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Article *in* Southeastern Naturalist · July 2024

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eDNA Illuminates Broader-Than-Expected Distribution of an Imperiled Freshwater Darter Species (Percidae: *Etheostoma striatulum*) in the Duck River, Tennessee

Adam L. Bajo-Walker[1,*], Kit Wheeler[2], and Carla R. Hurt[2]

Abstract - *Etheostoma striatulum* (Striated Darter) is an increasingly rare darter found in the middle-to-upper regions of the Duck River watershed in Tennessee. This rarity, coupled with a lack of collection and sampling, has put them in review for listing under the Endangered Species Act. Because of the Striated Darters' reclusive and cryptic behavior, conventional techniques tend to be less effective for detection, requiring more precise and sensitive methods. To provide information regarding distribution and occupancy within the Duck River, we used molecular detection of species-specific environmental DNA to test for presence of the species at historical sampling sites. Assessments indicate that Striated Darter is persisting in all tributaries of historical occurrence, with the addition of 4 new tributaries. However, given that 16 sites produced positive signals for less than a third of qPCR replicates, it appears that 60% of these populations are persisting at low detection levels. Detection was positively correlated with turbidity, which could be explained by their preference for lower-velocity habitats. Our results will assist with identifying critical-habitat locations for the update on the status of Striated Darter while also aiding in the standardization of eDNA-assay development for rare and cryptic species.

Introduction

Etheostoma striatulum Page and Braasch (Striated Darter; Fig. 1) is an uncommon darter species currently under review for protection under the Endangered Species Act. The known range of the Striated Darter is limited to 9 tributaries in the middle-to-upper region of the Duck River watershed, TN (Abernathy and Mattingly 2011). However, due to a lack of recent collection and sampling, knowledge of Striated Darter distribution and population dynamics is lacking. Agricultural and urban development are both continuing to increase within the watershed, which could potentially impact long-term persistence of Striated Darter (Elkins et al. 2019, Kuehne and Barbour 1983). Moving forward, management plans must clearly define the geographic distribution of the Striated Darter so that critical-habitat locations can be established, preventing further decline of populations.

Delineation of the current distribution of the Striated Darter is needed to identify and protect critical habitat more precisely. For stream fishes, current-distribution information is typically gathered using conventional techniques such as electrofishing and seining. However, these methods may be less effective for detecting benthic fishes like darters because of their affinity for taking shelter near or under rocks,

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Manuscript Editor: Keith Gibbs

enabling them to avoid most capture techniques (Eley et al. 1975). In addition, these conventional methods can also cause unintentional harm to target species and their eggs, other fishes, amphibians, insects, and mussels (Dwyer et al. 1993, Hayes et al. 1996, Reynolds and Kolz 2013, Snyder 2003).

To combat these obstacles, molecular tools have been developed to survey environmental DNA (eDNA) released from rare and cryptic species such as the Striated Darter to provide a more sensitive, economical, and non-invasive method of monitoring (Davy et al. 2015, Hinlo et al. 2018, Johnston and Janosik 2019, Lor et al. 2020, Paine et al. 2021, Spear et al. 2015). In aquatic environments, the release of eDNA from source organisms is altered through transport, dilution, settling into and resuspension from sediments, and overall degradation that can affect the detectability of a species (Barnes and Turner 2016, Turner et al. 2014) Some studies have indicated detection of eDNA can occur up to hundreds of meters and in some cases even to several kilometers (Mckelvey et al. 2016, Wilcox et al. 2016) away from the actual location of an organism, providing insight into the spatial distribution of an aquatic species. Over time, consistent monitoring paired with standardized eDNA sampling can deliver powerful information regarding temporal changes in distribution.

Detection and non-detection data derived from eDNA surveys can also be applied to an occupancy-model framework to estimate occupancy and detection

Figure 1. Photographs of *Etheostoma striatulum* (Striated Darter) collected in the Duck River watershed, TN. Top: nuptial male, 52 mm standard length, North Flat Creek. Bottom: non-gravid female, 48 mm total length, South Flat Creek. Photographs © Adam L. Bajo-Walker.

probabilities (Dorazio and Erickson 2018, Reid and Haxton 2020, Strickland and Roberts 2019). These models assess the spatial distribution and status of a species across a landscape and yield parameter estimates that are more reliable than naïve occupancy estimates; the models account for imperfect detection, which is common when working with small-bodied cryptic fishes and when presence–absence surveys such as eDNA sampling are used (Mackenzie et al. 2017). Surveys utilizing an eDNA approach provide detection and non-detection data across 3 nested, hierarchical sampling levels: sampling locations, water samples within a location, and qPCR replicates for a given water sample. Once collected, these data can then be applied to the statistical occupancy-model framework to estimate detection probabilities for each of the 3 levels (Dorazio and Erickson 2018). Parameter estimates from these models can also provide information on the efficiency of sampling protocols, allowing for improvement where needed, and provide guidelines for previously published assays or the development and application of new assays (Thalinger et al. 2021).

Here, we describe our development of an eDNA field protocol for the detection of Striated Darter DNA. Our specific objectives were to: (1) develop and optimize an eDNA monitoring assay for Striated Darter using singleplex quantitative polymerase chain reactions (qPCR), and (2) apply the assay to delineate the current distribution of Striated Darter in the Duck River watershed. We used these results to identify critical-habitat locations and to update the status of Striated Darter while also aiding the standardization of eDNA assays for rare and cryptic species.

Field Site Description

The Duck River (Fig. 2) is one of the most biologically diverse rivers in North America and one of the greatest freshwater biodiversity hotspots in the temperate region, with 155 species of freshwater fishes, 66 species of freshwater mussels, and 22 species of freshwater snails (Ahlstedt et al. 2017, Etnier and Starnes, 2001, Hubbs et al. 2011). Most of this diversity has been adversely affected by anthropogenic influences such as dam construction or poor land-use practices (Elkins et al. 2019). This area is composed of an array of rural landscapes, including pastures, row-crop agricultural fields, and woodlands, intermixed with moderately sized urban areas. The Duck River has also become the principal source of drinking water for communities within and around the watershed (Knight and Kingsbury 2007; TDEC 2005a, b). Transformation of agricultural land into urban environments is continuing and has potential consequences for the entire watershed and its biota.

The Duck River originates on the Highland Rim physiographic province in middle Tennessee and flows 457 km westward through 7 counties before reaching its confluence with the Tennessee River at Kentucky Lake. It is the largest river contained entirely within the State of Tennessee and drains ~7070 km^2 (TDEC 2005a, b), and has had some level of impoundment since the mid-1800s. Normandy Dam, located at river kilometer 401, was completed in 1976 for flood control and economic development purposes and does not produce electricity (Knight

and Kingsbury 2007). This dam and 2 low-head dams located on the main-stem Duck River between Shelbyville and Columbia, TN, restrict free-flowing regions of the Duck River to areas above Normandy Lake and below Columbia Dam. Due to these barriers, physiographic boundaries, and relatively large watershed sizes, researchers and agencies have commonly separated the river into the upper and lower Duck River drainages.

We sampled for the Striated Darter primarily within the upper Duck River drainage, with 24 of the sites being upstream of Columbia dam and downstream of Normandy Dam (Fig. 2). The remaining 6 sites are downstream of Columbia Dam, 4 of which are in the Big Bigby Creek system. We defined all sites by a 100-m reach that contained multiple habitat types (i.e., pool, glide, riffle, run), with the primary substrate being bedrock.

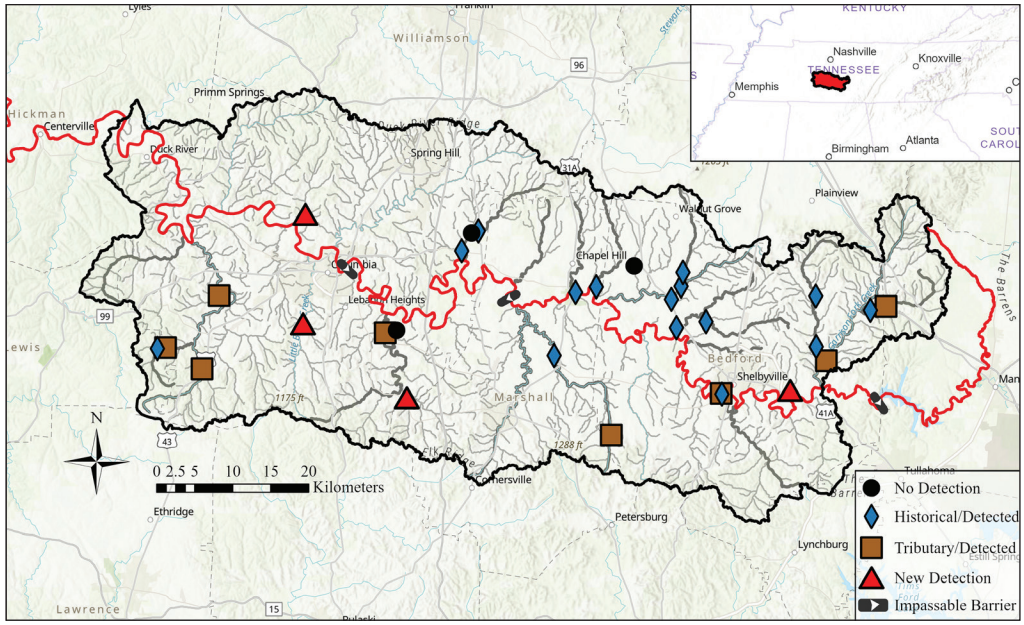

Figure 2. eDNA sampling sites (n = 30) for *Etheostoma striatulum* (Striated Darter) during 2020 and 2021 surveys. Area covered in the map represents the 12-digit hydrologic units associated with the known species distribution. The solid red line indicates the mainstem Duck River, which flows east to west. Black ovals with white triangles represent impassable barriers in the upper Duck River watershed. Blue diamonds (n = 15) are sites where our assay detected Striated Darter and where the species had been captured previously. Brown squares (n = 8) are sites where our assay detected the species, but where it had not been captured previously; these sites, however, are all within tributaries where Striated Darter had been captured previously (at other locations). Red triangles (n = 4) are sites where our assay detected Striated Darter, but where it had not been captured previously; these sites are all within tributaries without historical collections of the species. Black circles (n = 3) are sites where Striated Darter had not been caught previously and was not detected with our 16S assay. Information about previous captures compiled from Cook et al. (1996), Abernathy and Mattingly (2011), Wheeler et al. (2021), and unpublished collections by Tennessee Valley Authority, University of Tennessee, and Yale University.

Materials and Methods

Sampling and water filtration

We obtained 93 water samples across 30 sites (Fig. 2, Table 1) in the middle-to-upper region of the Duck River system in the summers of 2020 and 2021 following the procedure described by Mahon et al. (2010). We selected these particular collection sites because each one had been sampled during previous species surveys. We collected 3 water samples at each of 23 sites between 11 June and 6 August 2020 and at each of 7 sites on 9 August 2021 (Table 1). We sampled during baseflow conditions to reduce the effects of rain or drought events on detection. There were a few rain events that occurred in the summer of 2020, and we delayed sampling after these events until flow in the main-stem Duck River matched the median for that time period (Curtis et al. 2020). One site on South Flat Creek (SFLT-A; see Table 1 for site codes) was the only site sampled twice. We collected a total of 3 L (three 1-L subsamples) of water in Nalgene bottles at each site, along with one 1-L negative control, in a downstream-to-upstream direction at the 0-m, 50-m, and 100-m locations. Prior to sample collection, all bottles were sterilized with a 20% bleach solution for 10 min, thoroughly rinsed with tap water, and then autoclaved. To collect a sample, a bottle was held in front of the researcher, facing upstream, and then submerged just below the surface (depth of 5–15 cm) with the lid securely attached. After removal of the lid, water entered the bottle, and when it was filled except for 1 inch of headspace, we re-secured the lid while still holding the bottle below the surface. Negative control bottles containing deionized water were "collected" first at the most downstream end of the reach for each site and were treated as sample bottles to determine if cross-contamination occurred between sites in the field. We removed the lid for the negative control for 10 s while standing in the water to expose the sample to air, then securely reattached it. We then submerged negative control bottles as described above (apart from removing the lid underwater). Following collection, all bottles were dried of excess water using disposable paper towels, sterilized by wiping with a disposable paper towel soaked with 20% bleach solution, wiped down with a disposable paper towel soaked in deionized water, sealed with parafilm, and transported back to the laboratory on ice for filtration. We placed generated waste (paper towels, gloves, and excess parafilm) in a designated garbage bag for disposal that we kept separate from samples and sampling supplies.

Prior to filtering, we separated all filters (Whatman® 1.0-µm glass fiber filters; Cytiva, Marlborough, MA) into containers in a sterilized PCR hood for negative controls and field samples to reduce potential contamination of filters. We vacuum-filtered all water samples within 12 h and subsequently stored filters in 50-ml conical tubes at -20 °C until DNA extraction. All filtration equipment was decontaminated with a 20% bleach solution for 10 min and thoroughly rinsed with deionized water after processing the water from each sample. We performed sample filtration in a separate room from DNA extractions and qPCR set up to avoid potential contamination.

Table 1. List of sampling locations for 2020–2021 study of *Etheostoma striatulum* (Striated Darter) within the Duck River watershed. One site on South Flat Creek (Site #24) was sampled twice in 2020 due to a population estimate generated on 6 August 2021. Abbreviations: W. = West, N. = North, E. = East, S. = South; Br. = Branch; Cr. = Creek; Fk. = Fork; R. = River; DOC = date of collection. Site numbers and codes match those in other tables, text, and figures throughout manuscript.

Site #	Stream name	Site code	Location description	County	DOC	Latitude (°N)	Longitude (°W)
1	W. Fk. Bigby Cr.	WFBC	West Fork Road (Macedonia Church of Christ)	Lewis	06/11/2020	35.526101	87.295865
2	W. Fk. Bigby Cr.	SPRING	Mt. Joy Road, downstream of Macedonia Church	Maury	08/09/2021	35.527192	87.284101
3	Big Bigby Cr.	BBC	Hwy 43 Bridge	Maury	06/26/2020	35.502150	87.231023
4	Dog Br.	DOG	Roy Thompson Road Bridge	Maury	08/09/2021	35.588954	87.206523
5	Little Bigby Cr.	LBC	Albert Matthews Road Bridge	Maury	07/29/2020	35.557487	87.084690
6	Knob Cr.	KNB	Knob Creek Road Bridge	Maury	06/19/2020	35.687011	87.081367
7	Fountain Cr.	FNT	Blue Springs Road	Maury	07/22/2020	35.544940	86.965272
8	Silver Cr.	SLV	Bryant Road Bridge	Maury	08/09/2021	35.547790	86.948971
9	Globe Cr.	GLB	McKibbon Road Bridge	Maury	06/19/2020	35.469961	86.934173
10	N. Flat Cr.	NFLT-A	Hwy 99 Bridge	Maury	07/22/2020	35.641912	86.854265
11	Little Flat Cr.	LFC	Rally Hill Road Bridge	Maury	06/19/2020	35.662946	86.839750
12	N. Flat Cr.	NFLT-B	Hwy 431 Bridge	Maury	08/06/2020	35.665402	86.829986
13	E. Rock Cr.	ERC-A	Wade Brown Road Bridge	Marshall	07/29/2020	35.517811	86.720421
14	Duck R.	DUCK	Henry Horton State Park Canoe Launch	Marshall	07/22/2020	35.592511	86.689032
15	Wilson Cr.	WIL	Hwy 270 Bridge	Marshall	07/29/2020	35.599002	86.658906
16	E. Rock Cr.	ERC-B	Pickle Road Bridge	Bedford	06/26/2020	35.423594	86.637877
17	Clem Cr.	CLM	Intersection of Chapel Hill and Creekside Ln	Bedford	08/09/2021	35.623459	86.603783
18	N. Fork Cr.	NFC-B	Hwy 41 - Rt. 16 Bridge	Bedford	07/23/2020	35.583768	86.549963
19	Fall Cr.	FALL	Ben Williams Road Bridge	Bedford	06/11/2020	35.550234	86.542813
20	N. Fork Cr.	NFC-A	Unionville-Deason Road Bridge (North Fork Church)	Bedford	08/09/2021	35.599390	86.535830
21	Alexander Cr.	ALX	Barber Road Bridge	Bedford	06/11/2020	35.615697	86.532992
22	Hurricane Cr.	HUR	Old Nashville Dirt Road Bridge	Bedford	06/11/2020	35.557152	86.499903
23	S. Flat Cr.	SFLT-B	Hwy 64 Bridge	Bedford	07/16/2020	35.472174	86.478412
24	S. Flat Cr.	SFLT-A	Hwy 64 Bridge	Bedford	07/16/2020, 08/06/2020	35.471352	86.477391
25	Butler Cr.	BUT	Mullins Mill Road Bridge	Bedford	08/09/2021	35.477120	86.378384
26	Wartrace Cr.	WAR-B	Winnette-Ayers Park – Wartrace Dog Park	Bedford	06/11/2020	35.526871	86.340193
27	Wartrace Cr.	WAR-A	Hwy 82 Bridge	Bedford	07/16/2020	35.586784	86.340372
28	Garrison Fk.	GAR	Bugscuffle Road Bridge	Bedford	07/16/2020	35.510158	86.325737
29	Noah Fk.	NOA-A	Dr. Jackson Road	Coffee	07/16/2020	35.569363	86.261287
30	Noah Fk.	NOA-B	Luke Jacobs Lane Road	Coffee	08/09/2021	35.573926	86.238542

Assay design and validation

In silico. We designed a species-specific singleplex qPCR assay (i.e., 2 primers and 1 probe; Table 2) to target a small fragment of the mitochondrial 16S rRNA gene; 16S has been shown to amplify robustly across taxonomic groups and is sufficiently variable for species identification (Franklin et al. 2019, Shaw et al. 2016). In the summer of 2020, we collected 42 Striated Darters, representing 7 unique sites within the Duck River, and 54 individual darters representing 12 non-target species using a 1.2 m x 3.0 m (3.2-mm mesh) seine. We collected tissue samples by clipping the outer rim of the anal fin, or caudal fin if the individual was less than 30 mm total length, following the protocols from Moyer and Williams (2017). We sterilized all equipment with 90% ethanol between handling specimens and stored all fin clips in RNAlater™ (Invitrogen™) in the field before transporting them back to the lab, where we stored them in a -20 °C freezer until processing. We extracted genomic DNA from fin clips using an Omega Bio-Tek E.Z.N.A.® Tissue DNA Kit (Norcross, GA) following manufacturer protocols with the exception that we used molecular-grade water instead of elution buffer in the final step.

We developed a primer set to find highly conserved regions of the mitochondrial 16S rRNA gene for *Etheostoma* and *Nothonotus* species because 16S sequence information for all targeted species was not readily available for this study. We downloaded 28 *Etheostoma* sequences from GenBank (see Supplemental Table 1 in Supplemental File 1, available online at https://www.eaglehill.us/SENAonline/suppl-files/s23-2-S2869-Bajo-Walker-s1 and, for BioOne subscribers, at https://www.doi.org/10.1656/S2869.s1), aligned them in BioEdit, and manually assessed them to find highly conserved regions of 16S. We selected a primer set to amplify a 1598-bp region for *Etheostoma* species. This primer set (Table 2) successfully amplifies a 1598-bp region for species from *Etheostoma* and *Nothonotus* under the following PCR conditions: initial denaturation step of 95 °C for 2 min; 35 cycles of 95 ºC for 30 s, 59 ºC for 30 s, and 72 ºC for 30 s; and a final extension step of 72 ºC for 10 min. Each reaction consisted of a 10-µl volume that included 5 µl of Promega PCR Master Mix 2X (Promega, Madison, WI), 0.25 µl of each primer (20 µM), 3.5 µl of nuclease-free water (VWR), and 1 µl of template DNA.

Table 2. Summary of qPCR primers/probes developed and used to amplify a 167-bp region of the mitochondrial 16S rRNA gene for Striated Darter (*Etheostoma striatulum*) DNA. Primer set designed for amplifying members of *Etheostoma* and *Nothonotus* is given at the bottom of the table (EtNo_16S). TM = melting temperature, GC% = the percentage of the primer sequence comprised of G/C nucleotides. Start and stop refer to the specific position of the oligos within the gene.

Oligo	Sequence (5'-3')	Product size (bp)	Length (bp)	Start	Stop	T_M (°C)	GC%
EtST_16S_A		167					
EtST_16S_651F	CTAATTCTACCGGGCCATCC		20	651	671	61.65	55.00
EtST_16S_735P	ACGTGTGTACCTCGGAACGGACAT		24	735	759	68.09	54.17
EtST_16S_817R	GACAGCAAACCAAACAACCG		20	817	797	62.31	50.00
EtNo_16S		1598					
EtNo_16S_1F	TCTCCCTTACACTGAGAAG		19	1	20	57.60	47.00
EtNo_16S_1598R	TCAGACCGGAGTAATCCAGG		20	1598	1578	60.07	55.00

We PCR-amplified 7 Striated Darters representing 7 separate populations, and 19 individuals representing 19 non-target species using the primer set and thermal cycler conditions described in the previous paragraph. We performed Sanger sequencing on the amplified products using an ABI 3730XL automated sequencer platform (Molecular Cloning Lab, San Francisco, CA). We inspected and trimmed chromatograms using Sequencher 5.4.6 (Gene Codes Corporation, Ann Arbor, MI) and aligned sequences using ClustalW (Thompson et al. 1994) in BioEdit Sequence Alignment Editor (Hall 1999). All sequences generated for this study were accessioned in GenBank (OQ867967-OQ867992; see Supplemental Table 2 in Supplemental File 1).

We generated a consensus sequence from the 7 Striated Darter 16S sequences and then imported them into PrimerQuest (Integrated DNA Technologies, Coralville, IA) to identify primer- and probe-binding sites. We used the default parameters in PrimerQuest, except for amplicon length, which we altered to search for lengths of 75–300 bp. The majority of eDNA studies target DNA fragments varying between 90 and 120 bp because shorter DNA fragments persist longer in the environment to allow species detection (Jo et al. 2017, Rees et al. 2014, Wei et al. 2018); however, longer fragments may be required to differentiate between closely related, sympatric species (Díaz-Ferguson and Moyer 2014). We retained all candidate oligos (i.e., primers and probes) for the screening process.

We imported candidate 16S oligos into the BioEdit file containing the 26 sequenced individuals along with 7 additional sequences from GenBank for comparison to nontarget species. We then uploaded sequence files to MEGA-X (Kumar et al. 2018) to build pairwise-distance matrices to assess bp difference between oligos, non-target species, and the Striated Darter. Oligos that were retained had a minimum of 1 bp mismatch to non-target *Etheostoma* and *Nothonotus* species. We compared the resulting candidate oligos with the GenBank Non-redundant database using the Basic Local Alignment Search Tool for nucleotide sequences (BLASTn; Altschul et al. 1990) to assess similarity to nontarget sequences. We used 3 criteria from GenBank database to select candidate primers and probes for qPCR amplification: percent identity, e-value, and total score. We selected candidate oligos based on the highest combined scores of the 3 criteria mentioned above. We then retained resulting candidate oligos in BioEdit alignment files and removed the others.

In vitro. We tested primer candidates for amplification specificity and efficiency using tissue-derived DNA in end-point PCR (epPCR) reactions. Laboratory screening of primer pairs simultaneously tested for amplification efficiency across representatives from the 7 Striated Darter populations and specificity (i.e., failure to amplify non-target species) using DNA from 28 non-target taxa known to co-occur with Striated Darter in the Duck River drainage and 2 non-target taxa that are closely related (Table 3). *Etheostoma bison* (Buffalo Darter), *E. derivativum* (Stone Darter), *E. duryi* (Blackside Snubnose Darter), *E. nigrum* (Johnny Darter), *E. obeyense* (Barcheek Darter), *E. pseudovulatum* (Egg-mimic Darter), and *E. virgatum* (Striped Darter) were not included for specificity testing as we could not obtain

tissues. However, previous phylogenetic studies show that DNA samples used in this optimization step contained representatives from all subgenera of *Etheostoma*

Table 3. List of sympatric and closely related *Etheostoma* and *Nothonotus* species targeted for optimizing *Etheostoma striatulum* (Striated Darter) eDNA assay. Reference denotes sequences that were generated and used for optimization in this study, plus any additional sequences publicly available through GenBank. * denotes species that are not sympatric but that are closely related to Striated Darter (Near et al. 2011). ^ denotes species for which tissue for testing was not obtained.

Genus and species	Common name	Reference
Etheostoma		
E. basilare Page, Hardman, and Near *	Corrugated Darter	1
E. bison Ceas and Page ^	Buffalo Darter	0
E. blennioides Rafinesque	Greenside Darter	1
E. blennius Gilbert and Swain	Blenny Darter	1
E. caeruleum Storer	Rainbow Darter	1+2
E. cinereum Storer	Ashy Darter	1
E. crossopterum Braasch and Mayden	Fringed Darter	1
E. derivativum Page, Hardman, and Near ^*	Stone Darter	0
E. duryi Henshall ^	Blackside Snubnose Darter	0
E. flabellare Rafinesque	Fantail Darter	1+3
E. flavum Etnier and Bailey	Saffron Darter	1
E. histrio Jordan and Gilbert	Harlequin Darter	1
E. kennicotti (Putnam)	Stripetail Darter	1
E. luteovinctum Gilbert and Swain	Redband Darter	1
E. nigripinne Braasch and Mayden	Blackfin Darter	1
E. nigrum Rafinesque	Johnny Darter	0+1
E. obama Mayden and Layman	Spangled Darter	1
E. obeyense Kirsch ^*	Barcheek Darter	0
E. planasaxatile Powers and Mayden	Duck Darter	1
E. pseudovulatum Page and Ceas ^	Egg-Mimic Darter	0
E. smithi Page and Braasch *	Slabrock Darter	1
E. virgatum (Jordan) ^*	Striped Darter	0
E. zonale (Cope)	Banded Darter	1+1
Nothonotus		
N. aquali (Williams and Etnier)	Coppercheek Darter	1
N. camurus (Cope)	Bluebreast Darter	1
N. denoncourti (Stauffer and van Snik)	Golden Darter	1
N. rufilineatus (Cope)	Redline Darter	1
Percina		
P. burtoni Fowler	Blotchside Logperch	1
P. evides (Jordan and Copeland)	Gilt Darter	1
P. caprodes (Rafinesque)	Common Logperch	1
P. sciera (Swain)	Dusky Darter	1
Lepomis		
L. cyanellus Rafinesque	Green Sunfish	1
L. megalotis (Rafinesque)	Longear Sunfish	1
L. macrochirus Rafinesque	Bluegill	1
Micropterus		
M. dolomieu Lacepède	Smallmouth Bass	1
M. salmoides (Lacepède)	Largemouth Bass	1

recognized in the Duck River and included *Etheostoma smithi* (Slabrock Darter), the species most closely related to the Striated Darter (MacGuigan and Near 2019). The BLAST results also indicated that these species have a low chance of amplifying using these primer pairs.

We optimized annealing temperature for candidate primer sets using a gradient thermocycler in 10-µl reactions containing 5 µl of Promega Master Mix, 0.25 µl of each primer, 3.5 µl of nuclease-free water, and 1 µl of template DNA. Thermal cycling conditions were 95 °C for 2 minutes; 35 cycles of 95 °C for 30 s, 58–65 °C for 30 s, and 72 °C for 60 s; and a final elongation of 72 °C for 10 min. We visualized results in a 1% SB agarose gel stained with GelRed under a UV Gel Imager (UVP GelSolo; Analytik Jena, Jena, Germany). We tested primer candidates for cross-amplification of non-target species using 1–3 individuals for each species from Table 3, except for the 7 species that DNA could not be obtained from. The resulting candidate qPCR assay showed no amplification of non-target species, except for the Slabrock Darter. We Sanger sequenced amplified products from our representative Striated Darter and Slabrock Darter samples and confirmed them to be products from their representative species. The Slabrock Darter is not a sympatric species and is not known to persist in the same tributaries as the Striated Darter; Striated Darters occur 145 river kilometers farther upstream than the known upstream limit of the Slabrock Darter (Etnier and Starnes 2001). Thus, we assumed that this assay is specific to the Striated Darter.

We tested the resulting candidate qPCR assay (i.e., 2 primers and 1 probe) for specificity against Striated Darter and DNA from non-target species on a Quant Studio™ 3 Real-Time PCR system (Applied Biosystems, Waltham, MA), with the addition of a probe designed for the specific candidate assay, following methods from Paine et al. (2021) and Diaz-Ferguson et al. (2014). We standardized tissue-derived DNA from all species to a concentration of 15 ng/µl for assay-specificity testing and performed all reactions using a 10-µl volume reaction consisting of 5 µl of TaqMan® Environmental Master Mix 2x (ThermoFisher, Waltham, MA), 0.25 µl of each primer (20 µM), 0.125 µl of probe (10 µM), 3.375 µl of nuclease-free water (VWR), and 1 µl of template DNA. All qPCR reactions were performed using the following optimized profile: initial denaturation at 95 °C for 10 min; 55 cycles of 95 °C for 30 s, 64 °C for 16 s, and 72 °C for 60 s.

We used a synthetic gBlocks™ Gene Fragment (Integrated Gene Technologies, Coralville, IA, USA) based on the generated Striated Darter 16S consensus sequence, as the DNA template for qPCR standards to establish qPCR efficiency, limits of detection (LOD), and limits of quantification (LOQ). We estimated copy number for the gBlock™ stock by using the following formula:

$$\text{Number of copies (molecules)} = \frac{X \text{ ng} * 6.0221 \times 10^{23} \text{ molecules/mole}}{(N * 660 \text{ g/mole}) * 1 \times 10^{9} \text{ ng/g}},$$

where X is the amount of amplicon (ng), and N is the length of the double-stranded DNA amplicon.

We performed a 10-fold serial dilution using this estimate to generate an 8-point standard curve (5,000,000 to 1 copy/reaction). We used a curve-fitting analysis

outlined by Klymus et al. (2020) to calculate LOD and LOQ. We generated the reaction efficiency and R^2 (coefficient of correlation) for the standard curve from the calculated slope in the QuantStudio™ Design and Analysis software (v. 2.6.0; ThermoFisher). The assay did not amplify in any of the nontarget species, except for the Slabrock Darter, in silico or in vitro using epPCR or qPCR, and therefore we assumed it to be specific to the amplification of Striated Darter and suitable for all subsequent eDNA sampling.

We used 2 quality controls and repeated assays for each qPCR plate, which included 1 type of negative control, consisting of a molecular-grade water substitution for DNA, and a positive control. The positive control was from tissue-derived Striated Darter DNA of a known concentration equal to the median concentration of the standard curve (1 ng/µl). A positive control was used as an external standard on each qPCR plate to confirm that our assay was amplifying correctly and efficiently during each run.

eDNA extraction and qPCR amplification

We extracted DNA from filters using IBI Scientific Fecal DNA Extraction kits (IBI Scientific, Dubuque, IA) following manufacturer protocols. Extraction took place in a separate hood than qPCR prep and in a separate room than water filtration to avoid possible contamination. We employed an extraction blank during each round of DNA extractions and collected it by conducting the extraction process with all kit materials and chemicals but no filter placed in the spin column.

Using the optimized qPCR assay (Table 2), we ran 4 replicate qPCRs for each collected water sample in 96-well plates; thus, there were 12 total qPCR replicates for each sampling event at a site. To reduce the risk of false detections, we included field negative controls, extraction negative controls, no-DNA negative controls, and a positive genomic DNA control for each qPCR plate (see Supplemental Fig. 1 in Supplemental File 1). We ran samples alongside a dilution series of synthetic control DNA varying from 5,000,000 to 5 copies/reaction to enable quantification of DNA in unknown samples. For each qPCR replicate, we prepared 10-µl reactions containing 5 µl of TaqMan® Environmental Master Mix 2x, 0.25 µl of each primer (20 µM), 0.125 µl of probe (10 µM), 1 µl of IPC MM (10 X), 0.2 µl of IPC DNA (50 X), 2.175 µl of nuclease-free water (VWR), and 1 µl of template DNA. We assessed presence of Striated Darter DNA using a 96-well QuantStudio 3 and the optimized profile as detailed in the in vitro subsection above. We performed analyses of run data using QuantStudio Design and Analysis Software (v. 2.6.0). We used standard curve analysis and cycle threshold values to determine if a qPCR reaction resulted in a positive detection. We assumed that if at least 1 of the 4 qPCR replicates yielded a positive amplification, then a sample had a positive detection. Additionally, we assumed that a site was occupied by Striated Darter if at least 1 of the 3 water samples from a site had a positive detection. To confirm that the correct target fragment was amplified, we Sanger sequenced products to compare with our reference sequence database.

Statistical analyses

We used results from the qPCR replicates to fit multiscale occupancy models to estimate the probability of site occupancy (ψ), the probability of DNA being detected in a water sample given that DNA was at the site (i.e., sample occupancy [θ]), and the probability of detecting DNA in each qPCR replicate given that there was DNA in a sample (i.e., detection [*p*]). For each qPCR replicate, we used a binary variable to indicate DNA detection, where a value of 1 indicated a positive detection and a value of 0 indicated a non-detection. The collection of multiple samples at a site ($n = 3$) and the use of multiple qPCR replicates ($n = 4$ per sample, $n = 12$ total replicates per site) allowed the estimates of ψ, θ, and *p* through a Bayesian multiscale occupancy model using the package 'eDNAoccupancy' (Dorazio and Erickson 2018) in R (v 4.1.2; RStudio Team 2020).

We used the function 'occData()' (Dorazio and Erickson 2018) to compute matrices required by the model, based on detection of Striated Darter eDNA in the sample. To run a series of occupancy models that varied with respect to included covariates (see below), we used the function 'occModel()' (Dorazio and Erickson 2018). When fitting each model, we used 15,000 iterations and a 1000-iteration 'burn-in' period for calculating derived parameters. We assumed ψ, θ, and *p* to be constant across sites for the null model [i.e., ψ(.) θ(.)*p*(.)], and included covariates at all levels for the global model. We built alternative models wherein ψ, θ, and *p* varied as a function of environmental conditions that could potentially affect sample efficiency and detection. To avoid over-parameterizing models, we allowed no more than 2 covariates per parameter.

We considered different models where the potential effects of different covariates were included for estimating probabilities (i.e., ψ, θ, and *p*; Table 4). First, models allowed ψ to vary with discharge (CFS; ft^3/s), land cover (forest; %) or both. To incorporate discharge into models, we generated site-specific discharge estimates by averaging all recorded flow values during the time of collection from the nearest USGS streamgage downstream of each site (Holsopple 2021; see Supplemental Table 3 in Supplemental File 1). To account for stream-size differences between sample locations and streamgage locations, we calculated average flows by multiplying each flow value by the ratio of site-specific upstream drainage area (obtained using ArcGIS) to streamgage-specific upstream drainage area (USGS streamgage data). Further models also allowed ψ to vary with land-use patterns, as suggested in previous studies of Striated Darter (Abernathy and Mattingly 2011, Cook et al. 1996, Page and Braasch 1977). We considered proportions of 3 different land-use categories (forested, developed, and farmed) but were restricted to the forested category because farmed and developed categories were highly correlated to the forested category ($r = 1$, $P < 2.2 \times 10^{-16}$ and $r = 0.62$, $P = 0.001$, respectively), and they encompassed a smaller range among sites than forested land use. During data collection, we recorded values of turbidity (T; ntu) in the field using a YSI ProDSS water quality meter (Yellow Springs Instrument, Yellow Springs, OH; see Supplemental Table 3 in Supplemental File 1), and models included the effect of this covariate on θ, *p*, or both probabilities. We measured other water-quality

parameters, including temperature, pH, DO, conductivity, during the summer of 2020 but not in the summer of 2021 due to multiple probe malfunctions. Therefore, temperature, pH, DO, and conductivity were not included in generated models as 7 of the 30 sites would not have been included in the final estimates. We also generated a single model that considered the possibility that each estimated probability was constant across sampling sites.

We evaluated normality of all covariates by computing Shapiro–Wilk normality tests and constructing quantile–quantile plots in R using base R functions (v 4.1.2) and the 'ggpubr' package (Kassambara 2022), respectively. Using these assessments, we determined the site on the main-stem Duck River (DUCK) to be an outlier for most of the covariates, and therefore removed it from further analyses. Furthermore, complete covariate data were not available for 4 sites (SPRING, DOG, CLM, BUT), so we also removed these sites from further analyses. All occupancy models encompassed covariate data from the remaining 25 sites (see Supplemental Table 3 in Supplemental File 1).

Collectively, these different model structures resulted in 13 different models, which included null (i.e., all probabilities constant) and global (all covariates included) models. We calculated 2 criteria widely used for model evaluation for each of the models. Following Dorazio and Erickson (2018), the 2 criteria used included the widely applicable information criterion (WAIC) and posterior predictive loss criterion (PPLC); lower values indicate better model fit for both criteria. To assess the significance of parameter estimates for individual covariates, we examined 95% credible intervals of the generated posterior distributions; if this interval included zero, we considered the effect of the covariate to be non-significant (i.e., indistinguishable from zero).

Results

Assay design and specificity

Using the generated Striated Darter consensus sequence, a set of oligos were designed to amplify a 167-bp long fragment of the mitochondrial 16S rRNA gene. The forward primer (EtST_16S_651F; Table 2) showed high specificity to Striated Darter in silico with a minimum of 1 bp mismatch in all non-target *Etheostoma* and *Nothonotus* species other than Slabrock Darter, which was an exact match. The reverse primer (EtST_16S_817R) had similar specificity with a minimum of 1 bp mismatch in all non-target species, including Slabrock Darter. The probe-binding region for the assay (EtST_16S_735P) also had similar specificity with a minimum of 1 bp mismatch in all non-target species other than from Slabrock Darter, which was an exact match.

The BLASTn alignment of oligos against sequences in the GenBank database did not indicate similarity to other fishes, with less than a 90% match and an e-value greater than 11 for all results. Striated Darter was specifically amplified with the designed assay and was confirmed through in vitro assay validation with epPCR (see Supplemental Fig. 2 in Supplemental File 1). Although Slabrock Darter DNA also amplified with this assay, current distributional data for both species indicate

they do not co-occur in the same tributaries of the Duck River, with the Slabrock Darter primarily occupying waters much lower in the watershed (Etnier and Starnes 2001, Page and Burr 2011).

qPCR limits of detection

Effective LOD and LOQ of the 16S assay, corrected for 4 replicates, was determined to be 9 copies of target DNA per extraction (95% CI: 5.36–12.61) and 200 copies per reaction, respectively, using a set of serial dilutions with 16 replicates per dilution. All positive qPCRs had a cycle threshold (CT) below 42 cycles (see Supplemental Fig. 3 in Supplemental File 1). Low amounts of amplification were seen with CTs above 43 cycles, and these qPCRs were included in the subset of reactions sent for Sanger sequencing.

All sequences generated from positive qPCR amplifications were an exact match for the Striated Darter, and all negative control sequences produced non-specific products. These non-specific products can be attributed to primer-dimer formation and the result of low amounts of fluorescence due to probe cleavage with non-specific binding (Lahr and Katz 2009). All negative controls resulted in no positive amplification of either target or non-target DNA.

Field sampling

Using the 16S rRNA assay and qPCR data, Striated Darter was detected at 27 of the 30 sites in the Duck River drainage (Fig. 2), and all detections were confirmed via Sanger sequencing. Comparing results with past surveys (Abernathy and Mattingly 2011, Cook et al. 1996, Wheeler et al. 2021; unpublished collection records from Tennessee Valley Authority, University of Tennessee, and Yale University), the eDNA survey suggested Striated Darter presence in 4 tributaries where the species has never before been captured: Knob Creek (KNB), Little Bigby Creek (LBC), Globe Creek (GLB), and Butler Creek (BUT). However, population densities in the 4 newly documented tributaries may be low as rates of detection were reduced for these samples; Knob Creek produced 1 positive qPCR replicate out of 12, Little Bigby Creek had 4 out of 12, Globe Creek had 2 out of 12, and Butler Creek had 2 out of 12.The 3 sites where Striated Darter was not detected are in tributaries where Striated Darter has not been captured previously: Little Flat Creek (LFC), Clem Creek (CLM), and Silver Creek (SLV).

Across all water samples (3 per site; 1 site sampled twice) and qPCR replicates (4 per water sample), Striated Darter DNA was detected in 61 of the 93 water samples (66%) and in 150 of the 372 qPCR replicates (40%). However, variation in the strength of the eDNA signal across qPCR replicates for a given water sample was observed. Striated Darter DNA was amplified across all 4 qPCR replicates for 18 samples, across 3 of 4 qPCR replicates for 11 samples, across 2 of 4 qPCR replicates for 13 samples, and across 1 of 4 qPCR replicates for 19 samples. While Striated Darter DNA was detected at 90% of sampled sites, there was also considerable variation in strength of the eDNA signal among sites. More specifically, Striated Darter DNA was amplified in 1–4 qPCR replicates for 16 sites, in 5–8 qPCR replicates for 2 sites, and in 9–12 qPCR replicates for 9 sites.

Occupancy

Overall, the qPCR data suggested a naïve site occupancy probability of 0.90 (or 90%), and the results of the occupancy models suggested similarly high values of ψ (i.e., the probability that a site was occupied by Striated Darter); estimated values varied between 80 and 96% (Table 4). Among all models considered, only models where turbidity (T) was a covariate on θ (i.e., the probability that DNA was detected in a sample if it was present at a site) produced posterior estimates where the 95% credible interval did not overlap zero. Credible-interval results from models where T was a covariate for θ suggested a positive relationship between T and θ (Fig. 3).

However, there was not a lot of variation in the evaluation criteria among the models (WAIC = 95.56–99.04, PPLC = 124.88–127.15). These results suggest that no single model represented a dramatically better fit to the data than any other model. Even though there was a degree of variation in estimates of θ (min–max = 0.403–0.923) and *p* (min–max = 0.578–0.699), the interpretation of these ranges may not be meaningful given the non-significant effects of almost all included covariates. Instead, estimates of ψ, θ, and *p* that were not allowed to vary with the effects of covariates (i.e., null model) may be more reflective of actual probabilities associated with the Striated Darter and the methodological approach of this project.

Discussion and Conclusions

Results of this eDNA study offer valuable insights into the distribution of the Striated Darter, aiding managers in identifying key areas for habitat protection. Successful management and conservation of imperiled species hinge on streamlined monitoring and precise knowledge of their detectability and distribution.

Table 4. Set of models used in occupancy framework for eDNA detection probabilities. Model denotes formulations for each individual model in terms of estimated probabilities ψ (i.e., site occupancy), θ (i.e., sample occupancy), and *p* (i.e., detection). Term included in parentheses following estimated probabilities are covariates including forested land cover (forest; %), turbidity (T; ntu); and discharge (CFS; ft^3/s); (.) indicates constant value of associated probability in model structure. Values in ψ, θ, and *p* columns reflect posterior distribution means. WAIC = widely applicable information criterion; PPLC = posterior predictive loss criterion.

Model	WAIC	PPLC	ψ	θ	*p*
ψ(CFS), θ(.), *p*(.)	95.56	124.88	0.846–0.951	0.730	0.650
ψ(.), θ(.), *p*(.)	95.72	125.38	0.908	0.729	0.650
ψ(Forest), θ(.), *p*(.)	95.78	125.00	0.796–0.926	0.728	0.650
ψ(CFS), θ(T), *p*(.)	96.21	125.78	0.879–0.957	0.414–0.922	0.649
ψ(.), θ(T), *p*(.)	96.37	126.12	0.933	0.406–0.923	0.648
ψ(Forest), θ(T), *p*(.)	96.37	125.95	0.857–0.947	0.403–0.922	0.649
ψ(Forest + CFS), θ(T), *p*(T)	97.87	126.01	0.800–0.961	0.420–0.918	0.596–0.688
ψ(Forest), θ(T), *p*(T)	98.00	126.15	0.856–0.944	0.413–0.920	0.598–0.687
ψ(CFS), θ(T), *p*(T)	98.02	126.29	0.887–0.959	0.420–0.919	0.597–0.687
ψ(.), θ(T), *p*(T)	98.05	126.16	0.932	0.419–0.919	0.596–0.687
ψ(Forest), θ(.), *p*(T)	98.92	126.84	0.795–0.927	0.731	0.579–0.699
ψ(CFS), θ(.), *p*(T)	99.02	127.15	0.850–0.955	0.734	0.579–0.699
ψ(.), θ(.), *p*(T)	99.04	126.83	0.909	0.733	0.578–0.699

Acquiring these data poses many challenges, especially for elusive species like the Striated Darter. However, leveraging eDNA techniques and occupancy modeling can streamline this process, enhancing conservation efforts. Using these techniques, Striated Darters were detected at 27 of the 30 historical sampling sites in the Duck River Watershed. Seventeen of these 27 sites have some level of historic occurrence, whether that be a single voucher specimen or repeated captures over multiple years. At 6 of the 10 remaining sites with eDNA detections, Striated Darters have not been captured previously, but have been caught in other locations in those tributaries, indicating that these detections likely do not represent new populations. Positive eDNA detections at the remaining sites suggest the possibility of previously undocumented Striated Darter populations existing at low population densities in 4 new tributaries: Knob Creek, Little Bigby Creek, Globe Creek, and Butler Creek.

In aquatic environments, the release of eDNA from source organisms is altered through transport, dilution, settling into and resuspension from sediments, and overall degradation that can affect the detectability of a species (Barnes and Turner 2016, Turner et al. 2014). These factors can contribute to increased DNA degradation and dilution in a natural environment (Thomsen and Willerslev 2015); populations existing at low densities may go undetected as concentrations of eDNA

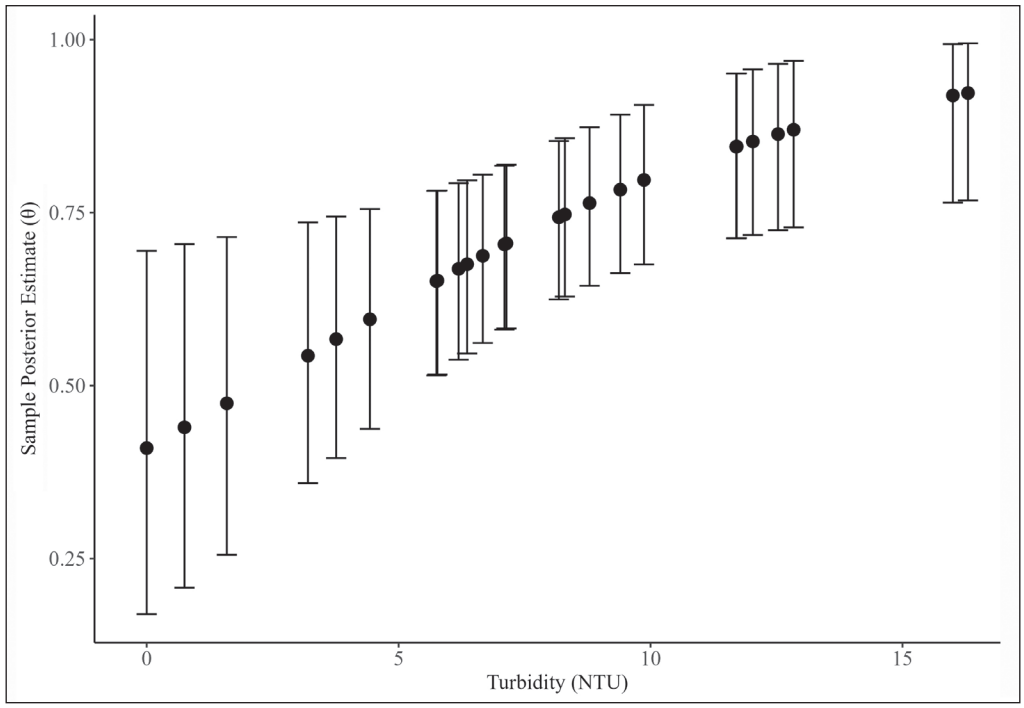

Figure 3. Relationship between turbidity and estimates of the probability of detecting *Etheostoma striatulum* (Striated Darter) DNA in a water sample given that species DNA is present at a site (i.e., sample occupancy θ). Mean posterior estimates for θ from the model [ψ(.), θ(T), p(.)] are represented by black dots and 95% credible intervals are represented by error bars on either side of each dot.

may not be high enough to detect (Fluker et al. 2010, Taylor et al. 2021). Based on data collected during a 2006 survey, Abernathy and Mattingly (2011) estimated a population density of 0.14 individuals/m^2 at SFLT-A, a site that has reliably produced higher numbers of Striated Darters since 2001. Future sampling efforts should target collections in areas with preferred habitat to improve the probability of detection, as Striated Darter populations may exist at very low densities (Abernathy and Mattingly 2011, Holsopple 2021).

Both biotic and abiotic factors can influence the detectability of targeted eDNA, which likely helps to explain the variation in detection observed in this study. Occupancy modeling can estimate detection probabilities when imperfect detection exists, and hierarchical occupancy models are especially suited for eDNA data because they can estimate probabilities at each level of the eDNA sampling hierarchy: site, sample, and PCR replicates (Katz et al. 2020, Ostberg et al. 2019, Thalinger et al. 2021). Although most of the covariates included in the models did not have a significant influence on final estimates, T did have a pronounced positive effect on sample occupancy (θ). From a biological perspective, this relationship makes sense given the resuspension of sediment and particles that elevates T in lotic environments is also likely to resuspend DNA fragments (Barnes et al. 2021). However, some studies have suggested that T may adversely affect the detection of eDNA (Fujii et al. 2019, Strickland and Roberts 2019), potentially due to (1) DNA degradation caused by longer or more challenging filtering processes, or (2) an increase in inhibitors present within the sample.

Wineland et al. (2019) found a significant reduction in the ability to detect *Cryptobranchus alleganiensis alleganiensis* (Sonnini de Manoncourt and Latreille) (Eastern Hellbender) eDNA with increasing T, supporting the idea that turbidity is playing a role in species detection. Although the detection of a species is largely dependent on size of filter being used to capture eDNA (Barnes et al. 2021), it is important to note that Eastern Hellbender usually selects habitats where siltation is low, oxygen levels are relatively high, and water clarity is also relatively high (Mayasich et al. 2003). In contrast, Striated Darters appear to select low-velocity habitats where fine sediment accumulates and is easily resuspended (Holsopple 2021). Page and Braasch (1977) described their collections of Striated Darter at the type locality (Wartrace Creek [WAR-A]) to be mostly under slab rocks in large-shallow pools, usually on bedrock and less frequently over gravel. Page (1980) further described their type locality as a pool with primarily bedrock, with large expanses of gravel and silt present along the east bank, and large flat stones scattered over an area encompassing about one-fourth of the pool.

Future studies should aim to explore the detection probability and occupancy of Striated Darter across various seasons, considering fluctuations in temperature and discharge throughout the year. Increased frequency of monitoring could also help capture the movement of the species effectively. This study highlights that conventional techniques may fail to detect certain populations, and the sensitivity of the assay developed here might reveal populations existing at low densities. Therefore, it is important to examine the spatial and temporal dimensions of Striated Darter DNA to comprehend the relationship between positive detections and

their respective populations. Additionally, the Striated Darter exhibits a short life cycle, with individuals typically spawning within their first year and rarely surviving beyond 1.5 years (Cook et al. 1996, Page 1980). Thus, it may be of interest to investigate whether eDNA detection is most successful before Striated Darter spawn in the spring, during spawning when gametes are abundant in the water column, or after spawning when populations include high abundances of both adults and juveniles. Researchers and managers are urged to prioritize these investigations, especially considering the ongoing federal review of the Striated Darter. Conducting manipulative experiments might become more challenging post-listing, underlining the urgency of these studies.

Although the distribution of the Striated Darter seems largely unchanged since their description, some populations appear to be persisting at low densities and are going undetected with conventional techniques, raising concern for the future trajectory of these populations. Verification of eDNA detections in both Knob Creek and Noah Fork are especially important as these tributaries could potentially be edge populations of the Striated Darter, which could serve as vital pieces of information regarding future management and protection of the species (Ginson 2012, Radinger et al. 2016, Turba et al. 2022).

Finally, we note the potential benefit of coupling conventional and eDNA surveys for management of imperiled species like Striated Darter. While the best way to confirm species presence will aways involve capture and identification of individuals, eDNA surveys offer a less-invasive technique that can be applied easily over large areas (Johnston and Janosik 2019, Lor et al. 2020, Shaw et al. 2016). Development of a functional eDNA assay is not trivial but, as demonstrated here, is possible even in speciose systems with closely related taxa. An example of a coupled sampling design that maximizes efficiency could involve eDNA surveys as an initial approach to delineate species distribution, or as part of a routine monitoring effort across a broad geographic area. More-targeted conventional sampling could then be used to confirm specific eDNA results, such as when the detection of a species is in new habitat, or to estimate vital demographic parameters such as population abundance or survival.

Acknowledgments

The authors thank the Tennessee Wildlife Resources Agency, the US Fish and Wildlife Service, and the Center for Management, Utilization, and Protection of Water Resources at Tennessee Technological University for the funding and support to complete this project. We also thank Dr. Robert Paine for help in the design and implementation of this project as well as Abbey Holsopple, Ryan Hudson, Emma Barnett, Maija Weaver, Fritz Hoogakker, and Brittany Bajo-Walker for field assistance and laboratory work.

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