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# Ultrastructural insights into the replication cycle of Salmon Pancreas Disease Virus (SPDV) using Salmon Cardiac Primary Cultures (SCPCs)

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## Data Availability Statement

The authors confirm that all the data supporting the findings of this study are available within the article itself. Additional EM images from the same study material may be available from the corresponding author (PN), upon reasonable request.

## Conflict of Interest

The authors confirm that there are no conflicts of interest.

## Abstract

Salmon pancreas disease virus (SPDV) has been affecting the salmon farming industry for over 30 years, but in spite the substantial amount of studies, there are still a number of recognised knowledge gaps, e.g. the transmission of the virus. In this work, an ultrastructural morphological approach was used to describe observations after infection by SPDV of an *ex vivo* cardiac model generated from Atlantic salmon embryos. The observations in this study and those available on previous ultrastructural work on SPDV, are compared and contrasted with the current knowledge on terrestrial mammalian and insect alphavirus replication cycles, which is deeper than that of SPDV both morphologically and mechanistically.

Despite their limitations, morphological descriptions remain an excellent way to generate novel hypotheses, and this has been the aim of this work. This study has used a target host, *ex vivo* model, and resulted in some previously undescribed features, including filopodial membrane projections, cytoplasmic stress granules or putative intra cytoplasmic budding. The latter suggests a new hypothesis which warrants further mechanistic research: SPDV in salmon may have retained the capacity for non-cytolytic (persistent) infections by intracellular budding, similar to that noted in arthropod vectors of other alphaviruses. In the notable absence of a known intermediate host for SPDV, the presence of this pattern suggests that both cytopathic as well as persistent infections may co-exist in the same host. It is our hope that the ultrastructural comparison presented here stimulates new research that brings the knowledge on SPDV replication cycle up to a similar level to that of terrestrial alphaviruses.

## Key words

Atlantic salmon, SPDV replication-cycle, ultrastructure, cardiac model.

## Introduction

The genus Alphavirus (*Togaviridae*) encompasses a diverse group of viruses infecting a wide range of invertebrate and vertebrate hosts worldwide (Lark L. Coffey, 2016). Alphavirus are predominantly arboviruses, with mosquito species being the most common invertebrate host along with ticks, biting fly and lice (Forrester et al., 2012; Powers et al., 2001b). The wide range of vertebrate hosts include humans and nonhuman primates, equids, pigs, rodents, birds, reptiles, amphibians and fish. The majority of alphaviruses species are therefore capable of replicating both in insect and vertebrate cell lines (Strauss & Strauss, 1994). Several members of the genus are responsible for debilitating conditions in humans and animals (Powers et al., 2001b). *Salmon Pancreas Disease Virus* (SPDV) is the first member of the genus isolated from fish, and an exception also within the genus in that it is not an arbovirus (Maclachlan & Dubovi, n.d.; Nelson, McLoughlin, Rowley, Platten, & McCormick, 1995; Olsen, Pemula, Braaen, Sankaran, & Rimstad, 2013; J. Weston et al., 2002).

SPDV also referred to as Salmonid Alphavirus (SAV), has been reported for over 3 decades affecting farmed salmonids, specifically Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), and brown trout (*Salmo trutta*) (Boucher, P., Baudin-Laurencin, 1994; McLoughlin & Graham, 2007; Villoing et al., 2000). SPDV has also been detected more recently in a non-salmonid marine species such as the common dab (*Limanda limanda*) (Bruno et al., 2014; Simons, Bruno, Ho, Murray, & Matejusova, 2016; Snow et al., 2010), suggesting the common dab may act as a wild reservoir for piscine alphavirus.

In farmed salmonids, SPDV is associated with two conditions: Pancreas disease (PD) of Atlantic salmon, first recognised in 1976 and described by 1984 (Munro ALS, Ellis AE, McVicar AH, 1984), and Sleeping Disease (SD) affecting rainbow trout and reported initially in France in 1994 (Boucher, P. Castric, J. and Baudin-Laurencin, 1994). The identification of an alphavirus as the aetiology of both PD

and SD took place soon after (Nelson et al., 1995; J. H. Weston, Welsh, McLoughlin, & Todd, 1999). At present, SPDV is taxonomically subdivided into 6 groups (referred to as “subtypes”), based on sequence differences (Fringuelli et al., 2008).

Mortality rates due to PD can vary widely between outbreaks, regions and subtypes (Aunsmo, Valle, Sandberg, Midtlyng, & Bruheim, 2010; Stien et al., 2020). However, low mortalities are relatively common, and the most common clinical sign in infected fish is chronic appetite reduction, with consequent weight loss. Additionally, a proportion of survivors may become “runts”, extremely thin fish which renders them unsuitable for the market (McLoughlin & Graham, 2007). This presentation is somewhat similar to many mammalian alphavirus infections such as Chikungunya, Venezuelan, Western, and Eastern equine encephalitis viruses (CHYKV, VEEV, WEEV, and EEEV, respectively), with relatively low mortalities but frequent chronic sequelae (Gérardin et al., 2008; Ronca, Dineley, & Paessler, 2016).

The direct horizontal transmission of SPDV is well documented (Atkins & Atkins, 2013). Also, the possibility of a vector role of the sea louse (*Lepeophtheirus salmonis*) has been investigated, a reasonable approach given all other members of the genus being arboviruses and that farmed Atlantic salmon is highly susceptible and affected by sea lice infection. However, despite positive RT-PCR detections in lice, no evidence to support viral replication within this host was found (Pettersen, Sandberg, & Santi, 2009). As a result of this, a putative vector role for sea lice, or indeed any relevant role for sea lice in SPDV dispersion remains hypothetical.

Understanding replication cycle events and the underlying mechanisms used by viral pathogens is essential for the study of host-pathogen interactions. A wealth of information is available on the replication cycle of the many terrestrial alphaviruses, particularly for the type reference Sindbis (SINV), Semliki-Forest virus (SFV), VEEV, Ross River virus (RRV) and CHYKV species (Freed & Martin, 2013; Jose, Snyder, & Kuhn, 2009). These advances on 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 (D. A. Graham et al., 2012; McLoughlin & Graham, 2007). A few previous studies have described SPDV ultrastructural morphogenesis *in vitro* (CHSE-214 cell line), and pathogenic aspects *in vivo* (fresh water salmon parr) (T K Herath, Ferguson, Thompson, Adams, & Richards, 2012; Tharangani K Herath et al., 2016). However, there have been no studies contrasting the knowledge available on the SPDV replication cycle with that of the model mammalian and insect alphavirus replication cycles.

This study used an *ex vivo* cardiac model isolated from Atlantic salmon embryos (P. A. Noguera et al., 2017; P. Noguera et al., 2018). Due to its isolation from the most affected host species and its being composed of cardiac tissue, one of the target organs of SAV infection, this model may help to address some of the limitations of *in vitro* work with immortalised cell lines of single cell type and different host lineage. The advantages and disadvantages of the use of immortalized cell lines versus primary cell

cultures remains a compromise between using tools readily available, which may have diverged from the original host, versus using tools that present with isolation challenges but are likely to be more similar to the original host (Pan, Kumar, Bohl, Klingmueller, & Mann, 2009). Care is required in interpreting results from cell lines, as they may not always replicate accurately the events noted in primary cell lines (Kaur & Dufour, 2012) and the serial passages required for maintenance may cause genotypic and phenotypic variations (Pan et al., 2009). Conversely, primary cultures may suffer degenerative changes after isolation, as has been reported for *ex vivo* cardiac cultures of zebrafish (Pieperhoff et al., 2014), degeneration that can have a direct impact in functional studies, particularly beyond a certain time threshold.

The current study used a descriptive ultrastructural morphological approach to gain insights into the SPDV replication cycle and, by comparison with the knowledge on terrestrial Alphavirus species, aimed to produce novel hypotheses. It is the author's hope that this stimulates further work into the mechanistic aspects of SPDV's replication cycle.

## **Materials and Methods**

Atlantic salmon embryonated eggs were obtained from AquaGen®. Salmon cardiac primary cultures (SCPCs) were isolated from these following previously described protocols (P. A. Noguera et al., 2017). In brief, embryos were removed from the shell, and disaggregated mechanically and enzymatically. The tissues were then centrifuged in culture media which was replaced before re-suspending the pellet, and placing the mixture on a plastic plate. Plates were incubated at room temperature and beating tissue aggregates were harvested from 24h and isolated into individual wells containing 0.5ml of medium, in x8 well chambered glass slides (Nunc® Lab-Tek II, Thermo-Fisher-USA). The culture medium used (L15, Lonza, UK) was supplemented with 10 % foetal bovine serum (Sigma-Aldrich, USA), 100 U/ml Penicillin and 0.1 mg/ml Streptomycin (Fisher Scientific, UK)). After isolation, the plates were placed in an incubator at 15°C for infection.

Infection was performed *in situ* at a dose of  $2.4 \times 10^4$  plaque forming units (PFU)/ml, using a field SPDV subtype 1 isolate (strain F07-220, provided in kind by Dr David Graham, formerly Agri-Food and Bioscience Institute -AFBI- Belfast, Ireland). The virulence and pathogenicity of F07-220 *in vivo* was previously reported (D. a Graham et al., 2011), and its infectivity *ex vivo* was noted during the development and application of the SCPCs model itself (P. A. Noguera et al., 2017; P. Noguera et al., 2018).

The infected medium was removed and replaced with new medium after 2h. Two to four SCPCs were sampled at 2, 3, 24, 48 and 72 hours post infection (hpi) and fixed *in situ* by adding 0.5 ml cold Karnovsky's fixative to each well (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M phosphate buffer pH 7.4).

After 30m at room temperature, fixed SCPCs were transferred to a 1.5ml Eppendorf tube with 0.1 M Phosphate buffer saline pH 7.4 (PBS) and placed on a rotator disk for 15m. This procedure was

repeated twice and followed by transfer to fresh buffer for short-term storage at 4°C. For post fixation, 1% OsO<sub>4</sub> 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). Staining was performed in an Ultrastainer AC20 (LEICA, Germany) with 0.5% uranyl acetate (Laurylab, France) and 3% lead citrate (Laurylab). Examination was conducted in a JEOL electron microscope JEM 1011 at 60 kV or JEM-1400 Plus at 120kV (JEOL, Japan).

## Results

Ultrastructural examination revealed SPDV-1 infection of SCPCs and replication, and viral particles of ~70nm of diameter were noted within cardiomyocytes, endothelial cells, fibroblasts and intraluminal leukocytes. Several features of the viral replication cycle were observed in cardiomyocytes and other cell types, such as endothelial cells, which are described below.

At 2-3hpi, viral particles were seen attached to the plasma membrane and undergoing internalization (Fig 1). In the former, viral particles were observed in close proximity with the cytoplasmic membrane (PM) surface and attached to it by a thin, poorly defined electron dense band. Viral internalization was characterized by PM invagination, with two distinct morphological patterns: with a sub-plasmalemmal spiky electron dense band (consistent with clathrin mediated endocytosis) (Fig 1 C-D), and without it (clathrin-independent endocytosis, suspected) (Fig 1 A-B). Viral attachment and internalization were also noted after 24hpi.

At 2-3hpi, intracytoplasmic viral particles were seen, free within the cytoplasm as well as within membrane-bound structures consistent with early (EE) (Fig 2 A-B) and late (LE) endosomes (Fig 2 C-D). Structures interpreted as EEs were devoid of debris and contained several viral particles, while LE were larger and contained numerous, viral particles admixed with loose debris. There were free intracytoplasmic viral particles associated with membrane fragments and in contact with LE membranes (putative release from endosomes) (Fig 2 D inset).

Structures consistent with spherules and cytopathic vacuoles type 1 (CPV-1) and type 2 (CPV-2) were seen from 3hpi and 24hpi, respectively (Fig 3). Formation of bulb-shaped membrane invaginations, consistent with spherules was observed from 3hpi in both the PM and cytoplasmic vacuoles (Fig 3 A-B). When associated with cytoplasmic vacuoles, spherules are the identifying feature for alphaviral CPV-1 (Froshauer, Kartenbeck, & Helenius, 1988a; Jose, Taylor, & Kuhn, 2017). In this study, CPV-1 were  $\geq 200$  nm wide, and were frequently located at the peri-nuclear region in proximity to the rough endoplasmic reticulum. Occasionally, CPV-1 presented a segmental stricture (Fig 3 C inset), and or had small numbers of moderately electron dense, 40-50nm spherical structures adjacent to them (nucleocapsids, suspected). Pleomorphic, round to ovoid membrane bound structures rimmed by an electron dense granular band with a variably electron dense centre, were noted from 24hpi (Fig 3 D). In these, the peripheral electron density is due to evenly distributed, ~25-30 nm electron dense particles.

These are consistent with an alternative presentation of CPV-2 recently described in SINV infected mammalian BHK-15 cells (Jose et al., 2017).

All of the above features have been previously described in the alphaviral replication cycle in mammalian cells. Additionally, from 24 hpi onwards, there were intracytoplasmic vacuoles that contained intraluminal structures consistent with virions, with and without debris (Fig 4 and Fig 5 B (arrow head)). These may be consistent with cytoplasmic vacuoles with internally budded virions as described in alphavirus replication in mosquito cell lines (Jose et al., 2017).

Viral budding at the PM was seen from 48 hpi, this was occasionally associated with presence of electron dense PM segments (Fig 5 A). Prominent filopodial PM projections were also noted, which were more frequently after 24 hpi, particularly on endothelial cells (Fig 5 B) and cytoplasmic, moderate electron dense loosely organised granular aggregates, associated with cytoplasmic adjacent viral particles at the periphery (Fig 5 C-D), consistent with previously described ribo-nucleoprotein stress granules (SG) (Kedersha & Anderson, 2007). Some features of reversible and irreversible cellular degeneration were recorded from 24 hpi onwards. These included e.g. mitochondrial swelling, loss of cristae, myelin figures, peroxisomes, nuclear apoptosis and cell detachment (not illustrated); all identifiable features which have been previously reported in SCPC after alphaviral infections (P. A. Noguera et al., 2017; P. Noguera, Collet, Klinger, Örün, & del Pozo, 2018).

## Discussion

Understanding the replication cycle events and underlying mechanisms used by viral pathogens is essential for host–pathogen interactions studies as well as for the development of preventive measures and/or targeted therapeutic strategies (Deeba et al., 2016; Jean Beltran, Federspiel, Sheng, & Cristea, 2017).

As zoonotic infectious agents, terrestrial alphaviruses (CHIKV, SINV and SFV) have been the focus of extensive studies. Given the variety of virus and host species in this genus there are some differences in replication mechanisms as well as temporal or spatial variations between different alphaviruses and/or their respective hosts. This is specially the case between vertebrate and invertebrate hosts. For example, notable differences in the timing and location of viral replication have been recently described by comparing SINV infected baby hamster kidney BHK-15, and mosquito *Aedes albopictus* C6/36 cell lines (Jose et al., 2017).

In salmon, ultrastructural features of SPDV infection and replication cycle have been described *in vitro* and *in vivo* using Chinook salmon embryo cells (CHSE-214) and Atlantic salmon juvenile fish, respectively (T K Herath et al., 2012; Tharangani K Herath et al., 2016). The work *in vitro* was performed using the type reference F93-125, a strain suspected to have adapted to culture (Christie et al., 2007; Villoing et al., 2000), whereas the *in vivo* work challenged the host in their fresh water phase using F02-

143, isolated from a 2002 outbreak (T K Herath et al., 2012; Tharangani K Herath et al., 2016). The study reported here used isolate F07-220, a subtype-1, 2007 field isolate from an outbreak in Ireland for which virulence and pathogenicity *in vivo* has been previously reported (D. a Graham et al., 2011). Its infectivity of *ex vivo* models was also noted during the development and application of the SCPCs model (P. A. Noguera et al., 2017; P. Noguera et al., 2018).

Several ultrastructural features of SCPCs infected with SPDV noted in this study align with those reported for other alphavirus species on vertebrate cell models. Briefly, these include viral attachment to cell surface suggesting binding to host cell receptors, formation of early (EE) and late (LE) endosomes after internalization, and presence of replication complexes involving membrane re-assembly and formation of spherules (Atkins & Atkins, 2013; Jose et al., 2009, 2017, (Kielian, Chanel-Vos, & Liao, 2010a; Marsh & Helenius, 2006)). There is also alignment with previous ultrastructural descriptions of SPDV in cell culture (CHSE-214) and *in vivo*, which also reported cytopathic vacuoles, and spherule formation within vacuoles (T K Herath et al., 2012; Tharangani K Herath et al., 2016). In the paragraphs that follow, we expand on our interpretation of the ultrastructural features of SPDV replication cycle in the SCPC model.

### *Entry mechanisms*

Most of the understanding of alphaviral cell entry has been obtained from studies focused on alphaviruses regarded as “Old World” alphaviruses, such as SINV and SFV. Comparably, much less attention has been received by other alphaviral species, although fundamental differences on the entry mechanisms have been suggested for other genus members such as VEEV (Kolokoltssov, Fleming, & Davey, 2006).

In this study, viral particles were observed during the process of attachment and internalization at 2-3hpi, and again at 24 hpi (Figs 1 and 2). At the latter time point this was observed simultaneous to later features of viral replication (e.g., CPV-2, budding), suggesting these instances of cell entry could have been a result of a re-infection process.

In this study, there were two morphological variants of attachment and entry, suggesting the possibility of two types of SPDV entry mechanisms into SCPCs. In the most frequent, viral attachment was associated with a moderately electron dense band below the PM, in a similar fashion to that reported for SFV (Helenius, Kartenbeck, Simons, & Fries, 1980). This finding is in line with the clathrin dependent endocytic pathway (CCP) (Fig 1 C-D), which is well described for alphavirus (Kielian et al., 2010a). Less frequently, we noted virions attached to the PM with no electron dense band, which can be consistent with clathrin independent (CI) viral entry (Fig 1 A-B). Pathways of clathrin-independent endocytosis for different molecules including viruses have also been reported, specifically for Chikungunya virus (Bernard et al., 2010; Mayor & Pagano, 2007).

Conventionally, enveloped RNA viruses exhibit a two-step entry mechanism involving receptor-mediated binding to host cell surface, followed by a low pH-triggered membrane fusion of the viral and



cell membranes that delivers the RNA into the cytoplasm (Kielian et al., 2010a; Más & Melero, 2013). These two steps may occur at the cell surface or after internalization of the virus, by endocytosis or some other route. However, even for reference prototypical alphaviruses the precise mechanisms of internalization are still not unequivocally defined (Carvalho, Silva, Oliveira, & Gomes, 2017). Currently, CCP endocytosis is thought to be the most common route and this is consistently reported for SFV (Kielian et al., 2010a) and CHIKV (Lee et al., 2013). However, CCP independent endocytosis has been shown to occur in CHIKV (Bernard et al., 2010) where there is also absence of caveolar vesicles (Leung, Ng, & Chu, 2011). Further, additional alternative paths such as virus genome injection directly through the cell plasma membrane may occur in SINV virus (Angel M Paredes, Davis Ferreira, Michelle Horton, Ali Saad, Hiro Tsuruta, Robert Johnston, William Klimstra, Kate Ryman, Raquel Hernandez, Wah Chiu, 2004; Wang, Hernandez, Weninger, & Brown, 2007). Seemingly, 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 (Vancini, Hernandez, & Brown, 2015; Vancini, Wang, Ferreira, Hernandez, & Brown, 2013). Importantly, the authors above also consider the possibility that the feature described as CI mechanism may represent an artefact and it is noteworthy that there are reports of the rapid post-internalization removal of the clathrin coat of virus-containing vesicles previous to delivery to early endosomes (Kielian, Chanel-Vos, & Liao, 2010b).

Overall, these findings highlight there is still a need for deeper understanding on the mechanisms of SPDV viral entry, using further mechanistic studies. Ultimately, this knowledge may be directly relevant for the potential development of rational antiviral therapies, for example exploiting compounds which could block critical steps of early infection events (Carvalho et al., 2017; Vancini et al., 2015).

#### *Post-internalization events: cytoplasmic translocation and replication*

Following internalization, abundant virus-laden vesicles were observed in our study from 2-3 hpi, consistent with cytoplasmic translocation of virus particles. These included endocytic vacuoles containing almost intact viral particles -consistent with early endosomes (EE)-, larger vacuoles containing a mix of debris and viral particles –consistent with late endosomes (LE)-, and free intracytoplasmic viral particles. Occasionally, features of endosome exit were noted (Fig 2 D).

The above is consistent with previous knowledge, where a process mediated by low pH in the LE has been described as necessary to allow the fusion of the viral and the endosome membranes. This fusion is necessary to enable release of the nucleocapsid core (NC) into the cytoplasm, where it will be disassembled (Jose et al., 2017). This disassembly is followed by translation, replication and transcription of the viral genome in both mammalian and arthropod insect replication cycles. Commonly, positive-strand RNA viruses replicate and transcribe their genomes in association with remodelled, intracellular, membrane arrangements such as single or double-membrane vesicles. However, even in thoroughly studied viruses, the exact site of RNA synthesis and the topological relationships between

membranes, vesicle content and cytoplasm are poorly defined (Kopek, Perkins, Miller, Ellisman, & Ahlquist, 2007).

Alphaviral RNA is known to replicate and transcribe in “replication complexes” (RC) formed from endosomal and lysosomal membranes by host and viral RNA proteins (Froshauer, Kartenbeck, & Helenius, 1988b; Jose et al., 2009). These RC are derived from host cell membranes, with PM, mitochondria and ER being frequently hijacked for that purpose by positive-strand RNA viruses (Romero-Brey & Bartenschlager, 2016). This process induces the formation of spherules, visualised as multiple endocytic processes resembling caveolae on host cell membranes. A CPV-I is formed as the replication spherules are internalized for cytoplasmic trafficking to the peri-nuclear region, and the vesicles are fused with lysosomes. The size of spherules is closely connected to the length of the replicating RNA template, and in SFV, a viral-genome template of 11.5 kb induced spherules of ~58 nm diameter, whereas a template of 6 kb yielded ~39 nm spherules (Kallio et al., 2013).

Consistent with the above, SPDV infection in our study induced the re-arrangement and remodelling of cell membranes into cytopathic vacuoles type I (CPV-I). The SCPCs infected with SPDV in our study showed spherules at the cell membrane and within peri-nuclear vacuoles, with a size of 35-55 nm, similar to the ~50 nm spherules reported in SINV infection (Jose et al., 2017). Additionally, we noted that in some CPV-1 some spherules had a neck-like narrowing which opened into the cytoplasm (Fig 2). A similar structure has been reported in other alphavirus, which may allow export of progeny RNA destined for translation or packaging and import of metabolites required for replication (Paul & Bartenschlager, 2013).

In the alphaviral replication cycle summarized by Jose et al. (2009 and 2017), formation of cytopathic vacuoles type II (CPV-II) precedes viral budding. The cellular secretory pathway plays an important role in the virus replication and assembly by transporting CPV-I and CPV-II to the PM. These CPV-II have been described as nucleocapsids radially attached to the cytoplasmic aspect of the membrane of a vacuole or to the outer and inner aspect of a vacuole lined by a double membrane (Jose et al., 2017). While such precise structures were not observed in this experiment, however, putative CPV-II structures in line with a recent description for CPV-II in a SINV infected vertebrate cell line (Jose et al., 2017) were observed. Variation of the electron density of the core of CPV-II was also noted in this study (Fig 3 D), which has been interpreted as differences between section planes as described in SFV infections on BHK-21 cells (Soonsawad et al., 2010). Interestingly, CPV-II structures as described by Jose et al (2009), have been reported in SPDV *in vitro* infection (T K Herath et al., 2012), and it is possible that these were not captured in this study.

In addition to the above, we noted a distinct type of cytopathic vacuole from 24hpi onwards, which featured intraluminal particles resembling virions, occasionally budding at the vacuolar membrane into its lumen (Figs. 4 and 5 B). This pattern may be consistent with intracytoplasmic budding, which has been described for the replication of alphaviruses in insect cell lines (Jose et al., 2017). In contrast with

that description the cytopathic vacuoles in our study did not present spherules in their membrane. It is also noteworthy that a similar type of vacuoles were illustrated in the report of a previous SPDV *in vitro* infection (T K Herath et al., 2012), without discussion of their potential relevance to the replication cycle of SPDV.

The relevance of this observation is that it suggests that events described previously for terrestrial alphaviruses infecting arthropod cell lines, may be also present in the SPDV replication cycle, which is a novel observation. This is more relevant in view that in arthropod cell lines these structures are linked to the ability of the virus to establish persistent infection. In mammalian cell lines, infection with terrestrial alphaviruses frequently results in acute cytolysis but in arthropod cell lines, the host cells survive the infection and can trigger antiviral pathways involving RNA interference (RNAi), which modulates arbovirus infections by gene silencing (Blair & Olson, 2015; Cerutti & Casas-Mollano, 2006; Cirimotich, Scott, Phillips, Geiss, & Olson, 2009). This may be in turn related to the establishment of persistent infections without cytopathic effects (Jose et al., 2017; Rupp, Sokoloski, Gebhart, & Hardy, 2015). The latter is remarkably interesting as it allows, based on the observations of the current study, to suggest that SPDV may have the capacity to establish both cytolytic and non-cytolytic replication cycles in its fish host, possibly facilitating persistent infection. Further, it is possible to hypothesize that factors that may regulate the cytolytic vs non-cytolytic replication cycle may involve tissue type and host genetic background. This may in turn be involved in the variability in mortality noted among sites, and the ability of this virus to persist in the population. Longitudinal studies in Scotland (D. a Graham et al., 2006) and Norway (Jansen et al., 2010), have reported sites without clinical PD with SPDV positive fish detected at slaughter. Extending beyond salmon, this mechanism may also explain non-lethal SPDV infections in other species like the common dab (*Limanda limanda*), which does not present with histopathological lesions (Bruno et al., 2014; Simons et al., 2016; Snow et al., 2010).

The family *Alphaviridae* has been suggested to originate in the aquatic environment, acquiring later the ability to infect warm-blooded vertebrates and mosquito vectors (Forrester et al., 2012), and that an ancestral alphavirus may have adapted to fish in the distant past, forming the SPDV lineage (Powers et al., 2001a). It has also been hypothesized that arboviruses are more constrained than single-host RNA viruses in their adaptability (Coffey et al., 2008). Put together, these features and information may be consistent with the hypothesis that SPDV may have retained the capacity to establish both cytopathic and persistent infections in the same host. In turn, this is a feature that, in the absence of an intermediate host, would be beneficial for SPDV transmission and survival.

### *Viral 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 assembled nucleocapsid core acquires a host derived envelope (Forsell, Xing, Kozlovskaya, Cheng, & Garoff, 2000). In this 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 envelope proteins accumulating below the PM (Fig. 5 A). 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 (Soonsawad et al., 2010).

In previous SPDV *in vitro* ultrastructural descriptions (T K Herath et al., 2012), 'fuzzy'-coated membranes in the Golgi apparatus and vacuoles were reported as more frequent in SPDV infected cells. Noting the current evidence for SFV, a potential interpretation for such structures is that these vesicles represent envelope proteins before translocation to the PM through the secretory pathway.

### *Additional observations*

The presence of clustered granular structures, consistent with stress granules (SG) in our study (Fig. 5 C-D) has not been previously reported for SPDV. These SG have been associated with stress events that severely repress translation, such as heat shock, oxidative stress or UV radiation (Anderson & Kedersha, 2008). Importantly, viral infection causes stress at multiple levels which can reduce host translation, and lead to the formation of stress granules (Reineke & Lloyd, 2013). Another way to view SGs is as a host response. In fact, some viruses counteract the assembly of SGs, suggesting their involvement in antiviral activity (White & Lloyd, 2012). Biochemically, SGs contain ribonucleoproteins (mRNPs) and stalled translation initiation complexes, where the packaging of cytoplasmic mRNA into discrete RNA granules regulates the gene expression, by delaying the translation of specific transcripts (Kedersha & Anderson, 2007). Alphavirus and other arboviruses 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 in turn, balances the levels of viral replication with the fitness of the vector host (Fros & Pijlman, 2016). For example, in SFV, SGs have been reported to occur transiently at early infection, and later disassemble with ongoing viral replication.

Another relevant feature described here for the first time for SPDV, is the presence of filopodial projections observed at the PM in endothelial cells (Fig. 5 B). Replication on endothelial cells, alongside in fibroblasts and migrating monocytes/macrophages, have been highlighted as pivotal for successful virus production in the early events of Chikungunya infections (van Duijl-Richter, Hoornweg, Rodenhuis-Zybert, & Smit, 2015). Filopodial projections have also been described for infection by terrestrial alphavirus in mosquito cell lines (Jose et al., 2017) and associated with viral dissemination in -C6/36, originally known as ATC-15 (Walker, Jeffries, Mansfield, & Johnson, 2014). Formation of long filopodial extensions contacting neighbouring, uninfected cells and mediating a cell-to-cell virus transmission have also been shown in SINV infected VERO cells, and described as a mechanism for alphavirus cell-to-cell transmission which may shield the virus from the host neutralizing antibodies (Martinez & Kielian, 2016).

### *Closing remarks*

The current study offers information extracted from a host specific *in vitro* model system which has provided morphological insights into aspects of the replication cycle of the virus not previously reported. SPDV has been affecting the salmon farming industry for over 30 years, and despite the substantial amount of studies along these years, there are still a number of recognised knowledge gaps that require further research – e.g. the transmission of the virus, (D. A. Graham et al., 2012; Izabela Deperasińska, 2018) or the uniqueness of SPDV, among the members of the genus, for not requiring a vector for successful transmission. To the best of our knowledge, this is the first study contrasting the knowledge available on the SPDV replication cycle to that of the models for mammalian and insect alphavirus, which are described at deeper level morphologically and mechanistically. While the authors are aware of the limitations of ultrastructural morphological descriptive work, we are also aware of its ability to create novel hypotheses. It has been our aim with this work to stimulate novel mechanistic research of the SPDV replication cycle, which may close our current knowledge gaps.

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## Figure captions

Fig 1: Ultrastructural pictures of Salmon cardiac primary cultures infected with SPDV subtype-1 at 2-3hpi (A, B, D) and 24 hpi (C), illustrating viral attachment and internalization. Also illustrated are viral particle measurements in A & D. In (A), note a moderately electron dense band between an extracellular virion and the cell surface (black arrow). In (A&B), there is invagination of the cytoplasmic membrane around two virions (black arrowheads), without sub-plasmalemmal electron dense material (clathrin independent endocytosis). In (C&D), there are examples of invagination of the cytoplasmic membrane with a sub-plasmalemmal band of electron dense material (white arrows, clathrin mediated endocytosis). In D, note the presence of a free intracytoplasmic viral particle (white arrowhead). Scale bars on A = 50 nm, C = 200 nm and B, D =100 nm.

New text Fig 2 A: Ultrastructural pictures of Salmon cardiac primary cultures infected with SPDV subtype-1 at 2-3hpi: viral cytoplasmic features after internalization. (A) Viral particles free within the cytoplasm (black arrowheads) and within an early endosome (black arrow). In (B), a larger early endosome contains several viral particles (black arrow). In (C) a late endosome containing at least one viral particle (arrow) admixed with electron dense debris. (D) Illustrates examples of putative release from endosomes Note a viral particle adjacent to a late endosome and apparently continuous with its membrane (white arrow), and adjacent free cytoplasmic particles (black arrowheads). Note close up of a viral particle detaching from a membrane fragment (inset). Scale bars on A, B and D = 70 nm, C = 100 nm.

Fig 3: Ultrastructural pictures of Salmon cardiac primary cultures infected with SPDV subtype-1 at 2-24hpi: spherules and cytopathic vacuoles. (A & B) membranous invaginations consistent with spherules (black arrowheads) are noted at the plasma membrane at 24hpi (A) and 3hpi (B). In A, there is also a viral particle in the process of endocytosis (black arrow). In (C), spherules are associated with a cytoplasmic vacuole (black arrow), cytopathic vacuole type I – (CPV-1), and are frequently noted adjacent to the nucleus (N) and rough endoplasmic reticulum (RER). Note the presence of lamellar bodies within the cytoplasm (black arrowhead). In (C) inset, there is a stricture at one pole of a CPV-1. From 24hpi (D), there are membrane bound intracytoplasmic structures with a rim of granular electron dense material (~25-30nm wide granules). Scale bars: A, C-D = 200 nm, B =100 nm.

Fig 4: Ultrastructural pictures of Salmon cardiac primary cultures infected with SPDV subtype-1 at 24-48 hpi: intracytoplasmic viral budding, suspected. In (A), an intracytoplasmic membrane-bound structure (cytopathic vacuole) contains a moderate number of intraluminal virions (black arrowheads) that are occasionally contiguous to its membrane. These are admixed with debris (black arrow). Please note a cytopathic vacuole type 1 with spherules (white arrow). In (B), a similar vacuole is noted, which contains intraluminal virions (black arrowheads) and is lined by several membranes. Scale bars on A-B = 500 nm.

Fig 5: Ultrastructural pictures of Salmon cardiac primary cultures infected with SPDV subtype-1 at 24-48hpi: viral exit and other features. Picture (A) illustrates viral budding at plasma membrane (black arrowheads). At 24hpi (B) there are rare filopodial expansions (arrow) of the plasma membrane, note also an instance of suspected intracytoplasmic viral budding (arrowhead) (C&D) Intra cytoplasmic clusters of loosely organised electron dense particles consistent with ribonucleoprotein stress granules (SG). These are surrounded by cytoplasmic viral particles (black arrowheads). Scale bars on A = 200, B = 100nm, C = 300 nm.