Abundance and distribution of early life stages of Asian carp in the Ohio River: Service Award Number F18AP00793

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)

Statement of Need:

The negative effects of Silver (*Hypophthalmichthys molitrix)* and Bighead Carp (*H. nobilis)*, also known as Asian 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). Others may be indirect and difficult to quantify, such as economic loss and negative social perception. Research investigating factors that lead to Asian 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 Asian carp reproduction in terms of timing, location, and environmental conditions. Asian 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, recent evidence suggests that 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 Asian carp are extremely mobile and effective sampling methods are still being determined; various habitats may require sampling with different gear types (Collins et al. 2017). In addition, the factors that promote successful reproduction and recruitment remain uncertain. Identifying these factors is critical in suppressing the spread of these invasive fishes into novel environments.

Confirmed Asian carp spawning events have been reported in tributaries (i.e. Wabash River) and as far upstream as J.T. Myers Locks and Dam, and signs of spawning (i.e. spawning patches) have been observed as far upstream as the Markland Pool for Silver Carp and the Meldahl Pool for Bighead Carp. Successful reproduction of *Hypophthalmichthys* spp. was detected at river mile (RM) 560 (McAlpine Pool) in 2015, and further upstream at RM 405.7 (Meldahl Pool) in 2016 (EA engineering, personal communication). This defined the leading edge of spawning in the Ohio River (EA Engineering, personal communication). 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 Asian carp (Strategy 2.8) and also monitor upstream range expansion and changes in distribution and abundance (Strategy 2.3). Results of sampling prior to 2019 determined the extent of recruitment as below Cannelton Lock and Dam, with the majority of young-of-year (YOY) and juvenile detections below Newburgh Lock and Dam in J.T. Myers Pool (Jansen and Stump 2017, Roth 2018). Because of the availability of potential nursery areas in Cannelton Pool, we wanted to conduct additional targeted sampling there in 2019 to insure that our previous sampling in the pool was not missing YOY Asian carp. 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 Asian 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 carps (Strategy 3.6.2). Additionally, the results of this project will help managers make informed decisions during future planning efforts regarding resource allocation for Asian carp deterrent and control strategies.

2019 Project Objectives:

1) Determine the extent of Asian carp spawning activity in the Ohio River above Markland Dam.
2) Identify tributaries of Newburgh, Cannelton, and McAlpine pools in which spawning occurs.
3) Determine the extent of Asian carp recruitment in the Ohio River.
4) Identify characteristics of potential Asian carp nursery areas when juvenile Asian carp are encountered.

Project Highlights:

- As of 2016, *Hypophthalmichthys* spp. larvae were collected at river mile 405.7 (Meldahl Pool).
- Eight sites below Markland Lock and Dam (RM 532) were sampled via ichthyoplankton tows in May and June, 2019, to evaluate potential spawning within tributaries; nearly all *Hypophthalmichthys* larvae and Asian carp type eggs were captured within the Ohio River proper.
- Six sites from RM 497 to RM 260 were sampled via ichthyoplankton tows multiple times from May 29 to July 30, 2019; one suspect Asian carp larvae was identified from Meldahl Pool and will be sent off for genetic testing.
- Targeted YOY trawling and electrofishing efforts were consolidated to primarily focus on Cannelton Pool; no YOY Asian carp were captured in Cannelton Pool.
- One sampling event at Hovey Lake captured 298 YOY Asian carp, demonstrating the lake's continued importance for Asian carp recruitment in the Ohio River Basin.
- Results continue to indicate the majority of recruitment remains in J.T. Myers Pool, and previous sampling suggests limited recruitment in Cannelton and Newburgh Pools.

Methods:

For analysis purposes and for the remainder of this report, the phrase "Asian carp" will be referring to Silver and Bighead carps (*Hypophthalmichthys* spp.) only. In addition, both "YOY" and "immature" are collectively referring to "juvenile" Asian 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 Asian carp are defined as fish greater than 400 mm with mature, identifiable gonads.

Ichthyoplankton tows:
To evaluate the extent of Asian carp spawning activity in the Ohio River above the Markland Dam, ichthyoplankton tows were conducted at sampling sites within the RC Byrd (N = 1), Greenup (N = 1), Meldahl (N = 2), and Markland (N = 2) pools five times from May 29 – July 30, 2019 . At each sampling site four tows were conducted: one tow at each the right-descending, middle, and left-descending portions of the Ohio River, and one tow either within the tributary or at the intake structure if the site was a previous EA Engineering larval sampling site.

To identify specific tributaries below Markland Dam in which Asian carp spawning occurs, ichthyoplankton tows were conducted at tributaries within Newburgh (N = 2), Cannelton (N = 4) and McAlpine (N = 2) pools at least twice from May 28 – June 20, 2019 during ideal spawning conditions. At each sampling site five tows were conducted: two tows within the Ohio River proper above the confluence of the tributary, and three tows within the tributary upstream of any potential Ohio River influence (based on water color and clarity changes).

For all tows, a conical ichthyoplankton net (0.76 m, 500 μm mesh) was deployed from the front of the boat. The boat was motored in reverse, pulling the ichthyoplankton net upstream for 3 minutes. The water volume sampled was recorded using a General Oceanics Flowmeter fitted to the ichthyoplankton net, and 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% ethanol (at an estimated ratio of nine parts ethanol to one part sample volume) for genetic testing and physical identification in the lab.

After 24 to 48 hours of storage, ethanol was replaced. At this time, a 50 mL sample of the original ethanol preservative was poured into a centrifuge tube and stored in a deep freezer. Ethanol samples were then sent to Whitney Genetics Laboratory for eDNA analysis. For specific details on the eDNA methods employed by the Whitney Genetics Laboratory refer to Appendix. Larval fish were sorted into non-Asian carp and suspected Asian carp species using Auer (1982). Furthermore, early developmental characteristics outlined by Yi et al. (1998) and Chapman (2006) were utilized to physically identify suspected *Hypophthalmichthys* larvae, advanced eggs and eggs from each sample. Asian carp larvae were identified by the presence of an eye spot, and suspected *Hypophthalmichthys* were differentiated from Grass Carp (*Ctenopharyngodon idella*) using myomere counts. *Hypophthalmichthys* larvae have 38 – 39 myomeres, whereas Grass Carp larvae have around 45. Suspected *Hypophthalmichthys* eggs were identified based on general size and the presence of large gelatinous membrane 5 – 6 mm in diameter. Suspected *Hypophthalmichthys* 'advanced eggs' were defined as the beginning of a yolk-sack larvae still contained within the gelatinous membrane.

Surface trawl:
As the Cannelton Pool is the suspected leading edge of Asian carp recruitment in the Ohio River, surface trawling effort was focused on tributaries within Cannelton Pool (N = 14). From July 2 – August 14, 2019, at least three trawls were conducted in each of the following tributaries: Blue River, Clover Creek, Deer Creek, Indian Creek, Little Blue River, Millstone Creek, Mosquito Creek, Oil Creek, Otter Creek, Poison Creek, Salt River, Spring Creek, Wolf Creek and Yellowbank Creek. Additionally, four trawls were conducted on June 19, 2019 at Hovey Lake within the J.T. Myers Pool to confirm continued recruitment of Asian Carp in the lake.

The surface trawl was 3.7 m wide, 0.6 m tall, and 5.5 m deep with 31.8 mm bar number 12 netting. The purse had an additional layer of 4.8 mm mesh (35 pound delta) bag 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 of the front 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 approximately 5 minutes before retrieving the net. In some cases, only trawls shorter than 5 minutes were possible. At the biologist's discretion, additional trawls were conducted at sites where either coverage was limited or juvenile Asian carp were suspected. All Asian carp were identified to species, measured to total length, weighed and sexed when possible.

Electrofishing:
As the Cannelton Pool is the suspected leading edge of Asian carp recruitment in the Ohio River, electrofishing effort was focused on tributaries within Cannelton Pool (N = 3) in August 2019. Specific electrofishing settings were 80 pulses per second at 40% duty cycle, and volts were adjusted based on water conductivity to achieve standard power goals and maximize Asian carp collection. Dippers specifically targeted all fish resembling Asian carp. All Asian carp were then identified to species, measured to total length, weighed and sexed when possible.

Environmental variables:

A suite of habitat variables were collected at each electrofishing and surface trawl site including: water temperature, Secchi disk visibility, conductivity, pH, dissolved oxygen, maximum depth, average depth, tributary width, and presence/absence of woody debris and aquatic vegetation.

Results:

Ichthyoplankton tows:

A combined total of 112 ichthyoplankton tows were conducted within the RC Byrd (N = 20), Greenup (N = 20), Meldahl (N = 40), and Markland (N = 32) pools. Above Markland Locks and Dam, no suspected *Hypophthalmichthys* eggs or advanced eggs were physically identified. However, one suspected Asian carp larvae was identified above Markland Locks and Dam from a sample taken in the Ohio River adjacent to the Scioto River on May 29, 2019 (RM 356.5; Figure 1). The preliminary eDNA testing did not detect that sample as positive for *Hypophthalmichthys* DNA, therefore the suspected larvae was sent for genetic testing to confirm the identification.

A combined total of 75 ichthyoplankton tows were conducted within the Newburgh (N = 15), Cannelton (N = 40) and McAlpine (N = 20) pools. Physical identification was completed for all samples taken in the three pools. Suspected *Hypophthalmichthys* larvae, advanced eggs and eggs were identified in samples from Salt River, Anderson River, Clover Creek and Deer Creek (Figure 1). With the exception of Deer Creek, all positive physical identifications came from samples taken in the Ohio River and not within tributaries. Deer Creek tributary samples contained seven suspected *Hypophthalmichthys* larvae on June 20, 2019. In total, 68 suspected *Hypophthalmichthys* larvae, 47 suspected advanced eggs and 136 suspected eggs were physically identified (Tables 1 – 4). A subsample of suspected *Hypophthalmichthys* larvae from each positive sample will be sent for genetic testing to confirm the identification.

Interpretation of the eDNA results can be found in Appendix. The eDNA results found using the mitochondria markers ACTM1 and ACTM3 were similar, so the remainder of the results will be described using only ACTM1. There was no clear relationship between the eDNA results and the number of suspected *Hypophthalmichthys* larvae, advanced eggs or eggs physically identified in the corresponding sample (Tables 1 – 3). For example, the sample with the highest mean copies of DNA (IN0011; Table 1) had no suspected *Hypophthalmichthys* larvae, advanced eggs or eggs present. Additionally, the two farthest upstream samples (Salt River in Cannelton Pool, and Scioto River in Meldahl Pool) where suspect Asian carp larvae were captured, did not result in positive eDNA detections.

Surface trawl:

Among the 14 tributaries sampled in the Cannelton Pool, 74 surface trawls were conducted for a total of 6.15 hours of sampling effort. No YOY or juvenile Asian carp were collected. In the J.T. Myers Pool, 4 surface trawls were conducted at Hovey Lake for a total of 0.2 hours of sampling effort. The catch included 298 YOY *Hypophthalmichthys* spp., which were suspected Silver Carp but could only be confidently identified to genus. Mean catch per unit effort (CPUE; ± SE) for YOY *Hypophthalmichthys* spp. was 1,490 ± 1,119 fish/hour. Size ranged from 18 – 36 mm TL, and on average (± SE) was 28 ± 0.15 mm.

Electrofishing:

Electrofishing was conducted at three sites within Cannelton Pool for a total of 0.75 hours of electrofishing. No juvenile Asian carp were captured. A total of 26 adult Silver Carp were collected, ranging from 597 – 956 mm TL. The overall catch rate was 34.67 Silver Carp per hour of electrofishing. Total electrofishing effort was lower than planned due to unexpected control box issues.

Environmental variables:

This year's sampling focused on identifying which tributaries within Cannelton Pool, if any, support YOY Asian carp. As no YOY Asian carp were detected by either electrofishing or surface trawls within Cannelton Pool, further analysis of environmental variables was not conducted.

Discussion:

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

With the incorporation of a more thorough ichthyoplankton sampling design to this project, we were able to provide the most up-to-date delineation of the extent of Asian carp spawning within the Ohio River. In 2018, ichthyoplankton sampling indicated the presence of Asian Carp "type" larvae as far upstream as the Kentucky River (RM 546) in McAlpine Pool. At the time we were unable to confirm if these Asian Carp "type" larvae were *Hypophthalmichthys* spp. or Grass Carp. In the 2019 ichthyoplankton sampling, the farthest upstream suspected Asian carp larvae was obtained from a sample taken in the Ohio River near the confluence with the Scioto River (RM 356.5, Meldahl Pool). There was only one suspect Asian carp in this sample, and the eDNA analysis did not return a positive result. This suspect Asian carp larvae will be sent to Whitney Genetics Lab to confirm the species, and the results will be shared with the working group upon receipt. All other suspected *Hypophthalmichthys* larvae were collected below McAlpine lock and dam. These detections confirm the continued spawning of *Hypophthalmichthys* spp. in the Newburgh and Cannelton Pools, and genetic testing by Whitney Genetics Lab will confirm the species. In Cannelton Pool, the most upstream suspected *Hypophthalmichthys* larvae was obtained from a sample taken in the Ohio River upstream of the confluence with the Salt River (RM 630), indicating that spawning could likely be occurring at the Falls of the Ohio just immediately downstream of McAlpine Lock and Dam.

The majority of suspected *Hypophthalmichthys* larvae, advanced eggs and eggs were collected in samples taken on June 20, 2019 (Newburgh and Cannelton Pools). As various stages of development were all collected, several different spawning events likely took place one to three days prior to June 20th. Suspected Asian carp larvae were also obtained from samples taken on May 29, 2019 (Meldahl Pool) and June 11, 2019 (Cannelton Pool), highlighting the prolonged spawning season of Asian carp, and the variety of environmental conditions in which they can spawn (Figure 2).

Environmental DNA results were inconsistent with the counts of suspected *Hypophthalmichthys* larvae, advanced eggs and eggs identified by morphometric characteristics. While eDNA may be useful for some applications, it should not be used as a tool to determine which ichthyoplankton tow samples contain Asian carp larvae or eggs. The presence of adult Asian carp in the reaches we sampled may have influenced the eDNA results. The mean copies of DNA were generally higher in samples with suspected *Hypophthalmichthys* larvae, so in the future eDNA may be useful as a tool to prioritize which ichyoplankton tow samples to physically identify using morphometric characteristics.

Results from juvenile sampling (surface trawling and electrofishing) in 2019 confirm the upstream extent of recruitment as defined in previous years (2016 – 2018). In 2017, several immature Asian carp (269 – 399 mm TL) were captured in Cannelton Pool, suggesting the extent of recruitment to be above

Cannelton Locks and Dam. However, no juvenile Asian carp have been captured in Cannelton Pool during sampling for Basin Framework projects since, despite extensive effort. There are likely habitats within Cannelton Pool tributaries serving as juvenile Asian carp nursery areas that we cannot sample with our current gears. As in previous years, the majority of juvenile Asian carp were collected from Hovey Lake in J.T. Myers Pool.

There has not been a strong spawning event or year-class since this project was initiated in 2016. Based on the potential Asian carp spawning in Meldahl Pool, as highlighted by the ichthyoplankton data, 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 Asian 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 that promote the reproduction and recruitment of Asian 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. In addition to this project, INDNR biologists aided KDFWR with the "Early Detection and Evaluation of Asian carp Removal in the Ohio River", and "Control and Containment of Asian carp in the Ohio River" projects.

Recommendation:

While the extent of Asian carp recruitment has been relatively stable, we suspect recruitment within Cannelton Pool might be occurring more often than our sampling is detecting. The use of alternative methods (i.e. seine hauls, mini fyke nets, backpack shocking, light traps) should be evaluated for detecting the presence of YOY Asian carp in waters where our current gears cannot sample. However, as the focused efforts on Cannelton Pool in 2019 were not effective at identifying specific tributaries as Asian carp recruitment areas, future efforts should expand to new or additional locations. Specifically, efforts should expand to include multiple pools again to ensure recruitment in other areas is not missed. Additionally, as Hovey Lake appears to be an important component of Asian carp recruitment in the Ohio River, quantifying the exchange of Asian carp at all life stages between Hovey Lake and the Ohio River should be a priority moving forward.

We recommend continued ichthyoplankton tows above Markland Locks and Dam in 2020 to continue monitoring the extent of Asian carp spawning in the Ohio River. We also recommend conducting targeted ichthyoplankton tows during ideal spawning conditions in Newburgh, Cannelton, and McAlpine Pools to begin identifying specific spawning locations. Additionally, we recommend conducting ichthyoplankton tows immediately above and below dams on the Ohio River, and to evaluate the use of Falls of the Ohio below McAlpine Locks and Dam as a spawning location.

In the future, morphometric characteristics should be used to identify *Hypophthalmichthys* larvae and eggs, and genetic methods should be used to confirm and narrow the identification down to species. If resources allow, eDNA methods could be employed to prioritize which ichthyoplankton tow samples to pick for physical identification. Additionally, we suggest any field staff involved in the physical identification of *Hypophthalmichthys* larvae and eggs be trained on larval fish identification.

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

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Table 1. Comparison of positive eDNA samples and physical identification results from ichthyoplankton tows collected by the Indiana Department of Natural Resources. Environmental DNA testing was replicated 8 times per sample. For suspect *Hypophthalmichthys* counts, 'Larvae' contained the presence of an eye spot and 38 – 39 myomere counts, 'Eggs' are based on general size and presence of large gelatinous membrane 5-6 mm in diameter, and 'Advanced eggs' are an intermediate stage where the beginning of a yolk-sack larvae is apparent but is still contained within the gelatinous membrane.

Sampling information					eDNA results (ACTM1)			Suspect *Hypophthalmichthys* (N)		
ID #	Sample date	Pool	Location	Transect type	Positive replicates (N)	Mean DNA copies	SD DNA copies	Larvae	Advanced eggs	Eggs
IN0011	28-May	Newburgh	Anderson River	Ohio River	8	1833.97	306.72	0	0	0
IN0021	20-Jun	Cannelton	Deer Creek	Tributary	8	1206.41	239.99	6	0	0
IN0027	20-Jun	Cannelton	Clover Creek	Ohio River	8	613.31	126.41	12	10	23
IN0034	20-Jun	Newburgh	Anderson River	Ohio River	8	221.85	42.19	10	2	13
IN0013	28-May	Newburgh	Anderson River	Tributary	8	164.80	38.35	0	0	0
IN0035	20-Jun	Newburgh	Anderson River	Ohio River	8	159.29	23.75	9	6	21
IN0015	28-May	Newburgh	Anderson River	Tributary	8	138.06	16.55	0	0	0
IN0025	20-Jun	Cannelton	Deer Creek	Ohio River	8	113.39	31.97	9	11	36
IN0024	20-Jun	Cannelton	Deer Creek	Ohio River	8	109.57	20.41	5	5	8
IN0012	28-May	Newburgh	Anderson River	Ohio River	8	65.71	19.93	0	0	0
IN0018	28-May	Newburgh	Little Pigeon Creek	Tributary	8	45.85	12.64	0	0	0
IN0026	20-Jun	Cannelton	Clover Creek	Ohio River	8	38.91	10.91	16	13	35
IN0023	20-Jun	Cannelton	Deer Creek	Tributary	8	32.18	13.45	1	0	0
IN0017	28-May	Newburgh	Little Pigeon Creek	Ohio River	8	31.63	6.26	0	0	0
IN0008	28-May	Cannelton	Clover Creek	Tributary	8	6.50	3.22	0	0	0
IN0016	28-May	Newburgh	Little Pigeon Creek	Ohio River	7	4.67	2.95	0	0	0
IN0010	28-May	Cannelton	Clover Creek	Tributary	8	4.48	2.71	0	0	0
IN0031	20-Jun	Newburgh	Anderson River	Tributary	8	4.14	2.47	0	0	0
IN0032	20-Jun	Newburgh	Anderson River	Tributary	6	3.88	2.13	0	0	0
IN0002	28-May	Cannelton	Deer Creek	Ohio River	7	3.60	2.31	0	0	0
IN0014	28-May	Newburgh	Anderson River	Tributary	6	3.47	1.91	0	0	0
IN0030	20-Jun	Cannelton	Clover Creek	Tributary	7	3.39	1.79	0	0	0
IN0004	28-May	Cannelton	Deer Creek	Tributary	8	3.22	1.99	0	0	0
IN0009	28-May	Cannelton	Clover Creek	Tributary	7	3.17	1.76	0	0	0
IN0007	28-May	Cannelton	Clover Creek	Ohio River	8	2.64	1.17	0	0	0
IN0028	20-Jun	Cannelton	Clover Creek	Tributary	2	2.28	1.01	0	0	0
IN0020	28-May	Newburgh	Little Pigeon Creek	Tributary	7	2.04	1.25	0	0	0
IN0005	28-May	Cannelton	Deer Creek	Tributary	6	2.02	0.81	0	0	0
IN0003	28-May	Cannelton	Deer Creek	Tributary	8	1.89	1.22	0	0	0
IN0033	20-Jun	Newburgh	Anderson River	Tributary	5	1.71	1.16	0	0	0
IN0022	20-Jun	Cannelton	Deer Creek	Tributary	3	1.67	1.02	0	0	0
IN0006	28-May	Cannelton	Clover Creek	Ohio River	4	1.63	0.46	0	0	0
IN0019	28-May	Newburgh	Little Pigeon Creek	Tributary	6	1.41	0.85	0	0	0
IN0029	20-Jun	Cannelton	Clover Creek	Tributary	3	0.86	0.06	0	0	0
IN0001	28-May	Cannelton	Deer Creek	Ohio River	1	0.63	NA	0	0	0

Table 2. Comparison of positive eDNA samples and physical identification results from ichthyoplankton tows collected by the Kentucky Department of Fish and Wildlife Resources. Environmental DNA testing was replicated 8 times per sample. For suspect *Hypophthalmichthys* counts, 'Larvae' contained the presence of an eye spot and 38 – 39 myomere counts, 'Eggs' are based on general size and presence of large gelatinous membrane 5-6 mm in diameter, and 'Advanced eggs' are an intermediate stage where the beginning of a yolk-sack larvae is apparent but is still contained within the gelatinous membrane.

Sampling information					eDNA results (ACTM1)			Suspect *Hypophthalmichthys* (N)		
ID #	Sample date	Pool	Location	Transect type	Positive replicates (N)	Mean DNA copies	SD DNA copies	Larvae	Advanced eggs	Eggs
KY0017	29-May	McAlpine	Indian Kentuck Creek	Tributary	8	83.41	22.17	0	0	0
KY0012	29-May	McAlpine	Kentucky River	Tributary	8	79.49	20.24	0	0	0
KY0018	29-May	McAlpine	Indian Kentuck Creek	Tributary	8	68.52	19.26	0	0	0
KY0014	29-May	McAlpine	Kentucky River	Ohio River	8	66.58	11.71	0	0	0
KY0009	28-May	Cannelton	Blue River	Ohio River	8	54.80	18.38	0	0	0
KY0015	29-May	McAlpine	Kentucky River	Ohio River	8	37.00	8.32	0	0	0
KY0020	29-May	McAlpine	Indian Kentuck Creek	Ohio River	8	22.05	5.74	0	0	0
KY0011	29-May	McAlpine	Kentucky River	Tributary	8	18.66	3.34	0	0	0
KY0013	29-May	McAlpine	Kentucky River	Tributary	8	17.09	5.25	0	0	0
KY0016	29-May	McAlpine	Indian Kentuck Creek	Tributary	8	9.36	4.32	0	0	0
KY0001	28-May	Cannelton	Salt River	Tributary	8	9.29	4.07	0	0	0
KY0002	28-May	Cannelton	Salt River	Tributary	8	9.12	4.09	0	0	0
KY0019	29-May	McAlpine	Indian Kentuck Creek	Ohio River	7	7.86	4.23	0	0	0
KY0037	12-Jun	McAlpine	Kentucky River	Tributary	1	4.13	NA	0	0	0
KY0003	28-May	Cannelton	Salt River	Tributary	8	3.92	2.04	0	0	0
KY0024	11-Jun	Cannelton	Blue River	Ohio River	6	2.70	0.96	0	0	0
KY0027	11-Jun	Cannelton	Salt River	Tributary	1	2.50	NA	0	0	0
KY0026	11-Jun	Cannelton	Salt River	Tributary	2	2.29	1.58	0	0	0
KY0005	28-May	Cannelton	Salt River	Ohio River	1	2.24	NA	0	0	0
KY0034	12-Jun	McAlpine	Indian Kentuck Creek	Ohio River	4	2.16	0.65	0	0	0
KY0030	11-Jun	Cannelton	Salt River	Ohio River	3	2.08	0.79	0	0	0
KY0010	28-May	Cannelton	Blue River	Ohio River	5	2.02	0.95	0	0	0
KY0028	11-Jun	Cannelton	Salt River	Tributary	3	1.78	0.41	0	0	0
KY0023	11-Jun	Cannelton	Blue River	Tributary	1	1.71	NA	0	0	0
KY0006	28-May	Cannelton	Blue River	Tributary	2	1.16	0.07	0	0	0
KY0007	28-May	Cannelton	Blue River	Tributary	1	0.94	NA	0	0	0
KY0008	28-May	Cannelton	Blue River	Tributary	1	0.92	NA	0	0	0
KY0004	28-May	Cannelton	Salt River	Ohio River	1	0.77	NA	0	0	0
KY0033	12-Jun	McAlpine	Indian Kentuck Creek	Tributary	0	NA	NA	0	0	0
KY0029	11-Jun	Cannelton	Salt River	Ohio River	0	NA	NA	1	0	0

Table 3. Comparison of positive eDNA samples and physical identification results from ichthyoplankton tows collected by West Virginia University. Environmental DNA testing was replicated 8 times per sample. For suspect *Hypophthalmichthys* counts, 'Larvae' contained the presence of an eye spot and 38 – 39 myomere counts, 'Eggs' are based on general size and presence of large gelatinous membrane 5-6 mm in diameter, and 'Advanced eggs' are an intermediate stage where the beginning of a yolk-sack larvae is apparent but is still contained within the gelatinous membrane.

Sampling information					eDNA results (ACTM1)			Suspect *Hypophthalmichthys* (N)		
ID #	Sample date	Pool	Location	Transect type	Positive replicates (N)	Mean DNA copies	SD DNA copies	Larvae	Advanced eggs	Eggs
WVU76	17-Jul	Greenup	Guyandotte River	Ohio River	8	555.45	84.85	0	0	0
WVU72	17-Jul	RC Byrd	Kyger Creek Plant	Ohio River	8	210.51	39.03	0	0	0
WVU68	26-Jun	Markland	Hogan Creek	Ohio River	8	162.39	18.37	0	0	0
WVU54	25-Jun	Greenup	Guyandotte River	Ohio River	8	21.69	8.28	0	0	0
WVU89	17-Jul	Meldahl	JM Stuart Plant	At structure	8	21.18	7.73	0	0	0
WVU92	17-Jul	Meldahl	JM Stuart Plant	Ohio River	6	4.87	3.30	0	0	0
WVU83	18-Jul	Markland	Hogan Creek	Ohio River	6	3.73	1.45	0	0	0
WVU90	17-Jul	Meldahl	JM Stuart Plant	Ohio River	4	2.77	1.87	0	0	0
WVU53	25-Jun	Greenup	Guyandotte River	Ohio River	6	2.68	2.18	0	0	0
WVU69	17-Jul	RC Byrd	Kyger Creek Plant	At structure	3	2.53	0.14	0	0	0
WVU65	26-Jun	Markland	Hogan Creek	At structure	1	2.24	NA	0	0	0
WVU67	26-Jun	Markland	Hogan Creek	Ohio River	3	2.09	1.01	0	0	0
WVU75	17-Jul	Greenup	Guyandotte River	Ohio River	2	1.91	0.90	0	0	0
WVU80	17-Jul	Meldahl	Scioto River	Ohio River	2	1.83	1.05	0	0	0
WVU84	18-Jul	Markland	Hogan Creek	Ohio River	1	1.71	NA	0	0	0
WVU79	17-Jul	Meldahl	Scioto River	Ohio River	3	1.64	0.95	0	0	0
WVU66	26-Jun	Markland	Hogan Creek	Ohio River	3	1.49	0.46	0	0	0
WVU73	17-Jul	Greenup	Guyandotte River	At structure	7	1.49	0.85	0	0	0
WVU77	17-Jul	Meldahl	Scioto River	At structure	1	1.38	NA	0	0	0
WVU71	17-Jul	RC Byrd	Kyger Creek Plant	Ohio River	3	1.27	0.21	0	0	0
WVU82	18-Jul	Markland	Hogan Creek	Ohio River	6	1.18	0.23	0	0	0
WVU62	25-Jun	Meldahl	JM Stuart Plant	Ohio River	1	1.16	NA	0	0	0
WVU70	17-Jul	RC Byrd	Kyger Creek Plant	Ohio River	3	1.12	0.38	0	0	0
WVU61	25-Jun	Meldahl	JM Stuart Plant	Ohio River	1	1.11	NA	0	0	0
WVU91	17-Jul	Meldahl	JM Stuart Plant	Ohio River	3	1.11	0.36	0	0	0
WVU63	25-Jun	Meldahl	JM Stuart Plant	Ohio River	2	1.09	0.71	0	0	0
WVU52	25-Jun	RC Byrd	Kyger Creek Plant	Ohio River	1	1	NA	0	0	0
WVU74	17-Jul	Greenup	Guyandotte River	Ohio River	3	0.79	0.51	0	0	0
WVU09	29-May	Meldahl	Scioto River	Ohio River	0	NA	NA	1	0	0

Table 4. Summary of positive eDNA replicates for both mitochondrial markers (ACTM1 and ACTM3), and counts of suspected *Hypophthalmichthys* larvae, advanced eggs and eggs by pool. Environmental DNA testing was replicated 8 times per sample. For suspect *Hypophthalmichthys* counts, 'Larvae' contained the presence of an eye spot and 38 – 39 myomere counts, 'Eggs' are based on general size and presence of large gelatinous membrane 5-6 mm in diameter, and 'Advanced eggs' are an intermediate stage where the beginning of a yolk-sack larvae is apparent but is still contained within the gelatinous membrane. Pools are listed from most downstream to most upstream.

		eDNA results			Suspect *Hypophthalmichthys* (N)		
Pool	Samples (N)	Total Replicates (N)	Percent positive replicates (ACTM1)	Percent positive replicates (ACTM3)	Larvae	Advanced eggs	Eggs
Newburgh	15	120	91%	91%	19	8	34
Cannelton	40	320	58%	60%	50	39	102
McAlpine	20	160	53%	54%	0	0	0
Markland	32	256	11%	11%	0	0	0
Meldahl	40	320	10%	10%	1	0	0
Greenup	20	160	21%	21%	0	0	0
RC Byrd	20	160	11%	11%	0	0	0

Figure 1. Map of study area including larval sampling sites. Red circles indicate locations where suspect *Hypophthalmichthys* larvae were captured, green square indicates locations where suspect Asian carp species was captured (pending genetic validation), and yellow triangles indicate sampled sites where no suspect Asian carp larvae were captured.

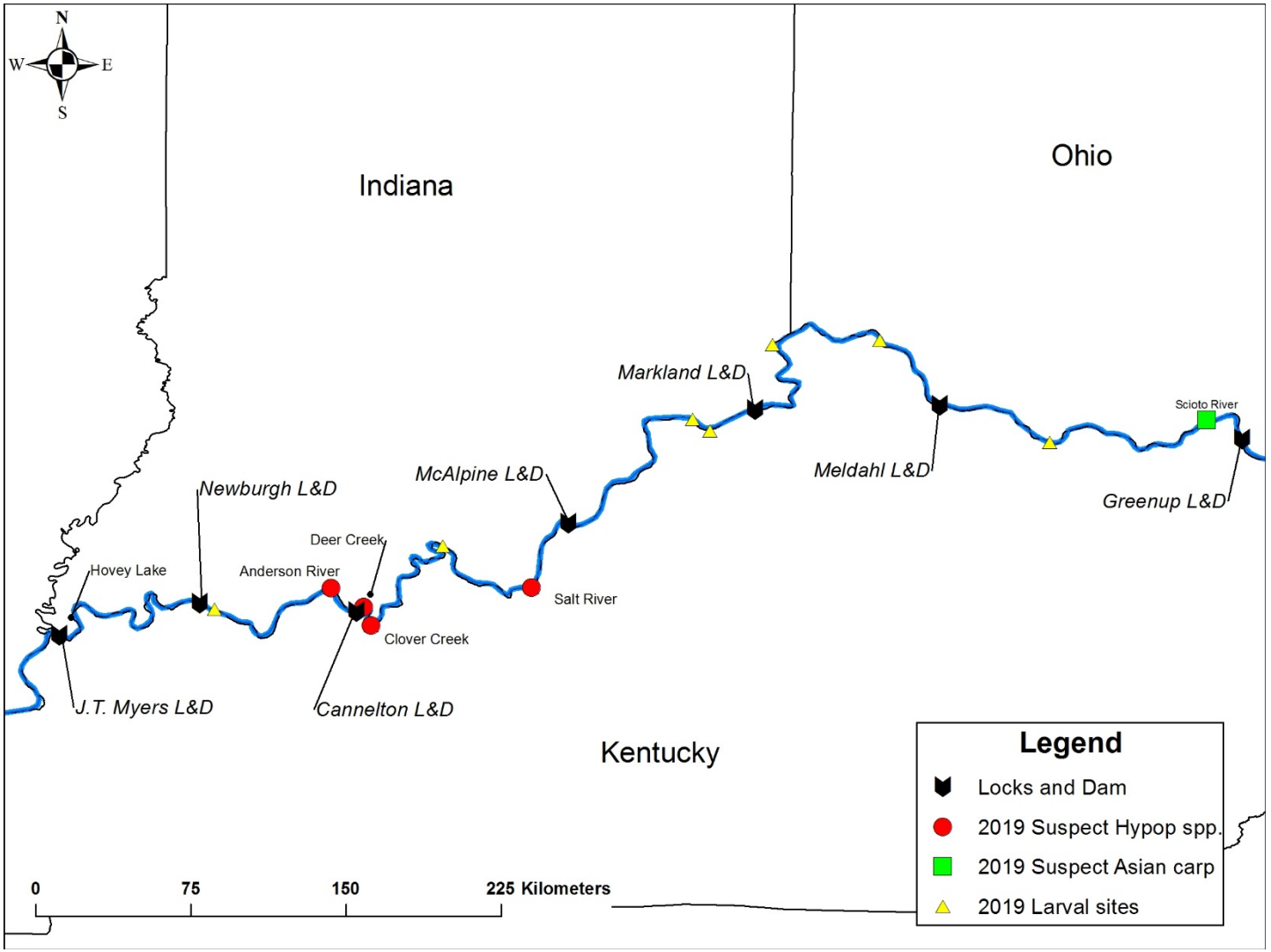

Figure 2. Timing of sampling events in Newburgh, Cannelton and McAlpine Pools in comparison to environmental characteristics of the Ohio River. One suspected *Hypophthalmichthys* larvae was collected on June 11, 2019 from the Ohio River upstream of the confluence with the Salt River. All other suspected *Hypophthalmichthys* larvae, advanced eggs and eggs were from samples taken on June 20, 2019 in multiple locations in Newburgh and Cannelton Pools. Gage height (ft) is from the USGS gauge below McAlpine Locks and Dam, and water temperature (F) is from the USGS gauge at Markland Locks and Dam.

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Screening Ethanol Used to Preserve Larval Tow Samples for Bigheaded Carps DNA

Case 10002 - Craig Jansen, Indiana DNR

Whitney Genetics Laboratory Report

By: Zeb Woiak

Samples (n=115) were received on 28 August 2019 at the Whitney Genetics Lab (WGL) by Zeb Woiak from Craig Jansen and assigned to case 10002. Samples were kept at -20°C until they could be processed by WGL lab staff.

Methods:

We used our laboratory's ethanol processing method (using all 50 ml of ethanol; modified from Fritts et al. 2018) which includes clean laboratory practices and appropriate contamination precautions. Samples were spun in a refrigerated centrifuge at 4200 rpm for 30 minutes, ethanol was decanted, and the pellet in the bottom of sample tubes were dried. Once samples were dried they were stored at -80°C until WGL staff could continue processing them. Samples were extracted using the gMax mini genomic DNA extraction (IBI Scientific; Peosta, IA, USA) with a positive (bluegill DNA) and negative control in each extraction batch. Quantitative polymerase chain reaction (qPCR) was done using two general Asian carp markers that detect two regions within the cytochrome oxidase I gene of both Silver and Bighead carp (collectively known as Bigheaded carps) mitochondria (ACTM 1 and ACTM 3; QAPP). Eight sample replicates for each loci were done per sample and any sample that had at least one PCR replicate test positive for either ACTM 1 or ACTM 3 was considered positive for Bigheaded carp DNA. Data were checked by eye and processed using Bio-Rad CFX manager software (version 3.1) with default settings and exported for further analysis in the R statistical package (version 3.3.2; R Core Team 2016). Minimum standard curve criteria (per QAPP) and expected control sample results were confirmed.

Results and Discussion:

Bigheaded carp DNA was detected in 92 of 115 samples (Tables 1-3). The ACTM 1 and 3 assay has a detection limit (LOD) of 1 copy/rxn and a limit of quantification (LOQ) of 10 copies of DNA/rxn. Because the lowest copy number with over 95% detection probability on the 5

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point standard curve used is 10 copies, the ACTM 1 & 3 assays cannot reliably quantify the starting copy number of DNA for sample replicates that resulted in less than 10 copies/rxn. Because DNA has to be present for the ACTM 1 & 3 qPCR reaction to occur, the detection down to one copy is reliable and indicates DNA was present (Tables 1-3). Regardless of DNA copy numbers quantified by the qPCR, detection of any Bigheaded carp DNA is considered a positive result; the number of positive replicates in a sample can be considered a measure of signal strength. Samples with more positive replicates are likely to have a higher concentration of source mitochondrial material.

References

Fritts, A.K., Knights, B.C., Larson, J.H. et al. 2018. Development of a quantitative PCR method for screening ichthyoplankton samples for bigheaded carps. Biological Invasions. https://doi.org/10.1007/s10530-018-1887-9

R Core Team (2016) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

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Table 1. IN samples with positive detections for Bigheaded carp DNA, negative samples are not presented. Positive reactions with at least one out of eight replicate qPCR assays include reactions with results above the limit of detection (LOD) of one copy of DNA (* denotes samples with mean starting DNA concentrations below the LOD). Mean and standard deviation of the starting DNA concentration per positive replicate are also included.

Sample	Total number of positive replicate rxns ACTM 1	Mean DNA Copies	Standard Deviation	Total number of positive replicate rxns ACTM 3	Mean DNA Copies	Standard Deviation
IN1*	1	0.63	-	2	0.48	0.26
IN2	7	3.60	2.31	7	3.64	2.22
IN3	8	1.89	1.22	8	1.94	1.21
IN4	8	3.22	1.99	8	3.19	1.88
IN5	6	2.02	0.81	6	2.16	0.91
IN6	4	1.63	0.46	4	1.67	0.53
IN7	8	2.64	1.17	8	2.64	1.08
IN8	8	6.50	3.22	8	6.28	3.05
IN9	7	3.17	1.76	7	3.14	1.69
IN10	8	4.48	2.71	8	4.70	2.70
IN11	8	1833.97	306.72	8	1961.01	313.50
IN12	8	65.71	19.93	8	68.95	19.97
IN13	8	164.80	38.35	8	168.46	41.48
IN14	6	3.47	1.91	6	3.42	1.95
IN15	8	138.06	16.55	8	147.41	17.14
IN16	7	4.67	2.95	7	4.68	2.84
IN17	8	31.63	6.26	8	32.00	4.92
IN18	8	45.85	12.64	8	47.74	12.46
IN19	6	1.41	0.85	6	1.43	0.92
IN20	7	2.04	1.25	7	2.28	1.52
IN21	8	1206.41	239.99	8	1257.26	211.33
IN22	3	1.67	1.02	3	1.75	1.14
IN23	8	32.18	13.45	8	33.64	13.83
IN24	8	109.57	20.41	8	116.91	16.76
IN25	8	113.39	31.97	8	123.45	30.44
IN26	8	38.91	10.91	8	41.86	12.30
IN27	8	613.31	126.41	8	654.69	115.34
IN28	2	2.28	1.01	2	2.48	1.25
IN29*	3	0.86	0.06	4	0.79	0.28
IN30	7	3.39	1.79	7	3.65	1.90
IN31	8	4.14	2.47	8	4.35	2.51
IN32	6	3.88	2.13	6	4.19	2.36
IN33	5	1.71	1.16	5	1.82	1.20
IN34	8	221.85	42.19	8	239.04	35.31
IN35	8	159.29	23.75	8	162.28	22.85

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Table 2. KY samples with positive detections for Bigheaded carp DNA, negative samples are not presented. Positive reactions with at least one out of eight replicate qPCR assays include reactions with results above the limit of detection (LOD) of one copy of DNA (* denotes samples with mean starting DNA concentrations below the LOD). Mean and standard deviation of the starting DNA concentration per positive replicate are also included.

Sample	Total number of positive replicate rxns ACTM 1	Mean DNA Copies	Standard Deviation	Total number of positive replicate rxns ACTM 3	Mean DNA Copies	Standard Deviation
KY1	8	9.29	4.07	8	9.40	4.33
KY2	8	9.12	4.09	8	8.87	3.79
KY3	8	3.92	2.04	8	3.97	2.07
KY4*	1	0.77	-	1	0.80	-
KY5	1	2.24	-	3	1.06	1.04
KY6	2	1.16	0.07	2	1.19	0.07
KY7*	1	0.94	-	2	0.83	0.33
KY8*	1	0.92	-	1	0.97	-
KY9	8	54.80	18.38	8	55.95	16.80
KY10	5	2.02	0.95	5	1.96	0.93
KY11	8	18.66	3.34	8	19.35	3.66
KY12	8	79.49	20.24	8	73.45	20.26
KY13	8	17.09	5.25	8	16.93	5.76
KY14	8	66.58	11.71	8	61.42	10.35
KY15	8	37.00	8.32	8	36.78	8.83
KY16	8	9.36	4.32	8	9.11	4.39
KY17	8	83.41	22.17	8	80.12	21.48
KY18	8	68.52	19.26	8	61.93	18.74
KY19	7	7.86	4.23	8	7.00	4.67
KY20	8	22.05	5.74	8	21.08	5.51
KY23	1	1.71	-	1	1.72	-
KY24	6	2.70	0.96	7	2.39	1.23
KY26	2	2.29	1.58	2	2.14	1.38
KY27	1	2.50	-	1	2.65	-
KY28	3	1.78	0.41	3	1.86	0.44
KY30	3	2.08	0.79	3	1.99	0.72
KY33*	0	-	-	1	0.60	-
KY34	4	2.16	0.65	4	2.22	0.74
KY37	1	4.13	-	2	2.24	2.65

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Table 3. WVU samples with positive detections for Bigheaded carp DNA, negative samples are not presented. Positive reactions with at least one out of eight replicate qPCR assays include reactions with results above the limit of detection (LOD) of one copy of DNA (* denotes samples with mean starting DNA concentrations below the LOD). Mean and standard deviation of the starting DNA concentration per positive replicate are also included.

Sample	Total number of positive replicate rxns ACTM 1	Mean DNA Copies	Standard Deviation	Total number of positive replicate rxns ACTM 3	Mean DNA Copies	Standard Deviation
WVU52	1	1.00	NA	1	1.04	NA
WVU53	6	2.68	2.18	6	3.17	2.78
WVU54	8	21.69	8.28	8	23.67	8.57
WVU61	1	1.11	NA	1	1.24	NA
WVU62	1	1.16	NA	1	1.33	NA
WVU63	2	1.09	0.71	2	1.13	0.67
WVU65	1	2.24	NA	1	2.54	NA
WVU66	3	1.49	0.46	3	1.64	0.63
WVU67	3	2.09	1.01	3	2.22	1.09
WVU68	8	162.39	18.37	8	161.97	16.74
WVU69	3	2.53	0.14	3	2.64	0.22
WVU70	3	1.12	0.38	3	1.32	0.29
WVU71	3	1.27	0.21	3	1.32	0.20
WVU72	8	210.51	39.03	8	219.06	37.27
WVU73	7	1.49	0.85	7	1.65	0.92
WVU74*	3	0.79	0.51	3	0.86	0.57
WVU75	2	1.91	0.90	2	2.00	0.87
WVU76	8	555.45	84.85	8	598.27	61.74