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REVIEW

Emergence of carp edema virus (CEV) and its significance to European common carp and koi *Cyprinus carpio*

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ABSTRACT: Carp edema virus disease (CEVD), also known as koi sleepy disease, is caused by a poxvirus associated with outbreaks of clinical disease in koi and common carp *Cyprinus carpio*. Originally characterised in Japan in the 1970s, international trade in koi has led to the spread of CEV, although the first recognised outbreak of the disease outside of Japan was not reported until 1996 in the USA. In Europe, the disease was first recognised in 2009 and, as detection and diagnosis have improved, more EU member states have reported CEV associated with disease outbreaks. Although the structure of the CEV genome is not yet elucidated, molecular epidemiology studies have suggested distinct geographical populations of CEV infecting both koi and common carp. Detection and identification of cases of CEVD in common carp were unreliable using the original PCR primers. New primers for conventional and quantitative PCR (qPCR) have been designed that improve detection, and their sequences are provided in this paper. The qPCR primers have successfully detected CEV DNA in archive material from investigations of unexplained carp mortalities conducted >15 yr ago. Improvement in disease management and control is possible, and the principles of biosecurity, good health management and disease surveillance, applied to koi herpesvirus disease, can be equally applied to CEVD. However, further research studies are needed to fill the knowledge gaps in the disease pathogenesis and epidemiology that, currently, prevent an accurate assessment of the likely impact of CEVD on European koi and common carp aquaculture and on wild carp stocks.

KEY WORDS: *Cyprinus carpio* · CEVD · Koi sleepy disease · Poxvirus · PCR · Aquaculture

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INTRODUCTION

Freshwater fish species, and particularly cyprinids, are an important global source of food. Common carp *Cyprinus carpio* is the cyprinid species most widely cultured and is among the most economically valuable species in aquaculture. According to statistics for 2013, European common carp production was 164 066 t (FAO 2015). Global production of the species increased by 92.5% in the 10 yr from 1994 to 2003 (from 1 535 905 to 2 956 211 t) but increased by only 59% in the next 10 yr from 2004 to 2013 (from 2 559 721 to 4 080 045 t; FAO 2015). As well as production for human consumption, common carp produced in aquaculture are also stocked into angling waters. Ornamental varieties of *C. carpio*, such as koi, produced for the pet fish market, represent one of the most expensive markets of individual freshwater fish (Rakus et al. 2013).

Disease is a factor that is likely to have slowed the growth in production of common carp and, in particular, the emergence of koi herpesvirus disease (KHVD) in the late 1990s. The disease caused major economic losses in countries worldwide. The rapid spread of KHVD was attributed to the international ornamental (pet) fish trade and regional koi exhibitions, and competitions where fish are displayed together in tanks or in ponds without quarantine considerations (Haenen et al. 2004). Less stringent disease checks of ornamental fish at border crossings increased the risk of spreading KHVD, and the herpesvirus was introduced into some countries with apparently healthy carrier fish before there was awareness of the disease and its consequences (Gilad et al. 2002, Haenen et al. 2004). Furthermore, other less easily recognised viral diseases can also be rapidly and carelessly spread by the same means.

Carp oedema disease was originally characterised in Japan in the 1970s, described as a viral oedema of juvenile carp and termed 'carp edema virus disease' (CEVD) (Murakami et al. 1976, Ono et al. 1986). The disease has also been referred to as 'koi sleepy disease' (KSD), since infected fish become lethargic and unresponsive (Miyazaki et al. 2005). The causal pathogen of CEVD has been shown to be a poxvirus by electron microscopy (Ono et al. 1986, Hedrick et al. 1997, Miyazaki et al. 2005) and sequence data (Oyamatsu et al. 1997b, T. Waltzek unpublished), and the disease is widespread across Japan where koi are cultured (Amita et al. 2002).

As was the case with KHVD, international trade in koi has undoubtedly led to the worldwide spread of CEVD, but the occurrence of disease outbreaks has often not been recognised; consequently, outbreaks were rarely reported. Unlike in KHVD cases, low mortality rates and the presence of secondary infections have made CEVD difficult to diagnose, particularly in common carp populations. However, a direct causal link between CEV and the disease outbreaks is often difficult to establish through lack of supporting evidence and is often confounded by immunosuppression and the presence of serious secondary bacterial and parasite infections.

The primary aim of this paper is to raise awareness, in diagnosticians and legislators, of the emerging threat of CEVD. The paper provides a review of the emergence of CEVD and the current global situation with special attention to the increasing detections in Europe. Recommendations on effective diagnostic techniques and possible strategies for prevention and control of CEVD in Europe are also given. Some of the information included in this paper was presented at a CEV workshop held on 12–13 January 2015 in Copenhagen, Denmark, organized by the European Reference Laboratory (EURL) for Fish Diseases in Denmark in collaboration with the Central Veterinary Institute (CVI) of Wageningen UR in the Netherlands (EURL 2015a). More recent information is also included from a workshop (Haenen et al. 2016) and meeting (EURL 2015b) held during the European Association of Fish Pathologists Conference in Gran Canaria, Spain, in September 2015.

DISEASE AETIOLOGY

Poxvirus particles have been observed in the cytoplasm of hypertrophic epithelial cells of the secondary gill lamellae by transmission electron microscopy (TEM; Fig. 1). Ono et al. (1986) described an enveloped virus, about 250–280 nm diameter, with a mulberry-like appearance. Hedrick et al. (1997) demonstrated ovoid virions, 360 nm in diameter, with 1 flattened surface, in the cytoplasm of infected gill epithelial cells. The rounded aspect of the virion was covered with projections (capsomeres) arranged symmetrically around a kidney-shaped nucleoid. Miyazaki et al. (2005) described mature virions in hypertrophied cells as ovoid, pleomorphic and 333–400 × 400–413 nm in size with an electron dense, dumbbell-shaped core.

Clinical signs of disease

As the name 'koi sleepy disease' suggests, the most consistent behavioural sign is pronounced lethargy

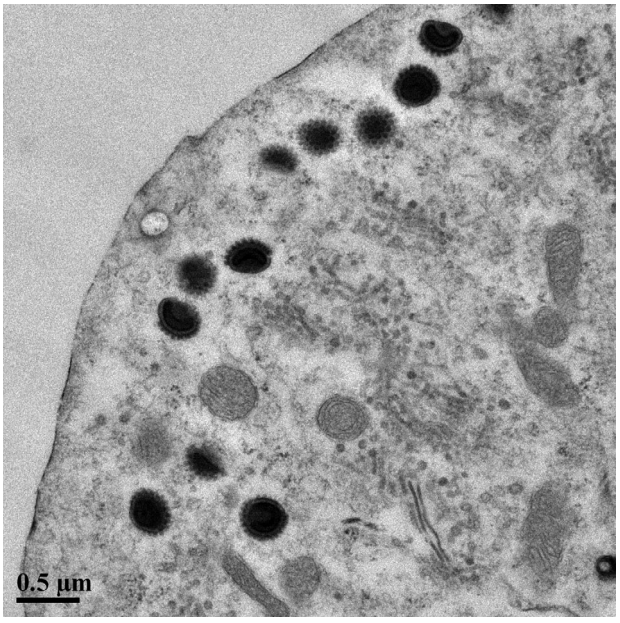

Fig. 1. Transmission electron photomicrograph of a koi *Cyprinus carpio* gill epithelial cell containing spheroid carp edema virus particles. Reproduced with permission from Hesami et al. (2015). Original publisher: University of Florida

and unresponsiveness. The affected fish will often lie motionless, sometimes on their side, on the bottom of the pond or tank for long periods, as if sleeping (Fig. 2). When disturbed by physical stimulation, the fish will swim for a short time and then resume their inactive state on the bottom of the pond (Miyazaki et al. 2005, Hesami et al. 2015, Lewisch et al. 2015). Diseased, juvenile koi also show inactivity but tend to congregate at the surface or around the margins of the pond (Oyamatsu et al. 1997a).

The disease occurs naturally in koi at water temperatures between 15 and 25°C with a cumulative mortality that may reach 75–100% in juvenile koi. At higher temperatures, fish die within 2 or 3 d of first showing lethargic behaviour (Hedrick et al. 1997, Miyazaki et al. 2005, Jung-Schroers et al. 2015, Swaminathan et al. 2016). In common carp and sometimes in koi, disease outbreaks also occur during periods of low water temperatures (6–10°C), and the course of the disease is more protracted, with lower mortality (Way & Stone 2013, Lewisch et al. 2015). External gross clinical signs, seen particularly in juvenile koi, include extensive erosions and haemorrhages of the skin with oedema of the underlying tissue (Miyazaki et al. 2005). Overproduction of mucus on the skin and gills is often observed (Pretto et al. 2015, Zhang et a. 2017). Other gross clinical signs, seen particularly in adult fish, include enophthalmia (sunken eyes, Fig. 3), anorexia, ulcers around the mouth and base of the fins, pale swollen gills (Fig. 4) and inflammation of the anal vent (Miyazaki et al. 2005, Haenen et al. 2014, Jung-Schroers et al. 2015, Swaminathan et al. 2016, Zhang et al. 2017).

The most severe microscopic changes are seen in the gill tissue, and these are described in the 'Diagnostic methods' section below. As a result of the gill damage, diseased fish are less active, display pronounced lethargy and die of anoxia (Miyazaki et al. 2005).

Parasitic, bacterial and viral infections have frequently been reported in CEVD cases (Haenen et al. 2014, Lewisch et al. 2015, Pretto et al. 2015, Way

Fig. 2. Common carp *Cyprinus carpio*, held in the Experimental Facility at Cefas, Weymouth, UK, showing carp edema virus disease signs of extreme lethargy. Affected fish often lie motionless, sometimes on their side, on the bottom of the tank for long periods, as if sleeping. Reproduced with permission from Way & Stone (2013). Original publisher: Cefas Lowestoft

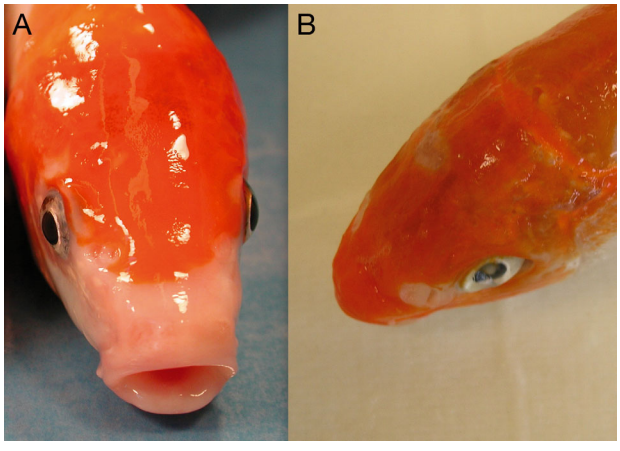

Fig. 3. Koi *Cyprinus carpio* displaying (A) normal eyes and (B) enophthalmia (sunken eyes) sampled during the first carp edema virus disease case in the Netherlands in 2013. Image courtesy of O. Haenen

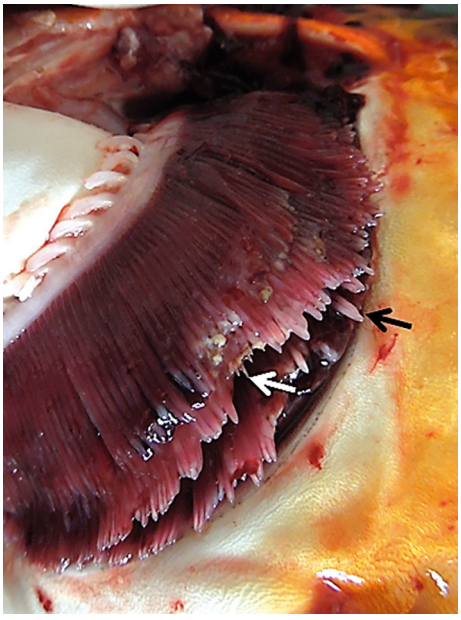

Fig. 4. Gills of carp edema virus-infected koi *Cyprinus carpio*. Swelling of the primary filaments (black arrow) and necrosis of gill tissue (white arrow) can be seen. Reproduced with permission from Jung-Schroers et al. (2015). Original publisher: BioMed Central

et al. 2015, Bachmann & Keilholz 2016, Swaminathan et al. 2016). Additionally, CEV has been detected in archive samples previously found positive for spring viremia of carp virus (SVCV) (Way et al. 2015) and cyprinid herpesvirus 1 (CyHV-1) (Pretto et al. 2015). In an Austrian survey of potential CEVD cases, multiple parasitosis has been reported; this includes infection with more than one of the following: *Trichodina* sp., *Gyrodactylus* sp., *Ichthyobodo necator*, *Dactylogyrus* sp., *Ichthyophthirius multifilis*, *Bothriocephalus* sp., *Capillaria* sp. and *Argulus foliaceous* (Lewisch et al. 2015, Haenen et al. 2016). Secondary bacterial infections have been reported in a number of CEVD cases, in particular infection with *Aeromonas* sp. (Jung-Schroers et al. 2015, Lewisch et al. 2015, Pretto et al. 2015). The high number and burden of concomitant or secondary infections may suggest that carp affected by CEVD are immunocompromised. Lewisch et al. (2015) suggested that the reduced immune status of the carp accounts for the severity of secondary infections and may explain the chronic nature of the disease during naturally occurring outbreaks. The gill damage induced by salmon gill poxvirus has been suggested to impair innate immunity and allow infections by secondary invaders (Gjessing et al. 2017). Immunosuppression also plays a role in the pathogenesis of mammalian poxvirus infection (Strayer 2012). However, many CEVD cases, reported in Germany and Japan, have not resulted in severe secondary infections (Murakami et al. 1976, Jung-Schroers et al. 2015). Additionally, in the Austrian CEVD survey, multiple parasitosis was also noted in carp mortality cases where CEV was not detected (Lewisch et al. 2015). Other factors, such as poor condition of the fish and poor water quality, may influence the severity of concomitant and secondary infections. To determine if CEV does impair the immune status of carp, experimental infections would need to be carried out under conditions where the other factors can be negated or controlled.

Molecular epidemiology

Members of the family *Poxviridae* exhibit linear dsDNA genomes ranging in size from 150–350 kbp. For instance, the genome of salmon gill poxvirus is about 240 kbp and encodes 206 protein-coding genes (Gjessing et al. 2015). The structure of the CEV genome is not elucidated yet. To date, only a partial sequence of the gene encoding P4a (a putative major core protein) has been used for molecular epidemiology studies. The available sequences initially suggested 2 main lineages of CEV. Lineage 1 contained detections from koi and lineage 2 detections from common carp (Way & Stone 2013). In a more recent analysis of CEV detections from Polish carp farms, 9 Polish isolates originating from traditional carp farms were assigned to genogroup I together with UK sequences from common carp. Five Polish isolates were assigned to genogroup II together with koi sequences from the UK and Japan. However, 2 of the sequences assigned to genogroup II were obtained from common carp maintained on traditional Polish farms. Furthermore, sequences detected in common carp from a further 3 sites were assigned to a distinct genogroup III and were reported to have links with imports of koi from Asia (Matras et al. 2017). This finding, and the assignment of sequences from common carp to genogroup II, may indicate the existence of distinct geographical populations of CEV, some of which infect both koi and common carp. A more recent report analysed 39 field samples from CEVD cases of different geographical origins, from koi and farmed carp (Adamek et al. 2017a). The phylogenetic analysis of a 357 bp fragment of the P4a gene supported the genetic distribution proposed by Matras et al. (2017) with a separation of CEV into genogroups I, IIa and IIb.

Emergence in Europe and current global situation

The first reported outbreak of CEVD occurred in Niigata prefecture in Japan in 1974 (Hosoya & Suzuki 1976). Subsequently, the disease was reported in a number of other prefectures, including Hiroshima and Saitama (Murakami et al. 1976, Murakami & Nemoto 1982, Suzuki & Fukuda 1987), and the virus is widespread in Niigata prefecture where koi are cultured (Amita et al. 2002). In the USA, an epizootic mortality in juvenile koi, associated with a poxvirus, occurred in 1996 in California (Hedrick et al. 1997). Subsequently, the disease has been seen sporadically, associated with outbreaks in imported and domestic koi, in 5 US states: in 2005 in Washington, North Carolina and Georgia; in 2010 in California and Georgia; and in 2014 in Florida (Hesami et al. 2015). The virus has also been detected on koi farms in Brazil, the first detection of CEV in the Southern Hemisphere (Hesami et al. 2015). Also, more recently, outbreaks of CEVD have been reported on koi farms in India and China (Swaminathan et al. 2016, Zhang et al. 2017).

The first published report of CEVD in Europe described detection by PCR of a CEV-like virus in imported koi carp showing typical signs and mortality in 2009 (Way & Stone 2013). The affected koi had been imported into Belgium, from Israel, by an ornamental fish wholesaler, and CEV-like virus was confirmed by the Cefas laboratory in the UK (D. Stone unpubl.). CEV was also confirmed by PCR in samples obtained from Japanese koi imported into Germany in 2009 (S. M. Bergmann unpubl.). Further detections of CEV-like virus were successful from KSD-affected koi imported into the UK in 2011 and in hobbyist ponds in the UK in 2012 and 2013. In all cases, CEV-like virus was detected by PCR and sequencing of the amplified product, but attempts to visualise the virus by TEM were not successful (Way & Stone 2013). In the Netherlands, CEV-like virus was detected in 2013 in juvenile and adult koi suffering high mortality and displaying KSD signs (Haenen et al. 2014). The koi had been imported from Japan 12 mo previously and began to show disease signs 2 mo after the introduction of more Japanese koi.

In the UK, early detections of CEV were from KSD-affected koi. In early spring and in autumn 2012, CEV was detected in common carp undergoing mortality and displaying clinical disease, from fishery sites in south-east England and the English Midlands (Way & Stone 2013). Importantly, outbreaks of CEVD in koi, seen at wholesalers and in hobbyist ponds in the UK, occurred within the temperature range reported in Japanese outbreaks of CEVD (15–25°C; Oyamatsu et al. 1997a, Amita et al. 2002), but CEV was detected in common carp during disease outbreaks at much lower temperatures of 6–9°C (Way & Stone 2013). However, exceptions to this trend have been observed. Disease outbreaks have occurred in common carp in Italy at 6–7°C but also at 23°C, and outbreaks in koi in the Netherlands have occurred at 6–9°C and also at 20–23°C (EURL 2015a).

In Austria, CEVD was diagnosed in spring 2014 in 2 unrelated cases of disease in hobby koi and in common carp imported from the Czech Republic (Lewisch et al. 2015). In both cases, disease signs were first observed at low water temperatures of 7–10°C. Also in spring 2014, in Germany, CEV was confirmed in koi from 6 hobby ponds that had all received koi earlier in the year from the same retailer (Jung-Schroers et al. 2015).

At the CEV workshop in Copenhagen in January 2015, fish disease diagnostic laboratories from the Czech Republic, France and Italy also reported detection of CEV during investigations of koi and common carp mortalities. CEVD cases reported from 10 laboratories in 9 EU member states, including information received after the workshop, concerning CEVD cases in Poland and Switzerland, are summarised in Table 1.

Detection of CEV in more recent carp mortalities has prompted a number of fish disease diagnostic laboratories to re-analyse archived DNA extracts, from previous unexplained carp mortalities. Archived DNA samples, held in laboratories in Austria, France, Germany, Italy, the Netherlands, Poland, Switzerland and the UK, have tested positive for CEV. Some of these archived samples were from cases investigated as long ago as 2004. More information on these cases is also given in Table 1.

DIAGNOSTIC METHODS

Best organs or tissues

When testing clinically affected fish, using light microscopy and TEM and by PCR-based methods, it is recommended to sample gill tissue. The virus appears to be most abundant in this tissue, as evidenced by the high levels of CEV genome detected in gill tissue (Swaminathan et al. 2016). In experimental virus transmission studies, gill tissue from CEV-infected common carp had statistically significant, higher, virus loads compared to other organs such as kidney, spleen, skin and gut (Adamek et al.

Table 1. Carp edema virus (CEV) disease in Europe: the current situation. Personal communications included in this table are taken from reports presented at the CEV workshop (12–13 January 2015; EURL 2015a) or reported to O. Haenen by members of the CEV network

Country	CEV cases/outbreaks & detections in archived samples	Case detail	Water temperature (°C)	Additional positive cases (year)	Reference
Austria	Koi, first case 2014	25% mortality in adult koi	~10	2 (2014)	Lewisch et al. (2015)
	Common carp, first case 2014; also detected in archived DNA from 2013	16% mortality in ~1 kg carp—all confirmed by PCR	7–15		
Czech Republic	Koi, first case 2014	Single mortality	15	Further cases in 2015 & 2016	Veselý et al. (2015), T. Veselý (pers. obs.)
	Common carp, first case 2013; also detected in archived DNA	~60% mortality in 2 yr old carp	13–14		
France	First case in 2013	Adult koi and common carp, 0–90% mortality; PCR and/or qPCR (gills)	Outbreaks in winter & May/July	4 (2014), 1 (2015)	Bigarré et al. (2016)
Germany	Koi, first case in 2009. First report in 2014	First report—spring outbreaks in koi, many cases from 1 wholesale source; up to 100% mortality	17–22;	Numerous linked and unlinked cases (2014–2016)	S. M. Bergmann (pers. obs.), Jung-Schroers et al. (2015), Bachmann & Keilholz (2016), M. Adamek (pers. obs.)
	Common carp, first case in 2015; also detected in 2016 and archived samples from ca. 2011	Clinical signs and losses up to 100% detected in spring and autumn of 2015 in 1–3 yr old carp	10–12		
Italy	Koi, first case 2015	Mortality in garden pond	14–16 (further 13–15)	Numerous outbreaks (2015/16)	Pretto et al. (2015), M. Abbadi & A. Toffan (pers. obs.)
	Common carp, first case 2014; also detected in archived DNA from 2010	Mortality (<10%) in 1–2 kg fish	23 2010 case: 6–7		
The Netherlands	Koi, first case 2013	High mortality in juvenile & adult fish	First 9 (further 20–23)	3 (2014)	Haenen et al. (2013, 2014), O. Haenen (pers. obs.)
	Common carp, first case 2014; also detected in archived DNA from 2004	Heavy mortality (450 carp in 1 wk) in adult fish	First 10 (further 6–10, 17–20)	3 (2014), 3 (2015), 1(2016)	
Poland	Koi, first disease outbreak with mortality 2015; detected in archived samples from 2013–15	5 cases from 1 wholesale source	16–19		M. Reichert (pers. comm.), Matras et al. (2016)
	Common carp, first case in 2013, numerous cases detected in archived samples from 2013–15	No mortality associated with detections. All confirmed by PCR	16–19		
Switzerland	Koi, detected in archived formalin-fixed, paraffin-embedded gill tissue from 2007	High mortality	January	2 (2016)	T. Wahli (pers. comm.)
	Koi, disease outbreaks 2016	No mortality	15–16		
UK	Koi, first case in 2009 (Belgian site), first UK case in 2011	High mortality in juvenile fish	>16	6 (2012), 7 (2013), 2 (2014), 1 (2015), 1 (2016)	Way & Stone (2013), Haenen et al. (2016)
	Common carp, first case in 2012; detected in archived samples from 2004, 1999 and 1998	High mortality in adult fish	6–9		

2017b). In CEVD-affected koi, a mean of 129 982 copies of CEV-specific DNA per 250 ng of extracted DNA has been reported (Adamek et al. 2016). In CEVD-affected common carp, a mean of 504 000 copies of CEV-specific DNA per 250 ng of extracted DNA has been reported (Adamek et al. 2017b).

Light microscopy and TEM

Low magnification examination by light microscopy of fixed and stained gill tissue sections from moribund carp can reveal 'clubbing' of gill filaments caused by proliferation of epithelial cells at the tips of the filaments (Ono et al. 1986, Miyazaki et al. 2005). Examination of gill tissue at higher magnification reveals hypertrophy and severe hyperplasia of the gill epithelial cells and fusion of adjacent lamellae (Fig. 5). Thickening and oedema of epithelial cells in the gill filaments and detachment of epithelial cells is also observed (Jung-Schroers et al. 2015). Oedematous changes are also noticeable at the bases of the gill lamellae (Miyazaki et al. 2005, Way & Stone 2013; see Fig. 5). Abnormal proliferation and sloughing of the epithelial cells of the gills and epidermis have also been reported (Hedrick et al. 1997). The cellular changes, mainly mitochondrial degeneration, seen particularly in cells of the lateral musculature, in hepatocytes and in renal epithelial cells, have been suggested to be the result of hypoxia (Miyazaki et al. 2005) caused by the severe damage to the respiratory epithelia. The cellular changes seen in other organs are not pathognomonic. The changes observed in the gill tissue are also not pathognomonic, as similar gill pathology is often seen in carp suffering from parasite or bacterial infections or KHVD.

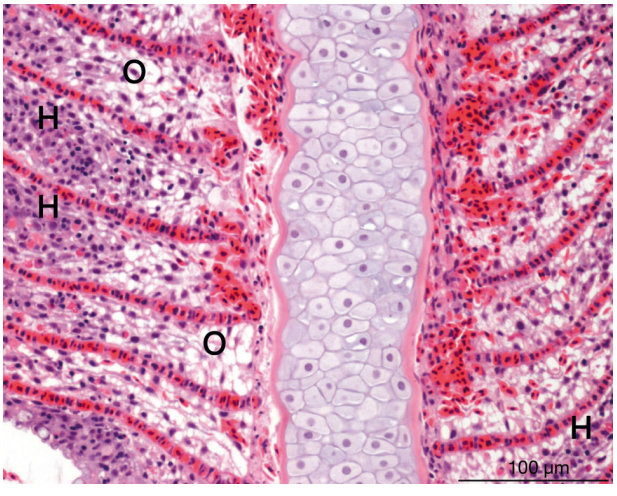

Fig. 5. Gill histopathology in common carp *Cyprinus carpio* showing carp edema virus-like disease signs. H: areas of hyperplasia; O: oedematous changes. Reproduced with permission from Way & Stone (2013). Original publisher: CEFAS Lowestoft

Examination of glutaraldehyde-fixed gill tissue from koi by TEM has revealed hypertrophied cells containing many mature virions within the cytoplasm, not only in the upper layer of hyperplastic epithelia of clubbed gill filaments, but also in the respiratory epithelia of gill lamellae (Miyazaki et al. 2005). However, some diagnostic laboratories have failed to observe virus particles in gill tissue by TEM examination, particularly in samples taken from common carp. This suggests that tissues need to be sampled and fixed for TEM at an optimal time in the virus infection cycle and may require extensive repeat sampling when investigating a suspected disease outbreak in a common carp population.

Virus isolation and detection

The poxvirus cannot be isolated in cell lines that are routinely used for isolation of SVCV and CyHV-3 such as EPC, FHM and CCB cells (Jung-Schroers et al. 2015, Lewisch et al. 2015). Japanese laboratories, and more recently an Indian laboratory, have attempted to isolate CEV in an extensive range of cell lines from different fish species, but none have proved susceptible to the virus (Oyamatsu et al. 1997a, Swaminathan et al. 2016). Primary, explanted, cultures of gill tissue enable replication of CEV over a period of 2 d, measured by mRNA expression; in the same study, CEV replication was not detected in fin tissue (Adamek et al. 2017b). Development of a cell line derived from carp gill epithelial tissue may provide the best opportunity to culture CEV *in vitro*.

PCR for detection of CEV

Detection and identification of cases of CEV in Japan were achieved using PCR primers designed by Oyamatsu et al. (1997b). In their virus survey of colour carp cultured in Niigata prefecture, Amita et al. (2002) used the Oyamatsu primers to successfully detect CEV in koi showing signs of KSD.

In some cases, the Oyamatsu primers produced non-specific products of a similar size to the expected product, making interpretation of the initial stages of the assay difficult. Therefore, in an attempt to improve reliability of detection, new primer sets were designed for both conventional and qPCR assays

based on an alignment of a large region of putative CEV sequence of the P4a gene generated at Cefas and an unpublished CEV sequence provided by T. Miyazaki. The conventional assay produces a clean product when screening diseased fish, and is capable of detecting a range of CEV-like sequences in koi and common carp (Way & Stone 2013). The qPCR assay has also proved useful for surveillance purposes and when screening formalin-fixed, paraffin-embedded archived material (Matras et al. 2017, D. Stone unpubl.). The improved PCR assays are now routinely used to screen tissue samples from koi or common carp mortalities at the Cefas laboratory and the method is detailed below.

DNA is usually extracted from 5 mg of tissue and suspended in 50 µl nuclease-free water. The conventional PCR is generally performed in a 50 µl reaction volume consisting of 10 µl reaction buffer (5× concentrated), 5 µl $MgCl_2$ (25 mM stock), 0.5 µl dNTPs (25 mM mix; Promega catalogue no. U1240), 0.5 µl forward primer (100 pmol μl^{-1} stock), 0.5 µl reverse primer (100 pmol μl^{-1} stock), 0.25 µl Go *Taq* polymerase (5U μl^{-1}; Promega catalogue no. M8305), 30.75 µl molecular biology grade water and 2.5 µl of extracted DNA (equivalent to DNA from 0.1 mg of tissue sample). Cycling conditions are 94°C for 5 min and then 35 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min followed by an extension step of 10 min at 72°C. The forward primer is CEV ForB (5′-ATG GAG TAT CCA AAG TAC TTA G-3′); the reverse primer is CEV RevJ (5′-CTC TTC ACT ATT GTG ACT TTG-3′). In some laboratories, the PCR mix is scaled down to a 25 µl reaction volume in 0.2 ml thin-walled PCR tubes.

For the second-round nested PCR, the same reaction conditions are used except that 0.5 µl of internal forward primer and 0.5 µl internal reverse primer (both 100 pmol μl^{-1} stock) are used and 2.5 µl of the first-round PCR product is added as a template. Cycling conditions are identical to the first round, with forward primer CEV ForB-Internal (5′-GTT ATC AAT GAA ATT TGT GTA TTG-3′); and reverse primer CEV RevJ-Internal (5′-TAG CAA AGT ACT ACC TCA TCC-3′). To visualise the amplification products (528 bp in the first round and 478 bp in the second round), 20 µl volumes of PCR product are electrophoresed on a 2% ethidium bromide stained agarose gel at 120 V for 20 min and visualised under UV light.

The qPCR assay is performed in a 20 µl reaction volume consisting of 1 µl each of 10 mM CEV qFor1 (5′-AGT TTT GTA KAT TGT AGC ATT TCC-3′) and reverse CEV qRev1 (5′-GAT TCC TCA AGG AGT TDC AGT AAA-3′) primers (500 nM final concentration), 1 µl of 4 mM CEV qProbe1 (5′-AGA GTT TGT TTC TTG CCA TAC AAA CT-3′) (200 nM final concentration), 10 µl TaqMan Universal PCR master mix (Thermo Fisher Scientific, catalogue no. 4304437), 5.1 µl nuclease-free water and 2.5 µl of the extracted DNA. The samples are initially held at 50°C for 2 min followed by 10 min at 95°C, and then 50 temperature cycles of 15 s at 95°C and 1 min at 55°C.

The Cefas primers and the above PCR assay methods have been used by other European laboratories to successfully amplify CEV DNA from infected tissue samples from koi and common carp (Lewisch et al. 2015, Pretto et al. 2015, Matras et al. 2017). Although the assays have not been validated for diagnostic sensitivity and specificity to OIE stage 3 validation, initial indications are that both the qPCR and conventional nested PCR assay share similar analytical sensitivities with a detection limit of 1–10 copies $reaction^{-1}$ or 4–40 copies mg^{-1} of starting tissue material (D. Stone unpubl.). In a very recent report of the comparison of 5 different PCR protocols, the end-point and quantitative PCR assays developed at the Cefas laboratory showed the highest analytical inclusivity and diagnostic sensitivity (Adamek et al. 2017a). The Cefas PCR primers are located in a less divergent fragment of the P4a gene and, as a result, the assays developed at Cefas are currently the most suitable protocols for detecting viruses from all known CEV genogroups (Adamek et al. 2017a).

MANAGEMENT AND CONTROL METHODS

There is currently no therapy against CEVD, and the virus may prove difficult to detect at sub-clinical levels in apparently healthy survivors. Therefore, control and prevention requires efforts on a number of levels. Diagnostic laboratories need to adopt the PCR techniques recommended above, so that they can reliably diagnose an infection with CEV. Equally important is to be able to declare freedom from the disease with a reasonable certainty, and this necessitates development of sensitive and reliable screening tests. Disease control measures that have been effective for KHVD should also be effective for CEVD. Effective approaches that have prevented introduction of KHVD are to source fish from reputable suppliers who carry out regular health checks, and/or to establish disease-free broodstock (Haenen et al. 2004). Principles of biosecurity and disease surveillance applied to the production of KHV-free koi in the ornamental fish industry can be equally applied

to CEVD, particularly as sensitive diagnostic tests become more readily available. The Ornamental Aquatic Trade Association has published guidance on good biosecurity practices that are available to both members and non-members (OATA 2015).

Good health management practices should be followed at all times to minimize the risk of disease outbreaks, including quarantining and testing new arrivals, avoiding crowding and stressful events, maintaining good water quality and providing good quality nutrition (Hesami et al. 2015). The suggested quarantine requirement is a minimum of 30 d at a constant temperature between 15 and 25°C and should be carried out in closed facilities to avoid CEV contamination from wild or feral carp. The overstocking of fish should be avoided, and this should include wild/feral carp, in open waters. In the Netherlands, some wild carp mortalities that tested CEV positive displayed anorexia, probably as a result of overcrowding (O. Haenen pers. obs.). If a disease outbreak is suspected, then immediate removal of fish showing disease signs will minimize disease transmission in the population (Hesami et al. 2015).

Regional koi shows where pet fish are transported to and from shows present a serious risk of disease spread. Koi exhibitors are advised to participate in koi shows in which fish from different sources are separated rather than shows where koi are placed together in the same tank. Most importantly, any fish returning from a show should be placed in quarantine before being returned to the general population. Also, as it is not known whether survivors of CEVD outbreaks become lifelong viral carriers, it is recommended that survivors are not taken to koi shows or cohabitated with naïve carp/koi (Hesami et al. 2015).

In Japan, outbreaks of CEVD are managed by holding the koi in 0.5% salt water following exposure to stress, such as transferring juvenile koi from earthen nursery ponds into cement-lined ponds for grading (Seno et al. 2003, Miyazaki et al. 2005). Salt reduces the toxicity of nitrite in the water and, as a result, is thought to reduce methaemoglobinaemia, a known stressor. Therefore, carp exhibit less stress in 0.5% salt water, and proliferation of CEV is reduced (Miyazaki et al. 2005). Some koi producers also avoid harvest during periods when water temperatures are permissive for the disease (15–25°C) (Hesami et al. 2015). However, CEVD outbreaks in both koi and common carp have occurred at 7–10°C (Lewisch et al. 2015), and research is needed to determine if the virus can be transmitted to naïve fish at these much lower water temperatures.

International legislation

There is a strong likelihood that CEV has already spread extensively via the ornamental fish trade. Without sensitive and reliable diagnostic methods to detect sub-clinical carrier fish, absence of the disease cannot be ascertained and therefore disease-free areas cannot be documented. When virus-free areas and batches of fish cannot be identified to a high degree of certainty, legislation is of little use to control and prevent incursion of a virus disease (Haenen et al. 2004). Based on the above, and also the difficulty in fulfilling Koch's and River's postulates for CEV, there is very little likelihood that CEVD would be considered for listing as a notifiable disease. However, this does not prevent governments instigating national disease control measures in an attempt to control the disease.

Potential impact of CEV/KSD on European aquaculture

The movements of fish pathogens with ornamental fish and the active international trade in live fish, including koi, have been recognised as a key pathway for the spread of emerging fish diseases (Hedrick 1996). Gilad et al. (2003) suggested that intensive fish culture, koi shows and regional domestic and international trading are the 3 main mechanisms that have contributed to the rapid global spread of KHVD. Gilad et al. (2003) also highlighted that, at that time, unrestricted movements of koi were commonplace, nearly all without health inspections or implementation of quarantine programmes at the wholesale or individual hobbyist level. Similar to the emergence of KHVD, it is likely that CEV has spread globally by the same mechanisms, and the detection of the virus in common carp is another example of unchecked transmission of a potentially serious disease from ornamental pet fish to consumption and recreational fish culture and to wild fish.

Evidence given in this paper suggests that CEV may be a significant disease agent in previously unexplained carp mortalities and, in particular, mortalities during spring months as carp emerge from overwintering. In England, spring carp mortality syndrome (SCMS) was first reported in the 1980s and first investigated by the Environment Agency and Cefas in the late 1990s. Some cases of spring mortality in carp were associated with CyHV-3 infection (KHVD), but in the majority of cases, the aetiology of SCMS remained unknown (Way & Stone 2013). CEV-

like virus was detected in a high proportion of archived DNA samples and at high levels, by PCR and qPCR, from suspected SCMS cases in England (Way et al. 2015). More recently, the virus has also been detected in gill tissue from archived, paraffin-wax embedded, formalin-fixed tissues, from SCMS investigations conducted over 15 yr ago (Haenen et al. 2016).

A number of unknown aspects of the disease pathogenesis and epidemiology currently prevent an accurate assessment of the likely impact of CEVD. The main knowledge gap is confirmation of the poxvirus as the primary causal agent of CEVD. This is problematical, because Koch's and River's postulates are difficult to establish for CEVD because of the inability to culture the poxvirus *in vitro*. Recent studies in Austria have shown that CEV infection can be transmitted to naïve koi and specific pathogen free common carp after 6 h of cohabitation. Typical clinical patterns and mortality were observed by Day 7 post infection (Haenen et al. 2016). More recently, a German study has reported that common carp, naturally infected with genogroup I CEV, and koi, naturally infected with genogroup II CEV, transmitted the virus to naïve common carp and koi (Adamek et al. 2017b). In the same study, different strains of common carp showed varying levels of resistance to CEV infection. In particular, the Amur strain was resistant to both genogroup I and II CEV and did not develop clinical CEVD (Adamek et al. 2017b). The Amur wild carp, of Asian origin, is also far less susceptible to KHVD than carp strains of European origin (Piačková et al. 2013). The Amur strain is recommended for breeding programmes to limit potential losses from both CEVD and KHVD, but these programmes would benefit from further research into the mechanisms of resistance to CEVD.

A further knowledge gap concerns persistence of the virus in the aquatic environment, risk of recurrence of infection and the possible trigger events that promote disease outbreaks. The stress apparent in carp during harvesting, transporting and re-stocking may play an important role in disease outbreaks. Adamek et al. (2016) reported imported koi with high copy numbers of CEV-specific DNA in gills that had not developed signs of CEVD. The authors suggested possible stress-related reactivation of a persistent CEV infection, similar to that suspected in fowlpox infections. In Austria, restocking was thought to play a crucial role in 2 disease outbreaks in common carp, with the onset of disease observed 2 wk after stocking. In 1 of these cases, the disease was seen almost exclusively in the newly introduced carp of 1.3–1.5 kg. In the SCMS cases investigated in England in 1998–1999, disease outbreaks often occurred 2–3 wk after the introduction of new stocks of carp (P. Bolton pers. comm.).

There are a number of knowledge gaps in the epidemiology of the disease, and one of these concerns the carrier status of previously exposed carp. Latency has not been described in poxviruses (Damon 2007), but, until recently, information on duration of persistence of CEV in affected fish was lacking. In cohabitation experiments, Adamek et al. (2017b) reported that in fish surviving clinical CEVD, CEV could not be confirmed by qPCR 1 mo after the last clinical signs were noted. Furthermore, these fish did not transmit CEV to naïve fish. The authors suggested that survivors of CEVD clear the virus and do not develop a persistent sub-clinical infection. However, the authors also suggested that the influence of stress needs to be explored in carp that have survived CEVD. The main site of replication of the virus, and one of the portals of entry, appears to be gill epithelial cells. In the above mentioned cohabitation study, across the tissues investigated, the gills were the main organ for CEV replication, with consistently high virus loads. However, more studies are needed to determine if there are other portals of entry and further sites of replication of the virus.

A further knowledge gap concerns transmission of CEV and the vectors involved. Water is very likely to be the main abiotic vector, particularly because virus is likely to be released by detachment or sloughing of epithelial cells from the gill filaments (Hedrick et al. 1997). Poxviruses are most commonly transmitted by direct contact or via fomites or by arthropod vectors (Damon 2007). Animal vectors and carp acting as carriers of the virus will potentially play a major role in the further spread of CEV to naïve populations, making future eradication of the disease more difficult.

The extent of the spread of CEV in Europe is not known, but the finding that CEV is associated with SCMS suggests that the virus could be widespread in carp populations in some EU member states. Lewisch et al. (2015) suggested that the low virulence and low mortality rate for adult carp, and chronic nature of the disease, may be responsible for the wide spread of the virus within fish populations. In Poland, as part of a KHVD surveillance programme, tissue samples were collected from 36 carp farms between 2013 and 2015. When the archived samples were later screened with the qPCR assay, 16 of the farms (45%) tested positive for CEV (Matras et al. 2017).

Basic studies are needed to improve virus purification methods and to find, and/or establish, cell lines

that will allow propagation of CEV. Progress in these fundamental research areas will greatly assist the development of diagnostic methods, allow pathogenicity studies, to include establishment of Koch's postulates and investigation of the antibody response in carp.

Development of alternative, non-molecular, diagnostic tests is hampered by the inability to culture CEV *in vitro*. The existing PCR-based assays appear to be sensitive and should be added to national fish health monitoring surveillance programmes in EU member states. However, these tests should be further optimised and validated for both koi and common carp samples to ensure reliable screening of apparently healthy *Cyprinus carpio* populations.

CONCLUSIONS

CEV has been present in several European countries since at least 2004, both in koi in closed facilities and in wild common carp. In the UK, a link has been established with SCMS, indicating that the virus has been present in UK common carp populations since 1998. There is evidence to suggest that CEV causes immunosuppression, resulting in the establishment of extensive secondary bacterial and parasite infections. More extensive investigations, including analysis of blood parameters in affected fish, are needed to confirm immune impairment. Koch's or River's postulates also need to be established to confirm that CEV is the primary causal agent of the disease with which it is associated. The extent of the emergence of CEVD in Europe is difficult to assess because European fish disease laboratories have only recently been able to reliably detect the causative virus. Many research groups are also attempting to establish cell lines in which the virus will propagate. The development of virus-susceptible cell lines will allow development of antibody-detection assays that provide an important tool for estimation of disease prevalence. Conventional and qPCR assays have been developed for detection of the virus, and these methods are described in this paper. Numerous knowledge gaps concerning disease epidemiology and pathogenesis currently do not allow an accurate assessment of the impact of the disease in Europe. Funding is needed for international research projects that will enable these issues to be addressed. An open CEV network exists, headed by Dr. Haenen and supported by the EURL for Fish Diseases, and this network includes the authors of this paper (EURL 2015a).

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