PESTSMART

Review of the literature on cyprinid herpesvirus 3 (CyHV-3) and its disease

Kenneth A McColl

Invasive Animals CRC

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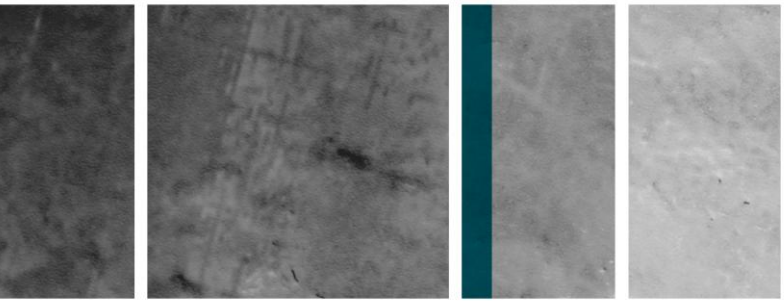
An IA CRC Project



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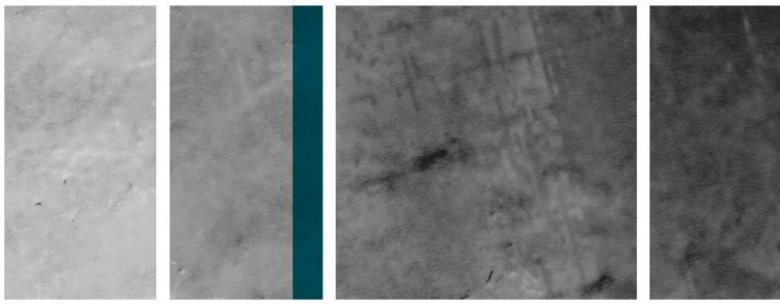

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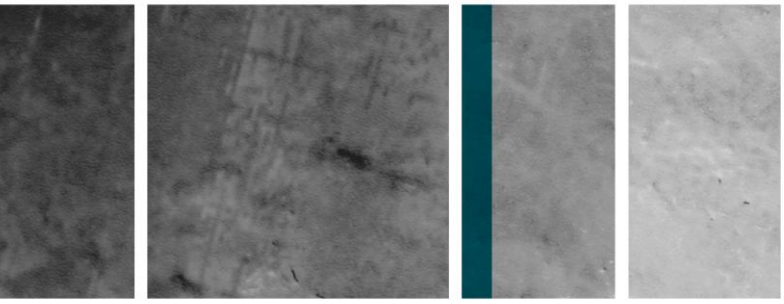
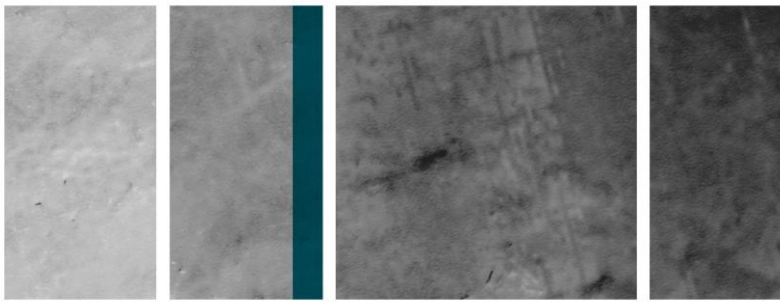




1. Introduction

A disease in carp that was associated with high mortality and that appeared to be of viral aetiology was first described in 1997 in Germany (Bretzinger et al, 1999), and from Israel and the USA in 1998 (Hedrick et al, 2000). There is evidence that CyHV-3 was actually present in carp in the UK in 1996 (Aoki et al, 2007). It has since spread to many other countries around the world. The aetiological agent was considered to be a herpesvirus (Koi herpesvirus, KHV, now known as CyHV-3), and, in experimental infections, was associated with up to 100% mortality in both common carp (*Cyprinus carpio carpio*) and koi carp (*C carpio koi*). Furthermore, CyHV-3 affected fish of any age in either species (Hedrick et al, 2000). The disease is now considered to represent a significant threat to the important carp industry, not only in Eastern Europe (Haenen et al, 2004), but worldwide where the common carp is the world's fourth most-farmed fish (Ronen et al, 2003).

2. Aetiology

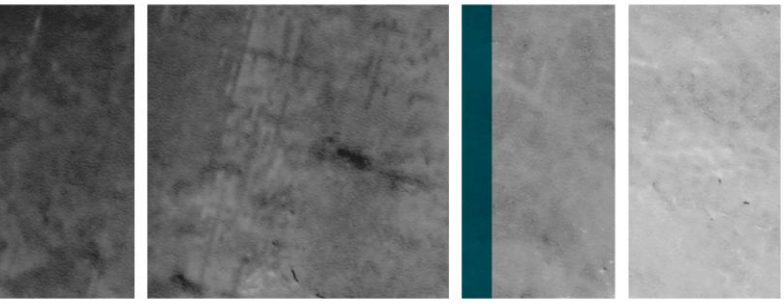
It was recommended that KHV be classified as CyHV-3 (Waltzek et al, 2005). Waltzek et al (2005) noted that herpesviruses have a double-stranded (ds) DNA genome located within an icosahedral capsid that is surrounded by a proteinaceous tegument and an outer envelope. The capsid is composed of 162 hexameric and pentameric capsomeres.

Despite the recommendation for the virus to be called CyHV-3, Ilouze et al (2006) noted that the International Committee on Taxonomy of Viruses had not yet officially named the virus. Unofficially, it has been called KHV, Carp interstitial Nephritis and Gill Necrosis Virus (CNGV), and koi herpes-like virus. However, they claimed that, while the virus looked like a herpesvirus, the large genome (larger than any known herpesvirus apart from the other cyprinid herpesviruses), and the presence of genes that encode peptides that are found in other large dsDNA viruses (but not herpesviruses), suggested that CyHV-3 may belong to a novel virus group.

There was a suggestion that CyHV-3 can be differentiated from another herpesvirus of cyprinids, Cyprinid herpesvirus (CHV, also known as CyHV-1), based on the age of affected fish (Gilad et al, 2003; 2004), ie, CyHV-3 affects fish of any age, while CyHV-1, that can also affect both subspecies of carp, generally only kills young fish upto 2 months old (Sano et al, 1985a,b; 1991).

It should be noted that CyHV-1 is also the cause of "carp pox" (Sano et al, 1985a,b), and upto 60% of fry surviving the acute form of the disease may develop epidermal tumours/hyperplastic papillomas (Sano et al, 1991; Hedrick et al, 1990). CyHV-1-like lesions are seen regularly in Australia, especially in koi carp (Fran Stephens, pers comm). This disease has been present for many years, although there appear to be no formal reports of its presence.

In general, CyHV-3 and CyHV-1 can be differentiated by (1) differences in cell-line specificity (CyHV-1 grows on EPC and FHM cyprinid cell lines); (2) type of cytopathic effect (CPE) in cell culture; (3) antigenic properties. Hedrick et al (2000) demonstrated that anti-CyHV-1



antiserum recognized CyHV-1-infected cells, but not CyHV-3-infected cells; and, (4) timing of lesions (CyHV-1 lesions occur when water temperatures are declining [Hedrick et al, 1990]). Note that goldfish (*Carassius* [*Cyprinus*] *auratus*), which are also cyprinids, are not affected by either of these viruses.

Hedrick et al (2000) and Gilad et al (2004) also mentioned another herpesvirus of cyprinid fish, goldfish haematopoietic necrosis virus (GHNV also known as CyHV-2). CyHV-2-lesions have been detected on a number of occasions, since about 2001, in goldfish in Australia (Stephens et al, 2004). Neither common carp nor koi carp appear to be susceptible to CyHV-2 (Hedrick et al, 2006).

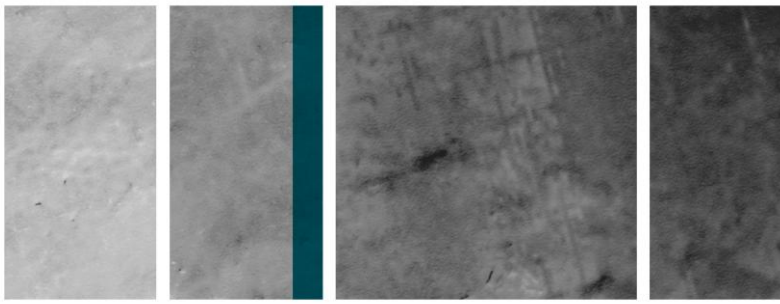
3. Molecular virology

Waltzek and Hedrick (2004) noted that the complete genome sequence was available for CyHV-3. Based on the sequences of four complete genes (two encoding proteins involved in DNA replication, and two encoding capsid structural proteins), Waltzek et al (2005) then proposed that KHV should be classified as CyHV-3. They found that CyHV-3 was most closely related to CyHV-1 and -2, and more distantly to IcHV-1 (channel catfish virus). Later, however, Waltzek et al (2009) slightly revised their view on the taxonomy of cyprinid herpesviruses (when reviewing that of teleost herpesviruses in general). Their conclusion was that there is strong support for the family Alloherpesviridae (comprising 13 fish and amphibian herpesviruses, HVs), and that 2 major clades are present within the family. The first contains cyprinid and anguillid taxa, while the second contains ictalurid, salmonid, acipenserid and ranid taxa (King et al, 2011).

Aoki et al (2007) finally published an analysis of the complete genome. They noted that herpesviruses have now been taxonomically classified into three families, each of which shows little sequence similarity. These are: F. Herpesviridae (includes herpesviruses of mammals, birds and reptiles); F. Alloherpesviridae (viruses of fish and amphibians); and F. Malacoherpesviridae (viruses of invertebrates). They demonstrated that the genome of CyHV-3 was 295 kilobase pairs (kbp), and had a 22 kbp direct repeat at each terminus. There are 156 genes in the genome (with 8 repeated in the terminal repeat, resulting in 164 genes in total). Some of the major characteristics of the genome are:

- CyHV-3 contains only 15 genes that have clear homologs in ictalurid herpesvirus 1 (IcHV-1), thus indicating the clear evolutionary distance between the two viruses.
- Only 13 (of these 15) show clear similarity to the ranid HVs.
- Conserved genes are located centrally in the CyHV-3 and IcHV genomes
- As in mammals, CyHV-3 encodes a protein that is related to a host interleukin-10 (IL-10, and therefore possibly is important in modulating the immune response).

More recently, Doszpoly et al (2011) have sequenced about half of the genome of acipenserid herpesvirus 2 (AciHV-2), and, in the process, they have suggested a new taxonomic grouping for the alloherpesviruses. They say there are now more than 20 that have been described (with only the herpesvirus infecting tilapia larvae said to belong to the F. Herpesviridae), and



that they should now be divided into three clades: (1) one containing CyHV-3 and AnguillidHV-1 (AngHV-1) (the *Cyprinivirus* genus, size range 249-295 kbp); (2) another containing the ranid herpesviruses, RaHV-1 and -2 (the *Batrachovirus* genus, size range 220-230 kbp); and (3) a further containing IcHV-1 and AciHV-2 (the *Ictalurivirus* genus, size range 130-174 kbp). By this scheme, the *Salmonivirus* genus (SalmonidHV-1, -2, -3) would be a sister group of the *Ictalurivirus* genus. Of course, this distribution does not match the taxonomy of the hosts, suggesting that host-switching of viruses has occurred.

One of the most significant advances in understanding the molecular virology of CyHV-3 was the production of a CyHV-3 bacterial artificial chromosome (BAC) clone (Costes et al, 2008). This will allow the study of the role of individual viral genes in the pathogenesis of CyHV-3, and will allow a systematic approach to the production of a recombinant vaccine. The same research group has followed up this achievement with a study in which they identified 40 structural proteins in the mature virion of CyHV-3 (Michel et al, 2010). Three were found to be capsid proteins, 13 envelope proteins, two were tegument proteins, and 22 were unclassified structural proteins. In addition, up to 18 cellular proteins may also be incorporated in CyHV-3 virions.

ORF81, which encodes possibly the most immunogenic membrane protein of CyHV-3 (Rosenkranz et al, 2008), is one of the few proteins for which a specific gene has been identified, although Aoki et al (2011) recently reported on the production of a monoclonal antibody (mAb) against CyHV-3 ORF68 protein (the mAb can be used in an indirect fluorescent antibody test (IFAT), or in immunohistochemistry).

4. Epidemiology

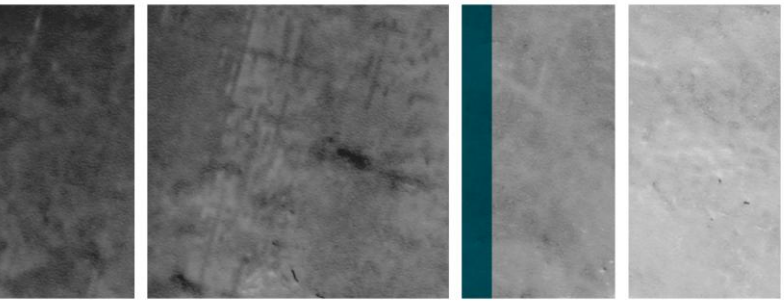
Common carp are important as a food source for humans in Asia, Europe and Israel (Gilad et al, 2003), whereas koi carp are purely ornamental fish. Common carp are native to eastern Europe and central Asia.

4.1 Molecular epidemiology

Three strains of CyHV-3 have been fully sequenced – USA (U), Israeli (I) and Japanese (J) – and >99% identity was found (Aoki et al, 2007). From this work, it was proposed that there were two lineages, J and U/I, and Kurita et al (2009) later confirmed a clear genetic distinction between Asian and European genotypes.

Kurita et al (2009) examined CyHV-3 sequences from many isolates from around the world. Based on their examination, they considered there were two genotypes, Asian and European, with the former having 2 variants and the latter having 7 variants. The Asian and European genotypes can most easily be differentiated by sequencing a particular fragment of the thymidine kinase (TK gene; C-terminal region).

The A1 Asian variant is found in all Asian countries, while the A2 variant has a more restricted distribution (parts of Indonesia and Taiwan). This relative lack of variation in Asian isolates suggests that CyHV-3 has spread in this region more recently than in Europe. There are seven



variants (E1-E7) in Europe, all closely related to each other, and all distinct from the Asian variants (the US isolate appears to be a European variant).

The greater diversity of CyHV-3 in Europe, together with the earliest record of identification (1996), suggests that the European genotype has been present for a longer time than the Asian genotype. Furthermore, it appears that the Asian CyHV-3s did not evolve from European isolates, nor did European isolates originate in Asia. Identification of an isolate that was intermediate between the Asian and European genotypes is now the "holy grail".

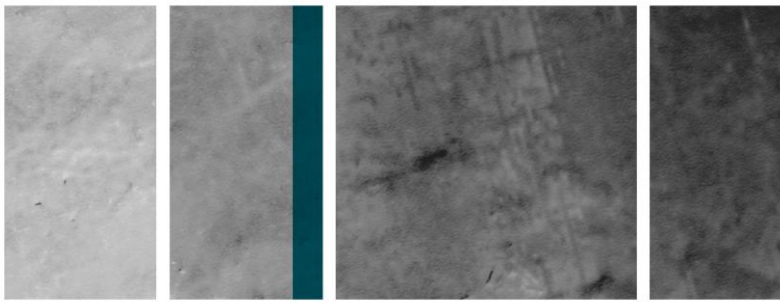
Sunarto et al (2011) found that a number of Indonesian isolates of CyHV-3 were, by analysis of the TK gene, more closely related to the Asian than the European lineage. The Israeli strain (and a single UK strain – G406) also grouped with the Asian strains, while the US strain grouped with the European strains (despite a theory that CyHV-3 entered the US from an infected Israeli koi). Based on marker I and II analysis, Sunarto et al (2011) also found that the Indonesian strains of CyHV-3 may represent a new intermediate lineage.

So, the bottom line with molecular epidemiology, is that there are European and Asian genotypes of CyHV-3, the European consisting of 7 variants and the Asian consisting of 2 variants (US and Israeli isolates are generally considered to be European, despite the findings of Sunarto et al, 2011). New isolates, wherever they are found, need to be characterized, however, because, as in Austria (Marek et al, 2010) where a new isolate was found to be most closely related to a Japanese strain, Asian isolates may be readily introduced into Europe (and vice versa). Presumably these events occur because of global transfer of fish infected with CyHV-3.

4.2 Distribution

Haenen et al (2004) documented the chronological appearance of CyHV-3 in various parts of the world. Retrospective investigations have shown that the disease was first recognized in 1996 in common and koi carp in the UK, but the first definitive outbreaks were described in Israel in 1998 (prototype strain is KHV-I). CyHV-3 has now been found in many countries of the world, including (Haenen et al, 2003, 2004):

- **Europe**: Belgium, Denmark, France (?), Austria, Switzerland, Poland, Luxembourg, Italy, Germany (Bretzinger et al, 1999), the Netherlands, Slovenia (Toplak et al, 2011) and Britain (Denham, [2003] summarised the situation; Taylor et al, [2010]) mapped the distribution, and suggested that CyHV-3-free geographical zones in England and Wales are likely to be difficult to designate).
- **Asia**: Philippines (?), Malaysia (?), Taiwan (Tu et al, 2004; Cheng et al, 2011), Japan (Sano et al, 2004), Indonesia (2002), China (the Chinese virus is said to be an Asian genotype [Dong et al, 2011]), Korea (Oh et al, 2001), Singapore (reported on AquaVetMed, 27 Sept, 2005). The initial outbreak in Japan occurred in farmed common carp, and was then transmitted to wild common carp (Uchii et al, 2009). An outbreak in wild carp in Lake Biwa is said to have killed 70% of the population (100,000 mortalities; Matsui et al, 2008).
- **Africa**: Sth Africa



- **North America**: USA (1998). Prototype strain is KHV-U. Now also found in Canada (Garver et al, 2010) in lakes in Ontario and Manitoba. These are the most northern discoveries of the virus in wild carp in Nth America.

It is now estimated that CyHV-3 has been reported from at least 30 different countries (Haenen and Olesen, 2009).

It is worth noting that the first cases were often not confirmed. It is not clear if the disease first arose in Israel, and then spread to other countries, or if it arose in a number of locations around the world, but was first *identified* in Israel (Promed, 5 Jan 2004). Perelberg et al (2003) provide details of the discovery, and spread, of the disease in Israel.

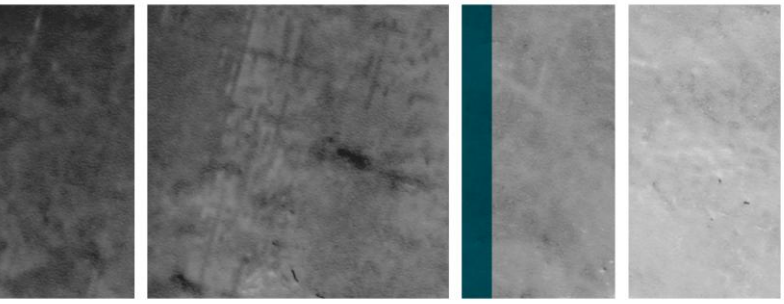
Gilad et al (2003) hypothesize that the virus has spread globally as a result of intensive fish culture, koi shows and local and international trade. On the whole, these activities tend to occur without any restrictions or inspections. Studies by Taylor et al (2011) predicted that movements of live carp were likely to be the main means of transmission of CyHV-3 between locations (in this case, in England and Wales). They suggested that transmission of virus by fomites (such as fishing gear) was of low probability (although the high frequency of the use of fishing equipment suggested that there would, nevertheless, be a risk with this activity). Alternatively, the stocking of imported ornamental fish appears to represent a high-risk activity for the introduction of CyHV-3.

4.3 Temperature susceptibility

The outcome of infection is very dependent on environmental temperatures, with losses tending to occur when water temperatures range from 18-25 ^{o}C (Gilad et al, 2003) or 17-26 ^{o}C (Haenen et al, 2004) or 17-28 ^{o}C (Ilouze et al, 2006). Gilad et al (2003) report that most studies suggest that viruses tolerate a wider temperature range for optimal growth *in vitro* compared with *in vivo*. Gilad et al (2003) suggested that 28 ^{o}C is possibly the maximum water temperature tolerated by the virus in koi. Gilad et al (2004) tested 13 ^{o}C, 18 ^{o}C, 23 ^{o}C and 28 ^{o}C in *in vivo* expts.

Certainly most natural outbreaks seem to occur in the spring, as water temperatures are increasing (Hedrick et al, 2000). It is presumed that the reasons for this are that (1) the immune response of fish is suppressed by the low water temps in the winter, and reactivation of the immune responses then lags behind the ability of the virus to replicate as waters gradually warm-up, and (2) in fish that are infected in the autumn, the virus becomes dormant until water temps begin to warm up again. This reactivation of virus does not occur after indefinite periods of dormancy however.

The Israelis, on the other hand, say that the disease appears in spring AND autumn, corresponding to the times when water temperatures range from 18 ^{o}C to 28 ^{o}C (Perelberg et al, 2003). Gilad et al (2003) found that fish infected at 13 ^{o}C did not die until the water temperature was shifted to 23 ^{o}C at 30 days post eexposure (pe). However, if the fish were held for 60 days at 13 ^{o}C, there was no mortality when the temperature was subsequently shifted to 23 ^{o}C.



Yuasa et al (2008) examined the susceptibility of common carp at three different temperatures, 16 °C, 23 °C and 28 °C. In 4.4 g carp, they found mortality to be 70%, 70% and 50%, respectively (and, at 16 °C, mortality did not begin until 21 days pe).

4.4 Survival of CyHV-3 in water and sediments

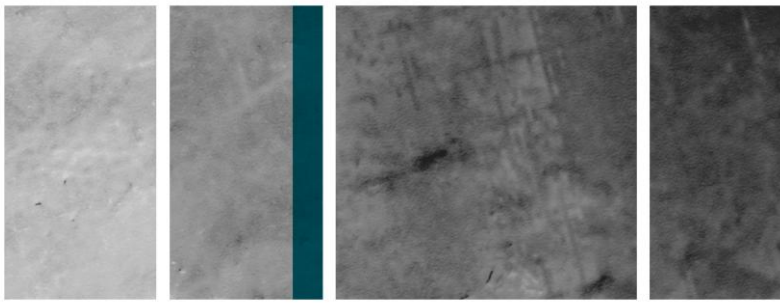
There have been some limited studies on the persistence of the virus in water (Perelberg et al, 2003). This group found that CyHV-3 in water (at an unspecified temperature, but later reported by Ilouze et al, 2006 to be 22 °C) caused mortality when fish were exposed immediately or within 4 hours of the virus being added to the water. However, after 21 hrs, the virus was no longer active. Work by a Japanese group (Shimizu et al, 2006) examined the survival of CyHV-3 in environmental water more thoroughly. They found that when virus persistence was tested in untreated water (or in autoclaved water mixed with untreated sediment), virus fell below detectable levels by 3 days. On the other hand, if the water was autoclaved or filtered, virus was still present at 7 days post inoculation.

Dishon et al (2005) suggested that the virus probably survives longer in fish droppings and in mud. They were able to detect viral DNA and viral antigen (Ag) in faeces by polymerase chain reaction (PCR) and ELISA, respectively, and they also demonstrated the presence of virulent virus in the faeces following inoculation of naive fish. Viral DNA could be detected in faeces as early as 4-8 days post infection (dpi).

Matsui et al (2008) reviewed the methods appropriate for the detection of CyHV-3 in natural freshwater environments. They concluded that there were no well-established methods at that time for CyHV-3. Haramoto et al (2007) used a special concentration procedure to try to identify CyHV-3 in river water in Japan. They were only able to identify CyHV-3 in 2/48 (4.2%) samples. What this meant in practical terms was not at all clear. Minamoto et al (2009) extended this work by examining both the distribution, and the amount of virus, in a Japanese river system immediately after a diagnosis was made of a mass mortality event in the river. They pointed out that detection of CyHV-3 DNA is not the same as detecting infectious virus. Furthermore, they hypothesized that CyHV-3 may have been introduced to the river in the spring or winter when the temperature was low. As the water temperature increased in spring, the virus became more active, and then, in summer, surviving fish became ‘immune’ carriers. As water temperature again fell in the autumn (from above permissive temperatures), virus was activated once again, and carriers began to release more virus into the river until, once again, the onset of lower water temps halted viral activity. So, virus was found in environmental water before, during and after an outbreak of disease.

Later, Minamoto et al (2011b) conducted a survey of 109 of the major rivers in Japan, and they found that, in 2008 (4-5 years after the initial outbreak of CyHV-3 in Japan), 93 of the rivers (90.3%) had CyHV-3 DNA present (minimum detectable dose of 50 copies per litre). Since they showed that viral DNA degrades quickly, their conclusion was that, within 5 yrs of the initial outbreak of disease, almost every river in Japan was contaminated with CyHV-3. Unfortunately, they had no data on carp density in each of the affected rivers.

Further work by Minamoto et al (2011a) identified that CyHV-3 could be demonstrated in plankton in outbreak waters. They examined both zooplankton and phytoplankton, and found the best correlation to be between rotifers (of the zooplankton component) and virus. They



surmised that plankton may be ingested by carp directly (when stirring-up the muddy bottom) or indirectly (by eating bivalves that have concentrated the plankton and virus).

There has been a report of CyHV-3 being found by nested PCR in freshwater mussels and in crustaceans from ponds with a history of CyHV-3 in common carp (Kielpinski et al, 2010). However, it is not at all clear from the work whether the virus actually infected these species, or whether they were simply acting as fomites.

While a number of workers checked for virus in water, Honjo et al (2012) were the first to demonstrate that CyHV-3 DNA could be found in the sediment at approximately 100 times higher concentration than water, suggesting that the sediment could be a reservoir of infection. They did not check the survival time of CyHV-3 in the sediment, but they noted that some enteroviruses have been shown to survive longer in sediment than in water. This suggests that the sediment could be a location for overwintering of virus.

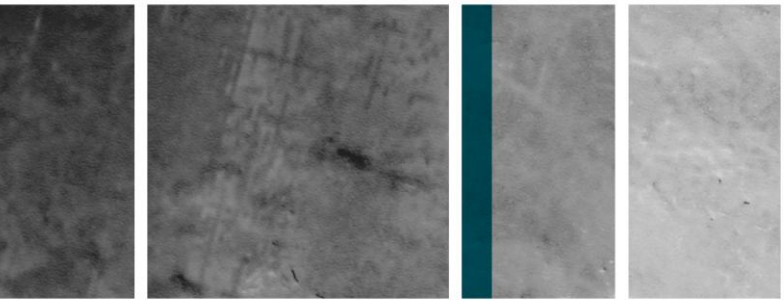
Uchii et al (2009) addressed the question of how CyHV-3 persisted in Japanese waters following the initial outbreak. They examined fish in Lake Biwa following the initial massive mortality. They tested surviving fish for viral DNA (by PCR) and for specific anti- CyHV-3 antibodies, and they demonstrated the presence of CyHV-3 DNA in 44% of antibody-positive individuals. Antibody-positive fish were always >300 mm (ie, mature). They concluded that CyHV-3 persists in surviving (mature) carp in a freshwater ecosystem, and that virus transmission (and mortality) occurs continually, usually in immature naive fish. Spawning would be a good time for virus transmission between these two groups of fish.

4.5 Transmission

Horizontal

Within the optimal temperature range of the virus, fish are susceptible to extremely low concentrations of virus (Gilad et al, 2003). For example, in infection trials conducted at optimal temperatures (18, 23 ^{o}C), most fish died following bath exposure to 12 or even 1.2 $TCID_{50}$/mL of virus. The mortality and "mean days to death"(MDTD) at various temperatures are shown in the accompanying Table.

Temperature (^{o}C)	$TCID_{50}$/mL = 12		$TCID_{50}$/mL = 1.2	
	Mortality	MDTD	Mortality	MDTD
28	17/20	7.7	17/20	9.2
23	19/20	9.2	20/20	9.9
18	20/21	18.2	17/19	23.6
13	0	NA	0	NA



Gilad et al (2004) note that mortality was first observed in koi at 5 days pe at 28 ^{o}C, at 8 days pe at 23 ^{o}C and at 14 days pe for 18 ^{o}C (and no mortality at 13 ^{o}C).

Yuasa et al (2008) examined shedding of virus from infected common carp that were held at 16 ^{o}C, 23 ^{o}C or 28 ^{o}C. Excreted virus was detected by co-habiting koi carp for 24 hr periods with the infected common carp. They demonstrated that virus was shed for extended periods (particularly at 16 ^{o}C), and that virus excretion from infected common carp began before mortality began (eg, at 16 ^{o}C, excretion began at 7 days pe, but mortality did not occur until 21 days pe). This suggests that there is potential for a high risk of transmission from fish that show no clinical signs of disease, and that therefore all newly-introduced fish into an aquarium should be held in isolation at 23 ^{o}C for several weeks.

Vertical

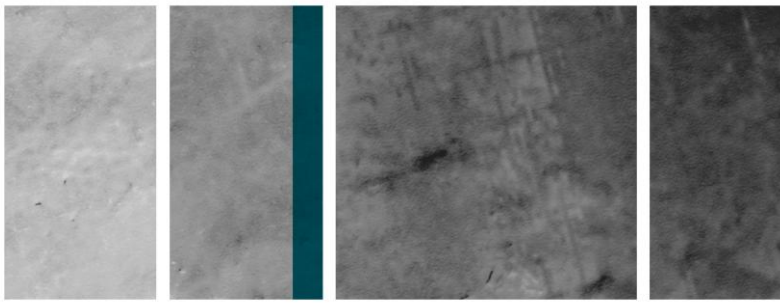
There appear to be no publications suggesting vertical transmission of CyHV-3.

4.6 Affected species

While common carp (*Cyprinus carpio carpio*) and koi carp (*C carpio koi*) are well-known for their susceptibility to CyHV-3, Marek et al (2010) also mentioned that ghost carp (*C. carpio goi*) are also a susceptible subspecies. They provided neither data nor a reference to support their statement however.

The Centre for Environment, Fisheries and Aquaculture Science (CEFAS) has done transmission studies with various cyprinid species, and has found that only carp are affected; they tested goldfish, tench and orfe (Haenen et al, 2003; Haenen and Hedrick, 2006). Bretzinger et al (1999) noted that sturgeon (*Acipenser* sp; Chondrostei) and goldfish (*Carassius auratus auratus*; O. Cypriniformes) remained unaffected when held with affected koi, while Perelberg et al (2003) found that tilapia (*Oreochromis niloticus*; O. Perciformes), silver perch (*Bidyanus bidyanus*; O. Perciformes), silver carp (*Hypophthalmichthys molitrix*; O. Cypriniformes), goldfish (*C auratus*; O. Cypriniformes), and grass carp (*Ctenopharyngodon idella*; O. Cypriniformes) were all resistant to disease after exposure to sick fish (note that Hedrick et al, 2006 stated that the silver perch that were used by Perelberg et al (2003) were American silver perch, *Bairdiella chrysoura* [also O. Perciformes], not Australian silver perch, *Bidyanus bidyanus*, as claimed by Perelberg et al). Furthermore, they also found that, even after cohabitation with infected carp, fingerlings of these resistant species did not transmit disease to susceptible carp. There is very little detail on how these susceptibility trials were conducted. Species whose susceptibility was being examined were exposed to virus by co-habitation with infected carp (5 infected carp per 500 L tank for 5 days). Direct inoculation (eg, intraperitoneal, IP) was not used. Crucian carp (*Carassius carassius*) are also apparently insusceptible (Ito in Haenen and Hedrick, 2006), although Bergmann reported on the susceptibility of both goldfish and crucian carp (in Haenen and Hedrick, 2006). Questions were raised about the latter work, however, since it was primarily based on PCR diagnosis.

Tinman reported that, after goldfish were inoculated with CyHV-3, not only were they unaffected, but they also failed to cause mortality in naïve carp following cohabitation (Haenen and Hedrick, 2006). However, this is a controversial issue, and there has been much debate over it. Sadler et al (2008) reported that CyHV-3 DNA could be detected in goldfish



that had been exposed to infected koi. However, their results could also be explained by the goldfish simply being mechanical vectors of the virus, ie, there was no evidence for replication of CyHV-3 in goldfish. El-Matbouli et al (2007b) also claimed that goldfish, after being exposed to infected koi, were then able to transmit the virus to naïve koi. This undoubtedly occurred, but the most likely explanation (based on the data in the paper) is that the fish were simply acting as fomites. Subsequent work by El-Matbouli and Soliman (2011) seemed to overcome previous doubts, and they demonstrated that goldfish could indeed be carriers of CyHV-3, that CyHV-3 actually replicated in the goldfish (without causing clinical signs of disease), and that the goldfish could disseminate the virus to susceptible carp (4 of 30 carp affected). No histopathological examination of the goldfish was undertaken.

Bergmann claimed that sturgeon, sheatfish, grass carp, bighead carp, silver carp, tench, vimba and different acipenseridae are infectable by CyHV-3. No (abnormal) clinical signs will occur in these species. It is claimed, however, that most of them can transfer the virus to carp or koi (Bergmann, cited in Haenen et al, 2007). Bergmann et al (2010a) confirmed that goldfish are indeed susceptible to infection, but that they do no develop disease (although no histopathology was done on the goldfish). Leucocytes were a very good tissue to identify the presence of virus (in both koi and goldfish), especially by PCR, IFAT and in situ hybridization (ISH). The authors also suggested that goldfish can spread the virus to naive koi carp.

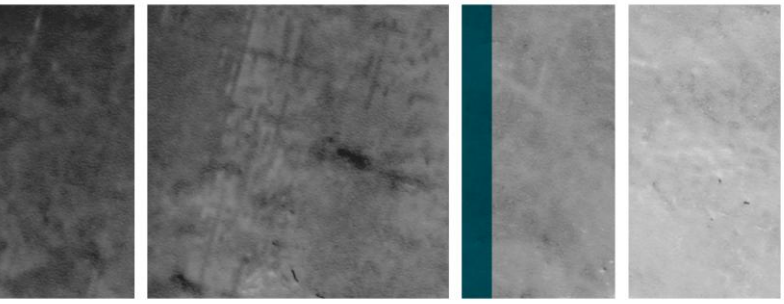
Kempter et al (2009) claimed that their studies on sturgeon demonstrated that these fish could act as carriers of CyHV-3. Only a small proportion of sturgeon was affected, and the ISH and IFAT were not entirely convincing (as a back-up for the initial PCRs). Once again, these sturgeons could simply be acting as fomites. This group claims that they now have a new list of farmed and wild species where the presence of CyHV-3 DNA can be demonstrated: goldfish, crucian carp, grass carp, bighead carp, silver carp, tench, wels catfish, vimba and Prussian carp.

4.7 Susceptibility of hybrids

Bergmann et al (2010b) have been among the first to publish the most convincing data supporting the view that CyHV-3 can be associated with fish other than *C. carpio*. They demonstrated mortality in carp x crucian carp hybrids (7 mths old; 90-100%) and carp x goldfish hybrids (18 mths old; 35-42%) following bath exposure at 10^4 $TCID_{50}$ of CyHV-3 per mL. Mortality continued until 20 dpi for these hybrids (cf. 8 dpi for the positive control carp), and there was slight variation in mortality depending on whether a European or Nth American isolate of CyHV-3 was used.

These findings contradicted those of Hedrick et al (2006) who suggested that hybrids were much less susceptible than pure carp (about 5% mortality in hybrids).

The susceptibility of carp-goldfish hybrids may be an important factor in determining the efficacy of CyHV-3 as a biological control agent in Australia. What is the prevalence of these hybrids in Australian waters, and how are hybrids detected? Yamaha et al (2003) introduced two PCRs, one specific for carp and the other for goldfish (GF). The PCRs were based on highly repetitive sequences of DNA that are unique to each species. So, if both PCRs are positive for a particular fish (using fin clips), the fish is a hybrid. They also found that males



of carp-GF hybrids are sterile (they were unable to check the reproductive capacity of female hybrids).

Haynes et al (2012) published the results of their work documenting the importance of carp-GF hybrids throughout the Murray-Darling Basin (MDB) in Australia. They used microsatellite markers to mark the distribution and prevalence of hybrids, and they found that approximately 3.3% of 1155 fish were hybrids. These included 23 fish that were identified phenotypically as hybrids, 14 wild carp of possible cryptic interspecies ancestry (ie, morphologically identified as carp, but shown to have ancestry from both carp and GF), and one wild GF. Mitochondrial analysis of the 23 hybrids demonstrated that 21 had a maternal GF (and two had a carp on the maternal side). The cryptic hybrids invariably were F2-generation or backcrosses (never F1-generation). The majority of carp-GF hybrids were also found in regions where good spawning sites were limited.

Haynes et al (2012) stated that, like carp, GF are very common throughout the entire MDB, and, in both cases, their breeding seasons overlap (with that of carp being slightly longer). Hybridization studies demonstrated that it was mainly male carp that mated with female goldfish, a fact that could be explained by: (1) larger male carp may produce more milt than smaller GF; (2) smaller male GF might be excluded from mating aggregations of the two species; (3) the slightly longer spawning season of carp might mean that fertile male carp are always around in the GF breeding season, whereas the reverse does not hold; and (4) limited spawning habitat, ie, hybrids are more likely to be found in transient water courses (eg high altitude waterways in Australia).

4.8 Age susceptibility

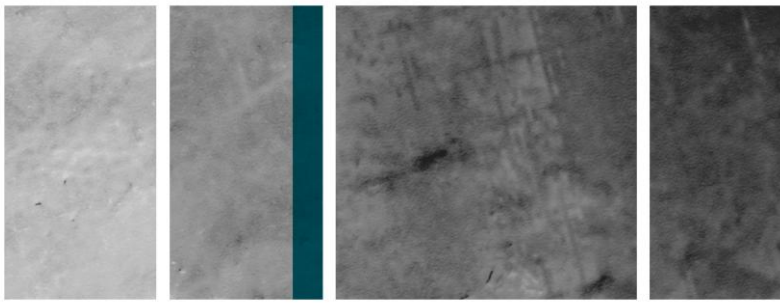
Perelberg et al (2003) demonstrated that >90% of 2.5 g and 6 g fish were killed following exposure to infected fish, whereas only 56% of 230 g fish were killed. They stated that young fish are more susceptible to disease than adults. Ishioka et al (2005) quoted others when they stated that ~70-100% of CyHV-3-infected carp will die. Sunarto et al (2005) suggest that 80-95% mortality can be expected.

Ito noted that although juvenile and adult carp are susceptible to CyHV-3, larvae are probably insusceptible (Haenen and Hedrick, 2006). This was followed by a study by Ito et al (2007) in which they demonstrated that larvae >2 cm were, in general, about 100% susceptible, larvae between 1 and 2 cm were of mixed susceptibility (66%), and larvae/fry <1 cm were generally insusceptible.

4.9 Carrier Fish

Carrier fish represent an important issue in terms of developing control measures. At the International Koi Herpesvirus workshop (2004) it was recommended that fish be held at 23-28 ^{o}C for 2-4 wks followed by a PCR check for CyHV-3.

Adkison et al (2005) quoted other work in saying that most outbreaks of disease due to CyHV-3 are associated with the movement of apparently healthy fish. They suggested that there is strong evidence for latency and a carrier state with CyHV-3, although “convincing laboratory transmission trials have yet to be reported”. They developed an antibody (Ab)-detection



ELISA that is capable of detecting specific Ab in fish for upto 1 yr following infection with CyHV-3, and they suggested that it provided a useful, non-lethal means of detecting carrier fish. Bergmann reported that 7 months after infection, virus could be found at the base of the gills and in kidney, spleen and leukocytes (Haenen and Hedrick, 2006).

Dixon claimed that CEFAS could establish a carrier state in koi carp by manipulating water temperatures (in Haenen and Hedrick, 2006; see also St-Hilaire et al, 2005). With this method, they held fish for 150 dpi, and, when the infected fish were reactivated with increased water temperatures, a number of them died, and all naïve cohabitating fish died too.

4.10 Virus activity in natural populations of carp

In most countries of the world, a major concern with CyHV-3 is the impact that it has on natural wild populations of carp. Diseases of wildlife are said to represent one of the greatest threats to diversity, and, for that reason, Uchii et al (2011) have invested a great deal of effort into understanding the epidemiology of CyHV-3 in wild carp, particularly in Lake Biwa.

Since the initial outbreak in Lake Biwa, where 70% of fish were killed within a few months (Matsui et al, 2008), there have been no further major outbreaks. Uchii et al (2011) have collected data that allow them to hypothesize that annual seasonal temperature shifts in the water, and annual carp reproductive behaviour are pivotal in controlling transmission of CyHV-3 among carp. The remaining key factor is that, in a natural setting like Lake Biwa, a proportion of adult carp are CyHV-3-genome positive, seropositive survivors of infection (and additional such survivors are added each year). In summary, CyHV-3 has evolved to take advantage of:

Seasonal variations in water temperatures

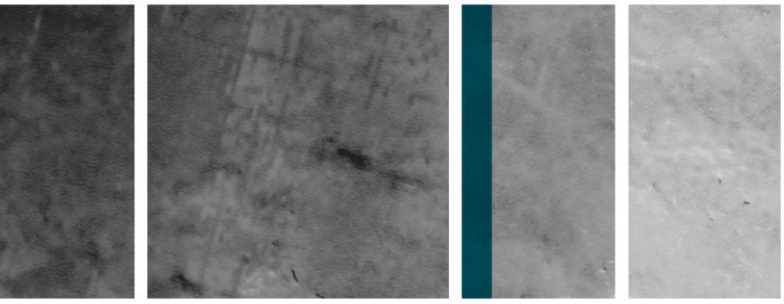
Theoretically, optimal temperatures for virus replication occur in Spring, Summer or Autumn, but, practically, Spring alone seems to be the most favourable time. Why is this?

Seasonal reproductive behaviour

Spring is when carp breed, and breeding results in both immunosuppression of adult fish (suppression of immune functions by sex hormones has been reported in carp), and aggregation of the fish at breeding locations. This leads to gatherings of susceptible hosts, together with seropositive adults that presumably introduce the virus. CyHV-3 spreads among fish at these sites, possibly by direct skin to skin transmission (Raj et al, 2011), and reaches high titres in the fish and in the water of the breeding sites. Presumably some (if not, many) of these fish die, while a few survive, and seroconvert. Cell-free virus in the water survives for a maximum of about 3 days (Shimizu et al, 2006).

So, important questions are: “Is the breeding season a relatively short and tight event, and is it likely that most virus has disappeared from the water by the time the fertilized eggs hatch?”

The Japanese have shown that larvae <1cm in size are not susceptible to the disease (Ito et al, 2007). Is this because they still carry passive antibodies? By the time they are >2cm in length, they are apparently fully susceptible, but, by this time presumably most virus has



disappeared from the water as the breeding season should be over. Is this whole process an adaptation by the host/virus in order to prevent annual massive losses of newly-hatched hosts (which would be detrimental to host and virus)?

These are questions that need to be addressed, regardless of whether the aim is to preserve a natural species in the wild (as in Japan), or to eliminate an introduced pest species (as in Australia).

5. Clinical signs

Hedrick et al (2000) reported that fish ceased feeding within 3 days pe, and became lethargic at 4 days pe. This was followed by nervous signs (shaking, twitching, uncoordinated movements, erratic swimming in shallow water). Death occurred within 3-4 d after the onset of these signs, ie, from about 7 days pe. Bretzinger et al (1999) stated that, in fish held at 20 ^{o}C, carp were normal one day, lethargic the next morning, and dead within hours, ie, very acute. "Gasping movements in shallow water, sunken eyes, pale patches on (the) skin and increased mucus secretion" were all noted by Pikarsky et al (2004).

Swollen (and necrotic) gill filaments may also be observed; in fact, Haenen et al (2004) claimed that "irregular discolouration of the gills" is the most consistent gross clinical sign of disease. Ilouze et al (2006) said that gill necrosis can appear as early as 3 dpi, and may be accompanied by an increase in the level of parasites and bacteria.

Interestingly, Tinman (in Haenen and Hedrick, 2006) reported that, in Israel, disease associated with CyHV-3 has become of lesser importance recently. Now, while erratic swimming may be seen, there is often no mortality.

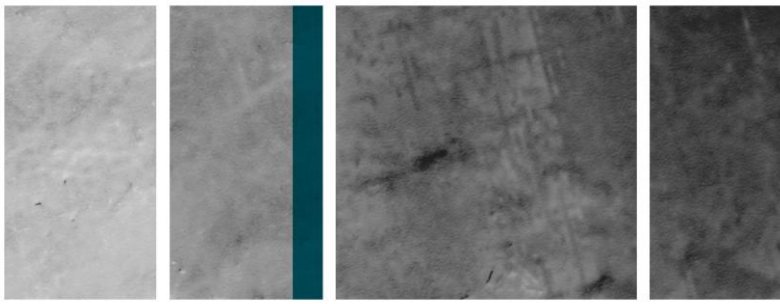
6. Pathogenesis

Water temperature directly affects the body temperature of fish which, in turn, affects viral infections of fish (1) directly, by affecting the rate of viral replication, and (2) indirectly, by affecting the host immune response (Gilad et al, 2003).

Hedrick et al (2000) believed that fish are infected via the gills, and virus then spread systemically. They believed that death is due to loss of normal function in the gills or kidneys, or possibly as a result of nervous involvement. They have seen inclusion bodies (IBs) in neurons (although not in their study). Gilad et al (2004) demonstrated a general spread of virus from the gills and skin, and they believed that loss of osmoregulatory function in the gills, kidney and gut (all of which were shown to contain virus) accounts for death of the fish.

Pikarsky et al (2004) conducted a reasonably comprehensive investigation of the pathogenesis of the disease in carp using a natural route of inoculation:

- PCR: viral DNA was found in blood and kidneys by 1 day pe, but was found in the brain, liver and spleen much later. Gray et al (2002) had earlier reported on the detection of CyHV-3 DNA by PCR in gill, gut, kidney, liver and brain (although they suggested that there may only be a low copy number in the latter).



- Immunofluorescence: fish with clinical signs of disease were shown to have large amounts of viral antigen in kidney (and much less in brain and liver).
- Histopathology: changes were observed in the gills from 2 dpi. These changes are now thought to be specific, while non-specific lesions (caused by secondary infectious agents) may become important later (following focal denudation of gill epithelium). Early kidney lesions are seen at 2 dpi.
- Immunohistochemistry: viral proteins were detected in the kidney by 2 dpi, in cells in the renal interstitium (presumably inflammatory cells); infected cells increased in number up to 10 dpi, at which stage viral proteins were also detected in renal tubular epithelial cells. Infected cells were also detected in the gills from 2 dpi (although there was also a problem on non-specific staining in gills).
- Electron microscopy: While Pikarsky et al (2004) did some E/M, perhaps the best E/M examinations were done by Miyazaki et al (2008) and Miwa et al (2007). The latter described the development of virions in cell culture, and they concluded that the process was the same as for mammalian herpesviruses.

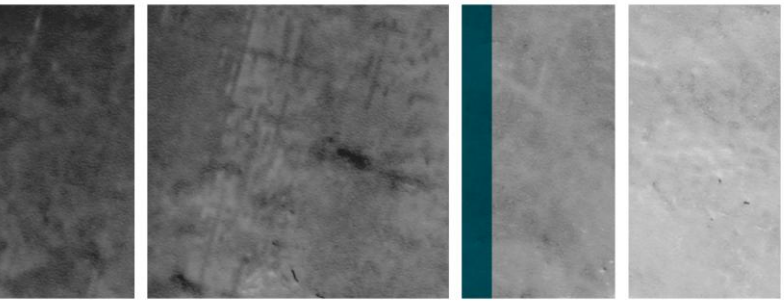
As a result of their work, Pikarsky et al (2004) stated that they still don't know how the virus enters the fish (gills or intestine), but that the early lesions in the gills suggested that these are the portal of entry. From the gills, they hypothesized that virus spreads rapidly to the kidneys (where it may be found in white blood cells). The gills were seen as the portal of entry, an important site of replication, and the site of release of virus (similar to respiratory viruses in mammals).

While early work suggested that the gills were the portal of entry of the virus, Costes et al (2009) demonstrated, through the use of bioluminescence studies, that skin may be the major portal of entry of CyHV-3 (as had already been demonstrated for Infectious Haematopoietic Necrosis virus). This would allow skin-to-skin transmission of virus between fish. This work was subsequently followed up by demonstrating the, seemingly contradictory, finding that skin mucus of carp actually inhibits CyHV-3 from binding to skin epidermal cells (Raj et al, 2011). They account for these more recent findings by suggesting that carp mucus only partially prevents virus binding to epidermal cells, or that virus entry into carp occurs on those parts of the fish that are uncovered by mucus.

Further work from the same laboratory, also using bioluminescence imaging (Fournier et al, 2012), found that virus could infect fish via the pharyngeal periodontal mucosa. The authors hypothesized that carp could be infected via the skin in infected water, or via the oral mucosa after feeding on infected tissues (eg, through cannibalism or necrophagous behaviour).

7. Persistence/Latency

Because disease due to CyHV-3 is restricted to those times when water temperatures are optimal for virus survival (18-28 ^{o}C), an important question is: how does the virus survive between seasons? Davidovich et al (2007) provided four possible answers: (1) CyHV-3 persists in carp and koi that have survived an infection; (2) the virus survives in excreted fish faeces;



(3) the virus may be transmitted by vectors such as birds, insects and parasites; and (4) CyHV-3 may be maintained in other fish species. This raises the question of the differences between "persistent" and "latent" infections.

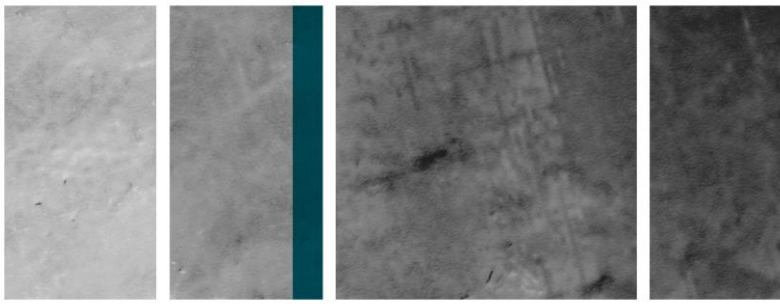
A latent infection may be defined as one where there is no production of infectious virus, but viral DNA may be detected in the affected tissue, and there is also limited gene expression of the viral genome. By contrast, in a persistent infection, infectious virus is still present and produced, although possibly at a very low level.

Gilad et al (2004), using a TaqMan assay, demonstrated low levels of viral DNA in the gill, kidney or brain of fish that survived infection with CyHV-3 (and appeared healthy). Samples from these fish were collected at 62-64 dpi. They believed that these observations, alone, did not prove latency (or carrier status); they wanted to demonstrate transmission of disease from these fish to naïve fish, or reactivation of virus in the potential carriers.

St-Hilaire et al (2005) conducted a more extensive investigation of latent infections in common carp. Their experimental protocol was particularly complex, but some of the key points from the work are: (1) they observed that a specific Ab detection assay [and perhaps a real-time PCR] was the best way to monitor for exposure of fish to CyHV-3 (VI and conventional PCR were not reliable for detecting sub-clinical infections); and (2) they claimed that their findings suggested that common carp can become persistently infected for up to 200 days, and that they can shed the virus and infect naïve fish during co-habitation experiments.

This group published a subsequent paper, using the same experimental set-up, in which they examined the antibody (Ab) response of carp in acute and persistent infections (St-Hilaire et al, 2009). Their specific anti-CyHV-3 Ab levels were checked prior to the experiment, but possibly the levels had fallen to below detectable levels (see Eide et al, 2011a,b). If the fish had been previously exposed, then the dynamics of the Ab responses would be associated with an anamnestic response rather than a primary response. This criticism also applies to the work of Perelberg et al (2008) who compare the kinetics, and levels, of CyHV-3-specific Ab in fish inoculated with wildtype (wt) and attenuated virus. The question here is: what was the immune status of those so-called 'naïve' fish that were exposed to wt virus? They could have been exposed previously, and their Ab titre had simply waned to below detectable levels. The work of Bergmann et al (2010a) suggests that the best way of determining the CyHV-3 status of a fish might be to conduct a PCR on the leukocyte fraction of the blood. It appears that Ab titres alone are not sufficient.

Dishon et al (2007) developed a cell culture system using CCB cells for studying CyHV-3 latency at permissive (22 ^{o}C) and non-permissive (30 ^{o}C) temperatures. A cell culture system removes the influence of the host immune response in controlling latency. Firstly, they demonstrated that CyHV-3 is stable at 30 ^{o}C (and at 40 ^{o}C, but not at 50 ^{o}C, for 30 min). They then found that CyHV-3 persisted in cultured cells that were infected at 22 ^{o}C (and held at that temperature until CPE appeared), and then maintained at 30 ^{o}C (CPE disappeared) for at least 30, but not 70, days (cells were passaged during this time). When the cells were then reverted to a permissive temperature, CPE reappeared. No viral DNA replication was apparent at the non-permissive temperature. However, while mRNAs associated with the enzymes of viral replication were quickly lost at the non-permissive temperature, some viral mRNAs



continued to be expressed for up to 15-17 days. The authors state that it was impossible to determine if these latter mRNAs were being continually transcribed (and involved in virus persistence; Ilouze et al, 2011), or whether they were simply long-lived mRNAs. When cultures were restored to a permissive temperature, genes involved in DNA replication (eg, polymerase) were among the last to be reactivated. Whether this is a model of latency is questionable (since it relies on non-permissive conditions for induction whereas true latency occurs under permissive conditions).

Eide et al (2011a, b) studied latency by examining six clinically normal koi (aged between 2 and 15 yo) that had probably been exposed to CyHV-3 in the past (two were serologically positive for CyHV-3 Ab, and three had been survivors of CyHV-3 outbreaks since 1998). White blood cells from all fish were positive for CyHV-3 DNA (although not at every sampling). Preliminary results suggested that B cells may be the primary site of latency. No CyHV-3 DNA was detected in the plasma. On the other hand, no CyHV-3 DNA was detected in gill or vent swabs of any fish, nor was virus isolated from any swab. These findings suggested latency rather than persistence. They also detected CyHV-3 DNA in a wide variety of other tissues; whether these were latently infected, or simply contained latently-infected white blood cells (WBC) is not clear.

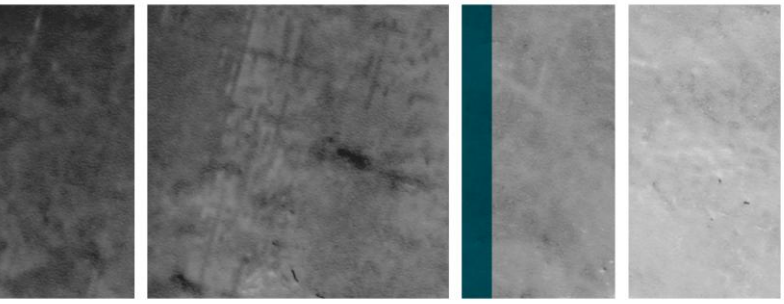
Eide et al (2011a) also examined WBC of 5 koi that showed no signs of disease, and that had no history of exposure to CyHV-3. All five were positive for CyHV-3 DNA (real-time PCR, and PCR plus Southern blotting). They suggested this implies that CyHV-3 is more widespread than expected, and that latently infected fish are best detected by PCR on WBC samples.

8. Immune Response

It is known that in carp, low environmental temperatures enhance non-specific cytotoxic cell activity, and decrease antibody production, while, at higher temperatures, the adaptive immune response may be enhanced, and is capable of dealing with virus that does not replicate although it still produces viral antigens (stated in Dishon et al, 2007).

Adkison et al (2005) demonstrated that hyperimmune serum (produced as a pool from 10 fish surviving CyHV-3 infection, and administered intraperitoneally) provided only partial, or no, protection from bath challenge with CyHV-3. They believed this is proof that other immune responses, including cell-mediated immune responses, are necessary for complete protection of fish. However, Perelberg et al (2008) demonstrated that virus, first incubated with serum from immune fish, *was* inactivated. They suggested that perhaps Adkison et al had insufficient Ab in their immune serum. Perelberg et al (2008) also showed:

- Fish that survive infection with wt CyHV-3 (or attenuated CyHV-3) at 22 ^{o}C begin to develop a detectable anti-virus titre by 7-14 days pe
- They have high titres of specific Ab 3-4 wk pe, and a high level of Ab is sustained for several months.
- Although the titre of Ab may wane, protection against challenge with wt virus is maintained for a long time. Challenge results in an anamnestic response that is sufficiently rapid to protect the fish.



Perelberg et al (2008) also showed that serum from an uninfected fish could inactivate virus, although the effect is far weaker compared with using specific immune serum. They suggested that this is consistent with the observation that aged (non-vaccinated) fish often have anti-virus antibodies that slowly increase with age.

On the other hand, Ito suggested that the detection of anti-CyHV-3 Abs in survivors of CyHV-3 outbreaks was an explanation for the absence of secondary outbreaks of disease (Haenen and Hedrick, 2006). Furthermore, Eide et al (2011a) demonstrated that koi that show no clinical signs of disease, and that have no history of being in a CyHV-3 outbreak, may still carry CyHV-3 in their WBC. It would seem possible that these fish may have serum Ab that is below the level of detection but that can still inactivate virus.

Kongchum et al (2011) investigated the hypothesis that genetic alterations in certain immune response genes might influence the susceptibility of fish to CyHV-3. Since a viral homologue of the host IL-10a gene (known as vIL-10 gene) has been found in the CyHV-3 genome, it is likely that this gene has an important role in helping the virus evade the host immune response. In fact, the authors found that there was a significant association between CyHV-3 resistance and a single nucleotide polymorphism (SNP) in the host IL-10a gene that caused a Gln/Glu substitution – fish with a GCG/GCG or GCG/CGA genotype were usually susceptible to infection, while those with a CGA/CGA genotype were usually CyHV-3-resistant. However, this correlation appeared to break down when similar studies were done on domesticated susceptible carp and a more resistant feral strain (Sassan) where the opposite results were found.

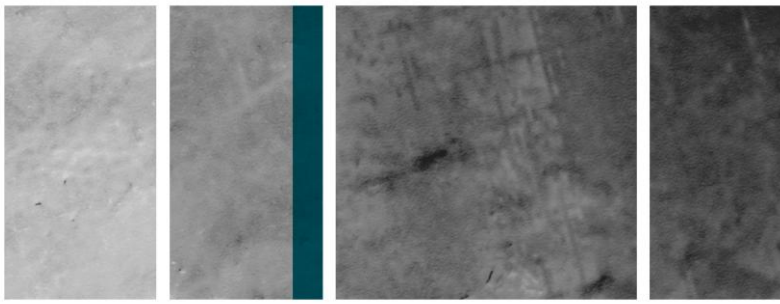
Van Beurden et al (2011) examined the vIL-10 from eel HV (anguillid HV-1) and from CyHV-3. They found that, whereas the *host* IL-10 gene had a similar structure of 5 exons and 4 introns across a variety of species (including humans), generally there were no introns in the viral homologue. CyHV-3 vIL-10 does, in fact, have one very small intron. The lack of introns suggests that viruses acquired the IL-10 gene from the host as a reverse-transcribed mRNA. They also demonstrated, from modelling exercises, that vIL-10s retain a high 3D homology with their hosts' IL-10.

Although AnHV-1 is closely related to CyHV-3, there was little sequence homology between the respective vIL-10s. Also worth noting is that CyHV-1 and -2 both lack a vIL-10; this may indicate an important role in pathogenesis. Resistance to CyHV-3 has also been reported to be linked to polymorphism in the MHC II gene (Rakus et al, 2009).

9. Pathology

9.1 Gross Pathology

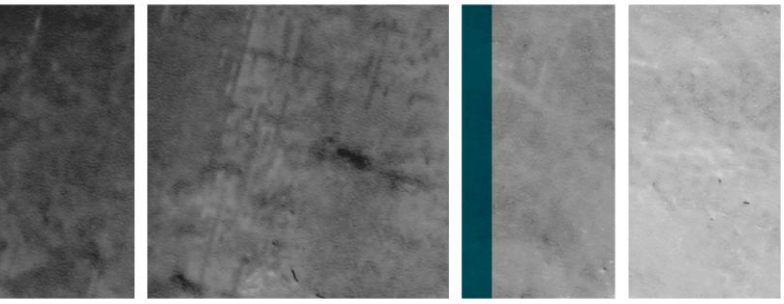
- Irregularly coloured gills (associated with branchial necrosis) with excess mucus on the gills.
- No other consistent lesions, although Bretzinger et al (1999) noted that there may be multifocal loss of the epidermis giving the skin a "sandpaper-like texture" (where the scales are exposed). Excess mucus on the skin is also a consistent lesion (Gilad et al, 2004).



9.2 Histopathology

According to Ilouze et al (2006), the "most prominent lesions" are seen in the gill, skin, kidney, spleen, liver and gastrointestinal tract.

- Gills:
    - Lesions seen as early as 2 dpi (Ilouze et al, 2006), and characterized by a mixed inflammatory cell infiltrate and loss of lamellae
    - From 6 dpi, there may be "complete effacement of the gill architecture" with a severe inflammatory response.
    - Moderate-severe hyperplasia and hypertrophy of the branchial epithelial cells.
    - Fusion of secondary lamellae.
    - Multifocal necrosis of branchial epithelium (with infiltration of lymphocytes into the primary lamellae, and eosinophilic granulocytes also in the epithelium of the primary lamellae [Bretzinger et al, 1999]).
    - Many bacteria are present at the necrotic tips of the gills, but, while they may contribute to the inflammation, Ilouze et al (2006) are adamant that the virus initiates the inflammatory response.
    - Pale I/N inclusion bodies in epithelial cells and leukocytes
- Gill rakers (Pikarsky et al, 2004).
    - Sometimes these changes are more readily seen than those in the gills (Ilouze et al, 2006).
    - Sub epithelial inflammation and congestion, with focal sloughing of surface epithelium.
- Kidney
    - Ilouze et al (2006) stated that, in addition to the gills, the most prominent changes were noted in the kidneys.
    - Multifocal necrosis in haematopoietic tissue, and, in some fish, in the tubular epithelium. Pikarsky et al (2004) revealed that the initial changes were a peritubular inflammatory infiltrate by 2 dpi, becoming heavier by 6-8 dpi. By 8 dpi there was also some feathery degeneration of tubular epithelium.
    - From 6 dpi, large cells with foamy distended cytoplasm (and possibly I/N IB) may be scattered among inflammatory cells in the interstitium.
- Oral epithelium
    - Hyperplastic with foci of necrosis
    - Some IB



- Spleen
    - Multifocal necrosis with possible IBs
- Pancreas
    - Multifocal necrosis with possible IBs and lymphocytic infiltration.
- Gut
    - Mild to moderate enteritis with possible IBs in the lamina propria.
- Liver (Pikarsky et al, 2004)
    - Mild inflammatory infiltrates in the parenchyma, and foci of necrosis (Perelberg et al, 2003)
- Brain (Pikarsky et al, 2004)
    - Focal meningitis

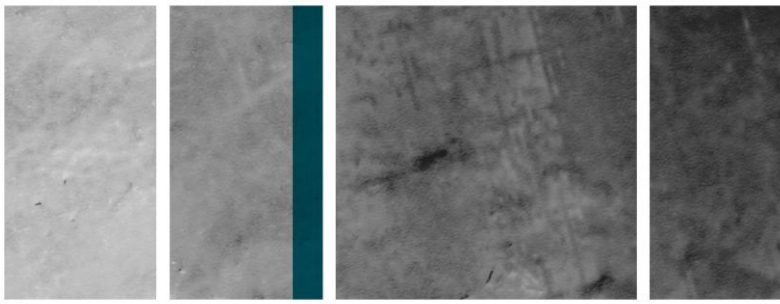
Herpesvirus particles may be seen by E/M in the gill lesions, and possibly in other sites also (Hedrick et al, 2000). Hedrick et al (2000) said that lesions in experimentally infected fish may be slightly different from those in a natural outbreak, eg, IBs seem to be more apparent in experimentally infected fish. Pikarsky et al (2004) noted that many of the later changes in the gills may be the result of secondary infectious agents.

10. Differential diagnosis

Bretzinger et al (1999) stated that, when skin lesions occur with CyHV-3, the lesions are quite different from a bacterial dermatitis, ie, they are not ulcerative. It should also be noted that secondary bacterial or parasitic infections may create confusion in the diagnosis.

Spring viraemia of carp (SVC) is an important differential diagnosis. It may cause mortality in common carp, koi carp and other fish species (cyprinids and ictalurids) but it can be differentiated on the following basis (Goodwin, 2003):

- SVC occurs at much lower temperatures (eg, 5-18 ^{o}C with a peak at 16 ^{o}C cf 21-26 ^{o}C for CyHV-3
- Gross signs for SVC are as for any septicaemia (exophthalmia, petechiae [externally and on necropsy], abdominal distention, bloody mucus from the vent) whereas for CyHV-3 the necrotic gill lesions are the only remarkable gross findings. While bacterial septicaemias may be confused with SVC, the latter usually occurs at much lower temperatures than bacterial infections.
- Histologically, SVC is associated with inflammation of the swim bladder, necrosis of hepatic blood vessels and parenchyma, pancreatitis, necrosis of haematopoietic tissue in the kidney, perivascular inflammation of the blood vessels in the intestine, and myocarditis (Roberts, 2001).



- For SVC *most* outbreaks occur in young fish (although mature fish may be affected), whereas for KHV any age fish may be affected.
- Tissue culture: SVCV grows readily at 22 °C on many cell lines resulting in CPE within 2-4 days. CyHV-3 only grows on CCB and KF cells, producing CPE after about 7 days.

Sleepy disease of koi (KSD) is another differential diagnosis. Clinical disease is very similar to CyHV-3 infection (lethargy, skin erosion, sunken eyes, and high mortality), and disease occurs between 15-25 °C. However, it is known that a 0.5% salt-water bath will prevent outbreaks of KSD (and, indeed, quarantining new fish in such a bath for at least 4 weeks will prevent introduction of this disease). The disease (which is probably the same as viral edema of carp, VEC) appears to be caused by a pox-like virus, carp edema virus, CEV (Miyazaki et al, 2008). The virus primarily affects respiratory epithelial cells.

A disease associated with mass mortality of common carp in the St Lawrence River, Canada was investigated very thoroughly, and, although CyHV-3 and SVC were considered as differential diagnoses, no definitive diagnosis was made (Monette et al, 2006). It should be noted that no PCRs were conducted, and samples for virus isolation (VI) were not optimal. But, no viruses were seen by E/M. A lymphocytic encephalitis was noted in a proportion of the fish.

11. Diagnosis

A number of papers describe case reports of CyHV-3, sometimes with other viruses also being involved (Body et al, 2000; Neukirch and Kunz, 2001; Neukirch et al, 1999).

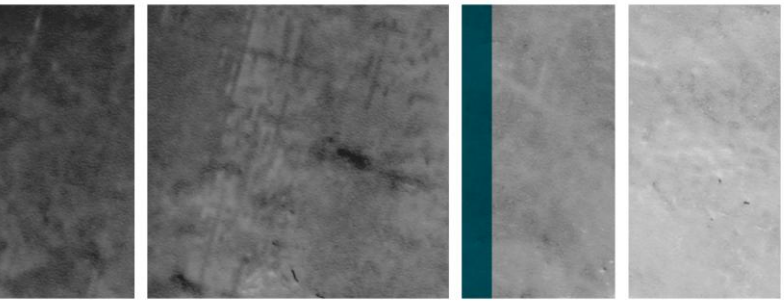
It is reported in Haenen and Hedrick (2006) that the Ab-detection ELISA in combination with a sensitive DNA detection method (PCR or LAMP, see 1.11.2) is the best method for detecting CyHV-3 at the population level. However, there are apparently major differences in current serological methods (and these need to be examined).

11.1 Virus isolation

CyHV-3 may be cultured in KF-1 (Koi Fin-1) cells (Hedrick et al, 2000), with CPE first appearing at 7-10 dpi. Hedrick et al also stated that while uninfected cells should be cultured at 25 °C, virus isolation attempts should be conducted at 20 °C. Pikarsky et al (2004) claimed that CPE can be seen as early as 3-5 dpi (with typical plaques by 4-6 dpi). These variations can probably be accounted for by differences in incubation temperature. Cells initially become enlarged, and then vacuolated before detaching (Pikarsky et al, 2004). Ilouze et al (2006) said that syncytia and vacuolated cytoplasm are also characteristic. Haenen et al (2004) stated that some labs have problems maintaining KF cells (getting pseudo-cytopathic effects).

Virus may also be cultured in CCB (Common Carp Brain) cells (Neukirch et al, 2001). Haenen et al (2004) said that a “readily identifiable CPE” may be seen in about 5-8 days at 26 °C.

A new cell-line (the KCF-1, koi caudal fin) was developed by the Chinese to characterize CyHV-3 isolated in their own country (Dong et al, 2011). They stated that 5 koi fin-derived



cell lines, the CCB line, and a common carp fin-derived line have all been developed for the culture of CyHV-3.

Hedrick et al (2000) said that a transient and focal CPE may also be seen in EPC cells. However, Bretzinger et al (1999) were unable to grow the virus on FHM, BF2, EPC or RTG cells, and Ronen et al (2003) also state that EPC cells are non-permissive. In addition, Way (2004) stated that the cytopathic effect seen on EPC cells is probably due to a paramyxovirus. Davidovich et al (2007) stated that CyHV-3 could be grown on CCB and KF-1 cells, and on silver carp and goldfish fin cells, but not on EPC, FHM nor CCO cells. Note that FHM cells were used to isolate CyHV-3 from moribund carp in New York State (Grimmett et al, 2006).

It is certainly interesting that the EPC cell line, which were derived from *Cyprinus carpio*, are not (uniformly) susceptible to CyHV-3. Davidovich et al (2007) stated that not only are they insusceptible to the virus, but they do not allow viral DNA synthesis or gene expression either. It has now been shown that EPC cell line is derived from Fathead minnow but the designation EPC is still used to differentiate them from the FHM cell line commonly used for isolation of fish viruses (Winton et al, 2010).

Gilad et al (2003) found that optimal growth of CyHV-3 in KF-1 cells was at temperatures between 15 – 25 ^{o}C. Greatest virus concentrations were in the cell-free fraction at 7 dpi in cultures held at 20 ^{o}C. This optimal temperature range corresponded with a similar range for CHV in EPC cells. Neither virus will grow at temps greater than, or equal to, 30 ^{o}C. Sunarto et al (2011) found that maximum virus concentrations in the supernatant fluid of a KF-1 infected culture were between 4 and 6 dpi.

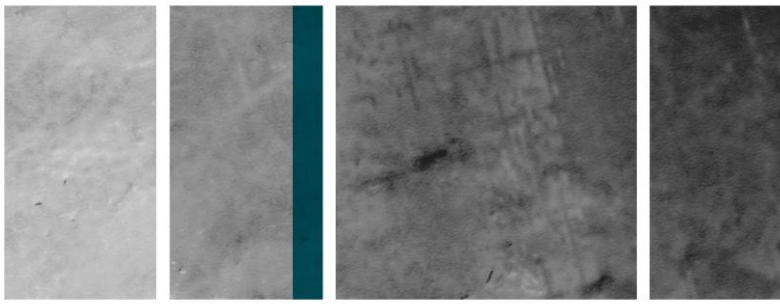
Gill, kidney and spleen are most frequently targeted for attempts at virus isolation (Gilad et al, 2004). Gilad et al (2004) noted that it is often difficult to isolate virus from fish that have been dead for a few hours, or fish that have been frozen. Freezing tissues may also hinder virus isolation (Gilad et al, 2002).

A number of studies have shown that, when CyHV-3 is harvested from KF-1 cells, it is still capable of producing disease in susceptible fish with a mortality (following inoculation) of 75-100% (Perelberg et al, 2003; Ronen et al, 2003; Pikarsky et al, 2004).

Yoshimizu reported that $10^{3.2}$ was the highest titre of CyHV-3 obtained in their laboratory (Haenen and Hedrick, 2006), however, Ilouze et al (2006) reported that titres of up to 2 x 10^{6} pfu/mL may be obtained.

11.2 Polymerase chain reaction / Loop mediated isothermal amplification (PCR/LAMP)

PCR is commonly used for diagnosis, and it is believed that virus isolation is much less sensitive for detection of virus than PCR (Haenen et al, 2004; Haenen and Hedrick, 2006)). Two conventional one-step PCRs were described initially. Gray et al (2002) cloned, and partially sequenced, two restriction enzyme fragments from CyHV-3, and then designed two one-step PCRs based on the sequences (yielding 365 and 290 bp products). These worked on tissues from infected fish. However, Yuasa et al (2005) noted that occasionally there were non-specific reactions with this PCR, possibly due to a single base error in the original published sequence of one primer. When the sequence was corrected, and the optimal



conditions determined for the PCR, it was found to be marginally more sensitive than the PCR of Gilad et al (2002). It was subsequently officially adopted for all CyHV-3 work in Japan.

Gilad et al (2002) developed a one-step assay that produced a 484 bp product, and that could differentiate CyHV-3 from CHV and CCV. This test was said to be more effective than VI on fish from outbreaks (especially if the samples had been frozen or were autolyzed). Neither of these conventional PCRs are capable of routinely detecting carrier fish.

Both of these PCRs are based on non-coding regions of the genome, and it is thought that more conserved regions of the genome may yield a more reliable PCR. Consequently, a third conventional PCR was developed based on the thymidine kinase gene (Bercovier, et al, 2005). TK is said to be essential for the virulence of herpes viruses. This PCR amplifies a 409 bp product, and is said to be 10-1000 times more sensitive than either of the original PCRs. Furthermore, unlike the original PCRs, the TK-based PCR is able to detect viral DNA in fish, even two months post-exposure, and it is at least as sensitive as virus isolation.

Two further conventional one-step PCRs have been developed (Ishioka et al, 2005), one based on the DNA polymerase and the other on the major envelope protein. They seem to work well, but are not as sensitive as the PCR of Bercovier et al (2005). El-Matbouli et al (2007b) described the first nested PCR for CyHV-3, and they claimed it is at least 10 times more sensitive than the PCR of Gilad et al (2002). However, they did not compare its sensitivity with the real-time PCR of Gilad et al (2004; see below). The nested PCR is based on the gene encoding the major capsid protein, and it differentiates CyHV-3 from the other cyprinid herpesviruses.

Bergmann et al (2010a) introduced another one-step PCR based on the major glycoprotein gene of CyHV-3. It appeared to provide a major increase in sensitivity compared with the tests of Gilad et al (2002) and Bercovier et al (2005), and this sensitivity applied to both European and Nth American isolates of CyHV-3.

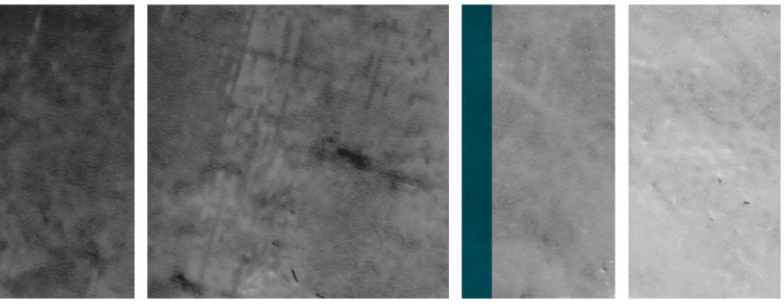
Therefore, a total of at least 7 conventional PCRs (one being a nested PCR) are available for CyHV-3 detection.

Dixon reported that tests on the efficacy of various PCRs showed that primer sets amplifying short fragments were the most consistent in identifying the presence of CyHV-3 DNA (Haenen and Hedrick, 2006). Furthermore, Dixon claims the PCR based on the TK gene is the most sensitive for detection of CyHV-3.

A TaqMan PCR has also been developed (Gilad et al, 2004). This PCR demonstrated high levels of viral DNA in gills and mucus initially, followed, after a lag phase of a few days, by high levels in liver, gut, spleen, kidney and brain. This quantitative PCR also showed higher levels of virus in fish held at higher temperatures. It should also be noted that freshly-dead moribund fish tended to have higher levels of virus than live fish that were sacrificed.

Gilad et al (2003) suggest that a PCR may eventually be developed in a more variable region of the genome that allows differentiation of isolates from different geographical sites.

Detection of carrier fish is particularly important for control of the disease, and PCR seems to be the method of choice at the moment. However, Adkison et al (2005) state that, because



detection of viral DNA by PCR is difficult beyond 64 days after initial exposure, there is a case for the use of a serological assay to detect potential carriers.

Doszpoly et al (2008) tried to develop a generic PCR for alloherpesviruses (based on the DNA polymerase gene). They wanted a PCR that would detect herpesviruses from all fish and amphibians. However, their final product, while useful, failed to detect CyHV-1 and at least one of the ranid herpesviruses.

Gunimaladevi et al (2004) used loop-mediated isothermal amplification (LAMP), and claimed that it was more sensitive for the detection of CyHV-3 than PCR (using a PCR, designed by them, based on the TK gene). El-Matbouli (2007 a) noted that the speed, and the absence of expensive equipment to run the test (only requires a water bath or a heat-block) were the great advantages of the LAMP test. However, the LAMP test appears to be no more sensitive than a one-step PCR (El-Matbouli et al, 2007a), although Cheng et al (2011) claimed that a commercial LAMP test was more sensitive than two PCRs that were used. They also suggested that the commercial test was more sensitive than the tests established by Gunimaladevi et al (2004) and Soliman and El Matbouli (2005). Soliman and El Matbouli (2005, 2009), and Yoshino et al (2006, 2009) have also reported on a LAMP test for CyHV-3.

Haramoto et al (2007) used a special concentration procedure to try to identify CyHV-3 in river water in Japan. They took 500 mL samples of river water, concentrated it, and then ran TaqMan PCRs. However, even with this procedure, they were only able to identify CyHV-3 in 2/48 (4.2%) samples taken from rivers known to contain CyHV-3-infected fish. What this meant in practical terms was not at all clear.

11.3 Immunochemistry/Immunohistochemistry

Note that CyHV-1, -2 and -3 all do share some common antigens (Adkison et al, 2005), but that various antibodies have now also been produced that are specific for CyHV-3.

Polyclonal and monoclonal antibodies have been produced by the Germans (Haenen et al, 2003; 2004), and Aoki et al (2011) also reported on the production of a mAb (against CyHV-3 ORF68 protein) that can be used in an IFAT, or in immunohistochemistry.

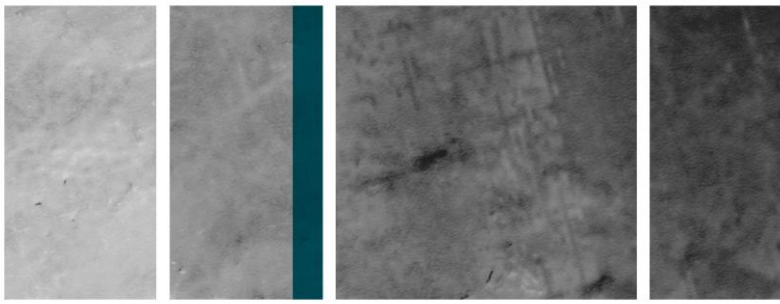
11.4 Electron microscopy

Viral particles with typical herpesvirus morphology have been observed in the tissues of CyHV-3-infected fish (eg, Hedrick et al, 2000). The inner capsid is ~100-110 nm in diam, while the envelope increases the size to ~170-230 nm (Hedrick et al, 2004).

11.5 Serology

Ronen et al (2003) developed an ELISA for the detection of anti- CyHV-3 antibodies. For fish that survived infection, a “distinct elevated titer” was first observed at 14 dpi with the peak titre occurring at 21 dpi. The titre remained elevated until at least 51 dpi (when the trials were terminated). Note that, in this case, titres were based on sera pooled from 15 fish.

Haenen et al (2004) noted that a reliable serological method for detection of CyHV-3 would be very valuable to allow non-lethal sampling of expensive breeding fish. Presumably the



ELISA developed by Ronen et al (2003) may be useful for this purpose, but Adkison et al (2005) developed another antibody-detection ELISA that can detect specific antibodies to CyHV-3 for up to 1 year after the last known exposure of fish to virus. This should be a very useful diagnostic tool for the detection of carrier fish.

St-Hilaire et al (2005) also developed an Ab-detection assay (they used an anti-carp Ig mAb obtained from Aqua Diagnostics). They stated (reported in Haenen and Hedrick, 2006) that their CyHV-3 Ab detection ELISA cross-reacts with Ab to CyHV-1, but that the cross-reactivity issue is easily overcome by dilution of the serum.

The test by Adkison et al (2005) appears to be the serological test of choice at the moment, and even this test is said to be more useful in identifying the Ab status of a population of fish rather than any individual within the population.

11.6 In situ hybridisation

The use of ISH as a diagnostic tool has been reported only infrequently (Bergmann et al, 2006, 2010).

11.7 Techniques for diagnosis

Presumptive: clinical signs, histopathology

Confirmatory: Virus isolation, PCR

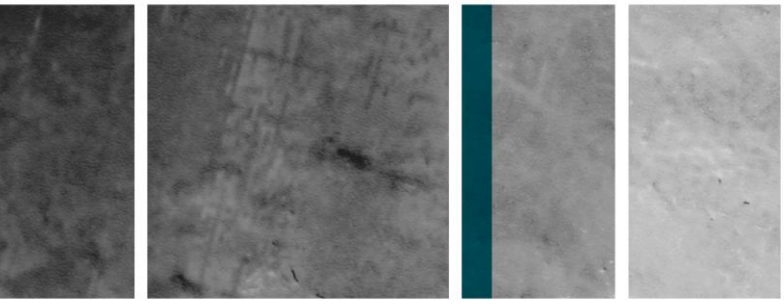
12. Control

12.1 Vaccines

Gilad found no protection was conferred by a formalin-killed (inactivated) vaccine (reported in Haenen et al, 2003). By contrast, Ronen et al (2003), apart from renaming the virus CNGV developed a live attenuated vaccine by serial transfer of the virus in cell culture. This was achieved by 26 passages of virus in KF cells (Ronen et al, 2003) (whereas up to 4 passes does not appear to affect the virulence). The vaccine is administered IP or by immersion.

Perelberg et al (2005) then went one step further by irradiating the attenuated vaccine in order to ensure there was no reversion to virulence. They also found that the vaccine virus remained active in the water (at permissive temperatures) for up to 2 hrs. Beyond 2 hrs, there was little/no protection of susceptible fish.

In an attempt to overcome the possible problem of reversion to virulence of attenuated vaccines, Yasumoto et al (2006) developed an orally-delivered liposome vaccine that was fused with formalin-inactivated CyHV-3 antigen. They claim that the vaccine was taken up in the posterior intestine of carp, and that it caused elevated levels of specific Ab against CyHV-3. With one strain of virus, vaccinated fish had a mortality of 23.1% (cf. 90.0% for unvaccinated fish), while with another strain the corresponding figures were 23.3% and 66.7%. Interestingly, they note that there is some natural anti- CyHV-3 antibody in unvaccinated carp (just as natural antibody to CyHV-1 [that cross-reacts with CyHV-3] has been reported in the US [Adkinson et al 2005]). It might be useful to attempt this work again using the recently-



defined ORF81, said to be the most immunogenic membrane protein of CyHV-3 (Rosenkranz et al, 2008) – see Molecular Virology.

In the case of recombinant vaccines, Fuchs et al (2011) developed a number of recombinant vaccines based on viral enzymes involved in nucleotide metabolism. All of the mutants remained replication-competent in tissue culture, and they afforded some protection for carp challenged with wild-type virus. But, the protection varied widely between trials suggesting that none of the vaccines were ideal.

Perhaps the most significant advance in the development of a recombinant vaccine was the production of a CyHV-3 bacterial artificial chromosome (BAC) clone (Costes et al, 2008). This will allow the study of the role of individual viral genes in the pathogenesis of CyHV-3, and will allow a systematic approach to the production of a recombinant vaccine. The same research group has followed up this achievement with a study in which they identified 40 structural proteins in the mature virion of CyHV-3 (Michel et al, 2010). Three were found to be capsid proteins, 13 envelope proteins, two were tegument proteins, and 22 were unclassified structural proteins. In addition, up to 18 cellular proteins may also be incorporated in CyHV-3 virions.

12.2 Anti-viral treatments

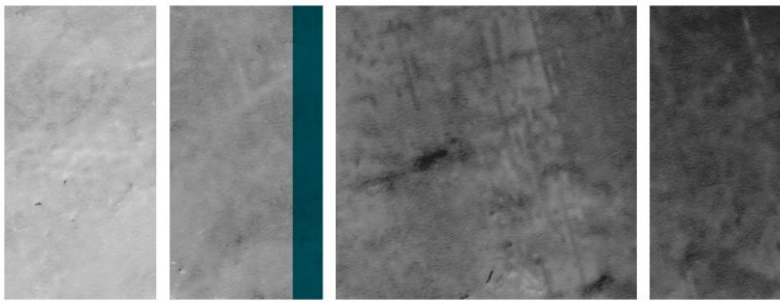
Kasai et al (2005) noted that control programs for CyHV-3 involve (1) quarantine, (2) removal of infected carp and (3) disinfection. To address the latter, they tested the virucidal effects of UV irradiation, heat treatment and disinfectants on CyHV-3. They determined the minimum virucidal dose of UV irradiation to be 4×10^3 μ Ws/cm^2. Virus was only partially inactivated when held at 40 ^oC for 0.5-5 min, whereas temperatures > 50 ^oC for 1 min resulted in complete inactivation. Iodophors, benzalkonium chloride solution and ethyl alcohol were all very effective within 30 s, whereas sodium hypochlorite required longer to be effective (note that, in the presence of organic matter, the effectiveness of sodium hypochlorite is markedly reduced).

Ilouze et al (2006) summarize conditions for inactivation of the virus:

- Infectivity abolished after 2 days at 35 ^oC, or 30 min at 60 ^oC
- pH < 3 or > 11
- In the presence of chloroform, 25% ether or 0.1% Triton

12.3 'Natural' vaccination

Besides the use of the attenuated vaccine, the Israelis (Ronen et al, 2003) also control the disease by exposing 3 month-old koi carp for 2-3 days at 23 ^oC to CyHV-3-positive older koi. Just before disease develops, the young fish are transferred to water at 30 ^oC for 30 days, at which point elevated levels of circulating anti- CyHV-3 antibodies are found. After this, the water temperature is again lowered to 24 ^oC, and they claim the procedure is highly effective (reducing mortality from 80-90% down to approximately 40%).



12.4 Crossbreeding for resistance

Shapira et al (2005) reported that crossbreeding between domesticated carp and resistant wild strains could confer a degree of resistance. They cited previous work as evidence that such a strategy works for other diseases of carp. The wild strain that they used was the Sassan strain of carp (*C. carpio haematopterus*), ie, a subspecies not normally recognized as being susceptible to CyHV-3 (although in their work, they did not specifically examine the susceptibility of pure Sassan strain fish). Whereas mortality in the domesticated carp varied from ~73-92%, one line of crossbreed carp had a significantly lower mortality of only 40%. Furthermore, the production levels of the crossbreds were as good, or better, than the domesticated breeds.

Similar studies were conducted by Dixon et al (2009). Their results encouraged some optimism that breeding for resistance may be possible but the results were certainly not unequivocal. They also cited other work that generated similarly marginal increases in resistance of hybrid strains.

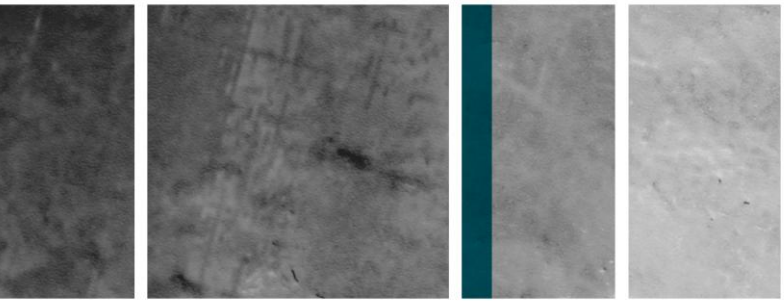
Also, see under "Epidemiology – Affected Spp" for further information about the resistance of cross-bred fish.

12.5 RNA interference (RNAI)

There have been no publications on the use of RNAi as a means for controlling CyHV-3 disease in commercial carp or koi. Rauch (2011) has presented a preliminary notice of intent at a meeting on CyHV-3.

12.6 Control practices

Sunarto et al (2005) discuss a number of control measures that were instituted at national, and farm, levels in Indonesia following the arrival of CyHV-3. For example, laws were introduced to prohibit the movement of infected fish from infected to non-infected areas. At the farm level, it was recommended that only CyHV-3-free seed and broodstock were used, but, if untested fish had to be introduced to a farm, then they should be quarantined for a minimum of 2 weeks at 18-28 ^{o}C. Where outbreaks occur, destocking, drying-out, liming of waterways, and disinfection of equipment are all required prior to restocking.



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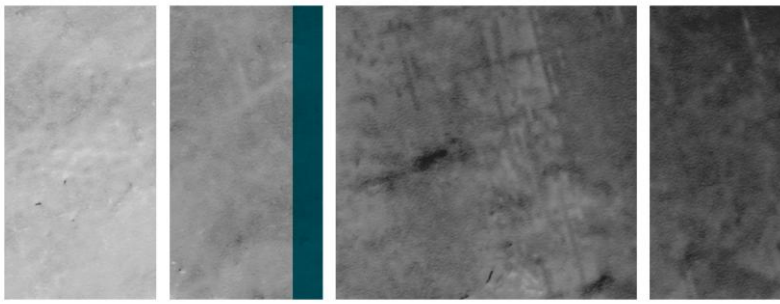

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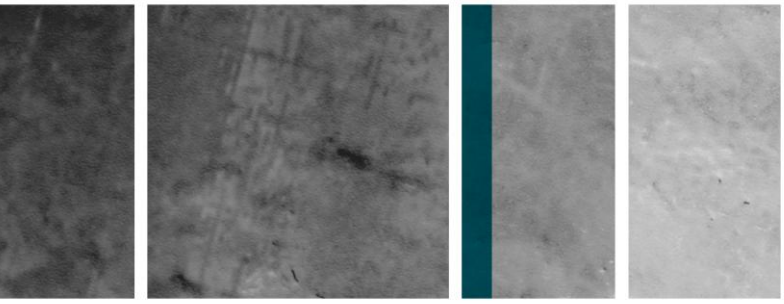

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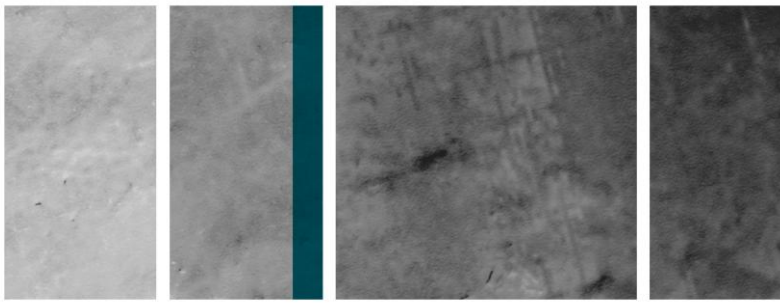

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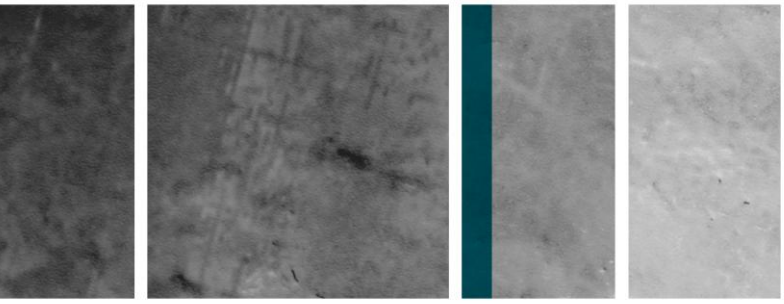

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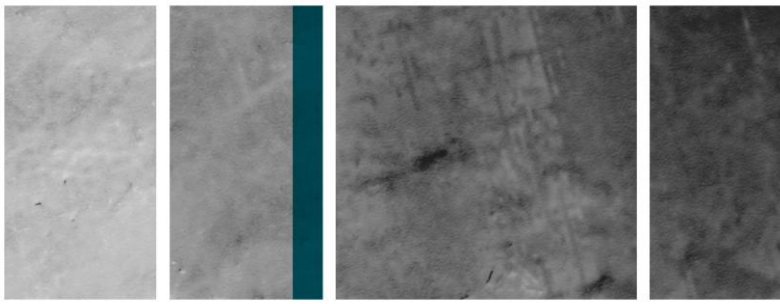

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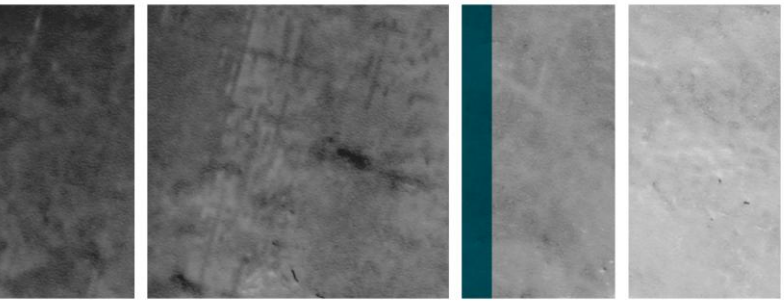

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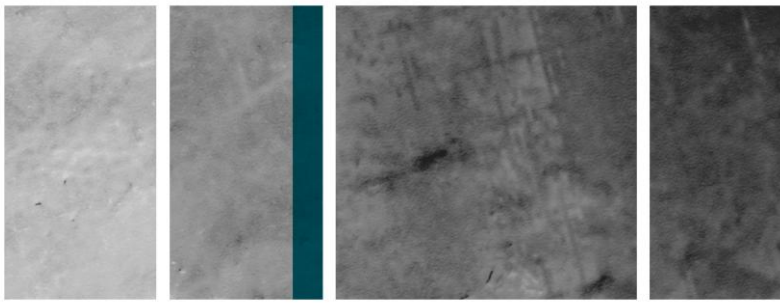

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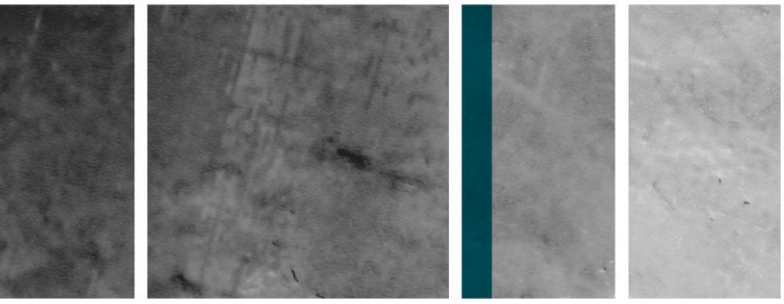

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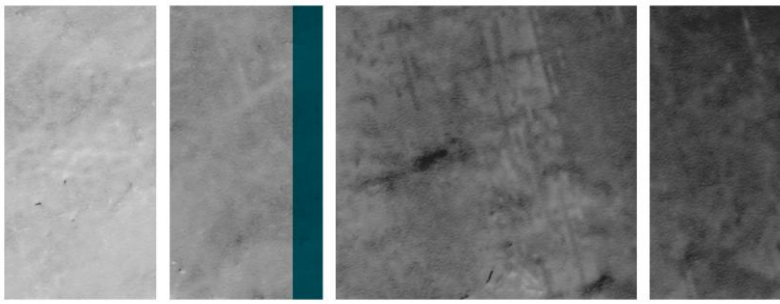

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