Pathogen Susceptibility of Silver Carp (*Hypophthalmichthys molitrix*) and Bighead Carp (*Hypophthalmichthys nobilis*) in the Wabash River Watershed

FINAL REPORT

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9/22/2014

Silver Carp (top) and Bighead Carp (bottom) caught in the Tippecanoe River, Photos by Alison Coulter

Executive Summary

The Pathogen Susceptibility of Silver Carp (*Hypophthalmichthys molitrix*) and Bighead Carp (*Hypophthalmichthys nobilis*) in the Wabash River Watershed project was undertaken to address the lack of available information regarding pathogens in the highly invasive Silver and Bighead Carps, collectively known as bigheaded carps. Very little is known about the prevalence and effects of parasites, bacteria and viruses on the health of invasive bigheaded carp populations in the United States or the effects of bigheaded carps on the disease risk profile for sympatric, native fish of the U.S.

The main objectives of this project were to conduct a systematic survey of parasites, bacteria and viruses of Asian carps and a representative number of native Indiana fish species in the upper and middle Wabash and the lower Tippecanoe Rivers, Indiana; to determine the susceptibility of Asian carps to a representative number of natural pathogens using *in vitro* approaches; and to involve anglers in the development of a cost effective state-wide surveillance program for documentation of viral diseases of fish.

Fish collection occurred from March 2013 to September 2013 in the Wabash River near river mile 293 and the Tippecanoe River at Oakdale Dam. The species collected included Silver Carp, Bighead Carp, Common Carp (*Cyprinus carpio*), Bigmouth Buffalo (*Ictiobus* cyprinellus), Smallmouth Buffalo (*Ictiobus bubalus*), Emerald Shiner (*Notropis atherinoides*), Channel Catfish (*Ictalurus punctatus*), Flathead Catfish (*Pylodictis olivaris*), Freshwater Drum (*Aplodinotus grunniens*), Gizzard Shad (*Dorosoma cepedianum*), Bluegill (*Lepomis* macrochirus), Largemouth Bass (*Micropterus salmoides*), Smallmouth Bass (*Micropterus dolomieu*), and Shovelnose Sturgeon (*Scaphirhynchus platorynchus*).

Fish (n=12/species) were bled and necropsied for the collection of tissues (spleen, liver, kidney, stomach and intestines). Data collected from each fish included total length (fork length for Shovelnose Sturgeon), body weight, sex, external parasites, pre-existing external injuries and presence and type of internal lesions. Internal lesions were present on individuals of 6 species: Shovelnose Sturgeon, Channel Catfish, Largemouth Bass, Smallmouth Bass, Bluegill, and Freshwater Drum. None of the Silver Carp or Bighead Carp had observable external parasites, injuries or internal lesions. DNA extracted from spleens and plasma from all fish was submitted to Purdue University's Genomics Core Facility for sequencing. Sequencing was performed on an Illumina sequencer using a MiSeq multi-indexed run. Sequencing results indicated the presence of bacterial DNA from *Pseudomonas fluorescens* and *Pseudomonas putida* in Silver Carp, and *Pseudomonas fluorescens*, *Pseudomonas putida*, and *Salmonella enterica* in Bighead Carp. DNA from two copepod species was also detected in both species, however no clear evidence of viral DNA was found. All stomach and small intestine contents removed during fish collection were examined for the presence of helminths. The most prevalent parasites identified were the monozoic tapeworm *Atractolytocestus huronensis*, present in over 70% of the Common Carp; the trematode *Leuceruthrus micropteri*, present in over 50% of the Smallmouth and Largemouth Bass; and the cestode *Megathylacoides giganteum,* present in 50% of the Flathead and Channel Catfish. Both Asian carp species were free of macroscopic, gastrointestinal parasites.

Overall, the bigheaded carps in our field study appeared to be in healthy condition and had fewer parasites and bacteria present than most of the native species and Common Carp. However, the *in vitro* study demonstrated high sensitivity of bigheaded carp cell lines to Largemouth Bass Virus, Golden Shiner Virus and Infectious Pancreatic Necrosis Virus, thus there are indications that bigheaded carps could be affected by or be carriers of some viruses common to the Midwestern U.S.

1. Background and Rationale

Asian carp, in particular Silver Carp (*Hypophthalmichthys molitrix*) and Bighead Carp (*Hypophthalmichthys nobilis*), are invasive fishes in the Midwestern United States (USFWS 2004) and other locations around the world (Shrank & Guy 2002). Silver and Bighead Carp, collectively known as 'bigheaded carps', were brought to the United States from eastern Asia in the 1970s to control plankton in aquaculture and wastewater treatment ponds (Conover et al. 2007). Eventually, both accidental and intentional introductions allowed these fishes to enter into local rivers. From there, they have spread throughout the Midwest and they are now established and reproducing successfully in the Mississippi, Missouri, Wabash and other rivers and their tributaries (Shrank & Guy 2002; Conover et al. 2007; Sampson et al. 2009; Coulter et al. 2013). Both exotic carp species have shown a remarkable ability to spread and increase in abundance, creating concern that they are negatively impacting ecosystems in which they have become established.

One way by which aquatic, invasive species negatively impact native fish communities is through the spread of pathogens (Kolar et al. 2005). However, very little is known about the susceptibility of bigheaded carps to parasites, bacteria and viruses in the United States. Similarly, nothing is known on the effects of bigheaded carps on the disease risk profile for sympatric, native fish of the U.S. The knowledge regarding the pathogen susceptibility of bigheaded carps is limited to data from fishes in their native habitat.

A detailed list of pathogens and other disease causing agents known to affect bigheaded carps in their native habitats is given in *Asian Carps of the Genus Hypophthalmichthys (Pisces, Cyprinidae) — A Biological Synopsis and Environmental Risk Assessment* (Kolar et al. 2005). According to Kolar et al. (2005), both species appear to be vulnerable to several types of bacteria, including commonly seen genera such as *Aeromonas* and *Edwardsiella*. Only one genus of fungi, *Saprolegnia*, is known to cause disease in Bighead Carp, while several genera of fungi are reported to cause disease in Silver Carp, including *Saprolegnia*, *Aspergillus* and *Aphanomyces* (Kolar et al. 2005). Additionally, the authors state that Bighead and Silver Carp can be parasitized by a large number of protozoans, including *Ichthyophthirius mulitifilis*; as well as trematodes, cestodes, including *Bothriocephalus acheilognathi* "Asian carp tape worm", and copepods. Spring Viremia of Carp is the only virus listed as causing disease in bigheaded carps (Kolar et al. 2005). About half of pathogens listed in the Kolar et al. (2005) summary affect both Silver Carp and Bighead Carp; however, there are many additional disease causing agents cited as affecting Silver Carp that are not cited as affecting Bighead Carp. It is unclear whether this discrepancy is due to Silver Carp being more susceptible to disease or a lack of thorough study on Bighead Carp's susceptibility to disease.

While the health of bigheaded carps is not of particular concern in the U.S. due to their status as invasive species, it is possible for pathogens they carry to affect native species in the waters in which they have been introduced and become established. Kolar et al. (2005) identified two parasites carried by Asian carp, including Silver and Bighead Carp that are thought to be particularly threatening to native North American fishes. Anchorworm (*Lernaea cyprinacea*) was found to affect Channel Catfish when they were kept in ponds with Bighead Carp. It can also affect salmonids, eels, and other carp species. Asian carp tape worm (*Bothriocephalus acheilognathi*), which was introduced to the U.S. through Grass Carp, has been known to spread to bait fish, such as Golden Shiners (*Notemigonus crysoleucas*) and Fathead Minnows (*Pimephales promelas*), as well as several endangered species, including Virgin Spinedace (*Lepidomeda mollispinis*), Woundfin Minnow (*Plagopterus argentissimus),* Colorado Pikeminnow (*Ptychocheilus lucius*), and Humpback Chub (*Gila cypha*) in the western and southwestern United States.

Thus, whether bigheaded carps act as carriers and/or hosts for exotic and native parasitic and infectious diseases is largely unknown. The prevalence of infectious diseases in bigheaded carps is also largely unknown. This is critical information that can be used by fishery managers for more accurate assessments of the impact of bigheaded carps on native fish populations, and the development of potential biological control programs for Silver and Bighead Carps.

2. Objectives

Due to the overall lack of information available regarding pathogens in the highly invasive bigheaded carps, we undertook this study with the following main objectives:

1. Conduct a systematic survey of parasites, bacteria, and viruses of Asian carps and a representative number of native Indiana fish species in the upper and middle Wabash and the lower Tippecanoe Rivers, Indiana;
2. Determine the susceptibility of Asian carps to a representative number of fish pathogens using *in vitro* approaches; and
3. Involve anglers in the development of a cost effective state-wide surveillance program for documentation of viral diseases of fish.

3. Results

I. Survey of Pathogens

The first objective was to conduct a survey of parasites, bacteria and viruses affecting Asian carps in the Wabash and lower Tippecanoe Rivers, which are part of the established range of these invasive species. This information about the susceptibility of Asian carps to exotic and native parasitic and infectious diseases and the prevalence of these diseases in Asian carps will contribute to more accurate assessments of the impact of Asian carp on native fish populations and the development of potential biological control programs for Asian carp.

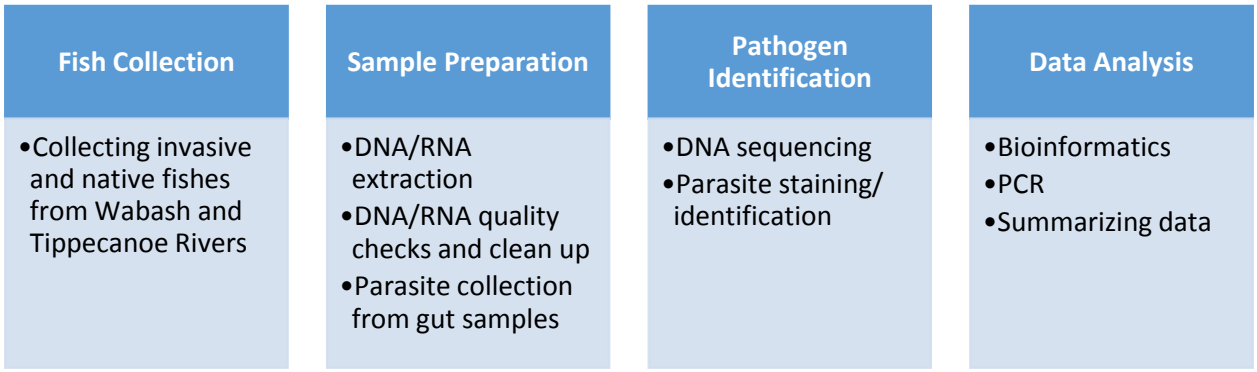

Figure 1: Overview of process.

Fish collection.- Fish collection occurred from March 2013 to September 2013 in the Wabash River near river mile 293 and the Tippecanoe River at Oakdale Dam. The invasive fishes collected included both Asian carp species of interest - Silver Carp and Bighead Carp - as well as Common Carp (*Cyprinus carpio*). The remaining species were all native fishes and included - Bigmouth Buffalo (*Ictiobus* cyprinellus), Smallmouth Buffalo (*Ictiobus bubalus*), Emerald Shiner (*Notropis atherinoides*), Channel Catfish (*Ictalurus punctatus*), Flathead Catfish (*Pylodictis olivaris*), Freshwater Drum (*Aplodinotus grunniens*), Gizzard Shad (*Dorosoma cepedianum*), Bluegill (*Lepomis* macrochirus), Largemouth Bass (*Micropterus salmoides*), Smallmouth Bass (*Micropterus dolomieu*), and Shovelnose Sturgeon (*Scaphirhynchus platorynchus*). See Table 1 for the number of fish collected for each species.

A combination of electrofishing, gill netting, fyke netting, and hook and line were employed during the collection period due to the variety of species sought. Silver Carp were primarily caught by electrofishing in a large borrow pit connected year-round to the Wabash River at West Lafayette, IN. All of the Bighead Carp were caught just downstream of the Oakdale dam on the Tippecanoe River, except for one small individual which was collected in a fyke net in the large borrow pit on the Wabash River. Bighead Carp proved difficult to collect due to their occurrence in areas, such as the Oakdale Dam, that are not amenable to gill netting or electrofishing and their low density relative to the Silver Carp. We collected and necropsied 134 fish, but were unable to obtain the target number of individuals (n = 12) for some species. All Shovelnose Sturgeon samples for the study were taken from fish sacrificed as part of the regular IDNR sturgeon monitoring, thus so no additional sturgeons were sacrificed for this project.

Table 1: Fish collected, March 2013-September 2013.

Family	Scientific Name	Common Name	Individuals Collected
Cyprinidae	*Hypophthalmichthys molitrix*	Silver Carp	12
Cyprinidae	*Hypophthalmichthys nobilis*	Bighead Carp	6
Cyprinidae	*Cyprinus carpio*	Common Carp	12
Cyprinidae	*Notropis atherinoides*	Emerald Shiner	12
Catostomidae	*Ictiobus cyprinellus*	Bigmouth Buffalo	6
Catostomidae	*Ictiobus bubalus*	Smallmouth Buffalo	6
Ictaluridae	*Ictalurus punctatus*	Channel Catfish	12
Ictaluridae	*Pylodictis olivaris*	Flathead Catfish	4
Clupeidae	*Dorosoma cepedianum*	Gizzard Shad	12
Sciaenidae	*Aplodinotus grunniens*	Freshwater Drum	12
Centrarchidae	*Lepomis macrochirus*	Bluegill	12
Centrarchidae	*Micropterus salmoides*	Largemouth Bass	7
Centrarchidae	*Micropterus dolomieu*	Smallmouth Bass	9
Acipenseridae	*Scaphirhynchus platorynchus*	Shovelnose Sturgeon	12

Tissue collection.- Fish were bled from the caudal vein using a 3 mL syringe fitted with 22 – 26G ½ - 1" needles depending on fish size. Blood was stored in heparinized vacutainers kept on ice until centrifuged for the collection of plasma (0.1 – 1 mL). Fish were then immediately euthanized by an overdose (≥ 250 mg/L) of the fish anesthetic MS-222 (Tricaine methanesulfonate) and necropsied in the field in a controlled environment within an enclosed trailer. Tissue samples (spleen, liver and kidney) were taken in the field with sterile equipment and immediately frozen in liquid nitrogen for later testing. The stomach and intestines were removed and stored on ice in the field. All samples were transferred to -20°C or -80°C freezers for storage in the Aquatic Ecology lab at Purdue University.

Body measurements.- Measurements of total length (fork length was taken for Shovelnose Sturgeon) and body weight are shown in Table 2.

Table 2: Physical measurements taken at time of collection, *Fork length.

Fish Species	Total Length, mm Range (Mean)	Body Weight, kg Range (Mean)
Silver Carp	440 - 816 (599.5)	0.84 - 4.98 (2.75)
Bighead Carp	630 - 895 (796.8)	2.36 - 8.47 (5.97)
Common Carp	243 - 800 (558.6)	0.20 - 6.10 (2.74)
Emerald Shiner	45 - 84 (67.7)	0.0001 - 0.005 (0.0002)
Bigmouth Buffalo	405 - 635 (535.3)	0.84 - 3.98 (2.55)
Smallmouth Buffalo	276 - 653 (418.7)	0.28 - 3.60 (1.20)
Channel Catfish	141- 650 (356.3)	0.06 - 2.88 (0.76)
Flathead Catfish	229 - 705 (491.5)	0.13 - 4.35 (1.86)
Gizzard Shad	163 - 388 (263.8)	0.03 - 0.34 (0.19)
Freshwater Drum	232 - 423 (341.3)	0.13 - 1.00 (0.48)
Bluegill	70 - 178 (123.7)	0.01 - 0.095 (0.043)
Largemouth Bass	146 - 311 (260.4)	0.04 - 0.42 (0.32)
Smallmouth Bass	161 - 335 (230.8)	0.06 - 0.43 (0.17)
Shovelnose Sturgeon	604 - 780 (706.8)*	0.83 - 1.78 (1.35)

Fish lesions.- Observations of internal lesions, external parasites, and injuries were noted in the field. Internal lesions were present on individuals of 6 species (Figure 2). External parasites ("black grub") were seen on several of the 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 preexisting wounds that appeared to be infected. None of the Silver Carp or Bighead Carp had any observable external parasites, injuries or internal lesions.

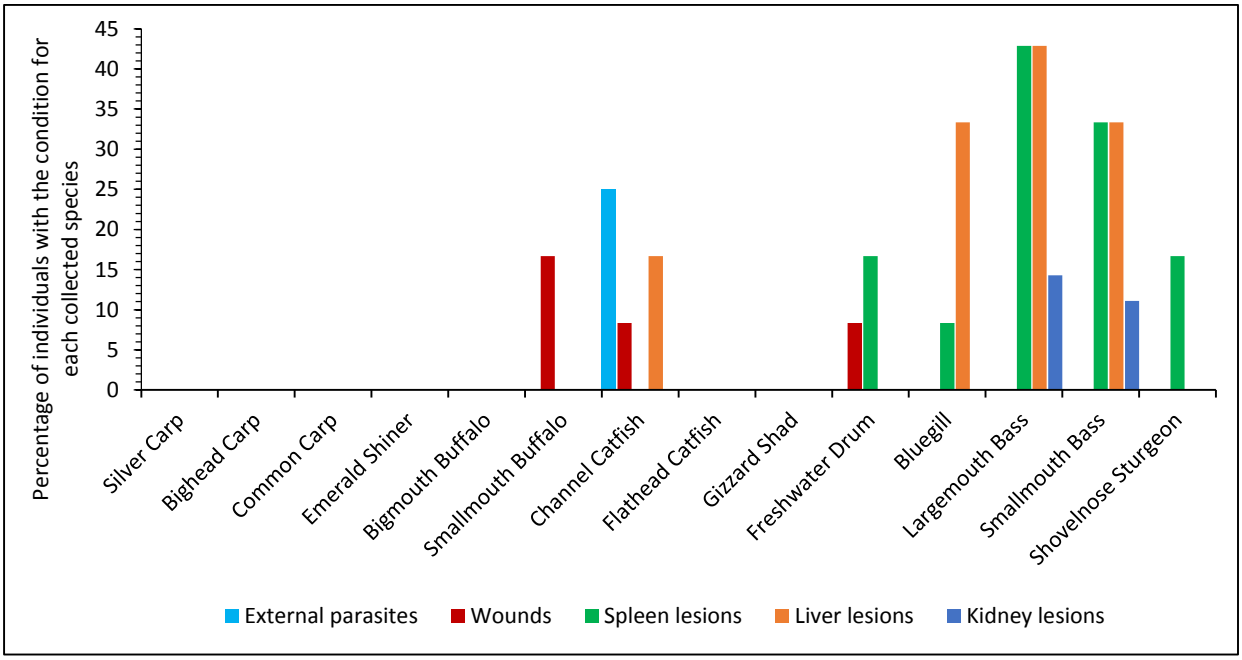

Figure 2: Frequency of external parasites, open wounds and internal lesions on necropsied fish.

Metagenomic analyses using next generation sequencing.- The analysis for the presence of bacteria and viruses was done through the DNA-based methods of polymerase chain reaction (PCR) and next generation sequencing (NGS). From September-November 2013, the preserved samples were processed to extract DNA and RNA. Genomic DNA was extracted from the spleen tissue samples using DNAzol Reagent (Invitrogen, Grand Island, NY) to be used in identifying bacteria present in the fish tissue. Viral DNA and RNA were extracted from the plasma using a ZR Viral DNA/RNA kit (Zymo Research, Irvine, CA).

Quality checks were performed on the DNA and RNA samples by Nanodrop and gel electrophoresis. Small amounts of residual chemicals from the extraction process were found, which is normal. However, due to the high sample quality required for NGS, additional sample cleanup by ethanol precipitation and/or ZR Clean & Concentrator kits (ZymoResearch, Irvine, CA) was completed to remove any trace amounts of extraction chemicals that would have interfered with sequencing performance.

In December 2013, DNA samples were pooled by species and submitted to Purdue University's Genomics Core Facility for sequencing. Emerald Shiners were omitted from sequencing, because their small size precluded the required amount of plasma and spleen DNA from being collected. Sequencing was performed on an Illumina sequencer (Illumina, Inc., San Diego, CA) using a MiSeq multi-indexed run which generated over 27 million raw reads. These short reads were assembled into longer pieces (contigs) using the ABySS (Assembly by Short Sequences) software (Simpson et al. 2009). The majority of these contigs sequences were from the DNA of the host fish and a smaller percentage were from pathogens present in the fish. Sequence read quality statistics from FASTQC software (The Babraham Institute, Cambridge, UK) were provided by the Purdue Genomics Core Facility.

We analyzed the assembled sequences using the program BLAST (Basic Local Alignment Search Tool) and sequence databases made available by the National Center for Biotechnology Information (NCBI). We used the blastn option for comparison of DNA contigs to known DNA sequences of bacteria, viruses, invasive carps and native fish. In order to process the large number of sequences, we made use of the high-throughput computing resources at Purdue University, mainly DiaGrid and the Radon computing cluster.

The majority of the contigs were from host fish DNA and matched with various zebrafish genes in the NCBI database. Zebrafish is a popular model species for genetic and other research and has high genetic similarity with our surveyed fish species. A smaller percentage of the reads were from pathogens. Table 3 and Table 4 show bacteria and parasite detections from the sequencing.

Table 3: Pathogens detected in Silver Carp and Bighead Carp by blastn.

Pathogen Species	Type	Fish Species
Pseudomonas fluorescens	Bacteria	Silver Carp, Bighead Carp
Pseudomonas putida	Bacteria	Silver Carp, Bighead Carp
Salmonella enterica	Bacteria	Bighead Carp
Lepeophtheirus salmonis, Salmon louse	Copepod	Bighead Carp
Caligus rogercresseyi, Sea louse	Copepod	Silver Carp, Bighead Carp

Table 4: Pathogens detected in native fishes and Common Carp by blastn, *Buffalo includes: Bigmouth Buffalo (*Ictiobus* cyprinellus) and Smallmouth Buffalo (*Ictiobus bubalus*).

Pathogen Species	Type	Fish Species
Pseudomonas fluorescens	Bacteria	Buffalo, Bluegill, Freshwater Drum, Flathead Catfish, Largemouth Bass, Smallmouth Bass, Shovelnose Sturgeon
Pseudomonas putida	Bacteria	Common Carp, Buffalo, Channel Catfish, Flathead Catfish, Bluegill, Freshwater Drum, Largemouth Bass, Smallmouth Bass, Shovelnose Sturgeon
Salmonella enterica	Bacteria	Common Carp, Channel Catfish
Lactococcus lactis subsp. *lactis*	Bacteria	Gizzard Shad
Lactococcus lactis subsp. *cremoris*	Bacteria	Gizzard Shad
Serratia marcescens	Bacteria	Common Carp
Serratia plymuthica	Bacteria	Common Carp
Staphylococcus aureus subsp. *aureus*	Bacteria	Common Carp, Smallmouth Bass
Acinetobacter baumannii	Bacteria	Channel Catfish
Bacillus cereus	Bacteria	Channel Catfish
Bacillus subtilis	Bacteria	Channel Catfish
Klebsiella pneumoniae	Bacteria	Channel Catfish
Lepeophtheirus salmonis, Salmon louse	Copepod	Common Carp, Freshwater Drum
Caligus rogercresseyi, Sea louse	Copepod	Common Carp, Buffalo, Flathead Catfish, Freshwater Drum, Largemouth Bass, Smallmouth Bass
Caligus clemensi, Sea louse	Copepod	Common Carp

All 5 of the pathogens found in Silver Carp and Bighead Carp were also found in the native species and/or Common Carp. However, there were 10 additional species/subspecies identified that were not found in either of the Asian carps (Table 4). In particular, Common Carp, Gizzard Shad, and Channel Catfish had numerous additional pathogens.

PCR.- PCR (Polymerase Chain Reaction) was used to further investigate the susceptibility of each species to pathogens and to assess rates of infection within each species by testing individual fish samples (non-pooled). We tested *Aeromonas salmonicida*, because it is very common and was one of our bacteria of interest at the start of the study. We also tested *Salmonella enterica* and *Pseudomonas putida*, because these bacteria were identified in the sequencing results as being present in Silver Carp, Bighead Carp, Common Carp and many of the native fishes. We also tested *Lactococcus lactis* subsp. *lactis*, because it was identified as being present in Gizzard Shad and was also found in the tissues (brian) of one Silver Carp in a large fish kill in the Mississippi River in 2011 (Khoo et al. 2014). Finally, we tested Largemouth Bass

Virus (LMBV), because early results from the *in vitro* study showed high sensitivity to it in bigheaded carp cells (see more on these results below).

For many of the detections in the sequencing results, we were unable to duplicate the results using PCR. Figure 3 is a side by side comparison of sequencing (Seq) and PCR results for *S. enteric, P. putida* and *L. lactis* subsp. *lactis*. We are unclear why there is this discrepancy. One possibility is that when matching sequencing reads to the DNA databases, BLAST will find an appropriate match without bias. This means, for example, that if a given sequencing read is from a gene shared by both freshwater lice and sea lice species, BLAST will not limit it's search only to lice species that are local to the area or parasitic to fish. The result is that it might return the wrong species or subspecies in a genera, such as is likely the case for *Lepeophtheirus salmonis* and *Caligus* sp. that were detected (see Table 3).

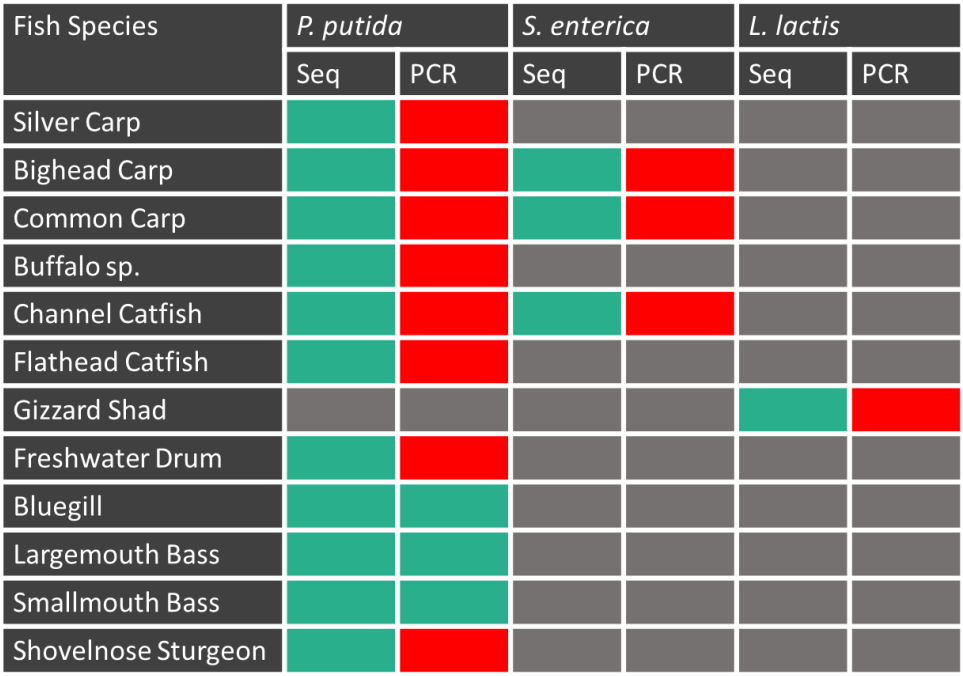

Figure 3: Comparison of results from sequencing and PCR. Green – bacteria detected, Red – bacteria not detected, Gray – blank.

We did detect *P. putida* in Bluegill, Largemouth Bass and Smallmouth Bass with PCR and most samples tested positive for a universal 16S bacterial primer (not shown). Neither *A. salmonicida* nor LMBV were detected in Silver Carp or Bighead Carp via PCR. All Centrarchidae species in the study were also screened for LMBV and the virus was not detected.

Parasites.- Between September-November 2013, stomach and small intestines removed during fish collection were unthawed and the contents extracted and examined for helminthes. Parasites were stored at room temperature in 70% ethanol and shipped to Dr. Mike Kinsella, a specialist in parasite identification. Most of the parasites were identified to species (Table 5).

The most prevalent parasites identified were the monozoic tapeworm *Atractolytocestus huronensis*, present in over 70% of the Common Carp; the trematode *Leuceruthrus micropteri*, present in over 50% of the Smallmouth Bass; and the cestode *Megathylacoides giganteum,* present in 50% of the Flathead and Channel Catfish. Both Asian carp species were free of macroscopic, gastrointestinal parasites.

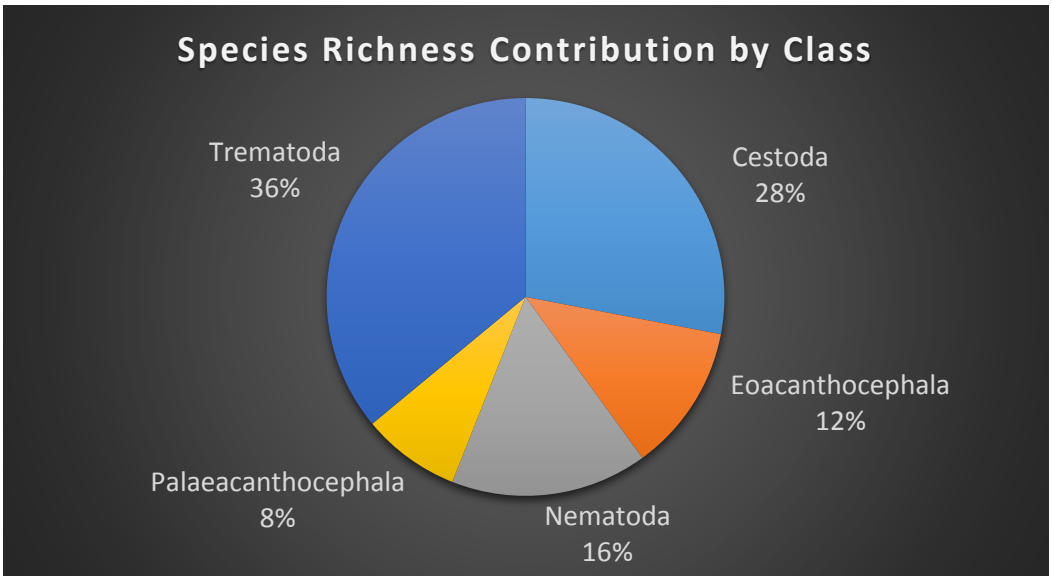

Figure 4: Parasite species richness by class for all fish combined.

Despite the relatively higher parasite load in the bass and catfishes, they nonetheless appeared to be not heavily burdened by the parasites and were active, strong fish. It is unclear why the bigheaded carps are not preferable hosts for local parasites, particularly given that Common Carp, the closest relative to bigheaded carps in our study, was one of the most affected species. Since many of these helminths have complex cycles requiring one or more intermediate hosts to become infective, it is possible that these intermediate hosts are absent from the areas Asian carp are inhabiting.

Table 5: Parasites identified from stomach and small intestines.

Fish Species	Location	Parasite Species
Silver Carp		None
Bighead Carp		None
Common Carp	stomach	*Atractolytocestus huronensis*
	small intestine	*Kwahia iowensis*
	small intestine	*Atractolytocestus huronensis*
Emerald Shiner	small intestine	larval nematode
Bigmouth Buffalo	small intestine	*Lissorchis attenuatum*
	small intestine	*Neoechinorhynchus australis*
	small intestine	*Camallanus ancylodirus*
Smallmouth Buffalo	stomach	*Monobothrium ingens*
	stomach	*Capigens singularis*
	small intestine	*Lissorchis gullaris*
	small intestine	*Capigens singularis*
Channel Catfish	small intestine	*Megathylacoides giganteum*
	small intestine	*Polylekithrum ictaluri*
	small intestine	*Dichelyne robustus*
Flathead Catfish	small intestine	immature digenean

	small intestine	*Dichelyne cotylophora*
	small intestine	*Corallobothrium fimbriatum*
	small intestine	*Megathylacoides giganteum*
Gizzard Shad	small intestine	*Tanaorhamphus longirostris*
Freshwater Drum	small intestine	*Acanthocephalus dirus*
	small intestine	encysted ascarid larva
Bluegill	small intestine	*Posthodiplostomum* sp.
	small intestine	*Dichelyne robustus*
	small intestine	*Homalometron armatum*
	small intestine	*Neodiplostomum americanum*
Largemouth bass	small intesti ne	*Neoechinorhynchus cylindratus*
	stomach	encysted nematode
	small intestine	*Echinorhynchus salmonis*
	small intestine	*Leuceruthrus micropteri*
	small intestine	*Proterometra macrostoma*
	small intestine	*Posthodiplostomum* sp.
	small intestine	*Proteocephalus ambloplites*
Smallmouth Bass	small intestine	*Leuceruthrus micropteri*
	stomach	*Leuceruthrus micropteri*
	small intestine	encysted nematode larva
	stomach	*Bothriocephalus* sp.
	small intestine	*Neoechinorhynchus cylindratus*
	small intestine	*Proterometra macrostoma*
	stomach	*Proterometra macrostoma*
	small intestine	*Camallanus* larva
Shovelnose Sturgeon	small intestine	*Skrjabinopsolus manteri*
	small intestine	*Acanthocephalus dirus*

II. *In Vitro* Studies of Pathogen Susceptibility

The second objective of this project was to determine the susceptibility of Asian carps to a representative number of fish pathogens using *in vitro* approaches. The original goal was to use *in vitro* methods to determine the sensitivity of bigheaded carps to the pathogens that we found in the survey. However, we had no positive matches for viruses from our sequencing reads. Therefore, we looked at bigheaded carp susceptibility to a representative set of viruses frequently seen in fish mortality events in the midwestern U.S. This work was conducted in collaboration with Eric Leis from the US Fish and Widlife Service La Crosse Fish Health Center in Onalaska, WI. The results from this work are preliminary and need further replication and more detailed studies.

Cell cultures.- Silver and Bighead Carp cell lines (skin, gill, fin and fry) were inoculated with Largemouth Bass Virus (LMBV), Bluegill Virus (BGV), Golden Shiner Virus (GSV), Infectious Pancreatic Necrosis Virus (IPNV), Channel Catfish Virus (CCV), Spring Virema of Carp Virus (SVCV) and Viral Hemorrhagic Septicemia Virus (VHSV). Cell lines were split and allowed to become confluent in a 24 well plate. The cells were then challenged with 0.1 mL of a high titer virus in duplicate wells and then incubated and monitored for viral

cytopathic effect. Incubation temperatures were 25°C for LMBV, CCV and BGV; 20°C for SVCV; and 15°C for VHSV, GSV and IPNV.

Results of the *in vitro* studies indicate potential susceptibility of Silver and Bighead Carp to several fish viruses common to the midwestern U.S. and not previously identified as affecting bigheaded carps. Notably, all cell lines from both species showed unusually high sensitivity to LMBV. This was unexpected, since LMBV is primarily a virus of centrarchids and generally only fatal to Largemouth Bass. In addition, all bigheaded carp cell lines were very sensitive to GSV and IPNV and showed some sensitivity to BGV. As expected, all cell lines also showed sensitivity to SVCV, previously noted by Kolar et al. (2005) as causing disease in both species. Somewhat surprisingly the Silver and Bighead Carp cell lines did not show sensitivity to VHSV. Given the range of species affected by VHSV, including Common Carp cell lines, we would have expected the Asian carps to also exhibit sensitivity to this virus.

III. Angler Collaboration for Disease Monitoring

The third objective of this project was to involve anglers in the development of a cost effective state-wide fish pathogen surveillance program, including testing the effectiveness of non-lethal, cost-saving methods of sample collection. The specific goals will be to demonstrate to anglers simple methods that they could use on their own to collect samples in a surveillance program and to gather information about anglers' willingness to participate in such a program. This outreach also allows us to dialogue with participants about the current status of Asian carp and encourage participants to engage in responsible practices regarding these fishes.

***Verifying filter paper method*.-** Although spleen, kidney, liver or caudal vein blood samples taken under sterile, controlled conditions and stored in freezers are commonly used to screen fish for the presence of pathogens, as we did in our pathogen survey; these methods are too time consuming and cumbersome to be practical for a recreational anglers disease monitoring program. For this reason, we suggest using a paper strip method that does not require much equipment or special sample preservation. We tested this method with blood samples from captive fish while affecting to create conditions similar to those available to anglers.

Three adult brood stock yellow perch (*Perca flavescens*) from the Aquaculture Research Lab at Purdue University were processed outdoors next to the facility. The fish were euthanized by placing them in a lethal solution (≥ 250 mg/L) of the fish anesthetic MS-222, which is not used by anglers, but does not affect blood pathogens. To collect controls, an area of the caudal peduncle was cleaned with alcohol (70% ethanol), fish were bled from the caudal vein using 3 mL syringes with 23G 1" needles and the blood was transferred from the syringe directly onto a clean filter paper. This control is used to verify that gill blood samples, used by anglers and having the potential for contamination from the gill surface, will give the same results as caudal blood samples commonly used by professionals.

Next the gill filaments were rinsed with pure water to remove excessive dirt or debris from the holding water. A clip was made in the gill filaments using a scissors cleaned with alcohol (70% ethanol). A clean filter paper was placed against the gill filaments to absorb blood flowing from the gills. A negative control with no blood was also used. Each used filter paper was placed in a new open Whirl-pak and allowed to air dry on-site. The negative control was already dry, but was left in an open Whirl-pak to ensure exposure to the same conditions as the samples. After drying and being left at room temperature for the day, the bags were closed and placed in a 4°C refrigerator overnight/for long term storage. This method requires anglers to use only basic cleaning methods during sample collection and have access to filter paper, clean water, alcohol and clean envelopes or whirl paks.

On the second day after collection, DNA was extracted from 4x4 mm sections of each filter paper using Tris-EDTA buffer-based extraction following the method of Bereczky et al. (2005). A PCR was run on the samples and controls using a universal bacterial primer and bacterial DNA in the blood could be recovered from all the filter paper samples. This confirms the utility of this method, which could also be applied for the detection of viruses, fungi and parasites.

Angler involvement.- As part of an extension component required for our PhD students, Kensey has been conducting outreach at the Oakdale Dam on the Tippecanoe River. The overall goal of the outreach is to dialogue with participants about the current status of Asian carp and encourage participants to engage in responsible practices regarding these fishes. In particular, she is giving demonstrations, using Silver Carp and other fishes, on how to easily collect blood samples with filter paper and also explaining how these can be used to help monitor diseases of fish populations.

She will be distributing a survey on Asian carp and fish disease monitoring to anglers. The survey covers the angler's experience level, beliefs about the impact of Asian carps on recreational fisheries and interest in citizen scientist approaches to fish disease monitoring. Specifically, it asks for feedback from the anglers regarding how they would feel about being involved in pathogen monitoring through a means such as taking blood samples on filter paper of the fish they catch and sending them to a lab.

IV. Conclusions

The goal of this project was three-fold: conduct a systematic survey of parasites, bacteria, and viruses of Asian carps and a representative number of native Indiana fishes; determine the susceptibility of Asian carps to a representative number of fish pathogens using *in vitro* approaches; and involve anglers in the development of a cost effective state-wide surveillance program for documentation of viral diseases of fish. The results of our survey of fishes in the Wabash and Tippecanoe Rivers indicate that Silver and Bighead Carp are not heavily burdened by many of the fish native pathogens. In particular, no endo-parasites were found in the stomach or small intestine of either species and there were no visual internal signs (lesions, etc.) of infection. DNA sequencing did produce a short list of pathogens for Silver and Bighead Carps (*Pseudomonas* sp., *Salmonella enterica*, copepods), but we were unable to confirm via PCR the presence of these particular bacteria in the samples. Sunfishes, catfishes and Common Carp had higher numbers of pathogens than buffalo species, Freshwater Drum, sturgeon and other carps. We did not find a pathogen unique to bigheaded carps that would be a potential candidate for use in a biological control program. Results of the *in vitro* studies, however, indicate potential susceptibility of Silver and Bighead Carp to several fish viruses, all of which also affect native fishes. Gill, skin, fin and fry cell lines from both species were highly sensitive to LMBV, GSV and IPNV and slightly sensitive to BGV and SVCV. Exposure studies with live bigheaded carp larvae might be useful for determining whether the fish are affected by the viruses or simply carriers. We developed and are currently testing a cost-effective and easy approach for fish disease monitoring involving anglers.

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