FINAL REPORT
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South Carolina Department of Natural Resources
October 1, 2014 – September 30, 2019

Project Title: Development and implementation of an environmental DNA (eDNA) monitoring tool for Blackbanded Sunfish (*Enneacanthus chaetodon*) populations in South Carolina and Georgia with determination of relative abundance, genetic health, and connectivity of extant populations

Summary - The fragmented nature of *E. chaetodon's* distribution and its apparent population declines are of conservation concern, particularly in the southern portion of its range. Accordingly, the species is designated as a Species of Greatest Conservation Need in both the South Carolina and Georgia State Wildlife Action Plans (2015). Our completed project represents a rigorous assessment of the current distribution, relative abundance, and population health of *E. chaetodon* throughout the southern portion of its range, providing critical baseline data for this species. The developed eDNA tool provides a time and cost-effective means for *E. chaetodon* monitoring in the remainder of its range as well as future monitoring in South Carolina and Georgia. Although project results provide justification for continued concern for populations in Georgia, South Carolina appears to represent a regional stronghold for *E. chaetodon* – suggesting the Carolina region is perhaps the most robust of the four historic population centers for this species. Overall, project results have provided substantially improved distribution and relative abundance data necessary for managers to protect *E. chaetodon* populations and their habitats. Additionally, genetic characterization of current populations throughout the range of *E. chaetodon* provides excellent baseline metrics of their genetic health as well as important information about population connectivity for guiding conservation priorities and actions.

The data collected during our project has provided guidance for the development of recommendations for additional conservation measures for *E. chaetodon* at both state and site-specific levels. We also updated *E. chaetodon* accounts for each state's SWAP, updated online resources with *E. chaetodon* data, and produced an information brochure for private landowners to promote conservation of *E. chaetodon* and their habitats. Data obtained will lead to better informed adaptive management of *E. chaetodon* and will be useful in monitoring current and future population impacts. While application of the direct project products will be restricted to management within the southeastern US, results of the research will also be relevant to management agencies in the northern portion of *E. chaetodon*'s range. Additionally, due to the frequent impacting of freshwater wetland habitats, *E. chaetodon* represents a valuable indicator species for these habitats; increased knowledge of distribution, connectivity, and population health of *E. chaetodon* within these systems is a valuable resource for effective conservation for these ecosystems.

Introduction – The Blackbanded Sunfish (*Enneacanthus chaetodon*) is a member of the North American Centrarchid family and is distributed in the Atlantic and Gulf of Mexico coastal plains from New Jersey to Florida (Lee et al. 1980). Typically, *E. chaetodon* populations are limited to quiet, shallow, heavily-vegetated blackwaters of herbaceous and forested wetlands. Historically, *E. chaetodon* has occurred primarily in four disjunct population centers, including central Florida, southern Georgia, the Carolinas, and the New Jersey region. Population surveys over the past two decades have failed to document the persistence of the Florida population center (Darden 2004, Tate & Walsh 2005) and

surveys within GA and western FL have provided evidence of only a few extant populations in the Georgia population center (Darden 2000, Bechler & Salter 2014, John Knight Florida Fish and Wildlife Commission, personal communication). *Enneacanthus chaetodon* was known from only three population areas in south-central Georgia, ranging from the St. Marys drainage west to the Aucilla River system. Surveys of six historic and 27 potential sites in Georgia during the early 2000s did not capture any *E. chaetodon* (Darden 2000). Over 70 locations in south Georgia have been repeatedly surveyed (250 total collections) for *E. chaetodon* between 2009-2012 using seines and traps (Bechler & Salter 2014), confirming one historic population and documenting a single newly detected population. While the discovery of a new population of the species is very promising, these results indicate the difficulty in detecting *E. chaetodon* using traditional survey methods. Within South Carolina, data on *E. chaetodon* occurrence showed a fragmented distribution along the upper coastal plain but directed survey efforts are lacking. However, a recent (2006-2011) statewide wadeable streams assessment in SC indicated a low frequency of occurrence (1.3% of sampled sites) and low mean density (0.005/100 m^2) in stream habitats for *E. chaetodon*.

Throughout its range, the fragmented nature of *E. chaetodon's* distribution has been noted, even from areas where appropriate habitat is present (Rohde et al. 1994), giving the impression of a species in decline particularly in the southern portion of its range. As such, the species was a high priority in both the South Carolina and Georgia initial State Wildlife Action Plans (2005) and continues to be designated as a high priority during ongoing SWAP revisions in both states. Conservation actions recommended in the SWAP plans included additional surveys of historic and potential sites, protection of known sites on private land, and determination of population genetic structure within the southeastern US. Based upon the limited number of populations, the extent of population fragmentation, and threats associated with invasive species and extreme drought, the Georgia state protection status of this species was changed from Rare to Endangered in 2006 (Freeman et al. 2009). Additionally, *E. chaetodon* was considered vulnerable in the southeastern United States (Warren et al. 2000) and range-wide (Jelks et al. 2008) in recent assessment carried out by the American Fisheries Society (AFS). The Southern Division AFS Blackbanded Sunfish Working Group (SD AFS Meeting 2010) prioritized needs for range-wide population status assessments, detection probability analyses for *E. chaetodon* collection methods, and population genetic diversity determination.

The collection of environmental DNA (eDNA) has become an increasingly popular approach for monitoring threatened, endangered, or invasive aquatic species. A primary benefit of eDNA detection is the ability to detect organismal presence within an area without sampling the organism directly which provides a substantial benefit for rare species and those that occur in low densities or are logistically difficult to detect. The earliest studies using eDNA targeted microbes in soil samples (Ogram et al. 1987), but more recently, environmental water samples from ponds, lakes, streams, and rivers have been used to document the presence of a variety of fish, invertebrates, amphibians, and reptiles (*e.g.*, Ficetola et al. 2008, Harvey et al. 2009, Jerde et al. 2011, Piaggio et al. 2013). Here, we report a rigorously-designed project using this developing technology as a means to survey suitable habitats for *E. chaetodon* which has become rare throughout the southern portion of its range (*i.e.*, Florida, Georgia, South Carolina).

Purpose and Objectives - The intent of our project was to provide a comprehensive and proactive assessment of *E. chaetodon* distribution, relative abundance, and genetic health of SC and GA populations. We achieved our goal through the development and application of a new eDNA tool combined with traditional surveys and population genetics. Our research has resulted in the development and implementation of improved management and conservation actions for *E. chaetodon*. Our specific SMART project objectives and their quantifiable metrics include:

(i) develop and test an eDNA detection tool for *E. chaetodon*:
   a. number of primers tested: 18
   b. number of species amplifying with primers: 15 species tested; selected primer set amplifies only *E. chaetodon*
   c. completion of laboratory experiments: completed
   d. eDNA sampling of four known *E. chaetodon* locations: completed
   e. analysis of test results to determine optimal eDNA sampling protocols: SOP developed

(ii) use the eDNA tool to conduct field surveys in appropriate *E. chaetodon* habitats throughout SC and GA:
   a. eDNA surveys conducted in a total of 60 sites: completed 61 site surveys
   b. number of water samples processed: 608
   c. number of amplifications: 13,240
   d. generation of distribution maps of positive detections: completed

(iii) conduct traditional survey methods in eDNA 'positive' field sites to provide a relative abundance index among sites and collect fin clips:
   a. number of sites surveyed: 14
   b. number of sites verified with *E. chaetodon* populations: 9
   c. generation of distribution maps and relative abundance metrics for GA and SC populations: completed
   d. number of fin clips sampled and archived: 193
   e. calculation of detection probabilities: completed

(iv) characterize the genetic health of extant *E. chaetodon* populations in SC and GA:
   a. optimization of microsatellite marker suite: completed
   b. number of genetic samples genotyped: 551
   c. completed genetic data analyses: completed

(v) develop and implement improved conservation and management actions to protect *E. chaetodon* populations and habitats:
   a. publish updated *E. chaetodon* distribution maps on SC and GA DNR websites: completed
   b. meet with SC and GA managers to determine appropriate updates for *E. chaetodon* conservation and management: completed
   c. produce updated *E. chaetodon* species accounts for SC and GA SWAPs: completed
   d. develop *E. chaetodon* conservation and management recommendations for public and private landowners, agencies, and scientific/conservation communities: completed
   e. update the SC Stream Conservation Planning Tool to model occurrence of *E. chaetodon* in SC, identify landscape/watershed factors important to their habitat, and allow public access to the Planning/Predictions tool from a SCDNR website: analyses completed
   f. number of project presentations: 12
   g. number of project publications: 3

Objective 1: Develop and test an eDNA detection tool for *E. chaetodon*. Specifically, our tasks for Objective 1 were to: (1) develop and optimize an eDNA tool capable of detecting *E. chaetodon* in environmental water samples; (2) conduct laboratory tests of the eDNA tool using experimental laboratory tanks containing *E. chaetodon*, closely related, and co-occurring species and conduct field tests of the eDNA tool at four localities in SC known to support populations of *E. chaetodon*.

Accomplishments

Task 1. *Develop an environmental DNA (eDNA) monitoring tool for E. chaetodon.*

A total of 18 primers pairs were designed and tested for PCR amplification and specificity to *E. chaetodon* (Table 1). Initial screening efforts comprised amplification reactions with positive control DNA (*E. chaetodon* DNA isolated from fin clip tissue) and DNA from the two most closely related species, *Enneacanthus obesus* and *Enneacanthus gloriosus*. A total of 648 amplification reactions were run and products were visualized with gel electrophoresis for the initial screening phase for PCR amplification efficiency and specificity to *E. chaetodon*. During this time, we determined that agarose gel electrophoresis was insufficient to reliably assess primer specificity which resulted in a transition to a qPCR platform for the remainder of the eDNA tool development process.

Table 1. Originally designed eDNA primer pairs. Primer names indicate the gene region targeted (CytB – cytochrome b gene, CR – control region) and base pair location on complete mitochondrial genome.

Primer Pairs	Primer Sequence 5' - 3'	Length (bp)	Frag. Length (bp)
CytB-F-1484x	TCTGTCTGCCGTCCCCTATATC	22	155
CytB-R-1496x	GGAGAAAGAGCAGGTGAATGA	21	
CytB-F-1499x	TCTCCATGAGACTGGCTCAAAC	22	129
CytB-R-1508x	AGGGAAGTTAAAGCAATAAGGAG	23	
CytB-F-1487x	GCAACAGCTTAGTACAGTGAATCT	24	256
CytB-R-1508x	AGGGAAGTTAAAGCAATAAGGAG	23	
CytB-F-1523x	CGCCTACGCTATTCTTCGTTCTAT	24	150
CytB-R-1533x	CAGAAGAGGAATTGTGTGAGC	21	
CytB-F-1510x	CCCTCCTTATTGCTTTAACTTCC	23	206
CytB-R-1526x	CATAAGGATAAGGATGGAGGCTAGA	25	
CytB-F-1523x	CGCCTACGCTATTCTTCGTTCTAT	24	214
CytB-R-1539x	GAAGGGATGTTCTACCGGTATTC	23	
CytB-F-1487x	GCAACAGCTTAGTACAGTGAATCT	24	132
CytB-R-1496x	GGAGAAAGAGCAGGTGAATGA	21	
CytB-F-1484x	TCTGTCTGCCGTCCCCTATATC	22	279
CytB-R-1508x	AGGGAAGTTAAAGCAATAAGGAG	23	
CytB-F-1470x	TCGAGGCCTTTACTATGGTTCATAC	25	172
CytB-R-1482x	GATATAGGGGACGGCAGACAGA	22	
CytB-F-1457x	ATCGCAACAGCCTTCTCCTC	20	319
CytB-R-1484x	AGATTCACTGTACTAAGCTGTTGC	24	
CytB-F-1528x	TCTAGCCTCCATCCTTATCCTTATG	25	160
CytB-R-1539x	GAAGGGATGTTCTACCGGTATTC	23	
CytB-F-1484x	TCTGTCTGCCGTCCCCTATATC	22	51
CytB-R-14855	CCCCAGATTCACTGTACTAAGC	22	

Primer Pairs	Primer Sequence 5' - 3'	Length (bp)	Frag. Length (bp)
CytB-F-14865	ATCGGCAACAGCTTAGTACAG	21	136
CytB-R-1496x	GGAGAAAGAGCAGGTGAATGA	21	
CytB-F-1529x	TCCATCCTTATCCTTATGGTTGTA	24	96
CytB-R-15342	GAGGGTTCAGAAGAGGAATTGT	22	
CR-F-1587X	CTCGATTAAATGACTGGCGAGAT	23	90
CR-R-1592X	TTTGATATTTGACGGGATGGT	21	
CR-F-1569X	AAACTATTCTTTGTTAGCGATTCTACAT	28	225
CR-R-1587X	CATGAGTTTGTGTGGTAGGTCTTA	24	
CR-F-1569X	AAACTATTCTTTGTTAGCGATTCTACAT	28	267
CR-R-1592X	TTTGATATTTGACGGGATGGT	21	
CR-F-1569X	AAACTATTCTTTGTTAGCGATTCTACAT	28	200
CR-R-1585X	ATCTCGCCAGTCATTTAATCGAG	23	

Using a SYBR green qPCR assay, we identified five priority eDNA primer pairs for the project (Table 2). These primer pairs were further screened using positive control DNA and DNA from 12 additional co-occurring species. Based on PCR efficiency and specificity, a single primer pair (CytB-F-14865 and CytB-R-1496x, Table 3), which amplifies a 136 base pair region of the mitochondrial gene cytochrome b, was selected for subsequent laboratory and field tests.

Table 2. Species tested with the five top candidate primer pairs. Positive amplification are shown with (+) and negative amplification are shown with (-). Bold primer pair indicates final selection for tool development.

	Primer pairs				
Species Tested	CytB-F-1484x / CytB-R-1496x	CytB-F-1487x / CytB-R-1496x	CytB-F-1470x / CytB-R-1482x	CytB-F-1484x / CytB-R-14855	**CytB-F-14865 / CytB-R-1496x**
Enneacanthus chaetodon	+	+	+	+	+
Enneacanthus gloriosus	-	-	-	-	-
Enneacanthus obesus	-	-	-	-	-
Acantharchus pomotis	-	-	-	-	-
Ameiurus natalis	-	-	-	-	-
Ameiurus platycephalus	-	-	-	-	-
Aphredoderus sayanus	-	-	-	-	-
Centrarchus macropterus	-	-	-	-	-
Esox americanus	-	-	-	-	-
Gambusia holbrooki	-	-	-	-	-
Umbra pygmaea	+	-	-	-	-
Lucania goodei	-	-	-	-	-
Fundulus chrysotus	-	-	-	-	-
Lepomis macrochirus	-	-	-	-	-
Etheostoma fusiforme	-	-	-	-	-

Results from a Primer-BLAST search of the complete nucleotide collection of Genbank, using the primer specificity stringency criteria of at least 1 total mismatch to unintended targets and including at least 1 mismatch within the last 5 base pairs at the 3' end, show *E. chaetodon* as the only target species for the final selected primer pair. Our PCR results when using the SYBR green assay were easy to interpret; however, several cases of high background signal and non-specific binding resulted in inconclusive data. SYBR green is an intercalating DNA dye, meaning that it binds to all double-stranded DNA; and, because eDNA isolations concentrate all DNA from environmental samples (potentially containing hundreds to thousands of species' DNA), background signal from SYBR green binding to the high concentrations of DNA (Figure 1) going into a PCR can reduce the sensitivity of the qPCR assay. Non-specific, non-homologous PCR product formation was also problematic in some cases during our initial testing of field and laboratory tanks. Dissociation curves produced after qPCR thermal cycling in these cases sometimes had multiple peaks, melt peaks within a degree or two of that of our target fragment, and melt peaks dissimilar from the target fragments peaks in negative controls (Figure 2).

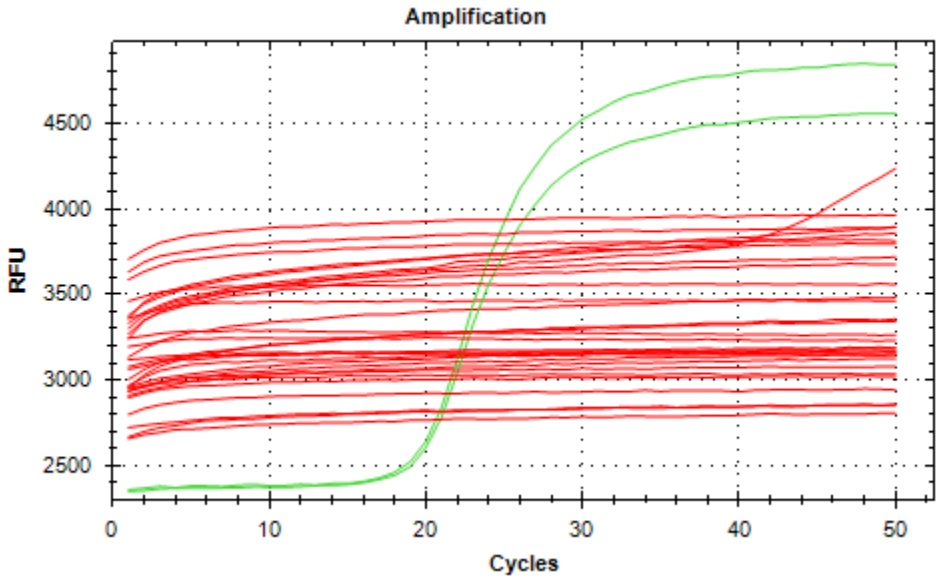

Figure 1. Amplification curves for field samples demonstrating high background signal using the SYBR green assay. Green curves represent positive control DNA; red curves represent reactions with eDNA from field samples.

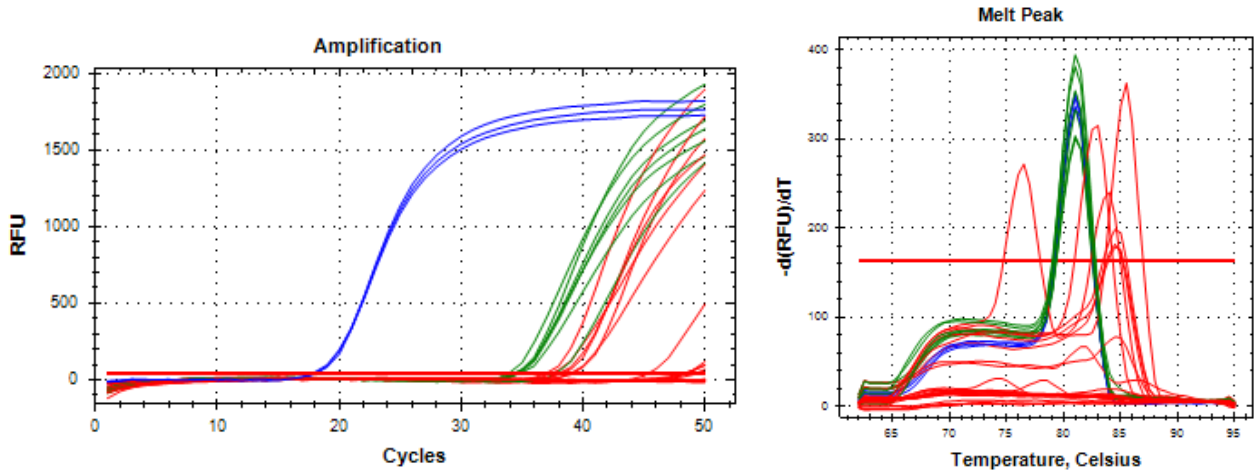

Figure 2. Amplification curves and melt peaks for field test samples using SYBR green qPCR assay. The blue curves are positive control DNA, the green curves are positive amplifications in field samples with proper melt peaks, and the red curves are positive amplifications in field samples with multiple or dissimilar melt peaks. The solid red line is an arbitrary threshold line.

To increase tool specificity and reduce background signal, we designed a hydrolysis probe to target a 31 base pair region (Table 3) within the 136 base pair fragment targeted by the optimal primer pair, CytB-F-14865/CytB-R-1496x. Hydrolysis probes exploit the exonuclease activity of specific DNA polymerases often used for qPCR. A hydrolysis probe comprises a DNA sequence specific to the PCR target, a 5' reporter fluorophore, and a 3' quenching molecule which absorbs the energy of the reporter fluorophore while in close proximity. During the elongation of the PCR target, the exonuclease activity of *Taq* DNA polymerase cleaves the reporter from the probe allowing the fluorophore to lose proximity with the quenching molecule thereby producing signal to be detected by the qPCR detection system. Therefore, only PCR products formed by the specific primer pair which also contain the exact DNA sequence of the hydrolysis probe will be detected. Sequences for both primers and the probe used in qPCR tests had multiple base-pair mismatches with closely related non-target species (Figure 3).

Table 3. Sequence data for primers and probe selected for E. chaetodon eDNA tool. The number of base pairs, guanine-cytosine content (GC %), and melting temperature (Tm) of primers and probe are reported in the right three columns.

Name	Sequence 5' - 3'	Base pairs	GC %	Tm (°C)
CytB-F-14865	ATCGGCAACAGCTTAGTACAG	21	47.6	58.1
CytB-R-1496x	GGAGAAAGAGCAGGTGAATGA	21	47.6	57.7
CytB-FAM-14884	FAM/CTCAGTAGA/ZEN/TAACGCTACCCTCACCCGATTC/IBFQ	31	51.6	67.3

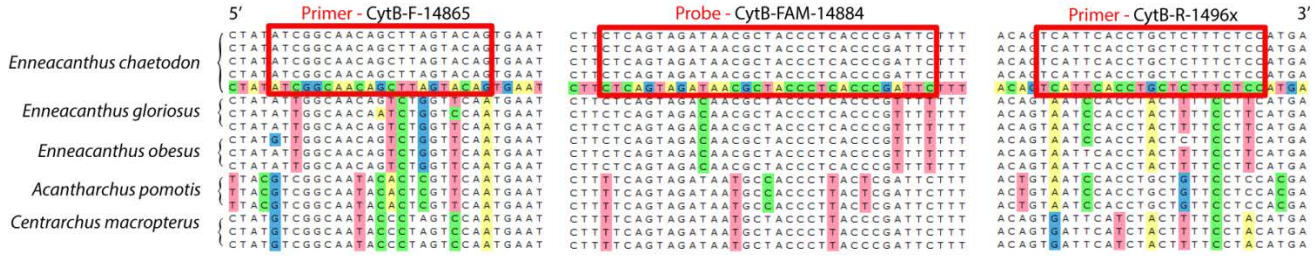

Figure 3. Multiple sequence alignments of primer and probe regions of cytochrome b for E. chaetodon and closely related species.

When comparing the SYBR green assay directly with the probe assay, we only had positive amplifications with the probe for samples that showed positive amplification with the SYBR green with proper dissociation curves (Figure 4). To further verify that we were not detecting non-specific products with the probe assay, we used capillary electrophoresis to separate PCR products for both putatively positive and negative samples for *E. chaetodon* eDNA. All samples showed various levels of non-specific product formation; however, only putatively positive samples had products produced at precisely the correct fragment length of 136 base pair. Non-specific product formation is common in PCR with high concentrations of magnesium, as our optimized protocol requires.

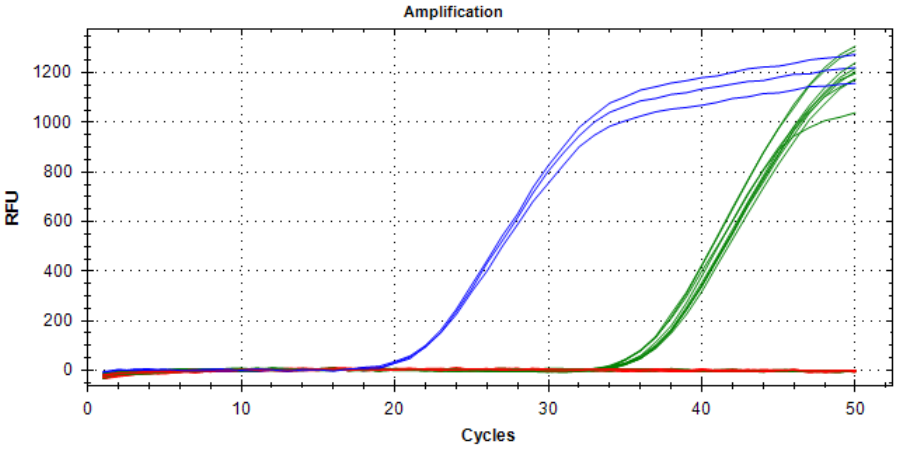

Figure 4. Amplification curves for the same field test samples used in Figure 2 using the hydrolysis probe assay. The blue curves are positive control DNA, the green curves correspond to the samples that showed proper melt peaks using the SYBR green assay, and the red curves (flat lines) correspond to the samples that showed multiple or dissimilar melt peaks using the SYBR green assay.

The limit of PCR detection and PCR efficiency was determined by amplifying serial dilutions of positive control DNA (freshly extracted *E. chaetodon* DNA from fin tissue) and evaluating a standard curve based on the critical detection levels (Cq values) at each PCR cycle. A 10-fold DNA dilution series ranging from 7ng/µl to 7 x 10^{-8} ng/µl was amplified using optimal primer/probe PCR conditions to develop the quantification curves and standard curve (Figure 5). Each dilution series was amplified in 12 technical replicate reactions resulting in a standard curve with $R^2 = 0.994$, a slope of -3.311, and efficiency (E) of 100.5% (90% < E < 105% indicate a robust reproducible qPCR assay). Dilutions of

DNA isolations amplified in 100% of technical replicates down to 7 x 10^{-5}ng/µl. Dilutions of 7 x 10^{-6}ng/µl amplified in ~70% of the technical replicates, dilutions of 7 x 10^{-7}ng/µl amplified in ~15% of the technical replicates, and dilutions of 7 x 10^{-8} did not consistently amplify. Given these results, we will run 8 technical replicate PCRs for field samples collected in the survey. It is important to note that the limit of detection results were produced using freshly isolated, high quality DNA template without impurities or inhibitory compounds often found in environmental samples.

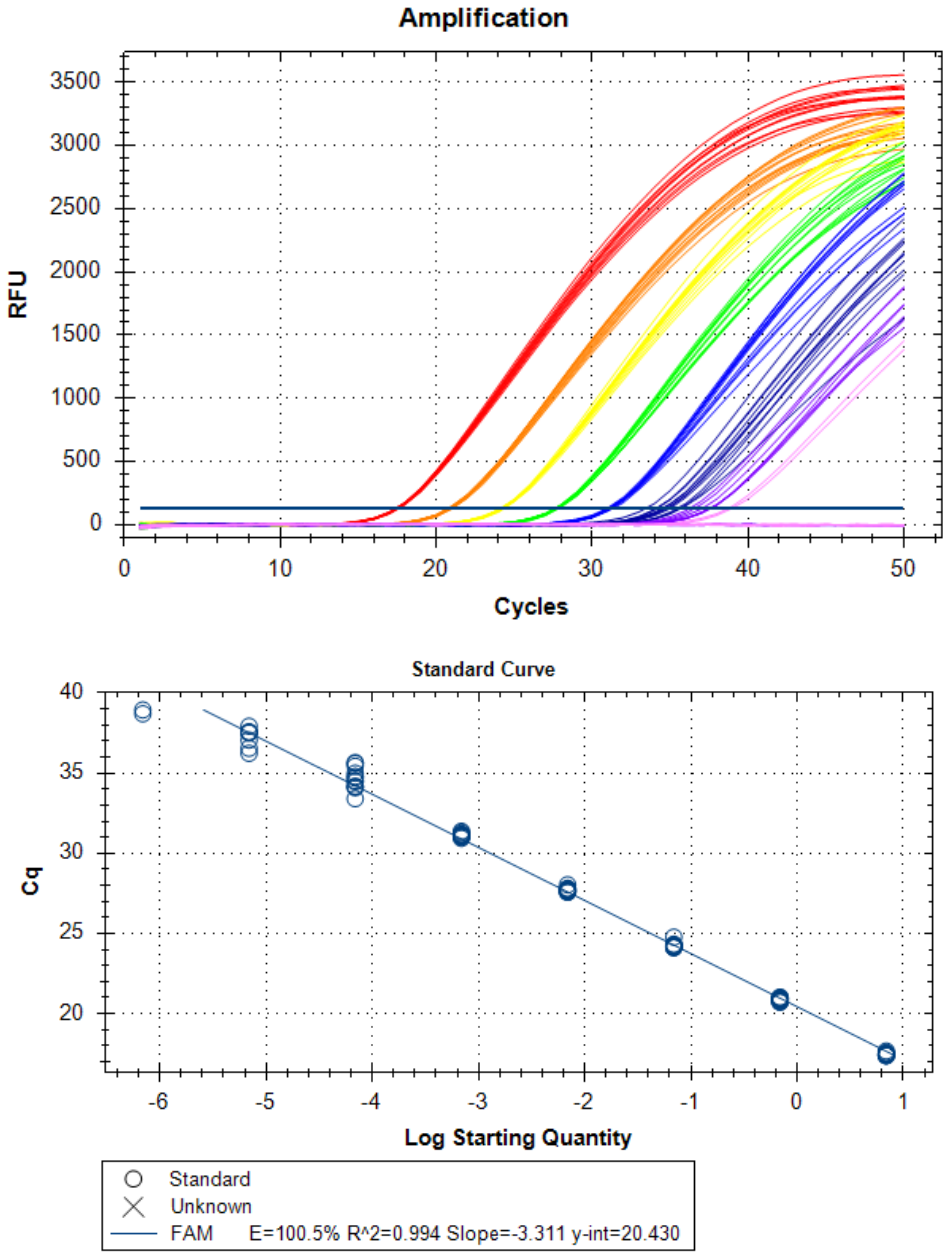

Figure 5. Amplification curves and standard curve for 10-fold dilutions of positive control E. chaetodon DNA using the hydrolysis probe qPCR assay. Amplifications curve for 10-fold dilutions have red lines depicting 1 X DNA isolations (~7.0 g/µL) and pink lines depicting 1:10,000,000 dilutions.

It is well documented that chemicals which inhibit PCR are common in environmental samples (e.g., Jiang et al. 2005; McKee et al. 2015), and while DNA extraction and isolation methods are designed to remove most impurities, often chemicals such as tannic and humic acids persist in the final DNA isolation (Alvarez et al. 1995; Koonjul et al. 1999; Takahara et al. 2015). Inhibition of PCR by environmental chemicals such as tannic and humic acids varies from minor deviations in PCR efficiency to complete amplification failure. Given that *E. chaetodon* are typically associated with tannic, black-water systems, we anticipated that PCR inhibition could be problematic for our project. In order to test for PCR inhibitors, we developed and tested a method where positive control DNA is added to amplification reactions containing DNA isolated from environmental samples. We found that PCR inhibitors were present in eDNA isolations using the MO-BIO Power Water isolation kits. In order to minimize the concentration of PCR inhibitors in our reactions, we tested a DNA isolation kit (MO-BIO Power Soil DNA isolation kit) with proprietary PCR inhibitor removal solutions which has previously been shown to perform well in the presence of PCR inhibitors in environmental water samples (Eichmiller et al. 2015). While the Power Soil kit improved the performance of our eDNA PCR assays, indications of inhibition were still present in the data. However, as magnesium concentrations in amplification reactions were increased, signs of PCR inhibition were eliminated. The best results occurred when using a proprietary multiplex PCR master mix from BioRad, the iQ™ Multiplex Powermix, which contains higher concentrations of magnesium than typical proprietary master mixes.

Our final optimized amplification reaction conditions are, in a total volume of 11 µl, 1 X iQ™ Multiplex Powermix (BioRad), 0.5 mM additional $MgCl_2$, 0.3 µM PrimeTime probe (IDT, Inc.), 0.9 µM forward and reverse primers, and 1 µL of eDNA isolation with thermal cycling as follows: 2 mins at 94°C followed by 50 cycles of 15 seconds at 94°C and 30 seconds at 62°C.

Task 2. *Test the developed eDNA tool in laboratory tanks and known E. chaetodon field populations.*

Previous studies have primarily adopted one of two approaches for eDNA capture in aquatic environments: filtration of large-volume water samples (e.g., Jerde et al. 2011; Goldberg et al. 2011; Jane et al. 2015) or centrifugation-based collection of suspended material in small-volume samples (e.g., Ficetola et al. 2008; Dejean et al. 2012; Thomsen et al. 2012). Both methods were evaluated for our project. eDNA was able to be collected using both methods; however, we concluded that centrifugation was not a viable option for the project for two main reasons. First, filtration equipment is relatively mobile when compared to centrifugation equipment. As many of our survey sites for the project are hundreds of miles from the SCDNR Charleston genetics lab where the samples will be analyzed, a water sample processing laboratory within close proximity is critical. Our filtration lab can be packed up for transport within a matter of hours and can be set for filtering in any room with a power source. Second, while both methods yielded eDNA, we were not able to effectively remove/circumvent PCR inhibitors in centrifuged samples. Filtering environmental water samples also captures PCR inhibitors; however, we were able to cut the filters into small pieces and add them to the MO-BIO Power Soil DNA isolation kit mentioned above, thereby reducing PCR inhibition.

Laboratory tests were initially conducted as a proof of concept for the eDNA tool developed in *Task 1* utilizing 12 gallon covered aquaria stocked with experimental densities of *E. chaetodon* ranging from 0 – 10 fish per tank (Table 4). Negative control tanks, with no fish, were maintained in the same room as the experimental tanks. Six tanks contained only *E. chaetodon*, 2 tanks contained *E. chaetodon* and closely related and co-occurring species, and 2 tanks contained only closely related and co-occurring species (no *E. chaetodon*).

Table 4. eDNA detections for DNA degradation experiment. Positive detection indicated by (+) and negative detection indicated by (-).

	Hours after *E. chaetodon* removed					
Number of *E. chaetodon* / tank	6	24	48	72	96	120
0	-	-	-	-	-	-
1	+	-	-	-	-	-
1	+	-	-	-	-	-
2	+	+	+	-	-	-
3	+	+	-	-	-	-
5	+	+	+	-	-	-
9	+	+	+	+	-	-
10	+	+	+	-	-	-

All tanks were maintained for ~ 8 weeks at ~22°C and each contained pouches of peat to mimic the acidic environment in which the eDNA field survey would occur. Tank water pH was maintained between 5.5 and 6.7. Weekly, 50 ml water samples were collected from the surface of each tank for eDNA analysis and filtered through 1.6 µm glass fiber filters. After filtration, filters were stored at -40°C until processed in the SCDNR Charleston genetics lab. Initial laboratory tests were conducted using the SYBR green qPCR assay with the optimal primer pair developed with three technical replicate PCRs for each tank sampled. The Cq values reported for SYBR green assays are not directly comparable to Cq values reported for the hydrolysis probe assays, which is demonstrated in the amplification curves of our positive control DNA. The SYBR green assay control DNA Cq values are consistently 1-2 units lower than what we see with the probe assay.

Due to technical difficulties involving PCR inhibition and centrifugation, the first two week's samples were not able to be analyzed using the optimized eDNA capture and isolation methods. At the 3 week time point (22 May 2015), *E. chaetodon* eDNA was prevalent in all tanks containing *E. chaetodon* and absent from all tanks without *E. chaetodon*. qPCR amplification curves (Figure 6) resulted in Cq values ranging from 25.8 – 31.9 cycles. The Cq values for the different experimental densities fell into three distinct groupings (Figure 6). Treatments with 8 and 10 *E. chaetodon* had Cq values between 25.8 – 26.3, treatments with 4 and 5 *E. chaetodon* had Cq values of 28.5 – 28.8, and treatments with 1 – 3 *E. chaetodon* had Cq values between 30.6 – 31.9. These Cq values were significantly correlated with the number *E. chaetodon* present in each tank ($R^2 = 0.89$; $p < 0.001$). Over time the relationship between Cq values and the number of *E. chaetodon* weakened (Figure 7); however, the expected general pattern of lower Cq values for tanks with more *E. chaetodon* and higher Cq values for tanks with fewer *E. chaetodon* persisted. Plots of Cq values through time show no distinct patterns of increasing or decreasing *E. chaetodon* eDNA (Figure 7) suggesting a relatively stable balance of DNA accumulation and degradation. While the complex dynamics of a DNA accumulation/degradation balance are not fully understood, environmental variables such as pH and temperature certainly play a role.

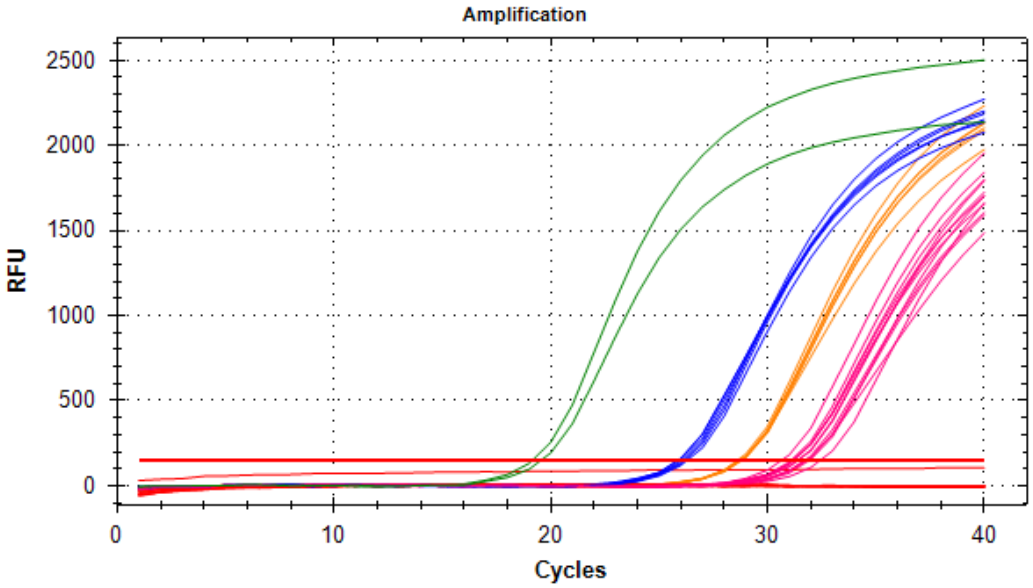

Figure 6. Amplification curves for experimental tank sampling on 22 May 2015 (week 3 of experiment). Green curves are positive controls, blue curves are tanks with 8-10 fish, orange curves are tanks with 4-5 fish, and pink curves are tanks with 1-3 fish, red lines show no amplification signal in control aquaria (both no fish and no E. chaetodon controls included).

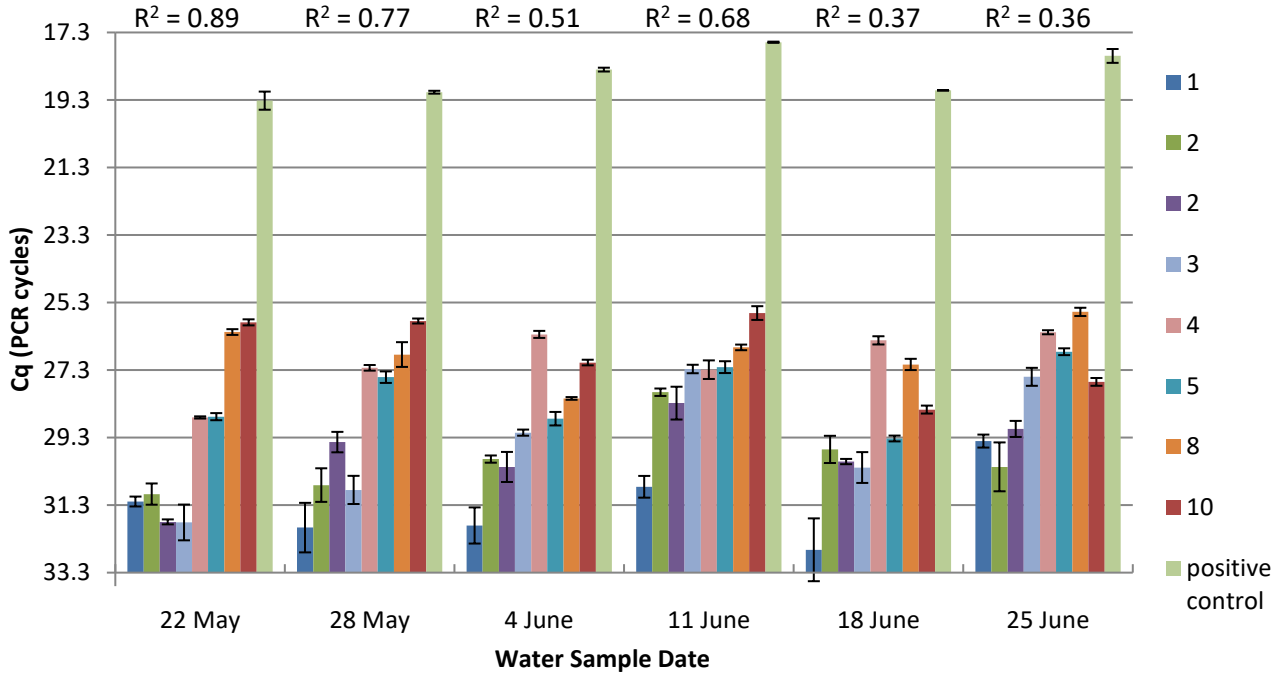

Figure7. Bar plots for average Cq value for 3 technical replicate qPCRs of eDNA collected during laboratory tests. Each bar represents a tank in the experiment; the number of E. chaetodon for each bar is shown in the legend. Error bars depict one standard deviation among replicates.

In order to better understand DNA degradation in controlled experimental conditions, we removed all *E. chaetodon* from the experimental tanks described above and sampled water for eDNA analysis until no *E. chaetodon* DNA was detectable with 3 technical replicate PCRs. Water samples were collected from each tank immediately before fish were removed; after fish were removed water samples were collected at 6 hours and then every 24 hours until no amplification was detected for 48 hours for all PCRs. The last detection of *E. chaetodon* DNA was at 72 hours after fish were removed (Table 4).

Due to weather and scheduling conflicts, our eDNA field survey in SC and GA was delayed from mid – late summer to fall. Therefore, we conducted a similar laboratory eDNA detection and degradation experiment at ~18°C in order to mimic the average water temperatures in areas where our survey sites were located. Conducting a 2nd eDNA laboratory experiment also allowed us an opportunity to use the qPCR hydrolysis probe assay for comparison to the SYBR green results. Prior to the start of the 2nd experiment, all tanks were drained and cleaned. Once tanks were refilled, seeded, and stabilized, water samples were collected to verify that no *E. chaetodon* eDNA was detectable. Two replicate systems of 3 tanks were stocked with high, medium, and low densities of *E. chaetodon* (8, 4, and 1 fish, respectively). Water samples were collected 24, 48 and 192 hours after the addition of fish to document the presence of *E. chaetodon* eDNA prior to removing fish, and *E. chaetodon* eDNA was detected in all tanks for all 3 experimental densities at each sampling time point. The results for the 2nd experiment were similar to the previous degradation experiment, with *E. chaetodon* eDNA being detected up to 72 hours after fish were removed. There were no positive eDNA detections after 72 hours (Table 5).

Table 5. eDNA detections for the 2nd laboratory experiment. Positive detection indicated by (+) and negative detection indicated by (-).

	Hours after *E. chaetodon* removed					
Number of *E. chaetodon* / tank	6	24	48	72	96	120
0	-	-	-	-	-	-
1	+	-	-	-	-	-
1	+	-	-	-	-	-
4	+	+	-	-	-	-
4	+	-	-	-	-	-
8	+	+	+	+	-	-
8	+	-	-	+	-	-

We conducted field tests of the eDNA tool at 4 localities where *E. chaetodon* are known to occur. At each sampling site, 10 x 2 L water samples were collected in reusable, glass bottles. All samples were collected in or near putative *E. chaetodon* habitat (i.e., submerged or emergent vegetation patches). Caution was taken to avoid cross contamination between bottles within a site and between sites. After water samples were collected, seine nets were used to collect *E. chaetodon* specimens; the presence of *E. chaetodon* was verified at all four sites. Water samples were kept on ice, returned to the lab, and filtered within 16 hours of initial collection. All water samples were filtered through 1.6 µm glass fiber filters and filtering protocols followed those outlined in the Quality Assurance Project Plan for eDNA Monitoring of Bighead and Silver Carps (USFWS 2013). Equipment controls were conducted

periodically during water filtration by filtering 2 L of MilliQ water. Water filtration time for a 2 L bottle varied from 5 – 60 minutes and required between 3 – 11 filters (mean 4.2 filters). These differences are likely attributable to varying quantities of particulate organic matter in the samples. After filtration, filters were stored at -40°C until processed in the SCDNR Charleston genetics lab. DNA was isolated from all filters using the optimized protocols developed in *Task 1*. Three technical replicate qPCRs were performed for filters from each bottle until (1) a positive *E. chaetodon* eDNA detection was documented or (2) all filters were tested.

A total of 40 bottles were collected and analyzed for *E. chaetodon* eDNA. Positive *E. chaetodon* eDNA detections were documented at all 4 "known" sites. At one of the "known" sites, all 10 bottles tested positive with our eDNA tool; and for the other 3 sites, 8 out of 10 bottles tested positive. No equipment controls showed positive amplifications. At site SC-2, where all bottles tested positive, 9 out of 10 tested positive on the first filter analyzed. Of those 9 that tested positive on the first filter, 6 amplified in all 3 technical replicate PCRs and the remaining 3 tested positive in 2 of the technical replicates. At site SC-1, where the fewest fish where found, only 1 technical replicate amplified for half of the bottles, 2 technical replicates amplified for 2 of the bottles, and 3 technical replicates amplified for one bottle. Interestingly, it was the 3rd filter analyzed for the bottle which had all 3 technical replicates test positive. The first two filters showed no signs of amplification. These data also suggest more than 3 technical replicates are necessary to detect *E. chaetodon* eDNA in the low concentrations encountered in the field; therefore, our protocol will include 8 technical replicates as indicated from the qPCR of serial dilutions of positive control DNA.

We conducted an additional sensitivity test of the eDNA tool developed. During the first year of the project, we conducted a limit of detection test using qPCRs on a series of 10-fold dilutions of freshly isolated total genomic DNA from *E. chaetodon* fin tissue. This limit of detection test allowed us to determine the efficiency of the qPCR as well as help us determine a reasonable number of technical qPCR replicates necessary to detect femtograms of genomic DNA. However, when surveying eDNA we expected DNA quality to be poor (i.e. eDNA is likely a composite of highly fragmented genomic DNA from multiple species). Therefore, we conducted a limit of detection test using only the target fragment of DNA found in *E. chaetodon* to estimate DNA copy number in the lower limits of our eDNA tool detection. A 136 bp synthetic DNA fragment (gBlock Gene Fragments, IDT) containing the exact target DNA sequence found in *E. chaetodon* was amplified in a series of 10-fold dilutions ranging from 1ng/µl to 1 x 10^{-12} ng/µl. DNA copy number was estimated using the following formula:

$$DNA\ copy\ number = \frac{DNA\ mass\ (g) * 6.022x10^{23}}{fragment\ length\ (bp) * 650}$$

In the series of 10-fold dilutions, DNA copy number ranged from > 6.8 billion copies/µl to < 1 copy/µl (Figure 8). Eight technical qPCR replicates were performed for each dilution and there were successful amplifications for all dilutions. Dilutions estimated to have >1 copy/µl amplified in all technical qPCR replicates. The 2 dilution series estimated to contain < 1 copy/µl amplified in only a single technical replicate each. The amplification curves for the 2 dilution series with > 1 copy/µl had nearly identical critical detection levels (Cq values) and therefore are likely examples of single copy DNA amplifications (Figure 8). These results are concordant with conclusions from our previous sensitivity test and we feel confident that 8 technical qPCR replicates are justified for each eDNA filtered water sample.

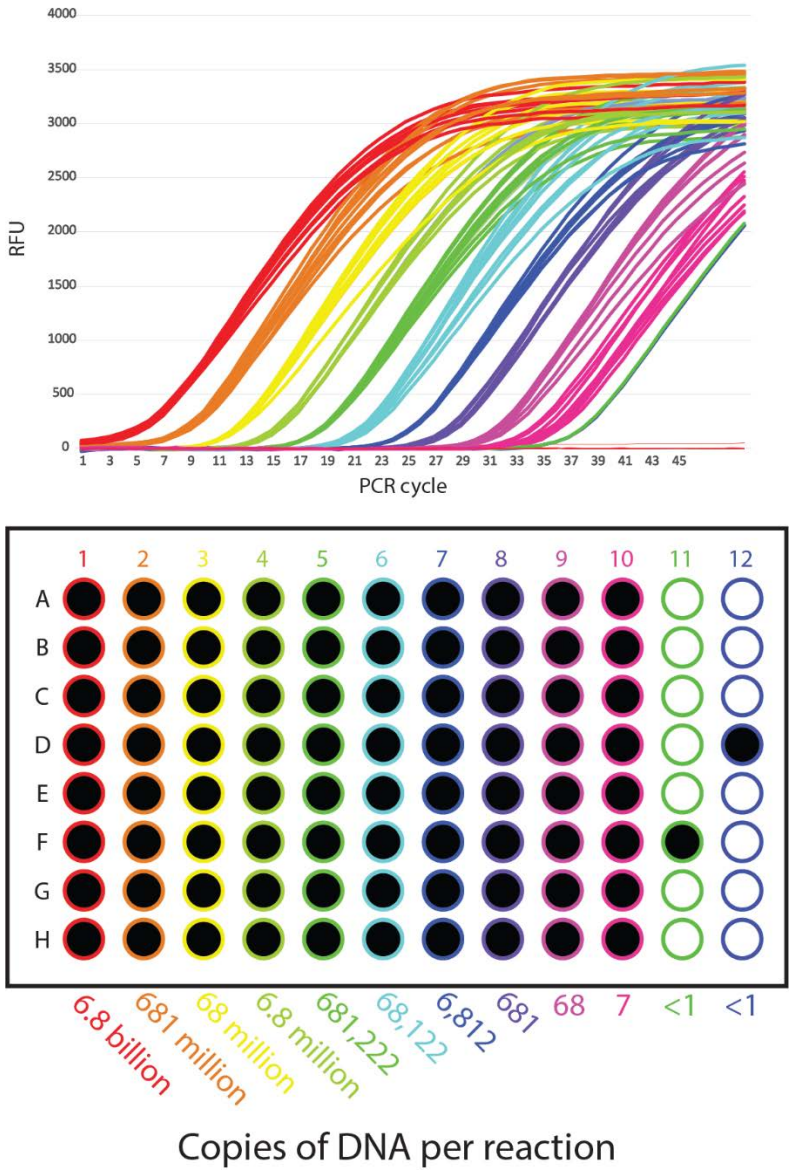

Figure 8. Sensitivity test of eDNA tool using 10 fold serial dilutions of the target mitochondrial DNA fragment. Quantification curves (top) and representation of qPCR plate (bottom). Black filled circles represent positive amplifications and open circles represent no amplifications.

We also conducted an additional laboratory test of the eDNA tool. Several of the selected field sites during the Georgia water sampling were characterized by pH values that were substantially lower (pH ~ 4) than those used in our previous laboratory testing trials. Therefore, following the completion of the field sampling, we conducted a 3rd round of laboratory experiments to verify that the results of our previous tests in terms of detection capabilities of the new eDNA tool and DNA degradation rates were valid under these lower pH conditions. Prior to the start of the 3rd experiment, all tanks were drained

and cleaned. Once tanks were refilled, seeded, and stabilized, water samples were collected to verify that no *E. chaetodon* DNA was detectable.

Four tanks were maintained at a pH of 4.0 (±0.1) for the duration of the experiment; three tanks were stocked with high, medium, and low densities of *E. chaetodon* (8, 4, and 1 fish, respectively) and one tank with no fish served as a negative control. Water samples were collected 6, 24, 48, 72, 96, and 168 hours after the addition of fish to document the presence of *E. chaetodon* eDNA. *Enneancanthus chaetodon* eDNA was detected in all 3 experimental densities and was absent from the negative control tank at each sampling time point. All fish were removed following the 168 hour sampling to evaluate DNA degradation in lower pH environments. The results from this experiment were similar to the previous degradation experiments, with *E. chaetodon* eDNA being detected up to 72 hours after fish were removed, although detection was sporadic after 24 hours. There were no positive eDNA detections after 72 hours (Table 6).

Table 6. eDNA detections for the 3rd laboratory experiment. Positive detection indicated by (+) and negative detection indicated by (-).

	Hours after *E. chaetodon* removed					
Number of *E. chaetodon* / tank	6	24	48	72	96	120
0	-	-	-	-	-	-
1	+	-	-	-	-	-
4	+	+	-	+	-	-
8	+	+	-	-	-	-

Significant deviations

Our proposal originally called for a comparison of centrifugation and filtration methods at all four known *E. chaetodon* locations. While samples for centrifugation were collected, they were not processed to the same extent as filtration samples, given our inability to remove PCR inhibitors from these samples. For our project, filtration was chosen as the optimal eDNA collection method and was used for all field and laboratory sample collections. Our original plan was to use the SYBR green qPCR assay for all eDNA tests. However, high background levels could obscure positive results, necessitating a transition to probe-based chemistry for qPCR-based eDNA detection in this project. The addition of the probe does not significantly increase the costs of our qPCR assay. In contrast, the inhibitors present in our samples required an alternative DNA isolation kit than originally proposed. The selected kit is substantially more expensive than the kits identified in our original proposal, but provides much more reliable results in the inhibited samples collected to date. Finally, we proposed a single, 3-week laboratory experiment to confirm positive detection of *E. chaetodon* in a controlled aquarium environment. We expanded on the proposed activities to include a substantially longer detection experiment (2 months), assessments of eDNA persistence time (after removal of fish), and tests of eDNA accumulation / persistence at a second (lower) temperature range and lower pH as a result of delays in our field sampling schedule. The two additional tasks conducted during Year 2 of the project

represented deviations from the original proposal, but have improved our confidence and interpretation capabilities during tool application in the field. No further testing of the tool is needed.

Objective 2: Use the eDNA tool to conduct field surveys in appropriate *E. chaetodon* habitats throughout SC and GA.

Accomplishments

Task 3 – *Conduct an eDNA survey of potential habitats in South Carolina and Georgia for presence of E. chaetodon populations.*

Site Selection

The site selection for the GA eDNA survey was based on a combination of historic collection information and a recent comprehensive survey for *E. chaetodon* conducted between 2011—2013 by Dr. Dave Bechler and his students (Valdosta State University). Bechler and Salter (2014) sampled 70 sites three to four times each by seining, dip netting and/or trapping, but only detected *E. chaetodon* at one historic site (Linton Lake) and in one new location (Fletcher's Lake). For the remaining 68 sites, we asked Dr. Bechler to rank sites with the best potential to contain an unknown population of *E. chaetodon*. Highly ranked sites contained diverse aquatic plant and fish communities and maintained flows (rivers) or water levels (lakes and wetlands) during recent drought periods.

A total of 31 sites received the top ranking and represented our initial site list; tier 2 classified sites were held in reserve as potential replacement sites if any of the top sites were either inaccessible or deemed inappropriate upon arrival. Additionally, as a positive control field test for GA, we also included the most recently identified *E. chaetodon* population in Fletcher's Lake as our 31st site. We were able to complete eDNA sampling at 28 sites that were top ranked and 2 sites that were classified as tier 2. Sites were distributed in six river systems (Alapaha, Aucilla, Satilla, St. Marys, Suwannee, and Withlacoochee) bracketing the known range of *E. chaetodon* in GA and included two historic sites in the upper Alapaha River where *E. chaetodon* were last detected before 1980. We completed the GA eDNA sampling during October and November 2015.

Due to the historic flooding events during October 2015 in SC, the SC survey was postponed until Year 2 of the project as exceptionally high water levels may have substantially reduced detection probabilities of the eDNA tool (e.g., via dilution or potentially as a result of *E. chaetodon* losses due to flooding). Because *E. chaetodon* exhibits a relatively contiguous distribution across the entire inner coastal plain of South Carolina, sample sites were primarily selected using a random selection process in order to objectively assess *E. chaetodon* occurrence and meet the statistical requirements for data analysis objectives. Sample sites in South Carolina included three historic localities for *E. chaetodon* and 27 randomly-selected sites. Historic sites included locations where *E. chaetodon* had been collected reliably with traditional gears in multiple years and in all cases as recently as 2013. Two of the historic sites were streams selected to assess eDNA dynamics in flowing channels with known *E. chaetodon* populations and the other was a large mill pond with outflow pool.

Random sites were selected from a statewide database of stream points containing a point for each 100-m stream and river segment (378,000+ points statewide). The statewide list was first filtered to include only points within the Southeastern Plains level-III ecoregion (Griffith et al. 2002), which nearly

exclusively represents the range of *E. chaetodon* in South Carolina. The list of all potential Southeastern Plains stream points was sorted by river basin to allow distribution of sites among basins, and was further filtered by watershed area to include only sites draining watersheds of 4 to 150 km², which corresponds to the small to moderate streams and swamps preferred by *E. chaetodon*. The stream points database represented all aquatic habitat types along the stream and river courses and therefore included natural and man-made impoundments (e.g. mill ponds), which are also known to support *E. chaetodon*. Finally, the site list was randomized to provide the rank order for site selection.

Sites were then plotted in an online mapping application and satellite imagery used to initially assess habitat types, suitability and accessibility. Sites plotting within the same drainage as existing, higher-ranking suitable sites were excluded in order to ensure independence of sample sites. Field reconnaissance was conducted in January 2016 to confirm site suitability (i.e. outwardly exhibiting potential *E. chaetodon* habitat) and obtain permission from property owners/managers for sampling. The final spread of sample sites represented all potential *E. chaetodon* habitat types, including flowing streams with vegetation, swamps, beaver impoundments (natural) and man-made impoundments (e.g. mill ponds; Table 7). These sites were distributed in all five major river basins in SC. Our eDNA surveys were completed at 30 sites during spring of 2016 under typical flow conditions.

Table 7. South Carolina environmental DNA (eDNA) sample sites for Blackbanded Sunfish (Enneacanthus chaetodon), with eDNA results status. Site type indicates randomly selected (R) or historic (H) localities from which E. chaetodon was known. The river basin code ACE refers to the Ashepoo-Combahee-Edisto basin.

Site ID	Type	Waterbody Name	River Basin	Water Sample Date	eDNA Status
SC-7	R	Jumping Gut Creek / Sanders Pond	ACE	4/12/2016	Negative
SC-8	R	Bridge Creek	Savannah	4/12/2016	Negative
SC-9	H	Hollow Creek	Savannah	4/12/2016	Negative
SC-10	R	Little Salkehatchie River	ACE	4/13/2016	Negative
SC-11	R	Toby Creek	ACE	4/13/2016	Negative
SC-12	R	Hercules Creek	ACE	4/13/2016	Negative
SC-13	R	Wells Branch	ACE	4/14/2016	Negative
SC-14	R	Miller Creek	Savannah	4/14/2016	Negative
SC-15	R	McNair's Millpond / Beaverdam Creek	Pee Dee	4/19/2016	Negative
SC-16	H	Naked Creek / Pledger Creek	Pee Dee	4/19/2016	Positive
SC-17	R	Flat Creek	Pee Dee	4/19/2016	Positive
SC-18	R	Beaverdam Creek	Pee Dee	4/19/2016	Negative
SC-19	R	Congaree Spring Branch	Santee	4/20/2016	Negative
SC-20	R	Big Beaver Creek	Santee	4/20/2016	Negative
SC-21	R	Cowpen Swamp	ACE	4/20/2016	Positive
SC-22	R	Fourth Creek	ACE	4/21/2016	Negative
SC-23	R	Flea Bite Creek	ACE	4/21/2016	Negative
SC-24	R	Woodard Millpond / Little Black Creek	Pee Dee	4/26/2016	Negative
SC-25	R	Big Bear Creek	Pee Dee	4/26/2016	Positive

Site ID	Type	Waterbody Name	River Basin	Water Sample Date	eDNA Status
SC-26	R	Wilkes Millpond / Mill Creek	Pee Dee	4/26/2016	Positive
SC-27	R	South Prong Swift Creek	Pee Dee	4/26/2016	Negative
SC-28	R	Reedy Creek	Pee Dee	4/27/2016	Negative
SC-29	R	Cypress Creek	Pee Dee	4/27/2016	Negative
SC-30	R	Lake Swamp	Pee Dee	4/27/2016	Negative
SC-31	R	Gantts Mill Creek	Savannah	4/28/2016	Negative
SC-32	H	Upper Three Runs	Savannah	4/28/2016	Positive
SC-33	R	Tylers Pond / Hunter Branch	ACE	5/4/2016	Positive
SC-34	R	Marrow Bone Swamp Creek	ACE	5/4/2016	Positive
SC-35	R	Cedar Creek	ACE	5/4/2016	Positive
SC-36	R	Little Pine Tree Creek	Santee	5/10/2016	Negative

eDNA Field Sampling

Ten eDNA water samples were collected from each site. After visually surveying the site, we identified habitats most likely to support *E. chaetodon*. Water samples were collected primarily from patches of aquatic vegetation or other cover types (undercut banks, logs) when aquatic vegetation was not present. We attempted to distribute sample points in areas of varying water depths, vegetation types or with respect to other site-level features that could affect the probability of detecting *E. chaetodon* when present. For example, at sites with a distinctive outflow (e.g., culvert or dam release), we selected at least one point near the outflow structure because eDNA originating at any habitats upstream could potentially be present. To increase the independence of each sample, points were separated by at least 5 m.

A total of 608 x 2 L surface water samples were collected across all of the sample sites. All water samples were taken prior to disturbing the area and caution was taken not to cross-contaminate samples within a site and samples between sites. All materials which were to contact water at a site prior to sample collection (waders, boots, etc.) were decontaminated with 10% bleach and rinsed with DI water between each site. During the eDNA survey, water quality characteristics were documented at the site level (61 localities across both states) and comprised temperature, pH, dissolved oxygen, conductivity, turbidity, and water color (Tables 8 & 9). Decimal degree GPS coordinates, time of sampling, average depth, average current velocity at 0.6 depth, presence of woody debris > 1 m long and 20 cm diameter, dominant substrate, percent floating vegetation, dominant floating plant species, percent submerged vegetation, and dominant submerged plant species were documented within a 0.7m x 0.7m (ca. 0.50 m^2) PVC grid at all individual water sampling locations. Each water sample site was also photographed (Figure 9).

Table 8. Habitat characteristics for eDNA sample sites in Georgia. Sites 1, 2, 3, 7, and 28 tested positive for E. chaetodon eDNA. Cur. = current, Vel = velocity, Dom. = dominant, Per = percent, and SAV = submerged aquatic vegetation.

Site No.	Site Name	Date	Ph	Turbidity (NTU)	Temp (°C)	Depth (cm)	Cur. Vel m/sec	Per. Float	Dom. Floating	Per. SAV	Dom. SAV
GA-1	Banks Lake	10/5/2015	5.22	0.59	22.3	73.1	0.004	30.3	*Brasenia*	47.5	*Myriophyllum*
GA-2	Grand Bay Creek	10/5/2015	3.93	0.07	19.6	44.46	0.011	12	*Lemna*	29.1	*Utricularia*
GA-3	Suwanoochee Creek	10/6/2015	4.01	0.64	19.25	44.5	0.075	42.5	*Utricularia*	7.78	Grass
GA-4	Arabia Bay, northern outflow	10/6/2015	3.6	1.29	19.65	24.36	0.041	0	No vegetation	29	*Sparganium*
GA-5	Guest Mill Pond	10/6/2015	4.98	1.55	21.7	90.94	0.01	16.2	Algae	38.5	*Cabomba*
GA-6	Brown's Pond	10/7/2015	5.12	ND	21.55	69.44	0	29.5	*Eichhornia*	33	*Cabomba*
GA-7	Private Farm Ponds	10/12/2015	5.58	3.98	24.25	39.66	0.031	54.3	*Nymphaea*	54.3	*Hydrocotyle*
GA-8	Suwannee River Borrow Pit	10/13/2015	4.27	1.1	20.9	52.5	0	43	Algae	53	*Panicum*
GA-9	Cypress Creek	10/13/2015	3.99	0.99	19.4	42.9	0.033	14	Algae	80.8	*Eleocharis*
GA-10	St. Marys River Borrow Pit	10/13/2015	5.79	7.41	21.2	46.38	0	50.8	*Myriophyllum*	50.5	*Myriophyllum*
GA-11	Mud Creek	10/14/2015	4.55	3.55	21.3	46.58	0.13	29	*Alternanthera*	20.1	*Alternanthera*
GA-12	Willacoochee Borrow Pit	10/19/2015	5.58	2.28	16.3	63.38	0	1.3	*Lemna*	13	*Alternanthera*
GA-13	Grand Bay WMA outflow	10/19/2015	5.29	0.98	14.9	42.8	0	27	*Lemna*	5.5	*Equisetum*
GA-14	Rays Mill Pond	10/19/2015	4.73	0.51	20	80.3	0.003	25.2	Algae	20	*Cabomba*
GA-15	Crevasse Pond	10/20/2015	6.57	5.04	18.5	86.9	0	14.5	*Nymphaea*	70	*Cabomba*
GA-16	Toms Creek	10/20/2015	3.57	0.52	18.2	26.52	0.078	13	Algae	42.5	Grass
GA-17	Suwanoochee Creek	10/21/2015	3.69	32.5	16.9	26.5	0.048	9	Algae	47.5	*Juncus*
GA-18	Lake Verne	10/21/2015	4.13	1.33	18.4	50.1	0	7.5	Algae	26	Grass
GA-19	Triangle Lake	10/21/2015	4.09	10.3	24.2	46.66	0	22	*Utricularia*	57.5	*Utricularia*
GA-20	Alapaha River wetland	10/22/2015	6.19	4.64	16.9	44.9	ND	0.2	Algae	7	*Sparganium*
GA-21	Lanes Mill Creek Wetland 1	10/26/2015	4.46	4.36	20.9	49.92	0	63.3	*Brasenia*	93.5	Grass
GA-22	Lanes Mill Creek Wetland 2	10/26/2015	4.18	3.08	21.1	63.22	ND	29.3	*Nymphaea*	47.5	*Utricularia*
GA-23	Copeland Rd. Wetland	10/26/2015	3.66	3.06	19.9	55.4	ND	40.5	*Nymphaea*	10	Algae
GA-24	Double Run Creek	10/27/2015	6.15	4.22	18.15	29.16	0	13	*Juncus*	47.5	Sedge
GA-25	Deep Creek Swamp	10/27/2015	6.37	7.8	18.7	37.58	0	45.5	*Nymphaea*	51	*Potamogeton*
GA-26	Heards Lake	10/28/2015	5.85	1.49	21.5	42.64	0	64.5	*Nymphaea*	57	*Utricularia*
GA-27	Barnes Pond	11/16/2015	6.05	4.3	14.6	62.38	0	66	*Nymphaea*	0	None
GA-28	Fletcher Lake	11/16/2015	5.64	3.8	17.5	55.18	0	7.22	*Hydrocotyle*	41.11	*Eleocharis*
GA-29	Botherment Branch	11/17/2015	3.67	1.57	16.3	46.4	0	46	*Nymphaea*	11.5	*Nymphaea*
GA-30	Suwannee Creek	11/17/2015	3.61	2.45	17.05	38.44	0.007	0	*n/a*	80.5	*Panicum*
GA-31	Mizell Lake	11/17/2015	3.69	2.54	19	75.3	0	0.4	*Utricularia*	13.7	*Panicum*

Table 9. Water quality conditions at South Carolina sample sites at the time of water (eDNA) sampling. For sites with discrete pond and outflow features, Sample Location indicates the position of the water quality measurements. Water color refers to the Borger Color System (BCS).

Site ID	Sample Location	Temperature (°C)	pH	Dissolved Oxygen (mg/L)	Conductivity (µS/cm)	Turbidity (NTU)	Water Color (BCS)
SC07	pond	16.2	4.95	8.02	25	3.20	120
SC08		17.4	4.13	8.35	14	6.20	120
SC09		18.6	4.19	9.29	17	2.42	92
SC10		16.2	5.82	8.85	49	4.36	91
SC11		16.8	5.94	8.12	43	6.01	91
SC12		17.0	6.35	8.46	55	4.90	91
SC13		16.3	6.03	8.69	41	4.52	91
SC14		16.8	6.48	8.86	58	3.62	120
SC15	outflow	18.3	6.44	7.57	69	1.55	91
SC15	pond	19.0	6.15	7.79	70	1.40	92
SC16	outflow	17.6	5.26	9.79	41	2.90	92
SC17		21.7	5.90	8.32	83	2.71	92
SC18		17.7	6.20	5.20	122	11.36	
SC19	pond	18.9	4.70	8.80	23	2.80	91
SC20		19.2	5.05	9.21	21	4.00	92
SC21		21.2	5.11	3.72	34	4.16	91
SC22		19.0	4.55	2.30	32	3.18	91
SC24	outflow	20.4	4.81	9.03	18	2.18	36
SC24	pond	19.1	4.45	4.10	19	1.20	36
SC25		19.5	5.19	5.85	32	14.73	39
SC26	pond	20.2	3.95	5.52	18	1.20	91
SC27	pond	23.9	4.12	5.81	18	14.70	91
SC28		20.5	5.90	6.29	84	4.90	91
SC29		20.1	4.31	5.84	52	8.64	91
SC30		20.9	5.61	4.60	86	3.53	48
SC31		20.8	6.07	7.05	53	7.09	91
SC32		19.4	4.77	9.37	18	2.95	93
SC33	outflow	22.4	4.51	5.45	20	3.24	36
SC33	pond	24.0	5.19	5.29	16	1.90	93
SC34	outflow	23.5	5.04	8.53	22	3.36	36
SC34	pond	22.3	4.84	5.40	23	2.30	36
SC35		23.0	5.22	7.92	23	2.99	92
SC36		19.0	4.47	8.63	19	1.94	91
Mean		**19.6**	**5.2**	**7.2**	**39.9**	**4.4**	

Figure 9. PVC quadrat used to assess aquatic vegetation coverage and other habitat variables at individual eDNA water sample locations.

Water samples were stored on ice and transported to the filtering laboratory to be processed. Valdosta State University was contracted to provide access to a laboratory to filter all GA water samples; all SC water samples were transported to the SCDNR Charleston genetics lab in Charleston SC for filtering. A total of 608 water samples were filtered following the optimized filtering protocols. Two of the 608 water samples from a single site in GA contained excessive amounts of organic matter and were not able to be fully filtered; following 2 hours of partial filtering, the remainder of the 2 water samples was discarded and the filters were retained for processing. After filtration of each water sample, dry filters were folded once and placed in 50 ml centrifuge tubes for storage at -40°C.

The eDNA analysis of all frozen filters occurred at the SCDNR Charleston genetics lab in a separate room from where water filtering occurred. Folded, frozen filters were cut into three strips using surgical grade stainless steel scissors and added to the MO-BIO Power Soil DNA isolation kit. Filters were only handled with clean gloves, forceps, and scissors. Gloves were removed and discarded immediately after handling each filter, and all scissors and forceps were soaked in 10% bleach for ~1-2 minutes after cutting and handling each filter. After the bleach soak, scissors and forceps were rinsed with ultrapure water and wiped dry with clean laboratory tissues. DNA isolated using the MO-BIO Power Soil DNA isolation kit was stored frozen in 100 µl of the proprietary storage solution provided with the kit. Amplification reaction protocols followed the optimized qPCR protocols. Eight technical qPCR replicates were conducted for each DNA isolation including both field sample filters and equipment control filters. Seven no-template control reactions and 1 positive control reaction were conducted for all qPCR master mixes. All eDNA isolations that were not positive for *E. chaetodon* DNA were further tested for PCR inhibition by replacing 1 µl of water with positive control DNA. For sites where PCR inhibition was detected, the results for that site were considered inconclusive.

We completed our eDNA laboratory analysis for all 31 sites visited in GA. A total of five sites had positive detections of *E. chaetodon* DNA (Figure 10) and the results for two additional sites were considered inconclusive due to signs of PCR inhibition. One of the five positive sites was the positive control site selected as our 31st GA locality. The positive detections of *E. chaetodon* DNA at the other four sites provides evidence of previously unknown *E. chaetodon* populations. Three of the positive eDNA sites are clustered in the Alapaha River system near Grand Bay Wildlife Management Area and Banks Lake National Wildlife Refuge; the fourth site is located just downstream of the Arabia Swamp (a conservation easement) in a Suwannee River tributary.

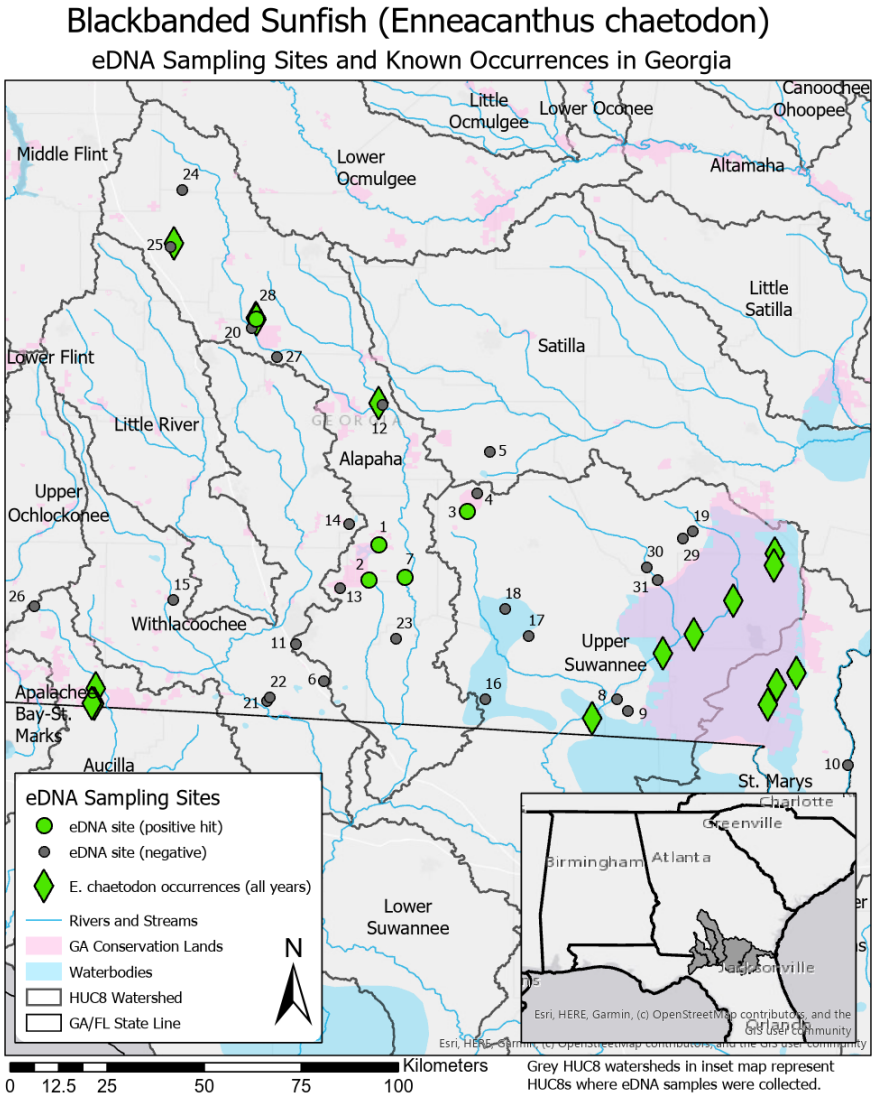

Figure 10. Known occurrences, positive eDNA sites and negative eDNA sites for E. chaetodon in Georgia. Site numbers correspond to data in Table 8.

All of the positive detections in GA, including the positive control site, show signals of low *E. chaetodon* DNA concentrations (Figure 11). More specifically, only 1 or 2 bottles per site had positive detections and only 1-4 technical qPCR replicates amplified per filter. Because the

eDNA tool is not designed to estimate abundance or biomass, we can only speculate as to why we are seeing such low levels of *E. chaetodon* DNA at the positive sites in GA. It may be that there are simply very few fish in these locations (i.e., smaller populations), or perhaps our sampling locations were not the exact source of the *E. chaetodon* DNA (i.e. *E. chaetodon* occupy adjacent waters and their DNA moves into our sampling location via water flow). Furthermore, fish behavior and environmental factors such as water temperature, pH, dissolved oxygen, conductivity and ambient light also play a role in the DNA accumulation/degradation balance.

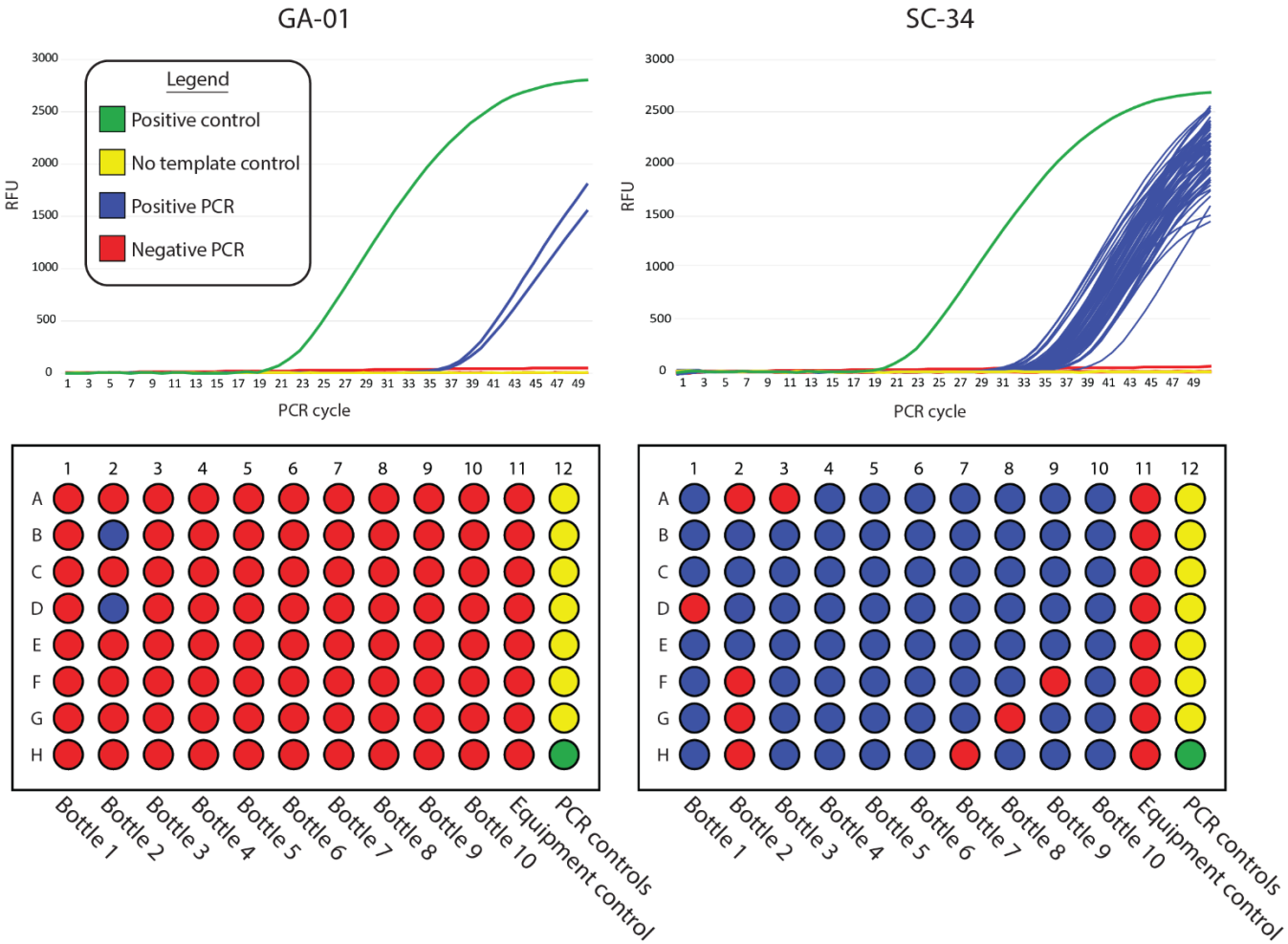

Figure 11. Examples of typical qPCR results for GA and SC. Quantification curves (top) and PCR plate maps (bottom) show positive eDNA PCRs (blue), negative eDNA PCRs (red), positive control PCRs (green), and no template controls (yellow).

All eDNA laboratory analyses on filtered water samples collected in SC were completed. *E. chaetodon* DNA was detected at nine of 30 (30%) sample sites in South Carolina—one site in the Savannah basin, four in the Edisto, and four in the Pee Dee (Figure 12). By site type, *E. chaetodon* was detected at seven of the 27 randomly selected sites (26%) and two of three historic localities (67%); the species was not detected at SC-9 (Hollow Creek) despite being

known from multiple collections at this location as recently as 2013. The detections at the randomly selections sites represent new localities occurrences in South Carolina.

Among DNA-positive sites in South Carolina, number of positive bottles (maximum = 10 per site) ranged from 8 to 10 (Table 10). DNA-positive sites represented a range of habitat types including flowing streams, swamps, beaver impoundments, and man-made mill ponds. The range of habitat types supporting *E. chaetodon* was illustrated by the frequency of mean current velocities (i.e. flow) among DNA-positive sites (Figure 13). Although the majority of positive sites were characterized by low velocities (<0.05 m/s), *E. chaetodon* was also detected in sites exhibiting velocities up to 0.37 m/s. Habitat conditions of all water sampling sites are summarized in Table 11. The positive detections in SC showed that relatively high concentrations of *E. chaetodon* DNA are present at the sites in SC. More specifically, the 8-10 bottles per positive site have shown strong signals of *E. chaetodon* DNA with most technical qPCR replicates amplifying for each filter. These high levels of DNA found in SC are in stark contrast to the results that we obtained for our GA survey, but are cautious to not relate DNA levels to abundance or biomass of *E. chaetodon* populations for reasons discussed above.

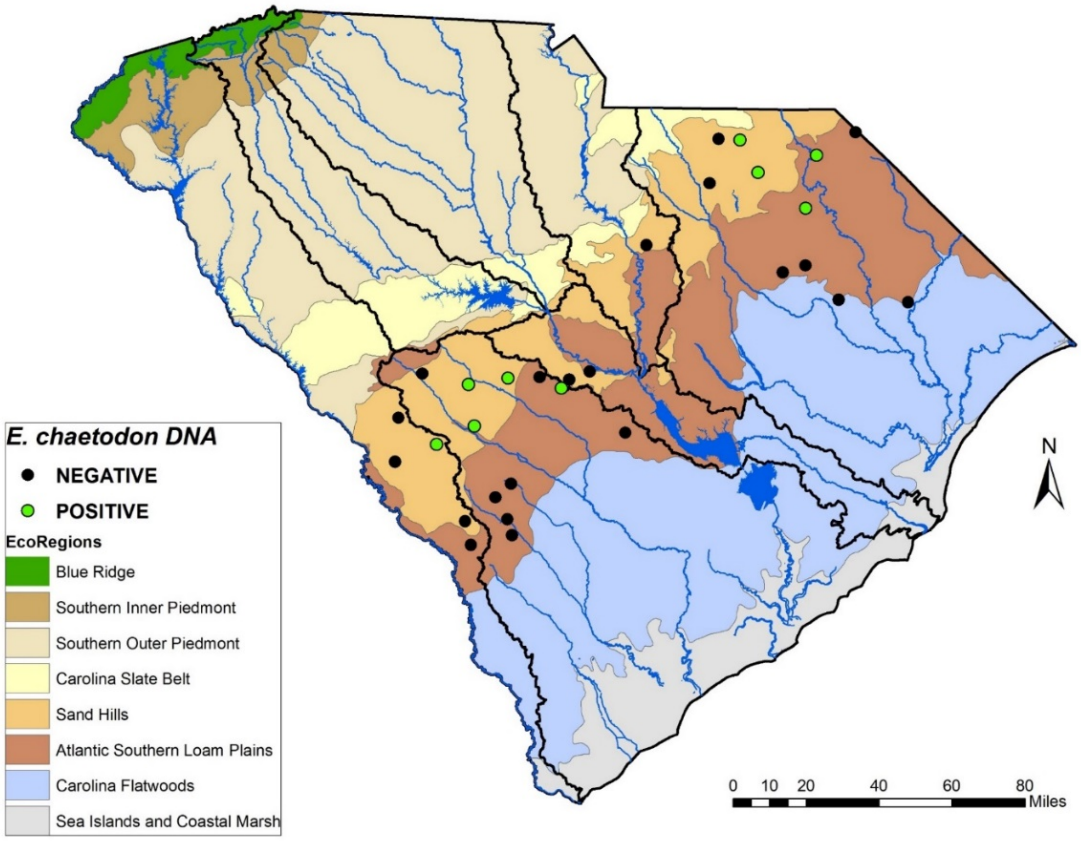

Figure 12. Map of environmental DNA (eDNA) sample sites for Blackbanded Sunfish (Enneacanthus chaetodon) in South Carolina. Black circles represent sites with no positive detections of E. chaetodon and green circles represent sites with positive detections.

Table 10. Enneacanthus chaetodon DNA detection summary among bottles within DNA-positive sites in South Carolina and Georgia. Ten samples (bottles) were collected per site.

Detection Results	**SC**	**GA**
Positive Sites	9	5
Positive bottles (total)	86	7
Negative bottles (total)	4	42
Range: Positive bottles per site	8 - 10	1 - 2

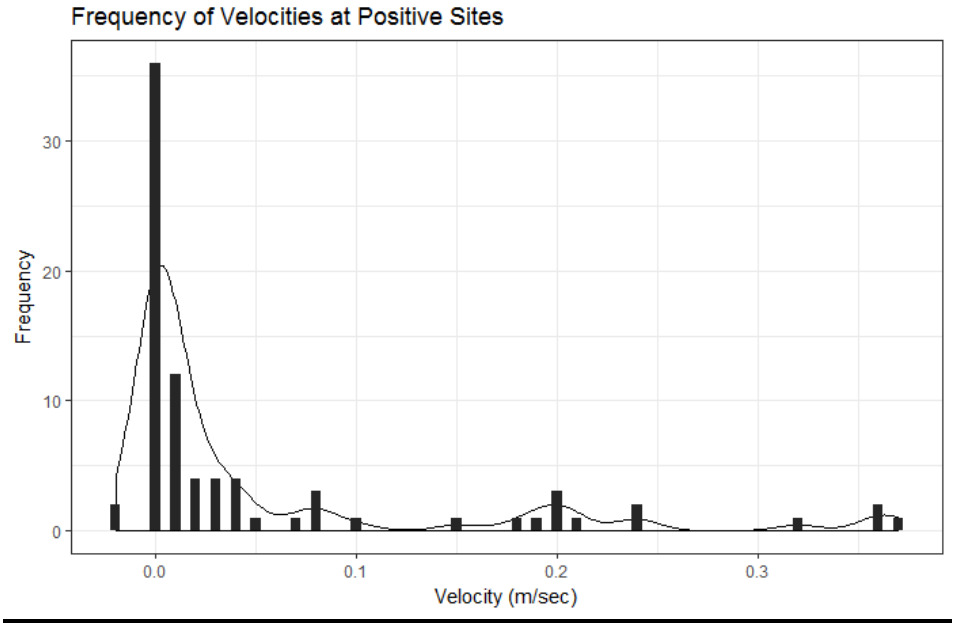

Figure 13. Frequency of mean current velocities from quadrat samples (10 per site) among the nine E. chaetodon DNA-positive sites in South Carolina.

Table 11. Summary of habitat conditions at South Carolina sites as measured by quadrat sampling at water collection locations (10 per site) in April-May 2016. Sites are grouped by DNA status (positive or negative).

Site ID	Mean Depth (m)	Mean Velocity (m/s)	Mean Floating Veg. Cov. (%)	Dominant Floating sp.	Mean Submerged Veg. Cov. (%)	Dominant Submerged sp.
DNA positive sites						
SC16	0.43	0.03	10.8%	Lily pads	38.0%	*Sparganium*
SC17	0.43	0.00	13.7%	*Brasenia*	48.5%	Fine Roots
SC21	0.31	0.01	6.1%	*Brasenia*, Lily	8.0%	Grass
SC25	0.44	0.00	1.6%	*Nymphaea*	44.4%	Naiad-like
SC26	0.43	0.02	0.0%		64.4%	Naiad-like
SC32	0.35	0.14	0.0%		52.5%	Canby's bulrush
SC33	0.27	0.02	16.0%	*Brasenia*	71.5%	Naiad, Grass
SC34	0.60	0.01	5.0%	Heart-leaf Lily	49.5%	Naiad-like
SC35	0.28	0.25	0.0%		29.8%	Fine Roots, Pondweed
Mean	**0.39**	**0.05**	**5.9%**		**45.2%**	
DNA negative sites						
SC07	0.30	0.07	3.3%	*Nymphaea*, *Brasenia*	33.8%	*Myriophyllum*, Naiad
SC08	0.48	0.12	0.0%		42.6%	*Sparganium*
SC09	0.46	0.21	0.0%		23.5%	*Sparganium*
SC10	0.39	0.17	0.0%		8.0%	Grass, Naiad
SC11	0.34	0.12	0.0%		15.5%	*Ludwigia*, *Sagittaria*
SC12	0.54	0.17	0.0%		7.9%	*Sparganium*
SC13	0.27	0.11	0.0%		35.9%	*Sparganium*
SC14	0.42	0.11	0.0%		8.5%	*Ludwigia*
SC15	0.43	0.00	13.4%	Lily	68.0%	*Cabomba*
SC18	0.30	0.03	0.0%		2.5%	Terrestrial
SC19	0.33	0.00	1.2%	Duckweed	38.8%	Naiad
SC20	0.32	0.14	1.4%	Duckweed	6.8%	Grass, *Myriophyllum*
SC22	0.49	0.00	35.3%	*Brasenia*, Lily	23.1%	Lily
SC23	0.31	0.00	0.0%		7.2%	Alligator weed-like
SC24	0.47	0.01	8.0%	*Brasenia*	76.0%	*Ceratophyllum*
SC27	0.35	0.00	14.3%	Lily	15.7%	Lizards tail
SC28	0.30	0.05	0.8%	Duckweed	31.1%	*Sagittaria*-like
SC29	0.95	0.00	0.0%		38.5%	Alligator weed
SC30	0.26	0.04	0.0%		27.0%	Naiad
SC31	0.40	0.10	0.0%		43.8%	*Sparganium*
SC36	0.39	0.07	0.0%		19.6%	Lizards tail, *Sparganium*
Mean	**0.40**	**0.07**	**3.7%**		**27.3%**	

Although we were confident in our qPCR results that yielded positive detections of *E. chaetodon* from our eDNA survey due to our rigorous probe and experiment development (as well as the field verification with actual fish collections in all of the SC positive sites), we took an additional molecular approach to confirm that the positive detections were indicative of presence of *E. chaetodon* DNA. The high concentration of magnesium in our amplification reactions often lead to the formation of non-specific PCR products; however, our probe-based qPCR assay allowed us to fluorescently detect only specific *E. chaetodon* target fragments formed during PCR even

though non-specific products were also being formed. As a result, our originally proposed confirmation method using Sanger sequencing would not be time or cost effective because of the high level of noise (i.e. multiple nucleotide calls at the same site) that would result from the sequencing of multiple PCR products from a single reaction. Therefore, in lieu of Sanger sequencing for target fragment verification, we used capillary gel electrophoresis to separate PCR products from a randomly selected subset of eDNA filter isolations (n=33) for sites in SC and all positive eDNA filter isolations (n=8) from GA sites. An identical temperature profile to our qPCR profile that was adapted for use in end-point PCR on 8 technical replicates of each randomly selected filter isolation before separating PCR products using capillary gel electrophoresis. The resulting chromatograms were scored for the presence or absence of a DNA fragment with the exact length of our eDNA target fragment (136 base pairs).

In both SC and GA the results from the capillary electrophoresis were concordant with those from the qPCR assays. In SC sites, the target fragment amplified in 97% (32/33) of the positive sites; the fragment did not amplify in 1 sample which also had the lowest non-zero percentage (1/8) of positive qPCR technical replicates (Figure 14). One filter isolation selected at random from the positive SC sites had zero positive qPCR replicates however we did have positive end-point amplification at that site. In GA sites, the target fragment amplified in 75% (6/8) of the positive sites; the fragment did not amplify in two samples, again these were samples with low numbers of positive qPCR technical replicates with signals of DNA near the limit of detection of the assay. The strong agreement of the number of technical replicates amplifying in each method (with qPCR generally outperforming capillary gel electrophoresis) provides support that the eDNA tool is properly detecting the presence of *E. chaetodon*.

Figure 14. Comparison between qPCR and capillary electrophoresis fragment length verification.

All hardcopy field datasheets and results of the eDNA analyses were proofed and incorporated into a relational database using Microsoft Access. All field collection data and eDNA results are stored in one location with reports generated for simple and quick display of all relevant data for a specific site of interest. Following completion of Task 3, the traditional field sampling for fish data and results were incorporated into the project Access Database as well.

Significant deviations

The timing of field eDNA surveys was delayed due to complications during the laboratory portion of the tool development. However, we felt the extra time was well spent to verify the function and efficiency of the tool prior to application in the field. The GA sampling was completed during the proposed project year; however, the historic flooding in SC during October 2015 further postponed our SC water sampling. Additionally, completion of this task was delayed due to the unexpected downtime in the SCDNR Charleston genetics lab due to evacuations and recovery associated with Hurricane Matthew in October 2016. The SC sampling was successfully completed during Year 2 of the project. We also modified the methodology from our proposed eDNA target verification, but the replacement methodology was more cost effective and reliable resulting in increased confidence of the verification tests of the eDNA detections.

Objective 3: Conduct a survey using traditional methods in eDNA-positive field sites to provide a relative abundance index among sites and collect fin clips.

Accomplishments

Task 4*: Survey positive eDNA sites using traditional methods.*

Fish Sampling – Georgia

We carried out traditional fisheries sampling in and near Georgia's positive eDNA sites to assess populations and collect *E. chaetodon* tissue samples for genetic analyses (Task 5). We utilized a variety of sampling methods including seining with 12 x 6 x 1/8" mesh seines, netting with 1/8" mesh dipnets, backpack electrofishing, boat electrofishing, and trapping. Trapping utilized pyramidal crayfish traps that have successfully captured *E. chaetodon* in other studies (Johnson and Barichivich 2004). Traps included three different funnels located on the bottom edge of each trap side and were lined with 1/8" mesh netting to prevent escapement (Figure 15a). Traps were set overnight with the top of the trap exposed to prevent suffocation of captured fishes during nocturnal oxygen sags (Figure 15b). We experimented with different baits, including red worms, frozen bloodworms, frozen brine shrimp, hot dogs, and clumps of locally collected aquatic plants that would presumably provide cover and foraging opportunities. Traps were typically set near different cover types, including patches of aquatic vegetation, woody debris, and live cypress trees. All species captured within traps were recorded and the number of *Enneacanthus spp.* were counted to assess their susceptibility to this gear type.

Figure 15. Design (a) and field deployment (b) of pyramidal crayfish traps used to survey fishes in potential E. chaetodon habitats in Georgia.

Sampling initially targeted the five positive eDNA sites. Since eDNA could have originated from a population hydrologically connected to but not within our eDNA sampling site, we conducted additional sampling at nearby waterbodies. Finally, in order to assess the status of Georgia's extant populations of *E. chaetodon* and collect more genetic samples for Task 5, we carried out additional fish sampling in Linton Lake and Okefenokee Swamp National Wildlife Refuge (OSNWR). We put forth considerable effort during surveys for *E. chaetodon* in Georgia using multiple gear types (Table 12). Despite our efforts, we were unsuccessful in sampling *E. chaetodon* at any of the five eDNA positive sites or sites nearby (Figure 16). We were also unable to capture any *E. chaetodon* in Linton Lake, but were able to collect 1 specimen in a trap set in Billy's Lake (OSNWR). During routine fish community monitoring in Okefenokee Swamp, Biologists from the Georgia DNR Fisheries Management Section captured 1 specimen from a new site near Billy's Lake and 6 specimens from Billy's Lake in 2018. Specimens were collected using boat electrofishing and fin clips were provided for genetic analysis.

Table 12. Traditional fish survey sites and sampling effort for E. chaetodon at positive eDNA sites, sites near positive eDNA sites and within historic localities in Georgia. Sites 1, 2, 3, 7, and 28 tested positive for E. chaetodon eDNA.

River System	Site Name	Survey Date	Seine Hauls	Boat Shock Minutes	Trap Hours	Dipnet Minutes
Alapaha	Banks Lake NWR (GA-01)	3/8/2017	60	70	504	289
Alapaha	Dixon Mill Pond (near GA-07)	2/22/2017	5			
Alapaha	Dixon Mill Pond (near GA-07)	12/22/2017		36	414	
Alapaha	Fletcher Lake, east side (GA-28)	2/21/2017	20		185	31
Alapaha	Fletcher Lake, east side (GA-28)	3/7/2017		73	112	
Alapaha	Fletcher Lake, east side (GA-28)	6/20/2019	12		163	
Alapaha	Fletcher Lake, east side (GA-28)	6/27/2019	11		87	
Alapaha	Fletcher Lake, west side (GA-28)	2/21/2017	30		344	297
Alapaha	Fletcher Lake, west side (GA-28)	3/7/2017			272	
Alapaha	Fletcher Lake, west side (GA-28)	6/20/2019	3		195	
Alapaha	Fletcher Lake, west side (GA-28)	6/27/2019			76	
Alapaha	Grand Bay Creek (GA-02)	2/23/2017	5		407	120
Alapaha	Private Farm Ponds (GA-07)	2/22/2017	50		502	270
Aucilla	Connell Creek	6/19/2018	22			40
Aucilla	Linton Lake	6/19/2018			400	50
St. Marys	Chesser Prairie (OSNWR)	12/21/2017			300	
St. Marys	Suwannee Canal (OSNWR)	12/21/2017			83	
St. Marys	Visitor Center Canal (OSNWR)	12/21/2017			111	
Suwannee	Billy's Lake (OSNWR)	12/6/2017		60	353	
Suwannee	Kingfisher Landing (OSNWR)	12/20/2017			446	
Suwannee	Suwannee River Borrow pit (GA-08)	12/6/2017				25
Suwannee	Suwanoochee Creek (GA-03)	3/9/2017	45		363	110
	Total		**263**	**239**	**5317**	**1232**

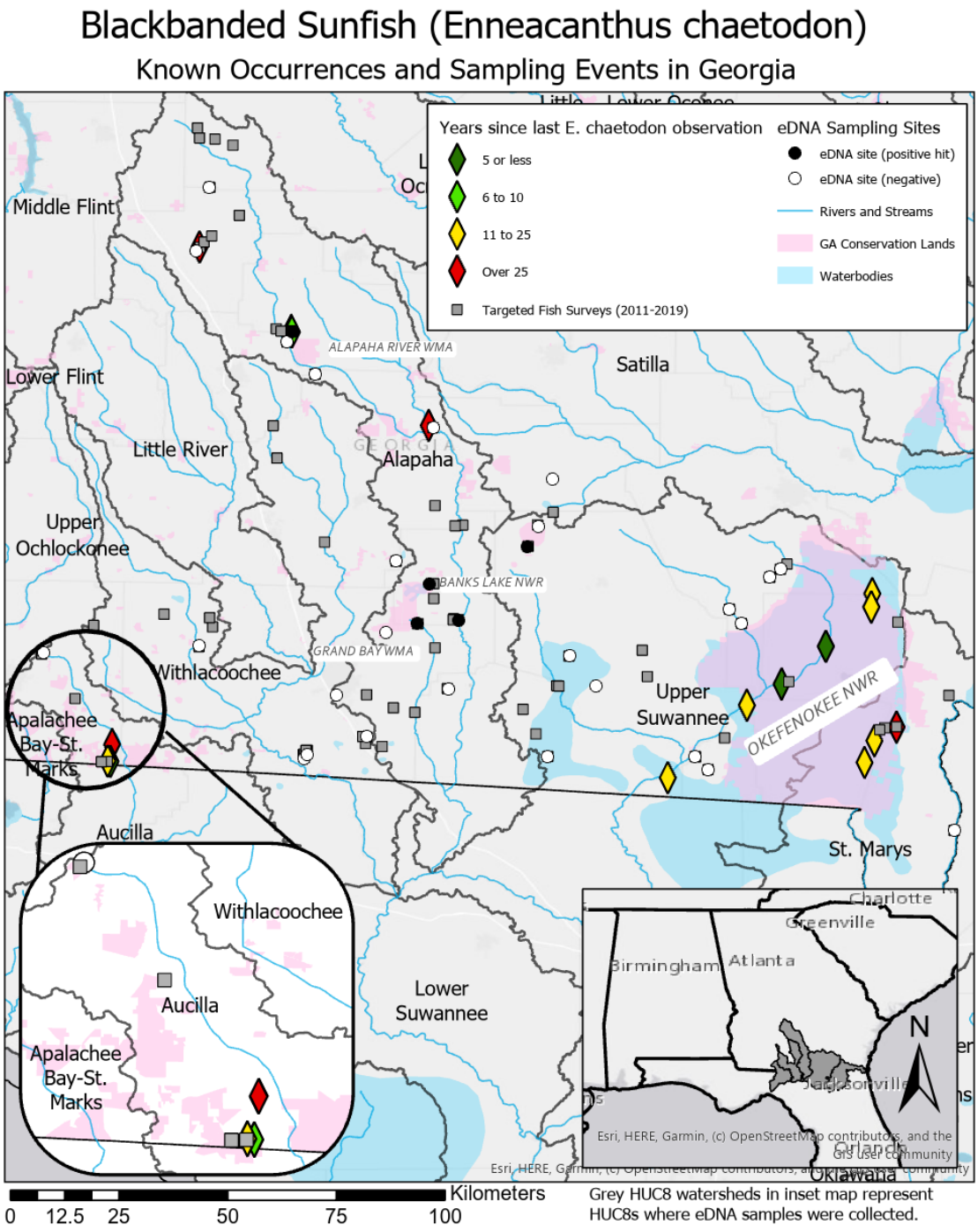

Figure 16. Targeted fish surveys for E. chaetodon during 2011-2018, including sites sampled for this project as well as sites sampled by Bechler and Salter (2014). Known occurrences are categorized by the most recent observation date.

Among the eDNA positive sites, Banks Lake, Fletcher's Lake, and the private farm ponds near the Alapaha River supported diverse fish communities and presumably suitable habitat that included submerged aquatic vegetation and typical water quality (Table 13). Suwanoochee Creek, in contrast, had limited submerged aquatic vegetation during eDNA sampling and consisted of stagnant pools when we completed our fish survey in spring 2017. Grand Bay Creek also had low aquatic vegetation coverage and fish diversity.

Table 13. Diversity of fishes captured during surveys for E. chaetodon at sites in Georgia.

	Banks Lake	Connell Creek	Dixon Mill Pond	Fletcher Pond East	Fletcher Pond West	Grand Bay Creek	Linton Lake	Private Farm Ponds	OSNWR Billy's Lake	OSNWR Chesser Prairie	OSNWR Kingfisher	OSNWR Suwannee Canal	OSNWR Visitor Center	Suwannee Borrow Pit	Suwanoochee Creek
Acantharchus pomotis			X												X
Ameiurus nebulosus	X		X												
Ameiurus sp.		X		X											
Amia calva	X		X	X				X	X	X	X				
Aphredoderus sayanus					X	X	X								
Centrarchus macropterus	X		X	X	X	X	X		X		X				X
Cyprinella leedsi								X							
Elassoma evergladei			X			X			X	X		X		X	X
Elassoma gilberti		X					X								
Elassoma okefenokee	X		X					X					X	X	
Elassoma sp.				X	X							X			
Elassoma sp. "okatie type"				X	X										
Elassoma zonatum		X													
Enneacanthus chaetodon									X						
Enneacanthus gloriosus	X		X	X	X	X	X	X	X	X			X	X	X
Enneacanthus obesus										X	X				X
Erimyzon sucetta	X	X	X	X				X	X	X					
Esox americanus			X												
Esox niger	X			X	X										
Esox sp.	X		X		X	X		X							X
Etheostoma fusiforme	X			X	X		X	X		X		X	X		
Fundulus chrysotus	X		X	X	X		X	X						X	
Fundulus lineolatus			X	X	X				X						
Gambusia holbrooki	X														
Gambusia sp.		X	X	X	X	X	X	X	X	X	X		X	X	
Heterandria formosa	X	X					X	X							

	Banks Lake	Connell Creek	Dixon Mill Pond	Fletcher Pond East	Fletcher Pond West	Grand Bay Creek	Linton Lake	Private Farm Ponds	OSNWR Billy's Lake	OSNWR Chesser Prairie	OSNWR Kingfisher	OSNWR Suwannee Canal	OSNWR Visitor Center	Suwannee Borrow Pit	Suwanoochee Creek
Labidesthes vanhyningi	X	X		X				X							
Lepisosteus platyrhincus	X			X											
Lepisosteus sp.				X											
Lepomis gulosus	X		X	X	X	X		X	X				X		
Lepomis macrochirus	X		X	X	X			X	X				X		X
Lepomis marginatus				X											
Lepomis microlophus				X	X										
Lepomis sp.				X			X				X				
Leptolucania ommata	X	X				X		X	X	X			X	X	X
Micropterus salmoides	X			X	X			X							
Micropterus sp.		X													
Notemigonus crysoleucas	X	X	X	X	X			X							
Notropis maculatus				X	X			X							
Pomoxis nigromaculatus	X		X	X	X			X							
Umbra pygmaea			X											X	X
No. Fish Species	20	10	18	24	18	8	9	18	11	8	5	3	7	7	9

Traps captured a wide diversity of fishes and amphibians, including greater siren (*Siren lacertina*) and two-toed Amphiuma (*Amphiuma means*). Traps captured 336 *E. gloriosus* and a single *E. chaetodon.* About one-third of trap sets included at least one *Enneacanthus* (overwhelmingly *E. gloriosus.* Table 14). The percentage of traps containing at least one *Enneacanthus* was higher for all bait types compared to un-baited traps and was highest for traps baited with brine shrimp or the combination of bloodworms and red worms. These results should be viewed cautiously due to un-equal sample size across trap type and because data were pooled across multiple sites and seasons. Redworms are much easier to use because they are widely available from bait stores and do not require freezing or refrigeration like brine shrimp and bloodworms. Hotdogs produced our largest catches of *Amphiuma* and may increase the chances that captured fishes will be preyed upon within traps.

Table 14. Number (percent) of trap sets containing at least one individual Enneacanthus of any species (gloriosus, obesus, or chaetodon, but primarily E. gloriosus). Traps were generally set overnight in patches of aquatic vegetation or cover and checked during the morning. Empty traps contained no fishes at all.

Bait	No. Traps	*Enneacanthus*	Empty
Un-baited	51	11 (21.6)	28 (54.9)
Plants	12	4 (33.3)	2 (16.7)
Brine Shrimp	21	10 (47.6)	7 (33.3)
Hotdogs	103	36 (35.0)	21 (20.4)
Bloodworms	158	58 (36.7)	53 (33.5)
Bloodworms+ Redworms	18	11 (61.1)	2 (11.1)
Total	**363**	**130 (35.8)**	**113 (31.0)**

Fish Sampling – South Carolina

Similar sampling techniques, including dip netting, seining, backpack electrofishing, and trapping, were used to sample the nine sites throughout SC that showed the presence of *E. chaetodon* eDNA. The effort for sampling these SC sites occurred in April 2017 and included 90 dip net-hours, 364 trap-hours, and over 5 hours of backpack electrofishing (Table 15). At each SC site, sampling effort was initially directed primarily toward the areas from which DNA-positive water samples were obtained, focusing on patches of aquatic vegetation and other dense cover types preferred by *E. chaetodon*. Sampling continued until at least 30 individuals of *E. chaetodon* were collected, or in cases where catch rates were low, when the presence of *E. chaetodon* was confirmed and multiple specimens collected. If sampling within the area covered by the water samples did not yield the target number of *E. chaetodon*, sampling was extended up to 300 m away from the water sampling area in an effort to locate additional *E. chaetodon* (i.e., SC-32a and SC-35a).

Table 15. Total fish sampling effort by gear at DNA-positive sites in South Carolina. Sites 32a and 35a were supplemental sites sampled upstream of sites 32 and 35, respectively. For the active gears (dipnetting and electrofishing), the values presented reflect total person-hours.

Site ID	Date	Trap-hours	Dipnet-hours	Backpack electrofishing hours
SC-16	4/5/2017	364.00	6.49	0.26
SC-17	4/3/2017		11.71	
SC-21	4/18/2017		10.02	
SC-25	4/4/2017		10.98	0.71
SC-26	4/4/2017		14.16	1.43
SC-32	4/17/2017		1.40	1.32
SC-32a	4/19/2017		11.73	1.30
SC-33	4/17/2017		5.10	
SC-34	4/17/2017		4.75	0.34
SC-35	4/18/2017		7.08	
SC-35a	4/18/2017		7.35	
Total		364.00	90.77	5.36

During fish collection, effort was made to measure microhabitat (depth, current velocity substrate and vegetation composition) at specific locations where *E. chaetodon* was collected. However, due to the number of collectors typically spread out over a large area and the relatively high *E. chaetodon* catch rates at many of the South Carolina sites, documenting specific locations was not possible and therefore effort was made to more broadly characterize habitat zones from which *E. chaetodon* was collected.

When *E. chaetodon* individuals were collected, the specimens were transferred to flow-through cages until the completion of sampling; then measured, photographed, fin-clipped for population genetics analyses, and released. Sanitized scissors were used to clip a small portion of the caudal fin from each fish that was placed in individual collection tubes containing 95% ethanol. Other fish and aquatic species were identified as sampling occurred and enumerated when possible to provide a characterization of fish assemblage associations with *E. chaetodon*.

We were able to collect *E. chaetodon* in the immediate area of the positive DNA samples at seven of nine sites in South Carolina, and found specimens farther upstream of the DNA detection area at the two other positive sites. Total catch (not standardized for sampling effort) ranged from 3 to 34 individuals (Table 16). Abundance showed an apparent relation to habitat type, with densely vegetated ponds exhibiting higher *E. chaetodon* densities and catch rates than natural swamps and streams.

Table 16. Blackbanded Sunfish (Enneacanthus chaetodon) total catch and catch per person-hour by gear at DNA-positive sites in South Carolina. Sites 32a and 35a were supplemental sites sampled upstream of sites 32 and 35, respectively.

Site ID	**Trap Total** *(catch/hr)*	**Dipnet Total** *(catch/hr)*	**Backpack Electrofishing Total** *(catch/hr)*	**Site Total** *(catch/hr)*
SC16	3 *(0.008)*	4 *(0.616)*	2 *(7.684)*	9 *(0.024)*
SC17		31 *(2.647)*		31 *(2.647)*
SC21		3 *(0.299)*		3 *(0.299)*
SC25		8 *(0.729)*	0 *(0.000)*	8 *(0.684)*
SC26		17 *(1.201)*	4 *(2.806)*	21 *(1.347)*
SC32		0 *(0.000)*	0 *(0.000)*	0 *(0.000)*
SC32a		19 *(1.620)*	1 *(0.768)*	20 *(1.535)*
SC33		34 *(6.667)*		34 *(6.667)*
SC34		30 *(6.316)*	4 *(11.726)*	34 *(6.678)*
SC35		0 *(0.000)*		0 *(0.000)*
SC35a		33 *(4.490)*		33 *(4.490)*
Total	**3** ***(0.008)***	**179** ***(1.972)***	**11** ***(2.053)***	**193** ***(0.419)***

At two sites where DNA-positive water samples were collected (SC-32 and SC-35), considerable fish sampling effort yielded no *E. chaetodon* from within 300 m of the water sampling area. In both cases, mill ponds with dense aquatic vegetation representing favorable *E. chaetodon* habitats were identified upstream of the water sampling area and were therefore sampled to determine if *E. chaetodon* was present with detectable eDNA potentially moving long distances downstream.

Site SC-32 (Upper Three Runs) is a typical Sand Hills stream with steady flow, predominantly sand substrate, and isolated patches of aquatic vegetation. This site was included as a known *E. chaetodon* location from which the species was collected annually with traditional sampling

methods including electrofishing and dip-netting from 2008 through 2013 (sampling effort not known beyond 2013). These stream systems generally support *E. chaetodon* in lower density than more heavily vegetated lentic environments; accordingly, detection of this species using traditional fisheries sampling methods is typically low in stream settings with limited or marginal habitat. Extensive electrofishing, dip-netting and seining at SC-32 from the eDNA-positive water sampling area to 300 m upstream did not yield any *E. chaetodon*. The nearest outwardly suitable habitat for *E. chaetodon* along the Upper Three Runs watercourse was a mill pond 2.56 rkm upstream of the water sample site, Tarrant Mill Pond (site SC-32a). Sampling in Tarrant Mill Pond yielded 20 *E. chaetodon*, suggesting that viable eDNA from the relatively dense population in the mill pond may have been traveling at least 2.5 rkm downstream in detectable concentrations. However, it remains uncertain what proportion of the positive eDNA detected at SC-32 originated from relatively few fish (i.e. low density) in the nearby stream environment that were simply not detected with traditional sampling compared to that originating from the relatively dense population in the mill pond farther upstream.

In a result mirroring that of SC-32, fish sampling at SC-35 (Cedar Creek) did not produce *E. chaetodon* in the area from which positive water samples were obtained—a braided but generally flowing, stream-like Sand Hills system. Fish sampling was therefore conducted in the first mill pond 1.97 rkm upstream, Rast Pond, where 33 *E. chaetodon* were collected. Together, these observations at SC-32/32a and SC-35/35a suggest that viable *E. chaetodon* eDNA may travel and remain detectable at least 2.5 rkm downstream of the primary DNA source population, under the conditions at the time of water collection and using the field and laboratory methods employed in this study. Nevertheless, these cases further illustrate the challenge of distinguishing the relative contribution of eDNA from nearby individuals occurring in low density versus that from more distant populations at higher densities, especially in complex coastal plain aquatic networks with highly variable habitats and flow regimes.

The lack of DNA detection at SC-9 (Hollow Creek) is interesting in that this was another stream setting at which *E. chaetodon* had been collected repeatedly and as recently as 2013 with traditional sampling (dip-netting), suggesting potential difficulty in eDNA detection where *E. chaetodon* density is relatively low and/or where stream discharge may dilute the DNA concentration.

A diverse assemblage of 35 fish species was documented altogether in association with *E. chaetodon* at the DNA-positive sites in South Carolina (Table 17).

Table 17. Fish species collected during E. chaetodon sampling at DNA-positive sites in South Carolina. Sites are grouped by river basin (SAV = Savannah; ACE = Ashepoo-Combahee-Edisto). Note: list primarily reflects presence of species observed incidentally during sampling targeting dense cover preferred by E. chaetodon; data are not available for SC-25 and SC-34.

Species Name	Common name	SAV	ACE				Pee Dee		
		SC 32	SC 21	SC 33	SC 35	SC 35a	SC 16	SC 17	SC 26
Chologaster cornuta	Swampfish								X
Anguilla rostrata	American Eel	X					X		
Aphredoderus sayanus	Pirate Perch	X	X		X		X		
Erimyzon oblongus	Creek Chubsucker						X		
Erimyzon sucetta	Lake Chubsucker	X						X	
Minytrema melanops	Spotted Sucker	X							
Acantharchus pomotis	Mud Sunfish	X	X				X		
Centrarchus macropterus	Flier		X			X	X		
Enneacanthus chaetodon	**Blackbanded Sunfish**		**X**	**X**		**X**	**X**	**X**	**X**
Enneacanthus gloriosus	Bluespotted Sunfish	X	X	X	X	X	X	X	X
Enneacanthus obesus	Banded Sunfish							X	
Lepomis auritus	Redbreast Sunfish	X						X	
Lepomis gulosus	Warmouth			X		X	X	X	
Lepomis macrochirus	Bluegill		X		X		X	X	
Lepomis marginatus	Dollar Sunfish	X		X		X	X		
Lepomis punctatus	Spotted Sunfish	X			X		X	X	
Micropterus salmoides	Largemouth Bass	X							
Elassoma evergladei	Everglades Pygmy Sunfish		X			X			
Elassoma zonatum	Banded Pygmy Sunfish		X	X					
Esox americanus	Redfin Pickerel	X	X	X	X	X		X	
Esox niger	Chain Pickerel	X	X	X		X		X	
Fundulus lineolatus	Lined Topminnow			X		X		X	
Ameiurus natalis	Yellow Bullhead	X	X	X	X		X		
Noturus gyrinus	Tadpole Madtom	X						X	
Noturus insignis	Margined Madtom				X				
Noturus leptacanthus	Speckled Madtom	X			X				
Nocomis leptocephalus	Bluehead Chub	X							
Notropis cummingsae	Dusky Shiner	X						X	
Notropis lutipinnis	Yellowfin Shiner	X			X				
Pteronotropis stonei	Lowland Shiner	X			X			X	
Semotilus atromaculatus	Creek Chub	X							
Etheostoma fricksium	Savannah Darter	X			X				
Etheostoma fusiforme	Swamp Darter			X		X	X	X	
Etheostoma olmstedi	Tessellated Darter	X							
Gambusia holbrooki	Eastern Mosquitofish		X	X	X	X		X	
Umbra pygmaea	Eastern Mudminnow		X						

Estimate of relative abundance

We were not able to calculate meaningful estimates of relative abundance of *E. chaetodon* in Georgia due to the capture of only a single individual in OSNWR during the project period. The largest known catches from single sampling events of *E. chaetodon* in Georgia's three population areas are: 6 from Linton Lake in 2012, 10 from Fletcher's Lake in 2011, and 9 from OSNWR in 2003. When viewed in context of the diverse fish communities documented during these sampling events, and hundreds of *E. gloriosus* we captured in sites where habitat was presumably suitable for *E. chaetodon*, our data indicate that the abundance of *E. chaetodon* in Georgia is relatively low.

We were able to provide relative abundance estimates for the sites in South Carolina where *E. chaetodon* was collected during the project period. As dip netting was the only collection method that successfully captured specimens at all South Carolina collection locations (Table 16), catch standardized for sampling effort calculated for this sampling gear can be used as an estimate of relative abundance among the sites. In South Carolina, relative abundances were lowest at sites SC-16, SC-21, and SC-25 (0.3-0.7 fish/hour) and highest at sites SC-33 and SC-34 (6.3-6.7 fish/hour).

Detection probability/occupancy modeling

We estimated detection probability and site occupancy for *E. chaetodon* in SC using the single-season models described by MacKenzie et al. (2002). Site occupancy (ψ) is the proportion of sites occupied within the overall study area, corrected for incomplete detection. Detection probability (p) is the probability of detecting a target species within a single eDNA water sample, given that it is present within the site. An important assumption of single-season occupancy models is that sites are closed to changes in occupancy during the entire survey season. Our sampling protocol helped satisfy this assumption because all water bottles collected from a site were sampled on a single day. The model also assumes independence among detections both within and between sites. We attempted to satisfy this assumption by collecting water samples from distinct habitat patches that were a minimum of 5 m from any other sample within a sample site. The closest sample sites in SC were 14 stream kilometers apart which increased the probability that sites represented independent occurrences. The eDNA sampling protocol design (10 water samples at each site) was incorporated into the model.

We hypothesized that detection would vary with a suite of microhabitat variables related to depth, velocity, substrate, and aquatic vegetation. However, since there was almost no variation in detection within sites, we did not include any covariates of p in models. We hypothesized that ψ would vary with elevation (elev), waterbody area (area), percent forest cover in the upstream catchment (forest), and coverage of submerged aquatic vegetation (csav). None of these predictor variables were strongly correlated (all Pearson's $r < 0.46$).

Models were analyzed using the occupancy-estimation procedure in Program MARK (White & Burnham 1999). We assessed models with all possible combinations of covariates, including the global (all covariates) and empty (no covariates) models, for a total of 16 models. All covariates were standardized to a mean of zero and standard deviation of one, which permits direct

comparison of parameter estimates. Model diagnostics in Program MARK indicated lack of fit for our global model (c-hat = 2.65, values greater than 1 indicate overdispersion), so models were ranked using the Quasi-Akaike's Information Criterion ($QAIC_c$). This approach accounts for overdispersion and small sample size (Burnham & Anderson 2002; MacKenzie et al. 2006).

Program MARK calculates model weights that range from 0 to 1, with the most plausible candidate model having the highest weight (Burnham & Anderson 2002). We selected models with weights (w_i) within 10% of the highest ranked model and included them in a confidence set for further interpretation. We compared different models within the confidence set by calculating the ratio of w_i values, which summarize the degree of evidence for one model over another (Anderson et al. 2000). We calculated cumulative detection probabilities, or the probability of detecting eDNA given a sampling effort of (N) water bottles, using the following equation: $1 - (1 - p)^N$. We used estimates of p from models without covariates to determine cumulative detection because they reflect average detection over the range of habitats sampled (Albanese et al. 2011).

DNA from *E. chaetodon* was detected within 9 of 30 (30%) sites using our eDNA sampling protocol. All 10 water samples tested positive for eDNA at 6 of these sites, 9 samples tested positive at 2 sites, and 8 samples tested positive at one site. The estimated probability of detecting *E. chaetodon* eDNA within a single water sample was 0.96 (SE = 0.02), with a 95% C.I. of 0.89-0.98. The cumulative probability of detection exceeded 0.99 after 2 water samples were collected. The model estimated an occupancy rate (ψ) of 0.30 (SE = 0.08), with a 95% C.I. of 0.16-0.48. All estimates are from the model without covariates and reflect average detection and occupancy over the range of habitats we sampled.

Eight of 16 occupancy models were retained in the confidence set for further interpretation (Table 18). The top model included no covariates and was 1.3 to 5.0 times more likely than the remaining models. There was relatively strong support for our second ranked model that included csav as a covariate of ψ and was 2.1 to 3.8 times more likely than the remaining models. The parameter estimate from this model indicated a positive relationship between ψ and csav (beta 0.95). However, the strength of this relationship was not precisely estimated (SE =0.48, 95% C.I. 0.03-1.89). A box plot illustrates higher csav at sites with detections but also high variability in csav at sites without detections (Figure 17). Parameter estimates for remaining covariates indicated weak to non-existent relationships, with confidence intervals overlapping zero.

Table 18. Model structure, weights, and number of parameters for the 8 occupancy models within the confidence set; an additional 8 models were also built but are not shown because of low model weights. Models are ranked by Quassi-AICc (QAICc), which is corrected for small sample size and accounts for overdispersion of the data. Coverage of submerged aquatic vegetation (csav), elevation (elev), forest cover in the catchment (forest), and watershed area (area) were included as covariates on occupancy (ψ). No covariates of ρ (detection) were included in any model because there was almost no variation in detection within sample sites.

Model	QAICc	Weight	Number of Parameters
ψ(.) *p* (.)	32.2	0.256	2
ψ(csav) *p* (.)	32.7	0.197	3
ψ(elev) *p* (.)	34.2	0.094	3
ψ(forest) *p* (.)	34.4	0.085	3
ψ(area) *p* (.)	34.5	0.081	3
ψ(elev, csav) *p* (.)	35.2	0.058	4
ψ(forest, csav) *p* (.)	35.3	0.054	4
ψ(area, csav) *p* (.)	35.4	0.052	4

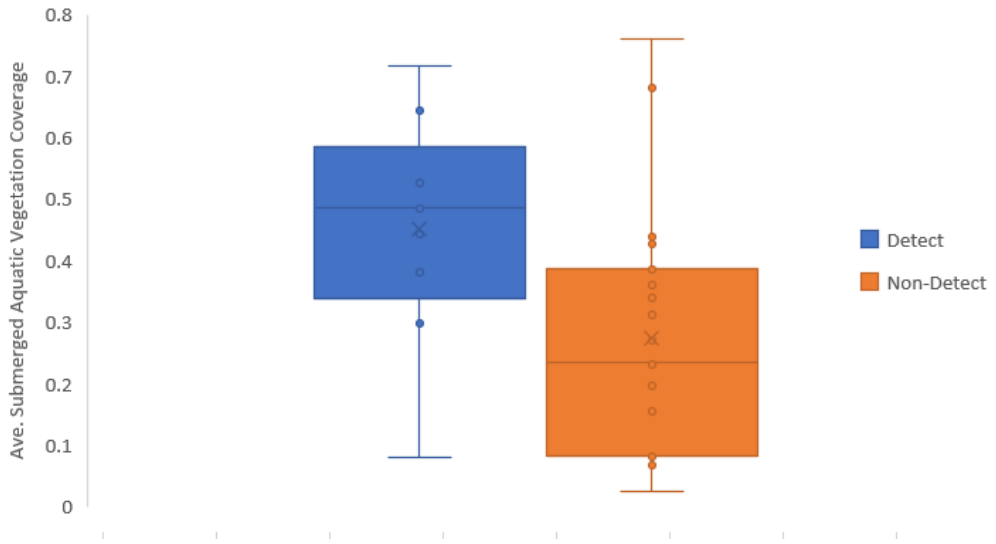

Figure 17. Average submerged aquatic vegetation coverage at sites where E. chaetodon were and were not detected using eDNA sampling in South Carolina.

Our analysis indicates a very high probability of detecting *E. chaetodon* using the eDNA sampling methodology we employed in the Coastal Plain of South Carolina. Our results reflect the environmental conditions, waterbody sizes, and *E. chaetodon* population densities and distributions within the sites that we sampled. Thus, while our results suggest that a single 2 liter

water sample may be sufficient to document the presence of *E. chaetodon*, more samples may be needed to document lower density populations. In addition, it may take more water samples to strategically sample a site. For example, we recommend collecting water samples in suitable habitats along with samples collected at upstream or downstream boundaries of the target area to gain a better understanding of the distribution of *E. chaetodon* within a sample site.

Our occupancy estimate ($\psi = 0.30$) was identical to the proportion of sites where we actually documented *E. chaetodon* eDNA (0.30). These results are consistent with the high detection probabilities estimated. If detection had been lower, there would have been a greater likelihood of not detecting *E. chaetodon* at sites where they occur. Collectively, our results indicate that our eDNA survey in South Carolina was not biased by incomplete species detection. The relationship between site occupancy and the coverage of aquatic vegetation was not precisely estimated, but does suggest that targeting sites with aquatic vegetation may be warranted.

Significant deviations

Outside of the carryover timing delays from Objectives 1 & 2, there were no significant deviations in Objective 3. We did conduct repeated sampling in GA to collect fin clips for genetic analyses. Due to the low number of positive eDNA detections within GA, the Occupancy Modeling was based only on the SC data only as the inclusion of the GA data would confound any occupancy patterns occurring within SC sites.

Objective 4: Characterize the genetic health of extant *E. chaetodon* populations in SC and GA: optimization of microsatellite marker suite, number of genetic samples genotyped, completed genetic data analyses.

Accomplishments

Task 5: *Characterize the genetic health of known and newly-discovered populations of E. chaetodon.*

The 193 fin clips available from the eDNA-associated collections were supplemented with all archived samples at the SCDNR's Genetic Tissue Collection and samples made available by collaborators in other states for a total of 551 samples of *E. chaetodon* across 38 different collection sites and eight states along the U.S. East coast (Figure 18, Table 19). Sample collection occurred across a wide temporal spread with samples generally collected in two time periods: early 2000s and 2015-2018. All genetic collections have been numbered geographically from north to south and do not correspond to project eDNA site numbers; a translation table is provided in Appendix A. DNA was isolated from fin clips and tissue samples using the Wizard SV Genomic DNA Purification System according to manufacturer's instructions and DNA was stored in a -20°C freezer for long-term storage prior to any downstream PCR.

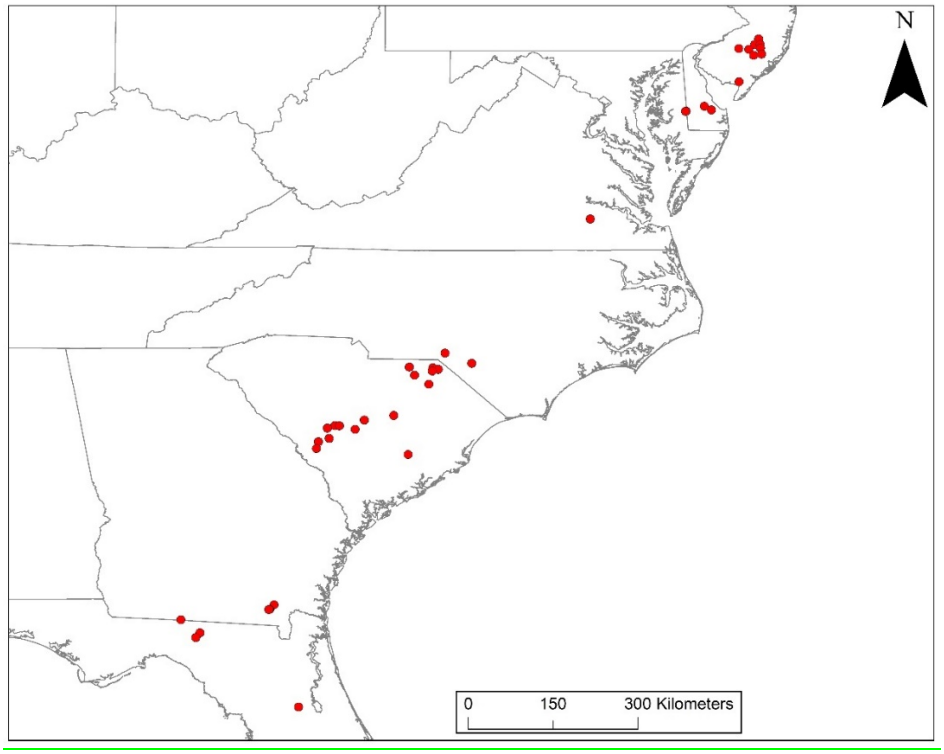

Figure 18. Distribution of all available E. chaetodon samples for genetic analysis.

Table 19. Summary by state and collection of all genetic samples available for the population genetic assessment of E. chaetodon. Multiple collections from a single site are listed separately in chronological order.

State	Collection	n	Year	State	Collection	n	Year
NJ	NJ01	4	2001	SC	SC17	8	2017
	NJ02	9	2001		SC18	10	2000
	NJ03	15	2001		SC19	18	2015
	NJ04	11	2001		SC20	9	2017
	NJ05	1	2001		SC21	21	2017
	NJ06	5	2001		SC22	31	2017
	NJ07	13	2001		SC23	3	2015
	NJ08	4	2009		SC24	34	2015
	NJ09	3	2009		SC25	4	2016
DE	DE10	1	2017		SC26	33	2017
	DE11	4	2009		SC27	34	2017
MD	MD12	3	2015		SC28	3	2017
		5	2016		SC29	34	2017
		5	2017		SC30	20	2017
	MD13	5	2009		SC31	9	2001
VA	VA14	1	2001		SC32	3	2015
NC	NC15	10	2000	GA	GA33	1	2017
	NC16	2	2002		GA34	1	2017
						6	2018
					GA35	2	2001
				FL	FL36	141	2017
					FL37	20	2018
					FL38	5	2015

Microsatellite Suite Optimization

Six microsatellite loci have been published and used for population genetic studies of *E. chaetodon* (Kercher 2001), including five specifically developed for *E. chaetodon* and one originally used in *Lepomis auritus*. To increase the power of our marker suite, we added the most polymorphic microsatellite markers from closely related genera *Ambloplites* (Eschenroeder and Roberts 2016) and *Archoplites* (Schwartz and May 2004). Seventeen total microsatellite loci were screened for amplification and polymorphism in *E. chaetodon* using singleplex PCR and agarose gel electrophoresis with nine showing successful amplification; however, after running hundreds of samples, one monomorphic locus (Ech33) showed inconsistent amplification across individuals and was dropped from any further analyses. The remaining eight microsatellite loci (Table 20) were optimized to run at a single annealing temperature, supplemented with a fluorescent label, incorporated into two groups for multiplex reactions in capillary gel electrophoresis, and retained for further analyses. All PCRs were performed on I-Cycler

thermocyclers (Bio-Rad) using the final optimized reaction profile: initial denature at 94°C for 2 minutes followed by 30 cycles of denaturing at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 65°C for 30 seconds, and a final extension at 65°C for 1 hour. Optimized PCRs occurred in 11 µL reactions containing 1× HotMaster Buffer, 0.2 mM dNTPs, 2.0 mM $MgCl_2$, 0.2 mg/ml BSA, 0.3 µM forward and reverse primers, and 0.03 U HotMaster *Taq* DNA polymerase, and ~5 ng DNA.

After PCR conditions were optimized, all samples were genotyped and the microsatellite suite was validated. Genotyping occurred on a Beckman CEQ 8000/GenomeLab GeXP (Beckman Coulter, Inc.). Both size standards (Genome Lab DNA Size standard kit 400) and reaction products were separated, with fragment size analysis performed with CEQ Fragment Analysis Software. All chromatograms were scored manually by two independent readers. Discrepancies between readers were resolved in conference, or samples were rerun to obtain an unambiguous genotype for all individuals. All loci were tested for Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) using the programs Arlequin and Genepop. All loci were in HWE across all collection locations ($p>0.065$) and none of the pairs of loci were significantly linked ($p>0.003$) after Bonferroni correction.

Table 20. Summary of optimized microsatellite loci used for the genetic structure and health of extant E. chaetodon populations.

Multiplex	Locus	Size Range	# alleles
1	Ech9	104-108	2
	Ech14	142-194	11
	RB20	248-264	5
	AinD212	253-257	2
2	Ech12	69-201	10
	Ech32	124-196	22
	AinA6	144-316	41
	AinA120	302-370	18

Genetic Population Structure

Prior to the characterization of the genetic health of *E. chaetodon* populations, samples were first evaluated on a spatial scale to determine the degree of differentiation in *E. chaetodon* across their range and determine realistic clusters of individuals for the estimation of genetic diversity metrics. The genetic population structure of *E. chaetodon* was evaluated across their range using pairwise comparisons of F_{ST} calculated in GenAlEx. In addition, a clustering model assignment was employed in the program Structure using a hierarchical approach with the assistance of the web-based software Structure Harvester to identify the most appropriate number of clusters (*K*) of each run. Simulations were run using sampling locations as a prior, with five replicates for each *K*, the length of the burn-in period = 50,000, and number of Markov chain Monte-Carlo reps after burn-in = 250,000. Sites that were strongly assigned to one population were removed from the data set and Structure was run iteratively on any clusters that included multiple

collection sites until *K*=1 was the most appropriate assignment for each cluster or until we reached a small geographic area (e.g. multiple sites within a small drainage clustering together). A PCA of major and minor genetic clusters was conducted using the *adegenet* package in the R programming environment (R Core Team 2012) to visualize genetic distance between these clusters. After the collection sites were assigned to the appropriate cluster, the genetic diversity of each cluster was calculated by estimating observed (H_O) and expected (H_E) heterozygosity, allelic richness (A), and inbreeding coefficient (F_{IS}) in GenAlEx.

Pairwise comparisons of F_{ST} and allelic frequency distributions were only performed on sites where at least five individuals were collected and successfully genotyped, which reduced the available sites for analysis to 22. Results indicated that *E. chaetodon* were highly isolated by collection site, showing elevated F_{ST} values between almost all sites, although a wide range was observed (mean: 0.290, range: 0.02–0.795; Table 21). Only two pairwise comparisons were not significantly different. In each of the instances where genetic distances did not differ from zero, *E. chaetodon* were collected from adjacent sites with a pair in MD and a pair in SC. These instances likely represent current or recent connection in those water bodies given their close proximity, allowing for gene flow between those sites.

Table 21. Pairwise F_{ST} (below diagonal) and probability (above diagonal) for E. chaetodon from each genetic collection with five or more individuals. Non-significant p-values (> 0.05) and corresponding F_{ST} values are highlighted red.

	NJ02	NJ03	NJ04	NJ07	MD12	MD13	NC15	SC18	SC19	SC20	SC21	SC22	SC24	SC26	SC27	SC29	SC30	SC31	GA34	FL36	FL37	FL38	
NJ02		0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	**NJ02**
NJ03	0.385		0.003	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.001	**NJ03**
NJ04	0.384	0.063		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	**NJ04**
NJ07	0.248	0.183	0.147		0.001	0.001	0.004	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.002	**NJ07**
MD12	0.775	0.405	0.391	0.392		**1.000**	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	**MD12**
MD13	0.795	0.418	0.404	0.405	**0.020**		0.002	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.007	**MD13**
NC15	0.317	0.237	0.227	0.193	0.404	0.412		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.001	**NC15**
SC18	0.373	0.282	0.272	0.243	0.451	0.459	0.100		0.001	**0.092**	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	**SC18**
SC19	0.321	0.255	0.245	0.210	0.387	0.394	0.062	0.053		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	**SC19**
SC20	0.379	0.294	0.292	0.252	0.461	0.469	0.111	**0.041**	0.049		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	**SC20**
SC21	0.403	0.261	0.240	0.244	0.446	0.453	0.127	0.146	0.127	0.194		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	**SC21**
SC22	0.476	0.338	0.336	0.322	0.526	0.534	0.137	0.133	0.092	0.129	0.202		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	**SC22**
SC24	0.489	0.358	0.349	0.320	0.560	0.568	0.159	0.164	0.117	0.154	0.215	0.104		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	**SC24**
SC26	0.259	0.220	0.209	0.171	0.372	0.380	0.090	0.097	0.073	0.102	0.139	0.133	0.102		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	**SC26**
SC27	0.425	0.314	0.306	0.266	0.497	0.507	0.131	0.150	0.117	0.151	0.192	0.144	0.167	0.107		0.001	0.001	0.001	0.001	0.001	0.001	0.001	**SC27**
SC29	0.500	0.332	0.325	0.327	0.569	0.577	0.224	0.265	0.221	0.277	0.227	0.299	0.272	0.190	0.286		0.001	0.001	0.001	0.001	0.001	0.001	**SC29**
SC30	0.422	0.308	0.296	0.279	0.500	0.507	0.181	0.201	0.170	0.218	0.183	0.199	0.198	0.133	0.185	0.122		0.006	0.001	0.001	0.001	0.001	**SC30**
SC31	0.541	0.374	0.362	0.365	0.615	0.624	0.256	0.285	0.249	0.303	0.228	0.280	0.276	0.206	0.267	0.158	0.055		0.001	0.001	0.001	0.002	**SC31**
GA34	0.424	0.332	0.322	0.313	0.567	0.577	0.270	0.251	0.245	0.264	0.300	0.334	0.322	0.170	0.314	0.360	0.291	0.368		0.001	0.001	0.004	**GA34**
FL36	0.499	0.314	0.318	0.343	0.535	0.542	0.238	0.228	0.200	0.256	0.224	0.209	0.218	0.182	0.240	0.298	0.220	0.278	0.301		0.001	0.001	**FL36**
FL37	0.435	0.295	0.288	0.272	0.496	0.503	0.149	0.168	0.125	0.181	0.163	0.153	0.165	0.136	0.164	0.253	0.174	0.226	0.275	0.093		0.001	**FL37**
FL38	0.545	0.415	0.397	0.362	0.655	0.667	0.263	0.250	0.231	0.273	0.269	0.281	0.277	0.187	0.251	0.393	0.283	0.369	0.404	0.280	0.214		**FL38**
	NJ02	**NJ03**	**NJ04**	**NJ07**	**MD12**	**MD13**	**NC15**	**SC18**	**SC19**	**SC20**	**SC21**	**SC22**	**SC24**	**SC26**	**SC27**	**SC29**	**SC30**	**SC31**	**GA34**	**FL36**	**FL37**	**FL38**	

The clustering model analysis employed in Structure allowed for all sites and individuals to be included in the analysis and indicated that the greatest differentiation occurred when *E. chaetodon* were assigned to four major groups with some collections showing admixture (Figure 18). These genetic clusters are generally associated with geographic areas, with a northern group (North), two southern groups (South A & South B), and a Florida group. Admixture was observed between South A & B (South A/B) and South A & Florida (South A/Florida, Figure 19). Additionally, PCA visualization (Figure 20) supports the Structure-identified major clusters as well as results indicating mixed ancestry between two major clusters at some sites. Furthermore, pairwise F_{ST} values were significant between all major clusters, though the mixed ancestry clusters generally showed lower F_{ST} values with the two clusters from which they were mixed (Table 22). Among major cluster differentiation for *E. chaetodon* ranged from 0.301 to 0.176 with an apparent isolation by distance pattern. The high level of F_{ST} values suggests low dispersal and gene flow are occurring among collection locations and major clusters. The collections showing admixture between the major groups appear to represent different ancestry patterns, with the South A/B collections indicating a closer genetic relationship to the South A cluster while the South A/Florida collections indicate similar genetic relatedness to both the South A and Florida clusters. Neither of these patterns would be anticipated based on their geographic proximities.

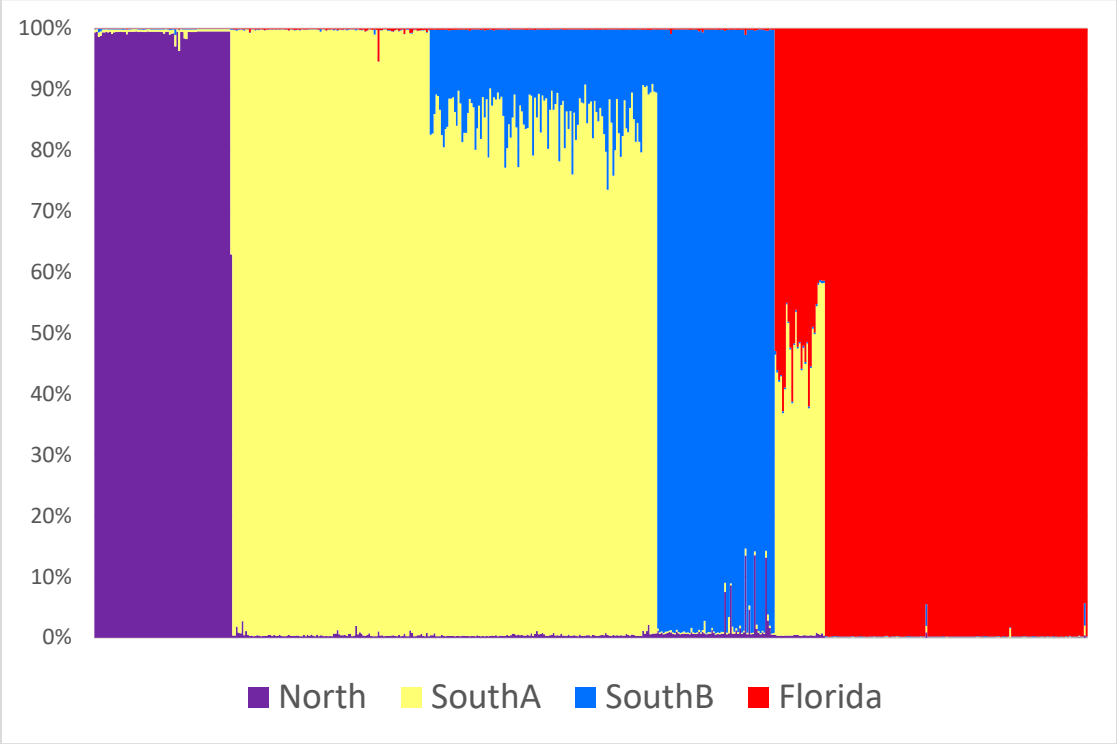

Figure 18. Output from Structure incorporating all collections and individuals that are displayed geographically with northern most (NJ) collections on the left and southern most (FL) collections on the right. Each vertical line represents an indivual's percent ancestry assigned to each of the four major clusters. Results are shown from an K=4 analysis with some collections showing admixture.

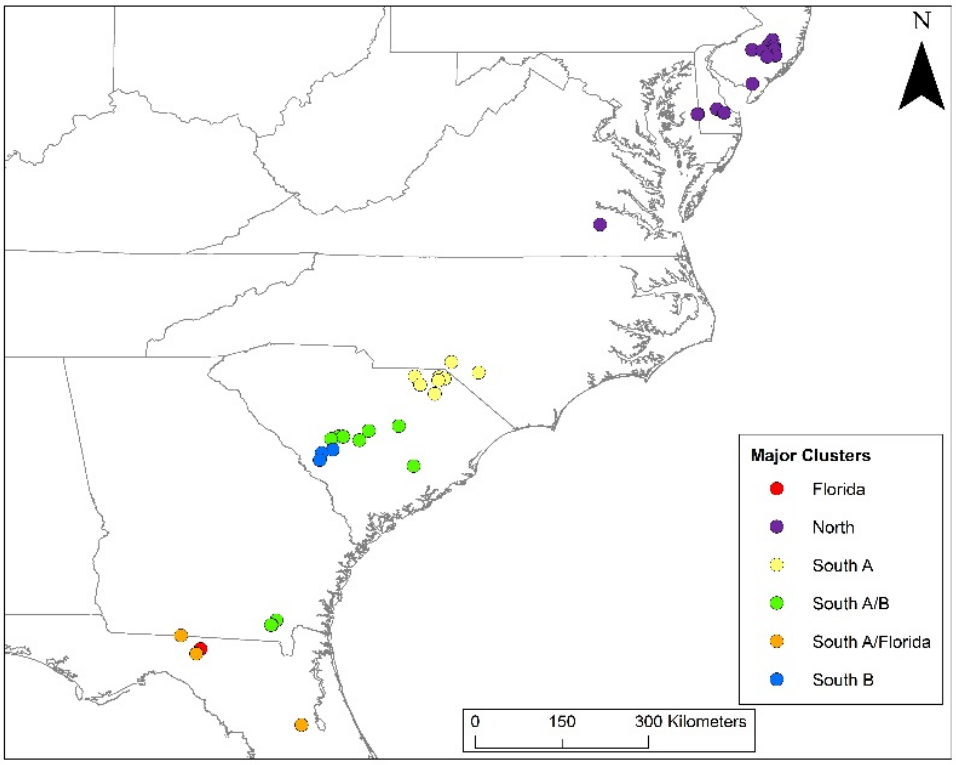

Figure 19. Geographic distribution of major genetic clusters of E. chaetodon by collection, with collections displaying admixture between major clusters given a unique color (i.e., South A/B, South A/Florida).

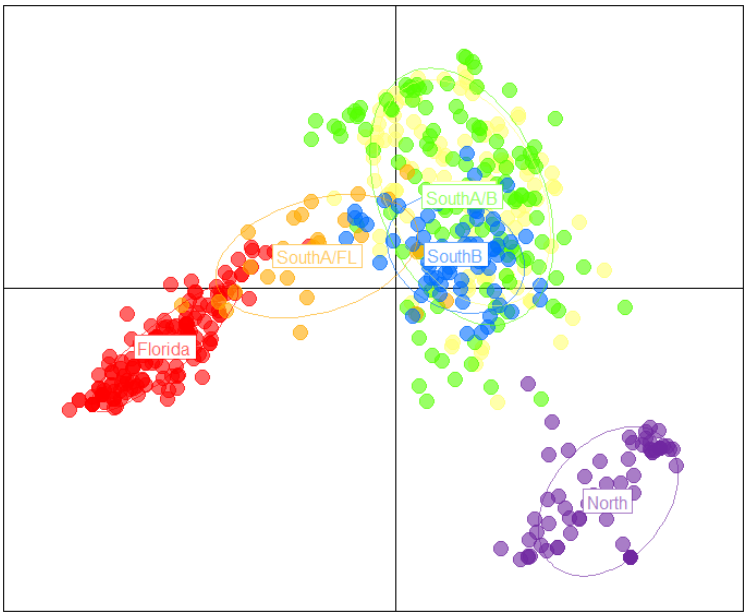

Figure 20. Principal Component Analysis (PCA) of axes 1 & 2, coded by the Structure-assigned major genetic clusters of E. chaetodon by collection. Collections displaying admixture between major clusters given a unique color (i.e., South A/B, South A/Florida).

Table 22. Pairwise F_{ST} (below diagonal) and probability (above diagonal) for E. chaetodon from each major cluster. All p-values are significant (<0.05).

	North	SouthA	SouthA/B	SouthB	SouthA/FL	Florida	
North		0.001	0.001	0.001	0.001	0.001	**North**
SouthA	0.176		0.001	0.001	0.001	0.001	**SouthA**
SouthA/B	0.177	0.036		0.001	0.001	0.001	**SouthA/B**
SouthB	0.261	0.145	0.138		0.001	0.001	**SouthB**
SouthA/FL	0.238	0.080	0.084	0.172		0.001	**SouthA/FL**
Florida	0.308	0.161	0.160	0.235	0.088		**Florida**
	North	**SouthA**	**SouthA/B**	**SouthB**	**SouthA/FL**	**Florida**	

Hierarchical Structure simulations of the northern collection sites revealed that *E. chaetodon* collected at two adjacent ponds in MD were strongly isolated relative to all other northern *E. chaetodon* and the remaining individuals were separated into three clusters for a total of four minor clusters in the northern region (Figure 21). Two sites in NJ and one site in VA were not strongly assigned to one population (showing a mixed population assignment) based on Structure results, but showed more association with cluster North03 in the PCA (Figure 22). The extreme isolation of the two MD ponds, which are approximately 0.6 km apart, relative to the other northern samples was expected after further examination of the genotypes for the MD fish. All of the *E. chaetodon* collected in the adjacent MD ponds had almost identical homozygous genotypes except for one individual genotype which diverged by one allele at one locus. The lack of variation in *E. chaetodon* at those sites is likely due to a small founding population and prolonged inbreeding in those locations causing a lack of genetic variation. The remaining northern clusters are generally separated by geographic space and river drainage.

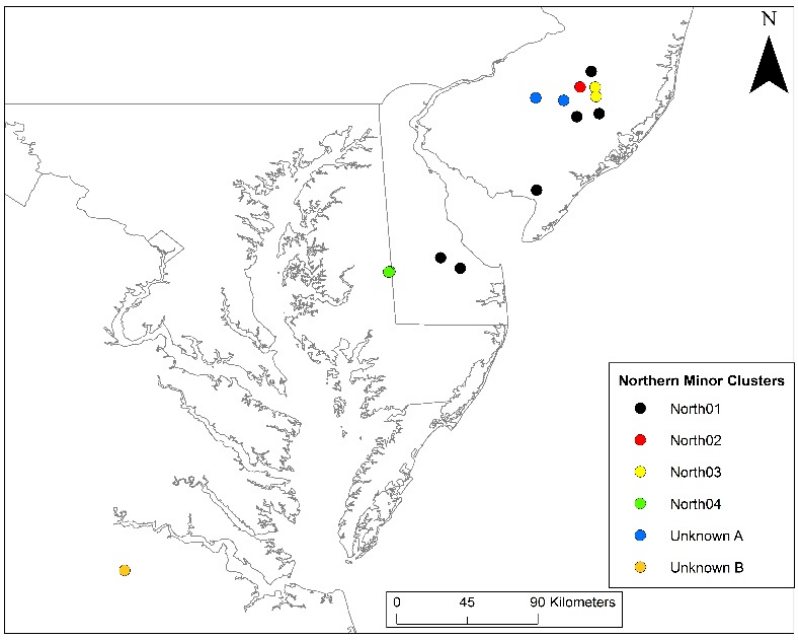

Figure 21. Geographic distribution of North minor genetic clusters of E. chaetodon by collection, with collections displaying admixture between major clusters given a unique color (i.e., Unknown A & B).

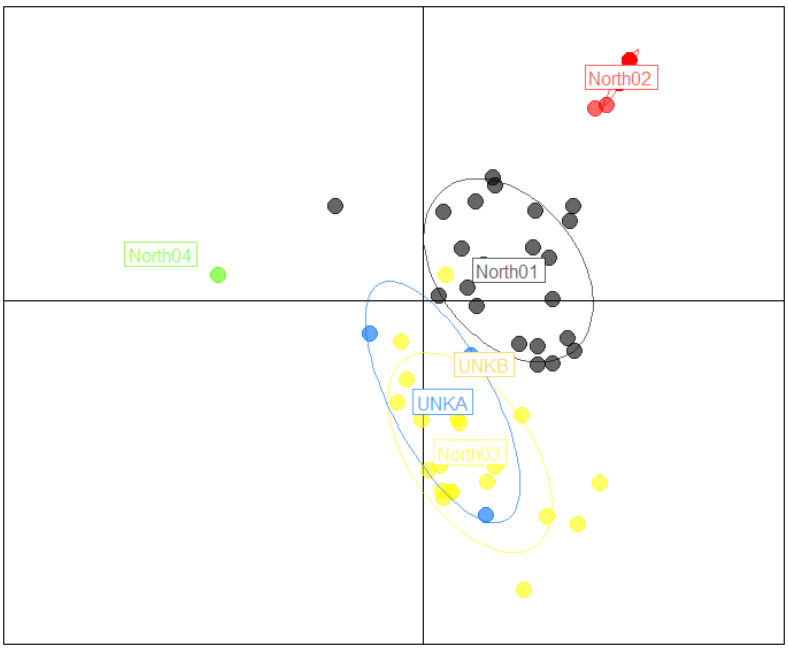

Figure 22. Principal Component Analysis (PCA) of axes 1 & 2, coded by the Structure-assigned North minor genetic clusters of E. chaetodon by collection. Collections displaying admixture between major clusters given a unique color (i.e., Unknown A & B).

In the south, *E. chaetodon* were separated into 13 minor clusters based on the hierarchical structure analysis (Figure 23). The two sites in Florida showed more divergence than the other southern sites (Figure 20) and therefore were displayed on a separate PCA (Figure 24). The remaining southern sites, despite showing significant population structure in Structure results, show mostly overlapping genotypes based on the PCA (Figure 25). One site in NC (Unknown C) showed a mixed assignment to multiple Structure clusters and was clustered amongst several of the SC sites in the PCA. These minor clusters are most likely approximating site level genetic differentiation and in most cases only represent a single site or two-three sites in close proximity. The overall results of the hierarchical Structure analyses supported 13 minor clusters and agreed with the pairwise comparison results in showing strong differences between *E. chaetodon* by collection sites.

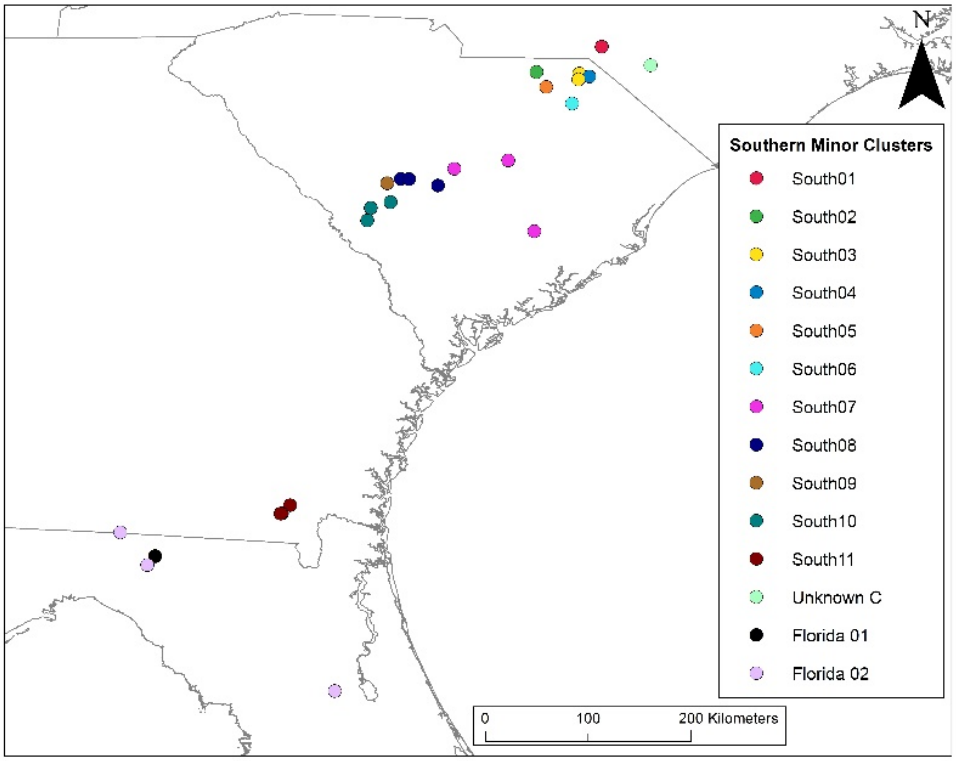

Figure 23. Geographic distribution of South A, South B, and Florida minor genetic clusters of E. chaetodon by collection, with collections displaying admixture between major clusters given a unique color (i.e., Unknown C).

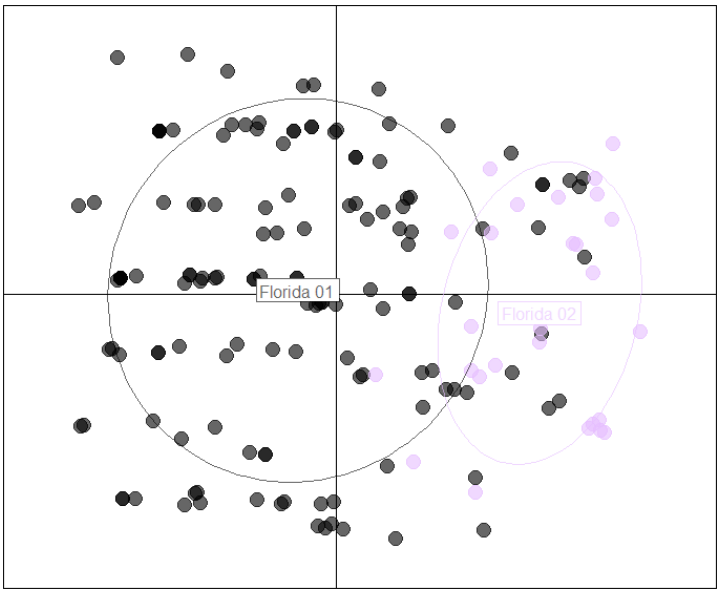

Figure 24. Principal Component Analysis (PCA) of axes 1 & 2, coded by the Structure-assigned Florida minor genetic clusters of E. chaetodon by collection.

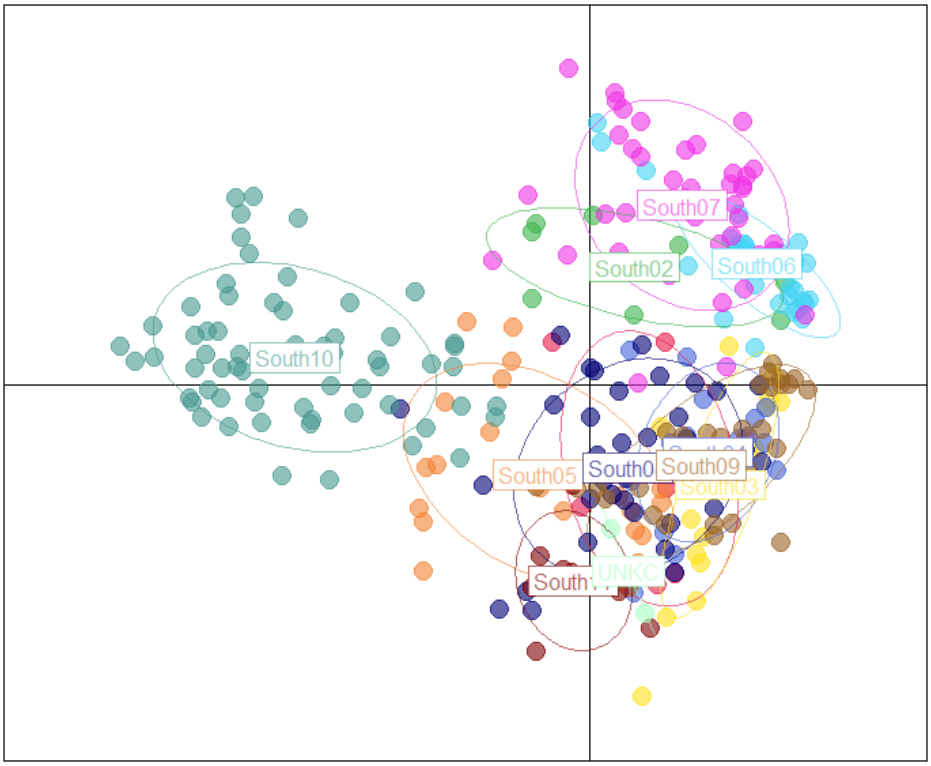

Figure 25. Principal Component Analysis (PCA) of axes 1 & 2, coded by the Structure-assigned South A & South B minor genetic clusters of E. chaetodon by collection.

Genetic Diversity

The genetic diversity of *E. chaetodon* was estimated across its range at both the level of both major and minor genetic clusters (Tables 23 & 24). At the level of the major clusters, overall genetic diversity is moderately low with indications of inbreeding occurring throughout its range ($F_{IS} \geq 0.10$). Expected heterozygosity was highest (0.57) in the South A cluster, occurring in northern SC and NC, and showed a decreasing trend southward to 0.42 in the Florida cluster. The North cluster exhibited the lowest diversity (0.35) as well as the highest levels of inbreeding (0.36).

Patterns at the level of the minor clusters indicates similar concerning patterns for all of the North clusters in terms of both diversity and inbreeding (Table 24). However, there are clear diversity differences among clusters in this area with two clusters having higher diversity (0.31-0.35) than the others. At the minor cluster level for the more southern groups, the decreasing diversity trend in the major clusters is not readily apparent. The South A and South B minor clusters consistently show higher levels of diversity (0.38-0.57) with lower indications of inbreeding. Although the Florida and South A/Florida minor clusters show higher diversity (0.42-0.49), both also indicate low levels of inbreeding. Most of the South A/B minor clusters show the lowest diversity in the southern region (0.31-0.39) that is comparable to the northern cluster diversities, with one cluster being an exception (South08) exhibiting one of the highest diversities (0.53).

Table 23. Summary of genetic diversity estimates of E. chaetodon across the range in major clusters (n=sample size, H_E=expected heterozygosity, H_O=observed heterozygosity, A=allelic richness, F_{IS}=inbreeding coefficient).

Major Cluster	n	H_E	H_O	A	F_{IS}
North	74	0.35	0.22	5.13	0.36
South A	106	0.57	0.46	11.13	0.20
South A/B	122	0.52	0.41	7.75	0.21
South B	63	0.46	0.39	5.88	0.14
South A/FL	27	0.49	0.44	4.75	0.11
Florida	141	0.42	0.46	3.25	-0.10

Table 24. Summary of genetic diversity estimates of E. chaetodon across the range in minor clusters (n=sample size, H_E=expected heterozygosity, H_O=observed heterozygosity, A=allelic richness, F_{IS}=inbreeding coefficient). N/A indicates insufficient heterozygosity to estimate an inbreeding coefficient since all individuals are nearly clones.

Major Cluster	Minor Cluster	n	H_E	H_O	A	F_{IS}
North	North01	22	0.35	0.30	4.0	0.14
	North02	9	0.09	0.07	1.4	0.19
	North03	21	0.31	0.36	2.9	-0.15
	North04	18	0.01	0.01	1.1	N/A
South A	South01	7	0.50	0.51	3.4	-0.01
	South02	8	0.38	0.38	3.0	0.01
	South03	19	0.48	0.46	5.0	0.03
	South04	18	0.57	0.60	6.1	-0.07
	South05	21	0.46	0.42	4.4	0.10
	South06	31	0.39	0.40	5.0	-0.04
South A/B	South07	40	0.39	0.34	5.4	0.13
	South08	40	0.53	0.52	6.3	0.01
	South09	34	0.36	0.37	3.0	-0.02
	South11	8	0.31	0.34	2.5	-0.11
South B	South10	63	0.46	0.39	5.9	0.14
Florida	Florida01	141	0.42	0.46	3.3	-0.10
South A/Florida	Florida02	27	0.49	0.44	4.8	0.11

The baseline data provided by the genetic characterization of *E. chaetodon* throughout their range provides a critical foundation for future conservation and management. The major clusters would be effective management units for the species, with minor cluster information providing important within-

unit variation to guide any restoration work. We also found no relationship between the relative abundance estimates and genetic diversity as the sites with the highest relative abundances occur in minor clusters showing high (South 08 & 10) and low (South 06 & 09) genetic diversities. Conversely, sites with low relative abundance occur in several clusters with high genetic diversity (South 03, South 08, Florida 01 & South A/Florida). Therefore, the genetic results support a strong recommendation for genetic evaluation of all sites prior to any relocation or supplementation programs for *E. chaetodon*. The short life history and often small-scale patchy distribution of *E. chaetodon* not surprisingly appears to result in small and isolated populations that is reflected in their lower genetic health metrics across their range. Although these metrics are on the low end for typical conservation targets, we found no decreasing trends in our dataset for this species from samples collected in the early 2000s when compared to those collected in 2015-2018.

Significant deviations

Timing of the completion of tasks for Objective 4 were impacted by the carryover timing delays as described for Objectives 1 & 2, as well as the federal government shutdown during the winter of 2018-2019 which required the physical relocation of both laboratory equipment and staff from the federal Hollings Marine Lab which houses the SCDNR Population Genetics Lab to SCDNR's Marine Resources Research Institute facility. However, all tasks were completed as proposed. In addition, we were able to supplement our project collection of genetic tissue samples from regional collaborators to expand the extend of our analysis of *E. chaetodon* population genetic structure and health.

Objective 5: Develop and implement improved conservation and management actions to protect *E. chaetodon* populations and habitats.

Accomplishments

Task 6 – *Update the SC Stream Conservation Planning Tool.*

In order to consider updating the Planning Tool, we must have a species distribution model that produces estimates of spatial distribution with reasonable confidence. After an assessment of the field-collected data, we found extremely low within-site variation in the data set that precluded us from meaningful microhabitat-based analyses. Therefore, we focused our *E. chaetodon* distribution modeling effort on among-site patterns to identify landscape and watershed factors that may be important predictors of their distribution across the landscape. Additionally, due to the low number of *E. chaetodon* collections in GA, model analyses were conducted only using the SC data, allowing for the possibility that predictions could be applicable for GA using the developed model.

We attempted to model two response variables. First was the binary presence or absence of *E. chaetodon* across South Carolina sites. The second was catch rates using dipnets (number of individuals per hour of effort; Table 16), which was the only method applied across all sites. Catch rate was a continuous variable and occurrence was a categorical variable.

Predictor variables were derived from an assessment and geodatabase (Wang et al. 2011, Wieferich et al. 2015) made under the National Fish Habitat Action Plan (NFHAP). The NFHAP hierarchical spatial framework and database provides spatial predictor data for catchments across the entire state, enabling us to extrapolate our models across unsampled areas of the state. The NFHAP spatial framework and database was created using the National Hydrography Dataset Plus (NHDPlus). The NHDPlus is a vector dataset describing hydrological networks and associated catchment spatial characteristics at a

spatial scale of 1:100,000. The smallest basic spatial unit of the NHD+ are fluvial networks represented by confluence to confluence stream reaches (flowlines). Within the NFHAP database, each flowline is attributed with predictor data at two spatial levels: 1) local catchment spatial attributes, and 2) network catchments spatial attributes. Local catchments are defined as the elevation-derived drainage boundary that has a 1:1 relation to a given NHD+ flowline. Network catchments are defined as the cumulative aggregation of local catchments that represent the entire upstream drainage boundary for a given NHDPlus flowline. Spatial predictor data attributed to each level includes a series of catchment-natural (physical) and human-disturbance factors that are known to influence stream characteristics and biota; predictors we used in constructing distribution models are listed in Table 25 (raw data are provided in Appendix B).

Table 25. Variables from the National Fish Habitat Action Plan used in construction of distribution models for E. chaetodon in South Carolina (Wang et al. 2011, Wieferich et al. 2015).

Variable	Description
comid	Common identifier in the NHDPlusv1
popdensity	Census 2000 average population density per local catchment (average population count/sqkm)
rx_cat_den	Local catchment road crossing density #/km²
slope	Mean catchment slope (degrees)
elev_mean	Mean catchment elevation (meters)
popdenC	Census 2000 average population density per entire catchment (average population count/sqkm) comfix density
rxcat_areaC	TIGER 2006 second edition. Road crossings (#)/entire catchment area (sqkm) comfix density
tri_denC	Toxic Release Inventory (EPA) sites (#)/entire catchment area (sqkm) comfix density
tot_wdc	Total water withdrawal mgal/year
lu11_URBc	NLCD 2011 Developed Land Low+Medium+High intensity, % entire catchment
lu11_AGc	NLCD 2011 Cultivated Crops, % entire catchment
lu11_FORc	NLCD 2011 Forested Land, % entire catchment
precipc	Network values calculated using upstream area weighted average of mean annual precipitation. (mm)
temp_meanc	Network values calculated using upstream area weighted average of mean annual air temperature (technically an average of the max/min mean annual air temperature). Degrees Celsius.
p_yield	Total anthropogenic phosphorous yield (kg/km/yr) from SPARROW models
n_yield	Total anthropogenic nitrogen yield (kg/km/yr) from SPARROW models
areasqkmc	Network catchment area (sqkm)
LC_HCI	Local catchment habitat condition index
NC_HCI	Network catchment habitat condition index

We used Random Forests (RF), an offshoot of Classification and Regression Tree approaches, to analyze occurrence and catch rates with respect to NFHAP spatial predictor variables (Breiman 2001, Cutler et al. 2007, Urban 2002). Machine learning techniques such as RF provide an alternative modeling paradigm to traditional statistics, where no a priori model is defined, and complex data structures (e.g., non-normal distributions, nonlinearity, interactions) are accommodated. Machine learning techniques use an algorithm to learn the relationship between the response and its predictors by identifying dominant patterns in the dataset (Breiman 2001, Elith et al. 2008). Random Forests represent an advance in machine learning techniques that have increased the accuracy and prediction power of single classification and regression trees by the creation of an ensemble of trees (Breiman 2001). Random forests are non-parametric, can handle both categorical and continuous data as either predictor and/or response variables, can handle high-order interactions, are insensitive to outliers, and can accommodate missing data by using surrogates (Breiman 2001, De'ath and Fabricius 2000, Urban 2002). Random Forests fit an ensemble of trees to a dataset, where each individual tree in the forest is built using a randomly selected bootstrap sample of the training dataset. In addition, only a random subset of predictor variables is considered for node and splitpoint selection (Amit and German 1997). In this way, two elements of randomness are injected into the procedure. Observations not included in the bootstrap samples (out-of-bag or OOB) are passed down their respective trees, and each tree's terminal nodes contain a predicted response to different combinations of observed values among predictor variable pathways with error estimates derived from the OOB sample predictions. Each tree has a 'vote' in the most important predictive variables to split on, and on the responses of different values of input combinations; and the majority of votes among the ensemble of trees 'wins'. Therefore, we can a) predict and rank variables that most strongly influence an outcome (variable importance), and b) isolate and examine the behavior of individual predictors on the outcome, while holding the effect of all other predictive variables constant (partial dependence). RF modeling was conducted by building 1000 trees using default values for other parameters in the *random Forest* package in the R programming environment (R Core Team 2012). RF models have known biases in variable importance selection for highly correlated predictor variables; therefore we conducted a preliminary screening of our abiotic variables to ensure highly correlated variables were not combined into a single model.

Results from both distribution and catch rate analyses were similar in their lack of fit. A series of predictor combinations were tested, and in every case the categorical distribution model of occurrence produced an overall OOB error rate exceeding 38%. In particular, the classification error for *E. chaetodon* presence exceeded 80%, whereas classification error for absence was 15-20%.

Regression RF for catch rates using the same predictor sets as in the categorical distribution modeling in all cases resulted in pseudo R^2 values below 0. The regression pseudo R-squared is calculated as 1 - MSE / Var(y); thus a negative R^2 may be interpreted as the simple use of the grand mean as a prediction of any sample is superior to the predictions of the tested model.

Because neither occurrence nor catch rates of *E. chaetodon* in South Carolina could be confidently modeled using the new data collected during the current project, we concluded that an update of the existing Conservation Planning Tool is not warranted at this time.

Task 7 – *Develop and implement management recommendations for E. chaetodon.*

Our project team recommends the following general measures to protect *E. chaetodon* populations throughout Georgia and South Carolina:

1. Management for native aquatic plant communities by preventing the spread of invasive plant species (e.g., http://stopaquatichitchhikers.org/), preventing run-off of fertilizers that can lead to eutrophication and algae blooms, and avoiding the use of aquatic herbicides in *E. chaetodon* habitat.
2. Protection and restoration of water quality through general watershed protection measures, including protection of wetland habitats and riparian buffers and best management practices for forestry and agriculture.
3. Protection of water quantity and natural hydrology through water conservation efforts, protection of instream flows, and maintenance of aquatic connectivity. Factors that could negatively affect aquatic connectivity include dams and poorly designed culverts that impede dispersal of aquatic species as well development (i.e., roadways, housing, etc.) that interferes with the inundation of floodplain habitats and wetlands.
4. Management for native fish communities by protecting water quality and habitat (see steps 1-3) and preventing the spread of non-native species. Although native sport fishes often co-occur with *E. chaetodon*, we do not recommend stocking sport fishes in the few known populations. Fishing with live fishes or crayfishes as bait should be avoided or carried out using legally harvested native bait species from the local watershed.
5. Protection of habitats with yet unknown but potential populations of *E. chaetodon*. Our eDNA tool can be utilized to assess areas with potential populations of *E. chaetodon* as part of the environmental review of projects that could negatively impact habitat, such as large developments and road construction projects.

Our project results and recommendations have been provided to State managers in both Georgia and South Carolina. We have also developed an online species profile to share these management recommendations with the general public through the Georgia Biodiversity Portal. The website also includes photographs, a range map, and other biological information. We have also created an online conservation status map that categorizes all occurrences of *E. chaetodon* in South Carolina and Georgia by date of last observation at different spatial scales such as watersheds or stream reaches (Figure 26). This tool also allows users with a password access to fine scale data that is not available to the general public. Additionally, we have created a conservation and management brochure for private landowners near *E. chaetodon* project sites to help promote the awareness of the species and its biology, our project results, and incorporation of the recommendations above into their land management practices (Appendix C). Due to differences in land ownership near project and known locations of *E. chaetodon* between Georgia and South Carolina, we focused the brochure to target South Carolina private landowners. With the reduced number of known *E. chaetodon* locations in Georgia, all landowners are known and the most significant one is USFWS (OSNWR) who is already managing the swamp for natural ecosystems. The project team has been in direct contact with all private Georgia landowners who have been informed in person about the project and the online data portal.

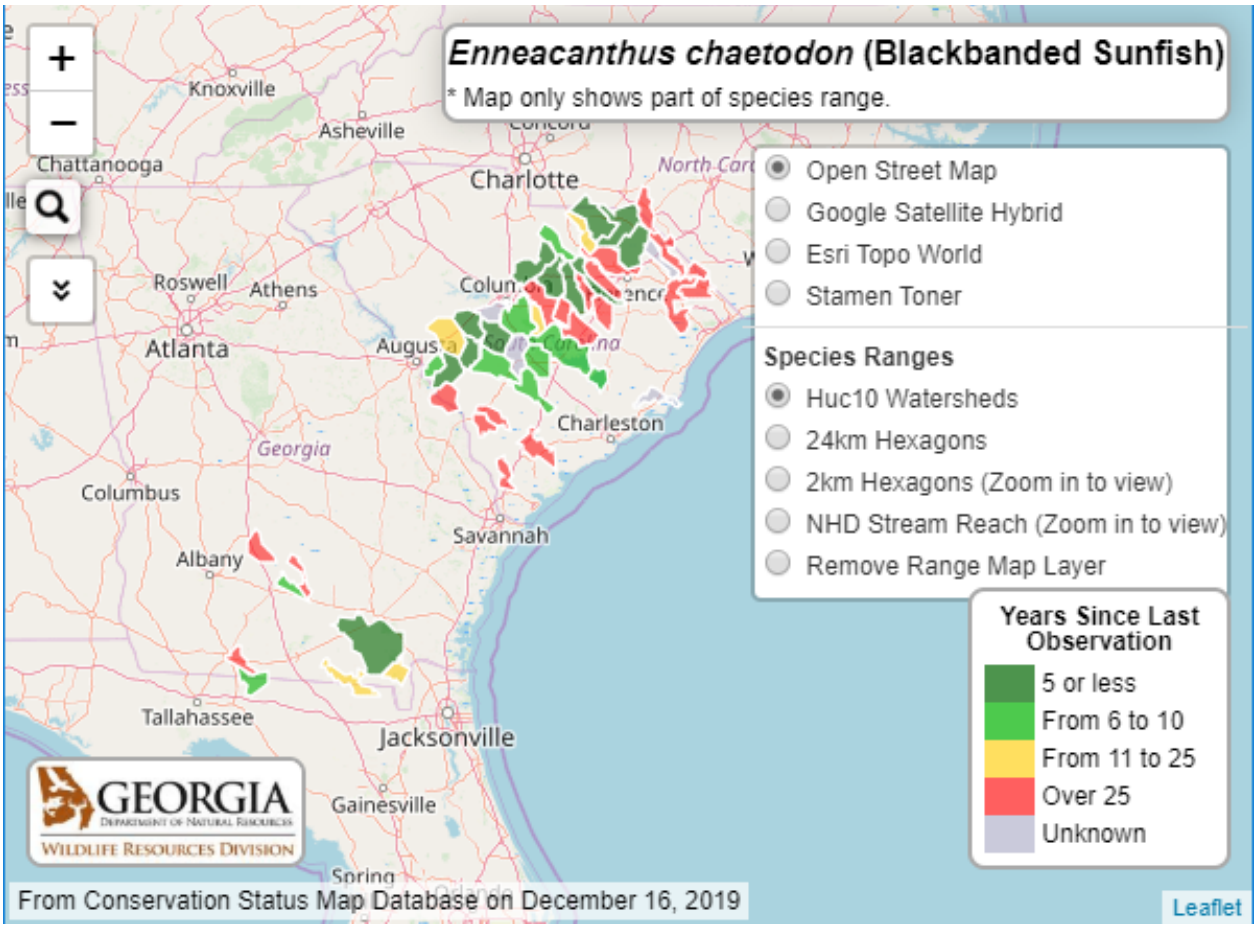

Figure 26. Conservation Status Map for E. chaetodon that categorizes different mapping units by the last observation date.

Specific Management Recommendations

While our project found that *E. chaetodon* populations in South Carolina are relatively abundant and appear more stable and genetically healthy than other areas, we recommend maintaining the current SWAP priority rankings as SC may represent a regional stronghold for *E. chaetodon.* Additionally, continued survey and monitoring of populations is recommended to ensure their status remains unchanged or improved in the future. No specific site recommendations have been developed for the South Carolina project sites. However, *E. chaetodon* in Georgia could benefit from site-specific conservation and management recommendations.

There are three primary population areas for *E. chaetodon* in Georgia: Okefenokee Swamp, the central and upper Alapaha River system, and the Aucilla River near the Georgia-Florida line. Each of these areas have different conservation and management needs and are discussed separately below. Given their occurrence in Atlantic Slope drainages north and south of Georgia, *E. chaetodon* could potentially occur in suitable habitats in Coastal Plain portions of the Savannah, Ogeechee, Altamaha, and Satilla drainages in Georgia. In 2019, we carried out additional eDNA surveys at 10 sites with suitable habitat in the Savannah and Ogeechee drainages. These samples have not yet been processed and represent application of our eDNA tool beyond the scope of the current project.

Okefenokee Swamp spans over 400,000 acres, making it one of the largest freshwater wetlands in North America (Edwards et al. 2013). Aquatic habitats are very difficult to survey due to water depth, dense aquatic vegetation, and the sheer size of the swamp. Despite this, *E. chaetodon* have been collected more frequently in Okefenokee Swamp than anywhere else in Georgia. This species has been collected from portions of the Swamp draining to both the St. Marys (eastern side) and Suwannee rivers (western side). The most recent records in Georgia were collected from the Suwannee side in and near the Billy's Lake area (Figure 27). The Georgia DNR Fisheries Management Section conducts annual monitoring of fish communities in the Suwannee portion of OSNWR and provides data for incorporation into Georgia DNRs rare species database. Additional surveys and monitoring are needed on the St. Marys portion of the swamp, where *E. chaetodon* has not been detected since 2001.

The OSNWR is protected by the U.S. Fish and Wildlife Service and managed for the protection and restoration of wildlife and natural habitats. The comprehensive conservation plan (CCP) for the refuge (USFWS 2006) lists *E. chaetodon* as a species of concern and also describes a long-term plan to restore natural swamp hydrology through breaching of the Suwannee River sill (currently underway). In addition to monitoring of OSNWR fish communities described above, the OSNWR Visitor Center is a key facility for the distribution of our *E. chaetodon* pamphlet and other public outreach. The OSNWR also manages Banks Lake National Wildlife Refuge that is discussed below.

Figure 27. Billy's Lake near E. chaetodon collection site within Okefenokee Swamp National Wildlife Refuge.

The most recent records of *E. chaetodon* in the Alapaha River system are from Fletcher's Lake (also known as Pleasure Lake locally, Figure 28), an impounded 90 acre Carolina Bay that is privately owned by several adjacent landowners. Despite our repeated efforts, we have not been able to collect any *E. chaetodon* in this locality since 2012. The lake continues to support a diverse native fish community, making it difficult to explain the lack of current records or the sparse detection of eDNA. We recommend continued monitoring using traditional fisheries surveys and eDNA sampling in Fletcher's Lake. However, it may be prudent to wait several years to allow for the local population, if extant, to increase to a level that can be detected by our methods. We also recommend additional surveys in suitable habitats near Fletcher's Lake. In support of this goal, but outside the scope of this project, we have recently collected additional eDNA samples from an impounded wetland on Alapaha River Wildlife Management Area, which is less than 2 miles south of Fletcher's Lake. These samples have not been processed yet.

Figure 28. Fletcher's Lake, where E. chaetodon was last collected in 2012. We detected E. chaetodon eDNA at this site but were not able to capture any specimens during the project period.

Aquatic habitats near Banks Lake and Grand Bay WMA (Figure 29) further south in the Alapaha River system merit additional effort to identify the exact source of *E. chaetodon* eDNA documented by our study. Banks Lake, with over 3000 acres of open water and wetland habitats (https://www.fws.gov/refuge/Banks_Lake/about.html) should be a target area for additional surveys.

This area was surveyed during our study and is sampled annually by the Georgia DNR Fisheries Management section using the same boat electrofishing methods utilized in OSNWR. However, the sheer size of the lake and complexity of habitat suggest that *E. chaetodon* may have gone undetected.

Figure 29. Banks Lake National Wildlife Refuge where we did detect eDNA but could not capture E. chaetodon during the project period.

Like Fletcher's Lake, we were unable to capture any *E. chaetodon* from Linton Lake or Connell Creek in the Aucilla River system. We documented extensive mats of non-native *Salvinia minima* in Connell Creek at Oak Grove Rd. and near the confluence of Connell Creek with Linton Lake (Figure 30). These mats of *Salvinia* were not present in Connell Creek during the surveys carried out by Bechler and Salter (2014). Dense mats of floating *Salvinia* can reduce oxygen levels and light penetration and compete with other native aquatic plants (Parys and Johnson 2013). While it is very difficult to eradicate invasive plants, measures to reduce their spread and promote native aquatic plant communities are warranted for all populations of *E. chaetodon*. We also recommend traditional surveys and eDNA sampling in the Aucilla River system. The Florida Fish and Wildlife Commission detected *E. chaetodon* in Lake Rachel and Sampala Lake in 2018-2019 (O'Conner et al. 2019), both of which are in the Aucilla River system near Madison, Florida. They also detected the species in the Aucilla River near the Georgia border in 2000.

Figure 30. Connell Creek in the Aucilla River system with extensive mats of non-native Salvinia minima.

An overall threat to *E. chaetodon* populations in Georgia is the distance between populations, reducing the probability of demographic support to rescue or recover small or locally extirpated populations. We are especially concerned about the loss of populations in the Alapaha River system, where we were not able to document any viable populations of *E. chaetodon* despite substantial sampling effort. A long-term goal for the Alapaha River system is to identify or establish at least one viable population of *E. chaetodon*. Potential introduction sites include the aforementioned impoundment on Alapaha River WMA and Banks Lake. Any reintroductions would follow protocols for captive propagation and translocation developed by George et al. (2012) and would require additional funding. Although we were not able to document *E. chaetodon* in Linton Lake, we are not recommending any reintroduction efforts in the Aucilla River system due the persistence of populations in the Aucilla River system in Florida.

Task 8 – *Disseminate results via interim/final reports, presentations at scientific meetings and peer-reviewed publications.*

All interim project reports have been completed and submitted on schedule to our project coordinator. Our team has consistently and continuously presented results during all years of the project at local, regional, and national scientific meetings – including meetings of the Southern Division of the American Fisheries Society, Southeastern Fishes Council, South Carolina Chapter of the American Fisheries Society, and SC DNR Marine Resources Research Conference. A total of 12 presentations have been shared to date by the project team. Additionally, a project press release was developed and released last

summer through both the SCDNR and GADNR media centers. Although we have not published the results from the project yet, we have three manuscripts in preparation including 1) eDNA tool development and optimization, 2) Distribution of *E. chaetodon* in Georgia and South Carolina using both eDNA and traditional survey methods, and 3) Genetic population characterization of *E. chaetodon* in the southeastern US.

Significant deviations

There have been no significant deviations for Objective 5.

Estimated Project Federal Cost: All project funds were expended to complete the project objectives.

Project Recommendations: The grant has been completed; close the grant.

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Appendices:

Appendix A. Summary of assignments of genetic collections and eDNA sites to major/minor genetic clusters.

Major Cluster	Minor Cluster	Genetic Collection	eDNA Site
North	North01	NJ01	
		NJ07	
		NJ08	
		NJ09	
		DE10	
		DE11	
	North02	NJ02	
	North03	NJ03	
		NJ04	
	North04	MD12	
		MD13	
	Unknown A	NJ05	
		NJ06	
	Unknown B	VA14	
South A	South01	NC15	
	South02	SC17	SC-25
	South03	SC18	
		SC20	SC-16
	South04	SC19	
	South05	SC21	SC-26
	South06	SC22	SC-17
	Unknown C	NC16	
South A/B	South07	SC23	
		SC24	
		SC32	
	South08	SC25	
		SC26	SC-35
		SC28	SC-21
	South09	SC27	SC-34
	South11	GA33	
		GA34	
South B	South10	SC29	SC-33
		SC30	SC-32
		SC31	
Florida	Florida01	FL36	
South A/Florida	Florida02	GA35	
		FL37	
		FL38	

Appendix B. Data from the National Fish Habitat Action Plan assessment used in distribution models of E. chaetodon in South Carolina.

Site ID	SC07	SC08	SC09	SC10	SC11	SC12	SC13	SC14	SC15	SC16	SC17
comid	10548646	22719289	22722061	20168868	20170300	20169896	20169944	22723061	9108956	9166640	9172544
popdensity	4.675953	30.10601	17.37054	27.36473	4.652966	1.958058	2.394798	1.863796	18.39828	7.392592	1.281402
rx_cat_den	0.103885	0	0.009349	0.038263	0.034398	0.035846	0.016547	0.009501	1.274697	0.012941	0.105463
slope	2.45686	3.303627	3.233023	1.286376	0.8823	1.465264	1.025753	2.377769	0.023523	1.555838	1.282337
elev_mean	153.55	109.38	84.73	71.2	61.59	52.39	44.08	49.06	64.69	36.21	45.82
popdenC	19.65832	126.8038	83.07563	26.69079	19.853	6.206352	3.447681	12.87763	18.39828	15.38192	4.684588
rxcat_areaC	0.311656	0.826881	0.813327	0.382629	0.292383	0.233	0.115831	0.294542	1.274697	0.310579	0.316389
tri_denC	0	0.055664	0	0	0	0	0	0	0	0.012941	0
tot_wdc	5.339921	53.06347	42.25254	12.5421	13.84051	12.11215	9.460421	4.869459	52.64932	100.4386	15.02744
lu11_URBc	0.055597	2.587939	1.047442	0.307461	0.082138	0.02382	0	0.035032	0	0.454274	0
lu11_FORc	33.63603	39.97003	22.52968	18.88639	26.24787	20.91607	34.06308	33.56431	2.029915	26.78138	13.59815
lu11_AGc	19.13454	3.953417	6.719614	23.14586	13.69495	15.24339	14.64145	3.961107	83.17308	28.48979	31.50651
precipc	1198.923	1222.057	1173.034	1162.49	1160.241	1166.228	1159.385	1151.139	1046.57	1112.864	1150.982
temp_meanc	17.04122	17.42163	17.56471	17.89555	17.9061	17.9689	18.02398	17.98129	16.93	16.77941	17.27346
p_yield	49.1	66.73	31.59	20.85	25.33	18.79	18.74	9.93	46.2	15.5	63.88
n_yield	238.26	499.82	213.69	250.08	235.93	216.56	189.71	117.96	471.67	208.65	449.96
areasqkmc	9.626	26.606	106.968	26.135	58.143	55.794	60.433	105.248	1.569	77.275	9.482
LC_HCI	4.8	4.4	4.5	5	4.4	4.4	5	4.75	3.2	5	4.4
NC_HCI	4.2	3.8	2	4	4.8	4.4	5	2.4	2	3.8	4.2

Appendix B. NFHAP model data (continued)

Site ID	SC18	SC19	SC20	SC21	SC22	SC23	SC24	SC25	SC26	SC27	SC28
comid	9174912	9680168	9680390	10538698	10540866	9607044	9171378*	9170284	9170986	9147866	9114442
popdensity	13.50775	5.873127	43.19434	4.353896	4.646772	4.225583	72.67324	5.982906	1.372572	0.353896	2.748578
rx_cat_den	0.076249	0.061271	0.037676	0.143916	0.146706	0	0.031503	0.048709	0.055185	0.164826	0.021545
slope	0.494859	3.772805	3.151103	1.715289	0.797905	0.373263	2.556962	2.440559	1.841227	2.520027	0.180923
elev_mean	39.26	58.38	73.63	108.1696	114.56	46.3	128.5	90.04001	65.03	128.29	14.78
popdenC	34.01447	30.72535	62.26449	4.353897	13.26798	6.091396	22.67086	6.347572	5.647182	1.563259	11.13674
rxcat_areaC	0.724361	0.183812	0.188381	0.143916	0.293413	0.261618	0.441042	0.535801	0.331108	0.247239	0.258534
tri_denC	0.190621	0	0	0	0	0	0	0	0	0	0
tot_wdc	138.503	69.09156	42.81866	7.445779	56.6508	229.9518	1.037639	4.873209	2.869673	2.108082	16.25577
lu11_URBc	14.30259	0	0.020432	0.046623	2.268582	0.19606	0.053197	0	0	0	0.288317
lu11_FORc	10.17444	32.44478	31.66928	30.40496	21.07113	16.34203	42.16038	47.33665	43.48524	52.93147	7.369079
lu11_AGc	19.32379	2.284844	1.995505	14.8195	24.20711	43.10082	7.554037	6.368236	9.491066	17.89624	37.70706
precipc	1156.446	1165.611	1174.268	1180.667	1192.753	1172.127	1156.632	1147.65	1139.582	1135.382	1226.669
temp_meanc	17.45111	17.87158	17.71088	17.65	17.4931	18.00191	16.72531	16.64536	16.74715	16.63828	17.43703
p_yield	95.03	13.07	13.07	35.36	35.36	42.99	30.17	23.56	12.22	49.56	41.71
n_yield	951.58	110.63	110.63	281	281	371.93	204.15	141.18	80.39	226.1	572.67
areasqkmc	26.23	16.321	26.542	12.937	20.449	103.204	31.743	20.53	18.121	12.134	92.831
LC_HCI	2.2	5	4.8	4.2	4	4.25	5	5	5	5	4.2
NC_HCI	2.4	4.4	5	5	4	2	4.2	4.8	4.8	4.4	4

***** Denotes original comid did not have NLCD land use data available at time of analysis. Next available downstream comid was used instead. Comid 917378 used land use data from 9170292

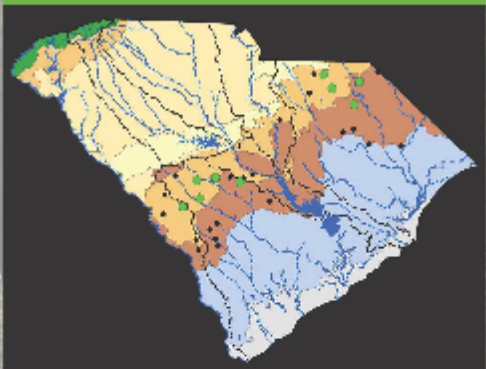
Appendix B. NFHAP model data (continued)

Site ID	SC29	SC30	SC31	SC32	SC32a	SC33	SC34	SC35	SC35a	SC36
comid	9174134	9150482	22722785	22721729	22723909*	10555900*	10540470	10540882	10541450*	9715981
popdensity	4.852764	3.281575	8.474998	42.83027	42.83027	7.422417	2.583913	2.38517	32.89143	102.191
rx_cat_den	0.220629	0.052487	0.192012	0.03617	0.03617	0.027624	0.34118	0.018998	0.042723	0
slope	0.181791	0.129682	1.888859	1.392053	1.392053	0.223301	1.697989	1.938411	2.160241	1.937167
elev_mean	30.57	42.08	64.3	111.5	111.5	72.66	126.31	120.32	129.67	65.71
popdenC	4.852763	12.97061	17.35066	34.27131	34.27131	3.31701	2.583913	17.52246	33.29381	68.81515
rxcat_areaC	0.220629	0.440894	0.192012	0.253192	0.253192	0.165741	0.34118	0.294461	0.576763	0.303046
tri_denC	0	0	0	0	0	0	0	0.009987	0.024	0
tot_wdc	52.1134	59.60383	3.614147	6.650524	6.650524	36.87108	18.58447	127.2349	50.3668	20.88185
lu11_URBc	0.098559	0.815268	0.388384	0	0	0	0	0.611437	0.925191	0.925191
lu11_FORc	17.65431	8.331322	39.12967	31.11852	31.11852	34.84306	15.78149	21.2502	20.08421	20.08421
lu11_AGc	21.39953	45.1715	2.436225	5.359353	5.359353	11.03578	4.113977	22.4891	17.0122	17.0122
precipc	1176.29	1158.485	1153.415	1201.892	1201.892	1186.903	1212.2	1231.487	1227.44	1079.574
temp_meanc	17.4895	17.31277	17.94915	17.5947	17.5947	17.54882	17.2964	17.31417	17.30835	16.64281
p_yield	24.76	42.11	0.31	16.98	16.98	20.24	25.01	38.49	38.49	27.27
n_yield	310.62	560.03	30.23	103.93	103.93	139.12	176.21	282.73	282.73	202.03
areasqkmc	18.13	95.261	10.416	27.647	27.647	36.201	5.862	105.277	46.813	19.799
LC_HCI	4.2	3.6	5	4.8	4.8	5	5	4.25	4.8	5
NC_HCI	4.2	4	5	4.8	4.8	5	4.8	2	4	4

* Denotes original comid did not have NLCD land use data available at time of analysis. Next available downstream comid was used instead. Comid 22723909 used land use data from 22721729, comid 10555900 used land use data from 10549598, and comid 10541450 used land use data from 10540412.

Appendix C. Conservation and management recommendation trifold brochure for South Carolina landowners. Outside panels shown here.

EASY WAYS TO HELP THE BLACKBANDED SUNFISH



Known locations of the blackbanded sunfish in SC (green dots). The black dots represent sites our team surveyed that tested negative for blackbanded sunfish.

Blackbanded sunfish are an indicator species, meaning their presence indicates a healthy environment. These guidelines can therefore help keep waters welcoming not just to blackbanded sunfish, but to many other types of wildlife as well:

- Maintain native plant growth around the water's edge, where small fish often rest and feed
- Avoid stocking nonnative fish or predators such as smallmouth bass, sunfish, or catfish, which can outcompete small native fish
- Avoid habitat loss: Leave blackwater ponds and swamps undrained, unimpounded, and undeveloped

RESEARCH PARTNERS

South Carolina Department of Natural Resources

Georgia DNR Wildlife Resources Division

U.S. Fish & Wildlife Service

Wildlife & Sport Fish Restoration

Valdosta State University

Hollings Marine Laboratory

LEARN MORE

SCDNR staff can provide technical assistance to landowners who wish to encourage blackbanded sunfish on/near their properties. For more information, please contact:

Dr. Mark Scott, Freshwater Biologist
(864) 986-6243
ScottM@dnr.sc.gov
http://bit.ly/SCDNRBlackBandedSunfish
http://dnr.sc.gov/water/envaff/aquatic

MEET THE BLACKBANDED SUNFISH

What we're learning about a rare fish found near your property

South Carolina Department of Natural Resources

Appendix C. Outreach trifold brochure (continued); inside panels shown here.

SOUTH CAROLINA IS KEY

The blackbanded sunfish is a little-known inhabitant of South Carolina's blackwater ponds, swamps, and streams. A relative of other sunfish (bream) and bass, these beautiful small fish feed on fly and mosquito larvae along shorelines with heavy vegetation.

Originally found from Pennsylvania to Florida, the blackbanded sunfish is now gone from many locations across its range, due in large part to the draining of ponds and swamps and runoff of nutrients and other pollutants.

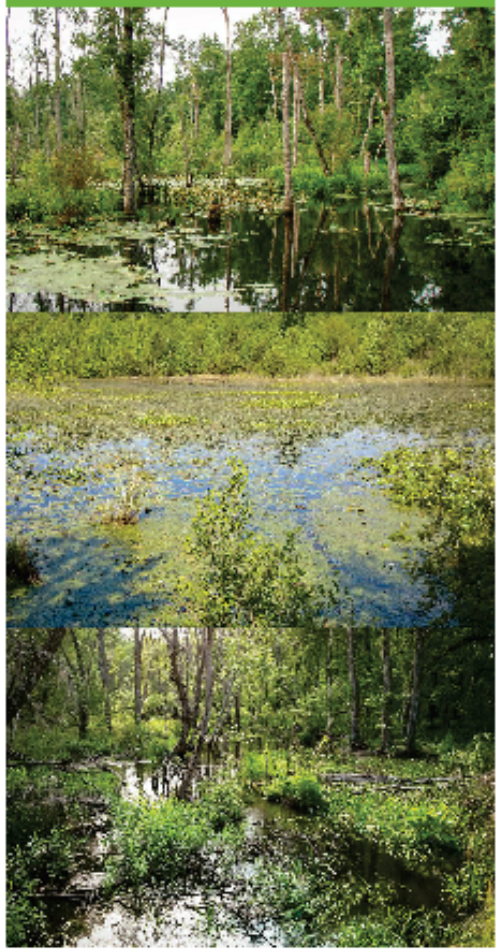
South Carolina is one of the last remaining strongholds for this fish, and we hope to keep their numbers healthy here. Using new technology, South Carolina Department of Natural Resources (SCDNR) biologists have confirmed a body of water near your property hosts a blackbanded sunfish population.

We're sharing what we've learned about this fish to help keep landowners informed and our state's waters and wildlife healthy for generations to come.

The silver dollar-sized blackbanded sunfish has six black bands and bright red fins on its underside.

SLOW-MOVING WATERS

The blackbanded sunfish can only thrive in shallow, quiet, acidic waters with aquatic plants, such as those seen below.



WHAT WE'VE LEARNED

The blackbanded sunfish is small and elusive, making it difficult to catch and study using traditional nets and traps. Several years ago, a team of SCDNR biologists set out to develop a new way to determine where these fish live in order to help them survive.

SCDNR's team and partners collected water samples at 60 swampy locations across South Carolina and Georgia, analyzing DNA traces left by shed scales and slime in the water. Nine sites in South Carolina and five in Georgia tested positive for blackbanded sunfish. Biologists later trapped live fish at all nine South Carolina sites, confirming the species' presence.

Our researchers are now working to learn more about these populations and talking to landowners about how we can keep South Carolina a center of freshwater fish diversity.

SCDNR biologist Matt Walker collects water samples at a South Carolina Sandhills location.