Inter-Research Science Publisher

Endangered Species Research
Volume 56: 27–39

Published January 23, 2025
https://doi.org/10.3354/esr01379

Occupancy of four endangered aquatic species on Bitter Lake National Wildlife Refuge, New Mexico, USA, using environmental DNA

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ABSTRACT: Environmental DNA (eDNA) has the potential to play an important role in surveys for rare and endangered aquatic species. eDNA sampling is non-invasive, and for small cryptic species that are difficult to survey, it may offer a viable, more efficient, and less expensive alternative than traditional techniques. We used eDNA for surveying 5 endangered spring-endemic species at Bitter Lake National Wildlife Refuge, Chaves County, New Mexico, USA. Water samples (n = 40) in spring systems were evaluated in July 2018 for presence of residual DNA for *Gambusia nobilis*, *Gammarus desperatus*, *Juturnia kosteri*, *Pyrgulopsis roswellensis*, and *Assiminea pecos*. We detected eDNA at 50% of sites for *G. nobilis*, 42.5% of sites for *J. kosteri*, 27.5% of sites for *P. roswellensis*, 20% of sites for *G. desperatus* and did not detect *A. pecos* eDNA at any of the sites. We also examined the habitat conditions that drive patterns in occupancy for these endangered species and developed thresholds for habitat parameters to inform conservation decisions. Salinity and dissolved oxygen influenced sample occupancy for *G. desperatus*, *P. roswellensis*, and *J. kosteri*, but only dissolved oxygen influenced sample occupancy for *G. nobilis*. Results highlighted the efficacy of using eDNA to monitor 4 of the 5 spring-endemic species and provided insight to the habitat preferences for each species, which will help drive conservation activities.

KEY WORDS: Endangered species · eDNA · Occupancy · Wetlands · Mollusks · Fish

1. INTRODUCTION

Bitter Lake National Wildlife Refuge (BLNWR) is located at the northern extent of the Chihuahuan Desert in southeastern New Mexico, USA. Designated a Ramsar Wetland of International Importance (Ramsar Sites Information Service 2010), BLNWR has an array of wetland types, each with their own unique environmental conditions that are largely driven by the Roswell Artesian Aquifer and the Pecos River (Land 2005, Land & Huff 2010). These wetlands were originally protected in 1937 to provide habitat for migrating and wintering waterfowl, but management and conservation issues on BLNWR have grown to include rare, often endemic, aquatic species that are reliant on its diverse wetland types and conditions.

Five rare species that occur in BLNWR wetlands and are also listed as endangered under the United States Endangered Species Act include 2 endemic springsnails, *Pyrgulopsis roswellensis* and *Juturnia*

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kosteri, 1 amphipod, *Gammarus desperatus*, 1 semi-aquatic snail, *Assiminea pecos*, and 1 spring fish, *Gambusia nobilis* (USFWS 1970, USFWS 2005). These species occur in or adjacent to (*A. pecos* utilizes wetlands margins) sinkholes, spring-runs, and other wetlands influenced by spring discharge and may occur sympatrically (Echelle & Echelle 1980, Cole 1981, Taylor 1987, Lang 1998, USFWS 2019). Current management and research of these species includes: monitoring population trends, which can be labor intensive, time consuming, and costly (Johnson et al. 2019, Beauchamp et al. 2021); establishing new populations in created and/or restored habitats (Beauchamp et al. 2021, Johnson et al. 2022); and planning and enacting measures related to maintenance of critical habitat (e.g. invasive plant removal, spring discharge monitoring) and endangered species recovery (USFWS 1983, 2019).

There is little information describing the current distribution of these species on BLNWR or what habitat conditions might drive their patterns of distribution. Foundational work conducted by Lang (1998, 1999, 2001, 2002) and Lang et al. (2003) at BLNWR reported descriptive associations of the habitat conditions where they sampled; these sample locations, although opportunistic, corresponded to the presence of the 4 invertebrates. Greater understanding of the habitat conditions that influence the occurrence and distribution of these 5 endangered species is needed to better focus management and conservation practices. Further, information regarding environmental conditions important to occurrence will allow managers to evaluate potential reintroduction sites both on and off the refuge within the species' historic ranges, and lead to targeted restoration efforts should an occupied site fall outside of suitable habitat conditions.

Environmental DNA (eDNA) is a modern tool used to detect fragments of DNA released or shed from organisms in their environment. A growing body of research suggests eDNA can be effective at detecting the presence and distribution of species, particularly those that are rare or occur in low densities (Jerde et al. 2011, Wilcox et al. 2013, Harper et al. 2018, Hinlo et al. 2018, Wood et al. 2019). This approach has the potential to lower monitoring costs and may be more sensitive and less disruptive compared to standard monitoring efforts, such as electrofishing or traditional trapping (Dolan & Miranda 2004, Miranda & Kidwell 2010, Wilcox et al. 2015, Evans et al. 2017). eDNA has been used to detect the presence of both aquatic vertebrates (Akre et al. 2019, Strickland & Roberts 2019, Mauvisseau et al. 2020, Villacorta-Rath et al. 2021) and macroinvertebrates (Goldberg et al. 2013, Mächler et al. 2014, Lor et al. 2020). Utilizing eDNA, particularly in aquatic systems, has been employed to evaluate the presence or absence of rare species (Villacorta-Rath et al. 2021), monitor for presence of invasive species (Robson et al. 2016, Pukk et al. 2021), gauge success of species reintroduction or removal efforts (Dejean et al. 2012, Rees et al. 2014, Rojahn et al. 2018), determine recolonization/immigration rates (Duda et al. 2021), and evaluate occupancy and detection probabilities (Hunter et al. 2015, Orzechowski et al. 2019, Strickland & Roberts 2019). Further, eDNA may have the potential to index abundance and biomass (Yates et al. 2019, Rourke et al. 2022).

Occupancy modeling provides a robust framework to examine the probability of occupancy of eDNA for target species while accounting for imperfect detection of DNA within the samples (MacKenzie et al. 2003, Nichols et al. 2008, Strickland & Roberts 2019, McClenaghan et al. 2020). Multi-scale occupancy modeling developed by Nichols et al. (2008) enables researchers to use a hierarchical approach to analyze eDNA samples by estimating the probability of occupancy at the site level (ψ), the conditional probability of occupancy of eDNA in a sample (θ), and the conditional probability of detecting eDNA in each quantitative PCR (qPCR) replicate (p). This hierarchical approach to modelling lends itself to eDNA sampling due to the typically nested design — multiple sites, multiple samples at each site, and multiple PCR replicates for each sample — and allows researchers to account for imperfect detection (p) of eDNA while including environmental factors that may influence the presence or absence of eDNA in the sample, given the site is occupied (θ). For example, multi-scale occupancy modeling with eDNA was used to analyze Burmese pythons *Python bivittatus* in the Florida Everglades (Hunter et al. 2015, Orzechowski et al. 2019) and Roanoke logperch *Percina rex* in Virginia and North Carolina (Strickland & Roberts 2019).

Our objectives were to: (1) evaluate if eDNA sampling techniques were effective at detecting residual DNA from 5 endangered species that occur in the wetlands on BLNWR, (2) examine habitat conditions that drive patterns in occupancy across BLNWR for the 5 endangered species, and (3) develop thresholds for habitat parameters that will inform conservation and management decisions. Based on prior monitoring of endangered aquatic and semi-aquatic species on the refuge, we expected to find eDNA from each of the species in at least some of the wetlands evaluated. Although we did not make predictions concerning

the occurrence of the 5 species addressed in this study, some may occur at low densities (*A. pecos*, Roesler 2016), be less common (*G. desperatus*, *P. roswellensis*, Johnson et al. 2019, 2022), or have patchy distributions (*A. pecos*, Roesler 2016; *G. desperatus*, Johnson et al. 2019) compared to other species.

2. MATERIALS AND METHODS

2.1. Study area

BLNWR is located in Chaves County, near Roswell, New Mexico, USA (Fig. 1). Much of the refuge lies in the Pecos River floodplain, and upland vegetation is characteristic of Chihuahuan Desert shrublands and grasslands (Griffith et al. 2006). Wetland habitats include springs, spring-runs, spring-ditches, sinkholes, marshes, salt flats, and managed wetlands (impoundments). Spring-runs are unmanipulated spring-fed systems. Spring-ditches are historic spring-runs that occur adjacent to large wetland impoundments but were modified (straightened and dredged) in the early 2000s for water control and delivery and to protect important spring habitat from the impoundments that can fluctuate in environmental parameters, principally salinity. Spring-influenced areas derive water almost entirely from the Roswell artesian aquifer system (Land 2005, Land & Huff 2010). Water characteristics (e.g. temperature, dissolved oxygen [DO], specific conductivity, and salinity) vary among spring systems due to the volume of water emanating from spring vents, exposure of water to differing soil-based minerals before or after it reaches the surface, and exposure of surface water to sunlight (MacRae et al. 2001, Gallo 2013).

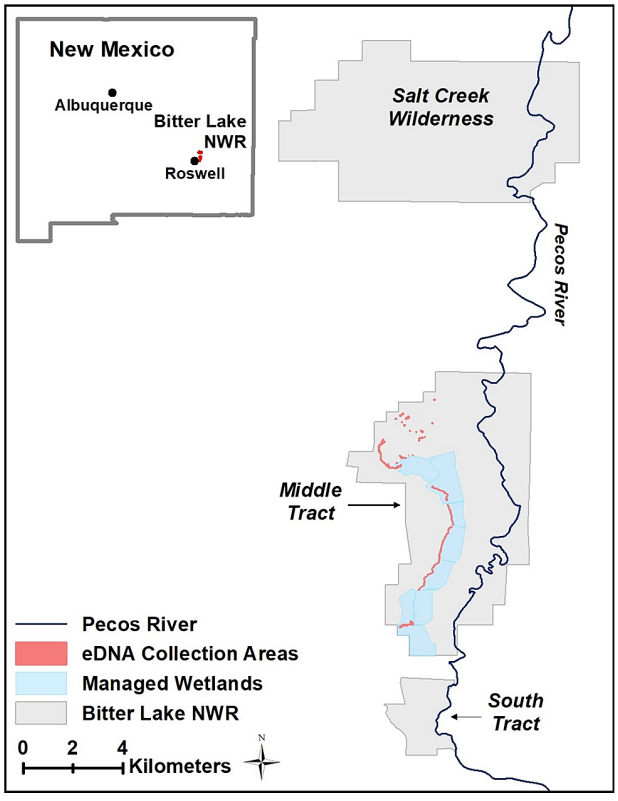

Fig. 1. Bitter Lake National Wildlife Refuge (NWR), New Mexico, USA, indicated by the red polygon in the inset map. Environmental DNA (eDNA) was collected during summer 2018

2.2. Field sampling

We sampled for eDNA at 40 sites (described below) on 9–23 July 2018 between 06:40 and 14:55 h. We stratified field sampling into 3 habitat types: sinkholes, spring-runs, and spring-ditches. We randomly sampled 24 sinkholes. Of those sinkholes, we collected water samples from 3 locations within each sinkhole at the magnetic 0°, 120° and 240° locations. If access was not possible at any of those locations, we chose the closest access point. For the spring-runs, we sampled at established monitoring sites (Beauchamp et al. 2021). Those sites were randomly selected (Sago Springs, 3 sites with 6 total sample locations) or had a stratified random sampling design (Bitter Creek, 7 sites with 22 total sample locations). For this analysis, nearby monitoring sites were combined into eDNA sampling sites (spring-run sections; n = 10). Lastly, 3 random sampling locations in each of 6 spring-ditch sites (n = 6) were sampled (Fig. 1); we moved 2 locations because water levels were too low to collect samples (these locations were randomly chosen on the day of sampling). We recorded water temperature, salinity, DO, and pH with a YSI Professional Plus water quality meter (Yellow Springs Instruments), and collection time at each sampling location, except at sinkholes where 1 environmental parameter was collected for all 3 sampling locations. In total we collected water samples at 116 sample locations (sinkhole samples, n = 70; spring-run samples, n = 28; spring-ditch samples, n = 18), with 2 sites receiving 1 sample, 1 site receiving 2 samples, and 1 site receiving 4 samples.

At each sampling location, we collected 1 l of water from the top of the water column in two 500 ml commercially produced sterile plastic water bottles (Nestle). Water samples were immediately stored on ice until processed. All sampling, transport, storage and

processing equipment was sterilized with 10% bleach solution or DNA OFF (Takara Bio) and flushed with distilled water, as well as UV-sterilized when possible.

2.3. Lab sampling

2.3.1. Primer development

We compiled publicly available sequences for target and similar non-target species for primary *in silico* gene region selection prior to primer design (Table S1 in the Supplement at www.int-res.com/articles/suppl/n056p027_supp.pdf). The Museum of Southwestern Biology, University of New Mexico, donated *Gambusia nobilis* fin tissue. For all other species (*Pyrgulopsis roswellensis*, *Juturnia kosteri*, *Gammarus desperatus*, and *Assiminea pecos*), we collected whole body tissues from BLNWR. We extracted DNA using Qiagen Blood and Tissue kit (Qiagen). We used Applied Biosystem 3130 Genetic Analyzer with broad range (11 invertebrate phyla) COI Folmer primers (Folmer et al. 1994), and in-house sequencing primers targeting sections of mitochondrial cytochrome b (cytb) for initial sequencing to amplify sequences of our target species for species-specific assay development. Alignments were created from the local sequences, as well as GenBank sequences for non-target species. We then developed qPCR primers and probes for the 5 target species (Table 1) manually using Benchling (Benchling Biology Software). Amplicons were between 100 and 157 base pairs in length (Table 1). Potential primers were tested for specificity *in silico* using alignments with local species (Table S1), and other potential species on GenBank via PrimerBlast. Primers were compared for primer–dimer formation both self and cross (for multiplexing purposes) using both PrimerBlast and IDT Oligo analyzer. Selected qPCR primers were tested for specificity against DNA extracted from target and non-target species and were optimized for concentration and thermal protocol using 6 member standard curve analysis with synthetic standards (Gblocks, IDT). The qPCR primer probe sets were field validated with water samples taken from 6 locations at BLNWR where target species are known to occur.

2.3.2. eDNA sample processing

We processed the 1 l water samples through 47 mm 1.5 µm pore glass fiber filter (Whatman plc) via vacuum filtration within 24 h of collection; filters were stored at −20°C for up to 7 d. We then extracted DNA from the filters using Qiagen Blood and Tissue kit (Qiagen). Extracted DNA was stored at −20°C. We ran 4 technical replicates of the extracted DNA on a Quantstudio 5 Real-Time PCR system (ThermoFisher). Total reaction volumes were 20 µl with 10 µl of Taqman Environmental Master Mix (ThermoFisher), 4 µl of template DNA, and 2 µl of each assay (1 µm primer, 500 nm probe reaction concentration). The remaining volume was DNA free water. Thermal cycle settings for the PCR run were optimized at 95°C 10 min (95°C 15 s, 60°C 30 s) × 50 cycles. All field and lab controls are described in Text S1. Synthetic DNA was used to establish standard curve and limit of detection.

2.4. Occupancy analysis

We used a multi-scale occupancy model (Nichols et al. 2008) to analyze eDNA observations by esti-

Table 1. Quantitative PCR primer and probe sequences and length for each target species

Species	Forward 5′-3′	Probe 5′-3′	Reverse 5′-3′	Length (bp)
Assiminea pecos	CGC CGG TGG TTC AGT TGA TT	TAG CTG GTG CGT CTT CTA TTT TG	AGG AAG ACG CTC AAA CTG CAT G	133
Juturnia kosteri	TTG GTT ACT TCC TCC AGC GCT T	TCT GCG GCA GTA GAA AGT GGT GTT	GAA CCG CCA GCA TGA GCC	120
Gambusia nobilis	GGG GCA GGA ACA GG TTG AAC TGT A	GCA ACT TAG CAC ACG CCG GG	GCC CGC TAG GTG AAA GGG AAA AAA TA	103
Gammarus desperatus	GCT GTG CGC AAC AG CTG CC	AGG GTA TAC AGT TCA GCC AGT TCC T	AGG TAG ACC AGA CAT GGC CTT TCC A	157
Pyrgulopsis roswellensis	GCA ACT GGC TTG TG CCG	GCT ATA TCT GGA GCA CCG AGC	AGT AGT AAT AAA GCA GGG GGT AGG	100

mating the probability of occupancy at the site level (ψ), the conditional probability of occupancy of eDNA in a sample (θ), and the conditional probability of detecting eDNA in each PCR replicate (p). Both occupancy parameters, ψ and θ, allowed us to model occupancy at 2 different spatial scales (ψ at the site-scale and θ at the sample-scale; Nichols et al. 2008, Dorazio & Erickson 2018). The sample-scale occupancy (θ) can be thought of as availability. Specifically, given the presence of eDNA at the site-scale, θ is the probability that eDNA is available to be detected at the sample-scale. We used the R statistical programing language (R Core Team 2022) and the package 'RPresence' (Hines 2006, MacKenzie & Hines 2022). For each species, we developed a suite of 16 models (Tables 2–5) to examine potential predictors of site occupancy, sample occupancy, and detectability. We included habitat type — sinkhole, spring-run, spring-ditch — as a predictor for site occupancy (ψ). Dissolved oxygen and salinity were included as potential predictors of sample occupancy (θ), given site occupancy, because they may be associated with environmental tolerance limits (survival) and therefore the population ecology of each species (Bednarz 1979, Seidel et al. 2010, USFWS 2019). Additionally, we included time of collection (which is highly correlated with water temperature and may be related to the availability and degradation of eDNA) (see Tsuji et al. 2017) as a predictor of eDNA detection (p). For each species we used Akaike information criterion corrected for small sample size (AIC_c) (Burnham & Anderson 2002) to compare competing models. We considered models competitive if $\Delta AIC_c < 2$ but excluded, as probable, models with uninformative parameters ($p > 0.157$; Burnham & Anderson 2002, Arnold 2010). We model averaged results from the retained competitive models.

Table 2. Occupancy models tested for *Gammarus desperatus*. Akaike information criterion corrected for small sample size (AIC_c) used for model selection (**bold** models were selected). Models are composed of probability of occupancy at the site level (ψ), the conditional probability of occupancy of environmental DNA (eDNA) in a sample (θ), and the conditional probability of detecting eDNA in each PCR replicate (p). In parentheses following each parameter are covariate effects included for each parameter (Sal: salinity; DO: dissolved oxygen). For each model, we give −2 × log-likelihood (−2log(*L*)), no. of parameters (*K*), AIC_c, difference in AIC_c compared to lowest AIC_c of the model set (Δ_i), and AIC_c model weight (w_i)

Model	−2log(*L*)	*K*	AIC_c	Δ_i	w_i
ψ(.) θ(Sal+DO) p(Time)	102.17	6	**116.72**	**0.00**	**0.414**
ψ(Habitat) θ(.) p(Time)	104.40	6	118.95	2.23	0.136
ψ(Habitat) θ(DO) p(Time)	101.75	7	119.25	2.53	0.117
ψ(Habitat) θ(Sal+DO) p(Time)	98.68	8	119.33	2.61	0.113
ψ(.) θ(Sal) p(Time)	108.54	5	120.30	3.59	0.069
ψ(Habitat) θ(Sal) p(Time)	103.17	7	120.67	3.95	0.057
ψ(.) θ(Sal+DO) p(.)	109.37	5	121.13	4.42	0.046
ψ(Habitat) θ(Sal+DO) p(.)	105.98	7	123.48	6.76	0.014
ψ(.) θ(DO) p(Time)	112.32	5	124.08	7.37	0.010
ψ(.) θ(.) p(Time)	115.07	4	124.21	7.50	0.010
ψ(.) θ(Sal) p(.)	115.67	4	124.81	8.10	0.007
ψ(Habitat) θ(Sal) p(.)	111.20	6	125.75	9.03	0.005
ψ(Habitat) θ(.) p(.)	115.43	5	127.19	10.48	0.002
ψ(Habitat) θ(DO) p(.)	115.40	6	129.95	13.23	0.001
ψ(.) θ(.) p(.)	126.59	3	133.26	16.54	0.000
ψ(.) θ(DO) p(.)	126.56	4	135.70	18.99	0.000

Table 3. Occupancy models tested for *Pyrgulopsis roswellensis*. Akaike information criterion corrected for small sample size (AIC_c) used for model selection (**bold** models were selected). Models are composed of probability of occupancy at the site level (ψ), the conditional probability of occupancy of environmental DNA (eDNA) in a sample (θ), and the conditional probability of detecting eDNA in each PCR replicate (p). In parentheses following each parameter are covariate effects included for each parameter (Sal: salinity; DO: dissolved oxygen). For each model, we give −2 × log-likelihood (−2log(*L*)), no. of parameters (*K*), AIC_c, difference in AIC_c compared to lowest AIC_c of the model set (Δ_i), and AIC_c model weight (w_i)

Model	−2log(*L*)	*K*	AIC_c	Δ_i	w_i
ψ(.) θ(Sal) p(Time)	**107.27**	**5**	**119.03**	**0.00**	**0.432**
ψ(.) θ(Sal+DO) p(Time)	**104.70**	**6**	**119.25**	**0.21**	**0.389**
ψ(Habitat) θ(.) p(Time)	108.94	6	123.49	4.45	0.047
ψ(Habitat) θ(Sal) p(Time)	106.14	7	123.64	4.61	0.043
ψ(.) θ(.) p(Time)	114.82	4	123.96	4.93	0.037
ψ(Habitat) θ(Sal+DO) p(Time)	103.78	8	124.43	5.39	0.029
ψ(Habitat) θ(DO) p(Time)	108.70	7	126.20	7.17	0.012
ψ(.) θ(DO) p(Time)	114.66	5	126.42	7.39	0.011
ψ(.) θ(Sal) p(.)	130.06	4	139.20	20.17	0.000
ψ(.) θ(Sal+DO) p(.)	127.66	5	139.42	20.39	0.000
ψ(Habitat) θ(Sal) p(.)	128.99	6	143.54	24.50	0.000
ψ(Habitat) θ(Sal+DO) p(.)	126.83	7	144.33	25.30	0.000
ψ(Habitat) θ(.) p(.)	134.76	5	146.52	27.49	0.000
ψ(.) θ(.) p(.)	141.03	3	147.70	28.66	0.000
ψ(Habitat) θ(DO) p(.)	134.38	6	148.93	29.89	0.000
ψ(.) θ(DO) p(.)	140.60	4	149.74	30.71	0.000

Table 4. Occupancy models tested for *Juturnia kosteri*. Akaike information criterion corrected for small sample size (AIC_c) used for model selection (**bold** models were selected). Models are composed of probability of occupancy at the site level (ψ), the conditional probability of occupancy of environmental DNA (eDNA) in a sample (θ), and the conditional probability of detecting eDNA in each PCR replicate (p). In parentheses following each parameter are covariate effects included for each parameter (Sal: salinity; DO: dissolved oxygen). For each model, we give −2 × log-likelihood (−2log(*L*)), no. of parameters (*K*), AIC_c, difference in AIC_c compared to lowest AIC_c of the model set (Δ_i), and AIC_c model weight (w_i)

Model	−2log(*L*)	*K*	AIC_c	Δ_i	w_i
ψ(Habitat) θ(Sal+DO) p(Time)	**120.48**	**8**	**141.13**	**0.00**	**0.450**
ψ(Habitat) θ(Sal+DO) p(.)	126.10	7	143.60	2.47	0.131
ψ(.) θ(Sal+DO) p(Time)	129.37	6	143.92	2.79	0.112
ψ(Habitat) θ(DO) p(Time)	127.00	7	144.50	3.37	0.083
ψ(Habitat) θ(.) p(Time)	130.38	6	144.93	3.80	0.067
ψ(Habitat) θ(Sal) p(Time)	127.44	7	144.94	3.81	0.067
ψ(.) θ(Sal+DO) p(.)	134.52	5	146.28	5.16	0.034
ψ(Habitat) θ(Sal) p(.)	132.70	6	147.25	6.12	0.021
ψ(Habitat) θ(.) p(.)	135.92	5	147.68	6.56	0.017
ψ(Habitat) θ(DO) p(.)	133.65	6	148.20	7.07	0.013
ψ(.) θ(Sal) p(Time)	138.50	5	150.26	9.14	0.005
ψ(.) θ(Sal) p(.)	143.63	4	152.77	11.65	0.001
ψ(.) θ(DO) p(Time)	159.07	5	170.83	29.71	0.000
ψ(.) θ(.) p(Time)	162.55	4	171.69	30.57	0.000
ψ(.) θ(.) p(.)	168.69	3	175.36	34.23	0.000
ψ(.) θ(DO) p(.)	166.61	4	175.75	34.63	0.000

Table 5. Occupancy models tested for *Gambusia nobilis*. Akaike information criterion corrected for small sample size (AIC_c) used for model selection (**bold** models were selected). Models are composed of probability of occupancy at the site level (ψ), the conditional probability of occupancy of environmental DNA (eDNA) in a sample (θ), and the conditional probability of detecting eDNA in each PCR replicate (p). In parentheses following each parameter are covariate effects included for each parameter (Sal: salinity; DO: dissolved oxygen). For each model, we give −2 × log-likelihood (−2log(*L*)), no. of parameters (*K*), AIC_c, difference in AIC_c compared to lowest AIC_c of the model set (Δ_i), and AIC_c model weight (w_i)

Model	−2log(*L*)	*K*	AIC_c	Δ_i	w_i
ψ(Habitat) θ(DO) p(Time)	**137.66**	**7**	**155.16**	**0.00**	**0.436**
ψ(Habitat) θ(Sal+DO) p(Time)	135.55	8	156.20	1.04	0.260
ψ(Habitat) θ(.) p(Time)	143.11	6	157.66	2.50	0.125
ψ(Habitat) θ(Sal) p(Time)	140.9	7	158.40	3.24	0.087
ψ(.) θ(Sal+DO) p(Time)	145.84	6	160.39	5.23	0.032
ψ(.) θ(Sal) p(Time)	149.13	5	160.89	5.73	0.025
ψ(Habitat) θ(DO) p(.)	148.1	6	162.65	7.49	0.010
ψ(.) θ(DO) p(Time)	150.96	5	162.72	7.56	0.010
ψ(Habitat) θ(Sal+DO) p(.)	145.99	7	163.49	8.33	0.007
ψ(.) θ(.) p(Time)	156.08	4	165.22	10.06	0.003
ψ(Habitat) θ(.) p(.)	153.55	5	165.31	10.15	0.003
ψ(Habitat) θ(Sal) p(.)	151.33	6	165.88	10.72	0.002
ψ(.) θ(Sal+DO) p(.)	156.29	5	168.05	12.89	0.001
ψ(.) θ(Sal) p(.)	159.58	4	168.72	13.56	0.001
ψ(.) θ(DO) p(.)	161.4	4	170.54	15.38	0.000
ψ(.) θ(.) p(.)	166.53	3	173.20	18.04	0.000

3. RESULTS

The primers we developed amplified the target tissue as well as water samples with known occupancy, and failed to amplify non-target species (Text S1, Fig. S1). Based on the results of our eDNA assay development, we rated this as Level 3 based on the scale presented in Thalinger et al. (2021). In summary, first we tested the assay on target tissue to demonstrate DNA amplification for the specific species, then we tested the assay on closely related non-target species to show species specificity, and lastly, we obtained positive detections using eDNA samples which signifies that the assay works in environmental conditions. At this level, positive detections indicate the target is present.

We detected *Gambusia nobilis* at 20 of 40 sites (50%), *Gammarus desperatus* at 8 sites (20%), *Pyrgulopsis roswellensis* at 11 sites (27.5%), *Juturnia kosteri* at 17 sites (42.5%), and *Assiminea pecos* at 0 sites; *A. pecos*, therefore, is not included in the statistical analysis. During sampling, water temperature averaged 24.5°C (SD = 3.6) and pH averaged 7.8 (SD = 0.4). Water salinity ranged from 3.3 to 134.2 ppt (mean = 30.2 ppt, SD = 34.8), and DO ranged from 3.9 to 119.1% (mean = 55.5%, SD = 27.8). The sample water's salinity and DO were uncorrelated (Pearson's r = −0.001, p = 0.994).

The most competitive models for *G. desperatus* (Table 2) and *P. roswellensis* (Table 3) did not have habitat type (sinkhole, spring-run, spring-ditch) as a predictor for site occupancy (ψ) of eDNA, while the most competitive models for *J. kosteri* (Table 4) and *G. nobilis* (Table 5) both had habitat type as a predictor for site occupancy. Site occupancy of eDNA was greatest in spring-run habitats for both *J. kosteri* and *G. nobilis* (Fig. 2). Site occurrence for *J. kosteri* eDNA ranged from 13.4% (95% CI = 2.8–45.4%) in sinkholes to 100% (95% CI = 99.9–100%) in spring-run habitats. Site occurrence for

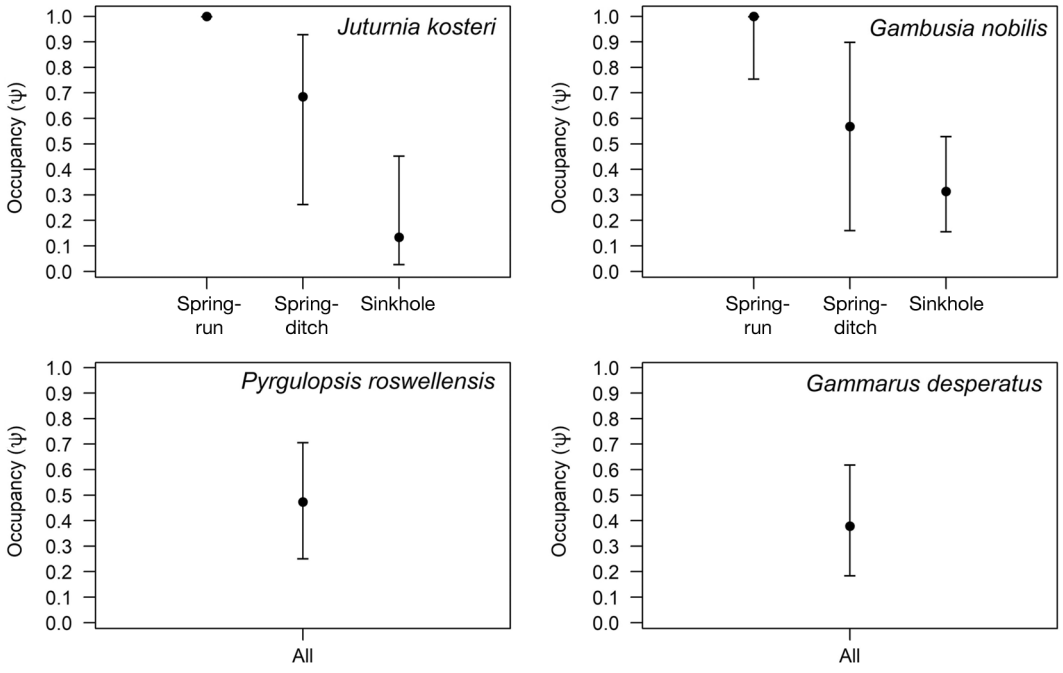

Fig. 2. Estimated site occupancy (ψ) of environmental DNA for 4 endangered aquatic species on Bitter Lake National Wildlife Refuge, New Mexico, USA, during summer 2018. Error bars represent 95% confidence intervals

G. nobilis eDNA ranged from 31.4% (95% CI = 15.7–53.0%) in sinkholes to 100% (95% CI = 75.5–100%) in spring-run habitats. For *P. roswellensis* and *G. desperatus*, site occurrence of eDNA was constant across habitat types at 47.3% (95% CI = 25.1–70.6%) and 37.8% (95% CI = 18.5–61.9%), respectively (Fig. 1).

Salinity and DO influenced sample occupancy (θ) for *G. desperatus*, *P. roswellensis*, and *J. kosteri* eDNA, but only DO influenced sample occupancy for *G. nobilis* (Table 1, Fig. 3). As DO increased, sample occupancy of *G. nobilis* eDNA increased, but an inverse relationship between sample occupancy and salinity was observed (Fig. 4). Time of collection affected eDNA detection for all species (Tables 2–5) such that detection of eDNA declined for samples collected later in the day (Fig. 5).

4. DISCUSSION

4.1. Overview

This study highlights the efficacy of eDNA biomonitoring of 4 US federally endangered species and using eDNA to quantify their occupancy on BLNWR. These results are consistent with the growing body of research validating eDNA as a useful tool for detecting rare, endangered species in freshwater ecosystems (e.g. Schmelzle & Kinziger 2016, Strickland & Roberts 2019, Piggott et al. 2021, Schmidt et al. 2021). This work further demonstrates the usefulness of combining eDNA presence/absence surveys with occupancy modeling in order to gain added information relevant to conserving threatened and endangered species (e.g. Schmelzle & Kinziger 2016, Harper et al. 2018, Strickland & Roberts 2019, Qu et al. 2020, Martel et al. 2021, Tingley et al. 2021). Specifically, our results quantified habitat preference for *Pyrgulopsis roswellensis*, *Juturnia kosteri*, *Gammarus desperatus*, and *Gambusia nobilis*. Finally, the work presented here adds to the evidence that while initial costs of eDNA assay development can be expensive, once developed, eDNA can be a cost-effective approach for sampling and detecting rare species (Evans et al. 2017, McInerney & Rees 2018, Fediajevaite et al. 2021).

The results of the occupancy modeling provide important insight to site and habitat suitability for each endangered species (Fig. S2). For this initial eDNA study, we decided on an 80% threshold in the sample occupancy (θ) modeling results to infer what habitat is likely preferred for each species, given the site is occupied. For example, *G. nobilis* eDNA had an 80% chance of occupancy in the sample when DO% was greater than 70% (Fig. 3), given the site was occupied. Additionally, *G. nobilis* eDNA had a high likelihood

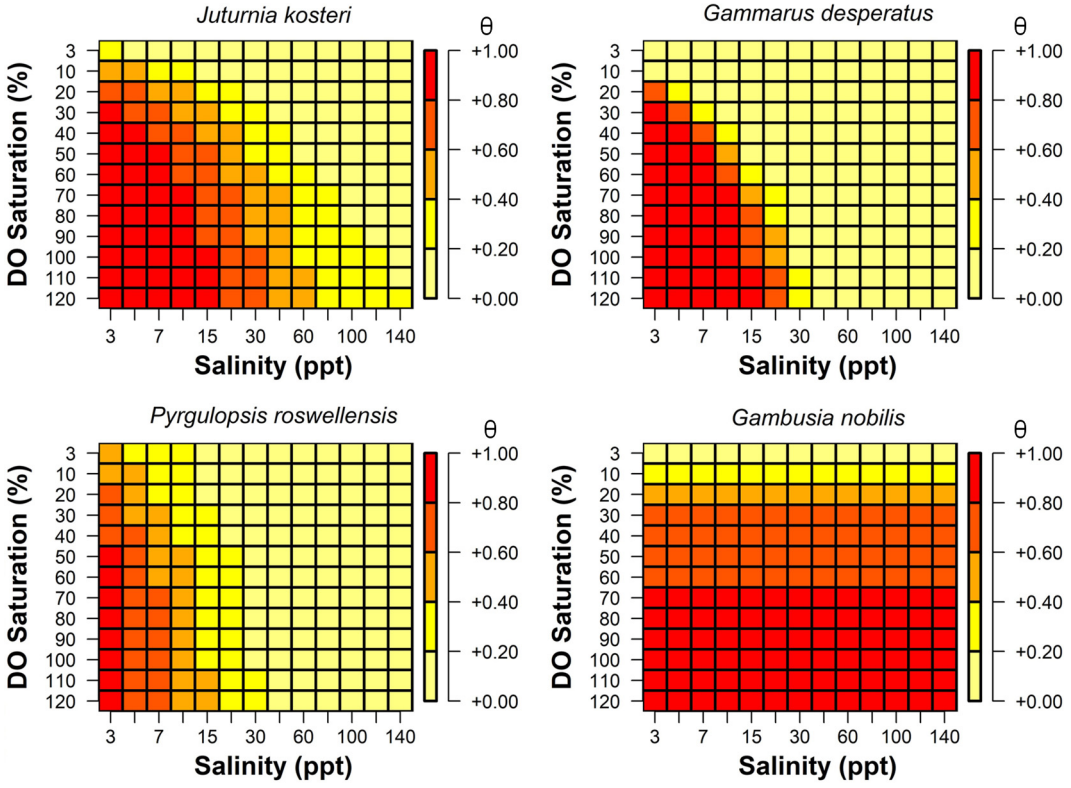

Fig. 3. Relationships between sample occupancy (θ) of environmental DNA and water salinity and dissolved oxygen (DO) for 4 endangered aquatic species on Bitter Lake National Wildlife Refuge, New Mexico, USA, during summer 2018

of site occupancy at spring-runs (Fig. 1). High salinity did not limit the likelihood of eDNA occupancy in the sample (Fig. 3). The high salt tolerance is not surprising for *G. nobilis*, as other species in the genus are salt tolerant; moreover, one study showed *G. nobilis* can withstand up to 100% sea water (~35 ppt) for 7 d (Chervinski 1983). Swenton & Kodric-Brown (2012) documented that *G. nobilis* preferred salinity of 10.4 ± 1.2 ppt and typically needed >6 mg l^{-1} of DO.

We found an 80% chance of site occupancy for *J. kosteri* and *G. desperatus* eDNA in the sample when DO was above 30% and salinity was low (<3 ppt), given site occupancy. In order to maintain the 80% threshold as salinity increased to 15 ppt (80% threshold), DO also had to increase. Interestingly, the likelihood of *G. desperatus* eDNA occurring in the sample sharply declined outside of these thresholds, while *J. kosteri* eDNA may be able to tolerate values outside of these thresholds (Fig. 3). *G. desperatus* did not have a habitat predictor for site occupancy, and overall site occupancy was low (37.8%). We hypothesize there is probably another factor that is not captured in this study that may be influencing *G. desperatus* occupancy. We have noticed during our biannual monitoring that *G. desperatus* occurs close to spring vents (Johnson et al. 2019, J. Beauchamp pers. obs.), and additional analysis examining *G. desperatus* occupancy related to distance to spring vents would be helpful. With *J. kosteri*, we found a 100% chance of spring-run habitat being occupied and a very high likelihood that a spring-ditch will be occupied (68%). Similar to our observations for *J. kosteri* eDNA occupancy, other species of *Juturnia* can exhibit salt tolerance, particularly at the lower end of the salt gradient (Rogowski & Stockwell 2006).

Finally, there was an 80% chance of occupancy of *P. roswellensis* eDNA in the sample when salinity was <3 ppt and DO was >50%, given site occupancy. The pattern of sample occupancy for *P. roswellensis* was similar to *J. kosteri* in that there appears to be some ability to tolerate values outside of the 80% threshold (Fig. 3). For example, there is a 60% chance *P. roswellensis* eDNA will occur in habitat that is <5 ppt salinity and DO >40%. Others have found DO is an important predictor of habitat for *Pyrgulopsis* (Malcom et al. 2005), and high levels of CO_2 in the water can lead to higher mortality (O'Brien & Blinn 1999). However, we were unable to find previous literature on the salinity tolerance for this species or genus.

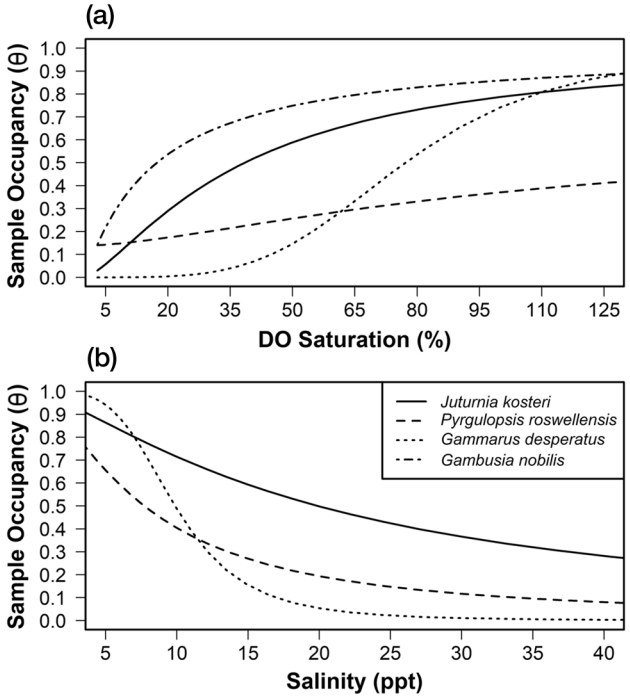

Fig. 4. Relationship (a) between dissolved oxygen (DO) and sample occupancy (θ) while holding salinity constant and (b) between salinity and sample occupancy (θ) while holding DO constant during environmental DNA sampling on Bitter Lake National Wildlife Refuge

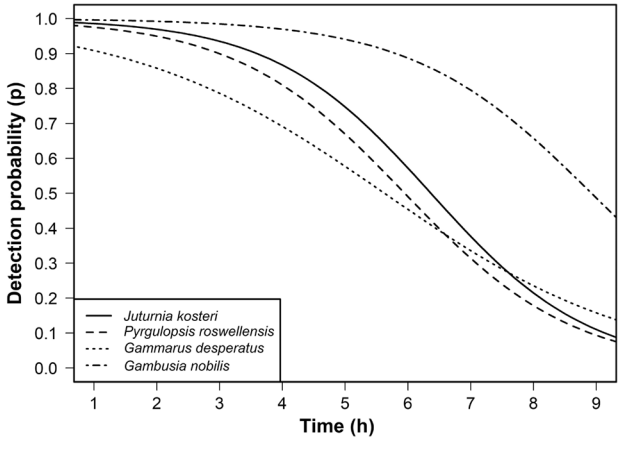

Fig. 5. Probability of detection of environmental DNA for 4 endangered aquatic species on Bitter Lake National Wildlife Refuge, New Mexico, USA, during summer 2018 declined as time of day increased

Along with understanding habitat preferences, we gained insight into improving monitoring designs using eDNA for these species. For example, we learned the impact that timing of sampling can have on detection probability of samples. Detection remained high for all 4 species until about 12:00 h local time, at which point the likelihood of detection decreased. Decreases in detectable concentrations of eDNA during afternoon hours may be related to behavior in aquatic species, particularly if they are less active during the day relative to night (Shiozuka et al. 2024). The behavioral patterns of species addressed in this study are poorly understood, but *G. desperatus* and *A. pecos* are assumed to be most active at night (USFWS 2005, 2019), and *P. roswellensis* is believed to be most active at twilight (USFWS 2024). These species potentially shed less DNA during the day (due to less activity), allowing for eDNA to degrade below detectable concentrations. Environmental DNA can be degraded by multiple avenues, including temperature (Strickler et al. 2015, Tsuji et al. 2017, Yu et al. 2022, Naef et al. 2023), UV exposure (Strickler et al. 2015, Kessler et al. 2020, but also see Mächler et al. 2018 as results are currently mixed), and water acidity (Strickler et al. 2015, Goldberg et al. 2018), among other conditions. For a more complete review, please see Harrison et al. (2019). The same pattern was shown for all 3 species of invertebrates, but *G. nobilis* detection had a longer lag time and never dropped below 0.4. A few possible reasons for this difference could be related to *G. nobilis* having a greater biomass, increased rate of DNA shedding, greater activity during the day, or increased mobility within habitats. As a result, we would recommend in these types of environments that all sampling in the summer occur before 1200.

Chance of detection remained above 50% for samples collected before 6 h post sunrise (Fig. 5). Therefore, no more than 5 PCR replicates would be needed to ensure >95% cumulative detection ($\log_{0.5} 0.05$), if present. Collection of samples before 4 h post-sunrise resulted in detection of approximately 80% (Fig. 5). Under these circumstances, only 2 PCR replicates would be needed to ensure 95% cumulative detection ($\log_{0.2} 0.05$), if present.

We developed, tested, and optimized a targeted assay for detection of 4 US federally endangered species that occur at BLNWR. Designing assays are of vital importance for species like those studied here as they can often be rare and difficult to detect. Importantly, the developed assays demonstrated specificity for the target species, but some limitations do exist. First, we initially targeted a fifth US federally endangered semiaquatic snail, *A. pecos*, which generally occurs in the plant litter and alkali crusts adjacent to spring-runs and sinkholes (Roesler 2016). We were able to amplify *A. pecos* DNA during primer development but were unsuccessful in detect-

ing any eDNA during field sampling. Abundance of *A. pecos* is thought to be low in suitable habitat, although population estimates are lacking (USFWS 2019). Further, new information (Sidhe et al. 2024) suggest *A. pecos* may not be as tightly associated with wetland margins as previously assumed; they may reach their highest abundance several meters away from wetland margins and potentially several cm deep in the substrate (Sidhe et al. 2024), which could limit their ability to shed DNA into aquatic environments. Second, we only sampled during the summer months, and it is possible that occupancy of the 5 endangered species could change seasonally, particularly for the highly mobile *G. nobilis*. Although the *P. roswellensis*, *J. kosteri*, and *G. desperatus* are rather sedentary, their abundance changes seasonally (Johnson et al. 2019). Presumably, the amount of eDNA in the water columns could also change seasonally, potentially altering our ability to detect eDNA. Third, although we sampled a variety of habitats, more sampling in the spring-ditch would improve occupancy estimates and should be explored further. Similarly, additional sampling of spring-influenced habitats on the refuge and expanding the number of environmental variables addressed in the effort might allow for stronger, more complex species-habitat relationships to be identified.

4.2. Management implications

The first and most immediate implication of this study is the ability to identify potential new sites suitable for translocation for each of these endangered species. Translocation is an important conservation step as it reduces the chances of a single catastrophic event extirpating these populations (Johnson et al. 2022). Using the approaches above, we can delineate suitable habitat within their historic range that may be currently unoccupied. Second, the eDNA assays developed here provide the possibility for long-term monitoring and could also be used as an alternative to traditional approaches of determining presence/absence. The use of eDNA can be cost effective (Fediajevaite et al. 2021) and, as seen here, capable of detecting 4 of the 5 endangered species addressed in this study. For rare invertebrates and fish, eDNA could be used to determine success or failure of translocation efforts, to conduct presence/absence assessments of habitats with unknown occupancy, to monitor currently suitable or occupied sites, and potentially determine the changes in populations over time.

Acknowledgements. We thank Taylor Bennett and Tim Huckaby for assistance in the field and the staff at the Southwestern Native Aquatic Resources and Recovery Center for processing samples. This research was supported by US Fish and Wildlife Service's Ecological Services Branch, and we thank Debra Hill and Vance Wolf for their support for this project. The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the USFWS. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the United States Government.

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Editorial responsibility: Brendan Godley, University of Exeter, Cornwall Campus, UK
Reviewed by: C. Vernesi, K. P. Phillips and 1 anonymous referee
Submitted: June 6, 2024; Accepted: November 22, 2024
Proofs received from author(s): January 10, 2025