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Viruses, Bacteria and Helminths of Invasive Carp: Insights from an In Vitro Assay and a Survey with Native Fishes in a Large Midwestern River

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Pathogen introductions associated with aquatic invasive species threaten ecosystems and biodiversity worldwide. Bigheaded carps (BHC), including Silver Carp *Hypophthalmichthys molitrix*, Bighead Carp *H. nobilis*, and their hybrids, are prolific, invasive pests in central US rivers. However, little is known about pathogen effects on invading BHC or how BHC affect the disease risk profile for native fishes in receiving ecosystems. We therefore conducted, from May 2013-December 2014, a systematic pathogen survey for BHC and native fishes in the Wabash River watershed, Indiana, USA. We found *Pseudomonas fluorescens*, *P. putida*, and *Salmonella enterica* DNA in BHC as well as native fishes, although none of these bacteria were exclusively present in BHC. DNA from other bacterial taxa was detected only in native fishes and Common Carp *Cyprinus carpio*. No gastrointestinal helminths were detected in BHC, although they were common in most native fishes examined. We also conducted *in vitro* studies on BHC tissues (skin, gill, fin, and fry) and found high sensitivity to Largemouth Bass virus, viral hemorrhagic septicemia virus, and infectious pancreatic necrosis virus. We conclude that BHC are not heavily burdened by bacteria, viruses and parasites in the invaded study ecosystems, although they do harbor native bacteria and show potential for high sensitivity to endemic viruses.

Key words: Aquatic invasive species, Bighead Carp, Metagenomics, Next Generation Sequencing, Silver Carp

Aquatic invasive species (AIS) threaten aquatic ecosystems and biodiversity through predation, competition, habitat alteration, and pathogen introduction (Moyle and Light 1996; Wilcove *et al*. 1998; Koehn 2004; Sato *et al*. 2010; Cucherousset and Olden 2011). AIS are nonetheless known to be the driving force behind native fish declines in some aquatic ecosystems (Hermoso *et al*. 2011). For example, predation by Nile Perch *Lates niloticus* led to the extinction of 200 fishes including endemic cichlids in Lake Victoria, Africa (Kaufman 1992; Witte *et al*. 1992). In the US, where many river systems have already been impacted by decades of overfishing, pollution, and habitat change (Karr *et al*. 1985), the pres-

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ence of AIS further stresses these ecosystems.

While certain AIS impacts are well-studied, little attention is given to introduction and transmission of pathogens by invasive fishes. Nevertheless, those impacts can be substantial (Prenter *et al*. 2004; Gozlan *et al*. 2005). Invasive Common Carp *Cyprinus carpio* introduced spring viremia of carp virus (SVCV) to the United States (Goodwin 2002). SVCV is associated with hemorrhaging and gill degeneration and is pathogenic to centrarchids, esocids, and other native US fishes (Goodwin 2002).

Silver *Hypophthalmichthys molitrix* and Bighead *H. nobilis* Carps and their hybrids, collectively referred to as bigheaded carps (BHC), are both invasive in the US (USFWS 2004). BHC were brought to the US from eastern Asia in the 1970s to control plankton first in municipal wastewater treatment ponds and later in aquaculture (Kolar *et al*. 2005; Conover *et al*. 2007). Since being introduced to local rivers, they have become established in the Mississippi, Missouri, Illinois, and Wabash Rivers and their tributaries (Schrank and Guy 2002; Conover *et al*. 2007; De Grandchamp *et al*. 2008; Sampson *et al*. 2009; Coulter *et al*. 2013). Increasing BHC populations in Midwestern rivers have created concerns that they are negatively impacting invaded ecosystems. For example, Silver Carp diets overlap with native Gizzard Shad *Dorosoma cepedianum* and Bigmouth Buffalo *Ictiobus cyprinellus* (Sampson *et al*. 2009), and they are expected to compete with juvenile sport and commercial fishes in the Great Lakes if they become established in the basin (Mandrak and Cudmore 2004). While there have been several studies on resource competition by invasive BHC (Schrank *et al*. 2003; Irons *et al*. 2007; Cooke *et al*. 2009; Freedman *et al*. 2012), knowledge of BHC pathogen susceptibility is primarily limited to data from their native ranges. A detailed list of pathogens and other disease causing agents known to affect BHC is provided in Kolar *et al*. (2005).

Whether invasive BHC are carriers or hosts for exotic and native diseases is critical information needed by aquatic resource managers for more comprehensive assessments of the BHC impacts on native fishes and the development of biological controls for BHC. Therefore, our first objective was to conduct a systematic survey of parasites, bacteria, and viruses of BHC and native fishes in the upper and middle Wabash and the lower Tippecanoe Rivers, Indiana, US. We sought to determine whether BHC in the study area carry pathogens that are also present in sympatric native fishes and hypothesized that BHC would harbor similar pathogens to Bigmouth Buffalo, due to niche overlap, and Common Carp, due to phylogenetic proximity. Our second objective was to determine the sensitivity of BHC to a representative number of fish viruses using *in vitro* approaches. This allowed us to test pathogens not present in our study area and to screen for high BHC sensitivity, which might indicate their potential use as a biological control. We hypothesized that BHC would be susceptible to viruses that are known to affect other cyprinids.

Materials and Methods

Fish collection

Fish were collected from March to September 2013 in the Wabash River near River Mile 293 and the Tippecanoe River at Oakdale Dam (Figure 1 and Table 1). The water temperature during this time ranged from ~15 to 28°C between April and September and from ~1 to 5°C from October to March (USGS https://waterdata.usgs.gov). A combination of electrofishing, gill netting, fyke netting, and hook-and-line was used to capture the full range of target species. The invasive fishes collected included both BHC species as well as Common Carp. The native species included: Bigmouth Buffalo, Smallmouth Buffalo *I. bubalus*, Emerald Shiner *Notropis atherinoides*, Channel Catfish, Flathead Catfish *Pylodictis olivaris*, Freshwater Drum *Aplodinotus grunniens*,

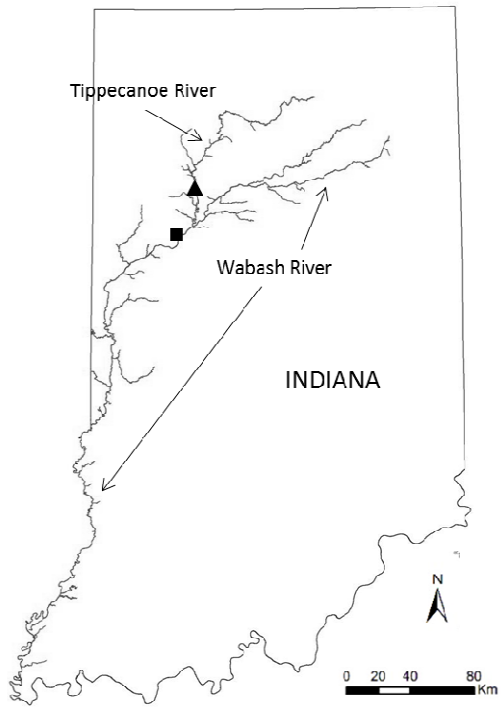

Fig. 1. Map of the Wabash River system in Indiana with West Lafayette/borrow pit (red dot) and Oakdale Dam (green dot).

Gizzard Shad, Bluegill *Lepomis macrochirus*, Largemouth Bass *Micropterus salmoides*, Smallmouth Bass *M. dolomieu*, and Shovelnose Sturgeon[1] *Scaphirhynchus platorynchus*. We collected and necropsied 134 fish, but were unable to obtain the target number of individuals (n=12) for some species (Table 1). Total length (mm) (fork length for Shovelnose Sturgeon) and mass (g) were measured and observations of internal lesions, external parasites, and injuries were noted in the field.

Sample collection

Fish were bled from the caudal vein using 3 mL syringes fitted with 22-26G ½-1" needles. For fish collected in the Wabash River and the attached borrow pit, bleeding occurred ≤8 h after collection. In the interim, fish were held in a 1.2×1.2×0.9 m mesh cage in the Wabash River. For fish collected from the Tippecanoe River, bleeding occurred ≤1 h after collection and fish were briefly held in a 19 L bucket of water or on a fish stringer. Blood was stored in heparinized vacutainers and kept on ice until centrifuged for plasma collection (0.1-1 mL). Fish were then immediately euthanized using an overdose (≥250 mg/L) of MS-222 (Western Chemical Inc., Ferndale, WA) and necropsied in the field in a controlled environment within an enclosed trailer. Spleens were removed in the field and immediately frozen in liquid nitrogen. Stomach and intestines were removed and stored on ice. Dissecting tools were sterilized between each necropsy with 70% ethanol and an ethanol lamp. Scalpel blades, surgical drapes, and gloves were changed between each fish and the work area was cleaned with 70% ethanol. Samples were stored in -20°C (gastrointestinal tracts) or -80°C (plasma and spleen) freezers in the Aquatic Ecology Laboratory at Purdue University.

Metagenomic analyses

Next generation sequencing (NGS) was used to screen for bacteria, viruses, and parasites from fish plasma and spleen tissue. Genomic DNA was extracted from spleen using DNAzol Reagent (Invitrogen, Grand Island, NY) and viral DNA and RNA were extracted from plasma using the PureLink Viral RNA/DNA Mini Kit (Invitrogen, Grand Island, NY) following manufacturer instructions. Quantification of the nucleic acids was carried out using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE), and samples with ratio of absorbance 260/280 nm >1.8 were used for further analysis. Viral RNA samples could not be processed further due to the presence of yeast tRNA used during the extraction. The high percentage of tRNA in the RNA samples would have swamped the sample RNA during se-

1) All Shovelnose Sturgeon samples for the study were taken from fish sacrificed as part of the annual IDNR Sturgeon monitoring effort; thus, no additional Shovelnose Sturgeons were sacrificed for this project.

quencing, resulting in little useable data.

DNA from the spleens and plasma were each pooled by species; however, Emerald Shiners were omitted from sequencing because their small size precluded the required amount of DNA from being collected. Bigmouth and Smallmouth Buffalo, both of genus *Ictiobus*, were pooled together to allow all samples to be run in one lane. The resulting 23 samples were submitted to the Purdue Genomics Core Facility for library construction and sequencing. Twelve libraries were constructed from spleen samples with TruSeq DNA PCR-Free Sample Preparation Kits (Illumina Inc., San Diego, CA) and 11 libraries were constructed from plasma samples with TruSeq Nano DNA Sample Prep Kits (Illumina Inc., San Diego, CA). A control (enterobacteria phage phiX174 library) was added to each sample library. Sequencing was performed on a MiSeq sequencer (Illumina Inc., San Diego, CA) using a 600-cycle, paired-end, multi-indexed run with the MiSeq Reagent Kit v3. Sequence read quality statistics from FASTQC software (Babraham Institute, Cambridge, UK) were provided by the Purdue Genomics Core Facility.

Raw reads were filtered to remove adapters and poor quality bases (Phred33 < 20) from 5' and 3' ends using Trimmomatic (Bolger *et al.* 2014), and a final minimum length of 30 bp was employed. There were on average, 1,273 single end reads (0.1%) created in each library from filtering and these were not used in further analysis. No other filtering was done. Reads from each library were assembled separately using ABySS (Assembly by Short Sequences) (Simpson *et al*. 2009) with k-mer=110, 90, and 70. The k-mer=110 assemblies were chosen for analysis based on their ABySS fitness score and analyzed using BLAST (Altschul *et al*. 1990) via DiaGrid (Purdue Research Foundation, West Lafayette, IN) and databases available through the National Center for Biotechnology Information (NCBI). Blastn was used to compare the contigs to known sequences in the nucleotide (nt) database (Benson *et al*. 2013). The 10 matches with highest E-value were filtered to retain matches with length ≥50 bases and e-value $\leq 10^{-5}$. Matches above this cutoff were considered acceptable. Matches below the cutoff were considered unreliable, due to a high likelihood of encountering such a match by random chance, and disregarded. From the acceptable matches, bacteria, viruses, and archaea matches were extracted and are presented in the results. The eukaryote sequences were then searched using Perl scripts for common and scientific names of fungi known to be harbored by and potentially pathogenic to fish (Noga, 2010). The remaining eukaryote sequences which represent host DNA were not used further. Additionally, matches to synthetic sequences in the database were ignored.

To verify the sequencing detections and provide additional screening, BHC spleen DNA was tested for *Aeromonas salmonicida*, *Salmonella enterica*, *Pseudomonas putida*, *Lactococcus lactis lactis*, and Largemouth Bass virus (LMBV) by PCR using primer sequences available in the literature (Table 2). Due to resource limits we were unable to run PCR on all organisms detected via NGS. Therefore, bacteria were selected based on detection in BHC via NGS (*A. salmonicida*, *S.enterica*, and *P. putida*) and on a previous report of a mortality event in BHC due to *L. lactis* (Khoo et al., 2014). LMBV was chosen because it is a DNA virus and an emerging pathogen (Noga, 2010). Native fishes and Common Carp were also screened for these pathogens using spleen DNA, if they were positive in the NGS results.

PCR was carried out using GoTaq Green Master Mix (Promega Corp., Madison, WI) and a T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) following manufacturer instructions. Each PCR reaction mixture (25 μL) contained 12.5 μL GoTaq Green Master Mix, 10 μM gene-specific primers (forward and reverse), 1 μg DNA template, and nuclease-free water. All reactions were performed under the following conditions: Initial template denaturation at 95°C for 2 min, 35 cycles of 94°C for 10 s, primer

Table 1. Fish collected from March-September 2013 and physical measurements taken at time of collection

Family	Scientific name	Common name	Individuals collected	Total length, mm range (mean)	Body weight, g range (mean)
Cyprinidae	*Hypophthalmichthys molitrix*	Silver Carp (SC)	12	440-816 (600)	840-4980 (2750)
Cyprinidae	*Hypophthalmichthys nobilis*	Bighead Carp (BC)	6	630-895 (797)	2360-8470 (5970)
Cyprinidae	*Cyprinus carpio*	Common Carp (CC)	12	243-800 (559)	200-6100 (2740)
Cyprinidae	*Notropis atherinoides*	Emerald Shiner (ES)	12	45-84 (68)	0.1-5 (0.2)
Catostomidae	*Ictiobus cyprinellus*	Bigmouth Buffalo (BF)	6	405-635 (535)	840-3980 (2550)
Catostomidae	*Ictiobus bubalus*	Smallmouth Buffalo (SF)	6	276-653 (419)	280-3600 (1200)
Ictaluridae	*Ictalurus punctatus*	Channel Catfish (CH)	12	141- 650 (356)	60-2880 (760)
Ictaluridae	*Pylodictis olivaris*	Flathead Catfish (FC)	4	229-705 (492)	130-4350 (1860)
Clupeidae	*Dorosoma cepedianum*	Gizzard Shad (GS)	12	163-388 (264)	30-340 (190)
Sciaenidae	*Aplodinotus grunniens*	Freshwater Drum (FD)	12	232-423 (341)	130-1000 (480)
Centrarchidae	*Lepomis macrochirus*	Bluegill (BG)	12	70-178 (124)	10-95 (43)
Centrarchidae	*Micropterus salmoides*	Largemouth Bass (LB)	7	146-311 (260)	40-420 (320)
Centrarchidae	*Micropterus dolomieu*	Smallmouth Bass (SB)	9	161-335 (231)	60-430 (170)
Acipenseridae	*Scaphirhynchus platorynchus*	Shovelnose Sturgeon (SS)	12	604-780 (707)[a]	830-1780 (1350)

[a]Fork length was measured for Shovelnose Sturgeon only.

Table 2. Bacteria and virus primers used in the PCRs

Primer	Sequences (5'-3')	Product size (bp)	Source
Aeromonas salmonicida	Aero-F CGTTGGATATGGCTCTTCCT Aero-R CTCAAAACGGCTGCGTA	416	Altinok *et al.* (2008)
Salmonella enterica	Se-F TGCGTATTCTGTTTGTCGGTCC Se-R TACGTTCCCACCATTCTTCCC	606	Baumler *et al.* (1997)
Pseudomonas putida	Pp-F CCAAAACTGGCAAGCTAGAGTAC Pp-R CATCTCTGGAAAGTTCTCTGC	380	Altinok (2011)
Lactococcus lactis lactis	Lac-F GCGYCCWGGGATGTATATYG Lac-R AAATTCRCGRCGWGGTTCAA	1825	Khoo *et al.* (2014)
Largemouth bass virus (LMBV)	LMBV-F GCGGCCAACCAGTTTAACGCAA LMBV-R AGGACCCTAGCTCCTGCTTGAT	248	Grizzle *et al.* (2003)
Bacterial 16S rRNA gene (universal primer)	16S-F GAGTTTGATCCTGGCTCAG 16S-R AGAAAGGAGGTGATCCAGCC	1541	Rainey *et al.* (1996)

annealing at 58°C for 30 s, product extension at 72°C for 1 min and final extension at 72°C for 5 min. PCR products were separated by electrophoresis on 1.5% (w/v) agarose gel in 1×TAE buffer using Mini-Sub cell GT cell (Bio-Rad Laboratories, Hercules, CA). The DNA fragments were visualized by ethidium bromide staining and photographed using the Image Lab software on the Gel Doc EZ system (Bio-Rad Laboratories, Hercules, CA) under UV transillumination.

Gastrointestinal helminths.—Stomach and intestines were thawed and cut open longitudinally and contents rinsed with tap water over a sieve. Contents were examined under a dissecting microscope and helminths (Trematoda, Cestoda, Nematoda, and Acanthocephala) were enumerated, placed in glass vials and fixed in 70% ethyl alcohol. Parasites were keyed to the lowest taxonomic level using Bray *et al*. (2008) and Chabaud *et al*. (1975:1978). Abundance, prevalence, and mean intensity were determined for each helminth species (Bush *et al*. 1997).

Cell cultures

Silver and Bighead Carp cells (skin, gill, fin, and fry) were inoculated with viruses commonly found in the upper Midwest: LMBV, Bluegill virus (BGV), Golden Shiner virus (GSV), infectious pancreatic necrosis virus (IPNV), Channel Catfish virus (CCV), SVCV, and viral hemorrhagic septicemia virus (VHSV). Isolates used for these assays are maintained at the USFWS La Crosse Fish Health Center, WI and included SVCV isolated from Common Carp from Pool 8 of the Upper Mississippi River (2007), LMBV isolated from Largemouth Bass from Pool 10 of the Upper Mississippi River (2008), BGV isolated from Bluegill from an aquaculture facility located in WI (2007), CCV isolated from Channel Catfish from an aquaculture facility located in IL (2011), IPNV isolated from Brook Trout *Salvelinus fontinalis* from Elton Creek, WI (2013), VHSV genotype IVb isolated from Muskellunge *Esox masquinongy* from Lake St. Claire, MI (2009), and GSV isolated from Muskellunge from an aquaculture facility located in WI (2014). Cells were rinsed with 0.05% trypsin, removed from the flask, and seeded on 24-well tissue cell culture plates. Once the cells were confluent, 0.1 mL of tissue cell culture medium known to contain each virus was added to the cells. Plates were incubated at appropriate temperatures (25°C for LMBV, CCV and BGV; 20°C for SVCV; and 15°C for VHSV, GSV, and IPNV) and monitored for the development of cytopathic effect (CPE) for up to 10 d.

Results

River survey

Upon capture, the majority of the fish collected for the study (Table 1 and Figure 2) appeared to be healthy, although several fish had external parasites

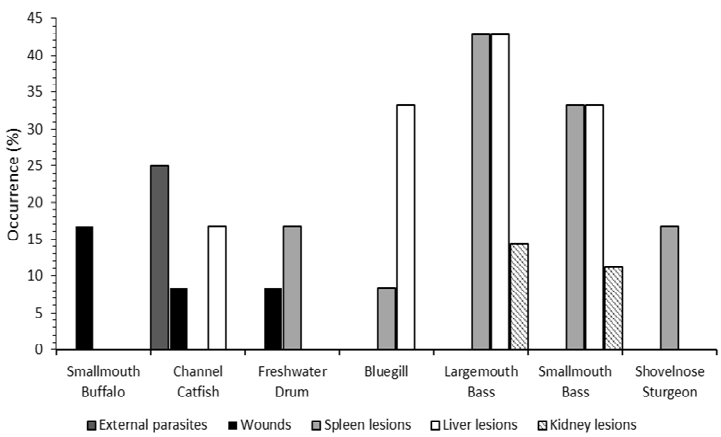

Fig. 2. Prevalence of external parasites, open wounds and internal lesions on necropsied fish. Collected silver carp, bighead carp, common carp, emerald shiner, bigmouth buffalo, flathead catfish, and gizzard shad had no observable external parasites, injuries, or internal lesions.

or pre-existing external wounds. Specifically, leeches (suspected *Piscicola* sp.) were collected on three Channel Catfish, primarily on the pectoral fins and the underside of the head. One Smallmouth Buffalo, one Channel Catfish, and one Freshwater Drum had significant pre-existing external wounds that appeared to be infected. This Freshwater Drum also had white nodules on several fins and necropsy of this fish revealed a pale spleen. Internal lesions were also present on individuals of six species. During necropsy, potential parasites were observed on the livers, kidneys, and/or spleens of two Channel Catfish, five Smallmouth Bass, four Bluegill, four Largemouth Bass, one Freshwater Drum, and two Shovelnose Sturgeon. None of the BHC collected had any observable external parasites, injuries, or internal lesions.

Metagenomic analyses

NGS generated 46,355,062 total raw reads (length= 300 bases) from spleen and plasma (Supplementary Table 1). ABySS assembly created 2,739,091 and 1,032,366 contigs for spleen and plasma, respectively. Of these contigs, 563,061 (20.6%) and 249,260 (24.1 %) had matches to the nt database with length ≥50 bases, e-value ≤10^{-}5. The remaining contigs did not have close matches in the nt database and were not identified. No fish-related pathogenic fungi were found searching BLAST output for common and scientific names via Perl script and satisfying cutoffs of length ≥50 bases, e-value ≤10^{-}5.

Bacteria and viruses comprised <1% of acceptable blastn matches for contigs against the nt database. The majority (i.e. >95%) of acceptable blastn matches were to Eukaryota and most of these aligned to sequences from fish taxa, especially model species (e.g. Zebrafish). A small percentage were not able to be assigned to a domain by BLAST. There were 5 bacteria species found in BHC (*Pseudomonas fluorescens*, *P. putida*, *Salmonella enterica*, *Caligus rogercresseyi*, and *Lepeophtheirus salmonis*), all of which were also found in the native species and/or Common Carp (Table 3). Ten additional species/subspecies were found only in the native fishes and/or Common Carp. In particular, Common Carp, Channel Catfish, and Gizzard Shad had multiple additional bacteria: five, five and two, respectively. *Pseudomonas* spp. and *C. rogercresseyi* were identified in the highest number of species (92% and 67%, respectively). Two *Lactococcus lactis* subspecies, two *Bacillus* spp., and *Staphylococcus aureus* were detected in Gizzard Shad, Channel Catfish, and Common Carp and Smallmouth Bass, respectively.

Cyprinid herpesvirus 3 (i.e. Koi virus) was detected in Common Carp, Freshwater Drum, and the pooled Bigmouth/Smallmouth Buffalo sample. No other viruses (except the Enterobacteria phage phiX174 control) were detected (using cutoffs of length ≥50 bases, e-value ≤10^{-}5) in the other study fish species.

Using PCR we detected *P. putida* in Bluegill, Largemouth Bass, and Smallmouth Bass with both PCR and NGS with prevalences of 55%, 43%, and 17%, respectively. However, we were unable to detect the same bacteria using PCR for most of the detections in the sequencing results. The bacterial 16S rRNA gene was detected using the universal primer in Silver and Bighead Carp samples, but none of the specific viruses and bacteria examined (*A. salmonicida*, *S. enterica*, *P. putida*, *L. lactis lactis*, and LMBV) were detected. The bacterial 16S rRNA gene was detected in all Smallmouth Buffalo and five of six Bigmouth Buffalo, but neither *P. putida* nor *S. enterica* were detected, although both bacteria were detected in the NGS results for the pooled buffalo sample. Similarly, we did not detect *P. putida* in Common Carp, Channel Catfish, Freshwater Drum, or Shovelnose Sturgeon, despite its detection in the NGS results for those fishes.

Gastrointestinal helminths

Parasites from the stomach and intestines were identified to species where possible. Twenty-five distinct taxa were identified (Table 4, Supplementary

Tables 2-3). Only six helminth species were present in more than one host species, but were generally restricted to one family. *Megathylacoides giganteum* was found only in the two ictalurid species, while *Leuceruthrus micropteri, Proterometra macrostoma, Posthodiplostomum* sp., and *Neoechinorhynchus cylindratus* were each found in multiple centrarchid species. The exception was *Acanthocephalus dirus* which was found in both Freshwater Drum and Shovelnose Sturgeon.

The most prevalent parasites identified were the invasive monozoic tapeworm *Atractolytocestus huronensis*, present in over 70% of the Common Carp; the trematode *L. micropteri*, present in over 50% of the Smallmouth Bass; and the cestode *M. giganteum*, present in 50% of the Flathead and Channel Catfish. Both Asian carp species were free of macroscopic gastrointestinal parasites (Table 4). The remaining 12 host species were generally not dominated by a single parasite. Seventy-five percent of the hosts had only low mean intensities (<10) of multiple parasites, and Gizzard Shad and Emerald Shiners each had only one individual helminth (Supplementary Table 2). The largest exceptions were Largemouth Bass (*Neoechinorhynchus cylindratus* intestine mean intensity=20) and Shovelnose Sturgeon (*A. dirus* intestine mean intensity=53). Unlike mean intensity, mean abundance varied less with ranges of 0.1-3.5 and 0.1-13.8 for stomach and intestines, respectively (Supplementary Table 3).

In vitro study

All viral isolates tested generated a cytopathic effect (CPE) in both Silver and Bighead Carp cell lines, except for CCV (Table 5 and Figure 3). All cell lines (skin, gill, fin, and fry) from both carp species showed high sensitivity to LMBV, with all inoculated cells undergoing rapid cell death. VHSV and GSV also

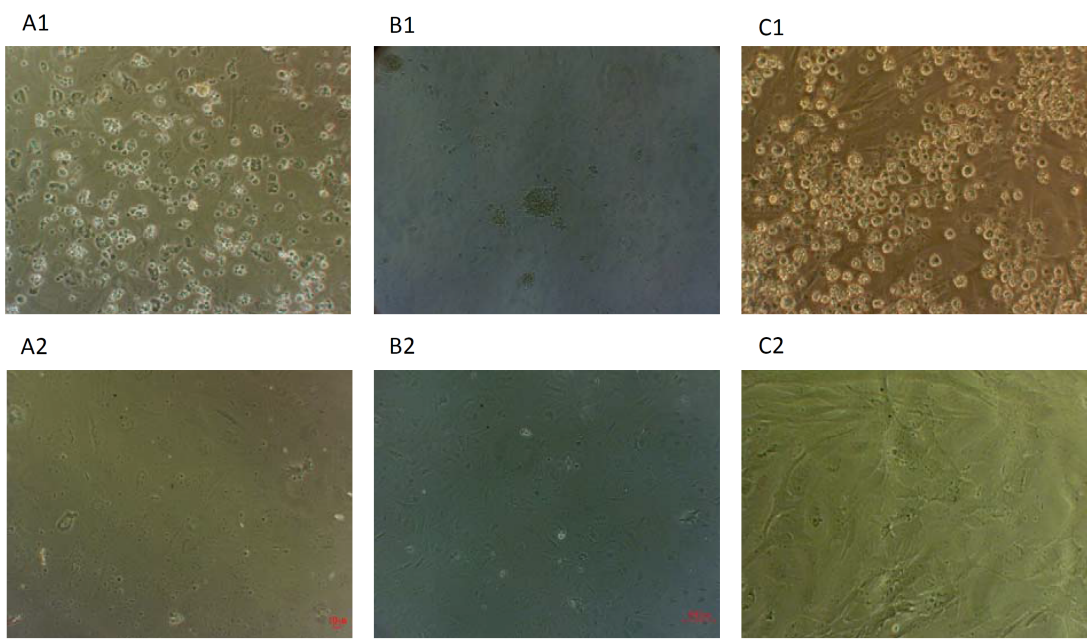

Fig. 3. Microphotograph showing cytopathic effects in several bighead carp cell lines. A1 – Viral hemorrhagic septicemia virus (VHSV) on fry at 18 hrs (20X), A2 – Negative control for VHSV (20X). B1 – Spring viremia of carp virus (SVCV) on fry at 18 hrs (10X), B2 – Negative control for SVCV (10X). C1 – Largemouth bass virus (LMBV) on gill at 18 hrs (20X), and C2 – Negative control for LMBV (20X). The scale for each magnification is shown in the bighead fry controls (A2 and B2).

Table 3. Organisms detected by blastn in pooled samples. "X" indicates organism detected in extracted DNA. Bigmouth and Smallmouth Buffalo, both of the genus *Ictiobus*, were pooled prior to sequencing

Order (Family)	Organism	SC[1)]	BC	CC	BF	CH	FC	GS	FD	BG	LB	SB	SS
Class Gamma Proteobacteria Gram –													
Pseudomonadales	*Pseudomonas fluorescens*	X	X		X		X		X	X	X	X	X
Pseudomonadales	*Pseudomonas putida*	X	X	X	X	X	X		X	X	X	X	X
Pseudomonadales	*Acinetobacter baumannii*					X							
Enterobacteriales (Enterobacteriaceae)	*Salmonella enterica*		X	X		X							
Enterobacteriales (Enterobacteriaceae)	*Serratia marcescens*			X									
Enterobacteriales (Enterobacteriaceae)	*Serratia plymuthica*			X									
Enterobacteriales (Enterobacteriaceae)	*Klebsiella pneumoniae*					X							
Class Bacilli Gram +													
Lactobacillales	*Lactococcus lactis cremoris*							X					
Lactobacillales	*Lactococcus lactis lactis*							X					
Bacillales (Bacillaceae)	*Bacillus cereus*					X							
Bacillales (Bacillaceae)	*Bacillus subtilis*					X							
Bacillales (Bacillaceae)	*Staphylococcus aureus aureus*			X								X	
Class Copepoda													
Siphonostomatoida (Caligidae)	*Caligus clemensi*			X									
Siphonostomatoida (Caligidae)	*Caligus rogercresseyi*	X	X	X	X		X		X		X	X	
Siphonostomatoida (Caligidae)	*Lepeophtheirus salmonis*		X	X					X				

[1)]Host species are abbreviated as follows: Silver Carp (SC), Bighead Carp (BC), Common Carp (CC), Bigmouth Buffalo (BF), Smallmouth Buffalo (SF), Channel Catfish (CH), Flathead Catfish (FC), Gizzard Shad (GS), Freshwater Drum (FD), Bluegill (BG), Largemouth Bass (LB), Smallmouth Bass (SB), and Shovelnose Sturgeon (SS).

Table 4. Prevalence (%) of helminths identified in the gastrointestinal tracts of hosts. Neither Bighead nor Silver Carp exhibited gastrointestinal helminths and were omitted from the table. Prevalence in stomach and small intestine are given, respectively, separated by a comma. "-" indicates no helminths were found or (for Common Carp only) the species has no stomach.

Helminth species	Host species											
	CC[1)]	ES	BF	SF	CH	FC	GS	FD	BG	LB	SB	SS
Cestoda												
Bothriocephalus sp.	-	-	-	-	-	-	-	-	-	-	11,-	-
Atractolytocestus huronensis	-,75	-	-	-	-	-	-	-	-	-	-	-
Proteocephalus ambloplites	-	-	-	-	-	-	-	-	-	-,14	-	-
Megathylacoides giganteum	-	-	-	-	8,50	50,25	-	-	-	-	-	-
Corallobothrium fimbriatum	-	-	-	-	-	25,50	-	-	-	-	-	-
Capigens singularis	-	-	-	33,50	-	-	-	-	-	-	-	-
Monobothrium ingens	-	-	-	33,-	-	-	-	-	-	-	-	-
Khawia iowensis	-,17	-	-	-	-	-	-	-	-	-	-	-
Trematoda												
Lissorchis attenuatum	-	-	-,33	-	-	-	-	-	-	-	-	-
Leuceruthrus micropteri	-	-	-	-	-	-	-	-	-	57,14	44,22	-
Proterometra macrostoma	-	-	-	-	-	-	-	-	-	29,-	22,11	-
Skrjabinopsolus manteri	-	-	-	-	-	-	-	-	-	-	-	17,42
Posthodiplostomum sp.	-	-	-	-	-	-	-	-	25,50	-,14	-	-
Homalometron armatum	-	-	-	-	-	-	-	-,8	-	-	-	-
Neodiplostomum americanum	-	-	-	-	-	-	-	-,8	-	-	-	-
Polylekithrum ictaluri	-	-	-	-	-,25	-	-	-	-	-	-	-
Lissorchis gullaris	-	-	-	-,17	-	-	-	-	-	-	-	-
Unidentified larval trematode	-	-	-	-	-	-,25	-	-	-	-	-	-
Nematoda												
Camallanus sp.	-	-	-	-	-	-	-	-	-	-	-,11	-
Camallanus ancylodirus	-	-	-,17	-	-	-	-	-	-	-	-	-
Dichelyne robustus	-	-	-	-	-,17	-	-	-	-,8	-	-	-
Dichelyne cotylophora	-	-	-	-	-	25,25	-	-	-	-	-	-
Unidentified larval nematode	-	-,8	-	-	-	-	-	17,42	-	14,-	-,11	-
Acanthocephala												
Neoechinorhynchus australis	-	-	-,17	-	-	-	-	-	-	-	-	-
Neoechinorhynchus cylindratus	-	-	-	-	-	-	-	-	-	-,43	-,22	-
Tanaorhamphus longirostris	-	-	-	-	-	-	-,8	-	-	-	-	-
Acanthocephalus dirus	-	-	-	-	-	-	-	-,8	-	-	-	17,17
Echinorhynchus salmonis	-	-	-	-	-	-	-	-	-	-,14	-	-

[1)]Host species are abbreviated as follows: Common Carp (CC), Emerald Shiner (ES), Bigmouth Buffalo (BF), Smallmouth Buffalo (SF), Channel Catfish (CH), Flathead Catfish (FC), Gizzard Shad (GS), Freshwater Drum (FD), Bluegill (BG), Largemouth Bass (LB), Smallmouth Bass (SB), and Shovelnose Sturgeon (SS).

Table 5. Sensitivity of Asian carp cell lines to seven common viruses

Cell line	SVCV	LMBV	BGV	CCV	IPNV	VHSV	GSV
Bighead Carp skin	+	+	+	-	-	+	+
Bighead Carp gill	+	+	+	-	+	+	+
Bighead Carp fry	+	+	+	-	+	+	+
Bighead Carp fin	+	+	-	-	-	+	+
Silver Carp skin	-	+	+	-	+	+	+
Silver Carp gill	+	+	+	-	+	+	+
Silver Carp fry	+	+	+	-	+	+	+
Silver Carp fin	+	+	+	-	+	+	+

Viruses are abbreviated as spring viremia of carp virus (SVCV), Largemouth Bass virus (LMBV), Bluegill virus (BGV), Channel Catfish virus (CCV), infectious pancreatic necrosis virus (IPNV), viral hemorrhagic septicemia virus (VHSV), and Golden Shiner virus (GSV). "+" indicates a cytopathic effect was observed and "-" indicates no observable cytopathic effect.

generated a CPE in all BHC cell lines, though the effect was not as rapid as observed with LMBV. Finally, CPE was generated by SVCV in all cell lines except Silver Carp skin cells, by BGV in all cell lines except Bighead Carp fin cells, and by IPNV in all cell lines except Bighead Carp skin and fin cells.

Discussion

BHC in the study area did not appear to be heavily burdened by native pathogens of the invaded ecosystems, although bacteria and ectoparasites were detected. In BHC, NGS identified: three Gram negative bacteria: *P. flavescens, P. putida,* and *S. enterica*; and two copepods *C. rogercresseyi* and *L. salmonis*. Both *C. rogercresseyi* and *L. salmonis* are ectoparasites, and although many precautions were taken, we cannot rule out contamination from the fish's exterior as a possible cause of the copepod detections. For the 12 fishes used in the metagenomic study, these five organisms were the most commonly detected and were each detected in at least one native fish. In fish, *Pseudomonas* spp. causes septicemia, fin erosion, and ulceration (Noga 2010); however, we did not observe any symptomatic BHC or external parasites on these invasive fishes. We were unable to confirm the presence of these bacteria via PCR. There are a few possible reasons for this discrepancy. PCR exhibits both high sensitivity and high specificity that can prevent detection of variants and new mutations of the target. On the other hand, NGS has the potential to identify pathogen DNA, including variants, in the presence of very low levels of pathogen DNA. Moreover, the lack of observable symptoms indicates that BHC were not heavily infected/infested, which could result in small quantities of pathogen DNA and low detection rates.

Cyprinid herpesvirus 3 was the only virus detected with high certainty. This virus might have been more readily detected with NGS due to its large double-stranded DNA (genome size=245-295 kb) (Michel *et al.* 2010). Detecting small viruses and RNA viruses proved to be more difficult. Interestingly, Common Carp is the only species known to be susceptible to this exotic pathogen (Michel *et al.* 2010), although it was also detected in this study in native Freshwater Drum and buffalo. Additional work is needed to determine whether Freshwater Drum and buffalo act as carriers of this virus and whether either species can become symptomatic.

The suspected parasites observed on the internal organs of the native fish during necropsy were likely white grub (*Posthodiplostomum* sp.) or tapeworm larvae. These observations were supported by our analysis of gastrointestinal helminths. Both Bluegill and Largemouth Bass had white grub on their intestinal tracts, while Channel Catfish and Smallmouth

Bass had adult cestodes. None of the Silver or Bighead Carp collected in our study had any observable intestinal parasites. This is consistent with results from an Illinois study that found no intestinal helminths in 53 necropsied Silver Carp (Wilcox 2013). Most fish species in the study had low mean abundance and mean intensity of gastrointestinal parasites and appeared healthy upon collection. Catfishes and bass were the most frequently afflicted with macroscopic, gastrointestinal parasites, but did not appear to be more affected than other species upon collection. Additionally, we identified one helminth of the *Bothriocephalus* genus in a Smallmouth Bass stomach, which we were unable to identify to species. However, *Bothriocephalus claviceps* and *B. cuspidatus* are native helminths commonly found in Smallmouth Bass (Hoffman 1999); therefore, this parasite was likely not the invasive Asian tapeworm.

One of the key steps in the ability of a virus to switch hosts is the ability of a virus to replicate in the cells of the new host species (Parrish *et al.* 2008), which would indicate that several viruses in this study have the potential to infect BHC. Several of these viruses have not previously been identified as affecting BHC, including LMBV, BGV, IPNV, and VHSV. All cell lines for both species exhibited a high sensitivity to LMBV. LMBV is primarily a centrarchid virus and is known to cause mortalities solely in Largemouth Bass. However, LMBV is also capable of causing CPE in a cell line produced from another cyprinid, the fathead minnow (USFWS and AFS-FHS 2014). Sensitivity to VHSV IVb and IPNV was not surprising given their broad range of hosts (Frattini *et al*. 2011; Munro and Midtylyng 2011). No sensitivity to CCV was found, but CCV is known to be highly host specific to Channel Catfish (Plumb 1978). *In vivo* studies are needed to determine if these viruses are able to infect live BHC, and whether BHC are symptomatic or asymptomatic carriers. Furthermore, when a new host successfully becomes infected with a virus, mutations generally occur which could cause changes in viral pathogenicity in the original host (Parrish et al 2008). Therefore, it is possible that changes could occur with the ability of these viruses to infect native fish after BHC species become established in areas known to harbor these fish viruses. The interaction between BHC and Midwestern fish viruses will be important to monitor as these invasive fish continue to expand their range.

The low prevalence of pathogens in the BHC included in our study area has both positive and negative implications for management of these invasive fishes. Low prevalence of pathogens in invasive BHC may point to a limited impact on native fishes via pathogen transmission. At the same time, limited susceptibility to local pathogens and the apparent relative good health of BHC compared to the native fishes we collected are concerning. Pathogens are generally an important mechanism of natural population control, and low susceptibility to local pathogens combined with a lack of natural predators for adult BHC in invaded ecosystems indicates a substantial need for greater for anthropogenic intervention in the absence of these natural control mechanisms.

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References

Altinok, I., Capkin, E., and Kayis, S.: Development of

multiplex PCR assay for simultaneous detection of five bacterial fish pathogens. Vet. Microbiol. 131: 332-338, 2008.

Altinok, I: Multiplex PCR assay for detection of four major bacterial pathogens causing Rainbow Trout disease. Dis. Aquat. Org. 93: 199-206, 2011.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J.: Basic local alignment search tool. J. Mol. Biol. 215: 403-410, 1990.

Bain, M.B., Cornwell, E.R., Hope, K.M., Eckerlin, G.E., Casey, R.N., Groocock, G.H., Getchell, R.G., Bowser, P.R., Winton, J.R., Batts, W.N., Cangelosi, A., and Casey, J.W.: Distribution of an invasive aquatic pathogen (viral hemorrhagic septicemia virus) in the Great Lakes and its relationship to shipping. PLoS ONE 5: article 10156, 2010.

Baumler, A.J., Heffron, F., and Reissbrodt, R. Rapid detection of salmonella enterica with primers specific for *iroB*. J. Clin. Microbiol. 35: 1224-1230, 1997.

Benson, D.A., Cavanaugh, M., Clark, K., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., and Sayers, E.W.: Gen Bank. Nucl. Acids Res. 41: 36-42, 2013.

Bolger, A.M., Lohse, A., and Usadel, B.: Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30: 2114-2120, 2014.

Bush, A.O., Lafferty, K.D., Lotz, J.M., and Shostak, A.W.: Parasitology meets ecology on its own terms: Margolis *et al*. revisited. J. Parasitol. 83: 575-583, 1997.

Bray, R.A., Gibson, D.I., and Jones, A. (eds.): Keys to the Trematoda, vol 3. CAB International, Wallingford, UK, 2008.

Chabaud, A.G.: Keys to genera of the order Spirurida. Part 1. Camallanoidea, Dracunculoidea, Gnathostomoidea, Physalopteroidea, Rictularoidea, and Thelazoidea. *In* Anderson, R.C., Chabaud, A.G., and Willmott, S. (eds). CIH keys to the nematode parasites of vertebrates, Commonwealth Agricultural Bureaux, Farnham Royal, Bucks, UK, 1975.

Chabaud, A.G.: Keys to genera of the superfamilies Cosmocercoidea, Seuratoidea, Heterakoidea, and Subuluroidea. *In* Anderson, R.C., Chabaud, A.G., and Willmott, S. (eds): CIH Keys to the nematode parasites of vertebrates, Commonwealth Agricultural Bureaux, Farnham Royal, Bucks, UK, 1978.

Cooke, S.L., Hill, W.R., and Meyer, K.P.: Feeding at different plankton densities alters invasive Bighead Carp (*Hypophthalmichthys nobilis*) growth and zooplankton species composition. Hydrobiologia 625: 185-193, 2009.

Conover, G., Simmonds, R., Whalen, M. (eds): Management and control plan for Bighead, Black, Grass, and Silver Carps in the United States. Asian Carp Working Group, Aquatic Nuisance Species Task Force, Washington, D.C., USA, 2007.

Coulter, A.C., Keller, D., Amberg, J.J., Bailey, E.J., and Goforth, R.R.: Phenotypic plasticity in the spawning traits of Bigheaded Carp (*Hypophthalmichthys* spp.) in novel ecosystems. Freshwater Biol. 58: 1029-1037, 2013.

Cucherousset, J., and Olden, J.D.: Ecological impacts of nonnative freshwater fishes. Fisheries 36: 215-230, 2011.

DeGrandchamp, K.L., Garvey, J.E., and Colombo, R.E.: Movements and habitat selection by invasive Asian carps in a large river. Transact. Am. Fish. Soc. 137: 45-56, 2008.

Frattini, S.A., Groocock, G.H., Getchell, R.G., Wooster, G.A., Casey, R.N., Casey, J.W., and Bowser, P.R.: A 2006 survey of viral hemorrhagic septicemia (VHSV) virus type IVb in New York State waters. J. Great Lakes Res. 37: 194-198, 2011.

Freedman, J.A., Butler, S.E., and Wahl, D.H.: Impacts of invasive Asian carps on native food webs. Final project report – Illinois-Indiana Sea Grant. 2012.

Goodwin, A.E.: First report of spring viremia of carp virus (SVCV) in North America. J. Aquat. Anim. Health 14: 161-164, 2002.

Gozlan, R.E., St–Hilaire, S., Feist, S.W., Martin, P., and Kent, M.L.: Biodiversity-disease threat to European fish. Nature 435: 1046, 2005.

Grizzle, J.M., Altinok, I. and Noyes, A.D.: PCR method for detection of Largemouth Bass virus. Dis. Aquat. Organ. 54: 29-33, 2003.

Hermoso, V., Clavero, M., Blanco–Garrido, F. and Prenda, J.: Invasive species and habitat degradation in Iberian streams: an analysis of their role in freshwater fish diversity loss. Ecol. Appl. 21: 175-188, 2011.

Hoffman, G.L.: Parasites of North American freshwater fishes. Cornell University Press, Ithaca, NY, USA, 1999.

Irons, K.S., Sass, G.G., McClelland, M.A., and Stafford, J.D.: Reduced condition factor of two native fish species coincident with invasion of non-native Asian carps in the Illinois River, U.S.A. Is this evidence

for competition and reduced fitness? J. Fish Biol. 71: 258-273, 2007.

Karr, J.R., Toth, L.A., and Dudley, D.R.: Fish communities of Midwestern rivers: a history of degradation. BioScience 35: 90-95, 1985.

Kaufman, L.: Catastrophic change in species–rich freshwater ecosystems. BioScience 42: 846-858, 1992.

Khoo, L.H., Austin, F.W., Quiniou, S.M., Gaunt, P.S., Riecke, D.K., Jacobs, A.M., Meals, K.O., Dunn, A. W., and Griffin, M.J.: Lactococcosis in Silver Carp. J. Aquat. Anim. Health 26: 1-8, 2014.

Koehn, J.D.: Carp (*Cyprinus Carpio*) as a powerful invader in Australian waterways. Freshwater Biol. 49: 882-894, 2004.

Kolar, C., Chapman, D., Courtenay Jr. W., Housel, C., Williams, J., and Jennings, D.: Asian carps of the genus *Hypophthalmichthys* (Pisces, Cyprinidae) − A biological synopsis and environmental risk assessment. U.S. Fish and Wildlife Service Report, 94400-3-0128, 2005.

Mandrak, N.E., and Cudmore, B.: Risk assessment for Asian carps in Canada. Available at http://www.dfo–mpo.gc.ca/csas/Csas/DocREC/2004/RES2004_103_E.pdf. Fisheries and Oceans Canada, Great Lakes Laboratory for Fisheries and Aquatic Sciences, Burlington, ON, USA, 2004.

Michel, B., Fournier, G., Lieffrig, F., Costes, B., and Vanderplasschen, A.: Cyprinid herpesvirus 3. Emerg. Infect. Diseases 16: 1835-1843, 2010.

Moyle, P.B., and Light, T.: Biological invasions of fresh water: empirical rules and assembly theory. Biol. Conserv. 78: 149-161, 1996.

Munro E.S., and Midtlyng, P.J.: Infectious pancreatic necrosis virus and associated aquatic birnaviruses. *In* Woo, P.T.K., and Bruno, D.W. (eds). Fish diseases and disorders vol 3: viral, bacterial and fungal infections, 2nd edition. CAB International, Cambridge, MA, USA, 2011.

Noga, E: Fish disease: Diagnosis and treatment (2nd edition). Wiley-Blackwell, Ames, IA, USA, 2010.

Parrish, C.R., Holmes, E., Morens, D., Park, E., Burke, D., Calisher, C., Laughlin, C., Saif, L. and Daszak, P.: Crosse species virus transmission and the emergence of new epidemic diseases. Microbiol. Molec. Biol. Rev. 72: 457-470, 2008.

Plumb, J.: Epizootiology of Channel Catfish virus disease. Mar. Fish. Rev. 40: 26-29, 1978.

Prenter, J., MacNeil, C., Dick, J.T.A, and Dunn, A.M.: Roles of parasites in animal invasions. TRENDS Ecol. Evolut. 19: 385-390, 2004.

Rainey, F.A., Ward–Rainey, N.L., Janssen, P.H., Hippe, H., and Stackebrandt, E.: *Clostridium paradoxum* DSM 7308T contains multiple 16S rRNA genes with heterogeneous intervening sequences. Microbiology 142: 2087-2095, 1996.

Sampson, S.J., Chick, J.H., and Pegg, M.A.: Diet overlap among two Asian carp and three native fishes in backwater lakes on the Illinois and Mississippi rivers. Biol. Invasions 11: 483-496, 2009.

Sato, M., Kawaguchi, Y., Nakajima, J., Mukai, T., Shimatani, Y., and Onikura, N.: A review of the research on introduced freshwater fishes: new perspectives, the need for research, and management implications. Landsc. Ecol. Eng. 6: 99-108, 2010.

Schrank, S.J., Guy, C.S., and Fairchild, J.F.:Competitive interactions between age-0 Bighead Carp and Paddlefish. Transact. Am. Fish. Soc. 132: 1222-1228, 2003.

United States Fish and Wildlife Service (USFWS): Asian Carp − Aquatic invasive species. Available at http://www.fws.gov/midwest/fisheries/library/fact–asian Carp.pdf, 2004.

USFWS and AFS–FHS (U.S. Fish and Wildlife Service and American Fisheries Society-Fish Health Section): Standard procedures for aquatic animal health inspections. *In* AFS-FHS blue book: suggested procedures for the detection and identification of certain finfish and shellfish pathogens, Accessible at: http://afsfhs.org/bluebook/bluebook-index.php, 2014.

Wilcove, D.S., Rothstein, D., Dubow, J., Phillips, A., and Losos, E.: Quantifying threats to imperiled species in the United States. BioScience 48: 607-615, 1998.

Wilcox, J.: Parasites of invasive carp and native fish in the Wabash River. Eastern Illinois University, Masters Thesis. Paper 1114, 61 pp. Available at http://thekeep.eiu.edu/theses/1114, 2013.

Witte, F., Goldschmidt, T., Wanink, J., Vanoijen, M., Goudswaard, K., Wittemaas, E., and Bouton, N.: The destruction of an endemic species flock: quantitative data on the decline of the haplochromine cichlids of Lake Victoria. Environ. Biol. Fishes 34: 1-28, 1992.

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