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Defining the current distribution of the imperiled Black-spotted Newt across south Texas, USA

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ABSTRACT

The Black-spotted Newt (*Notophthalmus meridionalis*) is a chronically understudied salamander species, with many aspects of its natural history, ecology, and distribution poorly known. Previous studies using traditional methodologies have had limited success documenting *N. meridionalis* on the landscape, detecting individuals at 6% (7 of 114) and 1% (2 of 221) of sites surveyed. A novel environmental DNA (eDNA) assay was designed and implemented with the goals of assessing the current distribution of *N. meridionalis* across south Texas, USA, and better understanding the conditions for positive eDNA detections. We conducted eDNA sampling and traditional surveys at 80 sites throughout south Texas. *Notophthalmus meridionalis* was detected at 12 localities in total: four localities using eDNA surveys, four localities using traditional methods, and four localities with both methodologies. eDNA detections were obtained from five counties, including one where *N. meridionalis* has never been reported and another where *N. meridionalis* has not been observed since the 1930s. eDNA detections were obtained in all four seasons, generally following moderate to heavy rainfall events. Our results support the increased use of eDNA surveys to detect rare and cryptic amphibians and to better understand the current distribution of this imperiled species.

1. Introduction

The Earth is in the midst of a biodiversity crisis and on the verge of a sixth mass extinction event (Catenazzi, 2015; Ceballos et al., 2015). Amphibians are among the most threatened global taxa (Stuart et al., 2004), with an estimate that ca. 32% of the amphibian species evaluated are threatened with extinction (IUCN, 2019). One species of conservation concern is the Black-spotted Newt, *Notophthalmus meridionalis* (Fig. 1), which is only found in south Texas, USA, and northeastern Mexico. The IUCN lists *N. meridionalis* as Endangered with a "decreasing" current population trend (Flores-Villela et al., 2008), and the Texas Parks and Wildlife Department lists *N. meridionalis* as Threatened (TPWD, 2020). Although Judd (1985) suggested that *N. meridionalis* should be listed as Threatened, the species holds no status with the U.S. Fish and Wildlife Service under the Endangered Species Act and is currently a candidate species for federal listing (USFWS, 2021).

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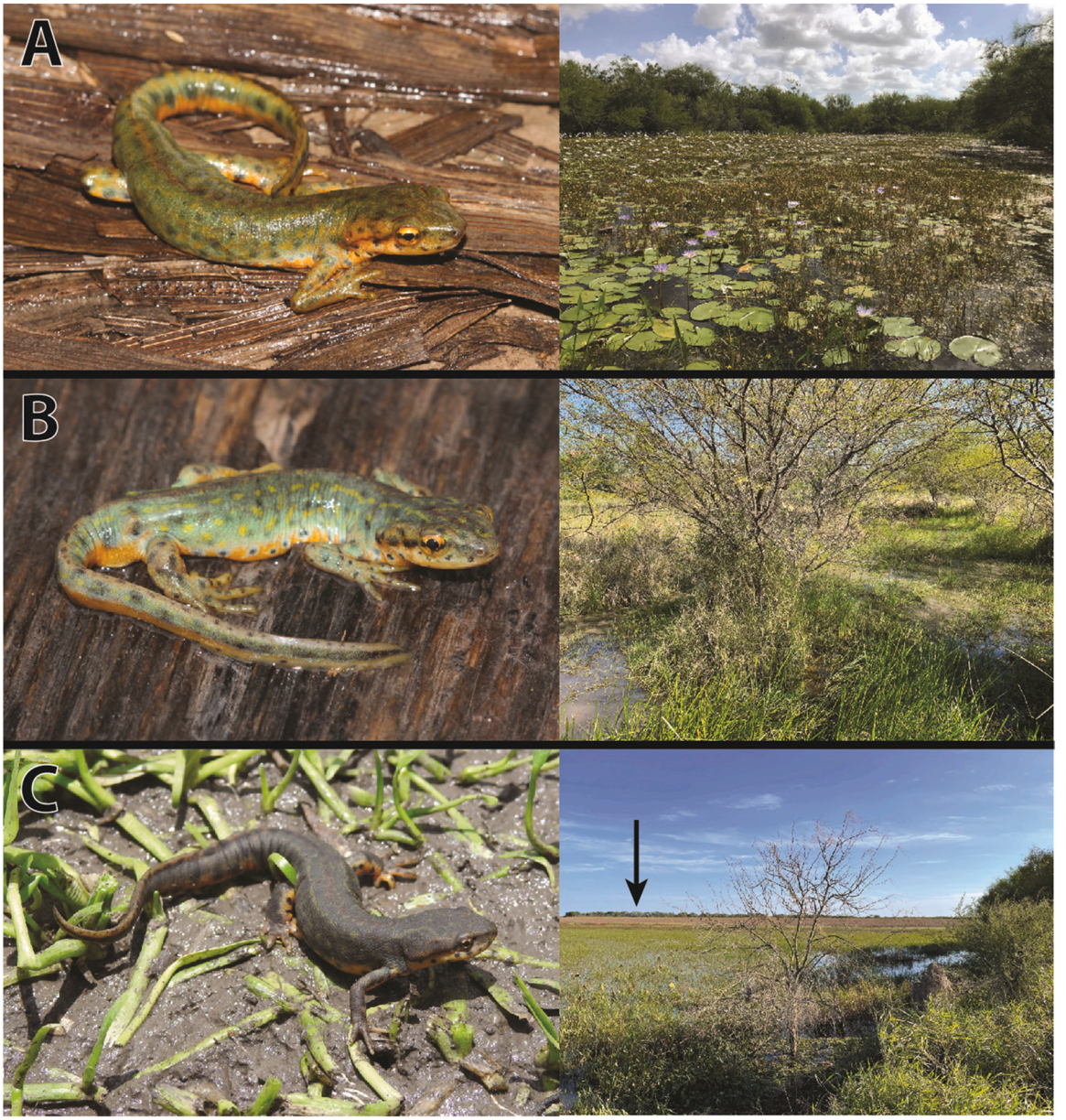

Fig. 1. Representative photos of Black-spotted Newts (*Notophthalmus meridionalis*) sampled during this study and their corresponding habitats: A) Adult male *N. meridionalis* (TNHC 116643 [DRD 6320]) from Site 73, Cameron County; B) Adult male *N. meridionalis* (TNHC 116644 [DRD 6610]) from Site 41, Willacy County; C) Adult male *N. meridionalis* (TNHC 116642 [DRD 5813]) from Site 52, Hidalgo County, with a black arrow indicating adjacent agricultural practices. Site numbers correspond to Table 1. TNHC = Biodiversity Collections, The University of Texas at Austin; DRD = Drew R. Davis Field Series.

A major threat to *N. meridionalis* is habitat loss and subsequent habitat fragmentation, which have been implicated as the primary drivers of biodiversity loss (Brooks et al., 2002; Wilson et al., 2016). South Texas has experienced significant habitat loss due to human activity (Jahrsdoerfer and Leslie, 1988; Fulbright and Bryant, 2002). In particular, >95% of native brushland in the Lower Rio Grande Valley has been cleared since the 1920s (Jahrsdoerfer and Leslie, 1988). The clearing of land for agriculture, rangeland, roads, and urban development has served to fragment suitable *N. meridionalis* habitat; the remaining tracts of native brushland exist patchily throughout the region, with most being maintained by federal and state agencies, non-governmental organizations, and private entities. Factors contributing to the decline of *N. meridionalis* are prevalent throughout the historic range of this species, which extended from Victoria County, Texas, USA, south to the state of Veracruz, Mexico, and up to 150 km inland from the Gulf of Mexico (Dixon, 2013; Petranka, 1998).

Throughout their range, *N. meridionalis* has been found in ephemeral wetlands, resacas, roadside ditches, and pools along both

Table 1
List of sites sampled for Black-spotted Newts (*Notophthalmus meridionalis*) as part of this study. The county, site category, and coordinates for each site are provided (coordinates are excluded for sites where the landowner did not consent to publishing this information). Sites are classified as "recent" (those where *N. meridionalis* was detected since 2000), "historic" (those where *N. meridionalis* was detected before 2000), "georeferenced" (sites based on historic *N. meridionalis* records that lack specific coordinates), or "other" (potential *N. meridionalis* sites within and just beyond the recognized range of this species in Texas). Site numbers correspond to those listed in Table 2 and Fig. 2.

Site #	County	Site Name	Latitude	Longitude	Site Category
1	Victoria	Coleto Creek at US Hwy 77	28.71199	-97.04293	Georeferenced
2	Victoria	Coleto Creek side channel, ca. 0.5 km SE of US Hwy 77	28.71146	-97.04239	Georeferenced
3	Goliad	Manahuilla Creek at US Hwy 59	28.67958	-97.32555	Other
4	Bee	Coastal Bend College, Beeville Campus, Lake Louise	28.43551	-97.75385	Other
5	Live Oak	Hilbert H. Kopplin Memorial Park	28.46476	-98.17814	Other
6	Live Oak	Live Oak County Park, pond SE of parking area	28.37257	-98.11606	Georeferenced
7	McMullen	Hwy 16 bridge over Nueces River	28.31076	-98.55787	Georeferenced
8	McMullen	ditch along Co Rd 624, ca. 2.6 rd km E jct Nueces River	28.17365	-98.68841	Other
9	Calhoun	Powderhorn WMA, midline fence pond	28.46752	-96.49008	Other
10	Calhoun	Powderhorn WMA, Bullrush Pond	28.45977	-96.44999	Other
11	Calhoun	Powderhorn WMA, dugout pond near barn at S entrance	28.43065	-96.49587	Other
12	Calhoun	Aransas NWR, Auto Loop Trail, ca. 0.6 rd km SW from observation towers	28.24829	-96.79416	Other
13	Aransas	Aransas NWR, ca. 0.2 rd km S jct Auto Loop Trail end and main road	28.28150	-96.81028	Other
14	Refugio	ditch crossing 1st St, ca. 0.1 rd km SW jct Cole	28.09421	-97.21234	Georeferenced
15	San Patricio	Welder Wildlife Foundation, Artesian Spring	28.12724	-97.39178	Other
16	San Patricio	Welder Wildlife Foundation, Pollita	28.12368	-97.37886	Other
17	San Patricio	Welder Wildlife Foundation, Big Lake	28.12055	-97.37287	Historic
18	San Patricio	CBBEP Nueces Delta Preserve, Wyatt Corrall	27.88942	-97.60838	Other
19	San Patricio	CBBEP Nueces Delta Preserve, wetland ca. 1.1 km SE Wyatt Corral	27.88015	-97.60139	Other
20	San Patricio	Live Oak Park pipeline pond	27.85838	-97.20405	Other
21	San Patricio	Live Oak Park, junkpile pond	27.85410	-97.20508	Other
22	Jim Wells	dugout pond along TX Hwy 359, SW bridge over Nueces River	28.03632	-97.86307	Other
23	Nueces	John J. Sablatura Park, flooded field along Agua Dulce Creek	27.79758	-97.82245	Georeferenced
24	Nueces	Pintas Creek at Co Rd 70	27.72945	-97.90458	Other
25	Duval	San Diego Creek W of TX Hwy 359	27.75987	-98.24115	Georeferenced
26	Kleberg	TAMU-Kingsville CKWRI, South Pasture pond	27.47093	-97.89059	Historic
27	Kleberg	417 S Co Rd 1120, Riviera, TX 78379; dugout tank	27.35159	-97.72819	Georeferenced
28	Kleberg	west side of Co Rd 1110 S, ca. 0.6 rd km N jct Co Rd 2300E	27.31603	-97.74364	Georeferenced
29	Kleberg	Audubon Outdoor Club of Corpus Christi, Louise Trant Bird Sanctuary, ditch near US Hwy 77	27.30135	-97.81564	Other
30	Kleberg	wetland E of jct of 3rd St and W Poplar Ave (in Riviera, TX)	27.29474	-97.81526	Georeferenced
31	Kenedy	East Foundation Santa Rosa Ranch, large pond S of Campo Viejo	27.18453	-97.87874	Other
32	Kenedy	East Foundation Santa Rosa Ranch, pond S of ranch HQ	27.16870	-97.86803	Other
33	Kenedy	East Foundation Santa Rosa Ranch, Escondido Pond	27.14583	-97.82238	Other
34	Kenedy	US Hwy 77, W side, ca. 12.0 rd km N Kenedy/Willacy county line	26.70689	-97.76932	Georeferenced
35	Kenedy	US Hwy 77, E side, ca. 8.5 rd km N Kenedy/Willacy county line	26.67494	-97.76772	Georeferenced
36	Kenedy	US Hwy 77, W side, ca. 1.0 rd km N Kenedy/Willacy county line	26.60713	-97.76729	Georeferenced
37	Brooks	G & M Glick Ranch, cattle pond ca. 5.3 rd km W entrance gate	26.93675	-98.21980	Other
38	Willacy	US Hwy 77, E side, ca. 1.6 rd km N jct La Chata gate #4	26.58250	-97.76800	Other
39	Willacy	US Hwy 77, E side, ca. 0.5 rd km S jct La Chata gate #4	26.56408	-97.77056	Other
40	Willacy	pond along Co Rd 398, ca. 0.7 rd km N jct Bay Ave	26.51099	-97.67220	Georeferenced
41	Willacy	East Foundation El Sauz Ranch, Newt Pond	26.50721	-97.49904	Recent
42	Willacy	East Foundation El Sauz Ranch, pond near TX Hwy 186	26.50530	-97.49881	Recent
43	Willacy	TX Hwy 186, N side ditch, ca. 1.1 rd km W East Foundation El Sauz Ranch gate near Huesos Tank	26.49621	-97.51921	Other
44	Willacy	Lower Rio Grande Valley NWR, Willamar Tract, NE pond	26.41781	-97.56106	Other
45	Willacy	Lower Rio Grande Valley NWR, Willamar Tract, S pond	26.40557	-97.56276	Other
46	Willacy	Grace Heritage Ranch, main pond	26.38820	-97.56434	Recent
47	Willacy	Grace Heritage Ranch, 60-acre pond	26.38094	-97.55993	Other
48	Starr	TNC Las Estrellas Preserve, dugout pond	26.47476	-98.87364	Other
49	Starr	Lower Rio Grande Valley NWR, San Francisco Banco Tract, canal	26.29453	-98.69356	Other
50	Starr	Lower Rio Grande Valley NWR, San Francisco Banco Tract, concrete spillover	26.28741	-98.69893	Other
51	Starr	Old Military Hwy, ca. 2.6 rd km ESE jct Co Rd 2360, pond #4	26.28044	-98.61979	Other
52	Hidalgo	Brushline Road, ca. 1.1 rd km S jct TX Hwy 186	26.49018	-98.05029	Recent
53	Hidalgo	Brushline Road, ca. 1.6 rd km S jct TX Hwy 186	26.48554	-98.05111	Recent
54	Hidalgo	ravine along W side of irrigation canal along 12th St, ca. 0.3 rd km W jct Jesus Flores Rd	26.42621	-97.99528	Other
55	Hidalgo	ditch along Jesus Flores Rd, ca. 0.4 rd km S jct 12th St	26.42128	-97.99273	Other

(*continued on next page*)

Table 1 (*continued*)

Site #	County	Site Name	Latitude	Longitude	Site Category
56	Hidalgo	La Joya Lake, entrance road pond	–	–	Georeferenced
57	Hidalgo	1222 Palm Ave, La Joya, TX 78560; SW corner of large wetland	26.23395	-98.45737	Other
58	Hidalgo	National Butterfly Center, wetland near Rio Grande	26.17091	-98.36708	Other
59	Hidalgo	Lower Rio Grande Valley NWR, Milagro East Tract, resaca	26.07055	-98.19347	Other
60	Hidalgo	Santa Ana NWR, Willow Lakes	26.08040	-98.14093	Georeferenced
61	Cameron	pond along Co Rd 2629 at jct with Co Rd 2845	26.34284	-97.82801	Other
62	Cameron	Laguna Atascosa NWR, Newt Pond	26.31062	-97.36507	Recent
63	Cameron	Laguna Atascosa NWR, pond ca. 0.2 km SW maintenance shop	26.22469	-97.34976	Other
64	Cameron	Laguna Atascosa NWR, Kidney Pond	26.22306	-97.36250	Historic
65	Cameron	Laguna Atascosa NWR, Scum Pond	26.22361	-97.36909	Recent
66	Cameron	Laguna Atascosa NWR, Prairie Trail, pond #3	26.19057	-97.40252	Other
67	Cameron	Laguna Atascosa NWR, Prairie Trail, pond #1	26.18155	-97.41661	Other
68	Cameron	Laguna Atascosa NWR, Prairie Trail, pond #2	26.17723	-97.41514	Other
69	Cameron	Laguna Atascosa NWR, pond along Buena Vista Dr, ca. 0.4 rd km N jct Co Rd 510	26.13513	-97.35100	Other
70	Cameron	pond S of Port Isabel High School Tarpon Stadium	26.07351	-97.24717	Recent
71	Cameron	Laguna Atascosa NWR, TX Hwy 100, crossing 3 A pond	26.07416	-97.37268	Other
72	Cameron	Los Fresnos High School, Agua Negra	26.08148	-97.47491	Other
73	Cameron	Palo Alto Battlefield National Historic Park, Crescent Tank	26.03528	-97.47407	Recent
74	Cameron	Palo Alto Battlefield National Historic Park, American Tank	26.03158	-97.46877	Recent
75	Cameron	Palo Alto Battlefield National Historic Park, dugout pond ca. 0.4 km SE visitor center	26.01446	-97.47650	Recent
76	Cameron	Resaca de la Palma State Park, resaca near Hunter's Trail	25.97577	-97.56607	Other
77	Cameron	Sabal Palm Sanctuary, boardwalk along N end of resaca	25.85182	-97.42056	Recent
78	Cameron	Sabal Palm Sanctuary, resaca blind	25.85050	-97.41920	Recent
79	Cameron	TNC Southmost Preserve, Siren Pond	25.85444	-97.39783	Other
80	Cameron	TNC Southmost Preserve, Black Willow Resaca	25.85481	-97.39424	Recent

small streams and large permanent water bodies (Bishop, 1947; Mecham, 1968; Fig. 1). Rainfall in south Texas is irregular, both seasonally and annually (Gutzler, 2013), and temperatures regularly exceed 37 °C (Jahrsdoerfer and Leslie, 1988); thus, wildlife within this semi-arid environment must be adapted to drought and heat. Rappole and Klicka (1991) reported that *N. meridionalis* is well-suited to the harsh climate that it occupies, persisting for long periods of drought in an inactive or semi-active state below the surface of the soil, utilizing burrows and cracks, and emerging following rainfall. The cryptic nature of *N. meridionalis* makes detection difficult, and traditional methodologies have had limited success detecting individuals on the landscape. Rappole and Klicka (1991) found individuals at 7 of 114 sites (ca. 6%) surveyed, and Judd (1985) found individuals at just 2 of 221 sites (ca. 1%) surveyed.

As a chronically understudied species, there is a critical need to understand more about the distribution of *N. meridionalis* and its habitat throughout south Texas. Previous records indicate that detections of *N. meridionalis* in Texas over the past two decades were limited to three southern counties (Cameron, Hidalgo, and Willacy) out of the 13 total counties with historic occurrence records. The combination of the numerous threats to this species and the cryptic nature of individuals result in an interesting research challenge: is *N. meridionalis* extirpated from much of its historic range, or are traditional survey methods insufficient for species detection?

One promising method to survey for cryptic species are environmental DNA (eDNA) assays, which involve the collection and identification of DNA from the environment that originated from an organism's shed skin, feces, urine, or saliva (Ficetola et al., 2008). The bi-phasic lifestyle of most amphibians can make eDNA a powerful tool for their detection by taking advantage of aquatic reproduction and aquatic larval stages. eDNA methods have been applied to detect the endangered, congeneric Striped Newt (*N. perstriatus*; McKee et al., 2015) and have been used to detect rare species in new localities (e.g., Sakai et al., 2019). eDNA analyses allow for the non-invasive detection of rare or cryptic amphibians through collecting, extracting, and amplifying target species DNA (Goldberg et al., 2016) and can be more sensitive, cost-effective, and less disruptive than traditional surveys (Dejean et al., 2012; Olson et al., 2013; Biggs et al., 2014; Ruppert et al., 2022). The use of nested PCR primers can help achieve greater specificity, in which a second round of PCR is performed with primers "nested" within the amplified region from the first round (Nix et al., 2010; Jackson et al., 2017). One downfall of eDNA methods includes false negatives, which can occur through failure to capture the targeted small fragments of DNA (Roussel et al., 2015) due to DNA degradation from UV-B radiation, high temperatures, and acidic conditions (Strickler et al., 2015). Concentrations of eDNA in a water body decrease following the removal of the species from a water body (Thomsen et al., 2012) and increase following reproduction (Spear et al., 2015; Buxton et al., 2017); thus, eDNA sampling must be planned in accordance with the presence and activity of the target species in the water body.

Here, we designed and implemented an eDNA assay and paired it with traditional sampling methods to detect *N. meridionalis* across south Texas, USA, with the goals of assessing the current distribution of this species and gaining a better understanding of the conditions in which positive eDNA detections can be obtained.

2. Methods

2.1. Sampling locations

A total of 80 sites were selected throughout and just beyond the historic range of *Notophthalmus meridionalis* across south Texas, USA, and were sampled from 2018 to 2021 (Table 1). With *N. meridionalis* activity expected to increase after rainfall (Mecham, 1968),

we collected eDNA samples following rainfall events whenever possible. Efforts were made to sample each site twice; however, due to unpredictable rainfall this was not always possible. Sampling locations were chosen by referencing a database of *N. meridionalis* occurrence records compiled from natural history collections and citizen science observations. The selection of sites was constrained due to the lack of publicly accessible lands in Texas (Schmidley et al., 2001), as well as the lack of suitable habitat (Jahrsdoerfer and Leslie, 1988). Sampling locations included wetlands, ponds, resacas, roadside ditches, and creeks across 19 counties (Fig. 1). Included within these sampling sites are 14 "recent" (post-2000) *N. meridionalis* localities, three "historic" (pre-2000) localities, 16 "georeferenced" localities based on historic occurrence records which lack a specific locality, and 47 "other" potential sites within and immediately beyond the current known range of *N. meridionalis* in south Texas (Table 1). Potential sites were chosen with a preference for ephemeral water bodies, which lacked large predatory fish and contained aquatic vegetation (Mecham, 1968; Rappole and Klicka, 1991).

2.2. Field protocol

At each site, water was collected from three locations to account for the heterogeneous distribution of eDNA and pooled in a sterilized bucket (Turner et al., 2014; Goldberg et al., 2016). The pooled water was poured over a 47-mm diameter Whatman Grade 4 cellulose filter (25–30 μm pore size) inside of a 250-mL filter cup and pumped through using a hand-operated fluid extractor. Filtration occurred in triplicate; up to 1 L of field-collected water was filtered three times per field site as recommended by Ficetola et al. (2008). The water bodies that *N. meridionalis* occupies are often turbid and suspended sediments can clog the filter. At times we were unable to filter the entire 1-L sample, and the final volume filtered was recorded. Before filtering field-collected water, 1 L of deionized (DI) water was filtered at each field site as a field control (blank). In total, each site visit yielded four filters: one field blank and three field samples. Filters were stored in 2-mL tubes with 700 μL of DNAzol, a DNA isolation and buffering reagent (Molecular Research Center Inc, Cincinnati, OH, USA). All filtration using the aforementioned equipment occurred on-site for immediate preservation. To prevent contamination among sites, nitrile gloves were worn, the filter cup and the bucket were sprayed with a 3.78% sodium hypochlorite solution (bleach), followed by a 100 g/L sodium thiosulfate solution to inactivate the bleach, and finally rinsed with DI water (Ruppert et al., 2022).

At each sampling location we also conducted a 30-min search for *N. meridionalis* through active dip-netting in the water and by searching under natural and artificial debris surrounding the wetland. Each *N. meridionalis* captured was photographed, swabbed, weighed, and measured. Genetic tissue samples (tail clips) were collected from all individuals, and a single individual from each unique site was collected, vouchered, and deposited at the Biodiversity Collections, The University of Texas at Austin (TNHC). Specimen handling and collection occurred under a Texas Parks and Wildlife Scientific Collecting Permit (SPR-1018-294), and all collecting activities followed an approved IACUC protocol (AUP #18-28).

2.3. Laboratory protocol

eDNA filter extraction occurred following an adapted GenCatch Blood and Tissue Genomic Mini-Prep Kit protocol (Epoch Life Science, Missouri City, TX, USA). The extraction protocol was modified at several steps (no LYS Buffer was added, 10 μL of 10 mg/mL Proteinase-K was used rather than 20 μL, 500 μL of 100% ethanol and EX Buffer were used rather than 200 μL, and the final elution volume was 100 μL). Although inhibition was not explicitly tested, inhibitor removal kits were shown to be essential for our study system (Supplemental Fig. 1), and a commercial inhibitor removal kit (Zymo OneStep PCR Inhibitor Removal Kit [Zymo Research, Irvine, CA, USA]) was used following DNA extraction. We acknowledge the potential for lower DNA yields due to the use of inhibitor removal kits and false negatives; however, our initial tests showed that inhibitor removal kits were necessary to achieve DNA amplification. Primers were designed based on the published mitochondrial genome for *N. meridionalis meridionalis* available on GenBank (accession numbers: MH367840.1, MH367841.1, MH367842.1, MH367843.1, MH367844.1). Initial and nested primers were designed to amplify a small segment (<200 base pairs [bp]) corresponding to the *N. meridionalis* cytochrome c oxidase subunit 1 (CO1) gene (Tsuji et al., 2019). The initial primers amplified a 181-bp segment (forward: 5′ GTAGACCTGAATGTGGACACC 3′; reverse: 5′ CTGTAAGCCCTCCCTCTGT 3′), and the nested primers amplified a 122-bp segment (forward: 5′ ACACCCGAGCCTATTTTAC 3′; reverse: 5′ GCCCATAGTATTGCAGCAT 3′) within the initial 181-bp segment. Primers were optimized in vitro, with an annealing step temperature gradient and a serial dilution of *N. meridionalis* tissue DNA extract. The primers were tested for specificity against 1 μL of ca. 1.0 ng/μL DNA of 32 potentially sympatric amphibians (Supplemental Table 1).

PCR was performed using a T100 ThermoCycler (Bio-Rad Laboratories, Hercules, CA, USA). For each reaction, 12.5 μL GoTaq G2 HotStart MasterMix (Promega Corporation, Madison, WI, USA), 0.5 μL of 10 μM forward and reverse primers, 6.5 μL of nuclease-free water, and 5 μL of extracted sample were added to a 0.2-mL PCR tube. To detect potential laboratory contamination or non-specific PCR products, a no template control (NTC) was run in conjunction with other samples, using 5 μL of nuclease-free water instead of extracted sample. No internal positive control was included in order to avoid potential contamination of samples due to the sensitivity of our nested PCR assay. The product from the initial round of PCR was purified with an Exo-CIP Rapid PCR Cleanup Kit (herein referred to as Exo-CIP; New England Biolabs, Ipswich, MA, USA) prior to use in the nested round. PCR conditions were as follows: 35 cycles of denaturation at 95 °C for 30 s, variable annealing temperature for 28 s, and elongation at 72 °C for 30 s. The annealing temperature was 55.5 °C for the initial primers and 53 °C for the nested primers. Following the completion of the nested PCR, 20 μL of the PCR product was run on a 2% agarose gel stained with GelRed Nucleic Acid Stain (Biotium Inc., Hayward, CA, USA) for 40 min at 100 V alongside a 50 bp GeneRuler ladder (Thermo Fisher Scientific, Waltham, MA, USA), and the gel was visualized using a UVP transilluminator. When samples produced at least two bands of the appropriate size (122 bp), the remaining 5 μL of PCR product from

Table 2
List of sampling sites, Black-spotted Newt (*Notophthalmus meridionalis*) detection results, date(s) sampled, water turbidity, and total volume filtered for each site visit. The "Detection" column indicates *N. meridionalis* sampling results: eDNA detection (eDNA), traditional detection (T), eDNA and traditional detection (Both), or no detection (No). Bold text indicates the date, turbidity, and total volume filtered ("L filtered") when positive eDNA detections were obtained. We were unable to collect eDNA from Site 7 because this site remained dry throughout the study. Volume and/or turbidity data are missing from sites 73, 76 and 78. An asterisk indicates sites where only one band of the correct size was obtained after PCR amplification, and therefore, did not reach the criteria for a full eDNA detection.

Site #	Detection?	Visit 1 Date	Visit 1 Turbidity	Visit 1 L filtered	Visit 2 Date	Visit 2 Turbidity	Visit 2 L filtered
1	No	4-Dec-20	light	3	–	–	–
2	No	30-Mar-20	moderate	0.9	–	–	–
3	No	4-Dec-20	light	3	28-Feb-20	light	1.77
4	No	6-Oct-20	none	3	3-Dec-20	light	3
5	No	29-Jun-20	none	3	–	–	–
6	eDNA	29-Jun-20	none	3	**3-Dec-20**	**light**	**3**
7	No	–	–	–	–	–	–
8	No	8-Nov-18	none	3	–	–	–
9	No*	27-Feb-20	light	3	6-May-21*	none*	3*
10	No	27-Feb-20	light	3	–	–	–
11	eDNA	**27-Feb-20**	**none**	**3**	6-May-21	none	3
12	No	26-Feb-20	light	1.33	6-Aug-20	high	0.75
13	No	26-Feb-20	moderate	3	6-Aug-20	high	1.15
14	No	28-Aug-20	none	3	–	–	–
15	No	30-Jan-20	none	2.6	–	–	–
16	No	31-Jan-20	high	0.15	–	–	–
17	No	29-Jan-20	light	1.18	7-May-21	light	3
18	No	13-Feb-20	none	3	6-Aug-20	light	3
19	No	13-Feb-20	none	3	6-Aug-20	light	3
20	No	7-May-21	high	3	–	–	–
21	No	30-Jan-20	moderate	1.95	–	–	–
22	No	16-Aug-20	light	3	–	–	–
23	No	21-May-20	light	2.55	3-Aug-20	light	3
24	No	21-May-20	light	3	3-Aug-20	light	3
25	No	29-Jun-20	light	3	–	–	–
26	No	24-Jul-20	light	3	27-Aug-20	light	3
27	No	26-Oct-18	none	2.35	12-Jul-20	light	3
28	No	21-Feb-20	high	1.39	–	–	–
29	No	21-Feb-20	light	1.38	18-May-20	light	2.9
30	No	18-May-20	moderate	1.5	3-Aug-20	very high	0.25
31	No	10-Feb-20	light	1.17	–	–	–
32	No	10-Feb-20	light	1.45	18-May-20	very high	0.2
33	No	21-Feb-20	high	2.5	18-May-20	high	1.5
34	No	31-Aug-20	light	2.6	–	–	–
35	No	26-Oct-18	none	2.6	16-Jun-20	none	2.2
36	No	13-Apr-20	light	2.5	28-Jul-20	high	2.5
37	No	20-Jan-20	high	0.25	24-Aug-20	very high	1.5
38	No	9-Apr-20	moderate	2.7	28-Jul-20	light	2.9
39	No	9-Apr-20	moderate	3	–	–	–
40	No	18-Jun-19	high	2.48	9-Apr-20	high	2.6
41	Both	**7-Apr-20**	**light**	**2.55**	14-May-20	high	1.05
42	No	7-Apr-20	moderate	3	28-Jul-20	moderate	3
43	No	27-Jun-19	high	3	14-May-20	light	3
44	No	1-Jun-20	moderate	2.75	18-Aug-20	light	2.75
45	No	1-Jun-20	moderate	1.45	18-Aug-20	none	3
46	Both	18-Jun-19	none	3	**27-Oct-20**	**moderate**	**3**
47	No	20-Nov-18	high	3	18-Jun-19	moderate	3
48	No	2-Feb-19	none	3	3-Nov-20	none	3
49	No	12-May-20	none	3	13-Aug-20	light	3
50	No	12-May-20	light	2.5	–	–	–
51	No	13-Aug-20	light	3	–	–	–
52	T	25-Jun-19	high	1.9	17-Jul-20	high	3
53	No	27-Jul-20	light	2.75	25-Aug-20	light	1.45
54	No	12-Feb-20	none	3	17-Aug-20	high	3
55	No	12-Feb-20	none	3	17-Aug-20	light	3
56	No	12-May-20	light	3	21-Aug-20	none	2.9
57	No	12-May-20	none	3	21-Aug-20	light	3
58	No	23-Oct-19	light	3	21-Aug-20	none	3
59	No	23-Jun-20	light	3	13-Aug-20	light	3
60	No	23-Jun-20	none	3	–	–	–
61	No	12-Feb-20	light	3	24-May-21	light	3
62	No	26-Jun-20	moderate	3	30-Jul-20	light	3

(*continued on next page*)

Table 2 (*continued*)

Site #	Detection?	Visit 1 Date	Visit 1 Turbidity	Visit 1 L filtered	Visit 2 Date	Visit 2 Turbidity	Visit 2 L filtered
63	No	25-Jun-19	moderate	3	–	–	–
64	eDNA	**19-Jun-19**	**light**	**3**	–	–	–
65	No	26-Jun-20	high	2.2	30-Jul-20	light	3
66	No	26-Jun-20	very high	0.8	23-Aug-20	moderate	3
67	No	11-Oct-19	moderate	3	–	–	–
68	eDNA	26-Jun-20	high	3	**23-Aug-20**	**light**	**3**
69	No	25-Jun-19	moderate	1.65	2-Jun-20	moderate	3
70	Both	27-Jul-20	high	3	**2-May-21**	**high**	**3**
71	No	25-Jun-19	light	3	27-Jul-20	light	3
72	No	11-Sep-19	high	3	–	–	–
73	No	29-Oct-18	–	–	14-Aug-20	none	3
74	Both	2-Nov-18	none	3	**14-Aug-20**	**none**	**3**
75	No	14-Aug-20	light	3	–	–	–
76	No	25-Jan-20	–	0.18	–	–	–
77	No	20-Feb-20	light	3	–	–	–
78	T*	19-Nov-20	light	2.07	4-May-21*	none*	3*
79	T	21-Dec-20	moderate	0.87	–	–	–
80	T*	20-Feb-20	light	3	4-May-21*	light*	2.8*

each technical replicate that produced a band of the correct size was pooled and purified using Exo-CIP. Then, 5 μL of purified PCR product and 5 μL of the reverse nested primer were sent to Eurofins Genomics (Louisville, KY, USA) for Sanger sequencing. Sequences >95% identical to published *N. meridionalis* sequences when searched using NCBI Blast (blast.ncbi.nlm.nih.gov/blast.cgi) resulted in a positive species detection. If only one band of the correct size was produced after nested PCR, samples were re-run.

3. Results

3.1. eDNA sampling

The initial and nested primers were tested for sensitivity and amplified *Notophthalmus meridionalis* tissue DNA extract at a concentration of 0.1 pg/μL. In the sympatric species test, the primers were shown to be specific to the genus *Notophthalmus*, only amplifying DNA extracted from the congeneric Eastern Newt (*N. viridescens*) and no other sympatric amphibians (Supplemental Table 1). However, amplified DNA of *N. viridescens* was easily distinguishable from *N. meridionalis* when sent for Sanger sequencing, and the area of sympatry between *N. meridionalis* and *N. viridescens* is minimal, if present at all (Dixon, 2013). Therefore, the amplification of *N. viridescens* DNA from field samples is unlikely.

We detected *N. meridionalis* eDNA in samples from eight sites (Table 2; Fig. 2). These eight sites included: Live Oak County Park, pond SE of parking area (Site 6; Tables 1, 2); Powderhorn WMA, dugout pond near barn at S entrance (Site 11); East Foundation El Sauz Ranch, Newt Pond (Site 41); Grace Heritage Ranch, main pond (Site 46); Laguna Atascosa NWR, Kidney Pond (Site 64); Laguna Atascosa NWR, Prairie Trail #2 (Site 68); pond S of Port Isabel High School Tarpon Stadium (Site 70); and Palo Alto Battlefield National Historic Park, American Tank (Site 74). *Notophthalmus meridionalis* eDNA was only detected one time (of two visits) at each of these sites (Table 2). These detections included four sites in Cameron County (Sites 64, 68, 70, 74), two sites in Willacy County (Sites 41, 46), one site in Live Oak County (Site 6), and one site in Calhoun County (Site 11). Four of these sites are "recent" *N. meridionalis* localities (Sites 41, 46, 70, 74), and one site is a "historic" locality site, with the last detection in 1938 (Site 64). *Notophthalmus meridionalis* has never been observed at the other three sites (Sites 6, 11, 68). Three additional sites did not meet our criteria for a positive eDNA detection because samples from these sites produced just one band of the correct size and zero bands upon subsequent PCR: Powderhorn WMA, midline fence pond (Site 9); Sabal Palm Sanctuary, resaca blind (Site 78); and TNC Southmost Preserve, Black Willow Resaca (Site 80). However, at two of these three sites (Sites 78, 80), *N. meridionalis* was physically detected, and as a result, both sites are considered *N. meridionalis* positive. The third site (Site 9) should be considered a potentially positive site as it is ca. 4.2 km from a *N. meridionalis*-positive site (Site 11).

3.2. Traditional sampling

During this study, 21 *N. meridionalis* were found at eight sampling locations using traditional methodologies (Table 2; Fig. 2). Sites where we physically detected *N. meridionalis* include: East Foundation El Sauz Ranch, Newt Pond (Site 41; Tables 1, 2); Grace Heritage Ranch, main pond (Site 46); Brushline Road, ca. 1.1 rd km S jct TX Hwy 186 (Site 52); pond S of Port Isabel High School Tarpon Stadium (Site 70); Palo Alto Battlefield National Historic Park, American Tank (Site 74); Sabal Palm Sanctuary, resaca blind (Site 78); TNC Southmost Preserve, Siren Pond (Site 79); and TNC Southmost Preserve, Black Willow Resaca (Site 80). Individual *N. meridionalis* were detected once at each sampling site, except for Site 46 where two individuals were found on two separate dates. These detections included five sites in Cameron County (Sites 70, 74, 78–80), two sites in Willacy County (Sites 41, 46), and one site in Hidalgo County (Site 52). Of these eight sites, one represents a new specific record of occurrence (Site 79). The most common method in detecting *N. meridionalis* was searching beneath cover objects along the shoreline (n = 11), followed by dip-netting (n = 9), and finally,

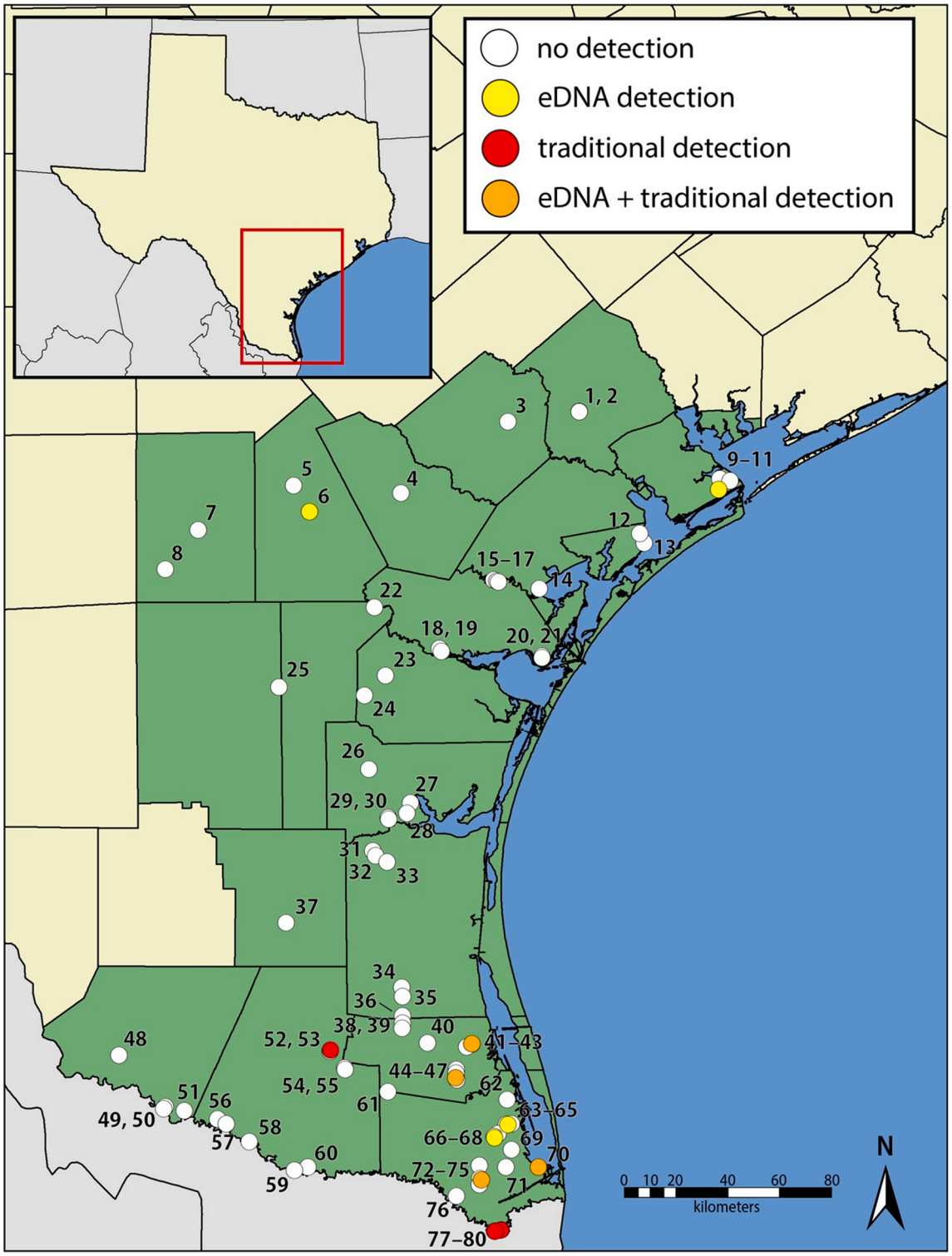

Fig. 2. Map of 80 sites sampled for Black-spotted Newts (*Notophthalmus meridionalis*) across south Texas, USA. Site numbers correspond to those listed in Table 1. Sampled counties (green shading), eDNA detections (yellow), traditional survey detections (red), and both eDNA and traditional survey detections (orange) are shown. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

capturing by hand in the water (n = 1).

3.3. Conditions for positive eDNA detections

eDNA detections of *N. meridionalis* were obtained from samples that were collected at various times throughout the year: August (n = 2), February (n = 1), April (n = 1), May (n = 1), June (n = 1), October (n = 1), and December (n = 1; Table 2). Out of our eight eDNA detections, two occurred 17–26 d following heavy precipitation (>10 cm; Brown et al., 2021) from Hurricane Hanna (Sites 68, 74; Table 1), five occurred within 8 d following moderate precipitation (2.5–7.6 cm; Sites 6, 11, 41, 64, 70), and one occurred with minimal measurable precipitation in the previous 30 d (<1.3 cm; Site 46). The water turbidity at the time of sample collection from seven of the eight sites with positive eDNA detections was classified as "none" or "light". At the remaining site (Site 70), the turbidity was recorded as "high"; however, a single *N. meridionalis* was observed in the water and the target volume of 3 L (in total) was filtered. At sites where higher levels of turbidity were recorded, the target volume was not often reached, which could have potentially hampered the ability to detect *N. meridionalis* eDNA. In addition, for samples from seven of the eight sites where positive eDNA detections were obtained (Sites 6, 11, 46, 64, 68, 70, 74), the target of 3 L was filtered. For the remaining site (Site 41), 2.55 L was filtered in total; however, eight individual *N. meridionalis* were observed in the water so the concentration of eDNA was likely relatively high.

4. Discussion

Overall, *Notophthalmus meridionalis* was detected at 12 sites across five counties (Fig. 2), and we detected *N. meridionalis* with both methodologies at four of these sites. Based on our results, a physical *N. meridionalis* detection did not always correspond with a positive eDNA detection and vice versa. We obtained several eDNA detections without detecting *N. meridionalis* through traditional methods, which have proved challenging in the past. In these instances, utilizing the eDNA assay likely expanded the detection window for this species. Additionally, the detections at sites where *N. meridionalis* had not previously been reported can serve to inform future sampling and potentially expand the known range of this species. Our results continue to support the efficacy of eDNA surveys to detect rare or cryptic amphibians (Goldberg et al., 2011; McKee et al., 2015; Brozio et al., 2017; Ruppert et al., 2022). The sites where *N. meridionalis* was observed and we failed to obtain a positive eDNA detection are confounding. In these instances, positive eDNA detections were more likely when *N. meridionalis* was captured in the water (2 of 3 sites) than when *N. meridionalis* was captured on the land, adjacent to the wetland (1 of 6 sites). The lack of detections when *N. meridionalis* was captured on the land could be due to the observed individuals not recently utilizing the aquatic habitat or that eDNA concentrations were below a detection threshold. Additionally, at two sites we were unable to collect an eDNA sample because the site had no water, and the individuals were observed beneath cover objects near the wetland basin. The discrepancy between traditional and eDNA detections underscores the importance of a dual approach as recommended by Thomsen et al. (2012) in order to minimize false negatives. Using only one methodology, our results would have produced eight (rather than 12) positive site detections. Considering the life history of *N. meridionalis*, future monitoring is recommended using both eDNA and traditional methodologies in order to maximize species detection.

We identified one new locality used by *N. meridionalis* through traditional methods (Site 79; Tables 1, 2) and three new localities through eDNA sampling (Sites 6, 11, 68). Most notable of these new localities are the positive eDNA detections obtained from Live Oak County Park, pond SE of parking area (Site 6), which is the first evidence of *N. meridionalis* occurring in Live Oak County since 1938 (Robinson et al., 2020), and Powderhorn WMA, dugout pond near barn at S entrance (Site 11) in Calhoun County, where *N. meridionalis* has never been reported. These detections represent the northernmost records of *N. meridionalis* in recent years, as all verifiable observations since 2000 are limited to the three southernmost counties in Texas (Cameron, Hidalgo, and Willacy). Given that eDNA analyses can occasionally give false-positive results (Darling and Mahon, 2011), future surveys (both traditional and eDNA) are needed to attempt to detect individuals at these locations as well as other nearby sites. Though we cannot completely rule out the possibility of false positives in eDNA detection, we observed no contamination in the field blanks nor NTCs, suggesting no contamination of samples in the field or the laboratory. Additionally, all three technical replicates for these northernmost sites resulted in a bright band of the appropriate size, and sequencing the PCR products from both sites resulted in a 100% match with published *N. meridionalis* mitochondrial sequences. The other sites that represent new records of occurrence are Laguna Atascosa NWR, Prairie Trail #2 (Site 68), which is ca. 7 km southwest of recent occurrence records of *N. meridionalis* at Laguna Atascosa NWR (Sites 64, 65), and TNC Southmost Preserve, Siren Pond (Site 79), which is ca. 0.4 km west of from another *N. meridionalis*-positive site (Site 80).

Notophthalmus meridionalis was not detected in 14 of the 19 counties sampled, including eight counties with historic records. Nearly half of the sites sampled in this study were in Cameron (n = 20), Hidalgo (n = 9), and Willacy (n = 10) counties. Cameron, Hidalgo, and Willacy counties were disproportionately sampled because *N. meridionalis* has been observed in these counties more recently (post-2000) and in greater abundance than other counties, which was revealed through our *N. meridionalis* occurrence database. Prior to 2000, there had been multiple confirmed observations of *N. meridionalis* from three additional counties: Kenedy, Kleberg, and San Patricio. Efforts were made to sample these counties thoroughly (Kenedy: n = 6 sites; Kleberg: n = 5; San Patricio: n = 7); however, we detected no *N. meridionalis* in any of these three counties, including at known ("historic") sites where previous collections were made. All other counties with historic *N. meridionalis* records are represented by either a single specimen (Aransas, McMullen, Nueces, and Victoria) or a single collection event (Duval, Live Oak, and Refugio). The other counties sampled (Starr, Brooks, Jim Wells, Bee, Goliad, and Calhoun) had no prior verifiable occurrence records of *N. meridionalis* but were along the periphery of their historic range in Texas and sampled in hope of detecting additional populations. The counties with minimal or no *N. meridionalis* observations were not sampled as thoroughly, as there was a lack of information available when identifying suitable sampling sites. Sampling in Starr County (n = 4 sites) was an exception. Starr County has been included in *N. meridionalis* range maps (e.g., Dixon, 2000, 2013), likely based on a

record published by Boundy (1994). However, the locality reported by Boundy (1994) for those Starr County specimens (Walker Lake) is in Hidalgo County. Additionally, there is an anecdotal report of *N. meridionalis* from southeastern Starr County (Irwin, 1993). Due to the lack of verifiable occurrence records, current distribution maps should exclude Starr County as part of the current recognized range of *N. meridionalis* but given the proximity to historic records in Hidalgo County, future survey efforts should continue in the region, particularly along the Rio Grande, in attempt to detect individuals. Future surveys should also focus on Kenedy, Kleberg, and San Patricio counties given the number of historic records and specific localities (e.g., Welder Wildlife Refuge, Big Lake [Site 17]; TAMU-Kingsville CKWRI, South Pasture pond [Site 26]) reported from these counties, as well as Live Oak and Calhoun counties in order to confirm *N. meridionalis* presence through the physical detection of individuals. Additionally, samples from Powderhorn WMA, midline fence pond (Site 9), produced one band of the correct size after nested PCR and should be investigated further considering the proximity to another site on Powderhorn WMA where *N. meridionalis* eDNA was detected (Site 11).

Rappole and Klicka (1991) provided the most comprehensive report on *N. meridionalis* distribution and using their results and personal communication from other biologists (e.g., A. Chaney, F. Judd, S. Labuda) they identified seven "metapopulation centers" in south Texas. Among these, we obtained a positive eDNA detection from two sites (Sites 64, 68) within the "Laguna Atascosa National Wildlife Refuge" metapopulation center. A positive eDNA detection and physical detection were obtained from Sabal Palm Sanctuary (Site 78) within the "Matamoros, Mexico–Brownsville" metapopulation center. Additionally, positive eDNA and traditional detections from TNC Southmost Preserve (Sites 79, 80) would likely fall within this metapopulation center as they are separated by <3 km. Our results indicate that breeding populations of *N. meridionalis* have persisted within these two areas for >30 yr. Possibly contributing to the persistence of this species is the conservation focus of the organizations that operate these sites. The remaining metapopulation centers were in Kleberg ($n = 4$) and Kenedy ($n = 1$) counties, where we obtained no positive *N. meridionalis* detections. These include "Vattmannville, TX", "TAMU-Kingsville CKWRI, South Pasture", "Riviera, TX", "US Hwy 77, 14.7–21.7 mi S of Armstrong, TX", and "FM 772, 1 mi S jct 628." Excluding "TAMU-Kingsville CKWRI, South Pasture", these sites are not operated by conservation-focused groups (roadside ditches and private property). Most notable, Rappole and Klicka (1991) reported that during their study, root-plowing occurred on private property within one of the "Vattmannville, TX" sites that caused "sure death to newts". During our study, many ponds and ditches along "US Hwy 77, 14.7–21.7 mi. S of Armstrong, TX" have been impacted by construction activities, which have caused erosion and siltation at two sampling sites (US Hwy 77, W side, ca. 1.0 rd km N Kenedy/Willacy county line [Site 36]; US Hwy 77, E side, ca. 1.6 rd km N jct La Chata gate #4 [Site 38]). Further, dirt roads running parallel to US Hwy 77 along this stretch of highway in Kenedy County are frequented by U.S. Customs and Border Protection (CBP) vehicles. These vehicles drag tires behind them to monitor foot traffic from illegal immigrants. Tire-dragging and the utilization of these dirt roads could serve as a direct threat to *N. meridionalis* and other wildlife along US Hwy 77. Whether any *N. meridionalis* populations remain at these other metapopulation centers identified by Rappole and Klicka (1991) should continue to be investigated.

The information on the conditions for positive eDNA detections from this study can be used to plan future eDNA sampling for this species and others with similar life histories. Generally, *N. meridionalis* reproduction and activity in the water are tied to rainfall events, which would provide the conditions for a positive eDNA detection (Buxton et al., 2017). Based on our results, eDNA sampling should occur within a few days of light or moderate precipitation events (ca. 1.3–7.6 cm) that cause wetlands to retain a small volume of water. However, for heavy precipitation from hurricanes or tropical storms, the best practice may be to wait for at least 14 d before eDNA sampling, as the target eDNA may be too dilute if immediately sampled. If rainfall events trigger reproduction, waiting at least 14 d may allow eDNA shed from reproductive events, eggs, and larvae to accumulate in high-volume sites. However, high temperatures typically observed during the tropical storm season and their effects on eDNA persistence must be considered (Strickler et al., 2015). Increased flow and runoff following heavy rainfall events can dilute eDNA concentrations (Curtis et al., 2021) as well as increase organic matter and suspended sediments, making eDNA detections more difficult to obtain (Buxton et al., 2017; Yaegashi et al., 2020). The water bodies that *N. meridionalis* occupy can be highly turbid, and sampling immediately following heavy rainfall events likely decreases the amount of water that can be filtered due to increased suspended sediment. Filtering larger amounts of water was necessary for our study as $\geq$85% of the target volume (3 L) was filtered in all eight of our positive eDNA detections. The filter pore size (25–30 μm) used in this study was larger than what is typically used in eDNA studies (Minamoto et al., 2016; Rourke et al., 2021) in order to maximize the volume filtered from the turbid water bodies in south Texas. Filtering more water can increase the amount of eDNA captured; however, smaller fragments of eDNA may not be captured with large filter sizes (Eichmiller et al., 2016). Ruppert et al. (2022) obtained positive detections in 98.2% of samples taken from known Lesser Siren (*Siren intermedia*) ponds using filters with 25–30 μm pores, lending support to the use of large pore sizes in eDNA assays. Future studies should continue to investigate the use of larger filter pore sizes considering the success of this assay, particularly in habitats with turbid waters.

5. Conclusion

The development and implementation of this assay was successful at detecting *Notophthalmus meridionalis* eDNA from field samples. In total, eDNA samples from 80 sites were analyzed, producing eight positive detections (10%). While not a direct comparison, these results are an improvement from previous efforts where Rappole and Klicka (1991) located *N. meridionalis* at ca. 6% of sites surveyed and Judd (1985) found *N. meridionalis* at ca. 1% sites surveyed. Using traditional methodologies, we found *N. meridionalis* at eight sites (10%) including four in which we also obtained positive eDNA detections. Altogether, *N. meridionalis* was detected at 12 of 80 unique localities (15%) spanning five counties across south Texas, USA. Five new *N. meridionalis* localities were reported as part of this study (eDNA: $n = 4$; traditional methods: $n = 1$), including one in Live Oak County, which is the first *N. meridionalis* detection in the county since 1938 and one in Calhoun County where there have been no previous confirmed *N. meridionalis* records. Accurately locating populations of *N. meridionalis* is essential to better understand the current distribution of this species across south Texas, especially

considering management and conservation decisions. The results of this study show the efficacy of this eDNA assay in detecting *N. meridionalis* across the landscape under various situations and environmental conditions, and this eDNA assay, in conjunction with traditional methodologies, can be successfully applied to monitor known populations as well to detect *N. meridionalis* at new localities. With proper timing of sampling, this eDNA assay holds the potential to help fill in the current knowledge gaps in *N. meridionalis* distribution throughout south Texas.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.gecco.2022.e02131.

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