Asian Carp Environmental DNA (eDNA) in the Osage River Basin

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INTRODUCTION

The overall goal of this study was to examine the extent of Bighead Carp (*Hypophthalmichthys nobilis*) and Silver Carp (*Hypophthalmichthys molitrix*) presence in tributary systems of Harry S. Truman Reservoir located in west-central Missouri using environmental DNA (eDNA) techniques. Truman Reservoir is the largest reservoir in Missouri, and its basin extends into several counties in Kansas (Figure 1). Several of the primary tributaries, including the Marais des Cygnes, the Marmaton, and the Little Osage Rivers, originate in eastern Kansas and are considered high quality rivers and streams with some of the highest quality mussel populations in the state (Wolf and Stark 2008). The filter feeding niche of Asian carp overlaps with these mussels (Kolar et al. 2007).

Silver carp were first noted in the lower Kansas River in 2005 and since this time the concern for the spread of both silver and bighead carp has grown as these species are now appearing throughout the central U.S. (Kolar et al. 2007). eDNA sampling in the St. Croix and Mississippi Rivers has provided evidence for the spread of bigheaded carps (*Hypophthalmichthys spp*.) into Minnesota (Hickox et al. 2011), and more recent studies have shown evidence of successful spawning of bigheaded carps in the Lower Missouri River (Deters et al. 2013) and phenotypic plasticity suited to invasion of a wide range of river conditions (Coulter et al. 2013). Tracking the initial spread of these species into other waterbodies is critical to the success of all management efforts, since the best management opportunities occur when population densities are low. Because of the size of the basin and the important need to quickly survey this drainage for the possible presence of silver and bighead carp, eDNA sampling was proposed for its relatively rapid, cost-effective, and high sensitivity potential for detection of these invasive fish (Goldberg et al. 2011).

When this study was first conceived, eDNA collection, extraction, amplification, and interpretation methodologies were relatively undeveloped for surface water monitoring of aquatic vertebrates, with five fundamental papers having been published (Ficetola et al. 2008, Dejean et al. 2011, Goldberg et al. 2011, Jerde et al. 2011, Thomsen et al. 2012). Since then, conventional polymerase chain reaction (PCR) and quantitative PCR (qPCR) assays have been codified by federal agencies (e.g., US Army Corps of Engineers 2012, US Fish and Wildlife Service 2014, US Fish and Wildlife Service 2015), and significant funding and effort have been undertaken to further understanding of the strengths and limitations of eDNA for monitoring through the Environmental DNA Calibration Study (ECALS) (Asian Carp Regional Coordinating Committee 2014). Ongoing research has also contributed significantly to understanding of the science and adoption of best practices for eDNA analyses, with numerous peer-reviewed journal articles (> 25 in the first 9 months of 2015 alone) and whole journal volumes dedicated to the subject (e.g., Biological Conservation volume 183). While the science is rapidly evolving, reviews by Goldberg et al. (2015), Thomsen and Willerslev (2015), and Rees

et al. (2014) summarize many of the recent advances in the science of eDNA for ecological applications.

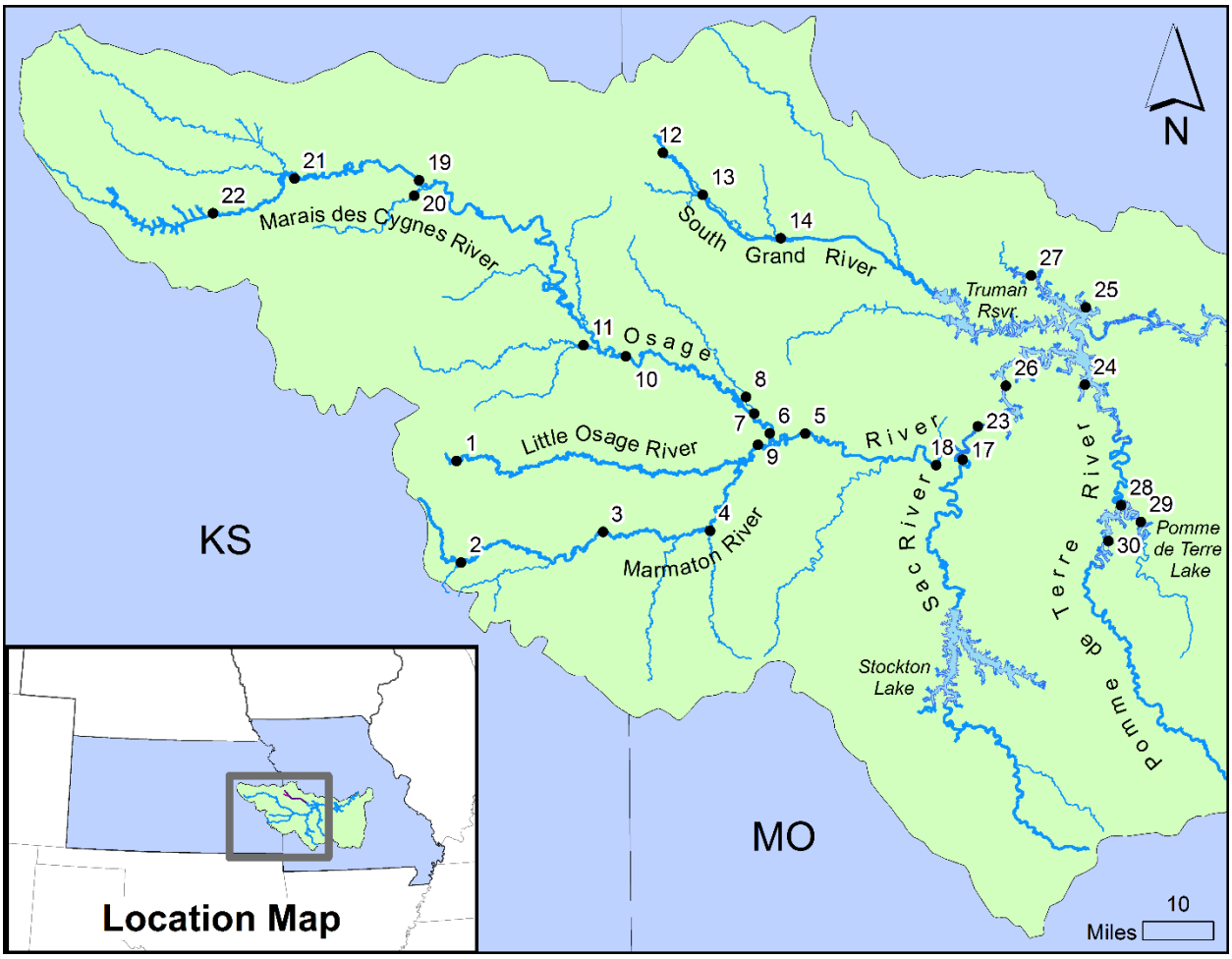

Figure 1. The Osage River watershed with sampling locations.

METHODS

Site Selection

Twenty-eight sites distributed across the Truman Reservoir basin were selected and sampled for eDNA in the fall of 2013 to determine the presence of silver and bighead carp (Figure 1, Table 1). Sites were selected along major tributaries and at major branches to give an idea of the potential directions that upstream migration may have occurred. Nine sites were on the major

western tributaries coming out of Kansas (i.e., 4 on the Marais de Cygnes, 3 on the Marmaton, and 2 on the Little Osage Rivers, respectively), five were on the Osage, three were on the South Fork of the Grand, and one was on the Sac. Eight sampling sites were on lakes with 3 on Pomme de Terre Lake and 5 on Truman Reservoir itself.

Table 1. Sampling locations by waterbody.

Waterbody	State	Sampling Site Numbers
Big Sugar Creek	KS	11
Little Osage River	KS	1
	MO	9
Marais des Cygnes River	KS	10, 19, 21, 22
Marmaton River	KS	2, 3
	MO	4
Miami Creek	MO	8
Middle Creek	KS	20
Osage River	MO	5, 6, 7, 8, 18
Sac River	MO	17
South Fork of the Grand River	MO	12, 13, 14
Pomme de Terre Lake	MO	28, 29, 30
Truman Reservoir	MO	23, 24, 25, 26, 27

Surface water samples were collected from bridges and by boat for subsequent extraction and amplification of environmental DNA from bigheaded carps (*Hypophthalmychthys nobilis* and *H. molitrix*) using previously published PCR markers by Jerde *et al.* (2011).

eDNA methods for this project were developed based on protocols that were still under development at the time of project initiation, including US Army Corps of Engineers protocols (US Army Corps of Engineers 2012) based on work by the Lodge lab at the University of Notre Dame (Jerde et al. 2011) and other methods as described in the literature (e.g., Coyne et al. 2005, Ficetola et al. 2008, Goldberg et al. 2011). These methods predate both the previous and current USFWS QAPP protocols (US Fish and Wildlife Service 2014, US Fish and Wildlife Service 2015) and the establishment of the Whitney Genetics Lab as the primary processor of USFWS Asian carp eDNA samples.

Field Methods

Prior to sampling, the site id, date, latitude, longitude, general water depth category (low, normal, high), water clarity (clear, turbid), and collection time were recorded at each sampling location. Each site was verified by topographic map, local signage and landmarks, and global positioning system (GPS) units to confirm concordance with the sampling design.

At each stream sampling location, three precipitation samples and one filtration sample were collected along transects perpendicular to the stream channel. For each of the three stations along the transect (left, center, and right when facing downstream), water was collected for precipitation samples by surface grab in a sterile container, and a sub-sample was collected in a new, sterile 50 mL centrifuge tube. After being filled, these tubes were immediately put on dry ice in a dark cooler. Water temperature of the first surface grab was also taken with a sterile thermometer and recorded at the first station. At the center station, an additional 2L grab sample for filtration was taken in a sterile container and placed immediately into a cooler with regular (wet) ice. Precipitation samples were returned on dry ice to the laboratory and stored at -80°C until processing. Filter samples were held on wet ice and processed within 16 hours of collection.

All collection containers were sterilized prior to use either by the manufacturer or by soaking in 10% bleach, followed by rinsing with reverse-osmosis purified water. After sterilization, containers were kept in sealed plastic bags until use in the field. Researchers donned new gloves and new sterile containers were used for each transect station, and waste materials were placed in sealed bags before returning to vehicles. After collecting samples at each site, all equipment used in the sampling effort, including sampling gear, boots, trailers and boats, were sterilized using a 10-minute exposure to 10% bleach solution before moving to a different stream (Prince and Andrus 1992, US Army Corps of Engineers 2012).

Prior to sampling, one sterile 2L container and three sterile 50 mL centrifuge tubes were filled with tap water to serve as field blanks for the day's sampling. Field blanks accompanied equipment to the sampling locations, were placed in coolers prior to and subsequently along with the day's field samples (2L in with wet ice and 50 mL tubes in with dry ice), and returned to the laboratory and stored along with the field samples. For the purposes of this experiment, field blanks also served as filtration blanks.

Laboratory Methods

Quality Control Procedures

Control blanks were introduced throughout laboratory processing to assess potential contamination. Each batch extraction was accompanied by an extraction blank (reverse osmosis water used as sample). Each batch evaporation was accompanied by a hood blank, and each PCR plate contained both a positive control (e.g., tissue extracted DNA) and a no template control (reverse osmosis water). Laboratory surfaces were cleaned with 10% bleach prior to use, between samples, and after use. Laboratory equipment was cleaned with either ultraviolet light and 70% ethanol (e.g., PCR and laminar flow hoods), commercial DNA remover (e.g., DNA away, ThermoScientific, Inc.) or 10% bleach. Tissue extractions, field sample extractions, low-copy template addition, and PCR and post-PCR processing were all performed in separate laboratories. PCR mastermix setup was performed in a UV sterilized, strictly no-template PCR hood with separate pipetters, tips, etc. Centrifuge tubes and filter pipet tips made of low adhesion plastics (VWR, Inc.; Corning, Inc.) were used throughout laboratory work. For samples that were detections, appropriate controls including field blanks, extraction blanks, and hood blanks were all run and the samples were run again for confirmation. For samples that were non-detections, 10% of controls were run to confirm no contamination. At least one field blank and one extraction blank were run for each batch of samples collected.

Filtration Samples

Filtration samples were processed by methods adapted from Jerde *et al.* (2011) and the US Army Corps of Engineers (2012). Prior to beginning work and between samples, the filtration apparatus and work space was cleaned and sterilized with 10% bleach and filtration start time was recorded. New gloves were donned for each sample and the outsides of tubes were cleaned and sterilized, then rinsed with sterile water. Sample volumes were filtered in aliquots through 0.45 um 934-AH filters in sterile, magnetic 500 mL filter cups at < 7 in Hg (US Army Corps of Engineers 2012). With turbid samples, small aliquot volumes were used, and when a filter was deemed clogged, the filter was folded, removed from the filtration apparatus, and placed in a sterile 50 mL centrifuge tube using sterile forceps. A new sterile filter was then placed on the filtration stage and filtration of the remaining sample volume was resumed. Multiple filters were frequently required to process the full 2L volume. Water samples were well mixed before pouring each aliquot into the filter cup. Centrifuge tubes were placed on dry ice once a filter was placed inside, and all filters for a particular sample were stored in the same centrifuge tube. Filters were held on dry ice in this manner until returned to the laboratory for storage at -80°C. Filtration end times were recorded to confirm processing within the 16 hour window from time of collection.

Filters were shipped on dry ice to the USFWS Whitney Genetics Lab facility for extraction and PCR amplification according to the 2014 version of the quality assurance plan (US Fish and Wildlife Service 2014).

Precipitation Samples

Precipitation samples were processed with methods adapted from Ficetola *et al.* (2008) and Coyne *et al.* (2005) and involved initial precipitation of field samples using ethanol and sodium acetate followed by a cetyltrimethylammonium bromide (CTAB) extraction using chloroform separation and precipitation of DNA with isopropanol and 5M sodium chloride. Centrifuge tubes used for sampling were certified for 25,000*x g*, DNase and RNase free, and free from plasticizers and biocides (VWR, Inc.), and pipette tips used were low-bind filter tips (VWR, Inc.).

For laboratory processing, precipitation samples were removed from -80°C storage and thawed on ice or in 4°C coolers for use. Once thawed, samples were well mixed and a 15 mL sample was added by sterile serial pipet to a sterile 50 mL centrifuge tube containing 33.5 mL of 95% ethanol and 1.5 mL of 3 M sodium acetate. Samples were then well mixed and incubated at 4°C for 24 to 48 hours.

After incubation, the samples were spun 3400*x g* for 35 minutes at 6°C, the liquid was decanted, and the pellet retained. After 5 minutes of drying, 700 µL of CTAB were added, the samples were vortexed briefly and then placed in a 60°C incubator. After 10 minutes, samples were removed and again spun at 3400*x g*, this time for 5 minutes at 20°C. The full contents of each tube were then transferred to low-bind microcentrifuge tubes containing 700 µL of 24:1 chloroform : isoamyl alcohol solution. The CTAB, chloroform, isoamyl alcohol pellet suspension was well mixed by shaking horizontally for 5 minutes, then centrifuged at 15,000*x g* to separate the suspension into phases. 500 µL of supernatant from each sample were transferred to new sterile tubes, and 500 µL of ice cold isopropanol were added, followed by 250 µL of 5 M NaCl. Samples were incubated overnight at -20°C to precipitate the extracted DNA.

The following day, samples were centrifuged at 15,000*x g* for 5 minutes, then the supernatant was removed and the pellets retained. 150 µL of 70% ethanol were added to wash each pellet, the sample again centrifuged at 15,000*x g* for 5 minutes, and the liquid again decanted. This ethanol wash, spin, decant process was again repeated, and the now cleaned pellets retained. After the second wash and decant, sample tubes were placed on their side in a UV-sterilized and 95% ethanol wiped laminar flow hood for evaporation of residual ethanol. The last drops of ethanol were removed via sterile cotton swabs carefully applied to avoid disturbance of the pellet. Once the ethanol was removed, the extracted pellets were eluted in 100 µL of 10 mM

Tris HCl buffer (pH 8.0) and 0.1 mM EDTA overnight at 4°C. Once eluted, sample DNA was well mixed and divided into aliquots for PCR analysis (held at -20°C) and long term storage (held at -80°C). Extracted DNA concentrations were quantitated using a Qubit dsDNA high sensitivity assay.

Polymerase Chain Reaction Conditions

Samples were amplified using assays reported by Jerde *et al.* (2011) (Table 2). Each PCR 25 mL reaction contained 0.75 u of Taq polymerase, 1*x* PCR buffer (Promega, Inc.), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2mM forward primer, 0.2mM reverse primer, and 5 µL of sample template. The thermocycle included an initial denaturation step of 2 minutes at 94°C, followed by 45 cycles of 94°C for 1 minute, 1 minute of annealing at either 50°C for *H. molitrix* or 52°C for *H. nobilis*, and 1.5 minutes of extension at 72°C. There was a final extension stage of 7 minutes at 72°C. Quality control measures were included as described previously. Each plate was run with a positive control (tissue extracted *Hypophthalmichthys* DNA) and a no template control (reverse osmosis water). PCR products were visualized by electrophoresis, and detection was determined by comparison with controls and amplicon size (191 for *H. molitrix*, 312 for *H. nobilis*) as determined by a concurrently loaded DNA ladder.

Table 2. PCR primers and amplicon sizes for the silver (*Hypophthalmichthys molitrix*) and bighead (*Hypophthalmichthys nobilis*) carp assays used in this study.

Target Species	Primer Designation	5' to 3' Sequence	Amplicon Size (bp)
H. molitrix	HMF-2	CCTGARAAAAGARKTRTTCCACTAATAA	191
	HMR-2	GCCAAATGCAAGTAATAGTTCATTC	
H. nobilis	HN203-F	TAACTTAAATAAACAGATTA	312
	HN498-R	TAAAAGAATGCTCGGCATGT	

RESULTS

Asian carp detections were confirmed in 3 locations for water samples collected by filtration and zero locations for samples collected by precipitation (Figure 2, Table 3). In addition, one filtration field blank (11/7/2013) tested positive for Asian carp DNA. All other field blanks and

extraction blanks had zero detections for Asian carp DNA. For all plates, positive controls were positive and no template controls were negative.

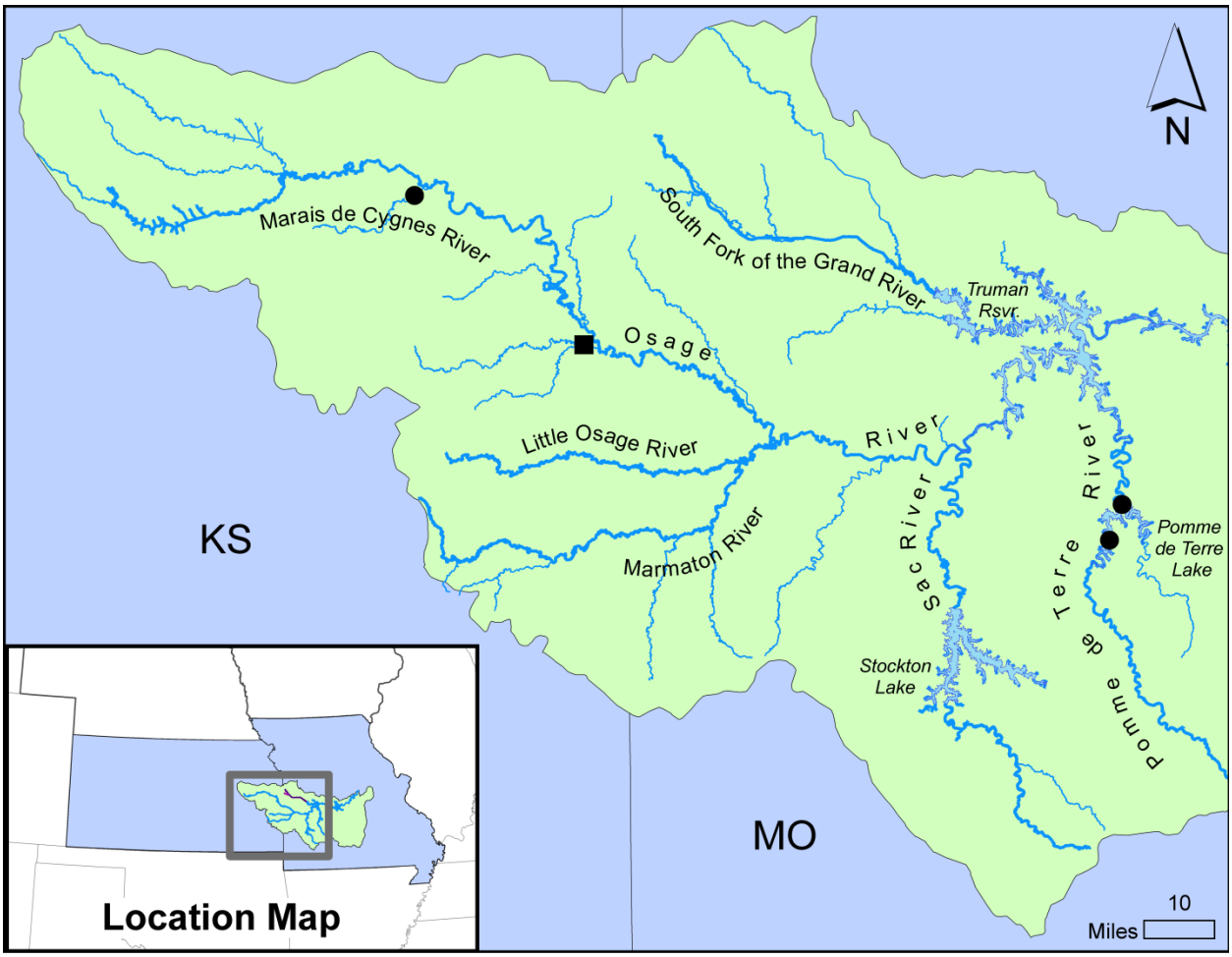

Figure 2. Sites with positive detections of *Hypophthalmichthys* spp.. eDNA in surface water samples. Closed circles indicate positive detection of *H. molitrix*. Closed square indicates positive detection of *H. nobilis*.

Table 3. Detection of Asian carp based on environmental DNA recovered from water column samples. Extraction and amplification of filter samples performed by USFWS Whitney Genetics Lab. Extraction and amplification of precipitation samples performed by KBS. "+" indicates a positive detection and "O" indicates non-detection. An asterisk indicates at least one initial positive detection that was not confirmed by subsequent PCR analysis.

Site	Site Name	Sampling Date	Filtration		Precipitation	
			H. molitrix	***H. nobilis***	***H. molitrix***	***H. nobilis***
1	Little Osage River	1/Oct/2013	O	O	O	O*
2	Marmaton River	1/Oct/2013	O	O	O	O
3	Marmaton River	1/Oct/2013	O	O	O	O*
4	Marmaton River	4/Oct/2013	O	O	O	O
5	Osage River	9/Oct/2013	O	O	O	O
6	Osage River	4/Oct/2013	O	O	O	O
7	Osage River	4/Oct/2013	O	O	O	O
8	Miami Creek	4/Oct/2013	O	O	O	O
9	Little Osage River	9/Oct/2013	O	O	O	O*
10	Marais des Cygnes River	24/Sep/2013	O	O	O	O
11	Big Sugar Creek	24/Sep/2013	O	+	O	O
12	South Grand River	26/Sep/2013	O	O	O	O
13	South Grand River	26/Sep/2013	O	O	O	O
14	South Grand River	26/Sep/2013	O	O	O	O
17	Sac River	9/Oct/2013	O	O	O	O
18	Osage River	9/Oct/2013	O	O	O	O
19	Marais des Cygnes River	24/Sep/2013	O	O	O	O
20	Middle Creek	20/Sep/2013	+	O	O	O
20	Middle Creek	24/Sep/2013	O	O	O	O
21	Marais des Cygnes River	8/Nov/2013	O	O	O	O
22	Marais des Cygnes River	20/Sep/2013	O	O	O	O
23	Truman Reservoir	7/Nov/2013	O	O	O	O
24	Truman Reservoir	7/Nov/2013	O*	O	O	O
25	Truman Reservoir	7/Nov/2013	O	O	O	O
26	Truman Reservoir	7/Nov/2013	O	O	O	O
27	Truman Reservoir	7/Nov/2013	O	O	O	O
28	Pomme de Terre Lake	8/Nov/2013	+	O	O	O
29	Pomme de Terre Lake	8/Nov/2013	O	O	O	O
30	Pomme de Terre Lake	8/Nov/2013	+	O	O	O

DISCUSSION

Molecular methods have become an essential tool for detection of organisms at low densities (Darling and Mahon 2011, Goldberg et al. 2015). Detection of invasive species by amplification of DNA recovered from the environment is one of the most established applications, with detection of the invasive bigheaded carps (*Hypophthalmichthys* spp.) being the prime example in the United States (Jerde et al. 2011). Significant efforts have been made over the past several years to increase the precision and reliability of the eDNA assays used for Asian carp (e.g., the Asian Carp Regional Coordinating Committee's eDNA calibration study, http://www.asiancarp.us/ecals.htm), and new eDNA methods are constantly being developed (Ficetola et al. 2008, Jerde et al. 2011, Turner et al. 2014, Deiner et al. 2015, Doi et al. 2015) and rigorously tested for quality assurance (US Army Corps of Engineers 2012, US Fish and Wildlife Service 2014, US Fish and Wildlife Service 2015).

Since bigheaded carps have been identified in the Osage River and Lake of the Ozarks (US Geological Survey 2015) and are speculated to be in Truman Reservoir, since they move upstream to spawn during high flow events in river networks (DeGrandchamp et al. 2008), and since they have proved to be effective invaders in many river networks of the central US (Kolar et al. 2007, DeGrandchamp et al. 2008, Hickox et al. 2011, Jerde et al. 2011, Deters et al. 2013), we developed a sampling plan to test for upstream movement of Asian carp in the Osage River basin. This basin is characterized especially in Kansas by streams with relatively high diversity of filter feeding freshwater mussels (Wolf and Stark 2008), which could be negatively impacted by invasion of large-bodied, filter-feeding carp.

Four confirmed *Hypophthalmichthys* records in the Osage Basin are listed in the USGS nonindigenous aquatic species database (US Geological Survey 2015). All were single caught *H. nobilis*, with one in 1998 near Schell City, MO on the Osage River above Truman Reservoir and the others below Truman in the Lake of the Ozarks in 1994, 2001, and 2011.

Using collection of eDNA by both filtration and precipitation, we found limited upstream movement of *Hypophthalmichthys* spp. in the Osage River basin. Filtration samples identified positive detections of *H. molitrix* in one western tributary (Middle Creek) and one southern lake above Truman Reservoir (Pomme de Terre Lake). *H. nobilis* detections by filtration were limited to one location (Big Sugar Creek) (Figure 2, Table 3). No records of *Hypophthalmichthys* have been reported previously at these locations. A confirmed positive detection was also made from a field blank accompanying samples taken from Truman Reservoir. However, concurrent field blanks taken for precipitation samples were non-detections.

Researchers noted that fishermen were cleaning fish and dumping bait at the Truman Reservoir ramp where the sampling crew launched their boat. Such cleaning could be one source of

contamination for the field blank, since juvenile carp are often difficult to distinguish from other wild caught bait species, and DNA from Asian carp carcasses has recently been shown to persist under certain conditions (Merkes et al. 2014). Pomme de Terre Lake is less than 25 river miles upstream from Truman reservoir along a relatively large tributary (Pomme de Terre River). Since the Pomme de Terre river flows unimpeded between the two waterbodies, the potential for upstream migration of wild fish from Truman Reservoir to below the dam at Pomme de Terre Lake is relatively high compared to other tributaries and waterbodies in the Osage basin given the short distance and unimpeded flow between the two. Moreover, Pomme de Terre Lake is very close (less than 30 miles by road) to both Truman Reservoir and Lake of the Ozarks, two popular recreational-use reservoirs in central Missouri. Transfer of eDNA from live fish, fish tissue, or eDNA between these lakes by fishermen is a distinct possibility.

Detection in Middle Creek is more surprising, given the upstream river mile distance from Truman Reservoir and multiple downstream sites with non-detection. Quality assurance procedures coupled with nondetections in associated controls suggest that this detection was likely not due to contamination during collection, extraction, or amplification. However, the site is located at a public bridge over Middle Creek. Therefore, one alternative potential source of DNA could be from debris dumped from the road. Further investigation is recommended to evaluate the presence of *Hypophthalmichthys* at the site.

H. nobilis DNA was detected in filtration assays at site 11, just upstream from the confluence of Big Sugar Creek with the Osage River (Figure 1, Figure 2, Table 3). This location is upstream from previous *H. nobilis* records on the Osage River (US Geological Survey 2015). However, sites 5, 6, 7, 8, and 10 occur between the mouth of Big Sugar Creek and the previous record, all with no detection of *H. nobilis* DNA. Since filtration, extraction, and amplification blanks were all negative, the detection of *H. nobilis* DNA in this sample suggests that *H. nobilis* DNA may be present in Big Sugar Creek at this site. Again, further investigation will be required to establish presence of actual fish.

The preponderance of nondetections in precipitation samples also suggests limited upstream movement of bigheaded carps in the Osage River Basin. Initial amplification of samples suggested *H. nobilis* DNA may have been present in samples from the Little Osage and Marmaton Rivers, however subsequent assays did not confirm detection (Table 3).

While the filtration samples were processed using updated markers (US Fish and Wildlife Service 2014), precipitation samples were processed using published conventional PCR markers (Jerde et al. 2011). These markers were found to cross-amplify (i.e., both primer sets amplify *H. molitrix* tissues and *H. nobilis* tissues), rendering species determination difficult. However, other published qPCR markers similarly do not differentiate between the species. Since both species are invasive, detection of either is significant. Recent research has suggested that inhibition can significantly affect amplification of environmental DNA samples (Alaeddini 2012, Goldberg et

al. 2015, Jane et al. 2015). Limited testing of inhibition in the samples collected for this study by method of standard additions (i.e., spiking with positive controls) suggested that some inhibition may be present. New molecular probe assays that test for inhibition in each replicate well (e.g., Turner et al. 2015) may provide better insight in this regard.

Asian carp are invasive and established in rivers of the central US, and in many cases, their upstream movement throughout free-flowing river networks is almost inevitable. Though impoundments may pose a barrier in some cases (e.g., the Clinton Reservoir dam on the Wakarusa River in Kansas), fish can still be introduced upstream by overtopping of low-head dams during high flow events (e.g., Coon Rapids Dam on the Mississippi River in Minnesota) or by other modes of transport (e.g., fishermen). For areas where Asian carp invasions pose potentially significant threats to native or current ecological resources, continued monitoring is recommended.

In this study we found that filtration of turbid waters as commonly found in the Osage River basin can be very time consuming and require multiple filters, sometimes 10 or more to pass 2L of water. For this reason, USFWS eDNA sampling may shift more to collection by precipitation for turbid waters like those found in the Osage River basin (Dr. Emy Monroe, pers. comm.), and the newly approved quality assurance plan includes a section for precipitation sampling that was not present when this project was initiated. Also, precipitation samples can be preserved immediately, thereby removing the 16 hour time frame constraints associated with filtration. Therefore, for the Osage Basin and similarly turbid waters, collection by precipitation may reduce both the time required for sampling and the time required for processing.

Significant advancements in the science and application of environmental DNA monitoring have been made. For example, eDNA of Asian carp has been shown to concentrate in sediments (Turner et al. 2015), and is speculated to be associated with clay and other resuspendable particles. Monitoring programs may benefit from sampling additional environmental compartments such as sediment. Likewise, new technologies such as digital droplet PCR (Doi et al. 2015), metagenomics analyses (Evans et al. 2015), and other biological markers such as RNA or proteins (Barnes and Turner 2015) may provide informative data on the extent or condition of invasive Asian carp in aquatic systems. The combination of continued vigilance and developing science are likely the best bet for limiting expansion of invasive Asian carp in the Osage River basin.

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