First detection of Cyclopterus lumpus virus (CLuV) in England, following a mortality event

Edwards M, Papadopoulou A, Gray J, Trani E#, Bignell J, Savage J, Joseph A, Wood G, Paley R and Stone D.

26th Annual Workshop of the NRLs for Fish Diseases, May 2022

Centre for Environment Fisheries & Aquaculture Science

25 YEARS
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Cefas

Cyclopterus lumpus
Lumpfish, Lumpsucker

- Delousing cleaner fish for salmon
- Susceptible to an ever-increasing range of diseases (see Erkinharju *et al*., 2021 for review).
- As production still relies on wild caught brood stock, potential vectors of serious diseases, both of salmon and lumpfish.

Mortality event

- Land based recirc facility in Dorset on south coast of England, imports screened eyed ova from Norway for hatching, on growing then shipping to Scotland
- Unusual behaviour including lethargy and inappetence and mortalities started end September 2021
- Daily mortalities of 1-3% reaching up to a total of 30%
- Across 4 separate units holding different life stages
- Mortality began in pre nursery (unit 3), fish were transferred to the nursery (unit 4) and mortalities continued there. Mortalities then followed in the ongrowing (unit 1) and hatchery (unit 2).

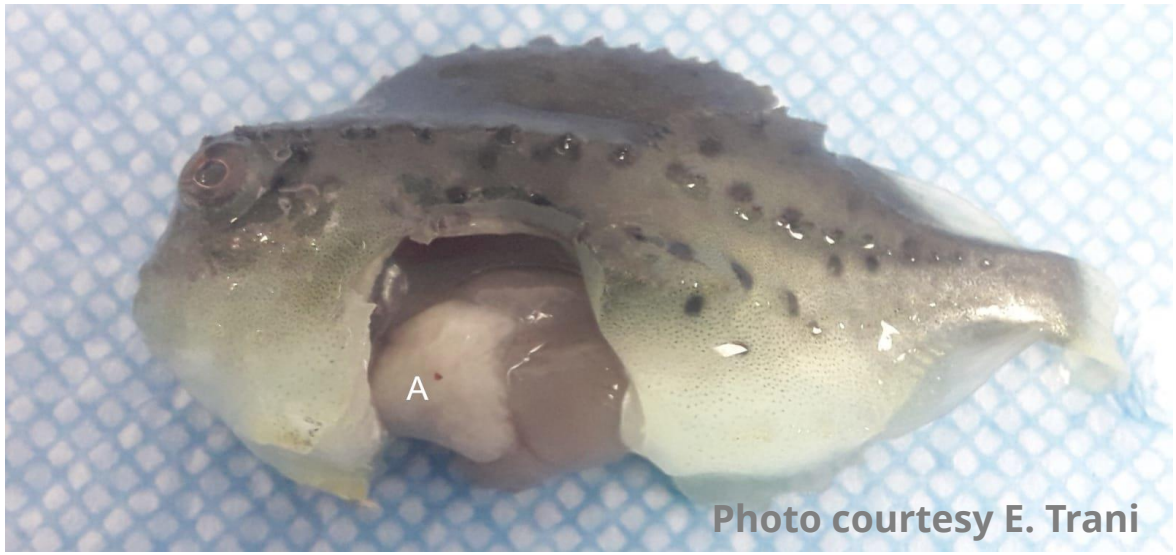
- Fish size and stocking densities were abnormally high
- Pre nursey had suffered from blocked filter and increased dissolved organic content

- Company veterinarian observed pale livers, lack of food in GI tract
- Suspected infection with CluV, presumptive diagnosis following positive RT-qPCR for CluV

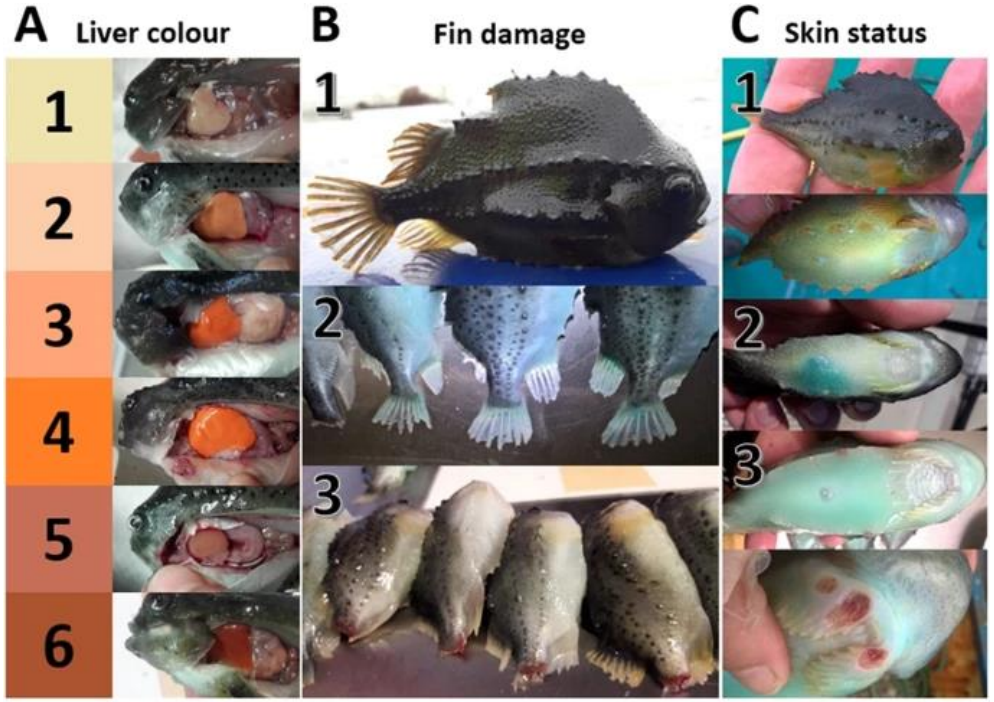
Mortality event

- FHI informed 1st Nov 2021
- 2nd Nov - full disease screen to rule out listed diseases (esp. VHSV) and confirm CluV
  - Histopathology, virology, bacteriology
  - Included kidney and liver target tissues amongst others
  - BF-2, EPC, CHSE-214 and E-11 cell lines, incubated at 15°C, 20°C and 25°C
  - Tested for VHSV, IHNV, IPNV, ISA, SAV, NNV, and CLuV
  - (Lumpfish totivirus and coronavirus not tested for)

Gross pathology

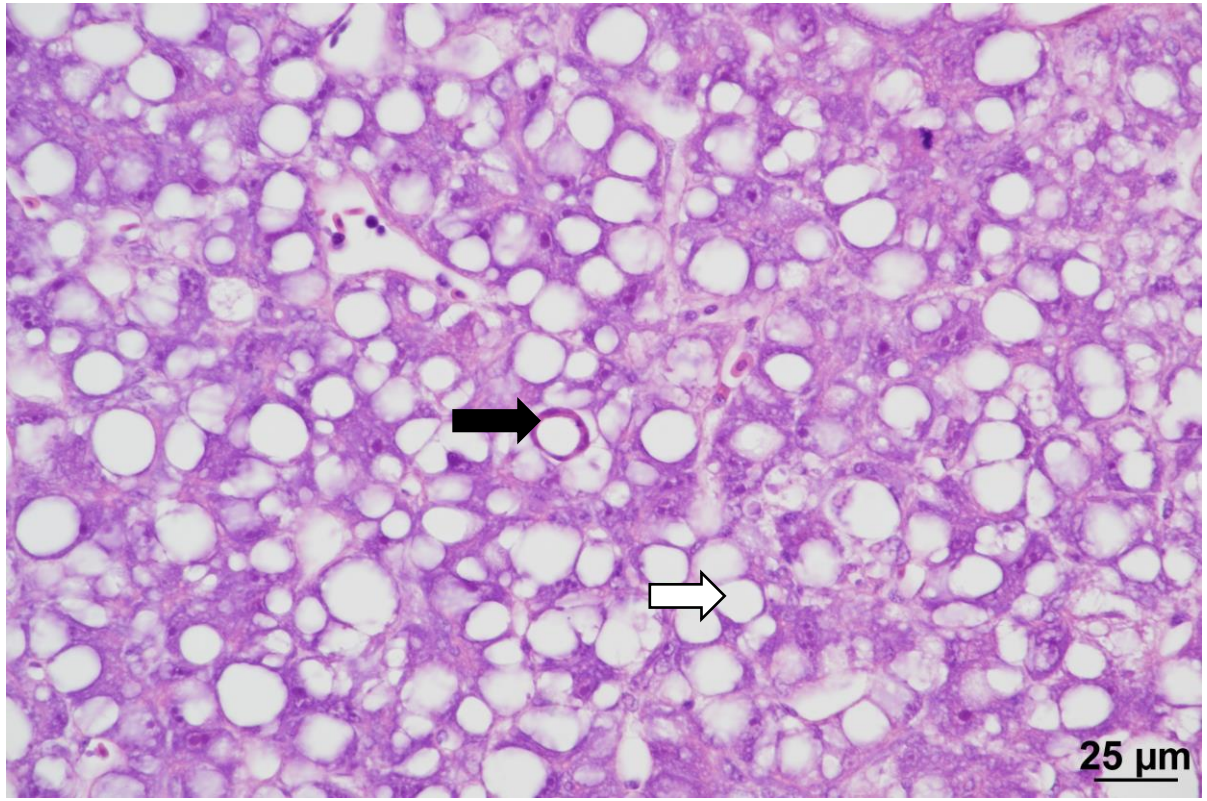


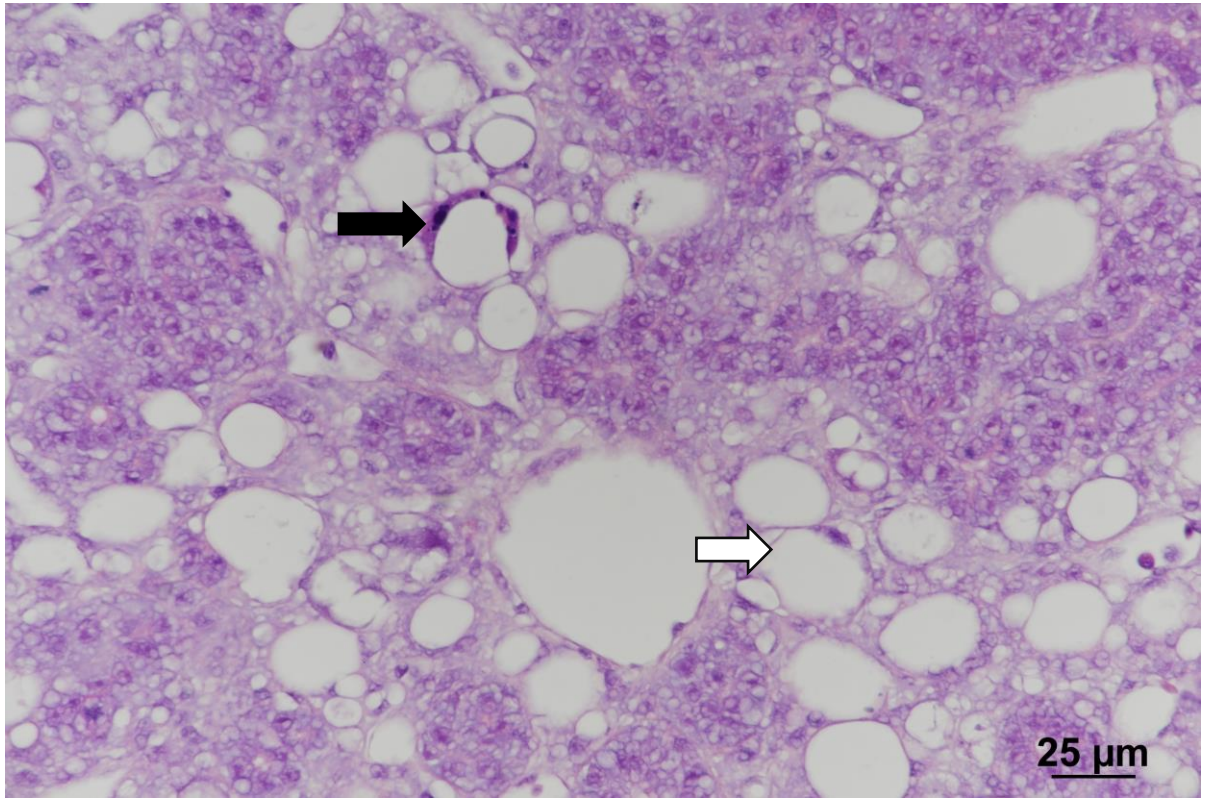
Enlarged, pale liver
Empty GI tract



Liver colour and health & welfare condition
From Eliasen et al 2020

Histopathology

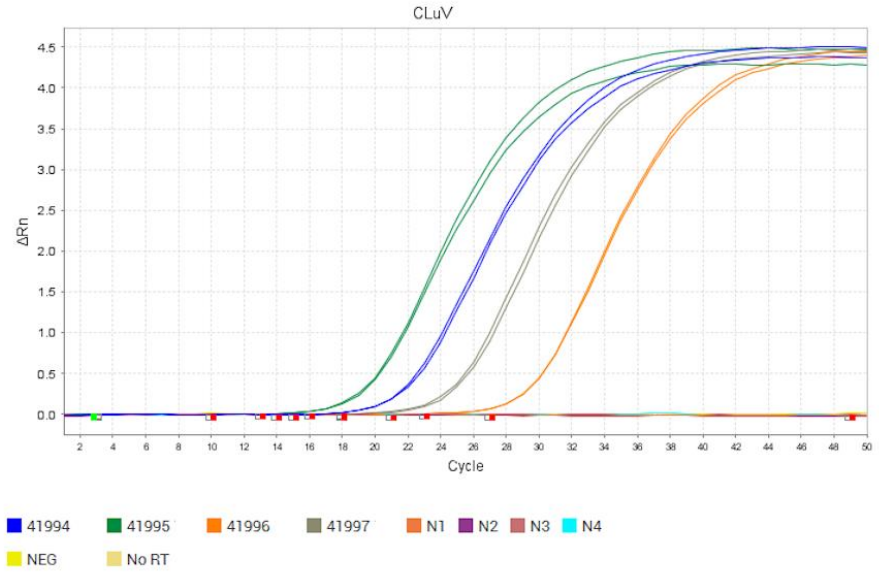




Apoptosis and macrovesicular steatosis

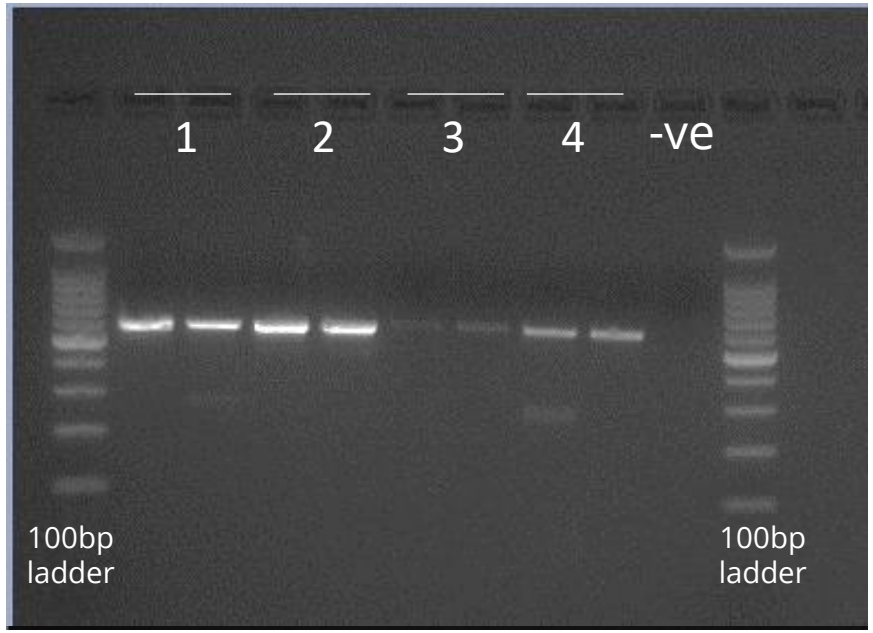
Molecular Diagnostics

- Listed diseases – EU/OIE protocols – all negative
- CLuV RT-qPCR (Skoge et al., 2018, - envelope protein)
  - All 4 units positive for CluV
  - Ct values typically ranged between 15 and 34,
  - occasionally as low as 9



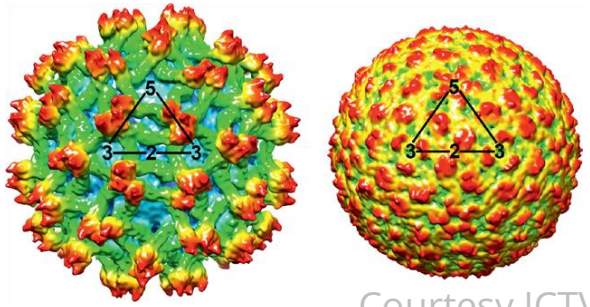
Molecular Diagnostics

- Conventional RT-PCR and sequencing for confirmation
- In-house designed assay to - NS5 polymerase gene
- 99.63% nucleotide identity to NS5 polymerase gene region of NC_040555.1 whole genome (submitted by Haugland et al., 2019).
- Two synonymous mutations
- Pending publication, for primer and assay information please contact david.stone@cefas.co.uk



568bp product

Cyclopterus lumpus virus (CLuV)



Courtesy ICTV

- Member of the family *Flaviviridae*
- Flaviviruses are; single-strand, positive-sense, RNA viruses
- Genome of approx. 9 to 13kbp long, which serves as both genome and viral mRNA
- Mosquito vectored Yellow fever, Dengue, Zika

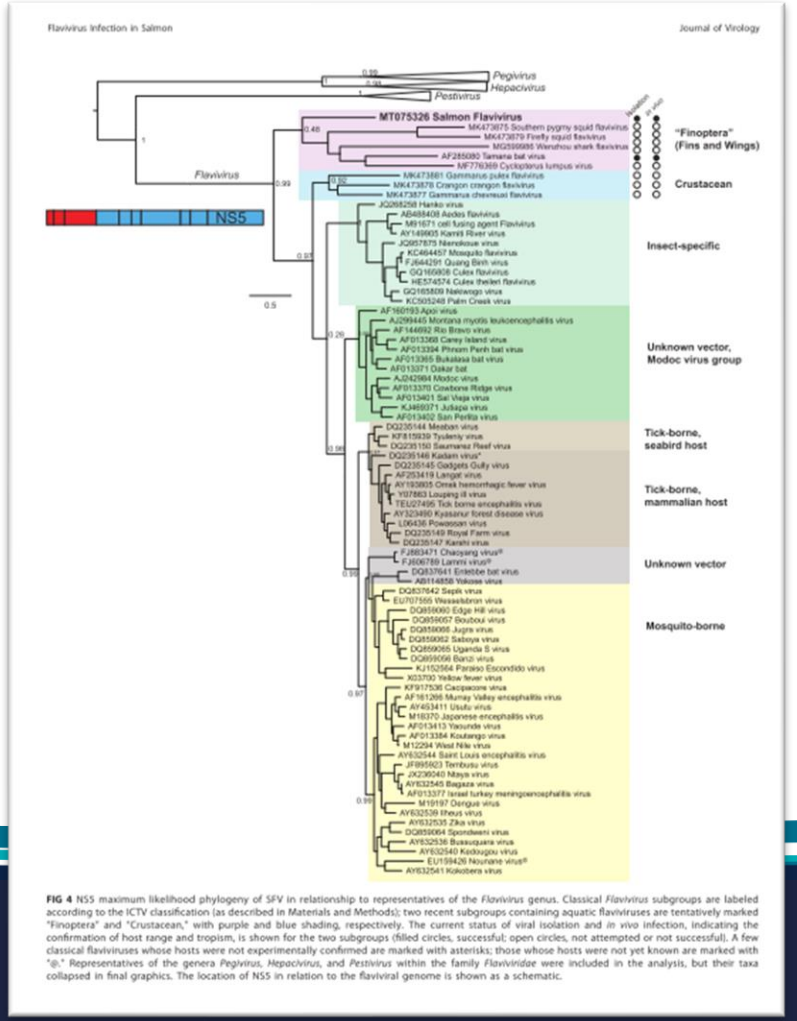
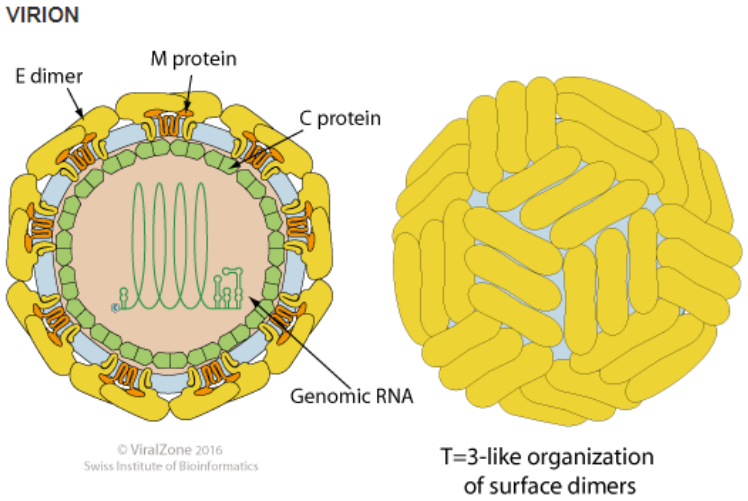
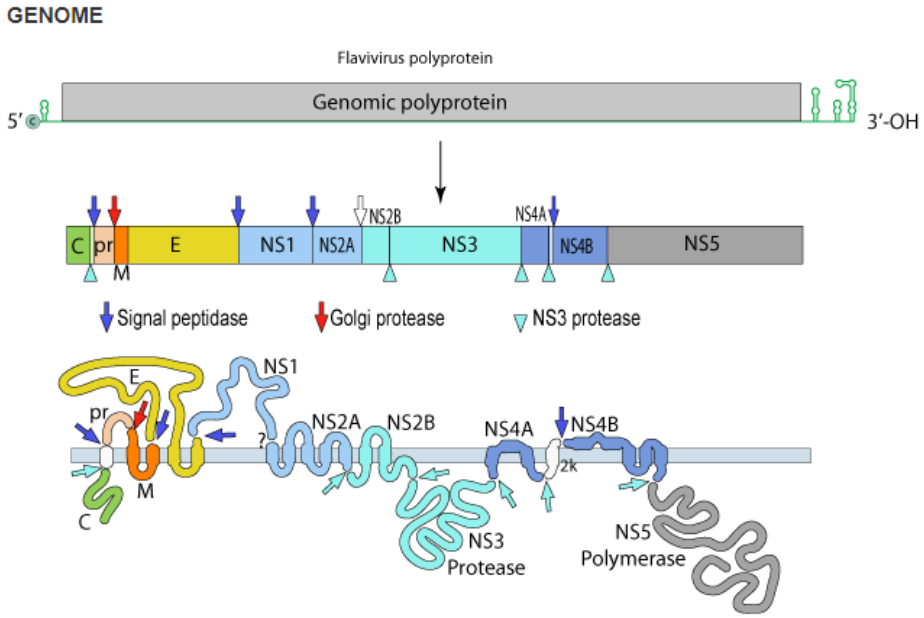


FIG 4 NS5 maximum likelihood phylogeny of SFV in relationship to representatives of the *Flavivirus* genus. Classical *Flavivirus* subgroups are labeled according to the ICTV classification (as described in Materials and Methods); two recent subgroups containing aquatic flaviviruses are tentatively marked "Finoptera" and "Crustacean," with purple and blue shading, respectively. The current status of viral isolation and *in vivo* infection, indicating the confirmation of host range and tropism, is shown for the two subgroups (filled circles, successful; open circles, not attempted or not successful). A few classical flaviviruses whose hosts were not experimentally confirmed are marked with asterisks; those whose hosts were not yet known are marked with "⊕." Representatives of the genera *Pegivirus*, *Hepacivirus*, and *Pestivirus* within the family *Flaviviridae* were included in the analysis, but their taxa collapsed in final graphics. The location of NS5 in relation to the flaviviral genome is shown as a schematic.

Flaviviridae



Enveloped, spherical, ~50nm diam., iscoahedral-like surface protein structure



Monopartite, linear, ssRNA(+) genome of about 9.7-12 kb. The genome 3' terminus is not polyadenylatec but forms a loop structure. The 5' end has a methylated nucleotide cap (to allow translation) or a genome-linked protein (VPg).

GENE EXPRESSION

The virion RNA is infectious and serves as both the genome and the viral messenger RNA. The whole genome is translated into a polyprotein, which is processed co- and post-translationally by host and vira proteases.

REPLICATION

CYTOPLASMIC

Courtesy Viral zone

Aquatic flaviviruses

- Only recently discovered in fish

Archives of Virology (2018) 163:679–685
https://doi.org/10.1007/s00705-017-3643-3

BRIEF REPORT

New virus of the family *Flaviviridae* detected in lumpfish (*Cyclopterus lumpus*)

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Abstract
In this study, we determined the complete coding sequence of a putative new member of the family *Flaviviridae*, named "Cyclopterus lumpus virus" (CLuV), which is associated with a serious disease in lumpfish (*Cyclopterus lumpus*). The virus was present in all tissues tested, but pathology was primarily observed in the liver and kidneys. CLuV shows low but distinct similarity to the unassigned Tamana bat virus (TABV). Unlike other known members of the family *Flaviviridae*, translation of the entire CLuV polyprotein is dependent on a − 1 ribosomal frameshift in the NS2A region.

The copepod parasite *Lepeophtheirus salmonis* is a cause of major expense in the production of farmed Atlantic salmon (*Salmo salar*) in Norway [1, 2]. One widely used approach to control this parasite is the use of commercially farmed or wild cleaner fish, i.e., wrasse species and lumpfish (*Cyclopterus lumpus*) [3, 4]. A range of different pathogens have been detected in wild and farmed lumpfish, including viruses, bacteria and parasites [5–14]. In 2015, a new disease emerged in culture facilities for lumpfish, resulting in more than 50% mortality among young fish. Preliminary studies of these lumpfish showed the presence of some bacteria and parasites, but also a pathology that could not be attributed to any known pathogens. To gain more knowledge about this emerging disease, total RNA was isolated from the most strongly affected tissues (liver and kidney) of one lumpfish, and the RNA was used for Illumina sequencing. The resulting RNA sequences included the complete coding sequence of a putative new virus of the family *Flaviviridae*. The new member of the *Flaviviridae* presented in this study, "Cyclopterus lumpus virus" (CLuV), shows some similarity to Tamana bat virus (TABV) but only a distant relationship to members of existing genera. This study describes the genome of CLuV and the histopathology associated with the newly reported disease.

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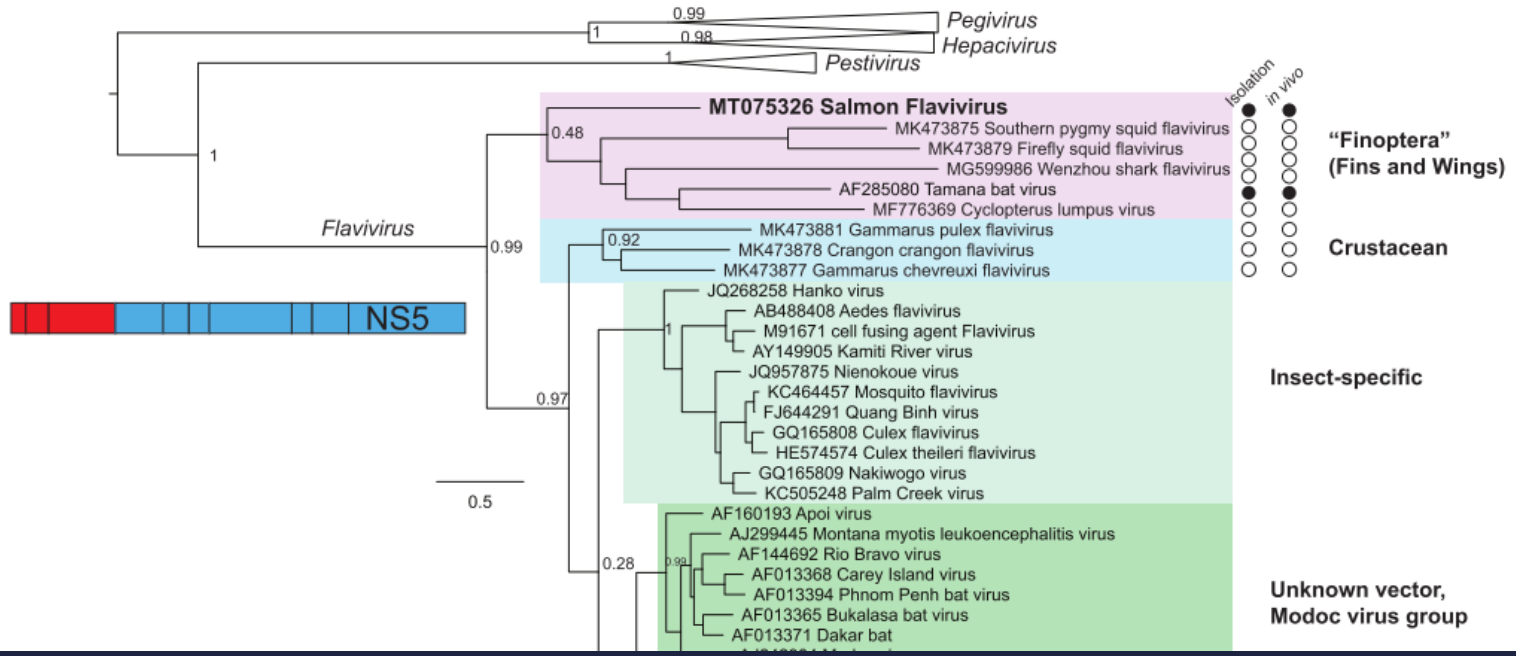
Lumpfish suffering from disease at a cultivation site in Western Norway were delivered live to the Fish Diseases Research Group at the Department of Biology, University of Bergen. A distinct finding during the dissection of the lumpfish was a pale and firm liver. Samples were taken from the gills, heart, liver, kidney and central nervous system (CNS) and stored in ethanol and in a modified Karnovsky fixative at 4 °C, or stored fresh at -85 °C. The fresh tissues were used for RNA extraction while the fixed tissues were processed and sectioned as described previously [15] and used for histology. Examination of the tissues from the moribund lumpfish revealed massive degeneration in the liver (Fig. 1A and B). The liver changes were associated with accumulation of large lipid droplets, which was also seen in the gills and kidney, but to a lesser extent (Fig. 1C and D).

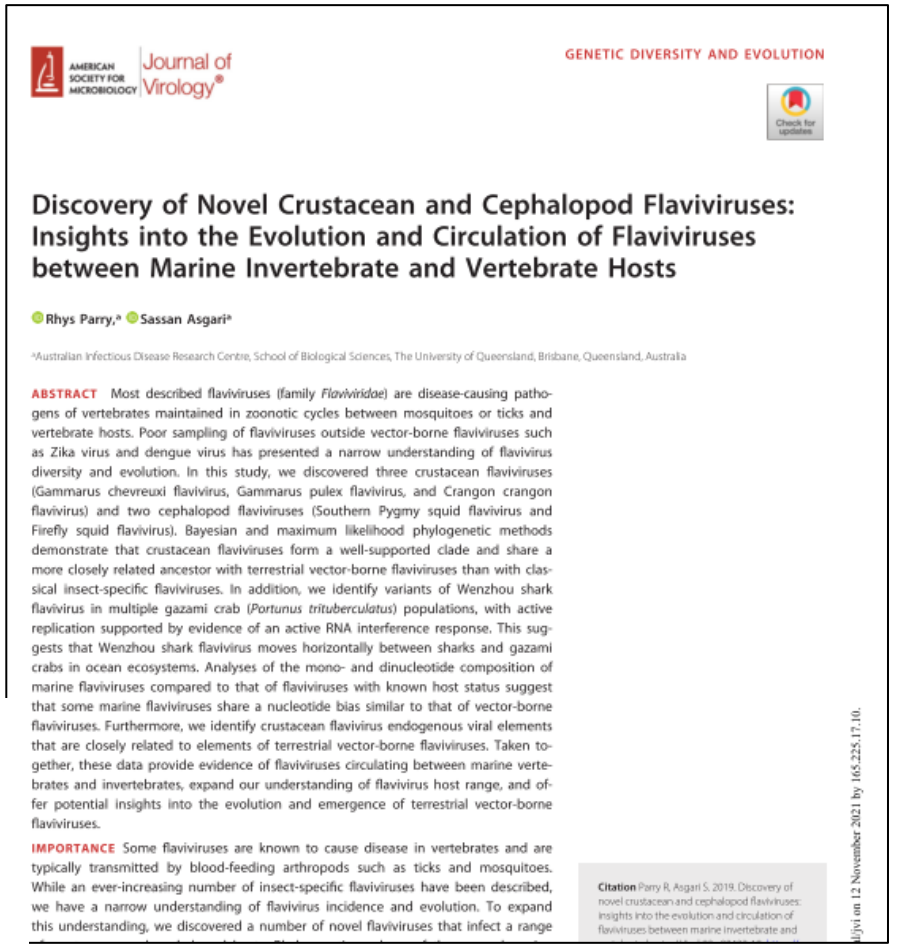
Attempts to cultivate the virus in CHSE-14 [16] and ASK [17] cells using sterile-filtered homogenates from the infected lumpfish as described previously [18] were unsuccessful. Therefore, infection studies using purified viruses could not be conducted and, hence, it could not be shown that CLuV was the cause of the described disease.

To determine the genome sequence of CLuV, liver and kidney samples from one lumpfish suffering from the putative new disease were homogenized in 1 ml of TRI Reagent® (Sigma-Aldrich) with a 5-mm bead for 7 min at 50 Hz in a TissueLyser LT (QIAGEN). Total RNA was extracted using a previously described protocol from [17], and one batch was kept at the laboratory for Sanger sequencing, while the other batch was sent to BaseClear for next-generation sequencing. Paired-end sequence reads (number of reads = 34,488,032) were generated using an Illumina

Aquatic flaviviruses

- Only recently discovered in fish
- But increasing diversity
  - Shark, crustacea, cephalopod molluscs, Chinook salmon

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GENETIC DIVERSITY AND EVOLUTION

Discovery of Novel Crustacean and Cephalopod Flaviviruses: Insights into the Evolution and Circulation of Flaviviruses between Marine Invertebrate and Vertebrate Hosts

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ABSTRACT Most described flaviviruses (family *Flaviviridae*) are disease-causing pathogens of vertebrates maintained in zoonotic cycles between mosquitoes or ticks and vertebrate hosts. Poor sampling of flaviviruses outside vector-borne flaviviruses such as Zika virus and dengue virus has presented a narrow understanding of flavivirus diversity and evolution. In this study, we discovered three crustacean flaviviruses (Gammarus chevreuxi flavivirus, Gammarus pulex flavivirus, and Crangon crangon flavivirus) and two cephalopod flaviviruses (Southern Pygmy squid flavivirus and Firefly squid flavivirus). Bayesian and maximum likelihood phylogenetic methods demonstrate that crustacean flaviviruses form a well-supported clade and share a more closely related ancestor with terrestrial vector-borne flaviviruses than with classical insect-specific flaviviruses. In addition, we identify variants of Wenzhou shark flavivirus in multiple gazami crab (*Portunus trituberculatus*) populations, with active replication supported by evidence of an active RNA interference response. This suggests that Wenzhou shark flavivirus moves horizontally between sharks and gazami crabs in ocean ecosystems. Analyses of the mono- and dinucleotide composition of marine flaviviruses compared to that of flaviviruses with known host status suggest that some marine flaviviruses share a nucleotide bias similar to that of vector-borne flaviviruses. Furthermore, we identify crustacean flavivirus endogenous viral elements that are closely related to elements of terrestrial vector-borne flaviviruses. Taken together, these data provide evidence of flaviviruses circulating between marine vertebrates and invertebrates, expand our understanding of flavivirus host range, and offer potential insights into the evolution and emergence of terrestrial vector-borne flaviviruses.

IMPORTANCE Some flaviviruses are known to cause disease in vertebrates and are typically transmitted by blood-feeding arthropods such as ticks and mosquitoes. While an ever-increasing number of insect-specific flaviviruses have been described, we have a narrow understanding of flavivirus incidence and evolution. To expand this understanding, we discovered a number of novel flaviviruses that infect a range

Citation Parry R, Asgari S. 2019. Discovery of novel crustacean and cephalopod flaviviruses: insights into the evolution and circulation of flaviviruses between marine invertebrate and

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Aquatic flaviviruses

- Only recently discovered in fish
- But increasing diversity
  - Shark, crustacea, cephalopod molluscs, Chinook salmon
- Significance in disease relatively poorly understood
- Research tools limited
- Epidemiological parameters similarly scarce

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First Isolation of a Novel Aquatic Flavivirus from Chinook Salmon (*Oncorhynchus tshawytscha*) and Its *In Vivo* Replication in a Piscine Animal Model

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ABSTRACT The first isolation of a flavivirus from fish was made from moribund Chinook salmon (*Oncorhynchus tshawytscha*) from the Eel River, California, USA. Following the observation of cytopathic effect in a striped-snakehead fish cell line, 35-nm virions with flaviviral morphology were visualized using electron microcopy. Next-generation sequencing and rapid amplification of cDNA ends obtained the complete genome. Reverse transcriptase quantitative PCR (RT-qPCR) confirmed the presence of viral RNA in formalin-fixed tissues from the wild salmon. For the first time, *in vivo* replication of an aquatic flavivirus was demonstrated following intracoelomic injection in a Chinook salmon model of infection. RT-qPCR demonstrated viral replication in salmon brains up to 15 days postinjection. Infectious virus was then reisolated in culture, fulfilling Rivers' postulates. Only limited replication occurred in the kidneys of Chinook salmon or in tissues of rainbow trout (*Oncorhynchus*

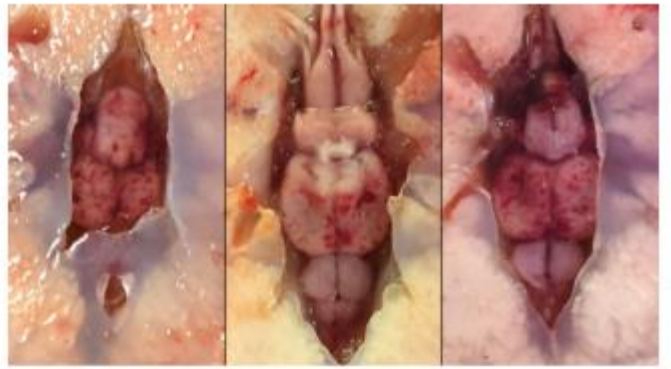

FIG 1 Examination of moribund Chinook salmon (*Oncorhynchus tshawytscha*) observed congregating along the banks of the lower Eel River, California, revealed petechial hemorrhages on the surfaces of the forebrain, optic lobes, cerebellum, and brain stem. The gross findings prompted investigation of a potential viral etiology as a cause of the clinical signs observed.

Citation Soto E, Camus A, Yun S, Kurobe T, Leary JH, Rosser TG, Dill-Okubo JA, Nyaoke AC, Adkison M, Renger A, Ng TFF. 2020. First isolation of a novel aquatic flavivirus from chinook salmon (*Oncorhynchus tshawytscha*) and its in vivo replication in a piscine animal model. J Virol 94:e00337-20. https://doi.org/10.1128/JVI.00337-20.

Editor Colin R Parrish, Cornell University

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Control

- Findings relayed to Aquatic Animal Health Policy Group (Defra, CVO).
- Decision to facilitate control by the industry (rather than emerging disease notification), given:
  - Time since initial discovery (EAFP, Tampere 2013 ?)
  - Understanding that CluV is reasonably prevalent in Norway, has been detected in Scotland (grey literature)
  - Robust screening process for lumpfish exports is in place by the industry involved
  - Discussion with receiving competent authority in Scotland
  - Intention to disseminate information by other means
- Initial designation removed once listed diseases not detected, with restriction on moving fish until some time after mortality had returned to base levels

Second detection

- In a linked facility in North Wales, also RAS
- Had received ~1million 1g fry from the Dorset site in early September (before mortalities started) from the same egg import.
- Mortality commenced late Dec 2022, vet notified Jan 2022 once 7d av. mortality exceded 0.8% per day (0.1-0.4% /d is typical baseline), daily rate peaked at 1.8% per day, totalled just over 10%
- FHI notified and visited 28th Jan 2022
- Gross pathology limited to liver, all else unremarkable
- CluV detected, identical sequence to index virus, no other apparent disease
- Environmental and management factors again likely played a role
  - fish had been graded on 10th Dec and vaccinated on 21st Dec
  - biofilter not fully mature – ammonia levels were elevated above high threshold of 0.4 mg/L for 8 of the preceding 10 days.
- Outbreak restricted to this one batch of nursery fish – hatchery, grow out and harvest holding tanks not affected.

Summary

- CluV detected (at high levels in some fish), despite recovering stocks
- Negative for all other diseases tested
- No significant bacterial infections
- No replication in available cell lines
- Role of environmental/husbandry conditions uncertain but likely
- Limited epidemiological data (endemicity unknown)

- **Future work**
- Publication (submission imminent)
- Establishment of additional susceptible cell lines (liver, kidney)
- Epidemiological survey – prevalence, geographical distribution, viral diversity
- Supporting vaccine development
- Using samples as model to test selective nanopore sequencing