First detection of Infectious Spleen and kidney Necrosis Virus (ISKNV) associated with massive mortalities in farmed tilapia in Africa

Short running tittle: First detection of ISKNV in farmed tilapia in Africa

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Abstract

In late 2018, unusual patterns of very high mortality (>50% production) were reported in intensive tilapia cage culture systems across Lake Volta in Ghana. Affected fish showed darkening, erratic swimming and abdominal distension with associated ascites. Histopathological observations of tissues taken from moribund fish at different farms revealed the presence of lesions indicative of viral infection. These included haematopoietic cell nuclear and cytoplasmic pleomorphism with marginalisation of chromatin and fine granulation. Transmission electron microscopy showed tilapia cells contained conspicuous virions with typical Iridovirus morphology i.e. enveloped, with icosahedral and or polyhedral geometries and with a diameter c.160 nm. PCR confirmation and DNA sequencing identified the virions in the tissues of the diseased fish as Infectious Spleen and Kidney Necrosis Virus (ISKNV). Samples of fry and older animals were all strongly positive for the presence of the virus by qPCR. All samples tested negative for TiLV and Nodavirus by qPCR. All samples collected from farms in the year prior to the mortality event were negative for ISKNV. The results suggest that ISKNV was the cause of disease on the investigated farms and likely had a primary role in the mortality events. A common observation of coinfections with *Streptococcus agalactiae* and other tilapia bacterial pathogens further suggests that these may interact to cause severe pathology, particularly in larger fish. The present results demonstrate that there are a range of potential threats to the sustainability of tilapia aquaculture that need to be guarded against.

Keywords: Tilapia; Africa; Tilapia Viral Diseases; fish virus; Infectious Spleen and Kidney Necrosis Virus; ISKNV;

Introduction

The farming of tilapia species (*Oreochromis* spp.) is one of the most important sectors in aquaculture worldwide with total global production estimated at more than 6 686 000 tonnes in 2016 (1). In Africa, production is still dominated by Egypt with more than 1 000 000 tonnes produced in 2017 (1). However, tilapia culture has become increasingly important in several other African countries, where it boosts the local economy and constitutes an affordable source of animal protein for human consumption.

In Ghana, Nile tilapia production in 2016 had reached more than 50 000 tonnes, from only 2 000 tonnes per year in 2006 (2) with more than 90% of the production derived from high stocking density floating cage systems in Lake Volta. However, as production systems have intensified in the area, the industry has been increasingly affected by a range of disease issues (3, 4).

In 2017 (3) conducted the first comprehensive disease investigation in tilapia farmed in Lake Volta Ghana. *Streptococcus agalactiae* multilocus sequence type 261 was shown to be a major cause of mortality for farmed Nile tilapia and a range of other bacterial and parasitic pathogens including *Aeromonas* spp., *Streptococcus iniae*, *Flavobacterium columnare* and a *Myxobolus* sp. were also detected.

Jansen et al. (4) conducted follow up studies, including a broad-ranging epidemiological investigation and suggested that mortalities caused by bacteria in Lake Volta were not a major concern for the local economy as farmers had managed to sustain losses by increasing production of fingerlings, treatment with antibiotics and use of autogenous vaccines.

Historically, bacterial infections were the major threat for the health of farmed tilapia (5). However, in recent years a number of viral diseases have emerged worldwide with devastating effects for the industry(6). Tilapia Lake Virus (TiLV) is considered the main viral challenge to

tackle, as it has spread to many producing countries, causing high mortalities in all production stages. Although TiLV has been detected elsewhere in Africa (7), the initial study by (3), diagnostic investigations undertaken by Ridgeway Biologicals Ltd. (Ramirez *et al*. unpublished data), or the more recent survey undertaken by (4), all failed to provide any evidence of TiLV in diseased tilapia reared in Lake Volta. Up until September 2018, with the exception of the detection of a nodavirus sequence by (3), no significant association between viral agents and tilapia mortality events have been demonstrated in Ghana.

From September 2018 to March 2019 outbreaks of disease with very high levels of morbidity and mortality (60-90%), were experienced in both vaccinated and unvaccinated tilapia by farmers in Lake Volta. In late-September 2018, a farm located below the lower dam in the region of Asutsuare, was the first to suffer episodes of massive acute mortalities (https://goo.gl/LmqbG2 and https://bit.ly/2NwDEbD). Approximately a week after the first report, a second farm located in the Akuse region (~5 km upstream of Asutsuare, but still below the lower dam) also experienced acute mortalities. By mid-October, multiple floating cage-based farms in the Dodi region (above the upper dam) reported losses of more than 10 tonnes per day https://goo.gl/yj4oT4. In late November, farmers that had been unaffected in the Asikuma region (downstream of Dodi but still above the upper dam) also started to suffer episodes of massive acute mortality. (**Figure 1**).

By the end of 2018, most tilapia farmers in Lake Volta had reported mortalities that they were not able to contain by increased production of fingerlings or treating with antibiotics (**Supplementary File 1**, https://bit.ly/2KZFFuw and https://goo.gl/cPmpSE). We report the results of a comprehensive disease investigation, conducted at two affected farms from two different regions of Lake Volta, to gain insights into the causes of these mortalities.

Figure 1 Map of lower region of Lake Volta in Ghana, West Africa. Red triangles indicate the regions in chronological order (A to D) where the outbreaks of mortality occurred.

2 Materials and Methods

2.1 Sampling

Farm 1, a medium size (approximately 600-800 tonnes per annum production) cage culture operation on upper Lake Volta, was visited on the 18/10/18. Samples from 10 fish (average weight 200g) were taken for bacteriology. In addition five sets of samples containing liver and brain from fish from different cages were collected for molecular diagnostics as detailed in **Table 1**.

Farm 2, a large cage culture operation (>2000 tonnes per annum), was visited for sampling on 28/11/18, 17/12/18 and 20/02/19. For the first two visits, moribund fish between 40-646g collected from cages on the main lake were sampled. In the first visit for bacteriology and virology on the second visit for virology only. For the third visit, responding to reports that they were now experiencing very heavy mortalities in their fry production units (>70%), samples of moribund fry and juveniles from both nursery cages on the main lake and from their onshore hatchery supplied with water pumped from the main lake, were analysed for virology. From this farm material was also taken for histological and molecular diagnostic investigations during the visits as detailed in **Table 1 and Supplementary File 2**.

All moribund fish from the visited farms were humanely euthanised with a lethal overdose of tricaine methanesulfonate 1,000 mg/g (Pharmaq, Hampshire, UK) followed by brain destruction prior to the necropsy.

2.2 Bacteriology

Samples for bacteriology were collected from the brain, liver, kidney and spleen with sterile cotton swabs and inoculated onto tryptone soya agar (TSA), Columbia blood agar (CBA),

109 Tryptone yeast extract salts agar (TYES) (Southern Group Laboratory, Corby, UK) and
110 cystine heart agar with 2% bovine haemoglobin (CHAH) (Becton Dickinson, Oxford, UK).

111 All inoculated agar plates were incubated at 28 °C for 24-72 hours. Colonies assessed as
112 significant based on occurrence and dominance were subcultured to purity on similar media.
113 Pure relevant isolates were initially identified by morphology and Gram staining. The partial
114 16S rRNA genes of the Gram negative isolates identified were PCR amplified and sequenced
115 using the method described by (8). Gram positive cocci forming chains were screened using a
116 *Streptococcus agalactiae* specific capsular typing multiplex PCR developed by (9). The
117 Gram negative isolates strains confirmed as *Aeromonas* spp. based on partial 16S rRNA gene
118 sequence analysis were further characterised based on partial *gyrB* sequencing analysis for
119 identification at the species level as described by (10).

120 **2.3 Histopathology and Electron Microscopy**

121 Tissues were fixed in neutral buffered formalin (NBF) for a minimum of 24 hr before being
122 placed in glycerol diluted 50:50 with phosphate buffered saline (PBS) for transportation to
123 Cefas. On receipt, tissues were rinsed in 70% alcohol and placed again in NBF for a final
124 period of fixation prior to processing using standard protocols in a vacuum infiltration
125 processor. Tissue sections were cut at a thickness of 3-4 µm on a Finnese® microtome,
126 mounted on glass slides and stained with haematoxylin and eosin using an automated staining
127 protocol. Stained sections were examined for general histopathology by light microscopy
128 (Nikon Eclipse E800). Digital images and measurements were obtained using the Lucia™
129 Screen Measurement software system (Nikon, UK).

130 For electron microscopy, small samples of tissues fixed in NBF as above were rinsed three
131 changes of 0.1 M sodium cacodylate buffer, followed by post fixation in 2.5% glutaraldehyde

in the same buffer for 1 hour prior to a second post fixation for 1 hour in 1 % osmium tetroxide in 0.1 M sodium cacodylate buffer. Subsequently, fixed tissues were dehydrated in an ascending acetone series acetone series and embedded in epoxy resin 812 (Agar Scientific pre-Mix Kit 812, Agar scientific, UK) and polymerised at 60 °C overnight. Semi-thin (1 µm) survey sections were stained with 1 % Toluidine Blue and examined by light microscope to identify areas of interest. Ultrathin sections (70-90 nm) of the targeted areas were placed on uncoated copper grids and stained with uranyl acetate and Reynold's lead citrate (Reynolds 1963). Grids were examined using a JEOL JEM 1400 transmission electron microscope and digital images captured using a GATAN Erlangshen ES500W camera and Gatan Digital Micrograph™ software.

2.4 Molecular diagnosis (viral and bacterial detection)

The samples collected for molecular diagnosis were washed twice in 750µl of sterile 1x PBS to remove the RNA-*later*® and homogenised. Total nucleic acids were extracted using nanomagnetic beads i.e. Genesig Easy DNA/RNA Extraction Kit (Primerdesign, Southampton, UK) and stored until further use.

Multiplex PCR for detection of Streptococcus spp.

Nucleic acids extracted were used as a template on a multiplex PCR (unpublished data) to confirm the presence of *Streptococcus* spp. that had been previously reported as fish pathogens including *Streptococcus agalactiae* (11), *Streptococcus uberis* (12) and *Streptococcus dysgalactiae* (13).

qPCR for detection of TiLV and Nodavirus

Nucleic acids were used for the detection of tilapia lake virus and nodavirus by quantitative PCR using the commercial kits: Path-TiLV-EASY and Path-Betanodavirus-EASY

(Primerdesign, Southampton, UK) in the platform Genesig q16® (Primerdesign, Southampton, UK) as per the protocols suggested by the manufacturer.

Conventional PCR for detection of megalocytiviruses

The Office International des Epizooties (OIE) reference PCR protocol for notifiable aquatic megalocytiviruses (Red Seabream Iridoviral disease / Infection spleen and kidney necrosis virus (14, 15)) with the modification proposed by (16) was initially used, to screen the samples of fish collected from visit 2 at Farm 2. For this, genomic DNA was extracted as follows: the RNA-*later*® was removed and the tissue samples weighed. Depending on the weight of the tissue available the samples were diluted in RTL buffer (Qiagen) to provide either a 1:10 w/v or a 1:5 w/v and homogenised per fish i.e. all the organs of each fish into an individual pool using Matrix A and the FastPrep apparatus (MP Biomedicals). Following homogenisation, the samples were diluted further with RTL buffer to give a 1:60 w/v homogenate and total nucleic acid was extracted from 300 µl of the clarified sample using the RNA tissue mini kit without DNase (Qiagen) and eluted in a 60 µl volume.

RT was performed at 37°C for 1 h in a 20 µl volume consisting of 1× M-MLV RT reaction buffer (50 mM) Tris pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM MgCl2) containing 1 mM dNTP, 100 pmol random primers, 20 U M-MLV reverse transcriptase (Promega, Southampton UK) and 4µl of the nucleic acid extracted above.

PCR was performed in duplicate in a 50 µl reaction volume with 2.5 µl of cDNA of total nucleic acid, 25 mM dNTPs, 1 x GoTaq® buffer (2.5 mM MgCl2 solution), 5 pmol of each primer (C1105 5′-GGTTCATCGACATCTCCGCG-3’ and C1106 5′-AGGTCGCTGCGCATGCCAATC-3’) and 1.25 units of GoTaq® DNA polymerase (Promega). The cycling conditions were as follows: 40 temperature cycles (1 min at 95°C, 1

178 min at 55°C and 1 min at 72°C) after an initial denaturing step (5 min at 95°C) followed by a
179 final extension step of 10 min at 72°C.

180 Amplified products were electrophoresed in a 2% (w/v) agarose/TAE (40 mM Tris-acetate,
181 pH 7.2, 1 mM EDTA) gel containing 1.0 μg ml-1 ethidium bromide at 120 v for 30 mins and
182 viewed under UV light.

183 PCR products were excised from the gel and the DNA was extracted and purified by ethanol
184 precipitation. Both strands of the PCR product were sequenced using the ABI PRISM Big
185 Dye Terminator v3.1 cycle sequencing kit and the same primers used for the amplification.
186 The forward and reverse sequences were aligned and a consensus sequence generated using
187 the CLC software (Qiagen). Generated consensus sequences were compared with sequences
188 from GenBank using BLASTN (17) and aligned using the MUSCLE application of the
189 MEGA software version 6 (18).

190 In addition, the OIE recommended PCR protocol for notifiable aquatic megalocytiviruses
191 (14) developed by (15) was used was used to screen total nucleic acids extracted from the rest
192 of the samples fixed for viral molecular analyses.

193 *qPCR for detection and quantification of megalocytiviruses*

194 The amount of virus present in the samples was also investigated by qPCR. For this the
195 homogenised tissues were subjected to total nucleic acids extraction (~20mg of each organ)
196 using the Genesig Easy DNA/RNA Extraction Kit (Primerdesign) as described earlier. The
197 extracted nucleic acids were tested using the commercial kit Path-ISKNV-EASY
198 (Primerdesign, Southampton, UK) in the platform Genesig q16® (Primerdesign) as per the
199 manufacturer instructions. This detects both red sea bream iridovirus and ISKNV variants. In

all cases fish were individually analysed either by pooling liver, spleen and brain or screening individual tissues.

Retrospective analyses of archived samples by qPCR

A total of 16 samples of archived tissue homogenates from 5 different farms (that included Farms 1 and 2) were retrospectively screened for ISKNV by qPCR with the commercial kit Path-ISKNV-EASY as described before. From these 7 had been collected during 2017 and the rest in March 2018 (**Supplementary File 5**). All the samples had been previously confirmed as negative for TiLV and Nodavirus using the commercial kits Path-TiLV-EASY and Path-Betanodavirus-EASY.

3 Results

3.1 Farm visits

On the first visit to Farm 2 on the 27/11/18, farm staff reported very high and ongoing mortalities (Figure 2) in fish bigger than 20g, including broodstock, but no significant losses in fingerlings. Losses reportedly peaked at about 670 crates (equating to approximately 40 tonnes per day) shortly after this visit on the 2/12/2018 **(Figure 2**).

Losses were so severe that accurate estimation was not possible, with more than 50 additional labourers recruited locally just to remove dead and moribund fish during the peak period. By the second visit to Farm 2, losses of ongrowing fish had reportedly declined back to the background 10-20 crates per day more typically observed i.e. less than 1-2 tonnes per day (**Figure 2**).

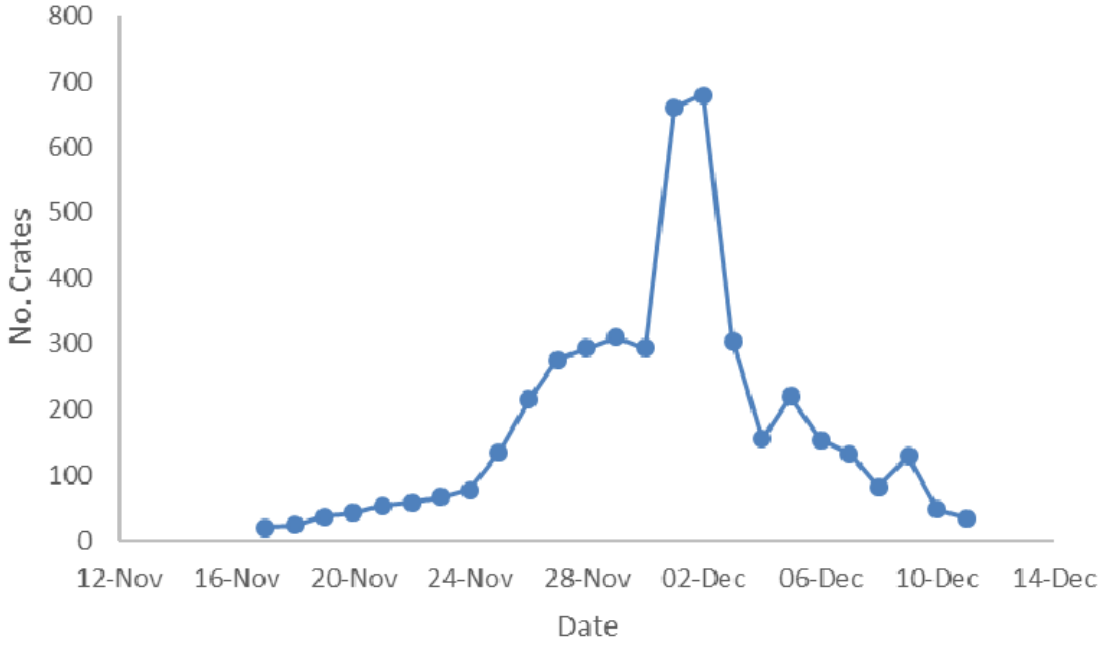


Figure 2. Number of crates of dead/ moribund fish removed from Farm 2 during period of maximum losses. Daily mortalities were estimated based on numbers of crates of rejected ongrowing tilapia collected each day by farm staff. Each crate typically contained approximately 60kg tilapia collected from the cages and rejected because they were either dead, moribund or displayed other adverse signs that prevented their sale.

During the first two visits to the farms, but particularly to Farm 2, diseased fish were observed swimming away from the school with erratic swimming i.e. on one side, in circles, lethargic, with no equilibrium, upside down etc. **(Supplementary File 3).**

Externally, the fish displayed a range of clinical signs, including skin nodules, frayed fins, loss of eyes, opaque eyes, loss of scales, exophthalmia, anorexia, decolouration or darkened skin, excess of mucous, skin haemorrhages and distended abdomen (**Figure 3)**. At necropsy, fish from both the first visits to Farm 1 and 2 presented with marked ascites, enlarged and haemorrhagic organs including the spleen, heart, brain, gills, but most notably liver and kidney. The gastrointestinal track was empty of solids but contained transparent fluid similar to that also seen in the peritoneal cavity.

In contrast to the earlier visits, when Farm 2 was visited on 20/02/19, there were very high and ongoing mortalities in the fry production systems (>70%). This was both in their onshore hatchery (supplied with water pumped from the main lake) where eggs were hatched and held until the fry were approximately 20g, and in the nursery cages on the main lake to which fry had been transferred. As with the larger fish sampled, affected fry showed erratic swimming behaviour, skin haemorrhages and severe ascites as the main clinical signs (**Figure 3)**.

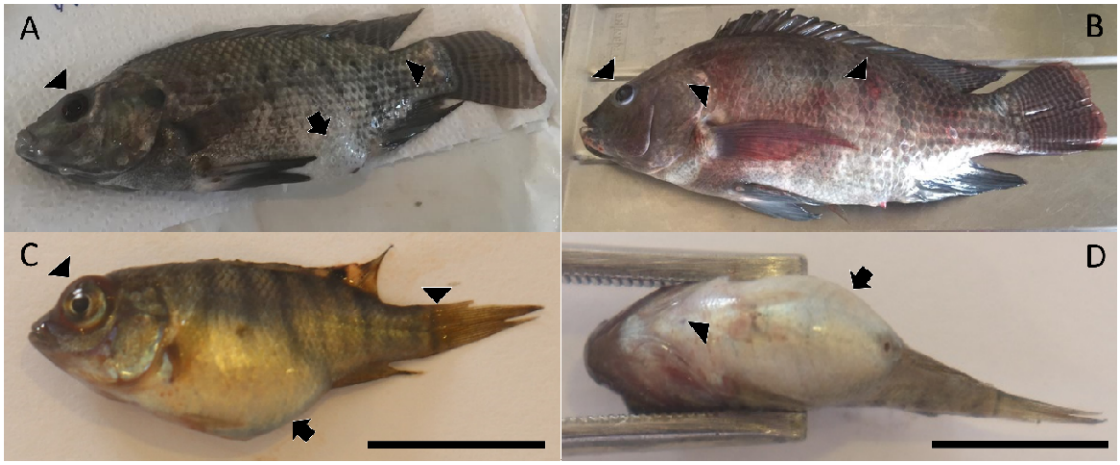


Figure 3 External lesions and clinical signs in diseased Nile tilapia in Lake Volta. A Ongrowing fish with emaciation slight ascites (arrow), endophthalmia (left arrow head) and skin purulent abseces (right arrow head). **B** Broodstock with microphthalmia left arrow head), skin haemorrhages (middle arrow head) and skin ulcers (right arrow head). **C** juvenile with exophthalmia (left arrow head), ascites (arrow) and loss of scales, excess of mucus and haemorrhages (right arrow head). Bar = 1cm. **D** Ventral view of juvenile fish presenting severe ascites black arrow and skin haemorrhages (arrow head). Bar= 1cm.

3.2 *Megalocytivirus* and bacterial infections in Farm 2 during period of high mortalities

Four out of the ten fish examined histologically showed mild tissue necrosis in the spleen and renal haematopoietic tissue with the presence of large numbers of cells showing relative eosinophilia cytoplasmic and nuclear pleomorphism with margination of chromatin in some affected nuclei suggestive of a viral infection diffused throughout the tissue **(Figure 4)**.

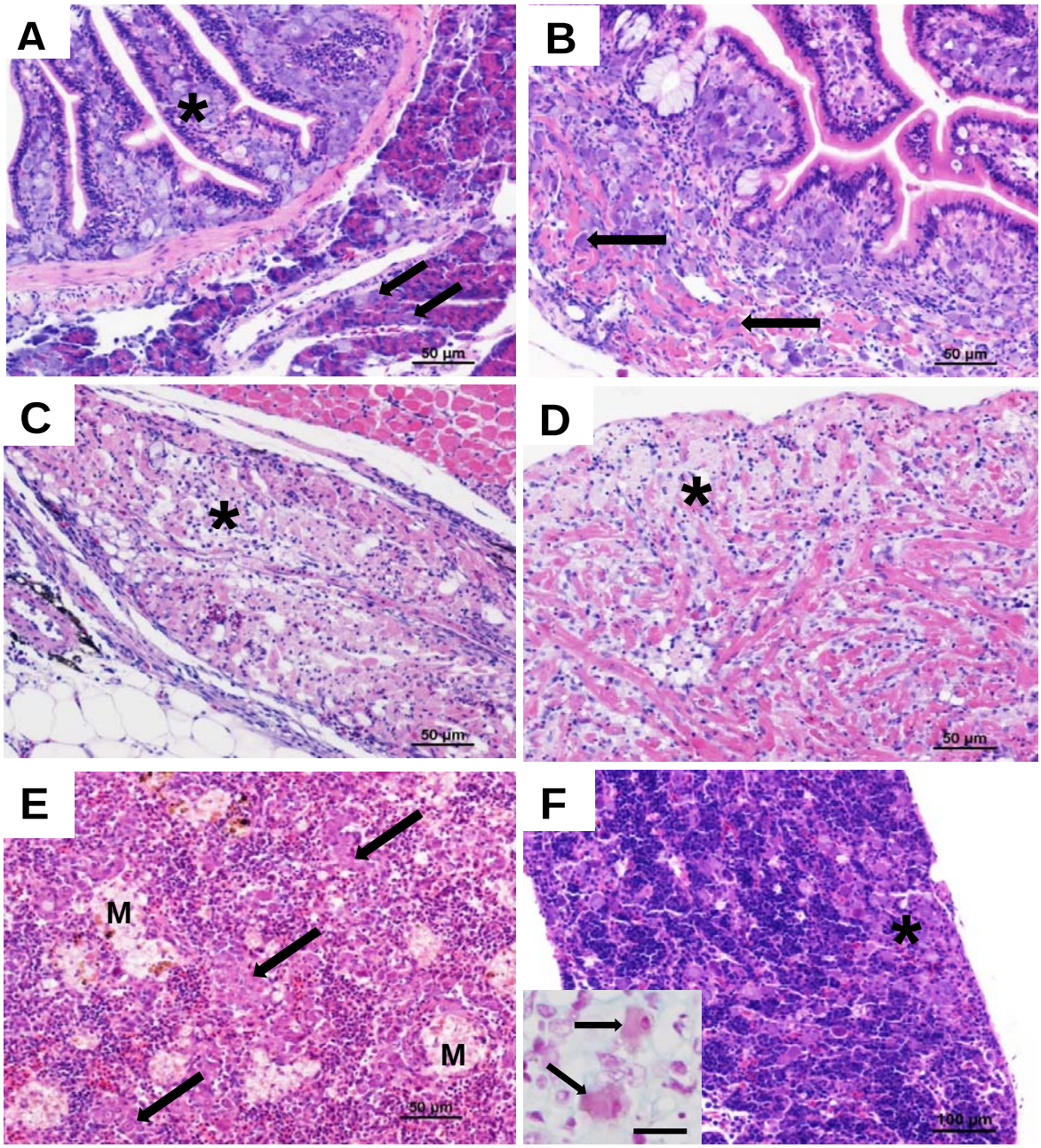


Figure 4 Histopathological cross sections of tissues from diseased Nile tilapia in Lake Volta. All sections stained with H&E unless otherwise stated. **A.** Section through the intestine of an infected fry showing the presence of large numbers of megalocytes in the lamina propria (*) but not affecting the mucosa or underlying muscularis. Note the presence of isolated megalocytes in the pancreatic acinar tissue (arrows). Bar = 50µm. **B.** Same fish as in A, showing infiltration and disruption of the muscularis associated with the presence of megalocytes (arrows). Bar=50 µm. **C.** Pseudobranch showing extensive necrosis (*). Bar=50µm. **D.** Heart showing necrosis of the ventricular muscle (*), particularly in the peripheral regions associated with inflammation. Bar=50µm. **E.** Spleen from a fish from Farm 2 during a mortality episode. Affected cells show pronounced eosinophilia and are distributed throughout the section (*). Numerous vacuolated macrophage aggregates are also present (M). Bar = 50µm. **F.** Pronephros showing extensive distribution of megalocytes without associated tissue necrosis (*). Bar=100µm. Inset shows pale staining of DNA in the cytoplasm of affected cells (arrows). Feulgen stain. Bar=25µm.

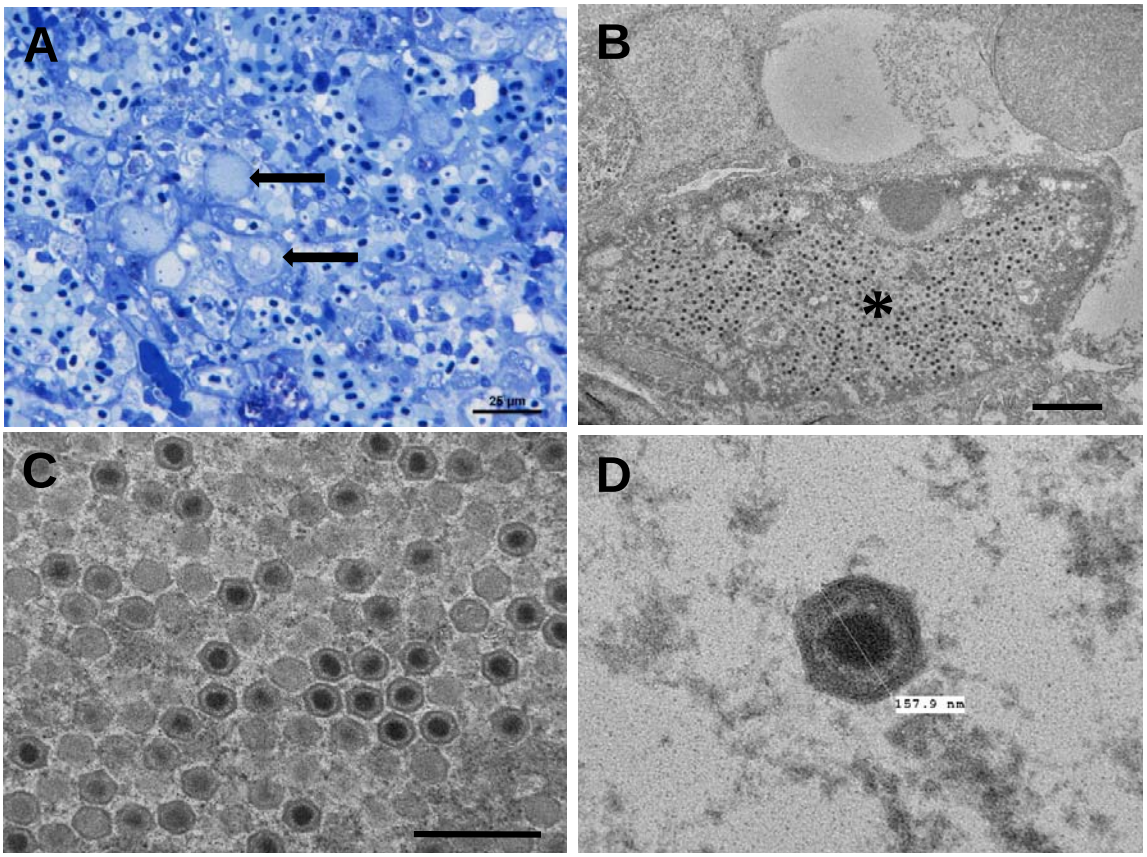
The ultrastructure of affected cells revealed the presence of conspicuous viral particles. Some cells showed the presence of numerous diffusely spread virions within the hypertrophied nucleus of affected cells, which also showed degradation of the nuclear membrane (**Figure 5)**. Virions were approximately 160 nm in diameter **(Figure 5D)**. Virion morphology showing icosahedral symmetry with an external double membrane and internal core was consistent with that of viruses from the genus *Megalocytivirus*. In other cases, the nucleus of affected cells appeared condensed and densely stained in histological and resin sections. TEM showed that in these cases the nucleus was tightly packed with virions in various stages of maturation and with some evidence of formation of 'arrays' **(Figure 5)**. In two of these cases, a concomitant Gram +ve bacterial infection was also present in the gill and liver. Incidental findings of gill parasites, myxozoan cysts and monogeneans, both present in low numbers as well as low grade epitheliocystis were observed. The brain of a single fish harboured small cysts containing necrotic debris. Gram and Ziehl-Neelsen staining did not demonstrate the presence of bacteria. Other tissues appeared normal. For the set of samples collected at the height of the mortalities from Farm 2 on 28/11/18, *Aeromonas jandaei*, *Aeromonas veronii* (from skin), *Streptococcus agalactiae* capsular type Ib biotype and *Edwardsiella tarda* (from liver and kidney) were recovered.

3.3 PCR and sequence confirmation of Megalocytivirus infection

Within the 7 individuals collected from Farm 2 on the second visit that were analysed with the protocol proposed by (16), a single fish (fish 1) was clearly positive by PCR for RSIV/ISKN and a second very weak product of the correct size was also seen in tissues from Fish 6 (**Supplementary File 4**) The consensus sequence generated from the PCR product from Fish 1 was confirmed as ISKNV sharing 100% nucleotide identity with ISKNV

294 accession no AF371960.1. In the phylogenetic analysis the sequence was assigned to the
295 same lineage as the bulk of the ISKNV sequences (**Figure 6)**.

296 The samples collected from Farm 1 and Farm 2 at the height of the mortalities were found to
297 be strongly positive when they were retrospectively tested using the (14, 15) recommended
298 PCR method.



299 **Figure 5 Micrographs of the kidney of diseased Nile tilapia in Lake Volta. A** Semithin
300 section of affected renal tissue showing characteristic cellular hypertrophy (black arrows).
301 Toluidine Blue stain. Scale bar = 25µm. **B.** Electron micrograph of an individual infected cell
302 with numerous viral particles (*). Adjacent cells appear uninfected. Scale bar = 2µm. **C.**
303 Numerous lightly stained developing virions with mature virions. The outer membranes and
304 central electron lucent core are clearly visible. Scale bar = 500nm. **D.** Mature icosahedral
305 virion showing detail of the outer caspid and inner membrane with central electron lucent
306 core.
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3.*4 qPCR results for megalocytivirus from ongrowing tilapia samples*

The qPCR results confirmed that all the fish sampled from Farm 1 in the Akuse region were positive for megalocytivirus. Also, as expected, the samples collected from the first Farm 2 visit during the peak of mortality (28/11/18), were also positive and presented the highest viral titres in grow out fish with some containing over 3 x 10^6 copies per sample reaction. In contrast, the samples collected from grow out fish during the second visit to Farm 2 had much lower viral copy numbers.

All the archived samples collected in 2017 and March 2018 were negative for ISKNV, when tested by qPCR **(Supplementary File 5)**.

3.5 Fry samples had characteristic megalocytivirus pathology and high copy numbers of virus

All the fry samples collected from Farm 2 on 20/02/19, when there were reportedly very high (>90%) losses in that part of their system, were positive for ISKNV by qPCR and these presented the highest titres in the study with some containing up to 1.5 x 10^7 copies per sample reaction **(Table 1)**. All fry showed moderate to marked histological and pathological features of infection with *megalocytivirus*. Splenic tissues were necrotic and associated with the presence of megalocytes characterised by light sometimes granular cytoplasmic basophilia and hypertrophied nuclei. Kidney also showed the presence of megalocytes but usually with only mild cellular necrosis. The lamina propria in the intestine of a single fish was packed with megalocytes **(Figure 4)**, although necrosis appeared to be absent and the epithelial layer remained intact. Gills showed only minimal focal necrosis, usually affecting the underlying connective tissues. In some fish the choroidal rete was affected with mild necrosis and variable numbers of megalocytes and most fish showed mild myositis with few

336 megalocytes in the skeletal muscle. However, a single fish showed extensive inflammation
337 and myofibrillar necrosis **(Figure 4)**. Connective tissues of the head and in particular around
338 the pharyngeal teeth were often infiltrated with megalocytes. Brain and spinal cord appeared
339 normal. Liver samples were not examined as they were used for virus quantification.

340

341 3.6 Other pathological observations

342 *Megalocytivirus* -like pathology was not observed in any of the samples taken from the
343 second visit to Farm 2, two weeks after the peak of mortalities had passed. As with the
344 samples taken at the first visit, there was evidence of bacterial infection in some individuals,
345 particularly fish 5, had marked bacterial infection of the spleen, liver and brain (meningitis).
346 All the samples were positive for the presence of *Streptococcus agalactiae* and negative for
347 *Streptococcus uberis* and S*treptococcus dysgalactiae* by PCR **(Supplementary File 6)**.

348 A range of different potential bacterial species, including *Aeromonas jandaei* and
349 *Plesiomonas shigelloides* were recovered from fish from Farm 1 **(Table 1)**, but not as pure
350 growths or high quantities, suggesting they had a limited role in observed disease in these
351 animals.

352 All the samples from Farm 1 and Farm 2 tested for TiLV and from Farm 2 for nodavirus by
353 qPCR were all negative **(Table 1 and Supplementary File 4)**.

354

355 The results for all the individual fish tested are shown in Supplementary File 2.

Table 1 Summary of sampling and results

Sampling date & Farm	No. of Fish and size	Farm observations and ‡clinical signs	Bacteriology	Histopathology	Virology Results[¥] [ ] – qPCR $_{RSIV/ISKNV}$ copy number/sample reaction range
18/10/2018 †Farm 1	n=5; 100-300g	Wide scale ongoing mortalities in on growing fish in cages.	*Aeromonas veronii*; *Aeromonas jandaei*; *Plesiomonas shigelloides*; *Chryseobacterium sp*; *Acinetobacter johnsonii*;	Not done	*qPCR $_{TiLV}$ 5/5 -ve *cPCRa $_{ISKNV}$ 4/5 +ve *qPCR $_{RSIV/ISKNV}$ 5/5 +ve [7.5 x 10^1 – 4.60 x 10^5]
28/11/2018 †Farm 2	n=14; 74-401g	Wide scale ongoing mortalities in on growing fish in cages (**Figure 2**). On shore fry production unit: no unusual mortalities.	*Aeromonas veronii; Aeromonas jandaei; Streptococcus agalactiae* capsular type Ib biotype 2 (non-haemolytic); *Edwardsiella tarda*	Evidence of Gram negative and Gram positive bacterial and viral# infection (including in same fish)	qPCR $_{TiLV}$ 14/14 -ve qPCR $_{NODA}$ 14/14 -ve *cPCRa $_{ISKNV}$ 12/14 +ve *qPCR $_{RSIV/ISKNV}$ 14/14 +ve [2.48 x 10^1 - 3.3 x 10^6]
17/12/2018 †Farm 2	n=7; 40-646g	Much lower mortalities in on growing fish in cages than previous sampling visit. On shore fry production unit: no unusual mortalities.	Not done	Evidence of bacterial infection but not of viral infection	qPCR $_{TiLV}$ 7/7 -ve qPCR $_{NODA}$ 7/7 -ve **cPCRb $_{RSIV/ISKNV}$ 1/7 +ve megalocytivirus, sequence = ISKNV** *qPCR RSIV/ISKNV 6/7 +ve [1 x 10^0 - 24 x10^0]
20/02/2019 †Farm 2	n=14; 6-9 cms	Limited mortalities in in on growing fish in cages. Very severe mortalities in on shore fry production unit.	Not done	Very severe virus-associated pathology (in tissue samples from all fish).	qPCR TiLV not done qPCR NODA not done cPCRa ISKNV 7/7 +ve qPCR RSIV/ISKNV 13/13 +ve [5.1 x 10^5 – 1.51 x 10^7]

† Farm 1: approx. 800 tonnes production per year cage unit on lake; Farm 2: approx. 2000 tonnes per year production ongrowing cage culture on lake and fry unit on land supplied with water pumped from lake.

‡ All moribund fish, including fry, presented similar signs: erratic swimming, lethargy, ascites, swollen, dark spleen, haemorrhagic livers and other organs.

Haematopoietic cell nuclear and cytoplasmic pleomorphism with marginalisation of chromatin and fine granulation.

¥ qPCR TiLV, qPCR NODA, qPCR RSIV/ISKNV: results of qPCR testing for tilapia lake virus, nodavirus and ISKNV respectively. cPCRa & cPCRb : conventional PCR using OIE recommended protocols by Kurita et al., 1998 (a) (15) and Rimmer et al., 2012 (b) (16) protocols, results in bold represent index finding, *represents retrospective testing post index finding.

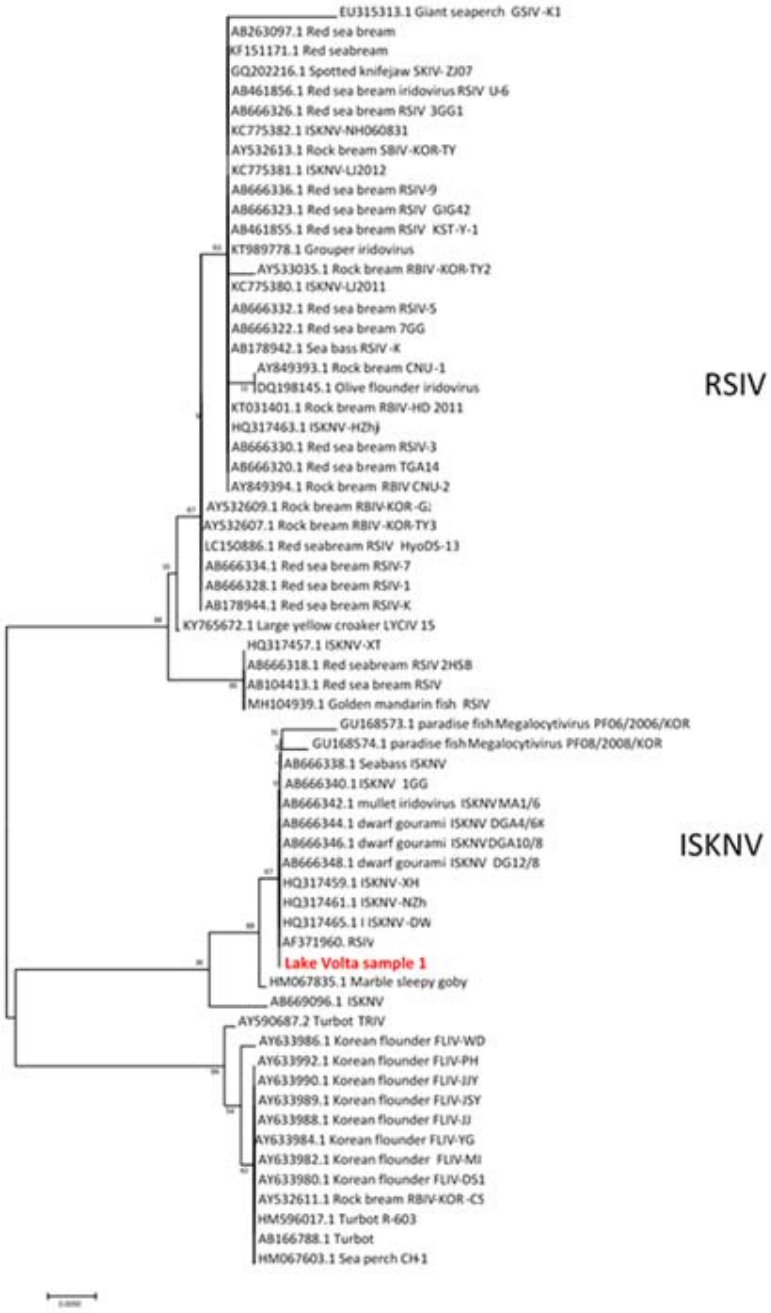

Figure 6 N-J tree showing the genetic relationship of partial MCP sequence from LV#1 to a range of RSIV, ISKNV and related Megalocytiviruses. The GeneBank accession numbers and the host species are included where available.

4 Discussion

The results suggest ISKNV had a significant role in the high mortalities experienced by the two farms that were investigated. Fish sampled from the second farm at the height of the disease outbreak showed severe clinical and pathological signs typically associated with infection by the virus (including visualisation of distinctive megalocytes with characteristic virions). Both, these fish and those sampled earlier from Farm 2, had extremely high copy numbers of RSIV/ISKNV *Megalocytivirus*-like virions and ISKNV was confirmed by gene sequencing.

Although it cannot be confirmed, without testing fish recovered from a range of the other affected farms, the results would be consistent with a recent introduction of the virus onto one or more farms prior to October 2018, that then extended upstream of the dam to other farms resulting in the unusual, widespread and significant mortalities observed. Firstly, there were no observations of typical ISKNV-associated pathology in any previous disease investigations, on the affected or other farms. Secondly, the limited PCR screening in this study of archived samples of diseased tilapia recovered from before the major mortality event including both farms, were all negative. Moreover, (3) also screened for the presence of RSIV/ISKNV by PCR in 2016, including samples from the affected farms, without detecting the virus, or associated pathology.

Iridioviruses are large icosahedral cytoplasmic double□stranded DNA viruses, which can infect a wide range of hosts, including invertebrates and poikilothermic vertebrates. The family *Iridoviridae* includes five genera: *Iridovirus, Chloriridovirus, Ranavirus, Lymphocystivirus* and *Megalocytivirus* (19). Fish pathogenic iridoviruses are representatives of *Ranavirus, Lymphocystivirus* and *Megalocytivirus* genera (20, 21). Infectious spleen and kidney necrosis virus (ISKNV), is a member of the genus *Megalocytivirus* (22), and causes

392 disease in a range of freshwater and marine fish species (Subramaniam, Shariff, Omar, &
393 Hair-Bejo, 2012; Whittington, Becker, & Dennis, 2010). ISKNV is closely related to red sea
394 bream iridovirus and both viruses are listed by the OIE as notifiable pathogens (14).
395 Although tilapia is not listed as a susceptible species by the OIE at present (14), recent
396 reports from the USA (23) and Thailand (24) suggest that it is a susceptible species likely to
397 suffer significant mortality. The change in the known host range of virus needs to be
398 communicated to the international community to prevent future transboundary spread through
399 movement of infected tilapia.

400 It was interesting to note that many of the ISKNV positive fish were actively co-infected with
401 *Streptoccocus agalactiae* and other bacterial pathogens, presenting severe bacteraemia /
402 meningitis, as well as ISKNV-associated pathology. The high mortalities on the farms in the
403 larger on-growing fish may well have been exacerbated by these coinfections.

404 Although the mortalities were initially confined to on-growing fish in cage culture systems,
405 the later observations of very high ISKNV associated mortalities in fry shows the virus likely
406 affects all life stages. As fry are often reared in onshore facilities below the dam and then
407 translocated to on-growing cages on the main lake, this may have been one of the routes that
408 disease was rapidly spread after it first emerged. Anecdotally, at the time of writing, farmers
409 on the sites visited report that mortalities in on-growing facilities have declined, while fry
410 production continues to be badly affected. It is possible that surviving fry have been exposed
411 to the virus and then protected against subsequent exposure. This suggests that immunisation
412 of fry, or use of previously exposed individuals, could represent a practical disease
413 management strategy. Vaccination as a control strategy may be used to control red sea bream
414 iridiodovirus (25, 26) and there are also encouraging reports of its potential effectiveness for
415 protection against ISKNV in other fish species (27).

Outbreaks of disease that cause significant morbidity and/or mortalities in an aquaculture operation is always a major concern. This is exacerbated when this appears to represent the incursion of a new agent into a system, or region (country or zone in a country) which has not previously been affected. A stark example of this is the epidemic of infectious salmon anaemia virus (ISAV) which reduced production by three quarters and resulted in severe economic and social crisis in the developing Chilean Atlantic salmon industry between 2007 and 2010 (28–30).

The Ghanaian authorities have for some time been concerned that the, to date, successful, expansion of its industry on Lake Volta may be affected by such disease incursions. Partly for this reason, and also to safeguard the genetic integrity of Lake Volta Nile tilapia strains, they have tried to limit the culture to locally reared Nile tilapia stocks. However genetic testing by the Ghanaian Fisheries Commission (Ziddah *et al*., Unpublished Observations) showed that fish on some of the farms on Lake Volta were likely of imported GIFT strain origin (31) or hybrids of GIFT and indigenous strains.

If farmers have been illegally sourcing broodstock from Asia and other areas, that would be an ideal method of translocating pathogens from one region to another. It should be noted though that ISKNV has also been detected in internationally traded freshwater ornamental species, theoretically posing another possible introduction route (32)

It is very possible that this is not the first time disease introduction has taken place in Lake Volta Ghana. The study by (3) showed that outbreaks of *S* . *agalactiae* investigated in 2016 were all caused by genetically indistinguishable isolates of ST 261, with closest genetic identity to Asian isolates. Discussions with affected farmers at the time suggested, that *S. agalactiae* associated mortalities were a relatively recent phenomenon, although the disease is now clearly endemic to all the areas in the Volta area. Other studies have shown that *S.*

agalactiae ST261 has likely been translocated around the world in association with farmed tilapia (33).

Although most attention to date has focussed on the emergence and spread of TiLV within the tilapia industry world-wide, these results also demonstrate that there is a range of other potential threats to the sustainability of tilapia aquaculture.

Conclusion

This is the first report of Infectious Spleen and Kidney Necrosis Virus (ISKNV) in farmed tilapia in Africa. ISKNV was found in co-infection with *Streptococcus agalactiae* and other bacterial pathogens in Lake Volta, Ghana. The correlations seen between the mortality events, histopathology and viral loads in the tissues suggest that ISKNV was a major cause of mortalities during the outbreaks. In general, the results support continued efforts to improve the biosecurity of the industry in Ghana. There is a clear need to strengthen domestic capability to rapidly diagnose and control emerging disease threats caused by ISKNV and other pathogens. Further work is also needed to map the distribution of the virus and its impact, including potential effects on wild fish species, and to implement practical control strategies.

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463 **References**

464 1. FAO. 2018. The State of World Fisheries and Aquaculture 2018 - Meeting the
465 sustainable development goals.

466 2. FAO. 2016. The State of World Fisheries and Aquaculture. Contributing to food
467 security and nutrition for all. Rome.

468 3. Verner-Jeffreys D, Wallis T, Cano I, Ryder D, Hayden D, Domozoro J, Dontwi J,
469 Field T, Adjei-Boteng D, Wood G, Bean T, Feis S. 2018. *Streptococcus agalactiae* Multi
470 Locus Sequence Type 261 is associated with mortalities in the emerging Ghanaian tilapia
471 industry. J Fish Dis 41:157–179.

472 4. Jansen MD, Cudjoe K, Brun E. 2018. Investigation of tilapia mortality in Ghana.

473 5. Plumb JA, Hanson LA. 2011. Tilapia bacterial diseases, p. 445–463. In Plum, JA,
474 Hanson, LA (eds.), Health Maintenance and Principle Microbial Diseases of Cultured Fishes,
475 Third edition. Blackwell Publishing Ltd, Iowa.

476 6. Machimbirike VI, Jansen MD, Senapin S, Khunrae P, Rattanarojpong T, Dong HT.
477 2019. Viral infections in tilapines: More than just tilapia lake virus. Aquaculture 503:508–
478 518.

479 7. Hounmanou YMG, Mdegela RH, Dougnon T V., Achoh ME, Mhongole OJ,
480 Agadjihouèdé H, Gangbè L, Dalsgaard A. 2018. Tilapia lake virus threatens tilapines farming
481 and food security: Socio-economic challenges and preventive measures in Sub-Saharan
482 Africa. Aquaculture 493:123–129.

483 8. Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, Glöckner FO.
484 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-
485 generation sequencing-based diversity studies. Nucleic Acids Res 41:e1.

486 9. Shoemaker CA, Xu DH, Garcia JC, Lafrentz BR. 2017. Capsular typing of
487 *Streptococcus agalactiae* (Lancefield group B streptococci) from fish using multiplex PCR
488 and serotyping. Bull Eur Assoc Fish Pathol 37:190–197.

489 10. Yáñez MA, Catalán V, Apráiz D, Figueras MJ, Martínez-Murcia AJ. 2013.
490 Phylogenetic analysis of members of the genus *Aeromonas* based on gyrB gene sequences.
491 Int J Syst Evol Microbiol 53:875–883.

492 11. Delannoy CM, Crumlish M, Fontaine MC, Pollock J, Foster G, Dagleish MP,
493 Turnbull JF, Zadoks RN. 2013. Human *Streptococcus agalactiae* strains in aquatic mammals
494 and fish. BMC Microbiol 13:41.

495 12. Luo X, Fu X, Liao G, Chang O, Huang Z, Li N. 2017. Isolation, pathogenicity and
496 characterization of a novel bacterial pathogen *Streptococcus uberis* from diseased mandarin
497 fish Siniperca chuatsi. Microb Pathog 107:380–389.

498 13. Abdelsalam M, Asheg A, Eissa AE. 2013. *Streptococcus dysgalactiae*: An emerging
499 pathogen of fishes and mammals. Int J Vet Sci Med 1:1–6.

500 14. World Organisation for Animal Health OIE. 2018. Chapter 2.3.8 Red sea bream
501 iridoviral diseaseManual of Diagnostic Tests for Aquatic Animals. OIE, Paris.

502 15. Kurita J, Nakajima K, Hirono I, Aoki T. 1998. Polymerase chain reaction (PCR)
503 amplification of DNA of red sea bream iridovirus (RSIV). Fish Pathol 33:17–23.

16. Rimmer, A. E., Becker JA, Tweedie A, Whittington RJ. 2012. 2012 Development of a quantitative polymerase chain reaction (qPCR) for the detection of dwarf gourami iridovirus (DGIV) and other Megalocytiviruses and comparison with the Office International des Epizooties reference PCR protocol. Aquaculture 358-359:15. Aquaculture 358–359:155–163.

17. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res 25:3389–3402.

18. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30:2725–9.

19. Jancovich JK, Chinchar VG, Hyatt A, Miyazaki T, Williams T, Zhang QY. 2012. Family Iridoviridae., p. 193–210. In King, AMQ, Adams, MJ, Carstens, EB, Lefkowitz. E.J. (eds.), Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, San Diego, CA.

20. Subramaniam K, Shariff M, Omar AR, Hair-Bejo M. 2012. Megalocytivirus infection in fish. Rev Aquac 4:221–233.

21. Whittington RJ, Becker JA, Dennis MM. 2010. Iridovirus infections in finfish-critical review with emphasis on ranaviruses. J Fish Dis 33:95–122.

22. Kurita J, Nakajima K. 2012. Megalocytiviruses. Viruses 4:521–538.

23. Subramaniam K, Gotesman M, Smith CE, Steckler NK, Kelley KL, Groff JM, Waltzek TB. 2016. Megalocytivirus infection in cultured Nile tilapia *Oreochromis niloticus*. Dis Aquat Organ 119:253–258.

525 24. Suebsing R, Pradeep PJ, Jitrakorn S, Sirithammajak S, Kampeera J, Turner WA,
526 Saksmerprome V, Withyachumnarnkul B, Kiatpathomchai W. 2016. Detection of natural
527 infection of infectious spleen and kidney necrosis virus in farmed tilapia by hydroxynapthol
528 blue-loop-mediated isothermal amplification assay. J Appl Microbiol 121:55–67.

529 25. Shimmoto H, Kawai K, Ikawa T, Oshima SI. 2010. Protection of red sea bream
530 Pagrus major against red sea bream iridovirus infection by vaccination with a recombinant
531 viral protein. Microbiol Immunol 54:135–142.

532 26. Nakajima K, Maeno Y, Honda A, Yokoyama K, Tooriyama T, Manabe S. 1999.
533 Effectiveness of a vaccine against red sea bream iridoviral disease in a field trial test. Dis
534 Aquat Organ 36:73–75.

535 27. Dong Y, Weng S, He J, Dong C. 2013. Field trial tests of FKC vaccines against RSIV
536 genotype Megalocytivirus in cage-cultured mandarin fish (*Siniperca chuatsi*) in an inland
537 reservoir. Fish Shellfish Immunol 35:1598–1603.

538 28. Vike S, Stian Nylund S, Nylund A. 2009. ISA virus in Chile: evidence of vertical
539 transmission. Arch Virol 154:1–8.

540 29. Mardones FO, Martinez-Lopez B, Valdes-Donoso P, Carpenter TE, Perez AM. 2014.
541 The role of fish movements and the spread of infectious salmon anemia virus (ISAV) in
542 Chile, 2007-2009. Prev Vet Med 114:37–46.

543 30. Godoy MG, Aedo A, Kibenge MJT, Groman DB, Yason C V., Grothusen H,
544 Lisperguer A, Calbucura M, Avendaño F, Imilán M, Jarpa M, Kibenge FSB. 2008. First
545 detection, isolation and molecular characterization of infectious salmon anaemia virus
546 associated with clinical disease in farmed Atlantic salmon (Salmo salar) in Chile. BMC Vet
547 Res 4:28.

548 31. Ponzoni RW, Nguyen NH, Khaw HL, Hamzah A, Bakar KRA, Yee HY. 2011.
549 Genetic improvement of Nile tilapia (*Oreochromis niloticus*) with special reference to the
550 work conducted by the WorldFish Center with the GIFT strain. Rev Aquac 3:27–41.

551 32. Jung-Schroers V, Adamek M, Wohlsein P, Wolter J, Wedekind H, Steinhagen D.
552 2016. First outbreak of an infection with infectious spleen and kidney necrosis virus (ISKNV)
553 in ornamental fish in Germany. Dis Aquat Organ 119:239–244.

554 33. Kawasaki M, Delamare-Deboutteville J, Bowater RO, Walker MJ, Beatson S, Ben
555 Zakour NL, Barnes AC. 2018. Microevolution of *Streptococcus agalactiae* ST-261 from
556 Australia indicates dissemination via imported tilapia and ongoing adaptation to marine hosts
557 or environment. Appl Environ Microbiol 84:e00859-1.