Massive mortality of Prussian carp *Carassius gibelio* in the upper Elbe basin associated with herpesviral hematopoietic necrosis (CyHV-2)

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ABSTRACT: From 22 May to 10 June 2011 massive mortality of Prussian carp *Carassius gibelio* was observed in alluvial Lake Řehačka close to the Elbe River in the Czech Republic. More than 1400 kg of dead fish were collected and no other fish species were affected. Further molecular and cytogenetic investigation of fish (n = 232) revealed that the Řehačka population of Prussian carp consisted exclusively of gynogenetic triploid females. The causative agent was identified by means of molecular and electron microscopy as a herpesviral hematopoietic necrosis virus (*Cyprinid herpesvirus* 2, CyHV-2). This is the first report of CyHV-2 from the Czech Republic and the second finding worldwide of CyHV-2 causing mass mortality of *C. gibelio*. Some other localities in the upper Elbe River basin where *C. gibelio* was affected are also noted. We assume that the massive wave of deaths of all female gynogenetic Prussian carp can be attributed to limited genetic variation and the favourable conditions for development of viral disease.

KEY WORDS: Cypriniformes · Herpesvirus · Triploid · Gynogenetic · Mortality

INTRODUCTION

The triploid biotype of Prussian carp *Carassius gibelio* Bloch 1782 is considered the most successful non-native fish in Europe. It easily becomes one of the dominant species in newly inhabited areas, and it has a severe impact on the environment and aquaculture (Economidis et al. 2000, Varadi et al. 2000, Özcan 2007, Lusková et al. 2010, Savini et al. 2010). The type of reproduction significantly facilitates spreading of all-female populations due to rapid multiplication realized through sperm-dependent parthenogenesis.

When the eggs of *Carassius gibelio* are inseminated by males of other species, the heterologous sperm triggers development but does not contribute significantly to the formation of the zygote (Gui & Zhou 2010). This is known as gynogenesis and it leads to all-female offspring, each of which is considered a clone of the mother (Lamatsch & Stöck 2009).

The first record of the invasive triploid form of *Carassius gibelio* in the Czech Republic was in the lower stretches of the Dyje River (Lusk et al. 1977). The population was derived from a Danubian invasion (Holčík & Žitňan 1978) and consisted exclusively of triploid gynogenetic females (Peňáz et al. 1979). Triploid Prussian carp subsequently invaded all 3 main hydrological systems of the Czech Republic (Lusk et al. 1980, 1998, Lusk 1986), and aquaculture activities were considered the key factor accounting for its spread (Slavík & Bartoš 2004). In the Elbe River basin, an all-female population of *C. gibelio* was recorded for the first time in 1989 (Kubečka 1989) and later became a natural component of all suitable habitats (Halačka et al. 2003). In the early 1990s, the

first males and diploids within the population of *C. gibelio* in the Dyje River alluvium (Danube basin) were found (Halačka et al. 2003, Lusková et al. 2004). In a relatively short time the former all-female triploid population changed to a diploid–polyploid complex with up to 43% males (Vetešník 2005). Similar situations were described in other European localities (Černý & Sommer 1992, Abramenko et al. 1998, 2004, Tóth et al. 2005).

Certain localities are now represented either by all-female gynogenetic populations or by the concurrent occurrence of fish with different ploidy and various proportions of males. However, low genetic variability in introduced all-female populations of Prussian carp is expected due to the low number of initial founders combined with the gynogenetic type of reproduction (Hänfling 2007). The all-female clonal populations could also be classified as vulnerable, since antigen recognition and killing by T-cells is genetically restricted by the major histocompatibility complex (MHC) (Somamoto et al. 2009). Lower tolerance to parasites has been reported in gynogens (Lively et al. 1990, Moritz et al. 1991, Poulin et al. 2000, Hakoyama et al. 2001).

In the present paper we report the rapid, massive and selective mortality of thousands of morphologically and genetically identified Prussian carp from Lake Řehačka with further investigation of ploidy level and sex ratio of the Prussian carp and identification of the causative agent. The concurrent occurrence of a massive kill of Prussian carp at several other localities in the upper Elbe River basin is also noted.

MATERIALS AND METHODS

Locality

Řehačka alluvial lake (50° 10′ 39″ N, 14° 48′ 27″ E) is situated close to the Elbe River. The lake covers 12.4 ha and represents an old oxbow of the River Elbe connected with an old flooded sand pit that is linked with the river by a pipeline connection. The water body is administered by the Czech Anglers Union, Local Organization Čelákovice, as a part of fishing district 'Labe 19 A' and includes common fish stock. According to local fishing statistics and an ichthyological survey (T. Daněk unpubl. data), the fish stock consists of: *Cyprinus carpio*, *Esox lucius*, *Sander lucioperca*, *Blicca bjoerkna*, *Tinca tinca*, *Anguilla anguilla*, *Silurus glanis*, *Abramis brama*, *Ctenopharyngodon idella*, *Aspius aspius*, *Perca fluviatilis*, *Gymnocephalus cernuus*, *Hypophthalmichthys molitrix*, *Hypophthalmichthys nobilis*, *Alburnus alburnus*, *Scardinius erythrophthalmus*, *Rutilus rutilus*, *Leuciscus idus*, *Rhodeus amarus*, *Ameiurus nebulosus* and *Carassius gibelio*.

Mortality evaluation

Dead fish were collected from the lake by members of the local organization of the Czech Anglers Union, and quantities were recorded with information on water temperature. Additionally, a survey was conducted of representatives of other local organizations by the Czech Anglers Union along the Elbe River, and the authors of this study personally carried out inspections of localities reporting mass mortalities of *Carassius gibelio*.

Fish identification and sex determination

Moribund and dead fish were identified morphologically according to Kottelat & Freyhof (2007) and additionally by sequencing of mitochondrial DNA of 8 specimens. The cytochrome *b* gene was amplified by the methods described in Rylková et al. (2010), using forward primer Kai_F (GAA GAA CCA CCG TTG TTA TTC) and reverse primer Kai_R (ACC TCC RAY CTY CGG ATT ACA) (Šlechtová et al. 2006). PCR products were subsequently sequenced in both directions (Macrogen) and aligned. Sequences were compared with the Genetic sequence database (GenBank) at the National Center for Biotechnology Information (NCBI) using the basic local alignment search tool (BLASTn) program.

The sex of fish was determined by inspection of gonads at autopsy of 200 dead fish and in specimens used in ploidy level determination.

Determination of ploidy level

Thirty-two moribund Prussian carp were used for ploidy level determination. The ploidy level was determined by computer-assisted image analyses using the measurements of mean erythrocyte nuclei area (MENA) as was proposed by Flajšhans (1997). The blood was obtained with a heparinised syringe from the fish heart, and blood smears were prepared as for conventional haematological examination, then air-dried, fixed in 90% ethanol, and stained with 4% Giemsa-Romanowski. Blood smears were then processed on a system consisting of microscope

(Nikon Eclipse 600, immersion objective 100×), analogue video camera (Hitachi HVC 20) and software (L.U.C.I.A version 4.71, Laboratory Imaging spol. sr. o.). The mean area of the nuclei was calculated from 200 erythrocytes for each specimen.

Moreover, the chromosome preparation according to Ráb & Roth (1988) was performed on 3 of 32 diseased fish. Nuclear suspensions were dropped on slides, stained with 4% Giemsa-Romanovski, and examined microscopically. The chromosome counts were realized on the 10 best metaphase plates per specimen. The ploidy levels of 3 specimens obtained by chromosomes counts were used as a reference for the ploidy level determination by MENA and possible differences were tested by *t*-tests using the program Statistica version 9.1. (StatSoft).

Identification of causative agent

Five moribund fish originating from 3 separate samplings (sample no. 1736/1 contained 1 fish collected on 27 May 2011; sample no. 1736/2 contained 3 fish collected on 30 May 2011; sample no. 1736/3 contained 1 fish collected on 5 June 2011) were frozen (−20°C) and transported to the laboratory. Standard pathological and parasitological examinations (Ergens & Lom 1970) were carried out, and an ELISA test for spring viremia of carp virus (SVCV) formerly known as *Rhabdovirus carpio* (RVC) was undertaken (Test-Line). Virological examination consisted of isolation on tissue culture and identification of the pathogen by electron microscopy and PCR.

Preparation of tissue homogenates

Pooled fish tissues were homogenized in a mortar with sterile sea sand, supplemented with Eagle's medium Tris MEM (minimal essential medium, Sigma), pH 7.6, enriched with 10% FBS (foetal bovine serum, GIBCO) and centrifuged (4°C, 1500 × *g*, 15 min). The supernatant was incubated overnight at 4°C with the addition of antibiotics (100 IU ml^{-1} of penicillin and 100 µg ml^{-1} of streptomycin) and afterwards used for cell line virological testing and for DNA extraction.

Isolation of viruses on tissue cultures

Monolayers (24 h) of bluegill fibroblast (BF-2), epithelioma papulosum cyprini (EPC), rainbow trout gonad (RTG-2) and fathead minnow (FHM) cell lines in 24-well plates (NUNC) were used for viral isolation. Cultures were inoculated with 3 serial tenfold dilutions of the examined samples and incubated at 15 and 23°C for 7 d. The cell lines were monitored by microscope every day for the development of cytopathic effect (CPE). If no CPE was observed, the cultures were frozen, thawed and subcultured for an additional 7 d. If CPE was not observed after the subculture, the samples were considered negative. Cell cultures exhibiting CPE were used for identification of viruses.

Electron microscopy

Supernatant from cell cultures displaying CPE were studied by electron microscopy. Subcultured samples were negatively stained with ammonium molybdate and examined using a Philips 208 transmission electron microscope (TEM) at 18000× magnification and an accelerating voltage of 90 kV.

DNA extraction

Tissue cultures with CPE were used for DNA isolation. The nucleic acid extraction was performed with a QIAamp DNA Mini kit (Qiagen) according to the manufacturer's instructions.

PCR and sequencing

Four primer pairs were used in this study; 2 pairs were used for a nested PCR on the thymidine kinase of CyHV-3 and 2 other pairs for a nested PCR on the DNA polymerase of cyprinid herpesviruses (Table 1). PCR products were subsequently sequenced in both directions using the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequences were aligned using BioEdit version 5.0.9 and compared with GenBank using the BLASTn program.

RESULTS

Mortality evaluation

More than 1400 kg of dead fish were removed from Lake Řehačka within the period 22 May to 10 June 2011. On 22 May only 30 kg were collected,

Table 1. Primer pairs used for identification of the virus. Nested PCRs targeted the thymidine kinase gene of herpesviral hematopoietic necrosis virus (CyHV-3) and the DNA polymerase gene of CyHV. One of 2 possible cycling conditions was used: (A) 40 cycles of 1 min at 95°C, 1 min at 55°C, 1 min at 72°C, and the reaction was preceded by 94°C for 5 min and finished at 72°C for 10 min, or (B) 30 cycles of 1 min at 95°C, 1 min at 55°C, 1 min at 72°C, and the reaction was preceded by 94°C for 5 min and finished at 72°C for 10 min. Centre for Environment, Fisheries & Aquaculture Science (CEFAS) protocol can be obtained at www.cefas.defra.gov.uk

Primer sequences (5′–3′)	Cycle conditions	Size (bp)	Source
CyHV-3 thimidine kinase			
Outer forward: GGG TTA CCT GTA CGA G	A	409	Bercovier et al. (2005)
Outer reverse: CAC CCA GTA GAT TAT GC			
Inner forward: CGT CTG GAG GAA TAC GAC G	B	348	CEFAS protocol
Inner reverse: ACC GTA CAG CTC GTA CTG G			
DNA polymerase gene of cyprinid herpesviruses			
Outer forward: CCC AGC AAC ATG TGC GAC GG	A	362	Jeffery et al. (2007)
Outer reverse: CCG TAR TGA GAG TTG GCG CA			
Inner forward: CGA CGG AGG CAT CAG CCC	B	339	Jeffery et al. (2007)
Inner reverse: GAG TTG GCG CAY ACY TTC ATC			

with the remaining fish collected on 27 May (700 kg), 30 May (300 kg), 1 June (300 kg), 5 June (100 kg), and the last dead specimens occurring on 10 June. Water temperature at the locality during this period was between 16.1 and 20.5°C. To our knowledge, no other fish species were affected besides *Carassius gibelio* and no newly dead fish were observed after 10 June.

The survey among representatives of the local organization of the Czech Anglers Union also revealed the occurrence of a selective kill of Prussian carp in 4 other localities within the upper Elbe Basin (Fig. 1, numbers 2 to 5; Table 2).

Identification and sex determination of fish from the Řehačka mortality event

All 232 investigated fish (200 examined grossly and a further 32 for ploidy determination) were females and were morphologically identified as *Carassius*

gibelio sensu Kottelat & Freyhof (2007). Eight fish that were also investigated genetically shared 1 haplotype of Cyt *b* mt DNA (final length of sequences consisted of 1027 characters). Sequence of the haplotype was compared in the program BLASTn that evaluates the percentage of sequence similarity (%S) and the percentage of sequence overlap (%O) with the reference sequence of *C. gibelio* from GenBank (Table 3).

Determination of ploidy level

Chromosome preparation of 3 of the 8 specimens (Table 3) revealed they were triploids with modal chromosome numbers of 156 (60% of investigated metaphases), 156 (50% of investigated metaphases), and 150 (60% of investigated metaphases). Ploidy level determination by MENA showed that all 32 investigated specimens were triploids with values of the nuclei area mean (± SD) ranging from 20.7 ± 2.2

Table 2. *Carassius gibelio*. Selective mortalities of Prussian carp in the upper Elbe Basin. Biomass: biomass of dead fish, which were removed from the locality by local organisations of the Czech Anglers Union during 2011. See Fig. 1 for locations of localities. nd: no data

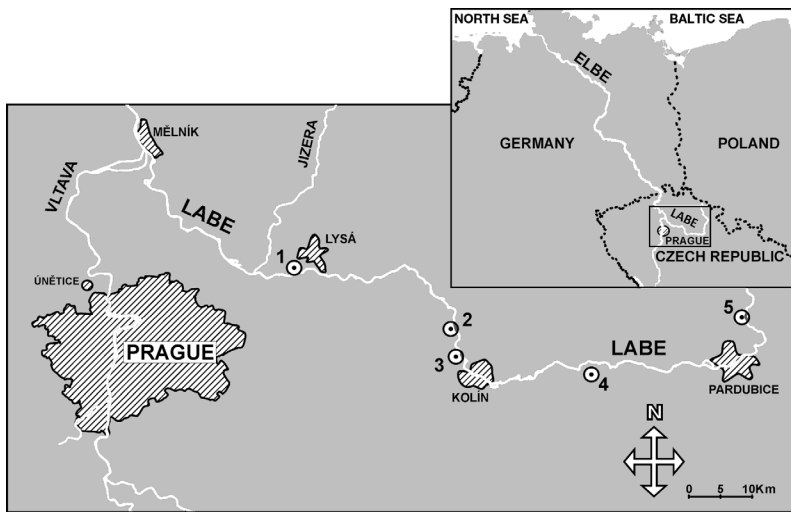| Locality number	Locality name	Period	GPS position	Biomass (kg)	% mortality
1	Řehačka	22 May–10 June	50° 10′ 37.956″ N, 14° 48′ 22.419″ E	>1400	>95
2	Pňov	30 June–5 July	50° 5′ 36.024″ N, 15° 9′ 11.413″ E	>700	nd
3	Nová Ves	30 June–25 July	50° 3′ 24.666″ N, 15° 9′ 35.738″ E	>5600	>95
4	Trnávka	1 June–11 June	50° 1′ 56.253″ N, 15° 27′ 48.229″ E	150	nd
5	Hrobice	5 June–19 June	50° 6′ 28.754″ N, 15° 47′ 23.408″ E	100	nd

Fig. 1. Localities with selective Prussian carp *Carassius gibelio* mortalities. Cases (numbers 1 to 5 in main map) are described in Table 2

Table 3. *Carassius gibelio.* Genetic and cytogenetic identification of 8 specimens of Prussian carp from Lake Řehačka (CgTL006, CgTL008, CgTL013, CgTL046, CgTL078, CgTL114, CgTL144 and CgTL113). Basic local alignment seach tool (BLASTn) comparisons used reference sequence from *Carassius gibelio* DQ399929 (Kalous et al. 2007). %S: percentage of overlap sequence similarity; %O: percentage of sequence overlap; MENA: mean erythrocyte nuclei area; Chro: modal chromosome number

Specimen	Morphological identification	Ploidy level		GenBank Accession No.	%S	%O
		MENA	Chro			
CgTL006	*Carassius gibelio*	3n	156	JN546055	100	98
CgTL008	*Carassius gibelio*	3n	156	JN546056	100	98
CgTL013	*Carassius gibelio*	3n	150	JN546057	100	98
CgTL046	*Carassius gibelio*	3n	–	JN546058	100	98
CgTL078	*Carassius gibelio*	3n	–	JN546043	100	98
CgTL114	*Carassius gibelio*	3n	–	JN546041	100	98
CgTL144	*Carassius gibelio*	3n	–	JN546040	100	98
CgTL113	*Carassius gibelio*	3n	–	JN546034	100	98

to $23.2 \pm 2.5 \ \mu m^2$. Comparison of values using a *t*-test confirmed no statistically significant difference (p > 0.05) between the 3 reference specimens and the remaining specimens (Fig. 2). All values are in agreement with those for triploid *Carassius gibelio* (Kalous & Petrtýl 2004).

Identification of causative agent of mortality

Diseased Prussian carp had pinpoint red foci at the base of their fins, red foci in the eyes, haemorrhaging of the gills, and some specimens showed pink-colored skin in the abdominal region and fins (Fig. 3). Internal organs were soft and reddened. Standard pathological and parasitological examina-

tion failed to detect parasites or evidence of spring viremia of carp. Given that the massive mortality was selective for *Carassius gibelio*, toxicosis was also excluded.

Isolation of viruses on tissue cultures and electron microscopy

Three separate fish samples (1736/1, 1736/2 and 1736/3) produced CPE on first passage in all 4 cell lines incubated at 23°C and in BF-2, FHM and RTG 2 cell lines incubated at 15°C. Cell cultures with clear CPE were examined by TEM, and viral particles morphologically similar to a herpesvirus were observed (Fig. 4). These samples were tested by PCR.

PCR and sequencing

Samples with CPE and herpesviral particles observed in TEM were investigated by nested PCR using primers specific for koi herpesvirus (CyHV-3) (Table 1). Negative results were obtained. Subsequently generic primers for the DNA polymerase gene of cyprinid herpesviruses were used (Table 1) and a primary product of 362 bp was obtained in 2 of 3 separate samples. The nested PCR resulted in a specific product of 339 bp in all 3 samples (Fig. 5). Products were sequenced and the sequences were deposited in GenBank (accession nos. JQ740764, JQ740765 and JQ740766). Nucleotide sequences were compared with GenBank using the BLASTn program and exhibited 100% identity with cyprinid herpesvirus 2 DNA polymerase gene (GenBank accession no. DQ085628.1) (Goodwin et al. 2006a).

DISCUSSION

The causative agent of a massive kill of Prussian carp was identified as CyHV-2, family *Alloherpesviridae*, genus *Cyprinivirus* (Davison et al. 2009). This virus shares morphological similarities with carp pox herpesvirus (CyHV-1) and koi herpesvirus
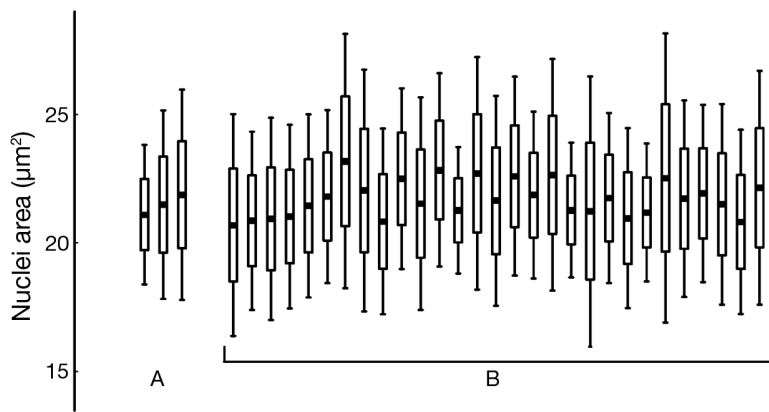
Fig. 2. *Carassius gibelio.* Obtained erythrocyte nuclei area of triploid reference specimens (A) and the remaining 29 specimens (B) from Lake Řehačka. Thick black line: mean; box: SD; whiskers: 1.96 × SD

Fig. 3. *Carassius gibelio.* Freshly dead fish from Lake Řehačka affected by CyHV-2 showing pinpoint foci at the base of fins and in the eyes

(CyHV-3), but it differs in the clinical manifestation, host range, antigenic properties, and growth characteristics (Waltzek et al. 2005). (CyHV-2) is a pathogen of goldfish *Carassius auratus* (Goodwin et al. 2006b, Jeffery et al. 2007), but recently it was also identified in *C. gibelio* in Hungary (Doszpoly et al. 2011). In the case of Lake Řehačka, all affected fish were *C. gibelio.* CyHV-2 is associated with mortality in at least 2 species of the genus *Carassius*—*C. auratus* and *C. gibelio*—but it seems not to be pathogenic for *C. carassius* (Jeffery et al. 2006) or for common carp *Cyprinus carpio* (Jung & Miyazaki 1995).

CyHV-2 was originally described in Japan (Jung & Miyazaki 1995) but it probably has a global distribution (Waltzek et al. 2009), with mortality reported in the United Kingdom (Jeffery et al. 2007), USA, Taiwan (Goodwin et al. 2006a) and Australia (Stephens et al. 2004).

The high mortality within goldfish can be attributed to their low genetic diversity (Rylková et al. 2010) since this species has experienced intensive selection during its breeding history (Balon 2004). We presume that some of the differences in the manifestation of the disease at Lake Řehačka in comparison to previously described symptoms (Jung & Miyazaki 1995, Stephens et al. 2004, Jeffery et al. 2007) could be influenced by the heterogeneity of affected species within the genus *Carassius.*
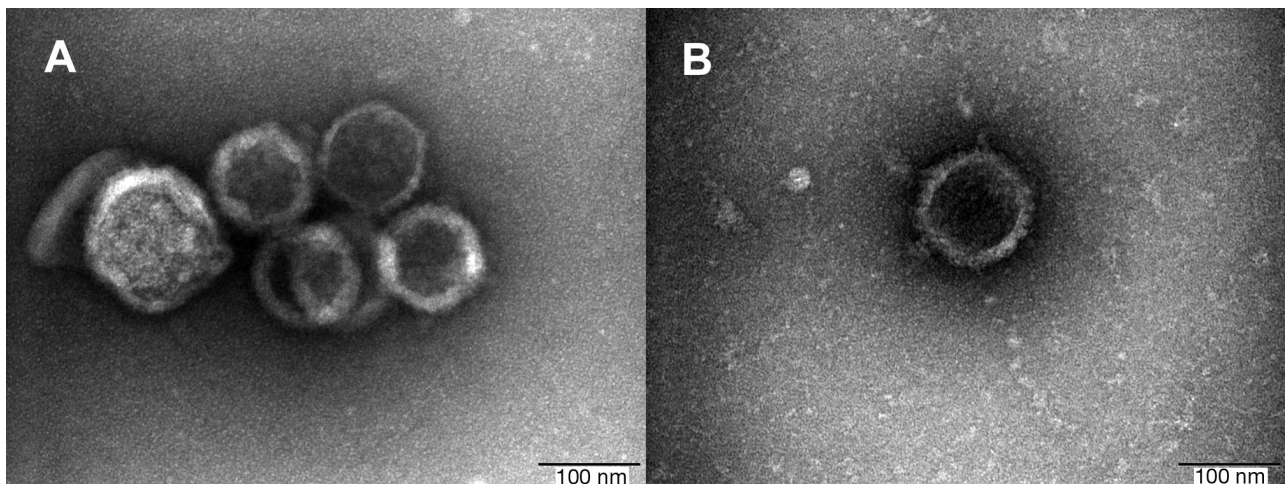

Fig. 4. Viral particles with a herpesviral morphology isolated in different cell lines and temperatures. (A) Rainbow trout gonad cell line, 15°C. (B) Epithelioma papulosum cyprini cell line, 23°C. Transmission electron microscopy; negative staining
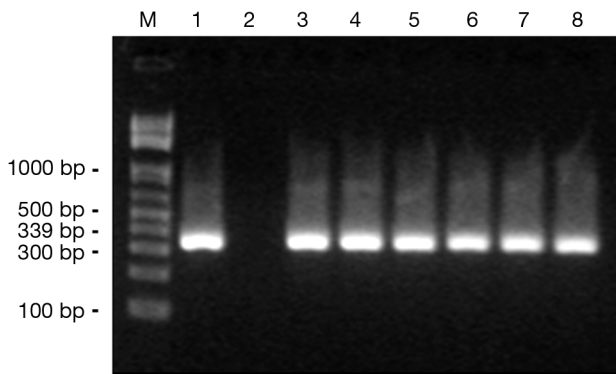
Fig. 5. Nested generic PCR of the DNA polymerase gene of CyHV. Lane M: mass ladder (TrackIt 1 Kb Plus DNA Ladder, Invitrogen); Lane 1: positive control; Lane 2: negative control; Lane 3: Sample 1736/1; Lane 4: Sample 1736/2; Lane 5: Sample 1736/3; Lanes 6 to 8: Same samples after virus multiplication in cell line RTG-2, 23°C

The natural populations of *Carassius gibelio* consist of clonal lineages with the sympatric occurrence of sexually reproducing individuals (Gui & Zhou 2010). In contrast, in newly inhabited areas the populations are characterized by the dominance of females that take advantage of rapid multiplication due to sperm-dependent parthenogenetic reproduction (Hänfling 2007). Our data suggest that the *C. gibelio* population from Lake Řehačka is gynogenetic since not a single male was found. Moreover, all investigated specimens were shown to be triploids, and only 1 haplotype of Cyt *b* mtDNA was shared among 8 sequenced fish.

Populations of asexually reproducing vertebrates are often considered to be less resistant to pathogens due to reduced genetic variability of the host (Neiman & Koskella 2009). In natural populations that reproduce sexually, usually only a fraction of individuals infected by viruses show symptoms of disease, and a significant part of the clinical variability observed within populations is explained by the host genetic background that plays an important role in the susceptibility to infections (Verrier et al. 2012). This phenomenon changes in a genetically uniform population caused by artificial selection. Within the common carp *Cyprinus carpio*, strains more or less susceptible to CyHV-3 have been identified (Shapira et al. 2005, Ødegård et al. 2010). High stock densities and low genetic variability can result in mass mortalities from virus, e.g. in koi carp (Hedrick at al. 2000), or even in introduced common carp in a natural environment (Garver et al. 2010). Animals that reproduce clonally face the same pop-

ulation breakdown possibility when infected by highly pathogenic viruses due to their obvious genetic similarity. Although we did not completely define the genotype diversity of Prussian carp in Lake Řehačka, only a few clonal lineages are likely to be present due to the bottle-neck effect associated with introduction and the gynogenetic type of reproduction. Our cytogenetic data showed 2 different modal chromosome numbers. The triploid biotype of *Carassius gibelio* is known to bear various numbers of chromosomes from 150 to 162 (Kalous & Knytl 2011). Particular clones are then usually characterized by a specific chromosome number (Zhou & Gui 2002). In the case of Lake Řehačka, there are at least 2 clones, although the cytogenetic data in the study are very restricted.

After the first dead fish appeared on 22 May, numbers rapidly increased within 5 d. One week after the peak of mortality, only a few newly dead specimens were found. Estimating mortality in natural waterbodies is very difficult. However, based on information from the local organization of the Czech Anglers Union and the complete lack of Prussian carp caught at the locality by anglers in the period 10 June 2011 to July 2012, we assume that all or nearly all of the *Carassius gibelio* in Lake Řehačka were eliminated by the pathogen during this short period.

The rapid progress of the pathogen was also reported in controlled conditions when fish began to die at 3 to 6 d post-inoculation and cumulative mortality ranged from 60 to 100%, depending on viral titre, within 13 d at 20°C (Jung & Miyazaki 1995). CyHV-2 is often present as an inapparent infection and could be widespread in nature (Goodwin et al. 2009). However, when infected fish are subjected to stress such as a temperature change, there is a greater probability of disease outbreaks.

We believe the stress/temperature hypothesis may explain the initiation of the disease outbreak at Lake Řehačka and other locations in the upper Elbe River because a sharp drop in water temperature occurred from 13 May to 16 May 2011. Additionally our data showed that the virus replicated well in the temperature range 15 to 23°C, which is in agreement with Jung & Miyazaki (1995) who noted the optimum for virus propagation ranging between 15 and 25°C.

We conclude that the massive wave of deaths of Prussian carp at Lake Řehačka and at other localities in the upper Elbe River basin can be attributed to limited genetic variation of Prussian carp and the favourable conditions for propagation of CyHV-2.

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