Vol. 131: 177–186, 2018
https://doi.org/10.3354/dao03296

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published November 20

First report of carp edema virus in the mortality of wild common carp *Cyprinus carpio* in North America

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ABSTRACT: Carp edema virus (CEV) is an unclassified poxvirus that infects skin and gill tissue to cause koi sleepy disease. In the USA, CEV was first detected in 1996 in a California koi wholesaler, and has since been reported sporadically only within imported and domestic koi. Common carp *Cyprinus carpio* are a non-native species now present in most waterways in the USA. In May 2017, >526 large adult common carp in spawning condition died in Mill Pond, Park Ridge, NJ, USA. The water temperature during the kill was 15°C and the affected fish displayed marked lethargy prior to death. The presence of CEV was confirmed by endpoint PCR, real-time quantitative PCR (qPCR), and transmission electron microscopy (TEM), making this the first report of CEV associated with a wild carp kill in North America. Phylogenetic analysis of a region of the 4a gene encoding the major core protein clustered the CEV strain among others in genogroup I, which includes CEV strains previously detected in common carp cultured in Europe. Gill histopathology included severe lamellar fusion and apoptosis in the interlamellar region and TEM identified cytoplasmic virions consistent in morphology with CEV in the branchial epithelial cells. Five months following the mortality, surviving fish were collected and screened for CEV by purifying and concentrating virus from the gills and testing with qPCR. No evidence of CEV was found, supporting previous studies showing CEV is not detectable in gills after abatement of clinical signs.

KEY WORDS: Carp edema virus · Poxvirus · Common carp · USA · Pathology

INTRODUCTION

Common carp *Cyprinus carpio* are hardy fish which have become established in most natural waters throughout the USA. Though their first introduction is disputed, the dissemination of common carp throughout the USA occurred when the US Fish Commission imported them from Germany in 1877, and were further spread by their use as baitfish (Nico et al. 2017). Two types of common carp occur in North America, the wild type introduced from Europe, which is considered an invasive species (referred to as carp from this point forward), and a colored variety known as koi imported for the ornamental trade (Balon 1995, Nico et al. 2017).

Despite their hardiness, diseases caused by viruses such as spring viremia of carp (SVC) and koi herpesvirus (KHV), which are reportable diseases to the World Organisation for Animal Health (OIE 2017), are particularly lethal to carp, which has led to mass mortality events both in wild carp populations and koi reared commercially (Plumb & Hanson 2011). In the USA, SVC has caused 2 major mortality events in wild carp populations, including one in Wisconsin that affected >10 000 kg mature fish (Goodwin 2009). KHV, known formally as cyprinid herpesvirus 3, is

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highly species specific and has caused mass mortality in koi (Hedrick et al. 2000) and wild carp populations in North America (Grimmett et al. 2006, Garver et al. 2010).

Carp edema virus (CEV) has recently emerged as a disease of concern to koi and carp aquaculture. CEV contains a large double-stranded DNA genome and has been aligned tentatively to viruses in the family *Poxviridae*. It was first detected in Japanese koi in the 1970s and derived its name from causing edematous skin lesions (Murakami et al. 1976, Oyamatsu et al. 1997a). It also causes gill pathology, severe lethargy, and high mortality rates more commonly reported as koi sleepy disease (KSD) (Miyazaki et al. 2005). While CEV was detected in carp in Europe in 2004 and now occurs in the UK, Germany, France, the Netherlands, the Czech Republic, and Austria, it may be traced back as early as 1998 associated with spring mortality syndrome (Way & Stone 2013, Haenen et al. 2014, Lewisch et al. 2015, Way et al. 2017). CEV was also detected in koi in South America in 2015 (Hesami et al. 2015), India in 2015 (Swaminathan et al. 2016), China in 2016 (Ouyang et al. 2018), and South Korea in 2017 (Kim et al. 2018). Despite it being detected in the USA in 1996, only sporadic mortalities have been documented between 2005 and 2014 in koi from Washington, North Carolina, Georgia, and Florida (Hesami et al. 2015), and until now there have been no reports of it affecting wild carp populations in North America.

Here we report the involvement of CEV in a mass mortality event of wild carp at a pond in Park Ridge, New Jersey, USA that occurred in May 2017. Sequence analysis of a region of the P4a major core protein gene was undertaken to identify the source of the CEV strain. To determine whether CEV persisted following the mortality, real-time quantitative PCR (qPCR) analysis of carp samples collected 5 mo after the event was undertaken.

MATERIALS AND METHODS

Fish kill observations and sample collection

Mass mortality of carp in Mill Pond located in Park Ridge, Bergen County, NJ, USA was reported on 23 May 2017 and investigated on 25 May 2017. Mill Pond is a shallow 9 acre pond fed by Pascack Brook that runs from New York State into New Jersey, through Mill Pond and terminating in Woodcliff Lake Reservoir (Fig. 1). At that time, the pond surface water temperature was 15°C with a ~10 mg l^{-1} dissolved oxygen level (100% saturation). On investigation, 30 dead carp were observed along the shoreline and pushed up against the dam on the south side of the pond where Pascack Brook flowed into Woodcliff Lake Reservoir. The various degrees of decomposition evident suggested that mortality had been occurring for ~1 wk. The carp ranged between 2 and 8 kg in weight, and no smaller dead carp were observed. No external lesions were apparent. While investigating the dead carp, the only evidence of morbidity was fish occasionally swimming slowly near the pond edges. A fish in only a mild state of decomposition (total length, 717 mm; weight, 5.29 kg) and a moribund fish (total length, 625 mm; weight, 3.95 kg) were collected for examination. The moribund fish, which displayed marked lethargy by swimming slowly and resting on the bottom near the shoreline, was caught in a landing net and euthanized with an overdose of tricaine methanesulfonate (MS-222). Fish were maintained on ice during transport to the Pequest Fish Health Laboratory, Oxford,

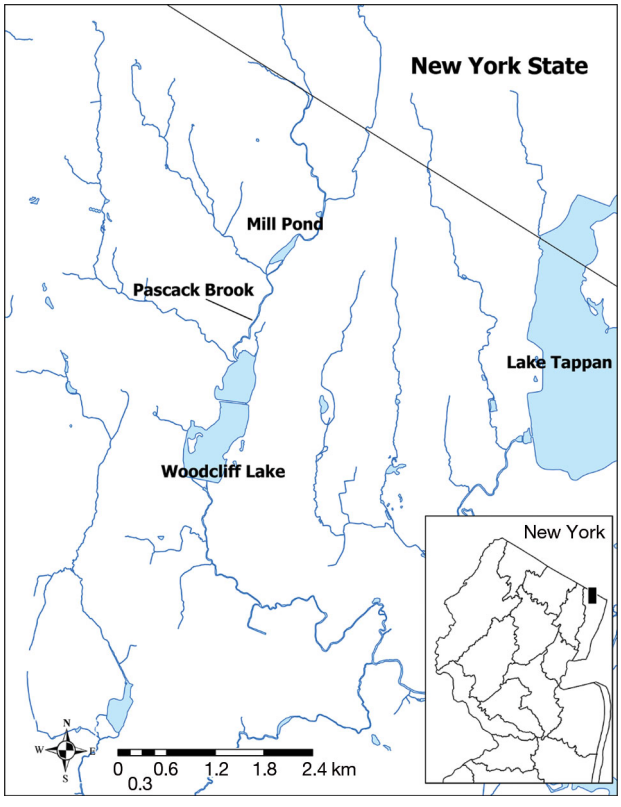

Fig. 1. Map showing the location of the fish kill, Mill Pond and Woodcliff Lake Reservoir, in northeastern New Jersey. Inset shows zoomed out view of central and northern New Jersey with borders indicating watershed boundaries; the boxed area is the location of the carp edema virus-associated mortality

NJ. During necropsy, samples of spleen, anterior and posterior kidney, reproductive tissue, liver, gastrointestinal tract, and gills were fixed in 10% neutral-buffered formalin (NBF) for histology and also stored at −80°C for molecular testing.

While mortality occurred in Mill Pond, dead fish washed over the dam wall into Pascack Brook and Woodcliff Lake Reservoir. As no moribund carp were reported in Woodcliff Lake Reservoir, all dead fish appear to have originated from Mill Pond. From 7 to 16 June 2017, 150 and 376 dead carp (total 526) were removed from Mill Pond and Woodcliff Lake Reservoir, respectively, due to the smell of the decomposing fish. No other fish species were found dead.

Histology and transmission electron microscopy

For histology, formalin-fixed tissue pieces were processed using routine methods and 4-µm-thick sections stained with hematoxylin and eosin were observed by light microscopy. Gill tissue fixed in 10% NBF was post-fixed overnight at 4°C in 2% glutaraldehyde in phosphate buffer. After washing in phosphate buffer, tissue was fixed in 1% osmium tetroxide for 2 h, followed by routine processing for transmission electron microscopy (TEM) and embedding in EMBED 812 resin (Electron Microscopy Sciences). Sections of 0.5 µm were stained with toluidine blue and examined for high-resolution light microscopy using a Carl Zeiss Axio-plan 2 microscope mounted with a Jenoptik digital camera. Ultrathin (80 nm) sections, cut and stained with 1% uranyl acetate in 50% ethanol and modified Sato's lead stain (Hanaichi et al. 1986), were examined using a Philips CM12 TEM fitted with an AMT-XR11 digital camera located at the Department of Pathology, Robert Wood Johnson Medical School, Rutgers University.

Gill tissue processing and DNA extraction

DNA was extracted from either 25 mg of gill tissue or from gill tissue processed using a modified protocol to purify and concentrate poxviruses (Hanson et al. 2006). In the modified protocol, 500 mg of frozen gill tissue was homogenized mechanically, suspended in 4.5 ml of Hank's balanced salt solution (HBSS) and homogenized for 30 s in a Whirl-Pak bag using a tissue stomacher (LabBlender 80, Seward Medical). The homogenate was centrifuged at 1000 × *g* for 5 min at 4°C and the supernatant was collected and centrifuged again at 16 000 × *g* for 35 min at room temperature. The supernatant was discarded and the pellet was resuspended in 80 µl molecular grade water. DNA was extracted from a 50 µl aliquot using a DNeasy Blood and Tissue kit automated on a QIAcube (QIAGEN) according to the manufacturer's instructions.

Endpoint PCR, sequencing, and phylogenetic analysis

Viral DNA was amplified by PCR in a 50 µl reaction containing 3 µl extracted DNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM each primer and 1.25 U *Taq* polymerase using a Veriti thermocycler (Applied Biosystems). A portion (5 µl) of the PCR product was analyzed by electrophoresis in a 1.2% agarose Egel (Invitrogen) and imaged under ultraviolet light. Gill DNA was initially screened for koi herpesvirus by endpoint PCR using 3 different protocols employing primers as listed in Table 1.

CEV DNA was detected initially using a nested PCR described by Oyamatsu et al. (1997b) and subsequently using a nested PCR targeting a sequence in the gene encoding the putative major core protein P4a (Matras et al. 2017, Table 1). Thermal cycling conditions used in each PCR were 95°C for 5 min, 35 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min, followed by 72°C for 10 min. The reaction was treated with ExoSAP-IT (Applied Biosystems) and diluted to ~3 ng μl^{-1}. Amplified DNA was sequenced in both directions in reactions employing 5 µM each PCR primer and BigDye Terminator V3.1 reagents (Applied Biosystems) using an ABI 3730xl DNA analyzer (Applied Biosystems) at GENEWIZ.

Sequences of the 506 nucleotide (nt) region of the P4a gene spanned by the first set of PCR primers were checked and aligned using BioEdit V7.2.5. (Hall 1999) and confirmed to be CEV by BLASTn analysis of GenBank sequences at the National Center for Biotechnology Information. Maximum likelihood phylogenetic analysis using the Tamura-3-parameter model (Tamura 1992) with a discrete Gamma distribution to model evolutionary rate differences among sites (5 categories [+*G*, parameter = 0.1013]) was performed using MEGA 7 (Kumar et al. 2016). The analysis included 1000 bootstrap runs of 42 related nucleotide sequences that had a minimum of 80% coverage with the CEV sequence (416 nt). The unrooted tree with the highest log likelihood (−1041.0664) is shown including bootstrap branch values (percentage of trees in which associated taxa clustered together) and branch lengths representing

Table 1. Sequences of primers/probe used in PCR tests to detect koi herpesvirus (KHV) and carp edema virus (CEV). E: endpoint PCR; Q: real-time quantitative PCR

Primer/probe	Sequence (5′–3′)	Use	Reference
KHV9/5F	GAC GAC GCC GGA GAC CTT GTG	E	Hedrick (2004), Gilad et al. (2002)
KHV9/5R	CAC AAG TTC AGT CTG TTC CTC AAC	E	
KHV Bercovier TK F	GGG TTA CCT GTA CGA G	E	OIE (2017), Bercovier et al. (2005)
KHV Bercovier TK R	CAC CCA GTA GAT TAT GC	E	
KHV Bercovier TK nested F	CGT CTG GAG GAA TAC GAC G	E	Garver et al. (2010)
KHV Bercovier TK nested R	ACC GTA CAG CTC GTA CTG G	E	
CEVF1	GCT GTT GCA ACC ATT TGA GA	E	Oyamatsu et al. (1997b)
CEVR1	TGC AGG TTG CTC CTA ATC CT	E	
CEVF2	GCT GCT GCA CTT TTA GGA GG	E	
CEVR2	TGC AAG TTA TTT CGA TGC CA	E	
CEV ForB	ATG GAG TAT CCA AAG TAC TTA G	E	Matras et al. (2017)
CEV RevJ	CTC TTC ACT ATT GTG ACT TTG	E	
CEV ForB-Internal	GTT ATC AAT GAA ATT TGT GTA TTG	E	
CEV RevJ-Internal	TAG CAA AGT ACT ACC TCA TCC	E	
CEV qFor1	AGT TTT GTA KAT TGT AGC ATT TCC	Q	Way et al. (2017)
CEV qRev1	GAT TCC TCA AGG AGT TDC AGT AAA	Q	
CEV qProbe1	AGA GTT TGT TTC TTG CCA TAC AAA CT	Q	

the number of substitutions per sequence. In another analysis, a 357 nt region of all 86 CEV P4a gene sequences available in GenBank was aligned to confirm strain assignment to CEV genogroups.

CEV persistence

On 25 October 2017, 31 carp in Mill Pond that survived the mortality event were captured by an electrofishing boat (Smith-Root). At this time, the pond water had a surface temperature of 16.3°C, reduced dissolved oxygen level (6.46 mg l^{-1}, 66.8% saturation), and a pH of 7.47. All carp captured were maintained alive in a well with flow-through lake water during their transfer to shore. Fish were then euthanized with an overdose of MS-222 and total length and weight data were recorded. The carp ranged from 17.5 to 71.5 cm (mean 54 ± 11.4 cm) in length and from 0.1 to 5.9 kg (mean 2.8 ± 1.4 kg) in weight. Most carp were considered adults except for the 17.5-cm-long fish that weighed only 96 g. Other fish species captured included channel catfish *Ictalurus punctatus*, largemouth bass *Micropterus salmoides*, white sucker *Catostomus commersonii*, bluegill sunfish *Lepomis macrochirus*, pumpkinseed sunfish *Lepomis gibbosus*, rainbow trout *Oncorhynchus mykiss*, and golden shiners *Notemigonus crysoleucas*.

A gill arch from each carp was dissected aseptically and transferred to a Whirl-Pak bag maintained on ice. To prevent cross-contamination of samples, dissecting instruments were sterilized in bleach, washed in water and ethanol, followed by flaming between each fish. Gill samples were maintained on ice during transport to the Pequest Fish Health Laboratory, where they were frozen at −80°C until processed.

CEV screening by real-time qPCR

For real-time qPCR testing for CEV, DNA was extracted either directly from 25 mg gill tissue or from pelleted material generated from 500 mg gill tissue processed as above to purify and concentrate poxviruses. DNA was extracted from each of the samples using the DNeasy Blood and Tissue kit automated on a QIAcube (QIAGEN) following the manufacturer's instructions. Extracted DNA samples (n = 62) frozen at −80°C were shipped on dry ice to the University of Florida in Gainesville, FL, for real-time qPCR analysis (Way et al. 2017). DNA was quantified by Qubit analysis, adjusted to 25 ng μl^{-1}, and 4 µl DNA (100 ng) was amplified by qPCR in a 20 µl reaction containing 1 × TaqMan Universal PCR Master Mix (Thermo Fisher Scientific), 100 µM each CEV forward and reverse primer and 5 µM CEV qPCR probe. Primers are listed in Table 1. The thermal cycling conditions used were 50°C for 2 min followed by 95°C for 10 min and 40 cycles of 95°C for 15 s and 55°C for 1 min using a Quart Studio 5 Real-Time PCE System (Thermo Fisher Scientific). All fish DNAs and CEV plasmid DNA standards prepared to a CEV DNA clone generated using a TOPO TA Cloning Kit #45-0030 (Invitrogen) were amplified in triplicate.

Any DNA that generated a cycle threshold (C_t) value were retested to confirm the result. Positive controls, including gill tissue samples of carp from the mortality event (known CEV-positives), and negative controls were included in the qPCR test.

RESULTS

Necropsy, histology, and TEM

Necropsy of the dead carp revealed that it was a female in spawning condition, with abundant eggs within the coelomic cavity. As internal organs and gills were discolored due to decomposition, no tissue samples were taken. Necropsy of the moribund fish revealed that it was a male in reproductive condition, with large, lobulated paired testes filling the body cavity. Gills were bright red with no grossly visible evidence of necrosis; microscopic examination showed increased mucus production and pale areas indicative of multifocal to diffuse lamellar fusion. Internal organs appeared normal. Notable gill histopathology included diffuse lamellar fusion throughout the gill tissue (Fig. 2A) and sloughing of epithelial cells (Fig. 2B). Fused lamellae contained hyperplastic epithelial cells and large numbers of cells undergoing apoptosis, with pyknosis, cytoplasmic condensation, and apoptotic bodies common throughout lesions (Fig. 2C–E). Focal lamellar degeneration with loss of pilar channel structure was evident in severely affected regions. Severe cell death of interlamellar cells formed rafts of dead cells and cellular debris within fused lamellae (Fig. 2F). Sloughing of epithelial cells was evident on the surface of lamellae and at interlamellar regions (Fig. 2G). Eosinophilic cytoplasmic inclusion bodies indicative of virus infection were observed rarely (Fig. 2H–J).

TEM identified immature, maturing, and mature virions in the cytoplasm of gill epithelial cells (Fig. 3).

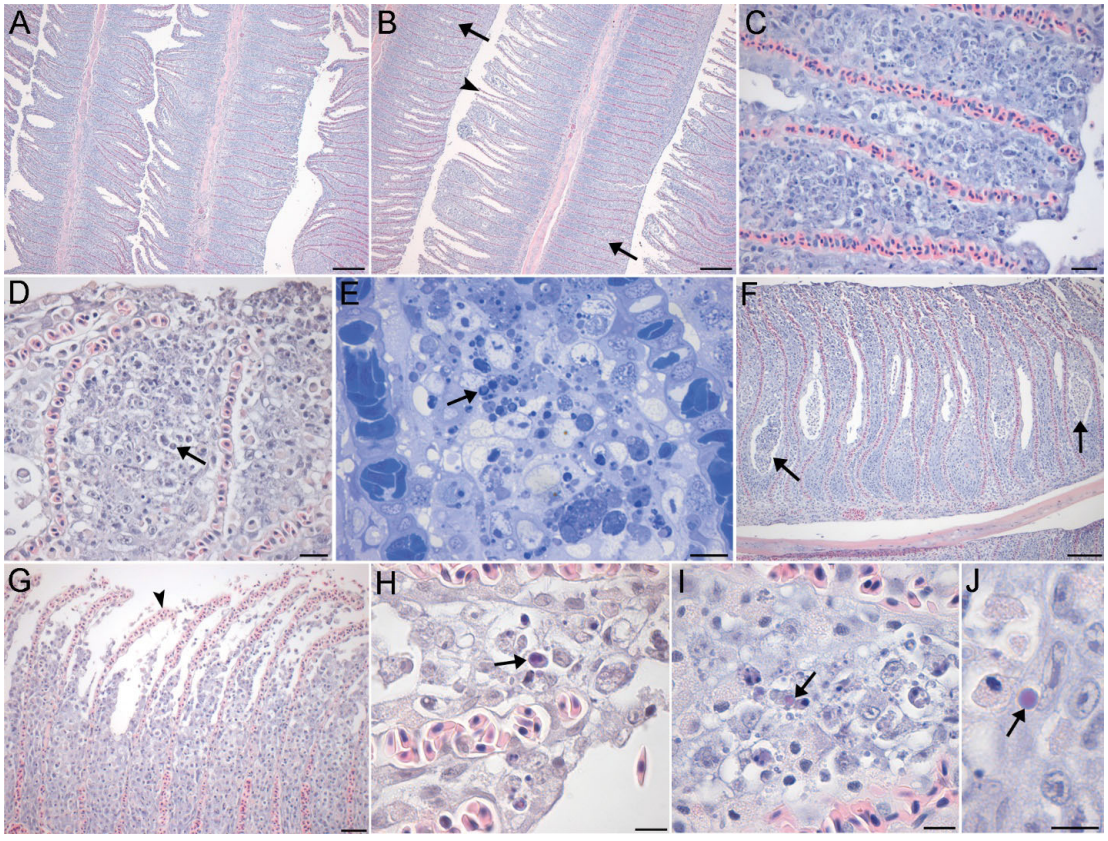

Fig. 2. Histopathologic lesions associated with carp edema virus, staining with hematoxylin and eosin unless otherwise specified. (A,B) Lamellar fusion attributed to epithelial hyperplasia (arrows) and sloughing of lamellar epithelial cells (arrowhead). Scale bars = 200 µm. (C,D) Fused interlamellar zone with extensive pyknosis, condensed cytoplasm, and apoptotic bodies (arrow). Scale bars = 20 µm. (E) High-resolution micrograph from resin embedded tissue showing large numbers of apoptotic bodies (arrow) within the interlamellar zone, stained with toluidine blue. Scale bar = 10 µm. (F) Nests of necrotic and apoptotic cells (arrows) forming in the interlamellar zone. Scale bar = 100 µm. (G) Sloughing of lamellar epithelial cells leaving exposed lamellae (arrowhead). Scale bar = 40 µm. (H–J) Rare occurrence of eosinophilic inclusion bodies (arrows) within cells. Scale bars = 10 µm

Immature virions were spherical (365–404 nm diameter) with a defined electron-dense membrane and an electron-lucent lumen containing loosely arranged material (Fig. 3A). Maturing virions had columnar-shaped projections on one side and increasingly electron-dense lumen contents (Fig. 3A). Mature virions were ovoid to spherical in shape (Fig. 3B–D) and 288–337 nm (mean; 313 ± 16 nm) long by 238–300 nm (mean; 273 ± 19 nm) wide. Mature virions had 10–13 columnar projections projecting from their surface that were 31–56 nm (mean; 41 ± 8.4 nm; n = 10) in length by 38–50 nm (mean; 40 ± 4.8 nm; n = 10) wide (Fig. 3B–D). In rare instances, columnar projections were observed up to 115 nm in length; however, whether this was due to the section plane or virion developmental state is unclear (Fig. 3C). Mature virions contained a unilateral concaved core that was most electron dense in the periphery and a single lateral body on the side opposite the columnar projections (Fig. 3C,D).

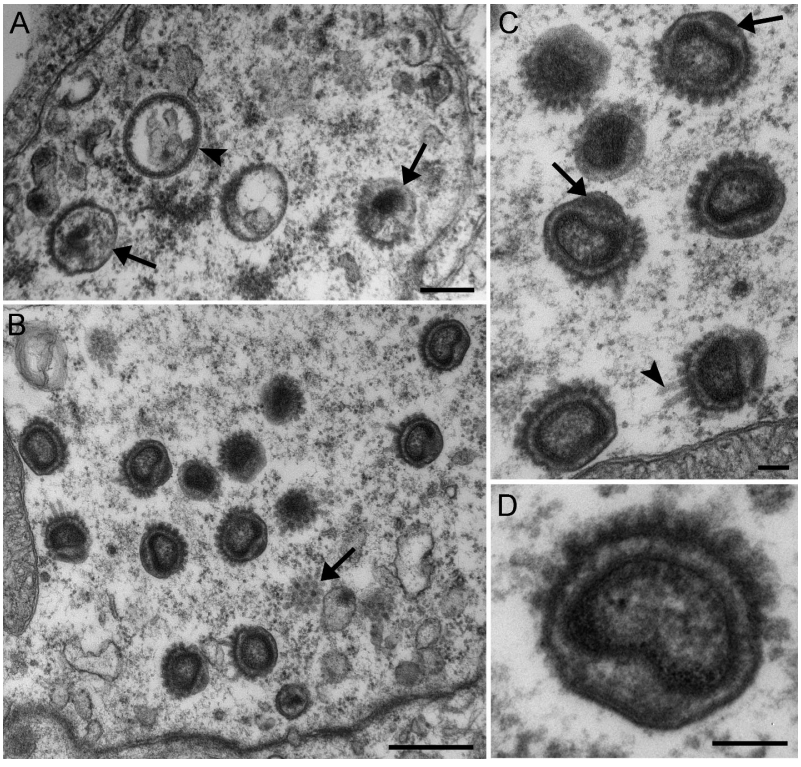

Fig. 3. Carp edema virus seen by transmission electron microscopy. (A) Immature (arrowhead) and developing viral particles (arrows). Note the formation of columnar units and the core on developing virions. Scale bar = 250 nm. (B) Mature virions in the cell cytoplasm. Note cross-sections of the columnar units (arrow) on the surface of the mature virus. Scale bar = 500 nm. (C) Mature virions with a single lateral body (arrows) and columnar projections. Note the long columnar units on one virion (arrowhead). Scale bar = 100 nm. (D) High magnification of a mature virion with columnar projections and a concaved electron-dense core. Scale bar = 100 nm

Diagnosis by conventional PCR and phylogenetic analysis

Gill DNA of the fish tested negative using 3 different PCR tests specific for KHV (data not shown). A CEV-specific nested PCR (Oyamatsu et al. 1997b) only yielded a visible 180 bp DNA product in the nested PCR. BLASTn analysis of the sequence of this DNA (accession no. MH397470) generated hits with sequences (between 92% and 87% identity) of 12 CEV strains including some detected in koi from India and China in 2015–2016 and South Korea in 2017 as well as in carp from Germany in 2014 and China in 2015. Repeat analysis of the gill DNA using an endpoint nested PCR (Matras et al. 2017) resulted in DNA products being detected in both the PCR and nested PCR steps of the test. BLASTn analysis of the 506 bp consensus sequence of the PCR product (accession no. MH397469) showed the highest (99%) identity to various CEV strains detected in pond-farmed carp from Poland.

As previously reported (Matras et al. 2017, Adamek et al. 2018), maximum likelihood phylogenetic analysis of a 416 nt sequence showed CEV strains to segregate into 2 major genogroups (I and II), with genogroup II forming 2 subgroups (IIa and IIb) (Fig. 4). The carp CEV strain examined here clustered most closely with genogroup I strains detected in carp raised for food in Poland and the UK, though these were not well supported as clades. When a trimmed 357 sequence contained within all 86 CEV sequences present in GenBank was analyzed, genogroup I was identified to comprise only carp strains (37 sequences) while genogroup IIa comprised 19 koi and 10 carp strains and genogroup IIb comprised 18 koi and 2 carp strains.

Testing for CEV persistence

For all 31 carp captured by electrofishing on 25 October 2017, real-time qPCR failed to detect CEV in DNA extracted either directly from gill tissue or from gill tissue processing to purify/concentrate CEV particles (data not shown). Gill DNA from the CEV-positive carp sampled dur-

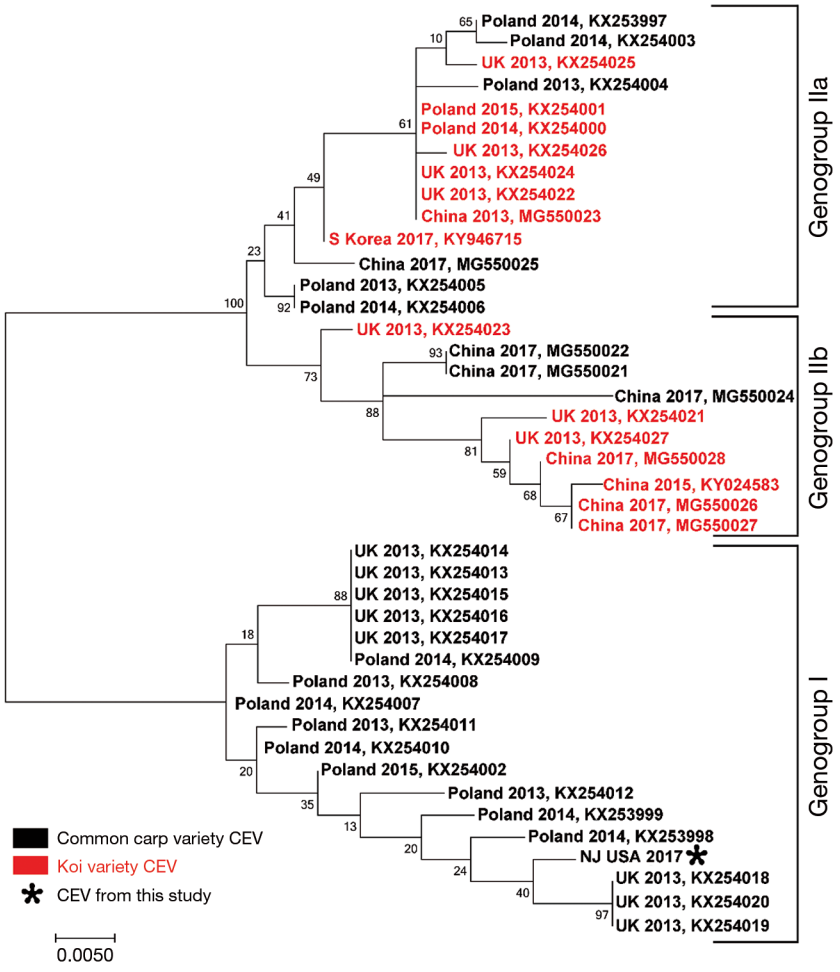

Fig. 4. Maximum likelihood tree of carp edema virus based on partial P4a sequences showing the carp edema virus sequence described herein grouping within genogroup I. Virus sequences from koi (red) and common carp (black)

ing the mortality event and used as a positive control generated a qPCR C_t value of 27.

DISCUSSION

This is the first study identifying CEV as the cause of mass mortality in wild carp in North America, as well as its first detection in the state of New Jersey, USA. As yet, however, the range of CEV in wild carp populations in the USA is unknown and the recent development of CEV-specific PCR tests will aid in elucidating the range of this virus in North America. Before this event, North American detections of CEV were limited to only imported koi populations in California, Washington, North Carolina, Georgia, and Florida (Hedrick et al. 1997, Hesami et al. 2015). As with KSD, the few carp observed here were swimming slowly near pond edges and appeared lethargic, with the one sampled fish observed to rest at the pond edge. However, those who witnessed the event suggested that most carp died in deeper water before floating to the surface several days postmortem making collection of moribund fish difficult. The finding of only large adult carp affected by this kill may be explained simply by the size distribution of carp inhabiting the pond, being composed mostly of adult carp in spawning condition. The electrofishing survey undertaken 5 mo after the mortality event showed the carp population in the pond contained mainly adults with only a single juvenile collected. As air temperatures fluctuated significantly in the 2 mo (April and May) preceding the carp kill, this would have caused water temperatures to also fluctuate significantly in the shallow pond; thus, temperature stress might have been a contributing factor. Moreover, with fish being in spawning condition, it is possible that spawning stress also contributed to acute CEV infections establishing. While at least 526 carp died, the total pond population was not estimated to determine the percent mortality related to the kill. However substantial, the detection of 31 mostly adult carp in the follow-up electrofishing survey indicated that some survived the event.

Unfortunately, only a single live lethargic carp collected toward the end of the mortality event proved to be suitable for clinical and molecular analysis. The gills of this carp had histopathology and virus particles consistent with CEV, and the presence of CEV DNA was confirmed using CEV-specific PCR tests, with no notable secondary infections. Importantly, only carp were affected in this kill, further supporting CEV being the causative agent due to its high specificity for this species. While these data clearly implicated acute CEV infection as the cause of the mortality event, the study of more fish would have assisted in confirming this beyond doubt.

Gill pathology such as epithelial hyperplasia, lamellar fusion, and cellular necrosis is not exclusive to

CEV and can be caused by environmental factors as well as some bacteria, parasites, or other viruses including KHV (Miyazaki et al. 2005, Way et al. 2017). Secondary infections can also complicate diagnosis as found previously with CEV (Lewisch et al. 2015) and salmon gill poxvirus (Gjessing et al. 2017). Gill histopathology in the index carp examined here had lesions mostly dominated by apoptosis, as evident by affected interlamellar regions appearing granular due to the presence of high numbers of apoptotic bodies and cells with pyknotic nuclei and condensed cytoplasm, all hallmarks of apoptosis (Cooper 2002). The high degree of apoptosis in the gill is not typical for other carp gill diseases, and perhaps this may be used as a histologic sign to differentiate CEV from other gill diseases, such as KHV. Cells within lesions also did not display nuclear heterochromatin margination as observed typically with KHV due to it replicating in the nucleus (Hedrick 2004). Moreover, unlike systemic KHV infections in which lesions develop in kidney, spleen, and other tissues (Hedrick 2004), lesions in the index carp were only evident in gill, consistent with the limited tissue tropism of CEV. While eosinophilic granular cells (EGCs) were evident in gill lesions, as reported previously in other CEV cases (Miyazaki et al. 2005, Kim et al. 2018), these are found commonly in varying numbers in overtly healthy fish; thus, their presence in relation to CEV infection is difficult to assess. Aside from the EGCs, there was little other evidence of inflammation in the gill lesions, possibly due to the widespread apoptosis, which, unlike necrosis observed commonly in KHV infections (Hedrick 2004, OIE 2017), does not elicit a pronounced inflammatory response (Cooper 2002).

While TEM rarely detected virus in CEV cases in carp (Way et al. 2017), viral particles consistent in morphology with CEV in epithelial cells nearby to, but never in, apoptotic cells were associated with lesions in the gills of the index carp in this case. It is possible that cell apoptosis limits virion maturation later in the virus replication cycle, but further research will be needed to explore its role in the disease pathogenesis. Considering that poxviruses elicit several anti-apoptotic factors to ensure the viral replication cycle can complete (Nichols et al. 2017), the high levels of apoptosis evident in the index carp were unusual. However, apoptosis is a strong indicator for salmon gill poxvirus infection, the only other fish poxvirus studied in any detail (Gjessing et al. 2015, 2017). Its role in the fish defense response and how it impacts the outcome of infection or differences in CEV genogroup virulence (Adamek et al. 2017b) appear worthy of investigation.

A nested PCR (Oyamatsu et al. 1997b) and an improved endpoint nested PCR (Adamek et al. 2017a, 2018, Matras et al. 2017) were used to detect CEV DNA. Primers employed in the nested PCR were designed to detect CEV strains infecting koi without regard for strains infecting carp (Adamek et al. 2017a). Using gill DNA from the index carp, a DNA product (180 bp) was only detected following nested PCR, but sequence analysis confirmed it to be most related (87–92% identity) to sequences of 12 CEV strains present in GenBank. It was likely, therefore, that primer–template mismatches reduced PCR amplification efficiency, as found previously with this test (Adamek et al. 2017a). In the revised nested PCR (Matras et al. 2017), DNA products were detected in both the PCR and nested PCR test components, and sequence analysis of the 506 bp region of the gene encoding the major core protein P4a confirmed the virus to be CEV and allowed more extensive phylogenetic analysis.

The partial major core protein P4a nucleotide sequence clustered the index carp CEV strain with genogroup I strains reported previously in European carp reared for food, and segregated strains into 2 primary genogroups, with genogroup II strains demarcated into genogroup subtypes IIa and IIb as reported previously (Matras et al. 2017, Adamek et al. 2018). The existence of 2 CEV lineages may be related to geographic or host species factors in their evolution. Interestingly, all koi CEV strains identified to date have clustered within genogroups IIa and IIb, while different carp CEV strains have clustered within either genogroup, suggesting the possible transfer of koi CEV strains to carp.

CEV has been suggested to have been introduced to the USA via imports of koi (Hesami et al. 2015). However, as a CEV genogroup I strain was detected in the wild carp examined here, its origin is more likely from introduced carp rather than koi that to date, have only been identified to be infected by CEV genogroup II strains. Further, prior to this report, genogroup I has only been reported from Europe, making this the first report of this genogroup outside of Europe. Irrespective of what genetic diversity exists among CEV strains present in koi and carp in the USA, its detection in wild carp reinforces the importance of biosecurity, with both hobbyists and commercial koi operations, to avoid any potential for contracting the virus from wild carp.

Important to the epidemiology of CEV is its fate in fish that survive a disease event. As the global spread of CEV is believed to have been promulgated by international movements of live koi and carp, virus

persistence in fish surviving a CEV epizootic has been considered a major risk factor for spreading infection. Contrary to this belief, however, no evidence of CEV was detected in the gills of carp examined here from the same pond 5 mo after the mortality event, consistent with other recent studies in which CEV was not detectable either 4 wk after signs of clinical disease abated in an experimental CEV challenge trial (Adamek et al. 2017b) or 6–11 mo after CEV clinical signs abated from a diseased koi population (Stevens et al. 2018).

Unlike herpesviruses such as KHV that can establish lifelong latent infections (Eide et al. 2011), poxviruses do not, but might establish low-level persistent infections within susceptible populations, for which there are examples in terrestrial animal species. While CEV may persist in gills and skin of carp and koi, this appears less likely due to the fast turnover of epithelial cells. Alternatively, poxviruses can also persist in the environment. Vaccinia virus, for example, which was used as a vaccine to eradicate smallpox in humans, is believed to have persisted in nature for at least 20 yr and re-emerged as problematic poxviruses of animals and humans including buffalopox in India and cantagalo virus in Brazil (Dumbell & Richardson 1993, Damaso et al. 2000). However, whether CEV can persist in aquatic environments or alternative hosts will also need to be explored further. The role of stress, such as incurred during fish transport, in activating dormant infections should also be investigated. In the carp mortality event examined here, there were no known records of recent carp or koi introductions. If not from such sources, it is possible that CEV was present in the resident population with acute infection being triggered by significant water temperature fluctuations combined potentially with reproductive stressors. However, further studies on the role of fish, vectors, and virus stability in the environment are needed to better understand the epidemiology of CEV.

Acknowledgements. We are grateful for the thoughtful input provided by Dr. Nick Phelps during the investigation of this case. We thank the Bureau of Freshwater Fisheries (N.J. Division of Fish and Wildlife), specifically Charles Sedor, Emily Powers, Ross Shramko, and Mark Boriek, for their assistance. We also thank the Animal Health Diagnostic Laboratory, N.J. Department of Agriculture, for their laboratory support and both Raymond Cywinski from Suez Canal, and Linda Quinn from the Bergen County Department of Health Services for their cooperation throughout the study. Funding for this study was provided by the Federal Aid in Sport Fish Restoration Act, Projects # FW-69-R18 and FW-69-R19, and the New Jersey Hunter and Anglers Fund.

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Editorial responsibility: Jeff Cowley, St. Lucia, Queensland, Australia

Submitted: May 30, 2018; Accepted: September 13, 2018
Proofs received from author(s): November 7, 2018