Abundance and distribution of early life stages of invasive carp in the Ohio River:

Geographic Location: Ohio River Basin

Participating Agencies: Indiana Department of Natural Resources (INDNR) Kentucky Department of Fish and Wildlife Resources (KDFWR), West Virginia University (WVU), United States Fish and Wildlife Service (USFWS), West Virginia Division of Natural Resources (WVDNR), Ball State University (BSU), Southern Illinois University (SIU)

Statement of Need:

The negative effects of Silver (*Hypophthalmichthys molitrix)* and Bighead Carp (*Hypophthalmichthys nobilis)*, also known as invasive carp, have been widely documented throughout their introduced range. These effects are numerous and varied in nature, some with direct implications to native biota (Irons et al. 2007, Sampson et al. 2009). Other effects, such as economic loss and negative social perception, may be indirect and difficult to quantify. Research investigating what factors lead to invasive carp range expansion is critical for the control of these invasive fishes, and mitigation of the deleterious effects they can cause.

Extensive research efforts have been directed toward invasive carp reproduction in terms of timing, location, and environmental conditions. Invasive carp exhibit a boom-and-bust pattern of reproduction, with strong year classes usually linked with large, sustained flooding and critical temperature ranges (DeGrandchamp et al. 2007). Although some understanding of their reproductive requirements exist, evidence suggests spawning of these species is possible over wider environmental ranges (Coulter et al. 2013), and in more habitats (i.e., tributaries) than previously thought (Kocovsky et al. 2012). Juvenile invasive carp are extremely mobile and may also elicit clumped distributions among static environments, requiring a variety of different gear types to effectively sample various habitats throughout the Ohio River (Collins et al. 2017; Molinaro 2020). In addition, factors promoting successful reproduction and recruitment remain uncertain. Identifying these factors is critical in suppressing the spread of these invasive fishes into novel environments.

Previous confirmed invasive carp spawning events have occurred in downstream tributaries (i.e., Wabash River) and as far upstream as McAlpine Locks and Dam (L&D), and physical signs of spawning (i.e., spawning patches) have been observed as far upstream as Markland Pool for Silver Carp and Meldahl Pool for Bighead Carp. Limited reproduction of *Hypophthalmichthys* spp. was detected by the presence of larvae at river mile (RM) 560 (McAlpine Pool) in 2015, and further upstream at RM 405.7 (Meldahl Pool) in 2016 (EA engineering, personal communication). Although these specimens were not genetically confirmed, this defined the leading edge of spawning in the Ohio River. To support the Ohio River Fish Management Team (ORFMT) Basin Framework objectives (ORFMT 2014), this project was initiated in 2016 in an effort to improve capabilities to detect early stages of invasion and spawning populations of invasive carp (Strategy 2.8) and also monitor upstream range expansion and changes in distribution and abundance (Strategy 2.3). Results of sampling prior to 2021 determined the extent of recruitment as below Cannelton L&D (Newburgh Pool), with the majority of young-of-year (YOY) and juvenile detections below Newburgh L&D in J.T. Myers Pool (Jansen and Stump 2017, Roth 2018).

In addition to the Basin Framework, this project directly supports the National Plan (Conover et al. 2007) by assisting in the forecast and detection of invasive carp range expansions (Strategy 3.2.4), determining life history characteristics (Strategy 3.3.1), and assembling information about the distribution, biology, life history, and population dynamics of Bighead and Silver Carp (Strategy 3.6.2). Additionally, the results of this project will help managers make informed decisions during future planning efforts regarding resource allocation for invasive carp deterrent and control strategies.

2021 Project Objectives:

1) Determine the extent of bigheaded carp spawning activity in the Ohio River above Markland Dam.
2) Identify tributaries and areas of the Ohio River in which spawning of bigheaded carp occurs.
3) Determine the geographic extent and locations of invasive carp recruitment in the Ohio River.
4) Identify characteristics of potential invasive carp nursery areas when juvenile invasive carp are encountered.
5) Estimate Hovey Lake recruitment potential and evaluate the feasibility of drain structure modifications to limit invasive carp recruitment from Hovey Lake.
6) Determine the propagule source of invasive carp in the Ohio River.

Project Highlights:

- Five sites were sampled above Markland Locks and Dam (RM 532) via ichthyoplankton tows in May, June, and July, 2021. Genetic analysis confirmed Silver Carp and Bighead Carp eggs from the Ohio River near Little Miami River (RM 463), shifting the invasion front upstream.
- Ichthyoplankton tows were used to determine egg and larvae present both upstream and downstream of four locks and dams on the Ohio River. *Hypophthalmichthys* larvae were identified both above and below Newburgh and McAlpine locks and dams.
- Several *Hypophthalmichthys* eggs and larvae were captured within the Kentucky River (RM 546), representing the first tributary of the middle Ohio River with confirmed invasive carp spawning.
- Targeted surface trawling during 2021 resulted in the first confirmed YOY invasive carp being captured in Cannelton Pool of the Ohio River, one pool further upstream than previous detections.
- Approximately 500 YOY invasive carp were sampled from Hovey Lake by Ball State University. Otoliths were pulled and are being aged for daily growth; results are expected in June, 2022.
- Southern Illinois University completed water chemistry analysis and expects to complete otolith microchemistry analysis in May, 2022. Some tributaries have distinct combinations of water Sr:Ca and Ba:Ca that may be useful for identifying fish from these sites.
- KDFWR collected a 180 mm Bighead Carp and 200 mm Silver Carp during a removal event in Craigs Creek of Markland Pool on September 1, 2021.

Methods:

For analysis purposes and for the remainder of this report, the phrase "invasive carp" will be referring to Silver and Bighead carps (*Hypophthalmichthys* spp.) only. In addition, both "YOY" and "immature" are collectively referring to "juvenile" invasive carp; "YOY" will be defined as fish less than 200 mm, and "immature" will define fish between 200 to 400 mm (likely 1 to 2 years old) which have undeveloped gonads and are not capable of spawning. Adult invasive carp are defined as fish greater than 400 mm with mature, identifiable gonads. Additionally, the term "suspect *Hypophthalmichthys*" is referring to an egg, advanced egg, or larvae with morphometric characteristics aligning with bigheaded carps, while the terms "suspicious egg/larvae" refers to specimens that do not have 100% of the morphometric characteristics of bigheaded carps but still warrant genetic confirmation.

Ichthyoplankton tows:
To evaluate the extent of invasive carp spawning activity in the Ohio River above Markland L&D, ichthyoplankton tows were conducted at sampling sites within the R.C. Byrd (N = 2), Meldahl (N = 1), and Markland (N = 2) pools at least twice from May 26 to July 14, 2021. At each sampling site, four tows were conducted: three within the Ohio River proper, and one within the tributary or at the intake structure if the

site was a previous EA Engineering larval sampling site. Additional tows were collected within Raccoon Creek because of the frequent collection of adult invasive carp there.

To identify specific tributaries below Markland L&D in which invasive carp spawning occurs, ichthyoplankton tows were conducted at tributaries within J.T. Myers (N=1), Newburgh (N = 2), Cannelton (N = 2), and McAlpine (N = 1) pools at least twice from June 7 to July 2, 2021 during ideal spawning conditions. Additionally, tows were conducted above and below (within five miles) the Newburgh, Cannelton, McAlpine, and Markland L&D's from June 6 to June 24, 2021. Three tows were conducted at each sampling site.

For all tows, a conical ichthyoplankton net (0.5 m, 500 µm mesh) was deployed from the bow of the boat. The boat was motored in reverse, pulling the ichthyoplankton net upstream for three minutes. The water volume sampled was recorded using a General Oceanics Flowmeter fitted to the ichthyoplankton net; depth (m) and water temperature (°C) were recorded using a boat-mounted depth sounder. All contents in the ichthyoplankton net were rinsed into a 500 µm sieve and preserved using 95% non-denatured ethanol (at an estimated ratio of nine parts ethanol to one-part sample volume) for physical identification in the lab. Suspect *Hypophthalmichthys* eggs and larvae were morphometrically identified (process outlined below) and a subsample were sent to Whitney Genetics Laboratory for genetic confirmation. For specific details on genetic identification results and methods employed by the Whitney Genetics Laboratory, refer to Appendix A.

Larval fish were initially sorted into non-invasive carp and potential invasive carp (suspicious) species using morphometric parameters provided by Auer (1982). Furthermore, early developmental characteristics outlined by Yi et al. (1998) and Chapman (2006) were utilized to physically identify suspect *Hypophthalmichthys* larvae, advanced eggs, and eggs from each sample (Figure 1). Invasive carp larvae were identified by the presence of an eye spot, and suspect *Hypophthalmichthys* were differentiated from Grass Carp (*Ctenopharyngodon idella*) and Black Carp (*Mylopharyngodon piceus*) using myomere counts. *Hypophthalmichthys* larvae have 38 to 39 myomeres, whereas Grass Carp larvae range from 43 to 45 myomeres and Black Carp have 40 and 41 myomeres. Suspect *Hypophthalmichthys* eggs were identified based on general size and presence of a large perivitelline membrane (5 to 6 mm in diameter). Suspect *Hypophthalmichthys* 'advanced eggs' were defined as the beginning of a yolk-sack larvae still contained within the perivitelline membrane. In most cases, suspicious eggs and larvae may not have every morphometric characteristic of invasive carp, however, due to their collection locations, several may have been vouchered and sent to Whitney Genetics Lab for genetic confirmation of species.

Surface trawl:
Surface trawling effort was focused on tributaries and embayments within J.T. Myers Pool (Hovey Lake, Hovey Lake drain, Highland Creek, Lost Creek), Newburgh Pool (two Borrow pits, Blackford Creek, Sandy Creek), Cannelton Pool (Deer Creek, Millstone Creek, Clover Creek, Sinking Creek, Yellowbank Creek, Poison Creek), and McAlpine Pool (Harrods Creek, Goose Creek, Fourteenmile Creek). From July 7 to September 2, 2021, a minimum of two trawls were conducted in each location. Additionally, 8 trawls were conducted between July 7 to September 1, 2021, at Hovey Lake within the J.T. Myers Pool to document potential recruitment of invasive carp within the lake.

The surface trawl measured 3.7 m wide, 0.6 m tall, and 5.5 m deep with 31.8 mm bar (number 12) netting. An additional layer of 4.8 mm mesh (35-pound delta) bag was attached externally to improve capture of small fishes. Additional foam floats were added to the top line of the trawl to provide extra buoyancy. Otter boards were 30.5 cm tall, 61.0 cm long, and each had a 12.7 cm diameter, 27.9 cm long "buoy style" PVC float attached to the top of the board allowing them to float. The trawl was deployed off the bow of the boat and attached with 24.4 m ropes. The boat was motored at 1.6 to 3.2 km per hour in reverse for five minutes before retrieving the net. In some locations it was not possible to complete five minutes of trawling, in which case sample time was documented. At the biologist's discretion, additional trawls were conducted at sites where either coverage was limited, or juvenile invasive carp were suspected. All invasive carp were identified to genus, measured to total length, and weighed.

Environmental variables:
A suite of habitat variables was collected at each surface trawl site including water temperature, water transparency, conductivity, pH, dissolved oxygen, maximum depth, average depth, tributary width, and presence/absence of woody debris and aquatic vegetation. Collection of environmental characteristics may determine preferred Ohio River tributaries for future invasive carp recruitment.

Hovey Lake recruitment:
YOY invasive Carp were collected from Hovey Lake to determine annual recruitment and YOY mortality. Targeted YOY sampling was conducted using boat electrofishing, seining (9.1 m wide x 1.8 m high, 6.35 mm mesh), and surface trawling (3.7 m wide x 4 mm mesh, 76 cm x 38 cm mullet doors, 30 m towline). Collected YOY were frozen, and otoliths were removed after thawing. Otoliths were mounted on glass microscope slides using cyanoacrylate glue. Otoliths were polished using lapping film (3M, St. Paul, MN) until the midplane was reached and circuli were visible. Otolith daily rings will be visualized using Otolith software (mediacy.com) that detects and measures growth lines. Daily growth data for cohorts of YOY will be summarized by collection date to identify if discharge of the Ohio River are linked to hatch dates. Additionally, in an attempt to quantify water levels and flow through the drain structure, HOBO (onset.comp.com) water level loggers were deployed in Hovey Lake near the drain structure, and in the drain just downstream from the drain structure in lake June 2021. An additional HOBO device was attached to a tree in the vicinity, to allow adjustment for variation in barometric pressure. Water level data will be compared to Ohio River water level at the J.T. Myers lock and dam, and the USGS gage at Henderson, Kentucky.

Microchemistry:
Water samples were taken from the Ohio River and tributaries from August to October 2021. Small tributaries that flow into J.T. Myers, Newburgh, Cannelton, McAlpine, and Markland pools were prioritized locations. Water samples were collected using a syringe filtration (0.45 μm pore size) technique and analyzed for Sr, Ba, and Ca concentrations. Additionally invasive carp otoliths were collected for microchemistry analysis. A total of 901 invasive carp were sampled and a single otolith from each was submitted to SIU for analysis; 107 from J.T. Myers Pool, 136 from Newburgh Pool, 280 from Cannelton Pool, 276 from McAlpine Pool, 27 from Markland Pool, 15 from R.C. Byrd Pool, and 60 from the Wabash River. Otoliths were embedded in epoxy, sectioned in the transverse plane, and sanded and polished to expose the otolith core. Sectioned otoliths were attached to glass microscope slides with double-sided tape in preparation for analysis by laser ablation-ICPMS.

Results:

Ichthyoplankton tows:
A combined total of 52 ichthyoplankton tows were conducted within the R.C. Byrd (N = 20), Meldahl (N = 8), and Markland (N = 24) pools (Table 1). A total of 380+ suspicious eggs and 18 suspicious larvae were sorted from tow samples in Markland Pool. There were 256 suspect *Hypophthalmichthys* eggs collected from Ohio River samples near the Little Miami River (RM 463), of which genetic analysis confirmed one Silver Carp egg and two Bighead Carp eggs. Of the 18 suspicious larvae from Markland Pool, five collected from the Ohio River near Hogan's Creek on 6/17/21 had morphometric characteristics of *Hypophthalmichthys* larvae, although they were slightly smaller than confirmed specimens encountered further downstream. Genetic analysis determined the Hogan Creek larvae in question were Grass Carp. In Meldahl Pool, two suspicious larvae and one suspicious egg were pulled from samples, although genetic

analysis could not determine species. One suspicious egg and one suspicious larva from the R.C. Byrd Pool were genetically verified as Grass Carp and Common Carp, respectively.

A total of 48 ichthyoplankton tows were conducted within the mainstem Ohio River in the J.T. Myers (N=6), Newburgh (N = 12), and Cannelton (N = 12) McAlpine (N = 12) and Markland (N = 6) pools (Table 2). No suspect *Hypophthalmichthys* larvae, advanced eggs, or eggs were collected above or below the Cannelton and Markland locks and dams. At McAlpine Locks and Dam, two genetically confirmed *Hypophthalmichthys* larvae were collected upstream, and four were collected downstream. Three additional suspicious larvae captured below McAlpine L&D were genetically identified as River Carpsucker and Grass Carp. At Newburgh Locks and Dam, 57 suspect *Hypophthalmichthys* larvae were collected upstream, as well as 19 suspect larvae and one suspect egg downstream. Seven larvae collected upstream of Newburgh L&D were genetically confirmed *Hypophthalmichthys*, while none of the samples collected downstream of Newburgh L&D were able to get a genetic reading.

An additional 42 ichthyoplankton tows were conducted in select tributaries of the Ohio River (Table 3; Figure 2). No suspect *Hypophthalmichthys* larvae, advanced eggs, or eggs were collected from the Green River in the J.T. Myers Pool. Little Pigeon Creek of Newburgh Pool was sampled twice; no suspect *Hypophthalmichthys* eggs or larvae were captured on June 8th, however, approximately 292,000 stage-38 *Hypophthalmichthys* larvae were collected on June 23rd. Four of these were genetically confirmed Silver Carp. One suspect *Hypophthalmichthys* egg was found in a sample from within the Salt River in Cannelton Pool, however, genetic analysis could not determine species. In the Kentucky River, genetically confirmed *Hypophthalmichthys* specimens collected included 3 Silver Carp eggs and 8 Bighead Carp larvae. One of the Silver Carp eggs from the Kentucky River was collected upstream of Dam 1.

Surface trawl:
Among the 6 tributaries sampled in Cannelton Pool, 28 surface trawls were conducted for a total of 2.3 hours of sampling effort. A total of 33 YOY invasive carp were collected in Cannelton Pool; six in Clover Creek and 27 in Millstone Creek. Additionally, nine surface trawls were conducted in Newburgh Pool for a total of 0.7 hours of sampling. One YOY was collected in a borrow pit near Owensboro, KY. In the J.T. Myers Pool, 20 surface trawls were conducted for a total of 1.6 hours of sampling effort and YOY invasive carp were only captured in Hovey Lake and its drain. A total of 1,110 YOY invasive carp were captured in eight surface trawls within Hovey Lake, and 21 were captured over four surface trawls within the drain. Mean catch-per-unit-effort (CPUE; ± SD) for YOY *Hypophthalmichthys* spp. in Hovey Lake was 1665 ± 256 fish/hour. Average length (± SD) of YOY *Hypophthalmichthys* spp. measured 32 ± 4 mm on July 7, 2021 and 76 ± 12 mm on September 1, 2021.

Hovey Lake recruitment:
Fourteen sampling events were conducted in 10 days of effort at Hovey Lake and the drain by Ball State University. Approximately 500 YOY *Hypophthalmichthys* spp. were collected from July 7 to September 4, 2021 (Table 4). Otoliths from these fish are currently being analyzed for daily growth. HOBO water level logger data was collected from June 30 to August 23, 2021. Water levels in Hovey Lake were higher than the water in the drain after July 11. The water levels in the lake and drain did not vary closely with the Ohio River water levels (Figure 4). Water temperature in Hovey Lake and the drain channel were similar during July and August 2021, and varied from 76 to 89 ˚F with highest temperatures when the Ohio River was low.

Microchemistry:
Several tributaries that enter the Ohio River from Markland Pool and downriver were sampled. The Ohio River water Sr:Ca differs from that of 18 tributaries that enter the Ohio River from Markland Pool and downriver. Ten tributaries had water Sr:Ca within the range of the Ohio River water Sa:Ca (Deer Creek, Sinking Creek, Oil Creek, Little Blue River, Blue River, Fourteenmile Creek, Indian-Kentuck Creek,

Laughery Creek, Hogan Creek, and Tanners Creek) (Figure 5). The water Ba:Ca of tributaries from Markland Pool and downriver strongly overlap with the range of water Ba:Ca for the Ohio River except for the Salt, Kentucky, and Licking rivers. Otolith microchemistry analysis is expected to be complete in May 2022.

Discussion:

Results of the sixth year of the Abundance and Distribution of Invasive Carp Early Life Stages in the Ohio River project offer the most up to date information on the extent of invasive carp spawning and recruitment in the Ohio River. Collective efforts of ichthyoplankton tows, targeted surface trawls, and electrofishing directly addressed Basin Framework Strategy 2.8 by improving capabilities to detect early stages of invasion and spawning populations of invasive carp. This project continues to provide data to describe our current understanding of the distribution of invasive carp recruitment. Moreover, knowledge acquired from this project directly informs planning efforts for future invasive carp deterrent, control, and other management strategies.

Prior to 2021, the leading edge of genetically confirmed invasive carp reproduction was near the Salt River (RM 630) in Cannelton Pool. Data collected in 2021 now confirms Bighead and Silver Carp spawning as far upstream as RM 463 near the Little Miami River, and therefore shifts the 'invasion front' further upstream. This suggests the tailwaters of Meldahl L&D may be a potential spawning site in the Ohio River. A relatively small river rise triggered this spawning event in mid-June. Additionally, 2021 marked the first year of confirmed spawning in a tributary of the middle Ohio River. Particularly, several eggs and larvae were collected from the Kentucky River. The Kentucky River is one of the larger tributaries of the Ohio River within our study area. These findings will be important to consider moving forward with management actions in the Ohio River basin.

Among the subsamples of suspected *Hypophthalmichthys* larvae and invasive carp-type eggs sent to Whitney Genetics Lab, most suspect larvae were confirmed to be Silver Carp. There were several Grass Carp, three River Carpsuckers, two buffalo spp., and one Common Carp larva genetically identified in our samples. As previously mentioned, we knew that not all eggs/larvae sent to Whitney Genetics Lab from 2021 had every characteristic of *Hypophthalmichthys* species, however due to the proximity of where some were collected, we wanted verification to be safe. Regardless, results affirm that invasive carp larvae can be readily and confidently identified by our trained biologist. Eggs remain inherently more difficult to discern and will likely need continued species confirmation through genetic methods.

In 2017, several immature invasive carp (269 – 399 mm TL) were captured in Cannelton Pool, suggesting the extent of recruitment to be above Cannelton L&D. Up until 2021, no YOY invasive carp had been captured in Cannelton Pool during sampling for Basin Framework projects, despite extensive targeted efforts in 2019 and 2020. However, in 2021 the first confirmed YOY Silver Carp were captured in Cannelton Pool, effectively confirming our previous notion that recruitment was possible there. In addition to the sampling efforts highlighted in this report, an additional 24 YOY Silver Carp, ranging between 60 to 96 mm, were captured via electrofishing in Oil Creek (Cannelton Pool) during otolith collection efforts as part of the Early Detection and Evaluation Project. During spring and early summer 2021, the Ohio River maintained a relatively low water level, possibly allowing any eggs that were spawned near McAlpine L&D to hatch and those larvae to escape drift prior to being washed out of Cannelton Pool. Also, 2021 was the first year with genetically confirmed eggs and larvae being found in McAlpine Pool, particularly with specimens being collected from the Kentucky River. This upstream spawning event could have contributed in the new YOY Silver Carp that were documented in Cannelton Pool.

KDFWR received reports of young-of-year (YOY) invasive carp being spotted in Markland Pool embayments. To investigate, KDFWR sampled Craigs Creek of the Markland Pool using a 100ft x 8ft shoreline seine with 1” mesh, as well as concurrent electrofishing efforts throughout the embayment. After two sampling runs with the seine at the Craigs Creek boat ramp, and 120 minutes of electrofishing, two adult silver carp were collected from the electrofishing effort – no juvenile or YOY carp were detected. However, during a separate removal event on September 1, 2021, a 180 mm Bighead Carp and a 200 mm Silver Carp were captured within Craigs Creek embayment suggesting that reports of small juvenile fish in the lower end of Markland Pool are valid. KDFWR aged the 200 mm juvenile Silver Carp at age-1, but was unable to accurately age the Bighead carp, possibly indicating a YOY fish. It is possible that reports of YOY invasive carp in Markland Pool was the result of a spawning event that occurred further upstream, as indicated by the collection of Bighead and Silver Carp eggs in mid-June of 2021. These findings suggest the establishment front for invasive carp in the Ohio River has shifted further upstream.

Most juvenile invasive carp encountered in 2021 were again collected near Hovey Lake in J.T. Myers Pool. Hovey Lake continues to demonstrate its importance as a nursery area for invasive carp recruitment in the Ohio River. The ongoing work at Hovey Lake will attempt to quantify the recruitment potential of Hovey Lake each year by determining YOY mortality rates throughout their first growing season. Due to unforeseen issues, the results for YOY mortality are still pending, but Ball State University is actively working to produce results. Additionally, they plan to define how and when conditions allow for invasive carp to move freely into the lake and suggest potential modifications to the drain structure (or operation of the structure) to limit YOY carp ability to traverse into Hovey Lake. Results from their work will be disseminated to the Ohio River basin project partners as soon as they are produced in a final report summarizing their 2021 work.

There has not been what we would consider a strong spawning event or year-class since this project was initiated in 2016. However, based on the presence of adult invasive carp as far upstream as R.C. Byrd Pool, the new findings of YOY Silver Carp in Cannelton Pool this year, and eggs and larvae collected in McAlpine and Markland pools, a highly successful spawning event could quickly shift the current known extent of recruitment to pools farther upstream. Therefore, the spatial and temporal variation in invasive carp recruitment in the Ohio River emphasizes the need for continued long-term monitoring with this project as well as others within the basin. Efforts in this project provide valuable insight into factors promoting the reproduction and recruitment of invasive carp, and ultimately range expansion. Results support several Basin Framework and National Plan strategies and will be used by biologists to mitigate the spread of these invasive fishes.

Recommendations:

We recommend continued ichthyoplankton tows above Markland Dam in 2022 to keep monitoring the extent of invasive carp spawning in the Ohio River. In doing so, an experimental design approach will be integrated into future sampling efforts to better quantify and track egg and larval presence through time and across varying river conditions. We also recommend continuing targeted ichthyoplankton tows during ideal spawning conditions in Newburgh, Cannelton, and McAlpine Pools to identify specific spawning locations. In 2021, we attempted to determine spawning potential at mainstem Ohio River locks and dams by sampling above and below them. Based on those results and internal discussions, we recommend modifying that approach in the future to gain more meaningful data. The likelihood of being below a dam at the right moment to capture eggs that were spawned at that location is slim. A suggested approach would be to develop sampling designs that will best inform the FluEgg model (i.e. well documented larval development status at various locations), and back-calculate to locate potential mainstem spawning areas in the Ohio River. Throughout future larval sampling events, biologists should continue to use best management practices to preserve the DNA integrity of the samples.

Based on genetically confirmed results from 2020 and 2021 samples, physical morphometrics have been successful in identifying *Hypophthalmichthys* advanced eggs and larvae from other native fish species. The identification of eggs can be more difficult and should still be verified via genetic analysis. The use of a measuring device on a microscope to determine if the perivitelline membrane is 5 to 6 mm will help in sorting between non-invasive carp and invasive carp-type eggs. We recommend the continued use of these methodologies, along with genetically confirmed subsamples to provide additional confirmation and to discern between Silver and Bighead Carp. Additionally, we suggest any field staff involved in the physical identification of *Hypophthalmichthys* larvae and eggs be trained on larval fish identification.

As suspected, the presence of adult invasive carp, abundant spawning sites, and suitable nursery habitat in Cannelton Pool allows recruitment to occur there. Because YOY invasive carp were captured in several locations within Cannelton Pool and two small invasive carp were captured in Craigs Creek within Markland Pool, we recommend that future targeted sampling be expanded to McAlpine and Markland Pools. Markland Pool contains several potential nursery areas; multiple tributaries have shallow and open embayment-type areas within them. New sampling locations in the pools should be prioritized based on previous sampling knowledge of locations where YOY are most likely to be found. As time allows, the use of alternative methods (i.e. seine hauls, mini fyke nets, backpack shocking, and light traps) may be necessary for detecting the presence of YOY invasive carp in waters where our current gears cannot effectively sample. Additionally, as Hovey Lake consistently appears to be an important component of invasive carp recruitment in the Ohio River, quantifying the exchange of invasive carp at all life stages between Hovey Lake and the Ohio River will continue to be a priority moving forward.

Other ongoing projects in the Ohio River basin are gathering data on presence of spawning patches on invasive carp; combining these data with information gathered through this project will help managers identify spatiotemporal patterns of invasive carp reproduction in the Ohio River. This information, along with recruitment patterns we have documented previously, can ultimately be used to identify sources of invasive carp population expansion throughout the basin, and help guide other ORFMT efforts such as deterrents and targeted removals.

Acknowledgements:

We would like to thank Zeb Woiak and staff at the USFWS Whitney Genetics Laboratory for their help processing egg and larval samples for this project.

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Table 1. Summary of ichthyoplankton tows collected by West Virginia University and West Virginia DNR. An asterisk (*) denotes genetically confirmed *Hypophthalmichthys* samples analyzed by Whitney Genetics Lab.

Sampling Information				Suspicious Samples to WGL (N)		Suspect *Hypophthalmichthys* (N)		
Pool	**Location**	**Transect Type**	**Tows (N)**	**Eggs**	**Larvae**	**Eggs**	**Advanced Eggs**	**Larvae**
Markland	Hogan's Creek	Ohio River	12	3	8	0	0	0
Markland	Hogan's Creek	Tributary	4	0	0	0	0	0
Markland	Little Miami	Ohio River	6	15	0	256*	0	0
Markland	Little Miami	Tributary	2	0	0	0	0	0
Medahl	Scioto River	Ohio River	6	1	1	0	0	0
Medahl	Scioto River	Tributary	2	0	1	0	0	0
R.C. Byrd	Kyger Creek	Ohio River	6	1	1	0	0	0
R.C. Byrd	Kyger Creek	At structure	2	0	0	0	0	0
R.C. Byrd	Racoon Creek	Tributary	12	0	0	0	0	0

Table 2. Summary of ichthyoplankton tows collected in the Ohio River proper by the Kentucky Department of Fish and Wildlife Resources and Indiana Department of Natural Resources. An asterisk (*) denotes genetically confirmed *Hypophthalmichthys* samples analyzed by Whitney Genetics Lab.

Sampling Information				Suspicious Samples to WGL (N)		Suspect *Hypophthalmichthys* (N)		
Pool	**Location**	**Transect Type**	**Tows (N)**	**Eggs**	**Larvae**	**Eggs**	**Advanced Eggs**	**Larvae**
J.T. Myers	Newburgh L&D Downstream	Ohio River	6	1	10	1	0	10
Newburgh	Newburgh L&D Upstream	Ohio River	6	0	15	0	0	15*
Newburgh	Cannelton L&D Downstream	Ohio River	6	0	0	0	0	0
Cannelton	Cannelton L&D Upstream	Ohio River	6	0	0	0	0	0
Cannelton	McAlpine L&D Downstream	Ohio River	6	0	9	0	0	4*
McAlpine	McAlpine L&D Upstream	Ohio River	6	0	2	0	0	2*
McAlpine	Markland L&D Downstream	Ohio River	6	0	0	0	0	0
Markland	Markland L&D Upstream	Ohio River	6	0	0	0	0	0

Table 3. Summary of ichthyoplankton tows collected in Ohio River tributaries by the Kentucky Department of Fish and Wildlife Resources and Indiana Department of Natural Resources. An asterisk (*) denotes genetically confirmed *Hypophthalmichthys* samples analyzed by Whitney Genetics Lab.

Sampling Information				Suspicious Samples to WGL (N)		Suspect *Hypophthalmichthys* (N)		
Pool	**Location**	**Transect Type**	**Tows (N)**	**Eggs**	**Larvae**	**Eggs**	**Advanced Eggs**	**Larvae**
J.T. Myers	Green River	Tributary	6	0	0	0	0	0
Newburgh	Little Pigeon Creek	Tributary	6	0	4	0	0	~292,000*
Newburgh	Anderson River	Tributary	6	0	0	0	0	0
Cannelton	Deer Creek	Tributary	3	0	0	0	0	0
Cannelton	Salt River	Tributary	9	1	0	0	0	0
McAlpine	KY River Dam 1 Downstream	Tributary	6	3	10	2*	1	10*
McAlpine	KY River Dam 1 Upstream	Tributary	6	2	0	1*	0	0

Table 4. Location, date, and number of invasive carp collected in July and August, 2021, by Ball State University in Hovey Lake and the Hovey Lake drain.

Location	Date	Number
Drain	7-Jul	13
Lake	7-Jul	~200
Lake	12-Jul	5
Lake	15-Jul	~50
Lake	20-Jul	~200
Lake	26-Jul	29
Drain	26-Jul	2
Lake	2-Aug	~50
Drain	2-Aug	8
Lake	9-Aug	15
Lake	17-Aug	1
Lake	23-Aug	20
Drain	4-Sep	8
Lake	4-Sep	1

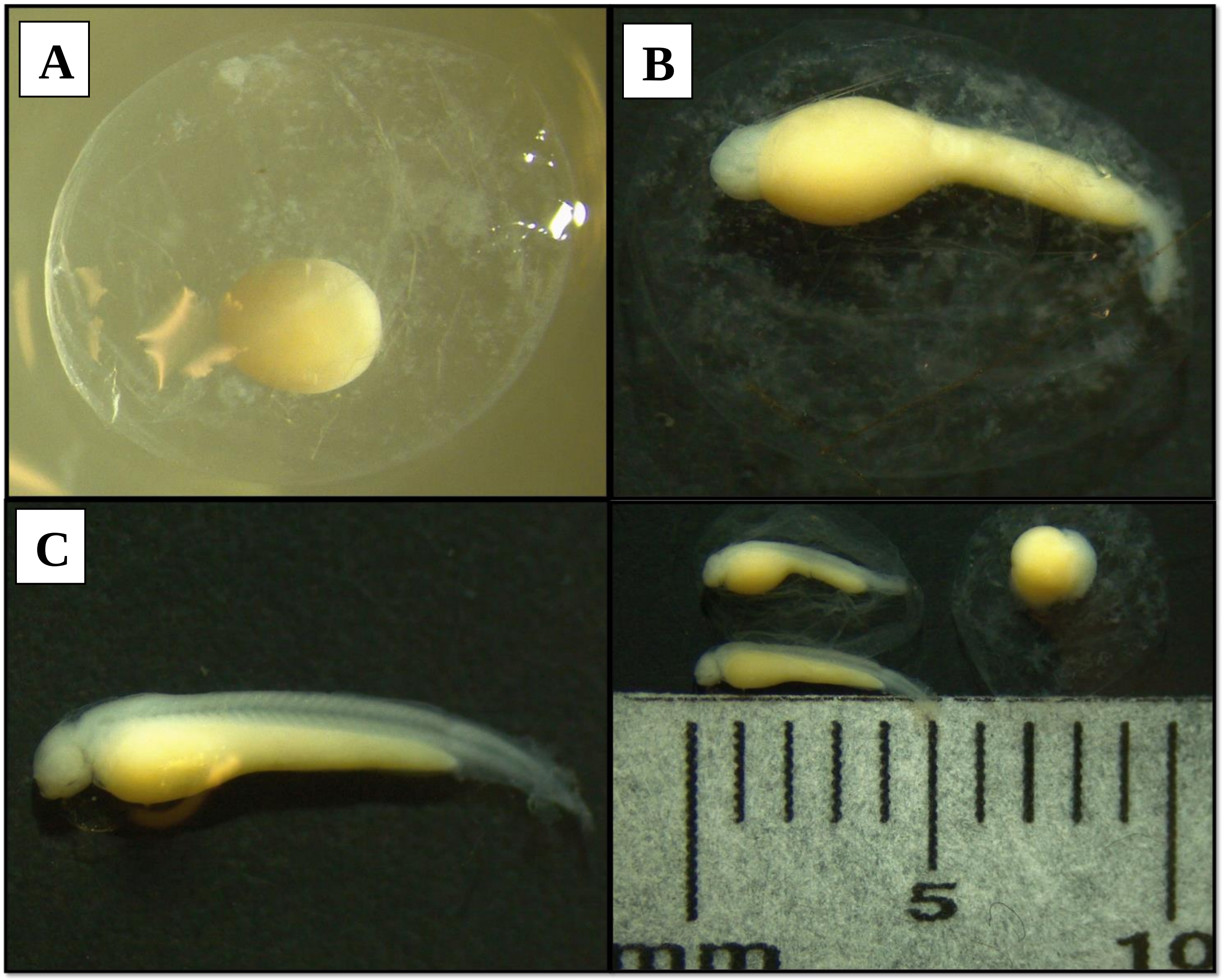

Figure 1. Developmental life stages of *Hypophthalmichthys* spp. with size comparisons. For the purposes of this report, pictures A, B, and C demonstrates specimens categorized as "eggs", "advanced eggs", and "larvae", respectively.

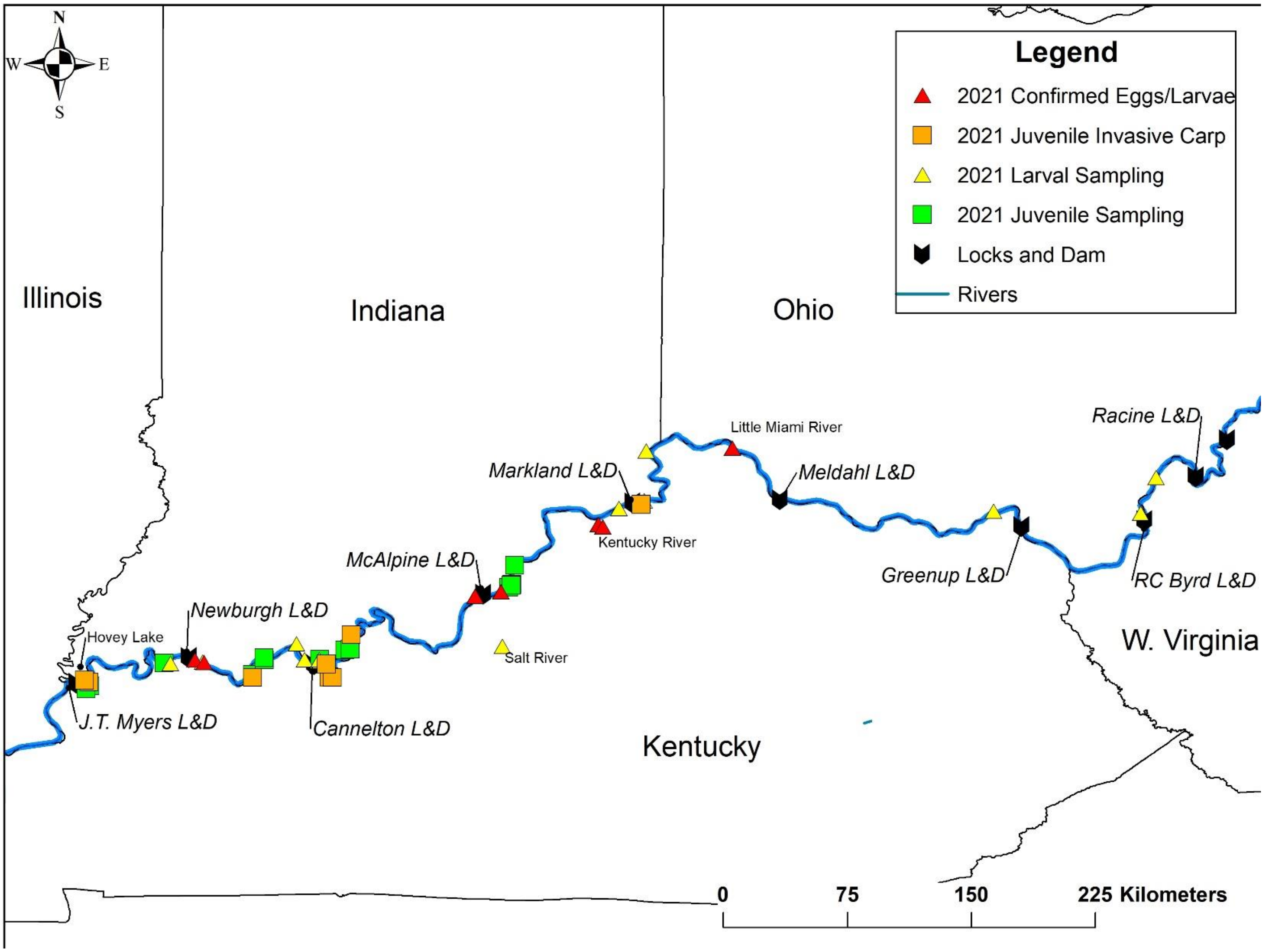

Figure 2. Map of study area including larval and targeted YOY sampling sites. Black icons denote a locks and dam, yellow triangles indicate larval sampling sites, red triangles indicate locations where genetically confirmed *Hypophthalmichthys* eggs, embryos, or larvae were collected. Green squares indicate locations where targeted YOY invasive carp sampling occurred, orange squares indicate locations where YOY invasive carp were collected.

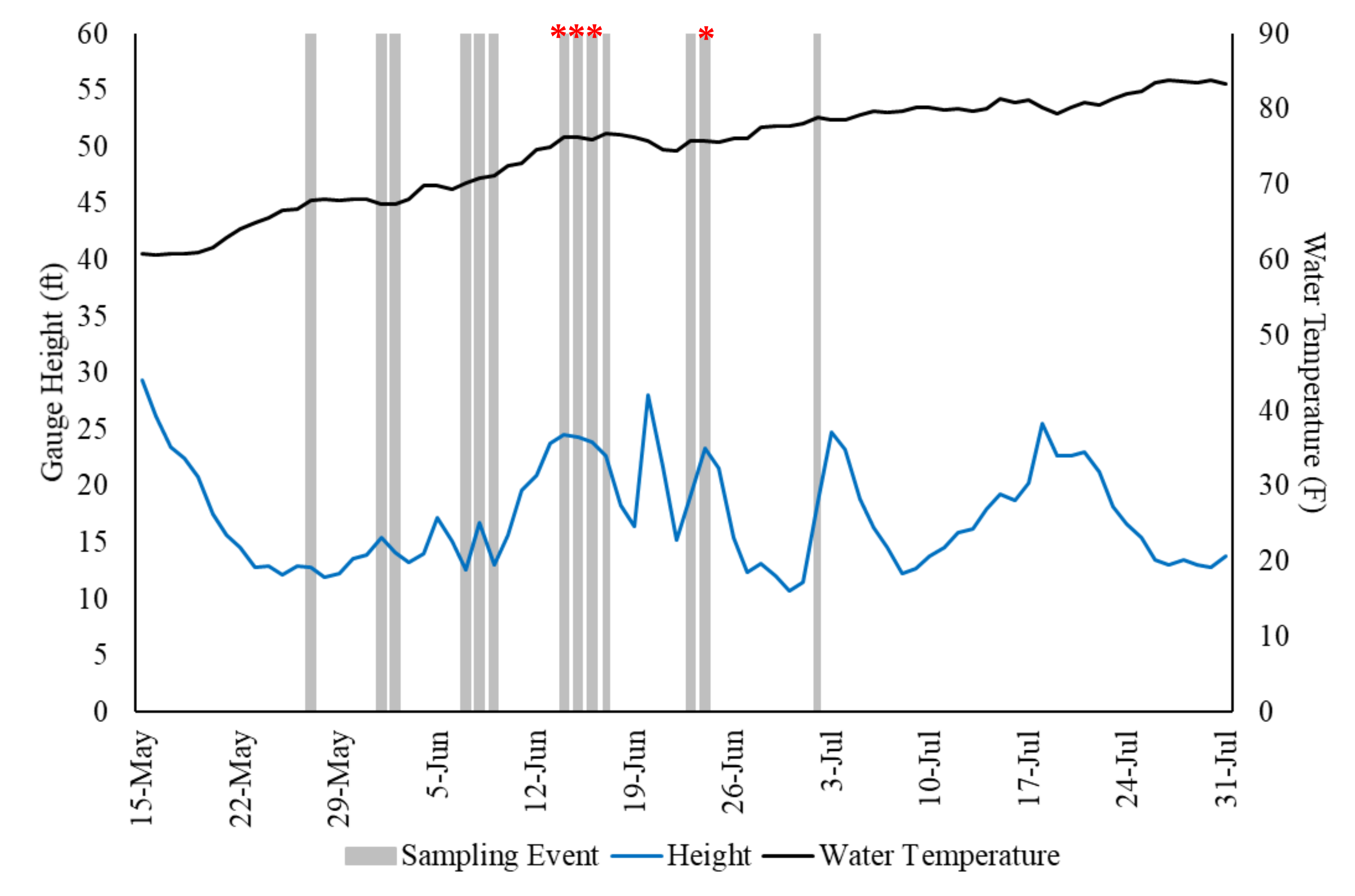

Figure 3. Larval sampling events across time in Newburgh, Cannelton, McAlpine, and Markland pools compared to environmental characteristics of the Ohio River. The red asterisk (*) indicates sampling events when genetically confirmed *Hypophthalmichthys* eggs or larvae were collected. Gauge height (ft) recorded by USGS below McAlpine Locks and Dam, and water temperature (F) is from the USGS gauge at Markland Locks and Dam.

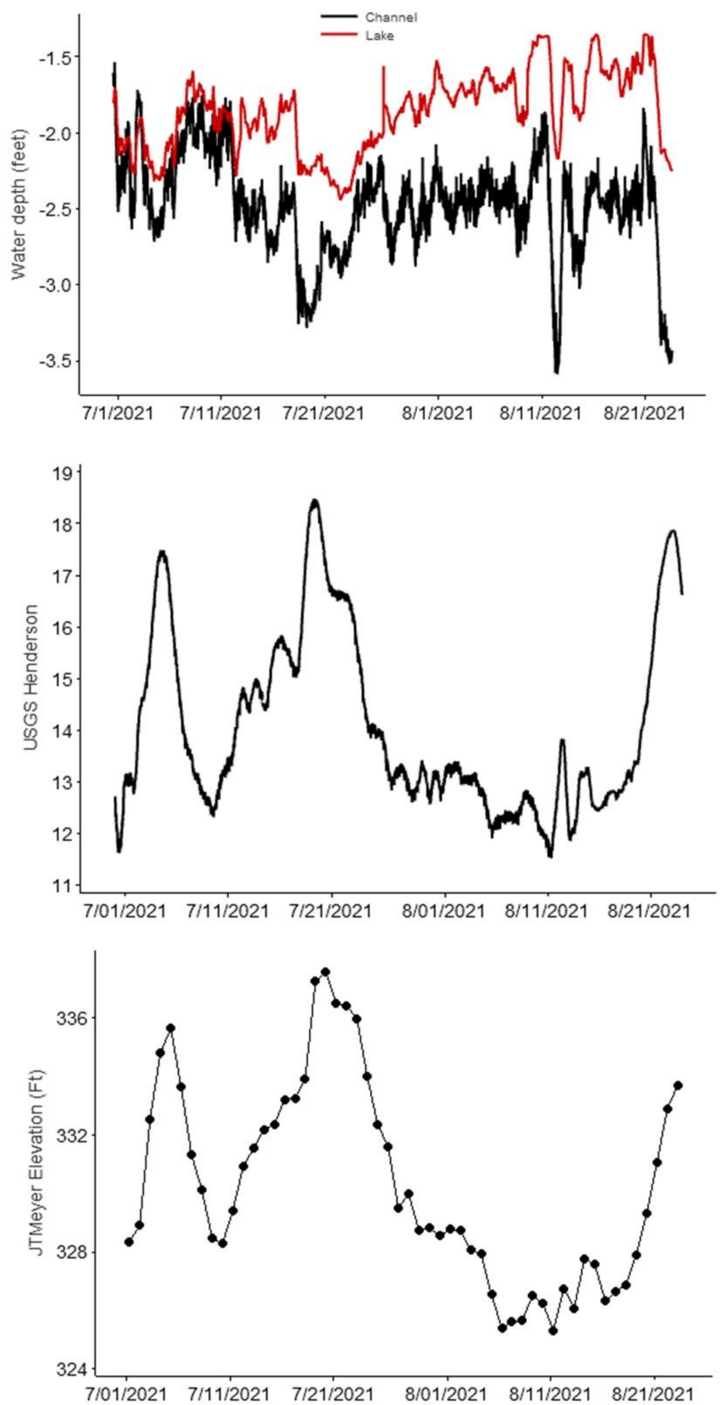

Figure 4 . Water levels for Hovey Lake and drain for July and August 2021, compared with water level of the Ohio River at the JT Meyer dam and the USGS gage at Henderson Kentucky.

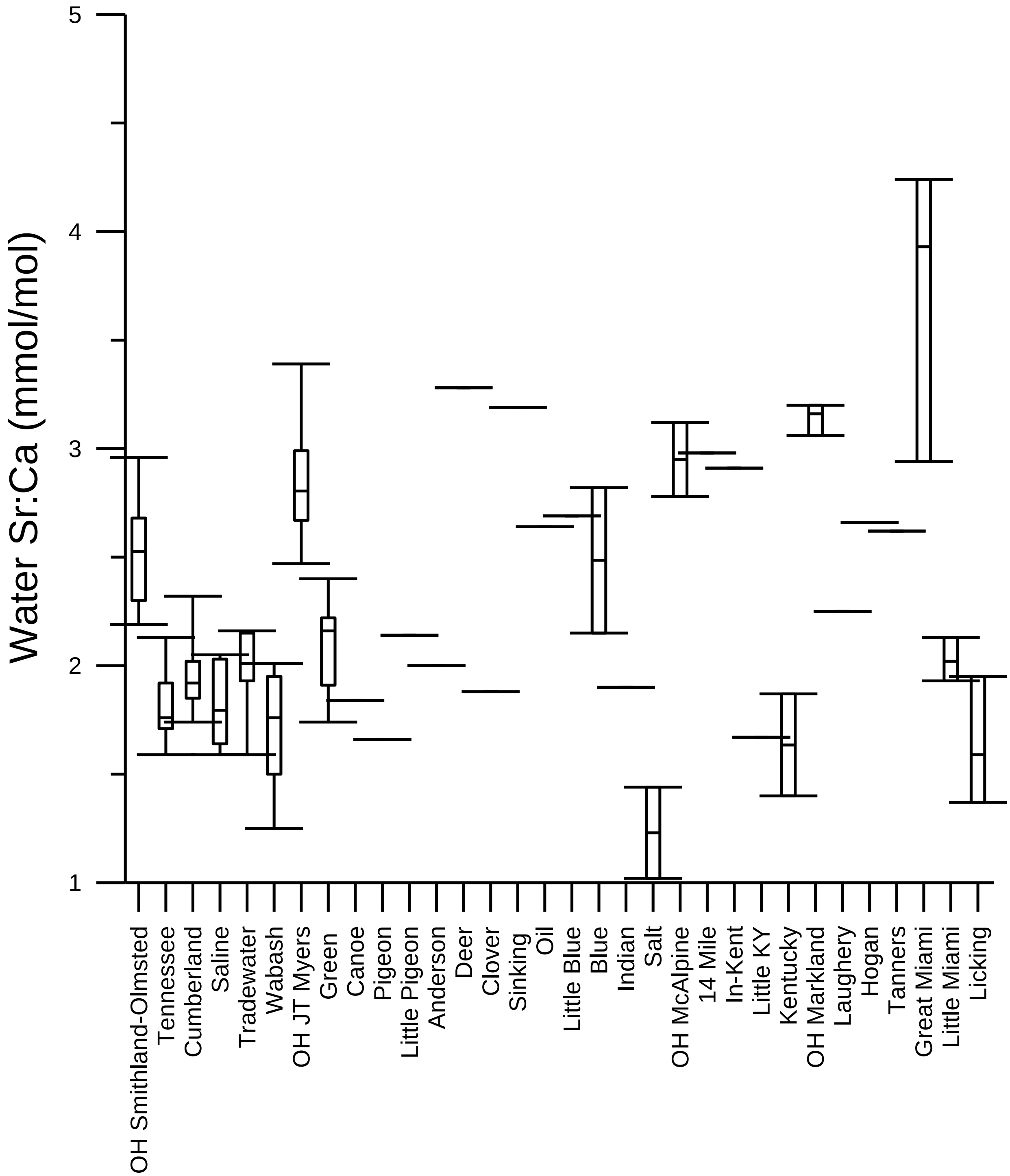

Figure 5. Boxplot of water Sr:Ca data from the Ohio River and tributaries that enter the river from Markland Pool and downstream, including data from 2021 water sample collections for this project and data from prior collections (when present).

Appendix A: Whitney Genetics Lab Report – 2021 Sampling.

WGL Report: March 11, 2022

Genetic Identification of Larval Fish

Indiana Department of Natural Resources
By: Zeb Woiak

Samples (n=88) were received on 12/8/22 at the Whitney Genetics Lab (WGL) by ZW from Craig Jansen. Samples were kept in a -20^{c} freezer until they could be processed by WGL lab staff.

Methods

We used our laboratory's standardized methods that are common in many core sequencing facilities. Samples were extracted using a modified Chelex-proteinase K method (Casquet et al. 2012) with a positive and negative control in the extraction batch. For DNA extraction procedures and all further analyses, clean laboratory practices and appropriate anti-contamination precautions were used.

Samples were sequenced first at the cytochrome c oxidase 1 gene (UCOI), which is commonly referred to as 'the barcode of life' (Ward et al. 2009) and has been sequenced for over 138,653 animal species specifically for the purpose of species-level identification. This gene was amplified with a cocktail of 4 primers that are universal to most fish species (Ivanova et al. 2007; Ward et al. 2005). A second assay was used to sequence the cytochrome b gene with a marker universal to all vertebrate species (UCYTB, Palumbi 1996). Amplification of UCOI and UCYTB were accomplished with the Platinum™ Green Hot Start PCR mix (Invitrogen™ Life Technologies, Carlsbad, CA) in 25-µl reactions, using primers (from references above) modified with M13 tags to streamline sequencing work. PCR products were cleaned up for sequencing with ExoSAP-IT® PCR Product Cleanup (Affymetrix, Santa Clara, CA) and then cycle sequenced in 1/16th BigDye Terminator v3.1 (Life Technologies, Carlsbad, CA) 20-µL reactions.

Clean-up of the sequences before analysis was done with BigDye Xterminator kits (Life Technologies) to remove un-incorporated bases. Sequence data was collected on an Applied Biosystems 3500XL Genetic Analyzer (Life Technologies). Sequences for each sample and each locus were edited by eye, trimmed, and aligned using Geneious DNA analysis software and compared to sequence data contained in GenBank using the Basic Local Alignment Search Tool (BLAST) for all sequences in NCBI GenBank. FASTA sequence files can be sent to you in a file which may be opened using Microsoft Notepad, if needed.

Results

50 of 88 samples successfully amplified at one or more genes (Table 1). 4 of the 50 samples could only be identified down to genus. This is a result of a lack of genetic diversity between the closely related species at the loci we analyzed with our markers. Samples with results for either one or both marker reactions, had control samples as expected, including the positive extraction and positive PCR controls. Extraction and PCR negative controls were clean, so there is no contamination issue. Sample failure may be due to low quality DNA or a failure in the sample processing. Please let me know if you have any questions or concerns.

References

Casquet J, C Thebaud, RG Gillespie. 2012. Chelex without boiling, a rapid and easy technique to obtain stable amplifiable DNA from small amounts of ethanol-stored spiders. Molecular Ecology Resources 12: 136–141.

Ivanova NV, TS Zemlak, RH Hanner, PDN Hebert. 2007. Universal primer cocktails for fish DNA barcoding. Molecular Ecology Resources 7:544-548.

Palumbi, SR. 1996. Nucleic acids II: the polymerase chain reaction. *In*: Hillis, DM, Moritz, C & Mable, BK, eds. Molecular systematics. Sinauer Publishing: Sunderland, MA, pp. 205-248.

Ward RD, TS Zemlak, BH Innes, PR Last, and PDN Hebert. 2005. DNA barcoding Austrailia's fish species. Philosophical Transactions of the Royal Society B: Biological Sciences 360:1847-1857.

Ward RD, R Hanner, PDN Hebert. 2009. The campaign to DNA barcode all fishes, FISH-BOL. Journal of Fish Biology 74:329-356

Table 1. Species identification results based on sequence data from cytochrome oxidase 1 (UCOI) and cytochrome b (UCYTB) mitochondrial loci. For each locus, percent sequence match is between the observed sequence and the reference sequence of the stated length (total base pairs) from the BLAST search. Table values for samples that failed to sequence were populated with "-".

	Universal COI				Universal CYTB				FINAL CALL	
Sample ID	% Match	Length (basepairs)	Accession Number	Species	% Match	Length (basepairs)	Accession Number	Species	Final ID	No. of Markers
1	-	-	-	-	-	-	-	-	-	-
2	99.7	654	MW649762	Hypophthalmichthys molitrix	99.6	730	MT002861	Hypophthalmichthys molitrix	Silver carp	2
3	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-
6	99.3	592	MW649762	Hypophthalmichthys molitrix	99.6	730	MT002861	Hypophthalmichthys molitrix	Silver carp	2
7	99.8	659	MW649762	Hypophthalmichthys molitrix	99.6	730	MT002861	Hypophthalmichthys molitrix	Silver carp	2
8	99.7	656	MW649762	Hypophthalmichthys molitrix	99.6	730	MT002867	Hypophthalmichthys molitrix	Silver carp	2
9	-	-	-	-	99.6	732	MT002861	Hypophthalmichthys molitrix	Silver carp	1
10	99.5	627	MW649762	Hypophthalmichthys molitrix	99.6	730	MT002867	Hypophthalmichthys molitrix	Silver carp	2
11	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-
15	99.5	633	MW649762	Hypophthalmichthys molitrix	99.6	730	MT002861	Hypophthalmichthys molitrix	Silver carp	2
16	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-	-	-

26	-	-	-	-	-	-	-	-	-	-
27	98.7	471	MT571861	Hypophthalmichthys molitrix	99.6	733	MT002861	Hypophthalmichthys molitrix	Silver carp	2
28	99.5	642	MT571861	Hypophthalmichthys molitrix	99.6	729	MT002867	Hypophthalmichthys molitrix	Silver carp	2
29	99.7	655	MW649762	Hypophthalmichthys molitrix	99.6	732	MT002861	Hypophthalmichthys molitrix	Silver carp	2
30	100	646	MT571861	Hypophthalmichthys molitrix	99.6	729	MT002861	Hypophthalmichthys molitrix	Silver carp	2
31	-	-	-	-	-	-	-	-	-	-
32	99.4/99.0	672/672	AY366087/NC_056404	Carpiodes carpio/Carpiodes cyprinus	99.2	733	JN053208	Carpiodes carpio	Carpoides spp.	2
33	99.5/99.1	658/658	AY366087/NC_056404	Carpiodes carpio/Carpiodes cyprinus	99.3	675	AB126083	Carpiodes carpio	Carpoides spp.	2
34	-	-	-	-	99.6	709	JN053185	Carpiodes carpio	River carpsucker	1
35	99.8	633	OL494288	Hypophthalmichthys nobilis	-	-	-	-	Bighead carp	1
36	99.8	630	OL494288	Hypophthalmichthys nobilis	-	-	-	-	Bighead carp	1
37	100	645	OL494271	Ctenopharyngodon idella	99.6	732	MN923201	Ctenopharyngodon idella	Grass carp	2
38	100	646	MW649762	Hypophthalmichthys molitrix	99.5	733	MT002867	Hypophthalmichthys molitrix	Silver carp	2
39	100	646	MW649762	Hypophthalmichthys molitrix	99.6	732	MT002867	Hypophthalmichthys molitrix	Silver carp	2
40	100	637	MW649762	Hypophthalmichthys molitrix	99.6	730	MT002861	Hypophthalmichthys molitrix	Silver carp	2
41	100	636	MW649762	Hypophthalmichthys molitrix	99.5	733	MT002861	Hypophthalmichthys molitrix	Silver carp	2
42	-	-	-	-	-	-	-	-	-	-
43	99.8	646	MT571705	Hypophthalmichthys nobilis	-	-	-	-	Bighead carp	1
44	-	-	-	-	-	-	-	-	-	-
45	98.5	668	KJ746963	Hypophthalmichthys nobilis	-	-	-	-	Bighead carp	1
46	99.5	655	MT571705	Hypophthalmichthys nobilis	-	-	-	-	Bighead carp	1
47	100	647	MW649762	Hypophthalmichthys molitrix	99.6	732	MT002861	Hypophthalmichthys molitrix	Silver carp	2
48	99.8	646	MT571705	Hypophthalmichthys nobilis	-	-	-	-	Bighead carp	1
49	99.1	447	OL494288	Hypophthalmichthys nobilis	-	-	-	-	Bighead carp	1
50	100	639	MT571861	Hypophthalmichthys molitrix	99.6	717	MT002861	Hypophthalmichthys molitrix	Silver carp	2
51	99.8	447	MT571705	Hypophthalmichthys nobilis	-	-	-	-	Bighead carp	1
52	99	381	MW341221	Hypophthalmichthys nobilis	-	-	-	-	Bighead carp	1
53	-	-	-	-	-	-	-	-	-	-

54	99.7	580	MH176328	Hypophthalmichthys nobilis	-	-	-	-	Bighead carp	1
55	100	636	OL494215	Hypophthalmichthys molitrix	99.6	732	MT002861	Hypophthalmichthys molitrix	Silver carp	2
56	99.4	641	KX145580	Macrhybopsis storeriana	-	-	-	-	Silver chub	1
57	-	-	-	-	-	-	-	-	-	-
58	-	-	-	-	99.6	729	MT002867	Hypophthalmichthys molitrix	Silver carp	1
59	-	-	-	-	-	-	-	-	-	-
60	99.8	643	MT571705	Hypophthalmichthys nobilis	-	-	-	-	Bighead carp	1
61	-	-	-	-	-	-	-	-	-	-
62	100	632	OL494289	Hypophthalmichthys nobilis	-	-	-	-	Bighead carp	1
63	99.8	637	OL494271	Ctenopharyngodon idella	99.6	724	MN923197	Ctenopharyngodon idella	Grass carp	2
64	99.2	479	MW649732	Ctenopharyngodon idella	99.6	730	MN923203	Ctenopharyngodon idella	Grass carp	2
65	99.8	552	AF544972	Ctenopharyngodon idella	99.6	722	MN923203	Ctenopharyngodon idella	Grass carp	2
66	99.7	667	MW649732	Ctenopharyngodon idella	99.6	732	MN923203	Ctenopharyngodon idella	Grass carp	2
67	99.7	642	MW649732	Ctenopharyngodon idella	99.6	732	MN923203	Ctenopharyngodon idella	Grass carp	2
68	-	-	-	-	99.6	720	MN923197	Ctenopharyngodon idella	Grass carp	1
69	-	-	-	-	99.6	730	MN923197	Ctenopharyngodon idella	Grass carp	1
70	-	-	-	-	99.6	716	MN923197	Ctenopharyngodon idella	Grass carp	1
71	100	633	OL494271	Ctenopharyngodon idella	99.6	726	MN923203	Ctenopharyngodon idella	Grass carp	2
72	-	-	-	-	-	-	-	-	-	-
73	100	658	MW649732	Ctenopharyngodon idella	99.6	733	MN923197	Ctenopharyngodon idella	Grass carp	2
74	99.6	541	MW649732	Ctenopharyngodon idella	99.6	730	MN923201	Ctenopharyngodon idella	Grass carp	2
75	-	-	-	-	-	-	-	-	-	-
76	-	-	-	-	-	-	-	-	-	-
77	-	-	-	-	-	-	-	-	-	-
78	-	-	-	-	-	-	-	-	-	-
79	-	-	-	-	-	-	-	-	-	-
80	-	-	-	-	-	-	-	-	-	-
81	-	-	-	-	-	-	-	-	-	-
82	-	-	-	-	-	-	-	-	-	-
83	100/99.7	641/641	KP306894/AP009316	Ictiobus cyprinellus/Ictiobus bubalus	99.5	732	FJ226283/FJ226259/FJ226364	Ictiobus niger/Ictiobus cyprinellus/Ictiobus bubalus	Buffalo spp.	2

84	-	-	-	-		-	-	-	-	-
85	99.7/99.3/98.7	573/573/600	JN026919/KX145263/AP009316	Ictiobus niger/Ictiobus cyprinellus/Ictiobus bubalus	99.3	716	FJ226283/FJ226364	Ictiobus niger/Ictiobus bubalus	Buffalo spp.	2
86	-	-	-	-	-	-	-	-	-	-
87	-	-	-	-	-	-	-	-	-	-
88	99.4	620	OL494229	Cyprinus carpio	100	732	MG570426	Cyprinus carpio	Common carp	2