies, while SAV1-4640 (SCO07-887) was isolated from a farmed Atlantic salmon on the west of Scotland (Snow et al., 2011; Matejusova et al., 2013) and was used for the *in vivo* experiment. The two isolates, referred as to F93 and 4640 in the following text, were propagated in chinook salmon embryo cells (CHSE-214) at 15 °C and were at 11th and 4th passage, respectively. Infections were performed using 2.79×10^8 TCID₅₀/ml for F93 (*in vitro* experiments) and 6.25×10^5 TCID₅₀/ml for 4640 (*in vivo* experiment).

2.2. RTG-P1 and luciferase assay

The rainbow trout transgenic RTG-P1 cell line (ATCC CRL 2829, Collet et al., 2004) was propagated in Leibovitz L-15 supplemented with 10% foetal bovine serum (FBS, PAA Laboratories, Pasching, Austria), 1% 200 mM L-Glutamine (Life Technologies, Paisley, UK), 0.5 mg/ml G418 (Sigma, Irvine, UK). Stock cultures were cultivated in 75 cm² flasks (Greiner Bio-One Ltd, Stonehouse, UK) at 23 °C. When the culture reached approximately 80% confluency and 24 h prior to the assay, the cells were detached with Trypsin-EDTA (Sigma, Irvine, UK) and seeded at a density of 10^5 ($\pm 10\%$) cells/cm² in 12-48- or 96-well plates (Greiner Bio-One Ltd, Stonehouse, UK) for the *in vitro* infection experiments, serum viraemia assay, or titration comparison, respectively. The cells were left to attach for 24 h before inoculation with SAV or serum. Serum showing strong RTG-P1 inducibility was inactivated by heating at 95 °C for 2 min and the inducibility measured again.

The medium was drained from the plate and 75 or 50 μ l of SteadyGlo Luciferase substrate (Promega, Southampton, UK) was added per well on a 12/48- or 96-well plate, respectively. The light emission was measured immediately over 10 s using a Wallac Victor³ 1420 Multilabel counter set up for luminescence (Perkin Elmer, Cambridge, UK) and the Relative Light Units (RLU) recorded. For each well containing infected cells, luciferase inducibility was calculated as the RLU value divided by the average RLU value of the triplicate uninfected or un-stimulated control cells. This will be referred as "inducibility" throughout the following text.

2.3. Experimental infection

Two hundred and sixteen (216) mixed-sex Atlantic salmon *Salmo salar* smolts weighing 200 ± 10 g were acclimatised in seawater at 14 °C for 2 wk prior the experiment at VESO Vikan (Norway). The stock population was tested for absence of known fish pathogens. Fish were randomly divided into 3 groups of 72 fish and assigned to 3 separate tanks holding 500 l, 2 replicates and one control, and maintained with a flow rate of 10.8 l/min with oxygen saturation of 70%. Fish were fed to appetite throughout, however food was withdrawn 12–24 h before handling. Thirty two fish from each tank were anaesthetised in a solution of tricaine methanesulphonate (MS222 Sigma, Irvine, UK) and tagged for easier identification prior to intraperitoneal injection of 200 μ l of 1.25×10^5 TCID₅₀ SAV1 4640 (2 tanks) or 200 μ l of tissue culture media for the control fish. These fish constituted the "shedders" for infection of the 40 cohabitant individuals. From each tank, four of the latter were sampled at 5, 8, 12, 16, 21, 30, 45 and 60 days post infection. All sampled fish were killed by overdose of anaesthesia and bled immediately into vacuainers containing sodium citrate (Greiner). Blood sample were stored overnight at

+4 °C before centrifuged at $3000 \times g$ for 10 min, sera removed and stored at –80 °C until processed.

2.4. Filtration experiment

RTG-P1 cells were seeded on 2×12 -well plates. The F93 inoculum *i.e.* the virus-containing supernatant was passed through a Microcon 100 kDa (Millipore, Watford, UK) column. The retentate is retained in approx. 50 μ l of medium after centrifugation. The volume of the retentate was adjusted to the same volume as the filtrate (500 μ l) by dilution with MEM (Life Technologies, Paisley, UK). One hundred (100) μ l of the inoculum prior to filtration, filtrate or retentate was added to triplicate wells (2 ml culture medium) and the luciferase assay was carried out after 6 days of incubation at 15 or 20 °C, as described above. In parallel, 100 μ l culture medium prior to filtration, filtrate or retentate obtained as described above was added to triplicate wells as uninfected controls.

2.5. Comparison between RTG-P1 inducibility test and end-point dilution titration

RTG-P1 and CHSE cells were seeded on to a 96-well Plate 24 h before use. F93 inoculum or fish serum (F64, F66, F69, or F70) was diluted serially 10-fold in RTG-P1 or CHSE culture media and 10 μ l was added to a monolayer of RTG-P1 or CHSE cells. The luciferase assays was performed 7 days later on the RTG-P1 plates whereas the CHSE plates were read for cytopathic effect under inverted microscope 14 days after.

2.6. Statistical analysis

Data were analysed using Minitab software package for Windows (Minitab, Inc.). The effect of time was tested by one-way analysis of variance and level of expression at a given time point was compared to the initial time point by Tukey's multiple comparisons.

3. Results

The Luciferase activity in the RTG-P1 uninfected control increased from 4.3 ± 0.4 to 18.5 ± 0.6 over the 12 days of sampling, reflecting the propagation of the cells and the intracellular accumulation of luciferase. There was a significant increase in the luciferase inducibility after infection with F93 at days 3, 5, 6, 8, 10 and 12 and 3–10 at 15 and 20 °C, respectively (Fig. 1). The luciferase inducibility

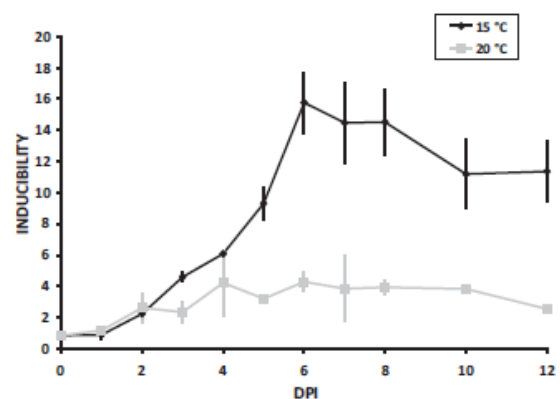


Fig. 1. Luciferase activity in RTG-P1 following infection with F93 isolate at 15 or 20 °C. Data represents the average inducibility (fold-increase compared to controlled sampled at the same time, $N = 3$) \pm STDEV.

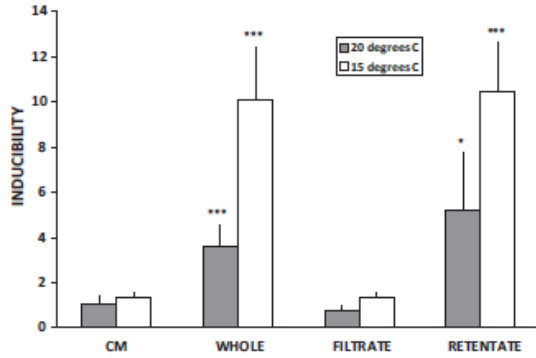


Fig. 2. Luciferase activity in RTG-P1 incubated for 7 days with F93 or culture medium unfiltered (whole), Filtrate or Retentate after passage onto a microcon 100kDa cartridge at 15 or 20 °C. Data represents the average inducibility (fold-increase compared to control sampled at the same time, $N=3$) \pm STDEV. Statistical significance of comparison between fractions and CM control is as follows: * $p < 0.05$; *** $p < 0.001$.

following infection with the F93 isolate peaked at day 6 and the maximal induction was 15.8 ± 0.2 at 15 °C. Similarly, at 20 °C, the inducibility was maximal at day 6 but was only 4.29 ± 0.68 (Fig. 1).

Viral particles were separated from molecules smaller than 100 kDa by filtration. The inducibility values above the negative control were 99.0% and 98.5% retained by the filter after 7 days incubation at 15 and 20 °C, respectively (Fig. 2). The filtrate did not significantly stimulate the cells compared to the culture medium control after 7 days incubation period. Following an incubation period of 24 h, the filtrate fractions were unable to significantly stimulate RTG-P1 over a period of 24 h neither at 15 °C not at 20 °C (data not shown). The inducibility values were 3.60 ± 0.53 , 5.22 ± 1.45 , 10.10 ± 1.34 , 10.44 ± 1.26 in the whole inoculum and retentate, at 20 and 15 °C, respectively (Fig. 2).

A comparison between the luciferase inducibility in RTG-P1 during 7 days incubation and the traditional end-point dilution titration method over a 14 days incubation period was carried out. The same 96-well plate format and dilutions of the F93 isolate inoculum were used (Fig. 3). Complete CPE in CHSE could be observed up to a 10^6 dilution whereas partial CPE (83.3%) could be detected at dilution 10^7 . In RTG-P1, significant increase in luciferase activity was detected up to a dilution of 10^4 (Fig. 3).

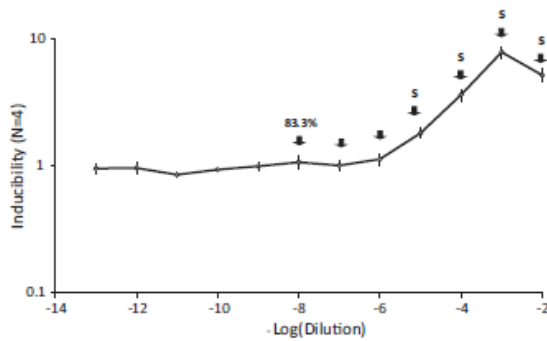


Fig. 3. Luciferase activity in RTG-P1 infected for 7 days with dilutions of F93 isolates. Arrows indicate CPE using the end-point titration method in CHSE performed in parallel after 14 days incubation. Data represents the average inducibility (fold-increase compared to controlled sampled at the same time, $N=4$) \pm STDEV. The symbol "s" indicates an inducibility significantly higher than 1 ($p < 0.01$).

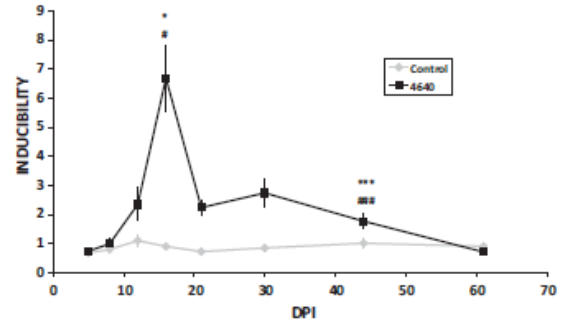


Fig. 4. Luciferase inducibility of RTG-P1 after incubation with serum from Atlantic salmon infected with 4640 isolates or mock-infected. Data represent average inducibility from 4 individuals (Control) or 8 individuals (4640) \pm STDEV. Statistical significance is as follows: comparison at given DPI between 4640 and CONT (* $p < 0.05$; *** $p < 0.001$) or between 4640 on given DPI and 4640 at DPI 5 (* $p < 0.05$; *** $p < 0.001$).

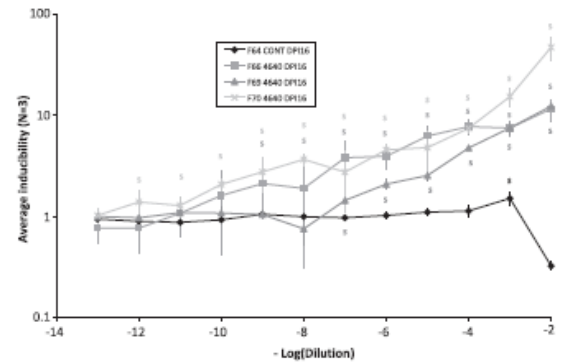


Fig. 5. Luciferase inducibility of RTG-P1 after incubation with serial dilutions of serum from Atlantic salmon infected with 4640 isolates (F66, F69 and F70) or mock infected (F64) collected 16 days after infection. Data represent the average inducibility ($N=3$). The symbol "s" indicates an inducibility significantly higher than 1 ($p < 0.01$).

After incubation of RTG-P1 cell with serum from fish infected with SAV1 4640, the luciferase inducibility peaked at day 16 post-infection giving an inducibility of 6.69 ± 1.11 . The values then dropped to levels not significantly different from serum from uninfected fish (Fig. 4). Heat inactivation of the serum taken from one infected fish at day 16 completely inhibited the inducibility from 8.63 ± 0.24 to 1.17 ± 0.06 , not significantly different from the negative control (Data not shown). Serial dilutions of serum from 3 infected fish and a control fish collected 16 days after infection were applied on the RTG-P1 cells. The inducibility obtained was significantly higher than the cell control (RTG-P1 incubated with medium) for F70 up to a dilution of 10^{12} (Fig. 5). Serum from the mock-infected fish showed a decreased of luciferase activity at the lowest dilution (10) suggesting serum cytotoxicity. The end-point titration after 14 days of incubation on CHSE cells was of the same samples showed CPE up to the dilutions 10^7 , 10^{13} , 10^8 and 10^{13} for the fish F64, F66, F69 and F70, respectively.

4. Discussion

This study investigated the use of the transgenic reporter fish cell line based on the Mx gene promoter (RTG-P1) to measure viraemia in serum as an alternative method to traditional cell-based

viral diagnostic. This is the first time that a transgenic fish cell line is used to detect the presence of viral pathogens prior to the development of cytopathic effect (CPE) (Olivo, 1996; Leland and Ginocchio, 2007). This novel method is currently the only method available to detect the presence of infectious viral particles in plasma or serum without any issue of cytotoxicity or lack of CPE in available diagnostic fish cell lines. Two well characterised SAV isolates were used in this study, the cell culture adapted F93 isolate (Weston et al., 2005) and a field isolate 4640 replicated in cell culture at low passage number (Snow et al., 2010). It is well documented that IFN activity is directly linked to viral replication through mechanism of detection of intracellular Replicative Intermediate (RI) double-stranded RNA (dsRNA) (Der and Lau, 1995; Matsumoto and Seya, 2008). Present results suggest that the induction of luciferase in RTG-P1 is dependent on viral replication.

The method is extremely easy to implement. The cells are seeded on 48-well plates and the supernatant containing the virus is added directly to the well 24 h later. A week later, the medium is quickly drained, the luciferase substrate added and the light emission read immediately in a single step procedure making the method cost effective. Virus titre is traditionally measured by end-point dilution assay on a permissive cell line. A common problem with some fish viruses is the lack of permissive cell lines making the dilution assay time consuming and the detection of CPE difficult and subject to interpretation. An important aspect of virulence studies is to focus investigations on isolates responsible for field outbreaks where cell-culture adaptation has been kept to a minimum i.e. no more than 10 passages (Bernoth, 1998). These types of isolates can be the most difficult to grow in available cell lines and making the present pre-CPE viral detection method particularly beneficial. In addition, it is often problematic to measure viraemia because of serum or plasma cytotoxicity (Fryer et al., 1965). Atlantic salmon serum or plasma is cytotoxic to CHSE, a fish cell lines widely used for viral diagnostic but not to RTG-P1.

In the present study, viraemia was found maximal 16 days after infection in a cohabitation experimental infection with a SAV subtype 1 isolate. In a similar challenge model, maximal viral genetic material load was found 3 weeks after infection with SAV of the same subtype in heart, gill, mucus and faeces (Graham et al., 2011), suggesting an early viraemia followed by infection of peripheral tissues. Interestingly, after a needle challenge using SAV2, the viraemia was also shown to be maximal 16 days after infection (Desvignes et al., 2002).

The traditional end-point dilution titration method with calculation interpolation of 50% endpoint (Reed and Muench, 1938), is normally based on the visual assessment of lytic CPE occurrence. Although less sensitive when using viral inoculum, the RTG-P1 inducibility method is objective and less time consuming. In the case of serum samples, the sensitivity of the RTG-P1 method is similar to the CPE method but the latter cannot be used reliably due to the cytotoxicity of serum for the cells used for viral diagnostic such as CHSE. In the present study, for comparison purpose the RTG-P1 test was carried out in the same format (96-well plate) as the end point titration. In some cases certain isolates fail to induce visible CPE in cell lines routinely used for diagnostic purposes (Graham et al., 2008) even if they are known to induce pathological development *in vivo*. The inability of some SAV isolates to reproduce a lethal *in vivo* infection in the laboratory (McLoughlin and Graham, 2007) makes the evaluation of virulence of different isolates very difficult. The use of RTG-P1 is based on the early induction of IFN in living cells and does not rely on the development and visual assessment of a cytopathic effect. In some cases, when CPE does not occur, an Indirect Fluorescent Antibody Test (IFAT) can be used as an alternative but the method is more laborious, time consuming compared to the cellular assay and it relies on the availability of a good antibody reagents. More routinely immuno-peroxidase staining is used

as a detection method (Schmidt et al., 1981) and recently, a novel method aiming at simplifying the titration method was described (Borisovich et al., 2008). However, these latter methods all require monoclonal antibodies reagent that are not always available for fish viruses.

SAV infection gave the highest response at 15 °C, close to the temperature of natural infection. Replication of alphaviruses such as Semliki Forest Virus (SFV) or Sindbis Virus generates partly intracellular dsRNA RI through the activity of RNA polymerase encoded by the nonstructural protein (nsP4) (Kääriäinen and Ahola, 2002). While the production of dsRNA in salmon cells infected by salmon alphaviruses has not been demonstrated we can reasonably assume that the life cycle of this class of viruses is well conserved. Therefore, the amount of RI in cells infected by SAV would be a good indicator of the replication activity. Every nucleated host cell is equipped with double stranded RNA receptors in different compartments. Intracellular dsRNA RI molecules are detected by the RIG-I/MDA5 pathway (Kawai and Akira, 2006) and/or the PKR pathway. The activation of the RIG-I/MDA5 and TLR3 pathways result in the phosphorylation, heterodimerisation and nuclear translocation of Interferon Regulatory factor (IRF)-3 and -7 molecules leading to the activation of the promoter of the α/β IFN genes (Kawai and Akira, 2006). In fish, functional genes encoding for TLR3, RIG-I, PKR and MDA5 have been isolated (Chang et al., 2011) suggesting that in RTG-P1 the mechanisms of IFN gene induction is similar to what is described for humans. The optimal response to the virus was seen at 15 °C and was considerably reduced at 20 °C suggesting that the temperature spectrum of SAV replication is narrow. This is in agreement with Graham et al. (2008) where TO cells infected by a SAV genotype 1 isolate at different temperatures only showed replication at 15 °C. Indeed, the production of dsRNA molecules directly depends on the activity of nsP4, encoded by the viral genome that is temperature dependent (Keränen and Kaariainen, 1979). As RTG-P1 itself can respond directly to salmonid IFN, it is crucial to distinguish between luciferase induction due to viral replication and to IFN molecules present in the SAV inoculum as it was propagated on CHSE-214, a salmonid cell line. Most of the IFN activity after 6 days of infection is due to molecules or particles with a molecular weight greater than 100 kDa. This demonstrates that the induction of the Mx promoter in RTG-P1 is due to viral particles and not to free interferon molecules that may have accumulated in the supernatant. Further evidence is given by the inability of the supernatant, unlike poly I:C, to stimulate fresh RTG-P1 over a short incubation time (24 h) suggesting that no detectable IFN activity is present in the supernatant. The ability to differentiate the stimulation of RTG-P1 by viral infection or by autocrine or paracrine IFN simply by the time of incubation make this system valuable in evaluating the replication potential of an isolate in a fibroblastic cell line. Other evidence is the ability of supernatant collected after 7 days of SAV infection in a salmonid cell line (CHSE-214) or in a non-salmonid cell line (BF-2) to stimulate RTG-P1 after 7d incubation (data not shown). Fish IFN being species-specific (Robertson, 2006) it cross-reacts within salmonids (CHSE/RTG-P1) but not between a centrarchid and salmonid species (BF-2/RTG-P1). The 7 day luciferase induction in both cases demonstrate that this is due to viral infection and not direct action of IFN molecules present in the inoculum. In addition, heat treatment completely inhibited the RTG-P1 induction demonstrating that this induction is dependent on viral replication and not on free interferon molecules or soluble RI that would not be destroyed by heat treatment.

It is not clear whether SAV infection is capable of inducing RTG-P1 to secrete a large amount of functional interferon molecules in the supernatant. In the present experiments, almost all the inducing agent is higher than 100 kDa and therefore free from interferon molecules, approx. 20 kDa in size. These results suggest that either SAV induces the Mx promoter directly without a secretion and

autocrine action of interferon or interferon is present as a complex in the culture medium with an apparent molecular weight greater than 100 kDa. The culture medium consists of 10% foetal bovine serum that contains potential transport proteins (BSA).

5. Conclusions

The method presented in this study is designed for measurement of viraemia using pre-CPE detection in a fish transgenic cell line. The example of SAV was used but preliminary data have shown that it may be also possible to detect viraemia from serum taken from Viral Haemorrhagic Septicaemia Virus (VHSV), Spring Viraemia of Carp Virus (SVCV) or Infectious Salmon Anaemia Virus (ISAV)-infected animals. The small volume of material required makes it compatible with non lethal sampling. In addition, this method does not rely on the visible observation of CPE nor on the genomic information required for virus identification making this method well suitable for the detection of unknown viral agents responsible of emerging diseases.

Acknowledgements

KHL benefited from the British Council within the International Association for the Exchange of Students for Technical Experience (IAESTE). The serum samples originated from an experimental infection funded by the transnational access activity of the EU FP7 network NADIR carried out in VESO, Viken, Norway. The authors are grateful to Anne Ramstad and Mari-Ann Finnanger from VESO for their help with the experimental infection. Part of the work is funded by the National Centre for the Replacement, Refinement and Reduction of Animals in research (NC3Rs, grant G1100675).

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Identification of a wild reservoir of salmonid alphavirus in common dab *Limanda limanda*, with emphasis on virus culture and sequencing

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ABSTRACT: Common dab *Limanda limanda* from Scottish and international waters were examined by quantitative real-time RT-qPCR for evidence of viral RNA consistent with salmonid alphaviruses (SAV). SAV prevalence in heart tissue varied between sampling sites and reached up to 17% in fish collected near the Shetland Islands, Scotland. Raw Ct values ranging from 22.31 to 39.45 were obtained from the SAV-positive tissue material using the nsP1 RT-qPCR assay. Bayesian-, likelihood- and distance-based phylogenetic analyses performed with the amplified partial E2 gene sequence dataset suggest that the dab-derived virus belongs to SAV Subtypes I, II and V. A single SAV subtype was identified from the majority of sampling sites, apart from Shetland, where Subtypes II and V were also identified. The presence of SAV RNA from common dab in regions detached from salmon aquaculture lends support to the hypothesis that common dab are bone fide wild reservoirs of SAV, independent of fish farming activity. There was no link between the occurrence of viral RNA, length and sex of the dab, water depth, or health status as recorded using the International Council for the Exploration of the Sea (ICES) guidelines. In addition, the histological changes recorded in dab could not, with certainty, be attributed to infection with SAV. Finally, and for the first time, this study demonstrated that the dab-derived SAV Subtype V virus could be successfully cultured in a salmonid cell line.

KEY WORDS: Common dab · Salmonid alphavirus · SAV · Wild fish · Virus culture

INTRODUCTION

Pancreas disease (PD) was first recorded in Scotland from farmed Atlantic salmon *Salmo salar* in 1976 (Munro et al. 1984). A similar condition termed 'sleeping disease' (SD) was subsequently described from freshwater-reared rainbow trout *Oncorhynchus mykiss* (Boucher & Baudin-Laurencin 1996). The 6 salmonid alphavirus (SAV) subtypes described (including SAV2/SDV) belong to 1 viral species. The name of the virus is salmon pancreas disease virus (SPDV) (Togaviridae), commonly called SAV. The 6 SAV subtypes have been distinguished using phylo-

genetic analysis with partial E2 and nsP3-gene sequence data (Fringuelli et al. 2008), providing evidence that some subtypes are dominant in certain geographical regions. For example, SAV II is the dominant subtype from Atlantic salmon reared in the Shetland Isles, whereas salmon from the northeast of Scotland and the Western Isles show Subtype V to be dominant (Graham et al. 2012). However, SAV II strains can now be found in saltwater (Graham et al. 2012), and, similarly, SAV I strains occur in freshwater salmonids (Lester et al. 2011). Salmonid alphaviruses do not require an arthropod vector to complete their life cycle, which is unusual compared to mammalian

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alphaviruses. Considerable existing data suggest that horizontal transmission represents an important route of spread of PD and that infection pressure has a strong effect on the probability of recording an outbreak in a single cohort (McLoughlin et al. 1996, Kristoffersen et al. 2009, Viljugrein et al. 2009). Jansen et al. (2010) reported that if infection occurs in a population, disease is highly likely to develop, assuming fish spend an adequate time in that environment. Through experimental studies, SAV has been shown to survive for 5.7 d at 10°C in sterile saltwater with organic loading (Graham et al. 2007). Moreover, viral RNA has been shown to persist in tissues for extended periods (Andersen et al. 2007, Christie et al. 2007, Graham et al. 2010, Jansen et al. 2010), potentially posing an infection risk to healthy fish.

Despite fallowing, re-stocking of salmonids at farms has resulted in re-infection (McLoughlin et al. 2003), suggesting that natural reservoirs of this virus may exist. This is supported by Karlsen et al. (2014) who suggested all subtypes diverged prior to the introduction of rainbow trout to Europe and, therefore, existed in a wild reservoir. A previous study using RT-qPCR detected a potential wild reservoir of SAV in several flatfish species, but failed to provide follow-up evidence in support of this result, e.g. successful isolation of the virus (Snow et al. 2010). The objectives of the current study were (1) to determine the prevalence of SAV subtypes in common dab from Scottish and international waters, (2) to culture any identified SAVs *in vitro* and (3) to examine common dab tissues from selected sampling areas for the presence of histopathological changes consistent with pancreas disease.

MATERIALS AND METHODS

Collection of wild common dab

Common dab *Limanda limanda* were collected from Cragmylie, Garron and Back o Garron (Stonehaven Bay, Kincardineshire, Scotland) (Fig. 1) using the MRV 'Temora' (10 m Blyth catamaran) between January 2009 and June 2010. Fish were collected using a demersal trawl, towed for short periods in 12 to 36 m of water across the 3 areas. The sampling plan, with respect to number of fish, was designed to achieve a 95 to 99% chance of detecting 1 or more infected dab per site. The fish were kept alive in a covered tank supplied with ambient temperature seawater for a maximum of 4 h before transportation from Stonehaven Bay to the laboratory (a distance of

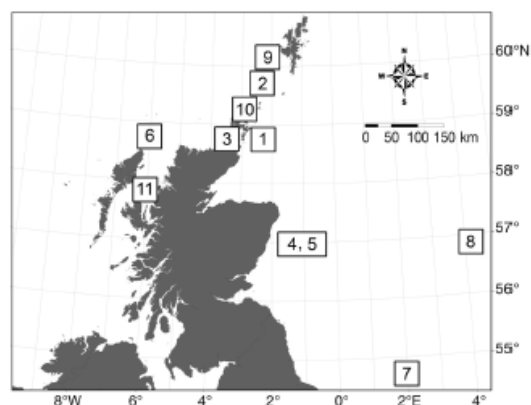


Fig. 1. Location of sampling sites (1–11) around Scotland

~24 km) in bins supplied with pumped air or oxygen. The fish were transferred to tanks that received natural seawater, which was mechanically filtered and ultraviolet irradiated, they were held in a bio-secure area where no infection studies were being carried out, and were examined within 4 d of arrival as detailed in the following subsection.

Common dab were also fished with a bottom trawl net for 30 min using the RV 'Scotia'. The same statistical approach was implemented for designing the sampling plan. Fishing was conducted in 7 areas around the Scottish coastline and in 2 areas in the northern North Sea, including international waters. The nominal centre of each trawl area is presented in Fig. 1. Common dab were laid flat in a blast freezer on board the vessel, and each fish was then individually bagged and labelled. The fish were returned to the laboratory and stored at -80°C until processed.

Tissue sampling

For molecular investigations, tissues from all fish (heart, kidney, brain and skeletal muscle) were placed into individually labelled cryotubes containing RNAlater (VWR International) and stored at -80°C. The samples of skeletal muscle did not include the scale layer. For virus isolation, heart and kidney tissues from individual dab were taken, stored in cryotubes and snap frozen at -80°C. For histopathological investigation, fish from Stonehaven Bay were examined following terminal anaesthetization, using tricaine methane sulphonate (MSS, Sigma) at 0.2 g l⁻¹, whereas fish frozen at other loca-

tions were allowed to thaw in the laboratory but were dissected while partially frozen. Signs attributed to epidermal hyperplasia/papilloma, lymphocystis, and acute and healing skin ulcers were recorded from all dab and prepared according to ICES standard quality assurance procedures (Bucke et al. 1996, Lang 2002). Hyperpigmentation on the ocular side was recorded according to the categories of the Biological Effects Quality Assurance Monitoring Programme (BEQALM): 1 being least severe, 2 being moderately severe and 3 being most severe. Total length (measured to the nearest centimetre below) was recorded for each dab (both frozen and freshly killed), and sex was noted externally from the shape of the gonad.

Tissues (skin including musculature, gills, heart, kidney, spleen, liver, pancreas, gut) were removed from anaesthetised fish (Stonehaven area only), fixed in 10% buffered neutral formalin solution for a minimum of 24 h and embedded in paraffin, and sections were stained with haematoxylin and eosin. A minimum of 30 fish from each of the 2 locations at Stonehaven Bay, including fish shown to be SAV positive by PCR, were examined histologically. Sections were reviewed without knowledge of the PCR results, and all tissues were assessed with a particular focus on pancreas, heart and red and white skeletal muscle, as these are the tissues most severely affected during SAV infection in salmonids (Christie et al. 2007, McLoughlin & Graham 2007). Histological changes attributed to PD include a severe loss of exocrine pancreatic tissue combined with chronic myositis, with red and white skeletal myocytic degeneration and hypercellularity within the heart (McVicar 1987, McLoughlin et al. 1996, Murphy et al. 1992). A SAV infection in dab, however, may not necessarily cause histological changes in the same tissues as those noted for salmon.

SAV RT-qPCR and sequencing

Several studies have shown heart tissue to be one of the most appropriate tissues for detecting SAV during all stages of infection (also persistent infections) (Graham et al. 2006, Christie et al. 2007, Andersen et al. 2007); for this reason, only heart tissue was processed for molecular testing. Approximately 5 mg of homogenised tissue was used for extraction of total RNA (MagAttract M48 RNA Tissue Kit, Qiagen) using the M48 BioRobot (Qiagen). Total RNA was eluted in a final volume of 100 µl. cDNA synthesis was performed using the Taqman Reverse Transcription Reagent Kit and random hexamers (Applied Biosystems) accord-

ing to Snow et al. (2010). For detection of the SAV target in heart tissue an nsP1 RT-qPCR assay was used, with a cut off Ct value of 39.3 (see Hodneland & Endresen 2006 for details on assay validation). RT-qPCR reactions were run on an ABI Prism 7000 detection system, using cycling conditions and the reaction set up according to Snow et al. (2010). Negative controls were routinely included at the stages of extraction, reverse transcription and RT-qPCR. RT-qPCR was performed in triplicate from each sample, with positives only being recorded where a raw Ct value below the assay cut off point was generated from all 3 independent reactions.

Partial E2 gene amplification was conducted in a final volume of 50 µl with 1 µl of cDNA tested SAV positive by RT-qPCR (as described above) according to Fringuelli et al. (2008). For cDNA exhibiting nsP1 RT-qPCR Ct values >33, a nested PCR was performed using the primers E2N Forward (5' AGG CCA CTG GCC ACT ACA 3') and E2N Reverse (5' GGA AAC CAA GGT TCC GTG 3') and 35 cycles of 94°C for 20 s, 50°C for 20 s and 72°C for 50 s, followed by second-round amplification according to Fringuelli et al. (2008). PCR product was either purified directly using ExoSAP IT (GE Healthcare) or excised from agarose gel and purified using MinElute (Qiagen). Approximately 10 ng of purified product was sequenced (GenomeLab DTCS Quick Start Kit, Beckman Coulter) in both directions on an automated CEQ8800 DNA sequencer (Beckman Coulter) using primers according to Fringuelli et al. (2008). Sequences were analysed using Sequencher software (Gene Codes).

Sequence and phylogenetic analyses

A single representative of each redundant dab E2 sequence type was retained for sequence analysis, which initially involved *blastn* searches (Altschul et al. 1990) against the non-redundant NCBI nucleotide database. The final dataset covered 55 sequences spanning 350 bp (Table 1), which was aligned using Clustal W (Thompson et al. 1994) within BioEdit Version 7.0.5.3 (Hall 1999). An established statistical test (Xia et al. 2003) performed in DAMBE V. 5.3 (Xia 2013) demonstrated an absence of mutational saturation for all codon positions. Prior to tree-building, the alignment was loaded into Mega 5.0 (Tamura et al. 2011) to identify the best-fitting model of nucleotide substitution by maximum likelihood (ML), which was K80+G (i.e. Kimura 1980, modelled assuming a gamma distribution of among-site rate variation).

Table 1. Details of salmonid alphavirus isolates from which a partial E2 gene sequence was used in the present study. AS: Atlantic salmon; RT: rainbow trout

Subtype	Virus	Isolation year	Country of origin	Host species	Accession number	
I	IRE F93125	1993	Ireland	AS	AJ316244	
	IRE F03123	2003	Ireland	AS	EF675548	
	SCO 08494	2008	Scotland	AS	KJ513287 ^a	
	IRE F02194	2002	Ireland	AS	EF675552	
	SCO 07887	2007	Scotland	AS	KJ513284 ^a	
	IRE F06182	2006	Ireland	AS	EF675549	
	SCO 10682	2010	Scotland	Dab	KJ513276 ^a	
II	FR S49P	1995	France	RT	AJ316246	
	FR EE37		France	RT	EF675578	
	NO SAV2011A	2011	Norway	AS	HE863662	
	FR	1999	France	RT	AJ238578	
	IT F04198	2004	Italy	RT	EF675590	
	SPA F0408	2004	Spain	RT	EF675587	
	SCO 02798	2002	Scotland	RT	KJ513286 ^a	
	ENG F04212	2004	England	RT	EF675585	
	SCO 031201	2003	Scotland	AS	KJ513285 ^a	
	SCO 09958	2009	Scotland	AS	KJ513283 ^a	
	SCO 10684/51	2010	Scotland	Dab	KJ513277 ^a	
	III	NO SAVR31/04	2004	Norway	AS	DQ122146
		NO SAVH05/01	2001	Norway	AS	DQ122141
NO SAVH21/03		2003	Norway	AS	AY604238	
NO SAVF29/03		2003	Norway	AS	DQ122127	
NO SAVT09/10/2		2009	Norway	AS	HM208123	
NO SAVH06/1/1		2006	Norway	AS	HM208094	
NO SAVH20/03		2003	Norway	AS	AY604235	
NO SAVT09/10/1		2009	Norway	AS	HM208122	
NO SAVF07/11/2		2007	Norway	AS	HM208092	
NO SAVF07/11/1		2007	Norway	AS	HM208091	
NO SAVH07/2/2		2007	Norway	AS	HM208098	
IV	SCO F06139	2006	Scotland	AS	EF675569	
	SCO F03209	2003	Scotland	AS	EF675565	
	IRE F91116	1991	Ireland	AS	EF675564	
	SCO F06243	2006	Scotland	AS	EF675566	
	IRE F0444	2004	Ireland	AS	EF675560	
V	SCO F0702	2007	Scotland	AS	EF675571	
	SCO F0693	2006	Scotland	AS	EF675567	
	SCO F05310	2005	Scotland	AS	EF675575	
	SCO F06267	2006	Scotland	AS	EF675574	
	SCO F0617	2006	Scotland	AS	EF675568	
	SCO 09913	2009	Scotland	Dab	KJ513288	
	SCO 10406	2010	Scotland	Dab	KJ513278 ^a	
	SCO 10407	2010	Scotland	Dab	KJ513279 ^a	
	SCO 10408	2010	Scotland	Dab	KJ513280 ^a	
	SCO 10525	2010	Scotland	Dab	KJ513281 ^a	
	SCO 10411	2010	Scotland	Dab	KJ513282 ^a	
	SCO 10684/17	2010	Scotland	Dab	KJ513267 ^a	
	SCO 10684/20	2010	Scotland	Dab	KJ513268 ^a	
	SCO 10648/27	2010	Scotland	Dab	KJ513269 ^a	
	SCO 10684/60	2010	Scotland	Dab	KJ513270 ^a	
	SCO 10684/70	2010	Scotland	Dab	KJ513271 ^a	
	SCO 10684/49	2010	Scotland	Dab	KJ513273 ^a	
SCO 10684/71	2010	Scotland	Dab	KJ513274 ^a		
SCO 10684/73	2010	Scotland	Dab	KJ513275 ^a		
SCO 10685	2010	Scotland	Dab	KJ513272 ^a		
VI	IRE F104596	1996	Ireland	AS	EF675547	

^aSequences generated in the present study

A Bayesian phylogenetic analysis was performed in BEAST V. 1.7 (Drummond et al. 2012), incorporating the best-fit substitution model, a relaxed molecular clock model (Drummond et al. 2006), a Yule Speciation prior (Gernhard 2008) and a UPGMA starting tree. The sequence data were split into 2 partitions (Codon Positions 1 & 2, separate from Position 3). The Markov chain Monte Carlo (MCMC) method was run for 100 000 000 generations, logging parameters every 1000 generations. Tracer (tree.bio.ed.ac.uk/software/tracer/) was used to confirm the convergence of the MCMC, evidenced by effective sample sizes >4000 for all parameters. A maximum clade credibility tree was generated using TreeAnnotator (Drummond et al. 2012), discarding the first 10% of trees sampled in MCMC. ML phylogenetic analysis was performed in Mega 5.0, using K80+G along with non-parametric bootstrapping (1000 replicates) to gain branch support values. A neighbour joining (NJ) analysis was also performed in Mega 5.0 using the composite ML+G model (Tamura et al. 2004), again with 1000 bootstrap replicates. Supporting neighbour joining and maximum likelihood phylogenetic analyses was performed with the 350 bp partial E2 gene sequence and are available as Fig. S1 in the Supplement at www.int-res.com/articles/suppl/q005p089_supp.pdf.

Virus culture

Heart and kidney tissue from individual dab were taken and stored in cryotubes and snap frozen at -80°C . Samples were thawed, diluted 1:5 in transport media (Liebovitz L-15, Lonza; 10% new born calf serum, Invitrogen; gentamicin at 50 mg l^{-1} ; polymyxin B at $10\,000\text{ U ml}^{-1}$, Sigma) at a final pH of 7.4 to 7.8, homogenised and clarified by centrifugation at 2000 g for 15 min. Samples were inoculated at 1:10 and 1:20 dilutions with a 3 h absorption

step onto 6-well plates containing monolayers of CHSE-214 cells (Fryer et al. 1965). The cell monolayers were at a 60 to 80% confluence, were 24 to 48 h old and were incubated at 15°C following inoculation.

Cultures were read every 7 d for appearance of cytopathic effect (CPE) and were sub-cultivated on Days 14 and 28. At Day 42, cell monolayers were scraped into RLT buffer (Qiagen) containing 0.1% β -mercaptoethanol (Sigma). The partial SAV E2 gene sequence was generated from the cell culture positive material as described above. Virus cultivation was not attempted if raw Ct values of less than approximately 36 were noted. Hence, tissue with low Ct values from SAV Subtype V positive fish were inoculated onto monolayers of CHSE-214 cells. Cultures were passed, and no CPE signs were observed.

RESULTS

Detection of SAV RNA in heart of common dab

All sampling sites, with the exception of Study Site 8 on the east coast of Scotland (Table 2, Fig. 1), revealed a positive detection of SAV in common dab *Limanda limanda*-derived RNA using the nsP1 RT-qPCR assay. The prevalence of SAV at the positive sampling sites varied between 0.8 and 16.8%

(Table 2). Generally, lower prevalence of SAV in common dab was detected on the east and southeast coasts of Scotland ($\leq 4\%$) compared to the west coast of Scotland, including the Shetland and Orkney Islands (Table 2). Negative controls applied during RNA extraction, cDNA synthesis and RT-qPCR did show detection of the pathogen target in any case.

Sequencing and phylogenetic analysis

A partial E2 gene was amplified from the SAV-positive dab from each sampled locality. Material from a minimum of 1 SAV-positive dab heart was sequenced for the partial E2 gene region, except for Sites 4 and 11 (Table 2). Three different subtypes of dab SAV were tentatively identified based on amino acid sequences: I (1 fish), II (1 fish) and V (19 fish) (Table 2). SAV nucleic acid variability among the individual study sites and within Site 9 was investigated, showing a 0.27 to 1.89% divergence in Subtype V originating from dab (Fig. 2). No amino acid substitutions resulted from the observed variation in the analysed partial E2 region.

Phylogenetic analyses using Bayesian, ML and NJ methods (Fig. 3) provided maximum statistical support for monophyletic SAV Subtypes III and II, using nomenclature introduced by Fringuelli et al. (2008).

Table 2. Prevalence of salmonid alphavirus (SAV), and summary information for common dab *Limanda limanda*. qPCR was conducted in triplicate, with positives only being considered if all 3 independent reactions showed a SAV target. Samples sequenced for the partial E2 gene are shown in **bold**. See Fig. 1 for the location of sampling sites. F: female; M: male (sex of SAV-positive fish is given in parentheses; Ct values for these individuals are given in the same order)

Sampling site	Date of collection	No. fish sampled	Male/female fish	No. of SAV-positive fish	Prevalence of SAV (%)	Ct values (average)	SAV subtype
1	Feb 10	49	18/31	3 (F/M/F)	6.1	37.82/37.77/ 34.41	V
2	Feb 10	45	12/33	3 (F/F/F)	6.7	35.52/37.43/ 36.43	V
3	Feb 10	96	67/29	5 (M/F/F/F/M)	5.2	34.3/ 27.1 /36.5/35.47/37.71	V
4	Mar 10	120	60/60	1 (F)	0.8	34.89	No sequence
5	May 10	118	11/107	1 (F)	0.8	34.74	V
6	Jul 10	77	44/33	1 (F)	1.3	36.64	V
7	Aug 10	119	60/59	1 (F)	0.8	36.43	I
8	Sep 10	120	72/48	0	0		
9	Oct 10	119	87/32	20 (F/F/M/M/F/F/F/M/F/M/M/M/M/F/M/F/F/F/M/M/M)	16.8	36.03/ 32.54 / 35.78 / 28.05 / 36.46 / 35.74/ 30.13 /32.96/33.75/ 37.51 / 36.24 ^a / 32.51 / 22.31 /39.45/ 26.46 ^b / 36.23 / 38.33 / 34.7 / 32.5 /35.6	II ^a V
10	Nov 10	120	44/76	3 (M/F/F)	2.5	37.09/37.52/ 36.07	V
11	Feb 12	40	23/17	2 (F/F)	5	32.5/31.12	No sequence

^aSAV Subtype II as identified based on partial E2 gene sequencing

^bCt value of the cultured samples

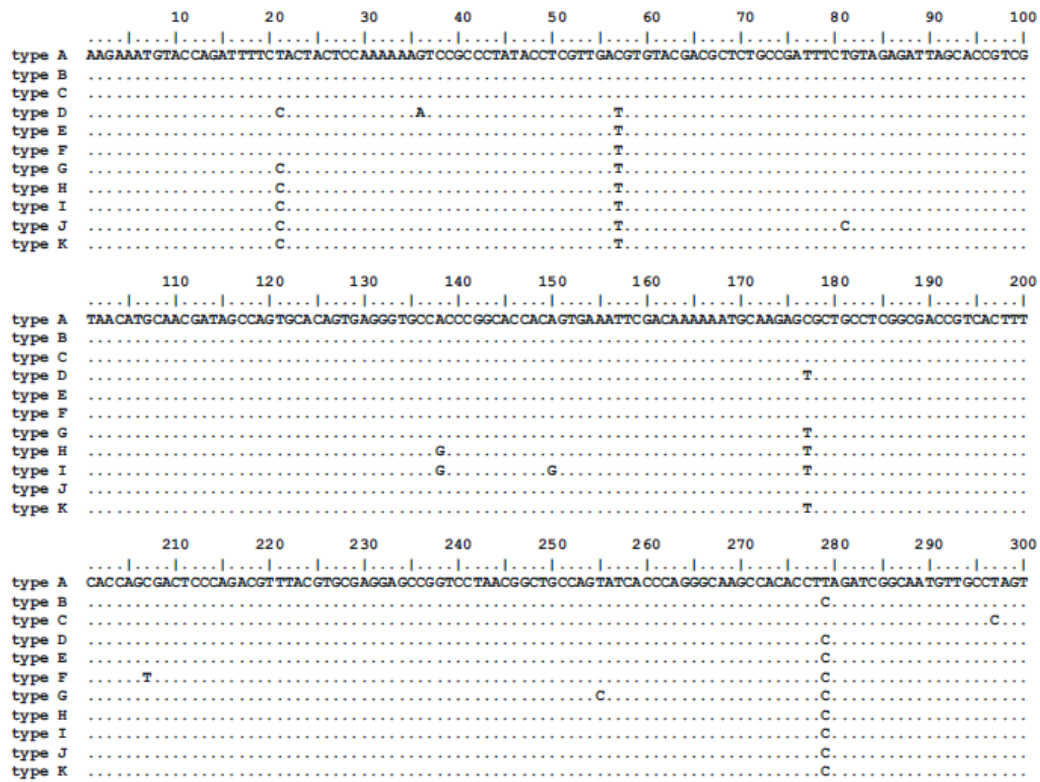


Fig. 2. Nucleic acid variability (type A to K) observed in the partial E2 gene of salmonid alphavirus Subtype V originating from common dab *Limanda limanda* observed in the present study. Numbers are base pairs

The split between SAV Subtypes III and II and all other SAV sequences received maximal statistical support as the true root of the tree under the Bayesian analysis (Fig. 3). While there is also strong support for monophyletic clades of characterised SAV sequence Subtypes I, IV and V, there is large statistical uncertainty surrounding their relationships (Fig. 3).

These analyses also confirmed that the dab 2010 684 51 isolate belongs to SAV Subtype II; this sequence forms the basal branch in a monophyletic cluster of sequences including marine isolates from Scotland and Norway (Fig. 3). Similarly, the dab 2010 682 isolate formed the most basal branch in the SAV Subtype I cluster (Fig. 3). In contrast, multiple dab SAV Subtype V sequences were clustered together with salmon Subtype V sequences (Fig. 3), a pattern consistent with relatively recent horizontal transmission of SAV.

Dab SAV isolation on CHSE-214 cell line

Total RNA was extracted from infected cells at Day 42, and the nsP1 RT-qPCR assay confirmed the presence of the SAV target, with an average Ct value of 21.40 from triplicate samples of individual fish.

Histological examination of wild caught common dab

There was no evidence of lesions that could be attributed specifically to that described for PD in the common dab examined from Stonehaven Bay, including those fish identified as positive for SAV RNA by RT-qPCR. Lesions associated with the heart occurred in 43% of the dab examined and included mild epicarditis involving the bulbous and a mild focal

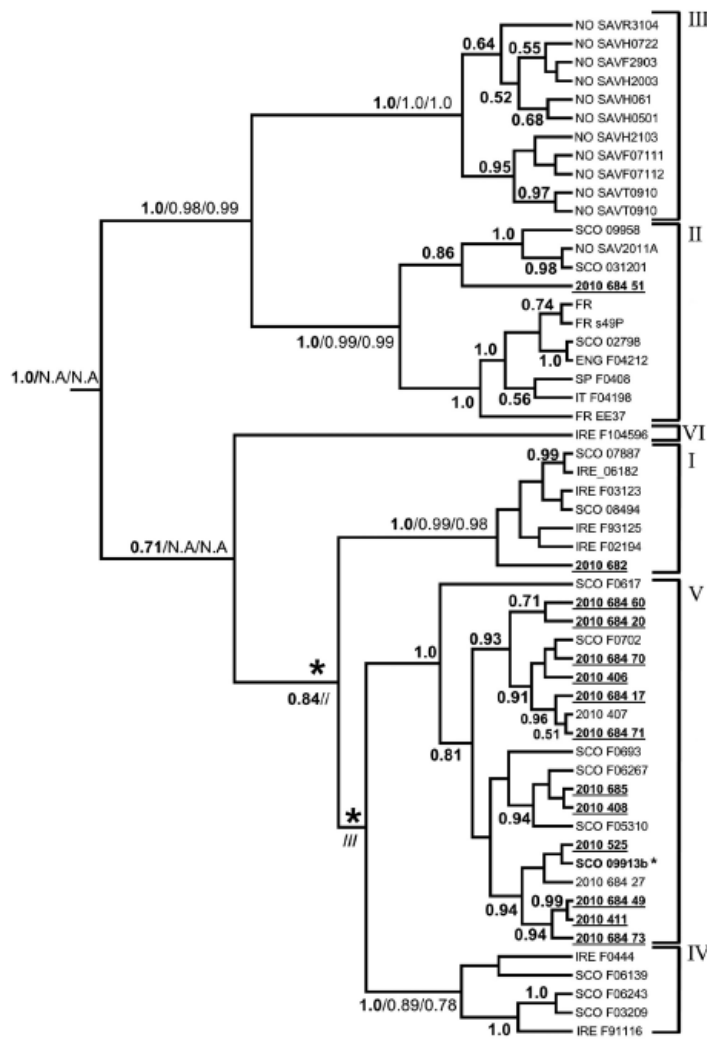


Fig. 3. Bayesian phylogenetic analyses performed with the 350 bp partial E2 gene sequence. The analysis was performed using the best-fitting model of nucleotide substitution (K80+G). The maximum clade credibility tree shown is based on 90 000 trees sampled from the MCMC chain after convergence was reached. Numbers at each node are support values from the different tree-building methods, ordered as: Bayesian posterior probability/ML bootstrap proportion/NJ bootstrap proportion (in all cases, only values >0.5 shown). Branch lengths are scaled to be relative in time according to an uncorrelated lognormal relaxed clock model. The asterisks on 2 internal nodes highlights the weak statistical support at branches separating defined subgroups I, IV and V. Bold underlined branch labels are sequences from dab isolates generated during this study

infiltration in the ventricle. Two dab showed mild infiltration into the red muscle tissue and extensive infiltration into the white muscle tissue, respectively. Nematodes were located in the pancreas and musculature in 26% of the fish examined.

DISCUSSION

This paper constitutes the first isolation of a salmonid alphavirus from a wild-caught non-salmonid marine fish species, namely the common dab *Limanda limanda*. A long time after the first isolation of SAV from farmed salmonids, the virus can now also be linked to subclinical infection in dab. The generally high Ct values reported in the current study are indicative of a low level of viral RNA in heart tissue. This confirms and extends previous work that identified the presence of SAV RNA in heart and kidney tissue collected from flatfish in Scottish coastal waters, but which failed to isolate the virus (Snow et al. 2010). Based on the presence of SAV in dab in a locality remote from salmonid aquaculture, we concluded that a reservoir of SAV in non-salmonid wild fish probably existed prior to the large-scale development of salmonid aquaculture in Scotland (Nash 2011). This is supported by a study on the evolutionary rate of SAV that suggested the location of a wild reservoir would be in or around the North Sea (Karlsen et al. 2014).

For SAV Subtype V, the present phylogenetic data are consistent with a history of repeated instances of horizontal transmission between dab and salmon. Wild reservoirs have

been well documented for a variety of viruses affecting marine fish farms, including viral haemorrhagic septicaemia virus (Snow et al. 2004, Gagné et al. 2007, Garver et al. 2013), piscine myocarditis virus (Poppe & Seierstad 2003, Böckerman et al. 2011, Tengs & Böckerman 2012) and piscine reovirus (Wiik-Nielsen et al. 2012). Nevertheless, the risk of transmission of viruses between wild and farmed fish is often difficult to ascertain (Kurath & Winton 2011). Fringuelli et al. (2008) reported identical sequences at individual farm sites over several years, implying that this is a slowly evolving virus. Nevertheless, this finding might also be explained by a consistent reservoir of infection associated with either environmental or anthropogenic processes. Common dab may play a role as a natural host for SAV, or, alternatively, the virus might be self-sustaining in aquaculture through farming practices; thus, areas of bio-security measures are required to control horizontal transmission (Kristoffersen et al. 2009). However, current practices of following sea sites would not support this theory. Graham et al. (2012) suggested that bio-security to prevent spread between sites has not been sufficient in the past, as SAV Subtype II strains from farmed marine Atlantic salmon shared 100% identity with some freshwater strains. Similarly, Lester et al. (2011) reported the isolation of SAV Subtype I from rainbow trout reared in freshwater. It is also plausible that amplification and spread of SAV from infected fish farms could lead to the establishment of potential reservoirs of re-infection through the complexities of trade (McLoughlin et al. 2003). The saithe *Pollachius virens* occurs in the vicinity of sea cages (Bruno & Stone 1990), and later studies have shown virus-neutralising antibodies, but have failed to record disease, suggesting that inter-species transmission from wild to farmed fish, or vice versa, can take place (Graham et al. 2006). Finally, in terrestrial alphaviruses, transmission of infection is linked to an arthropod vector; however, in the fish to fish transmission of SAV, as demonstrated by Boucher et al. (1995), no vector is needed as a routine route of infection, and none has been identified to date. Salmon lice have been suggested as a potential vector for SAV. Although SAV may be detected in salmon lice by PCR (Karlsen et al. 2006, Petterson et al. 2009), viral replication in lice and the consequent transfer of the virus to a new host has not been proven.

The discovery of viral RNA in areas remote from fish farming would suggest that common dab are natural carriers of the virus and that the viral RNA is not the result of fish farming activity. However, explanations are required as to why the prevalence of SAV

viral RNA was higher in some areas than in others, with a general trend of increasing prevalence towards the Shetland Isles. The distribution of SAV subtypes from farmed salmon by geographical region indicates that the northern Western Isles of Scotland have a higher number of Subtype V isolates than the Shetland Isles (Graham et al. 2012). However, these authors were aware of the fact that the number of strains available for analysis in each region would have varied.

These data might also be related to the kinetics of infection with SAV isolates of differing subtypes, as persistence of viral RNA in the tissues of farmed Atlantic salmon from which the virus was not isolated has been described (Christie et al. 2007, Graham et al. 2010, Jansen et al. 2010). It has also been noted that initial isolation of SAV can be complex, and a degree of cell-culture selection or adaptation may be necessary in some cases (McLoughlin & Graham 2007).

There was no histological evidence of PD in common dab from Stonehaven Bay. However, heart lesions were described in this species, which were independent of the presence of SAV RNA. The reason why lesions were located in this organ in what were believed to be healthy fish is unknown. We hypothesise that viruses other than SAV could be targeting this tissue and may have contributed to the lesions recorded, as no persistent subclinical SAV infection would be detectable by RT-qPCR. A comprehensive cohabitation challenge study by Graham et al. (2011) compared isolates from all 6 subtypes using parameters that included lesion score and virus load. In that study the authors demonstrated that there are differences between isolates with regards to replication and shedding dynamics; for example, Subtype V fish were found at a lower frequency than Subtype I and III fish, and virus load was initially lower than in the latter subtypes, but exceeded Subtype I at later times. From the dab examined in the current study only Subtype V was recovered from fish collected in Stonehaven Bay; hence, no comparable histology was available to examine the frozen dab that were PCR positive for Subtype I or II. As common dab are able to tolerate brackish waters and may enter freshwater river systems in coastal areas, they could encounter subtypes other than those frequently attributed to marine salmon. Snow et al. (2010) noted the occurrence of SAV RNA by RT-qPCR in pooled tissue from the long rough dab *Hippoglossoides platessoides*, common dab and the plaice *Pleuronectes platessa*; therefore, further research is required to understand the relevance of common dab and other flatfish as a wild reservoir of SAV and to assess the

potential of horizontal transmission with other wild or farmed fish. Such understanding would be advantageous in order to improve management practices on farms, as well as to interpret virus evolution and adaptation to new hosts.

Acknowledgements. The authors thank Finlay Burns and Paul MacDonald for helping to collect the common dab. Thanks are expressed to Mar Marcos-Lopez, Mark Fordyce and Nichola Still for helping to take the tissue samples, and to Stuart Wallace for preparing Fig. 1.

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Editorial responsibility: Dean Jerry, Townsville, Australia

Submitted: December 27, 2013; Accepted: March 16, 2014
Proofs received from author(s): April 17, 2014