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Examination of Amphibian Community and Environmental Relationships in South Texas Using Environmental DNA (eDNA)

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EXAMINATION OF AMPHIBIAN COMMUNITY AND ENVIRONMENTAL RELATIONSHIPS USING ENVIRONMENTAL DNA (eDNA)

A Thesis

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ABSTRACT

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Developing more efficient tools to assess amphibian biodiversity and understanding what environmental variables drive amphibian biodiversity are top priorities, as amphibians are facing extinction events across the globe. Environmental DNA (eDNA) surveys are a promising new tool to asses amphibian biodiversity. Throughout the study eDNA metabarcoding along with a targeted eDNA assay and traditional survey methods were used to provide information on amphibian community assemblages throughout South Texas. Water quality, habitat characteristics and soil composition data were collected and used to examine environmental relationships. eDNA metabarcoding detected significantly more amphibian taxonomic units compared to traditional survey methods. eDNA metabarcoding was less sensitive at detecting DNA from a rare-cryptic amphibian compared to a targeted eDNA assay. There were no significant groupings of amphibian communities, yet some environmental variables were found to be significantly correlated to amphibian community structure.

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CHAPTER I

VALIDATION OF ENVIRONMENTAL DNA (eDNA) METABARCODING ASSAY

Introduction

With the worldwide decline of amphibian populations, the need for characterizing amphibian community assemblages grows ever more important (Stuart et al. 2004, Wake and Vrendenberg 2008, Pimm et al. 2014). There have been mass amphibian extinction events across the globe driven by emerging infectious diseases, such as chytrid fungus and ranavirus (Wake and Vredenburg 2008, Barnosky et al. 2011, McLellan et al. 2014, Price et al. 2014). Amphibian species are also threatened by contamination from xenobiotics, habitat fragmentation and global climate change (Stuart et al. 2004, Wake and Vrendenberg 2008). Although a number of studies have examined community assemblages in the Amazon rainforest and other high diversity areas, there is a lack of information on the current amphibian community structure for many areas in the world (Thomsen et al. 2012, Junior and Rocha 2013, Hortal et al. 2015, Ficetola et al. 2019 Tavares-Junior et al. 2020). Without knowledge of current community assemblages there is no way to know what species have been extirpated or moved to more hospitable environments (Mazerolle et al. 2007, Thomsen et al. 2012, Hortal et al. 2015, Ficetola et al. 2019). The biphasic lifestyle, and potentially cryptic behavior, of most amphibians provides a challenge for characterizing community assemblages (Valentini et al. 2016, , Sasso et al. 2017,

Hauck et al. 2019). In addition, monitoring strategies need to consider the specific habitat and structural requirements of the target species as suggested by Goldberg et al. (2016). Therefore, the most effective tools for monitoring amphibian community assemblages needs to be determined.

Monitoring amphibian communities can be accomplished in multiple ways including a new promising technology, environmental DNA (eDNA). Traditional survey methods, such as audio strip transect surveys (Sasso et al. 2017), visual encounter surveys (Heyer et al. 2014) seining ponds (Judd 1985, Rappole and Klicka 1991) and pitfall traps (Heyer et al. 2014), have been used to detect amphibian species and characterize communities for decades. Traditional survey methods can be time and labor intensive as well as invasive to local habitats, as they require researchers to disturb habitats (Mazerolle et al. 2007, Goldberg et al. 2016). Previous studies (Bailey et al. 2004, Mazerolle et al. 2007, Heyer et al. 2014) have found that these traditional survey methods have potential bias towards more abundant and less cryptic species that may result in an incomplete understanding of the community assemblages. eDNA surveys rely on DNA that is shed from organisms during skin and mucous shedding, release of gametes and deposition of waste (Ficetola et al. 2008, Dejean et al. 2012, Valentini et al. 2016, Sasso et al. 2017, Ficetola et al. 2019) eDNA surveys can be conducted in a variety of mediums such as water (Ficetola et al. 2008), sediment (Parducci et al. 2013) and even fecal matter (Roffler et al. 2021, Tournayre et al. 2021). Aquatic eDNA surveys are common for amphibians in part because their DNA can disperse throughout the water column during reproduction and juvenile maturation (Dejean et al. 2012, Valentini et al. 2016, Sasso et al. 2017, Ficetola et al. 2019). Previous studies have shown that eDNA surveys by themselves are as or more sensitive at detecting species present in a water body, especially for cryptic taxa, when compared to

traditional survey methods (Valentini et al. 2016, Lacoursière-Roussel et al. 2016, Sasso et al. 2017, Robinson et al. 2022). eDNA surveys can be less invasive compared to traditional survey methods as eDNA surveys do not require researchers to disrupt habitat (Valenitni et al. 2016, Lacoursière-Roussel et al. 2016, Sasso et al. 2017) and can help reduce the chance of the unintended spread of pathogens from pond to pond (Phillott et al. 2010). In addition, eDNA surveys do not require the handling of organisms and less survey equipment is introduced into the water body. The increased detection capabilities of eDNA assays can provide more accurate occupancy details, univariate community metrics such as species richness and broad scale differences determined by multi-variate tests (Valentini et al. 2016, Lacoursière-Roussel et al. 2016, Gold et al. 2021, Wikston 2021). For example, Valentini et al. (2016) and Lacoursière-Roussel et al. (2016) found that eDNA surveys detected more species compared to traditional survey methods; the extra species detections from eDNA surveys resulted in more accurate occupancy data compared to the occupancy data from traditional survey methods.

Presently, there are two popular eDNA survey strategies; targeted assays (Ruppert et al. 2022, Robinson et al. 2022) and metabarcoding assays (Lacoursière-Roussel et al. 2016, Valentini et al. 2016). Targeted assays are designed to specifically detect a single species and only that species. Targeted assays can be useful for early detection and monitoring of invasive species as well as monitoring the presence of rare or cryptic species (Ficetola et al. 2008, Dejean et al. 2012, Voros et al. 2017). Targeted assays generally rely on qPCR or PCR and Sanger sequencing (Harper et al. 2018, Ruppert et al. 2022, Robinson et al. 2022). Metabarcoding assays are designed to detect groups of related taxa, such as all amphibians or even all vertebrates, and thereby are useful for examining community assemblages (Valentini et al. 2016, Sasso et al. 2017, Evans et al. 2017, Ruppert et al. 2019, Harper et al. 2020). Metabarcoding assays generally

rely on PCR and next-generation sequencing (NGS) (Valentini et al. 2016, Sasso et al. 2017, Evans et al. 2017, Ruppert et al. 2019, Harper et al. 2020). NGS is required for metabarcoding assays because qPCR and PCR and Sanger sequencing are designed around detecting one-specific sequence and metabarcoding assays produce a variety of unique sequences that can all be read individually using a next-generation sequencing platform (Valentini et al. 2016, Sasso et al. 2017, Evans et al. 2017, Ruppert et al. 2019)

Multiple factors can influence the sensitivity and confidence in the detections from eDNA surveys. For example, lab personnel can unintentionally transport DNA into a sample contaminating the sample and causing false-positives. (Roussel et al. 2015, Goldberg et al. 2016). DNA can also be introduced from the flow between bodies of water, contamination from field personnel and from the fecal matter of a visiting predator which could also provide a false positive (Roussel et al. 2015, Goldberg et al. 2016). Other factors that can influence detection probabilities in aquatic eDNA surveys include the timing of the survey as it relates to the activity of the species (Pilliod et al. 2014, Goldberg et al. 2016), water chemistry (Pilliod et al. 2014,Sasso et al. 2017) and UV intensity (Thomsen et al. 2012, Pilliod et al. 2014). There is also no current rule on positive detection limits of eDNA assays, with many studies having different thresholds for a positive detection (Harper et al. 2018, Bylemans et al. 2019, Robinson et al. 2022). Some previous studies (Harper et al. 2018, Bylemans et al. 2019) have had more than one threshold for their positive results, to help better convey which detections are more trustworthy and which detections require more sampling to be confirmed. Because of the false positive potential and the unclear positive detection limits, a positive result in an eDNA survey does not necessarily have the same weight as capturing a specimen at the same site (Roussel et al. 2015, Goldberg et al. 2016). To increase the confidence of positive eDNA results, negative field and

lab controls are used to examine contamination so that there is less uncertainty surrounding an eDNA result (Roussel et al. 2015, Goldberg et al. 2016). As with traditional survey methods, a negative eDNA result does not necessarily mean that a species is not present at a site (Roussel et al. 2015, Goldberg et al. 2016). Because of the factors that can influence eDNA assays some authors suggest that eDNA surveys need to be paired with traditional survey methods to have the best survey potential (Roussel et al. 2015, Goldberg et al. 2016, Yates et al. 2020).

There are limitations of both targeted and metabarcoding eDNA surveys; in most cases both surveys provide occurrence data rather than abundance, as there are some factors that can influence the estimation of biomass from eDNA surveys. Some previous studies have observed strong correlations of eDNA concentrations with actual species abundance (Thomsen et al. 2012, Takahara et al. 2012, Pilliod et al. 2013, Lacoursière-Roussel et al. 2016, Yates et al. 2021, Massey et al. 2021), but other studies observed weak correlations between eDNA concentrations and abundance (Bell et al. 2019) or provided evidence that complicates the correlation between population abundance and observed eDNA concentration (Fonseca 2018, Nichols et al. 2018, Beng and Corlett 2020). For example, metabarcoding primers can have a bias for certain species based on primer affinity and polymerases found in PCR master mixes can have an amplification bias towards certain sequence GC percentages, both of which can combine to skewed DNA concentrations observed among different species and thus inaccurate estimations of abundance (Fonseca 2018, Nichols et al. 2018, Beng and Corlett 2020). Inaccurate estimations of biomass could then lead to inaccurate interpretations of community metrics and misleading analyses as posited by Fonseca (2018), Nichols et al. (2018) and Beng and Corlett (2020).

South Texas is an area that could benefit from the application of an amphibian metabarcoding assay because the area has high amphibian diversity and multiple state and

federally protected species (Dixon 2013, Petersen et al. 2018, Duran 2021). South Texas is also under rapid industrial and domestic development; changing current habitats and causing population declines through increased habitat fragmentation, contamination from xenobiotics and road mortality (Garcia et al. 2001). The chosen study area of this thesis ranges from Cameron County to the northeast as far as Calhoun County, with the western limit being in the Starr County area (Fig. 1, Table 1). Traditional survey methods and targeted eDNA assays have been previously used within the study area to provide occurrence information for a few species of conservation concern, such as the Black-spotted newt (*Notophthalmus meridionalis*) (Cope 1880) (Judd 1985, Rappole and Klicka 1991, Bare 2018, Duran 2021, Robinson et al. 2022) and the Lesser Siren (*Siren intermedia texana*) Goin, 1957 (Judd 1985, Duran 2021, Ruppert et al. 2022). Yet, there is little information about amphibian community assemblages across the study area, so there is a need for more information. Although eDNA metabarcoding has not been used within the study area to characterize amphibian communities, it holds promise to provide new information at a variety of wetland sites. Providing information on amphibian communities throughout the study area and determining effective tools for characterizing amphibian communities will aid our understanding of amphibian community relationships across South Texas.

The study area within South Texas also is home to a variety of understudied and cryptic amphibians that have been difficult to detect using traditional survey methods. One such species is the Black-spotted newt (*Notophthalmus meridionalis*), which is a rare small-bodied salamander that spends a large amount of time underground and comes above ground to breed in water bodies (Mecham 1968, Judd 1985, Bare and Kline 2017). There have only been a few recent studies examining Black-spotted newt distribution (Judd 1985, Rappole and Klicka 1991,

Bare 2018, Robinson et al. 2022), which historically starts in South Texas and goes south into northeastern Mexico (Mecham 1968, Judd 1985, Rappole and Klicka 1991). Targeted eDNA assays have been successful at detecting Black-spotted newts (Bare 2018, Robinson et al. 2022), allowing for an updated distribution, with new detections in Calhoun County and the first detection in Live Oak County since the 1930s (Robinson et al. 2022). In contrast, eDNA metabarcoding has never been used to detect Black-spotted newts despite their potential to be an ideal species to test the sensitivity of eDNA metabarcoding for rare and cryptic species in comparison to targeted assays. Previous literature suggests that targeted eDNA assays offer increased detections for rare small-bodied organisms (Harper et al. 2018, Bylemans et al. 2019) compared to eDNA metabarcoding assays that have had varying levels of success (Klymus et al. 2017, Nester et al. 2020, Rojhan et al. 2021, Gold et al. 2021). Although, eDNA metabarcoding generally offers fewer detections than targeted eDNA assays (Harper et al. 2018, Bylemans et al. 2019, Nester et al. 2020), eDNA metabarcoding is still capable of detecting DNA from cryptic organisms (Harper et al. 2018, Bylemans et al. 2019). With continued optimization of eDNA metabarcoding assays, they could become a very useful tool in the future for detecting and monitoring rare small-bodied organisms (Jeunen et al. 2018, Bylemans et al. 2019, Schenekar et al. 2020).

The goals of this study were (1) to validate an eDNA metabarcoding assay on South Texas amphibian tissue samples, (2) to test the eDNA metabarcoding assay on field eDNA samples, (3) to provide information on amphibian community assemblages throughout the study area, (4) to determine if eDNA metabarcoding on its own can detect more taxonomic units than traditional survey methods and (5) to compare detections of Black-spotted newts using eDNA metabarcoding, a targeted eDNA assay and traditional survey methods to determine which tool is

most effective. The hypothesis for goal four is that eDNA metabarcoding will detect more taxonomic units than traditional survey methods. The hypothesis for goal five is that the targeted eDNA assay would be the most effective tool for detecting black-spotted newts.

Methods

Map of Study Area

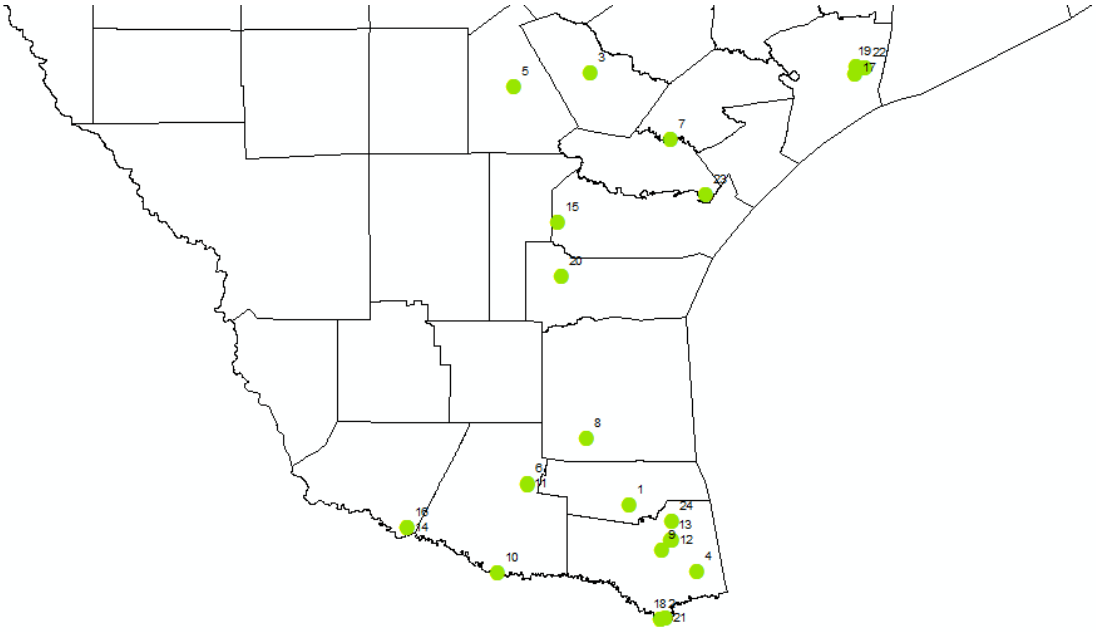

Figure 1. Map of study area. Green points show sites surveyed with corresponding site number found in Table 1.

Study Sites

Sites sampled consisted of 24 unique water bodies (Fig. 1, Table 1). Sites were sampled generally following rain events from 2020-2021. Sites were chosen based on being previously identified as potential, Black-spotted newt habitat by Bare (2018) and Robinson et al. (2022). See Fig. 2 for representative pictures of sites

Table 1. Information on sites sampled. Site number. county, site name, latitude and longitude, number of samples tested using eDNA metabarcoding and type of water body of sites.

Site Number	County	Site Name	Latitude	Longitude	Number of Samples	Date(s)	Eph/Perm
1	Willacy	Grace Heritage Ranch Main	26.38820	-97.56434	1	10/27/2020	Eph
2	Cameron	Sabal Palm Sanctuary Resaca Blind	25.85050	-97.41920	1	11/9/2020	Eph
3	Bee	Coastal Bend Community College	28.43551	-97.75385	1	10/4/2020	Eph
4	Cameron	PIHS Pond	26.07351	-97.24717	2	9/2/20, 5/2/21	Eph
5	Live Oak	Live Oak County Park	28.37257	-98.11606	2	12/3/20, 6/30/21	Eph
6	Hidalgo	Brushline North	26.49018	-98.05029	2	7/27/20, 6/7/21	Eph
7	San Patricio	WWR Big Lake	28.12055	-97.37287	4	1/29/20, 5/7/21 ,6/11/21, 7/1/21	Eph
8	Kennedy	HWY 77 GPZ	26.70689	-97.76932	2	8/31/20, 6/8/21	Eph
9	Cameron	LANWR Priarie trail #2	26.17723	-97.41514	1	8/23/2020	Eph
10	Starr	USFWS Kelly Unit Resaca	26.07055	-98.19347	1	8/13/2020	Eph
11	Hidalgo	Brushline South	26.48554	-98.05111	1	6/7/2021	Eph
12	Cameron	LANWR Kidney Pond	26.22306	-97.36250	1	5/26/2021	Perm

Table 1, cont. Information on sites sampled Site number. County, site name, latitude and longitude, number of samples tested using eDNA metabarcoding and type of water body of sites.

Site Number	County	Site Name	Latitude	Longitude	Number of Samples	Date(s)	Eph/Perm
13	Cameron	LANWR Scum Pond	26.22361	-97.36909	1	5/26/2021	Eph
14	Starr	Old Military Hwy Pond 4	26.2804	-98.61979	1	6/24/2021	Eph
15	Nueces	Pintas Creek	27.7295	-97.90458	1	6/8/2021	Eph
16	Starr	Old Military Hwy Pond 5	26.2801	-98.61991	1	6/9/2021	Eph
17	Calhoun	PHR Haybarn	28.4307	-96.49587	3	5/6/21, 6/10/21, 6/30/21	Eph
18	Cameron	Sabal Palm Sanctuary Side Pond	25.849	-97.41911	1	5/4/2021	Eph
19	Calhoun	PHR Midline Fence	28.4674	-96.48993	3	5/6/21, 6/10/21, 6/30/21	Eph
20	Kleberg	CKWRI South Pasture	27.4709	-97.89059	2	5/28/21, 6/8/21	Eph
21	Cameron	Southmost Black Willow Resaca	25.8548	-97.39424	1	5/4/2021	Eph
22	Calhoun	PHR Bullrush	28.4598	-96.44999	1	2/27/2020	Perm
23	Live Oak	Live Oak Pipeline Pond	27.8584	-97.20405	1	5/7/2021	Eph
24	Cameron	LANWR Newt Pond	26.3106	-97.36507	1	5/26/2021	Eph

Examples of Sites

Figure 2. Example of sites sampled. Top left is Southmost Black Willow Resaca (Site 21). Top right is Live Oak County Park (Site 5). Bottom left is PHR Haybarn (Site 17). Bottom right is Brushline North (Site 6).

Amphibian Species Native to South Texas

According to a review of the literature (Dixon 2013, Petersen et al. 2018, Duran 2021, Robinson et al. 2022) 33 amphibian species have possible distributions in the study area. Tissue samples for these amphibian species were gathered from museum specimens, collected within the study area when possible, or from specimens as close as possible to the study area, and tissue

extracts of each species were tested to validate the eDNA metabarcoding assay (Table 2). Taxonomic keys found in Dixon 2013 were used for identification.

Table 2. Scientific names, associated common names and taxonomic authorities for amphibians native to the chosen study area within South Texas

Species Name	Common Name	Taxonomic Authority	Specimen ID
Anaxyrus speciosus	Texas toad	Girard, 1854	DRD 5120
Scaphiopus couchii	Couch's spadefoot	Baird, 1854	DRD 5021
Rhinella marinus	Marine toad	(Wiegmann, 1833)	DRD 5405
Leptodactylus fragilis	Mexican white-lipped frog	(Brocchi, 1877)	DRD 6000
Gastrophryne olivacea	Western narrow-mouthed toad	(Hallowell, 1856)	DRD 5171
Incilius nebulifer	Gulf Coast toad	Girard, 1854)	DRD 7265
Hypopachus variolosus	Sheep frog	(Cope, 1866)	DRD 7255
Ambystoma mavortium	Barred tiger salamander	Baird, 1850	DRD 5176
Smilisca baudinii	Mexican treefrog	Duméril & Bibron, 1840	DRD 5801
Lithobates berlandieri	Rio Grande leopard frog	(Baird, 1859)	DRD 5122
Lithobates catesbeianus	Bullfrog	(Shaw, 1802)	DRD 5921
Rhinophrynus dorsalis	Mexican burrowing toad	Dumeril & Bibron, 1841	DRD 5990

Table 2, cont. Scientific names, associated common names and taxonomic authorities for amphibians native to the chosen study area within South Texas

Species Name	Common Name	Taxonomic Authority	Specimen ID
Siren intermedia texana	Rio Grande Siren	Goin, 1957	DRD 6034
Pseudacris clarkii	Spotted chorus frog	(Baird, 1854)	DRD 6019/8975
Notophthalmus viridescens	Eastern Newt	Rafinesque, 1820	DRD 5251
Acris blanchardi	Blanchard's cricket frog	Harper, 1947	DRD 5396
Scaphiopus hurterii	Hurter's spadefoot	Strecker, 1910	DRD 5493
Dryophytes chrysoscelis	Cope's gray treefrog	Cope, 1880	DRD 5553
Eleutherodactylus cystignathoides	Rio Grande chirping frog	Cope, 1877	DRD 5653
Gastrophryne carolinensis	Eastern narrow-mouthed toad	Holbrook, 1835	DRD 5920
Dryophytes squirellus	Squirrel treefrog	(Daudin, 1800)	DRD 5922
Dryophytes cinereus	Green treefrog	Schneider, 1799	DRD 5941
Lithobates sphenocephalus	Southern leopard frog	(Cope, 1886)	DRD 5942
Anaxyrus punctatus	Red-spotted toad	(Baird & Girard, 1852)	DRD 5967
Anaxyrus debilis	Green toad	(Girard, 1854)	TNHC 15368
Anaxyrus woodhousii	Woodhouse's toad	(Girard, 1854)	DRD 9500/ TNHC 55521
Spea bombifrons	Plains spadefoot	(Cope, 1863)	TNHC 26148
Dryophytes versicolor	Gray treefrog	(LeConte, 1825)	TNHC 26647

Table 2, cont. Scientific names, associated common names and taxonomic authorities for amphibians native to the chosen study area within South Texas

Species Name	Common Name	Taxonomic Authority	Specimen ID
Pseudacris streckeri	Strecker's chorus frog	Wright & Wright, 1933	TNHC 27211
Pseudacris fouquettei	Cajun chorus frog	Lemmon, Lemmon, Collins & Cannatella, 2008	TNHC 21090
Ambystoma texanum	Small-mouthed salamander	(Matthes, 1855)	DRD 5253
Lithobates areolatus	Crawfish frog	(Baird & Girard, 1852)	TNHC 14318
Notophthalmus meridionalis	Black-spotted Newt	(Cope, 1880)	DRD 5165

Tissue DNA Extraction

Approximately 30 mg of tissue was used for each tissue extraction, which was conducted using an EPOCH Life Sciences GenCatch Genomic DNA extraction kit following the manufacturers "tissue" protocol (cat #:1460250, GenCatch Blood & Tissue Genomic Mini-Prep Kit, Epoch Life Sciences, Missouri City, TX, USA) . The extracted DNA was then stored at -20°C.

Field Sample Collection

Field sample collection followed previous protocols from Ruppert et al. (2022) and Robinson et al. (2022). In summary, water was collected from 3-4 locations from the banks of a water body at a site to form a composite sample of the entire site. Once the composite sample was collected, totaling approximately 5L, it was brought back to the filtering equipment. To prevent contamination, all the equipment required for sample collection and water filtration was

sterilized with 50% bleach, was allowed to sit for 1 min before it was sprayed with 10% sodium thiosulfate and was rinsed with DI water to neutralize and remove the bleach. Water filtration equipment included a 250 ml filter holder (cat # XX1104700, MilliporeSigma, Darmstadt, Germany) connected to a fluid evacuator (cat # MV7400, Mityvac, St. Louis, MO, USA) that pulled water through the filter and a 1 L plastic liter container used for pouring the samples. Filters used for field sampling were 47-mm diameter Grade 4 (25-30µm pore size) Whatman cellulose (cat# 1004047, GE Healthcare, Chicago, IL, USA).

Prior to filtering the field eDNA sample for each site, 1 L of DI water was filtered as a field negative control to test if the field equipment was sterile. Following the field negative control, three 1 L field sample replicates from the composite water sample were filtered through the same apparatus. The field negative controls and field samples were each stored in a individual labeled 1.5mL snap top vial with 700µL of DNAzol (Molecular Research Center Inc, Cincinnati, OH, USA). Fresh gloves were used for each sample.

Field Visual Encounter Survey

A visual encounter survey was conducted throughout each site following the collection of the eDNA sample. Amphibians, reptiles and fish found during the visual encounter survey were noted and identified to the lowest taxonomic level possible. Methods from previous studies were selected to create the visual encounter survey (Judd 1985, Rappole and Klicka 1991, Mazerolle et al. 2007, Heyer et al. 2014). Field visual encounter surveys were conducted in a circular fashion around the water body and lasted 30 min or less depending on the size of the water body. Field surveys consisted of 2-3 crew members conducting dipnet surveys and visual inspection of the undersides of hard substrates. A dipnet survey conducted along the perimeter of the water body was done first. Dipnets were dragged along the substrate of the water body and checked for

amphibians every 4-5 steps and then the contents of the dipnet were then emptied before proceeding. Following the dipnet survey, any rocks, logs and cover boards around the water body were also flipped and inspected for amphibians.

Extraction of Field Samples

Field samples and field negative controls were extracted using an EPOCH Life Sciences Genomic DNA GenCatch Prep Extraction kit (Epoch Life Sciences, Missouri City, TX, USA) with a modified protocol from Robinson et al. (2022). Field negative controls were extracted and processed separately from field samples to avoid contamination. Following sample collection, the filter was left in DNAzol at room temperature for at least 3 d. After those 3 d, the centrifuge tubes containing the used filters and DNAzol were placed on a heat block at 55°C for 30 min. Then each centrifuge tube was vortexed for 30 s and centrifuged at 2400 rcf for 1 min. The filters were then squeezed into their centrifuge tube with clean forceps to collect any remaining DNAzol out of the filter prior to discarding the filters; new gloves were used for each sample during this step to prevent contamination and false positives between sites. DNAzol from each field sample was pooled and combined into a 5 mL centrifuge tube. Then 600 µL of DNAzol was pipetted out of the pooled field sample and placed into a fresh centrifuge tube; the remaining DNAzol from the pooled field sample was stored at room temperature. To the 600 µL of DNAzol, 10 µL of RNAase A was added, and the solution was incubated at 37°C for 10 min. After the solution sat at room temperature for 1 min, 10 µL of proteinase-K was added. . The solution was then vortexed and incubated at room temperature for 1 h, vortexing every 10-15 min during the incubation. After incubation, 500 µL of EX buffer was added and the solution was vortexed and incubated at 70°C for 20 min. During that incubation, 50 µL of sample elution buffer (EB) was preheated to 70°C. Following the 20 min incubation, the solution sat at room

temperature for 5 min. Then 500 µL of 100% ETOH was added to the solution and the solution was vortexed prior to the solution being passed through a GenCatch column and centrifuged at 5800 rcf for 2 min. This centrifugation step was repeated 2-3 times more until the entire volume of the solution passed through the GenCatch column. The column was rinsed by the addition of 500 µL of WS buffer, centrifugation at 5800 rcf for 2 min, addition of 500 µL WS buffer, and centrifugation for 2 min at 18000 rcf. The column was then moved into a new 1.5 mL snap cap tube and 50 µL of heated sample elution buffer was passed through the column. The DNA was then eluted by centrifuging the solution at 18000 rcf for 2 min, after which 50 µL of nuclease-free H_2O was pipetted onto the column, and any remaining DNA was eluted by centrifuging the solution at 18000 rcf for 2 min. A ZYMO One-Step PCR inhibitor removal kit or a Nuclease Spin inhibitor removal kit was then used following the respective manufacturer's protocol. The eluted DNA was stored at -20°C.

eDNA Metabarcoding Primer Validation

The Batrachia primer set (Table 3) developed by Valentini et al. (2016), that amplifies a portion of the 12s rRNA region, was tested against tissue samples of amphibians native to the study area (Table 2). PCR was conducted using a T100 ThermoCycler (Bio-Rad Laboratories, Hercules, CA, USA). Temperature gradients were used to determine annealing temperatures for PCR protocols. Two PCR protocols were validated; the 'original' PCR protocol: 2 min at 95°C, then 40 cycles of 20 s at 95°C, 30 s at 55°C and 30 s at 72°C followed by 7 min at 72°C and a 4 min hold at 4°C. The other PCR protocol was the 'optimized' PCR protocol: 2 min at 95°C, then 35 cycles of 20 s at 95°C, 20 s at 50°C and 10 s at 72°C followed by 7 min at 72°C and a 4 min hold at 4°C. Tissue extracts used for validation were diluted down to 1 ng/µL (Valentini et al. 2016). The PCR mixture for the validating species consisted of G2 Hot Start Master Mix (cat. #:

M7422, GoTaq G2 HotStart Master Mix Green, Promega, Madison, WI, USA), molecular grade water, 0.2 µM final concentration forward and reverse primers, 4 µM final concentration of human block (Valentini et al. 2016) and then 1ng of template was added to the mixture. A negative template control (NTC) consisting of the same reagent mixture with 1µL of molecular grade water, added in place of DNA, was run with each PCR cycle to test for contamination of lab reagents. Total PCR volumes were 50µL

Table 3. Batrachia primers and human block from Valentini et al. (2016)

ID	Sequence	Fragment Size
Batrachia FW	ACACCGCCCGTCACCCT	90bp
Batrachia RV	GTAYACTTACCATGTTACGACTT	90bp
Human Blocker	TCACCCTCCTCAAGTATACTTCAAAGGCA [SpC3]	N/A

Gel Electrophoresis and Sanger Sequencing for eDNA Metabarcoding Primer Validation

Gel electrophoresis was used to visualize each PCR product, a 2% agarose gel (40mL 1xTBE + 800mg agarose) with 2-4µL of GelRed Stain (cat. #: 41003, Biotium Inc., Hayward, CA, USA) at 100 volts for 40 min. Gels were then viewed in a UVP transilluminator (Fig. 3) . PCR products were purified using Monarch PCR Purification kits (cat. #: T1030L, New England Biolabs, Ipswich, MA, USA) following the manufacturer's protocol . PCR products were quantified using a QUBIT 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and samples with measurable DNA concentrations were sent for Sanger sequencing at Eurofins Genomics using their simple seq premixed sequencing protocol (SimpleSeq Service | Eurofins Genomics US) with 5µL of10µM forward primer and 5µL of purified PCR product. The resulting sequences were compared to sequences available on NCBI, with either the megablast

or blastn algorthims found on NCBI BLAST (Nucleotide BLAST: Search nucleotide databases using a nucleotide query (nih.gov). Purified PCR products that matched their respective species had a DNA concentration between 0.1 to 1.0ng/ µL of DNA. Due to the conserved nature of the 12s rRNA region some BLAST searches were limited to the respective species.

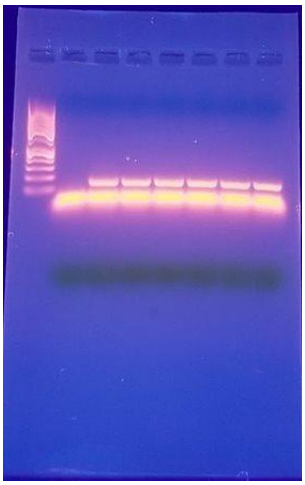

Figure 3. Example of visualized PCR product. 90 bp PCR product of tissue extracts amplified with the Batrachia primer set (Valentini et al. 2016). Left to right: 50 bp ladder, NTC, *Siren intermedia texana*, *Notophthalmus meridionalis*, *Rhinophrynus dorsalis*, *Anaxyrus speciosus*, *Smilisca baudnii*, and *Incilius nebulifer* .

eDNA Metabarcoding

The two validated PCR protocols were used with 50-µL reactions on extracted field eDNA samples. The only difference in the PCR mixture from the *in-vitro* validation PCR mixture was that the template volume for field samples was increased 10µL(1ng-30ng/µL of DNA). Field negative controls and NTCs were tested alongside field samples and received the same 10µL volume of template or molecular grade water as with field samples. All PCR products were visualized on 2% agarose gel electrophoresis with a UVP transilluminator and purified using the same kits as described previously. All field samples were purified using Monarch PCR Purification Kits (cat. #: T1030L, New England Biolabs, Ipswich, MA, USA).

Field samples were split into two next-generation sequencing (NGS) runs but the detections from each run were combined for analysis so that all possible data generated could be used for analysis (Chapter I appendix Table A2). All NGS was conducted at the Harvard Biopolymers Facility (Biopolymers Facility (harvard.edu). The initial run was a preliminary run and sequenced using a Miseq Nano platform with an expected sequencing depth of 100,000 reads per library. The main goals of this initial run were to validate the eDNA metabarcoding assay on field samples and get data on a small number of sites. Only the 'optimized' PCR protocol was used to amplify samples in the initial NGS run. The second run was sequenced with a Miseq V3 platform with an expected read depth of 330,000 reads per library, with the main goal of collecting more data on more sites. Both the 'original' and 'optimized' PCR protocols were used in the second NGS run. For both NGS runs all samples were diluted to a common concentration before being sent off for sequencing. Once the samples were received at the Harvard Biopolymers Facility, the DNA quality and fragment size were examined using an Agilent 4200 TapeStation instrument, with an corresponding Agilent TapeStation HSD1000 assay. Following confirmation of DNA quality and fragment size, samples for each run were moved to library prep for their respective sequencing platforms.

NGS Analysis

OBITools3 (Boyer et al. 2016, https://git.metabarcoding.org/obitools/obitools3) and Geneious Prime (v 2022.0.1) (Geneious Prime | Molecular Biology and Sequence Analysis Software) were used to analyze NGS data. Sequences were compared to a local *in-silico* generated reference database with a 97% match requirement. The local *in-silico* reference database was created using OBITools3 following the tutorial found at Wolf tutorial with the OBITools3 · Wiki · OBITools / OBITools3 · GitLab (metabarcoding.org) with modifications to commands so that the

reference database would be specific to Batrachia primers. Other primer specific modifications to the OBITools3 pipeline were done as needed (See chapter I appendix for full protocol). Sequences were matched to the family, genus or species level in OBITools3 based on the 97% match requirement. Sequences not identified by OBITools3 were exported to Geneious Prime and were compared to sequences on NCBI using either the megablast or blastn algorithms for further examination. If a sequence was assigned to a taxonomic unit that did not have a distribution within the study area it was examined further to determine if the sequence matched an organism with a possible distribution in the study area. The sequence was removed from further analysis if it was not able to be matched with a species native to the study area. Positive detections from megablast and blastn had a percent match ranging from 90%-96%. Ten reads per taxonomic unit was chosen as an initial threshold for a positive detection based on read numbers per Valentini et al. (2016). In three instances, a positive detection was also considered when a sample, that was amplified with replicates, had a low number of reads assigned to a taxonomic unit in each replicate *(i.e* 1-3 read(s) per replicate) as the detection was shown to be repeatable and previous literature has also considered low read numbers as a positive detection (Klymus et al. 2017).

Targeted eDNA Assay for Black-Spotted Newt (*Notophthalmus meridionalis)*

A targeted eDNA assay designed for Black-spotted newts (Robinson et al. 2022) (Table 4) was used to evaluate the sensitivity of the eDNA metabarcoding assay by comparing the number of Black-spotted newt detections from each field sample between each method. The Black-spotted newt PCR mixture consisted of GoTaq G2 Hotstart Master Mix (cat. #: M7422, GoTaq G2 HotStart MasterMix Green, Promega); molecular grade H_2O; 0.2 µM final concentration forward and reverse primers and 5 µL (1ng-30ng/µL of DNA) of a field sample for

25µL reactions and 10µL (1ng-30ng/µL of DNA) for 50µL reactions for the initial round and nested rounds. Field negative controls received the same sample volume. No template controls were included with every PCR run. The initial primers were used in the initial round and the nested primers were used in the nested round (Table 5). The initial PCR protocol consisted of 35 cycles of 95℃ for 30 s, 55℃ for 28 s, 72℃ for 30 s, followed by 72℃ for 5 min and 4℃ for 4 min. The nested PCR protocol consisted of 35 cycles of 95℃ for 30 s, 53℃ for 28 s, and 72℃ for 30 s, followed by 72℃ for 5 min and 4℃ for 4 min. The reaction volume, 25µL or 50µL, was dependent on the purification method. Initial round PCR products were purified using EXOCIP Rapid PCR Clean-up (25 µL; cat. #: E1050L, New England Biolabs) or Monarch PCR clean-up kits (50 µL; cat. #: T1030L, New England Biolabs) and 5µL for 25µL reactions and 10µL for 50µL reactions of the purified initial product was used for the sample in the nested round. For the nested round, NTCs received the purified product from the initial NTC, and field negative control received its own respective purified product from the initial round. Field samples were amplified in triplicate.

Two thresholds were used to determine a positive eDNA detection using the targeted eDNA assay. The "low" threshold was the successful amplification of at least one technical replicate of a field sample with no amplification in the field negative control or NTC and the sequence of that replicate would need to match Black-spotted newt on NCBI BLAST(Nucleotide BLAST: Search nucleotide databases using a nucleotide query (nih.gov) ;lowest percent match accepted was 94.4%) . The "high" threshold was successful amplification of at least two technical replicates of a field sample with no amplification in the field negative control or NTC and the sequence from a technical replicate would need to match Black-spotted newt on NCBI BLAST

(lowest percent match accepted was 94.4%). Successful replicates showing visible bands from gel electrophoresis and UV visualization were purified using either EXOCIP Rapid PCR Cleanup cat. #: E1050L, New England Biolabs) or Monarch PCR Purification Kits (cat. #: T1030L, New England Biolabs) and sent for Sanger sequencing at Eurofins Genomics (SimpleSeq Service | Eurofins Genomics US) using the simple seq premixed method. Sequencing composition consisted of 5µL of purified nested PCR product and 5µL 10µM nested RV primer.

Table 4. Targeted eDNA assay primer set. Forward and Reverse Primers for the initial and nested rounds of the targeted eDNA assay for Black-spotted newts developed by Robinson et al. (2021).

COI Primer ID	Sequence	Fragment Size
Initial Primers		
BSN COI FW 6	GTAGACCTGAATGTGGACACC	181 bp
BSN COI RV 6.1	CTGTAAGCCCTCCCTCTGT	181 bp
Nested Primers		
BSN COI FW 7.1	ACACCCGAGCCTATTTTAC	122 bp
BSN COI RV 7	GCCCATAGTATTGCAGCAT	122 bp

Statistical Analysis

For eDNA metabarcoding, the number of reads assigned to each taxonomic unit in each sample was summed together for each site. Read numbers were then transformed into presence/absence for comparison to field visual encounter surveys presence-absence data . Bar plots were then used to visualize the amphibian and non-amphibian detections of each method. The number of sites a

taxonomic unit was detected using eDNA metabarcoding and traditional surveys were totaled for comparison. Due to the varied resolution of detections between each method (e.g., a family-level detection from the traditional visual encounter survey vs a species-level detection from eDNA metabarcoding) a weighting scale was applied to all species-, genus-, and family-level detections so that a formal test of differences between eDNA metabarcoding and traditional survey methods could be conducted (See chapter 1 appendix for data matrix). Species-level detections were given a three, genus-level detections were given a two, and a family-level detection was given a 1. Lower taxonomic detections were given higher weights because they offer more finite information than higher-level taxonomic detections. To test whether one method or another detected more amphibian taxonomic units per site, the weighted detections for each site using eDNA metabarcoding and traditional visual encounter survey were respectively totaled. A Kolmogorov-Smirnov test (Kolmogorov 1933) and Levene's test (Levene 1960) for homoscedasticity were used to determine normality and homoscedasticity for the comparison of taxonomic units detected between eDNA metabarcoding and traditional survey methods. The data for that comparison were deemed not normally distributed ($p<0.05$) but homoscedastic ($p=0.634$) and so a Mann-Whitney U (Mann and Whitney 1947, Wilcoxon 1945) test was then conducted. All statistical analyses were conducted in SPSS (IBM v 28.0.00; Levesque 2007) and Primer e (v 7 Quest Research Limited; Clarke and Gorley 2006).

Results

Testing the Valentini Primers on Individual Amphibians Native to South Texas

Tissue extracts of 33/33 species successfully amplified and tissue extracts of 32/33 species were Sanger sequenced; only *Lithobates areolatus* was not able to be Sanger sequenced (Table 5).

Table 5. eDNA metabarcoding validation results. Linnean taxonomic name is in the leftmost column, followed by sequence percent match to its respective species is in the rightmost column.

Species Name	Sequence % Match
Anaxyrus speciosus	100.00
Scaphiopus couchii	100.00
Rhinella marinus	93.62
Leptodactylus fragilis	93.62[1]
Gastrophryne olivacea	100.00
Incilius nebulifer	96.00
Hypopachus variolosus	100.00
Ambystoma mavortium	98.00
Smilisca baudinii	97.50
Lithobates berlandieri	93.00[2]
Lithobates catesbeianus	100.00
Rhinophrynus dorsalis	97.83
Siren intermedia texana	92.53
Pseudacris clarkia	89.73
Notophthalmus viridescens	95.24
Acris blanchardi	98.15
Scaphiopus hurterii	97.37

Table 5, cont. Linnean taxonomic name is in the leftmost column, followed by sequence percent match to its respective species is in the rightmost column.

Species Name	Sequence % Match
Dryophytes chrysoscelis	97.44
Eleutherodactylus cystignathoides	100.00[1]
Gastrophryne carolinensis	95.24
Dryophytes squirellus	97.44
Dryophytes cinereus	97.44
Lithobates sphenocephalus	93.02
Anaxyrus punctatus	90
Anaxyrus debilis	95.35
Anaxyrus woodhousii	95
Spea bombifrons	97.56
Dryophytes versicolor	97.73
Pseudacris streckeri	97.06
Pseudacris fouquettei	97.06[1]
Ambystoma texanum	88.46
Lithobates areolatus	N/A
Notophthalmus meridionalis	97.62

[1] indicates that there was no published data on the 12s rRNAregion for respective species and the resulting sequence match is to other members of the genus. [2] indicates a genus level match even though there was published data for the 12s rRNA region. N/A indicates that a clean sanger sequence was not able to be produced.

Taxonomic Units Detected in Field Samples Using eDNA vs Traditional Visual Encounter Surveys

From the eDNA metabarcoding assay, 13 unique amphibian species and two unique genera (*Lithobates* and *Anaxyrus*) were detected (Table 6). From traditional survey methods alone, nine unique amphibian species, four unique genera and three unique families were detected (Table 6). eDNA metabarcoding detected almost all species, genera and families more

often at most sites compared to traditional visual encounter surveys with the exception of *Notophthalmus meridionalis*, *Lithobates berlanderi*, *Lithobates catesbeianus* and *Hypopachus variolosus*, were detected more often with traditional survey methods

Table 6. Amphibian taxonomic units detected using eDNA metabarcoding and traditional visual encounter surveys.

	Sites detected using eDNA metabarcoding	Sites detected using Traditional
Siren intermedia texana	15	1
Incilius nebulifer	16	7
Spea bombifrons	1	0
Notophthalmus meridionalis	1	3
Scaphiopus couchii	5	0
Anaxurus speciosus	3	2
Gastrophryne olivacea	14	0
Smilisca baudnii	1	1
Dryophytes squirellus	2	2
Rhinella marinus	2	0
Dryophytes cinereus	3	0
Lithobates sphenocephalus	4	0
Scaphiopus hurterii	1	0
Hypopachus variolosus	0	3
Lithobates berlandieri	0	6
Lithobates catesbeianus	0	1
Lithobates sp.	9	12
Anaxyrus sp.	8	2
Gastrophryne sp.	14	5
Scaphiopus sp.	6	2
Bufonidae	16	8
Microhylidae	14	7
Hylidae	6	5

eDNA metabarcoding provided more amphibian species- and genus-level detections for almost all sites (Fig. 4). In contrast, the traditional survey tended to provide mostly family- and genus-level detections (Fig. 5). There was a high amount of variation in the number of amphibian taxonomic units detected at each site using eDNA metabarcoding and traditional survey methods by themselves (Fig. 4-5). eDNA metabarcoding (Weighted Avg: 9.417 SE ±1.229) detected significantly more amphibian taxonomic units compared to traditional survey methods (Weighted Avg: 5.833 SE±1.064) (Mann-Whitney U=397.50, p=0.023).

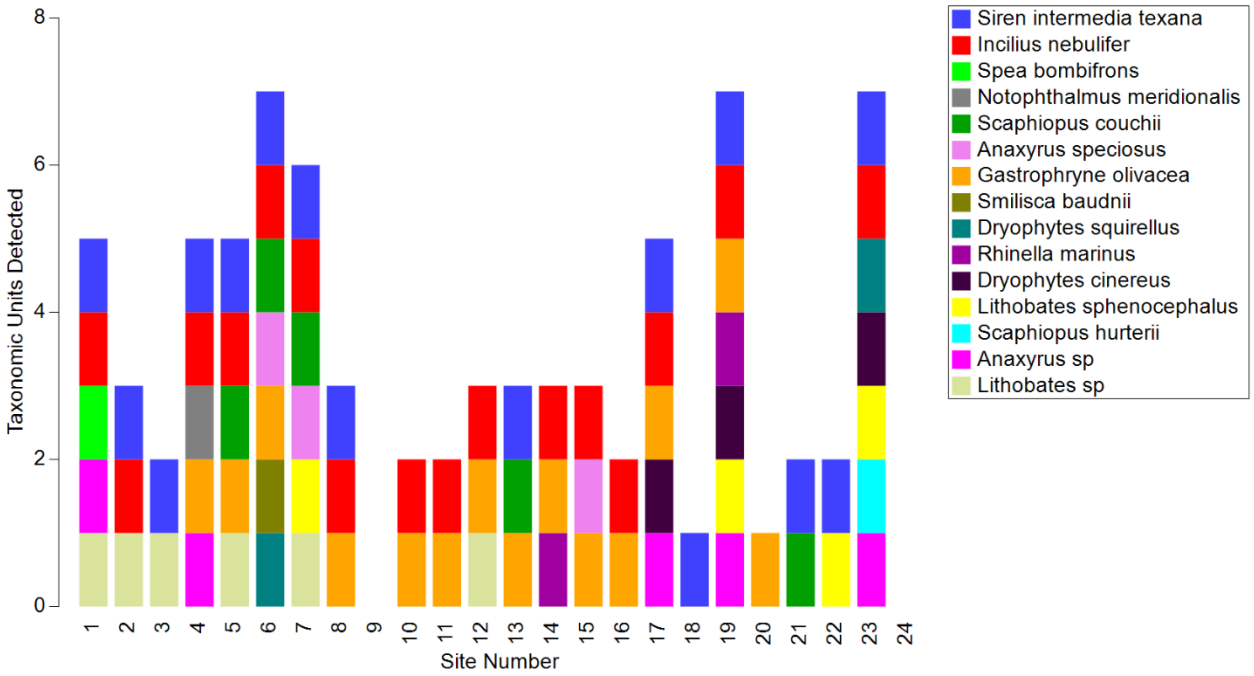

Figure 4. Amphibian taxonomic units detected at each site using eDNA metabarcoding. Detections are based on presence-absence data. Sites 9 and 24 had zero amphibian taxonomic units detected

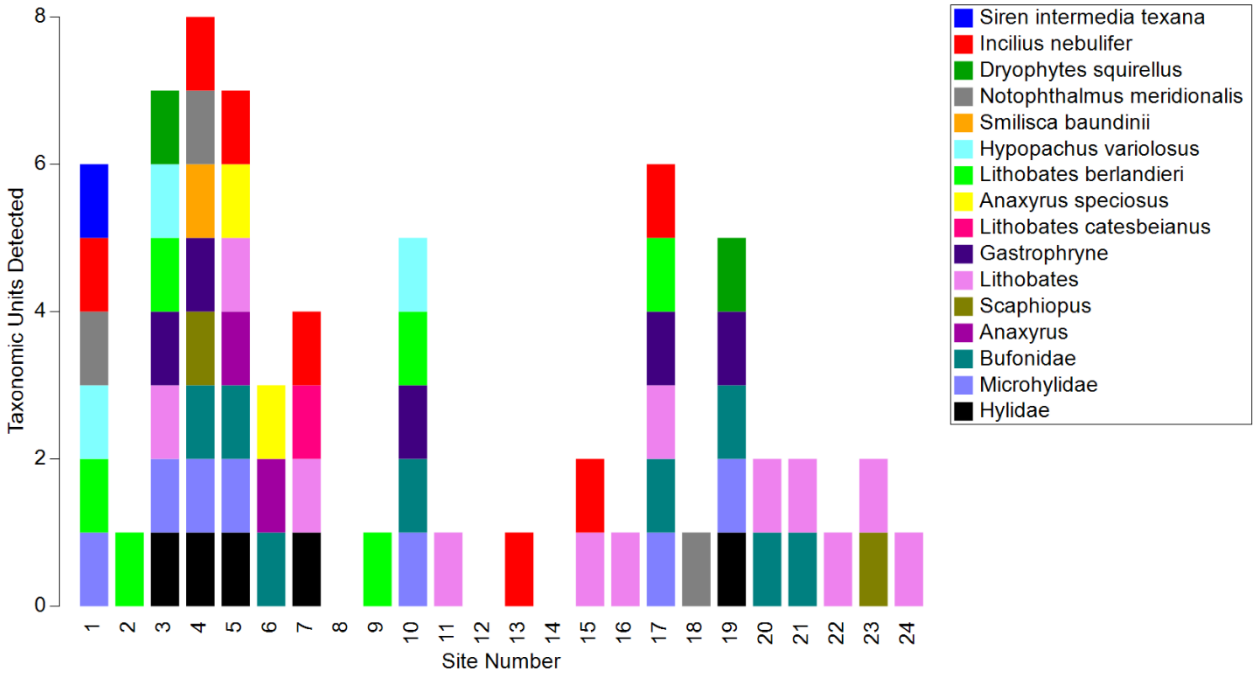

Figure 5. Amphibian taxonomic units detected at each site using the traditional visual encounter survey. Detections are based on presence-absence data. Sites 8, 12 and 14 had zero amphibian taxonomic units detected

Although the eDNA metabarcoding assay was not validated on taxa outside of amphibians, it was able to detect a variety of non-amphibian taxa in field samples (Table 7, Fig. 6-7). eDNA metabarcoding was again able to provide more species- and genus-level detections for non-target taxa compared to traditional survey methods (Table 7, Fig. 6-7). In many sites, non-target taxa represented the majority of taxonomic units detected using eDNA metabarcoding (Fig. 6). Twelve fish species, four fish genera, and the *Oreochromini* and *Stethaprioninae* families were detected using eDNA metabarcoding (Table 7). Six reptile species and the *Testudinoidea* family were detected using eDNA metabarcoding (Table 7). Using traditional visual encounter surveys, one species of fish, three genera of fish, and the *Centrarchidae* family of fish were detected. Six species and two genera of reptiles were also detected (Table 7).

Table 7. Non-amphibian taxonomic units detected using eDNA metabarcoding and traditional survey methods.

Taxonomic Unit	Class	Sites detected using eDNA	Sites detected using Traditional
Poecilia formosa	Fish	6	0
Poecilia latipinna	Fish	13	0
Gambusia affinis	Fish	15	2
Trachemys scripta	Reptile	1	0
Herichtys cyanoguttatus	Fish	2	0
Dormitator maculatus	Fish	3	0
Lepomis cyanellus	Fish	6	0
Cyrpinodon variegatus	Fish	7	0
Gambusia holbrooki	Fish	7	0
Poecilia mexicana	Fish	2	0
Fundulus grandis	Fish	1	0
Lepomis macrochirus	Fish	2	0
Menidia beryllina	Fish	1	0
Thamnophis proximus	Reptile	2	1
Regina grahamii	Reptile	1	0
Thamnophis marcianus	Reptile	2	0
Coluber constrictor	Reptile	1	0
Crotalus atrox	Reptile	1	0
Agkistrodon piscivorus	Reptile	0	1
Alligator mississippiensis	Reptile	0	1
Thamnophis saurita	Reptile	0	2
Holbrookia propinqua	Reptile	0	1
Oreochromini sp.	Fish	5	0
Thamnophis sp.	Reptile	5	4
Oreochromis sp.	Fish	3	0
Testudinoidea sp.	Reptile	3	0
Stethaprioninae sp.	Fish	3	0
Atractosteus sp.	Fish	1	0
Lepisosteus sp.	Fish	1	0
Ameiurus sp.	Fish	1	0
Gambusia sp.	Fish	22	8
Poecilia sp.	Fish	19	3
Kinosternon sp.	Reptile	0	1
Scincilla sp.	Reptile	0	2
Fundulus sp.	Fish	1	2
Holbrookia propinqua	Reptile	0	1

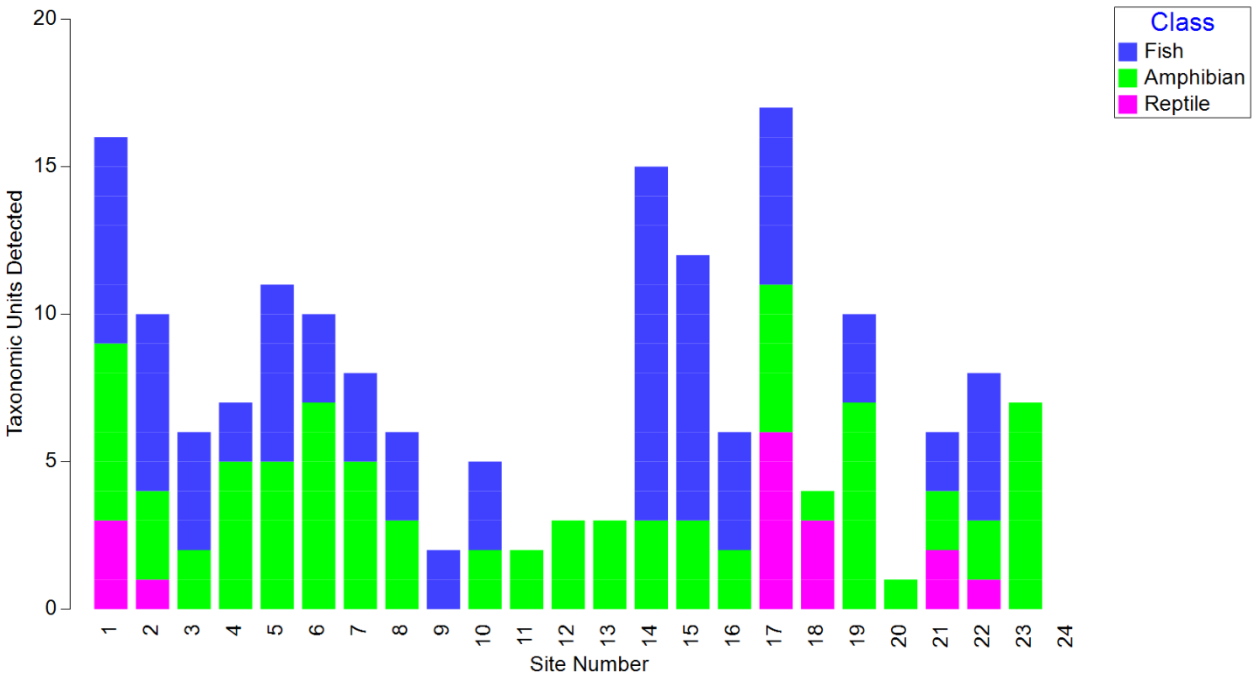

Figure 6. Non-amphibian taxonomic units detected at each site using eDNA metabarcoding. All presence/absence detections of fish, reptiles and amphibians are included.

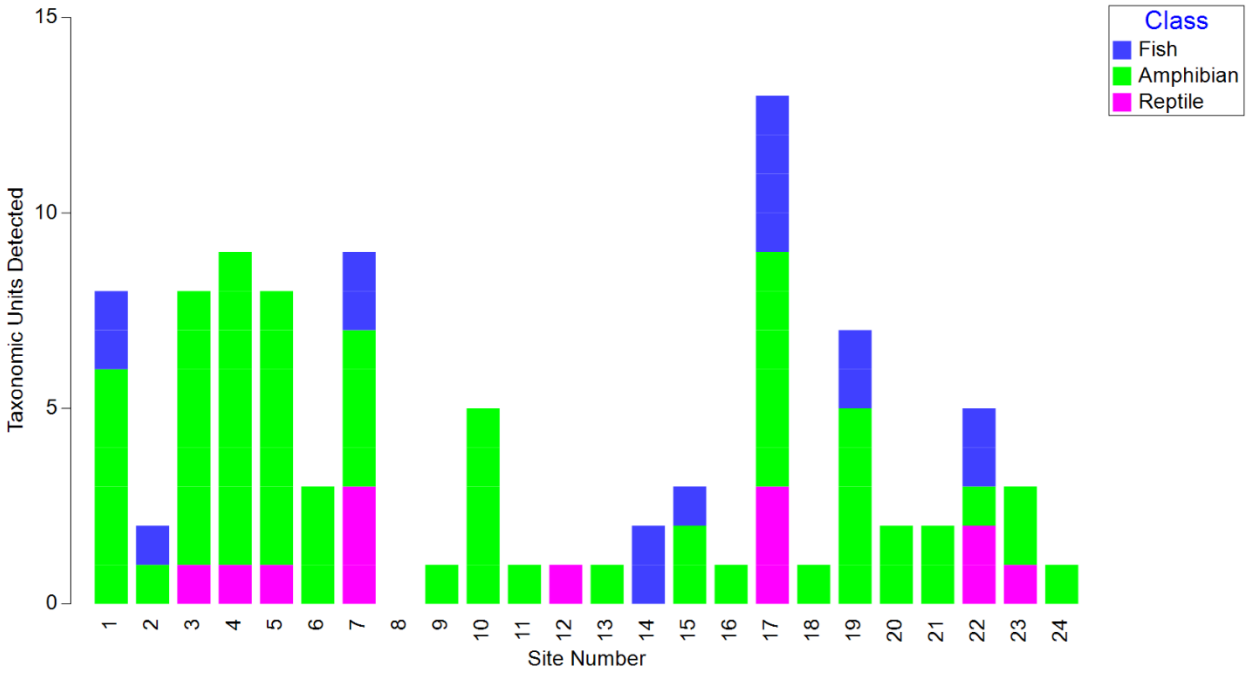

Figure 7. Non-amphibian taxonomic units detected at each site using traditional survey methods. All fish, amphibian and reptile presence/absence detections are included .

Detections of Black-Spotted Newts Using a Targeted eDNA assay, eDNA Metabarcoding, and Traditional Survey Methods

The targeted eDNA assay detected Black-spotted newts at the most sites overall 41.67% (n=10) of sites using the low BSN positive threshold and 25.00% (n=6) of sites using the high BSN positive threshold (Table 8). Traditional visual encounter surveys had the second highest number of detections, 12.5% (n=3), overall (Table 8). The eDNA metabarcoding assay had the lowest number of detections at 4.17% (n=1) of sites (Table 8).

Table 8. Black-spotted newt detections at all 24 sites. Detections are based on using eDNA metabarcoding, the targeted eDNA assay at low and high replicate thresholds and traditional visual encounter surveys.

Site #	Site Name	eDNA Metabarcoding	Targeted eDNA Low	Targeted eDNA high	Traditional
1	Grace Heritage Ranch Main		BSN+	BSN+	BSN+
2	Sabal Palm Sanctuary Resaca Blind				
3	Coastal Bend Community College				
4	PIHS Pond	BSN+	BSN+	BSN+	BSN+
5	Live Oak County Park		BSN+	BSN+	
6	Brushline North				
7	WWR Big Lake				
8	HWY 77 GPZ				
9	LANWR Priarie trail #2		BSN+	BSN+	
10	USFWS Kelly Unit Resaca				
11	Brushline South				
12	LANWR Kidney Pond				
13	LANWR Scum Pond		BSN+	BSN+	
14	Old Military Hwy Pond 4		BSN+	BSN+	
15	Pintas Creek				
16	Old Military Hwy Pond 5				
17	PHR Haybarn				
18	Sabal Palm Sanctuary Side Pond		BSN+		BSN+
19	PHR Midline Fence		BSN+		
20	CKWRI South Pasture				
21	Southmost Black Willow Resaca		BSN+		
22	PHR Bullrush				
23	Live Oak Pipeline Pond				
24	LANWR Newt Pond		BSN+		

Discussion

eDNA Metabarcoding Assay Validation and Comparison to Traditional Survey Methods

eDNA metabarcoding assay detected significantly more amphibian taxonomic units (U=397.50, p=0.023) compared to traditional survey methods with an almost twice as high weighted avg detection per site (9.417 SE ±1.229) compared to traditional survey methods (5.833 SE ±1.064). Most literature suggests that eDNA metabarcoding is as sensitive or more sensitive than traditional survey methods (Lacoursière-Roussel et al. 2016, Ruppert et al. 2019, Hallam et al. 2021, Sakata et al. 2021a, Sakata et al. 2021b). As hypothesized, the results of the present study support that eDNA metabarcoding is more sensitive than visual encounter surveys based on the significantly higher number of amphibian taxonomic units detected by eDNA metabarcoding than by the traditional survey method. However, the variation in amphibian taxonomic units detected at each site using both methods suggests amphibians detections is variable when using either method (Fig. 5-6). While out in the field it can be difficult to identify some tadpoles and metamorphs down to the species level without further analysis, which is why there were more family and genus level detections in the visual encounter survey and shows another potential advantage of eDNA metabarcoding as detections are based off of genetic match rather than morphology (Grosjean et al. 2015). Implementing other methods to the traditional survey such as call surveys, trapping or even pit fall traps could have made for a more robust traditional survey and resulted in more amphibians detected (Todd et al. 2007, Sasso et al. 2017). In some samples non-amphibian species accounted for the majority of detections (Fig. 6) and some amphibians could have been missed due to the ability of this primer set to amplify non-amphibian taxa. The biphasic lifestyle of most amphibians means they are not always going to be in the water, so the aquatic amphibian DNA concentration may be lower relative to other taxa.

For example, a previous study that used a microfluidic metagenomic eDNA approach to assessing aquatic biodiversity in a tributary in the Oregon Coastal Range found only 0.80% of total sequences were amphibian, while the rest of the sequences were attributed to other aquatic organisms (Hauck et al. 2019). In this study, likewise a high number of non-amphibian detections were observed. Thus amphibian DNA may be less abundant relative to other aquatic taxa, such as fish and microbes in some water bodies, so a consistent representation of amphibians using eDNA may be difficult without primers that have high affinity for amphibians. The variation in amphibian taxonomic units detected and the inability to obtain certain species-level detections (i.e. *Lithobates berlanderi*, *Lithobates catesbeianus*, *Hypopachus variolosus*) using eDNA metabarcoding suggests the need for primer refinement or pairing eDNA metabarcoding with other methods such as traditional surveys and targeted eDNA assays for a comprehensive community assessment, as traditional survey methods and targeted assays may help to detect species missed by eDNA metabarcoding, an idea that has been posited by previous studies (Coghlan et al. 2021, Wikston 2021). Using combined eDNA and traditional visual encounter surveys is beneficial because the latter can provide baseline information on taxa present at a site and thereby provide a comparison so that the effectiveness of eDNA assays can be determined (Wikston 2021). The unexpected detections of non-amphibian species do suggest a broader application of this primer set for broader community assemblage analyses of these sites, i.e. eDNA metabarcoding can be effective at characterizing a variety of taxa as shown by previous studies (Klymus et al. 2017, Bylemans et al. 2019, Roffler et al. 2021). The variety of taxa detected in this study emphasizes the importance of ephemeral ponds for wildlife in South Texas as previous studies have found that invertebrates (Pattillo et al. 1997, Tipton et al. 2012), reptiles (Boundy 1994, Duran 2021),wetland birds (Fulbright et al. 1990) and mammals

(Fulbright et al. 1990) can all use these ephemeral ponds for parts of their life history as well as for predation and refuge

The metabarcoding assay also provided information on amphibian community assemblages for each site, accomplishing another main goal for this chapter, and detected more species (13/33 amphibian species reported for the region) than traditional survey methods (9/33), further supporting that eDNA metabarcoding is more sensitive than traditional survey methods. The lack of detection of the other 20 amphibian species could have been due to a number of factors such as favoring ephemeral ponds, timing of the survey as well as overall DNA abundance. For example, sites used in this study favored potential habitat of Black-spotted newts, which are believed to mainly utilize ephemeral ponds, but other species of amphibians within the study area utilize different water-body types, so broadening the type of water body sampled may allow for a greater number of native amphibians to be detected (Dixon 2013, Petersen et al. 2018, Duran 2021). For detection of , species such as the Eastern Newt (*Notophthalmus viridescense*), Blanchard's Cricket Frog (*Acris blanchardi*) and the Red-spotted toad (*Anaxyrus punctatus*), which were all not detected, sampling in flowing systems may help to increase the number of unique species detected as these species can persist in slow-moving streams (Tipton et al. 2012). Because many of the species that were not detected, can persist in ephemeral water bodies the lack of detection may have been due a number of factors such as, the timing of the eDNA survey being when these undetected species are not as active or the eDNA survey was outside of the detectable time frame due to DNA degradation, or that the DNA of the species not detected was swamped by non-amphibian taxa DNA, or even random chance during the sampling. Having a balanced sampling design throughout the whole year found may also help to increase detection probabilities as many amphibians found within the study area are

active at different times of the year (Tipton et al. 2012, Dixon 2013, Petersen et al. 2018, Duran 2021) and most of the samples used in this study came from May-September (n=29) and only a handful of samples came from October-Feb (n=7). However, the May-September sampling range does overlap with potential breeding times of many amphibians found within the study area (Tipton et al. 2012), so time of sampling may not have influenced what species were detected but rather DNA degradation or aquatic DNA abundance may have influenced the lack of detection of certain species . DNA degradation rates and aquatic DNA abundance within these ephemeral ponds should be investigated further to better examine whether or not these two factors play a role in the detection of certain species.

The results found in this study support the growing evidence that many metabarcoding primer sets can have blind spots for certain taxa based on primer match and not just based on biomass (Harper et al. 2018, Nester et al. 2020, Scheneker et al. 2020, Gold et al. 2021). Although, the eDNA metabarcoding assay was validated on most tissue extracts (32/33) and on field samples, accomplishing two of the main goals for this chapter with both PCR protocols worked equally well on tissue extracts of most species, there were three troublesome (*Hypopachus variolosus, Gastrophryne carolinensis, Lithobates areloata*) species that required the redesign from the 'original' protocol to the 'optimized' protocol, In this study, the Batrachia primer set had a lower affinity, or a blind spot, for *Hypopachus variolosus, Gastrophryne carolinensis, Lithobates areloata* as tissue extracts of each species were difficult to amplify and Sanger sequence. *L. berlanderi* was also a species that was only able to get a genus-level match during the validation phase even though there is published data on the 12s rRNA region for *L.berlanderi*. *Lithobates berlanderi* and *L. catesbeianus*, which are larger-bodied abundant species, were species that were detected more often using the traditional visual encounter survey

compared to eDNA metabarcoding, indicating that the Batrachia primer set may have a lower affinity for those species as well. The Batrachia primer set obtained a number of genus-level detections for the *Lithobates* genus, however the number of detections was still less than the traditional visual encounter survey (Table 6). The difficulty of obtaining getting species-level detections for some species agrees with a previous study that had a similar issue with the Mifish Universal Teleost 12s rRNA primer set not being able to differentiate some species of gobies and rockfishes at the species level (Gold et al. 2021).

Comparison of Black-Spotted Newt Detections using eDNA Metabarcoding, Targeted eDNA assay and Visual Encounter Survey

The results of the Black-spotted newt detection comparison support the second hypothesis of this chapter, that the targeted assay will provide the most Black-spotted detections (low threshold 41.67% of sites; high threshold 25.00% of sites) and is consistent with the growing body of literature that targeted eDNA assays are more effective at detecting rare-small-bodied species compared to eDNA metabarcoding assays (Table 8) (Bylemans et al. 2019, Harper et al. 2018, Nester et al. 2020, Gold et al. 2021). Although eDNA metabarcoding assay detected more species than visual encounter survey, eDNA metabarcoding was less efficient at detecting the Black-spotted newts in field samples compared to the visual encounter survey, with the traditional visual encounter survey detecting Black-spotted newts at 12.5% of sites and eDNA metabarcoding detecting Black-spotted newts at 4.17% of sites (Table 8). (Table 8). Robinson et al. (2022) likewise had low detection of Black-spotted newts with traditional surveys (10%) and targeted eDNA assay (10%) of 80 sites surveyed. While the percentage of sites Black-spotted newts were detected was the same with each method in Robinson et al. (2022), the positive sites identified with each method were not the same, so they detected Black-

spotted newts at 15% of sites when they combined traditional surveys and the targeted eDNA assay. Using only traditional survey methods Judd 1985 found Black-spotted newts at 2/221 sites (0.9%) and Rappole and Klicka 1991 found Black-spotted newts at 7/114 sites (6%). This study detected Black-spotted newts at a higher percentage of sites using traditional survey methods and the targeted eDNA assay relative to Judd (1985), Rappole and Klicka (1991) and Robinson et al. (2022). The higher detection percentage was most likely due to surveying a smaller number of sites and each of the surveyed sites being previously identified as Black-spotted newt habitat, or potential habitat, by Robinson et al. 2022 .The results of the present study suggest that eDNA metabarcoding may be less efficient than traditional survey methods at detecting some rare-cryptic species, such as the Black-spotted newt. As found in Robinson et al. (2022) traditional survey methods can complement targeted eDNA assays as individuals that aren't in the water are more likely to be found with traditional survey methods. The combination of the two methods may be the most effective approach for Black-spotted newts and other rare-cryptic species. As mentioned previously the amplification of non-amphibian taxa using the eDNA metabarcoding assay may be one of the causes of the lowered sensitivity for rare-small-bodied organisms, which has been posited in previous studies (Bylemans et al. 2019, Harper et al. 2018, Nester et al. 2020, Gold et al. 2021). The higher amplification threshold for the targeted assay may represent more robust eDNA detections. While sites that only meet the lower threshold (Sites 19,21,24) for the targeted assay still should be considered but should be sampled further so that a more information can be gathered on black-spotted newt presence. Previous eDNA studies on rare cryptic taxa have considered amplification of 1/3,1/6 or even 1/12 technical replicates as potential positives and so samples that only meet the lower positive threshold should not be discounted immediately (Bylemans et al. 2019, Harper et al. 2018). Having a lower detection

threshold could help to show sites that have a smaller populations and thus less black-spotted newt DNA being present in the water body or sites where the eDNA survey was missed timed; if the eDNA survey was too early or too late surrounding reproduction and the DNA may have been too dilute or too degraded to amplify more than one replicate (Goldberg et al. 2016, Harper et al. 2018). Future studies could use this lower threshold and modify the methods to focus on re-sampling sites that met the lower threshold to confirm Black-spotted newt presence and examining how long Black-spotted newt DNA persists in a water body so that the effectiveness of eDNA sampling may be increased.

CHAPTER II

AMPHIBIAN COMMUNITY ANALYSIS AND ENVIRONMENTAL RELATIONSHIPS

Introduction

South Texas offers a unique area to look at amphibian community relationships as the eastern half is in the humid-subtropic climate classification and the western portion is on the edge of the semi-arid climate classification (Peel et al. 2007). The two climate types combined with many ephemeral, permanent, and flowing water bodies helps to support a wide variety of amphibian species (Dixon 2013, Petersen et al. 2018, Duran 2021). A portion of South Texas is also within the Rio Grande Delta; the local flora and fauna have uniquely adapted to the pulse flooding of the Rio Grande that occurred prior to the development of the upstream dams (Clover 1937, Everitt et al. 1996). Many water bodies in South Texas are close to the Gulf of Mexico, so the salinity of some water bodies can have coastal influences. Due to the hot temperatures, evaporation rates of most water bodies are high , thereby increasing the salinity. Sea level changes over the past thousand years may have also left certain areas with high salinity (Ricklis and Blum 1998). In addition, South Texas is an area that is under rapid development with much of the native landscape changing over the past century. For example, development for agriculture has removed much of the native habitat, increasing habitat fragmentation and changing the water quality across the landscape (Garcia et al. 2001). There is little detailed information about how amphibian community assemblages relate to one another across South Texas and the

environmental parameters that may affect community assemblages (Judd 1985, Bare 2018, Duran 2021, Ruppert et al. 2022, Robinson et al. 2022). There is also very limited information on community assemblages and environmental relationships of cryptic species, such as the Black-spotted newt, within the study area. A better understanding factors that may drive cryptic species persistence may help to conserve them (Bare 2018, Robinson 2021, Robinson et al. 2022). Broadly speaking amphibian community assemblages, and Black-spotted newt persistence, can be influenced by type of water body, water quality, habitat characteristics and soil composition (Hero et al. 2001, Vignoli et al. 2007, Tavares-Junior et al. 2020, Robinson 2021). Many of those environmental factors influence one another and can vary spatially because of a variety of site-specific characteristics that in turn can influence which species are able to persist at a site and thereby influence how similar the communities across the study area are to one another (Hero et al. 2001, Vignoli et al. 2007, Tavares-Junior et al. 2020). Understanding how similar communities are to one another is useful for making distinct community groupings; from those distinct groupings' inferences can then be made on what biotic and abiotic factors drive community structure, as well as which factors allow for Black-spotted newt persistence (Hero et al. 2001, Vignoli et al. 2007, Junior and Rocha 2013, Bare 2018, Tavares-Junior et al. 2020, Robinson 2021).

The type of water body (*e.g.* ephemeral, permanent or flowing), can drive amphibian community similarities across the study area, as different species have adapted to utilize the unique habitat provided by each type of water body (Gascon 1991, Tavares-Junior et al. 2020). The sporadic and intense rainfall in South Texas creates many quick-forming ephemeral ponds, yet there are more stable permanent water bodies and flowing water bodies throughout the study area that offer habitat year-round. Species affinities for water bodies can drive community

similarity within and among water-body types (Tipton et al. 2012). For example, some species such as *Acris blanchardi, Anaxyrus punctatus* and *Notophthalmus viridescens* can persist in slow-moving streams, whereas some species, such as *Lithobates catesbeianus,* can only persist permanent vegetated water bodies (Tipton et al.2012). On the other hand, some species, such as *Anaxyrus speciosus*, *Gastrophryne olivacea*, and *Incilius nebulifer,* can persist in almost any type of water body, so the community of each water body type may be more similar than among different water body types (Tipton et al. 2012, Dixon 2013**)** The rapid formation of ephemeral ponds allows for a greater turnover of resources relative to permanent and flowing water bodies, making high-quality nutrients and other resources available in a small area and in turn supporting a diverse amphibian community (Heyer et al. 1975, Hero et al. 1998, Hero et al. 2001, Tavares-Junior et al. 2020). The regular turnover of resources is one reason ephemeral ponds tend to be more diverse in their amphibian communities as compared to permanent ponds (Hero et al. 1998, Hero et al. 2001, Tavares-Junior et al. 2020). Another reason for the higher diversity of ephemeral ponds is the absence of predation pressure from large predators, such as large fish, that require a persistent water supply to become established (Hero et al. 1998, Hero et al. 2001, Junior and Rocha 2013, Tavares-Junior et al. 2020). Amphibian communities in ephemeral ponds are not without stress though, as previous studies have shown amphibians within ephemeral water bodies still face predation pressure from other amphibians, particularly during juvenile maturation, as well as from other taxa, such as predacious invertebrates, present in the pond (Heyer et al. 1975, Tipton et al. 2012, Junior and Rocha 2013). The amount of predation pressure and type of predator can then drive community assemblages and observed similarities between water bodies due to selective pressures (Heyer et al. 1975, Hero et al. 1998, Hero et al. 2001). The amphibian communities of ephemeral ponds are also subject to desiccation and must

seek refuge underground or under cover objects during dry periods (Vignoli et al. 2007, Tavares-Junior et al. 2020). Water body type and the site-specific characteristics related to the hydrology of different water bodies have been previously found to drive significant differences among amphibian communities in other parts of the world (Vignoli et al. 2007, Shulse et al. 2010, Junior and Rocha 2013, Mathwin et al. 2021) and so may be driving amphibian community similarities across the study area as well.

Vegetation also changes with water body type and different vegetation structures can influence amphibian community similarity across the study area. Vegetation structure creates areas of refuge and areas for reproduction and ovipositing; species have adapted to utilize these different habitat structures (Shulse et al. 2010, Tipton et al. 2012, Tavares-Junior et al. 2020). For example, species such as *Siren intermedia texana, Notophthalmus meridionalis* and *Notophthalmus viridescens* prefer densely vegetated water bodies, whereas treefrogs such as *Dryophytes chrysoscelis, Dryophytes cinereus and Dryophytes squirellus* prefer water bodies that have more emergent vegetation and vegetation surrounding a pond so that they have more areas for refuge and foraging (Tipton et al. 2012, Dixon 2013). Cortes-Gomez et al. (2013) found amphibian community composition at permanent water bodies in the Pacific rainforests of Columbia significantly differed among vegetation structures (mature forest, secondary forest, abandoned mixed farming areas) and that the type of canopy, the density of woody plants and leaf litter depth explained the most amount of variation among amphibian communities. Tavares-Junior et al. (2020) found that vegetation in and around ponds in Brazilian tropical rainforests was a significant environmental factor in driving amphibian community assemblages. Shulse et al. (2010) found that heavy aquatic vegetation abundance and anthropogenic disturbances in constructed wetlands were one of the main driving factors for salamander and Hylid abundance

in Northern Missouri and that many Ranid species favored habitats with lower aquatic vegetation abundance. Land use characteristics and vegetation of sampled sites used in this South Texas study area include undeveloped Sabal Palm Forest, water bodies adjacent to agricultural fields, water bodies in fragmented thorn scrub forests, and coastal wetlands, so the vegetation in and around study sites may be influencing amphibian community similarity across the study area as found in other parts of the world (Shulse et al. 2010, Tipton et al. 2012, Cortes-Gomez et al. 2013, Tavares-Junior et al. 2020).

Water quality parameters, such as salinity, suspended sediment, dissolved oxygen, hardness, alkalinity, pH and water temperature, can affect amphibian community similarity across the study area, because amphibians perform gas exchange and absorb minerals and contaminants through their skin making them very sensitive to water quality parameters. Higher salinity levels in aquatic environments can influence community assemblages by negatively influencing the osmoregulatory process which can reduce tadpole survival, delay metamorphosis, reduce size at metamorphoses and decrease species richness (Brock et al. 2005, Hopkins and Brodie 2015, Burraco and Gomez-Mestre 2016). Some species within the study area can persist in brackish water, such as the Marine toad (*Rhinella marinus)* and the Squirrel treefrog (*Dryophytes squirellus*), and thus are more likely to be present in sites with higher salinity relative to other species within the study area (Tipton et al. 2012). Some water bodies within the study area can also be subject to inflows of sediment because of their proximity to distributaries of the Rio Grande and to developments such as agricultural fields and suburban areas (Clover 1937, Relyea and Mills 2001, Wood and Richardson 2009, Adlassnig et al. 2013, Burraco and Gomez-Mestre 2016). Higher suspended sediment levels can reduce growth rates and survival to metamorphoses in amphibians, limiting the persistence of some species (Vignoli et al. 2007,

Wood and Richardson 2009, Cohen et al. 2012, Tavares-Junior et al. 2020). Dissolved oxygen levels play a critical role in respiration and every other physiological process in amphibians, so adequate dissolved oxygen levels are paramount for allowing amphibians to persist at a site (Wassersug and Seibert 1975, Vignoli et al. 2007 Tavares-Junior et al. 2020). Many minerals that make-up water hardness, such as calcium and magnesium, are essential micronutrients and allow for proper development and function of key physiological processes in amphibians; adequate water hardness levels can thereby help more species persist at a site (Wurts and Durburrow 1992, Stiffler 1993). A slightly alkaline aquatic environment can act as a buffer for acids, such as carbonic acid, coming into ponds helping to keep the pH balanced and more suitable for a wider range of species (Wurts and Durborrow 1992). However, high alkalinity can cause the pH to become too high which can become its own stressor on amphibians and thus limit the persistence of some species (Wurts and Durborrow 1992). Water temperature can influence many physiological processes such as the immune response and metabolic rate in amphibians; colder temperatures lessen the effectiveness of most amphibian physiological processes, while warmer temperatures tend to increase the effectiveness of most physiological processes in amphibians (Raffel et al. 2006, Terrel et al. 2013, Burraco and Gomez-Mestre 2016). Water temperature can be a result of location and seasonality but is also influenced by site specific factors, such as tree cover and water movement (Raffel et al. 2006, Terrel et al. 2013, Burraco and Gomez-Mestre 2016). In South Texas many of these water quality parameters may be significantly influencing amphibian community structure, as found in other parts of the world by Tavares-Junior et al. (2020), but no studies at this time have assessed the influence of water quality parameters on amphibian communities within South Texas.

The soils associated with water bodies can also influence community similarity across the study area. Soil can be particularly important because many amphibians burrow into the soil to seek refuge and may spend a large amount of time underground (Tipton et al. 2012, Dixon 2013). For example, many minerals found in the soil, such as boron, potassium and selenium, are essential micronutrients and adequate amounts can help support a robust community, but many of these minerals can also become toxic at higher levels, limiting the establishment of some species (Hamilton and Buhl 1990, Maier and Knight 1991, Howe 1998). Soil composition can drive community structure as well as some species have preferred soil types (Tipton et al. 2012). For example, some amphibian species found within the study area that prefer to burrow such as spadefoot toads (*Scaphiopus couchii, Scaphiopus woodhousi*) and even the Texas Toad (*Anaxyrus speciosus)* prefer sandier or loamier soils as they are easier to dig through than soils with high clay percentage (Tipton et al. 2012). Other soil properties, such as sand, clay, and organic matter percentage as while as pH and cation exchange capacity interact to influence the bioavailability of toxic compounds (Hooda and Alloway 1998, James et al. 2004). Sandier soils tend to absorb less contaminants making them more bioavailable in a water body which may result in many species not being able to persist at a site (Korte et al. 1976, Basta et al. 1993, Hooda and Alloway 1998, James et al. 2004). While soils with a higher clay and organic matter percentage tend to absorb more contaminants making them less bioavailable and allowing for more species to persist (Korte et al. 1976, Basta et al. 1993, Hooda and Alloway 1998, James et al. 2004). Higher soil pH and cation exchange capacity can also help reduce the bioavailability of contaminants, making for more suitable habitat (Lock et al. 2000, Lock and Janssen 2001, James et al. 2004). Soil composition directly affects infiltration rates and hydroperiod (Korte et al. 1976, Basta et al. 1993, Hooda and Alloway 1998, James et al. 2004) which can influence

community structure based on species-specific hydroperiod requirements (Vignoli et al. 2007, Shulse et al. 2010, Mathwin et al. 2021). Soil composition and mineral content can also affect vegetation abundance and structure in and around a water body (Colburn 2004, Ritchie and Olson 2015); vegetation structure, as mentioned in previous paragraphs, has been found to drive significant differences among amphibian communities (Vignoli et al. 2007, Cortes-Gomez et al. 2013, Tavares-Junior et al. 2020), so soil composition may be influencing amphibian community similarity within the study area based on site specific soil mineral availability and composition.

The type of water body, water quality, habitat characteristics and soil composition can not only influence broad scale community structure but the persistence of cryptic amphibian species such as the Black-spotted newt. Black-spotted newt reproduction is believed to be associated with rainfall events and recent studies suggest Black-spotted newts prefer ephemeral ponds for reproduction and maturation of juveniles (Mecham 1968, Rappole and Klicka 1991, Bare 2018, Robinson 2021, Robinson et al. 2022). Recent modeling also suggests that Black-spotted newt occurrence is negatively associated with ponds that are adjacent to paved roads and with ponds that have large fish, high salinity and large amounts of agricultural runoff (Rappole and Klicka 1991, Robinson 2021). Recent modeling also suggests higher soil copper levels and higher soil clay percentage are correlated to Black-spotted newt presence (Bare 2018, Robinson 2021). When not reproducing, Black-spotted newts are believed to burrow into the soil for refuge, so soil parameters may be important factors for their persistence (Rappole and Klicka 1991, Bare 2018, Robinson 2021). While ephemeral ponds are believed to be favored by Black-spotted newts, many other amphibians use these ponds for various stages of their life history, which can influence the persistence of the Black-spotted newt, so the amphibian community surrounding Black-spotted newts may be important (Fulbright et al. 1990, Petersen et al. 2018,

Duran 2021). For example, larger species of amphibians could predate juvenile newts, limiting their establishment (Fulbright et al. 1990, Tipton et al. 2012, Petersen et al. 2018, Duran 2021, Robinson 2021). Other amphibian species, particularly those that utilize explosive breeding, could out-compete juvenile and adult black-spotted newts for resources and thus limit their persistence at a site (Fulbright et al. 1990, Tipton et al. 2012, Petersen et al. 2018, Duran 2021, Robinson 2021). A better understanding of potential interactions with other amphibian species and what environmental factors drive Black-spotted newt persistence may help to better understand Black-spotted newt ecology, which may help to conserve the species.

Most of the water bodies that were sampled in this project were ephemeral, but each site could have differing habitat characteristics based on varied water quality and soil composition that can influence amphibian community similarities at sites across the study area. The goals of this chapter were (1) to examine amphibian community relationships across the study area, (2) to examine water quality, habitat characteristics and soil variables in relation to amphibian communities, (3) to determine if there were community relationships that were predictive of black-spotted newt presence, and (4) to examine environmental variables that might be correlated to black-spotted newt presence. The hypothesis for the first goal was that there would be distinct community groupings based on the potentially unique water quality, soil composition and habitat characteristics across the study area. The hypothesis for the second goal was that there would be environmental variables that were significantly correlated to amphibian community composition. The hypothesis for the third goal was that amphibian communities from ponds with Black-spotted newt presence would be significantly different from amphibian communities in ponds with no Black-spotted newts detected. The hypothesis for the fourth goal

was that soil characteristics and aquatic vegetation percent cover would be significant correlates for Black-spotted newt presence.

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Methods

eDNA Metabarcoding Field Detections, Targeted eDNA Assay and Traditional Survey Field Detections

The amphibian presence-absence detections from eDNA metabarcoding and the traditional visual encounter survey introduced in Chapter I were combined into one presence-absence dataset and used for this subsequent analysis. The high threshold detections from the targeted eDNA assay from Chapter I were included in this presence-absence dataset for subsequent analysis. Because the combined data had varied taxonomic resolution between sites (Chapter I), species level detections from all methods were transformed to the genus level where necessary so that the analysis could still retain the resolution and all detections used in the analysis carried the same weight. These genus-level detections for each site were then used to create the amphibian community dataset (hereafter referred to as the amphibian community dataset), which was then used for the subsequent analysis (See table A5 in chapter II appendix for detections used in analysis).

Water Chemistry and Habitat Characterization

Dissolved oxygen, conductivity and water temperature were measured from the water sample taken from each site, using a HACH HQ40D portable multi-meter water quality sonde (HACH, Loveland, CO, USA). pH and concentrations of ammonia, alkalinity and water hardness were measured using HACH water-quality test strips (HACH, Loveland, CO, USA). GPS

coordinates were collected for each site. Turbidity was estimated by using a visual ranking system that ranged from clear, low, moderate, high to very high turbidity. Clear indicating the substrate and aquatic organisms were easily visible, low indicated the substate was less visible and aquatic organisms were slightly difficult to see, moderate indicated that the substrate was difficult to see and aquatic organisms were only visible unless they were on the surface, high indicated that the substrate was not visible to the naked eye, very high indicated that the substrate, changes in depth and aquatic organisms were not visible at all. A habitat characterization was conducted for the water body and the surrounding area by visually estimating depth and the percent cover of floating, submergent, emergent vegetation and open substrate in increments of 10% throughout the water body, based on what was able to be seen during the visual encounter survey. Land use characteristics (i.*e.*undeveloped, residential, rangeland, road, row crop and combinations of above classifications when applicable) were also documented for each site.

Soil Composition Analysis

Soil samples reported in Robinson (2021) were also used for the subsequent amphibian community modeling. Soil samples were taken at three depths: surface, 30cm deep and 60cm deep. Soil samples were taken from at least two different points surrounding a water body, and pooled to create a composite sample. Composite soil samples were then sent to the Water and Forage Testing Laboratory at Texas A&M AgriLife Extension Soil Service for analysis. Soil parameters measured included pH, conductivity (umho/cm), nitrate (ppm),phosphorous (ppm), potassium (ppm), calcium (ppm), magnesium (ppm), sulfur (ppm), sodium (ppm), iron (ppm), zinc (ppm), manganese (ppm), copper (ppm), boron (ppm), sand percentage, silt percentage, clay percentage, organic matter percentage, textural class, Sodium Adsorption Ratio (SAR), Soluble

Sodium Percent (SSP). Four sites did not have any soil samples: PHR Bullrush pond (Site 22), Sabal Palm Sanctuary Side Pond (Site 18), Old Military Hwy Pond 5 (Site 16) and Live Oak Pipeline Pond (Site 23). These four sites were excluded from subsequent soil-related analysis. Southmost Black Willow Resaca (Site 21) did not have a 60cm sample; the surface and 30cm samples from Southmost Black Willow Resaca (Site 21) were used in the subsequent analysis.

Statistical Analysis

To examine amphibian community relationships among all sites, a Bray-Curtis resemblance matrix was created for the amphibian community dataset. A distance-based test of homogeneity of variance (PERMDISP) was used to determine the homoscedasticity of dispersions for the Bray-Curtis resemblance matrix; the resemblance matrix was deemed homoscedastic. A group average cluster ordination method with 9999 permutations was then conducted on the resemblance matrix to visualize site relationships. A SIMPROF test was also conducted with the cluster analyses to test for distinct community groupings. A cluster ordination and SIMPROF test were chosen to visualize and test for site differences because all sites did not have enough replicates required for other multi-variate tests such as ANOSIM or PERMANOVA (i.*e.* more than 5 repeat visits per site). All statistical analyses was conducted using Primer e (v 7 Quest Research Limited; Clarke and Gorley 2006)

Ammonia values for Sabal Palm Sanctuary Resaca Blind 11/9/20 and PHR Bullrush Pond 2/27/20 samples were not collected nor was a pH value for PHR Bullrush Pond 2/27/20, so a missing routine was used to estimate the missing values. Draftsmen's plots were then used to determine any correlations and skewness between water quality and habitat variables. Ammonia, floating vegetation percentage cover, and submergent vegetation percentage cover were skewed,

so a square-root transformation was applied. Following transformations, the data set was then normalized using the normalize routine in Primer-e. A principal component analysis (PCA) was then conducted on normalized water-quality and habitat characteristic data to visualize environmental differences among sites and determine what variables were driving variation among sites. The same PCA was displayed with black-spotted newt presence as the label to visualize environmental differences between Black-spotted newt positive (BSN+) and Black-spotted newt negative (BSN-) sites. To examine what environmental variables explained amphibian community patterns among all sites a Bio-Env+Stepwise (BEST) test was conducted using a Spearman rank coefficient with the site as the factor using the normalized water quality and habitat characteristic data and the amphibian community dataset. A BEST test was also run on the amphibian community dataset using black-spotted newt presence as a factor to examine what water quality and habitat characteristics explained patterns between BSN+ and BSN- amphibian communities, thus identifying what variables may be important for Black-spotted newts. A permutation test with 999 permutations was conducted along with each BEST test to test for the significance of correlates.

Draftsmen's plots were used to examine correlation and skewness in soil composition. Conductivity (umho/cm), nitrate, (ppm), sulfur (ppm). sodium (ppm) and organic matter percentage were skewed, so a square-root transformation was applied to each variable. Following transformations, the data set was normalized using the normalize routine in Primer-e. A PCA was conducted on the normalized soil composition data set to visualize soil composition among sites and to determine which soil variables were driving variation among sites. The same PCA was displayed with black-spotted newt presence as the label to visualize differences in soil composition among BSN+ and BSN- sites. To examine which soil variables explained patterns

among amphibian communities for each site, a BEST test was conducted for each soil sample depth and the amphibian community dataset using a spearman rank coefficient with the site as the factor; the variable combination that had the highest correlation value out of the three depths was chosen. A BEST test was also conducted for each soil sample depth and the amphibian community dataset using a spearman rank coefficient with black-spotted newt presence as the factor to determine which soil variables explained patterns between BSN+ and BSN- amphibian communities; the variable combination that had the highest correlation value out of the three depths was chosen. A permutation test with 999 permutations was conducted along with each BEST test to test for the significance of correlates.

To test if Black-spotted newt positive (BSN+) amphibian communities (n=7) differed from black-spotted newt negative amphibian communities (BSN-) (n=17), Black-spotted newts were first removed from the dataset so that they would not influence the analysis. A Bray-Curtis resemblance matrix was created for the amphibian community dataset with Black-spotted newts removed. PERMDISP was also used on this Bray-Curtis resemblance matrix with Black-spotted newts removed and the matrix was deemed homoscedastic. A bootstrapped (150 bootstraps) metric multidimensional scaling ordination (mMDS) was then conducted on the Bray-Curtis resemblance matrix with black-spotted newts removed to visualize differences between BSN+ and BSN- communities. A one-way unordered Analysis of Similarity (ANOSIM) was conducted using Black-spotted newt presence as an unordered factor as a formal test to examine if amphibian communities at BSN+ sites were significantly different from amphibian communities at BSN- sites. ANOSIM was suitable for testing differences between BSN+ and BSN- amphibian communities because there were more than five sites in each category, satisfying the requirements of the test.

Results

Amphibian Community Analysis

The hierarchical group average cluster of amphibian communities showed that all sites formed one large grouping with no significant sub-groupings (Pi: 1.96, p:0.436) (Fig 8)

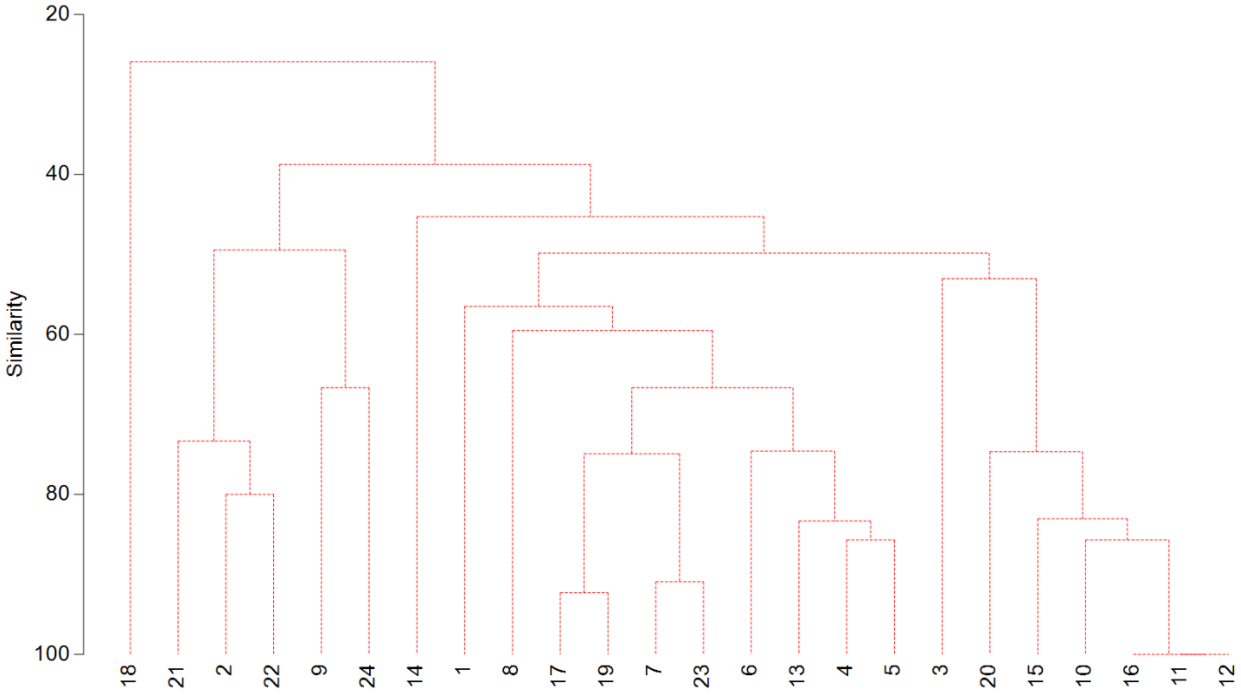

Figure 8. Group average hierarchical cluster analysis of amphibian communities. Detections used in analysis are from eDNA metabarcoding, the targeted eDNA assay (high threshold) and traditional visual encounter surveys. The site number is on the branch tips. Red lines indicate non-significant differences between groups.

Amphibian Community Environmental Relationships

The principal component analysis (PCA) showed that the major contributors to PC1 (21.8% of total variation) for water quality and habitat characteristics included longitude, dissolved oxygen, water temperature, substrate percent cover and emergent vegetation percent cover (Table 9). The PCA also showed that the major contributors to PC2 (19.4% of total

variation) were latitude, pH, conductivity, hardness and alkalinity (Table 9). BEST tests identified the variable combination with the highest correlation to community structure as dissolved oxygen (mg/L), floating vegetation percent cover, submergent vegetation percent cover, emergent vegetation percent cover and land-use characteristics with a non-significant ($p=0.825$) correlation value of 0.628. Some samples of the same sites are grouped close together, indicating that water-quality and habitat characteristics were similar between repeat visits (Fig. 9). Most samples that came from sites in the northern range are grouped at the bottom of the plot (Fig. 9). Most samples that came from the southern range are grouped at the top of the plot (Fig. 9). While there were two sites that were distinct from the rest of the group, Sabal Palm Sanctuary Resaca Blind (Site 2) and PHR Bullrush Pond (Site 22) (Fig. 9). Based on the PCA, most southern sites had higher submergent vegetation percent cover, while northern sites had higher floating vegetation percent cover (Fig. 9).

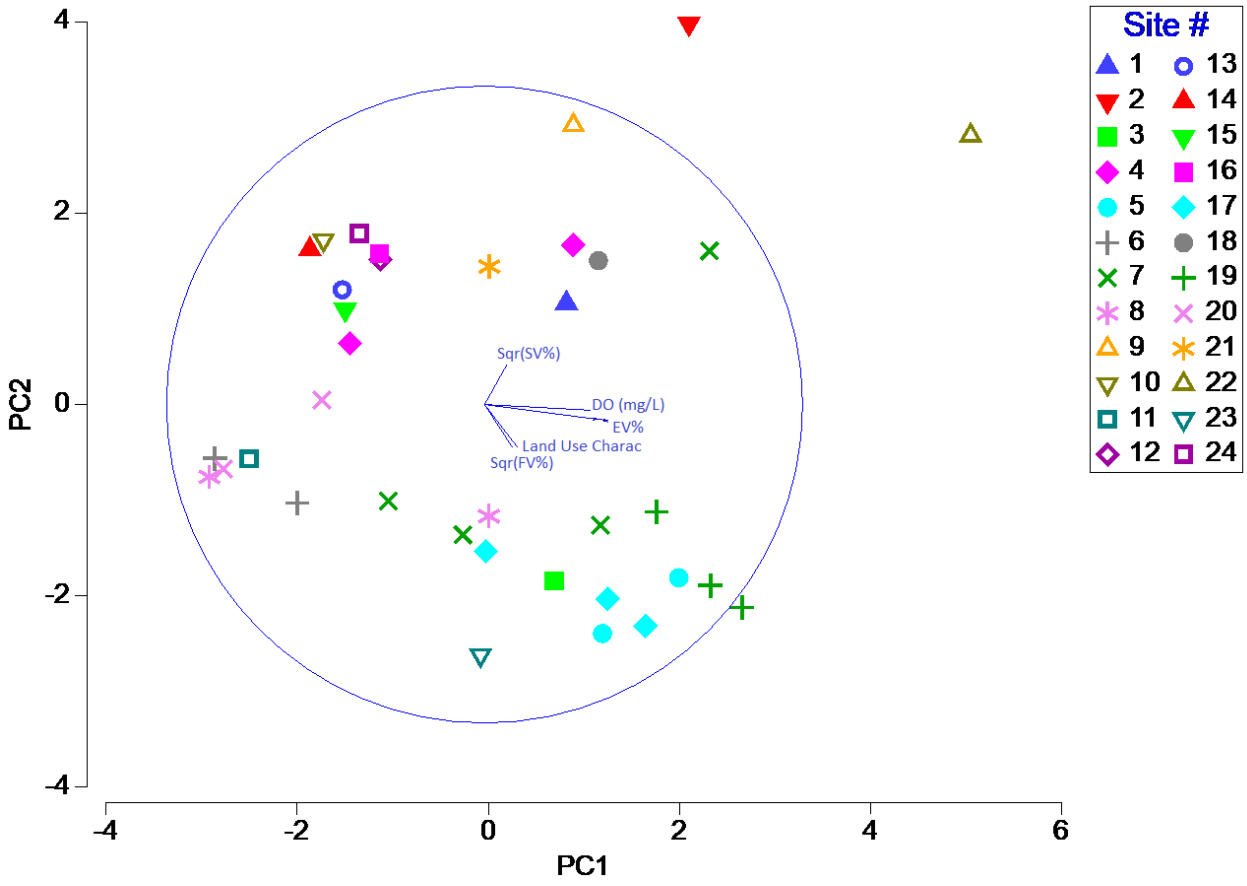

Figure 9. Principal component analysis of water quality and habitat data taken during eDNA sample collection. Refer to Figure 1 for site location. Variable vectors shown are explanatory variables identified by the BEST test. Sqr(SV%) indicates the transformed submergent vegetation percent cover, EV% indicates emergent vegetation percent cover, Sqr(FV%) indicates the transformed floating vegetation percent cover.

Table 9. Eigenvectors from PC1 and PC2 for all variables involved in the Water Quality and Habitat characteristics PCA**.** Sqr(Ammonia) indicates the transformed ammonia values**.** Sqr(SV%) indicates the transformed submergent vegetation percent cover, EV% indicates emergent vegetation percent cover, Sqr(FV%) indicates the transformed floating vegetation percent cover.DO (mg/L) indicates dissolved oxygen (mg/L).

Eigenvectors		
Variable	**PC1**	**PC2**
Latitude	0.258	-0.386
Longitude	0.33	-0.108
pH	0.078	0.328
Dissolved Oxygen (mg/L)	0.334	-0.018

Table 9, cont. Eigenvectors from PC1 and PC2 for all variables involved in the Water Quality and Habitat characteristics PCA**.** Sqr(Ammonia) indicates the transformed ammonia values**.** Sqr(SV%) indicates the transformed submergent vegetation percent cover, EV% indicates emergent vegetation percent cover, Sqr(FV%) indicates the transformed floating vegetation percent cover.DO (mg/L) indicates dissolved oxygen (mg/L).

Eigenvectors		
Variable	**PC1**	**PC2**
Temperature (°C)	-0.307	-0.172
Conductivity (µs/cm)	0.259	0.375
Sqr(Ammonia)	-0.252	0.131
Hardness (ppm)	0.027	0.481
Alkalinity (ppm)	0.129	0.474
Turbidity	-0.17	-0.113
Depth (m)	-0.254	0.135
Sqr(FV %)	0.088	-0.135
Sqr(SV %)	0.07	0.125
EV %	0.402	-0.053
Substrate %	-0.432	0.05
Land Use Characteristics	0.104	-0.135

The PCA for soil composition showed that the major contributors to PC1 (30.6% total variation) were copper, potassium, iron, sand percentage, conductivity, sulfur, boron and clay (Table 10). The PCA for soil composition also showed that the major contributors to the PC2 (23.0% total variation) were sodium absorption ratio, pH, sodium, zinc, soluble sodium percentage and magnesium (Table 10). Based on the PCA, most sites were grouped together in the plot with slight variation between depths (Fig 10). The variable combination identified by the BEST tests with the highest correlation to community structure was calcium, sulfur, sodium, manganese and boron with a significant ($p=0.024$) correlation value of 0.506.

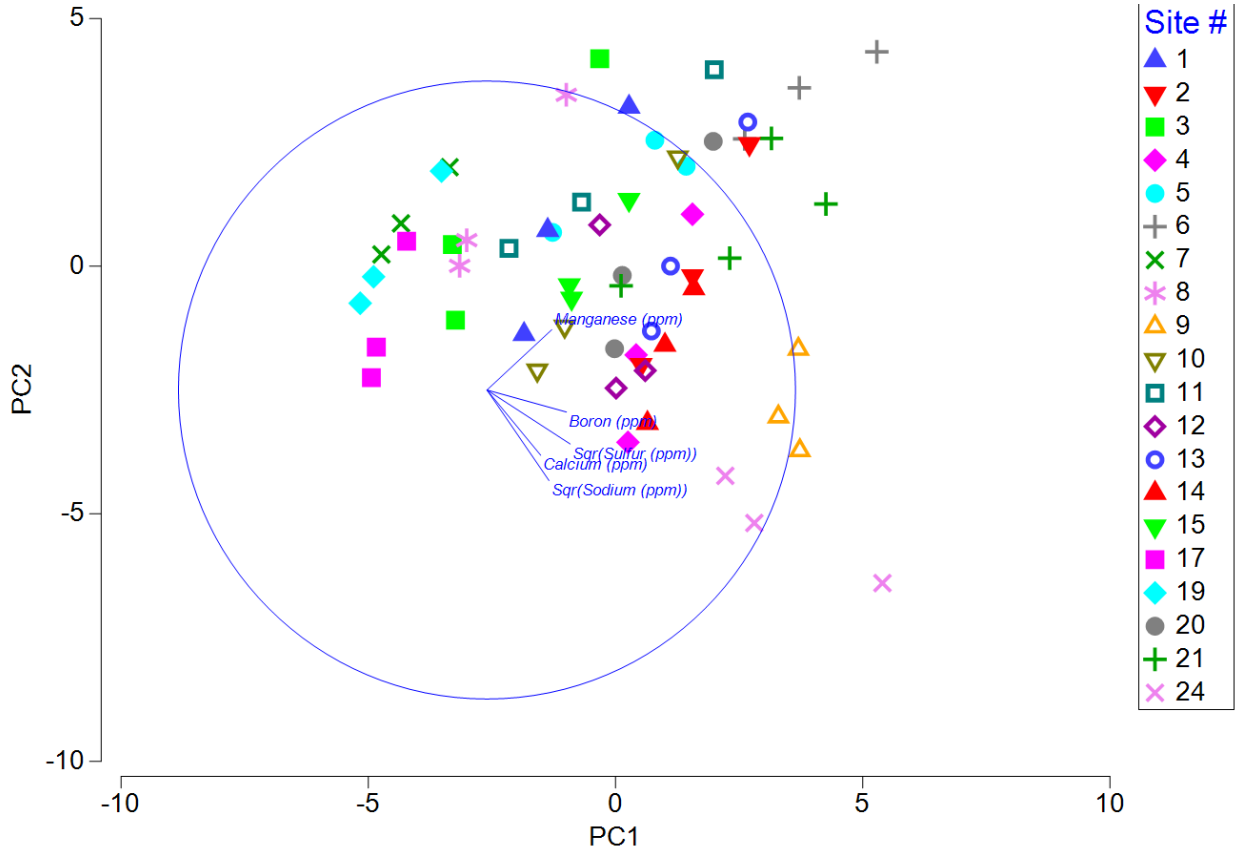

Figure 10. Principal component analysis of normalized soil data of sites sampled. Variable vectors shown are explanatory variables identified by the BEST test. Sqr(Sulfur ppm) indicates the transformed sulfur variable.

Table 10. Eigenvectors for all soil variables involved in soil composition PCA. Sqr at the start of a variable name indicates that it was square-root transformed prior to the PCA.

Eigenvectors		
Variable	**PC1**	**PC2**
Latitude	-0.245	0.08
Longitude	-0.133	-0.082
Depth (cm)	-0.096	-0.218
pH	-0.071	-0.272
Sqr(Cond. (umho/cm))	0.209	-0.207
Sqr(Nitrate (ppm))	0.159	0.22
Phosphorous (ppm)	0.194	0.241
Potassium (ppm)	0.291	0.083
Calcium (ppm)	0.174	-0.211
Magnesium (ppm)	0.262	-0.241
Sqr(Sulfur (ppm))	0.27	-0.175

Table 10, cont. Eigenvectors for all soil variables involved in soil composition PCA. Sqr at the start of a variable name indicates that it was square-root transformed prior to the PCA.

Eigenvectors		
Variable	**PC1**	**PC2**
Sqr(Sodium (ppm))	0.201	-0.293
Iron (ppm)	0.22	0.203
Zinc (ppm)	0.132	0.272
Manganese (ppm)	0.21	0.195
Copper (ppm)	0.275	0.134
Boron (ppm)	0.258	-0.071
Sand %	-0.281	0.085
Silt %	0.154	-0.167
Clay %	0.27	-0.016
Sqr(Organic matter %)	0.236	0.225
Textural class	-0.049	-0.091
SAR (Sodium Adsorption Ratio)	0.134	-0.316
SSP (Soluble Sodium Percent)	-0.032	-0.345

Black-Spotted Newt Amphibian Community Relationships

The ANOSIM of BSN+ and BSN- amphibian communities had a low R-value and revealed no significant difference between BSN+ and BSN- amphibian communities (R=0.139, p=0.089). The bootstrapped mMDS likewise revealed no distinct differences between BSN+ and BSN- communities on the bootstrapped mMDS plot (Fig. 11). However, the 95% confidence interval of the BSN+ sites was quite large, indicating higher variation for amphibian communities at BSN+ sites (n=7) relative to BSN- (n=17) sites (Fig. 11).

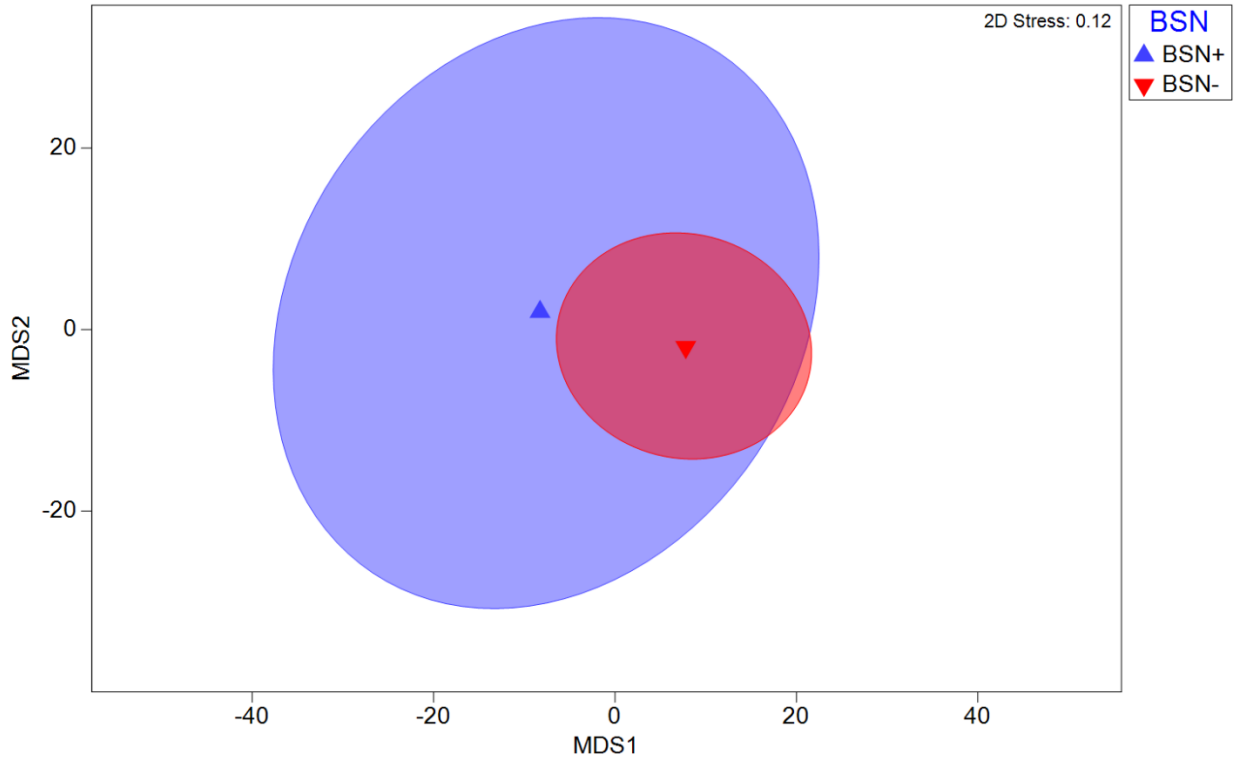

Figure 11. Bootstrapped metric multidimensional scaling (mMDS) ordination plot of black spotted newt positive (BSN+) and black-spotted newt negative (BSN-) amphibian communities. Detections used in this analysis are from from eDNA metabarcoding and traditional survey methods. Black-spotted newt detections were not included in the data matrix.

Black-Spotted Newt Environmental Relationships

For water-quality and habitat characteristic data, the PCA did not show any distinct groupings between most BSN+ and BSN- sites (Fig 12). BEST tests identified the variable combination that had the highest correlation to community structure between BSN+ and BSN- communities as dissolved oxygen (mg/L), conductivity (µs/cm), and submergent vegetation percentage cover with a low non-significant ($p=0.723$) correlation value of 0.247. The PCA showed that most of the BSN+ samples had higher conductivity and submergent vegetation percent cover relative to most of the BSN- samples, two BSN+ samples had the opposite pattern (Fig 12).

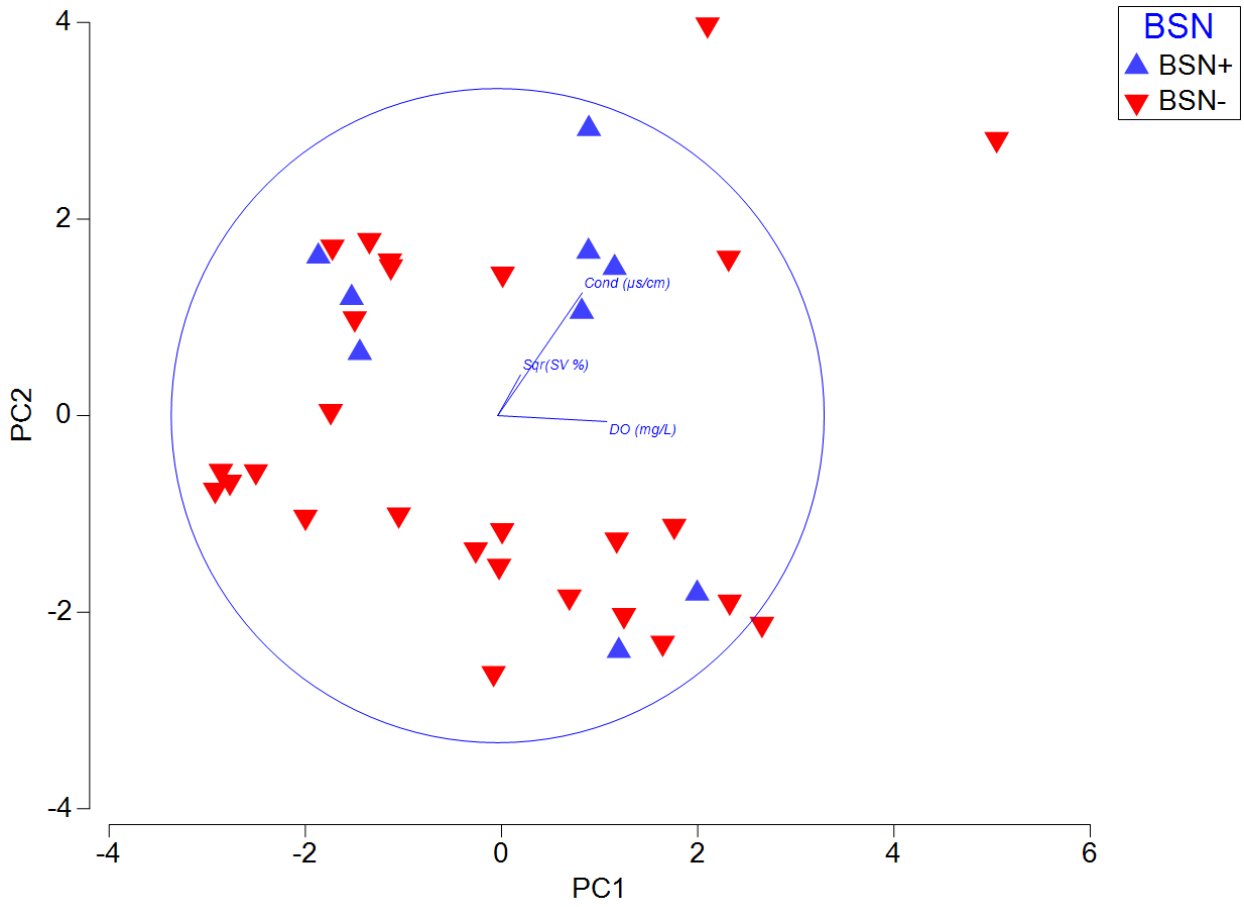

Figure 12. Principal component analysis of normalized water-quality and habitat data taken during eDNA sample collection using Black-spotted newt presence as the label. Variable vectors shown on plot are explanatory variables identified by BEST test. Sqr(SV%) indicates the transformed submergent vegetation percent cover. DO (mg/L) indicates dissolved oxygen (mg/L).

The PCA for soil data showed BSN+ and BSN- sites were intermixed with one another with no distinct groupings (Fig. 13). The soil variable combination identified by the BEST tests with the highest correlation to community structure between BSN+ and BSN- communities was the combination of iron (ppm), boron (ppm), sand percentage, textural class and sodium adsorption ratio with a significant (p=0.0064) correlation value of 0.695.

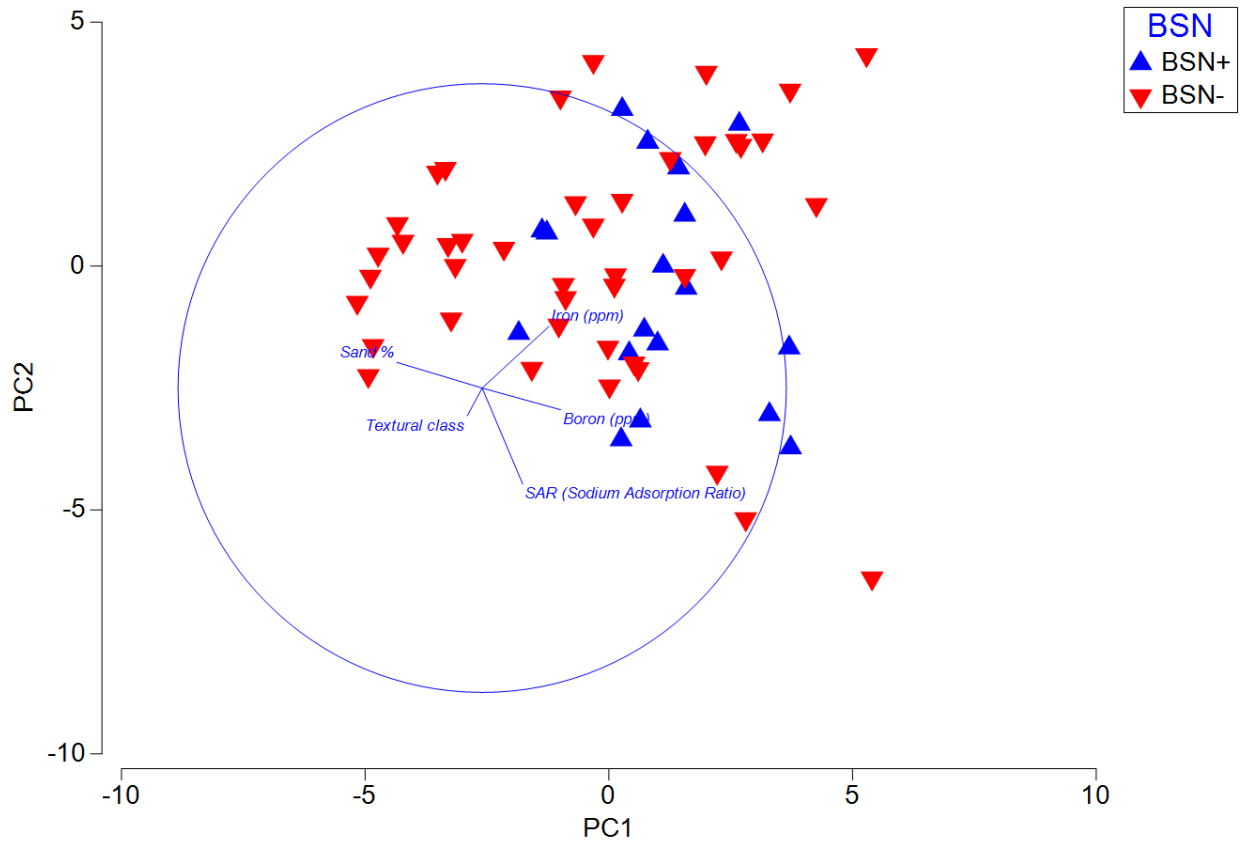

Figure 13. Principal component analysis of normalized soil data using Black-spotted newt presence as the label. Variable vectors shown are explanatory variables identified by the BEST test.

Discussion

Amphibian Community Relationships

The hypothesis that there would be distinct groupings of amphibian communities, was not supported because there were no distinct community groupings (Fig 8). The lack of significant groupings of amphibian communities among all sites could be due to favoring ephemeral water-bodies, as there are only so many species, within the study area, that are able to persist at ephemeral ponds (Tipton et al. 2012, Dixon 2013, Petersen et al. 2018, Duran 2021) making the potential community members of each site limited. To observe distinct differences in amphibian assemblages among sites may require including other types of water bodies (i.e.,

permanent ponds and flowing bodies of water) in future surveys. The likelihood of differences being found is likely higher because many amphibian species found within the study area were not detected in field samples in this study; those undetected species such as *Anaxyrus punctatus, Pseudacris streckeri* and *Acris blanchardi* are reported from permanent or flowing water bodies (Tipton et al. 2012, Dixon 2013, Petersen et al. 2018, Duran 2021). Because the dataset was in presence-absence form, the effect of such a strong transformation may have also led to the lack of significant differences among communities. However, presence/absence is a more conservative approach for eDNA surveys because estimating relative abundance from eDNA metabarcoding has some confounding factors, such as primer sets having different affinity for each species and PCR polymerases favoring sequences with specific GC content, that can skew observed DNA concentrations from the true biomass of a pond (Fonseca 2018, Nicholas et al. 2018).

Amphibian Community Environmental Relationships

The second hypothesis that there would be significant environmental correlates to amphibian community structure was not supported with regards to water-quality and habitat characteristics because BEST tests for water quality and habitat characteristics were not significant (p=0.825) despite dissolved oxygen, floating vegetation percent cover, submergent vegetation percentage cover, emergent vegetation percentage cover, and land-use characteristics together having high correlation (0.628). The lack of significant correlation for water-quality and habitat characteristic variables could indicate that there were other water-quality and habitat variables that were not measured that were more explanatory, such as habitat fragmentation, pathogen prevalence or even presence of contaminants. Habitat fragmentation has been linked to die-offs of salamander species in parts of the US (Farmer et al. 2017) and so could be influencing

persistence of other amphibians within South Texas. A ranavirus strain has been identified in a native amphibian the spotted chorus frog (*Pseudacris clarkii)* (Torrence et al. 2010) pathogen prevlance could also be significantly influencing amphibian community assemblages as seen in other parts of the world (Stuart et al. 2004, Wake and Vrendenberg 2008, Pimm et al. 2014). With the large amount of agriculture in South Texas these ephemeral ponds could be subject to runoff from pesticides and herbicides (Garcia et al. 2001), pesticides and herbicides have been found to negatively affect amphibian physiology (Frisbie and Wyman 1991, Brady and Griffins 1995, Muenz et al. 2006), and could be also influencing amphibian community structure within South Texas as well. The effect of habitat fragmentation, pathogen prevalence and contamination on amphibian community assemblages should be investigated further, so that the most explanatory water quality and habitat variables may be identified.

Although the water-quality and habitat characteristics variable combination identified by the BEST test was not significant, the combination of variables still offers some interesting inferences on what environmental variables may be driving amphibian community structure. Numerous species recorded in this study are known to be dependent on different vegetation types and overall habitat structure (Tipton et al. 2012). Because of species-specific dependency on vegetation types, having floating, submergent and emergent vegetation as well as land use characteristics as explanatory variables for amphibian community structure from site to stie makes logical sense. Floating, emergent and submergent vegetation percentage cover as well as land use characteristics combine to make different habitat structures and complexity in and around ephemeral ponds that can allow for different breeding sites for adult amphibians and different areas of refuge for tadpoles (Colburn 2004 Vignoli et al. 2007, Ritchie and Olson 2015, Tavares-Junior et al. 2020). For example, *Siren intermedia texana* prefer heavily vegetated water

bodies with a large amount of submergent vegetation (Tipton et al. 2012); as one of the most commonly detected amphibians, they could have had a large influence on those correlates being identified. In contrast, members of the *Dryophytes* genus can climb on emergent vegetation to forage and rest (Tipton et al. 2012), so *Dryophytes* detections may have also influenced these correlates being identified as well. Other reasons why aquatic vegetation percent cover is a logical explanatory variable for amphibian community structure could be due to most amphibians present in the community analysis laying their eggs on aquatic vegetation (Tipton et al. 2012);therefore, adequate amounts of aquatic vegetation can help successful reproduction and hatching of eggs. Without adequate amounts of aquatic vegetation amphibians would not have not be able to lay their eggs on anything and a amphibian community would not be able to persist, which further shows why aquatic vegetation is a logical explanatory variable. Submergent vegetation is also used by other aquatic species, such as aquatic invertebrates, which many amphibians found in the study area prey upon (Tipton et al. 2012).; adequate amounts of prey can also help to support a robust community Dissolved oxygen is another logical explanatory variable for amphibian community structure as it plays a major role in respiration and thereby every other physiological process in amphibians (Wassersug and Seibert 1975), so levels of dissolved oxygen may influence what species are able to persist at a site and thereby influence the community assemblage at each site.

The third hypothesis that there would be significant environmental correlates to amphibian community structure was supported with regard to soil composition by the BEST tests that found the most explanatory soil variables for amphibian community structure were the combination of calcium, sulfur, sodium, manganese, and boron with a correlation value of 0.506 ($p=0.024$). All BEST test correlates are essential micronutrients that support a variety of

physiological processes in amphibians as well as in the vegetation in and out of the water body they inhabit (Jordan and Ensminger 1959, Greenwald 1972, Wurts and Durburrow 1992, Stiffler 1993, Warne 2014). For example, manganese and calcium foster a slightly alkaline pH, which can reduce the bioavailability of contaminants and make for a more suitable habitat (Korte et al. 1976, Basta et al 1993, Hooda and Alloway 1998, Lock et al. 2000, Lock and Janssen 2001, James et al 2004). Manganese and calcium can also occupy the same binding sites as toxic compounds and thus can help increase the toxicity thresholds of amphibians (Bradley and Sprague 1985, Lauren and Mcdonald 1986, Wurts and Durburrow 1992, Linbo et al. 2009). The minerals identified by the BEST test can also influence how well vegetation can grow above ground, thereby affecting the percentage cover of emergent, submergent, and floating vegetation cover (Ritchie and Olson 2015, Colburn 2004), which were correlates identified in the water-quality and habitat characteristics BEST test. Because vegetation in and around ephemeral ponds does play a role in amphibian reproduction and offers areas of refuge, sites that have adequate soil nutrients support growth of vegetation that is important for a variety of species found within the study area (Colburn 2004, Vignoli et al. 2007, Tipton et al. 2012, Cohen et al. 2012, Ritchie and Olson 2015, Tavares-Junior et al 2020). Amphibians could be selecting habitats that have optimal soil mineral composition, and thus optimal vegetation profiles, or are not able to persist in habitats that do not have adequate vegetation profiles due to low soil mineral levels (Korte et al 1976, Basta et al 1993, Hooda and Alloway 1998, Lock et al 2000, Lock and Janssen 2001, James et al 2004). Although not all sites had all three soil sample depths, most sites did, so the BEST test is still robust and the variables identified are likely important correlates for future study.

Both PCAs showed environmental relationships among sites with many of the variables measured contributing a small amount of variation among all the sites to with PC1 and PC2 of the water-quality and habitat characteristics PCA accounting for 40% of the total respective variation and PC1 and PC2 in the soil composition PCA accounting for 56.3% of the total variation. In general, northern sites and southern sites formed groups based on the water-quality and habitat characterizes (Fig. 9), showing that water-quality and habitat characteristics within the study area could governed by latitude, which has been found on a large scale. For example, different latitudes have different rainfall amounts and temperatures that create different climate zones and vegetation structures (Peel et al. 2007). The patterns revealed in this study warrant further examination with a more finite approach to examining latitude as it relates to water-quality and habitat characteristics within the study area. In contrast, there was no distinct pattern of sites based on the PCA of soil samples, as most sites were clumped together with a few outliers, suggesting that the variation in soil composition among all sites is minimal at ephemeral ponds in this region (Fig. 10).

Black-Spotted Newt Community Analysis

No strong differences in Black-spotted newt positive (BSN+) and black-spotted newt negative (BSN-) amphibian communities were observed with the ANOSIM ($R=0.139$, $p=0.089$) or mMDS plot (Fig 11), thus the hypothesis that there would be distinct differences between BSN+ and BSN- communities was not supported. The lack of significant differences between BSN+ and BSN- sites could indicate that the surrounding amphibian community for all sampled sites was adequate for Black-spotted newts; meaning they could theoretically persist at any site, but they are only present at a select few sites for reasons that may be unrelated to the surrounding

amphibian community. Most sites used in this study came from ephemeral ponds that were previously identified as black-spotted newt sites or potential black-spotted newt sites by Robinson et al. (2022), which further supports that Black-spotted newts could persist at any of the sampled sites but factors outside of the amphibian community may be driving their presence. Based on the cryptic nature of Black-spotted newts and previous difficulties detecting them (Judd 1985, Rappole and Klicka 1991, Robinson et al. 2022) it could be possible that false negatives occurred at some of the BSN- sites, so the presence of Black-spotted newt should be investigated further at all sites. If more permanent water bodies or flowing water bodies were sampled, stronger differences between BSN+ and BSN- amphibian communities may have been present because Black-spotted newts are believed to prefer ephemeral ponds (Mecham 1968, Robinson et al. 2022) and some amphibian species, such as *Acris blanchardi* and *Anaxyrus punctatus*, found within the study prefer permanent or flowing water bodies over ephemeral water bodies (Tipton et al 2012). However, many non-amphibian species use these ephemeral ponds within the study area, such as predatory invertebrates (Pattillo et al. 1997, Tipton et al. 2012), reptiles (Boundy 1994, Duran 2021), wetland birds (Fulbright et al. 1990) and mammals (Fulbright et al. 1990), use these ephemeral ponds. To determine which community factors drive Black-spotted newt presence, one may also have to try to detect all potential taxa that could use these ponds because amphibians do not interact amongst themselves in a vacuum but experience predation and competition from other taxa. Other amphibians, such as bullfrogs, and different types of snakes and turtles have been found to predate the Black-spotted newt's congener the Eastern newt (*Notophthalmus viridescens*) (Tipton et al. 2012). The Eastern newt has been found to have toxic skin secretions, which the Black-spotted newt is believed to produce as well; if bullfrogs and certain snakes and turtles can tolerate Eastern newt toxic secretions they may be

able to tolerate Black-spotted newt skin secretions as well (Tipton et al. 2012) Potential amphibian and non-amphibian predators should be investigated further to better understand Black-spotted newt community interactions and the factors that limit Black-spotted newt persistence (Tipton et al. 2012).

Black-spotted Newt Environmental Relationships

The explanatory water-quality and habitat characteristics variables among BSN + and BSN- communities identified by the BEST test did not support the hypothesis that aquatic vegetation percentage cover variables would significantly correlate to Black-spotted newt presence; the combination of variables identified by the BEST was dissolved oxygen, conductivity and submergent vegetation percent cover with a non-significant low correlation value (0.247; p=0.723). Although the variable combination identified by the BEST test was not significant, a portion of aquatic vegetation was identified as a correlate and the other correlates in the group do offer some interesting inferences for future study. Dissolved oxygen and conductivity have been shown to influence many physiological processes for almost all organisms, so those variables likely influence black-spotted newt physiology as well (Wassersug and Seibert 1975, Brock et al. 2005, Hopkins and Brodie 2015, Burraco and Gomez-Mestre 2016). The BEST test correlates also agree with previous literature that suggests submergent vegetation is important for Black-spotted newts (Mecham 1968). The low BEST test correlation for water quality and habitat characteristics along with the lack of strong differences between BSN+ and BSN- on the PCA (Fig. 12) could also indicate there are other factors that were not measured in this study that influence their persistence such as habitat fragmentation or pathogen prevalence that have caused extirpations in other salamander species (Stuart et al. 2004, Wake and Vrendenberg 2008, Pimm et al. 2014, Farmer et al. 2017). For example, habitat

fragmentation is believed to be one of the major causes of expiration in a congener of the Black-spotted newt, the Striped newt (*Notophthalmus perstriatus*), which is found in Florida and Georgia (Farmer et al. 2017). Land development, such as roads, agricultural fields, or suburban developments, can isolate breeding populations of striped newts, and eventually, populations can die off on their own (Farmer et al. 2017). Something similar could be the reason why Black-spotted newts are so sporadically distributed in South Texas, which is under rapid development that has caused much of the habitat to change in the last few decades and thus could cause greatly limited connections among black-spotted newt populations (Garcia et al. 2001, Robinson et al. 2022). Black-spotted newt populations could have suffered isolated die-offs in recent decades in response to pathogens as has been reported for *Notophthalmus* species, which are susceptible to a number of different pathogens, i.e., ranaviruses, parasites, fungal and fungal-like pathogens, (Farmer et al. 2017, Jancovich et al. 2001; Green et al. 2002; Raffel et al. 2006, 2008, Duffus et al. 2008). A recent study found a novel ranavirus that uses the spotted chorus frog (*Pseudacris clarkii),* a potentially sympatric amphibian to the Black-spotted newt, as a host, so ranavirus could potentially be transmitted to Black-spotted newts and be detrimental to their persistence (Torrence et al. 2010). Although there is no documented evidence of pathogen causing die-offs of black-spotted newts within the study area, the potential for pathogens affecting Black-spotted newts should be investigated further.

Significant explanatory soil variables among BSN+ and BSN- communities identified by the BEST tests were iron, boron, sand percentage, textural class, and sodium adsorption ratio with a correlation value of 0.695 ($p=0.0064$), which supported the hypothesis that significant soil characteristics would be significant correlates to Black-spotted newt presence. Soil variables did have a much higher correlation value compared to the water-quality and habitat characteristics

correlations, which could suggest that soil parameters may play a more important role in black-spotted newt life history as they can spend a large period of time underground in the soil (Rappole and Klicka 1991, Bare and Kline 2017). However, there were no distinct patterns between BSN+ and BSN- sites on the soil composition PCA (Fig. 13), which could indicate there are other soil factors that were not measured driving Black-spotted presence. Nevertheless, the combination of variables identified by the BEST tests offer some interesting inferences. For example, iron and boron are essential micronutrients for many amphibians and thus may be important for black-spotted newts as well (Hamilton and Buhl 1990, Maier and Knight 1991, Howe 1998). How well sodium, another essential micronutrient, can absorb into the soil can influence its bioavailability, which may influence black-spotted newt physiology (Hamilton and Buhl 1990, Maier and Knight 1991, Howe 1998). The availability of soil minerals, such as iron, boron and sodium can also influence how well vegetation can grow in and around ephemeral ponds as previously mentioned (Colburn 2004, Ritchie and Olson 2015). Vegetation may play a role in black-spotted newt reproduction and could offer areas of refuge, so certain soil mineral ranges may suit the vegetation structure that black-spotted newts prefer (Mecham 1968). Sand percentage and textural class in soils combine to affect infiltration rate and thus hydroperiod (Lock et al. 2000, Lock and Janssen 2001, James et al. 2004). Hydroperiod may be important for black-spotted newts as a pond needs to remain filled long enough for juveniles to develop but not long enough to where larger predators may become established (Mecham 1968, Robinson 2021). Ephemeral ponds have been suggested as preferential Black-spotted newt reproduction sites because of the limited probability of larger predators becoming established, so sand percentage and textural class may be very important factors for Black-spotted newts (Mecham 1968). Sand percentage and textural class can also influence the bioavailability of contaminants, so black-

spotted newts could be selecting less contaminated sites or they are only able to persist in less contaminated sites (Lock et al. 2000, Lock and Janssen 2001, James et al. 2004).

CHAPTER III

SUMMARY AND CONCLUSIONS

The overall goals of this project were: (1) to validate an eDNA metabarcoding assay on tissue extracts and field eDNA samples, (2) to compare detection capabilities of eDNA metabarcoding with traditional visual encounter surveys and a targeted eDNA assay, (3) to provide foundational information on community assemblages, and (4) to examine foundational amphibian community and environmental relationships. Other goals included (5) examining community differences and important environmental variables for a species of conservation concern, i.e., Black-spotted newts. The eDNA metabarcoding assay was validated on almost all native amphibian species, amplifying tissue extracts of 33/33 species and Sanger sequencing 32/33 species with theoretical distributions within the study area. Difficulty amplifying and Sanger sequencing some tissue extracts could give evidence to some primer blind spots. A number of different amphibians, as well as many other taxa, were detected in field samples. The eDNA metabarcoding offered more species-level detections and detected significantly more amphibian taxonomic units compared to traditional visual encounter surveys. Although traditional surveys were able to identify some species that eDNA metabarcoding could not, eDNA metabarcoding was less efficient at detecting Black-spotted newts compared to traditional survey methods and the targeted eDNA assay. The most effective tool for detecting Black-spotted newts was the targeted eDNA assay. Therefore, to provide the most accurate characterization of community assemblages and thereby accurate community relationships,

methods such as eDNA metabarcoding, targeted eDNA assays, and traditional visual encounter surveys should be combined. The variety of taxa detected in field samples does also suggest a broader application of this eDNA metabarcoding primer set to other taxa. In the future, increasing metabarcoding primer specificity, using other genetic regions, and developing blocking primers for non-target taxa may help to further increase the number of amphibian taxonomic units detected using eDNA metabarcoding. Going forward, the logical next steps would be to develop a metabarcoding primer set that has the maximum taxonomic coverage and works equally well on all target species or to attempt to identify all potential taxonomic blind spots of a chosen metabarcoding primer set so that other tools can be used to supplement the metabarcoding assay. Tools such as targeted eDNA assays for rarer species or species that the metabarcoding primer set has a lower affinity as well as implementation of call surveys, camera trapping, or other less invasive traditional survey methods.

No significant amphibian community groupings among all study sites were detected in the analysis of the study data. The lack of distinct site groupings may be due to the focus on ephemeral water bodies in the range of the Black-spotted newt and the limited number of potential amphibians that can persist in those ephemeral ponds. However, there were a number of explanatory water-quality and habitat characteristics and soil parameters that were correlated with amphibian community structure, suggesting an interplay between water quality, habitat structure, and soil composition may influence amphibian community assemblages. Future avenues of study could include experiments to see the physiological effects of different water-quality parameters and soil composition with a variety of native amphibian species to determine the direct role water quality and soil composition have in local amphibian physiology and persistence.

No strong differences between black-spotted newt positive and negative amphibian communities were observed based on the bootstrapped mMDS plots and the ANOSIM. Soil variables had a higher correlation value relative to water quality and habitat characteristics, which could suggest that soil is more important for black-spotted newts. While all correlates identified by each BEST test were not significant, they still may help add to the number of environmental variables that are believed to influence Black-spotted newt persistence, such as a pond's distance to paved roads, soil clay percentage, soil copper percentage, salinity and agricultural runoff. Future avenues of study for environmental variables may include examining the effects of varying water quality and habitat profiles to see if Black-spotted newts have a preferred range or range of tolerance. Examining whole-organism effects of soil composition to tease apart how soil composition directly affects Black-spotted newts is another potential avenue of study.

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APPENDIX A

APPENDIX A

Supplementary Methods

OBITools3 Pipeline

OBITools3 was run using Ubuntu and the Windows subsystem for Linux. OBITools3 was activated using the source obi3-env/bin/activate command. The basic structure of the OBITools3 code is a command followed by an input file followed an output file,. OBITools3 uses a data management system (DMS) to house files during analysis. A DMS is identified by the .obidms ending. Each individual experiment, or NGS run, had its own DMS to make organization of data easier

Forward and reverse reads were imported and parsed out into an experiment specific DMS using the *obi import* command. Forward and reverse reads were then aligned using *obi alignpairedend* command. The quality of alignment for each sequence was assessed using the *obi stats -a score_norm* command. Alignment quality is given on a scale from 0 to 1, where 0 is the worst alignment and 1 would be a perfect alignment. Sequences with an alignment score of 0.80 and above were used for further analysis. That selection was done using the *obi grep -p "sequence['score_norm'] > 0.8"* command. The *obi ngsfilter* command was then used to identify and separate specific primers and primer tag combinations. The *obi ngsfilter*

command also trims the primers and tags from the sequence after it separates unique primer combinations. An NGS filter file was required for the *obi ngsfilter* command, the file needs to be in .txt format and needs to include sample number, primer tags, forward and reverse primer sequence and any extra information related to the sample (see table A1 for example of formatting)

Table A1. An example of an NGS filter file format

#exp	sample	tags	forward_primer	reverse_primer	extra_information
April_2021	sample_1	None:None	ACACCGCCCGTCACCCTGTAYACTTACCATGTTACGACT	T	Tissue_Extracts

Following the ngsfilter step, unique sequences were identified and dereplicated, to reduce computing time and power required, using the *obi uniq* command. Extraneous metadata was then removed using the *obi annotate* command. Sequences that had a count greater than 1 and a length less than or equal to 80bp will then be pulled from the data set using the *obi grep -p "len(sequence)<=80 and sequence['COUNT']>=1"* command. Sequences were then cleaned and removed of any errors resulting from PCR/Sequencing using the *obi clean -s*. If analyzing a tagged sample or individual sample the command is modified to *obi clean -s MERGED_sample*. Sequences were compared to the locally generated reference database with the minimum required match of 97% using *obi ecotag -m 0.97* command. Assignments were tallied and viewed using the *obi stats -c SCIENTIFIC_NAME* command. Data analyzed using OBITools3 was also exported to Geneious Prime for visualization and the BLAST function was used to confirm the taxonomic assignments of OBITools3 and provide more resolution on sequences not identified by OBITools3.

Reference Database Creation

A new directory called Batrachia_RD was made to house the downloaded sequences using the mkdir command. All publicly available sequences except for human and environmental microbe sequences were downloaded from EMBL into the Batrachia_RD directory using the following command; *wget -nH –cut-dirs=5 -A rel_std_*.dat.gz -R rel_std_hum_*.dat.gz,rel_std_env_*.dat.gz -m* ftp://ftp.ebi.ac.uk/pub/databases/embl/release/st. All sequences were be imported into a new directory, and parsed out into one file using the following command; *obi import –embl Batrachia_RD Val/embl_refs*. Following that step, the taxonomic information from NCBI was downloaded using the following command; *wget https://ftp.ncbi.nlm.nih.gov/pub/taxonomy/taxdump.tar.gz*. The taxonomy zip file was imported into the directory using the command; *obi import –taxdump taxdump.tar.gz Val/taxonomy/my_tax.* After that an in-silico PCR reaction was simulated for all downloaded sequences to see how primers bind to available sequences. The in-silico PCR reaction command used was *obi ecopcr -e 3 -l 50 -L 160 -F ACACCGCCCGTCACCCT -R GTAYACTTACCATGTTACGACTT –taxonomy Val/taxonomy/my_tax Val/embl_refs Val/Bat_refs*. As entered the command allows up to 3 errors along the sequence and the simulated fragment lengths range from 50bp to 160bp. After the in-silico PCR reaction, the simulated fragments were then checked to test if there is adequate taxonomic coverage for family, genus and species rankings using the command; *obi grep –require-rank=species –require-rank=genus –require-rank=family –taxonomy Val/taxonomy/my_tax Val/Bat_refs Val/Bat_refs_clean*. The reference database was then built with a minimum required match

percentage of 97% using the command *obi build_ref_db -t 0.97 –taxonomy Val/taxonomy/my_tax Val/Bat_refs_clean Val/Bat_db_97*.

Field Samples Used for NGS

Table A2. All field samples tested using eDNA metabarcoding. Site number is in the leftmost column followed by sample ID and date. Technical replicates per sample is in the second from the rightmost column. PCR protocol used is in the rightmost column. If a sample was amplified using both PCR protocols the number of technical replicates with each respective protocol is in prentices prior to the protocol name.

Site Number	Sample	Technical Replicates	PCR Protocol
1	Grace Heritage Ranch Main 10/27/20	2	Optimized
2	Sabal Palm Sanctuary Resaca Blind 11/9/20	2	Optimized
3	Coastal Bend Community College 10/4/20	1	Optimized
4	PIHS Pond 9/2/20	2	Optimized
4	PIHS Pond 5/2/21	2	(1)Original/(1)Optimized
4	PIHS Pond 5/2/21 W/O Human Block	2	(1)Original/(1)Optimized
5	Live Oak County Park 12/3/20	1	Optimized
5	Live Oak County Park 6/30/21	1	Original
6	Brushline North 7/27/20	1	Optimized
6	Brushline North 6/7/21	3	(2)Original/(1)Optimized
7	WWR Big Lake 1/29/20	1	Optimized
7	WWR Big Lake 6/11/21	1	Original
7	WWR Big Lake 7/1/21	1	Original
7	WWR Big Lake 5/7/21	1	Original
8	HWY 77 GPZ 8/31/20	1	Optimized
8	Hwy 77 GPZ 6/8/21	1	Original
9	LANWR Priarie trail #2 8/23/20	1	Optimized
10	USFWS Kelly Unit Resaca 8/13/20	1	Optimized

Table A2, cont. All field samples tested using eDNA metabarcoding. Site number is in the leftmost column followed by sample ID and date. Technical replicates per sample is in the second from the rightmost column. PCR protocol used is in the rightmost column. If a sample was amplified using both PCR protocols the number of technical replicates with each respective protocol is in prentices prior to the protocol name

Site Number	Sample	Technical Replicates	PCR Protocol
11	Brushline South 6/7/21	2	Original
12	LANWR Kidney Pond 5/26/21	1	Original
13	LANWR Scum Pond 5/26/21	2	(1)Original/(1)Optimized
14	Old Military Hwy Pond 4 6/24/21	2	Original
15	Pintas Creek 6/8/21	3	(2)Original/(1)Optimized
16	Old Military Hwy Pond 5 6/9/21	1	Original
17	PHR Haybarn 5/6/21	1	Original
17	PHR Haybarn 6/10/21	2	Original
17	PHR Haybarn 6/30/21	1	Original
18	Sabal Palm Sanctuary Side Pond 5/4/21	1	Original
19	PHR Midline Fence 5/6/21	1	Original
19	PHR Midline Fence 6/10/21	1	Original
19	PHR Midline Fence 6/30/21	1	Original
20	CKWRI South Pasture 5/28/21	1	Original
20	CKWRI South Pasture 6/8/21	1	Original
21	Southmost Black Willow Resaca 5/4/51	1	Original
22	PHR Bullrush 2/27/20	1	Original
23	Live Oak Pipeline Pond 5/7/21	1	Original
24	LANWR Newt Pond 5/26/21	1	Original

Supplementary Results

Table A3. Data matrix for eDNA metabarcoding detections used for comparison of taxonomic units detected

Site Number	Site Name	Siren intermedia texana	Incilius nebulifer	Spea bombifrons	Notophthalmus meridionalis	Scaphiopus couchii	Anaxyrus speciosus	Gastrophryne olivacea	Smilisca baudnii	Dryophytes squirellus	Rhinella marinus	Dryophytes cinereus	Lithobates sphenocephalus	Scaphiopus hurterii	Lithobates sp	Anaxyrus sp	Total
1	Grace Heritage Ranch Main	3	3	3	0	0	0	0	0	0	0	0	0	0	2	2	13
2	Sabal Palm Sanctuary Resaca Blind	3	3	0	0	0	0	0	0	0	0	0	0	0	2	0	8
3	Coastal Bend Community College	3	0	0	0	0	0	0	0	0	0	0	0	0	2	0	5
4	PIHS Pond	3	3	0	3	0	0	3	0	0	0	0	0	0	0	2	14
5	Live Oak County Park	3	3	0	0	3	0	3	0	0	0	0	0	0	2	0	14
6	Brushline North	3	3	0	0	3	3	3	3	3	0	0	0	0	0	0	21
7	WWR Big Lake	3	3	0	0	3	3	0	0	0	0	0	3	0	2	0	17
8	HWY 77 GPZ	3	3	0	0	0	0	3	0	0	0	0	0	0	0	0	9
9	LANWR Priarie trail #2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	USFWS Kelly Unit Resaca	0	3	0	0	0	0	3	0	0	0	0	0	0	0	0	6
11	Brushline South	0	3	0	0	0	0	3	0	0	0	0	0	0	0	0	6
12	LANWR Kidney Pond	0	3	0	0	0	0	3	0	0	0	0	0	0	2	0	8
13	LANWR Scum Pond	3	0	0	0	3	0	3	0	0	0	0	0	0	0	0	9
14	Old Military Hwy Pond 4	0	3	0	0	0	0	3	0	0	3	0	0	0	0	0	9
15	Pintas Creek	0	3	0	0	0	3	3	0	0	0	0	0	0	0	0	9
16	Old Military Hwy Pond 5	0	3	0	0	0	0	3	0	0	0	0	0	0	0	0	6
17	PHR Haybarn	3	3	0	0	0	0	3	0	0	0	3	0	0	0	2	14

Table A3, cont. Data matrix for eDNA metabarcoding detections used for comparison of taxonomic units detected

Site Number	Site Name	Siren intermedia texana	Incilius nebulifer	Spea bombifrons	Notophthalmus meridionalis	Scaphiopus couchii	Anaxyrus speciosus	Gastrophryne olivacea	Smilisca baudnii	Dryophytes squirellus	Rhinella marinus	Dryophytes cinereus	Lithobates sphenocephalus	Scaphiopus hurterii	Lithobates sp	Anaxyrus sp	Total
18	Sabal Palm Sanctuary Side Pond	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
19	PHR Midline Fence	3	3	0	0	0	0	3	0	0	3	3	3	0	0	2	20
20	CKWRI South Pasture	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	3
21	Southmost Black Willow Resaca	3	0	0	0	3	0	0	0	0	0	0	0	0	0	0	6
22	PHR Bullrush	3	0	0	0	0	0	0	0	0	0	0	3	0	0	0	6
23	Live Oak Pipeline Pond	3	3	0	0	0	0	0	0	3	0	3	3	3	0	2	20
24	LANWR Newt Pond	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table A4. Data matrix for Traditional survey detections used for comparison of taxonomic units detected

Site Number	Site Name	Hypopachus variolosus	Lithobates berlandieri	Incilius nebulifer	Siren intermedia texana	Notophthalmus meridionalis	Smilisca baundinii	Anaxyrus speciosus	Dryophytes squirellus	Lithobates catesbeianus	Lithobates sp	Gastrophryne sp	Scaphiopus sp	Anaxyrus sp	Microhylidae	Hylidae	Bufonidae	Total
1	Grace Heritage Ranch Main	3	3	3	3	3	0	0	0	0	0	0	0	0	1	0	0	16
2	Sabal Palm Sanctuary Resaca Blind	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
3	Coastal Bend Community College	3	3	0	0	0	0	0	3	0	2	2	0	0	1	1	0	15
4	PIHS Pond	0	0	3	0	3	3	0	0	0	0	2	2	0	1	1	1	16
5	Live Oak County Park	0	0	3	0	0	0	3	0	0	2	0	0	2	1	1	1	13
6	Brushline North	0	0	0	0	0	0	3	0	0	0	0	0	2	0	0	1	6
7	Welder Big Lake	0	0	3	0	0	0	0	0	3	2	0	0	0	0	1	0	9
8	HWY 77 GPZ	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	LANWR Priarie trail #2	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
10	USFWS Kelly Unit Resaca	3	3	0	0	0	0	0	0	0	0	2	0	0	1	0	1	10
11	Brushline South	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	2
12	LANWR Kidney Pond	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13	LANWR Scum Pond	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	3

Table A4, cont. Data matrix for Traditional survey detections used for comparison of taxonomic units detected

Site Number	Site Name	Hypopachus variolosus	Lithobates berlandieri	Incilius nebulifer	Siren intermedia texana	Notophthalmus meridionalis	Smilisca baundinii	Anaxyrus speciosus	Dryophytes squirellus	Lithobates catesbeianus	Lithobates sp	Gastrophryne sp	Scaphiopus sp	Anaxyrus sp	Microhylidae	Hylidae	Bufonidae	Total
14	Old Military Hwy Pond 4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15	Pintas Creek	0	0	3	0	0	0	0	0	0	2	0	0	0	0	0	0	5
16	Old Military Hwy Pond 5	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	2
17	PHR Haybarn	0	3	3	0	0	0	0	0	0	2	2	0	0	1	0	1	12
18	Sabal Palm Sanctuary Side Pond	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	3
19	PHR Midline Fence	0	0	0	0	0	0	0	3	0	0	2	0	0	1	1	1	8
20	CKWRI South Pasture	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	1	3
21	Southmost Black Willow Resaca	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	1	3
22	PHR Bullrush	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	2
23	Live Oak Pipeline Pond	0	0	0	0	0	0	0	0	0	2	0	2	0	0	0	0	4
24	LANWR Newt Pond	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	2

APPENDIX B

APPENDIX B

Amphibian Detections used for Community-Level Analysis

Table A5. Amphibian detections used for community level analysis. Site number is listed in the leftmost column, while amphibian taxonomic unit is in the top row. 1 indicates the amphibian taxonomic unit was detected at the site, 0 indicates the amphibian taxonomic unit was not detected at the site.

	Siren sp	Incilius sp	Spea sp	Lithobates sp	Anaxyrus sp	Notophthalmus [illegible]	Scaphiopus sp	Gastrophryne sp	Smilisca sp	Dryophytes sp	Rhinella sp	Hypopachus sp
Site 1	1	1	1	1	1	1	0	0	0	0	0	1
Site 2	1	1	0	1	0	0	0	0	0	0	0	0
Site 3	1	0	0	1	0	0	0	1	0	1	0	1
Site 4	1	1	0	0	1	1	1	1	1	0	0	0
Site 5	1	1	0	1	1	1	1	1	0	0	0	0
Site 6	1	1	0	0	1	0	1	1	1	1	0	0
Site 7	1	1	0	1	1	0	1	0	0	0	0	0
Site 8	1	1	0	0	0	0	0	1	0	0	0	0
Site 9	0	0	0	1	0	1	0	0	0	0	0	0
Site 10	0	1	0	1	0	0	0	1	0	0	0	1
Site 11	0	1	0	1	0	0	0	1	0	0	0	0
Site 12	0	1	0	1	0	0	0	1	0	0	0	0
Site 13	1	1	0	0	0	1	1	1	0	0	0	0
Site 14	0	1	0	0	0	1	0	1	0	0	1	0
Site 15	0	1	0	1	1	0	0	1	0	0	0	0
Site 16	0	1	0	1	0	0	0	1	0	0	0	0
Site 17	1	1	0	1	1	0	0	1	0	1	0	0
Site 18	1	0	0	0	0	1	0	0	0	0	0	0
Site 19	1	1	0	1	1	0	0	1	0	1	1	0
Site 20	0	0	0	1	0	0	0	1	0	0	0	0
Site 21	1	0	0	1	0	0	1	0	0	0	0	0

Table A5, cont. Site number is listed in the leftmost column, while amphibian taxonomic unit is in the top row. 1 indicates the amphibian taxonomic unit was detected at the site, 0 indicates the amphibian taxonomic unit was not detected at the site.

	Siren sp	**Incilius sp**	**Spea sp**	**Lithobates sp**	**Anaxyrus sp**	**Notophthalmus sp**	**Scaphiopus sp**	**Gastrophryne sp**	**Smilisca sp**	**Dryophytes sp**	**Rhinella sp**	**Hypopachus sp**
Site 22	1	0	0	1	0	0	0	0	0	0	0	0
Site 23	1	1	0	1	1	0	1	0	0	1	0	0
Site 24	0	0	0	1	0	0	0	0	0	0	0	0

BIOGRAPHICAL SKETCH

Sean Collins graduated from Allegheny College in 2018 with a B.S. in Biology and a Latin Minor. As an undergraduate researcher he examined how stress affected metabolic rate and disease resistance in red-backed salamanders. This undergraduate research experience sparked a passion for Sean and he realized it was something he wanted to do for the rest of his life. Eventually, he found his way down to the Kline Lab at the University of Texas at Rio Grande Valley where his passion for research continued to bloom and he received his M.S. in Agricultural, Environmental and Sustainability sciences in August 2022. Following his M.S. Sean will be starting a Ph.D program at Northern Arizona University in August 2022 on a project focusing on population genomics and genome-wide association of drought tolerance in Ponderosa pines. Outside of the research realm, Sean's other greatest passion is mountain biking and road cycling. Sean can be reached at collinssean604@gmail.com