Title: Development of biometric and environmental DNA standardized protocols for early detection and population assessment of aquatic invasive species for Arthur R. Marshall Loxahatchee NWR.

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***Executive Summary*—** Our project proposed to integrate standard fisheries data collection techniques to support inventory and monitoring programs for fish communities, including aquatic invasive species (AIS) of fishes, within National Wildlife Refuge (NWR) waters. We partnered with the Arthur R. Marshall Loxahatchee NWR (Loxahatchee NWR) for this pilot project. We designated geographically explicit sampling sites, sampled the fish community, analyzed data on fish species composition and biometrics including aquatic invasive species of fishes, provided a sub-study on largemouth bass which was a target species, and integrated molecular tools for validating species identification, and used environmental DNA (eDNA) for species detection.

Our first objective was accomplished. We described the fish community based on the results of boat electrofishing. Surveys were conducted at 102 sites in canals of Loxahatchee NWR between October 2011 and October 2012. These samples consisted of 15,440 individuals representing 34 species of fish. We found differences between seasons (i.e., October 2011, April 2012, and October 2012) and bank side sampled (i.e., Levee and Interior Marsh). These differences were found for species, number of individuals, and biomass. The metrics used for species richness were consistent and all in the good range, but had a declining trend. Non-native fish species comprised 15 percent of the fish community (i.e., five non-native species of the total 34 species). We identified three of these species as target species. Of the three target species, the bullseye snakehead (n=1 individual) and Mayan cichlid (n=2 individuals) were confirmed within the refuge's waters. We did not collect African jewelfish, which was the third target non-native fish species. The largemouth bass study determined that individuals ranged in total length (mm) 54-610 (mean= 268.3375, std.=95.21484) and standard length (mm) ranged from 53-530 (mean= 221.5588, std.= 81.23927). The total biomass for largemouth bass was 1183.05 kg. The mean CPUE was 47.38 LMB/ hour. Proportional Stock Density was balanced with a score of 43. Relative Stock Density was 14. There were two trophy size individuals (e.g., >600 mm total length).

We used molecular techniques to confirm the identification of fish species. We based preliminary species identification on field morphological traits and then challenged these identifications. A total of 106 tissue samples were analyzed. Thirty one of 105 (30%) sequences were verified by Barcode of Life Data System (BOLD). While BOLD categorized the remaining 74 sequences as unvalidated, they had a high (often >95%) sequence similarity to the field (morphological) species identification. We believe this technique is an important tool to consider for documenting the fish species present in National Wildlife Refuge waters.

The second objective was accomplished. We selected two AIS fishes, African jewelfish and Bullseye snakehead, and from aligned sequences for each species, specific mitochondrial cytochrome c oxidase I (*COI*) primers were developed. Primers AJFF3 and PROS2 amplified a 240 nt *COI* segment in African jewelfish. We used this 240 nucleotides (nt) segment of African jewelfish to develop primers AJFq3 and AJFR2Q2 along with probe PCOAJF6 (Table 1). For Bullseye snakehead, specific primers CMnewF1 and FishR1 amplified a 439 nt segment of *COI* from which primers FCM2 and Rcomp2C and probe P2CMCO1 were developed. Using serial dilutions of known amounts of DNA, we found that the lower limit of eDNA detection for African jewelfish was approximately 0.0002 ng/µL (R^2 = 0.89) at a PCR cycling threshold of

28.5-29 amplification cycles and the lower limit for Bullseye snakehead was approximately 0.005 ng/μL (R^2 = 0.94) at a cycling threshold of 22-23 amplification cycles.

Figure.1. Arthur R. Marshall Loxahatchee National Wildlife Refuge sign.

Introduction— The Arthur R. Marshall Loxahatchee National Wildlife Refuge (Loxahatchee Refuge) is located in Palm Beach County, Florida, which is the largest county east of the Mississippi River in terms of land area and is the largest agriculture producing county in the east in terms of dollar value. The Everglades Agricultural Area, which includes large sugar cane plantations, winter vegetables, sod farms, and cattle ranches, is located to the north and west. Rapidly expanding urban communities are home to nearly six million people, which live within two hours of the refuge (USFWS 2002).

In the 1940s, three water storage areas called Water Conservation Areas 1, 2, and 3 were constructed by the U.S. Army Corps of Engineers. This was accomplished through the construction of drainage canals and levees. Water Conservation Area 1 (WCA-1) is owned by the State of Florida and South Florida Water Management District, but managed by the U.S. Fish and Wildlife Service (USFWS or Service) as Loxahatchee NWR, a unit of the National Wildlife Refuge System. The remainder of the central and southern Everglades (WCA 2 and 3, and Everglades National Park) is located to the south (USFWS 2002).

In 1951, a license agreement between the State of Florida and the USFWS , under the authority of the Migratory Bird Conservation Act, enabled the establishment of the 143,238-acre Loxahatchee NWR in WCA-1. The license agreement was later amended to include the 1,604-acre Strazulla Marsh, which lies adjacent to WCA-1 on the northeast side. In addition to the licensed lands, the Service owns 2,550 acres to the east and west of the refuge interior. This acreage is sub-divided into four management compartments (A, B, C, D) and the 400 acre Cypress Swamp (USFWS 2002).

Figure.2. View of perimeter canal at Arthur R. Marshall Loxahatchee NWR

In total, the refuge currently includes 147,392 acres of northern Everglades habitat. In 1986, the refuge's name was changed from Loxahatchee National Wildlife Refuge to the Arthur R. Marshall Loxahatchee National Wildlife Refuge to honor former U.S. Fish and Wildlife Service employee and noted South Florida conservationist, Arthur Raymond Marshall (USFWS 2002).

As previously mentioned, Arthur R. Marshall Loxahatchee National Wildlife Refuge is surrounded by a high density of roads, canal systems, large urban center, and intensive agriculture. Canals within the Everglades ecosystem, including Loxahatchee NWR, disrupt natural sheet flow and species movement. Land use practices which are not properly conducted could result in alterations in hydrology, increased rates of erosion, impact species fitness, and provide a pathway for the introduction of nonindigenous/ non-native species. Reduced connectivity within a system can be the result of many structures. Roadways and levees disrupt contiguousness of riparian areas, are conduits for sediments and other pollutants, and in some instances create barriers to fish movements and fragment populations. In addition to the roadways and levees, Loxahatchee NWR receives agriculture runoff from adjacent areas. Our study proposed to describe the fish community.

The study site has both terrestrial and aquatic invasive species. The first objective was to survey aquatic habitats and describe the biometrics of the fish assemblage in collaboration with Arthur R. Marshall Loxahatchee National Wildlife Refuge Project Leader and the Florida Fish and Wildlife Conservation Commission. The landscape at the study site is heavily disturbed and

influenced by anthropogenic activities such as water level control. The influence of altered hydrology is apparent.

Site Description—The Arthur R. Marshall Loxahatchee National Wildlife Refuge is surrounded by an interconnected system of canals (Figure XX). The S-39 water control structure transfers water from these canals into the Hillsboro canal, outside Arthur R. Marshall Loxahatchee National Wildlife Refuge boundaries. There are a total of 58.4 miles (94 Km) of perimeter canals. The L-40 canal runs for 26 miles; L-36 canal is about 13 miles and the L-7 canal is approximately 16 miles.

Canal Waypoints, 1 km Interval

Waypoint	Latitude	Longitude
N001	26.68309	-80.36673
N002	26.67525	-80.36198
N003	26.66707	-80.35775
N004	26.65890	-80.35352
N005	26.65072	-80.34930
N006	26.64344	-80.34357
N007	26.63672	-80.33687
N008	26.63098	-80.32923
N009	26.62605	-80.32081
N010	26.62112	-80.31239
N011	26.61621	-80.30396
N012	26.61130	-80.29552
N013	26.60635	-80.28712
N014	26.60114	-80.27899
N015	26.59385	-80.27309
N016	26.58655	-80.26720
N017	26.57927	-80.26128
N018	26.57198	-80.25537
N019	26.56466	-80.24958
N020	26.55709	-80.24413
N021	26.54944	-80.23883
N022	26.54181	-80.23350
N023	26.53331	-80.23016
N024	26.52480	-80.22688
N025	26.51628	-80.22360
N026	26.50744	-80.22242
N027	26.49843	-80.22226
N028	26.48943	-80.22209
N029	26.48042	-80.22190
N030	26.47141	-80.22163
N031	26.46256	-80.22307
N032	26.45380	-80.22539
N033	26.44503	-80.22771
N034	26.43627	-80.23003
N035	26.42750	-80.23236
N036	26.41875	-80.23470
N037	26.40998	-80.23703
N038	26.40099	-80.23715
N039	26.39198	-80.23709
N040	26.38344	-80.23870
N041	26.37607	-80.24446
N042	26.36870	-80.25022
N043	26.36497	-80.25819
N044	26.36490	-80.26822
N045	26.36485	-80.27826
N046	26.36284	-80.28765
N047	26.35791	-80.29605
N048	26.35775	-80.30441
N049	26.36057	-80.31394
N050	26.36339	-80.32347
N051	26.36619	-80.33301
N052	26.36890	-80.34258
N053	26.37160	-80.35216
N054	26.37431	-80.36173
N055	26.37709	-80.37126
N056	26.38376	-80.37768
N057	26.39115	-80.38342
N058	26.39855	-80.38915
N059	26.40595	-80.39487
N060	26.41335	-80.40060
N061	26.42075	-80.40632
N062	26.42816	-80.41204
N063	26.43557	-80.41776
N064	26.44297	-80.42347
N065	26.45038	-80.42919
N066	26.45779	-80.43491
N067	26.46520	-80.44062
N068	26.47318	-80.44460
N069	26.48215	-80.44525
N070	26.49116	-80.44527
N071	26.50017	-80.44528
N072	26.50917	-80.44529
N073	26.51818	-80.44524
N074	26.52719	-80.44518
N075	26.53619	-80.44518
N076	26.54520	-80.44516
N077	26.55421	-80.44515
N078	26.56321	-80.44511
N079	26.57222	-80.44508
N080	26.58123	-80.44499
N081	26.59023	-80.44489
N082	26.59797	-80.44031
N083	26.60535	-80.43454
N084	26.61273	-80.42877
N085	26.62011	-80.42300
N086	26.62750	-80.41725
N087	26.63493	-80.41155
N088	26.64235	-80.40585
N089	26.64978	-80.40016
N090	26.65720	-80.39446
N091	26.66454	-80.38862
N092	26.67198	-80.38294
N093	26.67940	-80.37724
N094	26.68187	-80.36812

Figure. 3. The three canals that form the perimeter at LOX NWR were delineated into 1 Km segments with corresponding GPS waypoints (Lat/ Lon) identified for each start of the kilometer.

Justification/Need—

There is a need for decision support tools that could assist NWR manage issues related to Aquatic Invasive Species (AIS). It has been shown that the earlier AIS are detected in the invasion process, that more options will be available to the natural resource managers, and that costs will be lower in the initial introduction phase than costs associated with projects that try to remove established AIS. Our project goal was to provide tools to meet and support early detection and provide established monitoring approaches to be used for field deployment when detection has been made (i.e, traditional and genomic).

Figure. 4. Student Conservation Association Volunteer holding a Striped mullet that was collected during the electrofishing survey.

Two strategic components of the Service's Region 4 AIS program are coordination of effort and standardization of data. To date, these components are often lacking for AIS efforts throughout the region (including NWRs) and thus can often inhibit the effectiveness of AIS rapid response or long-term control strategies. To provide coordination and standardization, we proposed to integrate AIS data needs and approaches outlined by the following three strategic plans: Region 4 AIS Program, Gulf and South Atlantic Regional Panel on Aquatic Invasive Species, and the National Aquatic Species Task Force. Our project provided techniques and adaptive management measures for AIS that can be incorporated into management, comprehensive conservation, and detection/rapid response plans.

This project's other goal was to implement standard practices that best suit the conditions and objectives outlined by Loxahatchee NWR. We then built on standard monitoring practices by adding a proof of concept element to the project for environmental DNA (eDNA).

A technique new to monitoring and inventorying is environmental DNA (eDNA). Typical methods used in aquatic monitoring such as seining, dip netting, and shocking may not be possible and require multiple people and hours of effort. However, recent studies identify the usefulness of eDNA detection and its potential use as a monitoring tool. Environmental DNA refers to DNA fragments that a species leaves behind in the environment. Therefore, to test for the presence/absence of AIS, the eDNA can be collected from the water column and a known volume of water filtered on fine micron screens to trap the eDNA. The eDNA can then be extracted from the filter and species-specific eDNA can be detected via the polymerase chain reaction (PCR) with the aid of molecular markers specific to each species used to target known segments of the genome. A positive reaction for each species is identified by either visualization of the species-specific DNA segment via gel electrophoresis or by comparing the amount of species-specific gene copies that are PCR amplified to that of known controls.

Introduction--The objective of this portion of the study was to formulate and collect a standard set of biometrics for fishes, native and non-native, that is supportive of fisheries studies and for AIS. Data were collected for AIS of greatest concern to Arthur R. Marshall Loxahatchee National Wildlife Refuge NWR biologists (e.g., Mayan cichlid, African jewelfish, and Bullseye snakehead). We used biometrics to describe the fish community and establish metrics for long-term monitoring and surveillance. Biometric data included catch per unit of effort, species diversity, and biomass of native and non-native species.

Methods--We used a stratified random sampling design. This design considered unique sites, multiple visits to a site over time, seasonality, and bank side sampled. Twenty sites were sampled each quarter. Sites were selected using RANDOM.ORG, which is a random integer generator. The parameter was set for a range of numbers 1-94 and the limit was set at 120 random integers. Sites were selected based on the 1st 20 unique numbers, which corresponded to site numbers (e.g., site 20/ 20 Km was number 20). This approach also provided alternate sites in situations when a selected site could not be sampled (e.g., levee repair). A coin was flipped to determine bank selection (i.e., heads and tails equaled Interior Marsh or Levee side). All bank selections were determined at one time prior to any sample being taken. We sampled 20 Km quarterly. The L-7, L-39, and L-40 canals were divided into 1 km grids and each 1 Km segment was numbered 1-94 starting at the NE corner at S 5A (Twenty Mile Bend Area) of Arthur R. Marshall Loxahatchee National Wildlife Refuge. Site numbering continues sequentially moving south from S 5A (ACME 2, ACME 1, Ross' Structure, S39… S10D, S10E, G310, G251, and arrive at west side of S 5A) and continuing along the perimeter of the NWR. Each segment had a corresponding way point (i.e., latitude and longitude).

Each site was sampled using standard methodology and consistency in voltage output of using electrofishing boats equipped with a 9.0 GPP and certified operators. Generally, a four or five-person crew was used for each sampling trip. A two-person electrofishing team sampled the perimeter canal with boat-mounted electrofisher (pulsed DC, Smith-Root). The sampling segment consisted of a 1 km area adjacent to the pre-determined bank (e.g., Interior Marsh or Levee side). A single operator who is certified as an operator through U.S. Fish and Wildlife Service training course and MOCC certified was maintained throughout the study as the sole operator for the boat and electrofishing equipment. A single person netted fishes per kilometer. Netters were rotated every kilometer sampled. The net mesh was 1/8" and the net heads were on 8-10′ fiber glass poles. These crew members ranged in skill level, novice to experienced, and were not constant during course of study, that is, individual crew members changed during the course of the study. Effort was measured as shock time per segment in seconds, shock time for sampling reach in minutes, and total time at site in minutes. A second boat containing the remaining crew marked the endpoint of the kilometer, prepared datasheets, took water quality data (oxygen, temperature, salinity and conductivity), and prepared for measuring a weighing of fishes.

Fish were removed from the live-well, identified to species, sorted into individual buckets, and measured. LMB were measured for total length, standard length, and body weight. Body weight was taken on several scales based on the weight limit of the scale. Target AIS were measured for total length, standard length, and body weight. We used *Salter Brecknell ElectroSamson Digital hanging scale* (55lbs/ 25 kg x 0.02 kg), *Pesola Medio-line* (model 40310, 300 g), *Pesola Micro-line* (model 20060, 60 g), and *Pesola Micro-line* (model 20030, 30 g).

Data entry was performed in *MS EXCEL*. Information from field sheets was transcribed into spreadsheets. Additional statistical analysis was done in *EcoMethodology* and *Statistica*. Statistical analysis using *EcoMethodology* 7.2 software was done following Krebs (1999). Descriptive statistics were done using *Statistica* 64.

Figure. 5. Florida Fish and Wildlife Conservation Commission assisting with a sample on Loxahatchee National Wildlife Refuge.

We conducted two types of sampling during this project. We conducted quarterly sampling and seasonal sampling starting October 2011 and continuing until October 2012. Quarterly samples were taken during October, January, April, July, and October. Samples were taken at 21 sites in October 2011, 19 sites in January 2012, 22 sites in April 2012, 20 sites in July 2012, and 20 sites in October 2012 (n=102 samples). During quarterly sampling, a species list was generated, but no measurements were taken on native species, except Largemouth bass. Target AIS, Bullseye snakehead (*Channa marulius*), African jewelfish (*Hemichromis letourneuxi*), and Mayan cichlid (*Cichlasoma urophthalmus*), were identified and each individual was measured for standard length (mm), total length (mm), and weight (g). In addition, five individuals, when present, were preserved as a voucher. Each voucher collection had the following information collected: site number; date, collectors; species name; and a count of individuals in the collection. This information was put on the label then the label was placed inside the container with the specimen and a second label was attached to the outside of the container. We never obtained more than 2 individuals, so these methods were never used.

Seasonal Samples were taken in October 2011, April 2012, and October 2012. Samples were taken at 21 sites in October 2011, 22 sites in April 2012, and 20 sites in October 2012 (n=63 samples). These samples were more intensive than quarterly sampling and provided more information that described community composition and biomass between seasons. These samples provided species richness, overall fish biomass, AIS target % of biomass, AIS distribution, AIS length-weight relationship, catch-per-unit-of-effort for each species. All

individuals were counted and measured. We also collected water chemistry, but no analysis was completed at the time of this report.

Results for Sampling Site Distribution and Effort

The Arthur R. Marshall Loxahatchee National Wildlife Refuge canals were delineated into 94 one-kilometer segments. No samples were taken from the Interior Marsh surrounded by the perimeter canal, nor any other water body on the Refuge Property. As a result of the stratified random sampling design, site that was to be sampled, the numbers of times a site was sampled, the bank to be sampled, and when it was sampled were parts of the sampling design. Quarterly samples were taken during October, January, April, July, and October. Samples were taken at 21 sites in October 2011, 19 sites in January 2012, 22 sites in April 2012, 20 sites in July 2012, and 20 sites in October 2012 (n=102 samples). As the project progressed, we determined that some sites could not be sampled at the south end of the perimeter canal because it was too shallow and several sites were temporarily unavailable because of levee construction. Of a total 94 possible unique sites in the perimeter canal, 25 sites were not sampled at all during the October to October sampling periods. There were 42 sites sampled one time (n=42 samples), 21 sites were sampled twice (n=42 samples), and 6 sites were sampled 3 times (n=18 samples) during the October to October sampling periods (total n=102 samples). These results are shown in Table 1 and 2. Based on the sampling design, no site was sampled more than once during the same quarterly sampling period (i.e., no site was sampled two or more times during the same month). This consideration was made to avoid sampling recently disturbed sites. We considered bank side as a treatment. Bank side was described as Levee or Interior Marsh. Of the 102 sites, 58 (57%) sites were Levee side and 44 (43%) were Interior Marsh.

Table.1. Sites grouped by the number of times visited between October 2011-October 2012.

Number of visits	***Number of sites***	***Percent (%) of Samples***
n=1	42 (n=42)	41.18%
n=2	21 (n=42)	41.18%
n=3	6 (n=18)	17.64%
Total	***102***	***100%***

Table.2. The 27 sites with 2 or more visits and the month of the visits.

Site Number	***Number of Visits***	***Month of Visit***
4	2	October 2011, April 2012
6	3	October 2011, April 2012, October 2012
7	2	January 2012, April 2012
8	2	October 2011, October 2012
12	3	October 2011, July 2012, October 2012
13	2	April 2012, July 2012
17	2	July 2012, October 2012
18	2	October 2011, October 2012
19	2	October 2011, October 2012
20	2	October 2011, July 2012
30	2	January 2012, April 2012
31	3	April 2012, July 2012, October 2012
35	2	October 2011, July 2012
36	2	January 2012, July 2012
47	2	April 2012, July 2012
48	2	January 2012, October 2012
65	3	October 2011, July 2012, October 2012

68	2	January 2012, April 2012
71	2	October 2011, October 2012
74	3	January 2012, April 2012, July 2012
75	3	October 2011, January 2012, April 2012
76	2	January 2012, April 2012
79	2	January 2012, July 2012
80	2	July 2012, October 2012
84	2	January 2012, July 2012
86	2	January 2012, April 2012
90	2	April 2012, October 2012
Total	***60***	***NA***

Results of the Sampling effort

A total of 102 sites were sampled. Samples were made in October 2011, January 2012, April 2012, July 2012, and October 2012. Samples were taken at 21 sites in October 2011, 19 sites in January 2012, 22 sites in April 2012, 20 sites in July 2012, and 20 sites in October 2012 (n=102 samples). Pedal time was not reported for any site sampled in April (22 sites), so data analyses were done on 80 samples. A total pedal time of 193,885 seconds (~53 hours) were expended across 80 samples. Overall, Pedal time ranged from 1728-3105 seconds (mean=2423.56, std=261.57) at any single site. Pedal time in October 2011 ranged from 1728-2972 seconds (mean=2426.00, std.=319.89), January 2012 ranged from 2324-3105 seconds (mean=2622.15, std.=215.81), July 2012 ranged from 2052-2763 seconds (mean=2294.05, std.=182.11), and October 2012 ranged from 2104-2684 seconds (mean=2352.00, std.=194.57). We compared the total pedal time for differences among sampling months. There was an observed difference and this difference was a significant difference ($F(3, 76)=8.1059$, $p= 0.00009$). There was more pedal time in January than in October 2011, July 2012, and October 2012. We compared total pedal time for differences between bank sides. There was an observed difference but, this difference was not a significant difference ($F(1, 78)=1.9688$, $p= 0.1645$).

Species richness and diversity

We were interested examining species richness and diversity as it is integral to aquatic ecosystem health. We sampled 34 species, but no single sample contained all 34 species. Samples were made in October 2011, January 2012, April 2012, July 2012, and October 2012. Samples were taken at 21 sites in October 2011, 19 sites in January 2012, 22 sites in April 2012, 20 sites in July 2012, and 20 sites in October 2012 (n=102 samples). Our analysis was conducted on 34 species that we encountered (Appendix.1).

Of the 34 species, several species were non-native. We observed the Brown Hoplo regurgitated by largemouth bass on several occasions and this has been observed in other Florida waterways (pers. comm. Kelly Gestring, FFWCC). Of the identified fish species, 29 are native species and 5 species of introduced species. The introduced species were Brown Hoplo, Bullseye snakehead, Mayan cichlid, "Sailfin catfish", and "Tilapia". Several of these species may actually have multiple cryptic species or hybrids which we counted as a single species (i.e., "Sailfin catfish" and "Tilapia" are probably multiple species each).

This study found the number of species at a site being sampled ranged from 7-19. The lowest number of species at a site was seven and this occurred at one site which was site six in October

2012. The highest number of species at a single site was 19 and this occurred at three different sites which were sites 30, 31, and 38 during April 2012, April 2012, and July 2012, respectively. Species richness in October 2011 ranged from 9-14 (mean= 10.71, std=1.38), in January ranged from 8-14 (mean=11.47, std=1.712), in April ranged from 8-19 (mean=12.32, std=3.04), in July from 8-19 (mean=11.85, std=2.60), and in October 2012 from 7-14 (mean=9.85, std=2.11). Several species were not commonly sampled, but are believed to be widespread and common. Mosquitofish (*Gambusia spp.*) were netted and observed at multiple sampling sites and are assumed to be present throughout the perimeter canal. We suspect that Inland silversides are underrepresented in our samples. At least three species, Brown hoplo, Walking catfish, and Croaking gourami were not captured. This supports our species-area curve never reaching an asymptote. These observations, Brown hoplo observed during regurgitation and anecdotal observations on Walking catfish and Croaking gourami, would indicate that we did not capture 3/ 37 species, or 10%. Our samples contained a total of 15,440 individuals**.** Our Species-Area curve never reached an asymptote after 102 Km sampled and 34 species detected which is an indication of low species equitability (Figure.6).

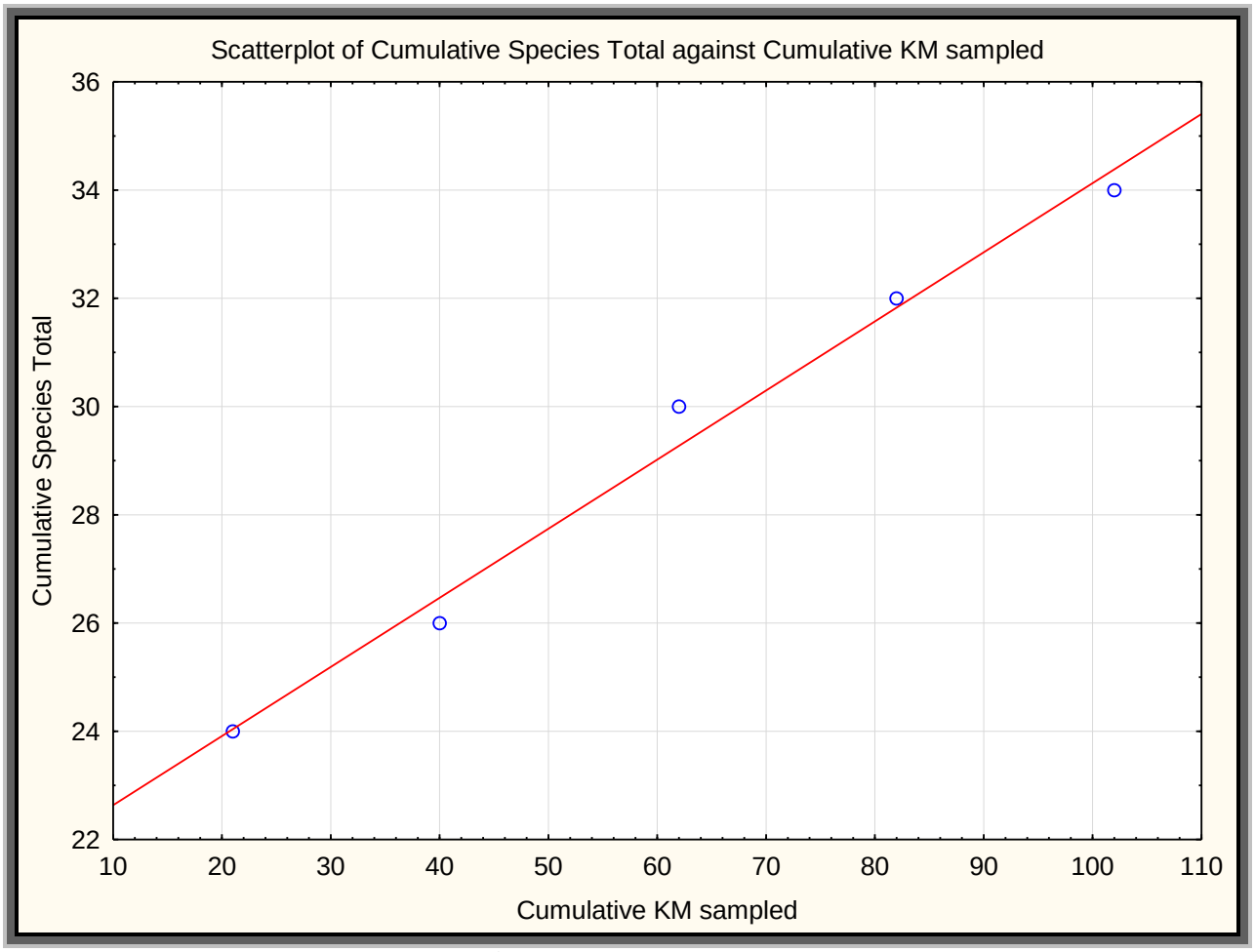

Figure.6. Species-Area curve represented by the number of species sampled in 110 Km.

We used a sub-set of these data to look at numerical abundance by species. We used seasonal samples (October 2011, April 2011, and October 2012) since all individuals were weighted and

would provide the best representation across most of the species. There were 25 species represented by a total 7,598 individuals in these three samples. Five out of the 25 species represented 84% of the total number of individuals in these Seasonal samples. These species are, in order of largest to smallest total number of individuals, Florida gar (25%), bluegill (24%), largemouth bass (17%), redear sunfish (10%), and bowfin (8%). Numerically, 49% of the individuals in these samples were either Florida gar or bluegill. We compared the number of species at a site for differences among sampling months. There was an observed difference and this difference was a statistically significant difference (F (4, 97)=3.8146, *p*= 0.0064) (Figure.7). There were more species sampled in April.

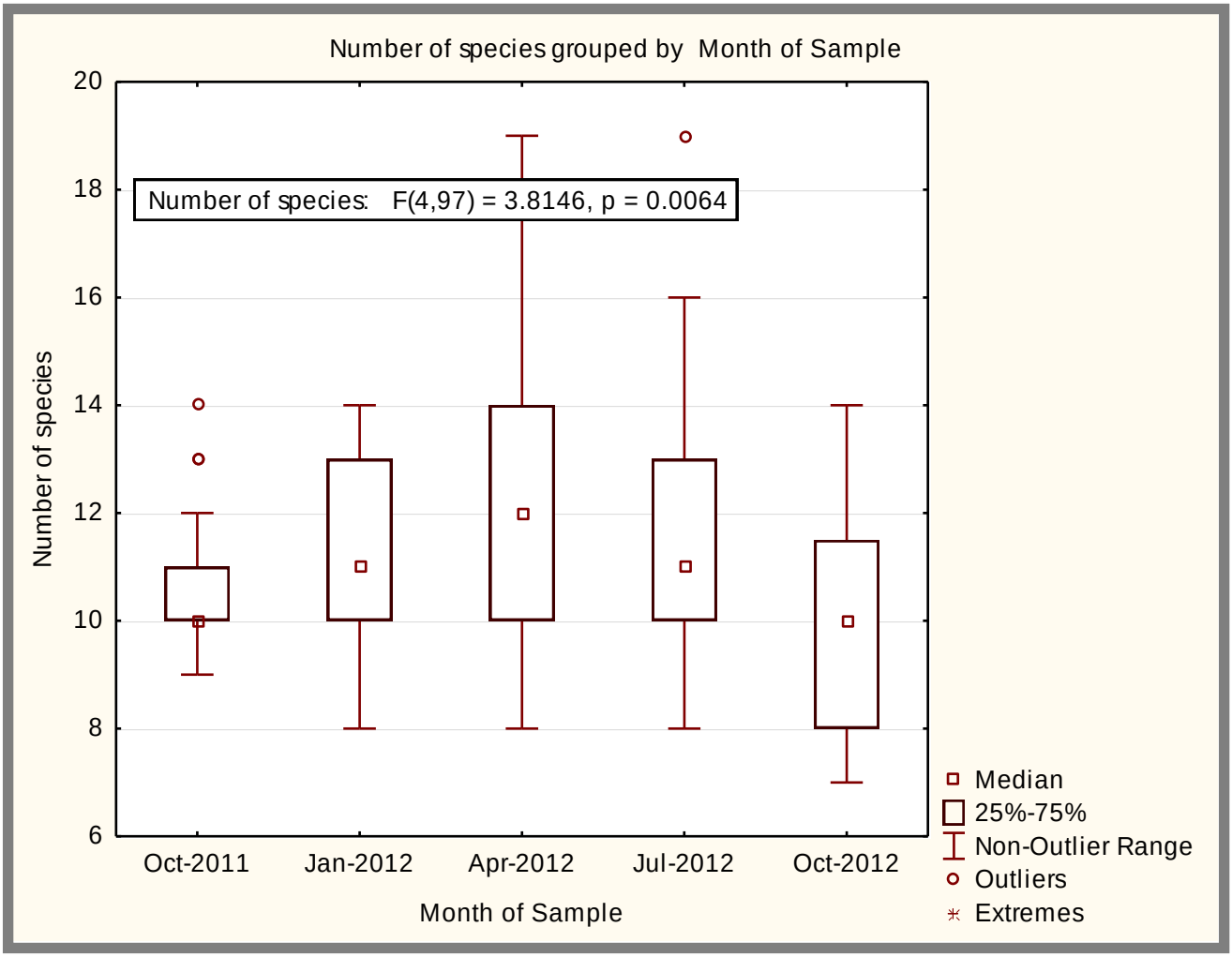

Figure.7. Box plot comparing number of species at a site by month.

We compared the number of species at a site for differences among seasonal samples (October, April, and October). There was an observed difference and this difference was a significant difference (F (2,60)=6.2711, *p*= 0.0034). There were more species observed in April than in October 2011 and October 2012. We compared the number of species at a site for differences between bank sides. There was an observed difference, but no significant difference (F (1,100)=1.0438, *p*= 0.3094).

Figure.8. Two species of gar, Longnose gar (lower) and Florida gar (upper), collected during this study.

Figure.9. Two species of gar, Longnose gar (right) and Florida gar (left), collected during this study.

We analyzed and described the fish community diversity using *Shannon-Weiner Diversity, Simpson's Diversity,* and *Brillouin's Diversity.* We used the software *Ecological Methodology* to analyze data for fish community diversity. We pooled score from all sites sampled in each quarterly sample for a single score. We provide a graph with these diversity indices plotted together (Figure.10).

Shannon-Weiner Index is an information index and results in the highest score when the community is diverse (e.g., high number of species) and the number of individuals for each species is equal across species. This measure is accounting for the change in abundance of rare species in the community. *Shannon-Weiner Diversity* score for October was 3.011, January was 3.007, April was 2.855, July was 2.752, and October 2012 was 2.629. The scoring is divided into 3 condition types: Good, Fair, and Poor. The category of "Good" is a score of 2.500 or higher. The category of "Fair" is a score of 1.500 and less than 2.499. The category of "Poor" is a score of below 1.499. The current study observed samples across seasons that scored a "Good".

Simpson's Index gives the probability of any two individuals which are drawn at random from a community would belong to different species. This measure is accounting for the change in abundance of species most often encountered in the community (i.e., the dominate species). *Simpson's Diversity* score for October was 0.840, January was 0.824, April was 0.808, July was 0.790, and October 2012 was 0.757. The scoring is divided into 3 condition types; Good, Fair, and Poor. The category of "Good" is a score of 0.75 or higher. The category of "Fair" is a score of 0.50 and less than 0.75. The category of "Poor" is a score of below 0.50. The current study observed samples across seasons that scored a "Good".

Brillouin's Diversity score for October was 2.984, January was 2.977, April was 2.839, July was 2.728, and October was 2.593. This result suggests a diverse fish community.

We examined ecological evenness using Simpson's Measure. *Simpson (1/D) Measure* score for October was 0.271, January was 0.246, April was 0.217, July was 0.190, and October was 0.179. These measures are indicative of a community that lacks evenness.

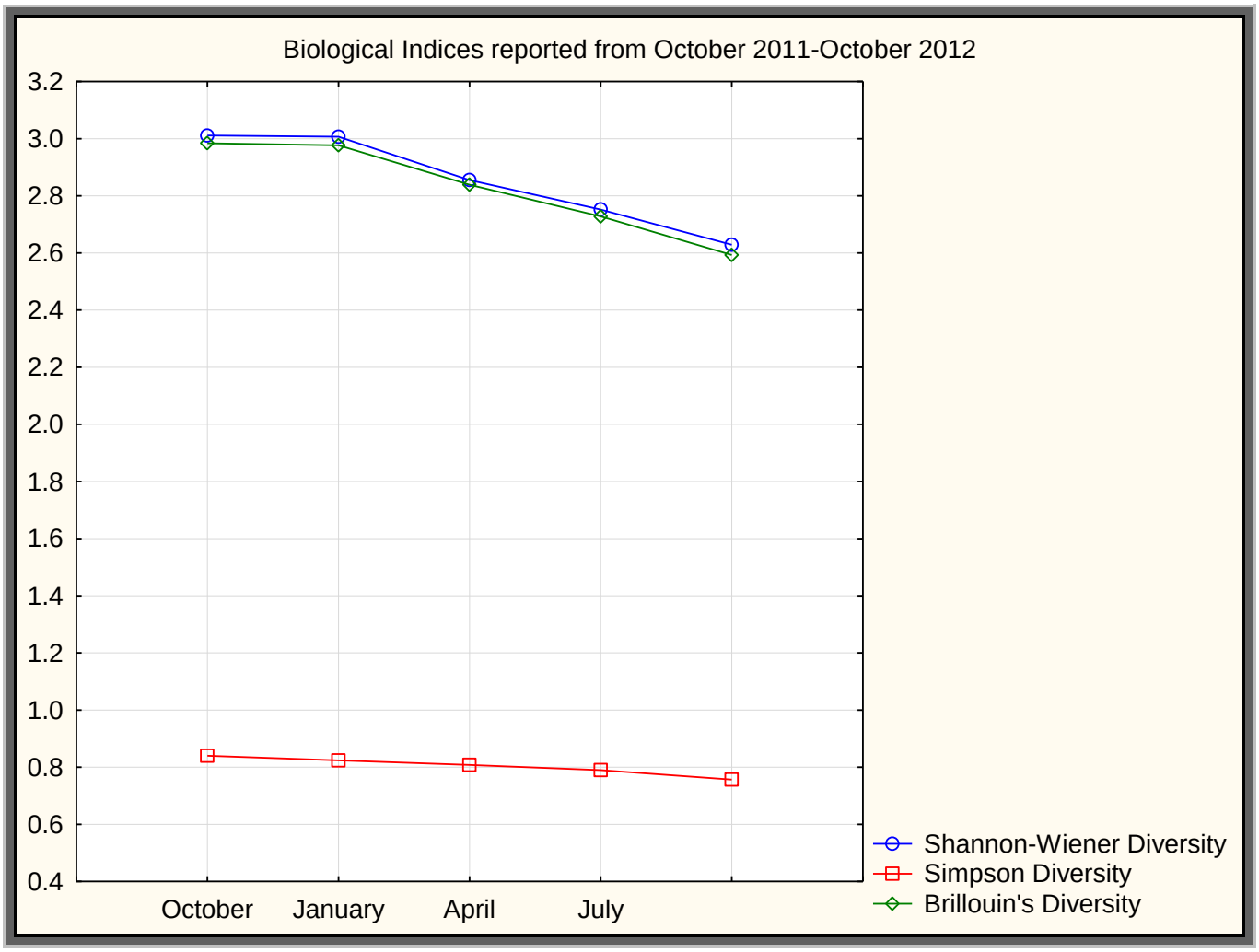

Figure.10. Graphics showing the three species diversity measures across sampling months.

We compared the total number of individuals at a site for sampling month. There was an observed difference and this was a significant difference (F (4, 97)=15.6203, *p*= 0.0000). There were more total number of individuals sampled in April, than any other month. We compared the total number of individuals at a site for difference between seasonal samples (October and April). There was an observed difference and this difference was a significant difference (F (2, 60)=30.70.98, *p*= 0.0000). There were more total individuals sampled seasonally in April than in October 2011 and October 2012. We compared the total number of individuals at a site for difference in bank sides. There was an observed difference, but this was not a significant difference (F (1, 100)=1.3291, *p*= 0.2517).

Biomass

One metric that can be tracked over time is biomass. This metric was measured seasonally. Individual species had varying amounts of biomass. We used a sub-set of these data for analysis of individual species contributions. We used seasonal samples (October, April, October) since all individuals were weighed and would provide the best representation across most of the species. Samples were made in October 2011, April 2012, and October 2012. Samples were

taken at 21 sites in October 2011, 22 sites in April 2012, and 20 sites in October 2012 (n=63 samples).

Total biomass measured in this study for all species, samples, and sites combined was 1,950.92 Kg. Cumulative biomass (all species/ site) sites in October 2011 ranged from 8.542-98.864 kg (mean= 41.69352, std=24.96478), in April ranged from 22.482-135.245 kg (mean=74.31932, std=23.71194), and October 2012 ranged from 8.5745-39.049 kg (mean= 20.18590, std=8.791944). All sites compared, cumulative biomass ranged from lowest sampled at site 20 in October 2011 (8.54 kg) and the highest biomass was sampled at site 4 in April 2012 (135.24 kg). A subset of data was used for examining seasonal biomass that did not include largemouth bass. There were 28 species 6,463 individuals and a total biomass of 1950.92 kg. There were five species that contributed 89% of the biomass in these Seasonal samples. These species are, in order of largest to smallest % of total biomass, bowfin (42.07%), Florida gar (33.86%), lake chubsucker (6.21%), and bluegill (5.85%).

Figure.11. Andy Jackson measuring the weight of a fish with a spring scale.

We compared total biomass for differences among sampling month. There was an observed difference and this difference was a significant difference (F (2, 1744)=244.4174, *p*= 0.0000). There was more biomass sampled in April. We compared the total biomass at a site by bank side. There was an observed difference and this difference was a significant difference (F (1, 1727)=4.2901, *p*= 0.0385). There was more biomass sampled on the Interior Marsh bank side.

Results for Fish Catch-Per-Unit-Effort (CPUE)

We expected, based on two separate and limited fish surveys, to encounter approximately 17-23 species and between 100-200 individuals to be collected per site from the Refuge (USFWS unpublished data 2008 and 2009). Samples were made in October 2011, January 2012, April 2012, July 2012, and October 2012. Samples were taken at 21 sites in October 2011, 19 sites in January 2012, 22 sites in April 2012, 20 sites in July 2012, and 20 sites in October 2012 (n=102

samples).

We compared CPUE for species by quarterly sample. There was an observed difference and this difference was a significant difference (F (3, 76)=3.8946, *p*= 0.0121). The trend was increasing CPUE from October 2011 to July 2012 with July having the highest CPUE. There was no effort reported for April. We compared CPUE for total number of individuals by quarterly sample. There was an observed difference and this difference was a significant difference (F (3, 76)=29.616, *p*= 0.0000). The trend was increasing CPUE across sampling months with highest CPUE in July. We compared CPUE for biomass by month sampled. There was an observed difference and this difference was a significant difference F (3, 76)=16.4906, *p*= 0.00000). The trend was decreasing CPUE for biomass with the highest CPUE in October 2011, then decreasing in January and July, then increasing in October 2012. We compared CPUE for number of species sampled by bank side sampled. There was an observed difference, but this difference was not a significant difference (F (1, 78)=0.4338, *p*= 0.5121). There was a higher CPUE for Interior Marsh.

We compared CPUE for number of individuals sampled by bank side sampled. There was an observed difference, but this difference was not a significant difference (F (1, 78)=1.5751, *p*= 0.2132). We compared CPUE for biomass by bank side sampled. There was an observed difference and this difference was a significant difference (F (1, 78)=4.364, *p*= 0.0400). There was a higher CPUE for Interior Marsh.

Figure.12. Dr. Edgardo Diaz-Ferguson netting fish from the electrofishing boat.

Largemouth Bass Biometric Study

The largemouth bass is an important recreational species at Loxahatchee NWR. The Managers at the NWR and the project staff worked on the development of a special project that would gather the appropriate data at the appropriate level of detail. Currently, there are fishing tournaments held at Loxahatchee NWR and there is interest in holding more. This study was designed to sample the LMB population at Loxahatchee NWR, provide our findings, and recommend management actions.

The current study collected information 3,317 individual bass from 102 sites. This was 21.48% of all fish sampled (n=15,440) sampled from October 2011 to October 2012. A subset of data (n=3289) was analyzed due to missing or incomplete data for individuals. Individual total length (mm) ranged from 54-610 (mean= 268.34, std.=95.21) and standard length (mm) ranged from 53-530 (mean= 221.56, std.= 81.24). Catch-Per-Unit-of-Effort (CPUE) was done on numbers of largemouth bass as well as largemouth bass total biomass.

Largemouth Bass Demographics and Analysis

Largemouth bass demographics were variable across samples. Largemouth bass total length ranged from 54-610 mm (mean= 268.34, std. 95.2148). We compared total length for largemouth bass in quarterly samples. There was an observed difference and this difference was a significant difference ($F(4, 3284)=75.5831$, $p= 0.0000$). The total length was greater in April 2012. We compared the largemouth bass total length by bank side. There was an observed difference and this difference was not significant ($F(1, 3268)=2.8394$, $p= 0.0921$). Largemouth bass total length was equal regardless of bank side. Largemouth bass standard length was plotted. The standard length ranged from 251-412 mm (mean= 221.5588, std. 81.2392). We compared standard length for largemouth bass in quarterly samples. There was an observed difference and this difference was a significant difference ($F(4, 3284)=79.6095$, $p= 0.0000$). The standard length was greater in April 2012. We compared the largemouth bass standard length by bank side. There was no observed difference ($F(1, 3268)=2.1435$, $p= 0.1433$). We compared the largemouth bass standard length by seasonal sample. There was an observed difference and this difference was a significant difference ($F(1, 1301)=52.2041$, $p= 0.0000$). Largemouth bass standard length was longest in April.

We analyzed data for October 2011, January 2012, July 2012, and October 2012 (n=20 samples each). The number of largemouth bass collected ranged from 17-52 (mean=25.55, std.=7.96) in October 2011, 22-66 (mean=37.40, std.=11.91) in January 2012, 9-66 (mean=27.45, std.=13.64) in July 2012, and 9-124 (mean=35.95, std.=27.28). The Catch-Per-Unit-of-Effort (CPUE) for 80 samples ranged from 12.41-186.05 LMB/ hour (mean=47.38 LMB/ hour, std.=26.39). CPUE for number individuals 20.89-76.59 LMB/ hour (mean=38.89, std.=13.99) in October 2011, 29.91-89.54 LMB/ hour (mean=51.89, std.=17.28) in January 2012, 14.25-99.57 LMB/ hour (mean=43.25, std.=21.37) in July 2012, and 12.41-186.05 LMB/ hour (mean=55.48, std.=41.96) in October 2012. CPUE for total biomass (kg) 3.91-32.14 (mean=14.16, std.=7.34) in October 2011, 7.08-45.05 (mean=21.82, std.=10.03) in January 2012, 1.68-19.07 (mean=9.35, std.=5.34) in July 2012, and 2.96-39.20 (mean=15.05, std.=10.18) in October 2012.

Largemouth bass total biomass for the study was 1183.05 kg. Individuals total biomass ranged 3.05-18.29 (mean=9.24, std.=4.11) in October 2011, 4.73-33.21 (mean=15.78, std.=7.05) in

January 2012, 1.06-11.63 (mean=5.88, std.=3.34) in July 2012, and 1.81-22.84 (mean=9.63, std.=6.11) in October 2012. We compared the largemouth bass total biomass by quarterly sample. There was an observed difference and this difference was a significant difference (F (4, 3284)=43.8241, *p*= 0.0000). Total biomass was highest in April. We compared the largemouth bass total biomass by bank side. There was an observed difference and this difference was not a significant difference (F (1, 3268)=1.1787, *p*= 0.2777). Largemouth bass total biomass was equal regardless of bank side.

Proportional Stock Density (Total Length mm) was balanced with a score of 43 (Table.3). It is the number of Quality size largemouth bass divided by the number of stock size largemouth bass. This is determined by taking the number of largemouth bass with a length of ≥ 201mm (≥8 inches) and dividing it by the number of largemouth bass individuals that had a length ≥ 301mm (≥12inches). The equation is (1076/2485=0.4329). Take the answer and multiply by 100 (0.4329*100=43). Balanced PSD ranges from 40-70.

Relative Stock Density for 351mm (Total Length mm) was balanced with a score of 19. This size is a Preferred size. RSD-351 is determined by taking the number of largemouth bass with a length of 351 mm (≥ 14 inches) and dividing it by the number of largemouth bass individuals that had a length ≥ 301. The equation for RSD-351 is (473/2485=0.19034). Take the answer and multiply by 100 (0.19034*100=19). Balanced PSD ranges from 10-40.

Relative Stock Density for 501mm (Total Length mm) was balanced with a score of 4. This size is a Memorable size. RSD-501 is determined by taking the number of largemouth bass with a length of 501 mm (≥ 20 inches) and dividing it by the number of largemouth bass individuals that had a length ≥ 301mm. The equation for RSD-501 is (94/2485=0.0378). Take the answer and multiply by 100 (0.0378*100=4). Balanced PSD ranges from 0-10.

Table.3. Largemouth bass biometrics for Proportional Stock Density (PSD)

Size Class (mm)	Approximate Size Class (~inches)	Number of fish in sample	Minimum Size (PSD) Total Length (mm)	% of sample
<51	2	0	Under stock	0%
51 to 100	4	98	Under stock	2.97%
101 to 150	5	224	Under stock	6.81%
151 to 200	6	482	Under stock	14.65%
201 to 250	8	587	Stock (200 mm)	17.84%
251 to 300	10	822	Quality (300 mm)	24.99%
301 to 350	12	603		18.33%
351 to 400	14	189	Preferred (380 mm)	5.75%
401 to 450	16	118		3.59%
451 to 500	18	72		2.19%
501 to 550	20	71	Memorable (510 mm)	2.16%
551 to 600	22	21		0.64%
601 to 650	24	2	Trophy (630 mm)	0.06%

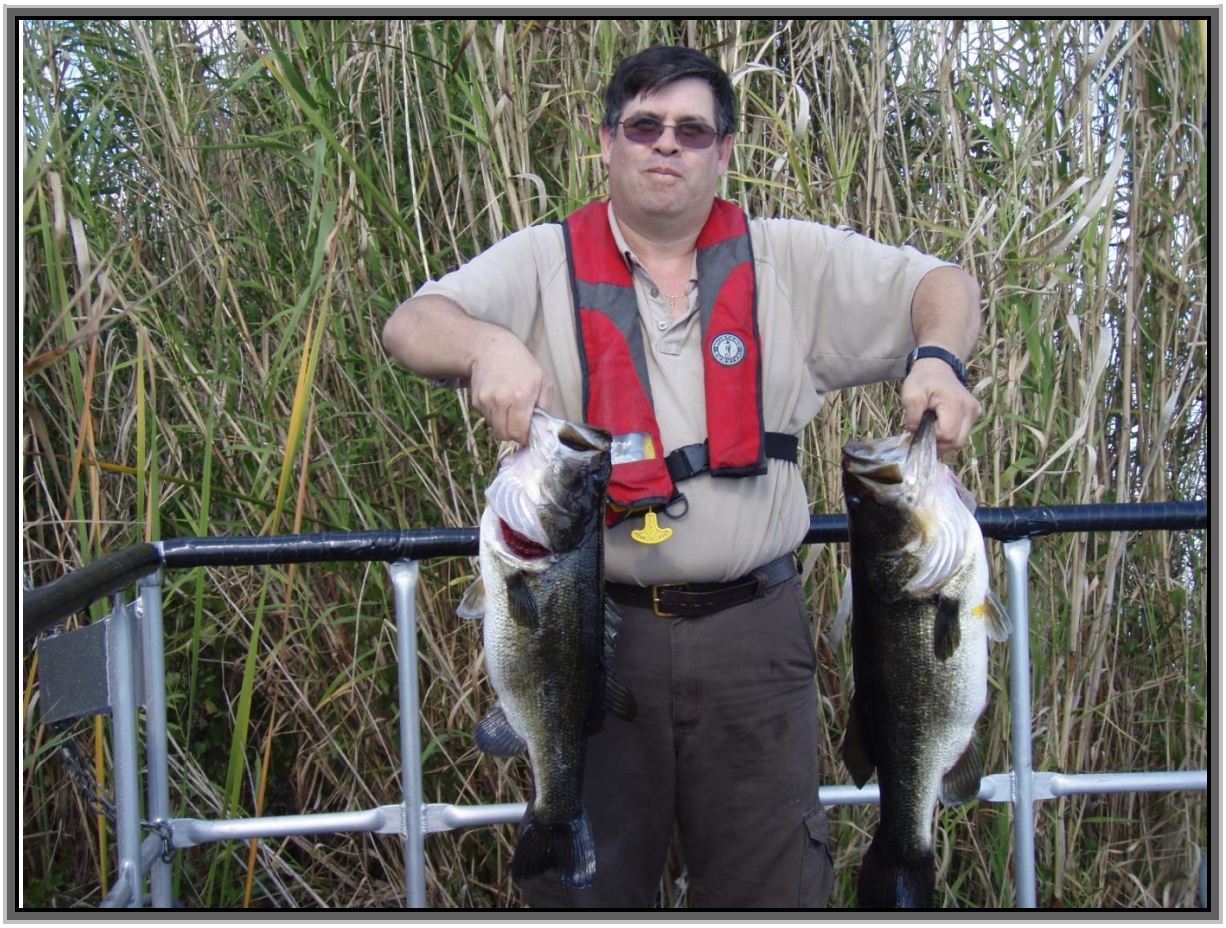

Figure. 13. Dr. John Galvez holding two largemouth bass.

Seasonal Differences in Largemouth Bass

We use a subset of these largemouth bass data to examine seasonal differences. These comparisons use largemouth bass sampled October 2011, April 2012, and October 2012. Largemouth bass total length was plotted seasonally. There was an observed difference and this difference was a significant difference (F (2, 1994)=78.7491, *p*= 0.0000). Total length (mm) was greater in April 2012, than either October sample. As expected, largemouth bass standard length also had a significant difference (F (2, 1994)=83.6893, *p*= 0.0000). Standard length (mm) was greater in April 2012. The largest fish were found in April. It follows that these fish were also the heaviest. Largemouth bass total biomass was plotted seasonally, and a significant difference was found for weight (F (2, 2017)=42.1718, *p*= 0.0000). Total biomass (g) was greater in April 2012.

Largemouth bass Catch-Per-Unit-of-Effort (CPUE) for number of individuals is reported for October 2011, January 2012, July 2012, and October 2012. Largemouth bass CPUE for number of individuals (n=80) ranged from 12.41-186.06 (mean=47.376, std.= 26.391045). ANOVA test resulted in an observed difference, but this difference was not a significant difference (F (3, 76)=1.7201, *p*= 0.1700). Largemouth bass CPUE for number of individuals by bank side is

reported for October 2011, January 2012, July 2012, and October 2012. There was an observed difference, but this difference was not a significant difference (F (1, 78)=1.5715, *p*= 0.2132). Largemouth bass CPUE for total biomass is reported for October 2011, January 2012, July 2012, and October 2012. There was an observed difference and this difference was a significant difference (F (3, 76)=16.4906, *p*= 0.0000). Largemouth bass CPUE for total biomass was highest in October 2011 and October 2012. Largemouth bass CPUE was determined for biomass by bank side. There was an observed difference and this difference was a significant difference (F (3, 78)=4.674, *p*= 0.0400). Largemouth bass CPUE for biomass was highest along the Interior Marsh.

Aquatic Invasive Species of Fish

Managers at Loxahatchee NWR have recognized that aquatic invasive species of fishes are an important component of the fish community and need to be a consideration in any management plan. As reported in the USGS 2001 *Summary Report of Nonindigenous Aquatic Species in U.S. Fish and Wildlife Service Region 4*, there are well over 300 aquatic invasive species (AIS) documented in the southeastern United States with more recognized introductions occurring every year. AIS are often introduced by various anthropogenic activities and can greatly impact the ecology of native species, as well as, the economy of the surrounding area, and human health (e.g., rat lungworm and venom from the lionfish). Invasive species are a management issue for USFWS NWR system. This management issue diverts resources from other program needs. Additionally, aquatic systems and aquatic invasive species are rarely, if ever considered in routine monitoring or management for aquatic conservation purposes, such as species diversity, ecosystem services, or resilience to aquatic invasive species introduction and establishment.

There were 5 introduced species (i.e, Brown Hoplo, Mayan cichlid, Bullseye snakehead, "Sailfin catfishes", and "Tilapia") encountered during this study. We also have heard of other species being collected. Two species not observed during this study were Walking catfish and Croaking gourami. Croaking gourami has been collected on the Refuge, but not in the perimeter canal. A walking catfish was collected in the perimeter canal (J. Galvez, USFWS, personal communication).

Figure. 14. Tom Sinclair taking length measurements on a "Sailfin catfishes" (Pterygoplichthyes spp.)

"Sailfin catfishes"

This study collected information 256 individual "Sailfin catfishes" from 59 sites (i.e., 58% of the samples). This is 1.66% of the total number of all individuals sampled (n=15,440) October 2011 to October 2012. A sub-set of data was used for length and weight analyses. "Sailfin catfishes" total biomass for 144 individuals was 110.84 Kg of 1,950.92 Kg (e.g., 5.68% of total biomass was AIS fishes).

In following sections, we describe the biometrics of "Sailfin catfishes" sampled between October 2011, January 2012, April 2012, July 2012, and October 2012. "Sailfin catfishes" total length was plotted for a sub-set of data which totaled 144 individuals. The total length ranged from 350-527 mm (mean= 439.8958, std. 39.7656). We compared the "Sailfin catfishes" total length by bank side. There was an observed difference, but this difference was not a significant difference ($F(1, 133)=0.3372$, $p= 0.5624$). "Sailfin catfishes" total length was almost equal regardless of bank side. "Sailfin catfishes" standard length was plotted for a sub-set of data which totaled 144 individuals. The standard length ranged from 251-412 mm (mean= 341.6944, std. 35.9635). We compared the "Sailfin catfishes" standard length by bank side. There was an observed difference, but this difference was not a significant difference ($F(1, 133)=0.5788$, $p= 0.4481$). "Sailfin catfishes" standard length was almost equal regardless of bank side. "Sailfin catfishes" body weight (g) was plotted for a sub-set of data which totaled 144 individuals. The Body weight (g) ranged from 200-1220 g (mean= 769.7638, std. 195.8752). We compared the "Sailfin

catfishes" body weight by bank side. There was an observed difference, but this difference was not a significant difference (F (1,142)=0.1615, *p*= 0.6884). "Sailfin catfishes" body weight was almost equal regardless of bank side.

Tilapia

The current study collected information on 91 individual Tilapia from 26 sites (25%). Tilapia abundance is 0.59% (i.e., 91 total Tilapia) of all fish sampled (n=15,440) October 2011 to July 2012. Analysis of length and weight was done on a sub-set of these data. There were 27 individuals that had a weight measured during this study. Tilapia total biomass for the 27 individuals was 24.980 kg, which is only 1.28% of the total biomass sampled in this study. Length and weight were measured for 27 individuals. Individuals ranged in total length (mm) from 189-442 (mean= 345.3703, std. 71.4310).

We compared the "Tilapia" total length by bank side. There was an observed difference, but this difference was not a significant difference (F (1, 25)=0.4184, *p*= 0.5236). We describe "Tilapia" standard length descriptive statistics. Standard length (mm) from 149-363 mm (mean= 278.7037, std. 60.2129). We describe "Tilapia" standard length by bank side. There was an observed difference, but this difference was not a significant difference (F (1, 25)=0.6005, *p*= 0.4457). We describe "Tilapia" body weight descriptive statistics. Body weight was measured (g) from 80-1650 g (mean= 947.4615, std. 458.9477). We describe "Tilapia" body weight by bank side. There was an observed difference, but this difference was not a significant difference (F (1, 25)=0.8987, *p*= 0.3522).

Mayan Cichlids

The current study collected information on 2 individual Mayan cichlids, one each of 2 separate sites (1.96%). The sites where Mayan cichlids were collected were 29 Km and 35 Km. Mayan cichlids abundance is 0.01% (i.e, 2 total Mayan cichlids) of all fish sampled (n=15,440). Mayan cichlid Total Body Weight was 354 g.

Bullseye Snakehead

The current study collected information 1 individual bullseye snakehead. It was collected at site 48 Km.

African Jewelfish

The current study collected no African jewelfish.

Discussion

This project integrated standard data collection techniques to support inventory and monitoring programs for fish communities, including aquatic invasive species (AIS) of fishes, within National Wildlife Refuge (NWR) waters. In addition, a novel approach for detecting aquatic invasive species was developed and implemented in the NWR waters. This project was a collaboration across Service programs, and a pilot study that provided inventory and monitoring protocols and tools for the Loxahatchee NWR with the expectation of establishing broad-scale (i.e., regional and national) standardized fish communities, including AIS fishes, inventory and monitoring protocols. Our approach provides answers to questions such as, does sampling location, timing, and amount effort matter; what species are present; what does the fish

community tell us about ecosystem health; and what about the largemouth bass population which is a management target species. Once developed, the standard methods and protocols can be used by staff of any National Wildlife Refuge or Field Station to assess fish communities, including AIS fishes. We recommend that the sampling approach used for this study be implemented.

As part of our biometric study, we set out to develop and implement a standardized sampling approach for fish communities with the intention to understand AIS component of these communities. Our results are baseline data which were collected to set expected distributions of fishes, describe fish assemblage attributes, examine the potential of seasonal differences(spatially and temporarily), describe aquatic invasive fish species biometrics, provide biometrics on largemouth bass as requested by the managers, and test sampling effort for species richness on Loxahatchee National Wildlife Refuge. Monitoring recommendations considered the current methods as well as information in the scientific literature. This study provides recommendations based on three assumptions about the fishery: 1) non-native fishes are not a primary management goal except in terms of eradication/ extirpation; 2) standardized monitoring is needed for the native fish community to assess its response to management actions such as changes in water levels, alterations in habitat, or changes in fishing pressures due to modifying fishing regulations; and 3) largemouth bass are an important part of the current fishery. Though no direct comparison should be made among historical studies and the current study, the current study used a stratified sampling design and had a comparable species list to historical studies. The study design made use of stratifying monitoring sites to achieve goals of the study which made efficient use of limited personnel and monies. It also eliminated the biases of previous studies associated with site and bank selections.

Our species list provides managers with areas and species that are critical to conservation planning. The few species encountered at only one site and represented by very few individuals need specific targeted surveys. An example of one such species is the American eel. Additional effort is needed to determine if the low level of American eel is an artifact of electrofishing or if there are barriers to movement and recruitment to this species. It maybe that the distribution and number of individuals are at a very low level and this is important for management and conservation of the species. Some species occurrences are probably biased by gear such as mosquitofish, Brook silverside, and Bluefin killifish. These species are surface oriented and all are small-bodied making the size of the net mesh important. Another important consideration is the non-native catfishes (“Sailfin catfishes” and Brown hoplo). There is a need to target and test gear types that may be more efficient at sampling these catfishes. It is also noteworthy that like the small-bodied fishes, there were no small individual non-native fishes. It may be important to conduct some samples at night, and target certain species and demographics to better understand the population and species interactions. Sampling results demonstrate a statistically higher CPUE when sampling in April and on the Interior Marsh bankside.

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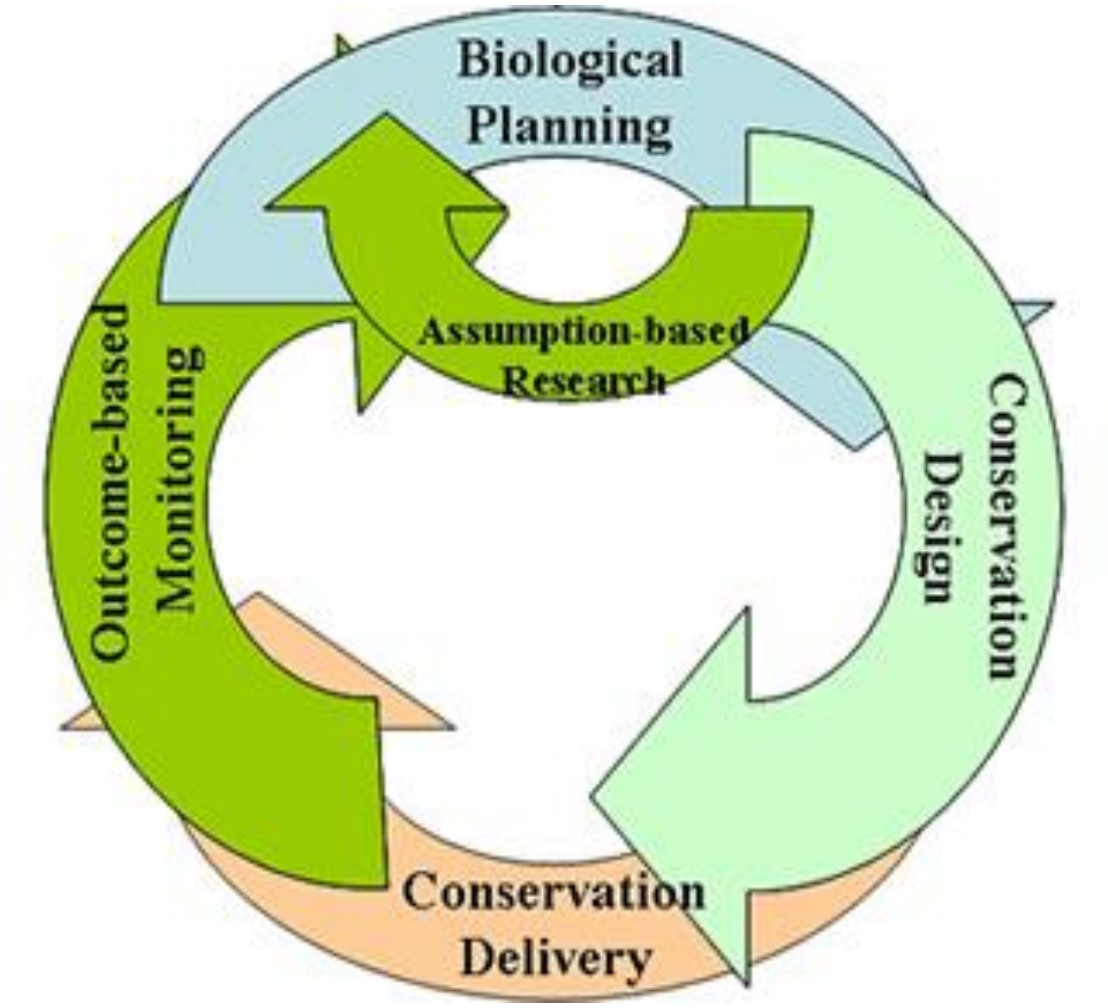
Management Recommendations for the Refuge

Our management recommendations are proposed with the purpose of assisting managers at Loxahatchee National Wildlife Refuge with understanding the biological integrity of the perimeter canal and when needed, make management decisions based on these recommendations to maintain or attempt to regain that biological integrity. In our analysis, we provided several different and complimentary approaches to monitoring this waterway. The biological diversity indices, target species, and community composition have been described. We recommend that these metrics be used to determine species and community responses to management actions and disturbances. The recommendations about sampling and timing are important for trend analysis and there must be a commitment to continue these samples over time. These data sets could be used to understand local management actions as well as larger landscape issues such as climate change. This information supports Strategic Habitat Conservation (SHC). We provide recommendations and linkages to SHC and the work that was accomplished in this study.

- Biological Planning: setting measurable biological objectives for our conservation targets (e.g., fish). We designed our study to provide data on seasonal changes, native and non-native species abundance, target species CPUE/ PSD, and other important metrics such as diversity indices.
- Conservation Design: involves combining geospatial data with biological information and models to create tools such as maps that evaluate the potential of habitats and locations in the perimeter canal. We provide a spatial structure and nomenclature for all studies to use going forward at the Refuge.
- Conservation Delivery: site-scale actions are coordinated and linked to landscape-scale habitat objectives and population outcomes. This project provides several metrics for native and non-native species. Other conservation delivery tools help to achieve biological outcomes through communication, environmental education, access to recreational opportunities, regulatory forums and processes, conservation policy development, and targeted law enforcement activities. We provide recommendations related to these tools as well.
- Outcome-based Monitoring: ensure that our work is adaptive – that we learn from our actions and improve our understanding of habitat and fish species in the perimeter canal. We will test our assumptions about how populations respond to stressors through future study designs as well as our use of continued monitoring of these sites through time. One such study that seems to be an immediate need is a tagging study using largemouth bass. This could use angler reports as well as information provided by this study to look at specific sites and overall movements of largemouth bass. Since this target species is an apex predator, changes to the habitat and species assemblage will result in changes in this species distribution, condition, and demographics. There is an abundance of information on this species and there are opportunities to integrate available information into management actions and monitoring. We provide a few recommendations below.



http://training.fws.gov/CSP/Resources/shc/shctraining.

Figure.15. Strategic Habitat Conservation.

1. We recommend that monitoring should be based on 20 stratified-randomly selected sites/ year and every 3-5 years there should be a more extensive project that is a repeat of this project (i.e., 20 sites sampled every quarter including seasonal samples and an annual repeated month, which in this study we repeated October, so n=100 samples). Sampling units should be the designated 1 km segments we developed. For these surveys, site selection should use the approach detailed in the study which we repeat here:
   1.1. Use the established site nomenclature and randomly-stratified selected sites (GPS defined sites). These sites have been delineated and can now be coordinated with other projects based on site nomenclature. Select 20 sites (i.e., 20 km sampled).
   1.2. Sample in April (spring-time March-April-May). Appears to be the most efficient time to sample based on species and numbers of individuals.
   1.3. Site selection should use a random number generator.
   1.4. It doesn't matter which bank is sampled when examining species diversity, so consider the bank that has better access, allows better boat movement, and greater visibility.
   1.5. Bank side does matter when comparing biomass (e.g., LMB biomass greater on Interior Marsh bankside). So consider what management objective is the focus of the sample and consider incorporating bank side into the sample design.
   1.6. Continue to use largemouth bass as a focal species. This apex predator is important recreationally as well as ecologically. Largemouth bass mean CPUE of 47.38 LMB/

hour (std.=26.39) and should be used as a baseline. Continue using a sample size of 20 sites (i.e., Km) to track the trend in CPUE of largemouth bass.

1.7. There are several biological diversity indices used in the current study and we recommend that these be used as part of long-term monitoring and trend analysis. Annual survey data should be used to examine trends in these metrics.

1.8. The suggested approach allows for the Refuge to track trends at individual sites over time, as well as look at composite scores at a suite of sites, over time.

2. There is a need to examine and describe the habitat on each bank side and mid-channel.
   2.1. Side scan sonar mapping should be conducted in the perimeter canal. This technology will provide a map of the habitat available below the surface of the water.
   2.2. Analysis of habitat and spatial distribution of fishes should be examined to understand species preferences, including habitats used by species. This needs to be informed by habitat mapping and specific species relationships need to be studied. Using tagged individuals and understanding their movements and areas used in the canal or interior marsh should be documented.
   2.3. Habitat description should include plant species, abundance, area of coverage, and native or native descriptors. Management actions would be the quantity of non-native aquatic plants pre and post treatment. Treatments should target the reduction of non-native species and monitor the changes in native plant species.

3. eDNA water samples should be taken at 20 sites using 3X3 sampling design (mid channel, right bank, left banks at 3 depths such as below surface, mid-water column, and just above the bottom).
   3.1. Revisit sites and sample adjacent sites in the perimeter canal where Mayan cichlids and bullseye snakehead were collected.
      3.1.1. Mayan Cichlids-The current study collected information on 2 individual Mayan cichlids, one each of 2 separate sites (1.96%). The sites where Mayan cichlids were collected were 29 Km and 35 Km. Mayan cichlids abundance is 0.01% of all fish sampled (n=15,440). Mayan cichlid Total Body Weight was 354g.
      3.1.2. Bullseye Snakehead-The current study collected information 1 individual bullseye snakehead. It was collected at site 48 Km
      3.1.3. African Jewelfish-The current study collected no African jewelfish. These results are based on intensive sampling. These species appear very limited or not present after 102 samples within the Refuge. All three of these species are established in canals adjacent to the Refuge.
   3.2. Samples should also be collected from the Interior Marsh and other ponds and ditches on the Refuge. The current study did not sample these areas which may harbor aquatic invasive species of plants and animals.
   3.3. New data should be compared to this studies data to determine increases or decreases AIS detections (i.e., in number of locations), abundance, or percent of the community composition.

4. Prioritize prevention and control for aquatic species and protecting aquatic habitats. We recommend a study to determine the effort needed to remove AIS fishes and that the Refuge should consider incorporating existing prevention efforts/ practices.

4.1. Consider including existing protocols for equipment and boat cleaning. It is important to interact with vehicles and vessels that visit the Refuge as well as boats leaving the Refuge. One such approach would be to put in a wash station and provide simple messages such as "Clean, Drain, and Dry". There are several such campaigns that could inform the design of a successful program.
4.2. We recommend educating anglers and other visitors through signage about fishing regulations and invasive species. Include in this education program responsible pet ownership information. The Refuge could invest in a kiosk where different messages could be posted (e.g., invasive plants and animals). An inexpensive first step would be to include invasive species information and events on the Refuge website.
4.3. Determine if and what types of live bait are being used. Consider limits on types of live bait. Bait bucket release is known to be the way that some fish species get moved and introduced into new waterways.
4.4. Consider a boater education event that demonstrates how to clean boats and identify high risk AIS. This could be an opportunity to engage user groups about the aquatic resources on the Refuge and management actions to protect those resources. We recommend engaging social scientists to design surveys and studies that analysis these efforts.
4.5. Work on best practices and messaging for the disposal of AIS caught by anglers. Information on who to call when they have an unusual fish or what to do with known AIS is needed. Consider invasive species fishing derbies. Reach out to angler groups and ECISMA to host fish round ups that target non-native species.

5. Study LMB and Brown hoplo interactions using lavage to measure the presence of Brown hoplos and determine importance/ selectivity/ preference of Brown hoplos as a prey item for largemouth bass.
   5.1. Brown hoplo abundance and distribution is not known in the perimeter canal and this information is needed.
   5.2. Observations suggest that large individual largemouth bass feed on Brown hoplos and this observation needs to be investigated to determine if there is a preference and selection of large individual largemouth bass to feed on Brown hoplos.
   5.3. It is important to understand the predator-prey interaction. Largemouth bass may be adapting to this new prey and any efforts to control or any changes in the Brown hoplo population may affect largemouth bass.
   5.4. The population dynamics (diet, age structure, reproduction, parasite load) need to be examined to assist with management actions.

6. It is recommended that the NWR use fish data to assess site conditions and inform management actions. There are seasonal differences and localized differences in the fish assemblage. Several species are in need of further study. American eel, bluespotted sunfish, Atlantic needlefish, and longnose gar need further study to understand their distribution and population dynamics.

7. Study should be conducted to understand the number of species of "Sailfin catfish" and "Tilapia". We suspect that the "Sailfin catfish" and "Tilapia" may actually be several cryptic species each. Our suspicion is being pursued through other studies that will assist the

Refuge. Further genetics work would be needed to determine the exact composition of these species or hybrids, but we suspect there are 2-3 species of Sailfin catfish that we have grouped under one species and there may be 2 species, plus hybrids of Tilapia.

7.1. "Tilapia" populations need to examined to determine how many species and whether hybridization is occurring.

7.2. "Sailfin catfish" populations need to examined to determine how many species and whether hybridization is occurring.

7.3. This information is critical to management or control projects targeting these species for control.

7.4. There is a need to assess impacts caused by these species. An assessment needs to be done to determine if the integrity of the levee is being compromised by "Sailfin catfish" burrows. Information related to the location, depth, and density of burrows is needed.

8. We recommend immediate action be taken to do depletions sampling for bullseye snakehead and Mayan cichlids. It appears that these two species are in very low numbers and immediate action to removal all individuals should be taken.

8.1. We recommend a concentrated effort at the sites of last known capture for both these species. Electrofishing effort should be approximately 100 hours; because that was the approximate effort we expended to collect these species. If this effort were to be expended in a smaller area where the species were collected, the result would be a better understanding of the abundance of these species. Once water levels rise it may become difficult to contain these species, so sampling in low water conditions would be best.

8.2. On the Loxahatchee NWR website homepage should be updated and accurate information about the non-native species should be displayed. The current information is at the bottom of the page and not connected with fishing. The information is about the Northern snakehead which is not found at Loxahatchee NWR. Since it is more likely that anglers will encounter non-native fish and be able to identify these species, we suggest that anglers should be the target audience. Information on non-native species should be accompanied by color photos and information on how to dispose or report these species.

9. The diversity indices indicate a system that is represented by common species that are represented by only a few individuals. When this information is combined with the evenness scores, the results show an unstable and declining species assemblage. We recommend a study that examines various water chemistry parameters at several stations in the perimeter canal at different seasons.

9.1. This type of study would address environmental stressors and lead toward ways to ameliorate its impacts on fishes. We suggest investigating water quality, water quantity, and habitat quality and habitat quantity. There are hydrologic measurements being made which should be used to understand if and how changes in hydrology through management are influencing the fish community.

9.2. Some fish species are more tolerant than other species to disturbance. It will be important to monitor the fish community and compare with this baseline study. Management will be better informed over time as trends are documented. The metrics that are used to mark and track these trends should be sensitive and be able to trigger management actions. Changes in biological diversity indices, CPUE, PSD or RSD, are

the type of metrics that inform the Refuge managers.

10. We recommend continued monitoring of largemouth bass and in addition to the current biometrics data collected, there should be a mark-recapture study conducted to understand largemouth bass movement and habitat utilization.
    10.1. We recommend that the baseline for mean CPUE be 47.38 LMB/ hour. If this measure were to decrease by greater than 1 std. (std.=26.39) then management actions should be triggered. Management actions should be coordinated with Florida Fish and Wildlife Conservation Commission.
    10.2. There is a small proportion of largemouth bass that are large (i.e., Preferred, Memorable, Trophy). We recommend that monitoring largemouth bass PSD and RSD continue annually. When the PSD score drops below 40 and/ or RSD drops below 10, then management actions need to be triggered. Exact management actions should be coordinated with Florida Fish and Wildlife Conservation Commission. Examples of management actions may be to set slot limits, adjust timing of fishing tournaments, or limit the number of tournaments.
    10.3. A study to understand largemouth bass movement and survival is needed. We recommend a mark-recapture study or a sonar tag study to understand largemouth bass movements and habitat usage in the perimeter canal.

11. We recommend the continued use of genomic tools to support management decisions. Specifically, the use of eDNA for surveillance of high priority AIS targets.
    11.1. Return to sites near the collection of the bullseye snakehead and Mayan cichlids and sample using eDNA protocols as stated in this study. There is also much more work that could be done to enhance this tool and its use for quantitative results as well as expand the current work so it provides a useful tool for different taxa such as amphibians and mollusks.
    11.2. Consider the use of metabarcoding techniques for fish community work. This would entail a pilot project which would use the baseline of species and detections as detailed in the current study. The metabarcoding work would analyze all material in the water and match sequences to known species. This could detect species that were not found with electrofishing. The pilot would have the goal of determining and refining the amount of samples needed and the usefulness of the reference databases.

Appendix.1. Species collected during this study

Species Common Name	*Species Scientific Name*	*ITIS Number*	*Integrated Taxonomic Information System (ITIS) Species link*
American eel	Anguilla rostrata	161127	http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=161127
Atlantic needlefish	Strongylura marina	165551	http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=165551
Black crappie	Pomoxis nigromaculatus	168167	http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=168167
Bluespotted sunfish	Enneacanthus gloriosus	168113	http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=168113
Bluefin killifish	Lucania goodie	165680	http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=165680
Bluegill	Lepomis macrochirus	168141	http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=168141
Bowfin	Amia calva	161104	http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=161104
Brook silverside	Labidesthes sicculus	166016	http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=166016
Brown bullhead	Ameiurus nebulosus	164043	http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=164043
Brown Hoplo	Hoplosternum littorale	679689	http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=679689
Bullseye snakehead	Channa marulius	166663	http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=166663
Chain pickerel	Esox niger	162143	http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=162143
Channel catfish	Ictalurus punctatus	163998	http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=163998
Dollar sunfish	Lepomis marginatus	168152	http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=168152
Florida flagfish	Jordanella floridae	165694	http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=165694
Florida gar	Lepisosteus platyrhincus	161098	http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=161098
Gizzard shad	Dorosoma cepedianum	161737	http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=161737
Golden shiner	Notemigonus crysoleucas	163368	http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=163368
Lake chubsucker	Erimyzon sucetta	163922	http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=163922
Largemouth Bass	Micropterus salmoides	168160	http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=168160
Longnose gar	Lepisosteus osseus	161094	http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=161094
Mayan cichlid	Cichlasoma urophthalmus	169802	http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=169802
Redear sunfish	Lepomis microlophus	168154	http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=168154
Redfin pickerel	Esox americanus	162140	http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=162140
Seminole killifish	Fundulus seminolis	165667	http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=165667
Spotted sunfish	Lepomis punctatus	168155	http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=168155
Striped mullet	Mugil cephalus	170335	http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=170335
Warmouth	Chaenobryttus gulosus	168139	http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=168139
Yellow bullhead	Ameiurus natalis	164041	http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=164041

Appendix.2. Site name and Latitude/ Longitude

Site Name	*LAT*	*LON*	*Site Name*	*LAT*	*LON*	*Site Name*	*LAT*	*LON*
N001	**26.68309**	**-80.36673**	**N034**	**26.43627**	**-80.23003**	**N067**	**26.46520**	**-80.44062**
N002	**26.67525**	**-80.36198**	**N035**	**26.42750**	**-80.23236**	**N068**	**26.47318**	**-80.44460**
N003	**26.66707**	**-80.35775**	**N036**	**26.41875**	**-80.23470**	**N069**	**26.48215**	**-80.44525**
N004	**26.65890**	**-80.35352**	**N037**	**26.40998**	**-80.23703**	**N070**	**26.49116**	**-80.44527**
N005	**26.65072**	**-80.34930**	**N038**	**26.40099**	**-80.23715**	**N071**	**26.50017**	**-80.44528**
N006	**26.64344**	**-80.34357**	**N039**	**26.39198**	**-80.23709**	**N072**	**26.50917**	**-80.44529**
N007	**26.63672**	**-80.33687**	**N040**	**26.38344**	**-80.23870**	**N073**	**26.51818**	**-80.44524**
N008	**26.63098**	**-80.32923**	**N041**	**26.37607**	**-80.24446**	**N074**	**26.52719**	**-80.44518**
N009	**26.62605**	**-80.32081**	**N042**	**26.36870**	**-80.25022**	**N075**	**26.53619**	**-80.44518**
N010	**26.62112**	**-80.31239**	**N043**	**26.36497**	**-80.25819**	**N076**	**26.54520**	**-80.44516**
N011	**26.61621**	**-80.30396**	**N044**	**26.36490**	**-80.26822**	**N077**	**26.55421**	**-80.44515**
N012	**26.61130**	**-80.29552**	**N045**	**26.36485**	**-80.27826**	**N078**	**26.56321**	**-80.44511**
N013	**26.60635**	**-80.28712**	**N046**	**26.36284**	**-80.28765**	**N079**	**26.57222**	**-80.44508**
N014	**26.60114**	**-80.27899**	**N047**	**26.35791**	**-80.29605**	**N080**	**26.58123**	**-80.44499**
N015	**26.59385**	**-80.27309**	**N048**	**26.35775**	**-80.30441**	**N081**	**26.59023**	**-80.44489**
N016	**26.58655**	**-80.26720**	**N049**	**26.36057**	**-80.31394**	**N082**	**26.59797**	**-80.44031**
N017	**26.57927**	**-80.26128**	**N050**	**26.36339**	**-80.32347**	**N083**	**26.60535**	**-80.43454**
N018	**26.57198**	**-80.25537**	**N051**	**26.36619**	**-80.33301**	**N084**	**26.61273**	**-80.42877**
N019	**26.56466**	**-80.24958**	**N052**	**26.36890**	**-80.34258**	**N085**	**26.62011**	**-80.42300**
N020	**26.55709**	**-80.24413**	**N053**	**26.37160**	**-80.35216**	**N086**	**26.62750**	**-80.41725**
N021	**26.54944**	**-80.23883**	**N054**	**26.37431**	**-80.36173**	**N087**	**26.63493**	**-80.41155**
N022	**26.54181**	**-80.23350**	**N055**	**26.37709**	**-80.37126**	**N088**	**26.64235**	**-80.40585**
N023	**26.53331**	**-80.23016**	**N056**	**26.38376**	**-80.37768**	**N089**	**26.64978**	**-80.40016**
N024	**26.52480**	**-80.22688**	**N057**	**26.39115**	**-80.38342**	**N090**	**26.65720**	**-80.39446**
N025	**26.51628**	**-80.22360**	**N058**	**26.39855**	**-80.38915**	**N091**	**26.66454**	**-80.38862**
N026	**26.50744**	**-80.22242**	**N059**	**26.40595**	**-80.39487**	**N092**	**26.67198**	**-80.38294**
N027	**26.49843**	**-80.22226**	**N060**	**26.41335**	**-80.40060**	**N093**	**26.67940**	**-80.37724**
N028	**26.48943**	**-80.22209**	**N061**	**26.42075**	**-80.40632**	**N094**	**26.68107**	**-80.36812**
N029	**26.48042**	**-80.22190**	**N062**	**26.42816**	**-80.41204**			
N030	**26.47141**	**-80.22163**	**N063**	**26.43557**	**-80.41776**			
N031	**26.46256**	**-80.22307**	**N064**	**26.44297**	**-80.42347**			
N032	**26.45380**	**-80.22539**	**N065**	**26.45038**	**-80.42919**			
N033	**26.44503**	**-80.22771**	**N066**	**26.45779**	**-80.43491**			

Appendix.3. Taxonomic confirmation of aquatic species for Loxahatchee National Wildlife Refuge via DNA barcoding

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Traditional species identification is reliant on hierarchical taxonomic identification keys of phenotypic characters; in contrast, DNA barcoding aims to provide an alternative method for species-level identifications using a species specific molecular tag derived from the 5′ region of the mitochondrial cytochrome c oxidase I (*COI*) gene (Hubert et al. 2008). In this technique, the specimen in question is sequenced using COI molecular tag, and the sequence compared to a known reference sequence that is coupled to a voucher specimen. If the unknown sequence shares high similarity to the voucher sequence, the unknown specimen is considered the same species as the voucher specimen. Despite the great promise of DNA barcoding, it has been controversial because the efficiency of the method hinges on the degree of sequence divergence among species. Species-level identifications are relatively straightforward when the average genetic distance among individuals within a species does not exceed the average genetic distance between sister species. As a result, DNA barcoding can become arduous for species that have diverged rather recently (i.e., average genetic distance among individuals within a species tends to exceed the average genetic distance between sister species) or for organisms that have undergone a recent hybridization event. Despite these potential pitfalls, studies have illustrated some straightforward benefits from the use of a standardized molecular approach for species identification (Hebert et al. 2003; Hebert and Gregory 2005). First, intraspecific phenotypic variation often overlaps that of sister taxa in nature, which can lead to incorrect identifications if based on phenotype only (Pfenninger et al. 2006; Moyer and Diaz-Ferguson 2012). Second, DNA barcodes are effective whatever the life stage under scrutiny (Caterino and Tishechkin 2006). Third, cryptic variation and often spectacular levels of undetected taxonomic diversity have been frequently reported (Hebert et al. 2004; Witt et al. 2006; Smith et al. 2007). Finally, DNA barcode libraries are fully available as they are deposited in a major sequence database,

and attached to a voucher specimen whose origin and current location are recorded (Hebert and Gregory 2005; Hebert et al 2004).

The Fish Barcode of Life Initiative (FISH-BOL; www.fishbol.org) is a DNA barcode reference library for all fish species derived from voucher specimens with authoritative taxonomic identifications (Ward et al. 2009). FISH-BOL allows for a fast, accurate, and cost-effective system for molecular identification of the world's ichthyofauna. The benefits of this work include facilitating species identification, flagging potentially previously unrecognized species, and enabling identifications where traditional methods are not applicable, such as for immature stages or body fragments. Herein, we provide DNA barcode confirmation of species identification for specimens collected from Loxahatchee National Wildlife Refuge.

Methods

Tissue samples (n = 106) were obtained by United States Fish and Wildlife biologists via boat electrofishing on waters in the Loxahatchee National Wildlife Refuge and placed in individually labeled vials containing 1 mL of 95% non denatured ethanol. All tissue samples were archived at the USFWS Conservation Genetics Laboratory in Warm Springs, GA. DNA was extracted from each tissue sample using the DNeasy Blood and Tissue kit (QIAGEN, Inc., Valencia, California). Final DNA templates were eluted in 200 uL of AE buffer (QIAGEN, Inc) and DNA concentration (50-400 ng/uL) estimated using a Nanodrop spectrophotometer (Thermo Scientific, Inc).

A partial coding segment of the mtDNA *COI* gene approximating 655 nucleotides (nt) in length was targeted. The segment of *COI* was amplified via the polymerase chain reaction (PCR) using universal FishF2 and FishR2 primers (Ward et al. 2005) in a 20 μL reaction volume containing approximately 100 ng/μL DNA, 0.5 × *Taq* reaction buffer (GoTaq Flexi, Promega,

Madison, WI), 3.0 mM $MgCl_2$, 0.25 mM of each dNTP, 0.50 μM of each primer, and 0.05 U Taq DNA polymerase (GoTaq, Promega, Madison, WI). Optimized thermal cycle conditions for *COI* were an initial 94 ˚C (2 min) denaturation followed by 35 cycles of 95 ˚C (1 min.), 55˚C (1 min.), and 72 ˚C (1 min.) and a final 72 ˚C (4 min.) extension.

PCR products were cleaned using the QIAquick Purification Kit (QIAGEN, Inc). Cycle sequencing was conducted following the Big Dye Terminator v3.1 protocol (Applied Biosystems, Inc., Foster City, CA) using the FishF2 primer under the following thermal cycle parameters: 25 cycles of 96˚C for 10 s, 50˚C for 5 s and 60˚C for 4 min. Cycle sequencing PCR products were purified using the BigDye XTerminator Purification kit (Applied Biosystems, Inc.), visualized on an ABI PRISM 3130 genetic analyzer (Applied Biosystems, Inc.), and analyzed with Sequence Analysis software v5.2 (Applied Biosystems, Inc.). Each sequence was then submitted to The Barcode of Life Data System (BOLD; http://www.barcodinglife.org) following the methods outlined by Ratnasingham and Hebert (2007). BOLD categorizes barcode records in its reference (search) library as either verified or unvalidated. Verified barcodes are defined as species with a minimum of three representatives and a maximum conspecific divergence of two percent. Unvalidated barcodes do not meet these criteria. Thus, for each sequence that we submitted to BOLD, we reported the percent sequence similarity given by BOLD and categorized the sequence as either verified or unvalidated. Often BOLD reported unvalidated sequences as having more than one species as a possible candidate. In these cases we reported all potential species return under the BOLD search criteria.

RESULTS/DISCUSSION

A total of 106 tissue samples were analyzed. One sample (USFWS 194) failed to PCR amplify even after repeated attempts and DNA re extraction. Sequence lengths of the remaining

samples were all approximately 500 nt long. No insertions, deletions or stop codons were observed in any sequence. The lack of stop codons is consistent with all amplified sequences being functional mitochondrial *COI* sequences. Thirty one of 105 (30%) sequences were verified by BOLD (Table 1). While BOLD categorized the remaining 74 sequences as unvalidated, they had a high (often >95%) sequence similarity to the field (morphological) species identification (Table 1).

CONCLUSIONS

Freshwater fish species can be efficiently identified or verified through the use of DNA barcoding; however, some species may be problematic due to high sequence similarity or the lack of validated sequence data in BOLD.

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Table 1. Species identification and confirmation using mtDNA *COI* barcoding. Percent similarity is the percent sequence similarity between the specimen of interest and that found in the Barcode of Life database. Verified barcodes are defined as species with a minimum of three representatives and a maximum conspecific divergence of two percent. Unvalidated barcodes do not meet these criteria. An asterisk represents individuals where field (morphological) and mtDNA barcoding results do not match.

USFWS#	Morphological identification	Bold results (Genus)	Bold results (species)	% Similarity	BOLD categorization
81	Brown Bullhead	*Ameiurus*	*nebulosus*	99.81	verified
82	Inland Silverside	*Labidesthes*	*sicculus*	98.43	unvalidated
85	Inland Silverside	*Labidesthes*	*sicculus*	97.00	unvalidated
86	Inland Silverside	*Labidesthes*	*sicculus*	98.87	verified
87	Inland Silverside	*Labidesthes*	*sicculus*	97.74	unvalidated
88	Chain Pickerel	*Esox*	*americanus; niger*	99.61	unvalidated
89	Chain Pickerel	*Esox*	*americanus; niger*	97.52	unvalidated
90	Chain Pickerel	*Esox*	*americanus; niger*	94.07	unvalidated
92	Dollar sunfish	*Lepomis*	*marginatus*	99.62	verified
93*	Dollar sunfish	*Enneacanthus*	*obesus; gloriosus*	97.61	unvalidated
94	Dollar sunfish	*Lepomis*	*marginatus*	98.89	verified
95	Bluespotted sunfish	*Enneacanthus*	*obesus; gloriosus*	99.25	unvalidated
181	Bluespotted sunfish	*Enneacanthus*	*obesus; gloriosus*	99.04	unvalidated
182	Bluespotted sunfish	*Enneacanthus*	*obesus; gloriosus*	99.43	unvalidated
183	Bluespotted sunfish	*Enneacanthus*	*obesus; gloriosus*	99.23	unvalidated
184	Inland Silverside	*Labidesthes*	*sicculus*	96.30	unvalidated
185	Bluefin Killifish	*Lucania*	*goodei*	100.00	verified
186	Yellow bullhead	*Ameiurus*	*natalis*	96.02	unvalidated
187	Mosquitofish	*Gambusia*	*holbrooki; affinis*	99.80	unvalidated
188	Mosquitofish	*Gambusia*	*holbrooki; affinis*	99.39	unvalidated
189	Mosquitofish	*Gambusia*	*holbrooki; affinis*	99.80	unvalidated
190	Black Crappie	*Pomoxis*	*nigromaculatus*	96.46	unvalidated
191	Brown Bullhead	*Ameiurus*	*nebulosus*	95.08	unvalidated
192	Tilapia sp. (?Nile)	*Oreochromis*	*sp.; aureus*	98.55	verified
193	Tilapia sp. (?Nile)	*Oreochromis*	*sp.; aureus; niloticus*	99.80	unvalidated
194	Chain Pickerel	unable to PCR			

195	Chain Pickerel	*Esox*	*americanus; niger*	99.61	unvalidated
196	Spotted Sunfish	*Lepomis*	*punctatus*	99.29	verified
197	Lake chubsucker	*Erimyzon*	*oblongus or sucetta*	97.68	unvalidated
198	Lake chubsucker	*Erimyzon*	*sucetta*	65.34	unvalidated
199	Lake chubsucker	*Erimyzon*	*sucetta*	90.40	unvalidated
200	Spotted Sunfish	*Lepomis*	*punctatus*	97.30	unvalidated
201	Golden shiner	*Notemigonus*	*crysoleucas*	98.28	unvalidated
203	Brown Bullhead	*Ameiurus*	*nebulosus*	99.25	verified
204	Brown Bullhead	*Ameiurus*	*nebulosus*	98.98	verified
205	Mosquito fish	*Gambusia*	*holbrooki; affinis*	99.79	unvalidated
206	Mosquitofish	*Gambusia*	*holbrooki; affinis*	99.80	unvalidated
207	Gizzard Shad	*Dorosoma*	*cepedianum; anale*	99.79	unvalidated
208	Sailfin catfish	*Hypostomus; Pterygoplichthys*	*plecostomus; pardali; disjunctivuss; joselimaianus*	99.47	unvalidated
209	Sailfin catfish	*Hypostomus; Pterygoplichthys*	*plecostomus; pardali; disjunctivuss; joselimaianus*	95.38	unvalidated
210	Lake chubsucker	*Erimyzon*	*sucetta*	95.52	unvalidated
211	Channel catfish	*Ictalurus*	*punctatus*	99.79	verified
212	Bluegill	*Lepomis*	*macrochirus*	99.48	verified
213	Bluespotted sunfish	*Enneacanthus*	*obesus; gloriosus*	99.41	unvalidated
214	Inland Silverside	*Labidesthes*	*sicculus*	97.98	unvalidated
215	Black Crappie	*Pomoxis*	*nigromaculatus*	96.00	unvalidated
216	Black Crappie	*Pomoxis*	*nigromaculatus*	97.00	unvalidated
217	Warmouth	*Lepomis*	*gulosus*	98.56	verified
218	Golden shiner	*Notemigonus*	*crysoleucas*	96.96	unvalidated
219	Golden shiner	*Notemigonus*	*crysoleucas*	98.45	unvalidated
220	Dollar sunfish	*Lepomis*	*marginatus*	99.40	verified
221	Black crappie	*Pomoxis*	*nigromaculatus*	96.00	unvalidated
826	Black Crappie	*Pomoxis*	*nigromaculatus*	97.00	unvalidated
827	Lake Chubsucker	*Erymyzon*	*oblongus; sucetta*	93.74	unvalidated
828	Yellow bullhead	*Ameiurus*	*natalis*	89.48	unvalidated
829	Gizzard Shad	*Dorosoma*	*cepedianum*	99.02	unvalidated

830	Gizzard Shad	*Dorosoma*	*cepedianum; anale*	99.82	unvalidated
831	Bowfin	*Amia*	*Calva*	98.75	verified
832	Bowfin	*Amia*	*Calva*	98.77	verified
833	Not given	*Micropterus*	*salmoides; floridanus*	99.40	unvalidated
834	Bowfin	*Amia*	*Calva*	99.05	verified
835	Largemouth Bass	*Micropterus*	*salmoides; floridanus*	99.65	unvalidated
16349	Not given	*Lepomis*	*gulosus*	93.13	unvalidated
16401	Largemouth Bass	*Micropterus*	*salmoides; floridanus*	98.42	unvalidated
16402	Dollar sunfish	*Lepomis*	*marginatus*	99.26	verified
16403	Redear	*Lepomis*	*microlophus*	99.82	verified
16509	Sailfin catfish	*Hypostomus; Pterygoplichthys*	*plecostomus; pardali; disjunctivuss;*	97.49	unvalidated
16510	Sailfin catfish	*Hypostomus; Pterygoplichthys*	*plecostomus; pardali; disjunctivuss;*	100.00	unvalidated
16511	Florida Gar	*Lepisosteus*	*oculatus; platyrhincus*	99.09	unvalidated
16512	Florida Gar	*Lepisosteus*	*oculatus; platyrhincus*	99.26	unvalidated
16513	Florida Gar	*Lepisosteus*	*oculatus; platyrhincus*	98.89	unvalidated
16514	Florida Gar	*Lepisosteus*	*oculatus; platyrhincus*	98.90	unvalidated
16515	Florida Gar	*Lepisosteus*	*oculatus; platyrhincus; osseus*	99.45	unvalidated
16516	Bowfin	*Amia*	*Calva*	98.49	unvalidated
16517	Bowfin	*Amia*	*Calva*	92.03	unvalidated
16518	Redear	*Lepomis*	*microlophus*	99.82	verified
16519	Redear	*Lepomis*	*microlophus*	97.83	unvalidated
16520	Gizzard Shad	*Dorosoma*	*cepedianum; anale*	99.81	unvalidated
16521	Gizzard Shad	*Dorosoma*	*cepedianum; anale*	95.67	unvalidated
16522	Warmouth	*Lepomis*	*gulosus*	93.57	unvalidated
16523	Warmouth	*Lepomis*	*gulosus*	99.08	verified
16524	Warmouth	*Lepomis*	*gulosus*	97.42	unvalidated
16525	Warmouth	*Lepomis*	*gulosus*	97.78	unvalidated
16526	Yellow bullhead	*Ameiurus*	*natalis*	87.83	unvalidated
16527	Tilapia sp. (?Nile)	*Oreochromis*	*sp.; aureus; niloticus*	99.81	unvalidated
16528	Golden Shiner	*Notemigonus*	*crysoleucas*	96.34	unvalidated

16529	Golden Shiner	*Notemigonus*	*crysoleucas*	97.52	unvalidated
16530	Bluegill	*Lepomis*	*macrochirus*	100.00	verified
16531	Bluegill	*Lepomis*	*macrochirus*	98.73	verified
16532	Bluegill	*Lepomis*	*macrochirus*	99.27	verified
16533	Bluegill	*Lepomis*	*macrochirus*	98.96	verified
16534	Bluegill	*Lepomis*	*macrochirus*	99.13	verified
16535	Redear	*Lepomis*	*microlophus*	97.79	unvalidated
16536	Redear	*Lepomis*	*microlophus*	95.66	unvalidated
16537	Redear	*Lepomis*	*microlophus*	99.43	verified
18746	Yellow bullhead	*Ameiurus*	*natalis*	95.83	unvalidated
18747	Mayan Cichlid (Whole specimen in Jar)	*Cichlasoma*	*urophthalmus*	99.65	verified
18748	Not given	*Oreochromis*	*aureus*	100.00	unvalidated
18749	Tilapia sp. (?Nile)	*Oreochromis*	*aureus*	100.00	verified
18750	Mayan Cichlid (Whole specimen in Jar)	*Cichlasoma*	*urophthalmus*	100.00	verified
18751	Yellow bullhead	*Ameiurus*	*natalis*	92.32	unvalidated
18752	Longnose Gar	*Lepisosteus*	*osseus*	98.11	unvalidated
18753	Not given	*Fundulus*	*catenatus*	89.41	unvalidated
18754	Spotted Sunfish	*Lepomis*	*punctatus*	99.63	verified
18756	Spotted Sunfish	*Lepomis*	*punctatus*	100.00	verified
18757	Striped Mullet	*Mugil*	*cephalus*	100.00	unvalidated

Appendix.4. Objective II. Development of environmental DNA methodology for early detection of aquatic invasive species in Loxahatchee National Wildlife Refuge.

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Summary of major findings

1) Species-specific primers for eDNA detection via traditional PCR agarose gel visualization and qPCR were developed for *Hemichormis letourneuxi* and *Channa marulius.*

2) Theoretical qPCR detection threshold levels for *H. letourneuxi* and *C. marulius* were approximately 0.0002 ng/uL (R^2 = 0.89) and 0.005 ng/uL (R^2 = 0.94) at a PCR cycling threshold of 28.5-29 and 22-23 amplification cycles, respectively

3) There was a positive and significant relationship between fish density and eDNA detection with detection probabilities ranging from 0.32-1.00 depending on fish density.

4) Environmental DNA persisted in controlled tank experiments for up to 24 days post removal of *H. letourneuxi* and *C. marulius* with minimal degradation, but between 24 and 31 days DNA concentration and 260/280 optical density readings decreased significantly.

5) The only significant (*P* = 0.0299) factor influencing DNA persistence in controlled tank experiments for the eight estimated abiotic parameters held over an eight day period was temperature. Degradation of DNA occurred between 25°C and 33°C

6) The use of qPCR for eDNA detection along with confirmation from direct sequencing of positive PCR reactions should provide a reliable method for the detection of *H. letourneuxi* and *C. marulius* when their densities are greater than threshold values and PCR inhibition is minimized.

INTRODUCTION

Environmental DNA (eDNA) refers to tissue fragments that a species leaves behind in the environment. Therefore to test for the presence/absence of an aquatic species, tissues suspended in the water column can be collected and a known volume of water filtered on fine micron screens to trap the tissue. The eDNA can then be extracted from the tissue on the filter and screened for the presence of a taxon (or taxa) of interest via the polymerase chain reaction (PCR) and molecular markers specific to the target taxon. Presence is typically confirmed by one or all of the following methods: agarose gel electrophoresis of PCR product, quantitative PCR (qPCR), or direct sequencing of the PCR product.

The basic technique outlined above, raises the possibility to monitor and detect representatives of target taxa in an environmental sample that are extremely rare and eliminate the extraneous noise generated by the multiplicity of non-target taxa. Thus eDNA as a monitoring method will have broad research and management applicability in freshwater, estuarine, and marine ecosystems for threatened and endangered species and for invasive species. However, recognizing this rare signal can be arduous because identification of the specimens requires both accuracy and sensitivity (Darling and Blum 2007; Dejean et al 2012).

The goal of this study was to explore the utility of eDNA detection methods for use with two (*Hemichromis letourneuxi and Channa marulius*) aquatic invasive species that are of concern to Loxahatchee National Wildlife Refuge. We achieved this goal with the following five objectives: 1) development of species specific molecular markers for *H. letourneuxi* and *C. marulius*), 2) estimation of the theoretical detection threshold levels for qPCR using known control amounts of DNA, 3) comparison of eDNA detection methods from aquarium trials where known numbers of individuals in known volumes of waters were used, 4) estimation of detection probabilities for

each eDNA detection method, and 5) assessing the influence of abiotic factors on eDNA persistence.

Considered a predatory cichlid fish, the African jewelfish, *H. letourneuxi*, was introduced in Florida during the early 1960s (Rivas 1965) and has expanded throughout Florida and has been observed in canals adjacent to Loxahatchee National Wildlife Refuge. To date there are no reports of this species in Loxahatchee National Wildlife Refuge. Regardless, *H. letourneuxi* has been thought to compete with native sunfishes (Schofield et al 2007) along with other non native species (Porter et al 2012) such as the spotted tilapia (*Tilapia mariae*), blue tilapia (*Oreochromis aureus*), the Nile tilapia (*O. nilotica*), Mayan cichlids (*Cichlasoma urophthalmus*) and the black acara (*Ci. bimaculatum*). The introduction of this predator represents a significant threat to aquatic species within the confines of Loxahatchee National Wildlife Refuge.

Very little is known regarding the interaction of *C. marulius* (bullseye snakehead) with native aquatic species; however, given the closely related congener *C. argus* (the northern snakehead) it is presumed to be a top predator, competing for habitat, spawning areas and prey with native species at the same trophic level. The species, which is native to Asia, was introduced to Florida during the early 2000s. Individuals of the species were first reported in the Loxahatchee National Wildlife Refuge in 2012 (J. Galvez, USFWS, personal communication). Like *H. letourneuxi* the introduction of *C. marulius* poses a potential threat to all levels of community structure for aquatic fauna in Loxahatchee National Wildlife Refuge.

Our investigation provides new genetic tools for aquatic invasive species detection in an effort to potentially reduce time and cost of traditional inventory and detection methods. This research also establishes a standard protocol for eDNA detection that can be used for detection and monitoring of other aquatic species across the United States.

MATERIAL AND METHODS

Collection and DNA extraction of tissue samples.

Tissue samples of *H. letourneuxi* (n= 10) and *C. marulius* (n = 19) were obtained by United States Fish and Wildlife (USFWS) biologists via boat electrofishing at Hillsboro canal (Broward county) FL and placed in individually labeled vials containing 1 mL 95% non denatured ethanol. All tissue samples were archived at the USFWS Conservation Genetics Laboratory in Warm Springs, GA. DNA was extracted from each tissue sample using the DNeasy Blood and Tissue kit (QIAGEN, Inc., Valencia, California). Final DNA templates were eluted in 150 µL of AE buffer (QIAGEN, Inc), which yielded DNA concentrations ranging from 50-150 ng/µL.

Molecular marker development

Molecular marker development is a critical first step in eDNA aquatic species monitoring and detection because the marker must be species-specific to ensure species detection. We targeted a partial coding segment of the mtDNA *cytochrome oxidase I* gene (*COI*). Specifically, 658 and 560 nucleotide (nt) segments of *COI* from *H. letourneuxi* and *C. marulius* were PCR amplified using *COI* primers known to amplify in these two species (Table 1). Reactions (20 µL reaction volume) contained 4 µL DNA (15-200ng), 2.0 µL of 5× *Taq* reaction buffer (GoTaq Flexi, Promega, Madison, WI), 2.5 µL $MgCl_2$ (25mM) 0.5 µL of each dNTP (1mM), 1 µL of each primer (10 µM each), and 0.20 µL *Taq* DNA polymerase (5 U/µL; GoTaq, Promega). Optimized thermal cycle conditions for *COI* were an initial 94˚C (5 min.) denaturation followed by 35 cycles of 95˚C (1 min.), 62˚C for *H. letourneuxi* or 56˚C for *C. marulius* (1.30 min.), and 72˚C (1 min.). An additional 7 min. extension at 72˚C was added at the end of the reaction. PCR products were visualized on 1% agarose gels, cleaned using the QIAquick Purification Kit (QIAGEN, Inc), and eluted with 30 µL EB buffer (QIAGEN, Inc). Cycle sequencing was conducted following the Big Dye Terminator v3.1 protocol (Applied Biosystems, Inc., Foster City, CA) using forward and reverse primers outlined above under the following PCR thermal profile: 25 cycles of 96˚C for 10

s, 50˚C for 5 s and 60˚C for 4 min. Cycle sequencing PCR products were purified using the BigDye XTerminator Purification kit (Applied Biosystems, Inc.) and then run on an ABI PRISM 3130 genetic analyzer (Applied Biosystems, Inc.). All sequences were imported into BioEdit Sequence Alignment Editor (Hall 1999), ends trimmed, and the remaining sequence aligned by eye. From the sequence data and that published in Genbank (four sequences of *H. letourneuxi* and two sequences of *C. striata*) we developed species-specific PCR primers for each taxon (Table 1). Primers were designed using Primer Express 3.0 (Applied Biosystems, Inc). Primer specificity was tested by comparing the selected primer sequences to all previously published sequence data using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast; default settings). We also tested for cross species amplification using *Micropterus salmoides* (fish), *Amblema neislerii* (freshwater mussel) and *Cittarium pica* (marine gastropod) as template DNA (PCR reaction conditions given previously).

***qPCR* primers and probe design**

While traditional agarose gel electrophoresis of PCR products can be used to detected species presence, qPCR is often more sensitive than traditional detection methods. This technique relies on the development of two primers and a probe internal to each species specific PCR primer set. Internal *COI* primers and probe sequence for each species were designed from *COI* alignments for *H. letourneuxi* and *C. marulius* using Primer Express 3.0 (Applied Biosystems, Inc) and corroborated by the online software program Genscript (http://www.genscript.com). As above primer specificity was tested by comparing the selected primer sequences to all previously published sequence data using the Basic Local Alignment Search Tool

Theoretical lower limit of detection

We used a qPCR standard curve analysis to determine theoretical threshold levels of DNA for qPCR detection. Specifically, we started with a 20 ng/µL sample for both species and performed a 1:10 serial dilution to $1{:}1x10^{-6}$. For standard curve analyses, we randomly selected three pure DNA samples per species (i.e., samples whose DNA was used for sequencing and marker development). Taqman assays (Applied Biosystems, Inc.) consisted of 20 µL reaction volumes and contained 4 µL of DNA solution from each dilution, 2.0uL of 5× *Taq* reaction buffer (Applied Biosystems, Inc), 2.5 µL $MgCl_2$ (25mM), 0.5 µL of each dNTP (1 mM), 1 µL of each primer (10uM each), 0.4 µL species specific probe (10 µM), 0.5ul AmpErase (Uracil-N-glycosylase), and 0.20 µL *Taq* DNA polymerase (5 U/µL; Amplitaq Gold, Applied Biosystems, Inc). All qPCR Taqman assays were run using the following thermal profile: 60˚C (1min), initial denaturation at 95˚C for 10min., followed by 40 cycles of 95˚C (15 s) and 60˚C (1 min.) Detection of DNA from each dilution and random sample was performed using a 7500 Fast Real Time PCR machine (Applied Biosystems, Inc.).

Field trials: proof-of-concept

For each species, 30 individuals were collected from Hillsboro canal (Broward County, FL), transported to a quarantine facility located at the USFWS Warm Springs Regional Fisheries Center (aquarium trials were conducted in March 2012 for *H. letourneuxi*, and June 2012 for *C. marulius*), and held at the quarantine facility during one week aquarium trials. Aquarium trials consisted of four treatments (0, 1, 3, and 6 individuals per gallon) with three replicates per treatment. Each treatment consisted of a 94.6 L aquarium filled with 75.7 L of water, an aquarium heater (set to 26˚C, which approximated the temperature of the canal water), and an air stone. After an initial acclimation period of three days, a 1 L water sample was taken from each aquarium on days 3, 5, and 7 of the experiment. Each water sample was treated with 1 mL of 3 M sodium acetate (pH 5.2) and 33 mL 95% non-denature ethanol for DNA preservation (see

Appendix 1). All samples were stored at 4˚C until DNA extraction was performed. At the end of each trial, all fish were weighed, euthanized with MS-222, and stored at -20˚C.

DNA extraction from water samples

Using a vacuum pump, we filtered each 1 L water sample through a sterile cellulose nitrate filter (0.45 μm). After filtration, filters were dried at 56˚C for 10 min. and DNA extracted using the protocol outlined by the Rapid Water DNA isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA). Extracted DNA was suspended in 70ul of buffer provided by the kit and quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc, Waltham, MA). For DNA yields lower than 15 ng/μL and optical density readings (260/230) lower than 1.5, standard ethanol precipitation of DNA was conducted (Sambrook et al. 1989) to increase DNA concentration. All template pellets were re-suspended with 25 μL AE buffer (QIAGEN, INC).

Detection of eDNA from aquarium trials and estimation of detection probabilities

There are various methods used to detect eDNA from water samples. We compared the utility of standard visualization via staining a PCR product in a gel to qPCR that detects and measures the number of gene copies during every cycle of the PCR. For each species, we performed PCR and qPCR from aquarium trials as outlined above and recorded either the presence or absence of the DNA template for each detection method. If presence was observed, then we sequenced each PCR product for species confirmation.

We performed generalized linear regression (probit link function) where the response variable was the observed presence or absence of eDNA for each water sample and continuous predictor variables were fish density (no. of fish/tank), DNA concentration of extracted water sample, and time of water collection (days). Note that we explored using a random intercept (data not shown) to account for dependence among each of the three replicate samples, but results were similar indicating dependence was not a significant problem. In some cases ANOVA

analyses (Sokal and Rohlf 1995) were used instead of generalized linear regression due to the lack of variability among treatments (see Results). We tested for significance of eDNA detection among the two competing methods using the Kruskal-Wallis rank sum test (Sokal and Rohlf 1995). All statistical analyses were conducted using the program S-Plus v7.0 (Insightful Corp., New York, NY)

We estimated detection probabilities for each eDNA detection method using the following logit back-transformed equation:

$$\text{prob.(PCR detect)} = 1/(1+\exp(-(\text{intercept} + \text{slope}*\text{density})))$$

where intercept and slope values were estimated for generalized linear regression (logit link function) using detection method as the dependent variable and density as the independent variable. Note that the above technique was done for each species and detection method except for *H. letourneuxi* qPCR due to the lack of variability in the datasets (see Results).

eDNA persistence

We performed several experiments to assess the influence of abiotic factors on eDNA persistence. First, upon completion of our aquarium trials, we removed all fish from aquaria and allowed any eDNA to persist in the water for up to 31 days post fish removal. Persistence of the DNA molecule was evaluated by removing 1 L of water at 7, 14, 24 and 31 days and testing for the incidence of eDNA using the DNA preservation, extraction, and qPCR approaches outlined above. Significance of factors (time, and density) on eDNA detection was assessed via ANOVA as implemented by S-PLUS. Second, at the end of the *H. letourneuxi* aquarium trials, we obtained 500 mL water samples from one tank (density of three fish, replicate II) and subjected these samples to four temperatures (8°C, 15°C, 25°C and 33°C). On days 0, 4, and 8 we estimated the following eight water quality parameters: pH, ammonia nitrogen, nitrate nitrogen, alkalinity, carbon dioxide, chlorine, hardness, and dissolved oxygen. Water quality parameters were

estimated using the Fresh Water Aquaculture Test Kit (LaMotte, Chestertown, Maryland) following the manufacturer's instructions. From these same samples, we extracted DNA, estimated DNA concentration (and 260/280 optical densities), and visually assessed DNA quality via agarose gel electrophoresis (methods outlined above). An ANOVA was performed to assess the significance of abiotic factors on DNA concentration and 260/280 optical density readings.

RESULTS

Molecular marker development

From aligned sequences for each species, specific *COI* primers for *H. letourneuxi* and *C. marulis* were developed (Table 1). Primers AJFF3 and PROS2 amplified a 240 nt *COI* segment in *H. letourneuxi*. We used this 240 nt segment of *H. letourneuxi* to develop primers AJFq3 and AJFR2Q2 along with probe PCOAJF6 (Table 1). For *C. marulius,* specific primers CMnewF1 and FishR1 amplified a 439 nt segment of *COI* from which primers FCM2 and Rcomp2C and probe P2CMCO1 were developed (Table 1). Note that the 5' and 3' end of each probe was labeled with florescent dyes 6-FAM and Tamra, respectively (Table 1). When sequences from the *H. letourneuxi* primer pair were subjected to the Basic Local Alignment Search, the only reported query to return both primer sequences was for *H. letourneuxi.* We found identical results for *C. marulius*. We observed no cross species amplification using fish, freshwater mussel, and gastropod DNA for PCR amplification.

Theoretical lower limit of detection

Using serial dilutions of known amounts of DNA, we found that the lower limit of eDNA detection for *H. letourneuxi* was approximately 0.0002 ng/µL (R^2 = 0.89) at a PCR cycling threshold of 28.5-29 amplification cycles (Fig. 1a). In contrast, the lower limit for *C. marulius* was approximately 0.005 ng/µL (R^2 = 0.94) at a cycling threshold of 22-23 amplification cycles (Fig 1b).

Detection of eDNA from aquarium trials and estimation of detection probabilities

For *H. letourneuxi,* average biomass per tank at different densities was 5.49 g (1 fish), 15.82 g (3 fishes) and 30.71 g (6 fishes). Average biomass for *C. marulius* was 44.46, 97.88, and 197.94 g for 1, 3, and 6 fish per tank, respectively. While there was no significant (both $P > 0.70$) difference among detection methods for each species, qualitative inspection of *H. letourneuxi* presence and absence data for aquarium trials indicated that qPCR was a more sensitive method for eDNA detection than standard PCR visualization on an agarose gel (Tables 2 and 3). At densities of one fish/tank for *H. letourneuxi*, all methods failed to detect the presence of eDNA in the water column (Table 2), but not for *C. marulius* (Table 3). For both species, generalized linear regression of traditional PCR detection method data indicated that there was no significant relationship between time and detection or DNA concentration (i.e., average DNA concentration of replicate water filtered samples) and detection; however, the relationship between density (no. fish/tank) and PCR detection was positive and significant (Tables 4 and 5). We had trouble fitting a generalized linear model to the *H. letourneuxi* qPCR data presumably because densities of 3 and 6 always resulted in detections, whereas densities of 1 never resulted in a detection (Tables 2 and 3). ANOVA analyses of these data indicated a positive and significant (sums of squares = 6.857; df = 1 $P < 0.0001$) relationship between density (no. fish/tank) and qPCR detection method.

For *H. letourneuxi,* we estimated detection probabilities for the traditional PCR eDNA detection method using the following logit back-transformed equation:

$$\text{prob.(PCR detect)} = 1/(1+\exp(-(-\text{intercept} + \text{slope}*\text{density}))),$$

given values estimated in Table 4. Detection probabilities were 0.32, 0.54, and 0.82 for 1, 3, and 6 fish/tank, respectively. In contrast, *H. letourneuxi* eDNA detection probabilities for qPCR and sequencing methods were 0.00, 1.00, 1.00 for 1, 3, and 6 fish/tank (Table 2). For *C. marulius,*

using the equation above and values given in Table 5, detection probabilities for the tradition PCR detection method were 0.42, 0.53, and 0.63 for 1, 3, and 6 fish per tank, respectively. Using the same equation but changing the intercept and slope values for the qPCR *C. marulius* data yielded detection probabilities of 0.46, 0.59, and 0.76 for 1, 3, and 6 fish/tank, respectively. Finally, all positive detections were confirmed to be either *H. letourneuxi* or *C. marulius* via sequencing of the detected PCR product except two *H. letourneuxi* samples (Table 2). For these samples, a positive (but faint) band was detected on the agarose gel; however, qPCR failed to detect the presence of *H. letourneuxi*, and sequencing of the PCR product was unsuccessful.

eDNA persistence

Environmental DNA persisted in the water column for up to 24 days post removal of *H. letourneuxi* and *C. marulius* with minimal degradation, but between 24 and 31 days DNA concentration and optical density (260/280) readings decreased (Table 6). We found a negative and significant relationship between DNA concentration and time, as well as, optical density readings and time (Table 7). The only significant (P = 0.0299) factor influencing DNA persistence for the eight estimated abiotic parameters held over an eight day period was temperature (Tables 8 and 9). Degradation of DNA occurred between 25°C and 33°C (Table 8; Fig. 2).

DISCUSSION

The use of genetic techniques to identify and monitor aquatic and terrestrial organisms has been shown to be an effective tool for many fields of biology (Taberlet et al 2012) including forensic science (Ogden 2008 and 2009), ecology (Valentini et al. 2009; Barbour et al. 2010), taxonomic identification (Moyer and Díaz-Ferguson 2012), and conservation biology (Godley 2009; Thomsen et al. 2011). Rather recently, genetic techniques have been introduced for species detection and monitoring in freshwater ecosystems (Ficetola et al. 2008; Mahon et al. 2010; Takahara et al. 2012). In particular, eDNA detection for aquatic invasive species has taken

center stage due to the high profile eDNA evidence implicating invasive bighead and silver carps may be in close proximity to or possibly above barriers that were intended to prevent their dispersal into Lake Michigan (Jerde et al 2011). The methodological advantage of using eDNA detection techniques is its presumed sensitivity for species detection (Jerde et al 2011; Thomsen et al 2011) and cost effectiveness (Goldberg et al 2011). For example, the use of such a technique to detect and monitor for aquatic invasive species invasion may be of great advantage to traditional detection methods since invasive species are usually reported at lower densities during early stages of their introduction (Harvey et al. 2009). Detection of aquatic species using eDNA methods appears to be a promising tool that can be incorporated into management, comprehensive conservation, and detection/rapid response plans for aquatic invasive species. Our study sought to develop eDNA techniques and provide proof-of-concept for use of this tool in monitoring for the presence of *H. letourneuxi* and *C. marulius* within the Loxahatchee National Wildlife Refuge.

Molecular marker development

Molecular marker development is a critical first step in eDNA aquatic species monitoring and detection because the marker(s) must be species-specific to ensure consistent species detection. We tested for species specificity by 1) comparing the selected markers to all previously published sequence data in Genbank (repository for sequence data), and 2) testing for cross-species amplification (or lack thereof) in other taxa. We had high primer specificity to deposited Genbank sequences of the target taxon and observed no cross-species amplification for species specific primer pairs. These observations imply that each primer pair should reliably amplify target species eDNA in water samples assuming that the DNA concentration extracted from water samples is above threshold levels. Although our primer pairs for each species appeared specific to the taxon in question, the potential for cross species amplification with

other taxa still remains. To reduce these potential risks requires an understanding of the genetic diversity for *H. letourneuxi* and *C. marulius* throughout their native ranges and is beyond the scope of this study. This is a topic of concern when basing management decisions on eDNA results. To avoid potential cross species amplification issues, we advocate sequencing of all eDNA samples tested as positive (via PCR or qPCR detection) for either *H. letourneuxi* and *C. marulius* within the Loxahatchee National Wildlife Refuge. Furthermore, the risk that primers developed in this study could amplify closely related congeners while unknown, should minimal because *H. letourneuxi* and *C. marulius* are non-native and have no closely related native taxa proximate to Loxahatchee National Wildlife Refuge. Thus any positive eDNA sample, if not the correct species, should at least identify other closely related (and invasive) *Hemichromis* or *Channa* species (or closely related congeners) in the Loxahatchee National Wildlife Refuge. On the other hand, if these markers are truly species specific, then any other *Hemichromis* or *Channa* species that has invaded Loxahatchee National Wildlife Refuge will go undetected using this technique. Our results suggest that the newly developed primer sets for *H. letourneuxi* and *C. marulius* should reliably amplify eDNA for these two taxa in water samples (assuming that the concentration of DNA from tissue is above threshold levels), but we urge caution when using these primers on other systems.

Theoretical lower limit of detection

Our observed theoretical lower limit of detection for qPCR and results from aquarium trials highlighted the sensitivity and accuracy of qPCR as method of eDNA detection. While Jerde et al. (2011) reported lowest detection of pure DNA extracts from Asian carp species at levels ranging from 3.30×10^{-8} to 7.25×10^{-11} ng/µL using traditional detection techniques, it is unclear how these numbers were derived. Standard curve analyses indicated that our detection threshold for *H. letourneuxi*, which was approximately 0.0002 ng/µL, was an order of magnitude

less than that of *C. marulius* (ca. 0.005 ng/µL). These results indicated that differing Taqman assay primers and probes have the potential to influence theoretical eDNA detection limits using qPCR and highlighted the importance of performing standard curve analysis for each taxon and for a variety of primers and probes so as to achieve the lowest possible limit of eDNA detection.

Detection of eDNA from aquarium trials and estimation of detection probabilities

Results from our aquarium trial experiments showed support that eDNA detection was positively correlated with target taxon density, which support the findings of Dejean et al (2011) and Takahara et al (2012). For *H. letourneuxi* there was a significant and positive relationship between density and eDNA detection. These findings also translated to an increase in detection probability (i.e., as fish density increased so did the detection probability). Detection of eDNA using traditional PCR visualization was never 100%; however, using odds ratios, we estimated that for *H. letourneuxi*, every 1 unit (fish) increase in fish density, the species was 1.58 times [exp(0.4564)] more likely to be detected. For *C. marulius,* every 1 unit (fish) increase translated to the species being 1.26 times [exp(0.2321)] more likely to be detected. In contrast, qPCR methods for *H. letourneuxi* produced reliable (100% detection) results for all but the lowest densities suggesting that the use of qPCR should be a reliable method for the detection of *H. letourneuxi* eDNA at densities greater than threshold values. For *C. marulius* there was also a significant and positive relationship between density and eDNA detection with every 1 unit (fish) increase translating to the species being 1.26 times [exp(0.2321)] more likely to be detected using the traditional PCR approach versus 1.32 times [exp(0.2652)] more likely using qPCR methods.

We successfully detected *C. marulius* eDNA at the lowest fish density aquarium trial, which was in contrast to that of *H. letourneuxi.* These findings seem counterintuitive given that the lower limit of qPCR detection for *H. letourneuxi* was less than that of *C. marulius;* however,

the average weight of *C. marulius* was greater than six times that of *H. letourneuxi* and might explain the discrepancy in detection at the lowest fish density between the two taxa. Also of disparity between taxa were detection probabilities for qPCR that were always less than one for *C. marulius*. The stark contrast to *H. letourneuxi* aquarium trials emphasizes some of the potential hurdles associated with eDNA detection from water samples. On day two of the *C. marulius* trials, we began to notice a fungal outbreak in all but the lowest density tanks. Actions of fungi in the environment are known to degrade DNA (Takahara et al 2012). Estimates of eDNA detection probabilities for *C. marulius* experiments; therefore, may be conservative having been potentially influenced by fungal degradation. Regardless, we still observed a positive and significant relationship between eDNA detection and density suggesting, like *H. letourneuxi*, that the use of qPCR (or sequencing) should be a reliable method for the detection of *C. marulius* eDNA (when the density is greater than threshold values).

Despite our findings from controlled aquarium trials, eDNA detection probabilities should be treated with caution because in more uncontrolled environments the probability of detection could also be influenced by environmental factors such as radiation, temperature, endogenous nucleases, fungi, density of microbial community, protracted DNA persistence after death, contaminants and poor protocol conditions (Goldberg et al. 2011; Takahara et al. 2012; Dejean et al. 2012). DNA persistence is defined as the continuance of DNA after the removal of its source (Dejean et al. 2011). Persistence of DNA in our study was observed to be between 24 and 31 days at an average temperature of 26˚C and similar to that of Dejean et al (2011) who reported that DNA persisted until 25 and 21 days for American bullfrog and Siberian sturgeon respectively. Other abiotic factors (e.g., pH, conductivity) may also influence DNA persistence (Thomsen et al 2011). Our controlled experiments; however, showed that the only factor influencing eDNA persistence and detectability was water temperature (given the range of

water quality parameters estimated in this study). The quality of DNA decreased drastically in experiments at temperatures of 33˚C.

In conclusion, our pilot study employing molecular techniques for aquatic invasive species detection shows great potential application for monitoring of *H. letourneuxi* and *C. marulius* in Loxahatchee National Wildlife Refuge. While more costly, we advocate qPCR techniques over that of the traditional eDNA detection (i.e., via visualization of PCR products on an agarose gel) because of the observed increased sensitivity of qPCR. Regardless of the detection method, all positive detections should be confirmed via sequencing of the PCR product to alleviate concerns about false positives. Finally, while abiotic factors other than temperature did not influence our results, PCR inhibition due to unknown abiotic factors in uncontrolled environments has the potential to greatly influence eDNA detection. To detect PCR inhibition, we recommend at least one water sample for every sampling location be spiked with DNA from the target taxon as a control for subsequent PCR, qPCR, and sequencing analyses (for a qPCR positive control we advocate using the lowest detectable DNA concentration found in standard curve analysis). These recommendation along with more controlled field data (i.e., sampling water from know locations where densities of the target taxon can be measured) and occupancy models should provide a rather cost effective and efficient detection method for *H. letourneuxi* and *C. marulius* in Loxahatchee National Wildlife Refuge.

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Table 1. PCR, sequencing and Taqman qPCR primers/probes used to amplify a segment of the mtDNA COI gene for *H. letourneuxi* and *C. marulius*.

Taxon	Name (direction)	Sequence (5`-3`)	Citation
H. letourneuxi	PROS1 (forward)	TTCTCGACTAATCACAAAGACATYGG	Sparks and Smiths (2004)
	PROS2 (reverse)	TCAAARAAGGTTGTGTTAGGTTYC	Sparks and Smiths (2004)
	AJFF3 (forward)	ATCCCCCTCTAGCAGGCAACCTCG	
	AJFq3 (forward)	CCCTCTAGCAGGCAACCTC	
	AJFR2q2 (reverse)	GTGGAGGGAGAAGATGGCTA	
	PCOAJF6 (probe)	6FAM-CCACGCCGGACCTTCCG TAGAC-TAMRA	
C. marulius	FishF1 (forward)	ACTTCAGGGTGACCGAAGAATCAGAA	Benziger et al. 2011
	FishR1 (reverse)	GATAAAGGATAGGATCTCCTCCAC	Benziger et al. 2011
	CMnewF1 (forward)	ATTGGCGCCCCTGACATAGCATT	
	FCM2 (forward)	ATTCTAATCACCGCCGTACTTCTT	
	Rcomp2C (reverse)	TCGGTCTGTGAGTAGCATTGTAA	
	P2CMCO1 (probe)	6FAM-CCTCTCACTCCCAGTACTAGCCGCCG-Tamra	

Table 2. Results of eDNA detection for *H. letourneuxi* using PCR and qPCR methods. All positive results were confirmed via DNA sequencing of PCR products. Average [DNA] is the average DNA concentration of water filtered samples for each treatment.

				Replicate (PCR)			Replicate (qPCR)			Replicate (sequence confirmation)		
Day	Treatment	No. fish/treatment	Ave. [DNA] ng/uL	I	II	III	I	II	III	I	II	III
3	I	0	21.6	-	-	-	-	-	-	-	-	-
	II	1	16.2	-	+	-	-	-	-	-	-	-
	III	3	49.5	+	+	-	+	+	+	+	+	+
	IV	6	25.9	+	+	-	+	+	+	+	+	+
5	I	0	19.6	-	-	-	-	-	-	-	-	-
	II	1	7.4	-	+	-	-	-	-	-	-	-
	III	3	22.9	+	+	-	+	+	+	+	+	+
	IV	6	6.7	+	+	+	+	+	+	+	+	+
7	I	0	12.25	-	-	-	-	-	-	-	-	-
	II	1	11	-	-	-	-	-	-	-	-	-
	III	3	19.41	+	+	-	+	+	+	+	+	+
	IV	6	24.2	+	+	+	+	+	+	+	+	+

Table 3. Results of eDNA detection for *C. marulius* using PCR and qPCR methods. All positive results were confirmed via DNA sequencing of PCR products. Average [DNA] is the average DNA concentration of water filtered samples for each treatment.

Day	Treatment	No. fish/treatment	Ave. [DNA] ng/uL	Replicate (PCR)			Replicate (qPCR)			Replicate (sequence confirmation)		
				I	II	III	I	II	III	I	II	III
3	I	0	0.56	-	-	-	-	-	-	-	-	-
	II	1	17.56	+	+	+	+	+	+	+	+	+
	III	3	36.7	-	+	-	+	+	+	+	+	+
	IV	6	33.4	+	-	-	+	-	-	+	-	-
5	I	0	1	-	-	-	-	-	-	-	-	-
	II	1	18.3	-	+	-	-	+	-	-	+	-
	III	3	70.16	-	+	-	-	+	+	-	+	+
	IV	6	47.25	+	+	+	+	+	+	+	+	+
7	I	0	0.66	-	-	-	-	-	-	-	-	-
	II	1	26.9	+	+	+	+	+	+	+	+	+
	III	3	36.1	+	+	-	+	+	-	+	+	-
	IV	6	133.9	+	+	+	+	+	+	+	+	+

Table 4. Generalized linear regression results of potential factors influencing eDNA PCR detection when visualized on an agarose gel for *H. letourneuxi* aquarium trials. Time was measured in days, density was number of fish per treatment, and DNA refers to average DNA concentration. Data are given in Table 2.

Value	df	Std.	Std. Error	*t*-value	*p*-value
(Intercept)		-2.12915	1.0419815	-2.04337	
time	32	0.145289	0.1657775	0.876411	0.387339
density	32	0.448196	0.1301641	3.443315	0.001623
DNA	32	0.012162	0.0142032	0.856259	0.398221
(Intercept)	34	-1.1904	0.3789917	-3.14096	
density	34	0.4564	0.1289573	3.539153	0.001186

Table 5. Generalized linear regression results of potential factors influencing eDNA PCR (visualized on an agarose gel) or qPCR detection for *C. marulius* aquarium trials. Time was measured in days, density was number of fish per treatment, and DNA refers to average DNA concentration. Data are given in Table 3.

Method	Factor	df	Value	Std. Error	*t*-value	*p*-value
PCR	(Intercept)	32	-1.5619	0.815608	-1.91501	
	time	32	0.197209	0.146304	1.347943	0.187142
	density	32	0.269219	0.132082	2.038272	0.049858
	DNA	32	-0.00214	0.007341	-0.29193	0.772221
	(Intercept)		-0.57341	0.324048	-1.76953	
	density	34	0.232094	0.099999	2.320956	0.02642
qPCR	(Intercept)		-0.81271	0.785807	-1.03424	
	time	32	0.07637	0.14395	0.530528	0.599409
	density	32	0.263352	0.139139	1.89272	0.067471
	DNA	32	0.000948	0.008023	0.118185	0.922443
	(Intercept)		-0.41456	0.320989	-1.29151	
	density	34	0.265235	0.10784	2.459517	0.02705

Table 6. Results from eDNA persistence trials. The concentration of extracted DNA from water filtered samples was [DNA] and the abbreviation OD represents optical density as measured by a spectrophotometer.

Taxon	Time	Density	[DNA]	260/280 OD
H. letourneuxi	7	1	15	1.9
	14	1	12	1.85
	24	1	10	1.6
	31	1	0	1.1
	7	3	17.5	1.7
	14	3	13.5	1.53
	24	3	10.2	1.5
	31	3	0	1.2
C. marulius	7	1	21.06	1.95
	14	1	8.06	1.93
	24	1	11.6	1.74
	31	1	0	0
	7	3	20.5	1.93
	14	3	17.4	1.89
	24	3	21.8	1.93
	31	3	0	0

Table 7. ANOVA results of eDNA persistence trials for the influence of time (days) and density (no. fish/treatment) on DNA concentration ([DNA]) and optical density (OD).

Measurement	Taxon	Factor	df	Sum of Sq.	Mean Sq.	*F*-value	*p*-value
[DNA]	*H. letourneuxi*	time	1	256.6158	256.6158	33.4883	0.002169
		density	1	2.205	2.205	0.28775	0.614662
		Residuals	5	38.3142	7.6628		
	C. marulius	time	1	311.6854	311.6854	6.955412	0.046125
		density	1	45.0301	45.0301	1.004867	0.362151
		Residuals	5	224.0597	44.8119		
260/280 OD	*H. letourneuxi*	time	1	0.4275148	0.427515	20.54664	0.006209
		density	1	0.0338	0.0338	1.62445	0.258491
		Residuals	5	0.1040352	0.020807		
	C. marulius	time	1	3.311	3.311	7.867693	0.037773
		density	1	0.002113	0.002113	0.00502	0.946263
		Residuals	5	2.104175	0.420835		

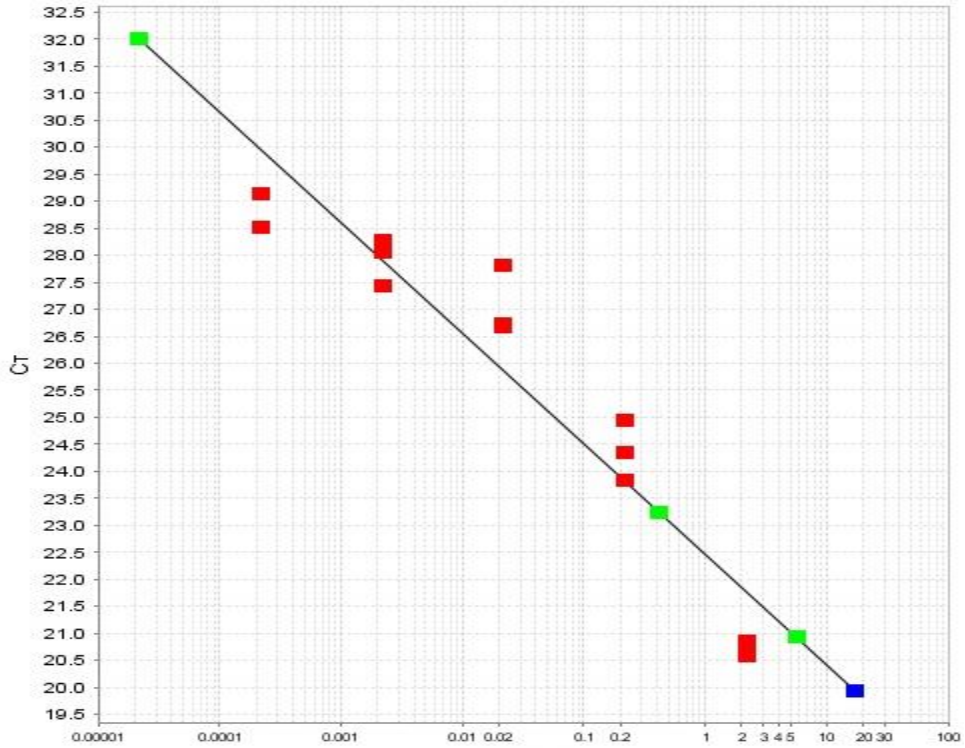
Table 8. Estimation of abiotic factors, DNA concentration ([DNA]), optical densities (260/280) and PCR eDNA detection (Detection). All abiotic parameters were recorded as ppm. Time and temperature (Temp) were recorded as days and ˚C, respectively.

Time	Temp	pH	Ammonia	Nitrogen	Alkalinity	CO_2	Chlorine	Hardness	O_2	[DNA]	260/280	Detection
0	8	7.5	1.5	0.05	20	3	12	20	5.8	22.9	2	+
0	15	6.5	0.4	0.05	23	2.5	15	18	5.6	21.6	2.05	+
0	25	6	0.8	0.05	15	2	14	12	5.9	24.9	2.06	+
0	33	5	0.6	0.05	30	2.5	16	8	5.4	27.8	2.07	+
4	8	6.5	1	0.05	20	3	12	8	5.81	50.4	1.9	+
4	15	5	1	0.05	18	2	16	8	5.8	50.9	1.92	+
4	25	6.5	1	0.05	26	2.5	16	8	5.4	38.6	1.89	+
4	33	5	0.6	0.05	30	2.5	17	8	5.4	2.6	1.23	-
8	8	6.5	3	0.05	20	3	10	8	5.4	47.3	1.86	+
8	15	6	2	0.05	19	3	15	8	5.6	47.9	1.88	+
8	25	6	1.5	0.05	24	2.5	14	10	5.3	35.7	1.84	+
8	33	8	2	0.05	25	2.5	13	10	2.4	2.3	1.13	-

Table 9. ANOVA results of eDNA persistence trials for the influence of time (days), temperature (Temp; ˚C), and abiotic factors on DNA concentration.

Factor	df	Sum of Sq.	Mean Sq.	F Value	Pr(F)
time	1	477.405	477.405	97.8153	0.064151
temp	1	2208.456	2208.456	452.4894	0.029906
ph	1	380.405	380.405	77.9409	0.071804
ammonia	1	94.997	94.997	19.464	0.141902
alkalinity	1	254.572	254.572	52.1592	0.087592
CO2	1	16.682	16.682	3.418	0.315652
Chlorine	1	13.129	13.129	2.69	0.348568
Hardness	1	213.939	213.939	43.8338	0.095434
O2	1	256.614	256.614	52.5775	0.087247
OD	1	93.202	93.202	19.0961	0.143217
Residuals	1	4.881	4.881		

A)



B)

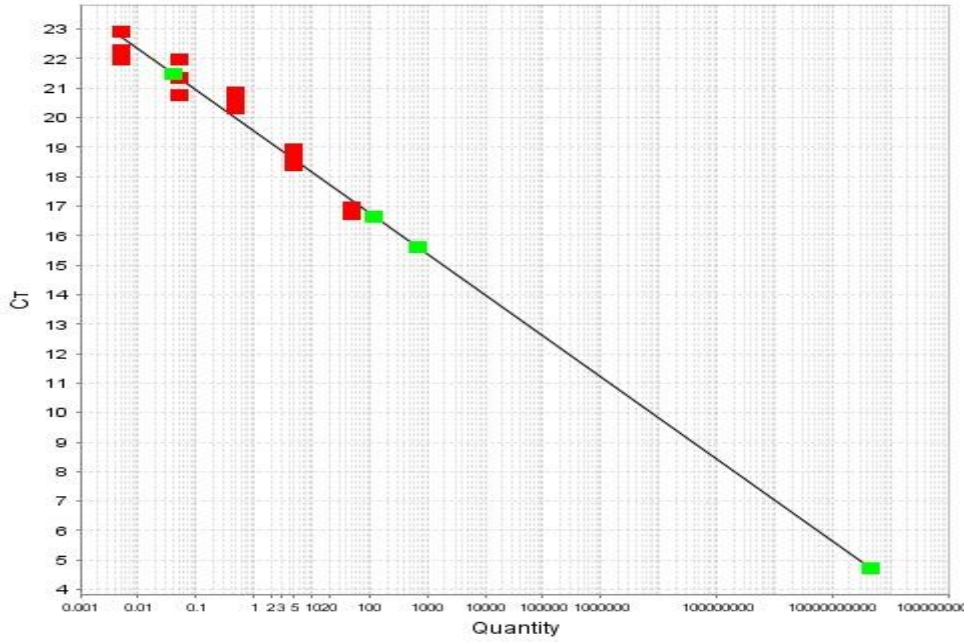


Figure 1. Graph of DNA quantity vs. PCR cycle threshold (CT) for standard curve analyses. A) results for *H. letourneuxi*; B) results for *C. marulius*. Red squares represent qPCR results for 1:10 serial dilutions of 20ng/uL template DNA (each dilution ran in triplicate). Blue or green squares represent random samples of DNA from tissue extracted samples.

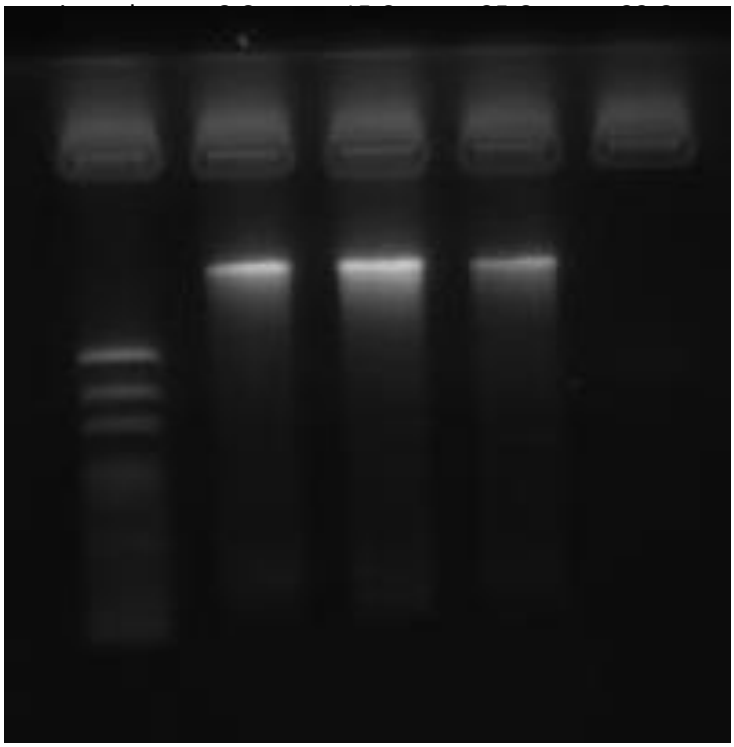


Figure 2. A representative 1% agarose gel image of stained genomic DNA from the DNA persistence study. From left to right the gel reads as follows: 1) 100 nt ladder; 2) genomic DNA whose tissue was held at 8˚C for four days; 3) genomic DNA whose tissue was held at 15˚C for four days, 4) genomic DNA whose tissue was held at 25˚C for four days; and 5) genomic DNA whose tissue was held at 33˚C for four days.

Appendix 1. eDNA preservation method for water samples

We conducted a controlled experiment to test the efficiency of ethanol and sodium acetate for preservation of tissue in water samples. Twelve water samples (1 L each) were collected from an aquaculture pond located at the USFWS Regional Fisheries Center, Warm Springs, GA. Each sample was treated with 10 mg of lyophilized tissue of our target species and six samples were treated with 3 M sodium acetate, pH 5.2 (1 mL) and 95% non denatured ethanol (33 mL). The remaining water samples went untreated as a control. Samples were stored either at room temperature or 4˚C for a period of 18 days before the DNA extraction. We estimated yield and quality of DNA extracted from water samples at day-9 and day-18 following the protocol establish in the Materials and Methods section.

Results from this experiment showed that DNA can be obtained from both preserved and unpreserved water samples; however, PCR detection of DNA in water samples was positive for all samples preserved with sodium acetate and ethanol.

Treatment	Time (Days)	Temp ˚C.	[DNA] (ng/µL)	PCR conf.	260/280 OD
NaOAc + EtOH - distilled water control	9	25	36	+	1.70
NaOAc + EtOH - pond water	9	4	95	+	1.83
NaOAc + EtOH - pond water	18	25	14.2	+	1.80
NaOAc + EtOH - pond water	18	4	91	+	2.00
No preservative - distilled water control	9	25	30	+	1.50
No preservative - pond water	9	4	86	-	1.30
No preservative - pond water	18	25	0.0	-	0.00
No preservative - pond water	18	4	16.5	-	1.20