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RESEARCH ARTICLE

The pathology of *Lates calcarifer* herpesviral disease—Disseminated intravascular coagulation explains mortality spikes

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Abstract

Lates calcarifer herpesvirus (LCHV) causes an emerging serious disease in aquaculture. Sudden drops in feed rates and mortality spikes exceeding 40%–50% often accompany LCHV infections in juvenile *L. calcarifer*, soon after transfer into sea cages. Affected fish have patchy white skin and fins, corneal opacity and frequently hang in surface water column like 'ghost' or 'zombie' fish. Fish have pale gills, fluid-filled intestines with yellowish casts, lipid depleted liver, enlarged spleen and kidney and reddened brain. Epithelial hyperplasia, apoptosis, marginated nuclear chromatin, amphophilic intranuclear inclusion bodies and the occasional multinucleated cells are observed in gills, skin, intestines, liver and kidney. These are often accompanied by lymphocytic-monocytic infiltration and extensive necrosis in gills, skin, kidney and intestines. Martius scarlet blue stains indicate presence of fibrin in vasculature in brain, gills, intestines, kidney and liver, or disseminated intravascular coagulation (DIC). DIC has been reported in human herpesviral infections. Multifocal lifting of intestinal epithelium with proteinaceous exudate and necrosis of several adjacent villi often progress to involve entire gut sections. Atrophied livers with accentuated lobules may progress to marked loss of hepatic acini. Multifocal dilated attenuated renal tubules are often accompanied by casts and marked protein loosing renopathy. This study on LCHV demonstrates that it can cause significant pathology and mortality.

KEYWORDS
aquaculture, disseminated intravascular coagulation, fish disease, herpesvirus, pathology, production losses

1 | INTRODUCTION

The last decade has seen the emergence of three novel species-specific viruses in Asian seabass or barramundi, *Lates calcarifer* Bloch viz. scale drop disease virus (SDDV), *L. calcarifer* herpesvirus (LCHV), a novel birnavirus and the infectious spleen and kidney necrosis virus (ISKNV), an iridovirus previously reported in multiple fish species (Chang et al., 2018; Chen et al., 2019; Dang et al., 2023; de Groof et al., 2015; Domingos et al., 2021; Dong, Jitrakorn, et al., 2017; Gibson-Kueh et al., 2012; Thanasaksiri et al., 2019). Most herpesviruses of fish are host specific and belong to the family Alloherpesviridae, with remarkably conserved virion structures, which include a host cell-derived viral envelop. Herpesviruses affecting freshwater fish include Ictalurid herpesvirus 1 in channel

catfish, Cyprinid herpesvirus 1 (carp pox), Cyprinid herpesvirus 2 (goldfish haematopoietic necrosis virus), Cyprinid herpesvirus 3 (koi herpesvirus) and salmonid herpesvirus (Hanson et al., 2011). It is possible that the enveloped virus observed in the first description of scale drop disease is the *L. calcarifer* herpesvirus (LCHV) in a co-infection with SDDV (Gibson-Kueh et al., 2012). LCHV was reported as most closely related to the Ictalurid herpesvirus 1, albeit with less than 60% homology (Chang et al., 2018). Notable characteristics of herpesviruses of animals are their distinct polyphyletic classification into 3 families, ability to persist in the host (latency), host specificity and subclinical infections (Hanson et al., 2011). Monoculture will favour a host specific virus and its successful transmission through horizontal spread in a population. Husbandry stress may additionally induce disease in intensive aquaculture. One of the most devastating fish kills was reported in wild pilchard in association with a herpesvirus, with dead fish reported along a 5000 km coastline in Australia and 500 km of the New Zealand coastline (Hyatt et al., 1997; Whittington et al., 1997, 2008). No other fish species were reported to be affected in this huge wild fish kills, again corroborating host specificity of herpesviruses. It was also reported that juvenile pilchards seen in vicinity of affected adult pilchards were apparently not affected.

Clinical signs such as skin or scale loss, lethargy and inappetence are rather non-specific, such that histopathological descriptions are important diagnostic tools in the recognition of novel diseases (Gibson-Kueh et al., 2012). Many pathogens are ubiquitous in the environment or endemic in fish populations, such that it is important to understand epidemiology and pathogenesis to guide management decisions. Viral diseases such as SDDV can cause significant mortalities of greater than 50%–70% (Domingos et al., 2021; Gibson-Kueh, 2012). In a study of SDDV and LCHV co-infections, SDDV loads spiked while LCHV loads remained low in diseased *L. calcarifer*, suggesting that SDDV is the primary pathogen (Domingos et al., 2021).

This study builds on the first histopathological description of LCHV by Dang et al. (2023), which describes the presence of intranuclear inclusion bodies in the liver, renal tubules, epithelium of skin and gill filaments, often with tissue necrosis. In this study, we investigate cases of high mortality in *L. calcarifer* tested positive for LCHV by PCR or qPCR from three different farm sites between 2019 and

TABLE 1 Clinical history of cases examined in this study.

Number of fish examined/Average bodyweight	Clinical history	Diagnosis[a]
Site 1		
$n=3$, 149 g	Nursery sea cage with 2% cumulative mortality 1 month post-stocking	PCR: LCHV +ve, SDDV −ve
$n=3$, 160 g	Nursery sea cage with 60% cumulative mortality 3 months post-stocking	PCR: LCHV +ve, SDDV +ve
$n=2$, 40 g	Land-based, seawater flow-through aquaculture system	Histopathology[b]
$n=1$, 4 kg	Diseased fish sampled from grow-out sea cage	PCR: LCHV +ve, SDDV −ve
$n=9$, 200 g	Nursery sea cage, 1 month post-stocking. Mortality of 0.7% at time of sampling. History of SDDV and LCHV in same batch, in seawater RAS nursery stage 2–3 months ago.	Histopathology[b]
Site 2		
$n=10$, 55 g	Mortality spikes of 40%, 2 weeks post-transfer into sea cages, with sudden onset of drops in specific feed rates from 3.4% to 1.8%. Patchy scale loss, white discoloration on fins, tail rot.	qPCR LCHV +ve
$n=5$, 47 g	Drop in specific feed rates from 1.9% to 1.4%. Patchy white discoloration on fins and tail.	qPCR LCHV +ve
$n=5$, 40–60 g	High mortality spikes. Patchy white discoloration on fins and tail. Bite marks on head in some fish indicate cannibalism.	qPCR LCHV +ve
$n=5$, 40–60 g	Clinically healthy fish sampled from batch with previous history of LCHV outbreak confirmed by qPCR.	qPCR LCHV −ve
Site 3		
$n=5$	Sampled late during disease outbreak. Multifocal scale loss, tail and fin rot.	qPCR LCHV +ve

[a]Other pathogens were absent in the cases in this study: ectoparasites based on wet mount microscopy, bacteria using routine plate cultures on TCBS and blood plates, or other viruses such as infectious spleen and kidney necrosis virus or scale drop disease virus using PCR.

[b]Histopathology suggests a herpesvirus infection, and observations were similar to that observed in fish from other cases in this study tested positive for *Lates calcarifer* herpesvirus using qPCR.

TABLE 2 Primers and probe used in this study.

Pathogen	Primer/probe	Primer sequences (5′→3′)	Product size (bp)
LCHV	Forward primer	TGCAAATGATGACGAAGAC	86
	Reverse primer	GCGACAGACCATCAACAA	
	Probe	TCACCGAACCCCGCATCA	
SDDV	Forward primer	TGGCCAGAGGAGGTGACTAT	225
	Reverse primer	ATCCTGACGACTTTCCACCG	

TABLE 3 LCHV viral loads per 200 ng of gill DNA extract from Batch 5 fish collected at two consecutive time points at Site 2, and late into a LCHV outbreak at Site 3.

Site/date sampled	Fish 1	Fish 2	Fish 3	Fish 4	Fish 5
B5 Site 2 14/09/21	1.55×10^{6}	1.04×10^{7}	2.68×10^{6}	$1.36E\times10^{6}$	3.25×10^{6}
B5 Site 2 16/09/21	7.92×10^{7}	1.15×10^{8}	3.02×10^{7}	6.67×10^{6}	4.27×10^{7}
Site 3	4.16×10^{8}	4.15×10^{8}	1.65×10^{9}	6.21×10^{8}	3.29×10^{8}

2022, to establish pathogenesis and guide future research and disease control.

2 | MATERIALS AND METHODS

2.1 | Background of cases examined and sample collection

The clinical history of cases examined are summarized in Table 1. Sites 1, 2 and 3 are separate distinct sites. At Site 1, juvenile *L. calcarifer* are kept in recirculating aquaculture systems (RAS) at 5–7 ppt salinity and acclimated to 30 ppt seawater in flow-through systems over 4–6 h. In order to manage mortalities due to viral disease, several cycles of heat treatment are carried out over a period of 5–7 days after acclimatization to seawater and before transfer to nursery sea cages (Michel, 2018). At 10–12 g, fish are vaccinated with a commercial Red Sea bream iridovirus vaccine (Aquavac™ Irido V, MSD), and autogenous vaccines against *Tenacibaculum maritimum* and *Streptococcus iniae*. Approximately 15,000 of these seawater acclimatized fish (90–120 g bodyweight) are stocked into nursery sea cages (6 × 6 × 7 m). At Site 2, fish are kept in land-based, seawater RAS nursery tanks, and at 10–12 g vaccinated with a commercial Red Sea bream iridovirus vaccine (Aquavac™ Irido V, MSD), and autogenous vaccines against *Tenacibaculum maritimum* and *Streptococcus iniae*. Approximately 10,000–15,000 fish (40–50 g bodyweight) are transferred into nursery sea cages (7 × 7 × 3 m). Fish are fed thrice daily at rates of 2.5%–4.5% of bodyweight. The samples from Site 3 were taken from nursery sea cages during a LCHV disease outbreak.

Following mortality events, fish samples were collected from land-based, seawater flow-through tank systems (Site 1), or nursery sea cages (Sites 1, 2 and 3) from fish farms at three different locations in Southeast Asia, between 2019 and 2022. The fish from all cases in this study were also examined using wet mount microscopy for ectoparasites on gills and skin, and PCR on kidney tissues for scale drop disease virus and infectious spleen and kidney necrosis virus (Kurita et al., 1998; WOAH, 2019), with negative results. Bacteria cultures from kidney on Thiosulfate-Citrate-Bile Salts-Sucrose Agar (TCBS) and blood agar plates showed mostly insignificant or no growth. The predominant pathogen detected was the *L. calcarifer* herpesvirus by PCR or qPCR. Tissues samples from fish freshly euthanized in AquiS™ immersion baths were fixed in 10% neutral buffered formalin for histopathological evaluation, and 95% ethanol for molecular analysis. Formalin fixed tissues were submitted to the Institute of Molecular and Cell Biology (IMCB), Singapore or Murdoch University Veterinary Histology Laboratory, for histoprocessing into haematoxylin and eosin (H and E) slides. Martius Scarlett Blue stain (MSB) was performed on selected wax block tissues to detect presence of fibrin.

2.2 | DNA extraction and primer design for LCHV and SDDV

DNA was extracted from gills for LCHV qPCR and spleen for SDDV PCR using the GF-1 Tissue Blood Combi DNA extraction kit (Vivantis), according to manufacturer's instructions. The extracted DNA was quantified using NanoPhotometer™ NP80 (Implen) and stored at −20°C until analysed. The specific primer pair and probe used were designed based on the major envelop protein (MEP) region of LCHV using Primer Express 3.0.1 (Applied Biosystems) and synthesized by Integrated DNA Technologies (Table 2).

2.3 | Molecular analyses for LCHV using qPCR and SDDV using PCR

The optimal annealing temperature for qPCR using the LCHV MEP primer sets (Table 2) was determined using gradient PCR at 54–62°C and an annealing temperature of 60°C used in this study. LCHV PCR reactions comprise 20 μL with 200 ng of DNA extracted from ethanol fixed gill tissues, 2× PrimeTime® gene expression master mix (IDT), and 200 nM of each primers and probe. Quantitative PCR cycling conditions were set with initial denaturation at 95°C for 3 min, followed

by 40 cycles of 95°C for 15 s and annealing at 60°C for 1 min using StepOnePlus™ Real-Time PCR System (Applied Biosystems). Plasmid containing MEP gene was used as positive control. Ten-fold serial dilutions of plasmid at 10^{10} to 10^{4} copies per µL in duplicates, was used to generate a standard curve to quantify LCHV viral loads in fish from batch 5 (Table 3). All samples were analysed in duplicates.

Scale drop disease virus PCR was carried out on spleen tissues using the primer set given in Table 2. SDDV PCR reactions comprise 25 µL with 200 ng of DNA extracted from ethanol fixed spleen tissues, 1× Buffer A, 4 mM dNTPs, 1.5 mM $MgCl_2$, 2.5 U of taq polymerase (all from Vivantis Technologies) and 200 nM each of forward and reverse primers. PCR was performed under the following conditions: 1 cycle at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, annealing at 53°C for 30 s, extension at 72°C for 45 s followed by final elongation step of 72°C for 5 min. Plasmid containing MCP gene was used as positive control. PCR products were loaded into agarose gel (1.0%) stained with 1× ViSafe Green gel stain (Vivantis) and run at 100 V for 30 min. Gels were visualized under the Blue light Transilluminator (Hercuvan).

3 | RESULTS

3.1 | Clinical signs and gross pathology in LCHV-infected *L. calcarifer*

Lates calcarifer herpesvirus disease outbreaks are typically observed in 40–150 g *L. calcarifer* (i) 7–10 days after acclimatization from brackish recirculating aquaculture systems (salinity 5–7 ppt) to seawater (30 ppt) in flow-through systems, in preparation for transfer to sea cages (Site 1), or (ii) 2–4 weeks after stocking into sea cages, when fish are kept in seawater throughout the nursery stage (Site 2). At Site 1, LCHV infections were more likely observed in nursery sea cages during rough weather, and cumulative losses can be up to 100%. Marked inappetence, with sudden drops in specific feed rates of up to 50% and mortality spikes often surpassing 40%–50% over a 3–6-day period were reported by the farms in this study (Table 1). Figure 1 shows a typical mortality spike in a cage suffering a LCHV disease outbreak at Site 2.

Affected fish often have multifocal to coalescing, patchy white discoloration of skin and fins, fin and tail rot, bilateral corneal opacity and are frequently found hanging in surface water column and described by farms as 'ghost' or 'zombie' fish (Chang et al., 2018). Diseased fish have pale gills often with excessive mucus, intestines filled with fluid and white or yellowish casts, red liver (lipid depletion), enlarged spleen and kidney and reddened brain (severe vascular congestion and thrombosis) (Figure 2). Other pathogens were absent in most cases: ectoparasites based on wet mount microscopy of skin scrapes and gills, bacteria using blood and TCBS plate cultures of kidney, or other viruses such as ISKNV or SDDV using PCR. Only one case included in this study had concurrent SDDV infection, as confirmed by PCR. Fish with a history of LCHV infections at the nursery stage and were subsequently tested negative using qPCR when in sea cages, were also included in this study (Table 1).

3.2 | Histopathology

The most prominent histopathological features of LCHV infection in *L. calcarifer* are epithelial hyperplasia, scattered single cell deaths

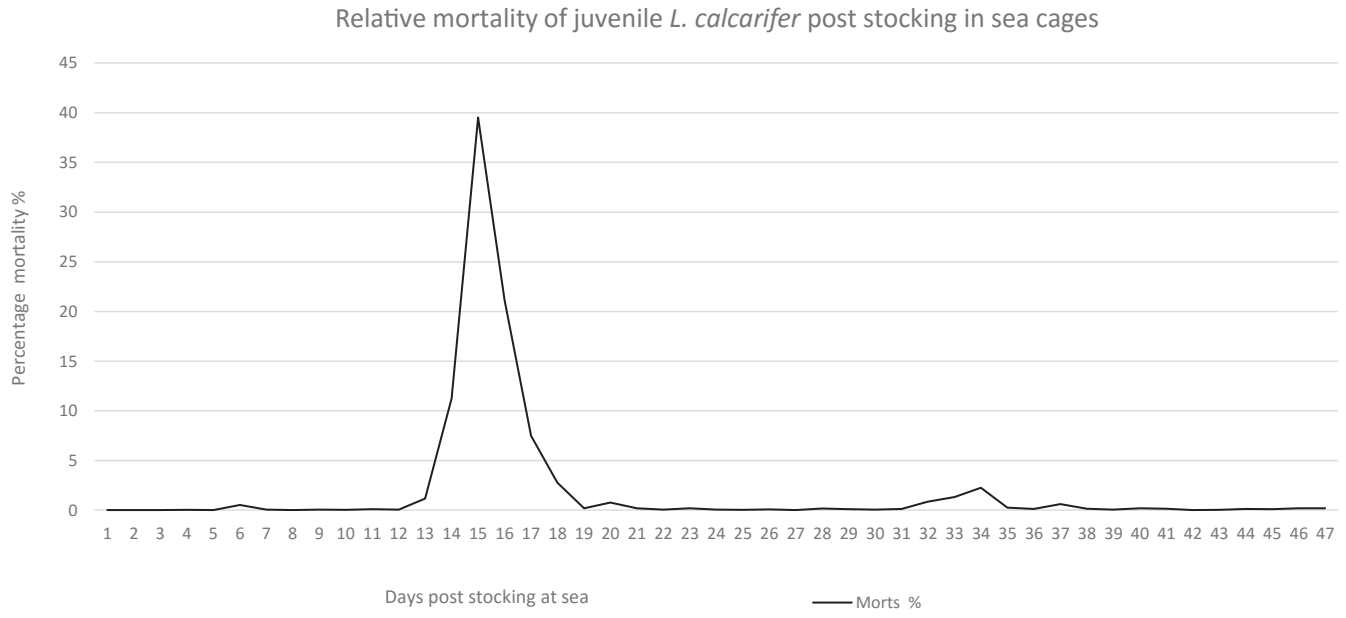

FIGURE 1 Mortality spikes are often reported in disease outbreaks associated with *Lates calcarifer* herpesvirus infection, with greater than 40%–50% mortality over a short span of 3–6 days. This graph is based on daily mortality records in one LCHV positive sea cage at Site 2. Mortality spikes may occur from 2 to 4 weeks post-stocking into sea cages. A second mortality spike (not shown) may be observed in the same sea cage.

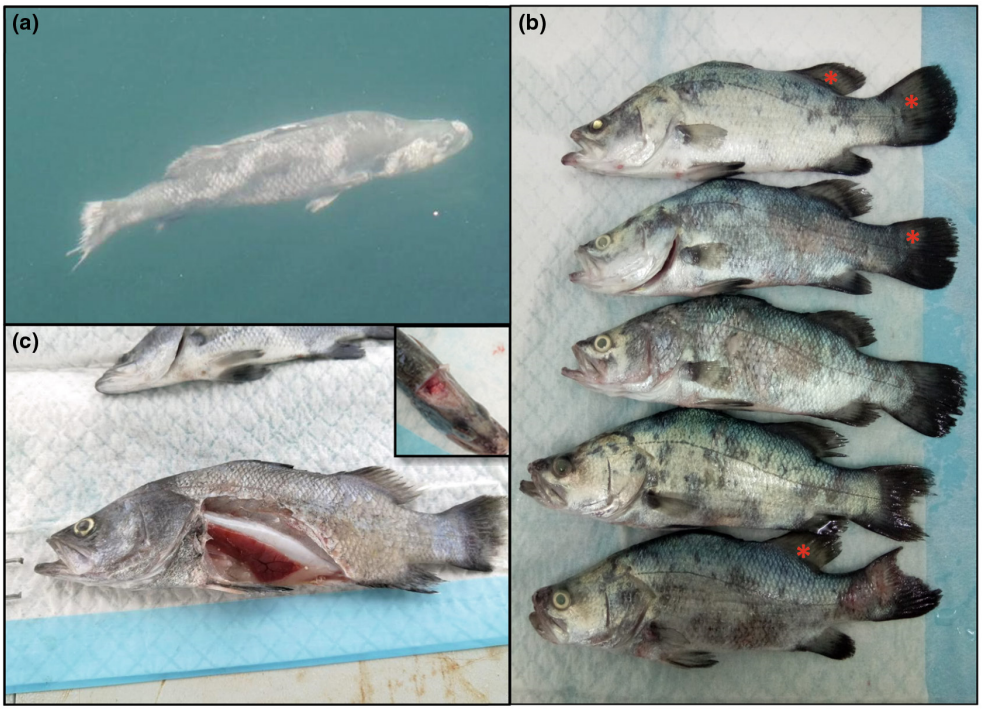

FIGURE 2 Clinical signs and gross pathology in LCHV-infected *Lates calcarifer*. (a) 'Ghost' or 'zombie' fish with patchy, dark and white skin discoloration, seen hanging in surface water column (this study), also described by Chang et al., 2018. (b) Fin and tail rot, patchy scale loss, and cloudy eyes are often seen in fish with LCHV infections. White discoloration on fins and tail (*) are likely areas with epithelial hyperplasia. (c) Fish often have patchy to diffusely red livers due to loss of lipid and glycogen stores, and fluid-filled intestines, with white to yellow casts (not shown). Inset shows fish with red discoloured brain, due to severe vascular congestion and thrombi formation.

(apoptosis), marginated nuclear chromatin, amphophilic (eosinophilic or basophilic) intranuclear inclusion bodies (INiBs) and the occasional multinucleated cells, in tissues of epithelial origin. These changes are often accompanied by lymphocytic-monocytic infiltration, disseminated intravascular coagulation (DIC) and extensive tissue necrosis in affected organs such as the gills, kidney, skin and intestines.

In the gills, there is multifocal to focally extensive interlamellar hyperplasia of hypertrophied epithelial cells and ionocytes (previously chloride cells). This is often accompanied by mild to moderate lymphocytic-monocytic inflammation, scattered apoptosis, INiBs and the occasional multinucleate cells (Figure 3a–c). Thrombosis, lifting of lamellar epithelium and expansion of lamellae by proteinaceous exudate, may involve single to several adjacent gill lamellae or filaments (Figure 3a–c). This may progress to extensive sheets of sloughed epithelium involving entire gill arches. Hyperplasia of epidermis of skin can be marked and is often accompanied by multifocal to coalescing necrosis, lymphocytic-monocytic infiltration, INiBs and occasionally neovascularization (Figure 3d).

In intestines, pathology can be mild in early LCHV infections, with the presence of palisading rows of basophilic mucosal epithelial cells, mild to moderate lymphocytic -monocytic infiltration, scattered INiBs and the occasional multinucleate cells (Figure 4a,b). The separation of mucosal epithelium from lamina propria by proteinaceous exudate and necrosis, can be multifocal to extensive sloughing of several adjacent villi, or involve entire sections of intestines (Figure 4c,d). In early-stage LCHV infections, the liver exhibits wispy cytoplasm indicating good lipid and glycogen stores, INiBs and the occasional apoptotic cells (Figure 5a). As the disease progresses, the lipid depleted liver takes on darker basophilic staining, with mild to moderate accumulation of fat vacuoles of variable size (macrovesicular lipidosis) (Figure 5b). Varying cell size (anisocytosis) is distinctive in the liver of LCHV-infected fish, with scattered apoptosis, hepatocytes with increased basophilia, INiBs and the occasional multinucleate cells (Figure 5b). These basophilic hepatocytes are often observed with eosinophilic, intranuclear inclusion bodies (Figure 5b inset). A more chronic disease in older fish is often accompanied by mild to moderate, chronic interstitial hepatitis (Figure 5c). There can be moderate to severe loss of liver parenchyma as the disease progresses, presumably from continual cell loss and regeneration, resulting in accentuated hepatic lobulation. This may go on to present as remnants of the liver parenchyma in between exocrine pancreatic tissues (Figure 5d), or liver with multiple, large cysts (not shown).

The presence of scattered apoptosis, basophilic cells and INiBs in a few renal tubules in early LCHV infection progress to multifocal, moderate to marked attenuation and dilation of renal tubules, often with apoptotic cells shed into the lumen as casts (Figure 6a,b). Protein losing renopathy can be marked and is often observed when there is also massive sloughing of epithelium in gills and intestines in affected fish (Figure 6a). The spleen shows variable loss of its red blood cell and lymphocyte population. This is often accompanied by moderate to marked, acute subcapsular

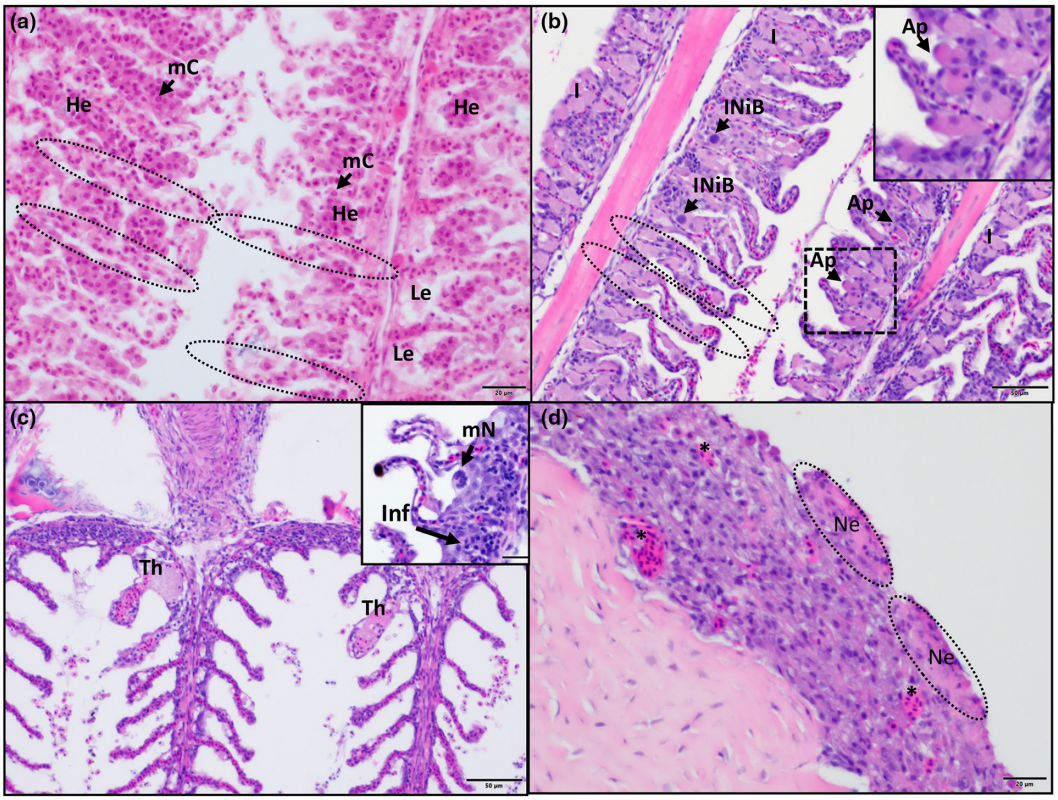

FIGURE 3 Gills in LCHV-infected *Lates calcarifer*. (a) Hyperplasia of epithelium between lamellae (He), with hypertrophied cells and marginated nuclear chromatin (mC). Lifting of epithelium by inflammatory cells and proteinaceous exudate is also observed in this gill section (Le). (b) Ionocyte (I) or chloride cell hyperplasia, apoptosis (Ap) and intranuclear inclusion bodies (INiB). Inset shows demarcated area with apoptotic cells (Ap) at higher magnification. (c) Thrombosis (Th) in isolated to several adjacent lamellae. The presence of intact squamous gill epithelium supports a systemic coagulopathy secondary to LCHV infection, rather than inflammation due to an environmental insult such as toxic algae (Gibson-Kueh & Uichanco, 2021). Inset: Epithelial hyperplasia is often accompanied by lymphocytic-monocytic infiltration (Inf) and the occasional multinucleate cells (mN). (d) Marked epithelial hyperplasia in skin is accompanied by neovascularisation (*) and multifocal necrosis (Ne). A common misdiagnosis of hyperplasia in gills is caused by inappropriate orientation of gills (Wolf et al., 2015). However, full length sections of gill lamellae (encircled) indicate that hyperplasia of ionocytes and epithelium observed are not due to sectioning artefacts. Scale bars (a), inset (c), (d) 20 μm; (b), (c) 50 μm.

inflammation, multifocal necrosis of ellipsoids and increased splenic haemosiderosis.

Marked, vascular congestion is often observed in the brain of LCHV positive fish. Multifocal encephalitis, with gliosis and neuronophagia is observed in the occasional fish. Positive red MSB staining of vasculature in multiple tissue sections is indicative of the presence of fibrin and disseminated intravascular coagulation (DIC). Fibrin is present within blood vessels in the gills, brain, liver, kidney and intestines of some LCHV positive fish selected for MSB stains (Figure 7a–d). Yellow MSB staining of red blood cells in vasculature in gills and brain suggest the absence of fibrin in some LCHV positive fish (Figure 7a & inset Figure 7c). The presence of fibrin within sloughed tissues in the gills, kidney and intestines showed that these changes occurred before death (antemortem) (Figure 7d inset).

In fish tested negative for LCHV by qPCR after an outbreak, epithelial hyperplasia and the single cell changes in tissues of epithelial origin are still present in the gills, skin, liver, kidney and intestines. Liver in these fish tested qPCR negative for LCHV after an outbreak (Table 1) were dark staining, indicating depletion of lipid stores and sloughed intestinal mucosa were observed in some fish. However, the marked vascular congestion frequently present in brain of LCHV qPCR positive fish were no longer observed in these fish tested negative for LCHV after an outbreak.

3.3 | LCHV viral load in diseased fish collected at different sampling points during an outbreak

qPCR assays detected variable loads of LCHV DNA in gills of same batch of fish collected from Site 2 at two different time points during a disease outbreak. LCHV qPCR on DNA extracted from gill tissues produced Cq values of 27.0–31.0 for fish collected on 14/09/2021, and 22.3–27.9 for fish collected on 16/09/21 during a disease outbreak at Site 2, and Cq of 17.1–20.3 for fish collected 2–3 weeks into a disease outbreak at Site 3. Samples with Cq values of >35 were considered negative for LCHV. Viral copy numbers were derived based on a standard curve generated using known copies of

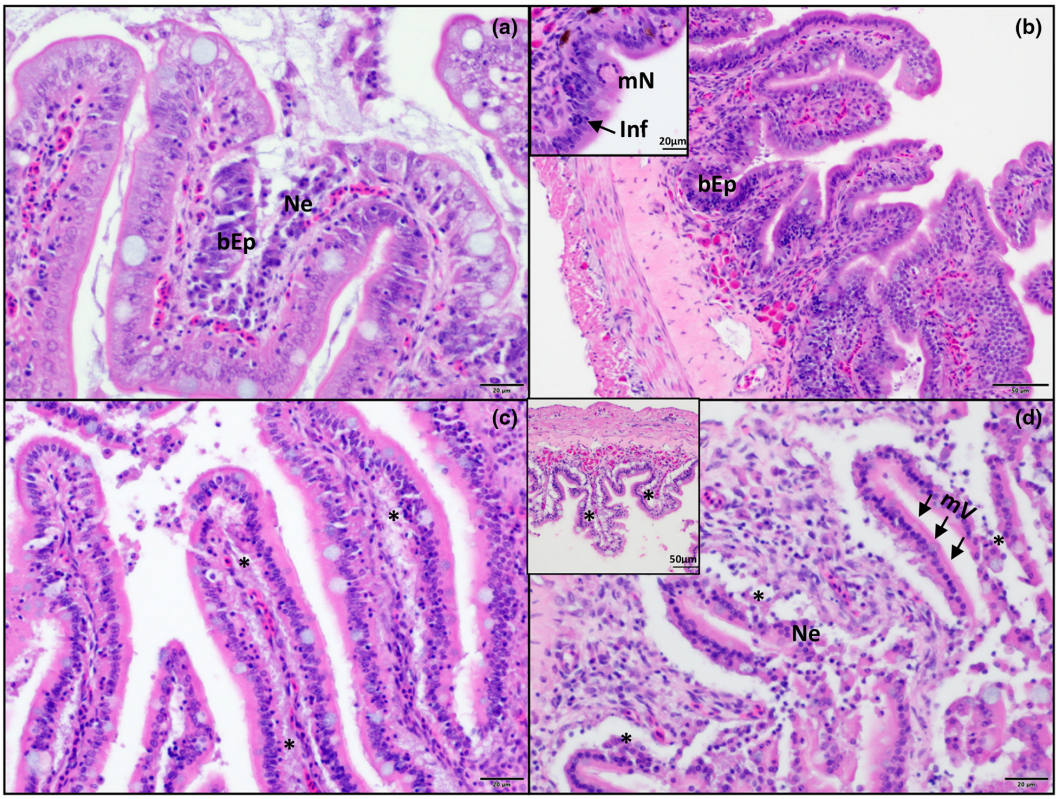

FIGURE 4 Intestines in LCHV-infected *Lates calcarifer* (a, b) Palisading rows of basophilic, intestinal mucosal epithelial cells (bEp) are often observed, possibly linked to upregulation of nuclei acid genesis in herpesvirus infected cells. This is accompanied by necrosis (Ne), lymphocytic-monocytic infiltration (Inf) and the occasional multinucleate cells (mN). (c) Intestinal mucosal epithelium is often observed to be lifted by proteinaceous exudate (*), mild in this section. (d) and inset. Detachment of intestinal mucosal epithelium (*) can be extensive and may progress to sloughing of mucosa involving entire segments of the gut. Intact microvilli or brush borders (mV) is evidence that samples are well preserved. Scale bars: (a), inset (b), (c), (d) 20 µm; (b), inset (d) 50 µm.

plasmids containing LCHV MEP gene. LCHV viral loads increase during a LCHV outbreak and were higher in fish sampled later during LCHV disease outbreaks (Table 3).

4 | DISCUSSION

In this study, viral load varies between fish sampled from the same batch at a sampling point, but appears to increase during a disease outbreak suggesting LCHV is the primary pathogen (Table 3). In an earlier study on co-infections in *L. calcarifer*, viral loads of SDDV spiked while LCHV remained low and pathology was consistent with a primary scale drop disease (Domingos et al., 2021; Gibson-Kueh et al., 2012). This study showed unequivocally that the *L. calcarifer* herpesvirus (LCHV) causes significant pathology, disseminated intravascular coagulation (DIC) and abrupt mortality spikes. These abrupt mortality spikes of greater than 40%–50% mortality over 3–6 days cannot be explained by the epithelial hyperplasia observed in gills or skin, or the scattered single cell changes such as basophilia, intranuclear inclusion bodies, or apoptosis in gills, skin, liver, intestines and kidney. Extensive sloughing of tissues in gills, kidney and intestines were initially thought to be post-mortem changes. This prompted the authors to stain selected tissue sections with MSB for fibrin, and the result was positive red staining in vasculature in gills, brain, kidney and intestines, suggesting widespread intravascular coagulation or thrombosis in some fish positive for LCHV by qPCR. Typically, blood clotting follows vascular damage, upon exposure of cellular transmembrane proteins, tissue factors and procoagulant phospholipids to plasma clotting factors. However, it has been shown that several herpesvirus including cytomegalovirus, and herpes simplex I and II can initiate thrombin production in the absence of vascular damage in host and maybe the earliest pathology initiated (Sutherland et al., 1997). The absence of vascular congestion in the brain of fish tested qPCR negative for LCHV after a disease outbreak suggests that LCHV viral loads are associated with the severity of the inflammatory response and possibly triggers the DIC observed. It would be fascinating to perform MSB stains on wax block tissues from the pilchard mass fish kills reported in Australia and New Zealand in 1995, as DIC may explain schooling pilchards dying in greater numbers if given chase and moribund fish jumping erratically when disturbed (Whittington et al., 1997). Erratic swimming was observed in a batch of rainbow trout just before death in a hobby farm in Western Australia one summer, and MSB stains showed presence of fibrin in blood vessels in brain (Gibson-Kueh, unpublished information).

High mortalities and gross pathology in LCHV-infected fish (this study) is similar to that described for Anguillid herpesvirus, Cyprinid herpesvirus and Acipenserid herpesvirus (Hanson et al., 2011; Plumb

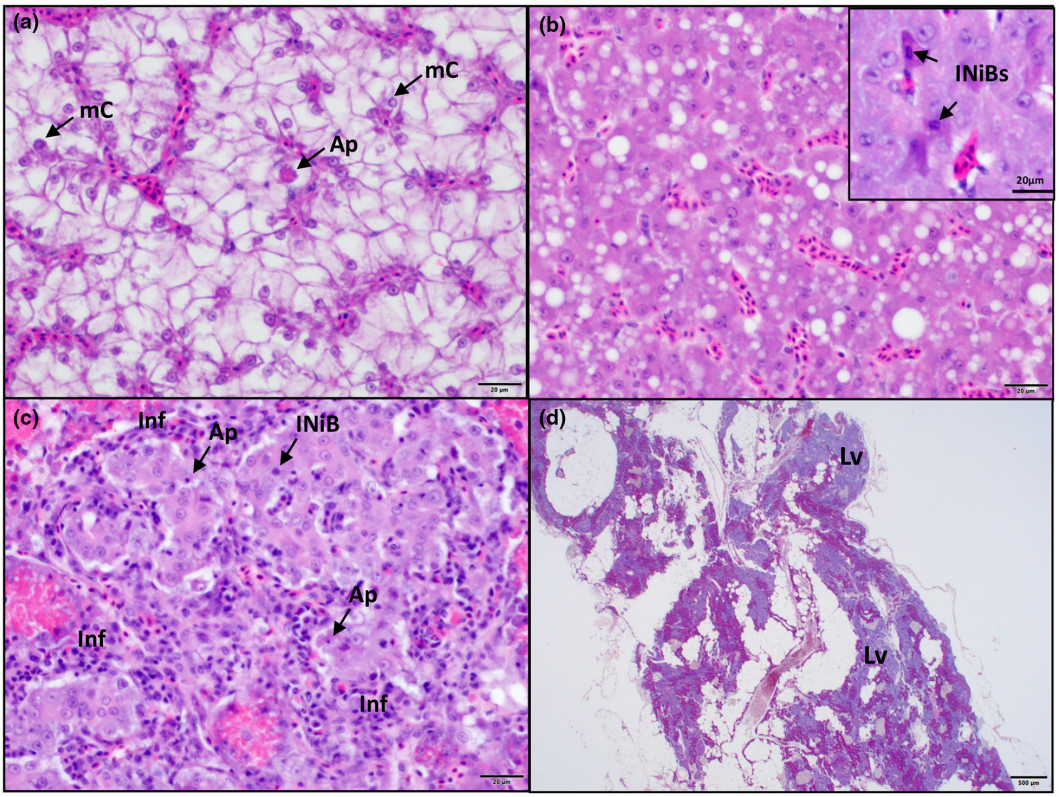

FIGURE 5 Liver in LCHV-infected *Lates calcarifer*. (a) Scattered hepatocytes with marginated chromatin (mC) and the occasional apoptosis (Ap), in early LCHV infection. Wispy, eosinophilic cytoplasm indicates good lipid and glycogen stores, and that fish was eating recently. (b) Note the uneven size of liver cell nuclei (anisocytosis), which suggests increased hepatocyte loss and regeneration, and darker staining parenchyma due to loss of lipid and glycogen stores. The accumulation of variable size fat droplets (macrovesicular lipidosis), maybe due to adipose fat mobilization, and/or inability to process fat due to energy deficiency from poor nutritional status. Inset shows basophilic liver cells similar to that observed in intestinal mucosa epithelium associated with LCHV infection, often with intranuclear inclusion bodies (INiBs). (c) Chronic interstitial hepatitis (Inf), with scattered apoptosis (Ap) and INiB. (d) Atrophied liver parenchyma (Lv), nested between exocrine pancreatic tissues. The livers in (c) and (d) are late stage progression of LCHV infections in 140–160 g *L. calcarifer*, 3 months post-stocking into sea cages. Scale bars: (a), (b), inset (b), (c) 20 μm; (d) 500 μm.

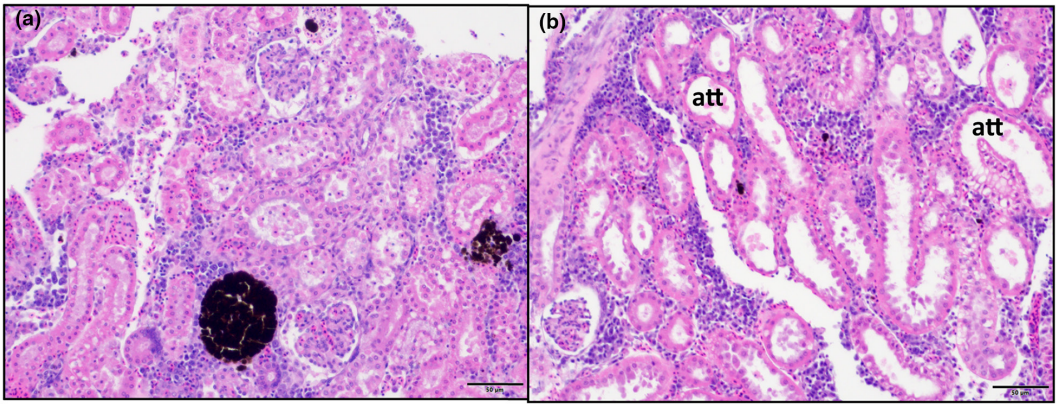

FIGURE 6 Kidney in LCHV-infected *Lates calcarifer*. (a) Moderate to severe, protein loosing renopathy with abundant eosinophilic, proteinaceous material and cellular casts within tubules and Bowman's space, are often observed in 40–50 g fish, 2 weeks post-stocking into sea cages. (b) Multifocal, moderate to marked attenuation (att) of renal epithelium and dilated tubules, are likely linked to continual cell deaths or apoptosis due to LCHV infections in 140–160 g fish, 1–3 months post-stocking into sea cages. Scale bars (a), (b) 50 μm.

et al., 1974). Pathology such as epithelial hyperplasia, and single cell changes in tissues of epithelial origin in gills, skin, liver, intestines and kidney including apoptosis, and intranuclear inclusion bodies are observed in this study in association with LCHV and were also described by Dang et al. (2023). Alloherpesviruses to which most fish herpesviruses belong are epitheliotropic (Hanson et al., 2011). Gill

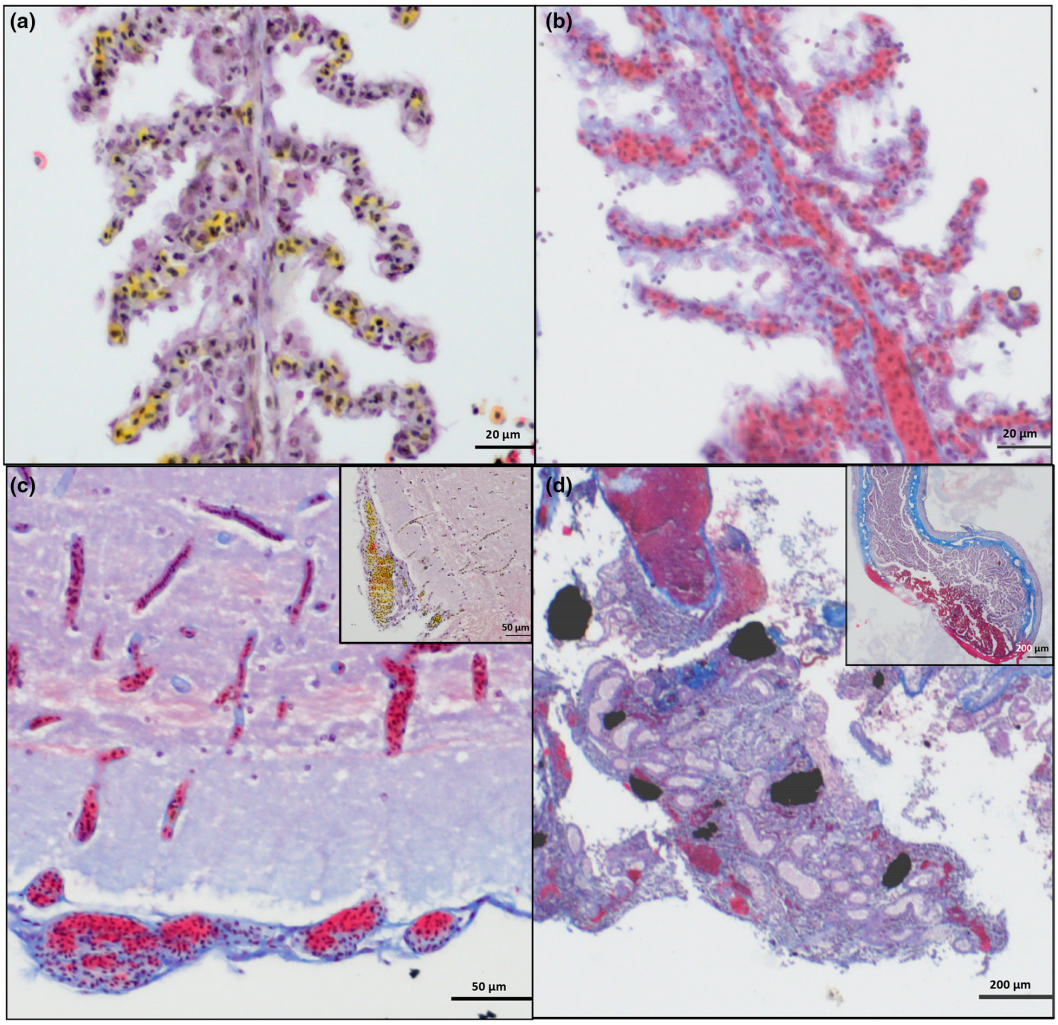

FIGURE 7 Martius scarlet blue (MSB) stain on *Lates calcarifer* tissues tested positive for LCHV by qPCR. Positive red staining for fibrin varies between fish sampled from the same batch of fish tested LCHV qPCR positive during an outbreak. Presence of intravascular fibrin is indicative of disseminated intravascular coagulation (DIC). (a) Red blood cells (RBC) stain yellow in gills, indicating the absence of fibrin. (b) Red staining with MSB indicate presence of fibrin in blood vessels or thrombi formation in gills. (c) Brain. Red stains within blood vessels indicate presence of fibrin. Inset - yellow staining of RBC indicating absence of fibrin in brain of different fish sampled at the same time point from same sea cage. (d) Kidney of same fish as brain in inset of (c) stain red within vasculature. Inset: The presence of fibrin as evidenced by red staining with MSB, showed that at least part of the sloughed intestinal mucosa occurred antemortem.

pathology (this study) is very similar to that described in the pilchard herpesvirus epizootic, with epithelial hyperplasia and hypertrophy and ionocyte (or chloride cell) hyperplasia between lamellae space, lifting of lamellae epithelium by proteinaceous exudate and lymphocytic-monocytic infiltration (Whittington et al., 1997). Sloughing of intestinal villi can be a common post-mortem artefact. The presence of intravascular fibrin based on MSB stains in intestinal sections with sloughed mucosa (Figure 7d inset) supports presence of antemortem tissue damage. Mild to severe autolysis of the gastrointestinal tract that was reported in pilchard herpesvirus mass fish kills, may not be completely due to autolysis (Whittington et al., 1997). Extensively sloughed intestinal mucosa observed in this study can explain the extreme and up to 50% drops in specific feed rates, and potentially be fatal as it can critically affect water absorption to combat dehydration in fish in a marine environment. The extensive thrombosis observed in brain (this study) may explain fish seemingly hanging in water column like 'zombies' as originally described (Chang et al., 2018). The patchy pattern of skin discoloration and scale loss in LCHV ('ghost' fish) is distinctive from skin and scale loss on the caudal third to half of the body associated with severe chronic, obliterative endarteritis due to SDDV (Domingos et al., 2021; Gibson-Kueh et al., 2012).

In LCHV-infected fish in this study, apoptosis and regeneration often results in anisocytosis in the liver, and continual loss of liver cells may progress to a point where remnants of liver parenchyma are nested between exocrine pancreas. The liver produces bile, which emulsifies fat to aid its digestion and processes fatty acids by tagging on lipoproteins to aid transport to adipose tissues for storage. As fat is often added to spare proteins as an energy source in fish feed, this can be expected to affect fish growth. Cells with increased basophilic cytoplasm correlate with upregulation of nuclei acid genesis

in association with a herpesvirus infection and were observed in intestinal mucosal epithelium, renal tubules and liver cells in this study. Increased cell basophilia was also described in gills of fish dying with Pilchard herpesvirus, often in association with lymphocytic-monocytic infiltration (Whittington et al., 1997). The maturation of naked nucleocapsids of cyprinid herpesvirus assembled in host cell nucleus is reported to take place in the cytoplasm, and this may explain the basophilic cells seen in herpesvirus infection (Miyazaki et al., 2008). The site of earliest pathology in LCHV infections suggest the entry of host via gills, skin and gut, with shedding likely from these same tissues into the water, where virus may survive for several hours (Hanson et al., 2011). Latency implies the importance of spread from brooder fish to fry, and the virus' ability to survive in water could facilitate horizontal spread to other cage mates.

Further research to determine a better correlation of viral loads to evidence of DIC may be useful in understanding how to manage mortality due to LCHV. The latency of herpesviruses and mild disease in some cases means that these viruses may be difficult to detect in batches of clinically healthy fish with low viral loads. Vaccination or genetic selection of *L. calcarifer* resistant to LCHV and SDDV remain possibilities, although this has not proven successful even for Cyprinid herpesvirus 3 (koi herpesvirus). Replication of Cyprinid herpesvirus 3 is temperature sensitive, with none occurring at temperatures above 30°C (Hanson et al., 2011). Therefore, heat treatment can be useful for controlling LCHV, but should be delivered during periods of active viral replication. Heat treatment when high viral loads are present with severe pathology may further exacerbate mortalities. Vertical transmission is thought to be important for Ictalurid herpesvirus (Hanson et al., 2011). qPCR and antibody tests in brooder fish may prove useful, to screen for LCHV negative fish.

Diagnosis using histopathology is based on the interpretation of observations based on a knowledge of disease processes and pathogenesis caused by specific pathogens. Vibrio infections are common opportunistic infections following disease outbreaks in the field. However, bacteria cultures from kidney of diseased fish in this study on blood plate or TCBS agar yielded mostly insignificant or no growth. Acute, diffuse (generalized) hydropic degeneration of renal tubules is described in *L. calcarifer* in associated with a toxin producing *Vibrio harveyi* (Dong, Taengphu, et al., 2017). In comparison, there is multifocal necrosis in renal tubules, with scattered pyknosis amongst relatively intact renal tubule epithelium in LCHV positive fish in this study (Figure 6). Moreover, the targeting of single cells and distinctively epithelium in kidney, gills, gut, skin and liver, is indicative of a primary herpesviral disease.

In conclusion, *L. calcarifer* herpesvirus can cause a severe disease in *L. calcarifer* with significant mortalities. The pathology described in this study gives a better insight into impact of the disease caused by LCHV and assist in their accurate detection in farmed *L. calcarifer*. Fish farms need to appreciate that LCHV infections likely occur weeks before first observations of clinical disease. What seems like acute mortality spikes are sequelae of tissue loss and intense inflammation triggered by these viruses, DIC in the case of LCHV and obliterative endarteritis by SDDV. Although some farms may use heat treatment to curb losses during a suspect viral disease outbreak, the complexity of pathogenesis that leads to mortality means that the timing of the heat treatment itself is critical to avoid further accentuating mortality rates (Michel, 2018).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

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